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A MICROCOMPUTER-CONTROLLED STOPPED-FLOW CLINICAL ANALYZER IN WHICH IMMOBILIZED ENZYME REACTION LOOPS ARE USED

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A MICROCOMPUTER-CONTROLLED STOPPED-FLOW CLINICAL ANALYZER IN WHICH IMMOBILIZED ENZYME REACTION LOOPS ARE USED

By

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ABSTRACT

A MICROCOMPUTER-CONTROLLED STOPPED-FLOW CLINICAL ANALYZER IN WHICH IMMOBILIZED ENZYME REACTION LOOPS ARE USED

By

Martin David Joseph

A microcomputer-controlled stopped-flow clinical analyzer (SFCA) has been developed for application in clinical analysis. The analyzer uses an immobilized enzyme to catalyze the conversion of a specific analyte to a specific product. The concentration of the product is determined spectrophotometrically in a follow-up indicator reaction, which takes place in the observation cell of the stopped-flow module. The separation of these two reactions permits the selection of optimum conditions for each, and also the use of either a reaction-rate method or an equilibrium method to monitor each reaction.

A procedure was developed for the immobilization of enzymes onto the interior surface of nylon tubing. Glucose oxidase was immobilized by this procedure, and showed only moderate loss of activity after several months of use. Immobilized alcohol dehydrogenase was used routinely for over a month.

A commercial stopped-flow instrument was modified to

incorporate the immobilized enzyme reaction loops into the stopped-flow mixing system. The sequencing of the SFCA and the acquisition of analog spectrophotometric data were placed under the control of a microcomputer. The interfaces were designed to function in harmony with software to allow a variety of sequencing schemes to be implemented. A mini-microcomputer hierarchical system was designed to facilitate modification of microcomputer software, and to permit sharing of the facilities of a minicomputer system among several microcomputers. The hierarchical system was used to divide computational tasks between the microcomputer and a more powerful minicomputer system.

The kinetics of the immobilized enzymes were studied. The Michaelis-Menten model of soluble enzyme kinetics was applied, and modifications to the model which attempt to account for diffusional effects were incorporated. The performance of the instrument in clinical analysis was evaluated. Adequate sensitivity was obtained by allowing the sample to react with the enzyme for as short a time as 2 min. Excellent agreement with accepted methods was obtained in analysis of serum samples.

To Betty

Matt and Meg

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CHAPTER I

INTRODUCTION

Analytical chemistry has played an important role in the field of medicine for a number of years. As man's knowledge of the relationship between the levels of certain substances in the body and disease increases, there is an ever increasing demand for accurate information about a patient's body chemistry. Also, with the greater focus on preventative medicine, the physician may soon be able to predict disease, based on body chemistry information, before the appearance of normal physical symptoms. In order to keep up with this ever increasing demand in the clinical laboratory, analyses must be carried out faster, more accurately, and at lower cost. Thus there is a real need for automation at every stage of clinical analysis, from sample preparation and introduction to data acquisition and the calculation of a final result.

Immobilized enzymes should play a key role in meeting the challenge. Enzymes, by their nature, have the ability to catalyze a particular reaction of a specific substance, even when the substance occurs in a complex matrix such as plasma or serum. When attached to an inert support, the enzyme can be reused, often hundreds or even thousands of times. The increased number of determinations per unit amount of enzyme can reduce greatly the cost of routine

enzymatic analysis. Problems of instability are often diminished when an enzyme is immobilized. Hence, immobilized enzymes are far superior in many aspects to their soluble forms.

This thesis describes the development of an automated stopped-flow clinical analyzer (SFCA), which makes use of an immobilized enzyme to catalyze a specific reaction of a specific substrate (analyte). The concentration of the product of the enzymatic reaction is determined in a stopped-flow spectrophotometer. The operation of the SFCA and data handling are under the control of a microcomputer. A hierarchical network was developed to allow fast and efficient communication between a more powerful minicomputer system and this and several other microcomputers.

Because the overall project involved work in several different areas of analytical chemistry, this thesis is generally divided according to the various sub-projects. Each chapter presents a particular aspect of the entire project as a complete unit, which can be read independently of the other chapters.

Chapter II presents a history of immobilized enzymes in analytical chemistry and includes a summary of current applications in the clinical laboratory. The enzyme immobilization procedure developed in this work for attaching glucose oxidase and alcohol dehydrogenase to nylon tubing is described in chapter III. These immobilized enzymes were employed in two different flow systems, the

commercial Technicon Auto Analyzer and the stopped-flow clinical analyzer. These flow systems are described in chapter IV.

Microcomputer instrumentation, for the control of the stopped-flow clinical analyzer and for the acquisition and processing of the spectrophotometric data, is described in chapter V. A hierarchical network system is also described. In chapter VI, instrumental aspects of the SFCA are evaluated, both from a theoretical basis and from actual observed characteristics. Sources of error are discussed.

Immobilized glucose oxidase and alcohol dehydrogenase are characterized in chapter VII. The traditional Michaelis-Menten model of enzyme kinetics is applied, and modifications to the model are included to describe diffusional effects. In chapter VIII, the potential of the SFCA in clinical analysis is evaluated. A proposed system which would contain multiple immobilized enzymes is discussed, and initial work on the immobilized enzymatic analysis of serum cholesterol is presented. Finally, a summary of this work and recommendations for future work are presented in chapter IX.

CHAPTER II

IMMOBILIZED ENZYMES IN ANALYTICAL CHEMISTRY

The development of immobilized enzymes is reviewed in this chapter in relation to the field of analytical chemistry. The first section reviews the methods of immobilizing enzymes. In the next section, an overview of analytical applications of immobilized enzymes is presented. Finally, some commercially available analytical devices which employ immobilized enzymes are presented.

A. Methods of Immobilizing Enzymes

Several methods have been developed for immobilizing enzymes onto a wide variety of inert supports. Several reviews (1-8) have recently appeared covering the topic in detail. The methods can be classified into four main categories: (1) adsorption, (2) cross-linking, (3) physical entrapment, and (4) covalent attachment. Some methods involve combinations of these four, and within each category many variations have been reported. The enzyme to be immobilized as well as the application should be considered when choosing a method.

1. Adsorption

Adsorption of the enzyme to an inert support is by far the simplest method of immobilization and usually does not involve the use of harsh reagents or conditions which might be harmful to the enzyme. Adsorption methods have been reviewed in depth by McLaren and Parker (9).

The earliest reported enzyme immobilization appeared in 1916 by Nelson and Griffin (10). The enzyme invertase was adsorbed onto charcoal, and its activity detected by measuring changes in optical rotation of solutions of cane sugar when the immobilized enzyme was added.

Adsorption methods have not become prevalent due to the ease with which the enzymes are desorbed, particularly in the presence of substrate (11,12).

2. Cross-Linking

Enzymes can be immobilized by cross-linking with low molecular weight multifunctional reagents. Covalent bonds between the enzyme and the reagent form intermolecular cross-links. Multifunctional reagents commonly used include diazobenzidine and its derivatives and glutaraldehyde. Reactive groups in the enzyme (4) include terminal amino and carboxyl groups and the substituents of some amino acid residues such as arginine (guanidyl substituent), lysine (amino), hystidine (imidazole), cysteine (sulphydryl),

serine (hydroxyl), tyrosine (phenol), aspartic acid (carboxyl), and glutamic acid (carboxyl).

When attempting an immobilization procedure which involves the formation of covalent bonds to the enzyme, bonds to a group in the enzyme's active site should be avoided. The amino acid residues located in the active sites of many enzymes have been compiled (13).

To avoid bonding to the active site in trypsin (14,15), the enzyme has been first polymerized with the N-carboxyl-anhydride of L-tyrosine before it is to react with various immobilizing polymers. The polytyrosyl chain which forms supplies an alternative site to the essential residues in the enzyme. Other approaches to protecting the active site involve performing the immobilization in the presence of the substrate or a competitive inhibitor (16,17). The presence of a species such as substrate which selectively binds to the active site may also result in enhanced activity of the immobilized enzyme due to stabilization in its active conformation (19). A review of cross-linking immobilization methods can be found in Melrose (4).

3. Physical Entrapment

When a polymer is formed in the presence of an enzyme, the enzyme can become physically entrapped within the polymer lattice. The polymer lattice can be so designed that the large enzyme molecules are not able to diffuse out, but the smaller substrate and product molecules are able to

diffuse freely in and out of the matrix. Reagents used for enzyme entrapment include polyacrylamides, silicone rubber, silica gel, and starch.

Cholinesterase (19) has been immobilized in a starch matrix and placed on a polyurethane foam pad. Enzyme activity was retained for at least 12 months. Hicks and Updike (20) immobilized glucose oxidase, catalase, lactate dehydrogenase, amino acid oxidase, and glutamic dehydgogenase by entrapment in polyacrylamide gels. These enzymes showed little loss of activity after three months.

The principal advantages of physical entrapment methods are the ease of preparation and the fact that the enzyme is left unperturbed. Entrapped in a cavity of the proper size, the enzyme is able to take on conformational changes which may occur during the catalysis. Enzymes entrapped within semipermeable microcapsules are considered to mimic best the environment of a living cell. An obvious disadvantage is that entrapment of enzymes which catalyze reactions of large substrate molecules will often result in little or no observed activity.

4. Covalent Attachment

Enzymes have been covalently attached to a wide variety of water-insoluble supports by numerous methods. Covalent attachment is the most common method of enzyme immobilization, since theoretically it offers the most stable and most versatile method (2). A representative

sampling of reports of covalent attachment is presented in this section.

Lactate dehydrogenase (24) has been attached to anion exchange cellulose sheets. Enzymes have been diazotized to cellulose particles (25) and to polyaminostyrene beads (26). Other supports include water-insoluble derivatives of trypsin (27) and carboxymethylcellulose (28). Weetall and Hersh (29-33) have described procedures for the covalent coupling of enzymes to inorganic materials with the aid of an intermediate coupling agent. They note that inorganic carriers have several advantages including immunity to microbial attack and immunity to changes in configuration over a wide pH range or under various solvent conditions.

Porous glass is a popular support for covalent attachment of enzymes. Using silane as a coupling agent, alkaline phosphatase (29), urease (30), trypsin, and papain (31) were covalently coupled to silica glass. The products were used in a packed column continuously for long periods of time without significant loss of activity. Lactate dehydrogenase and pyruvate kinase (34) were immobilized on glass beads by diazotization and used in packed bed reactors. Glucose oxidase has been covalently attached to controlled-pore glass and to NiO on a Ni screen through a silane coupling reagent (32).

Glucose oxidase (35), urease, and urate oxidase (39) have been chemically attached to polystyrene tubes and used in automated analysis. Goldstein (36) has developed a

synthesis of polyanionic and polycationic resins as supports for trypsin, chymotrypsin, subtilisin Novo, subtilisin Carlsberg, and papain.

L-Aspariginase has been attached to nylon tubing (38). Nylon has also been used as the support for immobilization of lactate dehydrogenase, maltate dehydrogenase, alcohol dehydrogenase (40), urease (41), trypsin (42), and glucose oxidase (43) for use in continuous flow analysis.

B. Analytical Applications of Immobilized Enzymes

Immobilized enzymes have tremendous potential as reagents in chemical analysis. Their major advantage over non-enzymatic methods is their specificity. As such, in many cases they can be used in conjunction with non-selective detectors such as pH, gas or ion-specific electrodes, thermistor probes, or certain non-specific photometric methods. This section describes some representative examples of analytical uses of immobilized enzymes, and is divided according to the type of detector employed.

1. Electrochemical Detection

a. Amperometric

One of the most common electrochemical detectors used in combination with immobilized enzymes is the oxygen

electrode. The so-called enzyme electrode, first reported by Updike and Hicks (43), is an elegant chemical transducer. Glucose oxidase is polymerized in a gelatinous membrane over a Clark type oxygen electrode and hence combines the specificity of the immobilized glucose oxidase with the electrochemical transducer to produce a portable probe for glucose determinations. Oxygen consumption during the conversion of glucose to gluconic acid is monitored by the current output of the electrode, held at a fixed polarizing voltage. About 30 s to 3 min is required for the diffusion processes to reach equilibrium.

Total serum cholesterol has been determined by immobilized cholesterol oxidase and cholesterol esterase (44). The enzymes were immobilized onto alkylamine glass beads and placed in a rotating porous stirrer near the platinum electrode. The hydrogen peroxide produced in the enzymatic reaction was measured amperometrically at +0.60 V vs. SCE. The reaction was monitored either by a rate method, by measuring the initial current change over a 2 min period, or after equilibrium had been attained, by measuring the total current change about 10 min after the start of the reaction. A precision of 1–3% relative standard deviation was obtained in equilibrium mode determinations. The immobilized enzymes were used in 200–300 analyses with only moderate loss in activity.

Immobilized glucose oxidase has been used in combination with an oxygen electrode in the continuous flow

analysis of glucose (37). Both equilibrium and reaction-rate methods were evaluated. The enzyme appeared to be stable for 200 days while analyzing over 1000 samples, and had a storage life in excess of one year.

Williams, Doig, and Korosi (48) have used a platinum electrode to monitor reactions of both glucose oxidase and lactate dehydrogenase entrapped between the electrode and a dialysis membrane.

b. Potentiometric

The ammonium ion electrode has been used extensively as a detector for the enzymatic conversion of urea to ammonium. Guibault and Montalvo (45) have prepared a urea electrode by immobilizing urease in a polyacrylamide matrix on a thin synthetic net. The net is placed over an ammonium ion-selective electrode. When the electrode is placed in a soluton containing urea, the urea diffuses to the urease membrane where it is hydrolyzed to ammonium ion. At pH 7, the ratio of ammonium ion to ammonia is approximately 100. The response is somewhat slow; after approximately 100 s, the potential is measured and found to be proportional to the urea concentration in the range 10 to 300 ppm. The urea electrode has been improved and further described by these workers (46,47).

Urea has also been immobilized over the gas diffusion membrane of an ammonia probe. Anfalt, Granelli, and Jagner

(49) used glutaraldehyde as a cross-linking agent to immobilize the enzyme. Papasthathopoulas and Rechnitz (50) measured urea by this technique directly in whole blood samples by standard addition. The electrode potential was measured approximately 5-6 min after immersion of the electrode and found to be proportional to the urea concentration.

Since the ammonia gas electrode responds to ammonia and not ammonium ion, the pH of the monitored solution must be basic enough to ensure that all ammonium ion is converted to ammonia. The urease-catalyzed reaction is however optimized at a somewhat neutral pH. Hence the separation of these two components permits selection of the optimum pH for each. Watson and Keyes (51) immobilized urease on a porous alumina support. Samples containing urea were injected into a continuously flowing buffer stream passing through the immobilized enzyme column. A sodium hydroxide solution was continuously added to the reagent stream as it left the column, which converted ammonium ion to ammonia (pH 11). The solution then came into contact with an ammonia gas electrode which was located downstream. Total conversion of urea took place in the enzyme column, and as such the method is immune to imprecisions arising from fluctuations in the rate of the enzymatic reaction caused by changes in solution composition and temperature.

In a similar technique, urease (52) was immobilized on controlled-pore glass using glutaraldehyde and used in

the continuous flow analysis of urea with an ammonia gas sensor as the detector.

An extensive mathematical treatment of the use of potentiometric sensors to monitor reactions of enzymes immobilized in semi-permeable membranes has been presented by Blaedel, Kissel, and Boguslaski (53).

Changes in solution pH that occur during certain enzymatic reactions has also been used as an indicator. May and Li (54) monitored the enzymatic hydrolysis of urea with a commercial pH stat. Urease was immobilized in hydrocarbon-based liquid-surfactant membranes by adding as aqueous urease solution dropwise to the membrane-forming solution. The enzyme-containing emulsion was added to a solution of urea, and the reaction monitored at pH 6.7 by recording the rate of addition of HCl.

A pH stat was also used to monitor reactions involving immobilized trypsin, chymotrypsin, subtilisin Novo, subtilisin Carlsberg, and papain (immobilized as described earlier) by Goldstein (36).

2. Spectrophotometric and Fluorometric Detection

Spectrophotometric and fluorometric detectors are commonly used to monitor immobilized enzymatic reactions, as the required instrumentation is found in almost all clinical laboratories. The measurement can be either direct or indirect. Direct measurement involves monitoring a

chromophore or fluorophore whose concentration changes during the reaction. Indirect measurement involves the conversion of a species (whose concentration is changing) into a chromophore or fluorophore. Both methods can be used to monitor the reaction continuously, or monitor the species after the reaction has proceeded for a specified time.

Newirth, et al. (34) monitored solutions of pyruvate (PYR), phosphoenolpyruvate (PEP), and adenosine-5'-diphosphate (ADP) with immobilized lactate dehydrogenase (LDH) and pyruvate kinase (PK). The LDH-PK assay system utilizes the change of absorbance at 340 nm due to the disappearance of reduced nicotinamide adenine dinucleotide (NADH).

$$PEP^{3-} + ADP^{3-} + H^{+} \xrightarrow{PK} PYR^{-} + ATP^{4-}$$

$$PYR^{-} + NADH + H^{+} \xrightarrow{LDH} lactate^{-} + NAD^{+}$$

In a continuous flow system, solutions were run through either one or both of two packed bed reactors containing the immobilized LDH and PK and on to a spectrophotometric flow cell. PYR solutions (0.01-0.1 mM), PEP and PEP-PYR solutions (0.01-0.1 mM in PEP), and ADP solutions (0.01-0.1 mM) were analyzed by the method.

The increase or decrease in NADH absorbance was used to monitor the immobilized enzyme catalyzed reactions of pyruvate, oxalacetate, and ethanol (40). The enzymes were immobilized onto nylon tubes and used in an automated

continuous flow analyzer. Substrate solutions pass through the immobilized enzyme tube and on to the flow cell for the determination of the NADH present. Each immobilized enzyme tube was used for at least 1000 analyses over a period of 20 days without any loss in activity.

The hydrogen peroxide produced in the enzymatic conversion of uric acid was monitored indirectly by Filippusson, et al (39). Urate oxidase was immobilized on nylon powder which was then packed into a column and used in an automated continuous flow analyzer. Samples were mixed with buffer and passed through the packed column where a certain fraction of the uric acid was converted to allantoin and hydrogen peroxide. After the sample left the column, HCl and KI were added to the reagent stream. The peroxide converted the iodide to triiodide. The absorbance at 349 nm due to triiodide was measured in a flow cell downstream, and was found to be proportional to the uric acid concentration in the sample. Calibration curve linearity extended from 0.01 to 0.10 mM uric acid. Over 400 analyses were performed with the immobilized enzyme over a 4 month period.

The hydrogen peroxide-iodide reaction has also been used to monitor the enzymatic reaction of glucose with glucose oxidase (35). The enzyme was covalently attached to a polystyrene tube and used in the continuous flow analysis of glucose solutions ranging from 0.5 to 10 mM.

Nessler's reagent was used to monitor ammonia production in the enzymatic conversion of L-asparagine (38).

The enzyme, L-asparaginase, was attached to a nylon tube. Solutions of the substrate passed through the tube, reagents were added to the departing stream, and passed through a flow cell. The flow kinetics of the system were studied, and it was found that at low flow rates the enzymatic reaction is largely diffusion-controlled.

The activity of trypsin aattached to nylon tubing has been determined spectrophotometrically (42). The hydrolysis of solutions of N-benzoyl-L-arginine ethyl ester (BAEE) was monitored by measuring the absorbance at 255 nm of the effluent relative to that of the solution entering the tube. The kinetics of the immobilized trypsin were studied by varying the flow rate of solution through the tube (and hence the residence time) and monitoring the extent of reaction.

3. Enthalpimetric Detection

The enzyme thermistor combines the specificity of an enzyme-catalyzed reaction with the generally non-specific detection of small changes in reaction solution temperature. Mosbach and Danielsson (63) developed the first enzyme thermistor by immobilizing trypsin and apyrase and monitoring, with a thermistor probe, the temperature changes which occurred during the enzymatic reaction. The change in thermistor resistance was recorded as a function of time. Linear calibration curves were obtained when the substrate

concentration was related to either the peak height, peak area, or the gradient at the inflection point. The system, however, required approximately 10 min to reach baseline after each sample.

Bowers and Carr (56) have presented an excellent treatment of the theory of immobilized enzyme thermochemical reactors. Samples containing urea were injected into a flowing buffer stream which passes through a reactor containing urease attached to porous glass. The temperature of the solution leaving the column was monitored with a fast thermistor and related to substrate concentration. Peak height, area, and width were studied as a function of sample concentration. Linearity in all three relationships were observed for urea concentrations up to 200 mM, above which complete conversion of substrate was not obtained in the column.

Urease immobilized on controlled-pore glass was also monitored enthalpimetrically in other reports (57,58). Cholesterol, glucose, lactose, and uric acid (59) were analyzed with immobilized enzymes in combination with thermal detecton. Similarly, hexokinase (59) was immobilized on controlled-pore glass and used in thermochemical analysis.

Enthalpimetric monitoring of the enzymatic reacton has the advantage of being free from several interferences which plague other detectors. The detector is, however, completely non-specific in that it responds to any

temperature change in the monitored solution. Another drawback to their use has been the somewhat slow analysis rate possible (due to slow response of the thermistor) and the large solution volume requirements. The use of non-compressable supports for the enzymes and the use of fast thermistors (56) has dramatically improved the situation.

C. Commercial Systems Which Employ Immobilized Enzymes

Immobilized enzyme reactors as well as complete analytical instruments which employ immobilized enzymes are rapidly becoming commercially available, and more are to appear. A review of commercially-available immobilized enzyme products has been prepared by Burns (60).

Owens-Illinois, Inc. (Toledo, OH) has patented a blood urea nitrogen (BUN) analyzer. The BUN analyzer has been evaluated by Watson and Keyes (51). Urease immobilized on a porous alumina support is used to convert the urea to ammonium ion. Samples are injected into a continuously flowing buffer stream which passes through the enzyme column. Sodium hydroxide is added to the reagent stream as it leaves the column and converts the ammonium ion to ammonia, which is then detected with an ammonia gas electrode located downstream. The column has been used in over 1000 analyses over a 3 month period.

Kimble produces a similar instrument, which has been

evaluated by Hanson and Bretz (61). A two-point calibration is recommended every 10 samples. Standard deviations of 1-3% were obtained on serum and plasma samples.

The "glucose oxidase reactor" can be purchased from Northgate Laboratories (Hamden, CT). The immobilized enzyme reactor has been incorporated in a Technicon AutoAnalyzer-II continuous flow system and evaluated by Leon et al (62). Over 25,000 samples were analyzed for glucose over a 74 day period with the reactor.

Technicon Corp. (Tarrytown, NY) is offerring "Autozyme" tubes for use with their AutoAnalyzer. Tubes containing immobilized enzymes (glucose oxidase, glycerol kinase, and co-immobilized hexokinase/glucose-6-phosphate dehydrogenase) are available. Analytical aspects of these products have been evaluated by Leon, et al (64). The immobilized glucose oxidase tube was used in the analysis of 20,000 samples over a 4 month period, during which time the activity decreased by only 30%. The shelf life (0°C) is estimated to be over one year. The immobilized glycerol kinase, which is a very unstable enzyme in its soluble form, was used to determine serum triglycerides. Serum ATP was determined by the co-immobilized hexokinase/glucose-6-phosphate dehydrogenase.

Chua and Tan (65) evaluated the Glucose Analyzer marketed by Yellow Springs Instrument Co. (Yellow, Springs, OH). Immobilized glucose oxidase on a membrane is placed over a platinum electrode, which senses hydrogen peroxide

produced in the enzymatic reaction. The current output is measured over a fixed time (approx. 45 s) early in the reaction, and related to glucose concentration in the sample. A digital readout displays the concentration in mg/dl. Approximately 300 analyses can be performed with each membrane.

CHAPTER III

IMMOBILIZATION OF ENZYMES ON NYLON

Glucose oxidase and alcohol dehydrogenase were immobilized onto the interior surface of nylon tubing for use in automated clinical analysis. Nylon tubing was chosen as the support because solution containing the analyte (substrate) can be quickly and easily brought into contact with the immobilized enzyme for conversion to product and then removed to determine the amount of product formed. Nylon tubing containing immobilized enzyme was used in both a continuous flow type arrangement and also in the discrete sampling stopped-flow clinical analyzer.

Development of the immobilization procedure involved repeated synthetic attempts at obtaining adequate activity of the immobilized enzyme. During the development of the procedure, substrate samples were analyzed by the immobilized enzyme on the Technicon Auto Analyzer continuous flow system described in Chapter IV. Each immobilization attempt was judged by its potential in a clinical laboratory environment, where large numbers of samples must be processed daily. A procedure reported by Inman and Hornby (66) was attempted first, but proved unsuccessful for reasons discussed below. This chapter describes the development of an immobilization procedure which is believed

to be applicable to a variety of enzymes.

Briefly, the enzymes were immobilized on nylon tubing of 1.5 cm and 0.87 mm inner diameters as follows. After removing amorphous nylon, the interior surface of the tubing was partially hydrolyzed with mild acid. Terminal carbons of glutaraldehyde molecules were then attached to the partially hydrolyzed nylon, after which the remaining terminal carbon bonded to primary amine groups of the enzyme.

This procedure results in enzyme molecules becoming attached to the support by a six carbon cross-link, which allows flexibility in the molecule and hence no steric hindrance to activity. Enzymes in which primary amine groups are not present at or near the active center could be immobilized by this procedure without loss of activity.

A. Initial Preparation and Hydrolysis of the Nylon

Amorphous nylon was first removed from the nylon tube by incubation in a 20% solution of calcium chloride in 80% methanol/20% water (v/v) solvent at 50°C for 20 min. When the tube was then rinsed with distilled water, a stringy white precipitate was purged from the tube. Apparently the $CaCl$ solution acts to remove sections of the nylon surface which are not totally a part of the polymeric structure. This should then result in a somewhat increased surface area for enzyme attachment as well as a more permanent support.

The remaining steps in the procedure were done by pumping the particular reagent through the tube with a peristaltic pump. By choosing pump tubing of appropriate inner diameter, the flow rate of reagent through the nylon tube can be selected from 0.015 to 4 ml/min.

Hydrolysis of the interior surface of the nylon tube was carried out by treating it with a mildly acidic solution. The surface hydrolysis is complete after 20 minutes of pumping 0.5 M hydrochloric acid through the tube at room temperature.

Early attempts at immobilization involved incubating the hydrolysis reaction at 40°C and using 4 M HCl. This invariably resulted in excess hydrolysis. The tube became so clogged that solution flow could not be maintained. Hydrolysis conditions were then made less severe by allowing the reaction to proceed at room temperature, after which solution could be easily pumped through the tube.

However, as shown in Figure 1, scanning electron microscope photographs of the interior surface of both virgin nylon tubing and tubing hydrolyzed under these conditions (4 M acid, room temperature) showed that the hydrolysis was so severe that sections of the tubing had been hydrolyzed nearly halfway through the tube wall. The extent of hydrolysis was also shown to be quite different in various sections of the tube wall.

Although the resulting structure could support immobilized enzymes, it proved to be too porous to be of any

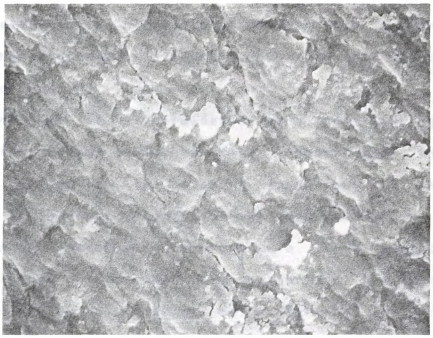
value in enzymatic analysis. Substrate and/or product molecules became trapped in the matrix and at times could not be removed by eluting for 30 minutes or more, which resulted in serious carry-over of these substances from one sample to the next and precluded its use in routine clinical analysis.

When the hydrolysis conditions were changed so 0.5 M HCl was used at room temperature, the problem of carry-over was completely eliminated. All traces of a previous sample could be completely removed by rinsing the enzyme loop with small portions of buffer solution. Presumably, the surface hydrolysis could be carried out even under milder conditions to attain good enzymatic activity.

B. Attachment of Glutaraldehyde

The bifunctional reagent glutaraldehyde is an ideal reagent for enzyme immobilization because both of its aldehydic carbons can be utilized in the formation of a cross-link between the support and the enzyme.

Substrate molecules must have free access to the active site on the enzyme molecule, and once they reach the active site, the enzyme molecule often must be able to take on conformational changes during the conversion of substrate to product. The product molecule must then be able to diffuse back into the bulk solution so that its concentration can be determined. Each of these steps may be



Inner Surface of Virgin Nylon (5000 diameters)

Inner Surface of Hydrolyzed Nylon (5000 diameters)

Figure 1. Scanning Electron Microscope Photographs of Virgin and Hydrolyzed Nylon Surface

hindered if the enzyme is directly bound to the support. Also, electrostatic interactions between the support and either the substrate or product species may slow the overall conversion rate.

The use of glutaraldehyde as a cross-linking agent leaves the enzyme somewhat less "immobilized" than if directly bonded to the support, and thus it can move somewhat in the near vicinity of the support wall.

In the procedure developed, a 12.5% solution of glutaraldehyde is slowly pumped through the partially hydrolyzed nylon tube, during which terminal carbons are covalently bonded to free nitrogens on the nylon wall, according to Figure 2. The remaining aldehyde groups on each attached glutaraldehyde molecule are then available for covalent bond formation with the enzyme. It is certainly possible that a significant fraction of the attached glutaraldehyde molecules will bond both aldehydic groups to the support and hence be unavailable to bond to the enzyme.

In the development of this procedure, it was found that the solution pH during the glutaraldehyde attachment is very critical, and must be maintained at 9.2. The glutaraldehyde solution in this procedure was buffered in 0.1 M tris(hydroxymethyl)methylamine. Final enzymatic activity was not detected when this reacton was performed at pH values of 8.5, 8.8, or 9.1. Apparently at a pH lower than 9.2, nitrogens on the hydrolyzed nylon are protonated and their reaction with the glutaraldehyde carbonyl is

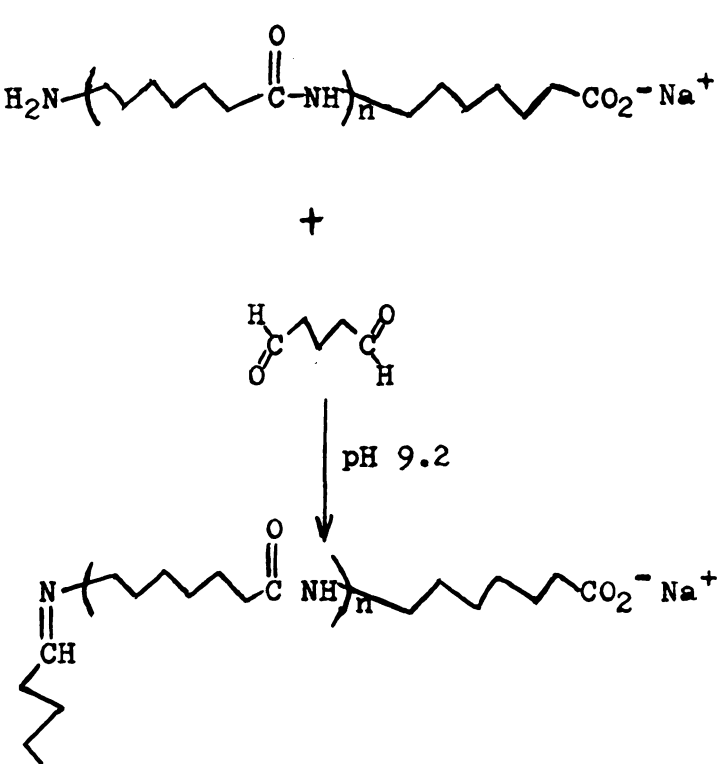


Figure 2. Attachment of Glutaraldehyde

hindered.

Glutaraldehyde is the most prevalent multifunctional reagent used in enzyme immobilization, yet its structure and chemistry are not well understood (3). It has been proposed that aqueous solutions of glutaraldehyde are largely polymeric (67), and also to the contrary that it exists mainly as the three hydrates shown in Figure 3 (68). The mechanism of the reaction of glutaraldyhyde with enzymes is similarly unresolved.

C. Immobilization of the Enzyme

After the glutaraldehyde attachment, the nylon tube is thoroughly rinsed with the tris buffer for 20 minutes. A solution of the enzyme (approx. 4 mg/ml) buffered at pH 6.2 is then slowly pumped through the tube in a closed loop at $0^{o}C$ (ice-water bath) for 4 h. During this time, primary amine groups on the enzyme, presumably, react with free aldehyde groups of the attached glutaraldehyde to form the structure shown in Figure 4. As can be seen, this structure allows the enzyme molecule flexibility and a certain degree of mobility.

The mechanisms involved in glutaraldehyde attachment and subsequent enzyme immobilization described here are only proposed mechanisms. The actual covalent linkages described have been proposed elsewhere in the literature (3), although

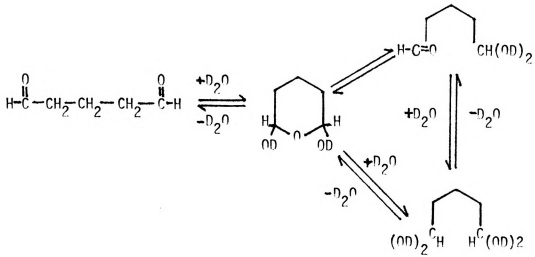


Figure 3. Proposed Aqueous Forms of Glutaraldehyde

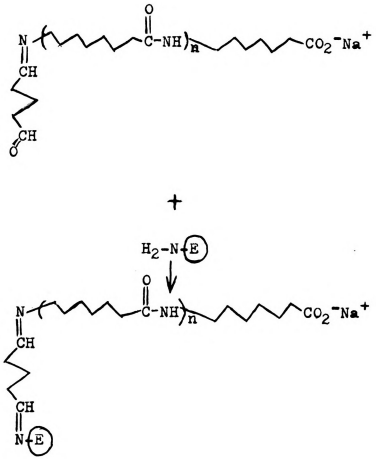


Figure 4. Attachment of the Enzyme

no definitive evidence has been put forth. It was not within the scope of this work to determine the mechanism of the immobilization procedure. Rather, this work involved the development of a somewhat empirical and functional procedure. Certainly, though, if the mechanism were better understood, reaction conditions could be intelligently chosen to maximize not only the amount of enzyme immobilized but also the resulting activity.

D. Specific Procedure for Enzyme Immobilization

The following is a detailed description of the immobilization procedure developed. Steps 2-9 were carried out by using a peristaltic pump to bring a constant stream of fresh reagent through the nylon tube.

1. Fill a 1.0 m length of the nylon tubing with a solution containing 20% CaCl in 80% methanol/20% water (v/v).

2. Rinse with 500 ml of dist. water at 8 ml/min.

3. Hydrolyze the surface by pumping a 0.5M HCl solution through the tube at room temperature for 40 min (8 ml/min).

4. Rinse with 500 ml of dist. water at 8 ml/min.

5. Pump a 12.5% solution of glutaraldehyde in 0.1M tris(hydroxymethyl)methylamine buffer, pH 9.2, through the tube at 2 ml/min for 20 minutes at 0°C.

6. Rinse with the 0.1M tris buffer at 0°C for 20 minutes.

7. In a closed loop, pump a solution containing 4 mg/ml of the enzyme per ml of 0.2M phosphate buffer at 0°C at 2 ml/min for 4 hours. The pH of the phosphate buffer should be chosen according to the particular enzyme to avoid possible denaturization.

8. Rinse with 0.1M NaCl at room temperature at 8 ml/min for 1 hour to remove any physically adsorbed enzyme.

9. Rinse and fill with buffer of appropriate pH, and store refrigerated (5°C) when not in use.

CHAPTER IV

FLOW SYSTEMS

Two types of flow systems have been employed in the implementation of the immobilized enzymes, and these flow systems are described in this chapter. The first system, a Technicon Auto Analyzer, uses continuoulsy flowing reagent streams to mix the sample with the desired reagents and then pass the solution through a transparent flow cell for spectrophotometric observation. Secondly, the stopped-flow clinical analyzer which was developed in this work is described. This system is a discrete sample analyzer which uses a commercial stopped-flow mixing system for reagent and sample mixing and spectrophotometric monitoring.

A. Technicon AutoAnalyzer

1. Description

The design concept of the Auto Analyzer was developed by Skeggs (69) in 1957. Solutions are pumped through flexible tubing at selected flow rates by a peristaltic pump. The flow rate for each reagent line is determined by the inner diameter of the tubing over which the pump rollers are driven at a constant rate. The reagent lines are joined

where desired by glass connectors to produce the desired configuration. A non-adjustable thermostatted heating bath can be used to incubate solutions only at 42°C as they pass through the bath.

2. Design for Immobilized Enzymatic Analysis of Glucose

The Auto Analyzer flow system was used in early studies of immobilized glucose oxidase. The enzyme was attached (as described in chapter III) to the inner surface of a coiled nylon tube, 2 meters in length and 1.5 cm inner diameter, and inserted into the flow system. For the analysis of glucose samples, the flow system components were arranged as shown in Figure 5.

In this configuration, the sample or blank solution was first mixed with the buffer solution and air bubbles were added to the flow stream. The solution then passed through the immobilized glucose oxidase tube which was immersed in the heating bath at 42°C. As the solution passed through the column, a fraction of the glucose was converted to gluconic acid and hydrogen peroxide. After the reacted solution left the column, a buffered solution containing potassium iodide and Mo(VI) was added to the flow stream, which then passed through a mixing coil. In the mixing coil, the hydrogen peroxide was completely reduced by the excess iodide, and a corresponding amount of triiodide

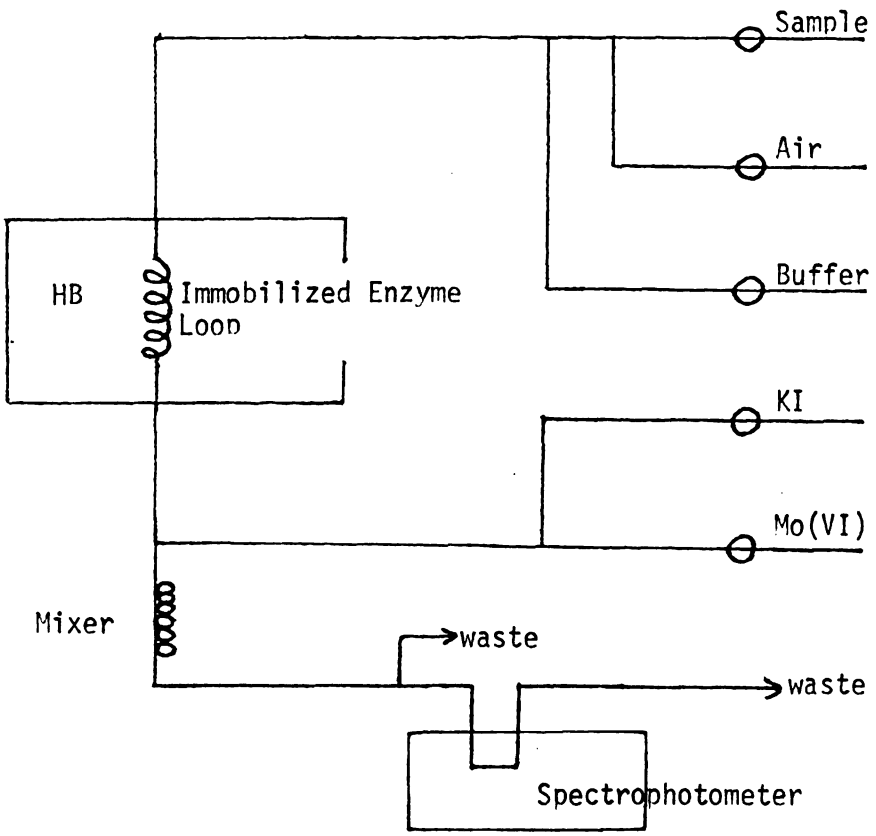


Figure 5. Auto Analyzer Configuration for Glucose Determinations

was produced. The Mo(VI) catalyzes the peroxide-iodide reaction. The air bubbles were then removed from the flow stream, as described later in this section, and the stream then passed through a quartz flow cell housed in a Beckman DB-G spectrophotometer. The absorbance of triiodide at 365 nm was recorded on a strip chart recorder as the triiodide solution passed through the flow cell. Either the peak height or the peak area of the absorbance vs time curve was related to the concentration of glucose in the sample.

3. Characteristics

Air bubbles are added at regular intervals to the flow stream in the AutoAnalyzer to isolate segments of the stream, which permits efficient mixing within each segment and also results in less axial dispersion of each sample plug as it flows through the system. The bubbles are removed from the stream just before it passes through the spectrophotometer to avoid large spikes in the response.

A major disadvantage of the AutoAnalyzer encountered in the study of the kinetics of the immobilized enzymes was the fact that it was difficult and time consuming to vary the enzymatic reaction time, even over a moderate range. The duration of the enzymatic reaction is controlled by both the volume of the column, which was fixed, and the flow rate through the column. However, by altering only the flow rate of the sample, other problems can result. The relative

amounts of each solution added to the flow stream (sample, buffer, air, iodide, and molybdate) are determined by their relative flow rates. Hence, although varying the flow rate of the sample line could have been used to vary the enzymatic reaction time, this would have also varied the relative concentrations of all other reagents. Since the iodide and Mo(VI) gave a small blank absorbance, the baseline would have to be reset each time the flow rate was changed. More importantly, the effectiveness of the buffer in pH control would have to be checked as its concentration was also changed. Similarly, the adequecy of the final iodide and Mo(VI) concentrations would have to be verified as well.

The mixing coil was very effective in mixing the column effluent with the indicator reagent. The mixer consists of 10 turns of glass tubing, so that each solution segment is inverted 10 times as it passes through the coil. The time spent in the mixer of course depends on its inner diameter and the flow rate through it; in the flow design used in these studies, the mixing time was typically 30 s.

The debubbler consists of an inverted tee inserted into the flow stream. The air bubbles and a small amount of liquid are drawn out the top of the tee by connecting the top to the pump. The proper flow rate for the debubbler was selected such that the bubbles were completely removed from the stream while at the same time only a small amount of liquid was removed. The debubbler was placed as near as

possible to the flow cell to avoid axial dispersion of the sample plug after the air bubbles had been removed.

In theory, the triiodide produced in the indicator reaction should pass through the quartz flow cell as a plug, and hence should then give a response curve in the shape of a gaussian peak. The flow rate of solution through the flow cell was typically 3 ml/min. The dimensions of the flow cell are 1 cm X 1 cm X 2 mm, with a total volume of 200 ul. Hence, if the chromophore moved into and out of the flow cell as a plug, each cross-sectional slice of the plug would reside in the cell for approximately 4 s. In practice, however, the sample plug was observed to require about two to three times that expected to completely pass through the cell, mainly due to inefficient design of the flow cell. A relatively large dead volume exists in the flow cell, especially in the corners, where solution can become trapped.

The slow passage of a plug of the chromophore through the flow cell made it difficult, if not impossible, to calculate the actual extent of the enzymatic reaction in the column. The peak area of the observed response could not be related to the integral of the chromophore concentration in the plug.

The peak height of the absorbance response curve, however, could be used to generate a linear calibration curve for glucose. However, due to the degree of axial dispersion and subsequent decrease in peak height of the

sample plug as it travelled through the flow system, the sensitivity was low. This is a fundamental disadvantage af the Auto Analyzer flow system when the peak height is the measured quantity. In clinical laboratories, the flow system is often designed so that the sample plug must flow over a long distance before reaching the flow cell, and often requires several minutes to complete the journey. Axial dispersion also limits the analysis rate, since samples must be introduced far enough apart to avoid overlap of the responses.

B. Stopped-Flow Clinical Analyzer

1. Description

The design of the stopped-flow clinical analyzer is illustrated in Figure 6. The components enclosed within the dashed line are part of a GCA-McPherson stopped-flow module. This particular module is well suited for automated operation, since no manual manipulation of valves is required. A single cycle can be initiated either from the front panel or with TTL-compatable signals to a plug on the rear panel.

The drive syringes (DS1 and DS2) are replaceable glass syringes, and the volume driven with each push is manually adjustable from 200 to 500 ul/syringe. The plungers are driven by a pneumatic cylinder which is driven

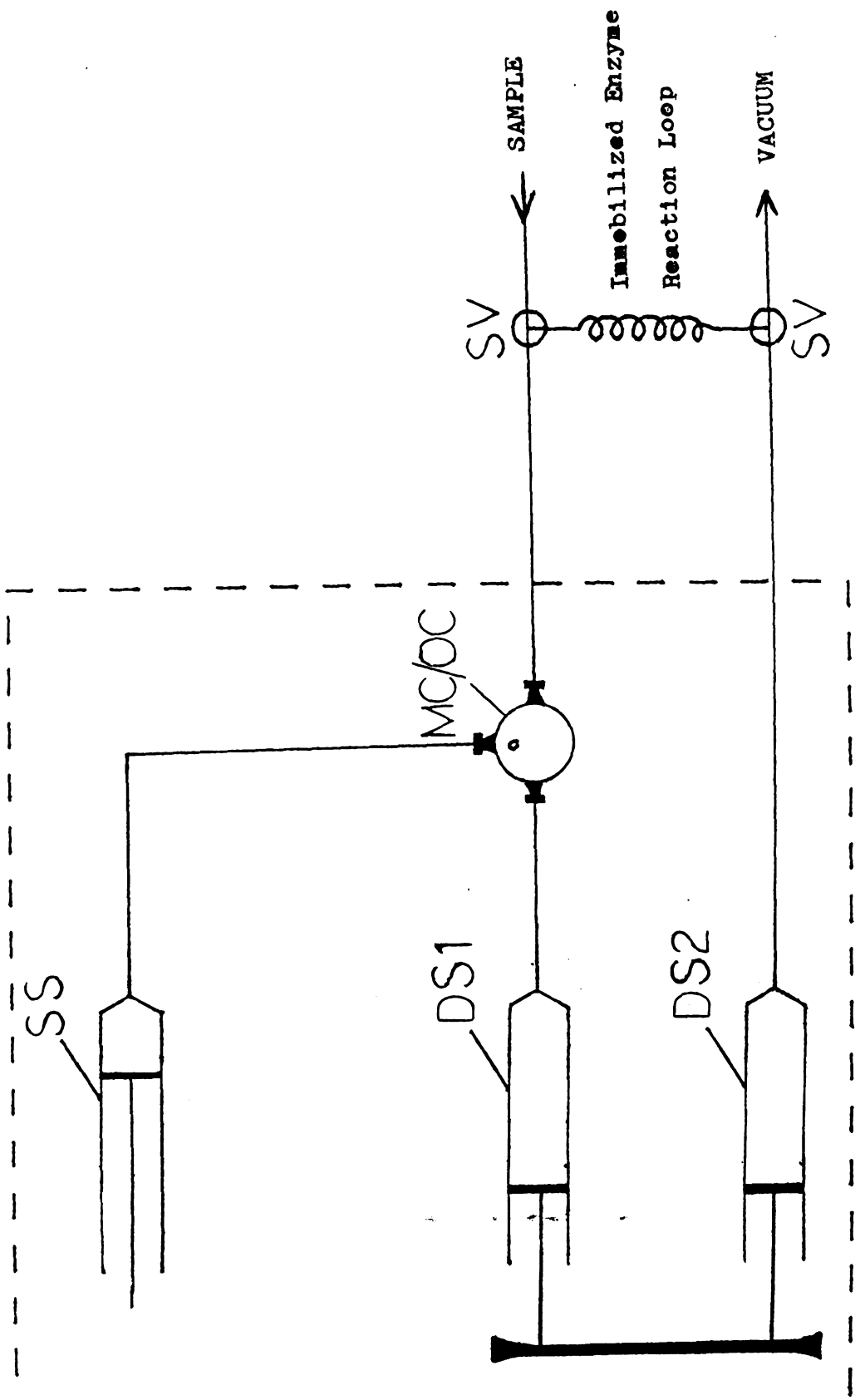


Figure 6. Stopped-Flow Clinical Analyzer

with 35-45 psi pressure supplied by an external air cylinder. The mixing chamber (MC) and observation cell (OC) are housed together in a Kel-F block mounted near the front of the module in the optical path. Solution is directed from the drive syringes to the mixing chamber/observation cell inlet through narrow diameter plastic tubing, and the connections are made with threaded fittings. The quartz observation cell is cylindrical in shape, measures 2 cm in length and 2 mm in diameter, and has a volume of 62.8 ul. The stop syringe (SS) is also replaceable glass, and the volume taken into the syringe before the flow stops is adjustable from 400 to 1000 ul. The dead time of the system is ~~5 ms (70).

The GCA-McPherson stopped-flow module was designed to be used with the Heath series modular spectrophotometer system. A complete stopped-flow spectrophotometer can be easily assembled by mounting the stopped-flow module with a light source module, monochromator, photomultiplier module, and a data collection system.

The GCA-McPherson module was modified to incorporate the immobilized enzyme reaction loop. As shown in Figure 6, one of the reagent lines was brought outside the module. This was done by installing HPLC tubing couplers (Altex) on the front panel of the module, and, on the inside the module, using short pieces of HPLC tubing to connect DS2 to one of the couplers, and the mixing chamber/observation cell entrance port to the other coupler. Outside the module,

short pieces of tubing were also used to connect the other ends of the two couplers to a pair of 3-way slider valves (SV) (Altex). The immobilized enzyme reaction loop was inserted between the valves, so that, when the 3-way valves were switched to the push position, the reaction loop was connected to the stopped-flow system. When the syringes were then driven, solution from DS2 forced the solution from the reaction loop into the mixing chamber for analysis.

The 3-way slider valves consist of a rectangular Teflon slider block mounted within the valve which directs the solution flow from the common line to either of the secondary lines. These valves were automatically switched in tandem by a pneumatic actuator/spring return mechamism. Tubing is connected to the valve ports by threaded fittings so that connections can be easily made.

The operation of the SFCA can be best described by the following typical sequence of events for the analysis of a substrate sample. Referring to Figure 6, the stopped-flow mixing system is first rinsed with the desired reagents. DS1 is filled with the indicator reagent and DS2 is filled with the push liquid (buffer). When this cycle is completed, the sample valves (SV) are switched to the fill position and the buffered sample is drawn into the reaction loop by the action of the applied vaccuum. When the loop has been thoroughly rinsed and filled, the sample valves are switched back to the push position, which stops the sampling process and marks the beginning of the enzymatic reaction.

After the enzymatic reaction has been allowed to proceed for a preset time, the stopped-flow syringes are driven. The push liquid in DS2 forces the reacted solution from the reaction loop through the connecting tubing into the mixing chamber, where it is rapidly mixed with the indicator reagent and driven into the observation cell. The indicator reaction then takes place in the observation cell. The indicator reagent converts one of the enzymatic reaction products into a chromophore, whose absorbance is monitored as an indicator of the amount of product produced in the enzymatic reaction.

2. Characteristics

The HPLC tubing and sample valves are designed to withstand a pressure of 500 psi, which is more than adequate for this system. These components are made of Teflon and are therefore chemically inert to the reagents used in these analyses. The sample valves are designed with zero dead volume.

When the immobilized enzyme loops are fitted with the threaded end fittings, they can be quickly and easily inserted or removed from the system, which simplifies replacement the reaction loop.

The design of the SFCA greatly facilitates a fundamental study of the kinetics of the immobilized enzymes. The volume contained within the reaction loop was

designed to be much greater than the combined volumes of the connecting tubing, mixing chamber, and observation cell. The push volume was adjusted so that the solution initially in the reaction loop completely replaced the solution that was previously in the observation cell. Thus, the reacted solution in the reaction loop could be accurately sampled and monitored in the observation cell, without the problem of axial dispersion prevalent in the Auto Analyzer flow system. The concentration of the enzymatic reaction product could be calculated from the measured absorbance, the molar absorptivity of the chromophore, and a knowledge of the stoichiometry of the indicator reaction.

The design of the SFCA allows one to monitor the enzymatic reaction and the indicator reaction separately, each by either a reaction-rate method or by an equilibrium method. This choice allows the use of an indicator reaction which may, for example, be slow, or have an unfavorable equilibrium constant, or undergo side reactions. Also, since the two reactions are physically separated, the reaction conditions can be drastically different in each, and can be chosen to yield the optimum sensitivity, accuracy, and precision from each reaction.

Another fundamental advantage of the SFCA design is that several immobilized enzyme reaction loops containing the same or different enzymes can be multiplexed into the stopped-flow mixing system. This could tremendously improve sample throughput.

The SFCA also is efficient in its use of reagents, since the volume of the stopped-flow syringes is small, and since reagents are consumed only when neccessary rather than continuously flowing.

A disadvantage with the present design of the SFCA is the lack of precise thermostatting. The entire system is held at room temperature. Any increase in the temperature of the reaction loop, mixing chamber, etc. must be dissipated into the surrounding air. In the stopped-flow module, thermostatted water is circulated in copper tubing which is in contact with a cast aluminum base. Thermostatting within the module is therefore dependent on heat transfer to and from the aluminum base via thermal conduction. Holler (71) showed that this design is somewhat inefficient. His studies demonstrated that temperature equilibrium of the solution in the observation cell is slow, and does not accurately track the temperature of the thermostatting bath.

CHAPTER V

MICROCOMPUTER INSTRUMENTATION

Computer technology, specifically microprocessors and microcomputers, has had a dramatic impact on analytical chemistry. With the aid of a computer, chemists are able to perform long and tedious experiments which were previously infeasible or highly prone to human error because of monotonous sequencing or massive data collection and processing. The high speed data acqusition and almost instantaneous feedback control offerred by computers have increased the amount of information that can be extracted from a measurement process. The use of a mini-and microcomputer as an aid to clinical analysis and the networking of computers in our laboratories are described in this chapter.

A. Overview

The microprocessor offers an inexpensive method of process control and data acquisition. It is highly flexible in application since it can be programmed to follow any sequence. With sufficient memory, the microcomputer can perform the entire analysis, including instrument set up, calibration, sampling, data collection, and calculation of a

final result (concentration, rate constant, absorption spectrum, etc.).

At the other extreme, microcomputers can be slaved to a larger computer (e.g. mini) and relegated to perform specific tasks. In this configuration, the minicomputer commands the slave micros to perform a task or to begin a certain sequence, receives information from the micros about the status of the experiment, and receives raw data (e.g. pH, temperature, etc.) collected by the slaves. The data are processed by the mini and displayed in a variety of formats such as lists, plots, etc.

In this work, a microcomputer was relegated to the tasks of instrument sequencing, acquisition of raw data corresponding to transmitted radiant power, and performance of simple calculations on the raw data. The partially worked-up data were then transmitted to a more powerful minicomputer for more complex calculations, display, and magnetic storage. A network was devised for allowing a user terminal and several microcomputers to share the facilities of the minicomputer.

Several network configurations (72) are possible for interconnecting the various devices (microcomputers and terminals) in our research laboratories to the minicomputer. Different networks involve varying levels of communication between the devices and varying degrees of hardware and software requirements. The point-to-point network shown in Figure 7 was implemented. In this configuration, access to

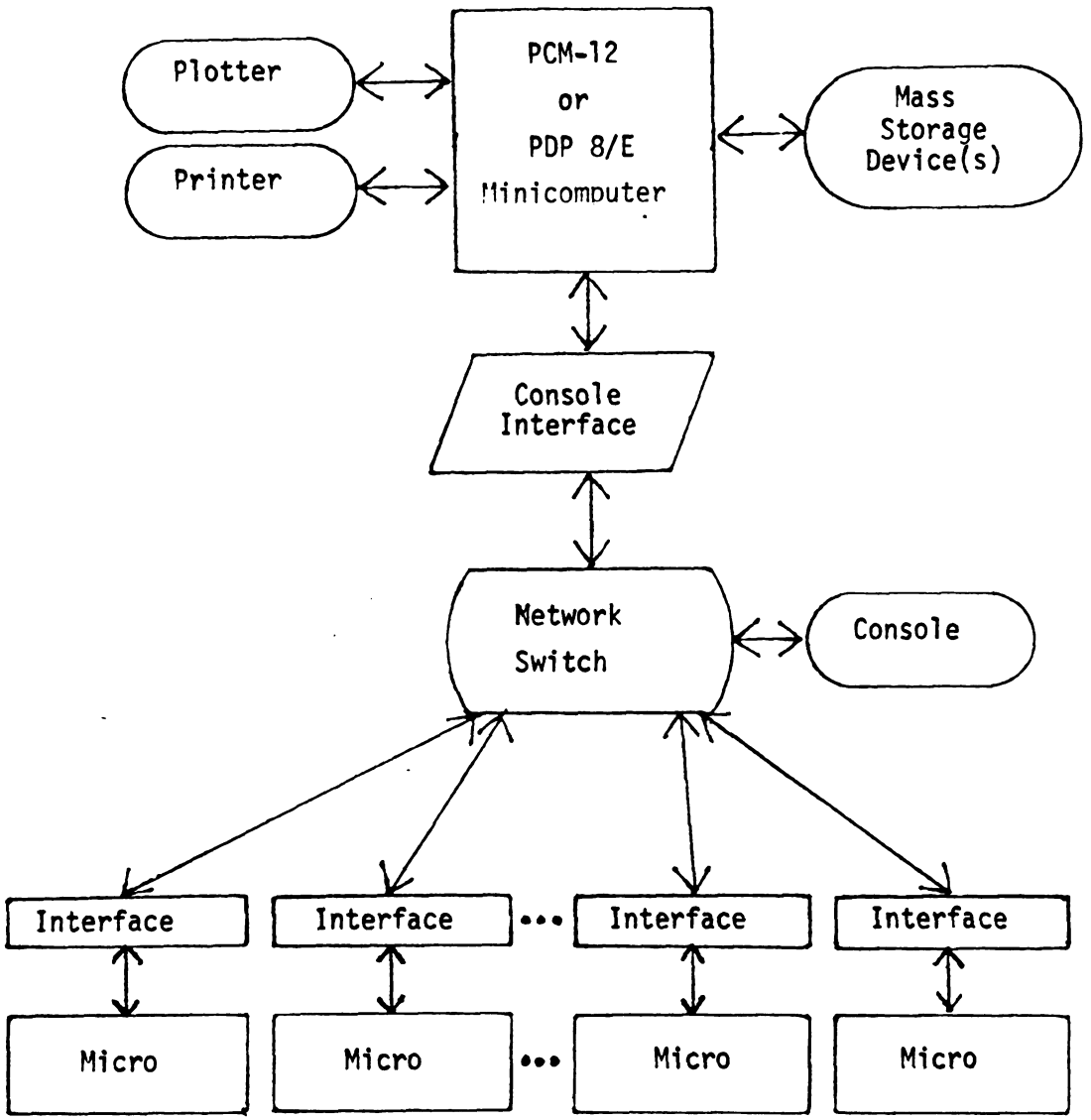


Figure 7. Point-to-Point Hierarchical Network

the minicomputer is controlled by a network switch, so that the facilities of the mini are time-sliced. When a particular device is switched to the mini, it is able to retain complete control for as long as desired, and releases control when its job is completed. The network switch then sequentially samples each device until it finds one which needs access to the mini.

The point-to-point configuration was chosen for several reasons. Because of the wide spectrum of experiments performed in our laboratories and the independence of each, communication between microcomputers is at present unneccessary. Since mini-to-micro communication is made through an asynchronous interface, no restrictions are made on the internal configuration of each microcomputer. The time-sliced sharing of the minicomputer can be implemented because of the typically short time requirements of each microcomputer. Once software has been developed, the mini-to-micro link needs to be made only to down-load the software into micro memory; a relatively much longer time is then consumed performing the experiment, during which the mini is accessable to other devices. Finally, this configuration is the simplest to implement both in hardware and software.

At present, either of two minicomputers can serve as the head of the point-to-point network. A PDP 8/e minicomputer (Digital Equipment Corp.) is configured with 16k of read/write memory, extended arithmetic element, real

time clock, RK05 cartridge disk drive, dual floppy disk drive, printer, plotter, and console. A second system is configured around a PCM-12 minicomputer (Pacific Cyber/Metrics) and includes 16k of RAM, dual floppy disk drive, printer, and console. The PCM-12 utilizes an Intersil 6100 microprocessor as the CPU. This processor uses virtually the same instruction set as the PDP 8/e, so the two minicomputers are software compatible. Software which does not make use of hardware unique to either system can be developed on either mini. Both operate under OS/8, the operating system developed by DEC for the PDP 8's.

B. The 6100 Microcomputer

1. Description

Control of the sequencing of the stopped-flow clinical analyzer and data acquisition from the photocurrent amplifier are accomplished with a twelve-bit microcomputer which utilizes an Intersil 6100 microprocessor. The central processor board, 4K RAM board, and the two serial interface boards were purchased (Pacific Cyber/Metrics, San Ramon, CA) and constructed from a kit. This particular microcomputer was chosen because the 6100 MPU uses virtually the same instruction set as the PDP 8/e in our laboratory, and, as such, software for the microcomputer can be created and developed on either the PDP 8/e or PCM-12 minicomputer

under OS/8 and then down-loaded into the microcomputer's memory for execution.

Software for SFCA execution is written either in FORTRAN-II/SABR or PAL8 (the PDP-8 assembly language) on the minicomputer, stored on a mass storage device (floppy disk or cartridge disk), and compiled and assembled under OS/8. The absolute binary program thus generated can then be down-loaded without further modification, via the asynchronous serial interface directly from the mini to the micro. This procedure greatly facilitates debugging the SFCA software since it can be quickly edited, assembled, and reloaded. It also has versatility in the SFCA operation, as a variety of routines for a variety of SFCA operating procedures can be quickly called up from mass storage and downloaded.

The SFCA micro-mini computer system is shown in Figure 8. Direct communication between the mini and micro is required only to transmit the desired binary program down to the micro, after which the link can be broken and the microcomputer operated in a stand-alone configuration. Optionally the link is later remade to transmit raw data acquired during an experiment back to the mini, where higher-level language routines can work up the data, list, or plot.

2. PROM Monitor Software

A general purpose routine to handle the down-loading of binary programs from the mini into micro memory was designed to operate in PROM in the microcomputer. This PROM

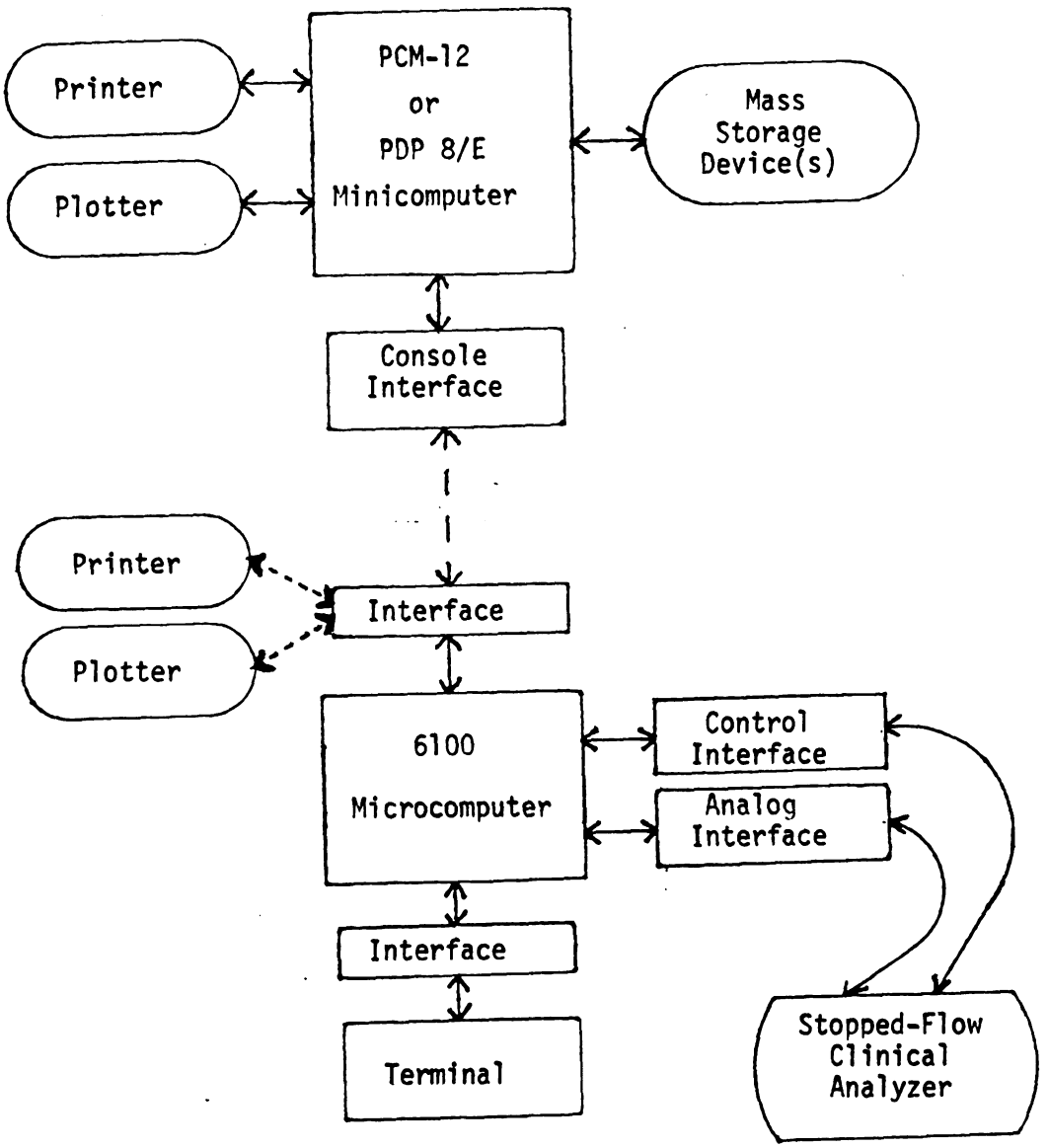


Figure 8. SFCA-Micro-Minicomputer System

Monitor routine serves three basic functions for the micro: (1) a transparent mode which serves as a software link between the microcomputer terminal and the minicomputer's main terminal port; (2) a binary loader for loading an incoming binary program into the micros mainframe memory; and (3) a RAM Monitor which allows the user, via commands typed at the keyboard, to examine and/or modify locations in mainframe memory, and to begin execution at a specified location.

a. Transparent Mode

The transparent mode provides a link between the user terminal and the minicomputer; in this mode the microcomputer is essentially transparent to the user. The transparent mode is entered automatically after power-up and initialization of the PROM Monitor. The program then monitors both of the micro's serial interfaces. Ordinary characters input at one port are immediately output to the other. This procedure continues until a special character is received at either port. These special characters cause the Monitor to chain to either the binary loader or RAM Monitor. The user can cause chaining to the RAM Monitor by typing CNTRL-A at the terminal, while the minicomputer can cause chaining to the binary loader by issuing leader-trailer code (octal 200).

A standard feature of the OS/8 Peripheral Interchange

Program (PIP), when outputting a binary program, is to begin and end the actual binary data with leader-trailer. This character is not otherwise normally output by OS/8 software, and hence the transparent mode is not unneccessarily interrupted unless the minicomputer is about to start transmitting a binary program. Similarly, CNTRL-A is not normally typed by the user when operating OS/8 software.

An important consideration in designing software for mini-micro communication (specifically with the operating system) was to be able to use the O/8 software without modification. In the particular case of the transparent mode routine, this meant that characters had to be input from the minicomputer and then output to the terminal at a fast enough rate that no characters would be missed. This was readily accomplished by operating the terminal interface at a higher baud rate than the minicomputer interface. Normally, for example, the terminal is operated at 2400 baud (240 characters/s) while the minicomputer interface is operated at 1200 baud (120 characters/s). As such, each transmission of a character to the terminal requires 4.167 ms, while character transmissions from the minicomputer require 8.333 ms. Once a character is received from the minicomputer, the next one cannot be completely transmitted before 8.333 ms has elapsed, and the micro software then has the additional 4.167 ms to check the input for a special character, which is more than ample time.

As expected, operation of both interfaces at the same

baud rate results in occasional missed characters.

The asynchronous interface between the micro and mini has the additional advantage of allowing the micro to run batch processes by communicating directly with OS/8 software without operator intervention. The micro can be viewed as a "virtual terminal" to the mini since the OS/8 software receives characters as if a terminal were present. Micro software can mimic the action of a user entering commands at a terminal by transmitting a sequence of characters. The routine needs only to wait for each character to be echoed by the mini before sending the next character. Commonly used command sequences can be stored in micro memory and later transmitted in this fasion.

b. Binary Loader

The binary loader secton of the PROM Monitor is automatically chained to when leader-trailer code is input from the minicomputer, a signal that the transmission of an absolute binary program is beginning. Approximately 60-70 frames of leader-trailer precede the actual binary code, so no binary data are missed during chaining.

The binary loader routine was adapted from a software front panel routine developed at Pacific Cyber/Metrics for their PCM-12 minicomputer. Their loader was modified by eliminating several unneccessary features so that it would fit in the 256-word PROM space along with the transparent mode and RAM Monitor.

First, the loader input device number was changed to 13 (the minicomputer interface). Second, extended memory loading was eliminated, since the micro at present has only one field (4K) of read/write memory. Finally, the action taken upon completion of the loading process was changed. The last two binary words of the transmission comprise the checksum of the transmission, which provides a crude check on the entire transmission. The loader calculates the checksum as the program is input and loaded, and then compares this with the input checksum. If the two are not identical, the loader halts. A halt indicates that an error has occurred. If the two are identical, the program is assumed to have been accurately input and loaded, and the loader then chains to the RAM Monitor.

c. RAM Monitor

This section of the PROM Monitor is chained to either after the successful completion of a binary load, or when the operator types a CNTRL-A at the terminal while in the transparent mode. The RAM Monitor serves the functions of examining and/or modifying microcomputer read/write memory, and beginning execution at a specified RAM address. The Monitor begins by typing a prompting dollar sign; this is repeated each time the Monitor is ready for terminal input.

After prompting the user, the Monitor expects to receive from the terminal four octal numbers followed by a control character; any other sequence is ignored. The

control characters are (1) "G" which instructs the Monitor to chain to the RAM address specified by the four octal numbers, and (2) "D" which instructs the Monitor to deposit these data at the last specified RAM address. Any other control character causes the contents of the specified RAM address to be displayed on the terminal. Examples of each are given below.

$1000/7000	RAM location 1000 now contains a 7000.
$7600D	The contents of location 1000 is changed to 7600.
$0200G	RAM execution begins at location 200.

When chaining to mainframe memory, the RAM Monitor first clears the CPU accumulator and link.

3. Operating Procedures

The combined functions of the PROM Monitor and its harmonious interaction with minicomputer software is best demonstrated by following a typical sequence for microcomputer execution of predeveloped software.

After initialization of the PROM Monitor, the asynchronous interface between the mini and micro is connected and OS/8 bootstrapped on the minicomputer.

A typical sequence for execution of a FORTRAN-II program (stored on the mass storage device) is then as follows:

	COMMAND	PROM ROUTINE	OPERATION
(1)	COM PROG1.FT	TM	The FORTRAN-II source file is compiled, assembled, and loaded into microcomputer memory.
(2)	SAVE SYS PROG1	TM	A CORE-IMAGE file of the program is created.
(3)	MAP PROG1	TM	The user finds out the actual entry point of the program.
(4)	R BIN4SV *PROG1<PROG1	TM TM	This creates an absolute binary file from the CORE-IMAGE file.
(5)	DOWNLOAD PROG1	TM,BL	The absolute binary program is transmitted (with leader-trailer) and loaded into microcomputer mainframe memory.
(6)	$1002G	RM	Execution is started at RAM address 1002, the starting address determined in step 3.

To simplify the procedure of executing software on the micro, the OS/8 Concise Command Language (CCL) program was modified to recognize the MAP and DOWNLOAD commands. MAP runs LODMAP, a program developed in our research group for determining the memory requirements and starting address of core-image files. DOWNLOAD runs PIP with the papertape punch (PTP) as the output device and the /B option to indicate that a binary file is to be transferred.

The sequence for executing a program written in PAL8 is somewhat simpler:

	COMMAND	PROM ROUTINE	OPERATION
(1)	COM PROG.PA	TM	The PAL8 source file is assembled into absolute binary and saved on mass storage.
(2)	DOWNLOAD PROG2	TM,BL	The binary file is transmitted and loaded.
(3)	$0200G	RM	Execution is begun at RAM address 200.

4. PROM Monitor Logic

A major portion of the design logic for the PROM Monitor board was modelled after the PCM-12 front panel logic. The circuitry consists of the neccessary logic and memory to provide the functions described above. The Monitor itself resides in three 256-word PROM's, and three 16-word RAM's are used for scratch-pad memory.

After power-up, the Monitor routine is initiated by first RESETting the CPU and then asserting a Control-Panel (CP) interrupt to the processor. The RESET is done by grounding the RESET line on the bus thruogh a momentary action switch on the board; this causes the 6100, after completing the current cycle, to clear the accumulator and link, to jam-set the program counter to 7777, and to HALT. A CP interrupt can then be asserted by grounding the CPINT line on the bus. The CP interrupt actually can be asserted even if the 6100 is RUNning, and therefore is logically not allowed if either an interrupt request or DMA request has been granted by the CPU.

The CP interrupt forces the 6100 into the RUN state (if HALTed). During instruction-fetch cycles, the CPU asserts the CPSEL bus line rather than MEMSEL so that instructions are fetched from the PROM Monitor memory rather than mainframe memory; the low 7 address bits are then decoded by the PROM's. PROM space extends from 7000 to 7377, while RAM space occupies addresses 0000 to 0017; hence if the most significant bit of the requested address is HIGH the PROM's are enabled and if LOW the RAM's are enabled.

The PROM Monitor routine chains to mainframe memory by executing the sequence ION (which does not actually enable interrupts) followed by a JMP I 0. The program counter is hence loaded with the contents of Monitor memory location 0 and, provided the CPU RUN/HALT flip-flop is in the RUN state, execution then begins at that address in mainframe memory. This RUN/HALT flip-flop is always forced into the HALT state after a RESET, executed just prior to the CP interrupt; the flip-flop is then toggled into the RUN state by Monitor logic when the CP interrupt switch is asserted. The CP interrupt switch triggers a 2 s pulse from a monostable to the RUN/HALT line on the bus, which toggles the flip-flop into the RUN state. The monostable pulse is used rather than the switch itself to debounce any switching transients.

B. SFCA Interfaces

1. General Considerations

The stopped-flow clinical analyzer is interfaced to the microcomputer through two wire-wrapped printed circuit boards; one is dedicated to the control functions and the other as a general purpose analog interface for the acquisition of analog data. The two basic functions were separated to minimize noise contributions from the control logic to the analog signals. Through a series of software commands, the interfaces can cause the instrument to open and close the sample valves, empty, fill, and push syringes in the stopped-flow module, digitize an analog voltage from the photocurrent amplifier, and output an offset voltage to the amplifier.

The interfaces latch each instruction from the data bus on the trailing edge of the LXMAR pulse. As shown in Figure 9, bits 3-8 of the instruction are then decoded as the device number; device 50 on the control interface and 51 on the analog interface cause the proper decoder outputs to be asserted LOW. This device number is combined with a DEVSEL LOW to produce COM50 and COM51 at the interfaces. The COM50 (control) and COM51 (analog) are then combined with bits 9-11 (the assignment bits) of the instruction to produce a specific action.

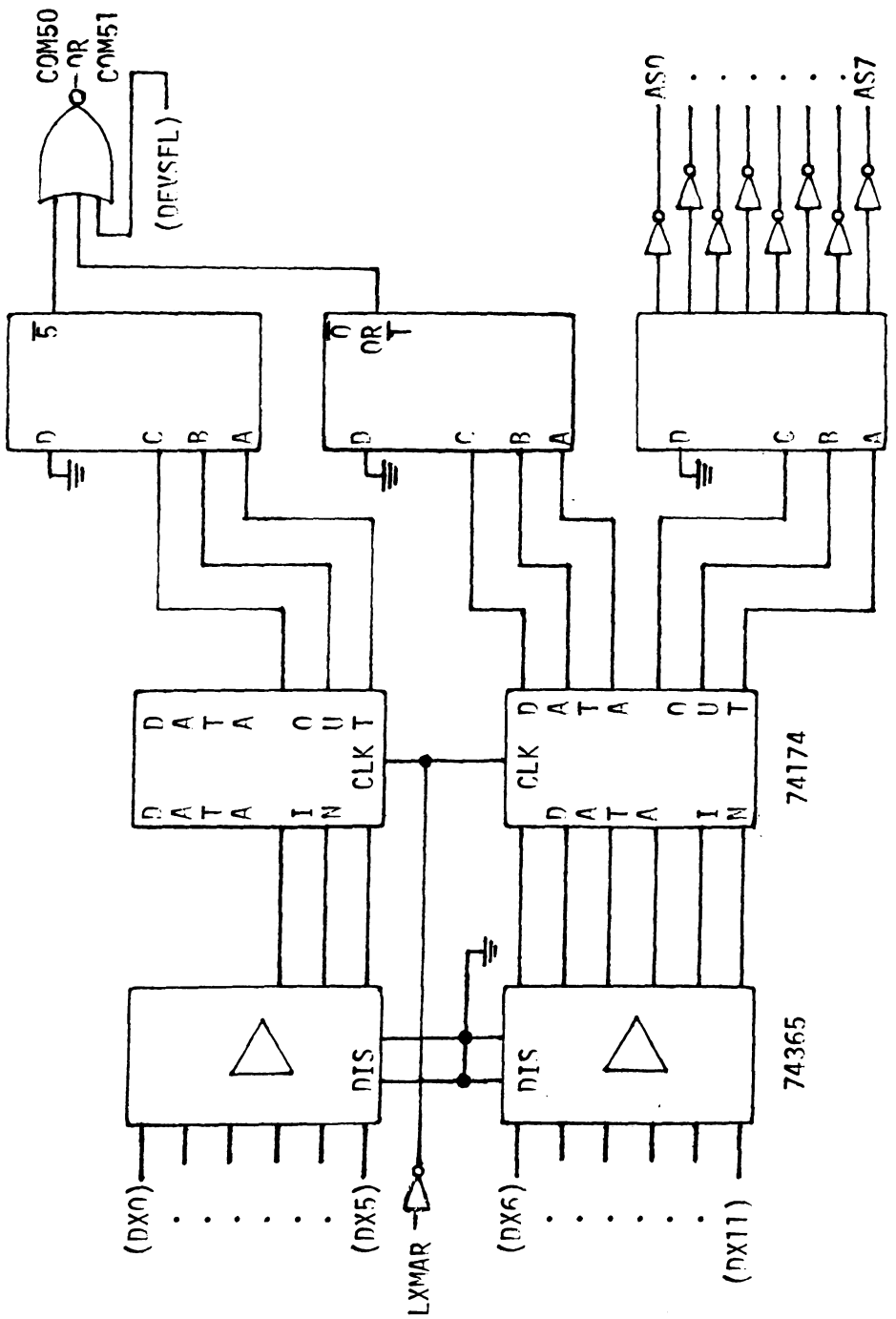


Figure 9. Schematic Diagram of Instruction Decoding Circuitry

2. Control Interface

The control interface handles the operation of the stopped-flow and sample valves. A summary of software commands to the control interface are given in Table 1. The folowing sections give details of the action of each instruction.

Table 1. Software Commands to the Control Interface

Command	Operation
6500	Open sample valves
6501	Close sample valves
6502	Empty stopping syringe
6503	Fill drive syringes
6504	Push drive syringes
6505	Skip the next instruction if flow has stopped
6506	RESET
6507	Read the 6-bit switch register

a. The Open and Close Sample Valve Instructions

The actual opening and closing of the sample valves is done by activating a solid state relay which then

switches AC line current to a solenoid valve controlling air pressure to the pneumatic actuator/ spring return mechanism on the sample valves themselves. Hence, the OPEN and CLOSE commands need only apply TTL HIGH and LOW levels across the control pins of the solid state relay. As shown in Figure 10, one half of a 7474 J-K type flip-flop is employed, the Q output being set HIGH by the 6500 command and LOW by the 6501. This Q output is cleared (set LOW - valves closed) by a RESET command (6506) or by the assertion of the RESET bus line.

b. The Syringe Control Instructions

The GCA-McPherson stopped-flow module contains the neccessary internal control logic to convert TTL-level signals from either the front panel or from a control port to a specific action in the module. The input signals are named CYCLE and DELAY and a summary of the function of these control signals is given in Table 2.

Table 2. Control Signals for the GCA-McPherson Stopped-Flow Module.

CYCLE	DELAY	FUNCTION
1→0	any	Empty stopping syringe
0→1	0	Fill drive syringes
1	1→0	Push drive syringes

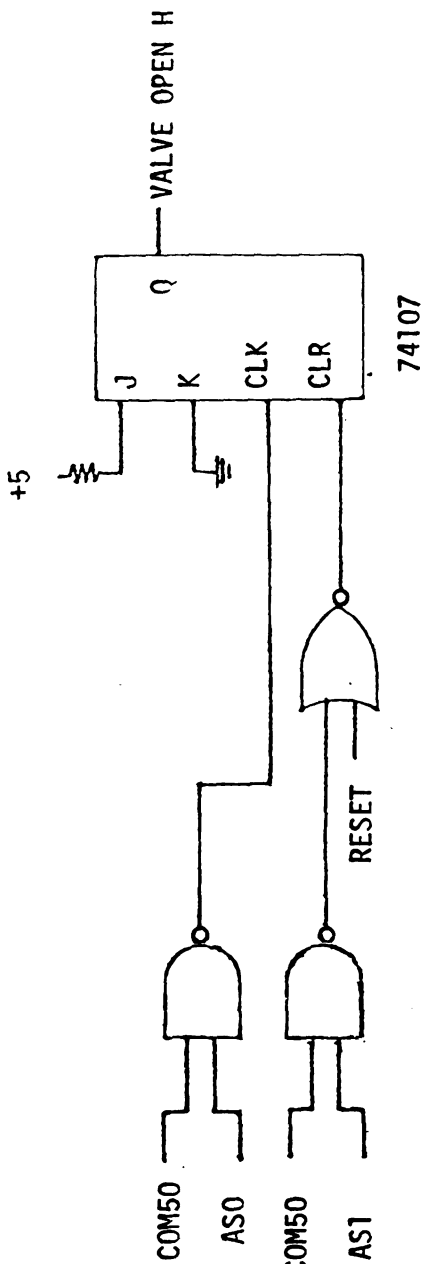


Figure 10. Schematic Diagram of Sample Valve Control Circuitry

J-K type flip-flops are used to generate the CYCLE and DELAY signals required for each of the three functions as shown in Figure 11. The actual inputs to the stopped-flow control logic are outputs of open-collector NAND gates, as the module operates on open-collector logic. A RESET command (6506) sets both CYCLE and DELAY signals HIGH which causes the stopped-flow module to complete any current cycle.

c. The Skip on Flow Stopped Instruction

An optical interrupter in the stopped-flow module generates a flow-stopped signal to the control interface. The OI is triggered when the drive syringes reach the ends of their travel, and the actual trigger point is adjustable. As shown in Figure 12, if this signal is TRUE and the 6505 instruction is executed, the interface directs the CPU to SKIP the next instruction by asserting the proper CONTROL and SKIP bus lines. The skip-on-flow-stopped instruction is used by the SFCA routines to determine when to begin data acqusition.

d. The Read Switch Register Instruction

A six-bit switch register located on the control interface board can be read into bits 0-5 of the CPU accumulator by the 6507 instruction. As shown in Figure 13,

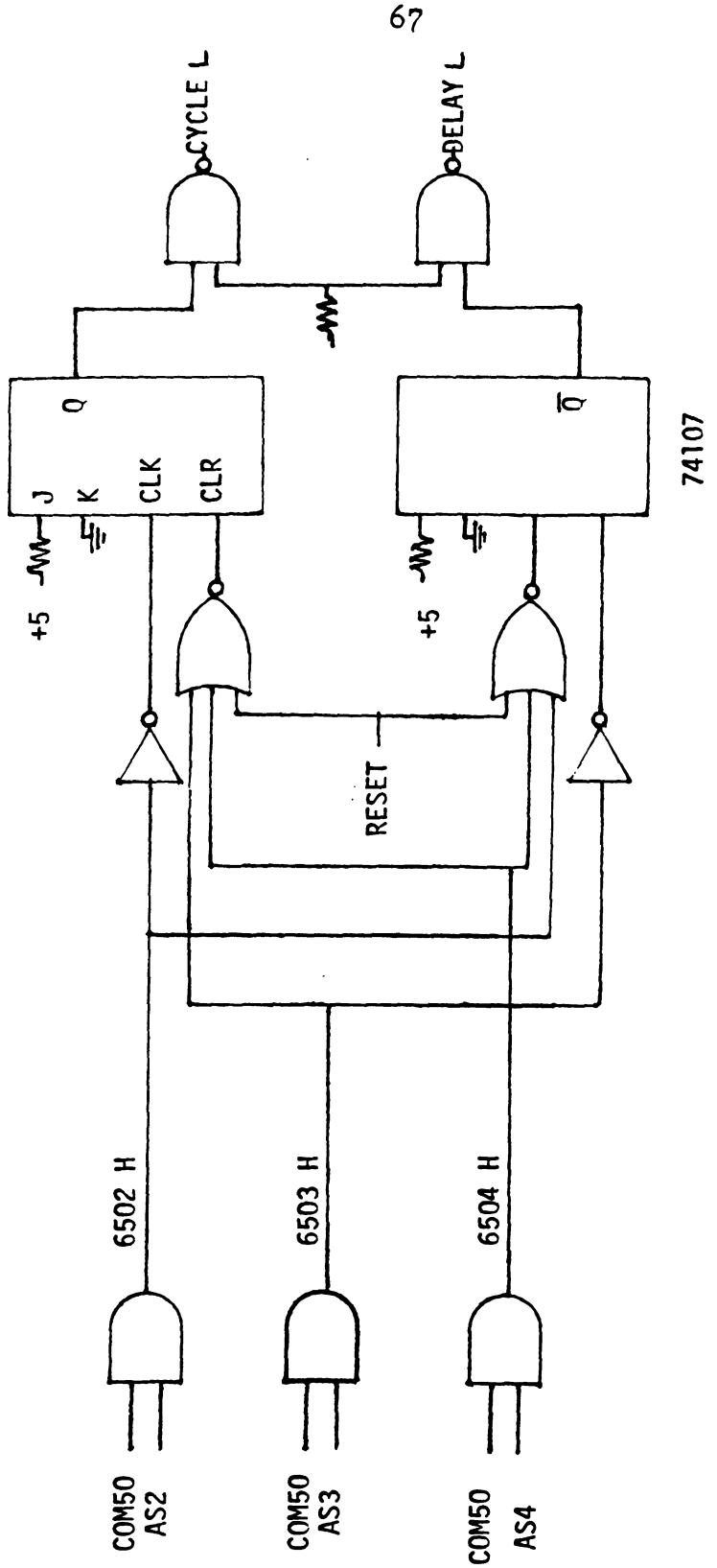


Figure 11. Schematic Diagram of Syringe Control Circuitry

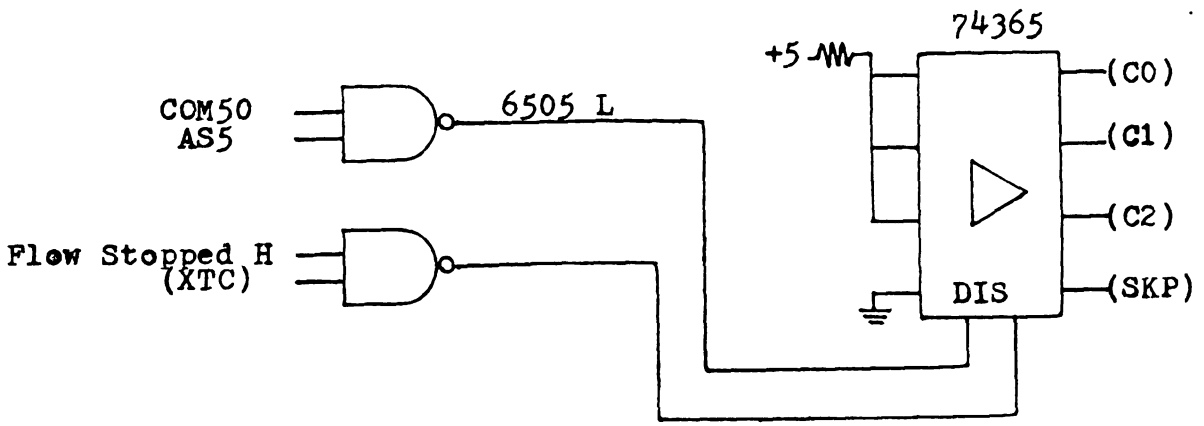


Figure 12. Schematic Diagram of Skip on Flow Stopped Circuitry

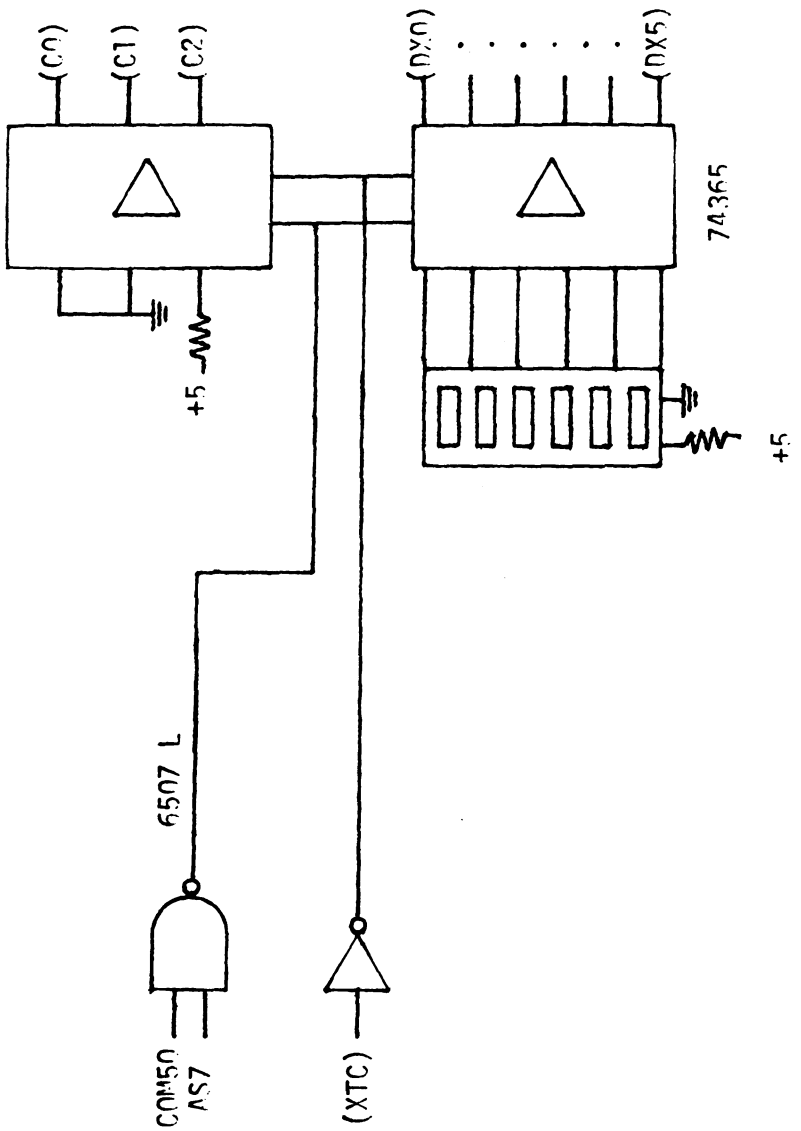


Figure 13. Schematic Diagram of Read Switch Register Circuitry

this instruction causes the interface to drive the contents of the switch register into the accumulator. During that part of the IOT cycle when XTC is LOW, the CPU samples the CONTROL lines to determine what action (if any) is to be taken. The combination of CONTROL lines shown in Figure 13 (C0 LOW, C1 LOW, C2 HIGH) causes the contents of the data bus to be jam transferred into the accumulator (i.e. the AC is first cleared). The data present at the switch register is driven onto the data bus during the last half of the IOT cycle (XTC LOW) and the required CONTROL lines asserted by enabling tri-state buffers.

The SFCA routines periodically sample this switch register to direct program flow.

3. Sequencing and Timimg

With the control interface instruction set, the operator can easily select any type of sequencing for the SFCA. The actual sequence of events as well as the time lapse between each event can be customized to the type of experiment being performed simply by changing the software, or even by designing software which allows user modifications during run time.

An obvious application of this versatility is in a case where the immobilized enzyme reaction rate is being studied. Here the time between sample valve closing (essentially the start of the enzymatic reaction) and syringe drive (the end) is controlled by a variable,

calibrated software timing loop. Even the time during which the sample valves are open can be selected at run time, since the actual sample volume drawn depends on this and the vaccuum pressure applied.

When making changes in the solution such as pH, type of buffer, ionic strength, or other parameters, it is desirable to rinse the reaction loop for an extended period of time. Ionic species and large molecular weight species may become trapped in the enzyme/nylon matrix. These can be eluted from the loop by drawing large volumes of the new solution through the loop.

It has been found that when filling the drive syringes with the sample valves closed (i.e. open to the flow system) solution in the reaction loop is perturbed due to the valve design in the stopped-flow module. It is usually possible to avoid this by filling the drive syringes during sample loop fill time, as the reaction loop is then isolated from the rest of the flow system. It may also be desirable to allow temperature equilibration of the reagents in the drive syringes after filling; this can be accomplished by extending the time between filling and pushing the syringes to suit the particular experiment.

4. Analog Interface

The analog interface serves the function of acquiring analog voltage data from the photocurrent amplifier.

Software commands recognized by this interface are summarized in Table 3.

Table 3. Software Commands to the Analog Interface

Command	Operation
6510	Initialize ADC conversion
6511	Skip the next instruction if conversion done
6512	Read the ADC output into the AC
6513	Set the DAC with the 12-bit word in AC

Instructions are latched and decoded as described earlier, with device number 51 being active. Each command is described in detail in the following sections.

a. The Convert Command

The twelve-bit analog-to-digital converter (ADC) begins a conversion after receiving a convert pulse of duration 100 ns to 2 us. Upon decoding a 6510 instruction, the interface triggers a 1 us pulse from a 74121 monostable to the A/D convert pin. Analog signals are brought to the interface by a coaxial shielded cable, and the shielding is connected to a large ground plane on the interface board to minimize noise.

b. The Skip on Conversion Done Command

Analog-to-digital conversions require typically 25 us, after which the ADC STATUS flag is asserted LOW to indicate the conversion is complete. As shown in Figure 14, if this flag is LOW (done) and a 6511 instruction is executed, the interface signals the CPU to skip the next instruction by asserting the SKIP bus line LOW and the CONTROL bus lines HIGH during the last half of the IOT cycle. The bus lines are asserted by enabling the appropriate logic levels through a tri-state buffer.

If the ADC STATUS flag is HIGH (busy) when the 6511 instruction is executed, the 74365 tri-state buffer is disabled, and the program flow falls through to the next instruction (usually a JMP back to continue testing the flag).

c. The Read ADC Command

After the ADC conversion is complete, the resulting 12-bit data word is read into the accumulator by execution of the 6512 command. As shown in Figure 15, this command causes the interface logic to enable three 74365 tri-state buffers, driving the ADC output onto the data bus at the proper time by asserting the CONTROL lines to the appropriate states. The combination of CONTROL line states shown in Figure 15 (C0 LOW, C1 LOW, C2 HIGH) causes the CPU

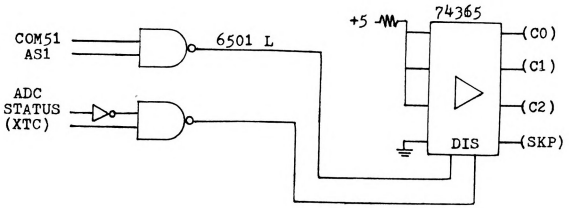


Figure 14. Schematic Diagram of Skip on Conversion Done Circuitry

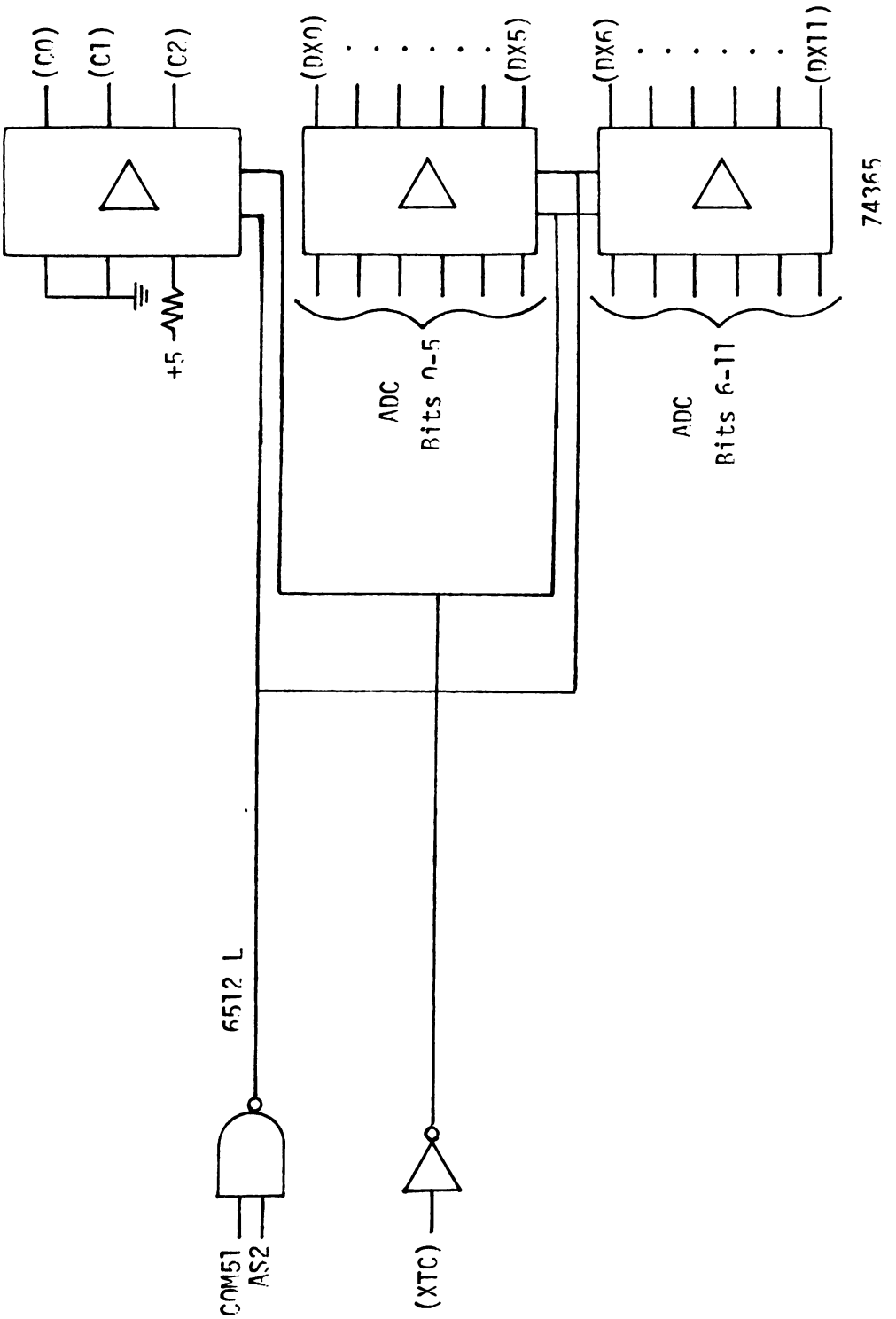


Figure 15. Schematic Diagram of Read ADC Circuitry

to jam transfer the contents of the data bus (in this case the A/D output) into the accumulator (i.e. the AC is first cleared).

d. The Set DAC Command

The digital inputs to the twelve-bit digital-to-analog converter (DAC) on the interface are set from the accumulator by executing the 6513 instruction. Upon decoding this instruction, the contents of the accumulator are latched by a pair of 74174 hex latches, the outputs of which then set the DAC as seen in Figure 16.

The DAC is typically used to output an offset voltage to the photocurrent amplifier; it can also be used to display digital data on a strip chart recorder, oscilloscope, etc.

C. SFCA Software

Software for the SFCA was designed to serve several functions. First, experimental parameters need to be determined according to the type of experiment to be performed; during the experiment the instrument is sequenced and raw data acquired; the operator occasionally needs to be notified when, for example, to change sample solutions, open or close the PM shutter, and so on; and finally evaluation

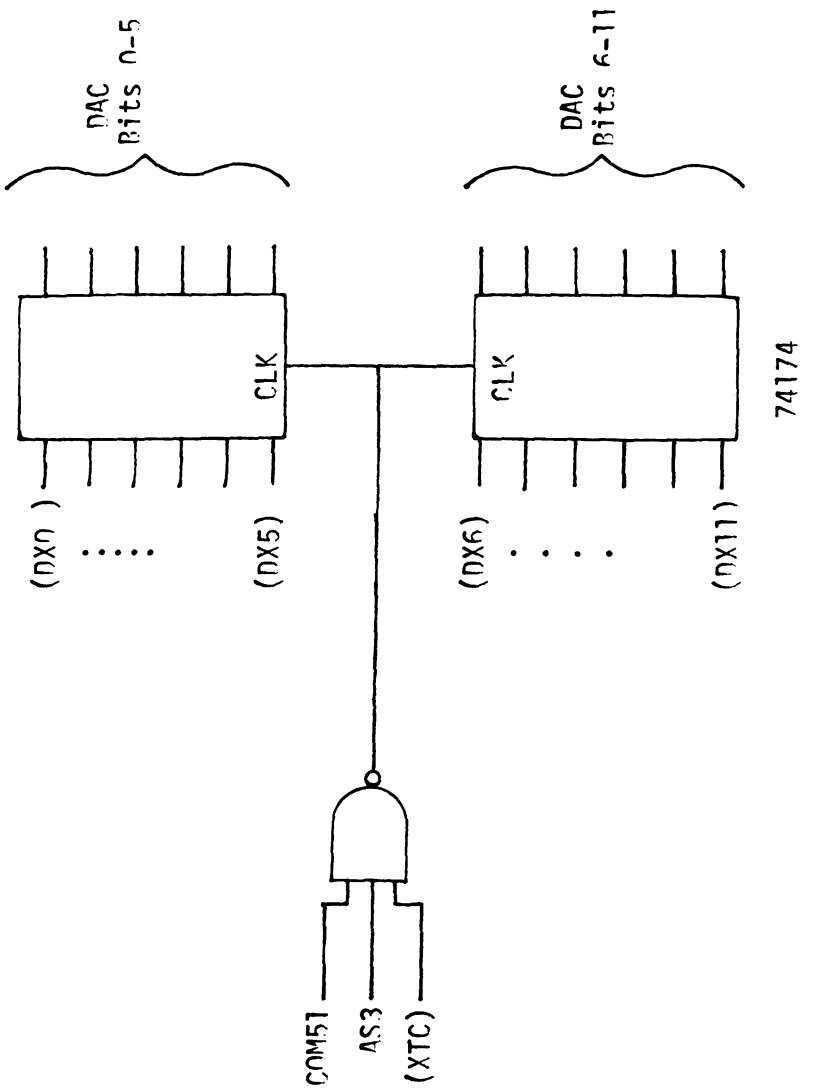


Figure 16. Schematic Diagram of Set DAC Circuitry

of the data involving calculation of average absorbances and statistics, plotting and/or printing the data, and storage on a mass storage device.

As these functions involve various degrees of complexity, they were divided into two groups: the more complicated, core-consuming routines were written in FORTRAN-IV for operation on either the PCM-12 or PDP 8/E minicomputer, while the simpler, less core-consuming routines were written in PAL8 for operation on the microcomputer. A few short testing programs were written in FORTRAN-II/SABR and executed on the micro to test individually some of the individual functions of the interfaces.

Data communication between the mini and micro are accomplished through a set of complementary subroutines written for that purpose. Actual listings of most of the programs are given in Appendix A, but a brief description of the software is presented here.

1. Microcomputer Software

The 6100 microcomputer used in these experiments has at present only 4096 words of read/write memory, which must accommodate both the software for controlling the SFCA and data acquisition as well as the raw data itself. Attempts were made to develop the software in FORTRAN-II/SABR, but were unsuccessful due to the relatively large memory

requirements of the language; even seemingly short routines quickly exceeded the available 4K. Therefore, a general purpose set of software modules were developed in PAL8, which allow the flexibility of executing a variety of experiments with only minor alterations. These routines will execute in much less than the available memory.

The 4K of memory (octal addresses 0000 through 7777) were allocated as follows. Page zero (000 through 177) is used to store certain experimental parameters and pointers to the commonly called subroutines, since data stored on page zero can be directly accessed from any other memory location. The executive routine occupies the next several core pages, typically locations 200 through 1777. The executive routine handles such functions as keeping track of which particular sample is currently being analyzed, requesting operator intervention if required, and sequencing the experiment by executing CALLs to the general purpose subroutines.

Most of the actual functions are carried out by these subroutines, called with or without arguments, which then RETURN to the executive. The general purpose subroutines occupy the 7 core pages following the executive, or 1400 locations. The remainder of the 4096 locations (typically more than 2K) are available for raw data, which consists of averaged ADC conversions for each sample stored during the experiment. These data can be optionally printed on the terminal or, at the conclusion of the experiment,

transmitted to the minicomputer for the more complex calculations. Actual program flow is described below.

The working parameters for a particular experiment are input either from the terminal or minicomputer. These include the total number of samples and the number of runs for each sample, the enzymatic reaction time for each, the delay time from flow stoppage to data acqusition, the data acquisition rate, and whether the indicator reaction is to be monitored continuously (for reaction rate analyses) or after equilibrium has been attained.

When the experimental parameters have been input, the dark current and 100% transmittance are sampled after the stopped-flow observation cell is flushed with the reference or blank solution. Zero and 100% T can then optionally be again sampled and updated after the completion of each run; program flow is directed to the scaling routines by setting a bit on the switch register.

Manual control of the SFCA can be obtained by typing an ALTMODE at the terminal any time the software is either waiting for terminal I/O or in a software timing loop. After gaining manual control the operator can cause all hardware functions to be performed be typing key letters at the terminal. A summary of these commands is given in Table 4.

Table 4. Manual Mode Commands and their Function.

Command	Function
O	Open sample valves
C	Close sample valves
E	Empty stopping syringe
F	Fill drive syringes
P	Push drive syringes
R	RESET
D	Set DAC from keyboard
A	Sample ADC
CNTRL-C	Exit to Monitor (transparent mode)
CNTRL-R	Return to calling program

When ready for each new sample, the executive routine prints the current sample and run number on the terminal and waits for any character to be typed before opening the sample valves. This feature can be disabled by setting a bit an the switch register.

The sequencing for each sample is then as follows. After opening the sample valves, a software timing loop is entered for the duration of the sample fill time while sample solution is drawn into the reaction loop. This sampling time is preset according to the solution uptake rate and the reaction loop volume and is determined empirically. The stop syringe is emptied and the drive syringes filled during sampling. The sample valves are then

closed, and the timing of the enzymatic reaction begun.

After the enzymatic reaction has proceeded for a preset time, the drive syringes are pushed. The program waits for the flow-stopped signal, and then delays the preset time before beginning data acqusition. If the indicator reaction is to be monitored after equilibrium, this delay is to the attainment of equilibrium, after which the ADC is sampled in rapid succession, averaged, and stored. The number of conversions to be averaged is preset by the operator.

When monitoring the rate of the indicator reaction, the delay after flow is stopped is to allow settling of the observed solution; incomplete mixing is often observed in the GCA McPherson module when mixing reagents of widely different concentrations. The ADC is then rapidly sampled, averaged, and stored as the first data point of a two-point fixed-time reaction-rate measurement. After delaying the preset time, the ADC is then again rapidly sampled, averaged, and stored as the second data point. The time lapse between the two data points is pre-determined according to the rate of the indicator reaction being monitored.

The entire data set can optionally be listed on the terminal at the end of the experiment, and finally transmitted to the minicomputer for further data analysis.

2. Data Transfer Subroutines

A protocol had to be developed for accurately transmitting data between the mini and micro so that the overall software scheme outlined above could be implemented. Simple FORTRAN READ and WRITE statements were tried first, but proved to be unreliable due to timing difficulties; typically the WRITE statement resulted in a very short time lapse between character transmissions, while the complimentary READ statememt involved a much longer time lapse between reading each character. The result was that characters were occasionally missed, since one or more characters had been sent before the previous one had been read.

The most succesful procedure developed requires that the receiving program indicate to the sending program that it is ready to accept a character by transmitting a special character. The sending program then merely waits until it receives this special character before transmitting each character, resulting in no loss of data.

3. Minicomputer Software

Complex calculations such as the use of logarithms and evaluations of standard deviations are more efficiently handled by a higher level language such as FORTRAN-IV (requiring much more memory than is available on the micro).

The FORTRAN-IV software which operates on the minicomputer accepts the raw data from the micro (averaged ADC conversions for each sample along with the 100% transmittance for each). The routines will then calculate the average absorbance and standard deviation for each sample and then display the information in a variety of formats and/or store the data on floppy disk or cartridge disk for later use. Calibration curve data can be called up from mass storage if calculation of an unknown concentration is desired.

The parameters for an experiment can be optionally set up by the minicomputer software. For example, if the kinetics of the immobilized enzymatic reaction are to be studied, the reaction time for each sample is varied between specified extremes while all other parameters remain constant; a calibration curve experiment requires that only the substrate concentration in each sample vary. The minicomputer software is able to set up the desired parameters and then transmit them to the microcomputer.

CHAPTER VI

EVALUATION OF THE STOPPED-FLOW CLINICAL ANALYZER

The potential of the SFCA as a useful analytical instrument was evaluated both by separate characterization of several of the individual elements of the system and also as an integral unit. This chapter describes the evaluation of the instrumuntal aspects of the unit and its fundamental potential in enzyme-catalyzed analysis. The two chapters following describe the characterization of the immobilized enzymes and an evaluation of the analytical capabilities of the SFCA.

A. Solution Volumes

A complete purging of solution from the immobilized enzyme reaction loop and filling with new solution requires drawing a volume equal to approximately three times the reaction loop volume. The volume contained in a 1.0 m length of the nylon tubing was determined by filling the tube several times with water and then weighing the combined fillings. The loop volume was thus determined to be 585 ul. Hence, during most of this study, 2-3 ml volumes of each new solution were drawn to ensure replacement of the old solution with the new.

Once the enzymatic reaction in the reaction loop has

proceeded to the desired extent, the solution is forced by the push liquid through connecting tubing into the stopped-flow mixing chamber and observation cell. The volume contained in the connecting tubing is kept small (approximately 80 ul) by using very narrow diameter tubing (0.8 mm i.d.). The volume of the observation cell is reported to be 63 ul (73). Hence, the push volume of each syringe must be enough to replace completely the total volume of 143 ul to ensure that solution in the observation cell is completely replaced by new solution with each push. The push volume must be not so large as to allow dilution of the observed solution by the oncoming push liquid itself.

The reagent syringes were adjusted to deliver 300 ul per syringe per push. The effectiveness of using this volume was tested by filling a duplicate reaction loop (of identical dimensions) with a dye (p-Nitrophenol) and monitoring the transmittance of a series of alternating distilled water and dye solutions pushed from the reaction loop.

A determination of the expected transmittance of the dye solution was made by successively pushing the dye from the reaction loop into the observation cell. Alternating then between the blank and dye solutions showed a transmittance reproducibly alternating between sample and reference, which verified that the 300 ul push volume was adequate.

The volume of solution taken up by the stop syringe

before flow is stopped is adjustable in the GCA-McPherson module. This volume was set so that the drive syringe plungers stopped just before reaching the ends of the syringes.

B. Sequencing

The best sequence of events involved for each sample or blank to be analyzed was empirically determined by observing each action of the sequence. It was noted that some solution is drawn from the reagent reservoirs through the observation cell by suction each time the stop syringe is emptied. Solution in the reaction loop is also disturbed when filling the drive syringes. As a result, it was decided that the syringes be operated while the reaction loop is being filled; switching the sample valves to the fill position isolates the reaction loop from the rest of the flow system, so possible contamination of the immobilized enzyme with harsh reagents in the drive syringes could be avoided.

Since all of these studies were made with all solutions at room temperature, it was not neccessary to await temperature equilibration of reagents in the drive syringes. The GCA-McPherson module relies on ambient air within the enclosed module to transfer heat to or from the glass syringes to thermostatted water circulating within copper tubing below the syringes. If temperature

equilibration of reagents in the syringes is desired, sequencing can be delayed after filling the syringes to allow for that.

As the 2-3 ml of solution (sample or blank) is drawn through the reaction loop by the vacuum, the stop syringe is emptied, and the drive syringes are filled. The sample valves are then switched to the push position, and timing of the enzymatic reaction is begun. At the end of the specified incubation period, the reagent syringes are driven. The push liquid forces the reacted solution from the loop into the stopped-flow mixing chamber and observation cell for analysis. After data acqusition, the cycle is repeated.

C. Indicator Reaction

An important feature of the SFCA is that the indicator reaction takes place in the stopped-flow observation cell. This allows the option of monitoring this reaction either by reaction-rate methods or after equilibrium has been attained. Another advantage of this arrangement is that, since the indicator reaction takes place outside of the immobilized enzyme reaction loop, drastically different reaction conditions can be used in each of the two reactions. An indicator reaction which may involve a strongly acidic or basic pH or other species which could otherwise interfere with the enzymatic reaction (in

fact even destroy its catalytic ability) can be utilized.

In choosing whether to use a rate or equilibrium method for the indicator reaction, some of the following points should be considered. Equilibrium methods have the advantage of simpler data acqusition and data analysis. One needs only to wait for equilibrium to be established, measure the absorbance of the chromophore, and compare it to the reference. The simple two-point fixed-time kinetic method requires two transmittance measurements a precise time interval apart and a more accurate measure of the reaction rate requires even more data and more data analysis. Reaction-rate methods are generally more sensitive to variations in the solution pH and temperature than corresponding equilibrium methods. Reaction-rate methods have the advantage of involving a relative measurement, the rate of change of the transmittance or absorbance with time. Problems such as light source drift and other spectral interferences which do not change during the measurement process do not affect a rate method, while they can cause serious errors in an equilibrium method.

Chemical interferences can also cause errors in an equilibrium measurement. For example, in enzymatic-based determinations which involve the monitoring of hydrogen peroxide produced in the reaction, sodium azide is often added to the reaction medium to inhibit the consumption of the peroxide by catalase (an impurity often present in commercial enzyme preparations). The iodide-peroxide

reaction then suffers from interference by the azide. The triiodide formed in the indicator reaction is slowly consumed, so the absorbance due to triiodide first quickly increases and then gradually decreases over the next few minutes.

By measuring the initial rate of formaton of the triiodide, errors due to the azide interference can be avoided. A two-point fixed-time rate method with two absorbance measurements a short time (1-5 s) after the flow stops should result in an accurate determination of the hydrogen peroxide present.

D. Sources of Error

Several factors play a role in the overall accuracy and precision of substrate determinations by the SFCA. The substrate in both sample and standard solutions must follow the same kinetics in the reaction loop. The product produced in both sample and standard must then react with the indicator reagents in an identical fashion. Similarly, all other elements of the detection system must behave identically for both solutions. The accuracy and precision in each of the transductions involved in the analysis are evaluated in this section, in the order that they occur.

1. Sampling

The introduction of the sample solution into the immobilized enzyme reaction loop can be complicated by several factors. The most important of these is the achievement of complete removal of the previous solution. As previously noted over-extensive hydrolysis of the inner surface of the loop in the immobilization process results in a strong affinity between the support-enzyme matrix and the substrate and/or product. This affinity is considered to be ionic in nature, although the addition of strong electrolytes to solution in one of these extensively hydrolyzed loops did not significantly improve the elution of substrate/product from the loop.

When the much milder hydrolysis conditions are used in the immobilization process, the problem of affinity disappears almost completely. Flushing the loop with 2-3 ml of the buffer solution (blank) results in no detection of product in the subsequent indicator reaction. It must be kept in mind however that the presence of certain species in the sample may inadvertantly interact to enhance the affinity of the matrix toward the substrate/product. Frequent sampling of a reference or blank solution should be carried out to monitor the complete replacement of solution in the sampling process.

Fluctuations in the vacuum pressure used to draw solution into the reaction loop cause variations in the actual volume of solution taken during the fixed-time

sampling period. Normally the time delay between opening and closing the sample valves is set at 5 s, which was previously shown to be adequate for complete solution replacement. The effectiveness of a 5 s sampling period should be periodically verified. Longer sampling times do not improve solution replacement but only add to the total solution volume requirements.

2. Enzymatic Reaction

Due to the relatively small amount of enzyme (i.e. activity) present in the reaction loop, the enzymatic reaction has been used in a fixed-time reaction-rate mode in all of these studies. That is, the enzymatic reaction is allowed to proceed in the reaction loop for a preset time, identical for both the sample and all standards. If this reaction time is kept short enough so that only a negligible fraction of the substrate is converted to product, the reaction will be psuedo-first order in substrate (see Chapter VII for a more detailed description of the immobilized enzyme kinetics).

Under these conditions it can be assumed that an equal fraction of the substrate in both sample and standard solutions will be converted. This assumption is critical if the amount of substrate in the sample is to be determined from a calibration curve, etc. The rate of the enzymatic reaction is, however, very sensitive to such variables as

solution pH, type of buffer, temperature, ionic strength, the presence of interferences, and others.

These parameters must be carefully monitored and controlled to be as nearly identical in both standards and sample as possible. Typically standard solutions are aqueous preparations of pure material, while the sample itself is extracted from a complex matrix such as blood or serum.

The magnitude of the error introduced by such changes as pH, temperature, and others can range from negligible to intolerable. The use of commercially available control samples in a variety of matricies is very helpful in evaluating and correcting for matrix effects.

3. Indicator Reaction

The amount of product formed in the enzymatic reaction is determined spectrophotometrically either by a reaction with a chromophore or with a reagent to produce a chromophore. By monitoring either the rate of appearance or disappearance of the chromophore or the total amount produced, and by knowing the stoichiometry of the indicator reaction, one can calculate the amount of product initially present. The transduction from enzymatic reaction product to chromophore is yet another step in the entire analysis scheme, and as such its contribution to the total accuracy and precision must be considered.

As previously described, the indicator reaction can be monitored in either a rate or an equilibrium mode. Errors introduced by variations in the rate of the indicator reaction will obviously affect a reaction-rate measurement much more than an equilibrium measurement. While differences in the solution pH, temperature, or other parameters can seriously alter the rate, the final position of the equilibrium may be slightly affected or even unchanged. It has been noted, however, that species may be present which can shift the position of the equilibrium. In this case reaction-rate monitoring will certainly be superior to equilibrium monitoring.

Other elements of the detection system (i.e. those involved in the absorbance measurement) must also be considered in evaluating the overall performance of each mode. These factors are described in the next section.

In this work, the hydrogen peroxide produced in the enzymatic reaction was determined almost exclusively by the peroxide-iodide redox reaction,

$$H_2O_2 + 3I^- + 2H^+ \xrightarrow{Mo(VI)} 2H_2O + I_3^- \quad .$$

The absorbance of triiodide is measured after equilibrium is attained; experimental data were actually acquired 30-40 s after the flow stops to improve precision (see Section D.4.b.).

The accuracy of determining hydrogen peroxide by this

method was evaluated by filling the reaction loop with hydrogen peroxide solutions of known concentrations, sequencing the stopped-flow to initiate the indicator reaction, and measuring the equilibrium absorbance. The absorbance was linear for concentrations of hydrogen peroxide ranging from 4.00 uM to 100 uM, which encompasses the range of peroxide to be determined in the enzymatic analysis of glucose.

4. Measurement of Absorbance

As previously mentioned, a variety of photometric errors can also contribute to the overall accuracy and precision. In this section the most important of these error sources are discussed.

a. Light Source Characteristics

Short term fluctuatons and long term drift are the major sources of error from a non-ideal light source (74). Most of this work involved monitoring the absorbance of triiodide at 365 nm, where both tungsten and deuterium continuum lamps are candidates as the source. The long and short term characteristics of each were evaluated.

The spectral output of each lamp at 365 nm (monochromator bandpass 3 nm) was recorded both by a strip chart recorder and by sampling the signal through the ADC

and averaging in the microcomputer. As expected, both lamps showed comparable long term characteristics, drifting on the order of 1-2% with a frequency of approximately 0.001 Hz. The short term characteristics proved to be significantly less serious for the deuterium source at 365 nm. In the frequency range 0.5 to 10 Hz, the deuterium lamp fluctuated less than 1%, the tungsten lamp operated in the optical feedback mode showed average deviations about the mean of 2-3%. As a result, the deuterium lamp was used almost exclusively in this work.

Errors due to the short term fluctuations were minimized by averaging multiple A/D conversions (typically 100) for each intensity measurement. Long term drift was compensated for by frequently running a reference or blank, and also by ensemble averaging multiple runs of each solution. Low frequency fluctuations of the source are not a serious problem in a reaction-rate measurement of the indicator reaction, since multiple intensity measurements are made over typically 1-10 s.

b. Physical Interferences

Variations in light throughput can be caused by particulates or air bubbles in the observation cell. Incomplete removal of precipitates formed during serum preparation causes erroneously low transmittance for the sample. Precipitates may also be formed during the

indicator reaction due to impurities in the sample or indicator reagents. Air bubbles may be inadvertently drawn into the drive syringes during filling and then be driven into the observation cell, which also causes decreased transmittance and erroneous results.

Incomplete mixing of reagents in the stopped-flow mixing chamber can create fluctuations in transmitted radiant power. This can be a serious problem in the GCA-McPherson module, especially when mixing two solutions of drastically different composition (e.g. different pH or salt concentrations). In this case mixing continues in the observation cell after the flow has been stopped and has been observed to continue for up to 30 s.

Incomplete mixing causes serious errors if the indicator reaction is monitored by a rate method; in an equilibrium determination, the measurement of the solution transmittance is usually delayed for 30-40 s after the flow stops to improve both accuracy and precision.

c. Spectral Interferences

The triiodide produced in the indicator reaction is monitored at 365 nm. The absorbance maximum for triiodide is actually at a somewhat shorter wavelength (349 nm); maximum sensitivity in this particular reaction is however obtained at 365 nm, since the Mo(VI) catalyst present has an absorbance which steadily decreases from 349 to 365 nm.

The spectrum of triiodide in the presence of Mo(VI) was recorded on a Cary-14 spectrophotometer, using a solution of Mo(VI) of equal concentration as the reference. The difference between triiodide in the presence of Mo(VI) and the Mo(VI) showed an absorbance maximum at 365 nm. The absorption band was found to be quite wide, so the monochromator slit widths of the SFCA subsequently were set to 1500 um (3 nm bandpass).

Stray light entering the stopped-flow module can be a serious problem, contributing 5-10% to the total light passing through the module under normal room lighting. This potential source of error was minimized by placing the stopped-flow module before the monochromator in the light path. In this configuration only stray light of wavelengths within the bandpass of the monochromator will be passed on to the detector.

d. Detector Noise

The major sources of imprecision arising from a photomultiplier tube are phtotcurrent shot noise, dark current noise, and Johnson noise (74). Photocurrent shot noise, arising from the random time distribution of photons striking the photocathode and the random intensity distribution of electrons reaching the photoanode, is proportional to the square root of the light intensity reaching the PM tube. Dark current noise and Johnson noise

are not a function of the light intensity. Since the signal is proportional to the light intensity, best signal-to-noise ratio is obtained, under shot-noise limited conditions, when the light intensity is a high as possible.

Detector noise was also minimized by sampling the photocurrent voltage repeatedly in rapid succession and averaging typically 100 samples for each data point. This essentially reduces the noise equivalent bandwidth of the measurement system for improved signal-to-noise ratio.

e. Photocurrent Amplifier

The response linearity and precision of the Keithley current amplifier were evaluated by using a precision current source as the input and monitoring the response with a digital voltmeter. The accuracy and precision were found to be better than 0.1% over a wide range of input currents (1 uA to 100 uA). Temperature variations may cause minor fluctuations in the transfer function. However, errors in the photocurrent amplification should not contribute significantly to the overall accuracy.

f. Digitization

The transfer function of the ADC was evaluated by using a precision voltage reference source as the input

analog voltage and monitoring the digital output over the complete range of +5 to −5 volts.

With the analog voltage held constant at near full scale (+5 V), the digital output of the ADC had an standard deviation of 0.018%. A variation of 1 in the least significant bit of the ADC would cause a deviation of 0.025% of full scale.

The ADC was then sampled over its entire dynamic range by averaging 100 conversions at each of 11 input voltages (measured with a digital voltmeter). A plot of the transfer function (digital output vs. analog input) had a slope of 409.37 (standard deviation = 0.19) and a Y-intercept of 2049.3 (standard deviation = 1.1). The expected slope is 409.5 and the Y-intercept 2047.

Long term drift of the ADC transfer function due to supply voltage drift and/or temperature variations is compensated for by frequent sampling of the reference photocurrent.

Individual digital data point acquisitions in this study were carried out by averaging 100 A/D conversions. Under these conditions, imprecision due to digitization errors should be negligible.

5. Summary of Error Sources

The relative contribution of each of these potential sources of error to the overall accuracy and precision of

the method are summarized in this section.

Probably the most important source of error is variation in the rate of the enzymatic reaction from sample to sample due to fluctuations in temperature, pH, the presence of activators and inhibitors, and other conditions. Carefully prepared aqueous standards can result in excellent precision, typically less than 1% relative standard deviation. Serum samples, however, can contain impurities which can seriously affect the accuracy of the determination.

The effect of carry over from one sample to the next can best be minimized by controlling the surface hydrolysis during the immobilization of the enzyme. Effective purging of each sample from the reaction loop can be easily checked from time to time by running a blank.

The indicator reaction used in these studies (iodide-peroxide) has been shown to be an accurate and precise monitor of the peroxide produced in the enzymatic reaction.

Photometric errors have been minimized both by frequent sampling of the dark and 100% transmittance and by averaging many ADC conversions for each data point. Errors caused by long term drift in the light source, current-to-voltage conversion, and analog-to-digital conversion were minimized by periodic sampling of the dark and reference photocurrents. Higher frequncy fluctuations in these elements were minimized by averaging typically 100 ADC samples for each data point.

CHAPTER VII

CHARACTERIZATION OF THE IMMOBILIZED ENZYMES

Glucose oxidase (GO) and alcohol dehydrogenase (ADH) have been immobilized on nylon tubing by the procedure described in Chapter III. A fundamental characterization of these immobilized enzymes is presented in this chapter. The Michaelis-Menten model of enzyme kinetics is applied to the system, and modifications to the model are included to take into account diffusional effects in the reaction loop. Data on the relative enzymatic activity as a function of pH are presented. Finally, the long term stability of the immobilized enzymes is considered.

A. Kinetics of the Immobilized Enzymes

1. Michaelis-Menten Model

The model of enzyme kinetics developed by Michaelis and Menten (75), although a simplified description of an often complex mechanism, can be applied to the kinetics observed in these studies. The model, as described in the following equations, assumes the reversible formation of an intermediate enzyme-substrate complex (ES), which then dissociates into the product (P) and the regenerated enzyme (E).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$ES \xrightarrow{k_2} E + P$$

The rate equation which describes the rate of appearance of the product with time is then

$$R_i = \frac{k_2 [E]_o [S]}{K_m + [S]} = \frac{R_{max} [S]}{K_m + [S]}$$

where Km is called the Michaelis constant of the enzyme, and is a rough measure of the dissociation constant of ES. Here $[E]_o$ is the initial enzyme concentration. Several assumptions are made in deriving the initial rate equation. The substrate concentration is assumed to be greater than the enzyme. The rate of the reaction is measured early in the reaction, before the build-up of product can cause any significant back reaction. Finally, the intermediate complex is assumed to be in rapid equilibrium with S and E or that ES is at steady state at any instant (Briggs-Haldane assumption (76)).

The initial rate equation derived under these conditions is illustrated in Figure 17 as a plot of the initial rate vs the initial substrate concentration. This curve was computer generated from the Michaelis-Menten equation. It can be seen that the initial rate is linear with S when the initial substrate concentration is much less than Km, and reaches a maximum when S is much greater than Km.

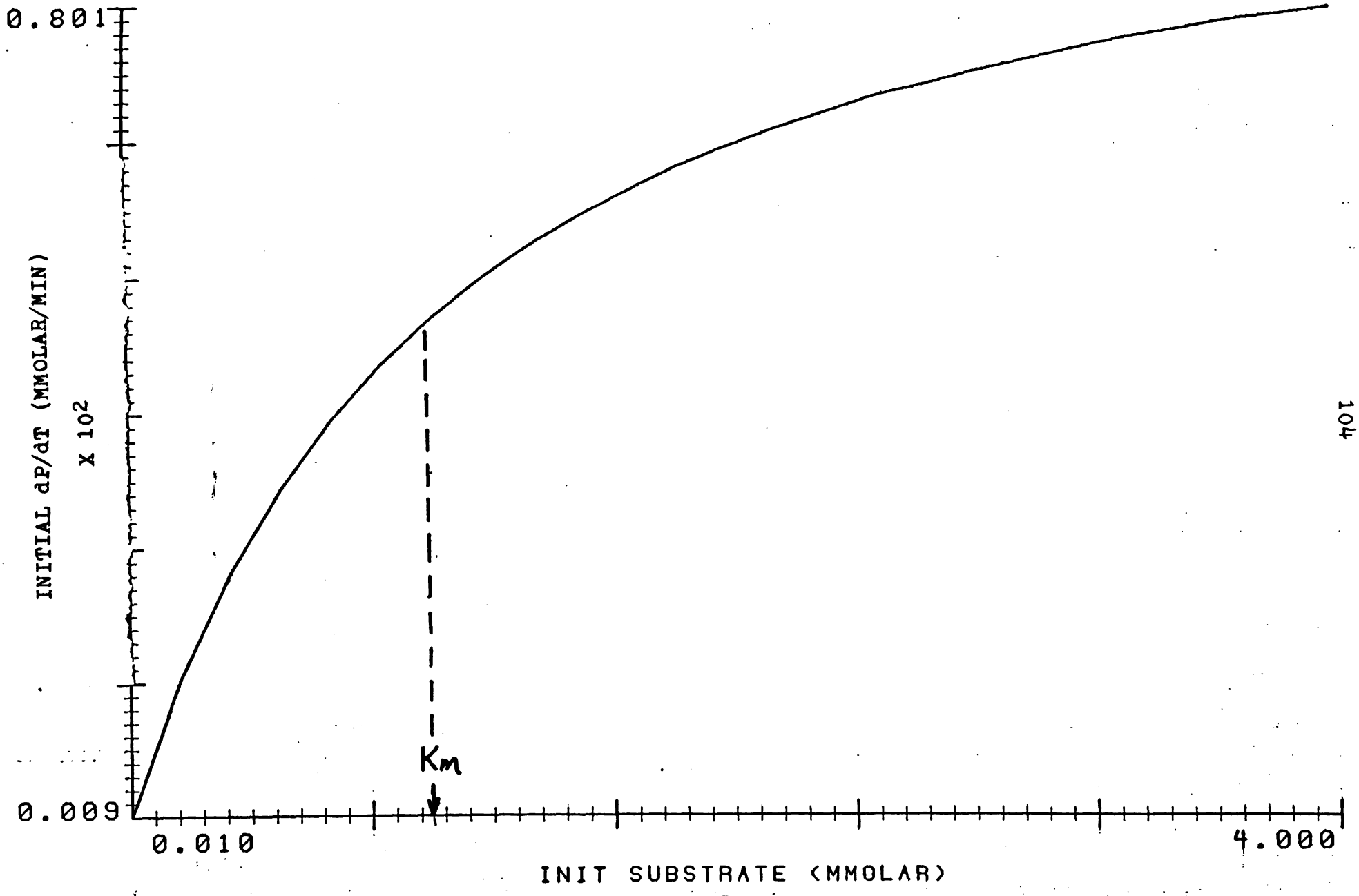


Figure 17. Computer Simulated Initial Rate Curve

a. Glucose Oxidase

In the specific case of immobilized glucose oxidase, the reaction is

$$\text{Glucose} + O_2 \xrightarrow{GO} \text{Gluconic Acid} + H_2O_2 \quad .$$

If the oxygen is present in excess and the glucose concentration is much less than the Michaelis constant, then the initial rate of appearance of H_2O_2 will be proportional to the initial glucose concentration.

A more detailed description of this reaction has been presented by Gibson, et al (77). Their work demonstrated that the enzyme first converts glucose to gluconic acid and is itself reduced in the process. In a second step, oxygen reacts with the reduced form of the enzyme to produce hydrogen peroxide and regenrate the oxidized form of the enzyme. This mechanism is illustrated below.

$$\text{Eox} + \text{Glucose} \xrightarrow{k_1} \text{Ered P1} \xrightarrow{k_2} \text{Ered} + \text{Gluconic Acid}$$

$$\text{Ered} + O_2 \xrightarrow{k_3} \text{Eox P2} \xrightarrow{k_4} \text{Eox} + H_2O_2$$

In this study, early experiments with immobilized glucose oxidase on the Auto Analyzer flow system (as described in Chapter IV), the injection of a 4 g/l glucose sample (30 s plug) through the GO column resulted in an extremely prolonged elution of hydrogen peroxide from the column, typically 30 min or more. On the basis of the

mechanism proposed by Gibson, some observations can be made. If the column had contained a large amount of active enzyme, enough in fact to convert nearly all of the glucose to gluconic acid, the first step would have then converted a fraction of the enzyme to its reduced form. Dissolved oxygen passing through the column would then react with the reduced enzyme, converting it back to its oxidized form as the oxygen itself was converted to hydrogen peroxide. Dissolved oxygen in a saturated solution (under ambient air pressure) is about 0.25 mM (78). The 4 g/l glucose solution was, however, 22.2 mM, or roughly 100 times as concentrated as the dissolved oxygen. It can then be concluded that, as the substrate plug passed through the column, a large fraction of the immobilized glucose oxidase was converted to its reduced form, and was then slowly converted back to its oxidized form as sufficient oxygen came into contact with the enzyme. This would give rise to the observed prolonged elution of hydrogen peroxide from the column, which would be expected to extend long after the substrate plug had passed through.

When air bubbles in the segmented flow stream were replaced with pure oxygen bubbles, the observed response, for the same 30 s plug of 4 g/l glucose, had a slightly greater peak height (0.29 vs 0.27 with air bubbles). The general shape of the response curve as well as the length of time required to reach the baseline was, however, not significantly different. A saturated solution under pure

oxygen (760 torr) is 1.275 mM in oxygen (78), which is still only approximately 5% of the glucose concentration in this sample; hence the same behavior is predicted.

Hornby, et al (35) employed immobilized glucose oxidase in a similar flow system. They noted an approximate two-fold increase in the maximum rate (Rmax, Figure 17) and over a two-fold increase in Km when air bubbles were replaced with pure oxygen. This behavior was not observed in the glucose oxidase column employed in this study. Hence, the column may have actually contained such a high degree of activity that the dissolved oxygen limited the extent of the reaction even during the short time of contact between the glucose and the enzyme.

The dissolved oxygen under ambient air (0.25 mM) may be rate-limiting in an enclosed reactor such as the immobilized glucose oxidase column. In the stopped-flow clinical analyzer, where the sample solution is incubated in the enclosed reaction loop, the dissolved oxygen restricts the total amount of hydrogen peroxide that can be produced. In this environment, only the oxygen initially present in the solution as it is drawn into the reaction loop is available to convert the enzyme from reduced to oxidized form.

The extent of reaction was studied as a function of the incubation time in the reaction loop. The results are shown in Figure 18 for a 500 ppm glucose solution at pH 6.30 and at room temperature. The initial rate of the reaction

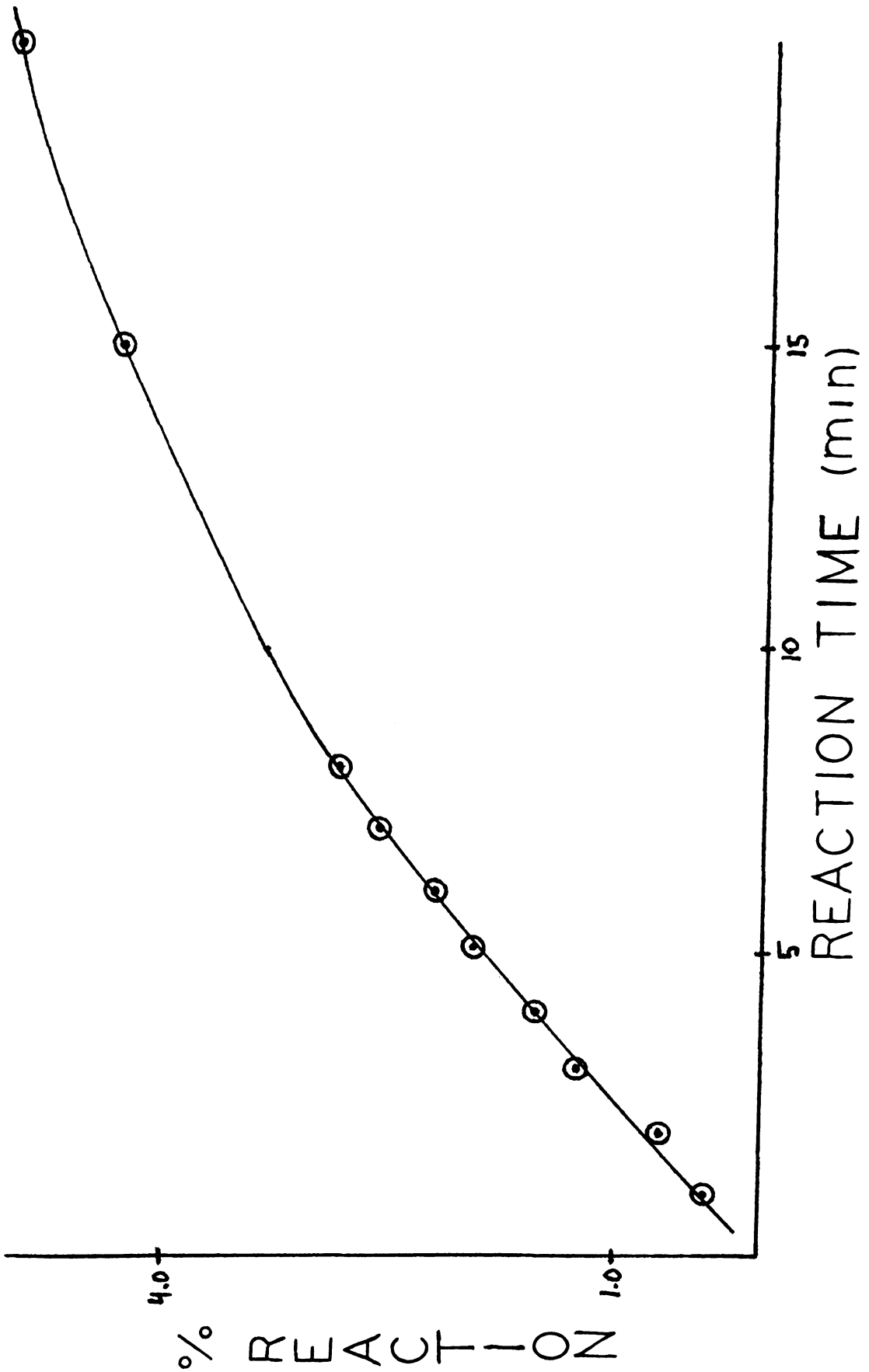


Figure 18. Glucose Progress Curve

was determined from the slope along the linear portion of the curve, early in the reaction, and was found to be 3.56 uM/min.

Lineweaver and Burke (79) rearranged the Michaelis-Menten rate equation to give

$$1/Ri = (Km/Rmax)x(1/Si) + 1/Rmax \quad .$$

By plotting the inverse of the initial rate of reaction as a function of the inverse of the initial substrate concentration, Km and Rmax for the enzyme can be determined from the slope (Km/Rmax) and the y-intercept (1/Rmax). The Lineweaver-Burke plot for immobilized glucose oxidase is shown in Figure 19. From the plot, Km was determined to be 19.0 mM (standard deviation 1.0) and Rmax was 333 uM/min (standard deviation 10). These Michaelis parameters were also determined for glucose oxidase immobilized on another column; Km was found to be 15.8 mM (standard deviation 1.0) and Rmax was 44.1 uM/min (standard deviation 1.5). The determined value of the maximum rate depends on the amount of active enzyme present, which is known to vary widely from column to column. The determined Km, however, is characteristic of the enzyme and should remain constant for the same enzyme in the same environment. Hornby, et al (35) reported a Km value of 15.0 mM for glucose oxidase immobilized on polystyrene tubes, and the value of 15 mM has also been reported for the soluble enzyme.

The value of Km corresponds to that substrate

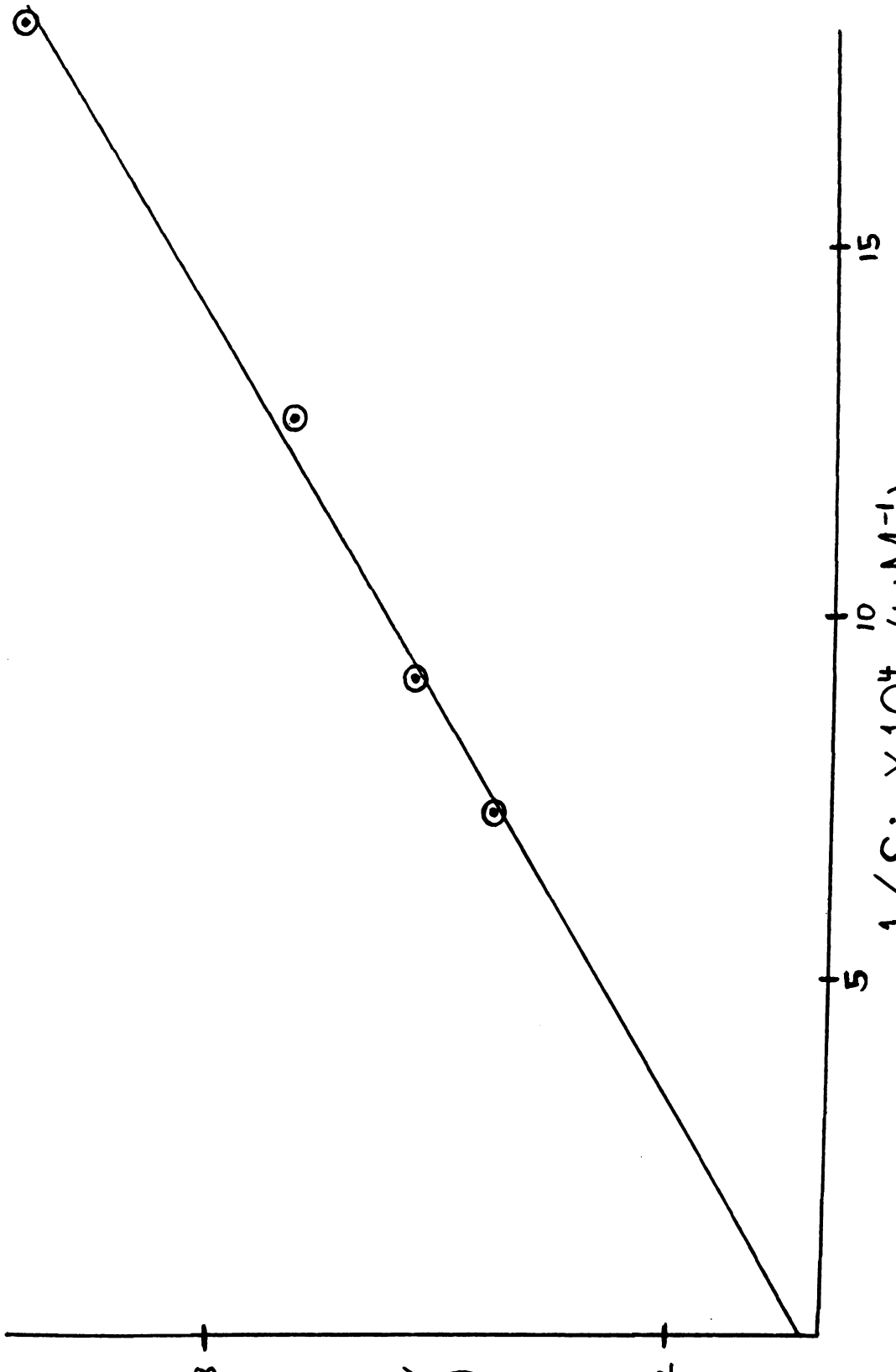


Figure 19. Lineweaver-Burke Plot for Glucose Oxidase

concentration which gives an initial rate equal to half the maximum initial rate. Km also gives an indication of the range of substrate concentration over which the enzyme is analytically useful, as demonstrated by the linearity of the calibration curve obtained over that range. A typical upper limit of the substrate concentration equal to one tenth Km is usually accepted. For glucose in this system, this limit is approximately 1.5 mM (270 ppm).

b. Alcohol Dehydrogenase

Immobilized alcohol dehydgogenase catalyzes the reaction

$$CH_3CH_2OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+$$

If the NAD is present in excess and the ethanol concentration is much less than Km for the enzyme, the rate of appearance of NADH in the initial stages of the reaction will be proportional to the ethanol concentration. The equilibrium lies far to the left (12); semicarbazide hydrochloride is added to the solutions to consume the acetaldehyde as it is formed. In the stopped-flow clinical analyzer, the enzymatic reaction was allowed to proceed in the reaction loop for a fixed time interval. This time interval was kept short enough that only a small fraction (typically 1-2%) of the ethanol was converted to product.

The absorbance at 340 nm due to NADH was then measured and related to the ethanol concentration.

The kinetics of the ADH catalysis are illustrated in Figure 20 as a plot of the observed percent reaction as a function of the reaction time for a 150 ppm ethanol sample at pH 7.7 and at room temperature. It can be seen from the plot that the slope is linear for approximately the first 8 minutes of the reaction, during which 1-2% of the ethanol is consumed. The small positive y-intercept is caused by NAD absorbance which was not present in the blank solution. The initial rate of the reaction, determined from the linear slope, was found to be 11.2 uM/min.

Some interesting facts about this reaction were revealed in deuterium- labelling experiments carried out by Fischer, et al (82). They discovered that the relative orientation of the ethanol and NAD molecules during the catalysis is always the same, and that it involves a direct transfer of a hydrogen atom from the ethanol to NAD. The reaction was thus shown to be stereochemically specific, always occurring with the same face of the NAD ring.

A Lineweaver-Burke plot for the immobilized ADH was carried out at a pH of 7.7, and yielded a maximum rate of 18.9 uM/min (standard deviation 1.2) and a Km value of 3.21 mM (standard deviation 0.11). Hayes and Velick (83) reported Km as 18 mM for soluble ADH at a pH of 7.9.

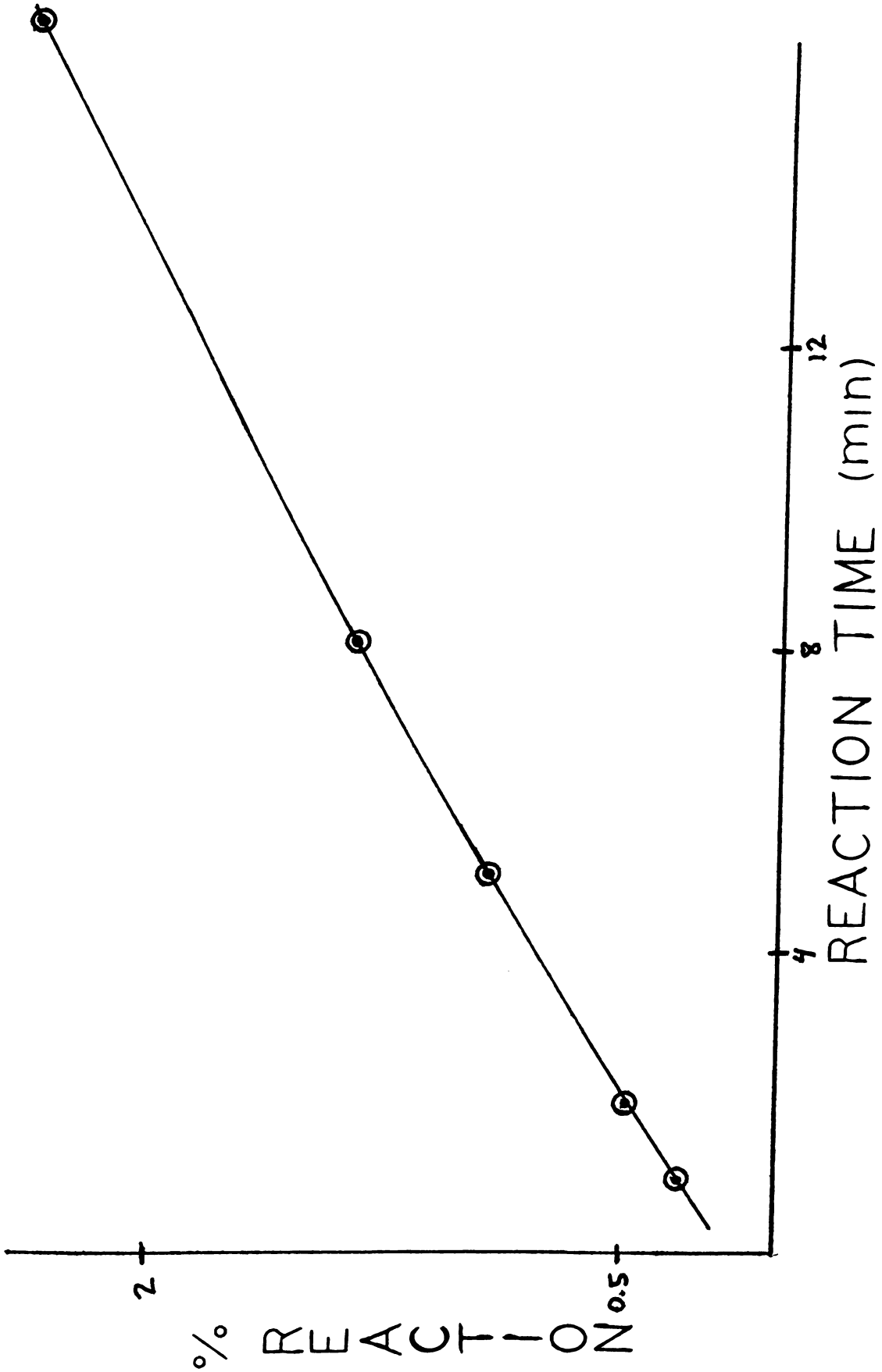


Figure 20. Ethanol Progress Curve

2. Diffusion Effects

In the stopped-flow clinical analyzer, the transport of reactants to the immobilized enzyme surface and the transport of products back into the bulk solution is totally dependent on diffusion. Although the inner diameter of the loop is only 0.86 mm, its diameter is significantly greater than the thickness of the immobilized enzyme layer. This results in a diffusion layer between the bulk solution and the surface if the rate of diffusion to the surface is less than the rate of the enzymatic reaction. The rate of the enzymatic reaction may in certain cases be much greater than the diffusion rate, in which case the overall conversion rate will be equal to the diffusion rate. As described by the Nernst-Plank equations (84), the diffusion rate across a diffusion boundary is proportional to the bulk solution concentration. Hence even if the reaction is diffusion-limited, the method will still be analytically useful since the observed rate will be proportioanal to the analyte concentration.

Hornby, Lilly, and Crook (80) have applied the Nernstian diffusion model to enzyme kinetics at a surface. The model accounts for both electrostatic and diffusion effects. The equation they derived is

$$R = \frac{R_{max}\, S_i}{(K_m + \frac{xR_{max}}{D})(\frac{RT}{RT - zxF\,\mathrm{grad}\psi}) + S_i}$$

where

R = observed rate

S_1 = initial concentration of substrate

Km = Michaelis constant

x = thickness of quasi-Nernst diffusion layer

Rmax = maximum rate

D = diffusion coefficient

R = gas constant

T = absolute temperature

z = charge on the mobile species

F = Faraday constant

gradψ = electric field gradient

Note that the equation is similar in form to the Michaelis-Menten equation. If either the substrate or the support is neutral (z=0), as in the case of both glucose and ethanol, the apparent Michaelis constant (Km') can be defined as

$$K_m' = K_m + \frac{xR_{max}}{D}$$

This equation demonstrates the effect of substrate transport on the observed Michaelis constant. From the equation, it can be seen that Km is enhanced with either increased Rmax (i.e. increased activity) or increased diffusion layer thickness (i.e. decreased diffusion rate). With Rmax constant, an increase in Km can be interpreted as a decrease in the initial rate of the reaction. Referring again to Figure 17, for Km to occur at a higher substrate concentration and Rmax to remain constant, the initial slope

must be decreased. It is also intuitively obvious that as the rate of diffusion becomes slower than the rate of enzymatic conversion, the overall rate will decrease.

From experiments performed in this study, it is not apparent which process is actually rate determining. The slope of the percent reaction vs substrate concentration curves showed a bending toward the concentration axis at high substrate concentrations, as predicted from the Michaelis-Menten model. However, the diffusion rate also increased as the bulk solution concentration was increased, and may have eventually become greater than the rate of the enzymatic reaction. When diffusion was no longer rate-limiting, the observed rate would then be determined by the enzyme kinetics, and the rate curve would still be predicted to take on the observed shape.

3. Induction Period

Enzymatic reactions usually often show an induction period between the initial contact of reagents and the appearance of the product species. In the case of soluble glucose oxidase, the production of hydrogen peroxide is often not detected for approximately 30 s after mixing. The duration of the induction period may be dependent upon several factors, and is known to vary inversely with the substrate concentration (85). If the induction period is caused by an orientation process between enzyme and

substrate (i.e. a build up of ES), it would follow that at higher concentrations the induction period should be shorter.

This phenomenon has been observed in the kinetics of the immobilized glucose oxidase reaction. In studies of the hydrogen peroxide produced as a function of reaction time (at a fixed substrate concentration), the observed curve does not pass through the origin but has a negative y-intercept (cf. Figure 18). The curve passes through the time axis (zero H_2O_2 detected) at approximately 20-30 s, which corresponds to the induction period. The magnitude of the x-intercepts (observed induction periods) for several different immobilized glucose oxidase columns were found to be quite varied, however, so no conclusions could be drawn.

The fact that the induction period is inversely related to the substrate concentration resulted in glucose calibration curves which had a small negative y-intercept (see Chapter VIII). For the more concentrated samples, the production of hydrogen peroxide actually occurred over a larger fraction of the fixed incubation period, since the induction period was shorter for these samples. This behavior was not observed in the immobilized ADH reaction.

B. pH Profiles

Figure 21 illustrates the pH profile of the immobilized glucose oxidase reaction. For this study, a

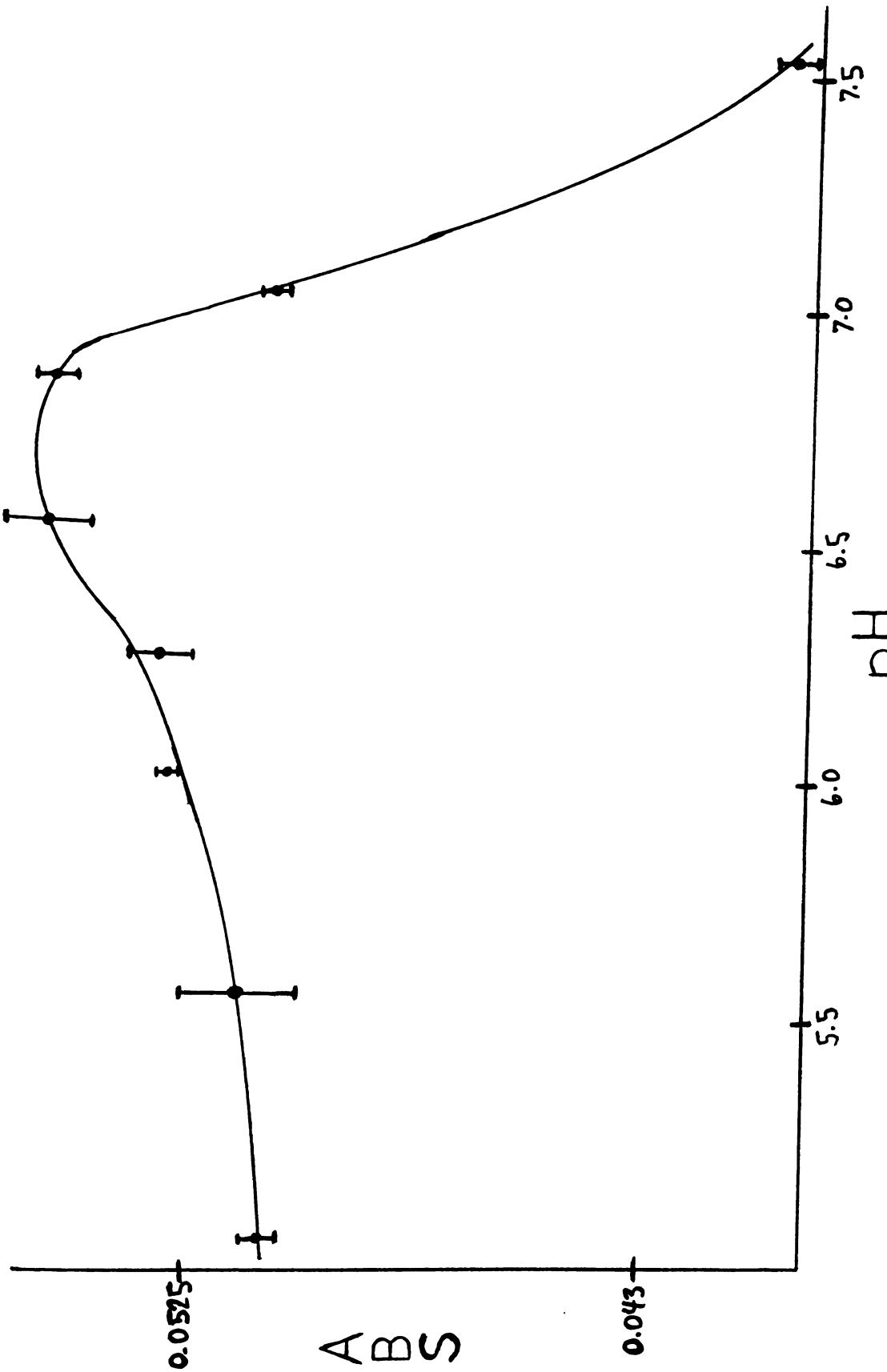


Figure 21. pH Profile of Glucose Oxidase

0.2 M phosphate buffer was adjusted to the desired pH by the addition of either NaOH or HCl and the final pH measured with a pH meter. This buffer was then used to dilute a stock solution of glucose to a final glucose concentration of 1.20 g/l.

The pH profile shows a typically broad, flat curve characteristic of glucose oxidase. The extent of reaction varied by only 8% from pH 5 to 7, and decreased dramatically above pH 7.5. Similar data have been presented by Bright and Appleby (86) for soluble glucose oxidase, which showed a pH maximum at 5.6.

The pH profile of the immobilized alcohol dehydrogenase reaction is shown in Figure 22. A pyrophosphate buffer (0.075 M) was adjusted to the desired pH and used to dilute a stock ethanol solution to a final ethanol concentration of 300 ppm. The solutions were incubated in the ADH reaction loop for 2.0 min at room temperature, after which the absorbance due to NADH was measured. Maximum activity was observed at a pH of 9.05

C. Long Term Stability and Storage Conditions

An important theoretical advantage of immobilized enzymes over their soluble forms is improved stability. The instability of enzymes in solution has been a major reason why enzymatic analysis has not become more prevalent in clinical laboratories. Glucose oxidase is an exception,

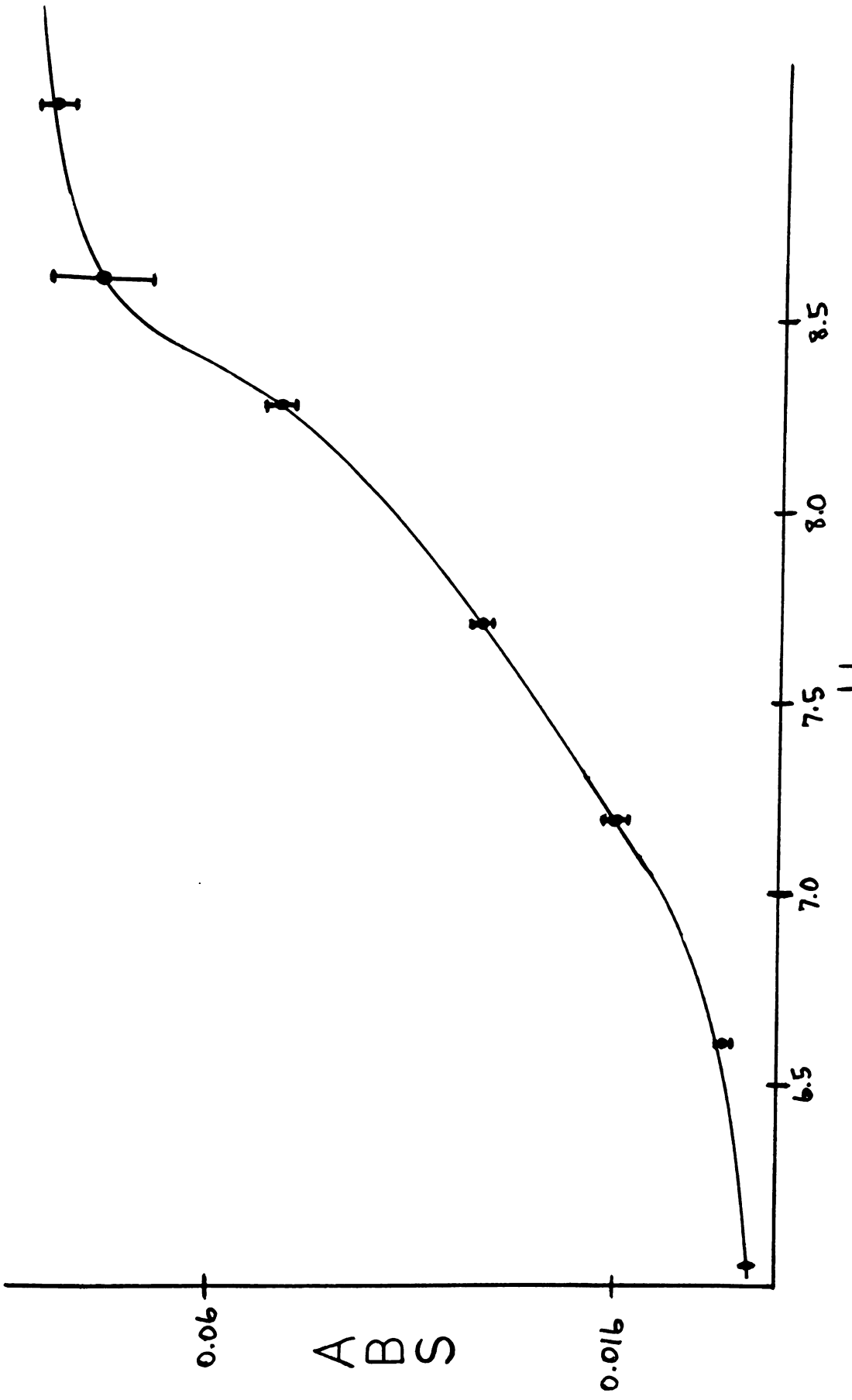


Figure 22. pH Profile of Alcohol Dehydrogenase

being one of the most stable enzymes in solution. Certain enzymes, however, denature so rapidly in solution that they have little value as analytical reagents.

In order to get an accurate evaluation of the stability of an immobilized enzyme, it should be subjected to continuous use, under the actual conditions of the analysis. This sort of rigorous testing was not possible in this work; however, an estimate of the stability of these immobilized enzymes under analysis conditions can be inferred from the stabilities observed here. The shelf life of these immobilized enzymes has also been evaluated.

When in use, the immobilized glucose oxidase and alcohol dehydrogenase loops were held at room temperature. All solutions drawn into the reaction loops were buffered at a mild pH to prolong the life of the enzymes. When the use of a loop was not anticipated for a few days or more, it was filled with a buffer solution and stored under refrigeration. However, if use was anticipated the following day, it was not refrigerated. As a result, the enzymes were exposed to room temperature for approximately half of their total lifetime.

For the immobilized glucose oxidase, a small decrease in activity was usually observed when the loop was left at room temperature overnight. Occasionally, the activity was observed to have remained constant or even slightly increased the following day, probably due to a slight increase in the room temperature. When left unused for one

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week at room temperature, the activity had decreased by only 4%.

When used in this manner, the immobilized glucose oxidase retained significant activity for over 6 months. During that time, an estimated 500 aqueous glucose samples were analyzed with the loop. Based on these observations, a conservative estimate of the life of the enzyme under continuous use would be about 3 months.

No significant loss of activity was detected in a glucose oxidase loop which had been filled with a buffer solution and stored refrigerated for nearly 4 months. When stored under these conditions, the shelf life of immobilized glucose oxidase is estimated to be in excess of one year.

Immobilized alcohol dehydrogenase was found to be far less stable than the glucose oxidase. In early studies of the ADH reaction, the sample and wash solutions were buffered at a pH of 8.7, near the observed pH optimum. Under these conditions, when the enzyme was subjected to continuous use, the activity steadily decreased from day to day, and activity could not be detected at all after one week. Either the enzyme had become slowly denatured at that pH, or possibly the enzyme had slowly become detached from the support.

Subsequently, ethanol analyses were carried out at a pH of 7.7. At this pH, the activity was only about 50% of the observed optimum, but the enzyme was found to be far more stable. Also, to further increase the life of the ADH,

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the loop was filled with a buffer solution of pH 6.5 when not in use. When used continuously under these conditions, the activity decreased by only about 3-4% from day to day, and the total lifetime of each loop was increased to about one month.

In choosing the pH and type of storage buffer for immobilized enzymes, one should consider both the effect of that pH on the enzyme itself and also its effect on the covalent bonds involved in its attachment to the support. These bonds may slowly break at certain pH values or in the presence of certain reactive species.

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CHAPTER VIII

PERFORMANCE OF THE INSTRUMENT AS A CLINICAL ANALYZER

Immobilized enzymatic analysis with the SFCA is evaluated in this chapter from the perspective of the clinical chemist. In order to be an effective clinical tool, especially in the demanding environment of a large clinical laboratory, the instrument must be able to withstand continuous use and still produce accurate analysis information. Data on the analysis of both glucose and ethanol samples are presented from this perspective.

First, the optimum analytical parameters are evaluated, and the clinical chemist's choice of either a reaction-rate or equilibrium method for both the enzymatic reaction and the indicator reaction is considered. Next, the linearity and precision of the response are evaluated, and possible interferences to the analyses are considered. The method of serum preparation is also considered. In the final sections, a proposed multi-reaction-loop configuration is evaluated in terms of its potential analysis throughput, and initial work on the enzymatic analysis of cholesterol is presented.

A. Optimum Analytical Parameters

The optimum working parameters used in the determination of both glucose and ethanol are presented in

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this section. An evaluation of instrumental variables, such as the optical parameters and the data acquisition rate, has been presented in chapter VI.

1. Reagent Concentrations

a. Glucose Determinations

For the determination of glucose, the solution in the immobilized enzyme reaction loop consisted of the sample diluted with a phosphate buffer. As was shown in the pH profile of immobilized glucose oxidase (see chapter 7, Figure 21), the extent of the enzymatic reaction was observed to vary about 1-2% for a pH change of 0.1 around pH 6.30. As a result, in order to minimize imprecision caused by fluctuations in the rate of the enzymatic reaction, the phosphate buffer must control the pH at 6.30 +/- 0.01 during the reaction.

To calculate the buffer capacity required to achieve this control, a worst case example is considered. In determining glucose samples, we allow the enzymatic reaction to proceed to approximately 1-2% completion. If the most concentrated glucose sample is to be 250 ppm glucose (1.39 mM), the maximum expected hydrogen ion consumption, based on a 5% reaction in the loop, is calculated to be 69.4 uM. (More concentrated samles were diluted with the buffer before analysis.) A calculation of the pH change of

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a 0.1 M phosphate buffer, consisting of a mixture of K_2HPO_4 and KH_2PO_4 at pH 6.30, upon the consumption of 69.4 uM hydrogen ion shows the change to be only 0.001 pH units. Note that this expected pH change is based on a 5% enzymatic reaction. In practice the reaction was allowed to proceed to at most 2% completion. Hence, the pH is not expected to be altered significantly during the enzymatic reaction, in a 0.1 M phosphate buffer of pH 6.30.

In the indicator reaction, the hydrogen peroxide produced in the reaction loop reacts with excess iodide in the presence of the Mo(VI) catalyst to produce triiodide:

$$2H^+ + H_2O_2 + 3I^- \longrightarrow I_3^- + 2H_2O \qquad (1)$$

The triiodide formed is also in equilibrium with iodine and the unreacted iodide:

$$I_2 + I^- \rightleftharpoons I_3^- \qquad (K_{eq} = 710) \qquad (2)$$

In order for the equilibrium absorbance of triiodide to be proportional to the hydrogen peroxide produced in the reaction loop, reaction (1) above must go to completion, and equilibrium (2) must lie far to the right. The latter requirement is met by the presence of an excess of iodide. Again using the worst case example of a 5% conversion of a 250 ppm glucose sample in the reaction loop, the maximum hydrogen peroxide concentration in the column effluent is found to be 69.4 uM. If the indicator reagent is made 0.05 M in iodide, the iodide concentration would essentially

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remain 0.05 M after a complete reaction with the 69.4 uM peroxide. This concentration of iodide is nearly a thousand-fold greater than the concentration of triiodide produced.

The concentration of ammonium molybdate in the indicator reagent was 0.102 M, as in a similar procedure from Malmstadt (88). Increasing the concentration of this catalyst had no effect on the equilibrium absorbance of the triiodide.

The indicator reagent was buffered at pH 6.30 with a 0.1 M phosphate solution for several reasons. First, the indicator reaction is somewhat pH dependent (1). Second, the Mo(VI) can undergo side reactions under certain pH conditions. Third, as noted previously, the GCA-McPherson mixer is not very efficient, and schlieren effects are observed in the observation cell when two solutions of moderately different concentrations are mixed.

In summary, for the determination of glucose, the enzymatic reaction was buffered at a pH of 6.30 with 0.1 M phosphate. The indicator reagent, which was mixed with the reaction loop effluent, consisted of 0.05 M KI, 0.102 M ammonium molybdate, and 0.1 M phosphate, pH 6.30.

b. Ethanol Determinations

In the determination of ethanol, the buffered sample, containing ethanol and NAD, was allowed to react in the

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reaction loop for a preset time, during which a fraction of the coenzyme NAD was converted to NADH. The concentration of NADH present at the end of the incubation period was used as a monitor of the reaction rate. Since the concentration of NADH could be determined directly by its absorbance at 340 nm, no indicator reaction was necessary. The stopped-flow system in this application served only to move the solution from the reaction loop to the observation cell for the absorbance measurement. The reagent drive syringe contained a buffer solution, which diluted the NAD by a factor of two in the stopped-flow.

From the stoichiometry of the ADH reaction, each conversion of an ethanol molecule to acetaldehyde is accompanied by the conversion of a molecule of NAD to NADH. The rate equation for the reaction is

$$R = \frac{R_{max}CS}{(K_c + C)(K_m + S)}$$

where S is the concentration of ethanol and C is the concentration of NAD (83). If the concentration of NAD is in excess and the reaction time is short enough so that its concentration remains essentially unchanged during the reaction, the rate equation simplifies to

$$R = \frac{R_{max}S}{K_m + S}$$

where C and Kc have been included in the constant Rmax. Hence, under these conditions, the initial rate will be proportional to the initial concentration of ethanol.

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A pyrophosphate buffer was used for the the ADH reaction. As described in chapter 7, the pH chosen for this reaction was the result of a compromise between the increased activity observed as the pH approaches 9, and the increased stability observed at lower pH values. A pH of 7.70 was chosen. Although the activity was only 50% of the optimum, the enzyme was stable for at least a month at this pH.

Hydrogen ions are produced in the ADH reaction by the following equation:

$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

To evaluate the efficiency of the buffer in controlling the pH during the enzymatic reaction, a worst case example of a 5% conversion of the most concentrated ethanol sample is considered. For a 150 ppm (3.26 mM) ethanol sample, a 5% reaction would produce 0.163 mM H^+. In a 0.075 M pyrophosphate buffer at pH 7.70, the addition of 0.163 mM of hydrogen ion will result in a shift of the pH to 7.69, which, according to the pH profile of ADH (chapter 7, Figure 22), should increase the rate of the enzymatic reaction by much less than 1%. Since in practice the enzymatic reaction was allowed to proceed to only 1-2% completion, the 0.075 M pyrophosphate buffer is quite adequate for pH control, as long as the ethanol samples are diluted to the 0-150 ppm range.

In summary, the sample solution consisted of a

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composite reagent of ethanol (0-150 ppm; more concentrated samples were diluted), NAD (0.50 mM), and pyrophosphate buffer (0.075 M, pH 7.70). Semicarbazide hydrochloride (0.075 M) was also present in the sample solution to consume the acetaldehyde as it formed.

2. Mode of the Enzymatic Reaction

The design of the stopped-flow clinical analyzer permits the enzymatic reaction to be monitored in either a reaction-rate mode or an equilibrium mode. A discussion of the theoretical advantages and disadvantages of each mode has been presented in Chapter VI. In this section, the alternatives are considered for applications in clinical determinations.

In evaluating the potential of this instrument in a clinical environment, one must consider the time involved in each analysis. It has been shown in these studies that a 2 min incubation period in the reaction loop resulted in approximately 2% conversion of substrate to product, for both the glucose oxidase and alcohol dehydrogenase catalyzed reactions. As predicted by the Michaelis-Menten model, reaction to completion would require a prohibitive amount of time. When the analysis time is crucial, the equilibrium mode is no longer a viable choice for enzymes immobilized on nylon tubing, unless an immobilization procedure which produces much more activity is developed.

Also, in the case of the glucose oxidase reaction, another restriction on monitoring the enzymatic reaction after equilibrium is the fact that dissolved oxygen in the sample solution enclosed in the reaction loop will limit the extent of the reaction.

3. Mode of the Indicator Reaction

Since the indicator reaction in the determination of glucose takes place in the observation cell of the stopped-flow module, it also can be monitored either continuously for reaction-rate analysis, or after equilibrium has been reached. Monitoring the reaction after equilibrium has several advantages, especially when considering analyses performed in a clinical lab. First, since the indicator reaction (iodide-peroxide) is fast (half life of a few seconds), the equilibrium method requires little or no more analysis time than the rate method. Also, the equilibrium method involves simpler data acquisition and calculation of the final result, although when done on a computer the difference is minimal. Finally, a reaction-rate method on this short a time scale is not feasible with the GCA-McPherson stopped-flow module as it is currently designed. Incomplete mixing in the mixing chamber results in the continuation of mixing in the observation cell after the flow has stopped. For slower indicator reactions, with half

lives of 30 s or more, reaction-rate monitoring may be superior. However, for the very rapid iodide-peroxide reaction used in this scheme, the equilibrium mode is better suited in a clinical application.

B. Linearity of Calibration Curves

1. Glucose Determinations

A calibration curve obtained for glucose using a 2.0 min incubation time is shown in Figure 23. A linear least-squares analysis of the data yielded a slope of 1.60x10(-3) A/ppm, with a relative standard deviation of 0.24%. The y-intercept was found to be -6.39x10(-3) with a relative standard deviation of 8.3%. The correlation constant was .099990. The linearity is shown to extend to 250 ppm glucose, which encompasses the expected concentration range in clinical samples.

2. Ethanol Determinations

The response linearity obtained for ethanol is illustrated in Figure 24. The absorbance data were obtained after the samples had been incubated for 2.0 min in the reaction loop. A linear least squares analysis of the data yielded a slope of 6.77x10(-4) A/ppm (relative standard

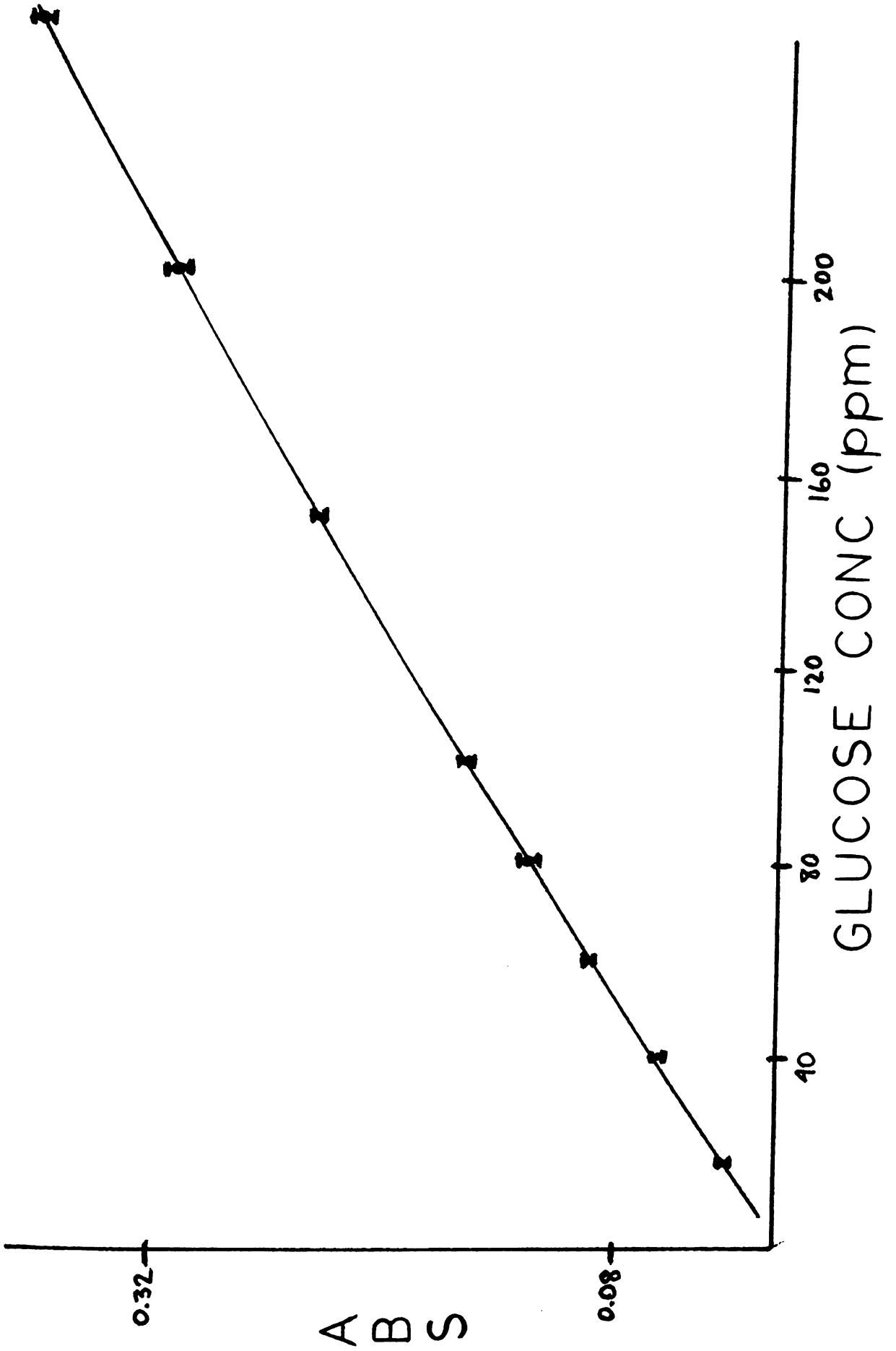

Figure 23. Calibration Curve for Glucose Determinations

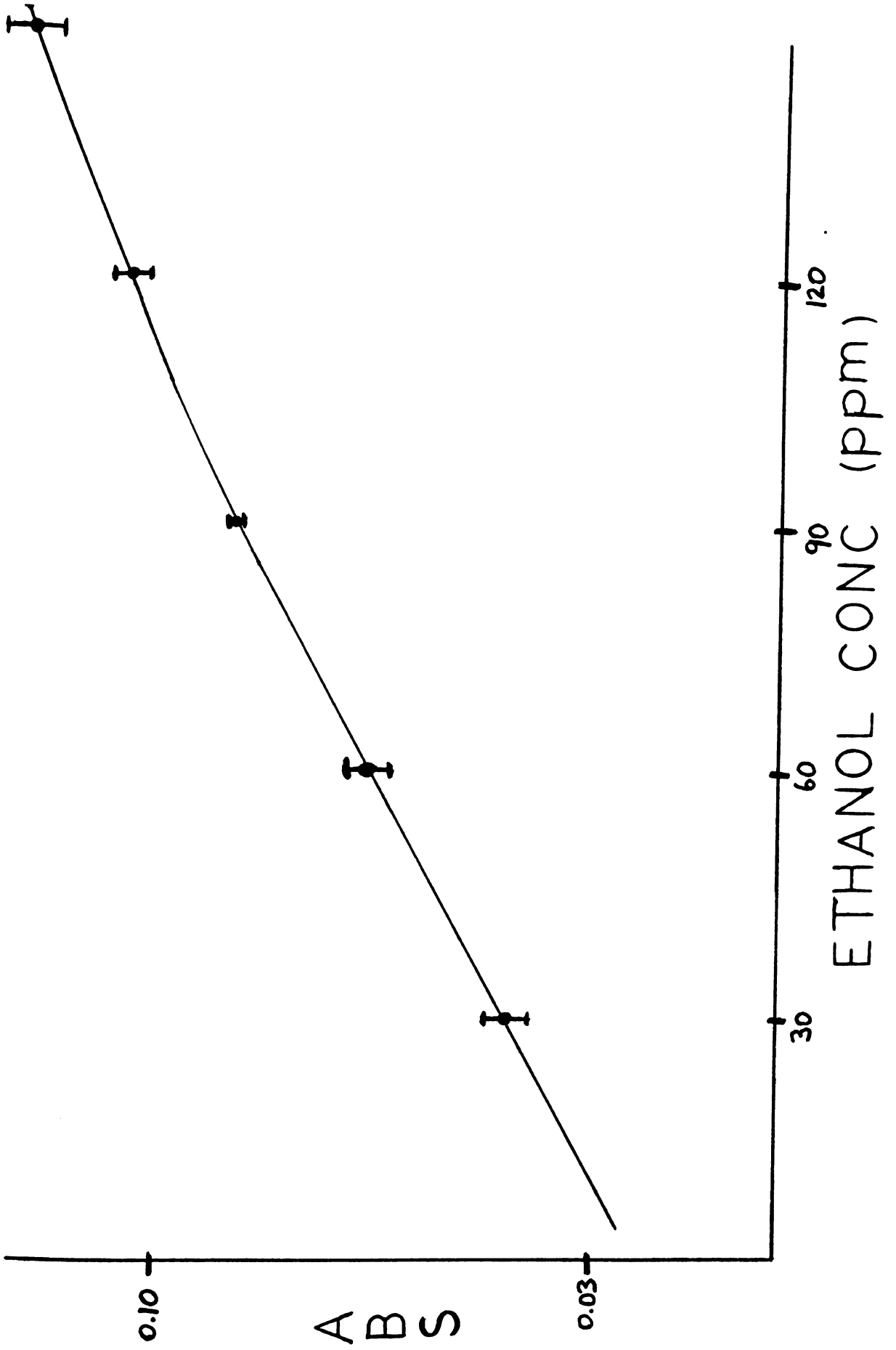

Figure 24. Calibration Curve for Ethanol Determinations

deviation 6.64%), and a y-intercept of 0.0273 (relative standard deviation 0.1%). The correlation coefficient was found to be 0.9942. Excellent linearity was obtained to about 100 ppm ethanol, above which the slope decreased slightly. This curvature at high substrate concentrations may be due to the fact that the substrate concentration was approaching the apparent Km for the immobilized enzyme, and the reaction was no longer pseudo-first order in ethanol. These data correspond to approximately 1% conversion.

C. Precision

To evaluate the precision of glucose determinations, a 200 ppm aqueous solution of glucose was analyzed 10 times. The relative standard deviation of the absorbance was found to be 2.24% (S/N = 45). For the determination of ethanol, the relative standard deviation of 6 determinations was 2.95% (S/N = 39). The precision of these methods is limited mainly by fluctuations in the rate of the enzymatic reaction caused by variations in room temperature.

D. Interferences

In the determination of serum glucose with immobilized glucose oxidase, Kunz and Stastny (37) evaluated some interferences which may hinder the analysis when a reaction-rate method is used. They found that oxalate and

fluoride ions, in concentrations up to 20 g/l, did not interfere, and also that albumin did not interfere in concentrations up to 100 g/l. Malmstadt and Hadjiioannou (88) found that oxalate, citrate, fluoride, and heparin did not interfere with a rate method for glucose which used soluble glucose oxidase. Similarly, Willis and Hill (89) noted that heparin, oxalate, and fluoride ions do not interfere with the ADH reaction. Thus, these substances are not expected to interfere in these determinations.

E. Serum Preparation

Since the protein substituent of serum samples absorbs at the monitoring wavelength and since other substituents in serum may interfere in the enzymatic reaction, it is necessary to deproteinize the serum samples before analysis.

First attempts at deproteinization involved the precipitation of the protein with a 10% trichloroacetic acid solution, followed by adjustment of the solution pH by the dropwise addition of concentrated NaOH and dilution with the buffer. It was found that the pH of the solution could not be accurately controlled or measured, since the volume was so small. As a result the final pH was imprecise, as demonstrated by large fluctuations in the observed absorbances.

The method of coprecipitation of the enzyme proved to

be most effective. In this method, 0.5 ml of serum was pipetted into a centrifuge tube, and chemically equivalent amounts of barium hydroxide and zinc sulfate (approximately 0.125 M each) were added. The required volume ratio of these two reagents was determined by titrating the $Ba(OH)_2$ with the $ZnSO_4$ to pH 7.0. The protein precipitated as the insoluble $BaSO_4$ and $Zn(OH)_2$ formed. After centrifugation, the supernatant was a clear solution, and was assumed to be free of the ions added. One ml of the supernatant was then pipetted into 3.0 ml of the phosphate buffer, mixed, and taken for analysis.

This method is attractive in that essentially all of the $Ba(OH)_2$ and $ZnSO_4$ are removed from solution via precipitation, which results in deproteinization without the addition of possible interferences and without any change in pH. Multiple analyses of serum samples prepared by this method showed excellent accuracy and precision, as shown in Table 2 of Appendix B.

F. Proposed Multi-Reaction-Loop SFCA

From a clinical standpoint, a major disadvantage of the SFCA as it is currently designed is that a great deal of the analysis time is spent waiting for the enzymatic reaction to proceed to the desired degree. It has been shown that excellent sensitivity can be obtained with these immobilized enzymes by allowing the sample to react for as

short a time as 2 min. In certain applications, the resulting throughput rate of 20-30 samples/hr is adequate. However, the throughput rate can be vastly improved if the stopped flow mixing system could be utilized to monitor one completed reaction while others were in progress.

The proposed multi-reaction-loop SFCA would optimize the use of the detection system by multiplexing several reaction loops into the stopped-flow mixing system. Two different configurations are possible. In one, the reaction loops would all contain the same immobilized enzyme, and would be used in the determination of one particular substrate. In another configuration, each loop would contain a different enzyme, and would be used to analyze rapidly for several different substituents of each serum sample. In either configuration, the initiation of the enzymatic reaction in each loop would be staggered, so that the detection system (stopped-flow spectrophotometer) would be available as each reaction was terminated.

The operation of the multi-loop system is described here for a system which contains six reaction loops. For the example, it is assumed that each enzymatic reaction is to be allowed to proceed for 2 min, and that the spectrophotometric detction of the reaction product requires 10 s. In this case, the reaction loops would be filled with sample at intervals of 20 s. If the first enzymatic reaction is started at t=0 s, the second would be started at t=20 s, and so on. At t=120 s, the reaction in the first

loop would be terminated by driving its contents into the stopped-flow observation cell. Then, at t=130 s, the first loop would again be filled with the next sample. At t=140 s, the reaction product in the second loop would be measured, and so on. This alternating sequence of push and fill would then continue at 10 s intervals. With this sequencing, samples could be analyzed at the rate of 180 per hr.

The proposed design for this six-loop SFCA is shown in Figure 25. The configuration is essentially the same as the single loop system with the exception of a pair of 6-to-1 valves located on each side on the reaction loops. The automatic switching of these 6-to-1 valves can be initiated by TTL-compatible signals generated by the microcomputer interface.

The sequencing of the 3-way sample valves and the stopped-flow module is exactly as previuosly described. The only addition to the entire sequence involves switching the 6-to-1 valves to the proper position before either filling a loop with sample or driving the reacted solution into the stopped-flow module to initiate the indicator reaction.

G. Initial Study of Cholesterol Determinations

The determination of serum cholesterol has become increasingly important because of the implied relationship between high cholesterol levels and cardiovascular disease

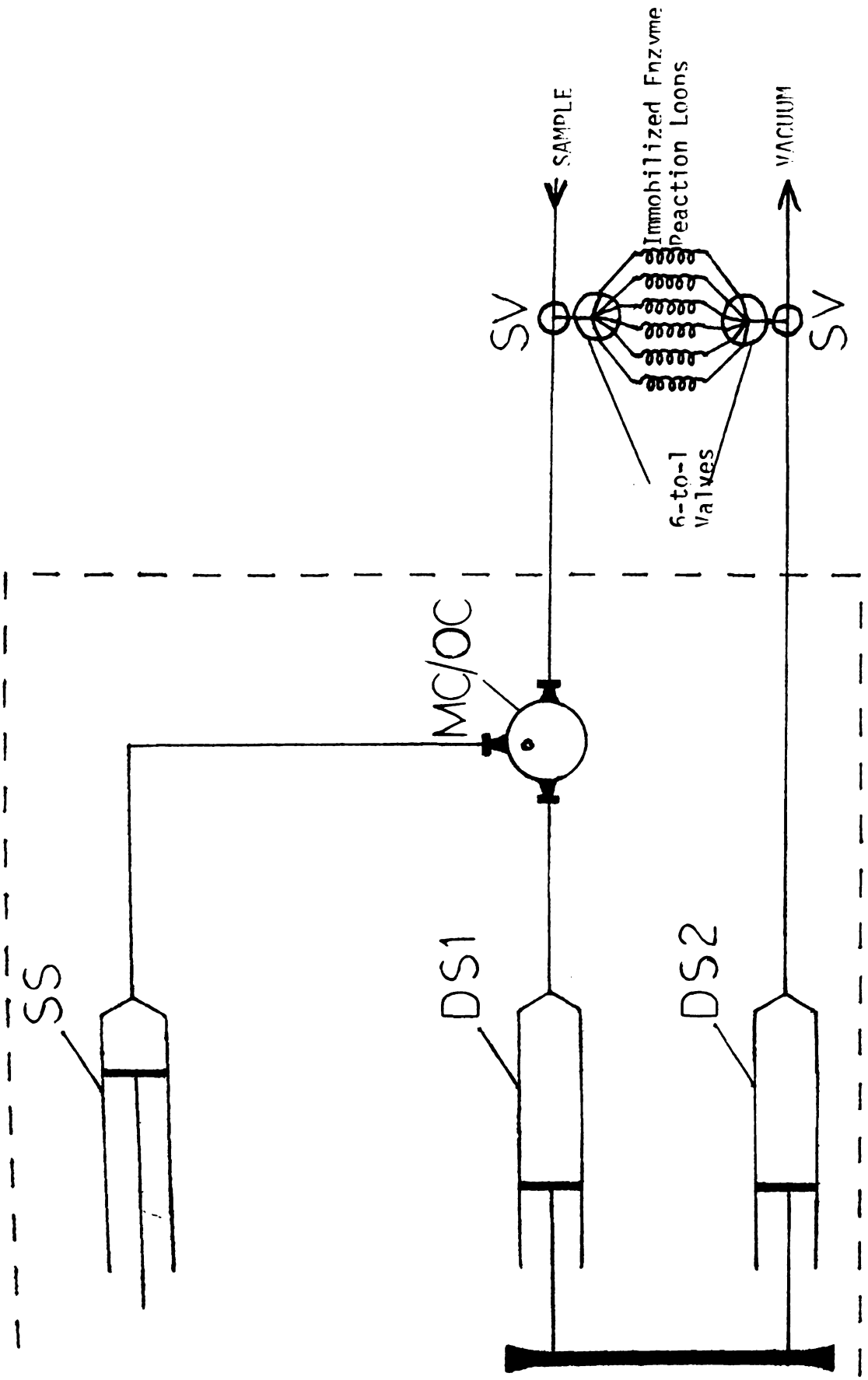

Figure 25. Proposed Multi-Reaction-Loop SFCA

(90,91), and also because abnormal cholesterol levels are of diagnostic value in several other diseases (92,93). Non-enzymatic determinations of serum cholesterol are in general use in clinical laboratories, but suffer from poor specificity, difficulty in standardization, unstable or corrosive reagents, and many interferences (94-97). Recently, the specificity of certain enzymes in the determination of cholesterol has been studied (98-100).

In the enzymatic determination of total serum cholesterol, the following sequence of reactions is used:

$$\text{cholesterol ester} + H_2O \xrightarrow[\text{esterase}]{\text{cholesterol}} \text{cholesterol} + \text{fatty acid}$$

$$\text{cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{cholest-4-en-3-one} + H_2O_2$$

The total cholesterol (cholesterol and cholesterol esters) has been indicated by the comsumption of oxygen (101), or by the reaction of the peroxide to produce a chromophore (100,102) or fluorophore (103).

In this study, the peroxide was monitored in the stopped-flow apparatus by allowing it to react with iodide in the presence of a Mo(VI) catalyst. Cholesterol oxidase (Miles Labs, Elkhart, IN) was immobilized on a nylon tube, by the procedure described in chapter III. However, the immobilized enzyme proved to be either highly unstable or had become detached, since activity lasted for only a few

days. Perhaps the immobilization at a different pH, or in the presence of cholesterol may improve the stability.

Studies of the peroxide-iodide indicator reaction in the cholesterol determination scheme demonstrated the versatility of the SFCA design. In reported procedures for the enzymatic determination of serum cholesterol, the reagents included sodium azide (approximately 1 g/l) to inhibit the consumption of peroxide by the enzyme catalase, which is an impurity in cholesterol oxidase preparations. Unfortunately, N_3^- is a serious interferent in the peroxide-iodide reaction. When N_3^- was present, the absorbance of triiodide in the indicator reaction was observed to increase rapidly over the first 5-10 s of the reaction, and then to decrease slowly over the next 5 min to approximately half the absorbance maximum.

The peroxide-iodide reaction was studied with and without the presence of azide, with the use of a stopped-flow spectrophotometer developed by Beckwith (104), and later modified by Notz (105), Holler (71), and Gall (106). This system was used because its mixing system is superior to that of the GCA-McPherson stopped-flow, and because fast data acquisition and analysis are computer controlled. Buffered solutions of hydrogen peroxide of known concentrations, with and without 0.015 M sodium azide, were placed in one reagent syringe, and a buffered solution of 0.5 M KI and 0.102 M Mo(VI) was placed in the other syringe. The buffer in both cases was 0.1 M phosphate, adjusted to a

pH of 7.50. After driving the reagents through the mixer into the observation cell, the reaction was monitored at 365 nm for 5 min. The data were then displayed, and initial slopes were calculated.

Plots of the observed initial slope (dA/dT over the first few seconds) with and without azide present vs the concentration of hydrogen peroxide are shown in Figure 26. The slope of the curve is only slightly decreased when azide is present, which demonstrates that the potential interference due to azide can be eliminated by the use of a reaction-rate method. In both cases, the linearity extends to 25 uM peroxide, which encompasses the range of peroxide concentrations to be determined in the SFCA. The peak height of the absorbance vs time curve could also be used to generate a linear calibration curve. Thus, by being able to observe the time dependence of the absorbance in the indicator reaction, the interference from azide can be eliminated. The microcomputer can either calculate the initial slope of the observed absorbance vs time curve, or simply on-line determine the peak absorbance, which occurred 12-15 s after the flow stopped.

This initial study demostrates the feasibility of serum cholesterol determinations with the stopped-flow clinical analyzer. In order to develop the procedure completely, the immobilization of cholesterol oxidase and cholesterol esterase would have to be studied further, in order to improve stability.

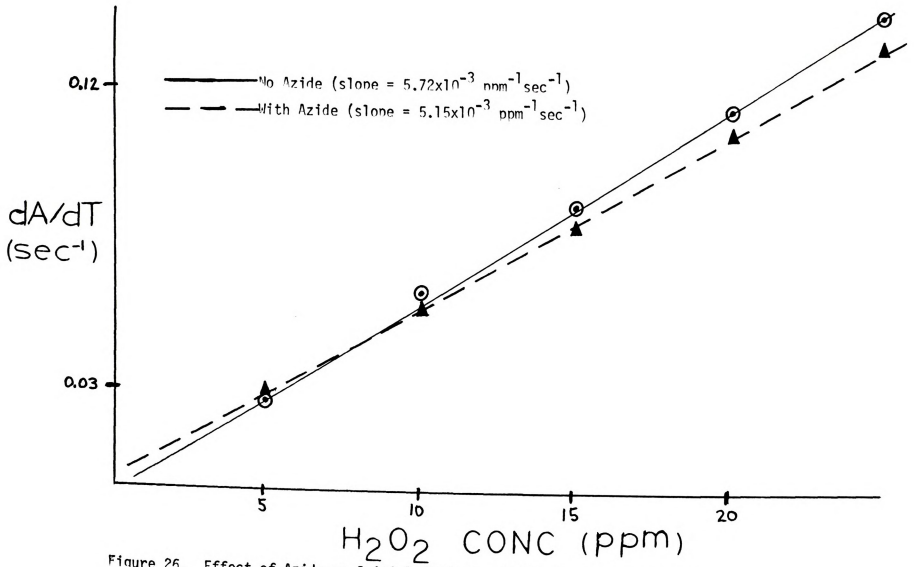

Figure 26. Effect of Azide on Initial dA/dt for Iodide-Peroxide Reaction

CHAPTER IX

SUMMARY AND FUTURE PROSPECTS

The major goal of this work was to demonstrate the potential of a stopped-flow analyzer in clinical analyses, and to this end the project was entirely successful. Rapid, accurate determinations of both glucose and ethanol were carried out on the SFCA with as little as 100 ul of serum. The technique uses microliter quantities of inexpensive reagents for each analysis, while the enzyme itself can be used in hundreds or thousands of determinations. Thus the main clinical objectives of speed, accuracy, and low cost have been realized.

Another important aspect of this research has been the application of computers to analytical chemistry. The 6100 microcomputer has great potential for completely automating the analysis scheme. The ability of the micro to share the facilities of a powerful minicomputer has been made possible by the development of a hierarchical network.

The development of the enzyme immobilization procedure was geared toward the discovery of a practical procedure. During this development and subsequent characterizations of the immobilized enzymes, some important aspects of the procedure were discovered, such as the effect of the hydrolysis conditions and the pH during the glutaraldehyde attachment. Certainly, if the entire immobilization sequence were better characterized,

conditions for each step could be intelligently chosen for each different enzyme.

The initial hydrolysis of nylon may be accomplished under very mild conditions, with comparable resultant activity. In these studies, nylon tubes were reused as supports after an enzyme had become inactive. The immobilization was started with the hydrolysis step. This hydrolysis presumably detached the previous enzyme and cross-link from the nylon wall, but did not significantly further hydrolyze the surface, since comparable activity could be attained. These tubes could possibly be reused indefinitely.

The presence of substrate or product, or a competitive inhibitor, during enzyme attachment may result in enhanced activity. Also, the time required to complete the entire procedure might be shortened tremendously by reacting the enzyme in the final step for a much shorter time. This reaction may actually be complete in an hour or less. It might also be instructive to determine the efficiency of this step, both in terms of the fraction of soluble enzyme actually attached, and the fraction of attached enzyme which is actually active. A similar study was carried out in our lab (107) for the immobilization of glucose oxidase on controlled-pore glass, where it was found that 44% of the enzyme in solution had been attached.

Since the rate of conversion in the reaction loop could be controlled by diffusion of substrate to the active

surface, a means of agitating the solution during this reaction might result in improved sensitivity.

The multi-reaction-loop system proposed in chapter VIII is expected eventually to burden the operator, since samples must be delivered to the loops frequently. An automated sample delivery system, such as a rotating turret, could be developed, and also placed under the control of the microcomputer. The potential throughput rate of the multi-loop system is also limited by the existing 4K of memory in the microcomputer. By expanding the memory, software for the micro could be written in FORTRAN-II, and be designed to carry out more, if not all, of the calculations. Calibration curve data for each loop could be stored in memory and recalled to calculate final concentration data, which then could be printed immediately on the console or lineprinter.

Another valuable addition to the 6100 microcomputer would be a real time clock. With the use of interrupts to signal a clock overflow condition, the CPU would be free to perform calculations or input/output operations during the execution of a timed operation.

The precision of the enzymatic reaction could be improved by thermostatting the reaction loop. For the glucose oxidase-catalyzed reaction, thermostatting the reaction at a temperature above room temperature (e.g. 37°C) would also result in enhanced activity (87). If the indicator reaction is monitored after equilibrium, the

temperature in the stopped-flow module is not critical.

The use of different indicator reactions warrants investigation. Deproteinization of serum samples could possibly be eliminated if the monitoring wavelength were such that serum substituents did not absorb. Fluorescence monitoring of the indicator reaction could also be implemented. Bostick and Hercules (108) have employed the chemiluminescence of luminol to detect hydrogen peroxide production in the glucose oxidase reaction.

Rapid characterization of a patient's blood chemistry could be carried out in a hospital emergency room or in a physician's office if the entire instrument could be miniaturized. The stopped-flow mixing system and detection system would be designed specifically for this application. The size could be drastically reduced, since a simple tungsten lamp, filters, and a PM tube would suffice. The dead time of the stopped-flow system would not be critical, so the syringe drive pressures and flow rates could be diminished to decrease size. The software for complete instrument sequencing, calibration, and calculation of the final results could be stored permanently in memory, and the microcomputer itself could also be miniaturized. If the instrument were made portable, it could be used in neighborhood mass screening programs.

REFERENCES

References

1. L. D. Bowers and P. W. Carr, Anal. Chem., 48, 544A (1976).

2. H. H. Weetall, Anal. Chem., 46, 602A (1974).

3. O. Zaborsky, "Immobilized Enzymes", CRC Press, Cleveland, 1973.

4. G. J. H. Melrose, Rev. Pure Appl. Chem., 21, 83 (1971).

5. D. N. Gray, M. H. Keyes, and B. Watson, Anal. Chem., 49, 1067A (1977).

6. K. J. Skinner, Chem. and Eng. News, Aug. 18, 1975.

7. G. R. Stark, "Biochemical Aspects of Reactions on Solid Supports", Academic Press, New York, 1971.

8. H. H. Weetall, Separ. Purification Methods, 2, 199 (1973).

9. A. D. McLaren and L. Packer, Advan. Enzymol. Relat. Areas Mol. Biol., 33, 245 (1970).

10. J. M. Nelson and E. G. Griffin, J. Amer. Chem. Soc., 38, 1109 (1916).

11. M. L. Green and G. Crutchfield, Biochem. J., 115, 183 (1969).

12. I. Langmuir and V. J. Schaefer, J. Amer. Chem. Soc., 60, 1351 (1938).

13. M. O. Dayhoff, "Atlas of Protein Sequence and Structure", National Biomedical Research Foundation, Maryland, 1969.

14. A. Bar-Eli and E. Katchalski, J. Biol. Chem., 238, 1690 (1963).

15. A. Bar-Eli and E. katchalski, Nature, 188, 856 (1960).

16. H. I. Silman, D. Wellner, and E. Katchalski, Isr. J. Chem., 1, 65 (1963).

17. H. Ozawa, J. Biochem., 62, 419 (1967).

18. S. A. Bernhard and G. L. Rossi, in "Structural Chemistry and Molecular Biology", (A. Rich and N. Davidson, eds.), Freeman Press, San Francisco and London, 1968.

19. E. K. Bauman, L. H. Goodson, G. G. Guibault, and D. N. Kramer, Anal. Chem., 37, 1378 (1965).

20. G. P. Hicks and S. J. Updike, Anal. Chem., 38, 726 (1966).

21. T. Wieland, H. Determan, and K. Buenning, Z. Naturforsch, 21, 1003 (1966).

22. A. D. McLaren and G. H. Peterson, Soil Sci. Amer. Proc., 22, 239 (1958).

23. P. Bernfeld and J. Wan, Science, 142, 678 (1963).

24. C. Wilson, Anal. Biochem., 31, 506 (1969).

25. M. A. Mitz and L. J. Summaria, Nature, 189, 576 (1961).

26. N. Grubhofer and L. Schleith, Naturwissenschaften, 40, 508 (1953).

27. A. Habeeb, Arch. Biochim. Biophys., 119, 264 (1967).

28. H. Weetall and N. Weliky, Jet Propulsion Laboratory Programs Summary No. 37-26, 4, 160 (1965).

29. H. H. Weetall, Nature, 223, 1969.

30. H. H. Weetall and L. S. Hersh, Biochim. Biophys. Acta, 185, 464 (1969).

31. H. H. Weetall, Science, 166, 615 (1969).

32. H. H. Weetall and L. S. Hersh, Biochim. Biophys. Acta, 206, 54 (1970).

33. L. S. Hersh, H. H. Weetall, and I. W. Brown, J. Biomed. Mater. Res., 3, 471 (1969).

34. T. L. Newirth, M. A. Diegelman, E. K. Pye, and R. G. Kallen, Biotech. and Bioeng., 15, 1089 (1973).

35. W. E. Hornby, H. Filippusson, and A. McDonald, FEBS Lett., 9, 8 (1970).

36. L. Goldstein, Biochim. Biophys. Acta, 315, 1 (1973).

37. H. J. Kunz and M. Stastny, Clin. Chem., 20, 1018 (1974).

38. P. S. Bunting and K. J. Laidler, Biotech. Bioeng., 16, 119 (1974).

39. H. Filippusson, W. E. Hornby, and A. McDonald, FEBS Lett., 20, 291 (1972).

40. W. E. Hornby, D. J. Inman, and A. McDonald, FEBS Lett., 23, 114 (1972).

41. P. V. Sundram and W. E. Hornby, FEBS Lett., 10, 325 (1970).

42. W. E. Hornby and H. Filippusson, Biochim. Biophys. Acta, 220, 343 (1970).

43. S. J. Updike and G. P. Hicks, Nature, 214, 986 (1967).

44. H. Huang, S. S. Kuan, and G. G. Guibault, Clin. Chem., 23, 671 (1977).

45. G. G. Guibault and J. Montalvo, J. Amer. Chem. Soc., 91, 2164 (1969).

46. G. G. Guibault and J. Montalvo, Anal. Lett., 2, 283 (1969).

47. G. G. Guibault and J. Montalvo, J. Amer. Chem. Soc., 92, 2533 (1969).

48. D. Williams, A. Doig, and A. Korosi, Anal. Chem., 42, 118 (1970).

49. T. Anfalt, A. Granelli, and D. Jagner, Anal. Lett., 9, 713 (1969).

50. D. S. Papasthathopoulas and G. A. Rechnitz, Anal. Chim. Acta, 79, 17 (1975).

51. B. Watson and M. H. Keyes, Anal. Lett., 9, 713 (1969).

52. G. Johansson and L. Ogren, Anal. Chim. Acta, 84, 23 (1976).

53. W. J. Blaedel, T. R. Kissel, and R. C. Boguslaski, Anal. Chem., 44, 2030 (1972).

54. S. W. May and N. N. Li, Biochem. and Biophys. Res. Com., 47, 1179 (1972).

55. R. S. Schifreen, D. A. Hanna, L. D. Bowers, and P. W. Carr, Anal. Chem., 49, 1929 (1977).

56. L. D. Bowers and P. W. Carr, Anal. Chem., 48, 544A (1976).

57. L. D. Bowers, L. M. Canning, C. N. Sayers, and P. W. Carr, Clin. Chem., 22, 1314 (1976).

58. B. Mattiasson, B. Danielsson, and K. Mosbach, Anal. Lett., 9, 217 (1976).

59. L. D. Bowers and P. W. Carr, Clin. Chem., 22, 1427 (1976).

60. J. A. Burns, Cereal Foods World, 21, 594 (1976).

61. D. J. Hanson and N. S. Bretz, Clin. Chem., 23, 477 (1977).

62. L. P. Leon, et al., Clin. Chem., 22, 1017 (1976).

63. K. Mosbach and B. Danielsson, Biochim. Biophys. Acta, 364, 140 (1974).

64. L. P. Leon, M. Sansur, L. D. Snyder, and C. Horvath, Clin. Chem., 23, 1556 (1977).

65. K. S. Chua and I. K. Tan, Clin. Chem., 24, 150 (1978).

66. D. J. Inman and W. E. Hornby, Biochem. J., 129, 255 (1972).

67. F. M. Richards and J. R. Knowles, J. Mol. Biol., 37, 231 (1968).

68. P. M. Hardy, A. C. Nicholls, and H. N. Rydon, J. Chem. Soc. D., 10, 565 (1969).

69. Skeggs, Am. J. Clin. Pathol., 28, 311 (1957).

70. F. J. Holler, S. R. Crouch, and C. G. Enke, Anal. Chem., 48, 1429 (1976).

71. F. J. Holler, Ph.D. Thesis, Michigan State University, East Lansing, MI, 1977.

72. R. E. Dessey, Anal. Chem., 49, 1100A (1977).

73. GCA McPherson Stopped-Flow Module Preliminary Operators Manual, GCA McPherson Co.

74. J. D. Ingle, Ph.D. Thesis, Michigan State University, East Lansing, MI, 1971.

75. L. Michaelis and M. L. Menten, Biochem. Z., 49, 333 (1913).

76. G. E. Briggs and J. B. S. Haldane, Biochem. J., 19, 338 (1925).

77. Q. H. Gibson, B. E. P. Swoboda, and V. Massey, J. Biol. Chem., 239, 3927 (1964).

78. J. Livingston, R. Morgan, and A. H. Richardson, J. Phys. Chem., 34, 2356 (1961).

79. H. Lineweaver and D. Burke, J. Amer. Chem. Soc., 56, 658 (1934).

80. W. E. Hornby, M. D. Lilly, and E. M. Crook, Biochem. J., 107, 669 (1968).

81. K. Kusai, Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ., 8, 43 (1960).

82. W. H. Fischer, A. Roedig, and K. Rauch, Liebigs Ann., 552, 203 (1942).

83. Hayes and Velick, J. Biol. Chem., 207, 225 (1954).

84. W. J. Moore, "Physical Chemistry", 4th Ed., Prentice-Hall, Englewood Cliffs, NJ, 1972.

85. H. V. Malmstadt and S. R. Crouch, J. Chem. Educ., 43, 340 (1966).

86. H. J. Bright and M. Appleby, J. Biol. Chem., 244, 3625 (1969).

87. R. H. Smith and J. Kilford, Int. J. Chem. Kinetics, 8, 1 (1976).

88. H. V. Malmstadt and S. I. Hadjiioannou, Anal. Chem., 34, 452 (1962).

89. F. W. Willis and J. B. Hill, Clin. Chem., 15, 91 (1969).

90. G. Pincherle, J. Chron. Dis., 24, 289 (1971).

91. D. Zilversmit, Am. J. Cardiol., 35, 449 (1975).

92. R. C. Neerhout, Clin. Chem. Acta, 41, 347 (1972).

93. C. Hirayama and T. Isira, Clin. Chem. Acta, 71, 21 (1976).

94. L. L. Abell and B. B. Levy, Stand. Methods Clin. Chem., 2, 26 (1958).

95. D. B. Tonks, Clin. Biochem., 1, 12 (1967).

96. W. D. Brown, Aust. J. Exp. Biol. Med. Sci., 39, 209 (1961).

97. B. Zak, Clin. Chem., 23, 1201 (1977).

98. W. Richmond, Clin. Chem., 19, 1350 (1973).

99. H. Flegg, Ann. Clin. Biochem., 12, 79 (1973).

100. C. C. Allain, L. S. Poon, and C. S. G. Chan, Clin. Chem., 20, 470 (1974).

101. A. Noma and K. Nakayama, Clin. Chem., 22, 336 (1976).

102. M. A. Pearse and S. H. Bodourian, Clin. Chem., 23 280 (1977).

103. H. Huang, J. W. Kuan, and G. G. Guibault, Clin. Chem., 21, 1605 (1975).

104. P. M. Beckwith, Ph.D. Thesis, Michigan State University, East Lansing, MI, 1972.

105. P. K. Notz, Ph.D. Thesis, Michigan State University, East Lansing, MI, 1976.

106. R. S. Gall, Current Research (1978).

107. P. Roth, unpublished results.

108. D. Bostick and D. Hercules, Anal. Chem., 47, 447 (1975).

APPENDIX A

Selected Program Listings

```
/+2     PARAMETER DEFINITIONS FOR SFCA     PAL8-V10A 12-JUL-78 PAGE 2


          /POINTERS TO FREQUENTLY CALLED SUBROUTINES

     0020           *20

00020  1200  DECIN,   XDECIN
00021  1335  TXOUT,   XTXOUT
00022  1400  TYPE,    XTYPE
00023  1600  READ,    XREAD
00024  1416  NOUT,    XNOUT
00025  1423  OCTOUT,  XOCTOU
00026  1450  DECOUT,  XDECOU
00027  1527  HDIV,    XHDIV
00030  2322  XHSUM,   HSUM
00031  2323  XLSUM,   LSUM
00032  2324  XAVCT,   AVCNT
00033  2200  SAMPLE,  XSAMPL
00034  2231  CRLF,    XCRLF
00035  2242  AVCLR,   XAVCLR
00036  2250  AVADD,   XAVADD
00037  2262  AVER,    XAVER
00040  2333  DELAY,   XDELAY
00041  2600  MONITR,  XMONIT
00042  2400  MANUAL,  XMANUA
00043  2000  SCALE,   XSCALE
00044  2120  CYCLE,   XCYCLE
00045  1631  IDATA,   XIDATA
00046  1707  ODATA,   XODATA
00047  1345  LST,     XLST

/+2     PARAMETER DEFINITIONS FOR SFCA     PAL8-V10A 12-JUL-78 PAGE 1

          /+2     PARAMETER DEFINITIONS FOR SFCA
          /
          /STOPPED-FLOW CLINICAL ANALYZER
          /SUBROUTINE ONE
          /
          /SFCA.S1 CONTAINS PARAMETER DEFINITIONS
          /    AND PAGE ZERO POINTERS
          /
          /SFCA.S2 CONTAINS FREQUENTLY CALLED SUBROUTINES
          /    WHICH LOAD BEGINNING AT THE PAGE FOLLOWING THE
          /    EXECUTIVE ROUTINE, AND OCCUPY 7 PAGES.
          /
          /DATA STORAGE BEGINS AT THE PAGE FOLLOWING SFCA.S2
          /    AND EXTEND TO LOCATION 7777.
          /
          /PGR: M.D. JOSEPH
          /DATE: 27-MAY-78
          /VER: 1B
          /FILE: SFCA.S1
          /
          /
     4400           CALL=    JMS I 0
     4421           WRITE=   CALL TXOUT
     4434           CR=      CALL CRLF

     6500           OPEN=    6500
     6501           CLOSE=   6501
     6502           EMPTY=   6502
     6503           FILL=    6503
     6504           PUSH=    6504
     6505           SFS=     6505
     6506           RESET=   6506
     6507           GETSR=   6507
     6510           CONVRT=  6510
     6511           SCD=     6511
     6512           GETAD=   6512
     6513           SETDA=   6513
```

```
                / EQUILIBRIUM MODE SFCA
                /
                /PGR: M.D. JOSEPH
                /DATE: 29-MAY-78
                /VER: 1C
                /FILE: SFCAEQ.EX
                /

                /PAGE ZERO DATA

        0150            *150

00150   0000    TSAMP,  0       /TOTAL NO. SAMPLES
00151   0000    CSAMP,  0       /-(CURRENT SAMPLE NO.)
00152   0000    TRUNS,  0       /TOTAL RUNS EA SAMPLE
00153   0000    CRUN,   0       /-(CURRENT)
00154   0000    RTIME,  0       /POINTER TO REACTION TIMES
00155   0000    EQTIME, 0       /DELAY TO EQUILIBRIUM
00156   0000    DATA,   0       /POINTER TO DATA TABLE
00157   0000    MDARK,  0       /-(DARK)
00160   0000    REF,    0       /REF-DARK
00161   0000    IFLAG,  0       /=0 FOR TTY SETUP
00162   3000    DATAB,  DATAT   /START OF DATA TABLE

        0170            *170
00170   0000            ZBLOCK 10       /REACTION TIMES

                        DECIMAL
        1750            ISAMP=1000      /NO. AVERAGES/DATA POINT
        0005            ISAMPT=5        /SAMPLE VALVE OPEN TIME (SEC)
        0002            IFILLT=2        /SYRINGE EQUILIBRIUL TIME
                        OCTAL
```

```
                0200            *200

00200   7300    START,  CLA CLL         /RESTART ENTRY
00201   4434            CR;CR
00202   4434
00203   4421            WRITE;  "E;"Q;"U;"I;"L;"I;"B;"R;"I;"U;"M;" ;"M;"O;"D;"E
00204   0305
00205   0321
00206   0325
00207   0311
00210   0314
00211   0311
00212   0302
00213   0322
00214   0311
00215   0325
00216   0315
00217   0240
00220   0315
00221   0317
00222   0304
00223   0305
00224   0240            " ;"S;"F;"C;"A;" ;"V;"4;"A;0
00225   0323
00226   0306
00227   0303
00230   0301
00231   0240
00232   0326
00233   0264
00234   0301
00235   0000
00236   4434            CR;CR
00237   4434
00240   4421            WRITE;"S;"E;"T;" ;"U;"P;" ;"F;"R;"O;"M;" ;"T;"T;"Y;"?;" ;0
00241   0323
00242   0305
00243   0324
00244   0240
00245   0325
00246   0320
00247   0240
00250   0306
00251   0322
00252   0317
00253   0315
00254   0240
00255   0324
00256   0324
00257   0331
00260   0277
00261   0240
00262   0000
00263   4423            CALL READ
```

```
/ EQUILIBRIUM MODE SFCA                          PAL8-V10A 12-JUL-78 PAGE 4-1

00264  1377            TAD (-"N
00265  7650            SNA CLA
00266  7001            IAC
00267  3161            DCA IFLAG        /=0 FOR TTY SETUP
00270  1161            TAD IFLAG
00271  7640            SZA CLA
00272  4441            CALL MONITR      /SET UP FROM MINI
00273  4434            CR
00274  4421            WRITE;"N;"O;".;" ;"S;"A;"M;"P;"L;"E;"S;" ;"(;"I;"-;"B;");0
00275  0316
00276  0317
00277  0256
00300  0240
00301  0323
00302  0301
00303  0315
00304  0320
00305  0314
00306  0305
00307  0323
00310  0240
00311  0250
00312  0261
00313  0255
00314  0270
00315  0251
00316  0000
00317  1161            TAD IFLAG
00320  7640            SZA CLA; JMP A1
00321  5327
00322  4420            CALL DECIN; 0            /GET NUMBER OF SAMPLES
00323  0000
00324  1323            TAD .-1
00325  3150            DCA TSAMP
00326  5331            JMP A2
00327  1376  A1,       TAD (TSAMP
00330  4445            CALL IDATA       /INPUT FROM MINI
00331  4421  A2,       WRITE;"N;"O;".;" ;"R;"U;"N;"S;" ;"E;"A;0
00332  0316
00333  0317
00334  0256
00335  0240
00336  0322
00337  0325
00340  0316
00341  0323
00342  0240
00343  0305
00344  0301
00345  0000
00346  1161            TAD IFLAG
00347  7640            SZA CLA; JMP A3
00350  5356
00351  4420            CALL DECIN; 0            /NUMBER RUNS EACH SAMPLE
00352  0000

                                                 PAL8-V10A 12-JUL-78 PAGE 4-2
/ EQUILIBRIUM MODE SFCA

00353  1352            TAD .-1
00354  3152            DCA TRUNS
00355  5775'           JMP P400
00356  1374  A3,       TAD (TRUNS               /INPUT FROM MINI
00357  4445            CALL IDATA               /PAGE ESCAPE
00360  5775'           JMP P400

00374  0152
00375  0400
00376  0150
00377  7462
       0400'           PAGE
```

```
00400  7300  P400,   CLA CLL
00401  1377          TAD (170        /INITIALIZE DATA
00402  3154          DCA RTIME
00403  1150  A4,     TAD TSAMP
00404  7041          CIA; DCA CSAMP
00405  3151
00406  4421  GETSEC, WRITE;"I;"E;" ;"T;"I;"M;"E;"(;0
00407  0311
00410  0305
00411  0240
00412  0324
00413  0311
00414  0315
00415  0305
00416  0250
00417  0000
00420  1150          TAD TSAMP
00421  1151          TAD CSAMP; IAC
00422  7001
00423  4426          CALL DECOUT
00424  4421          WRITE;");0
00425  0251
00426  0300
00427  1161          TAD IFLAG
00430  7640          SZA CLA; JMP A5
00431  5237
00432  4420          CALL DECIN;0            /ENZYMATIC INC TIME
00433  0000
00434  1233          TAD .-1
00435  3554          DCA I RTIME
00436  5241          JMP A6
00437  1154  A5,     TAD RTIME
00440  4445          CALL IDATA              /INPUT FROM MINI
00441  2154  A6,     ISZ RTIME
00442  2151          ISZ CSAMP
00443  5296          JMP GETSEC
00444  4421          WRITE;"E;"Q;" ;"T;"I;"M;"E;0
00445  0305
00446  0321
00447  0240
00450  0324
00451  0311
00452  0315
00453  0305
00454  0000
00455  1161          TAD IFLAG
00456  7640          SZA CLA; JMP A7
00457  5265
00460  4420          CALL DECIN;0            /DELAY TO FIRST DATA PT
00461  0000
00462  1261          TAD .-1
00463  3155          DCA EQTIME
00464  5267          JMP OPER
00465  1376  A7,     TAD (EQTIME
00466  4445          CALL IDATA              /FROM MINI
```

```
00467  7300  OPER,   CLA CLL              /OPERATE
00470  1150          TAD TSAMP
00471  7041          CIA; DCA CSAMP
00472  3151
00473  1377          TAD (170
00474  3154          DCA RTIME
00475  4421          WRITE;"R;"E;"-;"I;"N;"I;"T;" ;"D;"A;"T;"A;"?;0
00476  0322
00477  0305
00500  0255
00501  0311
00502  0316
00503  0311
00504  0324
00505  0240
00506  0304
00507  0301
00510  0324
00511  0301
00512  0277
00513  0000
00514  4423          CALL READ
00515  1375          TAD (-"N
00516  7650          SNA CLA
00517  5822          JMP SSCALE
00520  1162          TAD DATAB           /TO DESTROY OLD DATA
00521  3156          DCA DATA
00522  4433  SSCALE, CALL SCALE          /SET 0,100
00523  4434  SAMP,   CR                  /LOOP HERE FOR EA SAMPLE
00524  1152          TAD TRUNS
00525  7041          CIA; DCA CRUN
00526  3153
00527  6507  RUN,    GETSR               /HERE FOR EA RUN
00530  0374          AND (2000
00531  7640          SZA CLA
00532  4443          CALL SCALE          /OPTIONALLY RESET 0,100% T
00533  4421          WRITE;"S;"A;"M;"P;" ;0
00534  0323
00535  0301
00536  0315
00537  0320
00540  0240
00541  0000
00542  1150          TAD TSAMP
00543  1151          TAD CSAMP; IAC
00544  7001
00545  4426          CALL DECOUT                 /WRITE CURRENT SAMP NO
00546  4421          WRITE;" ;" ;"R;"U;"N;" ;0
00547  0240
00550  0240
00551  0322
00552  0325
00553  0316
00554  0240
00555  0000
```

```
/ EQUILIBRIUM MODE SFCA                    PAL8-V10A 12-JUL-78 PAGE 6-1

00556  1152          TAD TRUNS
00557  1153          TAD CRUN; IAC
00560  7001
00561  4426          CALL DECOUT              /AND RUN NO
00562  6507          GETSR
00563  0373          AND (4000
00564  7650          SNA CLA
00565  4423          CALL READ          /WAIT KB BEFORE OPEN SAMP VALVES
00566  7200          CLA
00567  4440          CALL DELAY;2
00570  0002
00571  5772          JMP I (CYC               /PAGE ESCAPE

00572  0000
00573  4000
00574  2000
00575  7462
00576  0155
00577  0170
       0600          PAGE
```

```
00600  4444  CYC,    CALL CYCLE              /COMPLETE CYCLE
00601  1155           TAD EQTIME
00602  3204           DCA .+2
00603  4440           CALL DELAY;0            /WAIT FOR THINGS TO SETTLE
00604  0000
00605  4433           CALL SAMPLE; ISAMP      /SAMPLE AND AVERAGE ADC
00606  1750
00607  1157           TAD MDARK               /SUBRT DARK CURRENT
00610  3556           DCA I DATA              /STORE IN THE TABLE
00611  6507           GETSR
00612  0377           AND (1000
00613  7640           SZA CLA          /IMMEDIATE OUTPUT?
00614  5227           JMP A8           /NO
00615  4421           WRITE;" ;"I;"-;"D;"=;" ;0
00616  0240
00617  0311
00620  0255
00621  0304
00622  0275
00623  0240
00624  0000
00625  1556           TAD I DATA
00626  4426           CALL DECOUT
00627  4434  A8,      CR; ISZ DATA
00630  2156
00631  7410           SKP; JMP I (DAOVFL      /CHECK DATA TAB OVFL
00632  5776
00633  1160           TAD REF
00634  3556           DCA I DATA              /STORE CURRENT REF
00635  2156           ISZ DATA
00636  7410           SKP; JMP I (DAOVFL
00637  5776
00640  2153           ISZ CRUN                /MORE RUNS THIS SAMP?
00641  5775'          JMP RUN          /YES
00642  2154           ISZ RTIME        /ADV POINTER
00643  2151           ISZ CSAMP        /MORE SAMPLES?
00644  5000           JMP              /YES
00645  4434  ALIST,   CR;CR
00646  4434
00647  4421           WRITE;"L;"I;"S;"T;" ;"D;"A;"T;"A;"?;0
00650  0314
00651  0311
00652  0323
00653  0324
00654  0240
00655  0304
00656  0301
00657  0324
00660  0301
00661  0277
00662  0000
00663  4423           CALL READ
00664  1374           TAD (-"N
00665  7650           SNA CLA
00666  5777           JMP I (NOLIST
```

```
00667  4434          CR;CR
00670  4434
00671  4421          WRITE;"S;"A;"M;"P;" ;" ;"I;"-;"D;"/;"R;"-;"D;0
00672  0323
00673  0301
00674  0315
00675  0320
00676  0240
00677  0240
00700  0311
00701  0255
00702  0304
00703  0257
00704  0322
00705  0255
00706  0304
00707  0000
00710  4434          CR;CR
00711  4434
00712  1162          TAD DATAB
00713  3156          DCA DATA                /RESET DATA PTR
00714  1150          TAD TSAMP
00715  7041          CIA; DCA CSAMP
00716  3151
```

```
00717  1373  SAMPL,  TAD (-6
00720  3370          DCA M6          /SET COUNT
00721  1152          TAD TRUNS
00722  7041          CIA; DCA CRUN
00723  3153
00724  4434          CR
00725  1150          TAD TSAMP
00726  1151          TAD CSAMP; IAC
00727  7001
00730  4426          CALL DECOUT
00731  4421  RUNL,   WRITE;" ;" ;0
00732  0240
00733  0240
00734  0000
00735  1556          TAD I DATA
00736  2156          ISZ DATA
00737  4426          CALL DECOUT
00740  4421          WRITE;"/;0
00741  0257
00742  0000
00743  1556          TAD I DATA
00744  2156          ISZ DATA
00745  4426          CALL DECOUT
00746  2153          ISZ CRUN
00747  7410          SKP
00750  5365          JMP A11
00751  2370          ISZ M6
00752  5331          JMP RUNL
00753  1373          TAD (-6
00754  3370          DCA M6
00755  4434          CR
00756  4421          WRITE;" ;" ;" ;" ;0
00757  0240
00760  0240
00761  0240
00762  0240
00763  0000
00764  5331          JMP RUNL
00765  2151  A11,    ISZ CSAMP
00766  5317          JMP SAMPL
00767  5245          JMP ALIST
00770  0000  M6,     0

00773  7772
00774  7462
00775  0527
00776  1653
00777  1000
       1000          PAGE
```

```
01000  4434  NOLIST, CR;CR
01001  4434
01002  4421          WRITE;"X;"M;"I;"T;" ;"D;"A;"T;"A;"?;0
01003  0330
01004  0315
01005  0311
01006  0324
01007  0240
01010  0304
01011  0301
01012  0324
01013  0301
01014  0277
01015  0000
01016  4423          CALL READ
01017  1377          TAD (-"Y
01020  7640          SZA CLA          /XMIT DATA TO MINI?
01021  5776          JMP I (START     /NO; RESTART
01022  4441          CALL MONITR      /SET UP MINI
01023  1162          TAD DATAB
01024  3156          DCA DATA
01025  1150          TAD TSAMP
01026  7041          CIA; DCA CSAMP
01027  3151
01030  1150          TAD TSAMP
01031  4446          CALL ODATA
01032  1152          TAD TRUNS
01033  4446          CALL ODATA
01034  1152  SAMPX,  TAD TRUNS
01035  7041          CIA; DCA CRUN
01036  3153
01037  1556  RUNX,   TAD I DATA
01040  2156          ISZ DATA
01041  4446          CALL ODATA       /SAMPLE-DARK
01042  1556          TAD I DATA
01043  2156          ISZ DATA
01044  4446          CALL ODATA       /REF-DARK
01045  2153          ISZ CRUN
01046  5237          JMP RUNX
01047  2151          ISZ CSAMP
01050  5234          JMP SAMPX
01051  4441          CALL MONITR      /TRANS MODE TO INIT MINI
01052  5776          JMP I (START

01053  7300  DAOVFL, CLA CLL          /DATA TABLE FULL (THRU 7777)
01054  1375          TAD (-3
01055  4441          CALL MONITR
01056  5776          JMP I (START

01175  7775
01176  0200
01177  7447
       1200          PAGE
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 10

        /+3     FREQUENTLY CALLED SUBROUTINES
        /
        /STOPPED-FLOW CLINICAL ANALYZER
        /SUBROUTINE TWO
        /
        /SFCA.S1 CONTAINS PARAMETER DEFINITIONS
        /   AND PAGE ZERO POINTERS.
        /
        /SFCA.S2 CONTAINS FREQUENTLY CALLED SUBROUTINES
        /   WHICH LOAD BEGINNING AT THE PAGE FOLLOWING THE
        /   EXECUTIVE ROUTINE, AND OCCUPY 7 CORE PAGES.
        /
        /DATA STORAGE BEGINS AT THE PAGE FOLLOWING SFCA.S2
        /   AND EXTEND TO THE END OF MEMORY.
        /
        /PGR: M.D. JOSEPH
        /DATE: 28-MAY-78
        /VER: 1B
        /FILE: SFCA.S2
        /
        /
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 11

        /GENERAL PURPOSE SUBROUTINES
        /
        /SUMMARY OF SUBROUTINE CALLS
        /==========================
        /
        /DECIN - KB INPUT A DECIMAL NUMBER IN THE RANGE 0-3999,
        /        RETURN THE OCTAL EQUIVALENT AN CALL+1
        /
        /        CALL DECIN
        /        OCTAL
        /        <RETURN>
        /
        /TXOUT - TYPE ASCII TEXT, PACKED ONE 8-BIT CHAR/WORD,
        /        TERMINATED WITH A ZERO
        /
        /        CALL TXOUT
        /        TEXT...
        /        0
        /        <RETURN>
        /
        /TYPE - TYPES CHAR IN AC, LEAVES AC UNAFFECTED
        /
        /        CALL TYPE (AC=CHARACTER)
        /        <RETURN>
        /
        /READ - KB INPUT AN 8-BIT CHARACTER, RETURNS IN AC
        /        ^C CAUSES JMS MONITR
        /        ALTMODE CAUSES JMS MANUAL
        /
        /        CALL READ
        /        <RETURN> AC=8-BIT CHARACTER
        /
        /NOUT - TYPES CHARACTER IN AC AS AN ASCII NUMERIC
        /        CHARACTER SHOULD BE IN THE RANGE 0-11(8)
        /        AC IS CLEARED BEFORE RETURN
        /
        /        CALL NOUT (AC=CHARACTER)
        /        <RETURN>
        /
        /OCTOUT - TYPES NUMERIC IN AC AS OCTAL
        /        RETURNS AC CLEARED
        /
        /        CALL OCTOUT (AC=OCTAL NUMERIC)
        /        <RETURN>
        /
        /DECOUT - TYPES CHARACTER IN AC AS DECIMAL NUMERIC
        /        RETURNS AC CLEARED
        /
        /        CALL DECOUT (AC=NUMERIC)
        /        <RETURN>
```

```
/SUMMARY OF SUBROUTINE CALLS, CONT
/================================
/
/SAMPLE - SAMPLES AND AVERAGES A/D UP TO 4096 TIMES
/        RETURNS AVERAGE IN AC
/
/        CALL SAMPLE
/        NO. POINTS TO AVERAGE
/        <RETURN> (AC=AVERAGE)
/
/CRLF - OUTPUTS CR,LF
/        RETURNS AC CLEARED
/
/        CALL CRLF
/        <RETURN>
/
/AVCLR - INITIALIZE DOUBLE PRECISION AVERAGER
/        RETURNS AC CLEARED
/
/        CALL AVCLR
/        <RETURN>
/
/AVADD - ADD AC TO ONGOING SUM
/        CLEARS AC
/
/        CALL AVADD (AC=INTEGER)
/        <RETURN>
/
/AVER - CALCULATE AVERAGE, RETURN AVG IN AC
/
/        CALL AVER
/        ERROR = AVERAGE > 7777
/        <RETURN) (AC=AVERAGE)
/
/DELAY - DELAY N SECONDS WHILE MONITORING KB
/
/        CALL DELAY
/        SECONDS TO DELAY (OCTAL)
/        <RETURN>
/
/MANUAL - MANUAL OPERATION OF SFCA
/        ^R FORCES RETURN
/
/        CALL MANUAL
/        <RETURN>
/
/MONITR - TRANSPARENT MODE AND ERROR HANDLING
/        ^A CHAINS TO *200
/        ^R FORCES RETURN
/
/        CALL MONITR
/        <RETURN>
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 13

        /SUMMARY OF SUBROUTINE CALLS, CONT
        /================================
        /
        /SCALE - OPTIONALLY SET DARK AND REFERENCE
        /        SAMPLES A/D ISAMP TIMES, AVERAGES, AND
        /        STORES AT MDARK, REF (PAGE ZERO)
        /
        /        CALL SCALE
        /        <RETURN>
        /
        /CYCLE - CYCLES SFCA THROUGH EMPTY, OPEN, FILL,
        /        DELAY(SAMP), CLOSE, DELAY(REACTION),
        /        WAIT FOR FLOW STOPPED.
        /
        /        CALL CYCLE
        /        (SAMP TIME = ISAMPT; REACT TIME = RTIME)
        /        <RETURN>
        /
        /IDATA - INPUT ONE 12-BIT WORD FROM MINI,
        /        STORE AT POINTER PASSED.
        /        AC RETURNED CLEARED
        /
        /        CALL IDATA (AC=POINTER)
        /        <RETURN>
        /
        /ODATA - OUTPUT ONE 12-BIT WORD TO MINI
        /        DATA IN AC; AC CLEARED
        /
        /        CALL ODATA (AC=DATA)
        /        <RETURN>
        /
```

```
                /DECIMAL INPUT
                /DECIMAL INTEGER (0-9999); RETURN OCTAL EQUIV
                /       (4096=0, 4097=1, ETC.)

01200  0000  XDECIN, 0                   /CALL DECIN; OCTAL; RETURN
01201  7200        CLA
01202  1377        TAD (-4
01203  3327        DCA N4
01204  1376        TAD (DCA DIG1
01205  3223        DCA STO
01206  3331        DCA DIG1;DCA DIG2;DCA DIG3;DCA DIG4
01207  3332
01210  3333
01211  3334
01212  3330        DCA OCT
01213  4421        CALL TXOUT; " ;"=;" ;0
01214  0240
01215  0275
01216  0240
01217  0000
01220  4423  GETD,   CALL READ
01221  4422        CALL TYPE
01222  4274        JMS CHK             /MAKE SURE IT'S A NUMERIC
01223  3331  STO,    DCA DIG1            /WILL CHANGE
01224  2223        ISZ STO
01225  2327        ISZ N4              /DO ONLY 4
01226  5220        JMP GETD
01227  4423        CALL READ           /NOW ALLOW A CR
01230  1375        TAD (-215
01231  7640        SZA CLA; JMP IERR           /NONE OF THE ABOVE
01232  5322
01233  4434  CVRT,   CALL CRLF
01234  1331  L1,     TAD DIG1
01235  7450        SNA; JMP L2
01236  5245
01237  1374        TAD (-1
01240  3331        DCA DIG1            /DECREMENT
01241  1373        TAD (1750
01242  1330        TAD OCT
01243  3330        DCA OCT             /ADD 1000(10)
01244  5234        JMP L1
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 15

01245  1332  L2,     TAD DIC2
01246  7450          SNA; JMP L3
01247  5256
01250  1374          TAD (-1
01251  3332          DCA DIC2
01252  1372          TAD (144
01253  1330          TAD OCT
01254  3330          DCA OCT          /ADD 100(10)
01255  5245          JMP L2
01256  1333  L3,     TAD DIC3
01257  7450          SNA; JMP L4
01260  5267
01261  1374          TAD (-1
01262  3333          DCA DIC3
01263  1371          TAD (12
01264  1330          TAD OCT
01265  3330          DCA OCT          /ADD 10(10)
01266  5236          JMP L3
01267  1334  L4,     TAD DIC4
01270  1330          TAD OCT
01271  3660          DCA I XDECIN     /PASS BACK TO CALLER
01272  2200          ISZ XDECIN       /POINT TO RETURN
01273  5660          JMP I XDECIN

01274  0000  CHK,    0
01275  1375          TAD (-215        /CR TERMINATES
01276  7450          SNA; JMP XCV
01277  5310
01300  1370          TAD (-48         /260 = LOWEST OCTAL
01301  7510          SPA; JMP IERR
01302  5322
01303  1367          TAD (-12         /271 = HIGHEST
01304  7500          SMA; JMP IERR
01305  5322
01306  1371          TAD (12          /RESTORE AND STRIP ASCII
01307  5674          JMP I CHK
```

```
/+3     FREQUENTLY CALLED SUBROUTINES      PAL8-V10A 12-JUL-78 PAGE 16

01310  1333  XCV,     TAD DIG3          /MOVE DIGITS ACCORDING TO
01311  3334           DCA DIG4          /HOW MANY WERE INPUT
01312  1332           TAD DIG2
01313  3333           DCA DIG3
01314  1331           TAD DIG1
01315  3332           DCA DIG2
01316  3331           DCA DIG1
01317  2327           ISZ N4
01320  5310           JMP XCV
01321  5233           JMP CVRT
01322  4421  IERR,    CALL TXOUT;"?;0
01323  0277
01324  0000
01325  4434           CALL CRLF
01326  5201           JMP XDECIN+1

01327  0000  N4,      0
01330  0000  OCT,     0
01331  0000  DIG1,    0
01332  0000  DIG2,    0
01333  0000  DIG3,    0
01334  0000  DIG4,    0

               /ASCII TEXT OUTPUT
               /ONE 8-BIT CHAR/WORD; 0 TERMINATES

01335  0000  XTXOUT, 0                 /CALL TXOUT; TEXT; 0; RET
01336  7200           CLA
01337  1735           TAD I XTXOUT
01340  2335           ISZ XTXOUT
01341  7450           SNA; JMP I XTXOUT  /0 TERM
01342  5735
01343  4422           CALL TYPE
01344  5336           JMP XTXOUT+1

               /LST - OUTPUTS CHARACTER IN AC TO LPT (DEV 14);
               /      LEAVES AC UNCHANGED

01345  0000  XLST,    0                 /CALL LIST (AC=CHAR); RETURN
01346  6146           6146
01347  6141           6141
01350  5347           JMP .-1
01351  5745           JMP I XLST

01367  7766
01370  7735
01371  0012
01372  0144
01373  1750
01374  7777
01375  7563
01376  3331
01377  7774
       1400           PAGE
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 17

        /TYPEOUT; CHAR IN AC; AC NOT CLEARED

01400 0000 XTYPE,  0
01401 6046         TLS
01402 6041         TSF
01403 5202         JMP .-1
01404 6031         KSF                 /CHECK KB
01405 5600         JMP I XTYPE
01406 3216         DCA XNOUT           /SAVE TEMP
01407 4423         CALL READ           /^S MEANS WAIT
01410 1377         TAD (-223
01411 7650         SNA CLA
01412 4423         CALL READ
01413 7650         CLA CLL
01414 1216         TAD XNOUT
01415 5600         JMP I XTYPE

        /NOUT OUTPUTS AC AS ASCII NUMERIC

01416 0000 XNOUT,  0                   /CALL NOUT (NUM IN AC); RETURN
01417 1376         TAD (260
01420 4422         CALL TYPE
01421 7200         CLA
01422 5616         JMP I XNOUT

        /OCTOUT OUTPUTS NUMERICS IN OCTAL

                                       /CALL OCTOUT (OCTAL IN AC); RET
01423 0000 XOCTOU, 0
01424 3247         DCA XO
01425 1247         TAD XO
01426 7006         RTL;RTL
01427 7006
01430 0375         AND (7
01431 4424         CALL NOUT
01432 1247         TAD XO
01433 7002         BSW
01434 0375         AND (7
01435 4424         CALL NOUT
01436 1247         TAD XO
01437 7012         RTR;RAR
01440 7010
01441 0375         AND (7
01442 4424         CALL NOUT
01443 1247         TAD XO
01444 0375         AND (7
01445 4424         CALL NOUT
01446 5623         JMP I XOCTOU

01447 0000 XO,     0
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 18

            /DECOUT CONVERTS OCTAL-DECIMAL AND OUTPUTS

01450  0000  XDECOU, 0                    /CALL DECOUT (OCTAL IN AC); RET
01451  3325         DCA TEMP
01452  3326         DCA DIG
01453  1325         TAD TEMP
01454  1374         TAD (-1750            /SUBTR 1000(10)
01455  7510         SPA
01456  4311         JMS XDOC
01457  2326         ISZ DIG               /INCR THOUS
01460  5254         JMP .-4
01461  1373         TAD (1750
01462  3325  XDOD,  DCA TEMP              /RESTORE
01463  4317         JMS TNUM
01464  1325         TAD TEMP
01465  1372         TAD (-144             /SUBTR 100(10)
01466  7510         SPA
01467  5272         JMP .+3
01470  2326         ISZ DIG               /INCR HUNDREDS
01471  5265         JMP .-4
01472  1371         TAD (144
01473  3325         DCA TEMP              /RESTORE
01474  4317         JMS TNUM
01475  1325         TAD TEMP
01476  1370         TAD (-12              /SUBTR 10(10)
01477  7510         SPA
01500  5303         JMP .+3
01501  2326         ISZ DIG
01502  5276         JMP .-4
01503  1367         TAD (12
01504  3325         DCA TEMP
01505  4317         JMS TNUM
01506  1325         TAD TEMP
01507  4424         CALL NOUT
01510  5650         JMP I XDECOU
01511  0000  XDOC,  0                     /DON'T SKIP IF ALREADY NEG
01512  1373         TAD (1750
01513  7500         SMA; JMP XDOD
01514  5262
01515  1374         TAD (-1750
01516  5711         JMP I XDOC
01517  0000  TNUM,  0
01520  1326         TAD DIG
01521  4424         CALL NOUT
01522  7200         CLA
01523  3326         DCA DIG
01524  5717         JMP I TNUM

01525  0000  TEMP,  0
01526  0000  DIG,   0
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 19

                /HDIV - DIVISION WHERE QUOT<1
                /RETURNS QUOT*1000

01527  0000  XHDIV,  0                /CALL HDIV;DIVIDEND;DIVISOR;OVFL;RET
01530  7200          CLA
01531  1374          TAD (-1750
01532  3357          DCA XHDC
01533  4455          CALL AVCLR
01534  1727  XHLOOP, TAD I XHDIV      /GET DIVIDEND
01535  7012          RTR;RAR
01536  7010
01537  0366          AND (777
01540  4436          CALL AVADD       /ADD TO SUMS 1000 TIMES
01541  5356          JMP XHDRET
01542  2357          ISZ XHDC
01543  5364          JMP XHLOOP
01544  2327          ISZ XHDIV
01545  1727          TAD I XHDIV      /GET DIVISOR
01546  7012          RTR;RAR
01547  7010
01550  0366          AND (777
01551  3402          DCA I XAVCT
01552  2327          ISZ XHDIV
01553  4437          CALL AVER
01554  7410          SKP              /ERROR RETURN
01555  2327          ISZ XHDIV        /NORMAL RETURN
01556  5727  XHDRET, JMP I XHDIV
01557  0000  XHDC,   0

01560  0777
01561  0012
01562  7766
01563  0144
01564  7634
01565  1750
01566  6030
01567  0007
01570  0260
01571  7555
       1600          PAGE
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 20

            /READ; 8-BIT CHAR RETURNED IN AC
            /^C CHAINS TO MONITOR (JMS)
            /ALTMODE CHAINS TO MANUAL MODE (JMS)

01600  0000  XREAD,  0
01601  6031          KSF
01602  5201          JMP .-1
01603  6036          KRB
01604  1377          TAD (-203       /^C CHAINS TO MONITOR
01605  7450          SNA; JMP XMON
01606  5214
01607  1376          TAD (-30        /ALTMODE CHAINS TO MANUAL
01610  7450          SNA; JMP XMAN
01611  5222
01612  1375          TAD (233        /RESTORE
01613  5600          JMP I XREAD
01614  1200  XMON,   TAD XREAD
01615  3230          DCA XRRET
01616  4441          CALL MONITR
01617  1230          TAD XRRET
01620  3200          DCA XREAD
01621  5201          JMP XREAD+1     /RETURN FROM MONITOR
01622  1200  XMAN,   TAD XREAD
01623  3230          DCA XRRET
01624  4442          CALL MANUAL
01625  1230          TAD XRRET
01626  3200          DCA XREAD
01627  5201          JMP XREAD+1
01630  0000  XRRET,  0
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 21

            /IDATA - INPUT 12 BITS FROM MINI

01631  0000  XIDATA, 0                  /CALL IDATA (AC=POINTER); RETURN
01632  3266         DCA XIDP            /STORE POINTER
01633  6131  XIDW1,  6131              /WAIT FOR SPECIAL CHARACTER
01634  5302         JMP XIKB            /ALSO WATCH KB
01635  6136         6136                /READ MINI
01636  1374         TAD (-100
01637  7640         SZA CLA             /SPECIAL?
01640  5233         JMP XIDW1           /NOPE
01641  1373         TAD (100
01642  6146         6146                /SEND IT BACK
01643  6141         6141
01644  5243         JMP .-1
01645  7200         CLA
01646  4270         JMS XIDIN           /GET 6 BIT BYTE
01647  7002         BSW
01650  3267         DCA XIDTEM
01651  4270         JMS XIDIN
01652  1267         TAD XIDTEM          /COMBINE BYTES
01653  3666         DCA I XIDP          /STORE
01654  4421         WRITE;" ;"=;" ;"(;0      / ( INDICATES INPUT FROM MINI
01655  0240
01656  0275
01657  0240
01660  0250
01661  0000
01662  1666         TAD I XIDP
01663  4426         CALL DECOUT         /ECHO TO TERMINAL
01664  4434         CR
01665  5631         JMP I XIDATA
01666  0000  XIDP,  0
01667  0000  XIDTEM, 0
01670  0000  XIDIN,  0
01671  6131  XIDW2,  6131
01672  5271         JMP .-1
01673  6136         6136                /READ MINI
01674  1374         TAD (-100
01675  7450         SNA                 /ANOTHER SPECIAL
01676  5271         JMP XIDW2           /YEP; LOOK AGAIN
01677  1373         TAD (100            /MUST BE DATA
01700  0372         AND (77
01701  5670         JMP I XIDIN
01702  6031  XIKB,  KSF
01703  5233         JMP XIDW1
01704  4423         CALL READ           /HE TYPED SOMETHING
01705  7200         CLA
01706  5233         JMP XIDW1
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 22

            /ODATA - SEND 12 BITS TO MINI

01707  0000  XODATA, 0                  /CALL ODATA (AC=DATA); RETURN
01710  3352         DCA XODTEM
01711  6131  XODW1,  6131               /WAIT FOR SPECIAL
01712  5345         JMP XOKB            /ALSO WATCH KB
01713  6136         6136                /READ IT
01714  1371         TAD (-101
01715  7640         SZA CLA             /SPECIAL?
01716  5311         JMP XODW1           /NOPE
01717  1370  XOPR,  TAD (101           /SEND IT BACK
01720  4337         JMS XOOUT
01721  6131         6131                /ANY RESPONSE YET?
01722  5317         JMP XOPR            /NOT YET
01723  6136         6136                /HERE IT IS
01724  1371         TAD (-101
01725  7640         SZA CLA             /SPECIAL AGAIN?
01726  5317         JMP XOPR            /NOT YET BUT HE WILL
01727  1352         TAD XODTEM          /OK - GET THE GOOD STUFF
01730  7002         BSW
01731  0372         AND (77
01732  4337         JMS XOOUT           /SEND FIRST BYTE
01733  1352         TAD XODTEM
01734  0372         AND (77
01735  4337         JMS XOOUT           /SECOND BYTE
01736  5707         JMP I XODATA        /RETURN
01737  0000  XOOUT,  0
01740  6146         6146
01741  6141         6141
01742  5341         JMP .-1
01743  7200         CLA
01744  5737         JMP I XOOUT
01745  6031  XOKB,   KSF
01746  5311         JMP XODW1
01747  4423         CALL READ           /HE TYPED SOMETHING
01750  7200         CLA
01751  5311         JMP XODW1

01752  0000  XODTEM, 0

01770  0101
01771  7677
01772  0077
01773  0100
01774  7700
01775  0233
01776  7750
01777  7575
       2000         PAGE
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 23

            /SCALE - OPTIONALLY SAMPLE 0, 100%

02000  0000  XSCALE, 0                    /CALL SCALE; RETURN
02001  4421  XDARK,  WRITE; "R;"E;"S;"E;"T;" ;"D;"A;"R;"K;"?;0
02002  0322
02003  0305
02004  0323
02005  0305
02006  0324
02007  0240
02010  0304
02011  0301
02012  0322
02013  0313
02014  0277
02015  0000
02016  4423          CALL READ
02017  1377          TAD (-"N
02020  7650          SNA CLA
02021  5245          JMP XREF
02022  4434          CR
02023  4421          WRITE; "C;"L;"O;"S;"E;0
02024  0303
02025  0314
02026  0317
02027  0323
02030  0305
02031  0000          CALL READ; CR
02032  4423
02033  4434          CALL SAMPLE; ISAMP      /AVERAGE ISAMP READINGS
02034  4433
02035  1750          CIA; DCA MDARK          /STORE -(DARK)
02036  7041
02037  3157          TAD MDARK; CIA
02040  1157
02041  7041          CALL DECOUT
02042  4426          CR
02043  4434          JMP XDARK
02044  5201

02045  4434  XREF,   CR
02046  4421          WRITE; "R;"E;"S;"E;"T;" ;"R;"E;"F;" ;"?;0
02047  0322
02050  0305
02051  0323
02052  0305
02053  0324
02054  0240
02055  0322
02056  0305
02057  0306
02060  0240
02061  0277
02062  0000
02063  4423          CALL READ
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 23-1

02064  1377            TAD (-"N
02065  7650            SNA CLA
02066  5316            JMP XSCRET
02067  4434            CR
02070  4421            WRITE;"O;"P;"E;"N;0
02071  0317
02072  0320
02073  0305
02074  0316
02075  0000
02076  4423            CALL READ
02077  4444            CALL CYCLE
02100  1155            TAD EXTIME
02101  3303            DCA .+2
02102  4440            CALL DELAY;0
02103  0000
02104  4423            CALL SAMPLE; ISAMP
02105  1750
02106  3160            DCA REF
02107  1160            TAD REF
02110  4426            CALL DECOUT
02111  4434            CR
02112  1160            TAD REF; TAD MDARK      /STORE REF-DARK
02113  1157
02114  3160            DCA REF
02115  5245            JMP XREF
02116  4434  XSCRET,  CR
02117  5600            JMP I XSCALE

               /CYCLE - ONE CYCLE OF SFCA

02120  0000  XCYCLE,  0                /CALL CYCLE; RETURN
02121  6502            EMPTY
02122  6540            OPEN
02123  6503            FILL
02124  4440            CALL DELAY; ISAMPT
02125  0005
02126  6501            CLOSE
02127  1554            TAD I RTIME     /GET REACTION TIME
02130  3332            DCA .+2
02131  4440            CALL DELAY; 0
02132  0000
02133  6504            PUSH
02134  6505            SFS
02135  5334            JMP .-1
02136  5720            JMP I XCYCLE

02177  7462
       2200            PAGE
```

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/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 24

                /SAMPLE A/D UP TO 4096 TIMES AND
                /RETURN THE AVERAGE IN AC

02200  0000  XSAMPL, 0                  /CALL SAMPLE; NPTS AVG; RET (AVG IN AC)
02201  7200         CLA
02202  1600         TAD I XSAMPL       /GET NPTS TO AVERAGE
02203  7041         CIA
02204  3231         DCA XCRLF
02205  2200         ISZ XSAMPL
02206  4435         CALL AVCLR         /CLEAR THE AVERAGER
02207  6510  SLOOP, CONVRT             /START A/D
02210  6511         SCD
02211  5210         JMP .-1            /WAIT FOR DONE FLAG
02212  6512         GETAD              /READ A/D
02213  7040         CMA
02214  4436         CALL AVADD         /ADD TO AVERAGE
02215  5223         JMP SERR1          /AVADD OVFL
02216  2231         ISZ XCRLF
02217  5207         JMP SLOOP
02220  4437         CALL AVER          /CALC THE AVERAGE
02221  5226         JMP SERR2          /AVER OVERFLOW
02222  5600         JMP I XSAMPL
02223  7240  SERR1, CLA CMA
02224  4441         CALL MONITR
02225  5600         JMP I XSAMPL
02226  7344  SERR2, CLA CLL CMA RAL
02227  4441         CALL MONITR
02230  5600         JMP I XSAMPL

                /CRLF OUTPUTS CR,LF

02231  0000  XCRLF, 0                  /CALL CRLF; RET (AC CLEAR)
02232  7200         CLA
02233  1377         TAD (215
02234  4422         CALL TYPE
02235  7200         CLA
02236  1376         TAD (212
02237  4422         CALL TYPE
02240  7200         CLA
02241  5631         JMP I XCRLF
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 25

            /DOUBLE PRECISION AVERAGER
            /MAX 4095 INTEGERS (0-4095 EACH)

02242  0000  XAVCLR,  0                  /CALL AVCLR; RET
02243  7200          CLA
02244  3322          DCA HSUM
02245  3323          DCA LSUM
02246  3324          DCA AVCNT
02247  5642          JMP I XAVCLR


02250  0000  XAVADD,  0                  /CALL AVADD (INTEGER IN AC); OVFL ERR; RET
02251  7100          CLL
02252  1323          TAD LSUM
02253  3323          DCA LSUM            /UPDATE LOW SUM
02254  7004          RAL                 /CARRY TO LINK
02255  1322          TAD HSUM
02256  3322          DCA HSUM            /UPDATE HIGH SUM
02257  2324          ISZ AVCNT
02260  2250          ISZ XAVADD          /NORMAL RETURN
02261  5650          JMP I XAVADD

02262  0000  XAVER,   0                  /CALL AVER; ERR (OVFL); RET (QUOT IN AC)
02263  7200          CLA
02264  1322          TAD HSUM
02265  3325          DCA HSUM2
02266  3326          DCA QUOT
02267  1324          TAD AVCNT
02270  7041          CIA; DCA MDIVS
02271  3320
02272  1323          TAD LSUM
02273  3321          DCA DIVD
02274  1321  AVLOOP, TAD DIVD
02275  1320          TAD MDIVS           /SUBTR DIVISOR
02276  7510          SPA
02277  5304          JMP BORROW          /MAY NEED TO BORROW
02300  3321          DCA DIVD
02301  2326  XAVRET, ISZ QUOT
02302  5274          JMP AVLOOP
02303  5662          JMP I XAVER         /OVERFLOW (QUOT>4095)
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V19A 12-JUL-78 PAGE 26

02304  3321  BORROW, DCA DIVD
02305  1320          TAD MDIVS
02306  7041          CIA
02307  1321          TAD DIVD
02310  7710          SPA CLA          /DON'T BORROW IF DIVD WAS ALREADY NEG
02311  5301          JMP XAVRET
02312  1325          TAD HSUM2
02313  7450          SNA              /ANYTHING LEFT TO BORROW?
02314  5327          JMP AVDONE       /NOPE
02315  1375          TAD (-1
02316  3325          DCA HSUM2        /DECREMENT HIGH SUM
02317  5301          JMP XAVRET       /CONTINUE

02320  0000  MDIVS,  0
02321  0000  DIVD,   0
02322  0000  HSUM,   0
02323  0000  LSUM,   0
02324  0000  AVCNT,  0
02325  0000  HSUM2,  0
02326  0000  QUOT,   0

02327  7300  AVDONE, CLA CLL
02330  1326          TAD QUOT
02331  2262          ISZ XAVER
02332  5662          JMP I XAVER      /RETURN
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 27

        /DELAY DELAYS N SECONDS WHILE MONITORING KB

02333 0000 XDELAY, 0                    /CALL DELAY; NSEC; RETURN
02334 7200         CLA
02335 1733         TAD I XDELAY         /GET N SECONDS
02336 2333         ISZ XDELAY
02337 7450         SNA; JMP I XDELAY  /0=NO DELAY
02340 5733
02341 7041         CIA; DCA XNSEC
02342 3364
02343 6031 XSEC,   KSF                  /CHECK KB
02344 5351         JMP XCOUNT           /NO
02345 4423         CALL READ
02346 1374         TAD (-222            /^R FORCES RETURN
02347 7650         SNA CLA
02350 5733         JMP I XDELAY
02351 1367 XCOUNT, TAD XMC2             /WAIT A SEC
02352 3370         DCA XDC2
02353 1365         TAD XMC1
02354 3366         DCA XDC1
02355 2366         ISZ XDC1
02356 5355         JMP .-1
02357 2370         ISZ XDC2
02360 5353         JMP .-5
02361 2364         ISZ XNSEC            /ENOUGH?
02362 5343         JMP XSEC
02363 5733         JMP I XDELAY

02364 0000 XNSEC,  0
02365 0240 XMC1,   240
02366 0000 XDC1,   0
02367 7775 XMC2,   -3
02370 0000 XDC2,   0

02374 7556
02375 7777
02376 0212
02377 0215
      2400         PAGE
```

```
                /MANUAL CONTROL
                /
                /COMMAND        FUNCTION
                /-------        --------
                /
                /  O            OPEN SAMPLE VALVES
                /  C            CLOSE
                /  E            EMPTY STOPPING SYR
                /  F            FILL DRIVE SYRINGES
                /  P            PUSH
                /  R            RESET
                /  D            SET D/A
                /  A            SAMPLE A/D
                / ^R            RETURN

02400  0000  XMANUA, 0                /CALL MANUAL; RETURN
02401  4434         CALL CRLF
02402  4421         CALL TXOUT;  "M;":;0
02403  0315
02404  0272
02405  0000
02406  4423         CALL READ
02407  1277         TAD (-215       /CR = CRLF
02410  7450         SNA; JMP XMANUA+1
02411  5201
02412  1376         TAD (-5         /^R FORCES RETURN
02413  7440         SZA; JMP XMA2
02414  5224
02415  4421         CALL TXOUT;"R;"E;"T;0
02416  0322
02417  0305
02420  0324
02421  0000
02422  4434         CALL CRLF
02423  5600         JMP I XMANUA
02424  1375  XMA2,   TAD (-61                  /CLOSE
02425  7440         SZA; JMP XMA3
02426  5240
02427  4421         CALL TXOUT;"C;"L;"O;"S;"E;0
02430  0303
02431  0314
02432  0317
02433  0323
02434  0305
02435  0000
02436  6501         CLOSE
02437  5201         JMP XMANUA+1
```

```
02440  1374  XMA3,   TAD (-1           /D
02441  7440           SZA; JMP XMA4
02442  5255
02443  4421           CALL TXOUT;"D:"/;"A;0
02444  0304
02445  0257
02446  0361
02447  0300
02450  4420           CALL DECIN;0
02451  0000
02452  1231           TAD .-1
02453  6513           SETDA
02454  5201           JMP XMANUA+1
02455  1374  XMA4,   TAD (-1           /E
02456  7440           SZA; JMP XMA5
02457  5271
02460  4421           CALL TXOUT;"E;"M;"P;"T;"Y;0
02461  0305
02462  0315
02463  0320
02464  0324
02465  0331
02466  0000
02467  6502           EMPTY
02470  5201           JMP XMANUA+1
02471  1374  XMA5,   TAD (-1           /F
02472  7440           SZA; JMP XMA6
02473  5304
02474  4421           CALL TXOUT;"F;"I;"L;"L;0
02475  0306
02476  0311
02477  0314
02500  0314
02501  0000
02502  6503           FILL
02503  5201           JMP XMANUA+1
02504  1373  XMA6,   TAD (-11          /O
02505  7440           SZA; JMP XMA7
02506  5317
02507  4421           CALL TXOUT;"O;"P;"E;"N;0
02510  0317
02511  0320
02512  0305
02513  0316
02514  0300
02515  6500           OPEN
02516  5201           JMP XMANUA+1
02517  1374  XMA7,   TAD (-1           /P
02520  7440           SZA;JMP XMA8
02521  5322
02522  4421           CALL TXOUT;"P;"U;"S;"H;0
02523  0320
02524  0325
02525  0323
02526  0310
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 29-1

02527  0000
02530  6504           PUSH
02531  5201           JMP XMANUA+1
02532  1372  XMA8,    TAD (-2          /R
02533  7440           SZA; JMP XMA9
02534  5346
02535  4421           CALL TXOUT;"R;"E;"S;"E;"T;0
02536  0322
02537  0305
02540  0323
02541  0305
02542  0324
02543  0000
02544  6506           RESET
02545  5201           JMP XMANUA+1
02546  1371  XMA9,    TAD (21          /A
02547  7440           SZA; JMP XMANUA+1
02550  5201
02551  4421           WRITE;  "A;"/;"D;"  ;"=;"  ;0
02552  0301
02553  0257
02554  0304
02555  0240
02556  0275
02557  0240
02560  0000
02561  6510           CONVRT
02562  6511           SCD
02563  5362           JMP .-1
02564  6512           GETAD
02565  7040           CMA
02566  4426           CALL DECOUT
02567  5201           JMP XMANUA+1

02571  0021
02572  7776
02573  7767
02574  7777
02575  7717
02576  7773
02577  7563
       2600           PAGE
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PALG-V10A 12-JUL-78 PAGE 30

            /MONITOR
            /TRANSPARENT MODE AND ERROR HANDLING

02600  0000  XMONIT, 0                    /CALL MONITR (AC<>0 MEANS ERROR); RET
02601  7440         SZA; JMP ERROR
02602  5245
02603  4434         CR
02604  4421         CALL TXOUT:207;"O;"N;" ;"L;"I;"N;"E;0
02605  0207
02606  0317
02607  0316
02610  0240
02611  0314
02612  0311
02613  0316
02614  0305
02615  0000
02616  4434         CALL CRLF
02617  7200  XMKB,   CLA
02620  6031         KSF
02621  5236         JMP XMINI
02622  6036         KRB
02623  1377         TAD (-201          /^A FROM KB CHAINS TO *200
02624  7450         SNA
02625  5776         JMP I (200
02626  1375         TAD (-21           /^R FROM KB FORCES RETURN
02627  7450         SNA
02630  5600         JMP I XMONIT
02631  1374         TAD (222           /RESTORE CHAR
02632  6146         6146
02633  6141         6141
02634  5233         JMP .-1
02635  5217         JMP XMKB
02636  6131  XMINI, 6131
02637  5217         JMP XMKB
02640  6136         6136
02641  6046         TLS
02642  6041         TSF
02643  5242         JMP .-1
02644  5217         JMP XMKB
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 31

                /ERROR CODES:
                /
                / -1            TOO MANY ARGS TO AVERAGER
                / -2            AVERAGE OVERFLOW
                / -3            DATA TABLE OVERFLOW
                / -4            SYNC ERROR ON TRANSMISSION
                /

02645  7001  ERROR,   IAC
02646  7450           SNA; JMP XADOVF
02647  5270
02650  7001           IAC
02651  7450           SNA; JMP XAVOVF
02652  5307
02653  7001           IAC
02654  7450           SNA; JMP XDAOVF
02655  5326
02656  7001           IAC
02657  7450           SNA; JMP XSYNC
02660  5346
                /
                /CHECK FOR OTHER ERROR ENTRIES HERE
                /
02661  4421           CALL TXOUT; "U; "E; " ; "@;0
02662  0325
02663  0305
02664  0249
02665  0000
02666  0000
02667  5203           JMP XMONIT+3

02670  4421  XADOVF, CALL TXOUT; "S; "U; "M; " ; "O; "V; "F; "L; " ; "@;0
02671  0323
02672  0325
02673  0315
02674  0240
02675  0317
02676  0326
02677  0306
02700  0314
02701  0240
02702  0300
02703  0000
02704  1200           TAD XMONIT
02705  4425           CALL OCTOUT
02706  5203           JMP XMONIT+3
02707  4421  XAVOVF, CALL TXOUT; "A; "V; "C; " ; "O; "V; "F; "L; " ; "@;0
02710  0301
02711  0326
02712  0307
02713  0240
02714  0317
02715  0326
02716  0306
02717  0314
```

```
/+3     FREQUENTLY CALLED SUBROUTINES     PAL8-V10A 12-JUL-78 PAGE 31-1

02720  0240
02721  0360
02722  0000
02723  1200           TAD XMONIT
02724  4425           CALL OCTOUT
02725  5203           JMP XMONIT+3
02726  4421  XDAOVF, WRITE; "D;"A;"T;"A;" ;"O;"V;"F;"L;" ;"@;0
02727  0304
02730  0301
02731  0324
02732  0301
02733  0240
02734  0317
02735  0326
02736  0306
02737  0314
02740  0240
02741  0300
02742  0000
02743  1200           TAD XMONIT
02744  4425           CALL OCTOUT
02745  5203           JMP XMONIT+3
02746  4421  XSYNC,  WRITE;"S;"Y;"N;"C;" ;"E;"R;"R;" ;"@;0
02747  0323
02750  0331
02751  0316
02752  0303
02753  0240
02754  0305
02755  0322
02756  0322
02757  0240
02760  0300
02761  0000

02774  0222
02775  7757
02776  0200
02777  7577
       3900           PAGE

       3000           DATAT=.
                      $      $      $      $      $      $      $
```

/+3 FREQUENTLY CALLED SUBROUTINES PAL8-V10A 12-JUL-78 PAGE 32

ALIST	0545	IFLAG	0161	XAVCLR	2242	XOKB	1745
AVADD	0026	ISAMP	1750	XAVCT	0032	XOOUT	1737
AVCLR	0035	ISAMPT	0025	XAVER	2262	XOPR	1717
AVCNT	2324	LST	0047	XAVOVF	2707	XREAD	1600
AVDONE	2327	LSUM	2323	XAVRET	2301	XREF	2045
AVER	0037	L1	1234	XCOUNT	2351	XRRET	1630
AVLOOP	2274	L2	1245	XCRLF	2231	XSAMPL	2200
A1	0327	L3	1256	XCV	1310	XSCALE	2000
A11	0765	L4	1267	XCYCLE	2120	XSCRET	2116
A2	0331	MANUAL	0042	XDAOVF	2726	XSEC	2343
A3	0356	MDARK	0157	XDARK	2001	XSYNC	2746
A4	0403	MDIVS	2320	XDC1	2366	XTXOUT	1325
A5	0427	MONITR	0041	XDC2	2370	XTYPE	1400
A6	0441	M6	0770	XDECIN	1200		
A7	0465	NOLIST	1000	XDECOU	1430		
A8	0627	NOUT	0024	XDELAY	2333		
BORROW	2304	N4	1327	XDOG	1511		
CALL	4430	OCT	1330	XDOD	1462		
CHK	1274	OCTOUT	0025	XEDC	1557		
CLOSE	6301	ODATA	0046	XHDIV	1527		
CONVRT	6510	OPEN	6500	XHDRET	1556		
CR	4434	OPER	0467	XHLOOP	1534		
CRLF	0334	PUSH	6504	XHSUM	0030		
CRUN	0153	P400	0400	XIDATA	1631		
CSAMP	0151	QUOT	2326	XIDIN	1670		
CVRT	1233	READ	0023	XIDP	1666		
CYC	0300	REF	0160	XIDTEM	1667		
CYCLE	0044	RESET	6506	XIDW1	1633		
DAOVFL	1053	RTIME	0154	XIDW2	1671		
DATA	0156	RUN	0527	XIKB	1702		
DATAB	0162	RUNL	0731	XLST	1345		
DATAT	3000	RUNX	1037	XLSUM	0031		
DECIN	0020	SAMP	0523	XMAN	1622		
DECOUT	0026	SAMPL	0717	XMANUA	2400		
DELAY	0040	SAMPLE	0033	XMA2	2424		
DIG	1526	SAMPX	1034	XMA3	2440		
DIG1	1331	SCALE	0043	XMA4	2455		
DIG2	1332	SCD	6511	XMA5	2471		
DIG3	1333	SERR1	2223	XMA6	2504		
DIG4	1334	SERR2	2226	XMA7	2517		
DIVD	2321	SETDA	6513	XMA8	2532		
EMPTY	6502	SFS	6505	XMA9	2546		
EQTIME	0155	SLOOP	2297	XMC1	2365		
ERROR	2645	SSCALE	0522	XMC2	2367		
FILL	6503	START	0200	XMINI	2636		
GETAD	6512	STO	1223	XMKB	2617		
GETD	1220	TEMP	1525	XMON	1614		
GETSEC	0406	TNUM	1517	XMONIT	2600		
GETSR	6507	TRUNS	0152	XNOUT	1416		
HDIV	0027	TSAMP	0150	XNSEC	2364		
HSUM	2322	TXOUT	0021	XO	1447		
HSUM2	2325	TYPE	0022	XOCTOU	1423		
IDATA	0045	WRITE	4421	XODATA	1707		
IERR	1322	XADOVF	2670	XODTEM	1752		
IFILLT	0002	XAVADD	2250	XODW1	1711		

APPENDIX B

A Stopped-Flow Clinical Analyzer in Which Immobilized Enzyme Reaction Loops are Used

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A Stopped-Flow Clinical Analyzer in Which Immobilized-Enzyme Reaction Loops Are Used

Martin D. Joseph, Daniel J. Kasprzak, and S. R. Crouch[1]

A stopped-flow clinical analyzer is described that makes use of a reaction loop containing immobilized enzyme(s) for the determination of the analyte/substrate. The analyzer has been evaluated by determining glucose with immobilized glucose oxidase. The stopped-flow mixing system was constructed at a current cost of less than $500. The analyzer separates the enzymatic reaction from a followup, spectrophotometric indicator reaction. This separation allows the enzymatic reaction to be used in either a fixed-time, kinetic mode or in an equilibrium mode. Likewise, the indicator reaction can be used in either mode. Results for glucose in blood serum indicate that good precision and accuracy can be obtained.

In recent years immobilized enzymes have become increasingly useful as specific catalysts for determining clinically important substrates (*1–4*). Immobilized enzymes are attractive as reagents in the clinical laboratory because they possess the usual specificity and sensitivity of soluble enzymes and can be re-used, often in hundreds or thousands of determinations, thus greatly reducing the cost of routine use of an enzymatic kinetic or equilibrium method. In addition, immobilized enzymes are often more stable than they are in solution; calibration is thus simpler and less frequently required.

In this report, a stopped-flow clinical analyzer is described that combines the advantages of immobilized enzymes with the speed, mixing efficiency, and ease of automation of the stopped-flow mixing technique. The system utilizes a simple, low-cost, stopped-flow sampling and mixing unit together with a reaction loop that contains the immobilized enzyme. The reaction loop is similar in principle to the sample loop described by Pardue et al. (*5, 6*) except that the enzyme-catalyzed reaction occurs in the loop. After a suitable incubation period in the loop, the sample solution is rapidly mixed by the stopped-flow unit with any desired reagent(s) and sent to an observation cell for spectrophotometric monitoring of the appropriate indicator reaction. The separation of the enzyme-catalyzed reaction from the indicator reaction provides extreme versatility. The enzyme-catalyzed reaction can be allowed to go to completion (equilibrium method) or it can be incubated for only a short, fixed time interval (kinetic method). Likewise, either the reaction rate or the equilibrium absorbance of the spectrophotometric indicator reaction can be used in the final measurement. Because of stopped-flow mixing, measurements can be made on time scales ranging from milliseconds to hours.

The semi-automated stopped-flow clinical analyzer described below illustrates the measurement principle and chemical flexibility of the system. We evaluated the analyzer by determining glucose in blood serum with a reaction loop containing immobilized glucose oxidase (EC 1.1.3.4). Possible extensions of the system to multiple determinations of the same substrate or to near-simultaneous determinations of multiple substrates are discussed.

Materials and Methods

Instrumentation

The instrumentation we used to determine glucose is schematically represented in Figure 1. This system, except for the enzyme reaction loop, was constructed completely from commercially available items. Two 1-ml gas syringes with threaded ends, *A* and *B* in Figure 1 (from Glenco Scientific, Houston, Tex. 77007), are used to deliver indicator reagent and push liquid, respectively. Valves *1* through *4* are 0.060-inch bore, three-way slider valves complete with accuator-return mechanisms *C* and *D* and solenoid controllers S_2 and S_3 (from Altex, Berkeley, Calif. 94710). The syringes are driven by a 2-inch stroke pneumatic cylinder, *PC*, with solenoid controller S_1 (Scovill, Wake Forest, N. C. 27587). Solutions are mixed by forcing them through *E*, a KEL-F tee (Altex). The indicator reaction is monitored in a 250-μl micro flow cell (*F*) with 10-mm light path (Thomas, Philadelphia, Pa. 19105). The flow cell is fitted into a single-beam uv–visible spectrophotometer (GCA McPherson EU-700, Acton, Mass. 01720). All tubing except for the enzyme reaction loop is 1.5 mm i.d., 3 mm o.d. Teflon with appropriate connector fittings (Altex).

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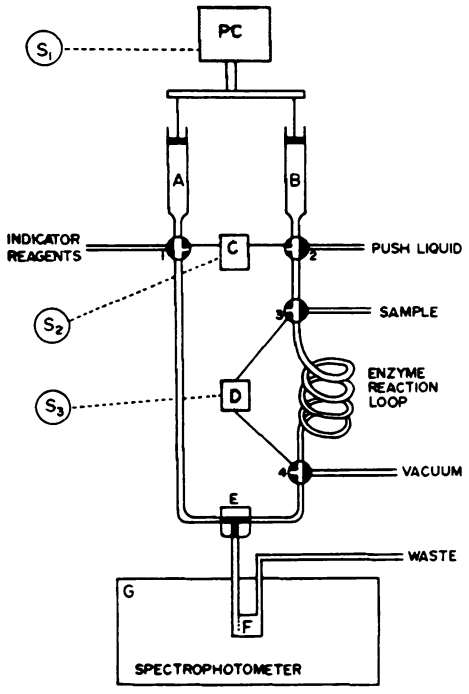

Fig. 1. Diagram of stopped-flow clinical analyzer

The heart of the system is the enzyme reaction loop, prepared as described below and fitted with plastic high-pressure liquid chromatography connectors. The enzymatic reaction that occurs in the loop for the determination of glucose is:

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

The extent of this reaction is determined outside the loop by allowing the H_2O_2 produced to mix with and rapidly oxidize iodide to triiodide in the presence of a molybdate catalyst (*7*). The indicator reaction is:

$$H_2O_2 + 3I^- + 2H^+ \xrightarrow{Mo(VI)} 2H_2O + I_3^-$$

Triiodide, measured spectrophotometrically at 365 nm, indicates the concentration of glucose in the sample. The indicator reaction is followed by an equilibrium method in this case, because the absorbance of triiodide is measured at equilibrium. The enzymatic reaction, however, is of the fixed-time reaction-rate type. The enzymatic reaction time is fixed by the incubation time of the sample in the loop. This time is kept short (2 min) to ensure that the enzymatic reaction is pseudo-zero order. This combination of reaction-monitoring methods exemplifies the flexibility of the system.

The actual function of the enzyme reaction loop in the system is best described in terms of a typical sequence of events for the determination of glucose in a sample.

First, syringes *A* and *B* are filled with KI/molybdate reagent and a push liquid (phosphate buffer), respectively, by simultaneous switching of valves *1* and *2* to the fill position and retraction of *PC*. Valves *1* and *2* are then switched to the "push-ready" position, as are valves *3* and *4* (positions shown in Figure 1). Next, *PC* is caused to push the contents of *A* and *B* through the system lines and out to the waste. This is repeated several times to remove air bubbles and fill all lines with solution. With *A* and *B* filled and valves *1* and *2* ready, valves *3* and *4* are simultaneously switched to the fill position, and a buffered glucose sample is aspirated into the enzyme reaction loop by a gentle negative pressure. Then valves *3* and *4* are switched to the "push-ready" position, which terminates sample introduction. The sample is incubated for a controlled time until *PC* is caused to push the contents of the enzyme loop through the mixer. Here the H_2O_2 produced by the enzymatic reaction is rapidly mixed with the KI/molybdate reagent, and the mixture is transported to the flow cell for observation and detection of I_3^-. The flow is stopped upstream when the drive syringes reach the ends of their travel.

The system described above was constructed at a cost of less than $500, not including the specific flow cell and spectrophotometer mentioned here, for which there are suitable substitutes. The instrument can be assembled within a day or two due to the ease with which the valves and tubing can be connected via plastic fittings rated at 3450 kPa (500 psi). The simplicity, low cost, and versatility of this system make it an attractive analyzer for clinical determinations.

Reagents

Buffer. A 0.2 mol/liter solution of dibasic potassium phosphate in de-ionized water was adjusted to pH 6.30 ± 0.02 by use of a Heath pH meter (Model EU-302A) by adding concentrated hydrochloric acid. This buffer was then used to prepare all other solutions except where noted.

Indicator reagents. A sodium molybdate stock solution, 90 g/liter, was prepared and allowed to equilibrate for 48 h. The iodide/molybdate indicator reagent was then prepared daily by adding a fresh solution of potassium iodide to molybdate stock, resulting in a solution containing, per liter, 18 g of sodium molybdate and 0.5 mol of iodide.

Glucose standards. A glucose stock solution was prepared by dissolving 1.00 g of anhydrous granular D-glucose and diluting to 1.0 liter with de-ionized water. This solution was allowed to stand at room temperature for 24 h to ensure complete mutarotation, and was then stored at 5 °C. Aqueous glucose standards were then prepared by appropriately diluting this stock with the buffer.

Deproteinization reagents. We deproteinized the serum samples by adding chemically equivalent amounts of barium hydroxide and zinc sulfate. A barium hydroxide solution (20 g/liter) was titrated with zinc sulfate (20 g/liter) to a pH of 7 to determine the required volume ratio of the two reagents.

Preparation of serum samples. A control serum ("Montrol"; Dade Division, American Hospital Supply Corp., Miami, Fla. 33152) was reconstituted according to the manufacturer's instructions, and 0.5 ml of the solution was pipetted into a centrifuge tube. The chemically equivalent amounts of barium hydroxide and zinc sulfate were added, and the contents of the tube were thoroughly mixed, centrifuged for 3 min, and 1.0 ml of the supernatant fluid was pipetted into 3 ml of the phosphate buffer. This solution was then drawn into the enzyme reaction loop for analysis.

Immobilization of glucose oxidase. A procedure reported by Inman and Hornby (*8*) was modified for immobilizing glucose oxidase on the inner surface of nylon tubing, 100 cm long and 0.86 mm i.d. Most of the immobilization steps were accomplished by slowly pumping the required reagent through the tube with the use of a Technicon AutoAnalyzer pump to provide a constant stream of fresh reagent. Amorphous nylon was first removed by filling the tube with a 200 g/kg solution of $CaCl_2$ in methanol and incubating at 50 °C. The nylon was then mildly hydrolyzed by pumping 1.0 mol/liter HCl through the tube at room temperature, followed by rinsing with water. Glutaraldehyde, 125 ml/liter in 0.1 mol/liter tris(hydroxymethyl)methylamine buffer, pH 9.2, was then attached to the hydrolyzed nylon at 0 °C, followed by rinsing with the buffer. An 8 g/liter solution of glucose oxidase (Sigma, Type II) in 0.1 mol/liter phosphate buffer, pH 6.3, was then pumped through the tube at 0 °C for 4 h in a closed loop, during which time covalent linkages were formed between the glutaraldehyde and free amino groups on the enzyme (*8*). Finally, the tube was rinsed with 0.1 mol/liter sodium chloride to remove any physically adsorbed enzyme. The enzyme reaction loop was filled with buffer and stored at 5 °C when not in use.

Results and Discussion

The analytical capabilities of the analyzer system were evaluated by determining glucose in aqueous samples and in blood serum, with use of a reaction loop containing immobilized glucose oxidase. The first generation analyzer we used in these studies was manually sequenced. That is, the solenoid controllers for each of the valves in Figure 1 were actuated in the appropriate sequence by a series of manual toggle switches, which applied or disconnected the ac line voltage from the controllers. The spectrophotometric readout was obtained from a strip-chart recorder, linear in transmittance. Thus, the final absorbance values recorded for I_3^- were obtained by converting chart-recorder readings from transmittance to absorbance. The temperature of the enzyme reaction loop was not controlled.

The optimum push volume of the stopped-flow system was determined to be 0.78 ml per syringe by filling a dummy reaction loop with dye (*p*-nitrophenol). This volume ensured thorough purging and refilling of the observation cell, but prevented significant dilution of the observed solution by the push liquid. The incubation time for the enzymatic reaction was fixed at 2 min. Shorter incubation times did not provide adequate precision because of decreased response and also because of imprecision in manual sequencing; longer incubation times only added to analysis time without greatly enhancing the signal-to-noise ratio. Hence, transmittance was directly read out slightly over 2 min after the sample was introduced into the reaction loop.

Table 1. Results for Aqueous Glucose Standards

Glucose concn, mg/liter	Absorbance, av	CV, %
20	0.0249	2.0
40	0.0610	3.0
60	0.0960	2.1
80	0.127	4.7
100	0.162	1.8
150	0.240	0.8
200	0.314	0.1
250	0.388	1.3

Slope = 1.60×10^{-3} *A* per mg/liter (CV, 0.24%)
Intercept = -6.39×10^{-3} *A*
Correl. coeff. (*r*) = 0.99990

A calibration curve for glucose was prepared in the range 20–250 mg/liter by running aqueous standards in triplicate. Table 1 shows the results. The observed negative intercept is expected in such a fixed-time method, because the enzymatic reaction is known to exhibit an induction period that is inversely proportional to glucose concentration (*9*), and hence a two-point calibration should be done periodically. The results in Table 1 indicate the excellent linearity and precision which may be obtained with this simple analyzer. Each determination requires only 1 ml of sample (about 100 µl of blood serum). The major sources of imprecision in this simple system appear to be variations in incubation time due to manual control, fluctuations in reaction rate due to variations in room temperature, and imprecision in reading the transmittance from the chart recorder.

For determining glucose in blood-serum samples, we prepared a two-point calibration curve, using the 20 and 200 mg/liter aqueous glucose standards. Two reconstituted Monitrol samples were analyzed, and the results (Table 2) agree well with the reported values. Note that the first two values reported for sample 1 were obtained on a different day from the second two values, although the same two-point calibration was used. Clearly, because of the high stability of the immobilized enzyme loop, the same calibration curve may be useful for several days. A reaction loop prepared earlier than the one used in these determinations showed excellent activity for six months before the enzyme was de-activated inadvertently by incubation with a highly acidic solution. Research is underway to determine more precisely the activity loss of immobilized enzyme loops with time under various conditions of storage and use.

Table 2. Results of Determination of Glucose in Serum

Glucose concn, mg/liter		
Determined	Reported	Difference[a]
Sample 1		
690		2.8
725		2.1
703	690,[b] 730[c]	0.9
673		5.2
Sample 2		
2006	2090[b]	4.0
2078		0.6

[a] % difference from the av reported value
[b] Beckman "Glucose Analyzer"
[c] American Monitor "Programachem"

In this study we have demonstrated the potential of the analyzer for the determination of glucose in serum samples. Although the system was evaluated by using one particular enzyme/substrate couple, many other clinically useful enzyme/substrate couples are obviously applicable. Versatility is inherent, not only in the enzyme chosen for the reaction loop, but also in the reagent chosen for the indicator reaction. Furthermore, any of several combinations of methods (kinetic or equilibrium) may be used to follow the separate enzymatic and indicator reactions. We are now undertaking investigations on the capabilities of this new system in the clinical laboratory. New immobilization techniques are being studied for bonding other, more expensive enzymes to various inert supports. The multiplexing potential inherent in the system will be used, to allow several reaction loops containing the same or different enzymes to be used nearly simultaneously, thereby minimizing the time lost in incubation and maximizing the use of one detection system. The entire system will then be automated by interfacing it to a minicomputer or microprocessor for sample handling, instrument control, and data acquisition and subsequent processing. A more extensive paper describing this work will be submitted at a later date.

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References

1. Weetall, H. H., Immobilized enzymes: Analytical applications. *Anal. Chem.* **46**, 602A (1974).

2. Bowers, L. D., and Carr, P. W., Applications of immobilized enzymes in analytical chemistry. *Anal. Chem.* **48**, 544A (1976).

3. Leon, L. P., Narayanan, S., Dellenbach, R., and Horvath, C., Immobilized glucose oxidase used in the continuous-flow determination of serum glucose. *Clin. Chem.* **22**, 1017 (1976).

4. Bowers, L. D., and Carr, P. W., An immobilized-enzyme flow-enthalpimetric analyzer: Application to glucose determination by direct phosphorylation catalyzed by hexokinase. *Clin. Chem.* **2**, 1427 (1976).

5. Sanderson, D., Bittikofer, J. A., and Pardue, H. L., Computer controlled stopped-flow studies—application to simultaneous kinetic analyses. *Anal. Chem.* **44**, 1934 (1972).

6. Mieling, G. E., Taylor, R. W., Hargis, L. D., et al., Fully automated stopped-flow studies with a hierarchical computer-controlled system. *Anal. Chem.* **48**, 1686 (1976).

7. Malmstadt, H. V., and Pardue, H. L., Quantitative analysis by an automatic potentiometric reaction rate method. *Anal. Chem.* **33**, 1040 (1961).

8. Inman, D. J., and Hornby, W. E., The immobilization of enzymes on nylon structures and their use in automated analysis. *Biochem. J.* **129**, 255 (1972).

9. Malmstadt, H. V., and Crouch, S. R., Systems for automatic direct readout of rate data. *J. Chem. Educ.* **43**, 340 (1966).