STOPPED-FLOW ANALYSIS: AUTOMATION AND APPLICATION

By

Roger Bernard Putt

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemistry

ABSTRACT

Stopped-Flow Analysis:
Automation and Application

by

Roger Bernard Putt

An improved microcomputer interface to a stopped-flow apparatus has been developed. The improvements consist of replacing an outdated PDP 8/e computer system with a sophisticated and flexible minicomputer/microcomputer arrangement; and developing a software package for instrument control, data acquisition and manipulation. The new interface utilizes a multi-user PDP 11/23 minicomputer for mass storage and high level data manipulation and a locally built microcomputer for direct control of the apparatus. Complete documentation of the software written in FORTH and FORTRAN IV is included.

The stopped-flow apparatus was applied to two kinetic studies to test the system performance and to gather fundamental data on an analytically useful reaction. The iron-thiocyanate reaction was used to determine the deadtime of the instrument and the precision and accuracy of rate measurement. The results show that the instrument performs satisfactorily after the recent modifications. Then the Griess reaction for nitrite analysis was studied to learn the kinetics of the reaction under the most common analysis

conditions. The results of pH and ionic strength experiments as well as the calculation of rate constants and individual orders are presented. A mechanism formulated from the kinetics data is also presented.

ACKNOWLEDGEMENTS

I would first like to thank Prof. Stan Crouch for providing the opportunity to work in his research group. His guidance and ability to answer innumerable questions were the keys to my success.

Special thanks should go to Bob Harfmann who willingly (?) helped with the preparation of many of the figures in the thesis. His invaluable assistance and unique sense of humor will not be forgotten.

The computer wizardry of Keith Trischan should also be acknowledged. Keith wrote much of the trickier software and helped me out of many predicaments which was a tremendous help in finishing on time.

Tom Atkinson should also be thanked for making MULPLT available and for answering questions on software and hardware.

I would especially like to thank both Kay Feaster and Janice Russell for typing assistance. Their expertise helped me stay on schedule.

Finally, to Kim, Marguerite, Pat, John, and Frank: thanks for being yourselves.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The stopped-flow method of analysis has gained popularity in recent years for its use in obtaining fundamental kinetics data, such as rate laws, rate constants and activation energies, as well as its potential as an analytical tool. Stopped-flow mixing is attractive for laboratory use because of the extremely small solution volumes (often only a few microliters) and the short analysis times required (a few seconds or less). The use of minicomputers and microprocessors facilitates the complete automation of the stopped-flow system to control sample preparation, instrument operation and data processing, thus decreasing analysis time while improving precision and accuracy.

In the chapters that follow, the automation of a stopped-flow mixing system and its application to a fundamental kinetic study are discussed. The automation involved up-grading an outdated computer/controller system (PDP 8/e) to a more sophisticated and flexible minicomputer/microcomputer arrangement using a PDP 11/23 minicomputer and a home-built microcomputer based on the Intel 8085A microprocessor chip. Complete documentation of the supporting software package for instrument control, data acquisition and manipulation is included.

The remaining chapters describe a brief system test using the pcpular iron/thiocyanate reaction and a

fundamental kinetic study of the Griess reaction for nitrite analysis using the most common reagent combination and reaction conditions. While previous studies of the Griess reaction have been carried out, the kinetics have not until the present been investigated with the most common reagents and reaction conditions used in most analytical procedures.

CHAPTER I

PRINCIPLES OF STOPPED-FLOW METHODS OF ANALYSIS

A. Reaction-rate methods of analysis

The technique of stopped-flow mixing is a sub-group of a broader class called reaction-rate methods of analysis. Rate methods use reaction kinetics instead of stoichiometries to obtain analytical results. The recent popularity of reaction-rate methods of analysis has resulted in the publication of numerous books and review articles on the subject (1-17).

Reaction-rate methods can be used for both analysis of compounds and fundamental research of reaction kinetics. When used for analysis purposes, the initial concentration of analyte, $[A]_o$, is desired. When conditions are chosen such that the reaction is either first-order or pseudo first-order in analyte A, the rate of disappearance of A with time is given by:

$$-d[A]/dt = k[A] \qquad (1)$$

where k is the first-order or pseudo first-order rate constant. Upon integration, one obtains the following relationship between the concentration of A at time t and

the initial concentration of A:

$$[A]_t = [A]_o \exp(-kt) \qquad (2)$$

When Eq. (1) and Eq. (2) are combined, one obtains an equation relating the rate of reaction to $[A]_o$:

$$-(d[A]/dt)_t = k[A]_o \exp(-kt) \qquad (3)$$

Eq. (2) can be used in one type of reaction-rate method of analysis, that is the determination of $[A]_o$ by the measurement of [A] at any time t. Eq. (3) is the basis of the other reaction-rate method which is better named a reaction-rate method because it deals with the change in the concentration of A with time, $\Delta[A]/\Delta t$. If $\Delta[A]/\Delta t$ is measured during the initial portion of the the reaction, then the analysis is called an initial reaction-rate method. The initial section of the [A] vs. t curve is nearly linear and in this case the exponential term in Eq. (3) is approximately unity. The validity of this statement is discussed by Crouch (11), Pardue (18), and Ingle and Crouch (19).

Reaction-rate methods, in particular initial reaction-rate methods, have several advantages. One advantage is that measurements can be made on a much faster time scale

than conventional equilibrium methods. Reaction-rate data can be obtained in many cases in a few seconds or less on a reaction with a half-life of several hours whereas equilibrium data could only be obtained after the reaction was completed. Thus reaction-rate methods are preferred for routine analyses.

Another advantage is that analytical data can be obtained from quite complicated reactions because the initial portion of the reaction is often simple and reproducible. Hence reactions with unfavorable equilibrium constants or that are nonstoichiometric can be used for analytical applications.

Reaction-rate methods also have increased specificity over equilibrium methods. In this case, reaction-rate methods take advantage of the difference in reaction rates of the various species rather than their thermodynamic differences. Complex mixtures can thus be analyzed by differential reaction-rate procedures which would be difficult in comparable equilibrium type methods where a mixture of products would be formed.

Perhaps the most important advantage of reaction-rate methods is that a relative measurement is involved. The absolute value of the measured parameter does not have to be accurate and as a consequence reaction-rate methods are free from interferences which do not affect the rate of change of the measured parameter even though the absolute value of

parameter may be affected. For example, in spectrophotometric measurements, interferences such as impurities, turbidity and cell imperfections can affect the absolute absorbance of a species at a certain wavelength. However, as long as these interferences do not change during the course of the reaction, valid reaction-rate data can be obtained.

Reaction-rate methods have disadvantages as well as advantages. One problem is that the reaction of interest must occur at a measurable rate for analytical data to be obtained. To be useful, the reaction half-lives should be longer than several milliseconds but shorter than several hours.

Obviously experimental conditions must be carefully controlled since reaction-rate methods depend on the accurate measurement of reaction rates. If pH, ionic strength, temperature, and size and shape of the reaction vessel are not taken into account, valid analytical results cannot be obtained.

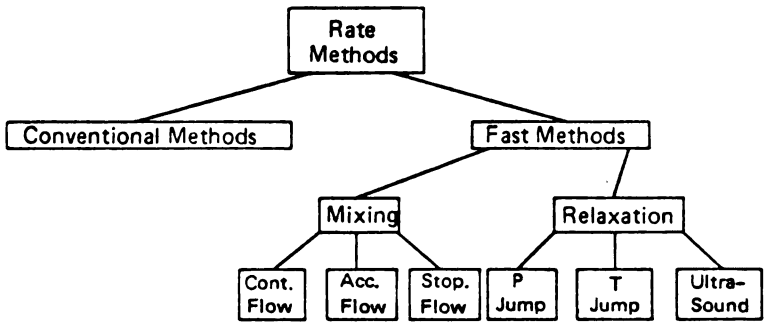
A final limitation is that reaction-rate methods have to contend with lower signal-to-noise ratios than equilibrium methods. Very sensitive detectors must be used because only a small fraction of the total signal is measured or a small change in the signal over a given length of time must be detected. Improvements in detector sensitivity and the use of computers for instrument automation have diminished the

limitations of reaction-rate methods. Thus for many reactions when experimental conditions are carefully controlled, the rate method of analysis can be a powerful analytical tool.

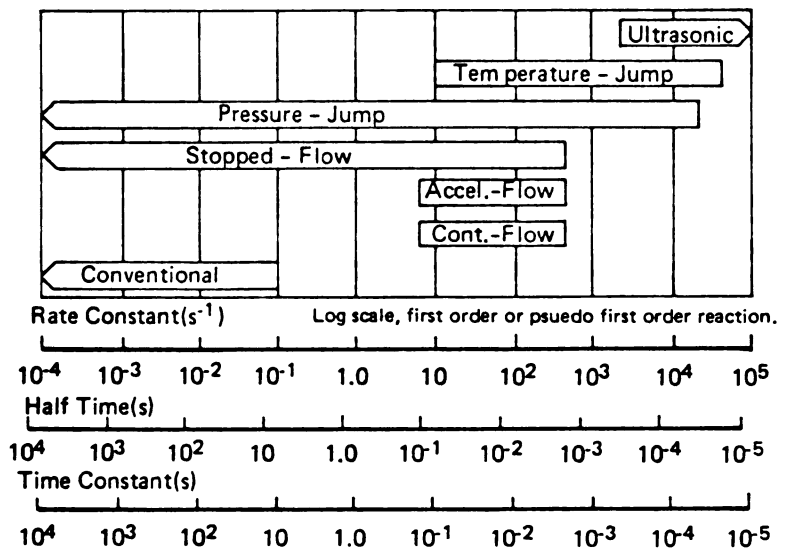
B. Classification of Methods

According to Figure 1(A), reaction-rate methods of analysis can be divided into conventional and fast methods. Although not clearly defined, the difference between conventional and fast can be explained by first stating that the time for mixing the reagents in the conventional method is on the order of 2 to 10 seconds which limits this method to reactions with first-order rate constants of approximately 10^{-1} sec^{-1} because the mixing time must be short relative to the half-life of the reaction. Thus fast methods involve reactions which are too rapid to be measured by conventional means i.e. reactions having half-lives of greater than approximately 10^{-1} sec. The range of reaction half-lives covered by conventional and fast methods is shown in Figure 1(B).

Fast methods can be further classified into mixing and relaxation methods. The mixing techniques include continuous-flow, accelerated-flow and stopped-flow methods. The flow methods all involve the mixing of separate solutions in a mixing chamber followed by observation of some



(A)



(B)

Figure 1(A). Classification of rate methods of analysis.
1(B). Time scale of rate methods.

Reprinted from Ref. 16, p. 171, by courtesy of Marcel Dekker, Inc.

physical property of the solution. The continuous-flow method involves allowing two solutions to flow from reservoirs through a mixer into a long tube. Since the flow velocity is constant, the distance along the tube is directly proportional to the time of reaction. The physical property can be measured at different points along the tube to obtain concentration versus time data. In the accelerated-flow method, the reagents are forced through the mixer at a rapidly increasing rate. The flow rate and some physical property of the solution are simultaneously measured from which are obtained concentration versus time data. The final flow method is stopped-flow which consists of taking measurements of the mixed solutions after suddenly stopping the flow. The stopped-flow method will be discussed in more detail in the next section. The above flow methods are each capable of utilizing reactions with half-lives of approximately 1 msec which has been made possible by the development of highly efficient mixers.

The remainder of the fast methods, called relaxation methods, consists of temperature-jump (t-jump), pressure-jump (p-jump) and ultrasonic absorption. Relaxation methods, as the name implies, deal with the relaxation of a chemical system to equilibrium after a sudden perturbation, the system originally having been at equilibrium. Extremely rapid reactions can be studied (half-life approximately 10^{-9} for ultrasonic absorption) because the reactants do not

require mixing. In general, relaxation methods are not analytically useful because the reactants are at equilibrium and thus initial concentrations would be difficult to obtain. On the other hand, flow methods are quite useful for analytical purposes, in particular the stopped-flow method which is discussed in the next section.

C. The Stopped-Flow Method

Figure 2 shows a schematic diagram of a typical stopped-flow system. The sequence of events of the instrument is under the direction of the controller which can be a manual sequencer, an electronic hard-wired sequencer, a minicomputer, or a microprocessor.

The analysis sequence begins with sample preparation which is normally carried out manually but can be performed by a reagent preparation system under the direction of the controller, resulting in a large time savings. The solutions are then drawn into drive syringes operated by pneumatic cylinders and are forced into a mixing chamber. After complete mixing, the solution flows through an observation cell into a stopping device which suddenly stops the flow based on a preset flow volume or time of flow. Immediately after the flow stops, a data acquisition system begins to collect absorbance vs. time data. After data processing to convert the data to the desired format (initial rate,

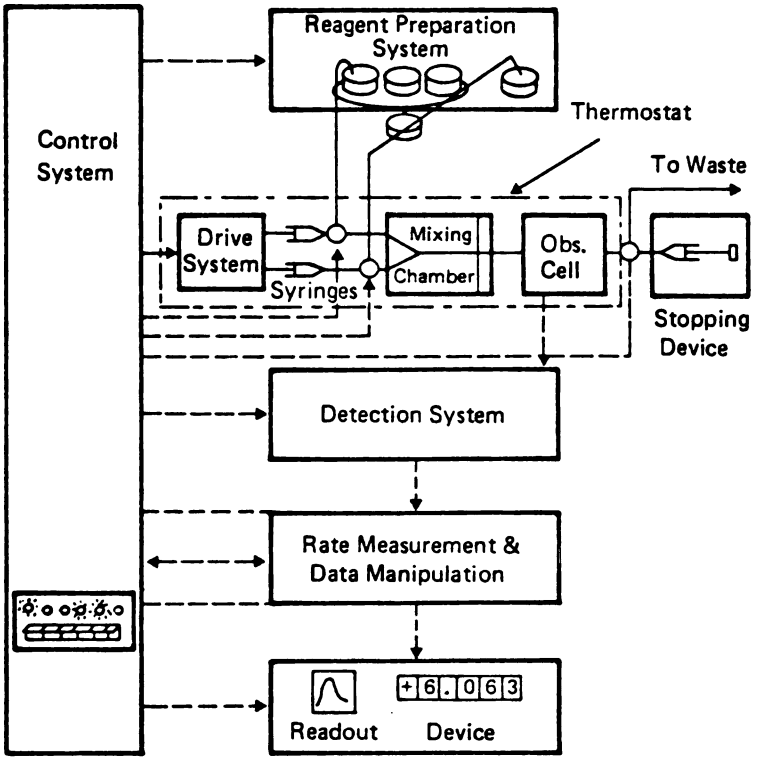


Figure 2. General diagram of stopped-flow system.

Reprinted from Ref. 16, p. 174, by courtesy of Marcel Dekker, Inc.

rate constants, etc.), the final results are sent to a readout device, such as a recorder, printer or plotter.

The stopped-flow instrument is usually thermostatted to a constant temperature to ensure that the measurement of the rate of the reaction is valid. Temperature variations upon mixing, as much as several degrees in extreme cases, can occur even though the instrument is thermostatted to a constant temperature and thus cannot be ignored when high precision work is performed.

Total automation can lead to significant time savings in many ways. Obviously reagent preparation and data manipulation are the most labor intensive steps and thus result in the greatest savings of time and labor when automated. Even the automation of mixing and stopping the flow can result in a large increase in efficiency. The advantages of automation are realized, particularly in stopped-flow analyses, by increased sensitivity due to improved data collection procedures, rapid data processing, and signal-to-noise enhancement procedures.

The stopped-flow method has several advantages over continuous- and accelerated- flow methods. The first is that the stopped-flow method requires a much smaller volume of solution compared to the continuous-flow method. A typical stopped-flow analysis, as well as an accelerated-flow method, uses less than a milliliter of solution compared to several milliliters to a few liters for the continuous-flow

procedure.

Each of the flow methods has limits placed on the reaction times which can be used for analysis. The continuous-flow method is limited by volume requirements and the minimum velocity for turbulent flow. Thus the upper limit for the half-life of the reaction is nearly 100 msec. The efficiency of mixing determines the lower limit of the reaction half-life. The range of reaction half-lives for the accelerated-flow method is 1 to 50 msec which is determined by the requirement that rapid observation must be made while the solution flow velocity is varied. The stopped-flow method has a much wider range of reaction half-lives: 1 msec to several minutes. As in the accelerated-flow procedure, the lower limit is determined by the efficiency of mixing, while the upper limit is determined by the stability of the detection system.

In the stopped-flow procedure, an entire reaction curve can be obtained from readings obtained in the observation cell. In contrast, readings must be taken from several different points along the reaction tube in the continuous-flow method in order to obtain comparable results. Finally, stopped-flow methods are insensitive to solution inhomogeneities because there is usually enough time to completely mix the solutions between stopping the flow and making observations. On the other hand, continuous-flow methods are very sensitive to solution inhomogeneities within the

reaction tube, especially with spectrophotometric detection. Also, because it is possible to obtain quantitative data shortly after the reaction has started in the stopped-flow method, interfering reactions can be avoided.

The efficiency of a stopped-flow mixing system is dependent upon the flow velocity, the mixer design and the distance between the mixer and the observation cell. A high flow velocity is necessary to generate turbulence and produce a high efficiency of mixing; however cavitation can occur at very high velocities. Thus, a compromise must be reached between flow velocity and mixing efficiency to obtain satisfactory results. Flow velocities of 1 to 30 msec have been reported.

The most useful performance measure of a stopped-flow instrument is the dead time, t_d, which is the difference between the time of initial contact of the reactants and the time at which they are stopped in the observation cell (20-22). The measurement of dead times is discussed in the literature (20, 21) Also related to the dead time is the mixing time, t_m, which is the time between initial contact of the reactant solutions and "complete" mixing. The dead time must be greater or equal to the mixing time because mixing must be complete by the time the solution is stopped in the observation cell. The time required for flow to cease once the stopping device has begun to impede flow is called the stopping time, t_s. For high precision work, t_s

must be much less than t_d.

Obviously, t_d must be small relative to the half-life of the reaction in order to follow the reaction from its initiation. Systems with low dead times must be developed to monitor rapid reactions when initial rates are desired (for analytical data and often in mechanistic studies). The relationship between dead time and the reaction rate is given by a rearrangement of Equation 3. Since the half-life, τ, of a first- or pseudo first-order reaction is given by $\tau = (\ln 2)/t$, it can be seen that for a first-order or pseudofirst-order reaction and $t/\tau \ll 1$,

$$(d[A]/dt)_t = (d[A]/dt)_o \exp(-0.693t/\tau) \sim (d[A]/dt)_o (1 - 0.693)\tau \qquad (4)$$

where:

$\tau = 0.693/k$ = half-life of the reaction, sec.

t = reaction time, sec.

$(d[A]/dt)_t$ = reaction rate at time t, mole l^{-1} sec^{-1}.

$(d[A]/dt)_o$ = initial reaction rate, mole l^{-1} sec^{-1}.

For the measured rate to be within 1% of the initial rate, the measurement must be complete within τ /69.3 sec of the reaction initiation.

If the rate cannot be measured at $t \ll \tau$, the analysis becomes quite complicated. A mathematical treatment of this condition is given by Crouch, et. al. (16) and will not be discussed here.

With careful consideration given to instrument design, t_d, t_m, and t_s can be minimized. Also, factors which affect the efficiency of a stopped-flow mixing system, such as character of flow, mixing chamber design, flow system volume, efficiency of the stopping device, and temperature, can be optimized.

CHAPTER II

HISTORY OF STOPPED-FLOW ANALYSIS

This review of past and present work in the field of stopped-flow analysis is divided into two parts. The first deals with a history of instrumental developments of stopped-flow equipment, concentrating on automation. The second covers applications of stopped-flow analysis to various chemical systems. This two-part review is followed by a chronological account of research done in stopped-flow analysis at Michigan State University leading up to the present work.

A. Instrumental Developments

Any review of flow methods of analysis must begin with the classic studies of continuous-flow by Hartridge and Roughton in 1923 (23, 24). These men laid the groundwork from which other flow methods were developed, in particular accelerated-flow and stopped-flow methods. They studied the rates of chemical reactions by allowing the reactants to flow from large reservoirs into a mixer and then into a long tube.

Observation of the absorbance of the solution at various points along the tube supplied them with concentration vs. time profiles.

Chance was the first to apply the continous-flow method by Hartridge and Roughton in the development of accelerated- and stopped-flow methods of analysis (25-28). Improvements were made in attainable flow velocity, sensitivity and speed of response of the photoelectric system, and in fluid economy. In Chance's experiments, the reactants were drawn into syringes then rapidly discharged through a tangential jet mixer to the observation cell. From data supplied by flow rates and the photoelectric detection system, concentration vs. time curves were obtained for various chemical reactions.

Many unique stopped-flow systems have appeared within the last ten years. A complete review of all systems is beyond the scope of this work so the reader is referred to several reviews on recent stopped-flow systems (8, 16, 17). The earlier systems of Chance and others are also reviewed elsewhere (29, 30).

A stopped-flow apparatus capable of both high speed and low speed operation was developed by Berger and co-workers (31). In the high speed mode, the dead time of the system was measured at 270 μsec, and the mixing time was about 90 μsec. An efficient mixer was developed using impinging jets which force the solutions over a hemispherical surface.

The system has a disadvantage in that at least 10 ml of each solution are required with about 1 ml of each reagent needed per trial.

Harvey has developed a general purpose system which could be used for educational and preliminary research purposes (32). The system is designed around a variable ratio principle using microliter quantities of solution. The relative volumes of the two reagents may be any one of seven ratios from 1:1 to 100:1 with a corresponding increase in dead times at the large ratios. The accuracy and precision of the ratios are good up to 20:1.

Caldin et. al. have developed an ideal system for research purposes (33). The most notable features are all glass construction, fiber optics light guides for flexibility and ease of interfacing to monochromators, and complete immersibility of the system for temperature control. The instrument has a low dead time of 3 msec and second-order rate constants as fast as 10^6 l $mole^{-1}$ sec^{-1} over a wide temperature range may be measured.

Another research apparatus similar to that of Caldin has been developed by Dye and co-workers (34, 35). The instrument is constructed entirely of quartz, Teflon, and Pyrex and can be used in double beam mode. The integral mixing and observation cell has two light paths of 0.2 and 2 cm with dead times of 2 to 6 and 6.0 msec for the short and long path lengths, respectively. The instrument is equipped

with a rapid scanning detection system capable of obtaining entire spectra during the course of the reaction. The apparatus has been interfaced to a PDP 8/I minicomputer (36), which controls data acquisition and manipulation. Data analysis programs may then be employed to display the data on a CRT display, send the data to a plotter, or punch the data on cards for more sophisticated data handling on a CDC 6500 computer.

A rapid-scanning detection system was also implemented in the apparatus of Wightman et al. (37). This system is similar to that of Dye with the differences being in the types of minicomputers, monochromators and stopped-flow mixers used.

Javier and co-workers (38, 39) developed the first stopped-flow system specifically designed for routine analytical use. The system consists of a motor-driven sample turntable, a stopped-flow spectrophotometer, and a digital ratemeter and readout. The system may be cycled continuously for averaging of multiple pushes on the sample solution. Typical relative standard deviations for single analytical results were 2 to 3% and averages of 10 cycles yielded RSD's of <1%.

An extremely sophisticated and totally automated system was developed by Pardue and co-workers (40, 41). The hierarchical computer system, consisting of an HP2100 minicomputer (42) and an Intel 8008 microcomputer (43),

controls the sample preparation, instrument operation, data acquisition, manipulation and display. The software package includes a foreground-background mode for the minicomputer and programmed interrupts to transfer control to the proper sequencing and data acquisition subroutines. The performance of the system has been evaluated by carrying out serial dilution experiments, mole ratio and continuous variation studies of metal ion complexes, a simplex optimization study, and a kinetics study of a well-known fast reaction.

O'Keefe, Malmstadt and co-workers (44, 45) have also designed a highly automated stopped-flow system. The system consists of several independent modules: a solution preparation system, a stopped-flow mixing system, a spectrophotometer and a controller, in this case a PDP 8/f (36) minicomputer. The operator may design and carry out equilibrium or kinetics experiments in either an investigative mode or a routine analytical mode. The advantages of this system are the speed with which solutions may be prepared and analyzed and the ease with which the investigator may move between the two principal modes of operation. The instrument has been applied to a fundamental study of the Jaffe reaction, which could be used for the routine analysis of creatinine.

Bonnell and Defreese (46) have developed a automated system using two microprocessors: one for control of the stopped-flow instrument and the other for operator

interaction and data manipulation. The two microprocessors operate independently which overcomes the limitations associated with low speed of microprocessors for measurement applications which also involve significant computation, e.g., reaction-rate methods. This instrument has been applied to the determination of phosphate and alkaline phosphatase activity and fundamental studies of bilirubin kinetics.

A stopped-flow system similar to that used in this project (designed by Beckwith, Crouch et al. (47)) was developed by Holtzman (48). This system is based on a commercial unit, MITS 8800b, which is a fully "burned-in" board that can be directly plugged in. The system also includes an INTEL 8080A LSI (43) microprocessor. Both fundamental kinetic studies and routine analyses can be performed on the instrument.

Clark and Schuster (49) have developed an automation package specifically designed for stopped-flow analysis. This is a simple, inexpensive system which is easy to operate. Raw data are stored on a mini-floppy diskette and the computer itself is programmed in BASIC with options for FORTRAN and PASCAL. One disadvantage is the slowness of obtaining a graph of the results on the printer. The entire system is comparable in price to a storage oscilloscope.

Within the last several years, a number of publications have appeared in the literature dealing with stopped-flow

analysis under high pressure (50-54). High pressure studies, via activation and reaction volumes in solution, are performed in order to answer fundamental questions about chemical systems (55). Current researchers have developed specialized equipment to study the relationship of pressure and rate constants for many different reactions; several examples of their work is presented below.

Isihara et al. (52) have developed a high-pressure stopped-flow apparatus with spectrophotometric detection which is used to follow fast reactions in various media at pressure $\leq$ 2000 kg/cm^2. The apparatus allows the measurement of activation volumes for reactions with half-lives longer than several milliseconds.

Smith and co-workers (50) developed a new design of the window-to-observation chamber seal for the Berger rapid stopped-flow apparatus and illustrated the performance of the apparatus by studying the reaction of $CaCl_2$ with EGTA (ethylenebis[oxyethylenenitrilo]tetraacetic acid). Incorporation of fiber optic light guides for the observation path makes the apparatus insensitive to vibration artifacts.

A simple, compact, easy-to-implement microprocessor ratemeter for kinetic determinations is described by Bonnell and Defreese (56) which is based on initial rate, fixed time and variable time principles. The ratemeter can be used with almost any transducer or instrument to monitor reactions in a wide variety of chemical systems. The

flexibility of the design was illustrated by the determination of phosphorous by stopped-flow analysis, glucose by amperometry, and alkaline phosphatase activity in control sera by visible spectrophotometry.

B. Applications

The stopped-flow mixing technique has been known for many years, particularly in biochemistry (57); however analytical uses of the technique have emerged only within the last 15 years. Stopped-flow methods have been recognized for several key advantages: large sample throughput for single component determinations (39, 47), applicability to a wide range of reaction rates and also to equilibrium methods (41, 44), and complete automation of the mixing process and data acquisition.

The number of analytical applications of stopped-flow methods of analysis has rapidly increased in the last several years. A brief account of only the most noteworthy applications over the last 15 years will be presented here.

The first analytical uses of stopped-flow mixing were by Pausch and Margerum (58) and by Javier and co-workers (39) in 1969. Pausch and Margerum studied the reaction between Pb(II) and the alkaline earth complexes of trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetate (CyDTA) in the differential kinetics determination of mixtures of Mg^{2+},

Ca^{2+}, Sr^{2+}, and Ba^{2+}. The detection limit of the method was as low as 10^{-6} M in metal ion with 10- to 100- fold excess of other metals.

Javier and co-workers (39) used stopped-flow mixing for the rapid determination of phosphate in the millisecond range. The reaction of phosphate with Mo(VI) in strong acid to yield 12-molybdophosphate was used with which it was possible to analyze thousands of samples per hour with relative standard deviations of 2 to 3%. The method was shown to be selective by determining phosphate in the presence of two common interferents: arsenate and silicate.

Willis et al. (59) have employed regression analysis in the simultaneous reaction-rate analysis of mixtures. They applied this procedure to the acid dissociation of alkaline earth complexes of CyDTA previously mentioned, obtaining relative standard deviations of usually below 2% with 7% being the worst case. From the beginning of the analysis to obtaining a printout of the results, the entire procedure takes only 3 minutes.

Willis et al. (40) also studied the well-known reaction of Fe(III) with SCN^-. These workers were able to accurately determine Fe(III) over a range of 100 to 500 μM. This reaction has been frequently used in performance measurements of stopped-flow systems as demonstrated by Beckwith and Crouch (47).

Sanderson et al. (60) studied the formation of complexes

of cysteine and thiolactic acid with nickel both individually and in mixtures. Relative standard deviations of these analyses range from 2 to 5%.

Stopped-flow mixing was also applied by Bishop, Everse, and Kaplan (61) in the determination of heart and muscle lactate dehydrogenases (LDH). They determined the total amount of LDH and the ratio of the activities of the isoenzymes by taking advantage of the inhibition of the two isoenzymes in pyruvate solutions. Errors in these analyses ranged from approximately 1 to 30%. This procedure has important uses as a rapid diagnostic aid in the treatment of myocardial infarction, liver disease, hemolytic anemia, and muscular dystrophy.

Rhee and co-workers (62) used the luciferase-luciferin system for the determination of ATP with a stopped-flow spectrometer. They utilized the initial light intensity produced by the reaction before emission due to the conversion of ADP to ATP by nucleoside diphosphokinase becomes appreciable. By this technique, ATP concentrations as low as 1.5pmoles were measured.

The enzymatic determination of glucose was studied by O'Keefe and Malmstadt (44). An automated spectrophotometer was used to obtain rate data on this relatively slow reaction between glucose and glucose oxidase-peroxidase reagent. Relative standard deviations for unknown solutions ranged from 0.6 to 9.1%.

Karayannis (63) has applied stopped-flow mixing to the classical method for the determination of ascorbic acid using 2,6-dichlorophenolindophenol (DCPI). A commercial stopped-flow instrument was used to measure the rate of the reaction of DCPI with ascorbic acid in 0.025 M oxalic acid at 522 nm. This rapid method can measure ascorbic acid down to 12.5×10^{-5} M with errors of 1 to 3%.

A reaction-rate method for the determination of trace levels of cyanamide has been developed by Nieman et al. (64). These workers utilized the reaction of cyanamide with sodium pentacyanoferrate(II) which forms a product absorbing at 530 nm. The procedure has a linearity over five orders of magnitude with a precision of 3.5% at the 95% confidence level.

Using a variation of the fixed-time method of Lee and Kolthoff (65) in conjunction with stopped-flow mixing techniques, Pelizzetti et al. (66) have developed a method to determine adrenaline, L-Dopa, and mixtures of the two substances. The compounds are individually determined by oxidation to corresponding o-benzoquinone derivatives while they are measured in a mixture by conversion to the corresponding aminochromes. Errors in these analyses are approximately 2%.

Ridder and Margerum (67) have developed a procedure to simultaneously determine mixtures of Zn^{2+}, Cu^{2+}, Hg^{2+}, and Cd^{2+} as well as mixtures of the catecholamines epinephrine,

norepinephrine, and L-Dopa. The mixtures of metal ions were analyzed by employing CyDTA in the reaction with the zincon complexes of the ions. The catecholamines were determined by conversion to their corresponding amine derivatives. The amine derivatives were subsequently reduced with ascorbic acid which was followed by the stopped-flow spectrometer at 480 nm.

Markley et al. (68) have applied stopped-flow mixing to reinvestigate reports of an intermediate prior to the acyl-enzyme complex in hydrolysis reactions of anilides catalyzed by trypsin and alstase. The evidence obtained does not support existance of such an intermediate. The construction of the stopped-flow apparatus designed for the cryoenzymolization used in this work that had novel features and was adaptable to a variety of spectrophotometers was described.

Stopped-flow mixing was also applied by Koupparis et al. (69) in the kinetic study of permanganate-oxalate reaction and the kinetic determination of Mn(II) and oxalic acid by the stopped-flow technique. Reaction-rate methods for the determination of Mn(II) in the range of 1-10 x 10^{-4} M and oxalic acid in the range of 0-20 μg mL-1 are reported. Precision for these analyses are on the order of 1-2%.

Thompson and Crouch (70) studied reactions catalyzed by enzymes immobilized inside open tubes using a stopped-flow instrument for direct and continuous monitoring. The results show that because the reaction occurs under static

conditions, the kinetics are controlled only by diffusion and the ingerent enzyme reaction rate. The kinetic constants for lactate dehydrogenase, convalently bound inside nylon tubing and in aqueous solution were determined and compared.

C. Evolution of Stopped-Flow Analysis at Michigan State University

The technique of stopped-flow mixing in the research group of Prof. S. R. Crouch was first investigated by P. M. Beckwith in 1972 (71). Beckwith developed the original stopped-flow instrument and applied it to various chemical systems. The instrument features a vertical flow system to minimize problems with air bubbles, pneumatically actuated valves for directing the liquid flow and dispelling waste solutions, and a spring loaded stopping syringe. Dead time and mixing efficiency of the instrument were also determined. A fundamental study was performed on the kinetics of formation of 12-molybdophosphoric acid in perchloric acid in order to investigate new methods of phosphate analysis.

P. K. Notz improved the stopped-flow mixing system with several design changes in 1975 (72). These design changes include thermostatting the instrument, and developing a novel mixer, high throughput quartz optics and an optical trigger system. The instrument was fully automated by

interfacing to a PDP 8/e minicomputer for control of valve operation and collection, analysis, display and storage of data. Also performed was a complete characterization of the mixing and spectrophotometer components to determine their individual effects on the system accuracy and precision. The instrument was applied to studies of the proton consumption by Mo(VI) in strongly acid solution, the dependence of the rate of formation of 12-molybdophosphate on nitric acid concentration, and the reactions of phosphate and silicate with Mo(VI).

F. J. Holler (73) further investigated the elements of stopped-flow mixing systems in 1977. A thorough study of temperature effects was performed in order to develop guidelines for minimizing these effects during mixing. A computerized version of the bipolar pulse conductance instrument was developed. The instrument is useful for conductometric analysis and was applied to the studies of the dehydration of carbonic acid and the reaction of nitromethane with base. Holler also designed a computer-controllable, stepping motor driven buret which can be used in a reagent preparation system.

C. C. Kircher, Jr., applied the technique of stopped-flow mixing to a study of heteropolymolybdate kinetics in 1982 (74). The kinetics of 12-molybdophosphate and 12-molybdosilicate formation, decomposition, and conversion to other heteropolymolybdates were monitored as a function

of phosphate, silicate, molybdate, hydrogen ion and heteropolymolybdate concentrations. Kircher obtained rate law equations, rate constants and chemical mechanisms for these reactions in nitric, perchloric, and sulfuric acids with the help of computer simulations from various chemical models and mathematical equations.

CHAPTER III

A MICROCOMPUTER CONTROLLED STOPPED-FLOW SYSTEM

In this chapter, a description of the modified stopped-flow instrument and its capabilities are presented. Included is a listing of the hardware involved as well as the functions, specifications and limitations of the equipment. The computer/instrument interface is discussed along with a description of the computer system and its functions. The chapter concludes with a complete documentation of the software package written for instrument control, data acquisition and manipulation.

A. A Modified Stopped-Flow Apparatus

Prior to this work, the stopped-flow apparatus was last improved by Notz (72) who characterized the components, developed a novel mixer, and by Notz and Holler (73) who interfaced the instrument to a PDP 8/e minicomputer. Figure 3 shows the current configuration of the stopped-flow system. Except for the new computer interface, the apparatus is essentially the same as that used by Notz and Holler. The system components are listed in Appendix A.

As seen in Figure 3, the instrument is mechanically controlled by solenoid actuated pneumatic cylinders. These

Figure 3. Block Diagram of the Modified Stopped-Flow Apparatus

A. Solenoid actuated pneumatic drive cylinder

B. Solenoid actuated pneumatic cylinder controlling inlet valve

C. Reagent drive syringes

D. Double 3-way reagent inlet valve

E. Port to reagent reservoirs

F. Mixing chamber

G. Observation cell

H. 3-way waste release valve

I. Spring-loaded stopping syringe

J. Port to drain

K. Solenoid actuated pneumatic cylinder controlling waste release valve

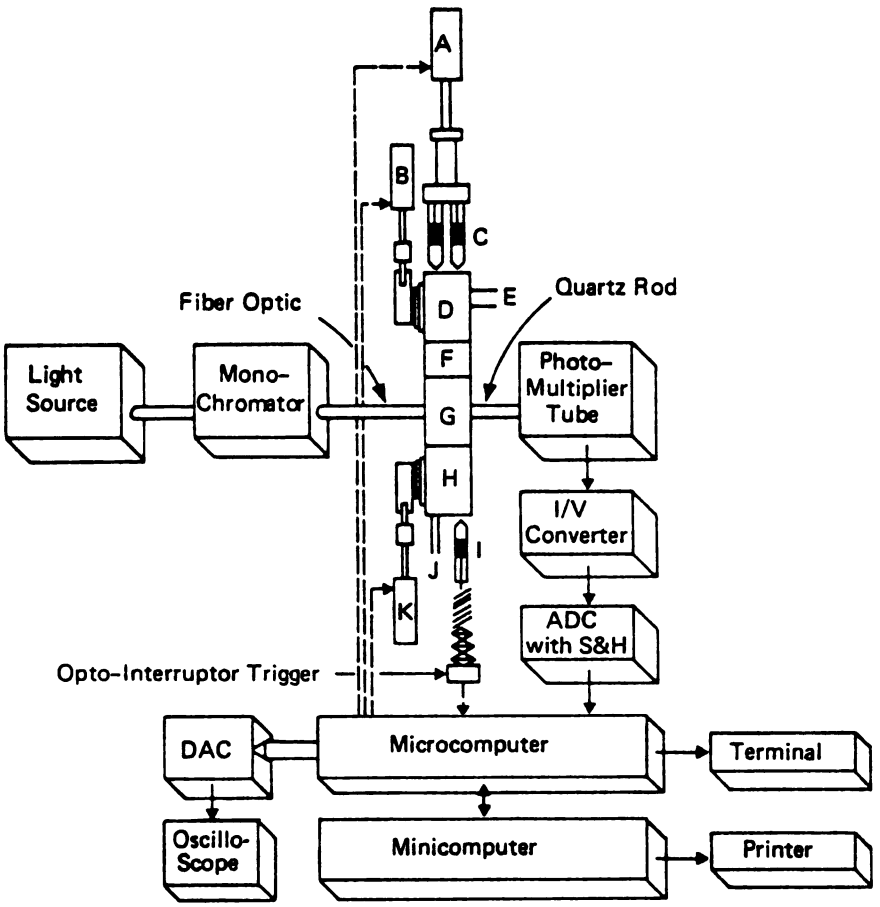

A
B
C
D
E
F
G
H
I
J
K
Fiber Optic
Quartz Rod
Light Source
Mono-Chromator
Photo-Multiplier Tube
I/V Converter
ADC with S&H
Opto-Interruptor Trigger
DAC
Microcomputer
Terminal
Oscillo-Scope
Minicomputer
Printer

Figure 3

cylinders, supplied with approximately 45 psi nitrogen, control the operation of the valves and syringes during an analysis. Cylinder A controls the drive syringes which, when actuated, are filled with separate solutions, one in each syringe. After the 3-way valve is switched to the proper position, under the control of cylinder B, the solutions are forced through the mixing chamber into the observation chamber. The flow is stopped by a spring-loaded stopping syringe after a preset volume of solution has flowed through the cell. The sequence of forcing the solutions through the mixing chamber and cell with a sudden stopping of the flow constitutes one push. The instrument is capable of five complete pushes with one filling of the syringes. The consecutive pushes are controlled by the combined action of the waste release valve and the stopping syringe. The waste valve, controlled by cylinder K, allows the solution to flow into the stopping syringe when in one position, thus depressing the spring as the flow is stopped. When turned to the other position, the force of the spring discharges the solution to a waste container. By alternating the position of the waste release valve after the system is pressurized, one can rapidly obtain reaction-rate data on several consecutive pushes.

The mixing chamber was specially designed by Notz (72). Efficient mixing is obtained by repeatedly separating and re-mixing the solutions as they are forced through the

chamber. This design replaced the more common mixer which utilizes tangentially offset jets to effect complete mixing. Jets mixers are prone to cavitation, however. The stopped-flow instrument has an advantage in that the two mixer types are interchangeable.

The detection system is comprised of a UV-visible scanning spectrophotometer and a photomultiplier tube (PMT). Fiber optics are employed for increased light through-put. The PMT is powered by a feedback-stabilized high voltage power supply in order to minimize drift which is a common problem with detection systems of this type (75). UV-visible spectrophotometry is the most common type of detection system, but many other optical and non-optical detectors have been employed (16).

The performance of the various stopped-flow components were not evaluated in this project. A complete performance characterization is given by Notz (73).

B. Microcomputer Interface

The computer/instrument interface utilizes a controller designed by E. Ratzlaff (76). The controller contains circuits and AC relays to control the valves and syringes of the instrument via solenoid actuated pneumatic cylinders. The controller also monitors the status of the opto-interrupter used to activate data acquisition. The

microcomputer interface will be discussed by first describing how the controller relays were connected to the solenoids on the instrument then describing the modified opto-interrupter circuit.

As shown in Figure 4, the controller contains four AC relays available for interfacing to the instrument. Associated with the relays are a series of output bits from a specific address which can be directly controlled with program commands. By "pulsing" the output bit associated with a certain relay, a TTL signal can be sent to the desired solenoid. Table 1 contains a list of output bit/relay pairs and also indicates how the relays were assigned to the various solenoids. Output bit B1 is the control bit for the auto/manual mode of the controller. If desired, the controller can be put into manual mode during the course of an analysis which allows the user to activate the solenoids manually by switches on the front panel of the controller. When in auto mode, the solenoids can only by activated by computer commands.

The controller circuit also contains a series of input, or "status" bits. These bits are used to monitor the status of the relays, the trigger, and the auto/manual mode of the controller. Table 2 contains the input bit assignments. Knowledge of the status of these components is useful for intelligent control of the instrument.

The instrument was originally connected to a controller

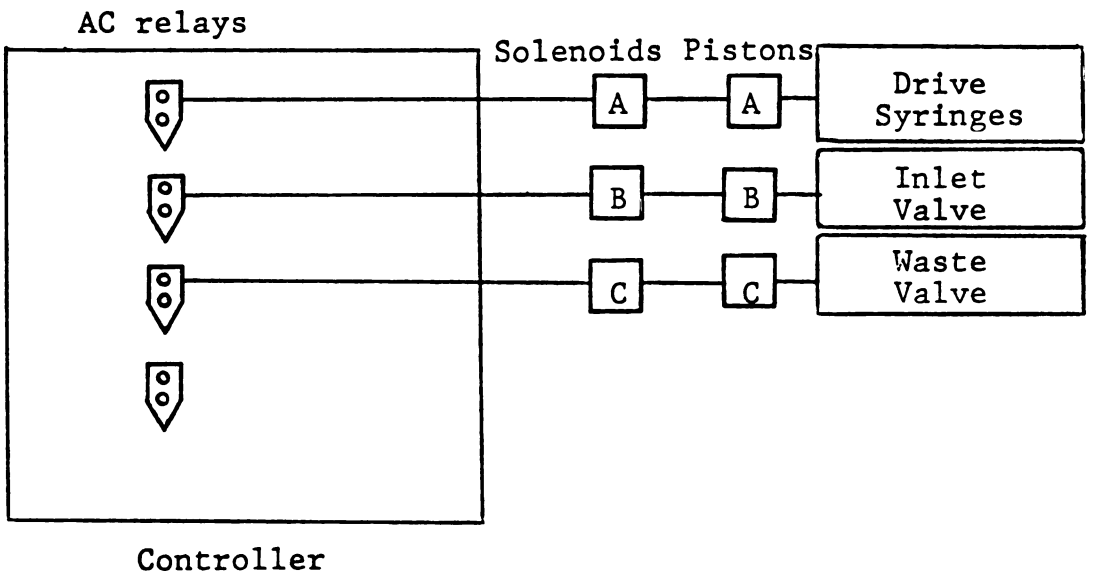


Figure 4. Controller interface.

Table 1. Output bit assignments

Output Bit	
B0	Relay 3
B1	AUTO/MAN
B2	Relay 4
B3	Relay 1
B4	Relay 2

Table 2. Input bit assignments

Input Bit	
C0	Relay 2
C1	Relay 3
C2	Trigger
C3	AUTO/MAN
C4	(Not Used)
C5	Relay 1
C6	Relay 4

designed by R. Balciunas (77). The wires connecting the controller to the solenoids were cut and reconnected to the new controller using Molex fittings. These fittings were then attached to the wires leading to the Balciunas controller so that the two controllers could be interchanged if desired. The Ratzlaff controller was connected to the progammable I/O port of the microcomputer.

An opto-interrupter is employed to monitor the instant the flow is stopped after which data acquisition begins. This is accomplished by blocking the light path of the interrupter by a metal bar connected to the stopping syringe and causing SIGNAL OUT to switch from high to low. The microcomputer continuously monitors the status of the interrupter and can thus activate data acquisition when the trigger status bit changes from high to low.

The lead to the opto-interrupter was also separated from the Balciunas controller and reconnected to the "TRIGGER IN" port of the Ratzlaff controller using Molex connectors. Figure 5 shows the pin assignments for the three wires from the opto-interrupter. The trigger circuit in the controller also had to be slightly modified to accept this opto-interrupter. After examining circuit diagrams for opto-interrupters, a 150Ω resistor was added to connect TRIGGER IN to ground to complete the circuit, as shown in Figure 6. Also a wire leading to an LED before the inverter was cut to prevent the in-coming signal from being pulled

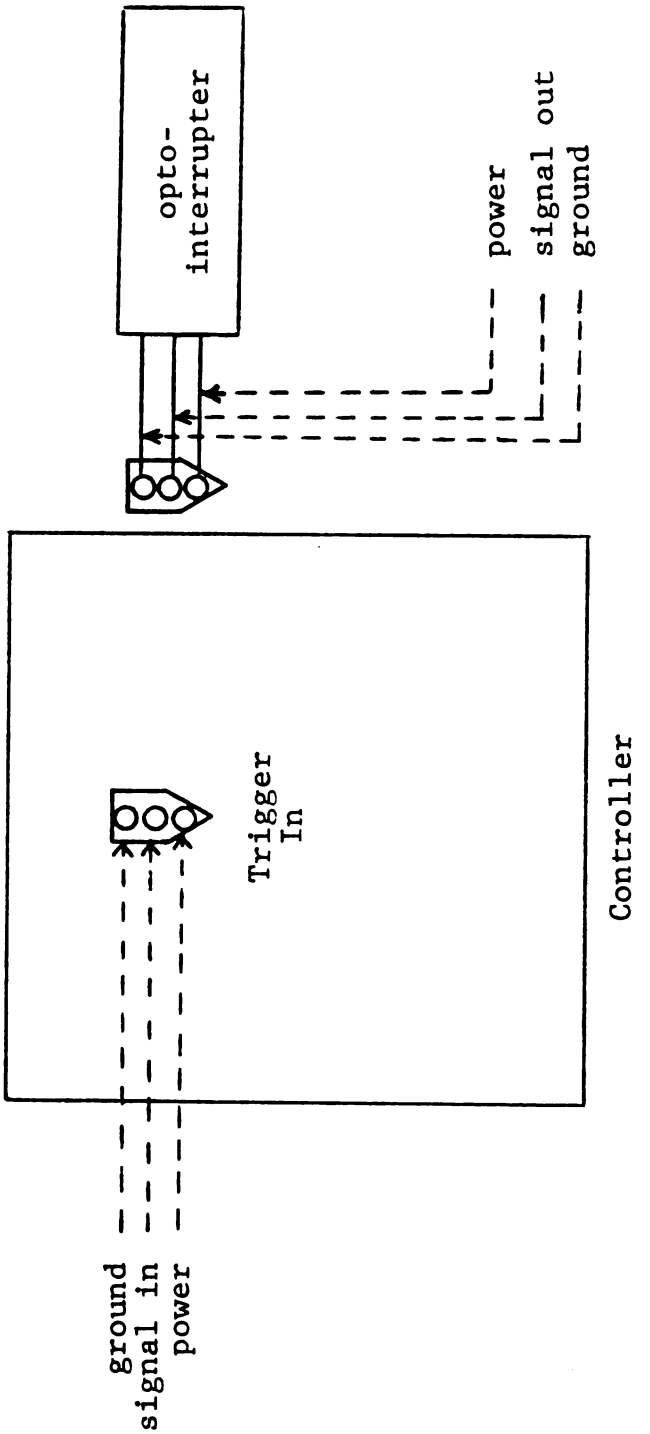


Figure 5. Opto-interrupter pin assignments.

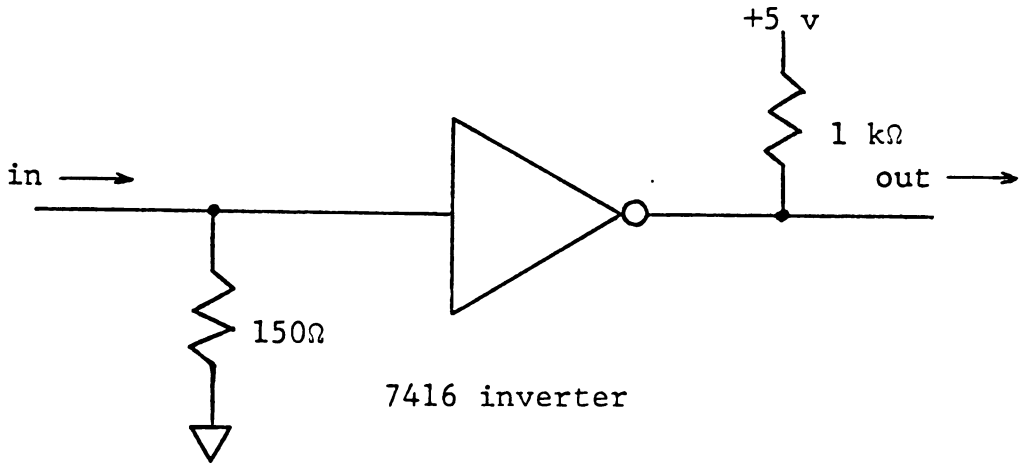


Figure 6. Controller circuit for opto-interrupter.

to +5 volts. The opto-interrupter is powered by +5 volts relative to ground and was thus properly connected to the source and ground tracks on the controller board. The third wire from the opto-interrupter is SIGNAL OUT and leads directly to the inverter. As mentioned before, the interrupter is designed to supply a low TTL signal when the light path is blocked.

The microcomputer used in this project was constructed by E. Ratzlaff (76) from a design by B. Newcome (78). The computer components are listed in Appendix A. Along with a terminal, the microcomputer provides direct control of the apparatus, collects the reaction-rate data, performs low-level data manipulation, and provides temporary memory space for the raw data.

One of the unique features of the microcomputer is the timer board which utilizes the Am9513 System Timing Controller (79). The timer chip contains an internal oscillator and associated frequency scaling circuitry plus five general-purpose 16-bit counters. Each counter is supported by control circuitry that allows it to be independently configured for a variety of tasks. The timer commands were incorporated into the instrument control software to provide timing and sequencing capabilities.

The microcomputer is tied in to a multi-user minicomputer system for mass data storage and sophisticated data manipulation and plotting. The minicomputer is a

DEC LSI 11/23 which uses the RSX-11/M operating system (36). Programs for instrument control, data acquisition, etc., are written on the microcomputer and serially transmitted to the minicomputer for storage. The programs are down-loaded to the microcomputer at run time thus the minicomputer acts as a pseudo-disk in this system. Although the terminal is directly connected to the microcomputer, the user can "talk" to either the micro or minicomputer during the course of an analysis.

C. Software for Stopped-Flow Analysis

Programs written for stopped-flow analysis must be capable of performing certain tasks: mechanical control of the apparatus, data acquisition, data manipulation and storage. The software written for the latest modified stopped-flow apparatus accomplishes all these tasks using a combination of programming languages described below.

Software for instrument control must be interactive, easily altered, and take up minimum memory space. For these reasons, FORTH (or more specifically, poly-FORTH) was selected as the language to be used on the microcomputer for direct instrument control. FORTH can be described as a high-level language, an assembly language, an operating system, a set of development tools, and a software design philosophy (80, 81). Incrementally compiled, it has the

ability to allow keyboard or disk definition of words that implement procedures. These words are built into a dictionary that can be called upon as new words are created using previously defined ones. The words can be strung together as in a sentence to perform complicated operations. FORTH is extremely easy to change using the editor and takes up much less memory space than other high-level languages. In FORTH, all machine facilities are fully available; thus the language appears to run much like the machine it is being used on. FORTH uses fixed-point arithmetic which makes it fast, especially for mathematical operations.

There are disadvantages to FORTH as well as advantages. All definitions must have unique names which can sometimes lead to difficulties when many words are developed for a particular application. Another disadvantage is that the FORTH executive is slow, which can be a problem during time-critical operations. Even though the words are compiled into the dictionary as they are defined, the executive spends a relatively large amount of time in routing commands. For time-critical steps, the words are defined in FORTH code (assembly) for the fastest possible operation. Also, the editor has limited capabilities which lengthens the time necessary to make program changes. Finally, the FORTH "program" is difficult to document because of the ease of making changes to the definitions.

The more sophisticated mathematical operations, such as

data conversion, were written in FORTRAN IV on the PDP 11/23. FORTH arithmetic uses a stack which forces the programmer to be aware of the relative position of the numbers involved in a mathematical operation. This aspect coupled with the fact that FORTH does not have many highlevel math functions built in, makes FORTH undesirable for these applications. FORTRAN, although not as interactive as FORTH, operates rapidly after compilation and is relatively easy to use for applications involving high-level mathematics.

The main purpose of this section is to document the software written for the stopped-flow instrument operation and associated data handling. The computer listings of all programs in both FORTH and FORTRAN are in Appendix B. The FORTH definitions will be discussed in order of their appearance in blocks 10 - 36 of MICRO.FTH as seen in the appendix. A detailed explanation of the function of each of the words will be presented; however the low-level FORTH words will not be discussed and can be found in the FORTH manual (80). The FORTRAN data conversion program will then be presented followed by a discussion of the overall analysis sequence.

1. Controller software

The initial FORTH software for controller operation was written by E. Ratzlaff (76). This software was modified to apply to the present system configuration. In general, these low-level FORTH words control the position of the

syringes and valves by sending a TTL pulse to the associated solenoids. The position of any of these devices is monitored by the microcomputer via the controller status bits.

In Block 10 of MICRO.FTH, the addresses for the various input and output ports are assigned names so that these values may be used in subsequent definitions. The ports are then specifically set as either input or output using SFSET which writes a command into the control address, CNTRLP. In this case, the control command sets B port for output and A & C ports for input. SFSET fetches the status bits from CPORT and puts them on the stack as a decimal number. Using the information in the CPORT value, the user can write commands to the desired solenoid using CHANGE, which writes two consecutive values to BPORT in order to simulate a sudden pulse. The first value, which is supplied by the programmer, is the decimal equivalent of the appropriate binary number corresponding to the desired output bit; the second value is zero. When CHANGE is executed, the solenoid receives a low-high-low signal.

SFSTAT, CHANGE and the logical commands, AND and IF, are used to define the next nine FORTH words. CKMAN checks if the controller is in manual mode by isolating the appropriate status bit. CKMAN is then used in all subsequent controller definitions which essentially allows the user to keep the controller in manual mode when an analysis is not being performed. The controller is put into either manual

or auto modes by the commands MANUAL or AUTO, respectively.

The words which change the position of the syringes and valves follow the same basic pattern. First, the controller is checked for manual mode then AUTO is used to put the controller into auto mode if it was previously in manual. SFSTAT then fetches the status bits to which an appropriate number is logically added using AND. IF tests the results of the addition in order to determine the position of the device being controlled, then CHANGE pulses the solenoid only when the IF statement is true. The controller is put back into manual mode after the operation is complete. Using this sequence, 3VINTAKE and 3VMIX turn the 3-way inlet valve to the intake and mix positions; FILL and PUSH fill and pressurize the drive syringes; and WVOPEN and WVCLOSE open and close the waste release valve.

Blocks 12 and 13 contain the software for timing the analysis sequence. As previously mentioned, the microcomputer contains a timer board built around the Am9513 chip which contains five independently programmable counters. Each counter can be controlled by writing commands to a specific address; and each can also store values in data registers by writing the values to a different address. These addresses were defined as constants and given the names CMD for the command address and DRG for the data register address. INIT initializes the counters by resetting the clock and loading the counters with the desired

values. Specifically, INIT performs the following functions: resets the Am9513 chip, loads all counters for start-up, selects an 8-bit bus, disables data pointer sequencing, sets Master Mode, sets the counter mode for counter 1, loads counter 1 with the value 10, and sets the counter mode for counter 2. For this application, counter 1 is set to count down repetitively from 10 to 0 at 100 Hz. Counter 2 counts down at 10 Hz using the output from counter 1. 1DELAY and 3DELAY are 1 and 3 second delays for sequencing the instrument operations. These delay commands are comprised of the following definitions: LOADC2, which is a means of loading the desired value into counter 2 load register; SET, which loads and arms counters 1 and 2; and TIME, which is an infinite loop that monitors the value in counter 2 hold register and exits when the value equals 1.

The timer commands are coupled with the instrument control commands to obtain the desired sequence of events. PREP prepares the stopped-flow apparatus for analysis by filling the drive syringes and leaving the 3-way inlet valve in the mix position. The length of the delay times were chosen to allow the apparatus to complete each operation before the next event occurs. RINSE is used to clean the system by using the previously defined words in a loop. Once the drive syringes are pressurized, the waste release valve is opened and closed a total of 6 times until the syringes are emptied.

TRGR also uses an infinite loop to test the trigger status bit for a low TTL signal. The status bits are fetched by SFSTAT and the third bit is isolated rwith a logical AND statement. This definition is used in the data acquisition routines discussed below.

2. Data acquisition software

The data acquisition routines utilize counter 4 of the Am9513 timer chip. The definition CLOCK sets counter 4 to count at 10 kHz with an active high Terminal Count (TC) pulse. The TC pulse is one of the types of counter outputs available to the programmer which sends a high TTL pulse to another device when the count in the counter load register reaches zero. In this case, counter 4 load register is loaded with the data acquisition rate stored in the variable PERIOD. This value is multiplied by 10 resulting in a maximum acquisition rate of 1 kHz if the value in PERIOD is 1. A separate clock command, .CLOCK, is used for the data acquisition delay. .CLOCK is defined in the same manner as CLOCK with the difference being the value in .PERIOD is not multiplied by 10. The delay routine, to be discussed further below, allows several milliseconds to elapse after the flow is stopped so that any mixing disturbances can be avoided.

The commands for data acquisition at the lowest level are written in assembly in order to obtain data at the fastest possible speeds. The timer directly controls the

data acquisition since the output from counter 4 is wired to an interrupt handler. Each pulse from counter 4 results in one voltage value being converted and stored via the commands WAIT and TAKE (82). WAIT is composed of a sequence of disabling and enabling the interrupt handler in order to avoid spurious noise which previously had caused problems in other applications using this microcomputer (76). TAKE converts and stores a digitized voltage value while simultaneously incrementing a variable, BFPTR, which holds the address of the variable used for raw data storage. MUX and SAMPLE control the 4 sample-and-hold ports by designating which of the ports are enabled as well as which have sample-and-hold capabilities. The commands 0 MUX and 0 SAMPLE designate all 4 input ports as enabled and having sample-and-hold.

The low-level data acquisition words are used in higher level FORTH definitions. ACQUIRE is an acquisition command using a DO loop to obtain the desired number of data points. First, the timer is set and counter 4 is loaded and armed. Then the number of data points are obtained from the variable NPOINT which defines the number of loops and thus the number of points taken. SFRUN was defined as a test loop for data acquisition and trigger operation. The word "DONE" was printed on the screen after the designated number of data points were taken and stored in the variable, B1.

SUM and AVERAGE are used to add a series of converted

values and compute an average. SUM simply takes a certain number of data points, divides each by 16, and stores the sum in the variable, B2. The values are divided by 16 because the ADC supplies 12-bit values with 4 leading zeros. AVERAGE fetches the value stored in B2, divides it by the total number of points in NPOINT, and stores the final result in B3.

GETLIGHT and GETDARK then use SUM and AVERAGE to obtain average values for 100% transmission and dark current. These two words are defined in essentially the same manner. The value in PERIOD is stored in a variable, X, so that the original value can be recalled after the operation is completed. The address for B1 is then stored in BFPTR and the values are acquired, averaged and stored in appropriately named variables.

ACQDLY supplies a millisecond delay before data acquisition begins. The timer is first set then BFPTR is loaded with the address for B1. Then data are taken as if in an actual analysis; however, the values are not used. The value for the desired millisecond delay is stored in DLY.

Data acquisition and instrument control commands are combined to define ANALYZE. This word is usually used after PREP during a typical analysis. When ANALYZE is executed, the drive syringes are pressurized and one push is completed by opening and closing the waste release valve. Then a DO

loop is used to perform a certain number of pushes and data acquisitions based on the value stored in NPUSH. The values are stored as separate datasets in the variable, DATA0 which can hold 4 datasets containing up to 100 16-bit values. Each dataset can be dealt with individually by incrementing into DATA0 by some multiple of 200 using ?DATASET. After data acquisition is complete, a final push is performed. The stopped-flow instrument is capable of a total of 6 pushes with the present configuration, but the first and last pushes are not used to acquire data. If a delay is desired, ACQDLY must be inserted in ANALYZE after the word TRGR. Since ACQDLY takes a certain amount of time to execute, it is often omitted for very fast reactions.

PR3 and PRDATA print the acquired data on the screen in different forms. PR3 prints the values stored in B1 as %T corrected for dark current. The values are scrolled across the screen in a column, separated by a short delay time using a DO loop. PRDATA prints the contents of the desired dataset from DATA0 as %T in the same manner as PR3.

DPREP increments through the total number of data points stored in DATA0 and divides them by 16 in order to eliminate the leading zeros as previously discussed. This definition uses nested DO loops to convert the acquired values by dataset. DPREP takes into account the number of pushes and the number of data points contained in each set.

The 4 datasets in DATA0 can be averaged point by point

using AVRG. The values, the total number being defined by NPOINT, are averaged and stored in the first 200 locations of DATA0, namely dataset 0.

Numeric input is made possible by the word JUST (83). The words necessary to define JUST are given in Block 20. JUST allows the input of values for the various parameters associated with an analysis. This command is used along with a prompt to which the user replies with a RETURN if the value is acceptable or with a new value as desired.

3. Data transfer software

Software was also written for the transfer of data from the microcomputer to the minicomputer (76). PREFLUSH transfers the data from DATA0 to the buffer by first emptying the buffer using the FORTH command, EB, erasing one block in the buffer, then transferring the data by dataset using <CMOVE. The address marking the beginning of buffer 1 is obtained by using the command, FIRST @ and the number of datasets transferred to the buffer is contained in the variable, #DAT. TRANSFER identifies the FORTH block number where the data will be stored, then sends the data to the PDP 11/23. TRANSFER consists of IDENTIFY which identifies the block which will hold the data, UPDATE which marks the block as modified and set for immediate transfer, and DDISK which renames the data file as a default file in 11SPEC so that the data may be serially transmitted to the minicomputer using the FORTH command FLUSH.

The block to be sent to the minicomputer can be identified with a string of up to 80 characters using LABEL (83). LABEL allows the user to enter an identifier string or press RETURN to accept a previous string. This command uses the FORTH word, PAD, which returns the beginning address of a scratch area used to hold character strings for intermediate processing.

The parameter values are placed in the buffer in a pre-defined arrangement so that the FORTRAN conversion program can properly locate the data. BLFILL, which makes use of SITE, is used to move the data into the buffer according to a 32X16 matrix. The value at the designated address is moved to a specific array position defined by SITE, which simply increments into the buffer using the address supplied by FIRST @. SITE requires two numbers on the stack before execution, the top number is the row and the second number is the column of the array. Since the positions remain constant, the conversion program can be easily written using the values found at these locations.

4. Analysis sequence

During a typical analysis sequence, the user is prompted to enter values for various parameters. These input routines follow a similar format: the first statement is a prompt which request the user to enter a value, the prompt is followed by a zero after which the address for the desired variable is put on the stack and JUST is executed.

The data routines are named NPTS, NPSH, DATRATE, TAKDLY, NDSTS, DBLOCK, and OPNUM which request input for the number of points, the number of pushes, the data acquisition rate, the length of the delay, the number of datasets, the block number, and the analysis option number, respectively. The corresponding prompt statements are named NPRMT, PUPRMT, DAPRMT, DLPRMT, DTPRMT, BLPRMT, and OPPRMT. As with the command, LABEL, the user may enter a new value or accept the present value.

The analysis option is selected by the word, GO, which contains the three options: .GO, 4AVE, and 8AVE. .GO prepares the instrument for analysis, performs the analysis, collects the data, and prepares the data for transfer to the minicomputer. 4AVE uses .GO followed by AVRG to average the four datasets and store the results in dataset 0. 8AVE averages eight datasets and stores the final result in dataset 0 by performing .AVRG which essentially averages 2 sets of 4 previously averaged datasets. Ensemble averaging of this type is employed to improve precision. Another data storage variable, .DATA0, is used in .AVRG to hold intermediate results.

Several high-level words were defined to develop an analysis sequence for stopped-flow mixing. The command, SF, prints some introductory comments and the Options List on the screen. The Options List contains information on six options available to the analyst: PAR, ANA, STO, RNS, BREAK,

and OPLIST. PAR allows numeric input of the desired parameter values in response to a series of prompts. The prompts are contained in the word, ?LOOK. ANA directs the user to obtain 100%T and dark current values then describes the three analysis options after which a prompt allows input of the desired option. STO prompts the user to enter several data storage parameter values then type SEND to upload the data to the minicomputer. SEND is comprised of three previously defined words, PREFLUSH, BLFILL, and TRANSFER, which prepare the data, move the data to the buffer, then transfer the data to the PDP 11/23. RNS gives the instruction to type CLEAN to rinse the system. CLEAN uses PREP and RINSE to send the instrument through the motions of an analysis without data acquisition. BREAK is not a definitions but is a key on the terminal which can be used to exit an operation at any time. Finally, OPLIST prints the Options List on the screen.

The information gathered in an analysis can be immediately observed on an oscilloscope using a background routine for digital-to-analog (DAC) conversion (83). A separate stack is created for this routine by the words, BACKGROUND and BUILD. An assembly word, TRYIT, was written to rapidly send the data from a particular storage variable to the DAC output. TRYIT is used in the definition SCOPE which is an infinite loop capable of continuously sending data to the scope during an analysis. SCOPE requires that the address

of the storage variable and the number of points be on the stack before execution. Hitting the BREAK key will disable the background task and thus SCOPE must be re-entered to reactivate the task. When the blocks are loaded to begin an analysis, 100 points from DATA0 are automatically sent to the scope.

5. Minicomputer data conversion software

After the data are acquired and transferred to the minicomputer, a FORTRAN data conversion program, CRUNCH, converts the digitized voltage values to absorbance. The computer printout for CRUNCH can also be found in Appendix B. This program puts the absorbance and time values in a form readable by the plotting routine, MULPLT, by assigning appropriate tags. The program requires that the values be in an existing RSX file, so the FORTH type file must be renamed as an RSX file before the conversion routine can be used. CRUNCH first uses EQUIVALENCE statements to handle the data in an array format. The data points and parameter values are read by the conversion program in exactly the same manner as the values were loaded into the block before transfer. The block description and parameter values are then printed on the screen, and the user is prompted to select which dataset is to be converted. The program then converts the data to absorbance and time pairs then inquires if another block is to be converted. If so, the program branches back and converts the next block. When all blocks

are converted, the program ends.

The data conversion/plotting sequence is aided by a short task routine called TAKE.CMD found in Appendix B. This routine streamlines the task of manipulating and plotting the data by automatically performing many functions which before were individually entered. TAKE.CMD renames the FORTH files as RSX files, runs the CRUNCH conversion program, initiates MULPLT to window a section of the absorbance vs. time curve, then performs a linear regression analysis on the data to obtain the slope of the line as well as a plot of the results. The routine then loops back to the beginning if another conversion is desired. A typical curve generated by this sequence is shown in Figure 7.

To begin an analysis, the FORTH blocks must be loaded into the microcomputer using the command, 9 LOAD. The word, SF, is immediately executed and the user is allowed to choose an analysis option. A unique feature of this style of programming is that the analyst is free to design the experiments in any order he choses because the program is not continuously running. The commands in the Options List may be executed at any time which gives the program great flexibility. After an analysis is complete, the data are sent to the PDP 11/23 and another analysis may be performed or the previously acquired data may be manipulated. The overall analysis sequence is summarized in a flow chart shown in Figure 8, which is the most common sequence used in

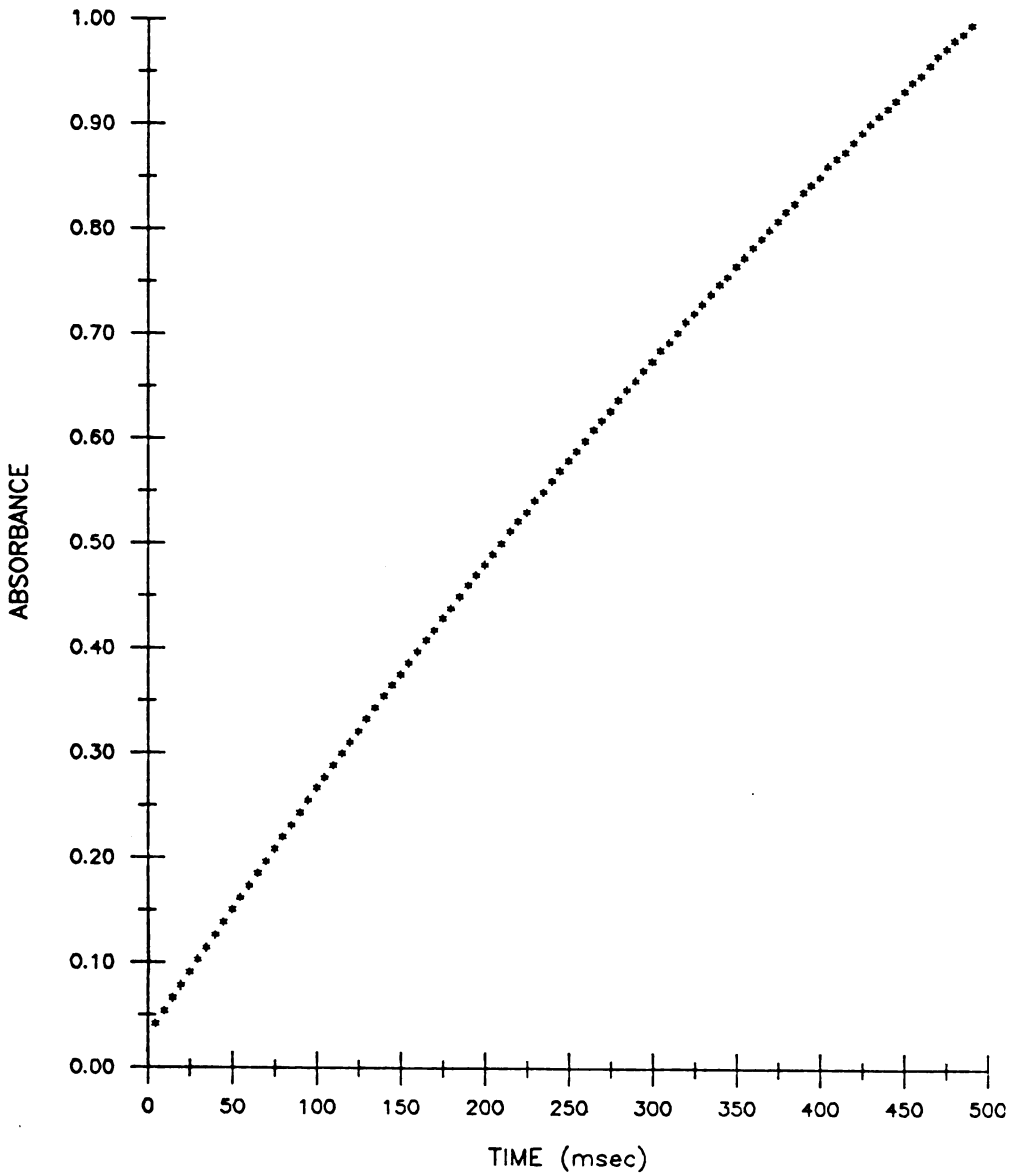
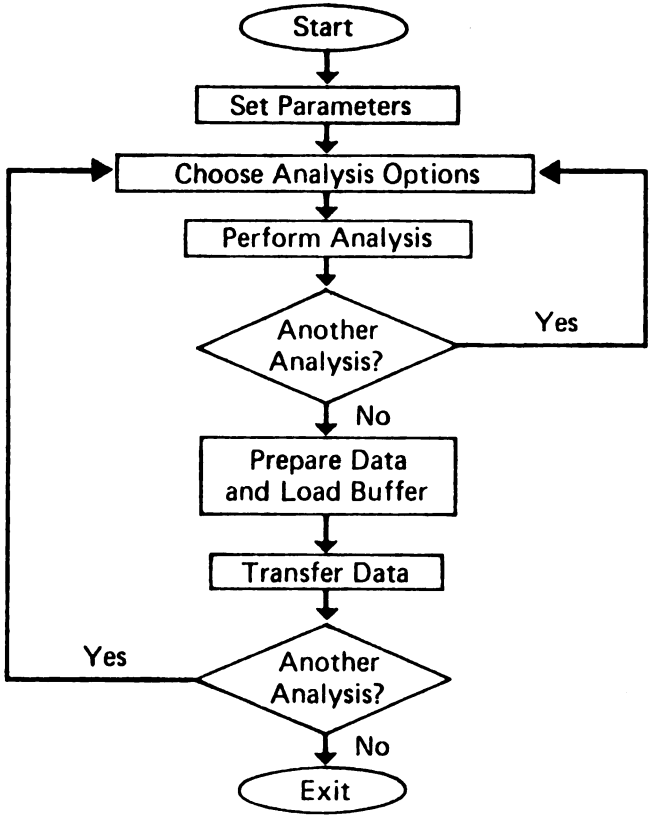


Figure 7. Typical absorbance vs. time curve.



Analysis Option . . .

- 4 pushes with no averaging
- 4 pushes with averaging
- 8 pushes with averaging

Figure 8. Analysis flowchart.

this project; however other formats are possible with this "open" programming design.

The software designed for this application contains enough flexibility to allow the user to easily obtain and manipulate valid analytical data; however, there are limitations. A maximum of eight pushes can be averaged to improve precision. If greater precision is required, the software would have to be changed to handle a larger number of pushes. Also, a maximum of 100 points can be acquired per run which limits the overall resolution of the results. Another limitation is that the data conversion program converts only one block at a time and so converting a large amount of data can be time consuming. The time for data manipulation was also increased because an interactive windowing function was not available. The linear portion of the absorbance vs. time curve had to be windowed using a special control key when using MULPLT which was difficult and unreliable. Finally, FORTH software is much slower than assembly and thus created problems during time-critical steps, especially during data acquisition. Assembly was used for the actual acquisition, however the FORTH words executed immediately before and after the assembly commands resulted in the loss of part of the reaction rate data. Valid data were still obtained, but attention must be given to the total elapsed time of the software written for rapid steps if a maximum amount of data is desired.

CHAPTER IV

KINETIC STUDIES

The modified stopped-flow apparatus and associated software was applied to two kinetic studies in order to evaluate the overall system performance and to acquire fundamental kinetic data on a useful analytical reaction. The chapter begins with a discussion of the iron-thiocyanate reaction used for a system performance evaluation followed by a presentation of a fundamental kinetic study of the Griess reaction for nitrite analysis.

A. Iron-Thiocyanate Reaction

The reaction of Fe^{3+} with SCN^- is a popular test reaction for the performance of stopped-flow instruments (84, 85, 40). It meets all the conditions for a good test reaction: 1) that the reaction has simple kinetic properties; 2) the reaction is accompanied by a significant change in the absorption spectrum; 3) the reaction velocity can be changed at will by changing one of the reaction conditions; 4) a half-life of less than 1 msec can be easily attained; 5) and reagents for the reaction are readily available and can be handled without difficulty. Another recommended reaction is

the reduction of 2,6-dichlorophenolindophenol by L-ascorbic acid at pH 2.0 (86).

The iron-thiocyanate reaction is actually reversible:

$$Fe^{3+} + SCN^{-} \rightleftharpoons Fe(SCN)^{2+} \qquad (5)$$

The following rate equation thus applies:

$$d[Fe(SCN)^{2+}]/dt = k_1 [Fe^{3+}][SCN^{-}] - k_{-1} [Fe(SCN)^{2+}] \qquad (6)$$

The reaction was used to measure the deadtime of the instrument, and to determine the accuracy and precision of the overall stopped-flow technique. The deadtime was measured using a graphical extrapolation technique (20); results were compared to previously determined values. The accuracy was checked by determining the rate of the FeSCN reaction and comparing measured rate constants to previous values. The precision was obtained by determining the rate of the reaction from 10 replicate runs then calculating the relative standard deviation.

1. Experimental

All solutions were prepared with reagent grade $FeCl_3$ $6H_2O$ and KSCN diluted with deionized distilled water (Millipore) in volumetric flasks. For all runs, the current

amplifier output was set so that the voltmeter reading was 9.90-9.95 volts (10 volts maximum). The gain was set to 10^7, the wavelength was 450nm, and the slit width was 2000 μm. For the accuracy and precision experiments, 100 points were taken over a range of 100 msec for each determination using 8 pushes with averaging. Roughly the first 20% of the absorbance vs. time curve was taken as the linear portion.

Each of the experiments follow the same analysis sequence: 1) down-load the software from the PDP 11/23 into the microcomputer; 2) enter the desired parameters when prompted; 3) place the sample inlet tubes into the solutions; 4) perform the analysis; 5) transfer the collected data to the minicomputer for further manipulation.

The solutions for the three experiments were prepared as follows:

Stock solutions: a 100 ml solution of 0.01M Fe^{3+} was prepared by dissolving 0.2706g $FeCl_3 \cdot 6H_2O$ in deionized distilled water (DDW). A 100 ml solution of 0.04M SCN^- was prepared by dissolving 0.3885g KSCN in DDW. The working solutions were prepared using an adjustable pipettor.

Deadtime calculation: $[Fe^{3+}]$ = 0.001M, $[SCN^-]$ = 0.004M, $[H_2SO_4]$ = 0.05M

Rate constant calculation: $[Fe^{3+}]$ = $1X10^{-4}$ to $1X10^{-3}$M (10 solutions), $[SCN^-]$ = 0.004M, $[H_2SO_4]$ = 0.05M

Precision study: $[Fe^{3+}]$ = $5X10^{-4}$M, $[SCN^-]$ = 0.004M,

$[H_2SO_4]$ = 0.05M

2. Results and Conclusions

The data conversion program accepts digitized voltage values and converts them to absorbance data which are plotted versus time using the MULPLT plotting routine. The linear portion of the curve is visually estimated by viewing the results on the graphics terminal. The desired portion is then windowed by setting the minimum and maximum of the x and y axes, replotting the curve and simultaneously pressing <cntrl> D to dump the windowed points into a data file. A linear regression analysis is then performed on the data to determine the slope of the line. Except for the deadtime calculation, this sequence was followed to obtain all rate data.

The deadtime was determined by the extrapolation technique (20) in triplicate and was found to be 5±1 msec. The deadtimes calculated by Beckwith (71) were also approximately 5 msec so the present value is in good agreement with previously determined deadtimes. The calculated value is small which indicates that modified stopped-flow apparatus is operating properly and can be used for quite fast reactions without introducing errors due to large deadtime effects.

The rate constant, calculated by determining the slope of the reaction rate vs. initial Fe^{3+} concentration curve and applying Eq. (3), is 273.4 M^{-1} sec^{-1}. The molar

absorptivity value at this wavelength is 4630 $\underline{M}^{-1}$ cm^{-1} (87). The results are summarized in Table 3 and shown graphically in Figure 9. This value is on the order of previously determined values (72), 200 - 400 $\underline{M}^{-1}$ cm^{-1}, although it is somewhat lower than expected. This deviation can be accredited to several factors: 1) a literature value for the molar absorptivity was used, 2) pH and ionic strength were not controlled in the present study. However, the fact that the value is fairly close to previous values proves that the system is providing valid rate data.

The results of the precision study are listed in Table 4. The relative standard deviation (RSD) was determined to be $\pm$1.474% which proves that the determination of reaction rates is satisfactorily reproducible. Previous precision studies on this apparatus have yielded RSD values of 1-3% (47).

B. Griess Reaction

Johann Peter Griess first described the formation of pigments from nitrosatable species (NS), nitrous acid, and coupling reagents(CR) in 1864 (88). The nitrosatable species are principally aniline derivatives and the coupling reagents are principally naphthalene derivatives. The Griess reaction is employed in the formation of diazo pigments in the dyestuff industry but is also extensively

Table 3. Data for FeSCN rate constant calculation

Initial $[Fe^{3+}]$ (μM)	Rate (abs. per. sec.)
100.0	.0241
200.0	.0483
300.0	.0703
400.0	.0991
500.0	.1230
600.0	.1500
700.0	.1740
800.0	.1990
900.0	.2260
1000.0	.2500

Slope = 2.532

Correlation = .9997

Rate Constant = 273.4 M^{-1} sec^{-1}

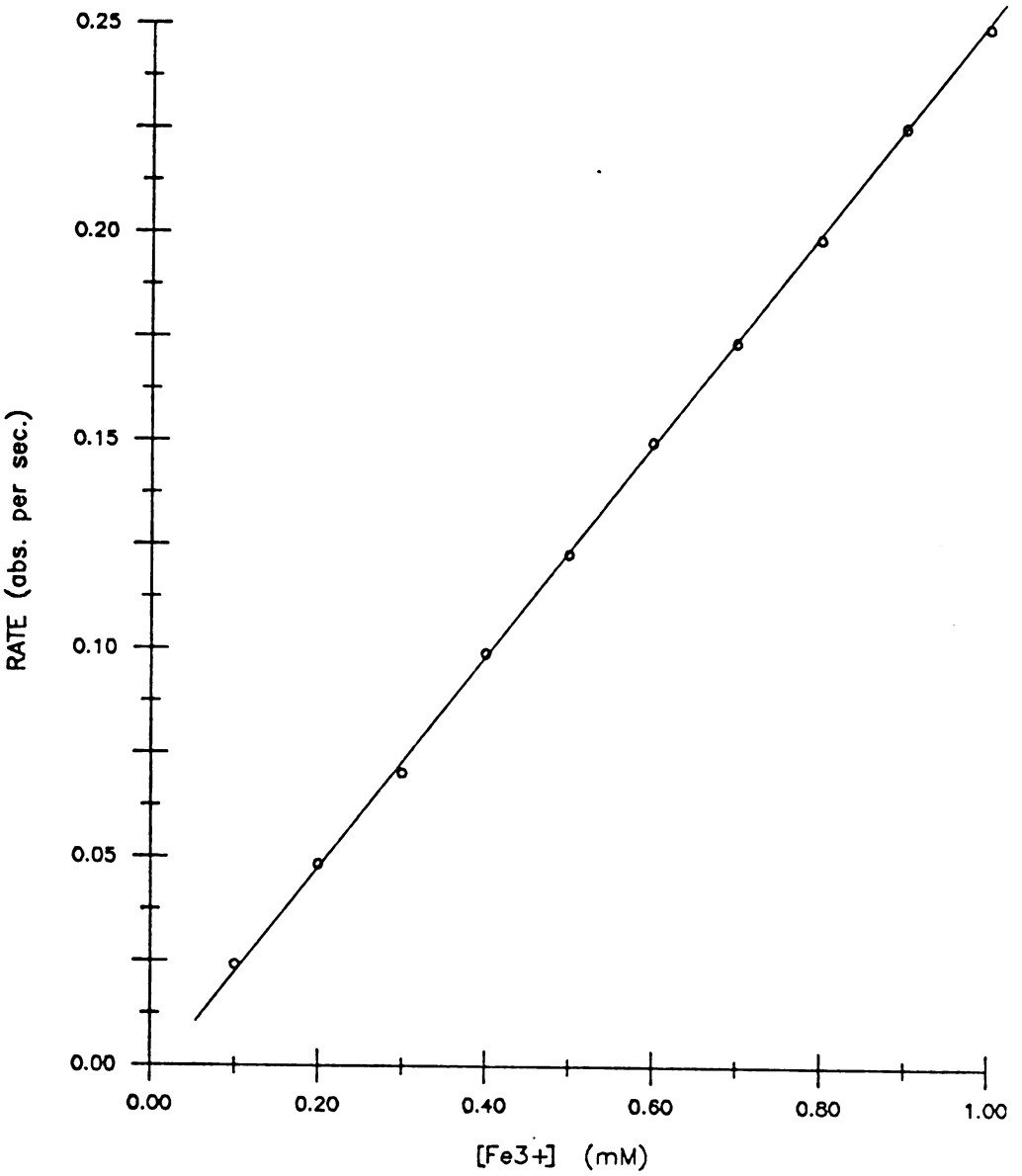


Figure 9. Dependence of rate of the iron-thiocyanate reaction on $[Fe^{3+}]$

Table 4. FeSCN precision study

Smpl	Rate (abs. per. msec)
1	0.1186×10^{-2}
2	0.1214×10^{-2}
3	0.1201×10^{-2}
4	0.1220×10^{-2}
5	0.1225×10^{-2}
6	0.1219×10^{-2}
7	0.1195×10^{-2}
8	0.1204×10^{-2}
9	0.1250×10^{-2}
10	0.1213×10^{-2}

$x = 0.1213 \times 10^{-2}$

$s = 1.788 \times 10^{-5}$

RSD = $\pm 1.474\%$

applied as a method for nitrite analysis, as first described by Griess in 1879 (89). Many different reagents for the Griess reaction have been employed under various conditions (90-92). The reaction occurs in three steps: nitrosation, diazonium ion formation, and coupling. The first and third steps are pH dependent and the second step is an internal rearrangement. In this study, the nitrosation and diazotization steps were not individually examined but were combined to constitute the first step. The coupling reaction was thus considered to be the second step.

The Griess reaction was recently studied by Fox in 1979 (93). In this case, several reagent combinations were employed under limited conditions in order to define their relative effects on pigment formation. Also, various factors critical to pigment production, such as pH, temperature, and concentration of reagents were quantitated. Fox used acetic acid to obtain a pH range of 2.75-2.85 based on a previous report which claimed that maximal pigment formation occurs in this range for the reagent combinations tested (92). The results of this study are valid for the conditions used, however these conditions are not the most commonly used conditions in practical analysis procedures.

Thus, a fundamental study has been performed to define the kinetics of the Griess reaction using the most common reagent combination and conditions. The reagents used most frequently for nitrite analysis are sulfanilamide (SAN) and

N-1-naphthylethylenediamine (NED). SAN has been shown to be one of the faster reacting nitrosatable species (93) and NED is used for its greater rapidity of coupling and increased acid solubility of the pigment formed (94). The analysis is most commonly performed in highly acidic solutions (approximately pH 1) for maximal speed of the diazotization reaction (95).

The study was proposed to define the kinetics of the Griess reaction by determining the orders, rate constants, and molar absorptivities of the individual steps, the effects of pH and ionic strength, and a probable mechanism. The results of these experiments, and the conclusions drawn from the data are presented below.

1. Experimental

All solutions were prepared using reagent grade sulfanilamide, N-1-naphthylethylenediamine dihydrochloride, and sodium nitrite diluted with acidified deionized distilled water (DDW). The acidified diluent was prepared by diluting 10 ml of 12 M HCl to 1 liter with DDW. The pH of the resulting solution was approximately 1.20.

A 5 mM nitrite stock solution was prepared by dissolving 0.3450 g $NaNO_2$ in 1 liter of the acidified diluent. SAN and NED stock solutions were prepared daily as they decompose on standing.

The experimental conditions were as follows: temperature = 25.0°C, gain = 10^7, slit width = 2000 μm, and for each determination, 100 data points were taken over 1 second using the analysis option of 8 pushes with averaging. The current amplifier output was adjusted so that the digital voltmeter reading was between 9.90 and 9.95 volts (10 volts maximum). The working solutions, which were prepared with an adjustable pipettor and 100 ml volumetric flasks, were allowed to equilibrate to 25.0°C in the water bath for approximately 30 minutes before analysis. Then the analysis sequence outlined in the FeSCN experimental section was followed. Any special conditions are described in the experimental sections that follow.

Determination of orders:

For the first step (diazonium ion formation), the rate dependence on the $[NO_2^-]$ was determined by running 10 reactions with limiting nitrite concentration and SAN in excess (100 X $[NO_2^-]$). For this study, $[NO_2^-]$ = 10 - 100 μM, and [SAN] = 10 mM. The diazonium ion formation was monitored at 310 nm (96).

For the second step (coupling reaction), the rate dependence was determined on both the nitrite and NED concentrations. The rate dependence on nitrite was determined by running 10 samples at limiting nitrite concentration, 10 - 100 μM, and [SAN] = [NED] = 10 mM. The rate dependence on NED was similarly determined by running 10 samples at

limiting NED concentration, 10 - 100 M, and [SAN] = [NO_2^-] = 10 mM. In order to determine the NED dependence, the SAN and NO_2^- were pre-reacted, for each concentration of NED, by mixing 10 ml of each solution at 25.0°C approximately 45 minutes before analysis. The wavelength used to monitor the coupling reaction was determined by scanning a typical reaction mixture containing the Griess pigment over the range of 500 to 600 nm on a Beckman DB-G spectrophotometer. The maximum was reached at 535 nm which exactly matches the literature value (93).

The overall orders were checked for each step by running typical reagent concentrations to obtain absorbance values which were then used to plot the integrated form of the rate equation versus time. For the first step, [NO_2^-] = 50 μM, and [SAN] = 10 mM. For the second step, [NO_2^-] = 20 μM, and [SAN] = [NED] = 10 mM.

Determination of rate constants:

The rate constant for the first step was determined by running a series of 10 samples at limiting nitrite concentration, 10 - 100 μM, with SAN in excess at 10 mM.

For the second step, the rate constant was determined against both limiting nitrite and limiting NED concentrations. In the case of limiting nitrite, [NO_2^-] = 10 - 100 μM, and [SAN] = [NED] = 10 mM. In the case of limiting NED, [NED] = 10 - 100 μM, and [SAN] = [NO_2^-] = 10 mM.

For the simultaneous reaction, a combined reagent

solution was prepared by mixing equal volumes of 20 m$\underline{M}$ SAN and 20 m$\underline{M}$ NED yielding 10 m$\underline{M}$ of each reactant. This reagent was then reacted with a series of nitrite solutions in the range of 10 - 100 $\mu\underline{M}$.

Ionic strength studies:

Ionic strength effects were determined using the same concentrations of reactants with varying ionic strengths. The ionic strength was increased by the addition of sodium chloride.

For the first step, [SAN] = 10 m$\underline{M}$, and $[NO_2^-]$ = 20 $\mu\underline{M}$. Six reactions were run over an approximate ionic strength range of 0.1 to 2.4 by adding 0, 0.5, 1.0, 1.5, and 2.0 g NaCl to 15 ml of each working solution.

For the second step, 10 m$\underline{M}$ SAN and 20 $\mu\underline{M}$ nitrite solutions were pre-reacted for 45 minutes at 25°C before the addition of NaCl over the identical range as in the first step. The pre-reacted solutions were then reacted with 10 m$\underline{M}$ NED solutions containing equivalent amounts of NaCl.

pH study:

The pH study was performed on the first step only. The concentrations of the reactants remained constant and the pH varied over the range of 0.8 to 3.0. In this experiment, [SAN] = 10 m$\underline{M}$, and $[NO_2^-]$ = 100 $\mu\underline{M}$. Six reactions were run at pH values of 0.8, 1.0, 1.5, 2.0, 2.5, and 3.0 by adjusting the pH of the working solutions with concentrated HCl and NaOH.

Determination of molar absorptivities:

The molar absorptivities were determined for the first and second steps, and for the simultaneous reaction by reacting appropriate solutions to completion, at least 45 minutes, then measuring the absorbance of the solutions with a Beckman DB-G spectrophotometer in a 1.0 cm cell. In each case, three reactions were run with [SAN] = [NED] = 10 m$\underline{M}$, and $[NO_2^-]$ = 2, 5, and 10 $\mu\underline{M}$, and the results were averaged. The first step absorbances were measured at 310 nm and the second step and simultaneous reaction absorbances were measured at 535 nm.

2. Results

Calculation of orders:

For each reaction step, the orders were calculated by plotting the logarithm of the reaction rate against the logarithm of the initial concentration of the limiting reactant, after dilution. The slope of the line directly gives the order of the reaction due to that reactant. The rationale for using logarithms can be explained mathematically by first considering the simple relationship between the rate and a reacting species:

$$\text{rate} = k[A]^n \qquad (7)$$

Taking the logarithm of both sides of the equation yields:

$$\log(\text{rate}) = \log(k) + n\log([A]) \qquad (8)$$

Thus, plotting the logarithm of the rate vs. the logarithm of the initial concentration of the reacting species gives a straight line with slope "n" which is the order of the reaction due to that species.

The complete results of the first and second step experiments for calculation of orders can be found in Table 5 which lists the logarithms of the rates and initial concentrations, the correlation values for the respective slopes, and the individual orders obtained. The results are graphically represented in Figures 10 - 12.

Overall orders:

The overall order for the second step (coupling reaction) was obtained by adding the individual orders for the nitrite and the NED, which equals 1.974. The overall order for the first step could not be calculated in this manner because the order for the SAN was not obtained (SAN is always in excess). However, a study was performed which confirmed that SAN and nitrite react stoichiometrically in the presence of acid (97). Thus the first and second steps were assumed to be second order overall and were verified by plotting the integrated form of the rate equation for a second order reaction vs. time. A straight line confirms a

Table 5. Results of order calculation experiments

First step (limiting $[NO_2^-]$)		Second step (limiting $[NO_2^-]$)		Second step (limiting [NED])	
log $[NO_2^-]$	log rate	log $[NO_2^-]$	log rate	log [NED]	log rate
-2.301	-1.304	-2.602	-.4162	-2.301	-.5511
-2.000	-.8499	-2.301	-.0796	-2.000	-.2240
-1.824	-.6714	-2.125	-.0300	-1.824	-.0156
-1.699	-.5491	-2.000	.1424	-1.699	.1113
-1.602	-.4363	-1.903	.2350	-1.602	.2124
-1.523	-.3951	-1.824	.2997	-1.523	.2827
-1.456	-.3150	-1.757	.3651	-1.456	.3627
-1.398	-.2570	-1.699	.4040	-1.398	.4201
-1.347	-.2155	-1.648	.4672	-1.347	.4739
-1.301	-.1544			-1.301	.5189
slope	1.096		.9056		1.068
correlation	.9688		.9901		.9994

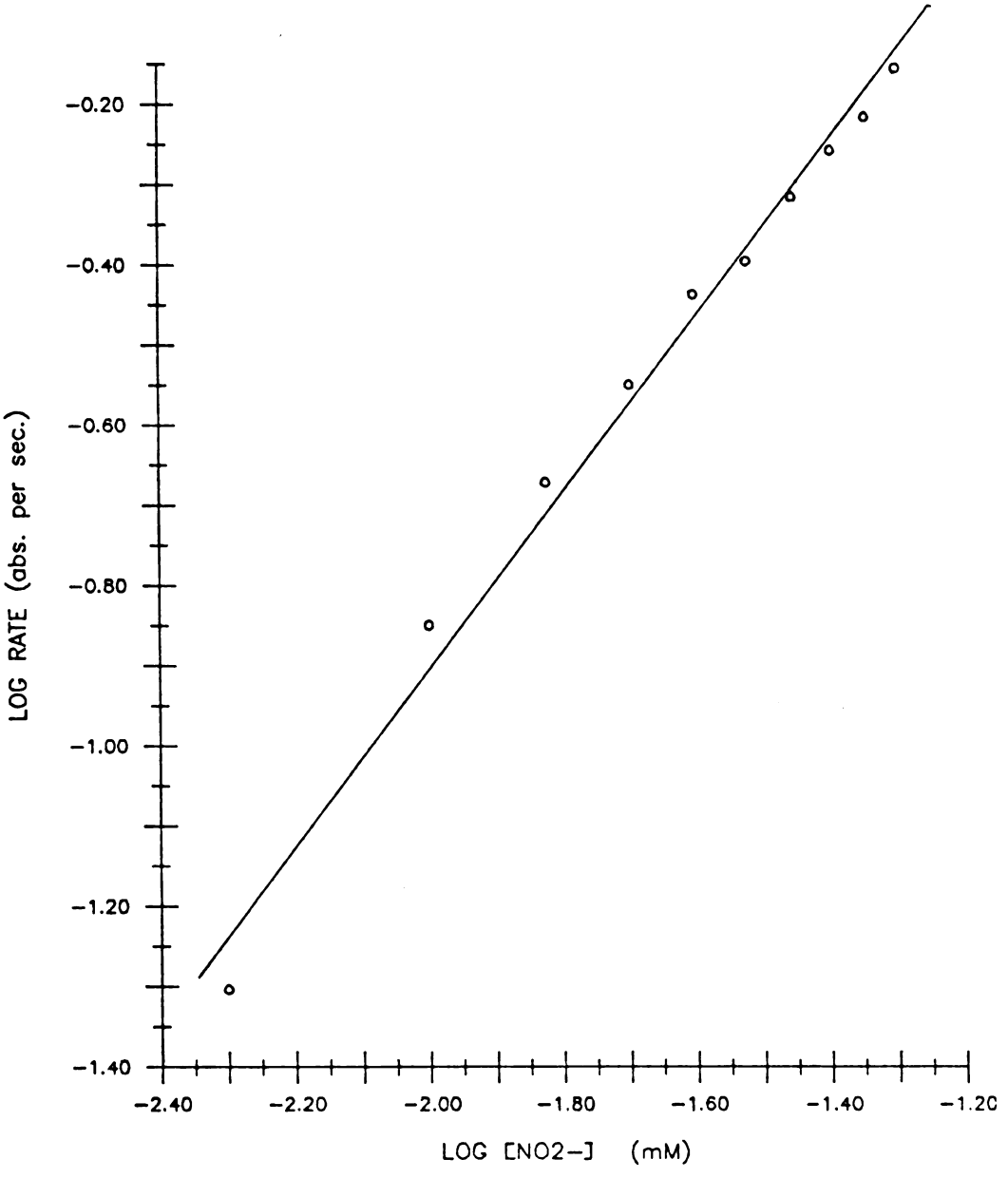


Figure 10. Calculation of order of the diazonium ion formation step due to NO_2^-.

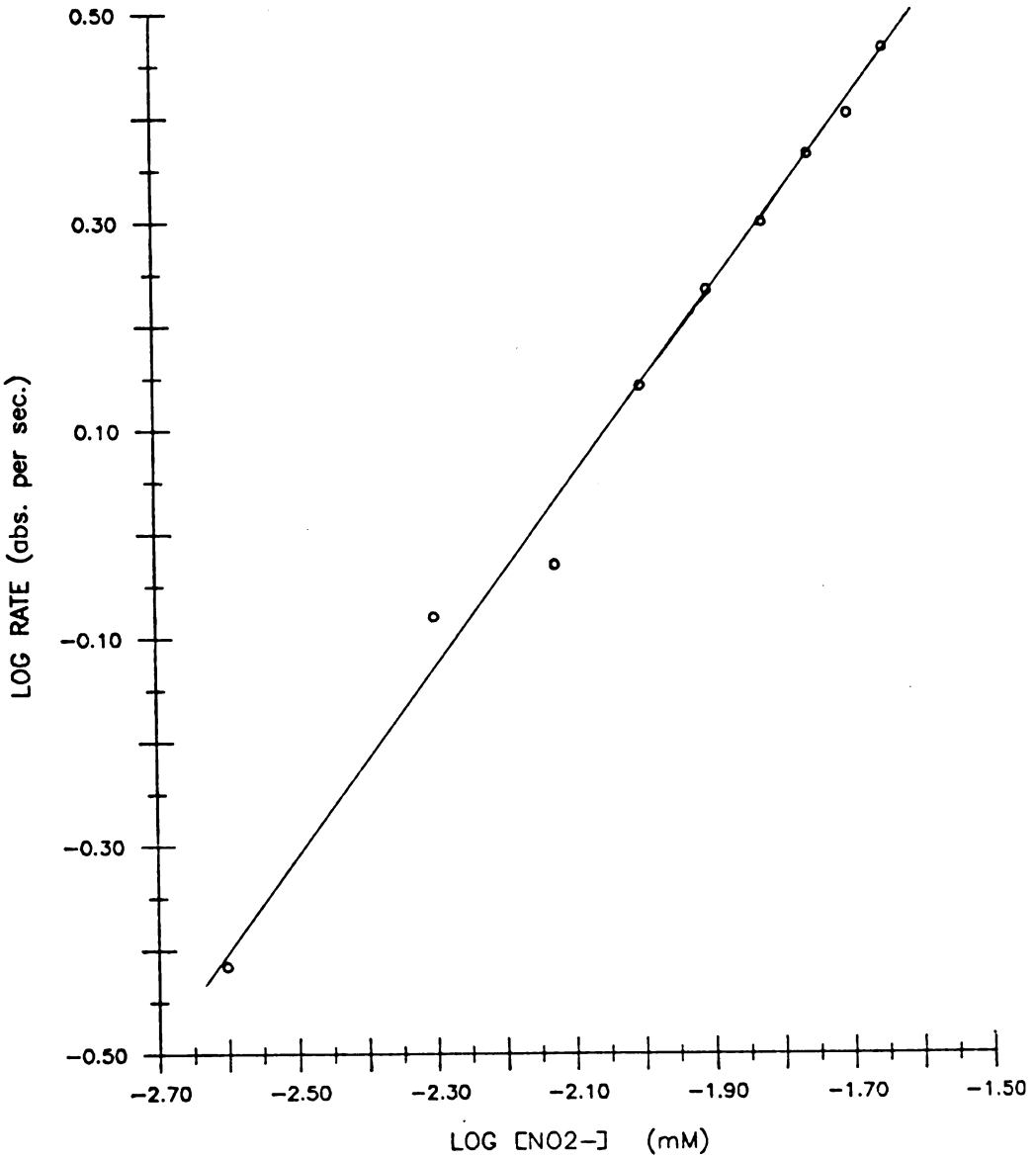


Figure 11. Calculation of order of the coupling step due to NO_2^-.

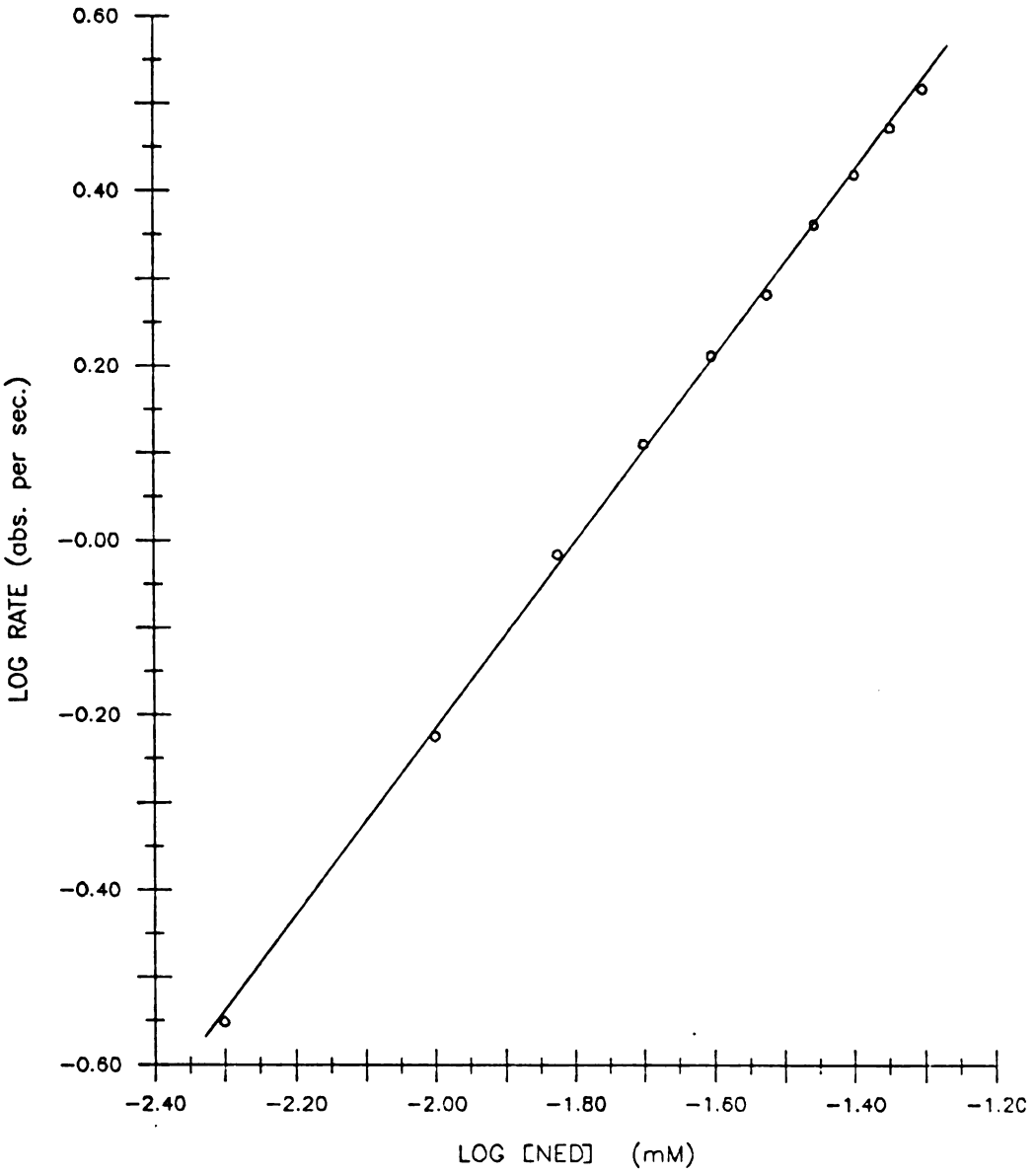


Figure 12. Calculation of order of the coupling step due to NED.

second order reaction. The integrated rate equation for a second order reaction, A + B $\longrightarrow$ products, takes the form:

$$\frac{1}{b-a}\left[\ln\left(\frac{a}{a-x}\right)-\ln\left(\frac{b}{b-x}\right)\right] = kt \qquad (9)$$

Where a = A_o, b = B_o, and x = the number of moles of A reacted at time = t. When the left-hand side is plotted against time, a straight indicates a second order reaction. The results of the overall order confirmation experiments for the first and second steps are listed in Table 6 and shown in Figures 13 and 14. The results indicate that the first and second steps are second order overall.

Calculation of rate constants:

For the first step, second step and simultaneous reaction, the rate constants were calculated using the slope of the reaction rate vs. initial concentration curve and the molar absorptivities for each step. These values were inserted into the rate equation for the desired step, which was solved for the rate constant, k. An example calculation is shown below:

For first step, limiting $[NO_2^-]$,

$$k = \frac{dA/dt}{[SAN]} = \frac{13910}{\varepsilon b\,[SAN]} = \frac{13910}{(2350)(2)(5x10\text{-}3)} = 592.0\ \underline{M}^{-1}\ s\text{-}1$$

The concentrations used in all calculations were initial concentrations after dilution, from both pre-reaction and

Table 6. Data for confirmation of overall order

First step		Second step	
time (msec)	value of integ. equation	time (msec)	value of integ. equation
20	113.3	20	8.962
40	122.8	100	34.24
60	131.9	200	65.04
80	141.2	300	95.37
100	149.0	400	124.8
120	156.4	500	154.4
140	165.5	600	182.2
160	172.8	700	208.7
180	180.6	800	234.8
200	188.6	900	259.9
		1000	281.9
correlation	.9986		.9979

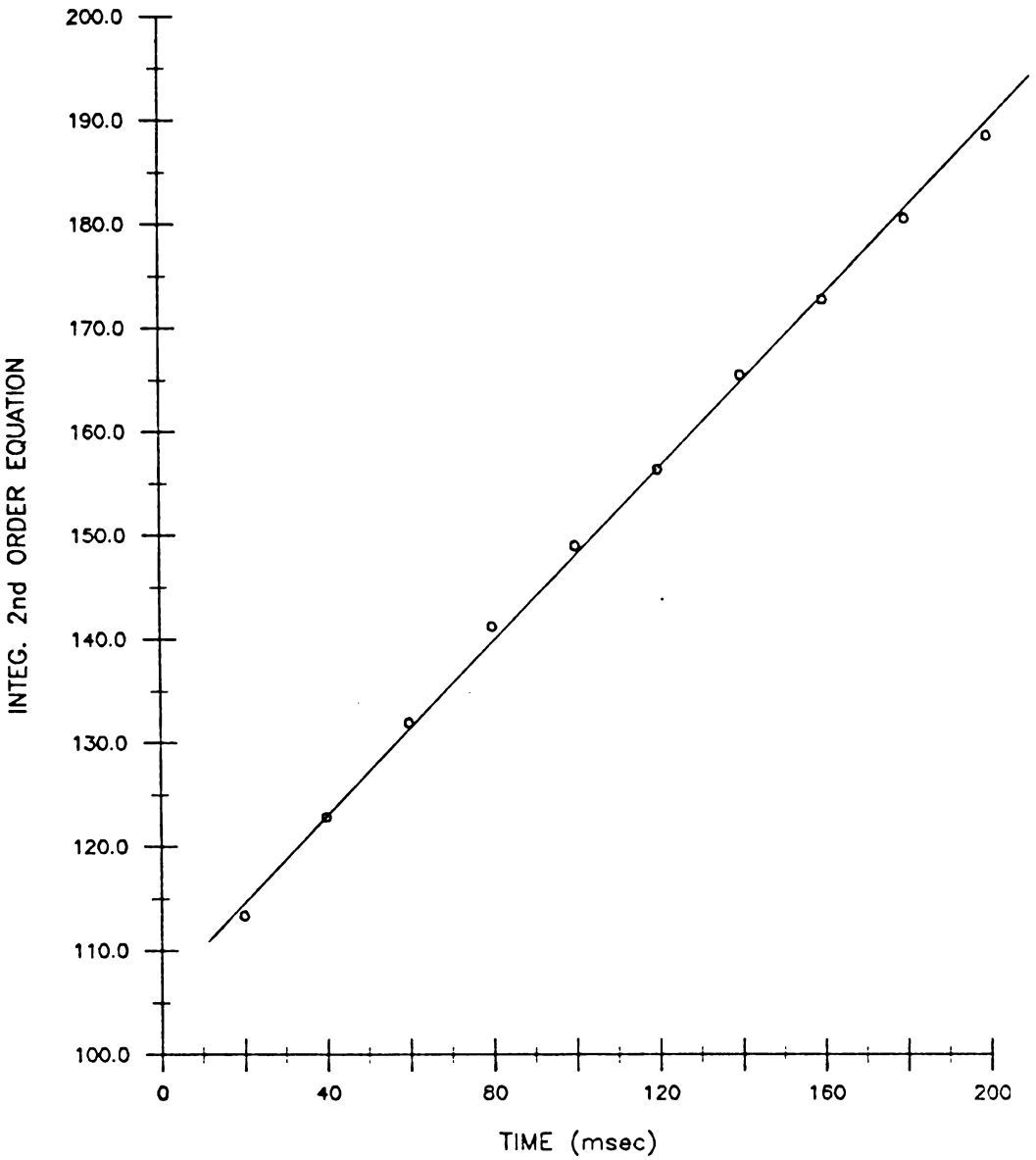


Figure 13. Confirmation of order of the diazonium ion formation.

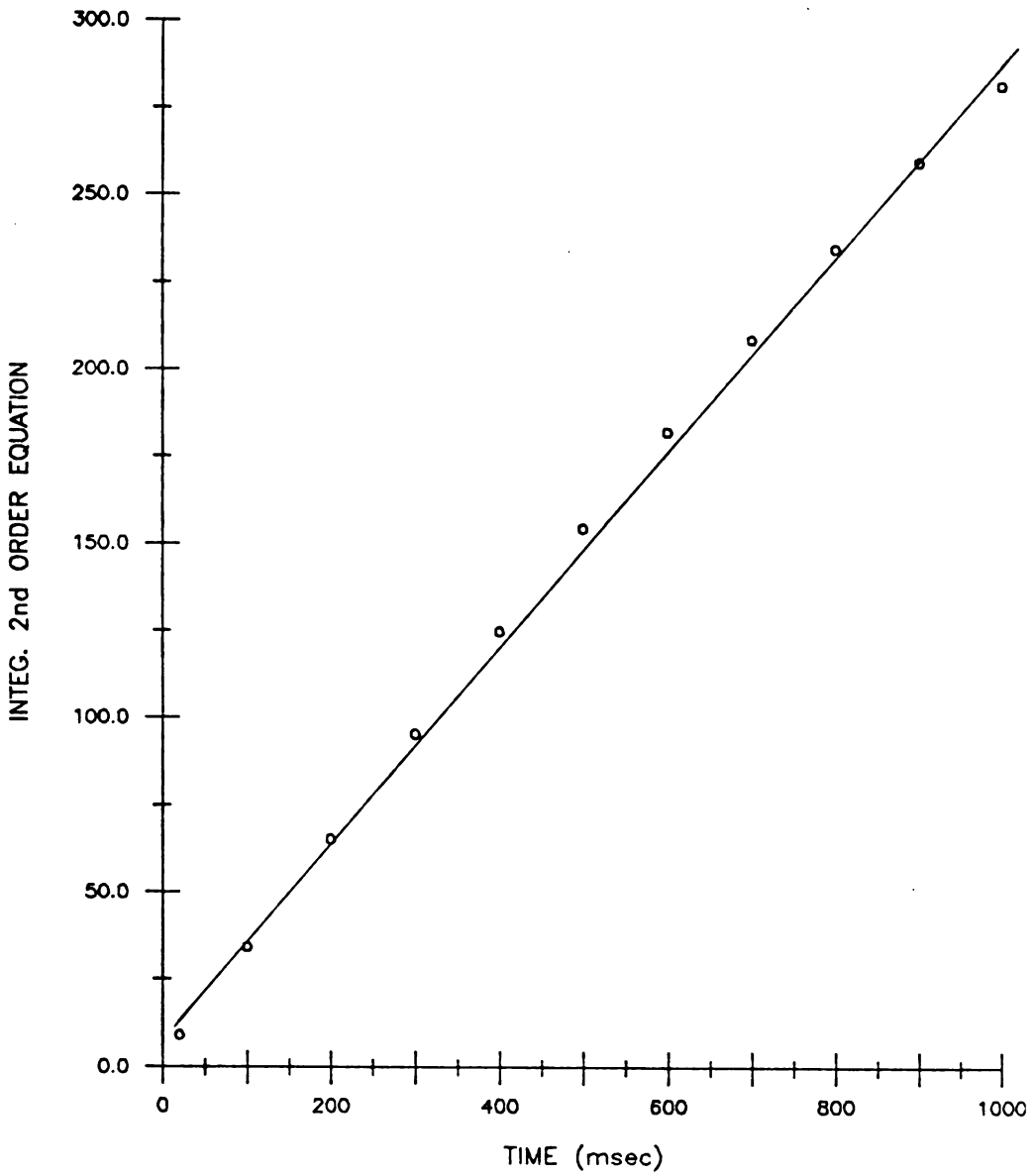


Figure 14. Confirmation of order of the coupling reaction.

mixing by the stopped-flow apparatus. The data from the rate constant experiments are listed in Table 7 which gives the rates obtained for each reaction step, the slopes and associated correlation values, and the calculated rate constants. The results are also graphically shown in Figures 15 - 18.

Ionic strength studies:

The effects of high ionic strength on the first and second steps are shown in Figures 19 and 20. The raw data are listed in Table 8. The ionic strength values were calculated as the sum of the ionic strength from the acid diluent (0.12 M) and any added NaCl. The first point in each of the curves is the rate without NaCl added which serves as a reference point to observe the change in the rate as the ionic strength increases. The results indicate that increasing ionic strength has very little effect on the rate of the reaction as seen by the small change in the overall rate in each case.

pH study:

The effect of varying the pH in the diazonium ion formation step is shown in Figure 21 with the raw data listed in Table 9. Increasing the pH from 1.0 to 3.0 dramatically decreases the observed rate which indicates that lower pH values are more advantageous for analytical applications of the Griess reaction because of the higher speed of the first step.

Table 7. Rate constant data for Griess reaction

First step (limiting $[NO_2^-]$) $[NO_2^-]$ (μM)	rate	Second step (limiting $[NO_2^-]$) $[NO_2^-]$ (μM)	rate	Second step (limiting [NED]) [NED] (μM)	rate	Simultaneous reaction (limiting $[NO_2^-]$) $[NO_2^-]$ (μM)	rate
5.0	.0496	2.5	.3835	5.0	.2811	5.0	.0347
10.0	.1413	5.0	.8326	10.0	.5970	10.0	.0387
15.0	.2131	7.5	.9333	15.0	.9648	15.0	.0535
20.0	.2824	10.0	1.388	20.0	1.292	20.0	.0665
25.0	.3662	12.5	1.718	25.0	1.631	25.0	.0808
30.0	.4026	15.0	1.994	30.0	1.935	30.0	.0914
35.0	.4842	17.5	2.318	35.0	2.305	35.0	.1031
40.0	.5534	20.0	2.535	40.0	2.631	40.0	.1125
45.0	.6088	22.5	2.932	45.0	2.978	45.0	.1204
50.0	.7008			50.0	3.303	50.0	.1322
slope	13910		124500		67320		2260
correlation	.9968		.9943		.9968		.9936
rate constant	592.0 $M^{-1}s^{-1}$		240.4 $M^{-1}s^{-1}$		259.9 $M^{-1}s^{-1}$		21.5 $M^{-1}s^{-1}$

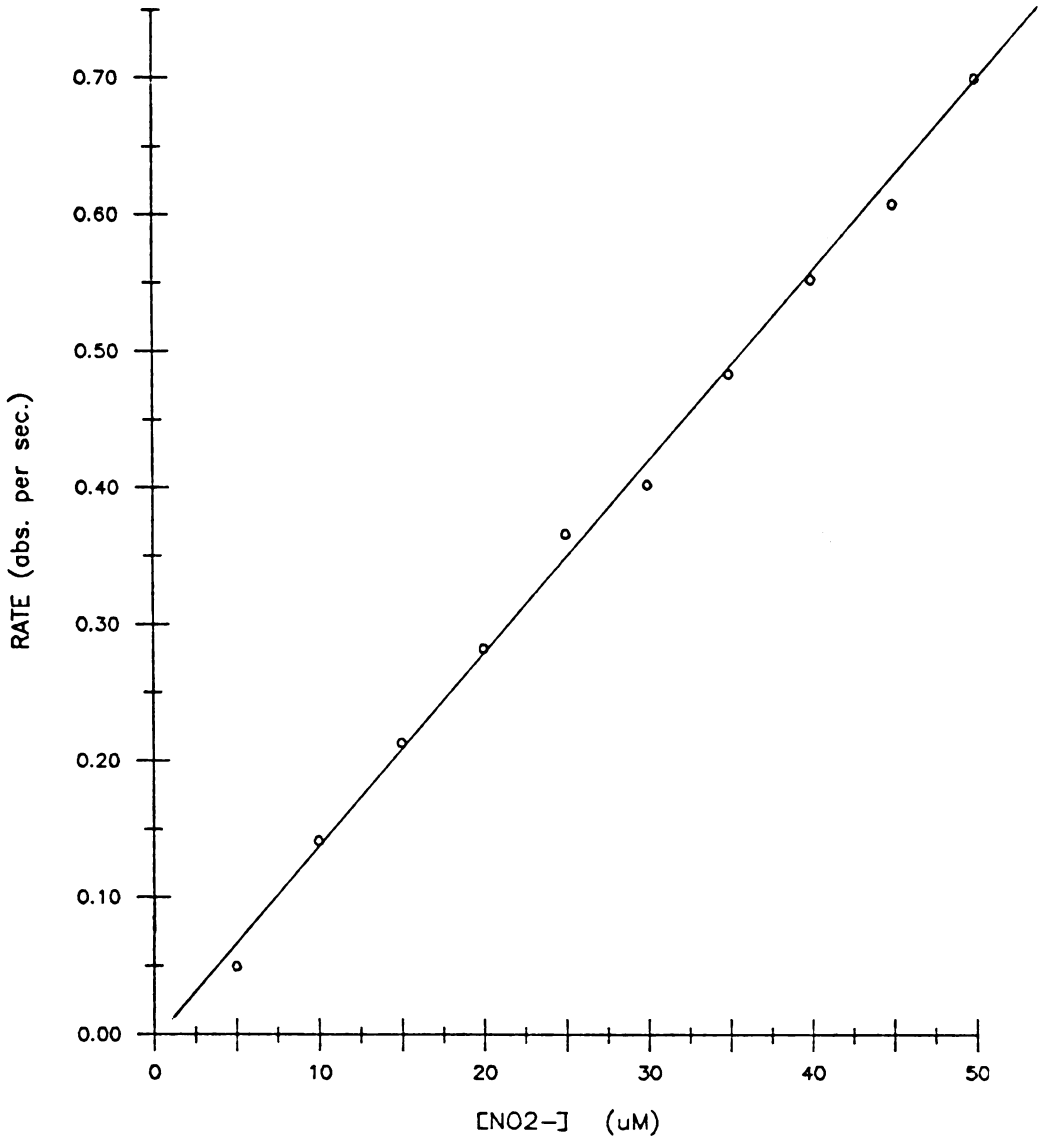


Figure 15. Dependence of the diazonium ion formation rate on $[NO_2^-]$.

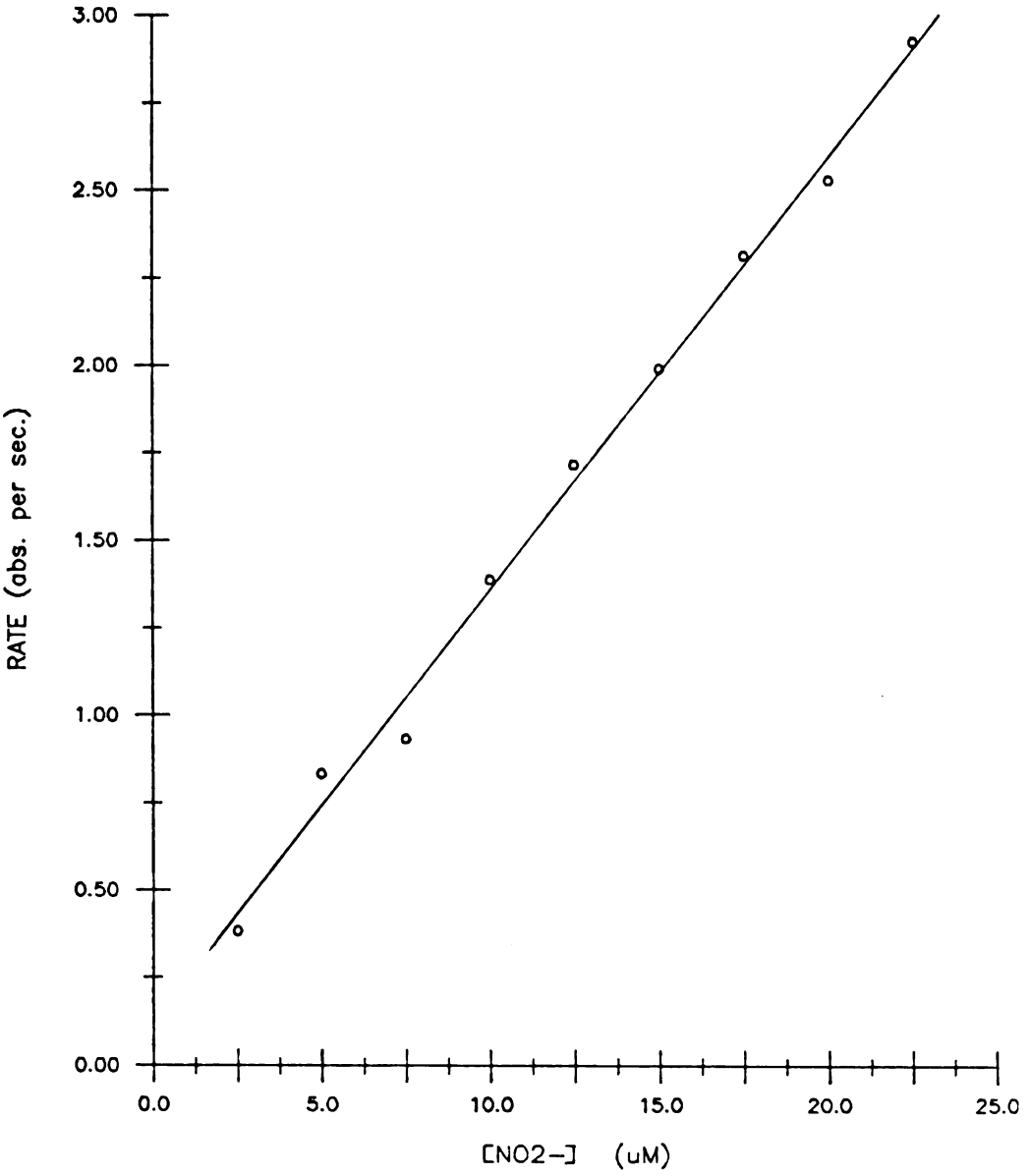


Figure 16. Dependence of rate of the coupling reaction on $[NO_2^-]$.

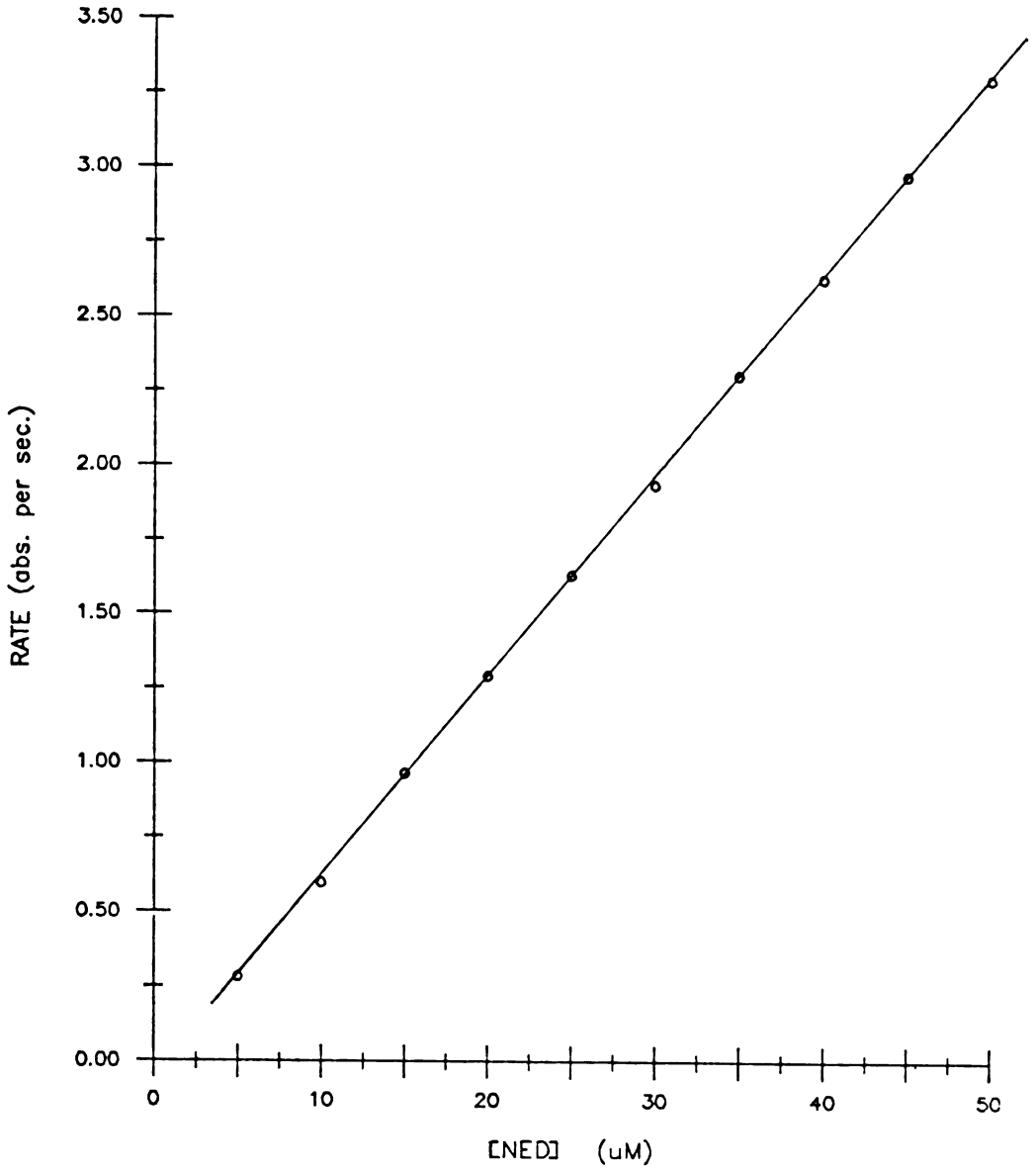


Figure 17. Dependence of rate of the coupling reaction on [NED].

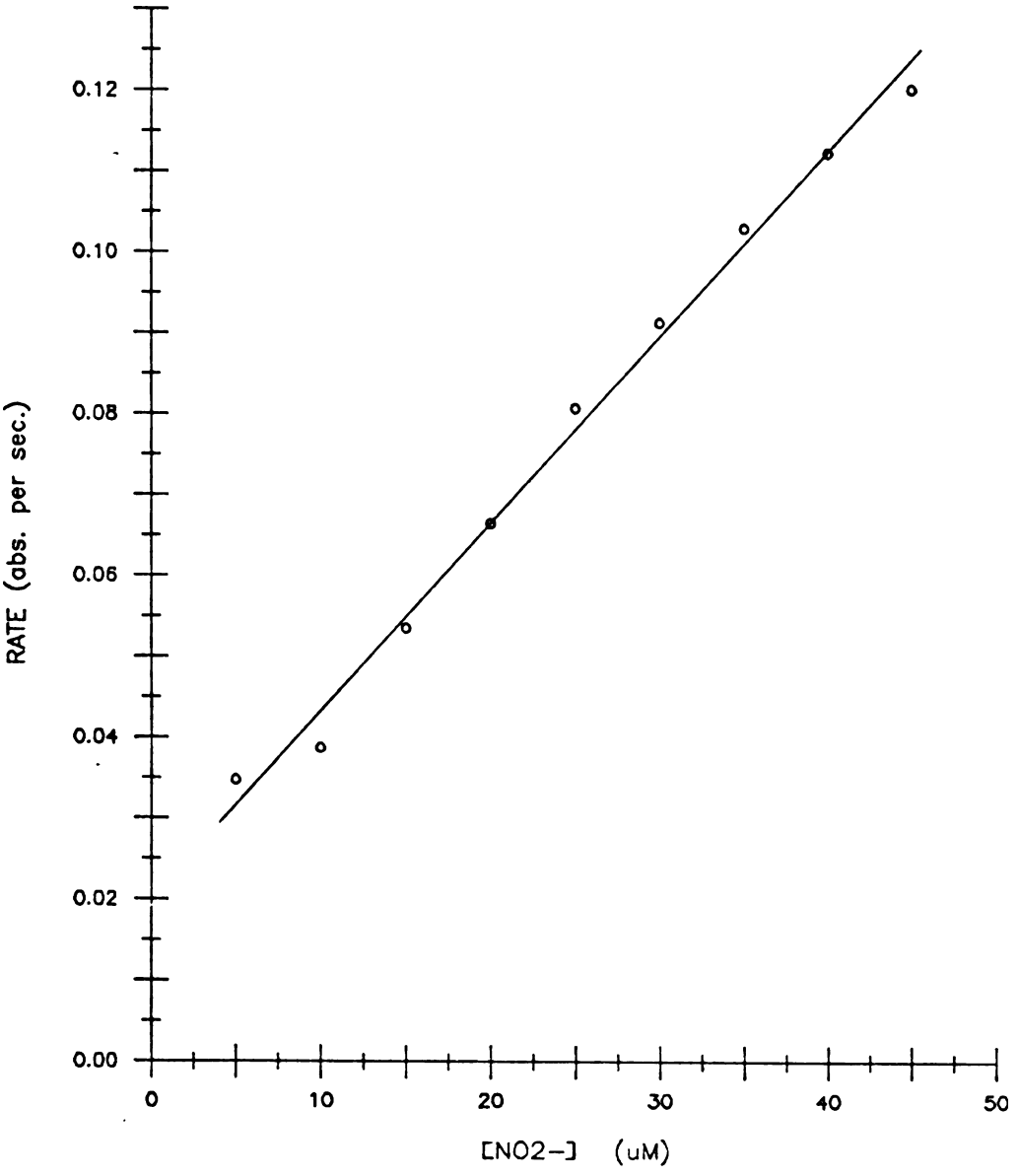


Figure 18. Dependence of the simultaneous reaction rate on $[NO_2^-]$.

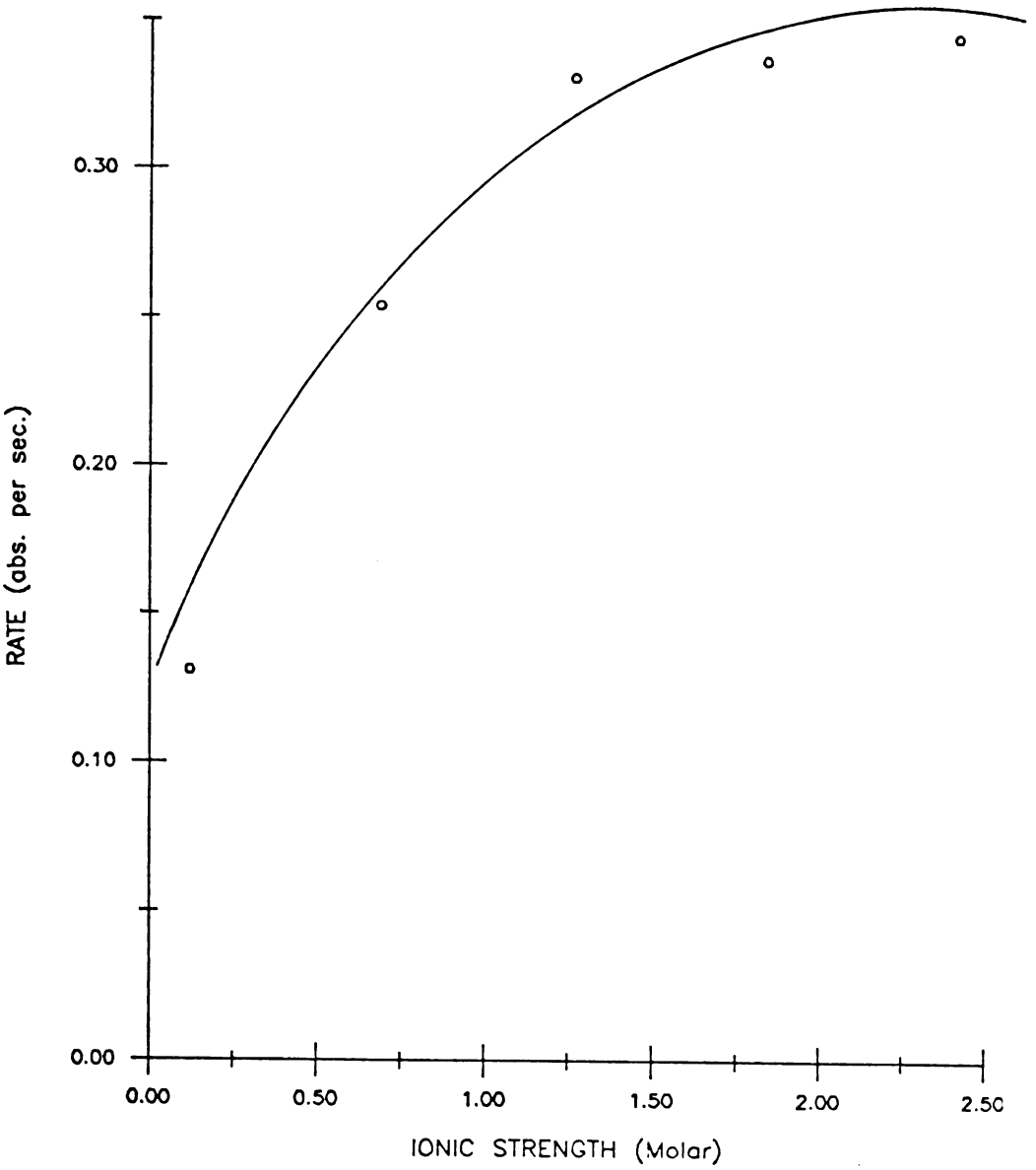


Figure 19. Effect of ionic strength on rate of diazonium ion formation.

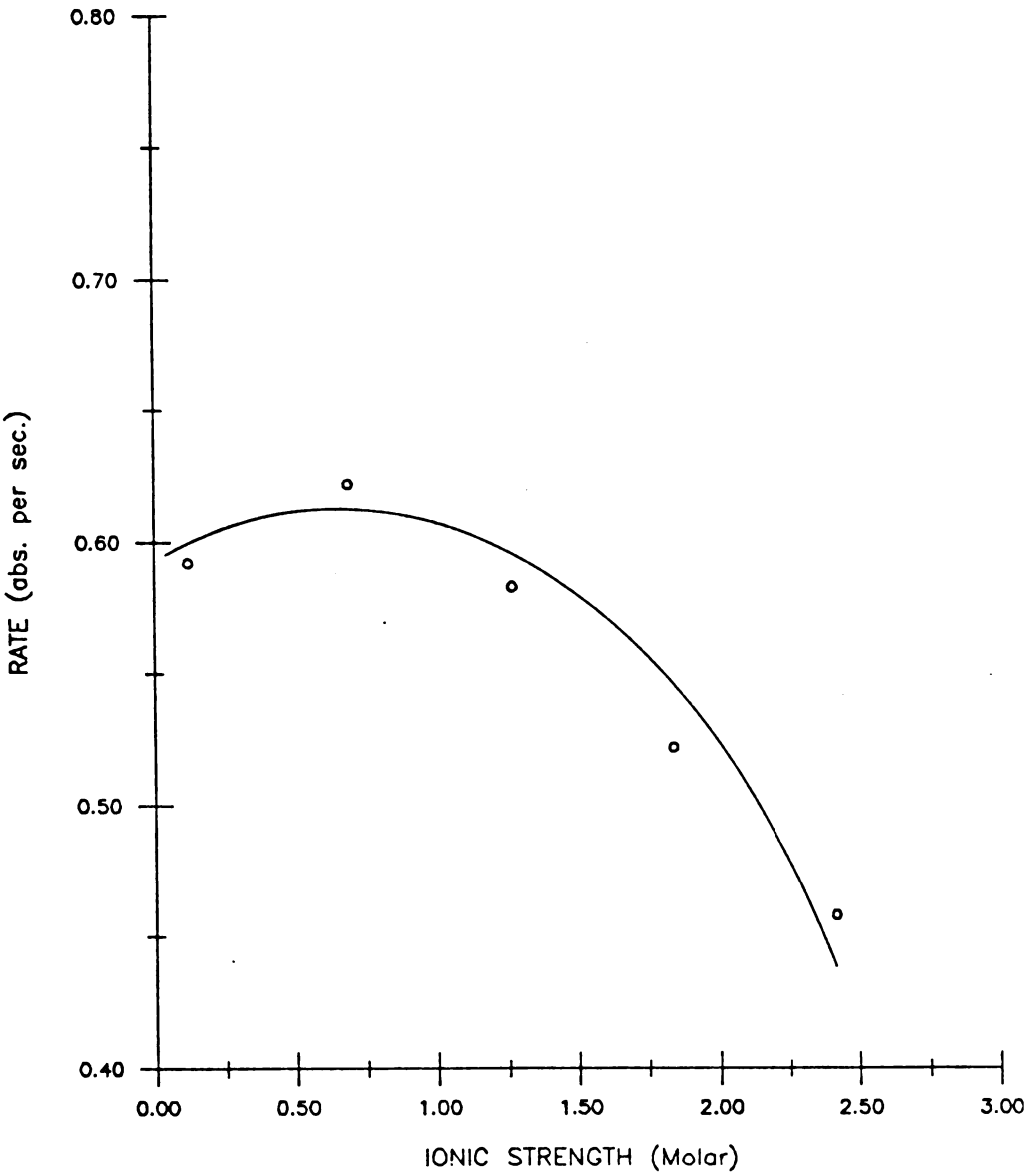


Figure 20. Effect of ionic strength on the coupling reaction.

Table 8. Results of ionic strength studies

First step ionic strength (M)	rate	Second step ionic strength (M)	rate
.120	.1310	.120	.5920
.690	.2540	.690	.6220
1.27	.3310	1.27	.5830
1.84	.3370	1.84	.5220
2.42	.3450	2.42	.4580

Table 9. Results of pH study

pH	rate
0.8	1.459
1.0	.9009
1.5	.3006
2.0	.07545
2.5	.02587
3.0	.00580

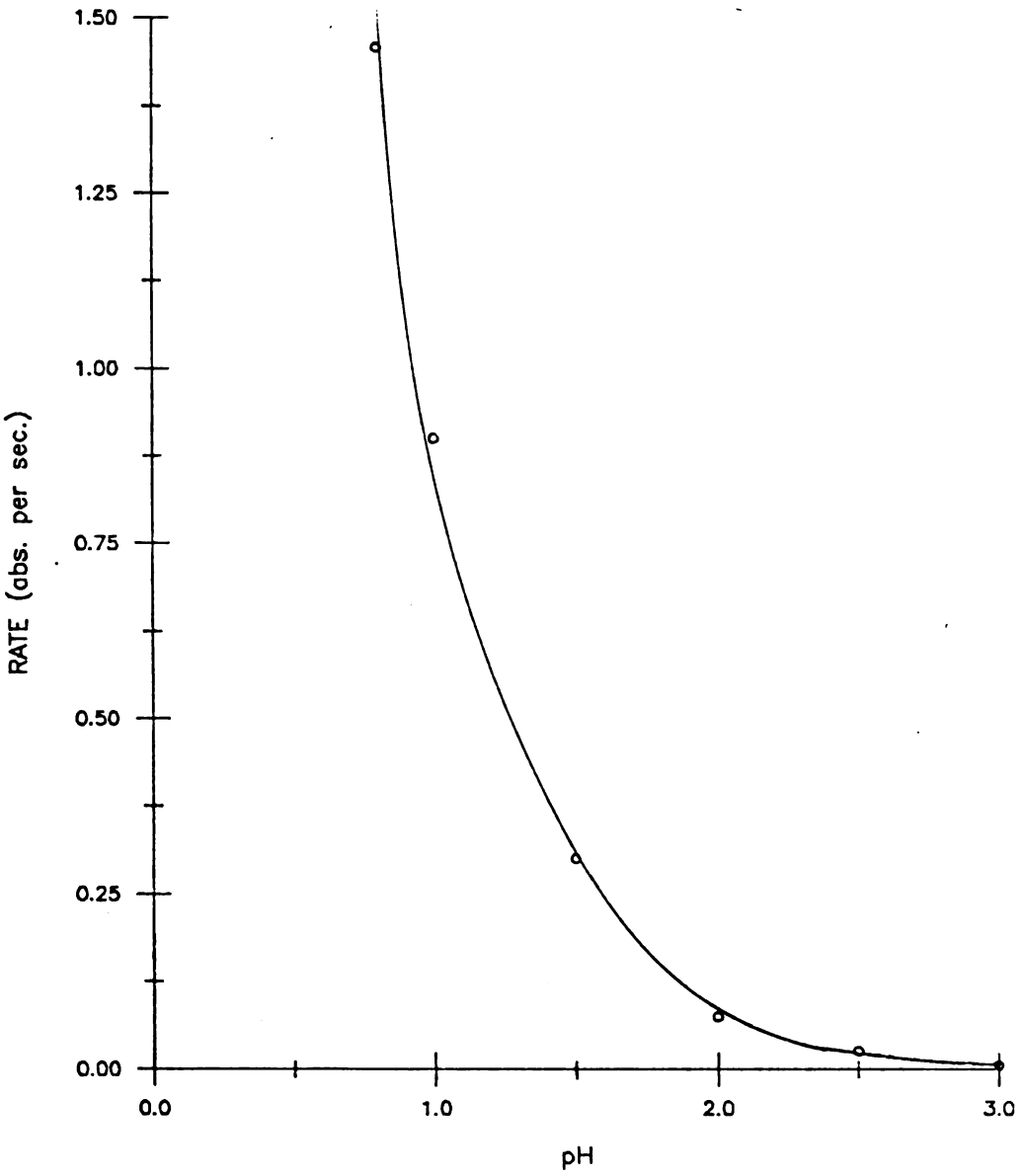


Figure 21. Effect of pH on diazonium ion formation.

Calculation of molar absorptivities:

Table 10 lists the results of the equilibrium experiments performed to determine the molar absorptivities for each step. Again, the initial concentrations reported are after dilution. The high value for the second step exemplifies the utility of the Griess reaction for the determination of low levels of nitrite.

3. Discussion

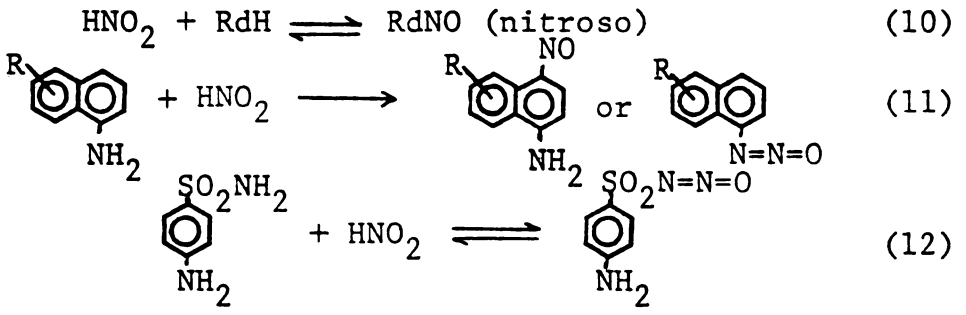
The results of the above experiments will be discussed separately and then a general mechanism will be presented which was formulated from the preliminary data obtained. The discussion will include any assumptions made in calculating the results as well as the sources of error in the experiments.

The orders calculated for the individual reactants for the first and second steps were not integral values, but were within 10% in each case. These errors are relatively large and are beyond the expected errors involved in stopped-flow experiments. These deviations may be due to competing side reactions which alter the rate dependence on the reactants involved. Fox (93) cites various side reactions which take place resulting in deviations from the expected orders. The most common are those which result in a loss or reversible binding of nitrite, and which result in

Table 10. Molar absorptivity results

First step			Second step			Simultaneous reaction		
$[NO_2^-]$ (μM)	Abs.	ε	$[NO_2^-]$ (μM)	Abs.	ε	$[NO_2^-]$ (μM)	Abs.	ε
1.0	.0022	2200	0.5	.0269	53,800	1.0	.0110	11,000
2.5	.0066	2640	1.25	.0630	50,400	2.5	.0269	10,760
5.0	.0110	2200	2.50	.1278	51,100	5.0	.0482	9,640

a loss of the diazonium ion:



These reactions may occur under the conditions of this study, but specific experiments were not performed to obtain proof for this assumption. Since these side reactions are not the main contributors to the overall observed rate, they are not major interferences, especially when the data are obtained from the initial portion of the reaction.

The relative magnitudes of the calculated rate constants are similar to literature values (93). At pH 1.0, it was expected that the diazonium ion formation would be faster than the coupling reaction and the results uphold this assumption. In calculating the rate constants, it was assumed that the steps were second order overall, and that the competing side reactions do not enter into the calculations. The two rate constants calculated for the second step with different reactants at limiting concentrations in each case are similar in magnitude which should be true. The rate constants should, of course, be constant regardless of which species is at limiting concentration. Since the constants are within 10%, the accuracy of determining rate constants is satisfactory.

The rate constant for the simultaneous reaction is much lower than those for the first and second steps. The rate for the simultaneous reaction cannot be slower than either step unless one of the steps is biphasic. It has been shown elsewhere (93) that the diazonium ion formation using SAN is indeed biphasic with the first phase being considerable faster than the second phase. In this case, the side reaction was the formation of a nitroso sulfonamide group in SAN, shown in Eq. (12). This intermediate then decomposed to yield the pigment directly or reformed the SAN. Under the conditions of the present study (pH 1.0), the low value of the rate constant for the simultaneous reaction indicates that SAN is exhibiting similar biphasic behavior. The rate constant for the first step is much higher because the data were obtained before the second phase was observed and so it is concluded that the simultaneous reaction must be controlled by the second phase of the biphasic reaction.

The absorbance vs. time curves for the simultaneous reaction experiments showed abnormal "humps" in the initial portion of the curves, as seen in Figure 22, compared to the usual curve shape shown in Figure 7. These abnormalities cannot be caused by mixing alone because they span approximately 100 msec which is much greater than the mixing time of the instrument (< 5 msec). They can be explained as the result of the various competing reactions occuring before steady state is reached. More detailed experiments would

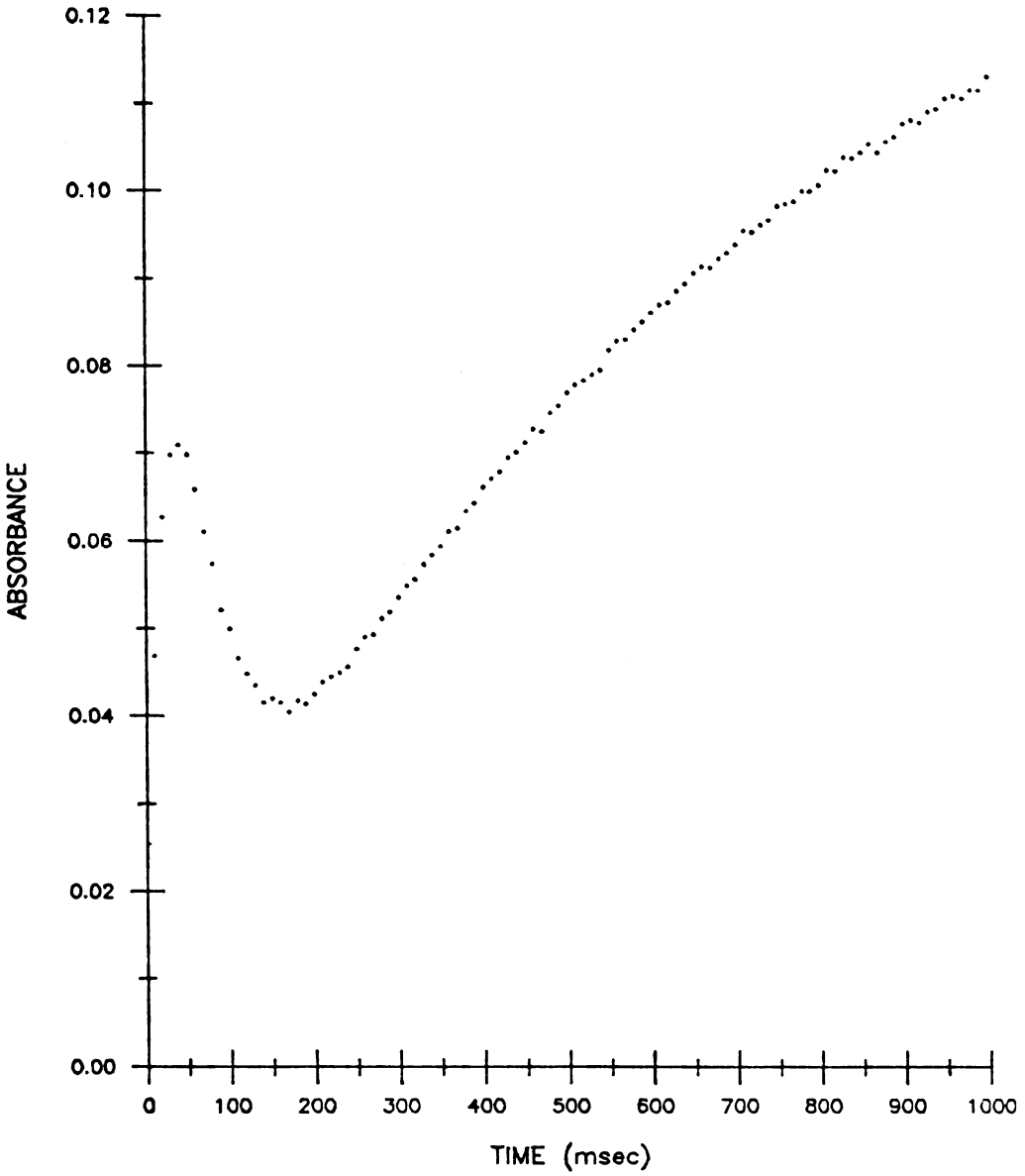


Figure 22. Experimental results of the simultaneous reaction.

have to be performed to model the reaction sequence in this case. The data were obtained from these curves immediately after steady state was reached which is a probable source of error in the calculation of the rate constant because the first 100 msec were masked by the initial hump.

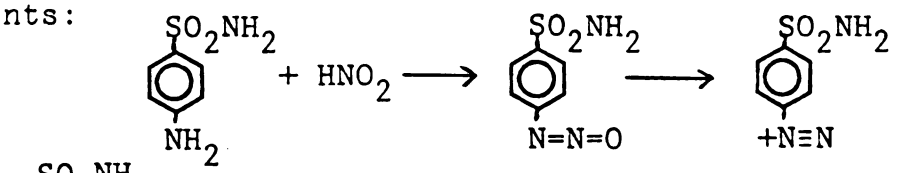
The ionic strength studies show that in each step the reaction must be between an ion and a neutral molecule because of the very small change in the rate as the ionic strength increases. One would expect a substantial ionic strength effect if each of the reactants were charged. The first step shows a slight increase in the rate which indicates that the SAN is probably protonated to some extent in the highly acidic solution. Ionic strength effects can also be observed on reactions involving uncharged species if a charged intermediate is formed. The second step curve shows a slight increase in the beginning which may indicate a charged intermediate; however the effect is not pronounced.

The results of the pH study performed on the first step show that the speed of the diazonium ion formation is increased by lowering the pH. Fox (93) states that pH 2.75-2.85 is the optimal pH range but apparently the first step rate is quite slow in this range. Certainly for analytical purposes, efficiency is improved when the reactions are rapid; thus pH 1.0 is preferred.

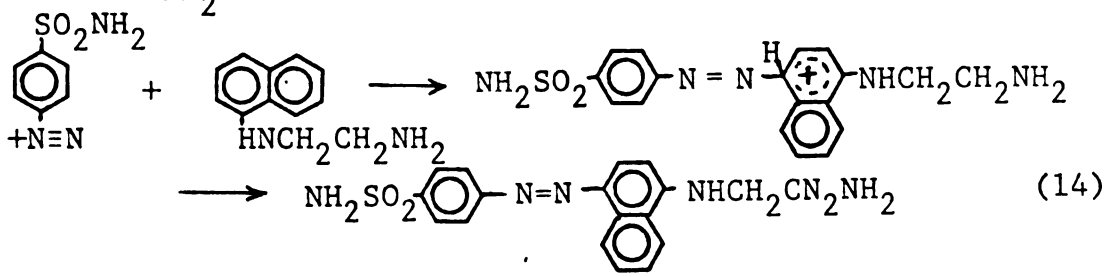
The molar absorptivity experiments are the only equilibrium experiments performed in this study. Errors may arise

in these calculations from incompleteness of the reactions or from fading of the pigment formed. It was decided that 45 minutes was enough time to complete each reaction at 25°C; however, in that time the pigment fades slightly and reduces the absorbance. These errors were not quantitated under these conditions, but were assumed to be relatively small and are thus carried over into the rate constant calculations.

A general mechanism is formulated from the above experiments:



(13)



(14)

The first step does not indicate the biphasic nature of SAN because it is not known if the nitrososulfonamide intermediate is in direct equilibrium with the SAN or if it is part of a complete side reaction which leads to pigment formation. The nitroso intermediate shown in the first step is the most common species for reactions of this type (93).

The second step is simply electrophilic aromatic substitution. The intermediate is stabilized by dispersal of the charge over the aromatic ring. Loss of hydrogen then leads directly to the final product. The ethylene diamine group

is para directing so that the most likely product is the one indicated in the above mechanism.

The mechanism does not indicate any side reactions which may be taking place because specific data are not available to define their role in the overall mechanism. Under the conditions of this experiment, which are the most common conditions for the Griess reaction, the mechanism appears to be similar to that obtained in Fox's study which in general is the stoichiometric formation of the diazonium ion followed by the direct substitution reaction to produce the pigment.

CHAPTER V

CONCLUSIONS AND FUTURE RESEARCH

A microcomputer interface to a stopped-flow apparatus has been developed which improves the flexibility of the instrument and facilitates the acquisition of valid analytical data. The microcomputer/minicomputer arrangement provides direct control of the instrument as well as access to high-level data processing and mass storage. The microcomputer uses an advanced microprocessor chip, The INTEL 8085A, which increases the speed and flexibility of the apparatus.

The interface could be improved by increasing the memory space of the microcomputer in order to facilitate the handling of large amounts of data. One of the most labor intensive steps in an analysis is converting and transferring the data from the microcomputer to the minicomputer, especially when a long experiment has been performed. If the raw data could be stored in the microcomputer after the appropriate manipulation, then a simple program could be written to sequentially load the data into the buffer and transfer it to the minicomputer, which would greatly streamline the analysis sequence. The microcomputer memory can be expanded to 32K if desired which would be useful if a lengthy study is anticipated.

A software package was developed which provides mechanical control of the stopped-flow instrument, and handles data acquisition, transfer, and manipulation. Written in FORTH and FORTH code, the software gives the user the freedom to design experiments as desired because of the "open" format of the language. The programs are not continuously running because each definition is executed individually by the analyst, which permits the option to manipulate data from other experiments or make software changes as needed. FORTH software is extensible, so the definitions could be used to custom-design higher level programs for specific applications, either routine analyses or investigative studies. The software was designed in a general fashion so that it could be used for a wide variety of future experiments. The data conversion program was written in FORTRAN which is used to convert raw voltage data to absorbance. In contrast to the FORTH software, the data conversion program is continuously running and is not easily changed. However, it is simpler than FORTH software when mathematical operations are involved.

Although the FORTH software is general enough to be used for other applications, the flexibility could still be improved. A maximum of 8 pushes can be averaged and a maximum of 100 data points can be taken with the current program. These limitations were imposed in order to conserve memory space and to keep the software simple. If

higher precision or more data points are desired, then the definitions would have to be changed to accept these improvements. Also, data smoothing or other more sophisticated averaging programs would be useful additions to the software package. Also, an interactive windowing routine would be very useful in determining the slope of the absorbance vs. time curve. The MULPLT routine is time-consuming and unreliable, so a program written specifically for this purpose on the PDP 11/23 would be a valuable improvement. A final improvement to the FORTH software would be the automatic data transfer routine mentioned previously. This routine would be simple to write with the already existing data transfer words.

The FORTRAN data conversion program could also be improved by adding the option of converting more than one block of data at a time. The program was initially written with the intent of keeping the blocks of data separate so that the results would be easier to identify and interpret. Converting and labeling each block individually is quite time consuming; thus the ability to convert a series of blocks with proper identification would be advantageous.

The overall system performance was tested by using the iron-thiocyanate reaction. The deadtime of the instrument was found to be low enough that very fast reactions could be employed without complications. The rate constant of the FeSCN reaction was on the order of previous values but is

slightly low due to an incorrect molar absorptivity value (or wavelength calibration) or a lack of proper pH and ionic strength control. Also, the precision of measuring the reaction rate was satisfactory. Thus, the stopped-flow system is operating correctly and is supplying valid analytical data.

The preliminary kinetic data from the Griess reaction study indicate that the mechanism is similar to that reported in the literature, under different conditions, with each step second order and nitrite first order throughout the reaction sequence. The ionic strength studies also support this mechanism. The small change in the rate with increasing ionic strength indicates that each reaction step is between a charged species and a neutral molecule. The pH and molar absorptivity results prove why these reagents and conditions are preferred for nitrite analysis. The rate of the first step is dramatically faster at pH 1.0 compared to pH 3.0 and the high molar absorptivity of the pigment under these conditions shows why the Griess reaction is so sensitive for nitrite analysis. Finally, the rate constants are of the same relative magnitude as literature values.

Further work on the Griess reaction under these conditions should include defining the interaction of the side reactions which may be causing the deviation of the individual orders from integral values. These competing reactions were observed in another study under a different set of

conditions but specific experiments were not performed in this study to take them into account. The overall mechanism was formulated based only on the available data; thus including the competing reactions would provide a more detailed picture of the reaction sequence. Another project would be to model the simultaneous reaction to explain the abnormal humps observed in the absorbance vs. time curves. The main intent of this study was to learn the kinetics of the reaction by examining the individual steps, so the simultaneous reaction was not studied in great detail. These deviations from the normal curves are thought to be the result of the various competing reactions occurring before steady state is reached. It would be useful to study this reaction in more detail to learn the roles of the various reactants in the overall mechanism when they are present simultaneously. Also, the biphasic behavior of SAN under these conditions should be investigated. Knowledge of how SAN reacts at pH 1 would be useful to further understand the overall reaction mechanism.

LIST OF REFERENCES

LIST OF REFERENCES

1. H. B. Mark, Jr. and G. A. Rechnitz, Kinetics in Analytical Chemistry, Wiley-Interscience, New York, 1968.

2. K. B. Yatsimerskii, Kinetic Methods of Analysis, Pergamon Press, Oxford, 1966.

3. G. A. Rechnitz, Anal. Chem., 38, 513R (1966).

4. G. A. Rechnitz, Ibid. 40, 455R (1968).

5. G. G. Guilbault, Ibid., 40, 459R (1968).

6. G. G. Guilbault, Ibid., 42, 334R (1970).

7. R. A. Greinke and H. B. Mark, Jr., Ibid., 46, 413R (1974).

8. R. A. Greinke and H. B. Mark, Jr., Ibid., 48, 87R (1976).

9. M. M. Fishman and H. F. Schiff, Ibid., 44, 543R (1972).

10. H. A. Mottola, Crit. Rev. Anal. Chem., 4, 229 (1975).

11. S. R. Crouch, in Computers in Chemistry and Instrumentation (H. D. Mattson, H. B. Mark, Jr., and H. C. McDonald, eds.), Dekker, New York, 1973, pp. 107-207.

12. H. V. Malmstadt, E. A. Cordos, and C. J. Delaney, Anal. Chem., 44, 26A (1972).

13. H. V. Malmstadt, C. J. Delaney, and E. A. Cordos, Ibid., 44, 79A (1972).

14. H. V. Malmstadt, C. J. Delaney, and E. A. Cordos, Crit. Rev. Anal. Chem., 2, 559 (1972).

15. H. B. Mark, Jr., Talanta, 19, 717 (1972).

16. S. R. Crouch, F. J. Holler, P. K. Notz, and P. M. Beckwith, Appl. Spect. Rev., 13(2), 165-259 (1977).

17. H. V. Malmstadt, Topics in Automated Chemical Analysis, Vol. 1, Holsted Press, New York, 1979, p. 95.

18. H. L. Pardue, in Advances in Analytical Chemistry and Instrumentation, Vol. 7 (C. N. Reilley and F. W. McLafferty, eds.), Wiley-Interscience, New York, 1968, pp. 141-207.

19. J. D. Ingle and S. R. Crouch, Anal. Chem., 43, 697 (1971).

20. J. E. Stewart, Flow Deadtime in Stopped-Flow Measurements, (Durrum Application Notes No. 4), Durrum Instrument Corp., Sunnyvale, CA 94303.

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

22. J. I. Morrow, Chem. Instrum., 2, 375 (1970).

23. H. Hartridge and F. J. W. Roughton, Proc. R. Soc. London Ser. A, 104, 376 (1923).

24. F. J. W. Roughton, Discuss. Faraday Soc., 17, 116 (1954).

25. B. Chance, J. Franklin Inst., 229, 455 (1940).

26. B. Chance, Ibid., 229, 613 (1940).

27. B. Chance, Ibid., 229, 737 (1940).

28. B. Chance, Rev. Sci. Instrum., 22, 619 (1951).

29. E. F. Caldin, Fast Reactions in Solution, Wiley, New York, 1964.

30. B. Chance, Investigation of Rate and Mechanisms of Reactions, Vol. VIII, Part II (S. L. Friess, E. S. Lewis, and A. Weissberger, eds.), Wiley-Interscience, New York, 1963, pp. 728-748.

31. R. L. Berger, B. Balko, W. Borcherdt, and W. Friauf, Rev. Sci. Instrum., 39, 486 (1968).

32. R. A. Harvey, Anal. Biochem., 29, 58 (1969).

33. E. F. Caldin, J. E. Crooks, and A. Queen, J. Phys. E., 6, 930 (1973).

34. J. L. Dye and L. H. Feldman, Rev. Sci. Instrum., 37, 154 (1966).

35. R. B. Coolen, N. Papadakis, J. P. Avery, C. G. Enke, and J. L. Dye, Anal. Chem., 47, 1649 (1975).

36. Digital Equipment Corporation, Maynard, Massachusetts.

37. R. M. Wightman, R. L. Scott, C. N. Reilly, R. W. Murray, and R. J. Burnett, Anal. Chem., 46, 1492 (1974).

38. A. C. Javier, S. R. Crouch, and H. V. Malmstadt, Ibid., 40, 1922 (1968).

39. A. C. Javier, S. R. Crouch, and H. V. Malmstadt, Ibid., 41, 239 (1969.

40. B. G. Willis, J. A. Bittikofer, H. L. Pardue, and D. W. Margerum, Ibid., 42, 1340 (1970).

41. G. E. Meiling, R. W. Taylor, L. G. Hargis, J. English, and H. L. Pardue, Ibid., 48, 1686 (1976).

42. Hewlett-Packard, Inc., Palo Alto, California.

43. Intel Corporation, Microcomputer Systems Division, Santa Clara, California.

44. K. R. O'Keefe and H. V. Malmstadt, Anal. Chem., 47, 707 (1975).

45. B. W. Renol, K. R. O'Keefe, and H. V. Malmstadt, Ibid., 48, 661 (1976).

46. I. R. Bonnell and J. D. Defreese, Ibid., 52(1), 139 (1980).

47. P. M. Beckwith and S. R. Crouch, Ibid., 44, 221 (1972).

48. J. L. Holtzman, Ibid., 52, 989 (1980).

49. D. D. Clark and S. M. Schuster, Comput. Chem., 4(1), 51 (1980).

50. P. D. Smith, E. E. Beile, and R. L. Berger, J. Biochem. Biophys. Methods, 6(2), 173 (1982).

51. K. Ishihara, S. Funahashi, and M. Tanaka, Inorg. Chem., 22(2), 194 (1983).

52. K. Ishihara, S. Funahashi, and M. Tanaka, Rev. Sci. Instrum., 53(8), 1231 (1982).

53. M. Sasaki, F. Amita, and J. Osugi, Ibid., 50(9), 1104 (1979).

54. R. Van Eldik, D. A. Palmer, R. Schmidt and H. Kelm, Inorg. Chim. Acta., 50(2), 131 (1981).

55. T. Asano and W. J. LeNoble, Chem. Rev., 78, 407 (1978).

56. I. R. Bonnell and J. D. Defreese, Anal. Chim. Acta., 134, 189 (1982).

57. A. N. Schechter, Science, 170, 273 (1970).

58. J. B. Pausch and D. W. Margerum, Anal. Chem., 41, 226 (1969).

59. B. G. Willis, W. H. Woodruff, J. R. Frysinger, D. W. Margerum, and H. L. Pardue, Ibid., 42, 1350 (1970).

60. D. Sanderson, J. C. Bittikofer, and H. L. Pardue, Ibid., 44, 1934 (1972).

61. M. J. Bishop, J. Everse, and N. O. Kaplan, Proc. Natl. Acad. Sci., U.S.A., 69, 1761 (1972).

62. S. G. Rhee, M. I. Griefner, and P. B. Chock, Anal. Biochem., 66, 259 (1975).

63. M. I. Karayannis, Anal. Chim. Acta, 76, 121 (1975).

64. T. A. Nieman, F. J. Holler, and C. G. Enke, Anal. Chem., 48, 899 (1976).

65. T. S. Lee and I. M. Kolthoff, Ann. N.Y. Acad. Sci., 53, 1093 (1951).

66. E. Pelizzetti, E. Mentasti, E. Pramauro, and G. Giriaudi, Anal. Chim. Acta, 85, 161 (1976).

67. G. M. Ridder and D. W. Margerum, Anal. Chem., 49, 2090 (1977).

68. J. L. Markley, F. Travers, and C. Balny, Eur. J. Biochem., 120(3), 477 (1981).

69. M. A. Koupparis, and M. I. Karayannis, Anal. Chim. Acta, 138, 303 (1982).

70. R. Q. Thompson and S. R. Crouch, Anal. Chim. Acta, 144, 155 (1982).

71. P. M. Beckwith, Ph.D. Dissertation, Michigan State University, 1972.

72. P. K. Notz, Ph.D. Dissertation, Michigan State University, 1975.

73. F. J. Holler, Ph.D. Dissertation, Michigan State University, 1977.

74. C. C. Kircher, Jr., Ph.D. Dissertation, Michigan State University, 1982.

75. P. S. Loach and R. J. Loyd, Anal. Chem., 38, 1709 (1966).

76. E. Ratzlaff, Ph.D. Dissertation, Michigan State University, 1982.

77. R. T. Balciunas, Ph.D. Dissertation, Michigan State University, 1981.

78. B. Newcome, Ph.D. research in progress, Michigan State University.

79. Advanced Micro Devices, Inc., Sunnyvale, California.

80. L. Brodie, Starting FORTH, Prentice-Hall, Inc., Englewood Cliffs, NJ, 1981, p. 3-5.

81. R. E. Dessy, Anal. Chem., 55(6), 650A (1983).

82. E. Ratzlaff, original software, Michigan State University.

83. K. Trischan, original software, Michigan State University.

84. J. F. Below, Jr., R. E. Connick, and C. P. Coppel, J.A.C.S., 80, 2961 (1958).

85. M. W. Lister and D. E. Rivington, Can. J. Chem., 33, 1572 (1955).

86. B. Tonomura, H. Nakatani, M. Ohnishi, J. Yamaguchi-Ito, and K. Hiromi, Anal. Biochem., 84, 370 (1978).

87. M. W. Lister and D. E. Rivington, Can. J. Chem., 33, 1572 (1955).

88. J. P. Griess, Philos. Trans. R.S. (London), 679 (1864).

89. J. P. Griess, Ber. Dtsch. Chem. Ges., 12, 426 (1879).

90. E. Sawicki, T. W. Stanley, J. Pfaff, and A. D'Amico, Talanta, 10, 641 (1963).

91. L. Kamm, D. F. Bray, and D. E. Coffin, J. Assoc. Off. Anal. Chem., 51,140 (1968).

92. R. N. Fiddler, Ibid., 60, 594 (1977).

93. J. B. Fox, Jr., Anal. Chem., 51(9), 1493, (1979).

94. A. C. Bratton and E. K. Marshall, Jr., J. Biol. Chem., 128, 537 (1939).

95. A. F. Hegarty, in The Chemistry of Diazonium and Diazo Groups, (S. Patai, ed.), John Wiley & Sons, New York, 1978, p. 511.

96. C. Patton, Ph.D. Dissertation, Michigan State University, 1982.

97. F. Allam and A. H. Agiza, Bull. Fac. Agr. Cairo Univ., 80, 22 (1956).

APPENDICES

APPENDIX A

SYSTEM HARDWARE

Stopped-flow system components:

Component	Manufacturer
Light source, Model EU-701-50	GCA McPherson Instrument Co.
Monochromator, Model EU-700	GCA McPherson Instrument Co.
Quartz flexible fiber optic bundle	Schott Optical Co.
Photomultiplier tube, Model 1P28	RCA Corporation
Power supply, Model EU-42	Heath Co.
Current amplifier, Model 427	Keithley Instruments, Inc.
Thermostatting unit, Model E52	Haake Co.
Oscilloscope, Model D40	Tektronix
Stopped-flow apparatus	P. M. Beckwith (71)

Microcomputer components:

Central Processing Unit (CPU): Intel 8085A microprocessor (43)
Memory: 32K maximum, presently 10K ROM, 14K RAM
Programmable Interrupt Controller (PIC)
Dual Universal Synchronous Asynchronous Receiver Transmitters (USART): two RS-232 communication ports
Timer board: Advanced Micro Devices 9513 System Timing Controller (79)
Analog input: four sample-and-hold (S/H) inputs multiplexed into a 12-bit analog-to-digital converter (ADC)
Analog output: two port (x,y) digital-to-analog converter (DAC)

APPENDIX B

STOPPED-FLOW SOFTWARE

Table 11. FORTH Analysis Software

```
L MICRO.FTH/E/BL 9 11 LP :

Block Number:  9
0     ( DIRECTORY)
1     ( BLOCKS 10 - 11:   : SF CONTROLLER COMMANDS)
2     ( BLOCKS 12 - 13 : CLOCK SET-UP FOR DELAYS)
3     ( BLOCK 14 : SF OPERATION)
4     ( BLOCK 15 : CLOCK SET-UP FOR DATA ACQUISITION ROUTINE)
5     ( BLOCKS 16 - 19 : DATA ACQUISITION ROUTINE)
6     ( BLOCKS 20 - 21 : NUMERIC INPUT)
7     ( BLOCKS 22 - 23 : DATA TRANSFER)
8     ( BLOCKS 24 - 25 : INPUT ROUTINES)
9     ( BLOCK 26 : ANALYSIS OPTIONS)
10    ( BLOCKS 27 - 29 : SF ANALYSIS SEQUENCE)
11    ( BLOCKS 35 - 36 : DAC OUTPUT)
12
13    10 29 THRU          35 36 THRU
14    SF
15

Block Number:  10
0     ( SF CONTROLLER COMMANDS)
1     HEX B000 DECIMAL CONSTANT APORT
2     APORT 1+ CONSTANT BPORT
3     APORT 2+ CONSTANT CPORT
4     APORT 3 + CONSTANT CNTRLP
5
6     : SFSET 153 CNTRLP C! ; ( set B for output, A & C for input)
7     : SFSTAT CPORT C@ ; ( get status bits)
8     : CHANGE BPORT C! 0 BPORT C! ; ( pulse output bit)
9     : CKMAN SFSTAT 8 AND ; ( check for manual - isolate status bit)
10
11    : MANUAL CKMAN NOT IF 2 CHANGE THEN ; ( put into manual)
12    : AUTO CKMAN IF 2 CHANGE THEN CKMAN IF CR
13      ( put into auto but check if computer is disabled)
14      ." ERROR - COMPUTER DISABLED" CR THEN ;
15

Block Number:  11
0     ( SF CONTROLLER COMMANDS CONTINUED)
1
2     : 3VINTAKE CKMAN AUTO SFSTAT 1 AND IF 16 CHANGE
3       THEN IF MANUAL THEN ;   ( turn 3-way valve to intake position)
4     : 3VMIX CKMAN AUTO SFSTAT 1 AND NOT IF 16 CHANGE
5       THEN IF MANUAL THEN ;   ( turn 3-way valve to mix position)
6
7     : FILL CKMAN AUTO SFSTAT 32 AND IF 8 CHANGE
8       THEN IF MANUAL THEN ; ( fill drive syringes)
9     : PUSH CKMAN AUTO SFSTAT 32 AND NOT IF 8 CHANGE
10      THEN IF MANUAL THEN ; ( pressurize drive syringes)
11
12    : WVOPEN CKMAN AUTO SFSTAT 2 AND IF 1 CHANGE
13      THEN IF MANUAL THEN ; ( open waste valve)
14    : WVCLOSE CKMAN AUTO SFSTAT 2 AND NOT IF 1 CHANGE
15      THEN IF MANUAL THEN ; ( close waste valve)      SFSET
```

Table 11 (Cont'd.)

```
L MICRO.FTH/E/BL 9 29 LP :

Block Number:  12
0     ( CLOCK SET-UP)
1     45561 CONSTANT COMAND
2     45560 CONSTANT DATAREG
3     : CMD COMAND C! ; ( write counter commands to CMD)
4     : DRG DATAREG C! ; ( write data register values to DRG)
5
6     OCTAL
7     : INIT      337 CMD   ( reset 9513)
8                 137 CMD   ( load all counters for startup)
9                 347 CMD   ( select 8-bit bus)
10                350 CMD   ( disable data pointer sequencing)
11                 27 CMD 340 DRG 321 DRG   ( set Master Mode)
12                  1 CMD  40 DRG  17 DRG   ( set ctr mode for ctr 1)
13                 11 CMD  12 DRG   0 DRG   ( load counter 1 with 10)
14                  2 CMD   0 DRG   0 DRG   ( set ctr mode for ctr 2) ;
15                                                    DECIMAL

Block Number:  13
0     ( CLOCK SET-UP CONT.)
1     OCTAL
2     : LOADC2 12 * 12 CMD  DRG 0 DRG ;
3       ( multiply value by 10 then store it in counter 2 load reg.)
4     : SET 143 CMD ;
5       ( load and arm counters 1 and 2)
6     : TIME BEGIN 243 CMD 22 CMD DATAREG C@ DATAREG C@ OR 1 = END ;
7       ( monitor value in ctr 2 hold reg., when = 1, exit loop)
8     : DELAY SET TIME ;
9       ( delay for n sec. based on value stored in ctr 2 load reg.)
10    : PDR DATAREG C@ . ;
11      ( print contents of selected data register)
12
13    : 1DELAY 1 LOADC2 SET TIME ; ( delay for 1 second)
14    : 3DELAY 3 LOADC2 SET TIME ; ( delay for 3 seconds)
15      DECIMAL                          INIT

Block Number:  14
0     ( MORE SF STUFF)
1     : PREP 3VINTAKE 1DELAY FILL 3DELAY 3VMIX 1 DELAY ;
2       ( prepare SF for analysis : fill drive syringes, leave
3       3-way valve in mix position)
4     : RINSE PUSH 1DELAY 6 0 DO WVOPEN 1 DELAY WVCLOSE 1DELAY LOOP ;
5       ( rinse cycle - no data acquisition)
6     : TRGR BEGIN SFSTAT 4 AND NOT END ;
7       ( trigger loop - tests for active low TTL signal)
8
9
10
11
12
13
14
15
```

Table 11 (Cont'd.)

```
L MICRO.FTH/E/BL 9 29 LP :

Block Number:  15
0     ( CLOCK SET-UP FOR DATA ACQUISITION ROUTINE)
1
2     VARIABLE PERIOD           1 PERIOD !
3       ( memory location to store data acquisition rate [msec])
4
5     : CLOCK 4 CMD 33 DRC 13 DRC 12 CMD PERIOD @ 10 * DUP 255 AND
6       DRC 65280 AND 256 /MOD DRC DROP ;
7
8       ( set otr 4 to count at 10 KHz.  Active high TC pulse.
9       Value in  PERIOD[*10] will be stored in otr 4 load register)
10
11      VARIABLE DLY  ( data acquisition delay)       0 DLY !
12      VARIABLE .PERIOD     10 .PERIOD !
13    : .CLOCK 4 CMD 33 DRC 13 DRC 12 CMD .PERIOD @ DUP 255 AND
14      DRC 65280 AND 256 /MOD DRC DROP ; ( count at 10 KHz)
15

Block Number:  16
0     ( DATA ACQUISITION ROUTINE)
1     HEX   B1C0 CONSTANT STCONV
2           B1C8 CONSTANT ADSTAT
3           B1C1 CONSTANT BOTH
4           B1D8 CONSTANT MUXADR
5           B1D0 CONSTANT HOLDER          DECIMAL
6     HEX   C9 2864 C! DECIMAL ( RST 6.5 HANDLER)
7     HEX   C9 2868 C! DECIMAL ( RST 7.5 HANDLER)
8     CODE MUX H POP L A MOV MUXADR STA NEXT JMP 0 MUX
9     CODE SAMPLE H POP L A MOV HOLDER STA NEXT JMP 0 SAMPLE
10    VARIABLE B1 200 ALLOT
11    VARIABLE BFPTR
12    CODE TAKE DI 13 # A MOV SIM STCONV STA EI HLT
13    15 # A MOV SIM EI BOTH LHLD XCHG BFPTR LHLD E M MOV H INX
14    D M MOV H INX BFPTR SHLD NEXT JMP
15    CODE WAIT DI 27 # A MOV SIM EI HLT 15 # A MOV SIM EI NEXT JMP

Block Number:  17
0     ( DATA ACQUISITION STUFF)
1       VARIABLE NPOINT
2       100 NPOINT !
3     : ACQUIRE CLOCK 104 CMD NPOINT @ 0 DO WAIT TAKE LOOP ;
4       VARIABLE B2 2 ALLOT
5     : SFRUN B1 BFPTR ! PUSH 1DELAY WVOPEN TRGR ACQUIRE WVCLOSE
6       CR ." DONE" CR ;
7     : SUM 0 0 B2 2! NPOINT @ 2* 0 DO B1 I + @ 16 /MOD SWAP DROP
8       B2 2@ ROT M+ B2 2! 2 +LOOP ;
9       VARIABLE B3
10    : AVERAGE B2 2@ NPOINT @ M/ B3 ! ;
11      VARIABLE LIGHT
12      VARIABLE X
13    : GETLIGHT  PERIOD @ X !  ( save old value)
14      B1 BFPTR ! 10 PERIOD ! ACQUIRE SUM AVERAGE B3 @ LIGHT !
15      X @ PERIOD ! ;  ( recall old value)
```

Table 11 (Cont'd.)

```
L MICRO.FTH/E/BL 9 29 LP :

Block Number:  18
0     ( DATA ACQUISITION STUFF)
1       VARIABLE DARK
2     : GETDARK   PERIOD @ X !  ( save old value)
3       B1 BFPTR ! 10 PERIOD ! ACQUIRE SUM AVERAGE B3 @ DARK !
4       X @ PERIOD ! ;  ( recall old value)
5     : PR3 NPOINT @ 2* 0 DO B1 I + @ DARK @ - 16 /MOD 100 LIGHT @
6       */ 10 U.R DROP 5000 0 DO LOOP CR 2 +LOOP ;
7       VARIABLE NPUSH                4 NPUSH !
8       VARIABLE DATA0 800 ALLOT
9     : ACQDLY .CLOCK 104 CMD B1 BFPTR ! DLY @ 0 DO WAIT TAKE LOOP ;
10    : ANALYZE PUSH 1DELAY WVOPEN 1DELAY WVCLOSE 1DELAY NPUSH @
11      0 DO I WVOPEN TRGR 200 * DATA0 + BFPTR ! ACQUIRE WVCLOSE
12      1DELAY LOOP WVOPEN 1DELAY WVCLOSE ;
13
14
15

Block Number:  19
0     ( DATA ACQUISITION STUFF)
1       VARIABLE DSET
2     : ?DATASET 200 * DATA0 + DSET ! ;
3     : PRDATA ?DATASET NPOINT @ 2* 0 DO DSET @ I + @ 16 /MOD
4       100 LIGHT @ */ 10 U.R DROP 5000 0 DO LOOP CR 2 +LOOP ;
5     : DPREP NPUSH @ 0 DO I ?DATASET NPOINT @ 2* 0 DO DSET @
6       I + @ 16 /MOD SWAP DROP DSET @ I + ! 2 +LOOP LOOP ;
7       VARIABLE 1DATA  VARIABLE 2DATA  VARIABLE 3DATA
8     : 1DATA DATA0 200 + ;
9     : 2DATA DATA0 400 + ;
10    : 3DATA DATA0 600 + ;
11    : AVRG NPOINT @ 2* 0 DO DATA0 I + @ 1DATA I + @ + 2DATA I
12      + @ + 3DATA I + @ + 4 / DATA0 I + ! 2 +LOOP ;
13
14
15

Block Number:  20
0     ( NUMERIC INPUT)
1     : 2@ACCEPT PAD 10 EXPECT PAD C@ IF 32 PAD BEGIN 1+ DUP C@ IF
2       0 ELSE C! 1 THEN END PAD 1- NUMBER PUNCT @ 0= IF 0 THEN
3       ROT 2! ELSE DROP THEN ;
4     : FT DUP C@ DUP 48 58 WITHIN 0= IF >R 2DUP SWAP - R> 2SWAP ROT
5       THEN 32 = ;
6     : RIGHTOF. PAD C@ IF PUNCT @ IF PAD DUP 1- BEGIN 1+ FT END
7       SWAP - ROT - SWAP DROP 1- ELSE 0 THEN ELSE -1 THEN ;
8     : /DIGIT 0 BASE @ M/MOD ROT ROT BASE @ M/MOD ROT ROT ;
9     : # /DIGIT NUMERAL HOLD ;
10    : #S BEGIN # 2DUP D0= END ;
11    : NPLACE >R (# R> DUP 0 > IF 0 DO # LOOP ELSE DROP THEN
12      44 HOLD #S SIGN #> TYPE SPACE ;
13    : D.N >R SWAP OVER DABS R> NPLACE ;
14    : 2@PUTGET >R DUP 2@ R> D.N 2@ACCEPT RIGHTOF. ;
15
```

Table 11 (Cont'd.)

```
L MICRO.FTH/E/BL 9 29 LP :

Block Number:  21
0     ( NUMERIC INPUT)
1       CREATE TEMP 0 , 0 .
2     : JUST DUP @ 0 TEMP 2! SWAP TEMP OVER 2*PUTGET DUP 0< IF
3       DROP 2DROP ELSE TEMP 2+ @ SWAP ROT 2DUP = IF 2DROP ELSE
4       2DUP > IF DO 10 / 1 /LOOP ELSE SWAP DO 10 * 1 /LOOP
5       THEN THEN SWAP ! THEN ;
6
7
8
9
10
11
12
13
14
15

Block Number:  22
0     ( DATA TRANSFER WORDS)
1     : LBFPTR FIRST @ BFPTR ! BFPTR @ 1024 ERASE ;
2     : INCDAT 200 * DATA0 + ;
3     : INCBF 200 * BFPTR @ + ;
4     : .DDISK ." GO.FTH" ;
5       VARIABLE #DAT                0 #DAT !
6     : PREFLUSH EB LBFPTR #DAT @ 0 DO I INCDAT I INCBF NPOINT @ 2*
7       (CMOVE LOOP ;
8     : DDISK 11SPEC 25 BLANK ['] .DDISK DUP 3 + SWAP 2+ C@ 11SPEC
9       SWAP MOVE ;
10      VARIABLE #BLO                1 #BLO !
11    : TRANSFER #BLO @ IDENTIFY UPDATE DDISK FLUSH ;
12      CREATE IDSTRNG 80 ALLOT       IDSTRNG 80 BLANK
13    : LABEL CR ." Enter identifier:" CR IDSTRNG 80 TYPE CR PAD
14      80 2DUP BLANK EXPECT PAD C@ IF PAD IDSTRNG 80 (CMOVE THEN ;
15

Block Number:  23
0     ( DATA TRANSFER WORDS)
1     : SITE ( position line --)  ) 64 * SWAP 2* + FIRST @ + ;
2
3     : BLFILL            IDSTRNG 0 13 SITE   80 (CMOVE
4                         NPOINT  0 15 SITE    2 (CMOVE
5                         NPUSH   1 15 SITE    2 (CMOVE
6                         PERIOD  2 15 SITE    2 (CMOVE
7                         DLY     3 15 SITE    2 (CMOVE
8                         LIGHT   4 15 SITE    2 (CMOVE
9                         DARK    5 15 SITE    2 (CMOVE ;
10
11
12
13
14
15
```

Table 11 (Cont'd.)

```
L MICRO.FTH/E/BL 9 29 LF :

Block Number:  24
0     ( INPUT ROUTINES)
1     : NPRMT CR ." Number of data points per push (100 max.)"
2       8 SPACES ;
3     : NPTS NPRMT 0 NPOINT JUST ;
4     : PUPRMT CR ." Number of pushes per fill (4 max.) " 15 SPACES ;
5     : NPSH PUPRMT 0 NPUSH JUST ;
6     : DAPRMT CR ." Data acquisition rate (1 data pt. per n msec)"
7       4 SPACES ;
8     : DATRATE DAPRMT 0 PERIOD JUST ;
9     : DLPRMT CR ." Delay before data acquisition (msec)"
10      14 SPACES ;
11    : TAKDLY DLPRMT 0 DLY JUST ;
12
13
14
15

Block Number:  25
0     ( MORE INPUT ROUTINES)
1     : DTPRMT CR ." Number of data sets sent to 11/23" 17 SPACES ;
2     : NDSTS DTPRMT 0 #DAT JUST ;
3     : BLPRMT CR ." Data storage block number" 25 SPACES ;
4     : DBLOCK BLPRMT 0 #BLO JUST ;
5     : ?LOOK NPSH NPTS DATRATE TAKDLY ;
6
7
8
9
10
11
12
13
14
15

Block Number:  26
0     ( ANALYSIS OPTIONS)
1       VARIABLE .DATA0 200 ALLOT
2       VARIABLE ANOPT              1 ANOPT !
3     : .AVRG NPOINT @ 2* 0 DO DATA0 I + @ .DATA0 I + @ + 2 /
4       DATA0 I + ! 2 +LOOP ;
5     : .GO PREP ANALYZE DPREP ;
6     : 4AVE .GO AVRG ;
7     : 8AVE .GO AVRG DATA0 .DATA0 200 CMOVE .GO AVRG .AVRG ;
8     : GO ANOPT @ DUP 1 = IF .GO ELSE DUP 2 = IF 4AVE ELSE
9       3 = IF 8AVE THEN THEN THEN ;
10    : OPPRMT CR ." Enter analysis option number     " ;
11    : OPNUM OPPRMT 0 ANOPT JUST ;
12
13
14
15
```

Table 11 (Cont'd.)

```
L MICRO.FTH/E/BL 9 29 LP :

Block Number:  27
0     ( SF ANALYSIS SEQUENCE)
1     : PAR CR CR ." Enter the desired values or press return" CR
2       ." to accept default values: " CR ?LOOK ;
3     : ANA CR CR ." Complete the following steps before analysis"
4       CR CR
5       ." 1.  Obtain a 100%T reading.  Make sure the PMT output" CR
6       ."     has stabilized.  Type GETLIGHT. " CR CR
7       ." 2.  Obtain a dark current reading.  Close the shutter" CR
8       ."     until the DVM reads 0.00.  Type GETDARK. " CR CR
9       ." There are 3 analysis options : " CR CR
10      ." 1.  4 pushes (1 filling) with no averaging. " CR CR
11      ." 2.  4 pushes (1 filling) with averaging.  The data " CR
12      ."     are stored in dataset 0 . " CR CR
13      ." 3.  8 pushes (2 fillings) with averaging.  The data " CR
14      ."     are stored in dataset 0. " CR CR OPNUM
15      CR CR ." Type GO to begin analysis. " ;

Block Number:  28
0     ( SF ANALYSIS SEQUENCE CONT.)
1     : STO CR CR ." Respond to the following prompts before "
2       ." storing data : " CR CR NDSTS DBLOCK LABEL CR CR
3       ." Type SEND to transfer data sets to the 11/23. " CR ;
4     : SEND  PREFLUSH BLFILL TRANSFER ;
5
6     : RNS CR CR ." Type CLEAN to rinse system. " CR ;
7     : CLEAN PREP RINSE ;
8
9
10
11
12
13
14
15

Block Number:  29
0     ( USER FRIENDLY COMMENTARY)
1     : OPLIST CR 15 SPACES ." OPTIONS LIST" CR CR
2       ." 1.  Set parameters :  Type PAR" CR
3       ." 2.  Analyze :  Type ANA" CR
4       ." 3.  Store data :  Type STO" CR
5       ." 4.  Rinse system :  Type RNS" CR
6       ." 5.  Exit program :  Hit BREAK key " CR
7       ." 6.  List options :  Type OPLIST " CR ;
8
9     : SF CR CR ." WELCOME TO THE STOPPED-FLOW ANALYSIS PROGRAM" CR
10    CR ." This program controls the stopped-flow apparatus and " CR
11      ." allows the user to collect, prepare, and transfer "  CR
12      ." data to the 11/23 for further data manipulation. "
13    CR CR OPLIST ;
14
15
```

Table 11 (Cont'd.)

```
L MICRO.FTH/E/BL 35 36 LP :

Block Number:  35
0     ( DAC STUFF)
1       45536 CONSTANT YDAC
2       45552 CONSTANT XDAC
3     : BACKGROUND CREATE )R OVER + DUP HERE + 2+ 2+ , SWAP HERE +
4       2+ , R) + ALLOT ;
5     : BUILD OPERATOR @ OVER @ 96 MOVE 2@ DUP OPERATOR @
6       1+ ! 5 + ! ;
7       60 60 60 BACKGROUND 1BACK 1BACK BUILD
8       VARIABLE BKSTAT     0 BKSTAT !
9       VARIABLE STNUM 2 ALLOT
10
11
12
13
14
15

Block Number:  36
0     ( BACKGROUND WORD FOR BLOCK OUTPUT TO SCOPE)
1
2     CODE TRYIT STNUM 2+ LHLD STNUM L@A A D MOV
3     BEGIN M A MOV 240 # ANA A E MOV H INX M A MOV
4     H INX 15 # ANA E ORA RRC RRC RRC RRC XDAC STA D DCR 0= END
5     NEXT JMP
6
7    : SCOPE  STNUM 2! 1BACK ACTIVATE BEGIN TRYIT PAUSE BKSTAT @ END
8       BEGIN STOP 0 END ;
9
10    DATA0 100 SCOPE
11
12
13
14
15
```

Table 12. FORTRAN Data Conversion Software (CRUNCH.FTN)

```
      EQUIVALENCE  (DATA(1,1),IDSET0(1)),(DATA(5,4),IDSET1(1))
      EQUIVALENCE (DATA(9,7),IDSET2(1)),(DATA(13,10),IDSET3(1))
      EQUIVALENCE  (DATA(1,14),ID(1))
      BYTE  INSPEC(32),OUSPEC(32),ID(80)
      INTEGER  DATA(32,16),IDSET0(100),IDSET1(100)
      INTEGER  IDSET2(100),IDSET3(100)
      DIMENSION  DSET0(100),DSET1(100),DSET2(100),DSET3(100)
      INTEGER  NPOINT,NPUSH,PERIOD,DLY,X,L,K,M
      REAL  LIGHT,DARK
      NN=1
      WRITE(5,850)
850   FORMAT(/' STOPPED-FLOW DATA CONVERSION PROGRAM'/
     +' This program accepts digitized voltage values sent from'/
     +' the microprocessor and converts them to absorbance.'/
     +' The absorbance and time values are put into a form'/
     +' readable by MULPLT by assigning the following tags:'/
     +' DATASET0=RD, DATASET1=AA, DATASET2=BB, DATASET3=CC.')
      WRITE(5,851)
851   FORMAT(' One block of data is converted at at time by'/
     +' reading the values from an existing RSX input file.'/
     +' The output filename may be specified as a new or'/
     +' existing file, as desired.')
600   WRITE(5,900)
900   FORMAT(' ENTER INPUT FILE  '$)
      READ(5,901)LEN,INSPEC
901   FORMAT(Q,32A1)
      INSPEC(LEN+1)=0
      WRITE(5,902)
902   FORMAT(' ENTER OUTPUT FILE  '$)
      READ(5,903)LEN,OUSPEC
903   FORMAT(Q,32A1)
      OUSPEC(LEN+1)=0
10    OPEN(UNIT=3,NAME=INSPEC,CARRIAGECONTROL='LIST',
     +     READONLY,TYPE='OLD',ERR=290)
      OPEN(UNIT=4,NAME=OUSPEC,FORM='FORMATTED',
     +     ACCESS='SEQUENTIAL',TYPE='NEW',ERR=291)
550   DO 100 I=1,16
110   READ(3,800,ERR=110,END=999)(DATA(J,I),J=1,32)
800   FORMAT(32A2)
100   CONTINUE
      DO 15 J=1,100
      DSET0(J)=FLOAT(IDSET0(J))
      DSET1(J)=FLOAT(IDSET1(J))
      DSET2(J)=FLOAT(IDSET2(J))
      DSET3(J)=FLOAT(IDSET3(J))
15    CONTINUE
      NPOINT=DATA(1,16)
      NPUSH=DATA(2,16)
      PERIOD=DATA(3,16)
      DLY=DATA(4,16)
      LIGHT=FLOAT(DATA(5,16))
      DARK=FLOAT(DATA(6,16))
      WRITE(5,860)ID,NPOINT,NPUSH,PERIOD,DLY,LIGHT
     +  ,DARK,INSPEC,OUSPEC
860   FORMAT(/' BLOCK DESCRIPTION'/' ',80A1,/
     +' ',I5,' points per push'/' ',I5,' pushes per fill'/
     +' ',I5,' msec acquisition rate'/' ',I5,' msec delay'/
     +' ',F5.0,' (100%T reading)'/' ',F5.0,' (dark current)'/
     +' ',32A1,' = Input file'/' ',32A1,' = Output file')
      K=0
      WRITE(5,750)
750   FORMAT(/' Enter 0 to convert all data sets'/
     +  ,' Enter 1 to convert a single data set')
      READ(5,751)M
751   FORMAT(I1)
```

Table 12 (Cont'd.)

```
      IF(NN.EQ.0)GOTO 20
      WRITE(4,860)ID,NPOINT,NPUSH,PERIOD,DLY,LIGHT
     +,DARK,INSPEC,OUSPEC
20    IF(M.EQ.0)GOTO 50
25    WRITE(5,700)
700   FORMAT(/' Enter the number of the dataset'/
     +,' to be converted (0 thru 3).  Enter 4 to exit')
      READ(5,701,END=999)L
701   FORMAT(I1)
      L=L+1
      GOTO(50,60,70,80,90),L
50    X=0
      K=K+1
      DO 500 I=1,NPOINT
      Y=1/(DSET0(I)/LIGHT-DARK/LIGHT)
      Y=ALOG10(Y)
      X=X+PERIOD
      WRITE(4,950)FLOAT(X),Y
950   FORMAT('RD',2G15.6)
500   CONTINUE
      IF(K.EQ.NPUSH)GOTO 90
      IF(M.EQ.0)GOTO 60
      GOTO 25
60    X=0
      K=K+1
      DO 501 I=1,NPOINT
      Y=1/(DSET1(I)/LIGHT-DARK/LIGHT)
      Y=ALOG10(Y)
      X=X+PERIOD
      WRITE(4,951)FLOAT(X),Y
951   FORMAT('AA',2G15.6)
501   CONTINUE
      IF(K.EQ.NPUSH)GOTO 90
      IF(M.EQ.0)GOTO 70
      GOTO 25
70    X=0
      K=K+1
      DO 502 I=1,NPOINT
      Y=1/(DSET2(I)/LIGHT-DARK/LIGHT)
      Y=ALOG10(Y)
      X=X+PERIOD
      WRITE(4,952)FLOAT(X),Y
952   FORMAT('BB',2G15.6)
502   CONTINUE
      IF(K.EQ.NPUSH)GOTO 90
      IF(M.EQ.0)GOTO 80
      GOTO 25
80    X=0
      K=K+1
      DO 503 I=1,NPOINT
      Y=1/(DSET3(I)/LIGHT-DARK/LIGHT)
      Y=ALOG10(Y)
      X=X+PERIOD
      WRITE(4,953)FLOAT(X),Y
953   FORMAT('CC',2G15.6)
503   CONTINUE
      IF(K.EQ.NPUSH)GOTO 90
      IF(M.EQ.0)GOTO 90
      GOTO 25
90    WRITE(5,760)
760   FORMAT(/' DATA CONVERSION COMPLETED')
      WRITE(5,775)
775   FORMAT(/' Do you want to convert another block'/
     +' of data? (Yes=1, No=0)    ')
      READ(5,751)N
      IF(N.EQ.1)GOTO 120
```

Table 12 (Cont'd.)

```
      CLOSE(UNIT=3)
      CLOSE(UNIT=4)
      STOP
120   WRITE(5,776)
776   FORMAT(/' New input file? (Enter 1)'/
     + ' Present input file? (Enter 0)     ')
      READ(5,751)NN
      IF(NN.EQ.0)GOTO 550
      CLOSE(UNIT=3)
      CLOSE(UNIT=4)
      GOTO 600
      STOP
290   WRITE(5,989)INSPEC
989   FORMAT(' ERROR OPENING '32A1' RE-ENTER'$)
      STOP
291   WRITE(5,989)OUSPEC
999   STOP
      END
```

Table 13. Analysis Task Routine (TAKE.CMD)

```
.ENABLE SUBSTITUTION
.IF P1 NE "" .GOTO 10
.5:
.ASKS P1 ENTER RSX FILENAME
.ASKS P2 ENTER FORTH BLOCK NUMBER
.10:
FPIP C GO.FTH/E/BL 'P2' 'P1' /R
RUN CRUNCH/TASK=CRNCH
.WAIT CRNCH
MUL
@REGPLOT
PIP FOR007.DAT;*/DE
.ASK YESNO DO YOU WANT ANOTHER RUN
.IFT YESNO .GTO 5
.EXIT
```