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APPLICATION OF AN AUTOMATED STOPPED-FLOW SYSTEM IN CLINICAL ANALYSIS

By

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ABSTRACT

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By

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Stopped-flow mixing with spectrophotometric detection has several desirable features that makes it a suitable technique for fast clinical analysis. Mixing times are as low as a few ms, which enables analysis to be done in very short times by fast reaction-rate methods. A typical application is the reaction-rate determination of serum total protein by the biuret method. With the stopped-flow method reported here, the analysis time was 10 s for each sample, which is a 100-fold decrease when compared with the normal equilibrium method. Preliminary kinetics studies of the biuret reaction were performed with a vidicon detector and the results provide a starting point for future mechanistic investigations.

The short mixing time of stopped-flow systems is also advantageous for equilibrium-based applications. Serum albumin reacts very rapidly with bromcresol green. By taking the equilibrium measurements early (2-6 s after mixing), interferences from other slower reacting components

are greatly reduced.

The automated stopped-flow system was also adapted for serum lipid analysis. Highly specific enzymatic methods of analysis for serum triglyceride and cholesterol were used, and the results obtained compared to other standard methods. Serum lipoprotein analyses were also performed. The complete serum lipid profile, including the increasingly popular serum high density lipoprotein (HDL) analysis, can be completed in about half an hour. Again the analysis time is reduced, in this case by at least 10-fold, in comparison to other prevalent methods.

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"And we know that all things work together
for good to them that love God . . . . ."
Romans 8:26.

As one goes through the disciplinary process of formal education, it is sometimes hard to remain convinced of this. These last five years have not been altogether smooth sailing. The days spent grinding out problem sets, the nights lost debugging programs, the hours spent brooding over mechanical problems and experimental failures, not to mention all the despair, expasparation and frustration that accompany these. But through it all, this particular knowledge has been my mainstay, bouying me up between those scattered moments of eurekas, making it possible for me to culminate my schooling in this volume.

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I. INTRODUCTION

With the advancement of instrumentation and fast analytical methods, the clinical sciences are becoming more and more chemically orientated. Massive screening chemical tests are being performed for individual patients nowadays, as evidenced by the popularity of the Technicon continuous flow analyzers that can do six, twelve, or even more tests on each sample. Because of the constant demand for higher throughput and 'stat' analysis, together with the increasing use of enzymatic methods, reaction-rate methods of analysis are becoming more important than ever in clinical chemistry. It is not impossible that in the near future enough tests may be performed on each individual so that everyone would have his or her own normal ranges and no longer would it be necessary to compare test results with the population's average. The large volume of tests performed will certainly require a much faster throughput rate than the typical 120 samples/hour rate of most current instruments.

The stopped-flow mixing system, although used primarily as a research tool until now, has the potential of being a very fast and versatile analyzer. It can measure reaction half-lives from milliseconds to hours. Required reagent volumes are as low as 100 μl, and the instrumentation is easily automated. The only problem that has prevented wider applications of it in clinical chemistry

is the lack of research in examining the applications of this fast mixing system.

In this thesis, the stopped-flow system was applied to routine analyses. Chapter 2 is a general discussion on some basic concepts about fast kinetics and the characteristics of several types of automated clinical chemistry analyzers. Then the automated stopped-flow system is described.

Chapter 3 presents a reaction-rate analysis of the protein biuret reaction that cuts analysis time by more than 100-fold when compared to conventional methods.

Chapter 4 describes an equilibrium method that allows serum albumin to be determined with bromcresol green within 2 s. Most of the interfering reactions are also reduced simultaneously through the use of the rapid equilibrium method.

Then we move on to the important area of lipid analysis. Triglyceride analysis is described in Chapter 5, and cholesterol analysis in Chapter 6. These lipid analyses use slow enzymatic reactions so some means of treating the problem of the long analysis times had to be devised in order to utilize stopped-flow mixing advantageously. Furthermore, the problems of formulating suitable aqueous standard solutions were also investigated.

Chapter 7 concentrates on lipid profiling and analyses of lipoproteins. The analysis of high density lipoprotein has generated increasing interest recently, and its significance as an indicator of coronary heart disease has created a large demand for this test. The technique of getting a total lipid profile using serum triglyceride, cholesterol, and lipoprotein concentrations was investigated. We are able to report a rapid procedure that cuts the normal analysis time six to forty fold.

Finally, in Appendix A, we report an application of the simplex technique in regression analysis and differential rate analysis. Although we have not applied the technique to actual clinical tests, it appears to have great potential in differential analysis of complex mixtures, especially those involving enzymes. A well documented super-modified version of the simplex program written in FORTRAN IV language is also included in this section.

We conclude that the stopped-flow system has great potential in the field of clinical chemistry. Additional comments on the future trend of research in methodology may be found in the last chapter.

II. BACKGROUND

The first part of this chapter describes the commonly performed clinical tests and the most used clinical-chemical instruments. Next, a summary of kinetic methods of analysis is presented, followed by a brief description of the automated stopped-flow system used in this research and its role in clinical chemistry.

A. Clinical Analysis and Instrumentation

The clinical field offers many exciting research opportunities for the analytical chemist who enjoys making meaningful measurements. While some may still regard quantitative chemical measurements merely as tools, many physicians and scientists are sharing with the analytical chemist a deep concern for accuracy, precision, and speed. Undoubtedly, the most recent trend is the increasing use of automated instruments to replace manual methods of analysis, and a wide variety of automated analyzers and methods have been reported. But, the emphasis of this dissertation is on automated wet chemistry only, and instrumental techniques such as automated flame spectrometry and chromatography are not discussed.

1. Common Clinical Tests

The number of different chemical tests performed in a clinical laboratory has risen from fewer than thirty in 1950 to more than one hundred in the past few years (23). The twelve most popular models of chemical analyzers provide 27 different routine tests that they can perform (See Table 2-1). Many factors determine what tests are to be performed routinely. First, they must serve as indicators of diseases, preferably with a one-to-one correspondence. For example, the use of the enzyme glucose oxidase for serum glucose analysis is a good test for diabetes mellitus. Second, the analysis time (both total work time and technician time) must be as short as possible. Tests such as serum Na^+ and K^+ using flame photometry require less than one minute and thus are performed routinely as stat tests. Third, the tests should be simple. Excellent reference methods such as the Kjeldahl test for protein nitrogen involve complicated distillation and multiple-step reactions and, therefore, most laboratories prefer the simpler, but less precise blood urea nitrogen (BUN) test. Finally, low cost, availability, and sample size requirements are also important factors that govern the choice of routine methods.

Recent research and development of new clinical chemical methods make increasing use of enzymes, which

Table 2-1. Routinely Performed Clinical Tests.

	DSA 560	SMA 12/60	ENI GeMSAEC	Mark X	DIGECON 1011	AM. MONITOR KDA	MICROSTAT	SMAC	ROBOT CHEMIST	DUPONT CLIN. ANAL.	STAC	GILFORD 3500
ACP						*						
ALP	*	*	*	*	*	*	*	*	*	*	*	*
ALBUMIN	*	*	*		*	*	*	*	*	*	*	*
ALCOHOL	*											
AMYLASE						*			*			
BILIRUBIN	*	*		*	*	*	*	*	*	*	*	*
BUN	*	*		*	*	*	*	*		*	*	*
Ca^{++}	*	*			*	*	*	*		*	*	*
CHOLESTEROL		*		*	*	*	*	*	*	*	*	*
Cl^{-}	*				*	*		*				*
CO_2								*				*
CPK	*		*		*	*		*		*	*	*
CREATINE	*		*		*	*	*				*	*
IRON	*				*	*		*	*			*
GLOBULIN				*								
GLUCOSE	*	*		*	*	*	*	*	*	*	*	*
α HBDH	*		*		*						*	*
K^{+}								*				*
LDH	*	*	*		*	*	*	*		*	*	*
Na^{+}								*			*	
PBI	*			*								
PO_4^{-3}	*	*	*	*	*	*	*	*		*	*	*
PROTEIN	*	*	*	*	*		*	*	*	*	*	*
SGOT	*	*			*	*		*		*	*	*
SGPT	*		*		*	*	*	*	*	*	*	*
TG						*	*	*				
URIC ACID	*	*		*	*	*	*	*	*	*	*	*

are very specific, as they become more available. With the development of multi-test systems such as the Technicon SMAC, more laboratories conduct screening programs as these systems allow much information to be gathered from a small sample. Simultaneously there is increased concern for greater precision in routine testing on a day-to-day basis and between laboratories (23). Moreover, there is a growing recognition for the need of referee methods, which would establish the "true" constituents of serum more exactly than is possible with the present routine daily production methods (23). It is hoped this will help to shift the emphasis from a concern for more economic service to one for improved quality in terms of proper standardization procedures and development of meaningful quantitative measurements.

2. Automated Systems

Commercially available automated systems for wet chemical methods have developed along three major directions. They are continuous-flow systems, centrifugal analyzer systems, and discrete-sample systems.

a. Continuous-flow Analyzers - L. T. Skeggs (10) conceived the continuous-flow principle in 1957 and Technicon Instruments Corporation later introduced the first AutoAnalyzer. This system received wide

acceptance in the clinical chemistry laboratory, and the newest model (SMAC) is computerized to do multiple tests simultaneously on the same sample within a few minutes.

The AutoAnalyzer uses a peristaltic pump and tubing of various sizes to carry fixed-volume proportions of samples and reagents continuously through the system. This eliminates the operations of pipetting, weighing and measuring of reagents. Air bubbles in the stream physically separate samples into segments to minimize the carry-over from the previous samples and to reduce the wash time. Glass tubing coiled in different lengths allows heavier liquids to fall through the lighter ones to produce uniform mixing. And, to avoid manual deproteinization of the samples, the AutoAnalyzer dialyses them through a semipermeable membrane.

The AutoAnalyzer has a modular design, which makes general maintenance and any necessary modifications relatively simple (7). The major component modules are the sampler, the proportioning pump and manifold, the dialyzer, the heating bath, the flow cells, and the colorimeter. Newer modules include a computer and detectors such as the flame photometer, fluorometer, and ion-selective electrodes. To change from one chemical test to another, different cartridges can be plugged in, thus expanding the system to run a maximum of 40 different tests if desired (7). Finer tubing, smaller flow cells, and

electronic debubbling help to reduce the wash volume tremendously, so that a sample volume of only 450 µl is now needed for a 20 test run (70).

Despite the wide adoption of the continuous-flow method, it still has its drawbacks. For example, the construction of the flow system makes it inconvenient to run single chemical tests, and often the laboratories would therefore do 12 or 20 different unnecessary tests on each sample. Then, because the system uses single-point or two-point fixed time reaction-rate methods to determine enzyme activities, blanks and the shape of the reaction curve have to be ignored, which could be a major source of error for abnormal samples. The system is also not suitable for studying very fast and very slow reactions. Finally, the initial cost of the system is high so that smaller laboratories often cannot afford it.

b. Centrifugal Analyzers - The concept of using centrifugal force to transfer and mix liquids in a centrifuge and do multiple sample analyses was introduced by N. G. Anderson at Oak Ridge National Laboratories (21,71). This system, which also has a modular design, consists of an analyzer, a control module, a rotoloader and a digital computer with all its accessories. The analyzer houses the spinning rotor, a monochromator, and a photosensor. The control module, as the name suggests,

controls the reaction time, and the mixing, washing and drying processes, while the rotoloader automates sample preparation and makes additions.

The rotor consists of an outer ring containing a number of cuvets formed by compressing the inert spacer between two plates of optically transparent material, and a removable central transfer disc of Teflon containing three concentric rows of cavities. Acceleration of the rotor causes any sample and reagent placed in the inner cavities to move outward, first to the outermost row of cavities and then into the cuvet. The transfer mixes the sample with the reagents. To monitor the reaction, one only needs to measure the transmittance change of the mixture inside the cuvet. Each rotor usually has 16 channels and the first one normally holds the reagent blank. The computer gathers the data and processes it immediately. As a final step, the rotor automatically cleans itself after each run (21,8).

The three commercial instruments currently available in the United States are the GeMSAEC by Electronucleonics Inc., the Rotochem by American Instrument Co., and the CentrifiChem by Union Carbide Co. (8,22).

The centrifugal analyzer can perform most of the commonly requested clinical chemistry tests, and is especially suitable for determining enzyme activities, because of its multi-point continuous monitoring approach,

its highly sensitive temperature control, and its precise metering of sample and reagents. No reagent blank is necessary because the mixing only takes three seconds, and by extrapolation, one can use the sample as its own blank.

Newly designed transfer discs, improved sequences of rotation (23), and disposable rotors (8) have eliminated the cumbersome process of sample preparation and many of the carry-over problems. But again, the relatively high cost of centrifugal analyzers has prevented many laboratories from purchasing one.

c. Discrete Analyzers - Many other instruments have been built for clinical chemical analyses. Some can perform as many tests as a SMAC system, some only do enzymatic analyses, and others can only run one or two specific tests. Yet, they all have one thing in common: each processes the specimen separately, generally by steps mimicking the conventional manual procedures.

For all their simplicity and lack of elegance, discrete sample analyzers have their distinct advantages. First, one does not have to worry about cross-over contamination and, since each step in the procedure is independent, one can easily trace the source of any trouble. Finally, these analyzers generally require lower volumes of reagents and samples. But, as a trade-off, one must

put up with a much lower throughput rate and a greater probability of breakdown, due to the mechanical complexity of discrete analyzers.

Since many such systems are available, it is impossible to review each one of them here. But, Table 2-2 does give a brief summary description of the larger systems. For more detailed descriptions, reference to the books (7-9) and reviews (15-18) listed in the bibliography should be made.

B. The Discrete Stopped-flow System and Kinetic Methods

Chance pioneered the development of the stopped-flow system in 1940 (24). Since then many sophisticated elaborations of this system have appeared. Some examples of these are the computer-controlled systems developed by Crouch, *et al.* (25), by Dye, *et al.* (27), and Pardue's fully automated system (194) that can even prepare solution automatically. A recent review by Crouch *et al.* (29) compares many of the home-made and commercial systems and describes them in detail.

The stopped-flow technique for rapid mixing of chemical reagents was originally designed to allow the study of fast reactions with half-lives as short as a few milliseconds. It has not been until recently that scientists have investigated its potential for other kinds of analyses. There are several advantages that

Table 2-2. Popular Models of Discrete Chemical Analyzers.

Instrument	Description	Throughput Per Hour	Vol. Serum Needed	Reference
1. Dupont ACA	Reagent Packs, sequential	100 tests	20-500µl each	8,11,12
2. Vickers M-300	Parallel 10 reactions console	300 specimens	400µl per 20 tests	8,11
3. Hycel Markx	Selection of up to 10 different tests, performed simultaneously by push buttons, test tube reactions, 30 min DWELL TIME	400 specimens or	0.1 ml	8,13,9
4. Coulter Chemical Analyzer	1-22 different tests in parallel, dedicated modules for different tests	60 specimens	15-40 µl	8
5. Clinicard	Prepackaged disposable curvets which are supplied with a program card, sequential 30 different tests, good for 'STAT' test	60 specimens	25-200 µl	8
6. Beckm n DSA 560	Sequential, disposable plastic sample containers (Q cups) horizontal tray transport mechanism, pneumatic drives	160 tests	5-50 µl	7,9,11
7. Am Optical Robot Chemist	First automated system, 24 channels conveyors	135 specimens or 3000 tests.	20-300 µl	9

make the stopped-flow method an attractive tool. First, it is a highly versatile system, for not only can it provide analytical data on reactions that occur in a matter of a few milliseconds, but it is also suitable for studying slow reactions. For example, by using kinetic measurements one can use it to study the initial stage of reactions. By waiting for the reaction to go to completion, one can also use it to make equilibrium measurements. Therefore, unlike the continuous-flow systems that have a limited range of reaction half-lives the stopped-flow analyzer features rapid throughput of information and the study of reactions over a wide range of half-lives. Secondly, the system can be fully automated. Using minicomputers or microprocessors to control the processes of solution preparation, data acquisition and data manipulation speeds data throughput, improves accuracy and increases precision. And finally, the small sample volume required (a few microliters) makes it attractive for routine clinical measurements where sample size is often severely limited.

This section describes briefly the stopped-flow system in our laboratory and some fundamentals of kinetic analysis.

1. The Automated Stopped-flow System

Beckwith (4) first built the pneumatic driven stopped-flow system, and Notz (5) interfaced it with a PDP 8/e minicomputer.

Figure 2-1 shows a diagram of the automated stopped-flow system. This system can be operated both manually and automatically.

Operation begins by turning on the delivery valve (A), which connects the reagent and sample reservoirs to the delivery syringes, while closing off the remainder of the flow system. The syringes then draw in reagent and sample solutions, and the delivery valve then turns to connect the syringes to the mixing chamber and the observation cell. Air pressure is applied to the drive syringe piston, when the waste valve (B) opens and connects the flow system to the stopping syringe. The syringe drive piston rapidly strokes forward and forces the solutions through the mixing chamber, observation cell, and stopping syringe. The flow stops when the spring-loaded stopping syringe comes to rest against a mechanical stop. At the same time, an optical interrupter sends out a pulse which signals the end of flow and triggers the computer to begin gathering data. When the measurement is finished, the waste valve opens to the drain and

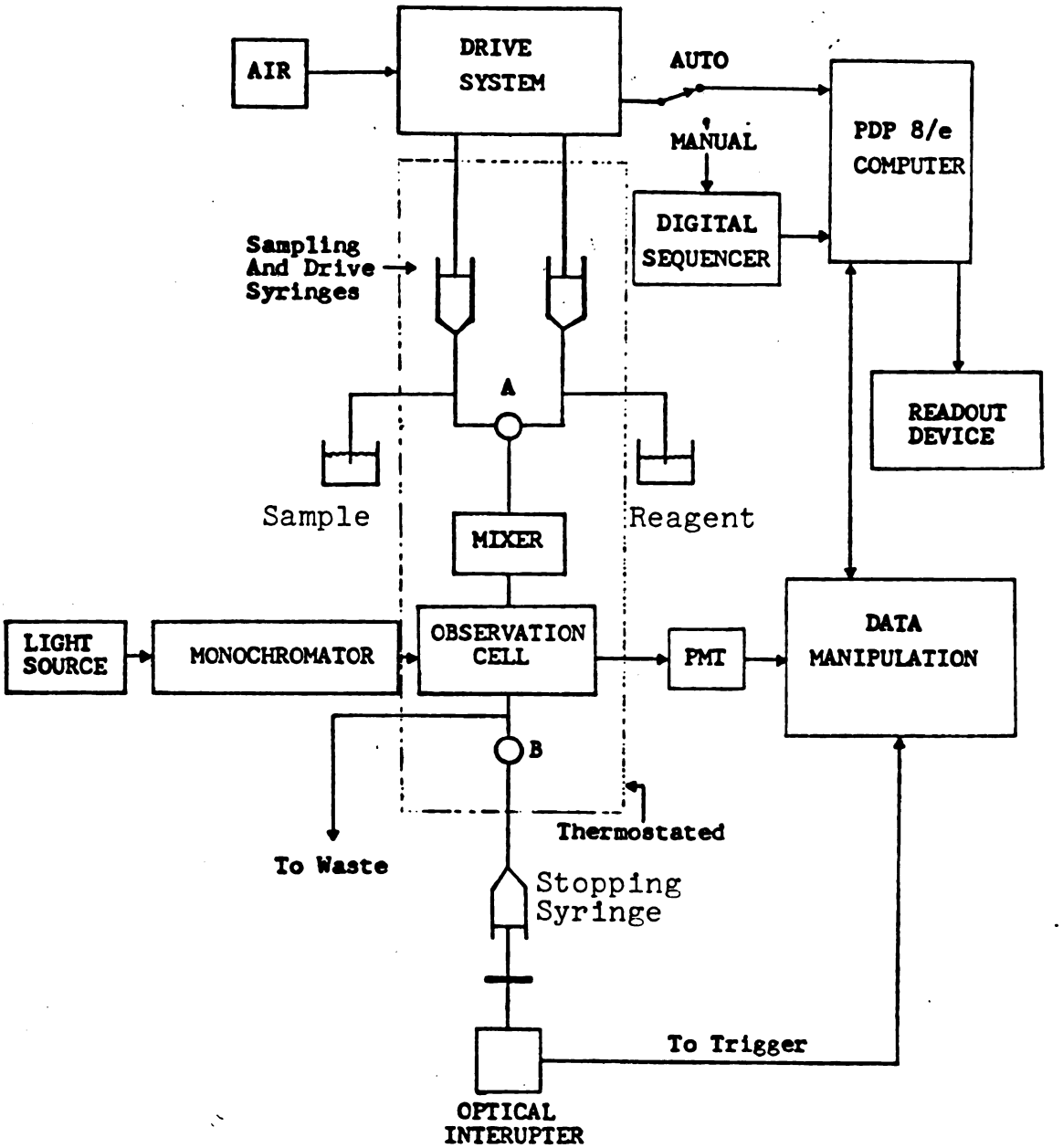


Figure 2-1. The Automated Stopped-Flow System.

the stopping syringe forces the old solution out the drain port (4). The valves then reset and run the next sample. Interactive FORTRAN programs are available to analyze the data. Results are stored on magnetic media, or the printer types out a copy of the results. Circulating water acts as the system's thermostat, keeping the temperature of the observation cell slightly above that of the drive syringes and the mixing chamber, so that it will match the temperature of the solution after mixing (6).

The design of the mixer and the flow rate ensure turbulent flow and efficient mixing. The rapid stopping of the solution completes the mixing and provides back pressure which prevents cavitation (5). Table 2-3 lists some other important information about the system. The dead time of the system (5±1 ms) dictates the practical limit on the fastest reaction which can be observed. However, for most clinically important reactions, the dead time imposes no serious problem.

2. Reaction-Rate Method of Analysis

With the advancement of electronics and instrumentation, reaction-rate methods, previously difficult to do experimentally, are now becoming routine. Several books (36-38), review articles (39-41), and papers (42-44) on the theory and application of these methods have been

Table 2-3. Important Properties of the Stopped-Flow System.

Dead Time	5±1 ms
Mixing Time	~5 ms
Stopping Time	$\leq$ 1 ms
Volume of Sampling Syringe	2.35 ml
Volume of Each Push (6 pushes/fill)	0.39 ml
Volume from Mixer Entrance to Exit of Observation Cell	0.16 ml
Pathlength of Cell	1.94 ± 0.01 cm

published.

The biggest advantage of the reaction-rate method compared to equilibrium methods is its greatly reduced experimental time. It is possible to measure the rate of change of the concentration of reactants or products immediately after mixing without waiting for the reaction to go to equilibrium. Since the measurement can be completed before significant side reactions occur, and because only relative rate measurements are made, many of the interfering factors are thus eliminated. Stopped-flow analyses often employ reaction-rate methods. By simply adjusting the experimental conditions, one can obtain first or pseudo-first order reaction conditions. For example, consider a simple reaction:

$$A + R \xrightarrow{k_A} P$$

where k_A is the rate constant, A is the substrate, R the reagent in excess, and P the product. The rate of disappearance of A can be expressed as

$$\frac{d[A]}{dt} = -k_A[A] \qquad (1)$$

If Equation (1) is integrated from time t = 0 to time t = t, we have

$$[A] = [A]_0 \exp(-k_A t) \qquad (2)$$

If Equation (2) is substituted into (1),

$$\frac{d[A]}{dt} = -k_A[A]_0 \exp(-k_A t) \qquad (3)$$

when $t = 0$, $\exp(-k_A t) = 1$

therefore,

$$\frac{d[A]}{dt} = -k_A[A]_0 \qquad (4)$$

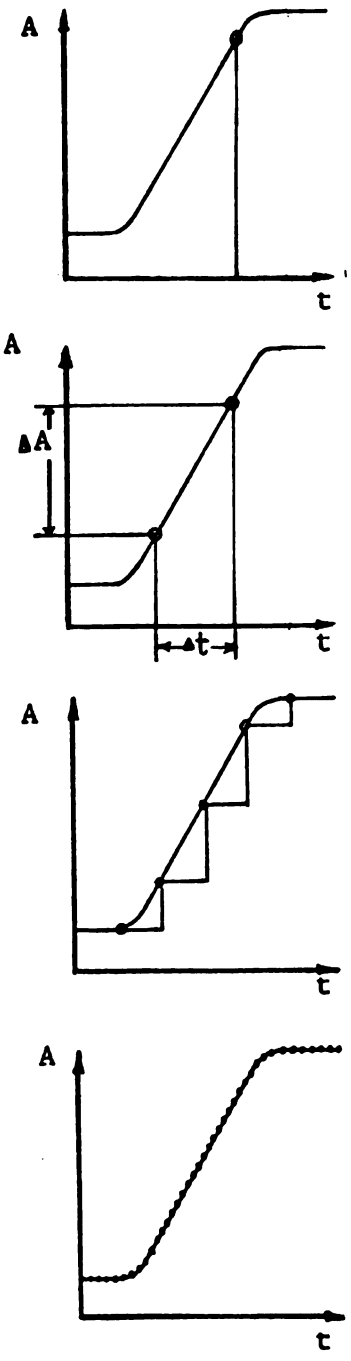
Equation (2) suggests that the initial concentration of the reagent $[A]_0$ can be obtained by measuring the concentration of A at any time t. $[A]_0$ can also be obtained by measuring the rate of change of A at time t, [Equation (3)]. But Equation (4) is the basis of the most commonly used rate method. By measuring the reaction rate at the very beginning so that the time is approximately equal to zero, the exponential term approaches unity and thus the reaction becomes pseudo-zero order in time. This is known as the initial rate method, and requires that the measurement be made while the reaction curve (the exponential concentration vs. time curve) is still linear. For 1% accuracy in the initial rate measurement, the measurement should be completed before the relative concentration changes by 5% for the fixed time method (31), and by 2% for the variable time

method (32).

In the last few years, more clinical chemists have been using rate methods mainly due to the extensive use of enzymes (33). At the same time however, this widespread use of rate methods has also brought about ambiguity in the terminology. Pardue has suggested a comprehensive classification which has helped to clarify the terms somewhat (34).

Equilibrium methods were defined as those methods based upon reactions that go to completion, and rate methods as those based on measurements made while the reaction is approaching completion.

The commonly used direct response rate methods can be classified as in Figure 2-2. The simplest is the one-point method wherein the signal is measured at some predetermined time after the reaction has been initiated. This method does not provide any information on the blank or the shape of the response curve. For the two-point method, there are two different approaches. The fixed-time approach measures the total signal change over a predetermined fixed time interval, while the variable-time approach measures the total time required for the signal to change a predetermined amount and relates it to the analyte concentration. The two-point method has an advantage over the one-point method in that it compensates for sample and reagent blank, but errors due to



1. <u>One-Point Method</u>
   Simple, no information on blank or shape of curve

2. <u>Two-Point Method</u>
   Fixed-time or variable-time, simple, no information on curve shape

3. <u>Multi-point Method</u>
   A) Delta Method
      Suitable for 5-10 data points

   B) Regression Method
      Suitable for large number of data points

Figure 2-2. Classification of reaction-rate method.

non-linearity of reaction curves caused by induction periods or substrate depletion still occur.

When three or more data points are collected either in the fixed-time or the variable-time modes, it is defined as the multiple-point method. At least two different approaches within this class can be identified. The "delta method" takes multiple absorbance data at fixed time intervals and computes values of A called "deltas". Delta values of the same magnitude are used to compute the substrate concentration. In the "regression method", one collects many data points and then uses a mathematical algorithm, such as the linear least-squares method, to obtain the slopes from the linear region. An advantage of the regression method is its applicability to both linear and nonlinear responses. The first and second derivatives of A vs. t help to locate the linear region of the response curve, and overlapping groups of data will give many values of the slopes for averaging. It appears that with the help of computerized data acquisition and analysis, the collection of large amounts of data is no longer a laborious task, and the regression multiple-point method, properly implemented with statistical analysis, is the preferred method.

Reaction-rate methods can be very rapid and show a high degree of freedom from interferences, which are important advantages in clinical analysis. The method is

also more specific than the equilibrium method so that separation steps can often be avoided. Differential analysis of complex mixtures can also be performed by taking advantage of the different reaction rates of two or more reactants with a common reagent (49-52).

But there are some rather complex problems in using reaction-rate methods. Rate methods usually require carefully controlled experimental conditions, such as temperature, pH, ionic strength, size and shape of the reaction vessels, etc. Minor changes in these conditions can change the reaction rate and thus the result. The half-life of the reaction must be greater than the mixing time of the instrument, and the precision also decreases with shorter observation time (42). On the other hand, reactions with half-lives longer than a few hours are also not practical for routine analysis. For selected procedure, however, the overall advantages of rate methods can outweigh the disadvantages.

3. Differential Reaction-rate Methods

Rarely does one find the sample for analysis in pure form, devoid of any other substances that might interfere in the determination of the sought after substance. So one must find some means to eliminate the interferences. Separation of the target sample component from interfering

substances is often too time consuming, or even impractical. In some cases, by judiciously selecting the experimental conditions, one can alter the reaction rates of different components within the sample so that they are sufficiently different from each other to allow one or more of the closely related components in the mixture to be analysed without prior separation (45-48).

Mark et al. discussed the various methods for analysing mixtures whose reactants have large rate differences (45), and Papa (36) and Mottola (40) have evaluated the more commonly employed methods.

In clinical analyses, we are mostly interested in first and pseudo-first order reactions with respect to total reactant concentration, or enzymatic activities. Several differential rate methods exist for the study of these reactions. The graphical extrapolation method (52) and the method of Roberts and Regan (54) are very useful, but the method of proportional equation is of particular interest here (53). Generally this method requires less time than the other approaches, while it does not require prior knowledge of the total initial concentration of reactants. Most important of all, it is a mathematical method which is therefore easily adaptable for automation (40).

This method is based on the principle of constant fractional life, which is applicable to most simple first

and pseudo-first order reactions. When a substance reacts with constant fractional life, its initial concentration is directly proportional to that of the product at any given time. Consider the following first or pseudo-first order reaction:

$$A + R \xrightarrow{k_A} P \tag{5}$$

The concentration of the product at any given time t is

$$[P]_t = k_A(1 - e^{-k_A t})[A]_0 \tag{6}$$

or

$$[P]_t = G_A[A]_0 \tag{7}$$

where

$$G_A = k_A(1 - e^{-k_A t}) \tag{8}$$

The proportionality constant G_A is dependent on the rate constant k_A and the time t. As long as the factors that affect k_A (such as temperature, pH, and ionic strength) do not change and the time t is fixed, G_A remains constant. In Equation (7), the concentration of the product at any given time is therefore proportional

to the initial concentration of A. Very often an electrical signal S_t is observed instead of P_t. This signal is proportional to P_t by a factor n, and Equation (7) becomes

$$S_t = G_A n[A]_0 \tag{9}$$

or

$$S_t = K_A[A]_0 \tag{10}$$

and K_A becomes the new proportionality constant. If there is a binary mixture (A + B) and the two different components react by first order kinetics to give the same product P, the concentration of the product at any time t_1 can be given by

$$[P]_{t1} = G_{A1}[A]_0 + G_{B1}[B]_0 \tag{11}$$

where G_B is the proportionality constant for substance B. At a different time t_2,

$$[P]_{t2} = G_{A2}[A]_0 + G_{B2}[B]_0 \tag{12}$$

Thus, to determine the proportionality constants G_{A1} and G_{B1} experimentally, one can measure the concentration

of the product at time t_1 for known amounts of pure A and pure B under the same experimental conditions as that of the mixture.

$$[P]_{t1} = G_{A1}[A]_0 \tag{13}$$

$$[P]_{t1} = G_{B1}[B]_0 \tag{14}$$

To find G_{A2} and G_{B2}, keep the experimental conditions constant and change the observation time to t_2. Substitution of the four constants back into Equation (11) and (12) will allow the concentrations $[A]_0$ and $[B]_0$ to be obtained on an unknown sample.

The method is applicable to mixtures that contain more than two substances (38).

With the help of a computerized on-line system, 100 or more data points can be obtained during the course of reaction. One can set up 100 or more proportional equations and solve for the unknown concentrations $[A]_0$ and $[B]_0$. Appendix A of this thesis describes the use of multiple proportional equations in some first and pseudo-first order reactions found in clinical analyses.

C. Application of the Stopped-flow System in Clinical Analysis

Traditionally, only the few scientists who study the kinetics of rapid reactions use stopped-flow mixing systems. It was not until 1969 that Pausch and Margerum (49), and Javier *et al.* (64), first reported its use for analytical purposes. And it was only after Pardue *et al.* had evaluated their discrete sampler stopped-flow mixer system in 1977 (1) that its use in clinical analysis appeared in a clinical journal. Two reasons account for this. First, instrument companies have been slow to realize the potential of this system as an analytical instrument. Therefore, very few commercial instruments are available. Also, the stopped-flow systems that are on the market are so inconvenient to use, they are in no way comparable to the "home-made" versions. Second, not enough clinical methods have been developed for recognition of the advantages of such a system and hence its adoption as a routine analytical tool.

1. Experimental Criteria and Problems

An ideal clinical chemistry analyzer should be inexpensive, simple to operate, have a high sample throughput rate, and if possible, be portable. It should be easily automated, with no carry-over problem. It should

give precise and accurate results, and be capable of doing a wide variety of tests. The sample volume for analysis should be as small as possible, and finally, the reagents inexpensive.

The advantages of the stopped-flow system in monitoring fast reactions, as discussed earlier, include high throughput rate, high precision and accuracy, easy automation, good mixing and a large reaction time range. In addition, it is also suitable for equilibrium (end-point) analyses. In fact, Pardue et al. (1) have just reported that attaching a sample loop to the system facilitates further measurements for equilibrium methods.

Most of the instruments currently used for kinetic analyses in clinical laboratories are designed for relatively slow reactions, usually requiring anywhere from a few seconds to a few minutes for a complete measurement. Thus they may not be adequate for such tasks as the LHD isoenzymes determination by Everse et al. (59), or the biuret protein reaction which is reported later. For the former, the first set of measurements must be taken within 0.2 s, while the measurement time for the latter is about 0.1 s. Pardue has already suggested that instruments with mixing and measurement capabilities that require approximately 0.01 s would be highly applicable in clinical chemistry (1).

To further reduce the sample size when using stopped-

flow systems, one can use new cells and mixer designs, or add zero dead-volume valves and fittings to the system. And, to cut reagent costs drastically, especially those needed for enzymatic reactions, one can employ reusable immobilized enzyme reaction loops such as those developed in our laboratory (2). The tremendous improvements in detection systems in recent years allow measurements with high sensitivity. Dual-channel and multichannel systems, such as the rapid scanning monochromator and vidicon have already been useful for fundamental studies of biochemical systems (67). For turbid samples, other non-optical detectors based on thermal or electrical principles can be used (68,67).

Since no system is perfect, there still remain some problems in using the stopped-flow instrument described here. One of the major criticisms made of this system is the lack of sample preparation and initial separation, like that found in the autoanalyzer. But with the possibility of adapting one of the recently reported automatic solution makers onto the stopped-flow analyzer, one of the most time consuming steps in the process would no longer be a problem (1,29).

A second problem with using this system is that of carry-over, which exists because at present the observation cell is not disposable. This makes repeated rinsings between samples necessary. It seems, however, that by

incorporating Pardue's proposed sample loops, this problem too can be eliminated (1).

A third disadvantage of this system is its inability to perform multiple tests in parallel due to its discrete mixing characteristics. One must remember, however, that a stopped-flow system is not designed for this, but is rather a 'stat' analyzer.

And finally, sample and reagent are mixed in a 1:1 ratio in the stopped-flow system. Therefore dilutions must be made to obtain favorable experimental conditions. However, in some cases excessive dilution causes undesirable problems, such as turbidity or slow reaction rate.

Despite the above limitations, the stopped-flow system can be made applicable to many kinds of wet chemical analyses done in clinical laboratories, and as is shown throughout this dissertation, it has uniquely advantageous features when compared with other existing systems.

2. Analyses Reported

Applications of the stopped-flow system in clinical analysis to date have been few, and they are all listed in Table 2-4. Most of these involve a reaction-rate method with adjustment of the experimental conditions so that the reactions occur under pseudo-first-order conditions. Enzymatic reactions are especially suitable

Table 2-4. Clinical Applications of the Stopped-flow System.

Substrate	Chemical Reagents/Reaction	Comments	Ref.
1. ATP	Firefly luciferin-luciferase		56
2. ALP	P-nitrophenyl-phosphate		65
3. ADRENALINE	Conversion to O-Benzoquinones		57,58
4. ASCORBIC ACID	2,6-Dichlorophenolindophenol		60,61
5. BILIRUBIN	P-Diazobenzene sulfonic acid	Kinetic	66,1
6. Ca^{++}	CDTA Complex	Differential Kinetic	49-52
7. CHOLESTEROL	Cholesterol Ester Hydrolase, Cholesterol oxidase	Equilibrium	1
8. CREATININE	Jaffe-Reaction	Kinetic	6
9. GLUCOSE	Glucose Oxidase-Peroxidase	Kinetic	63
10. LDH	L-Lactate, NAD^+	Kinetic, Enzymatic	1
11. LDH ISOENZYMES	Pyruvate, $NADH^+$	Differential Kinetic	3,59
12. L-DOPA	Conversion to O-Benzoquinones		54,57
13. PHOSPHATE	12-MPA in Acidic Solution	Kinetic	64.25

for this system, because of the kinetic measurements required. The differential technique used to determine isoenzymes is unique, and seems to be an interesting one to apply in the investigation of other isoenzyme systems (3,59). So little has been done in exploiting the stopped-flow system for routine clinical tests, that the inherent advantages of the system have not been exploited. But, with increased use, these will gradually emerge to make their contribution to the clinical sciences. The major portion of this research deals with exploiting the potential advantages of stopped-flow mixing for important clinical analyses.

III. SERUM PROTEIN ANALYSIS

A. Introduction

One of the most often employed yet valuable clinical tests is the determination of total blood serum protein. The total protein and the ratio of individual protein fractions of a patient may change independently of each other, due to any one of three causes: impaired protein synthesis, pathological loss or diminished intake of fluid, or an absolute increase in protein content (8). Consequently, the analysis of a patient's serum for total protein is done routinely, and the results are used to decide if any additional subfraction protein analysis is necessary. This chapter describes a kinetic biuret determination of total serum protein using the stopped-flow mixing system, which has the advantages of extremely short measurement times as well as small sample volume requirements. Because the stopped-flow system can obtain data almost instantaneously after mixing, a significant improvement in precision over other methods is obtained using the fixed-time rate method because of the speed of the biuret reaction.

1. The Biuret Reaction

As Table 3-1 shows, there are many methods available for the determination of serum protein. The physical methods based on UV absorption or refractive index are simple and non-destructive, but lack accuracy (8). The Kjeldahl method has high precision and accuracy, but the slow digestion and distillation processes involved make it unsuitable for routine analysis of large numbers of samples (8,76). Sensitive methods such as the Folin and Ciocalteu method are either unnecessarily complex or are based on estimation of a side chain such as tyrosine, which may yield serious errors when the composition of a serum sample is abnormal (77-79). In view of this, the biuret method, which is not as sensitive as the Folin and Ciocalteu method, is nevertheless sensitive enough for serum protein analysis, and is least affected by variations in protein compositions. Thus, it is the most widely used method in clinical laboratories for protein determinations.

All proteins contain a large number of peptide bonds. When a protein solution is treated with Cu^{2+} ions in an alkaline medium, a purple colored complex of unknown composition is formed between the Cu^{2+} ion, the carbonyl and the imine group of the peptide bonds. An analogous reaction takes place between the Cu^{2+} ions in alkaline medium and the organic compound biuret (See Figure 3-1),

Table 3-1. Methods for the Determination of Total Serum Protein.

Method	Reaction	Comment	Ref.
1. Kjeldahl	Measures total nitrogen content of sample	Used commonly as the standard method	7
2. Biuret	Reaction of protein peptide bonds with alkaline Cu^{2+} ions to produce blue complexes (A540)	Low sensitivity 0.5-.5 gm/dL	3
3. Folin-Ciocalteu	Reaction of the phenol groups of the serum protein with phospho-tungstomolybdate in alkaline solution to give blue complexes (A650)	Good sensitivity 0.3-1.5 mg/dL not specific, error if serum is abnormal	8
4. F. C. Lowry	Combine the biuret reagent with the F.C. reagent	50-100 times more sensitive than biuret, (0.03-0.1 gm/dL) more specific than F.C. interferes by some buffers	9
5. UV Absorption	Peptide bonds absorbs at 215 nm, aromatic rings of tyrosine absorbs at 280 nm	Rapid but limited in accuracy, non-destructive of sample	1
6. Refractive Index	Refractive index increases by an amount directly proportional to the conc. of solute in dilute solutions	Rapid but limited in accuracy, suitable for non-pigmented, non-turbid sera only	1

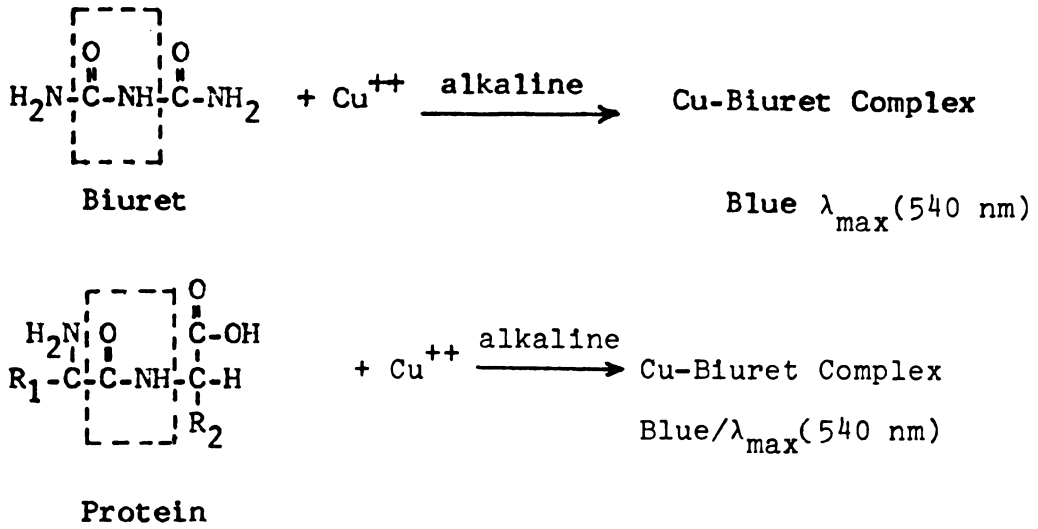


Figure 3-1. The biuret reaction.

which gives the reaction its name. The biuret reaction only takes place between the cupric ion and compounds which have at least two NH_2CO- groups, two NH_2CH_2- groups, or two NH_2CS- groups. These groups must be directly joined to another similar group, or joined via a carbon or nitrogen atom. Therefore any dipeptides or amino acids present in the serum will not react and will not interfere with the protein analysis. The actual mechanism of the reaction is not known, but it is hypothesized that each Cu^{2+} ion is coordinated to four to six nearby peptide linkages (8,72,85). The intensity of the color produced is proportional to the number of peptide bonds present, which is in turn proportional to the concentration of the protein.

2. Preliminary Observations

The biuret method introduced by Kingsley in 1939 (72) is well known, and many modifications of the method have been published (73-75). In fact, quite often it is taught to students in clinical biochemistry courses.

The standard procedure involves mixing blood serum or a protein standard solution with the biuret reagents. The color is allowed to develop for 30 minutes, and the absorbance at equilibrium is then measured not later than 60 minutes after mixing. No data points are taken during the 30 minutes waiting period. Because of the

long waiting period in the usual equilibrium biuret procedure, a preliminary investigation of the kinetics of the reaction was undertaken in order to determine if a reaction-rate method might be applicable. The standard biuret procedure was repeated, with absorbance values taken as a function of time during the 30 minute period before equilibrium. Typical absorbance-time curves are shown in Figure 3-2a. From this preliminary experiment two important observations were made.

The first observation was that the reaction did not follow first or pseudo-first order kinetics, so that the log absorbance vs. time plot was a curve instead of a straight line, as shown in Figure 3-2b. The second one was even more interesting. Figure 3-2c shows that straight lines were obtained when the absorbance of each standard was divided by the first standard and plotted against time. This suggests that one may obtain an analytical curve long before the reaction reaches equilibrium. Thus, the 30 minute wait may be eliminated by using a reaction-rate method. Since the earliest time observable using manual mixing and a conventional spectrophotometer was 2 minutes, it was decided to study this reaction further with a stopped-flow mixing system.

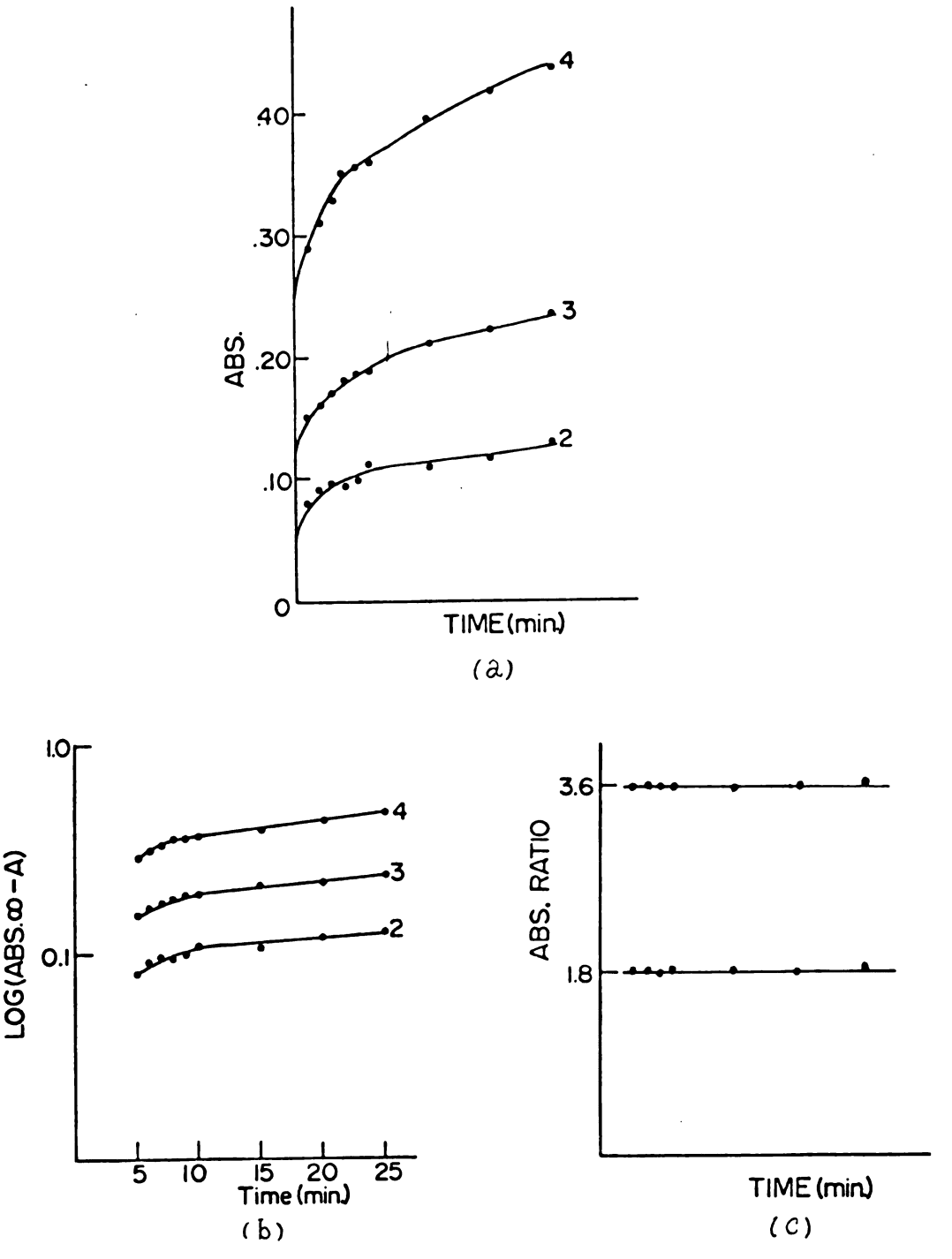


Figure 3-2. Preliminary observations of the biuret reaction.

3. Protein Analysis Using Reaction-Rate Method

A survey of the literature showed that Hatcher and Anderson (83), then Koch, Johnson, and Chilcote (74) have all reported various reaction-rate methods for determining total serum protein with the centrifugal analyzer. According to Koch *et al.*, their method was reasonably precise and rapid (90 s per run). But Savory *et al.* (75) reported that the method was relatively imprecise in their hands and was subject to errors in the analysis of sera from patients with dysproteinemias. Thus, we decided to try another kinetic approach, namely the stopped-flow mixing method, hoping to develop a more reliable, precise and rapid procedure.

B. Stopped-flow Method

1. Reagents and Sample Preparation

The biuret reagent, which contains copper sulphate and tartrate in alkaline solution, was prepared according to a modified formulation of Cornell *et al.* (84).

Biuret reagent. The working reagent contained cupric sulfate (2.4 g L^{-1}), sodium potassium tartrate (8 g L^{-1}), sodium hydroxide (48 g L^{-1}), and potassium iodide (1 g L^{-1}).

Blank reagent. The blank contained sodium potassium tartrate (8 g L^{-1}), sodium hydroxide (48 g L^{-1}), and

potassium iodide (1 g L^{-1}).

Standards. Standards were dilutions of human serum protein standards (Dade Division, American Hospital Supply Corp., Miami, FL 33152; Cat. No. B5158) in saline solution (NaCl, 9 g L^{-1}).

Quality Control Sera. Lyophilized control serum samples (Moni-trol I, Cat. No. B5103; Moni-trol II, Cat. No. B5113, Dade Division, American Hospital Supply Corp., Miami, FL 33152), were reconstituted according to the manufacturer's instructions and diluted 30 fold with saline solution before use.

All reagents were filtered through a 5.0 μm disposable filter (Millipore Corp., Bedford, Mass., 01730) before use. The biuret reagent was stored in plastic bottles in the dark, and all protein solutions were freshly prepared from vacuum sealed prepackaged bottles stored at 2-8°C.

2. Automated Stopped-flow System

Reaction-rate measurements were made on the computer controlled stopped-flow spectrophotometric system described in Chapter 2. All data were acquired and processed by a PDP 8/e minicomputer (Digital Equipment Corp., Maynard, MA 01754). Iteractive computer programs are written by Notz (PNSF3, PNF103) and by Gall (RGMAIN) in our group. Notz's programs control the sequencing

of the stopped-flow mixing system and the filling and rinsing of the drive syringes. They also perform the tasks of data acquisition and processing. Gall's program analyzes the data collected. It displays absorbance and rate on the CRT as a function of time. The slope of the absorbance-time curve as well as the maximum rate can also be calculated and displayed.

The rates are obtained by 9 point first derivative, quadratic, Savitsky and Golay smooth on the absorbance-versus-time data. If the reaction is not pseudo-first order, the maximum rate is obtained. If the reaction is pseudo-first order, the initial portion of the rate curve is linear and the operator can identify the linear portion and obtain the average initial rate with the program. The stopped-flow system was thermostated at 25°C. Spectrophotometric measurements were made at 540 nm.

3. Initial Rate Studies

Figures 3-3a-c show the absorbance-time curves for the formation of the biuret product from human serum protein standards. It is apparent that the biuret-protein reaction proceeds quite rapidly. The total observation times for the 3 runs shown in Figure 3-3 were 100s (Figure 3-3a), 10s (Figure 3-3b), and 0.1s (Figure 3-3c). As can be seen from Figure 3-3c and 3-3d, the

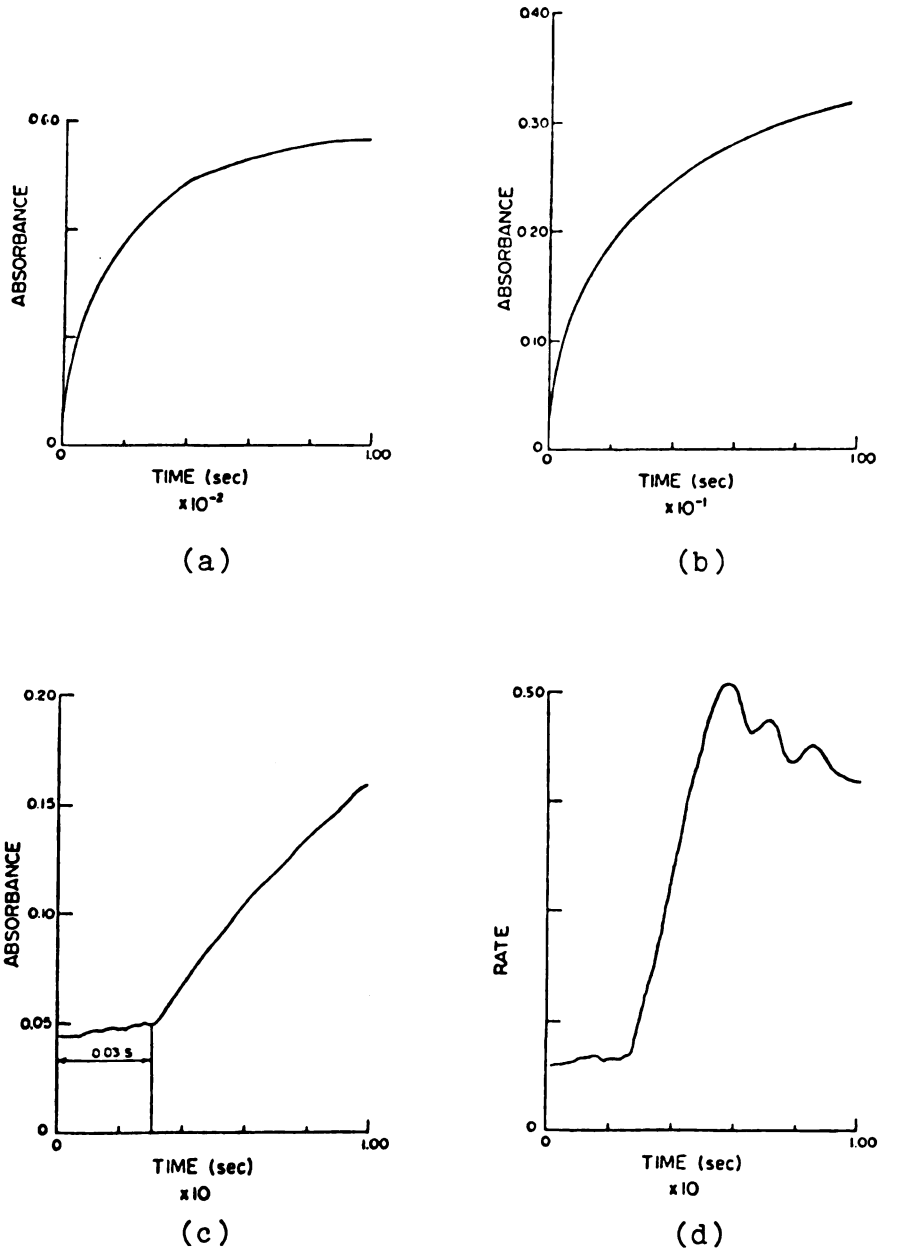


Figure 3-3. Initial rate studies of the biuret reaction. (a) Δt = 100s; (b) Δt = 10s.

reaction rate reaches a maximum before 0.1 s. No other instrument could be used for studying the biuret reaction in so short a time scale. In fact, the desirable feature of obtaining A_0 for the sample-reagent mixture without running a separate blank, can be done by the stopped-flow system. Although Koch et al. (74) did the experiment under slightly different conditions (on a CentrifiChem analyzer), they took their first data point at 3s after the reaction began, and called that their blank. But as can be seen in Figure 3-3b, the reaction has proceeded to a substantial extent after 3 s.

Fixed-time reaction-rate analysis of compounds is usually limited to those substances undergoing first-order or a pseudo-first order reaction (32,82). The biuret reaction, although not a simple first-order reaction, is suitable for fixed-time reaction-rate analysis, as shown in Figure 3-4. Here, a standard curve for human serum protein using a two point fixed time method is shown.

For the initial quantitative studies, we used two approaches: the two-point method, and the maximum rate method. In the two-point method we took data at time t_0 and at 0.1s, and used the difference in absorbance, ΔA, in the standard curve. Then we also measured the maximum rate and used it the with the other approach. Figure 3-4 presents the resulting standard curves. As can be seen, the two-point method yields more precise results.

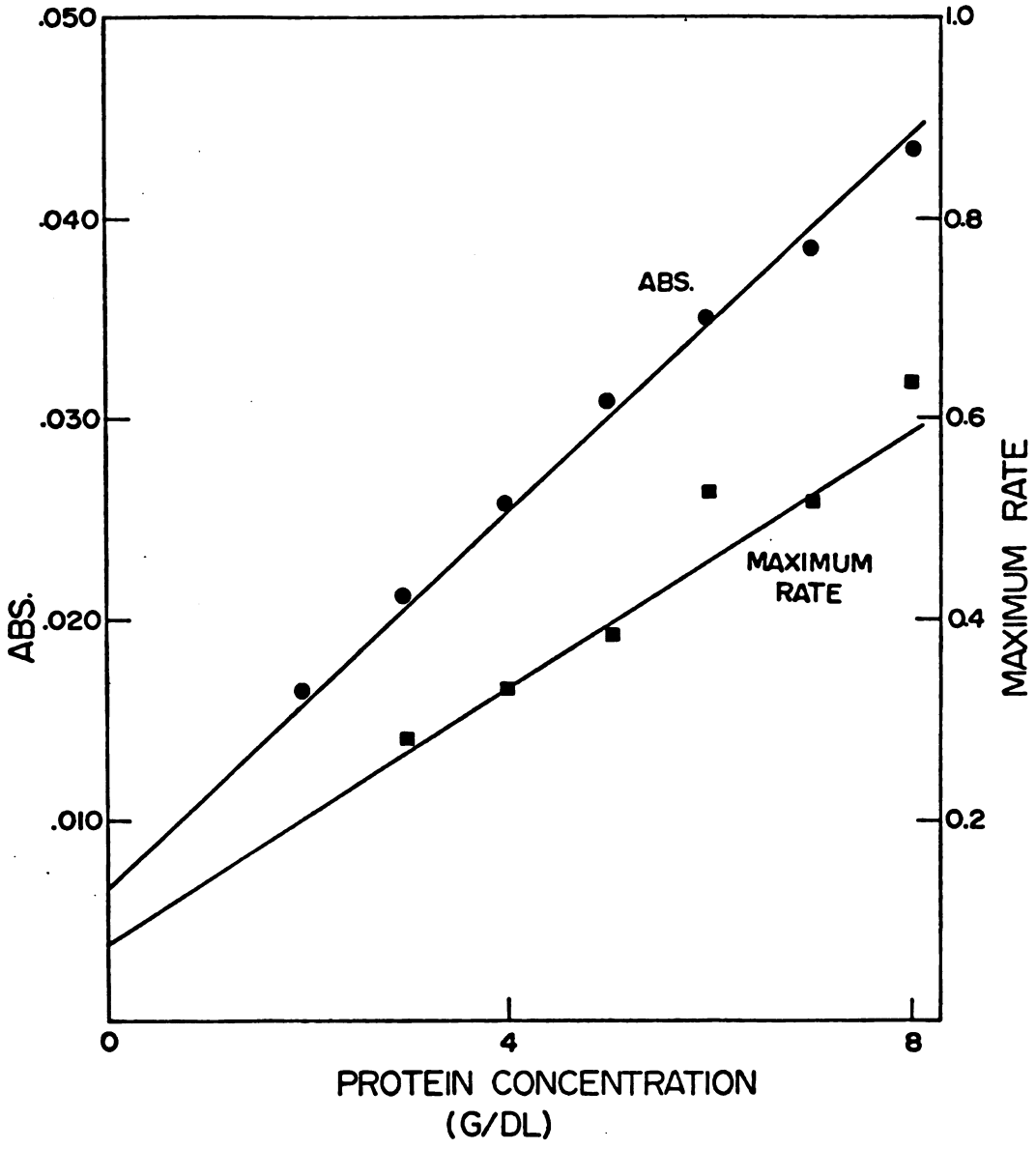


Figure 3-4. Standard curves for two reaction-rate methods used in the biuret reaction.

The statistics of the calibration curve are summarized in Table 3-2. Note that the precision is only fair. For the three different series of standard solutions, the relative standard deviation was as high as 5.8%.

The maximum rate method was even less precise, as can be seen in Figure 3-3d. The maximum rate does not last for a very long time period, and the relatively high noise level in the derivative curve causes imprecision.

Since the biuret reaction is rapid, a longer measurement time, Δt, was used in subsequent trials. Longer Δt values give greater absorbance changes and thus better precision due to less instrumental noise and a smaller error in ΔA. A measurement time of 10s was chosen as a compromise between precision and analysis time. It was found that the precision improved greatly with a 10 s measurement interval. Figure 3-5 shows that the standard curve is linear up to 8 g/dl serum protein concentration. The statistics of the plot are shown in Table 3-3. Note that the maximum relative standard deviation obtained was 1.4%, which is equal or better than any equilibrium method.

4. Experiments with Human Control Lyophilized Sera

To test our method with actual serum samples, three lyophilized sera (Moni-trol I, Patho-trol, and Moni-trol

Table 3-2. Results for Total Serum Protein Standards. (t = 0.1 sec)

Protein Concentration (gm/dL)	Absorbance (Average)	Relative Std. Deviation %
8	0.0454	1.4
7	0.0381	2.6
6	0.0365	5.8
5	0.0329	5.1
4	0.0246	2.2
3	0.0202	1.6

Slope = 4.86×10^{-3}

Intercept = 6.22×10^{-3} A

Std. Error of Estimate = 1.57×10^{-3}

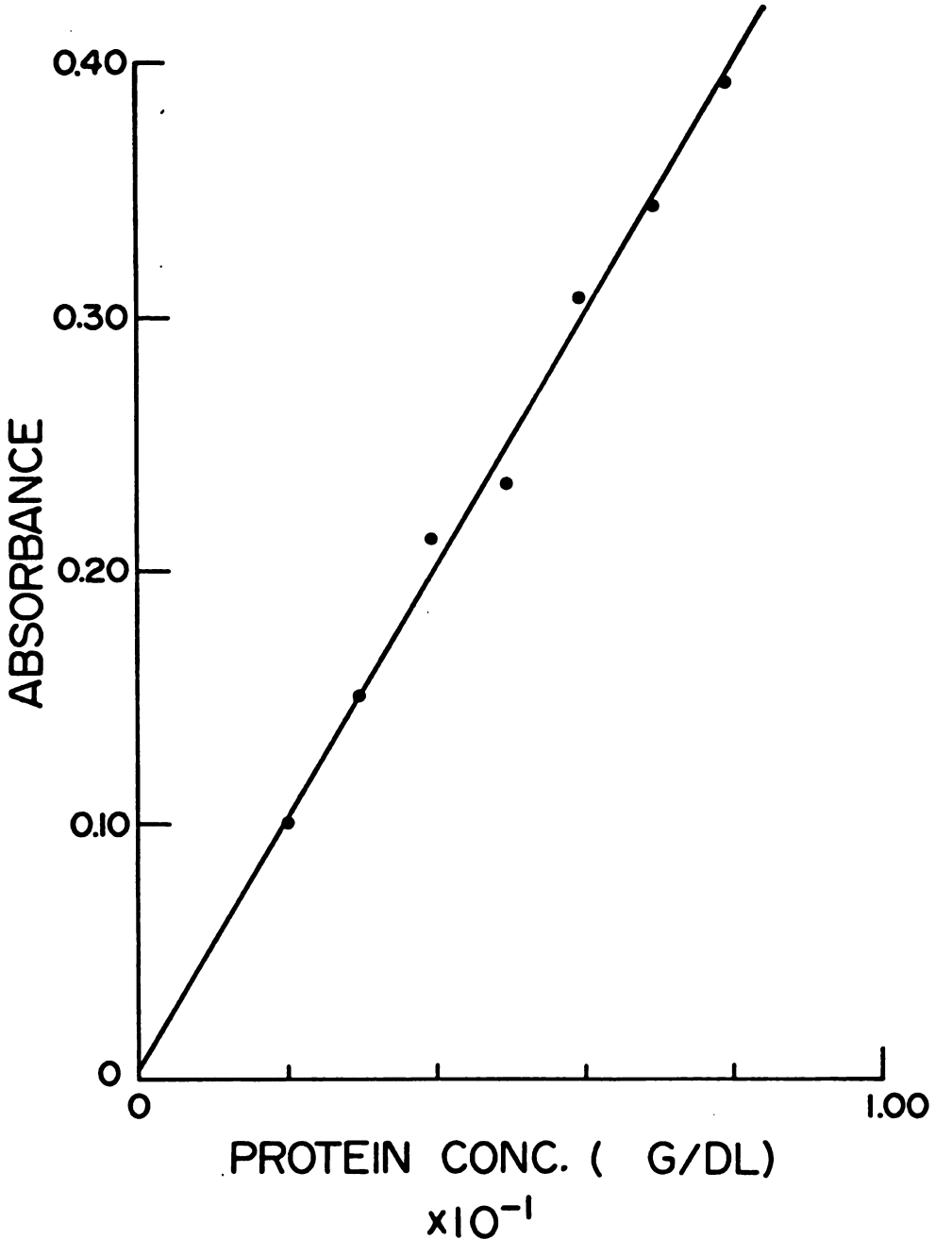


Figure 3-5. Standard curve for serum protein, Δt = 10 s.

Table 3-3. Results for Total Serum Protein Standards. (t = 10 sec).

Protein Concentration (gm/dL)	Absorbance (Average)	Relative Std. Deviation %
8	0.393	1.3
7	0.344	0.2
6	0.308	0.1
5	0.235	1.3
4	0.213	0.1
3	0.151	1.4
2	0.101	1.4
Slope	$= 4.85 \times 10^{-2}$	
Intercept	$= 6.96 \times 10^{-3}$ A	
Std. Error of Estimate	$= 9.57 \times 10^{-3}$	

II, DADE, Division American Hospital Supply Co., Miami, FL 33152) were used as controls. The manufacturer of these sera gives assigned values of total protein obtained by replicate analyses using well-established methods. A comparison of the results obtained here by the fixed-time rate method with those obtained by other methods is presented in Table 3-4. Note that the other reported rate method using the CentrifiChem analyzer gives relatively low results when compared to results from equilibrium methods. The precision is also poorer, and is especially noticeable with the Moni-trol I serum. As reported previously, the 0.1 s fixed-time method gave relatively poor precision, but we found the values still fell within the acceptable range provided by the manufacturer. The 10 s fixed-time method gave good accuracy and precision. An abnormal serum (Patho-trol) was also analyzed using the 10 s fixed-time method. As shown in Table 3-4, the results we got were slightly higher than the value reported by the manufacturers, but still within the allowable range.

5. Comparison with the Equilibrium Method

To further confirm the reliability of the results, the same control sera were analyzed using an equilibrium biuret method. A Beckman DB-GT spectrophotometer with a

Table 3-4. Results of Determination of Total Human Serum Protein.

	Reported Values		Observed Values	
	Mean (g/dL)	Range	Δt=0.1s	Δt=10s
Moni-trol I				
1. Improved biuret	7.0±.06	6.7-7.3		
2. CentrifiChem	6.5±.35	5.8-7.2	6.79±.15	6.79±.01
3. SMAC	6.8±.10	6.5-7.1		
4. Mark X	7.3±.10	7.0-7.6		
Moni-trol II				
1. Improved biuret	5.3±.07	5.0-5.6		
2. CentrifiChem	4.5±.05	4.2-4.8	5.35±.85	5.14±.04
3. SMAC	5.2±.08	4.9-5.5		
4. Mark X	5.6±.10	5.3-5.9		
Patho-trol				
1. Improved biuret	5.3±.12	5.0-5.6		5.85±.08
2. Technicon AA II	5.9±.05	5.6-6.2		

1 cm path length cuvet was used. The reagents and samples were mixed, and the color was allowed to develop for 30 minutes before the absorbances at 540 nm were measured. Dilutions were necessary for the more concentrated samples to produce a linear standard curve, which is shown in Figure 3-6. The results obtained were compared with the results from the rate method and are listed in Table 3-5. The equilibrium method gave practically the same results as the rate method. The differences found for the Moni-trol I and Moni-trol II samples were less than 1%, while the Patho-trol sample had about 7% difference.

6. Choice of Protein Standards

Human serum protein was used as the standard in the stopped-flow biuret procedure. Bovine serum albumin is often the preferred standard material in equilibrium methods because it is much cheaper and readily available in pure crystalline form (81). However, Koch *et al.* (74) have reported that it reacts faster than human serum protein with the biuret reagent. We repeated their experiment with the stopped-flow analyzer and observed the same result (Figure 3-7). However, careful analysis of the data revealed one other insight. It was found that the rate of reaction of the human serum protein was about 0.65 times as rapid as the BSA. The human serum protein standard used contains 5 g albumin and 3 g globulin per

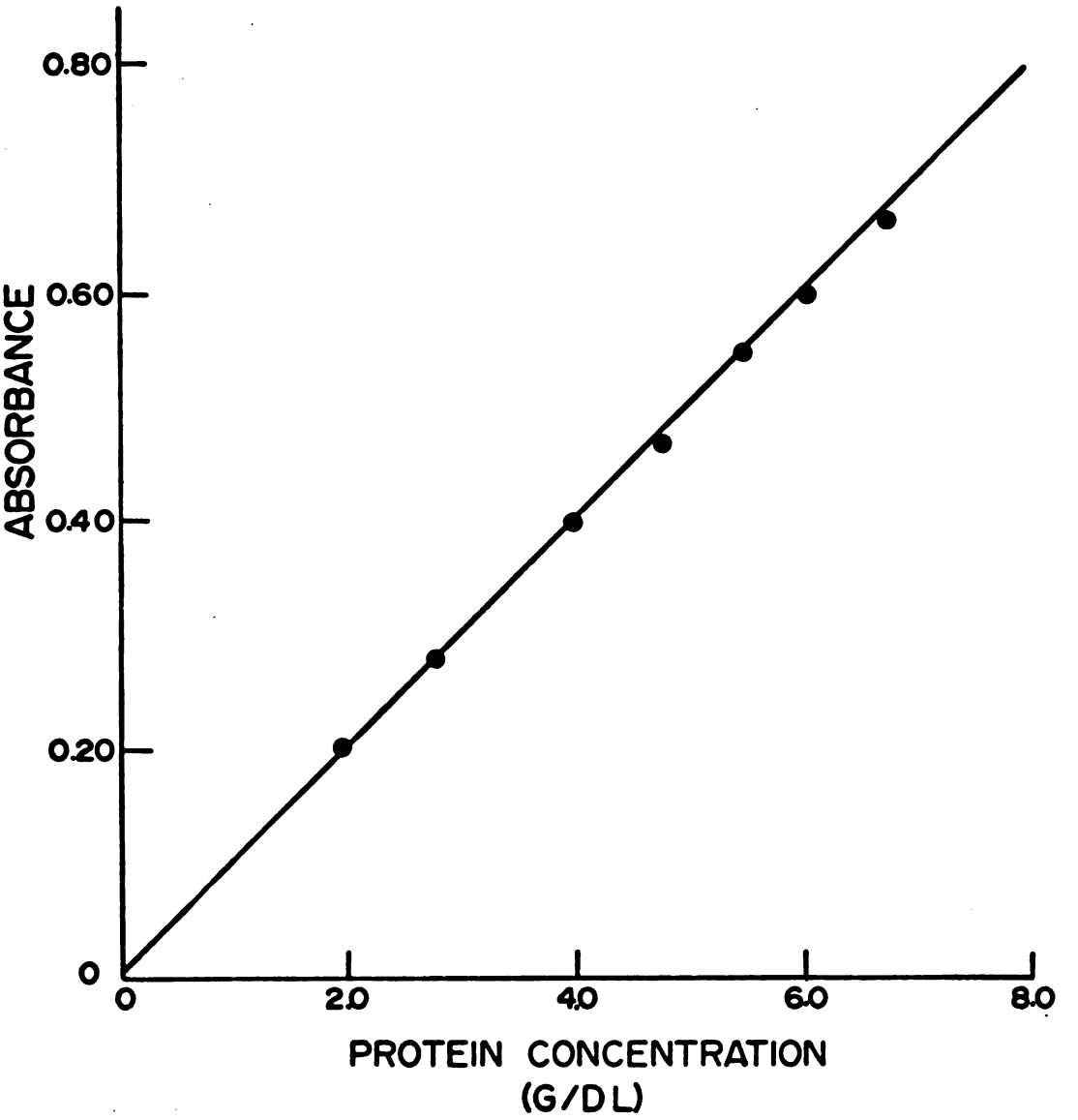


Figure 3-6. Standard curve for serum protein using the equilibrium method.

Table 3-5. Comparison of the Reaction Rate Method with the Equilibrium Method.

	Rate Method[a]	Equilibrium[b] Method	Difference
Moni-trol I	6.79 ± 0.01	6.77 ± 0.06	0.02
Moni-trol II	5.14 ± 0.04	5.05 ± 0.04	0.09
Patho-trol	5.85 ± 0.08	5.40 ± 0.05	0.45

[a]Average of 6 determinations on the same sample with 10 second measuring time.

[b]Average of 3 determinations on the same sample with 30 minutes waiting period.

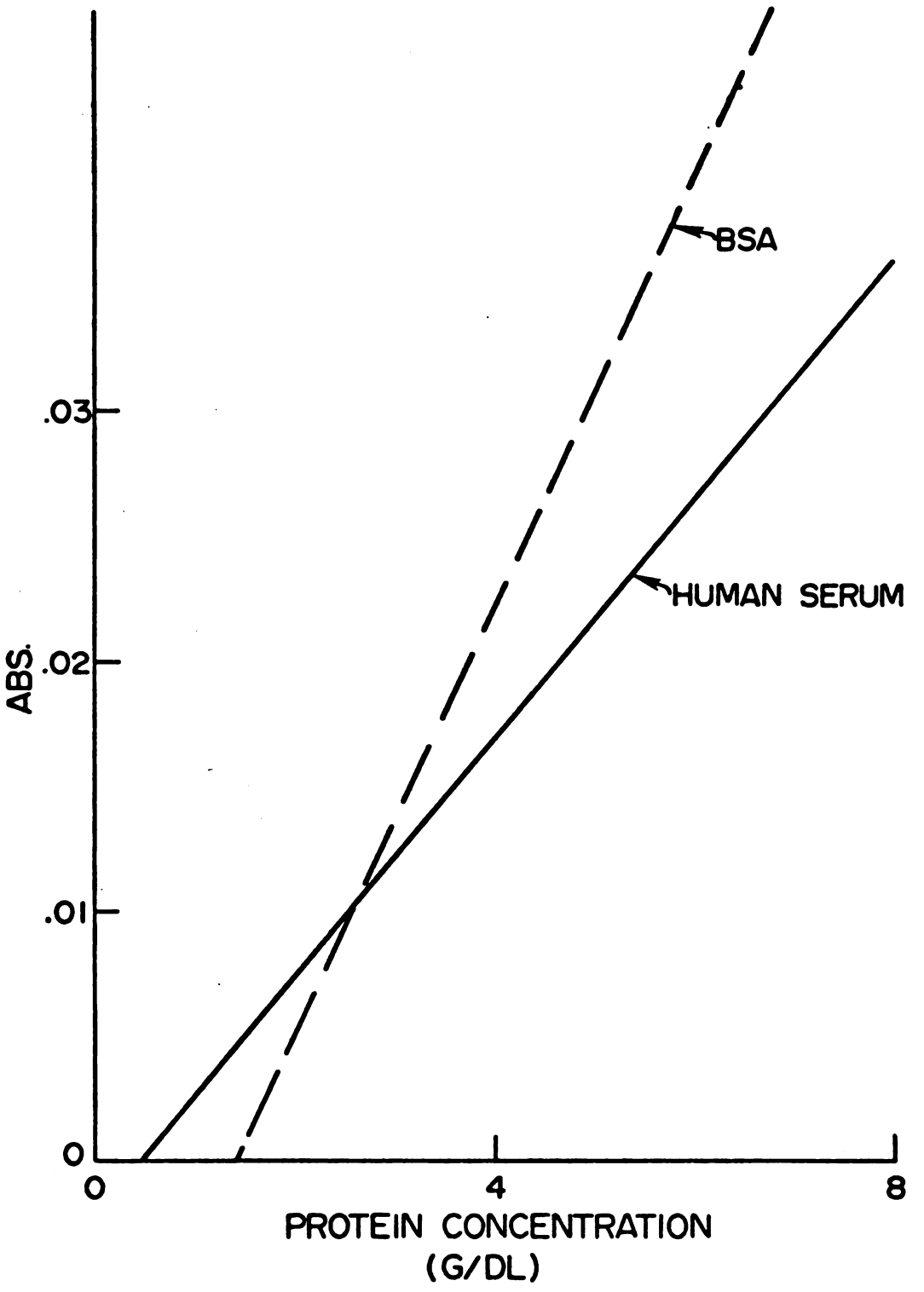


Figure 3-7. Comparison of rate of reaction between BSA and human serum protein.

100 ml solution, which means that albumin makes up 62.5% (W/W) of the total protein in the solution. As can be seen from Table 3-6, there is a consistent similarity between the two ratios (0.625 and 0.65). Hence, we wondered if the rate difference observed between the human and the bovine proteins could be caused by the percentage of albumin and, furthermore, if the rate were dependent on the albumin concentration.

If albumin actually reacts much faster than globulin, this fact might have been easily overlooked by other experimenters, because no one has studied the rapid kinetics of the biuret reaction (<1 s after mixing).

If this were true, differential rate analysis could be done on a serum sample to determine albumin and total protein simultaneously. One measurement could be taken very early in time to determine albumin and a second measurement could be made much later in time to determine total protein. Albumin is a clinically important substance and is determined routinely (See Table 2-1 in Chapter 2). Thus, if a simultaneous determination of both the total protein and the albumin concentration were possible, it would be most valuable.

In the first experiment, bovine serum albumin was added to bovine serum globulin in different proportions and their reaction rates with the biuret reagent were obtained. For all the mixtures, both albumin and globulin

Table 3-6. Comparison of Rates of Reaction of Albumin From Different Sources.

Protein Concentration	Rate Ratio[a]	% Albumin[b]
8 gm/dL	0.647	
5 gm/dL	0.650	0.625
4 gm/dL	0.654	

[a] $\text{Rate Ratio} = \frac{\text{Rate of Human Albumin (62.5\% Albumin)}}{\text{Rate of BSA (100\% Albumin)}}$

[b] $\frac{\text{Albumin Concentration}}{\text{Total Protein Concentration}} = \frac{5 \text{ gm/dL}}{8 \text{ gm/dL}} = 0.625$

had original concentrations of 6 g/dL, but the two were mixed in varying amounts, ranging from 0% albumin to 100% albumin. Table 3-7 shows the results, from which one can see that no significant difference in rate exists. This indicates that bovine albumin reacts with essentially the same rate as bovine globulin.

To double check these results, BSA, pure human serum albumin, and 65% human serum albumin standard solutions of the same total protein concentrations were studied, and the results are shown in Table 3-8. These indicate clearly that BSA reacts faster than the human serum albumin, while the percent of albumin in human serum does not affect the rate significantly.

Thus, the idea of simultaneous determination of total serum protein and albumin is not feasible. The experiments performed, however, indicate two important guidelines for the reaction-rate determination of total protein. First, human serum protein should be used in the rate method because of the faster reaction with BSA. Second, since the protein composition of the human serum does not affect the biuret reaction, one should be able to use human serum protein of any composition as a standard.

Table 3-7. Biuret Reaction with Different Albumin Compositions.

Albumin Content[a]	Abs. at 0.1 sec
100%	0.028
75%	0.025
50%	0.028
25%	0.028
0%	0.030

[a]6 gm/dL albumin + 6 gm/dL globulin.

Table 3-8. Biuret Reaction with Albumin From Different Sources.

Albumin	0.1 sec	100 sec
1. 8 gm/dL BSA	0.040	0.065
2. 8 gm/dL Human Serum (95% Albumin)	0.032	0.053
3. 8 gm/dL Human Serum (62.5% Albumin)	0.033	0.055

7. Studies with a Vidicon Detector

No attempt was made to determine the biuret reaction mechanism. But we used the biuret reaction to test a new micro-computer controlled vidicon rapid scanning stopped-flow system, designed by Carlson and Enke at Michigan State University (86). Several interesting observations were made, that may be of help in future studies of the mechanism of the biuret reaction.

Figure 3-8 shows the spectral changes with time for the biuret reaction with human serum protein. The original scan covered a range of 565 nm-845 nm recorded with the vertical scan frequency at 25 kHz with 102 channels per scan frame, which results in a scan time of 4.08 msec/ frame. The spectral changes which occurred from the time of mixing through 5 s were recorded.

The spectrum with the large peak centered at about 575 nm represents the Cu-protein complex, which has an increasing absorbance with time. One interesting observation is shown in Figure 3-9. A slight wavelength shift of the peak occurs very early in time. The absorbance maximum shifts rapidly from 610 nm to 560 nm in the first second after mixing, while the shift after 2 s becomes quite insignificant. The same spectrum in Figure 3-8 shows two isosbestic points at about 690 nm and 802 nm. The existence of these isosbestic points indicates

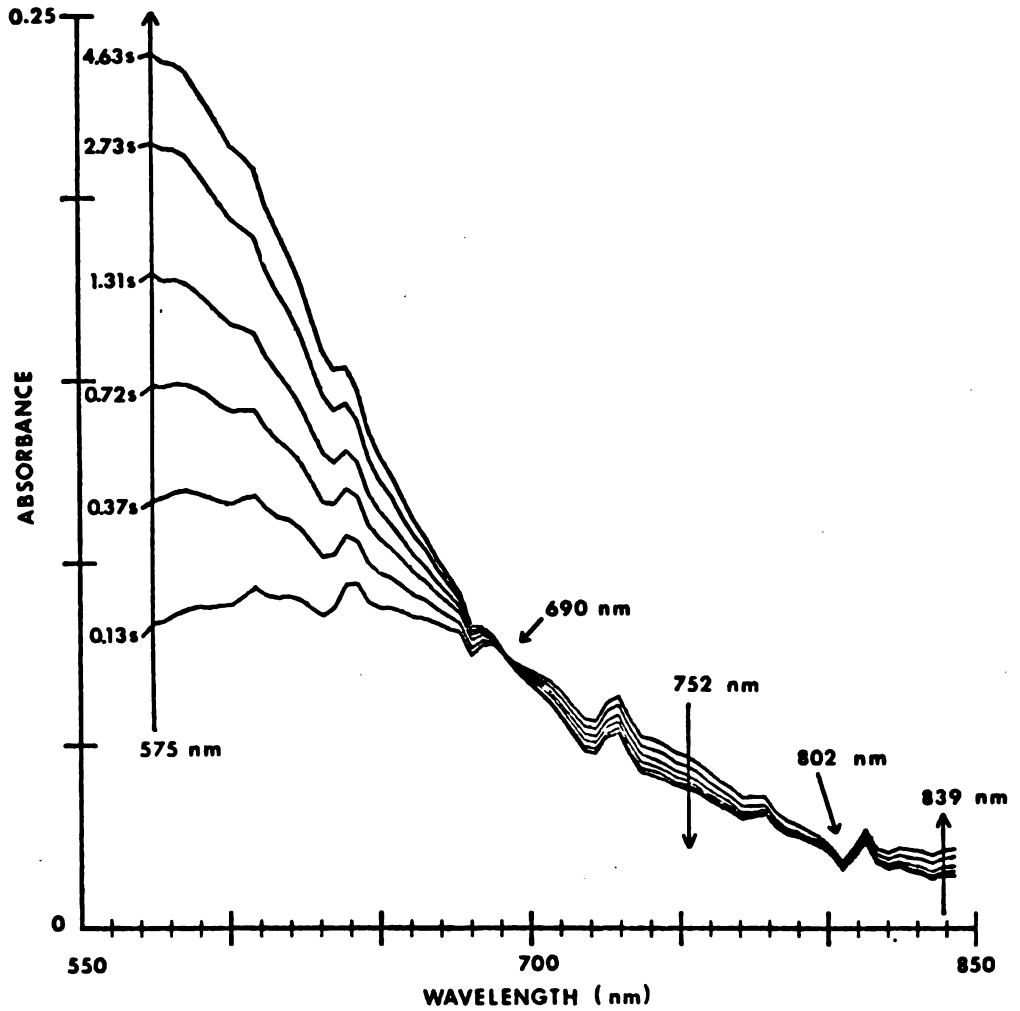


Figure 3-8. Time scan of the absorption spectra during the human protein-biuret reaction.

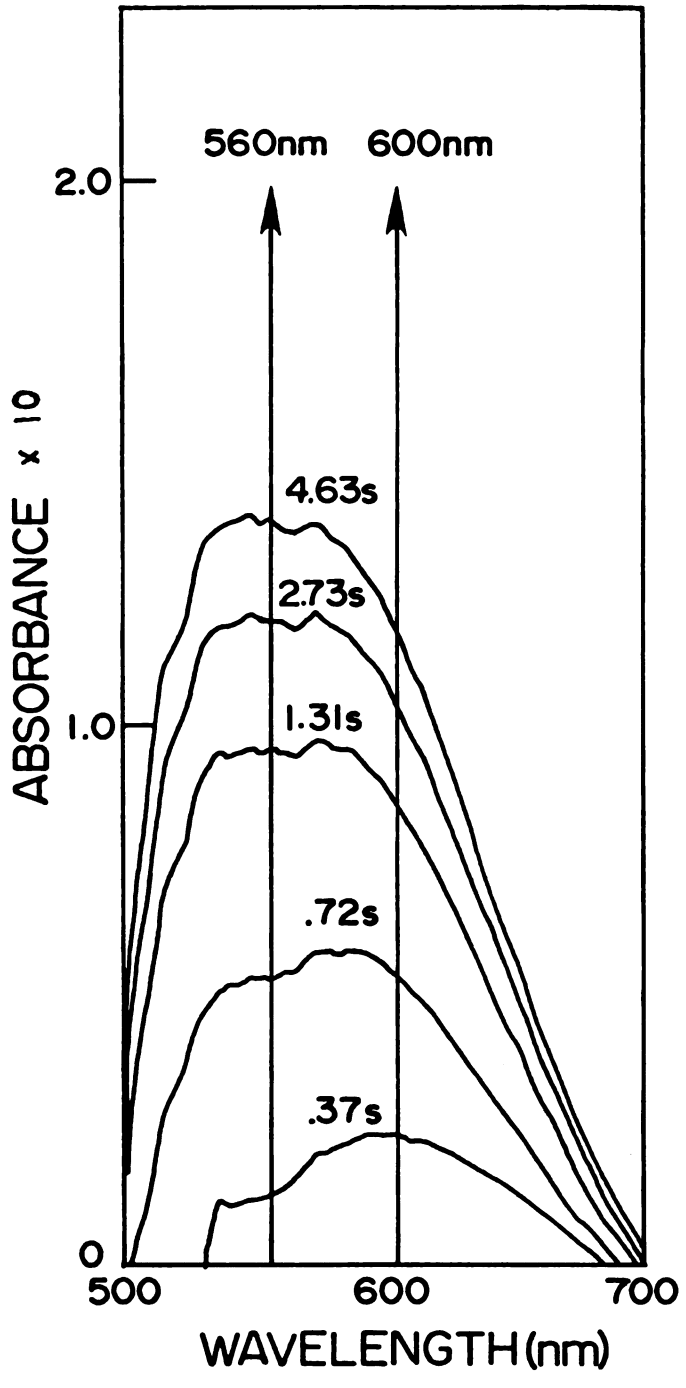


Figure 3-9. Apparent wavelength shift of the biuret reaction.

that there is probably an interfering species that absorbs in the IR region. Figure 3-10 shows the absorbance change with time of the 752 and 839 nm bands. The band at 752 nm decreases with time, while the one at 839 nm increases with time. In order to identify the interfering species, the biuret-human protein reaction was allowed go to completion (about 25 min) and obtained the absorption spectrum shown in Figure 3-11. A comparison of the spectrum with Figure 3-8 indicates that the absorption band of the Cu-protein complex extends well beyond 850 nm, and the increase in absorbance with time of the 839 nm band is thus due to the Cu-protein complex as well. Therefore, the decrease in absorbance in time at 752 nm must be caused by an interfering species.

We identified this species as the Cu-tartrate complex present in the biuret reagent. Figure 3-12 shows the absorption spectrum of the Cu-tartrate complex, with the biuret blank used as a reference. The spectrum has its maximum absorbance at 680 nm, but the absorbance diminishes beyond 800 nm. The amount of Cu-tartrate complex decreases during the reaction, which causes the net decrease in absorbance at 752 nm. Since the Cu-tartrate complex does not interfere beyond 800 nm, the observed increase in absorbance with time at 832 nm must be due to the Cu-protein complex. One other interesting observation is shown in Figure 3-13. The disappearance rate of the Cu-tartrate

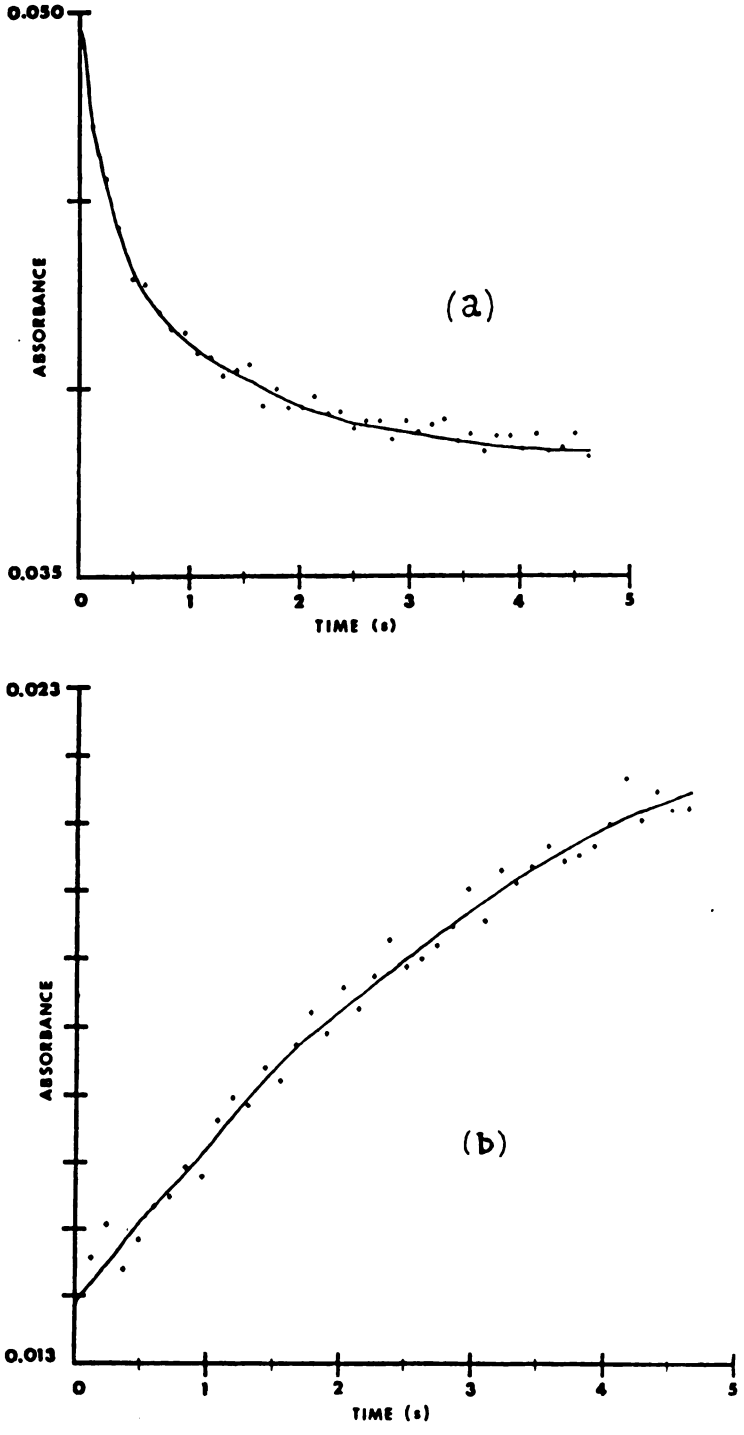


Figure 3-10. Absorbance versus time for the biuret reaction with human serum. (a) 752 nm; (b) 839 nm.

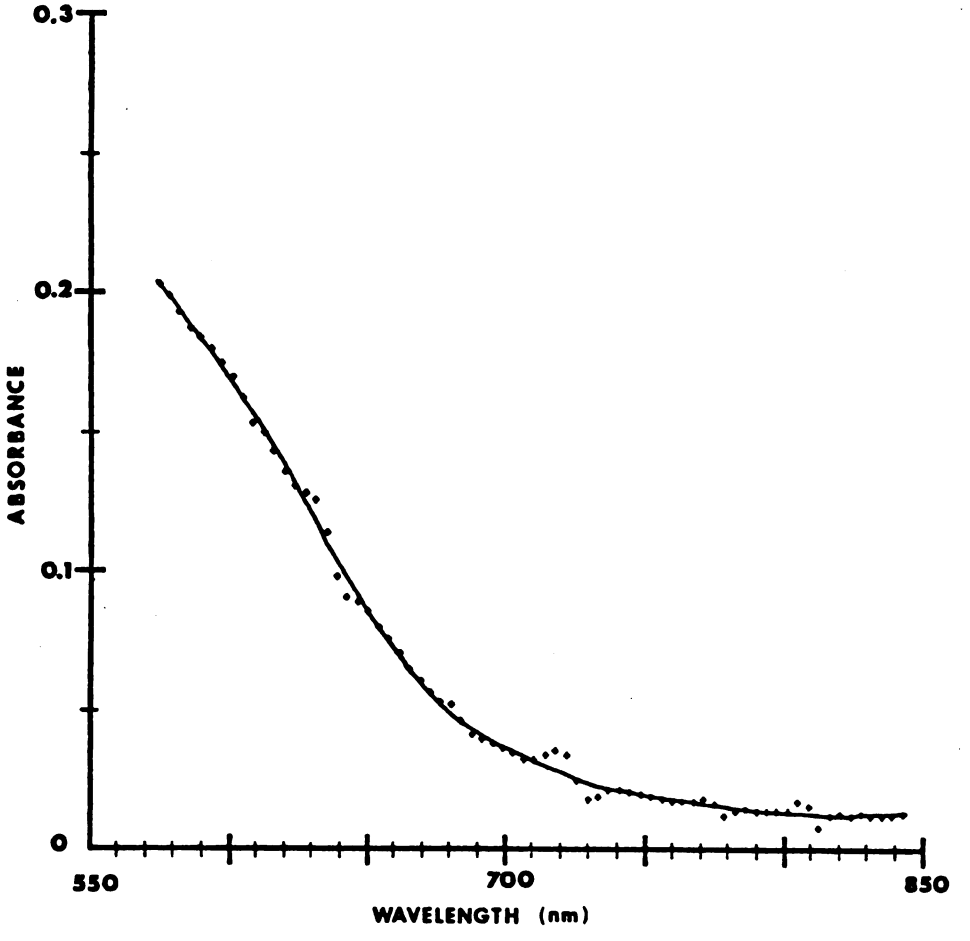


Figure 3-11. Absorption spectrum of the copper-protein complex from the biuret reaction with human serum after about 25 minutes.

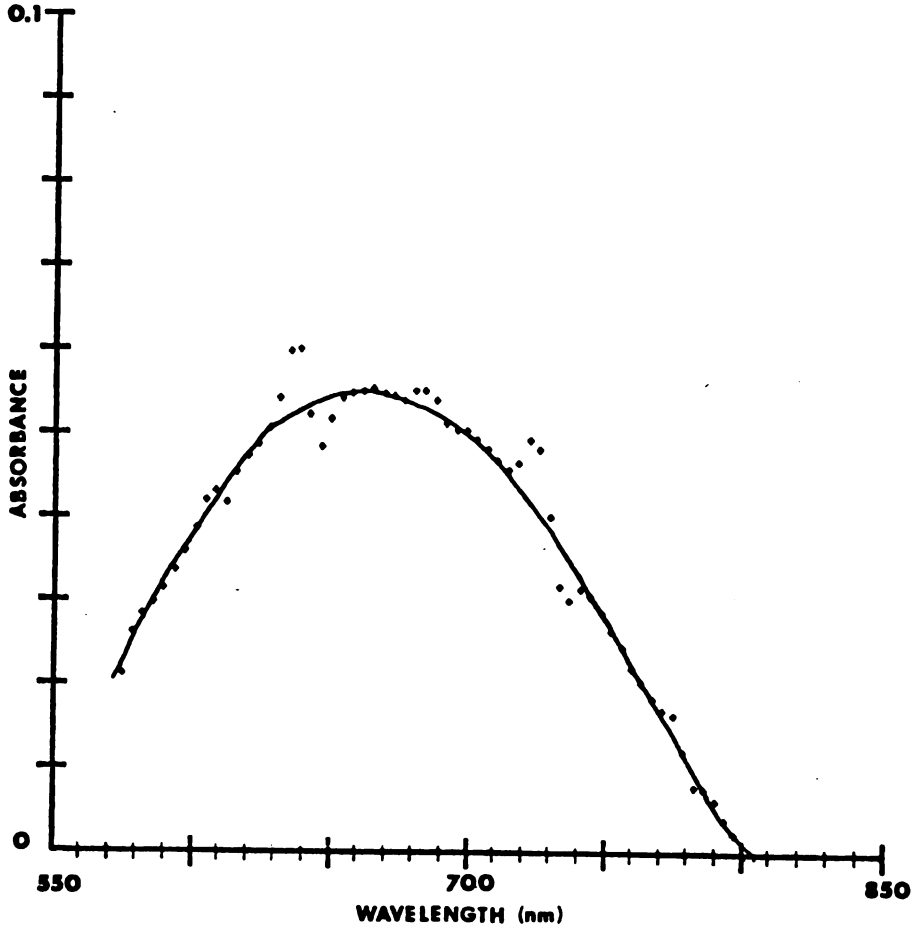


Figure 3-12. Absorption spectrum of the biuret reagent.

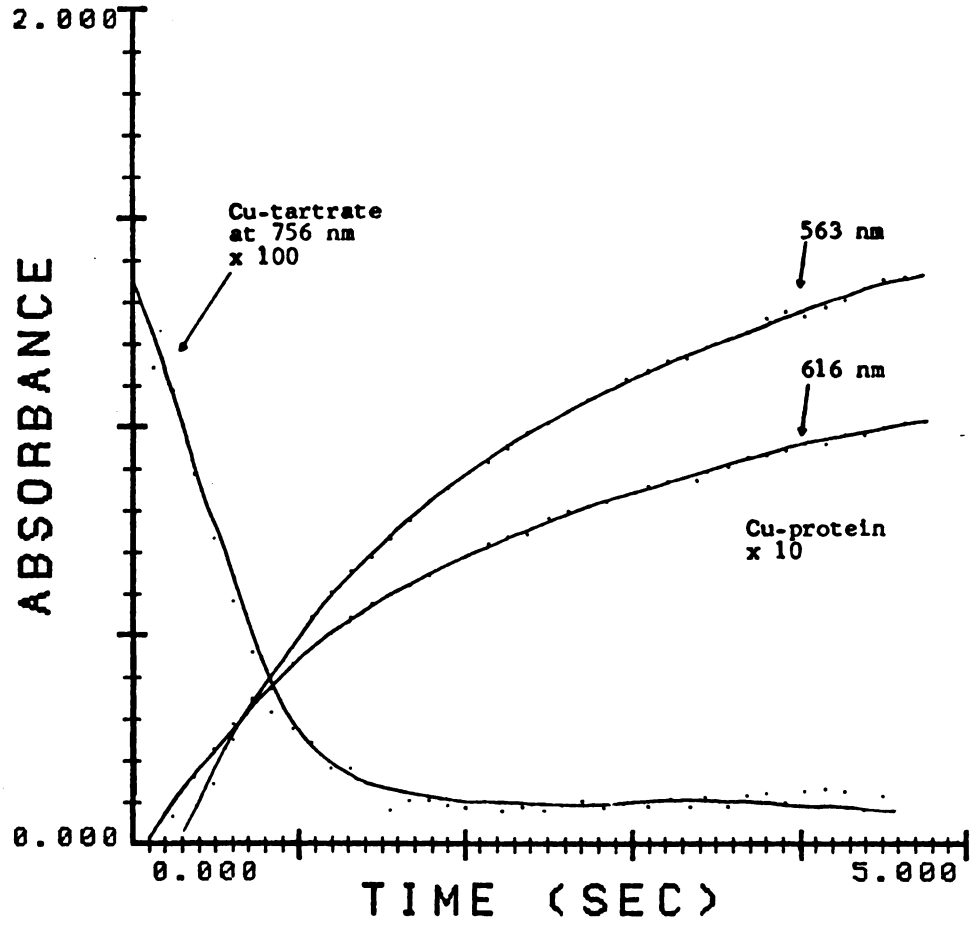


Figure 3-13. Rate studies of the biuret reaction.

complex is slightly faster than the appearance rate of the Cu-protein complex, which may account for the rapid wavelength shift observed at the 575 nm region during the first second after mixing.

Earlier in this chapter it was seen that BSA reacts more rapidly with the biuret reagent than does human serum protein, but proteins from the same source react with the same rate. We attempted to find out if there was any difference in the time dependent spectra by making up a bovine serum albumin and a bovine serum globulin solution with similar concentrations. Figure 3-14 shows the time dependent spectrum for BSA and Figure 3-15 for BSG. No significant differences can be found between these two spectra, which again suggests that albumin and globulin react similarly.

When these spectra are compared to the spectrum shown in Figure 3-8, it can be seen that the reaction profiles are significantly different in appearance from the human serum protein profile. Only one isosbestic point is observed at 690 nm, and the decreasing absorption band at about 775 nm extends beyond 800 nm. We checked the equilibrium absorption spectrum of the Cu-BSA complex, and Figure 3-16 shows that there is essentially no absorbance above 800 nm. This suggests that there is probably an additional interfering species that has a wavelength maximum at a longer wavelength than the Cu-tartrate

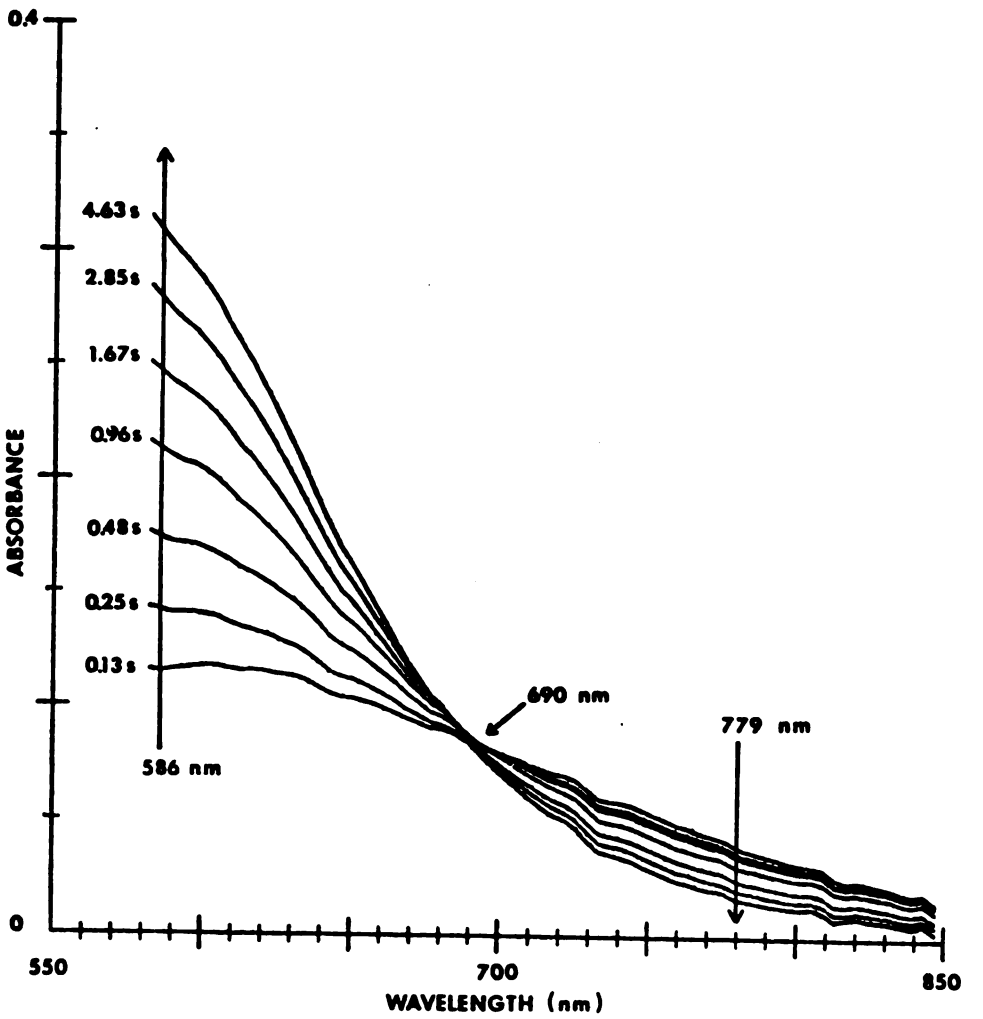


Figure 3-14. Time scan of the bovine albumin-biuret reaction with a vidicon detector.

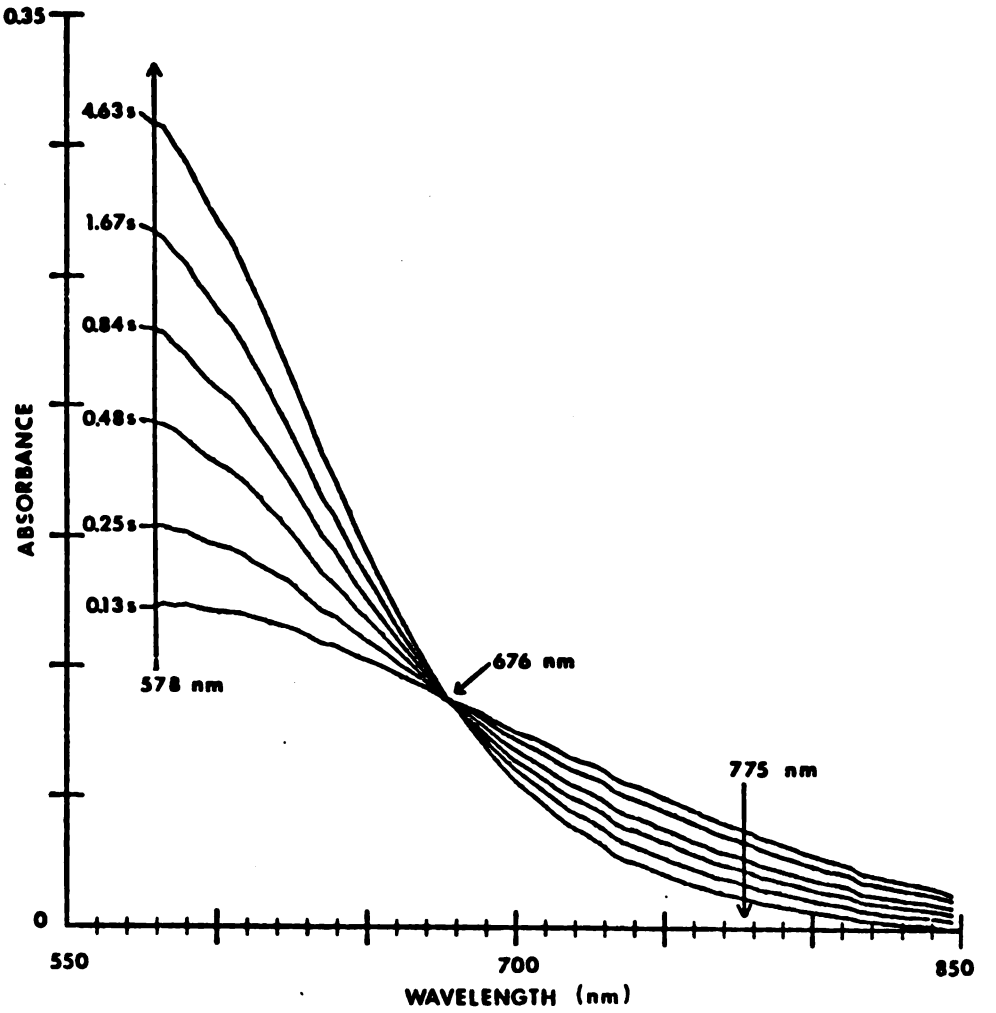


Figure 3-15. Time scan of the bovine globulin-biuret reaction with a vidicon detector.

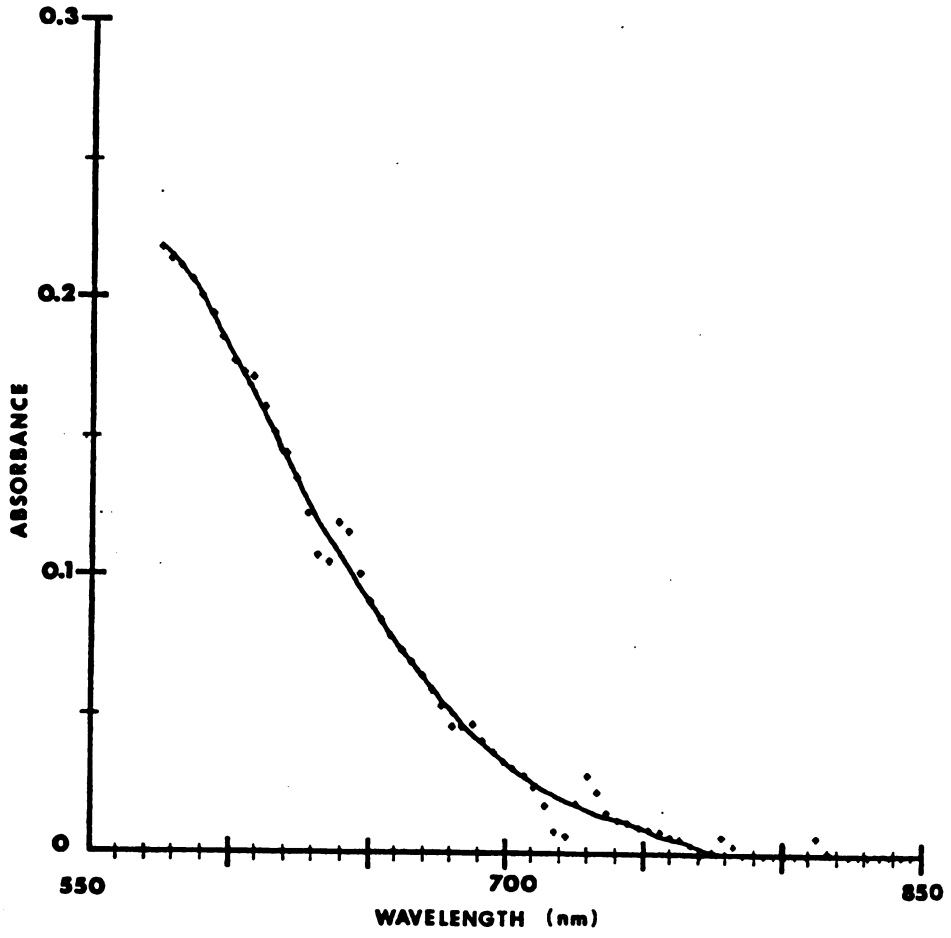


Figure 3-16. Absorption spectrum of the copper-protein complex from the biuret reaction with bovine serum albumin after about 25 minutes.

complex. This may be an intermediate that appears quickly after mixing and then disappears during the reaction. This could account for the decreasing band beyond 800 nm and at the same time off-setting the interference of Cu-tartrate at 575 nm. This may also account for the fact that the initial rate of the biuret-bovine protein reaction is faster than that of the biuret-human protein reaction.

Carlson suggested that the rate curve should be investigated at wavelengths below 550 nm in the future in order to identify the unknown interfering species (86). Rate studies will help in understanding the reaction rate difference between the different sources of protein, and in this way perhaps the cheaper and more readily available BSA can be used as a standard material in the kinetic biuret method.

C. Conclusions

It has been demonstrated that the unique capabilities of the automated stopped-flow analyzer enable the determination of total serum protein by the biuret method in 10s. The reaction-rate method is found to be rapid, simple, and precise. The method gave the same magnitude of precision as the equilibrium method, but used less sample volume (15 µℓ *vs.* 70 µℓ undiluted serum), and was considerably less time consuming (10s *vs.* 30 min).

Because it is a relative measurement, the reaction-rate approach should decrease greatly any interferences caused by turbidity, hemolysis, or other background noises. The highly automated system simplifies sample and data handling, and the continuous monitoring of the reaction enables the operator to detect any abnormal errors that may occur during the reaction.

A vidicon detector has been used to show that some kind of an intermediate is probably involved in the biuret reaction, and any future studies concerning the mechanism of the reaction should be performed in the early phase of the reaction. The stopped-flow system is undoubtedly a good instrument for studies of this kind.

IV. SERUM ALBUMIN ANALYSIS

A. Introduction

Albumins are water soluble proteins which are also soluble in dilute and moderately concentrated salt solutions. They are carbohydrate-free molecules with a molecular weight of 65,000. Normal serum from a healthy adult contains about 3.5-5.0 gm of albumin per 100 ml. A low serum albumin level is an important cause of edema. In most infectious diseases, liver problems, carbinomatosis, and congestive heart failures, albumin levels as low as 2.3 to 3.2 g/100 ml are common (8).

Currently there are four major types of procedures in serum albumin determination: the biuret method after precipitation of globulin by salt (92); the dye-binding method with bromcresol green, methyl orange, or 2-(4'-hydroxyazobenzene)-benzoic acid (HABA); electrophoresis (93); and the immunochemical method (94). The dye-binding method with bromcresol green (BCG) is most popular because it is simple to use and easily automated (87). However, it tends to give overestimations of the serum albumin, especially when the concentration is low (88-90). Consequently, the IFCC* expert panel on protein recommends its use should be limited to screening (91). We looked

*International Federation of Clinical Chemistry.

into this problem and have now presented a possible solution in this chapter.

1. Bromcresol Green Dye-binding Method

All proteins, especially albumins, tend to react with many chemical species by means of electrostatic or Van der Wall's forces, and by hydrogen bonding. In fact, most hormones, bilirubin, fatty acids, and many drugs are first bonded to the albumin and then transported through the body. Many anionic colored dyes also have this protein-binding property. Bromcresol green is currently the most popular method for determining serum albumin. The method was first introduced by Rodkey in 1965 (95), and then modified by Doumas et al. (96), who used a nonionic surfactant Brij-35, and reduced the absorbancy of the dye blank. This immediately raised the sensitivity of the method, prevented turbidity and made the standard curve linear (96). Doumas and Straumfjord have summarized the advantages and disadvantages of this method (97). The advantages are the small sample size, the availability and stability of the bromcresol green, the high precision, the lack of interference from bilirubin, salicylate, or hemoglobin, the ease of automation, and the negligible interference from globulins. The disadvantages are the increase of color produced by the serum with time (pure albumin produces color

instantaneously) and the yet undetermined specificity of bromcresol green.

2. Proposed Solutions

Because proteins other than albumin also react with BCG, considerable overestimation occurs, particularly when the concentration of albumin is low. In fact this has been one of the major criticisms of the method (98). Gustafsson (99) postulated that BCG reacts with serum proteins in two steps. The first is an immediate reaction within the first minute which is mainly due to the albumin present. This is followed by a slower reaction of some 30 minutes, which is caused by some "acute phase reactants", probably due to the presence of other proteins or reactants in the serum whichhave a slower reaction rate with BCG (99). Webster (100) investigated the immediate reaction (30s) using a conventional spectrophotometer, and found that his results were 0.3 g/100 ml higher than those obtained by electrophoresis. This difference was independent of the albumin content of the serum. Therefore, he suggested that 0.3 g/100 ml albumin concentration should be subtracted from the raw data to make them reliable (100). Both Gustafsson and Webster tried to get estimates of the absorbance contributed by the fast reacting albumin, by taking data as near to the initial mixing time as possible. Gustafsson also

tried to estimate the absorbance at 0 min by extrapolating the recorder curve to the time of mixing (99).

In this work, the albumin-BCG reaction was investigated with the stopped-flow system. We took advantage of the fast mixing feature of the stopped-flow system and took data as near to the initial mixing time as possible. By doing so the rapid albumin-BCG reaction could be monitored and the approach to equilibrium observed. Also, it was found possible to ascertain when other slower reacting proteins began to interfere significantly. We then compared our results with data from other conventional methods.

B. Stopped-flow Method

1. Reagent and Sample Preparation

The reagents described by Doumas et al. (96) were adapted to fit the stopped-flow system. The constituents of the reagents are listed as follows:

Succinate buffer. Dissolve 11.9 g of succinic acid in 800 ml of water, adjust pH to 4.0, dilute to 1 liter with water, store at 4°C.

BCG stock solution. Dissolve 0.419 g BCG (Sigma Co., St. Louis, MO 63178) in 10 ml 0.1 N NaOH, dilute to 1 liter, store at 4°C.

Brij-35 solution (30%). Dissolve 3 g Brij-35 (polyoxyethylene lauryl ether, Sigma Co., St. Louis, MO 63178) in 10 ml water with warming.

BCG working solution. Dilute 100 ml sotck BCG with 300 ml succinate buffer, add 1.6 ml 30% Brij-35 solution, adjust pH to 4.20, store at 4°C.

Standards. Standards were dilutions of human serum albumin (7.9 g/dl, diluted 200 fold with saline) obtained from Dade (American Hospital Supply Corp., Miami, FL 33152). Quality control sera (Moni-trol I and Moni-trol II) were obtained from the same source, reconstituted according to the manufacturer's instructions and diluted 200 fold with saline solution before use.

2. Rate Studies

Pure human serum albumin was mixed with BCG reagent, and the reaction at 25°C was monitored at 632 nm. Figure 4-1 shows the absorbance-time curves. The reaction proceeds rapidly and reaches equilibrium in less than 0.5 s for a 16 g/dl albumin standard solution and less than 2 s for the least concentrated albumin preparation. The reaction was monitored for another 200 s, but no apparent changes in absorbance occurred.

Next, a control serum (Moni-trol I) was prepared, for reaction under the same experimental conditions. This reaction proceeded rapidly at first, but slowed down after

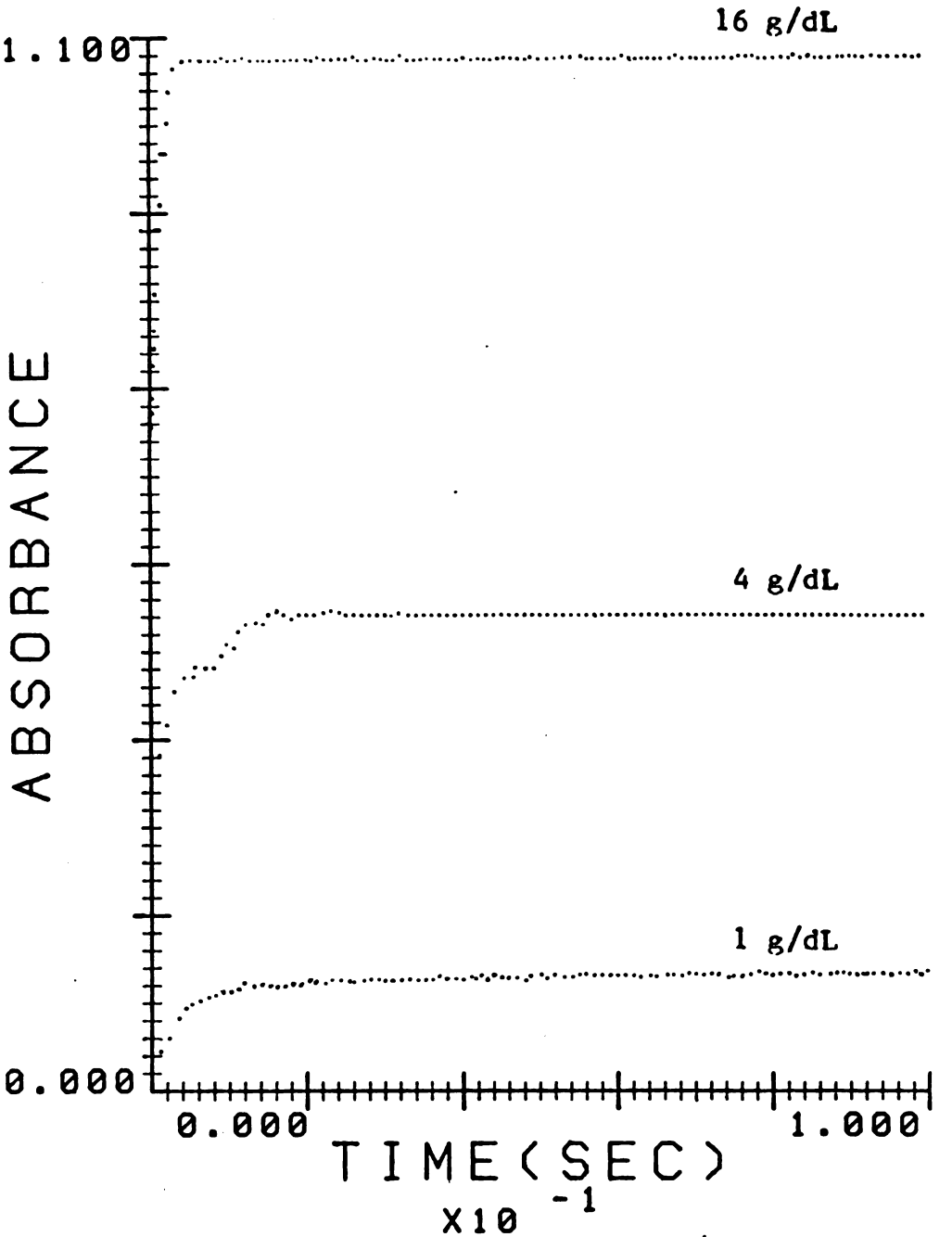


Figure 4-1. Reaction of BCG with pure bovine albumin.

2 s (Figure 4-2). The second slower phase of the reaction continued for about 30 min.

Finally, the reaction of a serum whose albumin concentration was beyond the linear range was also observed, and Figure 4-3 shows the results of this experiment. From this figure it can be seen that the reaction approached equilibrium very rapidly, and there is also a rather evident breaking point, which demarks a slower reaction about 6 s after mixing.

These rate studies indicate that there is indeed a second slower reaction in the overall serum-BCG reaction. This means that measurements must be made early in time if error is to be avoided. Prior to 1976, most procedures employed a reaction time of 5 to 30 min. Webster later tried to measure the "immediate reaction" within 30 s, but found that there was still a small positive difference between his results and those obtained from electrophoresis (100). Our results indicate that for the working concentration range of serum albumin (1.0 g/dl-6.0 g/dl) an observation time of 2-5 s after mixing should be used to minimize interference from non-albumin materials.

3. Experiments with Human Control Lyophilized Sera

A standard curve was prepared with diluted human albumin standard solutions (7.9 g/dl, Sigma, St. Louis, MO). At first we prepared the BCG reagent as before,

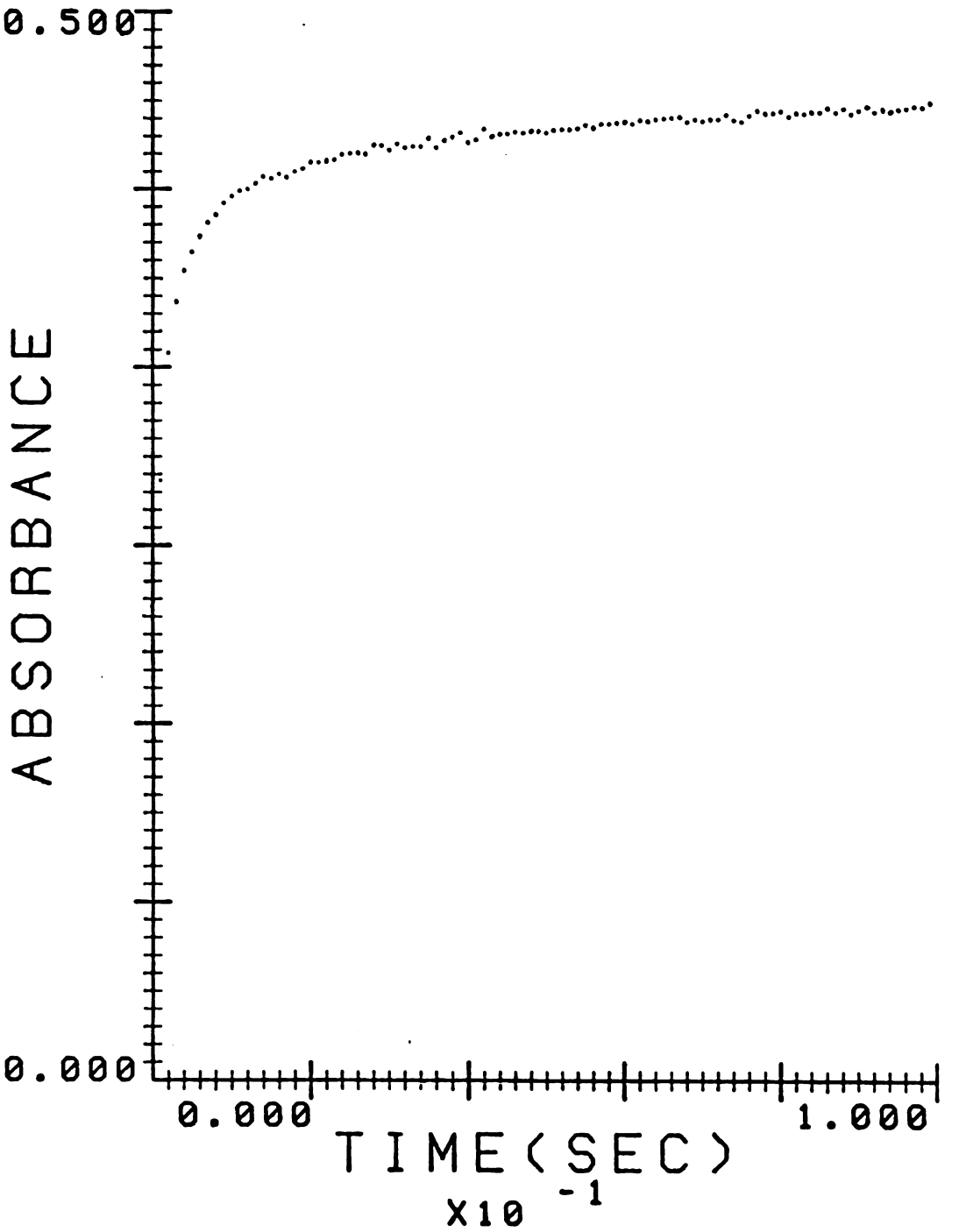


Figure 4-2. Reaction of BCG with serum albumin.

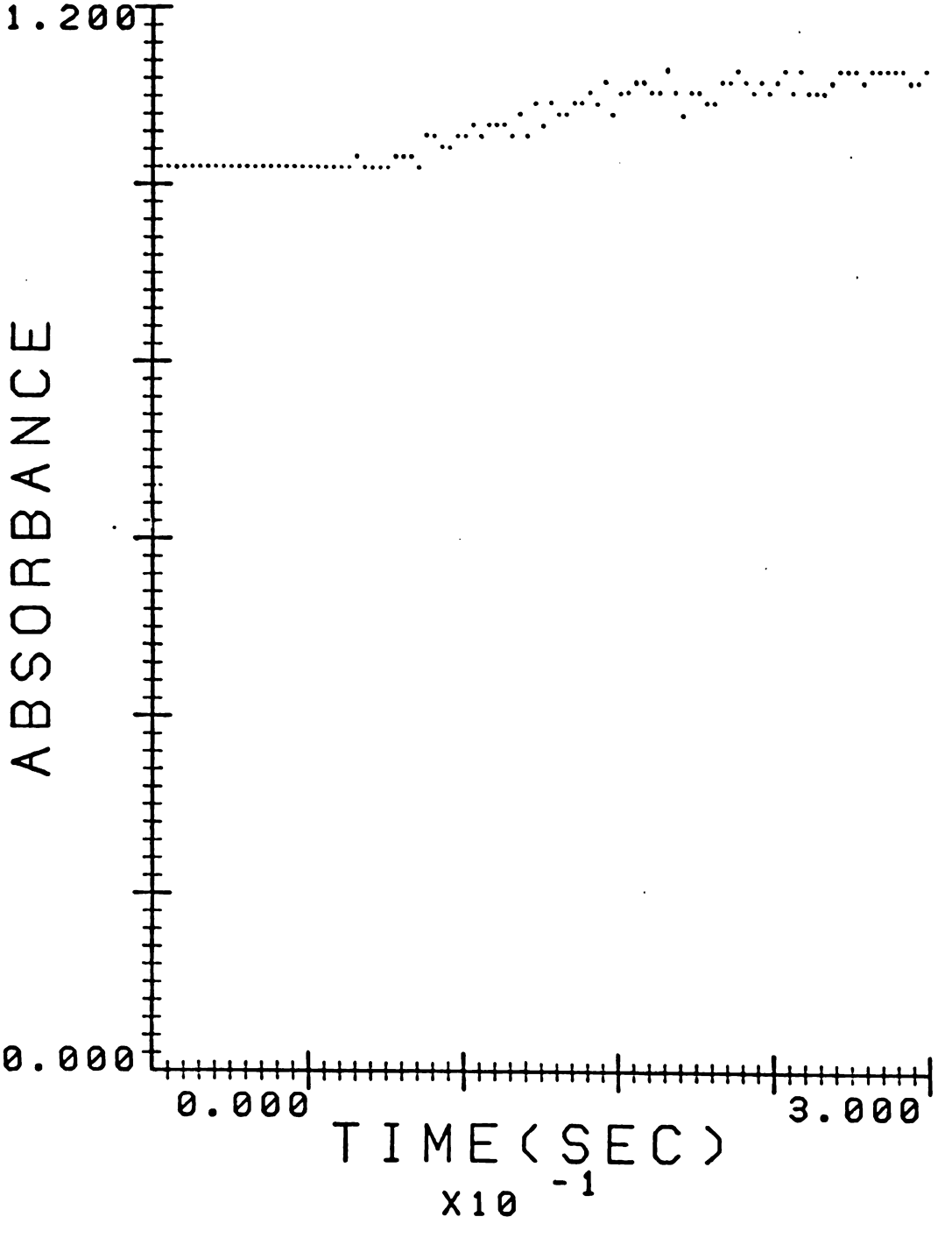


Figure 4-3. Reaction of BCG with serum albumin of abnormally high concentration.

but the Brij-35 caused minor mixing problems in the stopped-flow system. Thus, we decided to dilute all the solutions by a factor of two with saline solution. The sensitivity was unaffected and a linear standard curve up to an albumin concentration of 5.9 g/dl. was obtained. Table 4-1 gives the relative standard deviation of the data and the standard error of estimate of the curve.

We then followed the manufacturer's instructions and prepared 1:200 dilutions of three lyophilized sera: Moni-trol I, Moni-trol II, and Patho-trol from Dade. A fixed-time equilibrium method was used for the determinations with a 2 s measurement time. The measurement time of 2 s was chosen because our rate studies had shown that the BCG albumin reaction for the most dilute solution is completed in less than 2 s after mixing, while no significant amount of interference began until 6 s after mixing.

The results of our analyses are compared with the values provided by the manufacturer in Table 4-2, and it can be seen that our estimates of albumin concentrations within the normal range (3.5-5.9 g/dl) are lower than those obtained by other methods. For Moni-trol I, our results were 0.29 g/dl lower, and for the less concentrated Monitrol II, the difference was even greater ($\approx$0.52 g/dl). This is consistent with the fact that a decrease in serum albumin concentration is often accompanied by an increase

Table 4-1. Determination of Human Serum Albumin.

Concentration (g/100 ml)	A (Δt=2 sec)	RSD
1.38	0.118	3.0%
2.06	0.147	2.0%
3.16	0.197	1.4%
4.10	0.230	2.4%
4.51	0.255	2.0%
5.90	0.312	1.2%

Slope = 0.0428

Intercept =0.059

Std. Error of Est. = 3.78×10^{-3}

Table 4-2. Serum Albumin Determination with Different Methods.

	Stopped-Flow Method		Other Methods[a]				
	Mean	RSD	1	2	3	4	5
Moni-trol I	4.11[b]	3.0%	4.4	4.7	4.4	4.4	---
Moni-trol II	2.58	1.3%	3.0	3.1	3.3	3.1	3.2
Pathotrol	4.14	2.7%	---	---	4.7	---	4.1

[a]1 = DUPONT ACA
2 = CentrifiChem
3 = Technicon SMAC, or AAII
4 = Hycel Mark X
5 = Salt Fractionation, biuret method

[b]All concentrations expressed in mg/100 ml.

in globulin concentration, which further increases the magnitude of the slower reaction.

In fact, all our results were lower than those from other automated BCG methods, which seems to indicate that our method is less susceptible to overestimations of albumin concentrations. Webster (98) reported that his results were consistently higher than those from electrophoresis by 0.3 g/dl. Ferreria and Price said their analysis using bromcresol green gave results that were on the order of 0.5 g/dl higher than those obtained through immunoprecipitation (94). Thus based on these, one can safely assume that the manufacturer's reported values should be about 0.3-0.5 g/dL higher than the actual value, especially when their data are gathered from discrete and continuous-flow systems, some of which were measured 30 min after mixing.

C. Conclusions

We took advantage of the fast mixing feature of the stopped-flow system and were able to find conditions for determining serum albumin concentrations with bromcresol green, with the least amount of overestimation. Our rate studies suggest that a fixed-time equilibrium method with a measurement time of 2 s gives the most precise results with least interference from other proteins in the serum. Studies with lyophilized control sera show

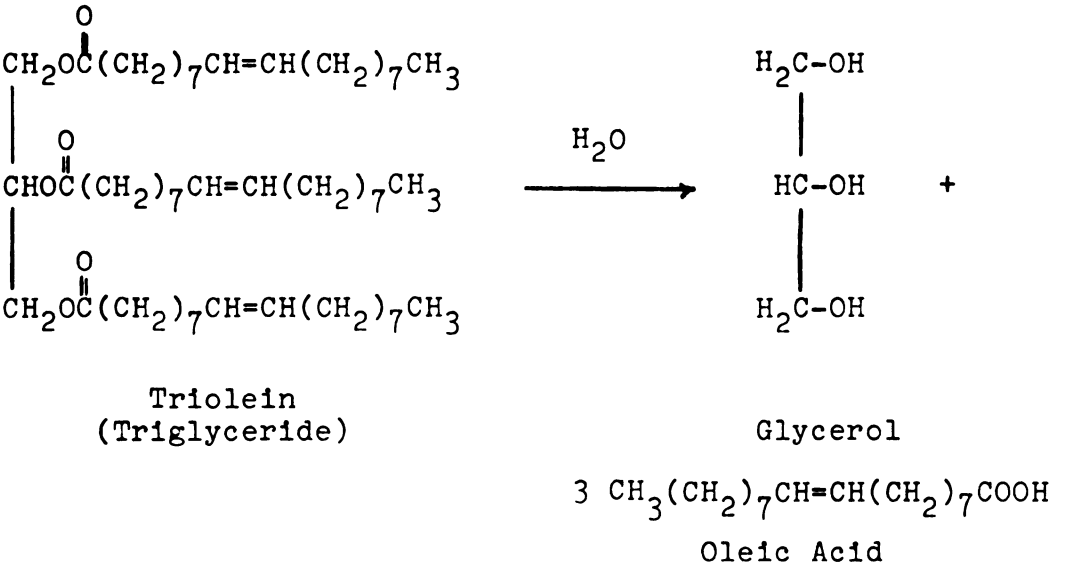
that our method consistently gives lower values than the other automated techniques which use BCG. Electrophoresis experiments should now be done on the same samples whenever the necessary equipment is available. This would further validate our results from the stopped-flow method.

V. SERUM TRIGLYCERIDE ANALYSIS

It is known that serum triglyceride levels increase in patients with atherosclerosis (114) and diabetes meilitus (115). Thus the assay of serum triglyceride is an important clinical test for detecting these diseases. Serum triglyceride is also determined routinely in lipoprotein phenotyping, which is described in Chapter VII. In this chapter, the stopped-flow system is shown to be useful in the determination of serum triglyceride. By monitoring the initial rate of an enzymatic reaction, results are obtained rapidly, and an early absorbance reading A_0 can be determined without the problems experienced in adapting the enzymatic procedure for the centrifugal fast analyzer (106).

A. Methods of Analysis

Many of the natural lipids are glyceryl esters of fatty acids, and triglycerides are the most common. Complete hydrolysis by strong acids, alkalis, or enzymes yields free glycerols and fatty acids as shown below for triolein.



Triolein
(Triglyceride)

Glycerol

3 $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Oleic Acid

The triglyceride level in blood rises to its maximum within 4 to 6 hours after digestion of food. For reliable results, blood specimens should be drawn after the patient has fasted for 12 hours.

Two colorimetric methods have been used extensively for serum triglyceride determination. Van Handel and Zilvermit (101) extracted the serum sample with chloroformmethanol, removed the phospholipids, hydrolyzed the triglycerides in alkaline solutions, and then oxidized the glycerol to formaldehyde. The final product is pink, with a visible absorbance proportional to the original triglyceride concentration. The Hantzsch condensation method (102) uses similar extraction and hydrolysis steps, but the final product is the yellow diacetyl lutidine species. This same product is also the basis of

fluorometric procedures (104).

These methods yield precise and accurate results, but they are cumbersome and time consuming because of the extraction and the chemical hydrolysis. On the other hand, hydrolysis of triglyceride by enzymes eliminates many of these complexities.

Currently there are two such methods. Bucolo and David's lipase hydrolysis of triglyceride (105) is at present the preferred method. It involves the following sequence of reactions:

1. Triglyceride hydrolysis

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{FFA}$$

2. Glycerol reaction sequence

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \alpha\text{-GP} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}$$

where

ADP = Adenosine diphosphate,

ATP = Adenosine triphosphate,

FFA = Free fatty acids,

GK = Glycerol Kinase,

α-GP = α-Glycerol phosphate,

LDH = Lactate dehydrogenase,

NAD = Nicotinamide - adenine dinucleotide,

NADH= Nicotinamide-adenine dinucleotide, reduced,

PEP - Phosphoenolpyruvate, and

PK = Pyruvate Kinase.

Lipase has been shown to hydrolyze preferentially the esters of the primary hydroxyl groups of glycerol (112). Thus, in order to complete the hydrolysis, a non-specific esterase (α-chymotrypsin) was added. When both enzymes are present, the hydrolysis is completed after 10 minutes at 30°C. The free glycerol then reacts as shown in the above equations resulting in the oxidation of NADH. The total decrease in absorbance at 340 nm is proportional to the concentratin of glycerol liberated from triglycerides and the free glycerol in the sample (free glycerol is usually subtracted as a blank).

The method has been used on the centrifugal analyzer (106,107), and on the Gilford 3500 (110). Previous serum extraction is not needed. Thus, the analysis is much simpler than those techniques which require an extraction step. However, phosphatase contamination by the enzymes introduces a competing reaction that makes the final value appear higher than what it is, and a "reagent blank" run is necessary (105). Free glycerol present in the sample also requires a sample blank determination.

Stinshoff et al (109) prescribed a correction factor

for free glycerol that follows a normal distribution:100 mg/l can be subtracted from each total triglyceride value instead of running a sample blank. A less empirical way to avoid running a blank is to use kinetic methods. Lehnus and Smith (110) reported using a Gilford 3500 system to measure the change in absorbance during a 14 second measuring time after a lag time of 30 seconds. The result was as good as the manual "end-point" method (110). Chong-Kit (106) and Tiffany et al. (107) used the centrifugal analyzer and made determinations by rate methods in less than 3 minutes.

B. Stopped-flow Method

Although the centrifugal analyzer can determine serum triglyceride rapidly, Chong-Kit reported that the inability to take an early absorbance reading A_0 was a principal problem in adapting the enzymatic procedure for the GeMSAEC (106). The mixing time for the GeMSAES is about 20 s, during this time, there is an appreciable amount of reaction, and the authors had to extrapolate back to zero time to correct for this. The stopped-flow system with its ability to obtain absorbance data very earily in time, should eliminate this problem.

1. Reagent Preparations

1. "Stat-Pack" Triglyceride Kit (Calbiochem, Post Office Box 12087, San Diego, CA 92112). Vial A was reconstituted with 80 ml of distilled water, and 0.25 ml of undiluted serum or standard glycerol was added to this solution. The contents were then mixed with Lipase in Vial C, and incubated at 30°C for 10 min. The Glycerol Kinase in Vial B was also reconstituted with 15.0 ml of distilled water.
2. Stock glycerol standard.
   Carefully weigh out 1.041 g of 99.8% pure glycerol (Drake Brothers, Menomonee Falls, WI 53051), and dilute to 1 liter. This is equivalent to 1000 mg triolein per liter.
3. Working standard solution.
   Dilute the stock glycerol standard with saline to make working standard solutions.

2. Rate Studies

The enzymatic reaction was adjusted to follow pseudo-first order kinetics. All analyses were carried out at 30°C, and the reactions were monitored at 340 nm for 60 s. Figure 5-1 shows the absorbance-time curves. There is a lag phase of ∿15 s, followed by a linear decrease in

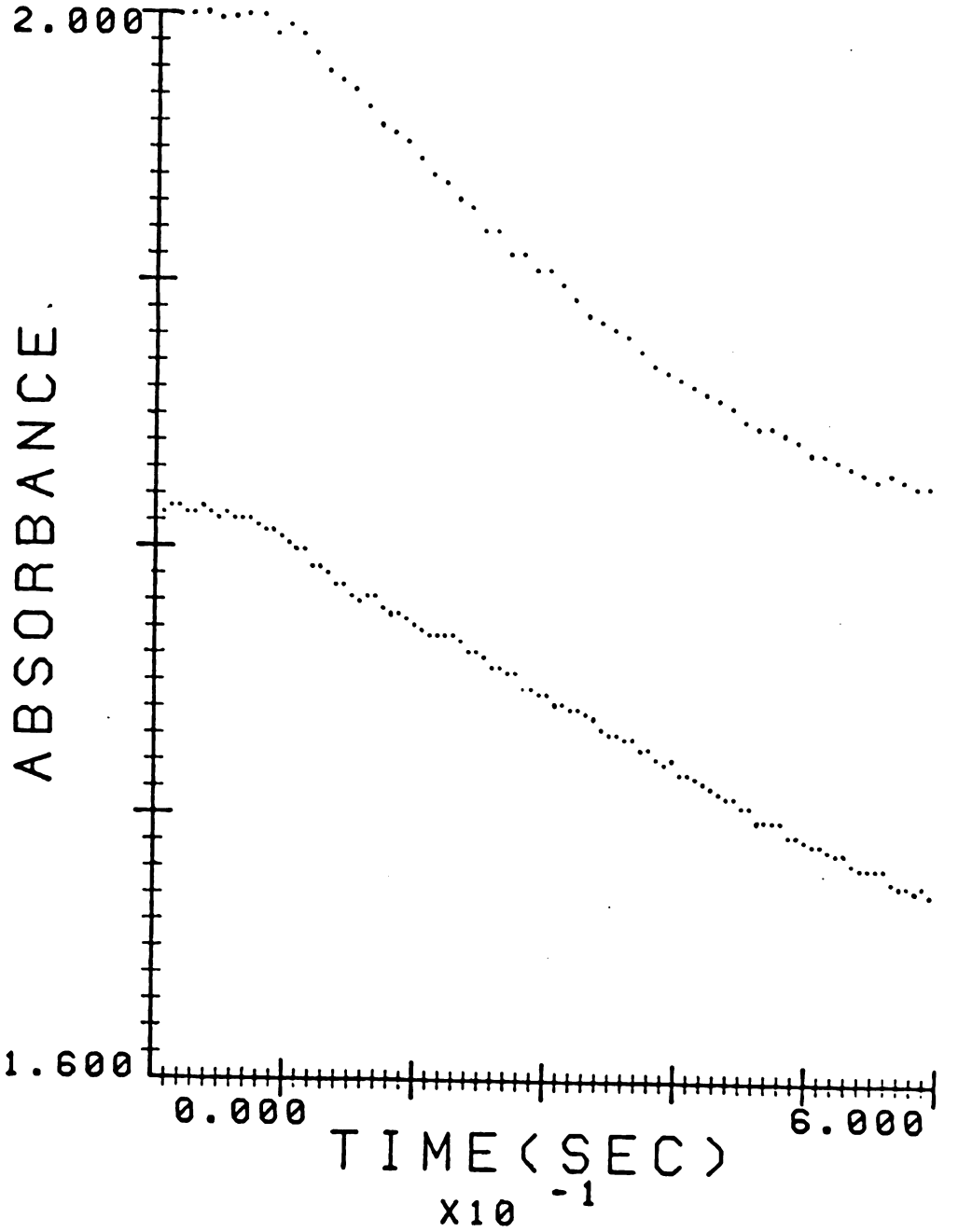


Figure 5-1. Enzymatic reaction of serum triglyceride.

absorbance that lasts from 20-40 s. After this, the rate of reaction begins to decrease.

A standard curve can therefore be prepared in two different ways. By measuring the constant rate in the initial linear portion of the reaction, one can easily get the initial reaction rate for each glycerol standard. Alternately, if the analyzer can give early absorbance readings - e.g., less than 1 s after the reaction has begun - then these may be considered as the initial absorbance reading A_0 in a fixed-time rate method.

Standard curves were prepared with both methods, and it was found that they produce similar results with excellent linearity up to 200 mg/dl. Figure 5-2 shows the calibration curve by the initial rate method. Table 5-1 gives the statistics of the standard curve using actual rate, as well as the precision of the stopped-flow rate method. In particular, it was found that being able to monitor the reaction in more than one way can minimize error caused by unsual samples. For example, in one case, the serum sample used had an exceptionally large amount of suspended particles, which caused excessive noise in the initial rate measurement. The fixed-time method saved us from repeating the analysis.

For both methods, there was no need to run separate serum blanks, nor was there any need to obtain A_0, as the first data point was taken 10 ms after mixing. The

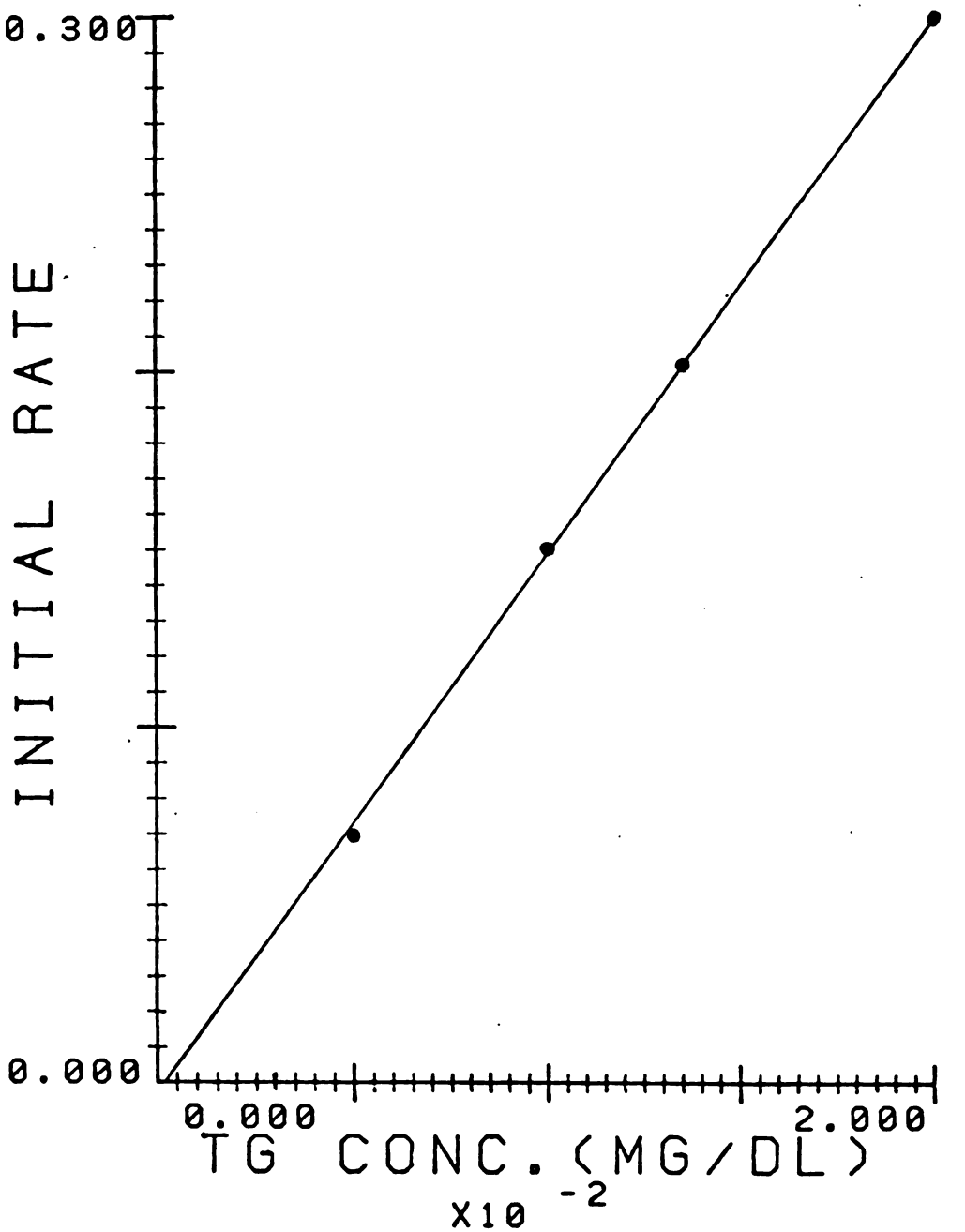


Figure 5-2. Standard curve for enzymatic analysis of serum triglyceride.

Table 5-1. Determination of Serum Triglyceride.

Concentration (mg/dL)	Rate	RSD
50	0.70	4.1%
100	0.15	2.4%
200	0.30	3.8%

Slope = 1.53×10^{-3}

Intercept = -0.005

Corr. Coeff. = 0.9997

reaction times, and lag phases for the reactions are determined by the concentration of the coupling enzymes. For a general discussion of this, the article by Scopes (116) should be consulted.

3. Experiments with Human Serum

The accuracy of the stopped-flow initial rate method was assessed by comparing the results obtained with reported values of a control lyophilized human serum (Moni-trol I). This comparison is shown in Table 5-2. Our results appear to be comparable with those of the reported values, and since we did not run a separate serum blank sample, 10 mg/dL should be subtracted from the results to correct for the presence of free glycerol as suggested by Stinshoff et al. (109). The 124 mg/dL triglyceride concentration is consistent with the average value obtained by the other methods.

The imprecision of the triglyceride analysis as can be seen from Table 5-2, may also be dependent on the dynamic blank caused by phosphatase contamination of the lipase in the commercially prepared reagent, as reported by Tiffany et al. (107). We tested this with a blank reaction without glycerol, and found its contribution insignificant for the batch of reagent we used.

Table 5-2. Serum Triglyceride Determination with Different Methods.

Method	Mean Concentration (mg/dL)	S.D.
DUPONT ACA	124	16.82
BIODYNAMIC	133	3.61
GEMINI	130	2.80
SMAC	93	4.81
HYCEL MARK X	140 (150)*	2.80
	Ave. = 124	
Stopped-Flow	124 (134)*	4.95

*Values not corrected for serum blank (free glycerol), all other values are corrected for free glycerol.

C. Conclusions

The enzymatic triglyceride reaction is relatively slow but has proved to be satisfactory with manual methods. Although our adaptation of it for the stopped-flow system did not improve the sample throughput rate, or decrease the sample volume requirements significantly, we were able to determine A_0 at an early time period, which allowed more effective use of the fixed-time rate method. Furthermore, it was found that the stopped-flow method is at least as efficient and viable as other methods for the routine analysis of triglyceride.

VI. SERUM CHLOESTEROL ANALYSIS

A. Introduction

The importance of cholesterol (and cholesterol esters) has long been recognized in clinical medicine. Serum cholesterol levels and their dietary intake have often been related to altherosclerosis, resultant strokes, cardiovascular diseases, etc. (8,117). New diagnostic approaches, such as hyperlipoprotein phenotyping, have considerably increased the interest in cholesterol determinations (118).

A host of different methods to determine serum cholesterol fills the literature. These have been reviewed so that even the interferences and sources of variation to which these methods are prone have also been reported (119-121). In the last 90 years, most analysts have preferred the iron-surfuric acid reaction and the Liebermann-Burchard reaction as the methods of analysis (8,122, 124). However, the recent introduction of enzymes as reagents to determine cholesterol has focused attention on the limitations of the strong acid approach (124).

The principle of the enzymatic procedure is presented in Figure 6-1. Cholesterol esters are first converted to free cholesterol by the enzyme cholesterol esterase. The free cholesterol is then oxidized by cholesterol oxidase, and the H_2O_2 produced is reacted with

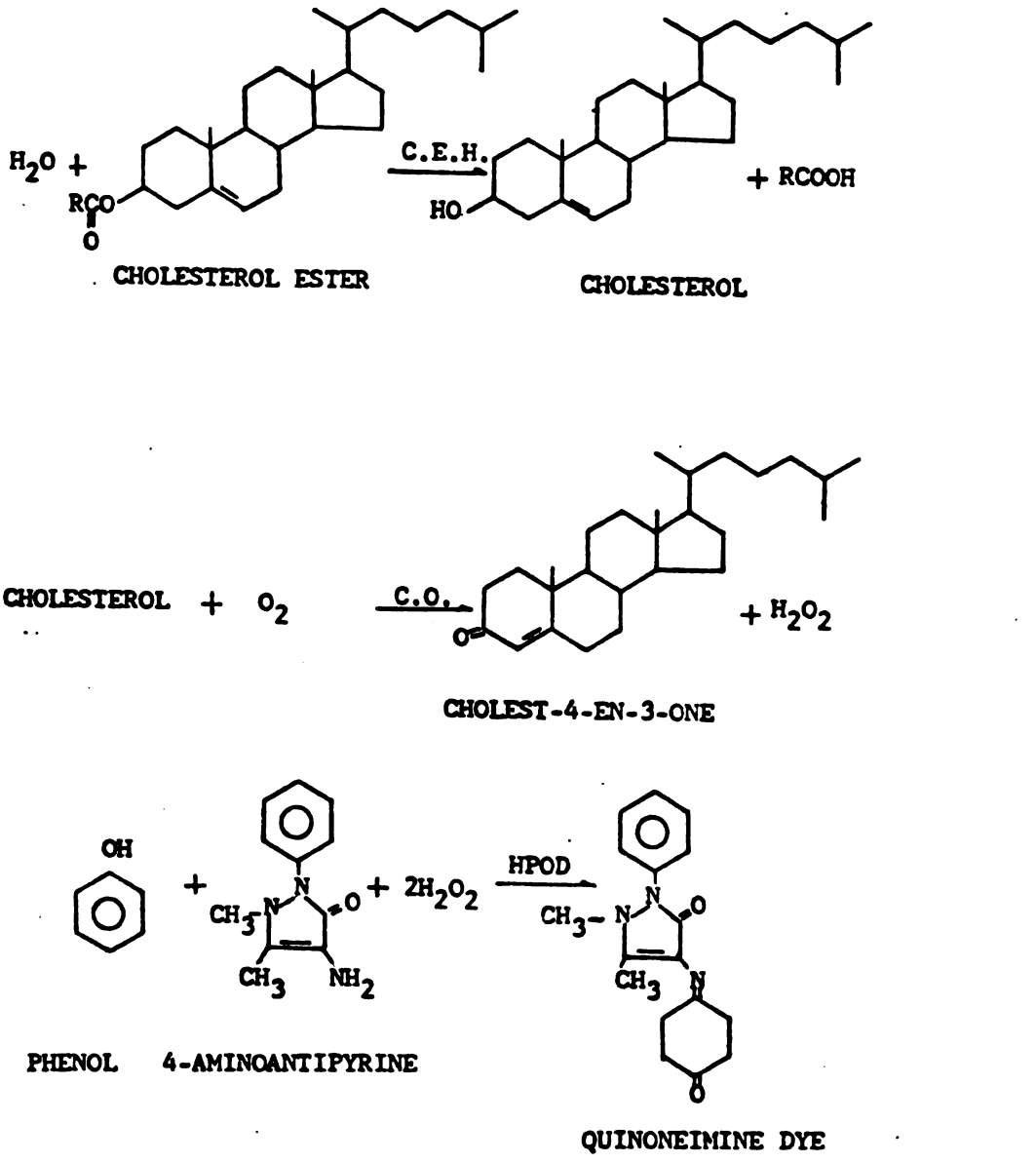


Figure 6-1. Enzymatic reaction of serum cholesterol.

4-aminoantipyrine and phenol to form a quinoneimine dye that absorbs at 510 nm.

The specificity of the enzymatic procedure is undoubtedly an important reason for its popularity. The enzymatic procedure is much simpler to implement when compared with the other reference methods (122,125). Furthermore, results are as precise and accurate as those obtained with other methods. All these factors make it a good procedure to use for routine analysis. The typical sample analysis time varies from 12 min. to 5 min., depending on whether a CentrifiChem analyzer (128) or a DuPont ACA analyzer (129) is used.

In this chapter results of serum cholesterol determinations with the stopped-flow system are presented. Two rather significant problems which must be solved in order to get satisfactory analytical results were discovered. They are the slow rate of the enzymatic reaction, which makes short analysis times difficult, and the difficulty in preparing suitable aqueous cholesterol standard solutions. We did not attempt to study the reported effect of interference by bilirubin (131) because the serum samples we worked with were all normal and contained insignificant bilirubin concentrations.

B. The Stopped-flow Method

1. Reagent Preparation

Cholesterol is not readily soluble in water, and as a suitable aqueous primary standard for cholesterol is lacking, we are seriously handicapped in our efforts to make quantitative measurements of the steroid by non-extracting procedures. The organic solvents, such as ethanol or 2-propanol used for non-aqueous standard solution preparations (8) inhibit enzymatic activity and are therefore unsuitable for our purposes (126).

As for the commercial aqueous cholesterol standards, the "Presicet" standards produced by Boehringer Mannheim Corp. (Indianapolis, IN 46250) are the most popular. The kit contains cholesterol standard solutions of 50, 100, 150, 200, 300, and 400 mg/dl, combined with a solubilizer and a preservative. Due to the unique 1:1 reagent to sample volume requirement of the stopped-flow system, we had to dilute the cholesterol standards 50 fold with 0.2% phenol solution so that the reagent solution contains all the buffers and enzymes and the sample solution contains the cholesterol sample. However, upon dilution, the solutions turned turbid, and thus a new method to prepare aqueous standard solutions for stopped-flow use was sought.

Solubilizer formulations that have been proposed

for aqueous standards are 25% Triton x-100, 12% Triton x-100 with unique mixing, and 15% Triton x-100 with 7% albumin (127). The 25% Triton x-100 preparation was found to be too viscous for efficient stopped-flow mixing, and several attempts to dissolve pure cholesterol in 15% Triton solution failed. We experimented with an alcoholic-aqueous mixture and prepared the standard solutions as follows:

2-propanol, reagent grade (Fisher Scientific Co., Fair lane, NJ 07410)

Cholesterol (Sigma, St. Louis, MO 63178).

Triton x-100 (Research Products International Co., Elk Grove Village, IL 60007).

To prepare 25 ml (500 mg/dl) cholesterol standard solution, dissolve 0.125 g cholesterol in 1.0 ml 2-propanol. Dilute to 25 ml with 15% Triton x-100, and warm in a water-bath at 60°C until the solution turns clear. Then prepare 50 to 400 mg/dl cholesterol standards by proper dilutions with 15% Triton x-100. This preparation procedure gave clear standard solutions in the normal concentration range, and the final 2-propanol content was small enough ($\sim$0.08%) that the enzymatic reaction was not inhibited.

2. Rate Studies

We modified a commercial cholesterol Auto/STAT kit (Pierce Chemical Co., Rockford, IL 61105) working reagent preparation by carefully transferring 2 bottles (12.2 gm each) of enzyme reagent to a bottle containing 450 ml phenol reagent. After reconsitution, this solution contains the enzymes peroxidase, cholesterol oxidase, cholesterol esterase, 4-aminoantipyrine, phenol, a stabilizer and a buffer. The substrate reagent was prepared by diluting cholesterol standards or human serum samples 50-fold with the phenol reagent provided. The two solutions were mixed in the stopped-flow system at 30°C, and the absorbance was measured as a function of time at 510 nm.

Initially the reaction was monitored for 100 s, as shown in Figure 6-2. There is a lag phase of about 20 s, and from approximately 35 to 55 s, the reaction becomes pseudo first-order. As can be seen in Figure 6-3, the rate is constant during this period. All the sera behaved similarly, and the rate was found to be proportional to the serum cholesterol concentration. However, the reaction with aqueous cholesterol standards was found to behave quite differently. It has been reported earlier that both alcohol and Triton x-100 retard the overall reaction rate (126,127), and we confirmed this observation in our studies. Figure 6-4 shows the rate of reaction with

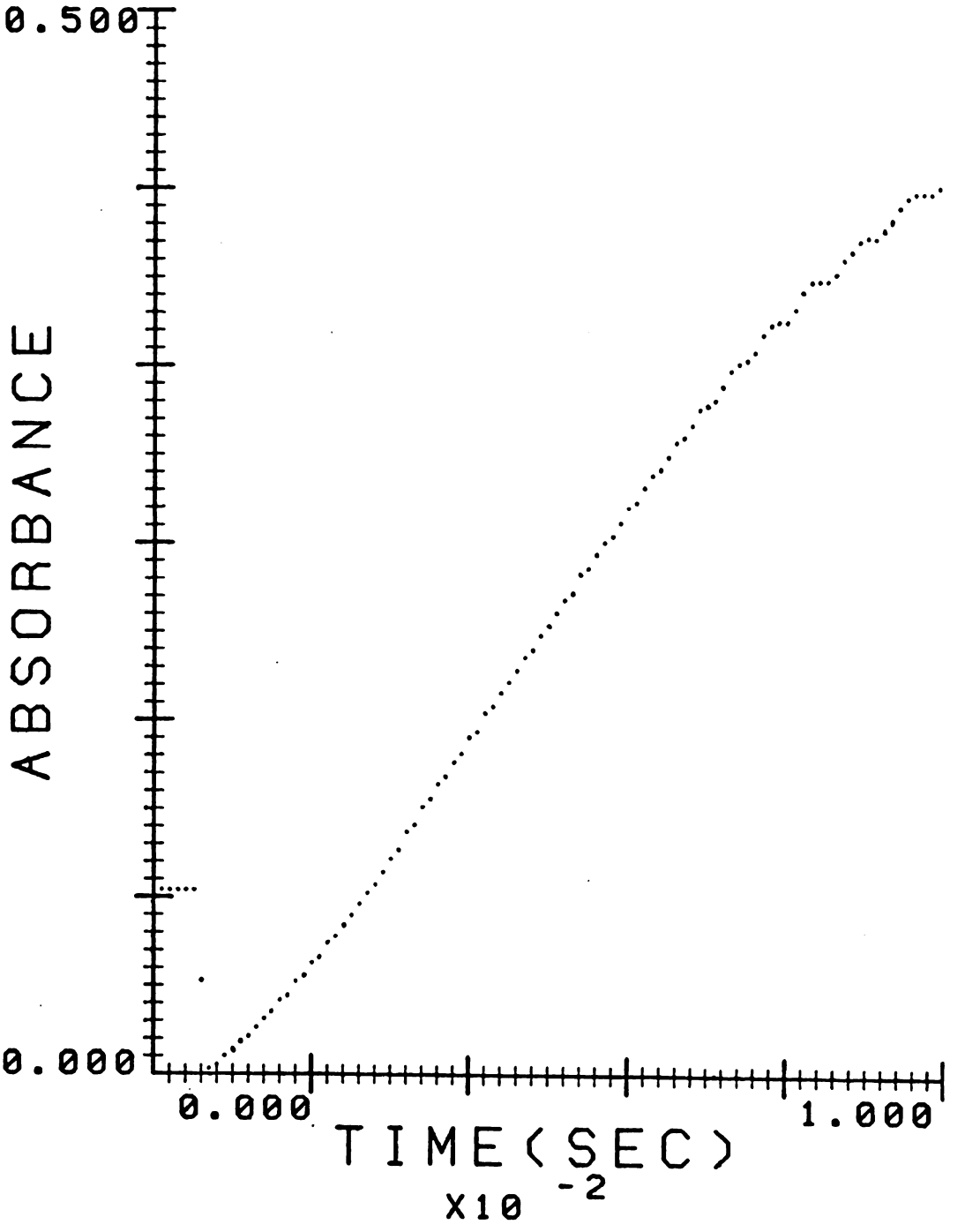


Figure 6-2. Enzymatic cholesterol reaction, Δt=100s.

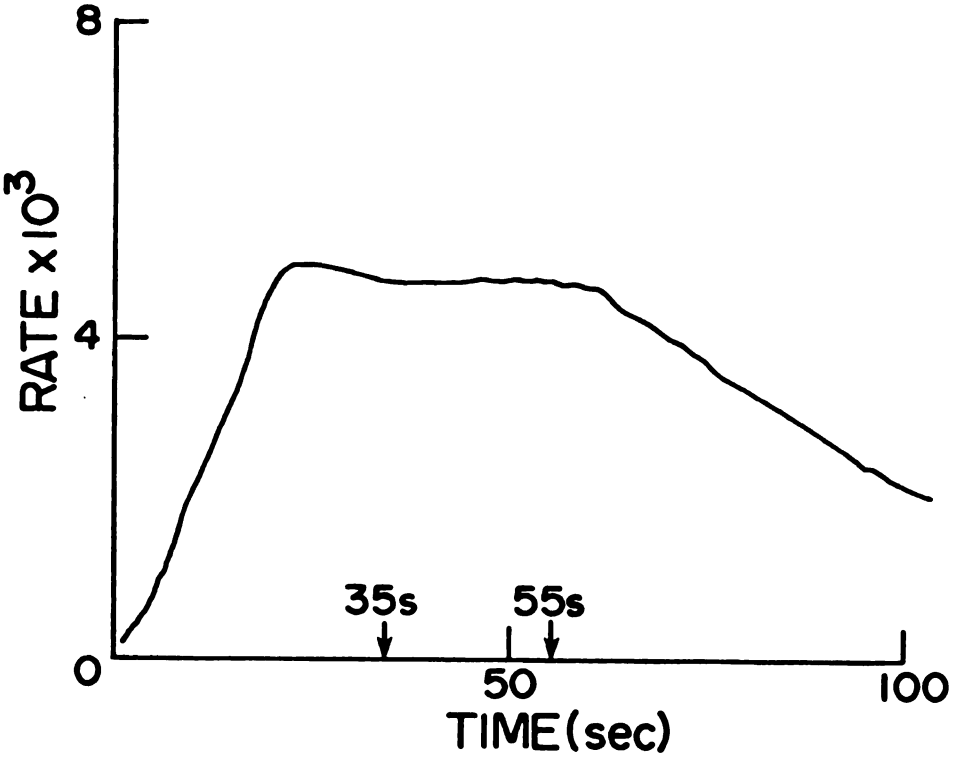


Figure 6-3. Reaction rate of enzymatic cholesterol vs. time (serum cholesterol).

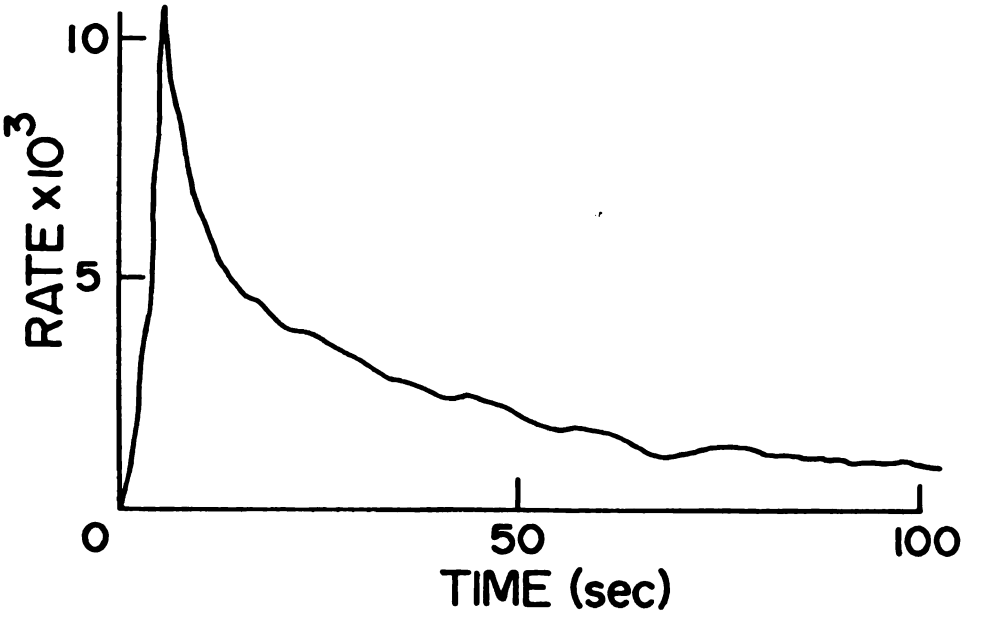


Figure 6-4. Reaction rate of enzymatic cholesterol vs. time (pure cholesterol dissolved in an aqueous solvent).

respect to time for a cholesterol standard. We noticed a rapid increase in rate in the first 10 s, which then decreased quickly. This is very different from the rate of cholesterol reaction in the serum samples as shown in Figure 6-3, and it was thus difficult to obtain a standard curve for cholesterol using the reaction-rate method.

Table 6-1 shows a representation of the magnitude of rate differences. Serum 3 is a fresh serum sample that has a reported cholesterol value of 190 mg/dl. The 200 mg/dl standard solution was prepared in our laboratory, while the Choles-trol was a commercially prepared aqueous standard (Sigma Co., St. Louis, MO 63178). Both aqueous standards gave a relative rate of 0.0031 absorbance unit per second, whereas the serum sample gave a much faster rate. This makes standardization based on the reaction-rate measurements of aqueous cholesterol standards rather difficult.

It was found, however, that if data are collected after the reaction reaches equilibrium (≈15 min), a linear standard curve can be obtained with the same samples as shown in Figure 6-5. Using the equilibrium method, we found that our observed values are on the average 3% lower than the values reported in general. Table 6-2 shows some of the results obtained. Moni-trol II is a control serum that has a normal cholesterol concentration of 239 mg/dl, and samples 1, 2, and 3 are fresh serum

Table 6-1. Cholesterol by Reaction-rate Method.

Sample	Concentration (mg/dL)	A (100s)
Serum #3	190	.416
Standard	200	.318
Choles-trol	195	.310

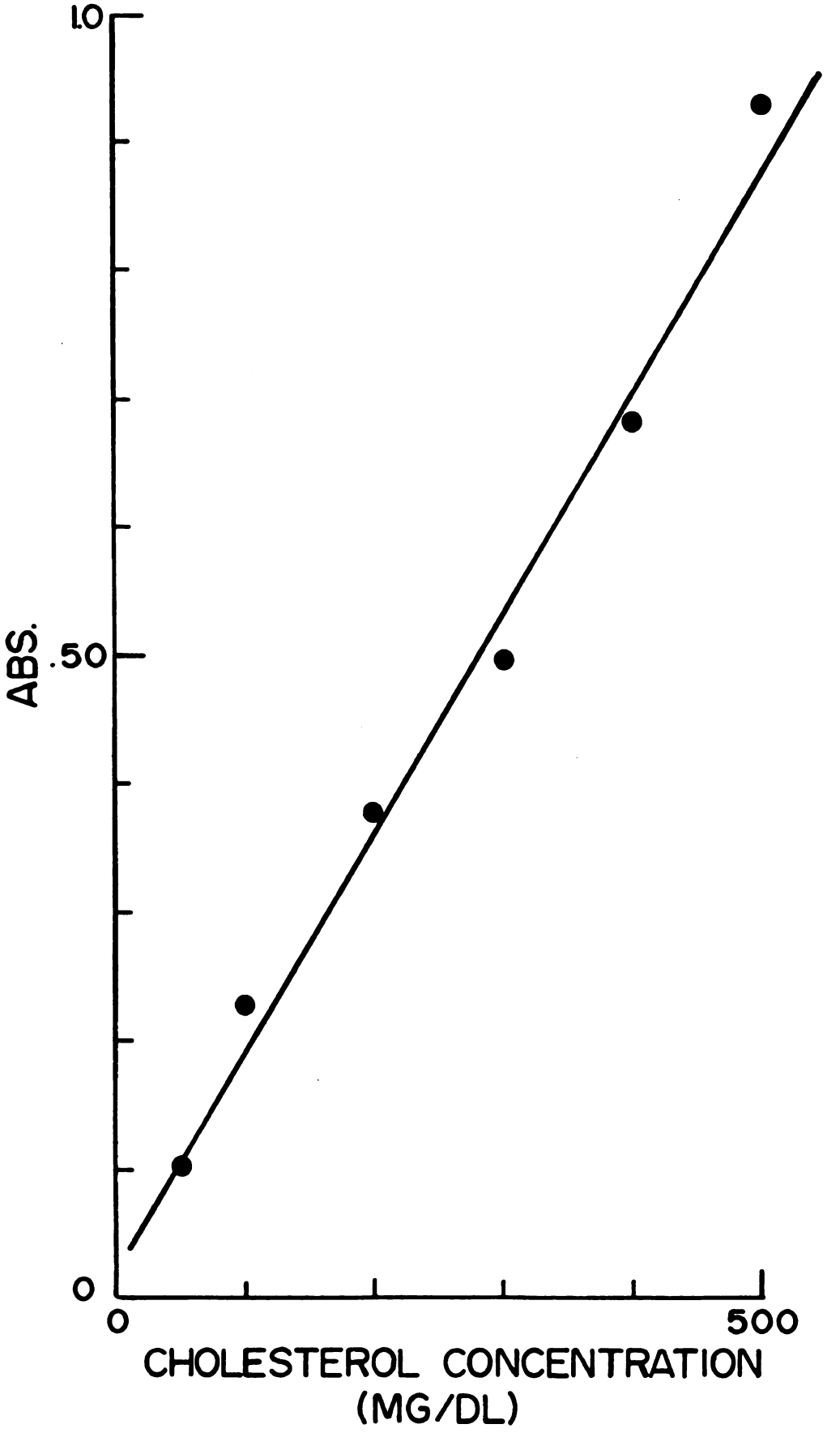


Figure 6-5. Standard curve for enzymatic cholesterol analysis.

Table 6-2. Cholesterol by Equilibrium Method.

Sample	Reported (mg/dL)	Observed (mg/dL)	% Diff.
Moni-trol II	239	230	3.8
Serum #1	225	219	2.7
Serum #2	260	251	3.5
Serum #3	193	190	1.6

samples obtained from Sparrow Hospital. The precision of our equilibrium method was found to be 1.9% RSD, and the maximum difference from the reported values was 3.8%.

C. Conclusions

What we have found about the reaction rate of aqueous cholesterol standards helps to explain why most reported cholesterol methods use the equilibrium approach. At present, serum cholesterol can be determined with the reaction-rate method in 100 s or less, but pooled sera standardized against a reference method must be used as standards. The problem with the aqueous cholesterol standard has illustrated one of the disadvantages of the stopped-flow system. Because sample and reagent are mixed in a 1:1 ratio, dilutions must be made to obtain favorable experimental conditions. In the case of the cholesterol standards, excessive dilution causes the undesirable problem of turbidity.

In order to use an aqueous cholesterol standard for the reaction-rate method, a solubilizer for the cholesterol must be found that does not affect the rate of the enzyme reaction. Abele et al. (126) used sodium deoxycholate (SDC) to stabilize cholesterol in an aqueous system. They have shown that the SDC solution was free of interference with the enzymatic procedure, but like Pesce et al. (128), they measured the absorbance change of the

reaction after a 12 min waiting period. Thus, they increased drastically the normally fast analysis times of the CentrifiChem centrifugal analyzer. Furthermore, Abele et al. did not mention any rate studies of the enzymatic reaction using their solubilizer. Therefore, it may be worthwhile to investigate this system further in the future.

Because of the difficulties involved in obtaining an aqueous standard solution for reaction-rate analysis, more emphasis should be placed in the future on searching for an enzymatic system that reacts faster. Rautela and Liedtke (129) reported a new enzymatic system in 1978. Instead of using phenol, they used N,N-Diethyaniline-hydrochloride as part of their chromogen system. With Triton x-100 as surfactant and sodium cholate as emulsifier, they were able to decrease the time required for complete reaction to 150 s.

There is still another approach to decrease the sample analysis times with the kind of aqueous cholesterol system we have available now. The immobilized enzyme loop system developed in our laboratory (2) can be used with the stopped-flow system. Dr. Marty Joseph (2) has worked on developing an immobolized cholesterol oxidase loop. Although the work needs further refinement, the technology for immobilization is available (130). If a multi-loop system can be developed for the serum

cholesterol analysis, one can first mix the sample and the reagent, then draw the mixture into one of the many immobilized enzyme loops with an inert solvent, so that while waiting for the reaction to go to completion, one simply has to switch to another loop for the next sample. After the reaction reaches equilibrium, the final mixture can be pushed to the mixing cell for reaction with the chromogen and then into the observation cell for data collection. The 15 min spent on waiting for the completion of the reaction can therefore be eliminated by a multiple, parallel immobilized-enzyme loop approach.

VII. SERUM LIPOPROTEIN ANALYSIS

A. Introduction

Numerous studies have demonstrated an association between serum lipids and atherosclerosis (132,136-138). Elevated levels of serum cholesterol and low density lipoprotein have been shown to cause cholesterol ester deposits in the artery walls, which eventually leads to atherosclerosis (133). Fredrickson and his colleagues separated serum lipoproteins by electrophoresis and worked out a classification system for hyper-lipoproteinemias (134) on which much of dietary management and drug therapy depends.

Liquid profiles are obtained by quantitative techniques. Standard methods, which use a preparative ultracentrifuge or electrophoresis, take 6 to 16 hours for each analysis. The purpose of our work here was to develop a reaction-rate method with the stopped-flow system so that a complete lipid profile may be done in less than half an hour.

1. Lipoprotein Profile and Hyperlipoproteinemia

There are three major components of serum lipids: phospholipids comprise the largest fraction (70% phosphatidylcholine, 20% sphingomyellin), cholesterol is the

second largest group (about 75% esterfied to fatty acids), and glycerides (almost all as triglycerides) comprise the third fraction (135). Most of these lipids are associated with lipoproteins. The serum lipoproteins are a family of neutral lipids (primarily triglycerides or cholesterol esters), surrounded by a layer of phospholipid and protein. These particles can be divided into four categories: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The function, composition, and physical properties of each are summarized in Table 7-1 (136,145,166). In general, the higher the protein content, the higher the density of the lipoprotein. The primary function of the lipoproteins is lipid transportation. Abnormal concentration of any fraction will create lipoproteinemias.

Fredrickson and coworkers (134) have classified hyperlipoproteinemia into six phenotypes. The characteristic lipoprotein fractions and electrophoretic patterns are shown in Figure 7-1. A frequency study by Jones (137) indicated that the bulk of hyperlipoproteinemias in medical practice are of types II and IV, while the odd ones, types I, III, and IV, are sufficiently rare to be of numerical importance.

High serum LDL or cholesterol levels have been used as an indicator of hyperlipidemia for the past two

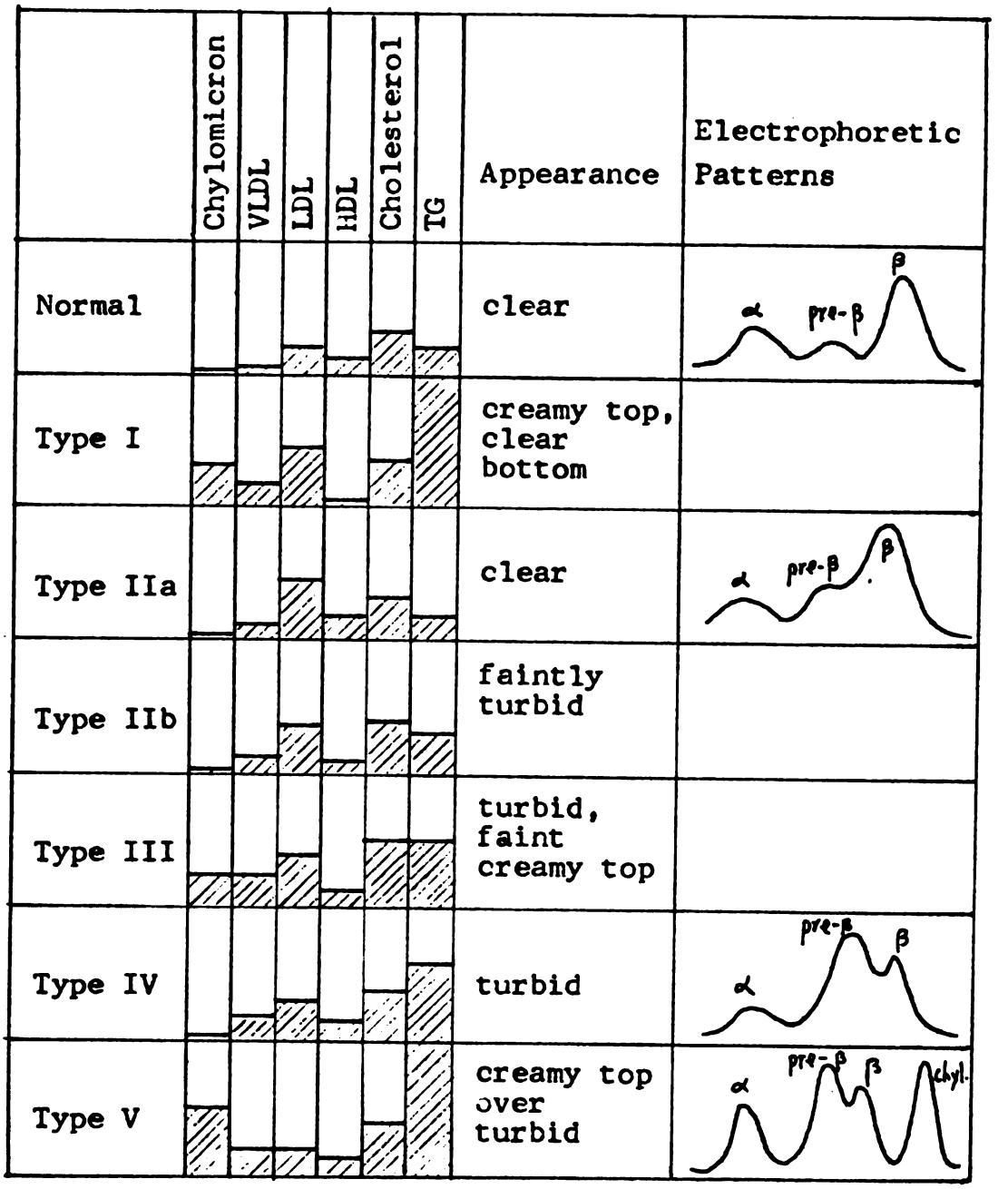


Figure 7-1. Characteristic lipoprotein fractions and electrophoretic patterns.

decades. Meanwhile there was relatively little interest in the role of the high density protein (HDL), which ordinarily carries about 20% of the total plasma cholesterol. In recent years, however, there has been an increased interest in HDL levels and their significance in the development of coronary heart diseases (139). Analysis of HDL will certainly replace that of LDL as an indicator of coronary heart diseases in the future.

2. Methods of Analysis

Lipid profiles are performed to determine serum or plasma cholesterol and triglycerides, after which lipoproteins are then measured by an analytical ultracentrifuge or through electrophoresis (140-146). The former is more quantitative but takes 8 to 16 hours for each analysis (140). The most recently developed instrument of this kind is the Beckman lipoprotein profiling system, which uses a portable air driven ultracentrifuge with an oxygen sensitive electrode as detector (143). Cholesterol determinations in HDL, LDL, and VLDL can be made with 0.5 ml sample within 3 hours.

The second method involves electrophoresis of lipoproteins and is generally performed on paper or agarose gel. When done on paper it takes about 10 hours (141), but when agarose gel is used, the analysis time is reduced to 3 hours (142). Because lipoprotein electrophoresis

is difficult to control, particularly with regard to uniform sample application and irregular dye uptake by lipids, it is only used in pattern recognition. In fact, Iammarino (138) has suggested that the method be discontinued as a routine procedure due to its high cost and because it offers no additional information to the data generated by lipid analysis.

Helena Laboratories announced their new line of HDL electrophoresis instruments early in 1978 and claimed that their new method was both rapid and precise. They used a specific enzyme reagent instead of the conventional Oil Red O dye in developing the electrophoregrams, and thus were able to reduce the total analysis time to 1 hour. But, a careful analysis of their data showed that relative standard deviations as high as 25% were reported in their quantitative analyses (144).

In this chapter, a stopped-flow method is reported that requires only about 1/2 hour to perform and can avoid the expense of using ultracentrifugation or electrophoresis equipment.

B. Stopped-flow Method

1. General Scheme

In order to use the stopped-flow mixing technique for lipoprotein determinations, it was necessary to find

a method to separate the three different lipoprotein fractions without using either ultracentrifugation or electrophoresis. Thus, several procedures were investigated.

Burstein and Scholnick (148,152) described several useful chemical precipitation methods for the separation of plasma lipoproteins. Fredrickson et al. (156) used a combined chemical precipitation and preparative ultracentrifugation method to separate the lipoproteins. Later they reported a modified method which totally eliminated the need for an ultracentrifuge (149). However, their method cannot be used for patients with substantial degrees of hyperglyceridemia (149). Wilson and Spiger (151,155) reported a dual precipitation method as an improvement of the above, but upon comparison with the ultracentrifuge method, it was also found to be inappropriate for serum with high level of triglycerides.

Our proposed method is shown in Figure 7-2. The goal is to estimate the relative concentrations of VLDL, LDL, and HDL along with the values for total cholesterol and total triglycerides, such that the sample can be classified into different phenotypes. Precise quantitation of the HDL fraction is important, especially in light of its increasing significance as a correlative index of coronary heart disease. The serum total cholesterol and total triglyceride concentrations are first determined. If

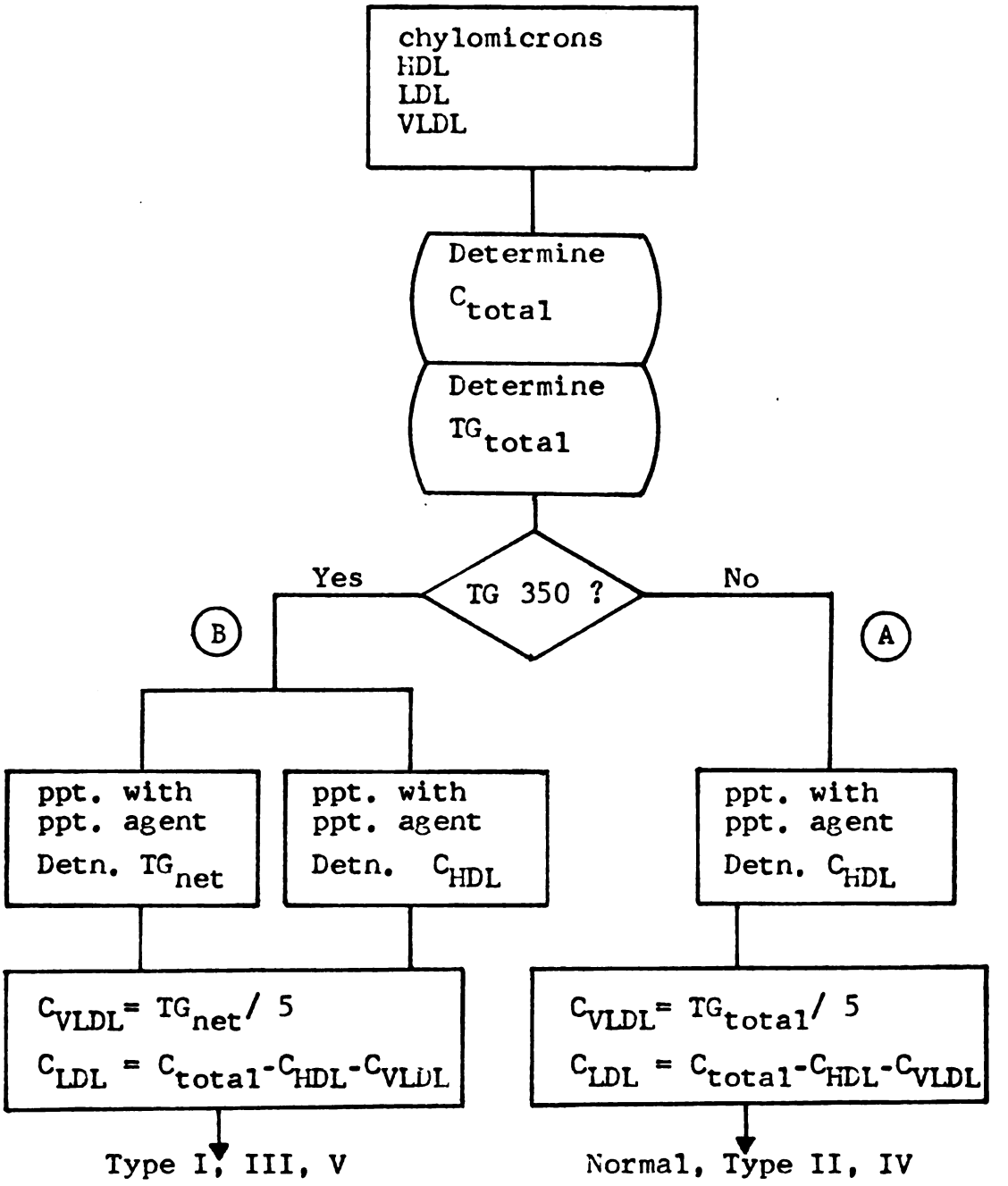


Figure 7-2. Proposed serum lipoprotein analysis procedure.

the concentration of triglyceride is normal, precipitation agents are added to completely precipitate chylomicrons, LDL, and VLDL. The cholesterol level of the HDL fraction left in the supernatant is determined, and the cholesterol concentrations of VLDL and LDL can be estimated as suggested by Fredrickson (149). If the triglyceride level is high, one additional precipitation is performed (148), and the estimation is done by a modified equation as shown in Figure 7-2.

The proposed method has several advantages over other methods. First and most important, total serum cholesterol, HDL, and triglyceride concentrations are known quantitatively. This allows estimation of lipoprotein concentrations without separation by electrophoresis or ultracentrifugation. Secondly, the total analysis time is short. Finally, the whole procedure can be easily automated.

2. Choice of Precipitation Agent

Serum lipoproteins are known to form insoluble complexes with polyphosphates and detergents (152), with polycations (161,150), and with polyanions (153-160). The polyanions were first shown to give complete, immediate, and selective precipitates by Burstein (148, 152), and the Lipid Research Clinic Program of the NIH

adapted a polyanion method (heparin-Mn^{++}) that is currently widely used in the United States. A mixture of LDL and VLDL can be prepared by precipitation with heparin and either $MnCl_2$ alone or $MgCl_2$ plus sucrose. Other polyanions may also be used either with dextran sulfate and $MnCl_2$, or with sodium phosphotungstate and $MgCl_2$. It was found that the lower the protein:lipid ratio, the easier is the formation of an insoluble lipoprotein-polyanion-metal ion complex, but the nature of the lipid moiety does not affect precipitation at all (148). The nature of the lipoprotein-polyanion-metal ion interation can be explained by assuming that the electrostatic interaction between the positive charges of the proteins and negative charges of the polyanion cause the polyanions to combine with the protein moiety of the lipoproteins to form soluble complexes. This is easily proved by agarose electrophoresis (163). These compounds are then precipitated by divalent cations.

The heparin-Mn^{++} precipitation process has been shown to correlate well with the accurate but time consuming ultracentrifugation method (153,155,159,160). However, it has several undesirable problems. Not all heparin preparations are equally efficient, and thus the completeness of VLDL and LDL precipitation must be checked each time a different lot of heparin is used (158). Also the Mn^{++}-heparin procedure requires that the sample be

placed in an ice-bath for 30 min. after the precipitating reagents are added. Sera with triglyceride concentrations of more than 400 mg/dL may require prior dilution before precipitation (153,164). If an enzymatic technique is used to measure HDL cholesterol, the Mn^{++} sometimes forms a visible precipitate with the enzyme reagents and also contributes a variable and significant blank (157).

The application of Mg^{++}-dextran sulfate as the precipitation agent has recently been adapted for routine lipoprotein quantification (158). There has not been any further report on the reliability of the procedure, and therefore this method was not studied here.

Lopes-Virella *et al.* (157) described a routine method using Mg^{++}-sodium phosphotungstate. Values thus obtained compared well with the ultracentrifugation method, and the method does not have theproblems experienced with the Mn^{++}-heparin method described (157). Personal communication with a local clinical laboratory confirmed the reliability of the method (165). Pierce Chemical Co. (Box 117, Rockford, IL 61105) manufactured a HDL-cholesterol rapid STAT kit in 1977 that uses the phosphotungstate-$MgCl_2$ method. This kit also has unique features that make it best suited for adaptation to the stopped-flow system. Therefore, we chose this as our precipitation agent. Details of the reagent have been presented in the cholesterol chapter.

3. Separation of the Lipoprotein Fractions

The polyanion-metal precipitation method is very efficient in separating HDL from the other fractions. The HDL fraction remains in the supernatant while the chylomicron, VLDL, and LDL fractions are precipitated. Electrophoresis studies have shown that the precipitation is selective and complete (148). In order to further separate the remaining fractions quantitatively, ultracentrifugation must be used. In fact, the Lipid Research Clinic program recommended using ultracentrifugation to separate VLDL from LDL and HDL before separating the HDL from the other fractions by precipitation (166).

Friedewald *et al.* (149) described a simple method to estimate the concentration of VLDL and LDL. Their procedure is very helpful in the interpretation of lipoprotein profiles, and it is based on the following relationships:

$$C_{VLDL} = TG/5$$

$$C_{LDL} = C_{total} - C_{HDL} - C_{VLDL}$$

where TG = serum triglyceride concentration, and

C = cholesterol concentration in the HDL, LDL, and VLDL fraction

all units are expressed in mg/dL.

These equations are based on two observations: (1) the ratio of the mass of triglyceride to that of cholesterol in VLDL is apparently relatively constant and about 5:1 in normal subjects; and (2) when chylomicrons are not detected, most of the serum or plasma triglycerides are in the VLDL. The equations are fairly accurate when the TG level of the serum is less than 400 mg/dL, making it suitable for normal and type II patients (see Table 701). But the use of the ultracentrifuge remains necessary with the much rarer type I, III, and IV hyperlipoproteinemia.

Dual-precipitation methods have been proposed. Detergents such as sodium dodecyl sulfate were used to separate the VLDL fraction (154,155). However, incomplete aggregation of VLDL by sodium dodecyl sulfate and the long incubation time reported do not justify the adaptation of these methods in place of the ultracentrifuge.

A modified procedure, which was listed earlier in Figure 7-2, is proposed in this work. The total cholesterol and triglyceride levels are first determined. If no elevation of the serum triglyceride level is observed, the HDL and VLDL, LDL fractions are separated by the Mg^{++}-phosphotungstate method using Friedewald's equations to calculate each concentration. If TG is larger than 350 mg/dL, an extra precipitation step is taken to determine the net concentration of TG present in VLDL before

Table 7-1. Physical Properties of Serum Lipoproteins.

	Approximate % Composition				Density (g/ml)	Electrophoresis Fraction	Function
	Protein	TG	Cholesterol	Phos. Lipid			
Chylomicron	2	84	7	7	0.95	Origin	Transport dietary TG and cholesterol
VLDL	9	54	19	18	0.95-1.006	Pre-β	Transport TG synthesized in the liver
LDL	21	11	45	22	1.006-1.063	β	Transport endogenous cholesterol to cells
HDL	50	4	22	22	1.063-1.21	α	Transport endogenous cholesterol back to liver

calculating the concentration in the different fractions. In our final lipoprotein profile, we will have total cholesterol concentration, total triglyceride concentration, HDL cholesterol concentration, and an estimate of the concentrations of all 4 different fractions of lipoproteins. This information combines the best of the old (Fredrickson's phenotyping) and the new (C_{HDL}) indices for a complete profile.

Finally we have to consider the physical method of separating the HDL in the supernatant from the VLDL and LDL in the precipitate. Traditionally, the sample was mixed and centrifuged in a table-top centrifuge (1500 x g, 4°C) for 30 minutes. But, since Burstein reported that the precipitation process is immediate (148), precious time is lost in the centrifugation process.

Warnick and Albers (160) used ultrafiltration in their attempt to clear up the HDL containing supernatant after centrifugation for 30 minutes. The flow rate was quite acceptable (1 mL/min). We explored the possibility of using ultrafiltration to replace centrifugation, thus cutting the total analysis time by 30 minutes or more.

4. The Ultrafiltration Method

Fresh serum samples from fasting patients in Sparrow Hospital were analyzed. To each 1.0 mL serum sample, 0.1 mL phosphotungstate-Mg^{++} precipitating reagent (Pierce,

Rockford, IL) was added, making the final concentration of $MgCl_2 \cdot 6H_2O$ 1% (W/V) and phosphotungstic acid 0.4% (W/V). Three samples were then centrifuged at 1000 x g for 20 minutes. The clear supernatants were aspirated out, and the cholesterol contents of these HDL fractions obtained by the Pierce manual method using an HDL/Cholesterol Rapid STAT kit (product #44040,44002).

Another three samples were processed as follows: a 0.22 um Millipore filter (Millipore Corp., Redford, Mass. 07130), 25 mm in diameter, was placed on the lower support of a Swinnex filter holder, and a silicone gasket was put on the filter. Then an AP 15 and an AP 20 glass depth prefilter (22 mm in diameter) were fitted into the gasket. The upper unit of the Swinnex holder was tightened over the lower support to form a complete filtration assembly. Figure 7-3 shows the filtration assembly. The 1.0 mL precipitated suspension is then forced through the filter assembly from a 10 mL syringe, with a flow rate of approximately 1 mL/min. The clear supernatant was then collected for HDL cholesterol analysis.

The results obtained are presented in Table 7-2. The difference in observed values between the two methods is compatible within experimental error. From these results, it seems reasonable to conclude that ultrafiltration is a separation technique which is at least comparable to centrifugation. The filtration process

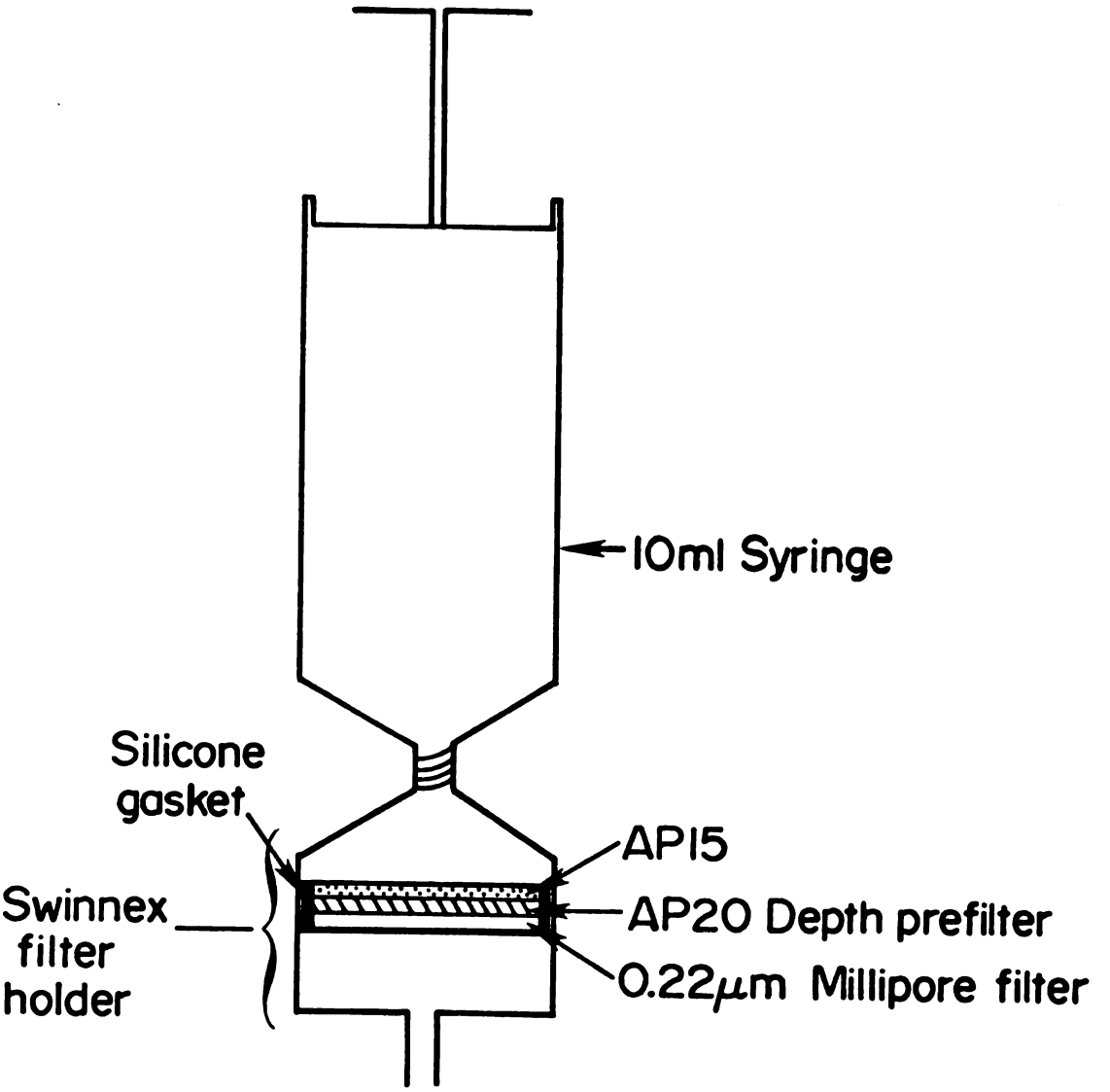


Figure 7-3. The Complete Filtration Assembly.

Table 7-2. Comparison of the centrifugation method with the ultrafiltration method.

Centrifugation		Ultrafiltration	
HDL Conc.* (mg/dL)	RSD	HDL Conc. (mg/dL)	RSD
55.6	3.6%	54.8	4.3%

*Average of 3 runs, total serum cholesterol conc.=230 mg/dL.

takes 1 minute instead of 20 minutes, and the filtration assembly is disposable if necessary. Ultrafiltration has the additional benefit of eliminating possible turbid supernatants that are sometimes found with the centrifugation method. Therefore, this separation technique was used in all further studies.

5. Experiment with Fresh Human Serum

Lipoproteins are not stable complexes and therefore fresh serum samples are required in serum lipoprotein analysis. We obtained three fresh serum samples from Sparrow Hospital for analysis. The total triglyceride and cholesterol concentrations were determined as follows:

Glycerol standards for serum triglyceride analysis were prepared as described in Chapter 5. A standard curve was prepared by using the initial rate method. All analyses were carried out at 30°C, and the reactions were monitored at 340 nm for 60 s. For each sample, 0.25 mL human serum was added to vial A of the "Stat-Pack" reagent kit and was reconstituted with 8.0 mL of distilled water. Details of the procedure can be found in Chapter 5.

Cholesterol was analyzed with the equilibrium method. Aqueous cholesterol standards were prepared from pure cholesterol as described in Chapter 6. The Pierce cholesterol Auto/STAT kit was used for the working reagents.

Human serum samples were diluted 50-fold with the 2% phenol reagent provided. The two solutions were mixed in the stopped-flow system at 30°C, and the absorbance was measured at 510 nm 15 minutes after mixing.

The results for the total cholesterol and total triglyceride determinations are listed in Table 7-3. Since all the triglyceride levels were found to be below 350 mg/dL, we proceeded to follow Scheme A of Figure 7-2 and added 0.1 mL phosphotungstate-Mg^{++} reagent to 1.0 mL of each serum sample.

The separation of the precipitate was then performed by ultrafiltration, and the cholesterol content of the filtrates were determined. The results are again shown in Table 7-3.

Using Fredrickson's equation, discussed earlier, concentrations of cholesterol in the different fractions of lipoprotein were calculated, and they are listed in Table 7-4. The three samples we obtained all showed values that were within the normal range. If the serum were type II abnormal, the C_{LDL} concentration should be very high, whereas if the samples were type IV, C_{VLDL} would be elevated. We therefore conclude that all three serum samples are normal. Using the same samples, a cellulose acetate plate electrophoresis study was performed with a procedure developed in cooperation with Dr. Hans Lillevik of the Michigan State University Biochemistry

Table 7-3. Lipid Analysis on Three Human Sera.

	RSD
Serum #1	
HDL = 48 (Norm = 40-75)	5%
Chol = 215 (Norm = 150-250)	1.9%
TG = 81 (Norm = 30-150)	3.8%
Serum #2	
HDL = 62 (Norm = 40-75)	
Chol = 251 (Norm = 150-250)	
TG = 115 (Norm = 30-150)	
Serum #3	
HDL = 69 (Norm = 40-75)	
Chol = 190 (Norm = 150-250)	
TG = 45 (Norm = 30-150)	

Table 7-4. Lipoprotein Analysis on 3 Human Sera.

Equation

$$C_{VLDL} = TG_{total}/5$$

$$C_{LDL} = C_{total} - C_{HDL} - C_{VLDL}$$

Results

	C_{VLDL}	C_{LDL}	C_{HDL}
Serum #1	16	155	48
Serum #2	23	166	62
Serum #3	9	122	69
Normal range	(0-40)	(62-185)	(40-75)
Type II range	(0-78)	(173-840)	(18-82)
Type IV range	(6-356)	(28-231)	(15-74)

Department (See Appendix C for details of the procedure).

The resultant electrophoretic pattern is shown in Figure 7-4. The normal lipoprotein pattern confirmed our stopped-flow results.

HDL analysis has become increasingly important in the past two years. Unlike electrophoresis data, our results give quantitative concentrations of serum HDL. Since the normal range of HDL is not very large (40-75 mg/dL), a less precise method would create false positive or negative results. The stopped-flow method gives results with better than 4% RSD at this low concentration range, which is comparable to the currently available manual methodology.

C. Conclusions

Although we did not encounter any abnormal samples in our limited sampling, we have shown that the stopped-flow method is feasible for obtaining a total lipoprotein profile (total cholesterol, total triglyceride, HDL, and phenotype of serum) in about 30 minutes. Analysis time can easily be reduced to 15 minutes if the reaction-rate method of cholesterol analysis is used. At this moment, we have to use commercially prepared enzyme reagents, and the reagent cost for each complete analysis is almost $3.00. This high cost can be reduced by developing multiple-loop immobilized systems as described in the

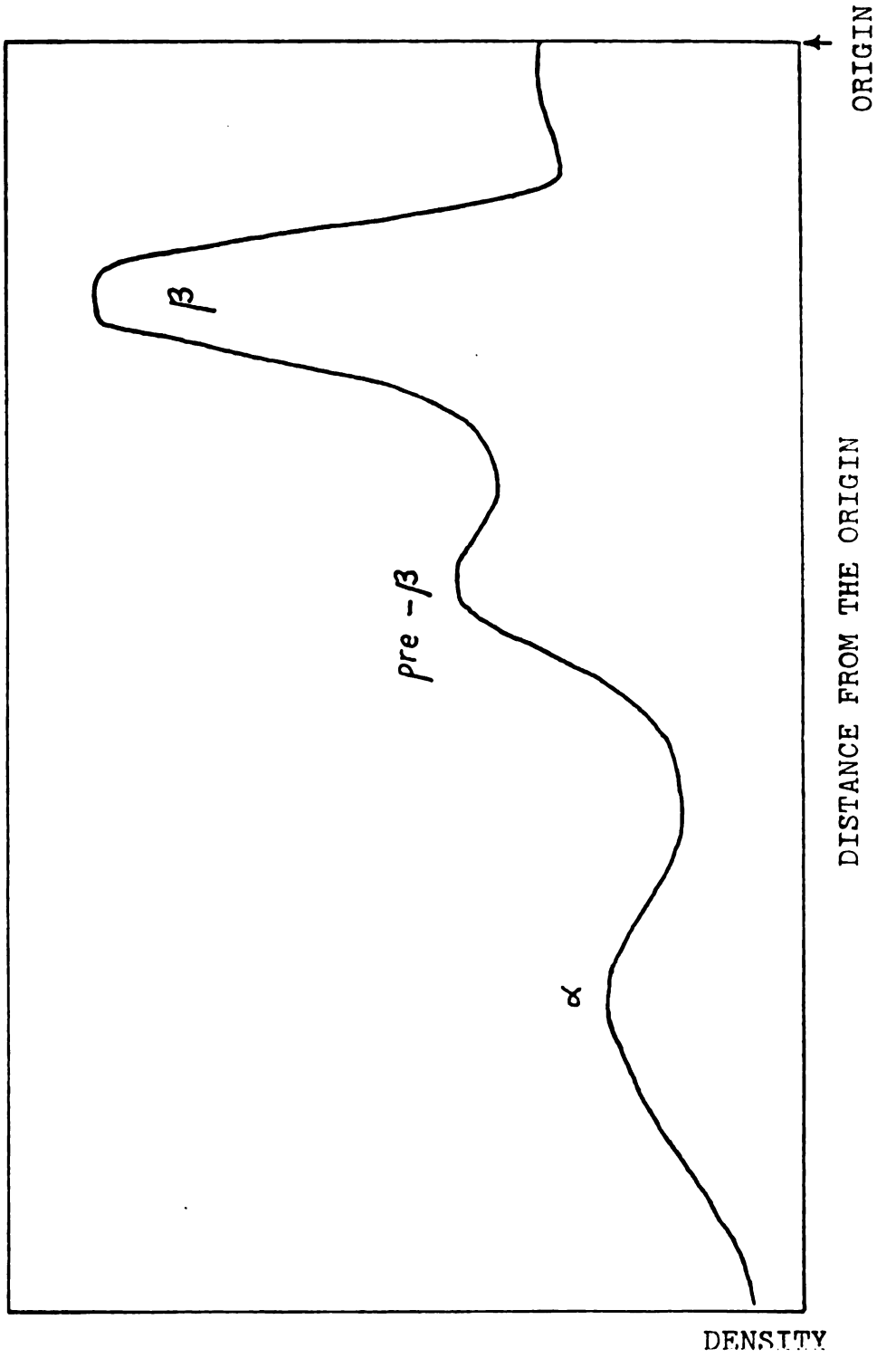


Figure 7-4. Electrophoretic pattern for serum sample.

cholesterol chapter, because then the expensive enzymes would be reusable. The sample throughput rate would also be further increased with this arrangement. The whole process can be totally automated when the solution preparation and the filtration procedures are brought under computer control.

VIII. FUTURE DIRECTIONS

The experiments performed in this thesis have illustrated several unique characteristics of the stopped-flow system in clinical applications. We found the system excellent for routine clinical analyses in many ways. The throughput rate is generally increased using the reaction-rate method. The fast mixing characteristic of the system allowed investigation of the initial rates of rapid reactions with ease. The same feature also enabled us to study fast reactions, such as the bromcresol green-albumin reaction, and in the process devise a way to eliminate most of the interfering reactions.

On the other hand, several undesirable problems were encountered. Several tests became uneconomical because of the necessity to rinse the stopped-flow system between samples with expensive reagents. We also met with turbidity and some mixing problems mainly due to the requirement of using a 1:1 sample to reagent volume ratio. While the results presented demonstrated some of the analytical capabilities of the stopped-flow system, there are probably many more routine chemical analyses that have not been investigated by reaction-rate methods but are adaptable to the system.

Pinnell and Northam reported a bromcresol purple (BCP) method for analyzing serum albumin. They claimed

that the dye is specific to albumin, thus eliminating the interfering problem to which BCG is susceptible (190). Furthermore, they reported that the BCP reaction with serum is an instantaneous one, making it very worthwhile for future investigations with the stopped-flow system.

Enzymatic analysis of uric acid was done with a centrifugal fast analyzer (189). The reaction is relatively fast, and the use of an early reading blank-corrected approach was found to be desirable (189). The uricase/catalase/aldehyde dehydrogenase-coupled procedure is also ideally suited for adaptation to the stopped-flow system.

The Jaffe reaction for serum creatinine appears to follow pseudo-first-order kinetics (192) and this routinely determined substrate can be easily analyzed by the reaction-rate method using the stopped-flow instrument.

Another commonly determined substance is serum alcohol. Jung and Ferard described a specific and precise enzymatic reaction which reached equilibrium after only 4 min (193). No stopped-flow method for serum alcohol has been reported yet.

One may also try to apply the simplex method described in Appendix A in differential rate analysis for mixtures. Pelizzetti et al. reported a kinetic determination of adrenaline, L-dopa and their mixtures with a

stopped-flow system (191). The simplex technique should be superior to the single-point method of Lee and Kolthoff they were using.

Another approach in research should be directed towards developing the immobilized enzyme loops as mentioned in Chapters 6 and 7. Successful adaptation of these loops onto the stopped-flow system would mean an increase in throughput rate and a decrease in cost per analysis.

Referring back to Table 1 in Chapter 2, one can see that we have only explored a small fraction of the clinical tests routinely performed. The largest unexplored area is the analysis of enzymes in serum. The stopped-flow system is especially suited for this kind of analysis because all procedures for serum enzyme analysis involve reaction-rate measurements; and the amount of enzyme present in serum can be measured only indirectly in terms of its activity (or rate of reaction) with its substrate. Commonly determined enzymes such as SGPT, SGOT, LDH, CPK, ACP and ALP all fit into this category.

Another important area for further work is the determination of isoenzymes. Kaplan et al. (59) were the first to apply the stopped-flow system in a differential determination of LDH isoenzymes. They pinpointed the exact source for abnormal levels of LDH enzyme and used the result as a sensitive test for myocardial infraction.

Their method was superior to conventional separation methods such as electrophoresis because analysis time was drastically reduced. Bostick and Mrochek (58) reported a procedure for creatine kinase MB isoenzyme using differential kinetics with a centrifugal analyzer. This should also be suitable for the stopped-flow system.

Pardue had demonstrated that the stopped-flow system could have an attractive throughput rate even for conventional types of equilibrium analyses (1). Thus, the stopped-flow system seems to be applicable to virtually all types of wet chemical analyses performed routinely in clinical laboratories. And in addition, it has a capability for making fast measurements that are not possible with any of the conventional instruments. We feel the stopped-flow system would make an excellent 'stat' analyzer in clinical chemistry. Further research would be centered around methodological development, complete automation of the system, and the decreasing of the dead volume in the system to minimize carry-over problems. Automated sample preparation, valve sequencing, and simple data processing can be brought under microprocessor control. Chemically inert and inexpensive high pressure liquid chromatography valves and fittings that have small dead volumes can be used in an improved design of the system. The continuous development in analytical methods should bring about wider acceptance of the system in the clinical chemistry field.

APPENDICES

APPENDIX A

APPLICATION OF THE SIMPLEX METHOD IN DIFFERENTIAL KINETIC ANALYSIS

A. Introduction

A clinical chemist is often faced with the difficult task of determining the fastest way to handle several variables which are often known to interact. Spendley et al. (171) introduced an idea in 1962 for tracking optimum operation conditions called the simplex optimization method. Essentially it searches for the optimum using empirical feedback strategy. Compared to other optimization methods, it requires fewer experiments for each move. It is also more compact, involves simple computations, and is particularly attractive for automation.

Nelder and Mead (172) modified the original fixed-size simplex method. Later Long applied it to analytical chemistry (174). Recently it has been applied in clinical chemistry (176). Several excellent reviews about the simplex optimization method have been published (167,169, 175,195), and at least one paper has indicated its possibility in regression analysis (187).

With the advent of high speed computers, linear least square analysis has been used in routine statistical analysis of data (179,180). However, more complex problems

such as non-linear or arbitrary functions sometimes require special constraints upon the data collections; or a user may face miserable failure (when an exasperated computer finally print out a message of defeat), or even disastrous failure (when the computer and user think they have found the answer) when improper procedures are employed. Matrix inversion is usually required in the curve fitting process and can lead to serious round-off errors in the calculation if handled improperly (185,186).

A powerful general purpose curve fitting program called 'KINFIT' was designed by Dye and Nicely (182) and was very successful using CDC 3600 and 6500 computers as well as IBM 7094 and 360-75 computers. In the process of doing differential kinetics analysis in our laboratory, the need for a simple and computationally more compact program to be used with a 16K PDP 8/e minicomputer became evident. This need stimulated an investigation of the simplex method in curve fitting. This idea is explored in this chapter, and experiments with computer-simulated data are reported to demonstrate the feasibility of the idea.

B. The Super-Modified Simplex

1. General Description

The Nelder and Mead modified simplex has been used widely, but the procedure sometimes fails in stochastic

or noisy environments due to premature simplex contractions, adherence to false ridges, or reluctance to approach the boundary constraints (178). Denton et al. (178) developed a super-modified simplex (SMS) procedure that increased the utility, reliability, and efficiency of the Nelder and Mead version. We further modified the SMS and applied it in least-square analysis as described later. The rules of the Nelder and Mead modified simplex are briefly reviewed below to facilitate the understanding of the SMS (169, 172).

A simplex is a n-dimensional geometric figure containing n+1 vertices. For example, the initial figure of a two dimensional simplex is a triangle with vertices $V_B V_N V_W$, as shown in Figure A-1. These three vertices were measured and vertex V_B was found to be the best response, V_W the worst response, and V_N the next-to-worst response. $\overline{P}$ is the centroid of the face remaining after the worst vertex has been eliminated (169). The optimum in the response surface can be found by one of several different moves as listed in Table A-1. To eliminate the worst response V_W, Table A-1 again provides us with several measures: it can be replaced by V_R through reflection, by V_E through expansion, or by V_{CR} or V_{CW} through contraction. A failed contraction occurs when the response at either V_{CR} or V_{CW} is worse than the response at V_R or V_W, respectively. When this happens, a "Massive Contraction" move is then made, each leg of the simplex is contracted to one half

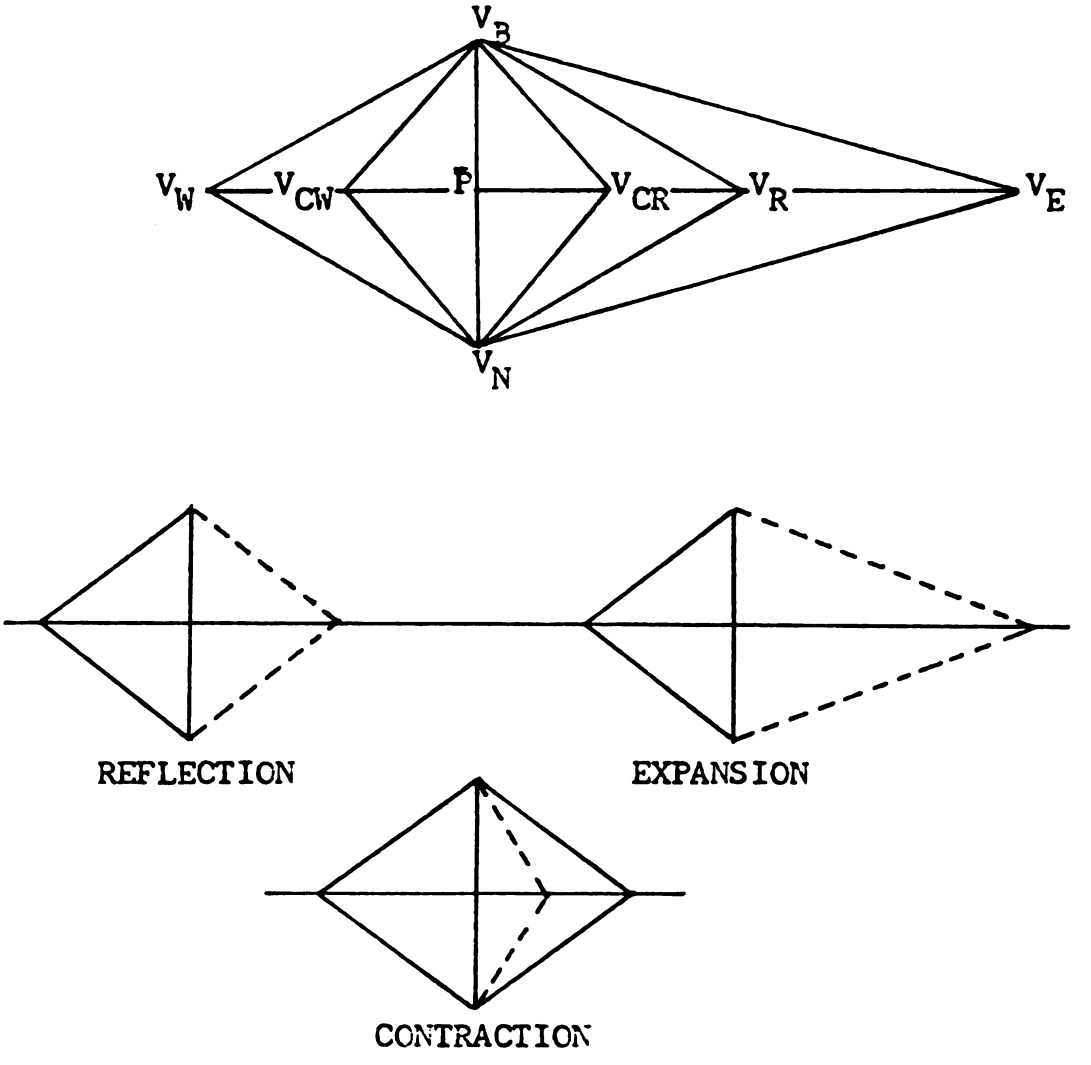


Figure A-1. Basic moves of a simplex.

Table A-1. Basic Moves of a Simplex.

$V_R = \overline{P} + (\overline{P} - V_W)$	
$V_E = \overline{P} + \gamma(\overline{P} - V_W)$	γ = expansion coeff.=2
$V_{CR} = \overline{P} + \beta(\overline{P} - V_W)$	β = contraction coeff.=0.5
$V_{CW} = \overline{P} - \beta(\overline{P} - V_W)$	

its present length towards V_B, forming a smaller new simplex V_BV_N,V_W, or V_BV_N,V_R. The responses are then determined and the optimization process continued. Boundary violations are handled by assigning a poor response to the vertex that violates the boundary and the new vertex is determined by a C_W contraction. A flow chart of the modified simplex is shown in Figure A-2. Reflection is always the first move, followed by expansion or contraction, while massive contraction is used only when the contraction has failed to give a better response than the original worst response.

2. Procedure for the Super-Modified Simplex

The flow chart for the super-modified version is shown in Figure A-3. The initial vertex and the responses are determined as before. A reflection is made to determine the new response R_{V_R}. Now, instead of expanding or contracting with a fixed coefficient, a second order polynomial curve ($y=Ax^2+Bx+C$) is fitted through the responses at V_W, $\overline{P}$, and V_R. If the optimum sought for is a maximum, and the slope A is negative (i.e., the curve is concave down), the maximum can be located within the interval by evaluation of the derivative of the curve. The response of this new vertex can then be determined, and after the safety and boundary checks, the optimization process can be continued. If the curve concaves up, no maximum can

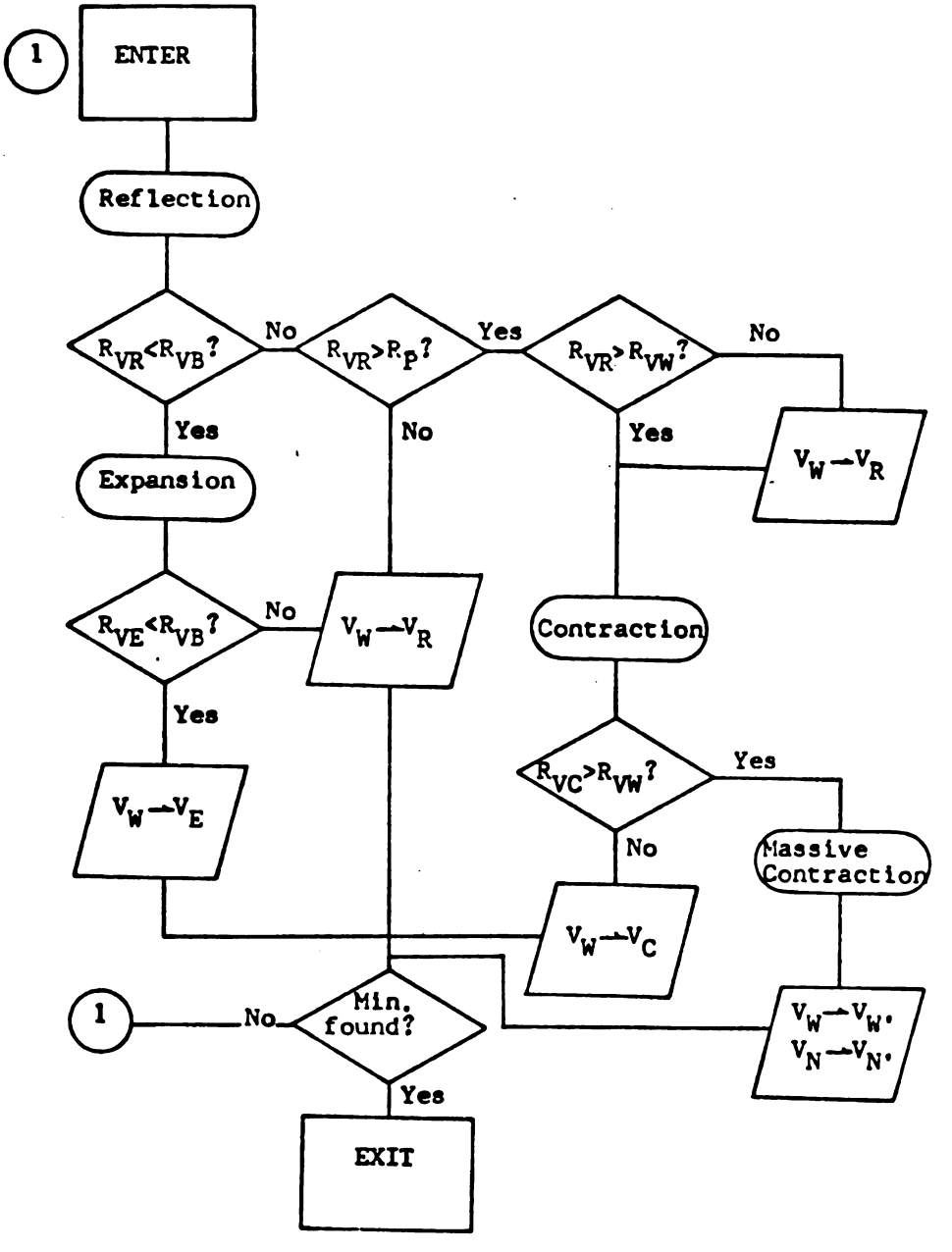


Figure A-2. Procedure for the modified simplex.

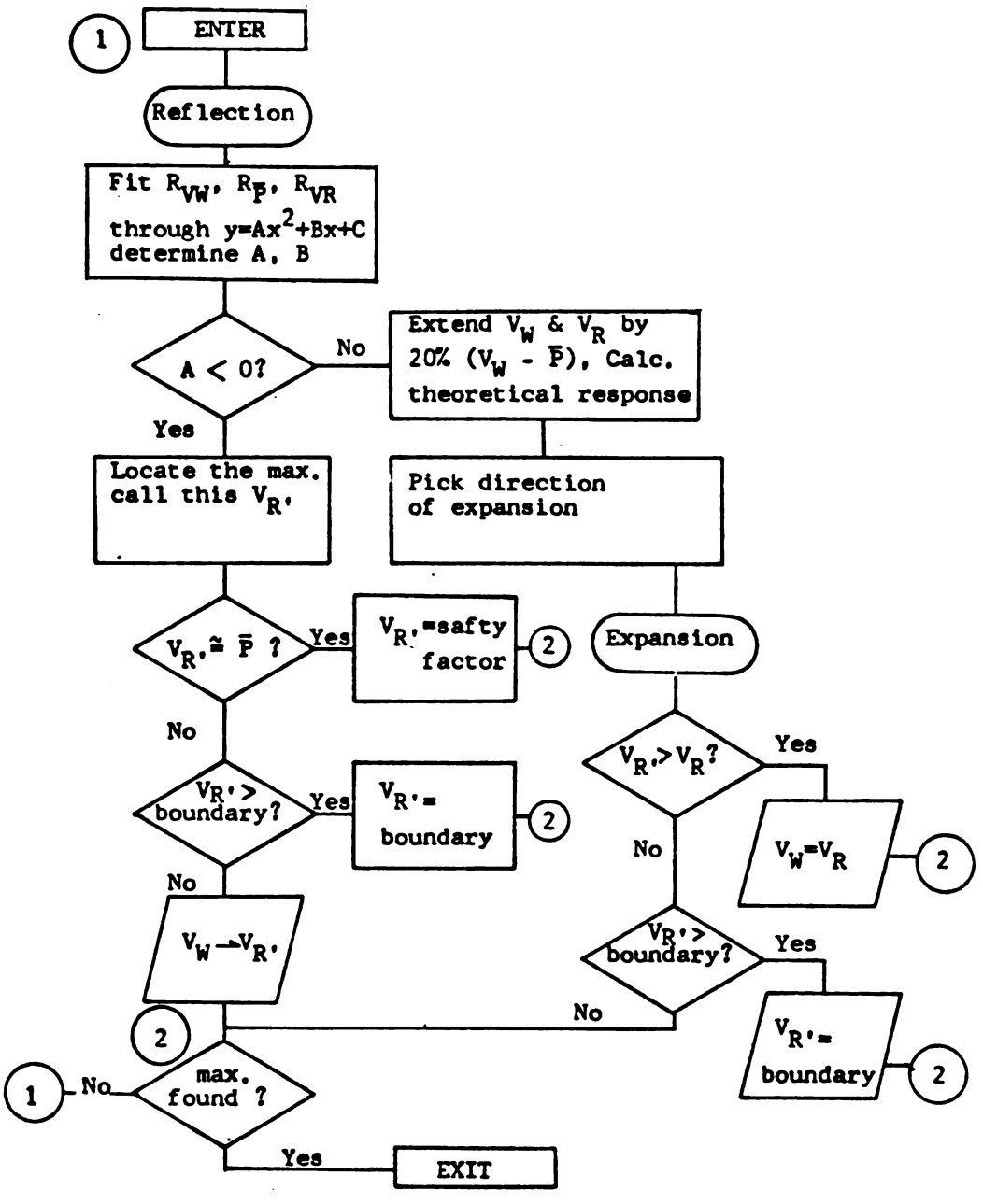


Figure A-3. Procedure for the super-modified simplex.

be located. The curve is then expanded a percentage of $(V_W-\bar{P})$ and the extended interval boundary producing the highest predicted response is chosen as the new vertex location. Further expansion can be made by testing the slopes at these extended boundaries, deciding upon the direction of expansion, and expanding according to the magnitude of the slope.

The SMS procedure thus allows the simplex to locate a new vertex more freely and move much faster towards the optimum by checking a larger response surface during each move by curve fitting. Although additional calculations are required, there is virtually no delay in experimental time since all calculations are performed by the mini-computer.

A subroutine SMSMPX.F4 was written following the algorithm described. It chains to other subroutines that input parameters in an interactive mode, print out results in several modes, and plot the simplex after each iteration as desired. A detailed description of the program, as well as the working program written in the FORTRAN IV language, is listed in Appendix B.

3. Design of the SMS Algorithm

This section describes the basic factors considered while designing the algorithm of the SMS subroutine.

i) Fitting a second degree polynomial curve - Instead of expanding or contracting with a fixed coefficient in each move, 3 points $(V_R, V_W, \bar{P})$ are fitted to the equation

$$y = Ax^2 + Bx + C$$

therefore we have three equations as follows:

$$y_1 = Ax_1^2 + Bx_1 + Cx_1^0$$

$$y_2 = Ax^2{}_2 + Bx_2 + Cx_2^0$$

$$y_3 = Ax_3^2 + Bx_3 + C_{x3}^0$$

Solving for the unknowns A, B, and C by matrix methods, we have

$$A = (y_1x_2+x_1y_3+y_2x_3-y_3x_2-x_3y_1-y_2x_1)/D$$

$$B = (x_1^2y_2+y_1x_3^2+x_2^2y_3-x_3^2y_2-y_3x_1^2-x_2^2y_1)/D$$

$$C = y_1-Ax_1^2-Bx_1$$

where

$$D = x_1^2x_2x_3^0+x_1x_2^0x_3^2+x_1^0x_2^2x_3-x_3^2x_2x_1^0-x_2^0x_3x_1^2-x_3^0x_2^2x_1$$

$$= x_1^2x_2+x_1x_3^2+x_2^2x_3-x_2x_3^2-x_3x_1^2-x_2^2x_1$$

We can simplify the equations by letting $x_1 = -1$, $x_2 = 0$, and $x_3 = 1$ as the x coordinates for the 3 responses. The resulting equations are

$$A = 1/2(y_1+y_3-2y_2) = 1/2(R+W-2C)$$

$$B = 1/2(y_1-y_3) = 1/2(R-W)$$

where R = response at V_R;

W = response at V_W;

C = response at $\bar{P}$.

Finding A and B in this manner, we can proceed to find the shape of the curve as described in the next section.

ii) Determination of minimum and maximum - The second derivative of the function $Y = Ax^2 + Bx + C$ was calculated to see if it is at a minimum or a maximum. If the second derivative is positive, the function curves upward and there is a minimum. If it is negative, the function curves downward indicating a maximum. And if the value is zero, it means that there is an inflection, which may be a maximum, or a minimum, or neither.

iii) Expansion coefficient - The next step is to decide how much further we are going to expand or contract the vertex V_R. Take the example of a maximization process.

If no maximum is found, the polynominal curve is then expanded further by a factor in order to investigate more portions of the response surface. The magnitude of this factor can be decided by looking at the slope of the new vertex at the extended boundary. Denton et al. (12) suggested that the greater the slope (indicating rapid approach to the optimum location), the smaller the required expansion coefficient; and conversely, the smaller the slope (indicating remoteness from the optimum location), the larger the required expansion coefficient. We found that there is a problem with this concept. It is difficult to define a "large slope" as such, because the value of a slope depends on the units one is using. An alternate way to attack this problem is to look at the ratio of the old and the new responses and decide upon the size of the expansion coefficient accordingly. In an optimization process for the maximum, if the ratio is large (indicating rapid approach to the optimum location), use a small expansion coefficient; but if the ratio is small or is close to 1, we want to expand more to explore new grounds. The following equation was found to work quite well,

$$x = \text{new response/old response}$$

$$y = \text{expansion coeff.} = -2.75x + 6.75$$

iv) Safety factor - Due to the adaptation of the curve fitting method, the new vertex may occur at or very near the centroid. This will reduce the dimensionality of the process and may terminate the progress of the simplex. Therefore a small safety interval is placed between the centroid and the new vertex at the beginning of the search. We picked 0.2 out of 1.0 absolute scale to be this safety factor. As illustrated in Figure A-4, when the new vertex is located within this range, we set it equal to 0.2. Use of the safety interval is unnecessary when approaching the termination of the optimization search and can easily be eliminated near the optimum.

v) Boundary violation - When V_R is located outside of the boundary constraint, we simply set the point at the boundary which is intersected by the vector connecting V_W and V_R as the effective V_R, we call this new vertex $V_{R'}$. The polynomial curve is then fitted through the responses of V_W, $\bar{P}$, and $V_{R'}$, and the search continues. This process is illustrated in Figure A-5. The method prevented the old problem of reluctance to approach the boundary constraint reported in the modified version of simplex (178).

vi) Initializing a new simplex - Usually the first question that confronts a user of the simplex method is

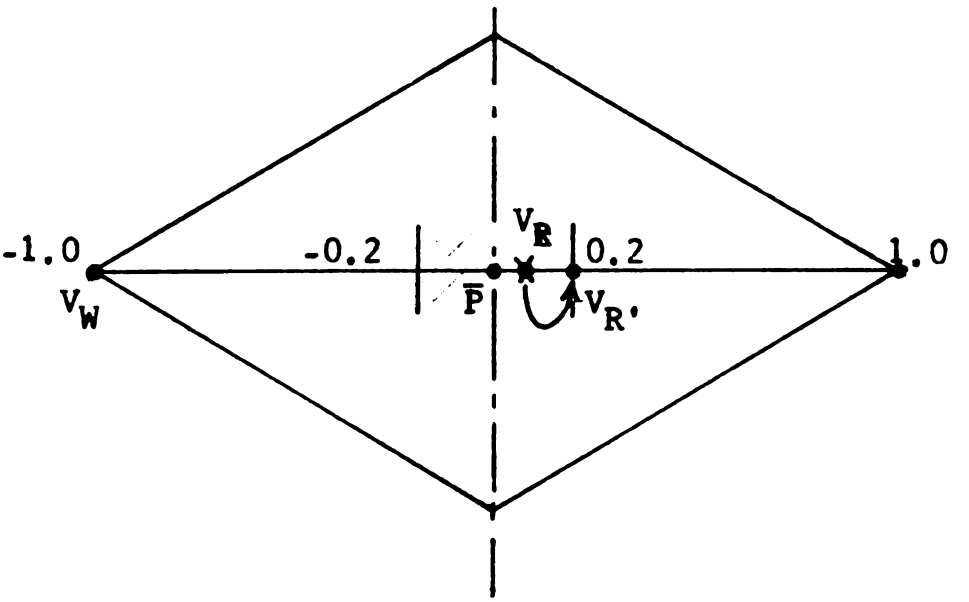


Figure A-4. Safety factor in SMS.

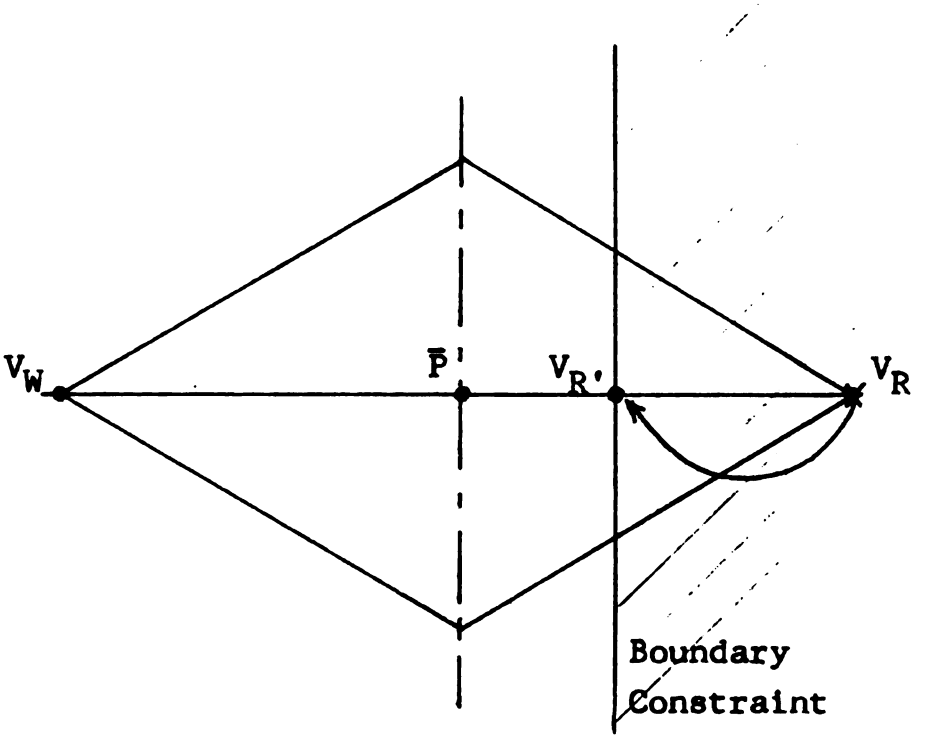


Figure A-5. Boundary violation in SMS.

how to design the initial simplex. Long (174) described in detail the construction of initial simplexes of up to ten factors. Instead of selecting the step size and the initial vertex randomly, a systematic algorithm is used. The following is an example of a two dimensional simplex.

1) Choose the initial vertex (V_i);

2) Choose step size (S_i);

3) Calculate p_i and q_i with N = number of factors;

$$p_i = (S_i/(N*2^{1/2}))((N+1)^{1/2}+N-1)$$

$$q_i = (S_i/(N*2^{1/2}))((N+1)^{1/2}-1)$$

4) Calculate the 3 vertices using p_i and q_i.

$$V_1 = (x_1, y_1)$$
$$V_2 = (x_1+p_1, y_1+q_2)$$
$$V_3 = (x_1+q_1, y_1+p_2)$$

A subroutine called VERTEX is written to help automatic calculation of the initial simplex. The user just has to type in the step size and the initial vertex to start the simplex process. This program can be found in Appendix B.

vii) Convergence and global optimum - There are many ways to check for convergence. Examples are methods based on objective function, zero slope, quadratic fit, variance of responses at simplex vertices, or predetermined number

of iteration steps (181). We found that using a small pre-assigned residual value to stop the optimization process provided satisfactory results. To assure that the optimum is global, two or more different regions of the factor surface are used as starting points. If the same optimum is obtained, the optimum is indeed the global one.

C. Simplex as a Curve Fitting Method

A generalized equation we used in our experiment is:

$$A \xrightarrow{k_A} P$$

$$B \xrightarrow{k_B} P$$

where k_A and k_B are pseudo-first-order rate constants. At any time t_i, the concentration of product in terms of the initial concentration $[A]_0$ and $[B]_0$ is

$$[P] = [A]_0(1 - e^{-k_A t_i}) + [B]_0(1 - e^{-k_B t_i})$$

In terms of observed absorbance,

$$Y_i = \varepsilon b\{[A]_0(1 - e^{-k_A t_i}) + [B]_0(1 - e^{-k_B t_i})\}$$

The objective is to determine $[A]_0$ and $[B]_0$ by obtaining the best fit of the observed Y_i values in response signal

and by using an optimum set of data points.

The data are n pairs of observations (Y_i and t_i, where i=1 to n) taken in a fixed period of time. The difference in the observed absorbance values and the calculated absorbance values are the residuals (μ_i). In random data, μ_i will be centered about the average ($\bar{\mu} = 0$) with a standard deviation σ_i. We want to minimize the sum of the residual square with the least square method.

If all σ_i are equal, we have the following equation:

$$\sum_{i=1}^{n} \mu_i^2 = \text{minimum}$$

If the σ_i are not equal, a weighting factor ω_i is used. One weighting method is shown in the following:

$$\sum_{i=1}^{n} \omega_i \mu_i^2 = \text{minimum}$$

where

$$\omega_i = 1/\sigma_i^2$$

The effect of ω_i is to de-emphasize imprecise data points. The detailed treatment concerning the principle and application of linear-least square regression analysis can be found in most statistics text books.

We have taken into account the fact that the simplex

method is computationally compact because there are few multiplicatcns, no divisions, and no matrix calculations at all. The super-modified simplex has also been shown to be efficient and reliable.

D. Tests with Simulated Data

We proceeded to generate simulated data with the computer and studied the factors that affect the speed, precision, and effectiveness of using the simplex method in regression analysis.

1. Simple Mathematical Models

Least square regression analyses on 3 equations shown in Table A-2 were performed. LLSQ is a general linear least square method available from most statistical text books. CRFIT is a non-linear curve fitting routine adapted from Bevington's book (177). It uses a combination of gradient search and linearization technique. Simplex performs equally well as the traditional methods; in the non-linear case in which there are 6 different unknown parameters to fit, the simplex routine found the optimum faster than the CRFIT method and in the case of poor initial estimates, it has a lower rate of failure to find the global maximum point. These initial studies with simulated data gave encouraging evidence that simplex is suitable for simple

Table A-2. Regression Analysis with the Simplex Method.

Equation #1

$$y(x) = a_1 x + b$$

Actual	LLSQ	Simplex
a_1=20.0	20.0	20.0

Equation #2

$$y(t) = a_1(1-e^{-k_A t}) + a_2(1-e^{-k_B t})$$

Actual	CRFIT	Simplex
a_1=0.100	0.099	0.100
a_2=0.200	0.200	0.199

Equation #3

$$y(x)=a_1 \exp -0.5\left(\frac{x-a_2}{a_3}\right)^2+a_4+a_5x+a_6x^2$$

Actual	CRFIT (good int. est)	Simplex (good int. est)	CRFIT (poor int. est)	Simplex (poor int. est)
a_1=208.2	207.99	210.70	40.81	162.6
a_2= 56.05	56.04	56.03	50.2	56.5
a_3= 3.56	3.84	3.56	1.59	3.55
a_4=186.6	185.8	186.2	227.5	245.4
a_5= -2.30	-2.27	-1.92	-4.24	-0.59
a_6= 0.0096	0.0093	0.0098	0.025	0.051

regression analyses. We performed a series of tests on Equation 2 in Table A-2 and used that as a guideline on selecting optimum performance conditions and present them in the following sections.

1) Effect of ratio of rates, rate constants, and concentrations on error - We ran a series of tests which contained ratios of rates from 0.006 to 250, rate constant ratios from 1.2 to 10, and concentration ratios from 0.005 to 25. A total of 88 runs were made. The initial estimates of concentrations A and B were up to 40 times different from the actual concentrations. Four different starting points were used in each run to ensure that the optimum obtained were global. Simulated noises, generated by the computer, was added to study its effect on error.

The data are tabulated and presented in Figure A-6. With the equations used, we found that the ratio of the rate constants k_A and k_B should be larger than 1.2. Any thing smaller produced a large error even with noise-free data. In our example, reagent A reacts faster than reagent B, and the error on determining the concentration of A is affected both by the rate constant k_A as well as the initial concentration of A. The product of the concentration and the rate constant gives rate A, and as can be seen in Figure A-6, a small rate A/rate B ratio gives large error.

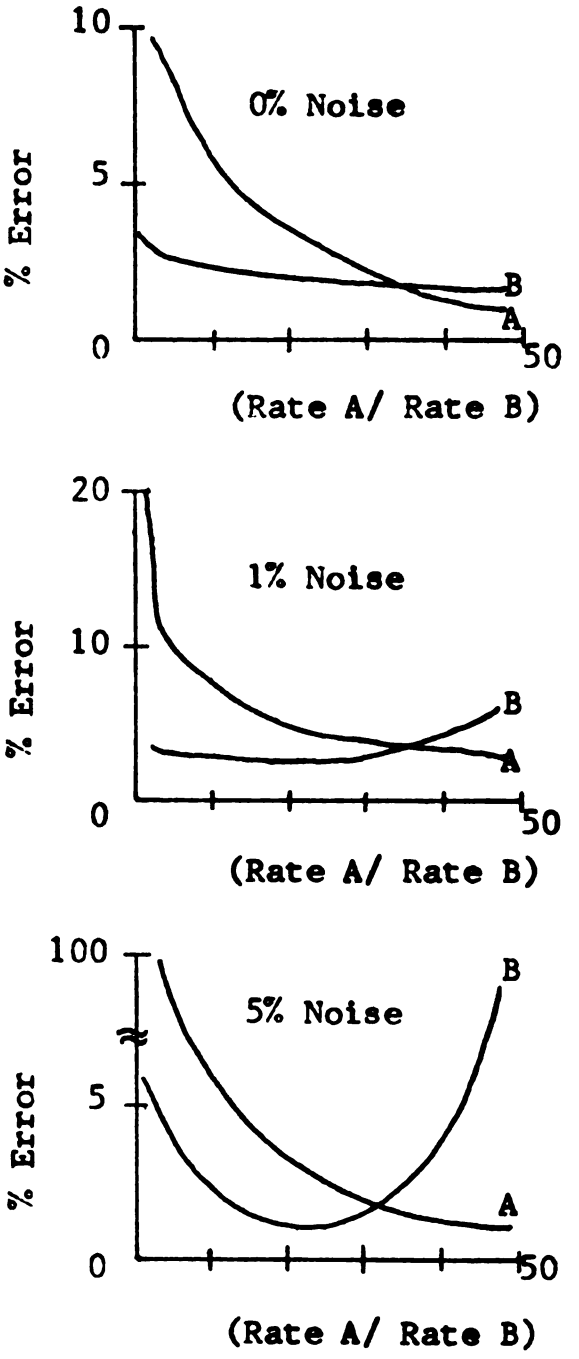


Figure A-6. Effects of rate differences and noise on error.

An excessive noisy response surface sometimes causes false convergence of a search at local optima. We found that the simplex method worked well up to a 5% noise level. The simplex method is less prone to the noise problem than the conventional curve-fitting methods due to 2 reasons: (1) if the differences in the responses are large compared to the size of noise, the simplex will move in the proper direction; and (2) if the difference in the responses are smaller than the noise, the simplex may move in the wrong direction. However, a wrong move will probably yield a lower response that would cause the simplex to proceed again towards the optimum.

ii) Effect of initial simplex step size on efficiency - There had been varied opinions on how to choose a suitable starting point for the simplex method. Long discussed the effect of step size on the efficiency of the simplex method (174). He pointed out that too large an initial step could cause the simplex to miss the optimum; but this would only apply to fixed-size samples. An initial large step size is usually an advantage, since the optimum is approached more rapidly.

We studied the same problem with the super-modified simplex and found that it is indeed advantageous to start with a large step size. Figure A-7 shows four randomly selected initial simplex vertices. The smallest step size

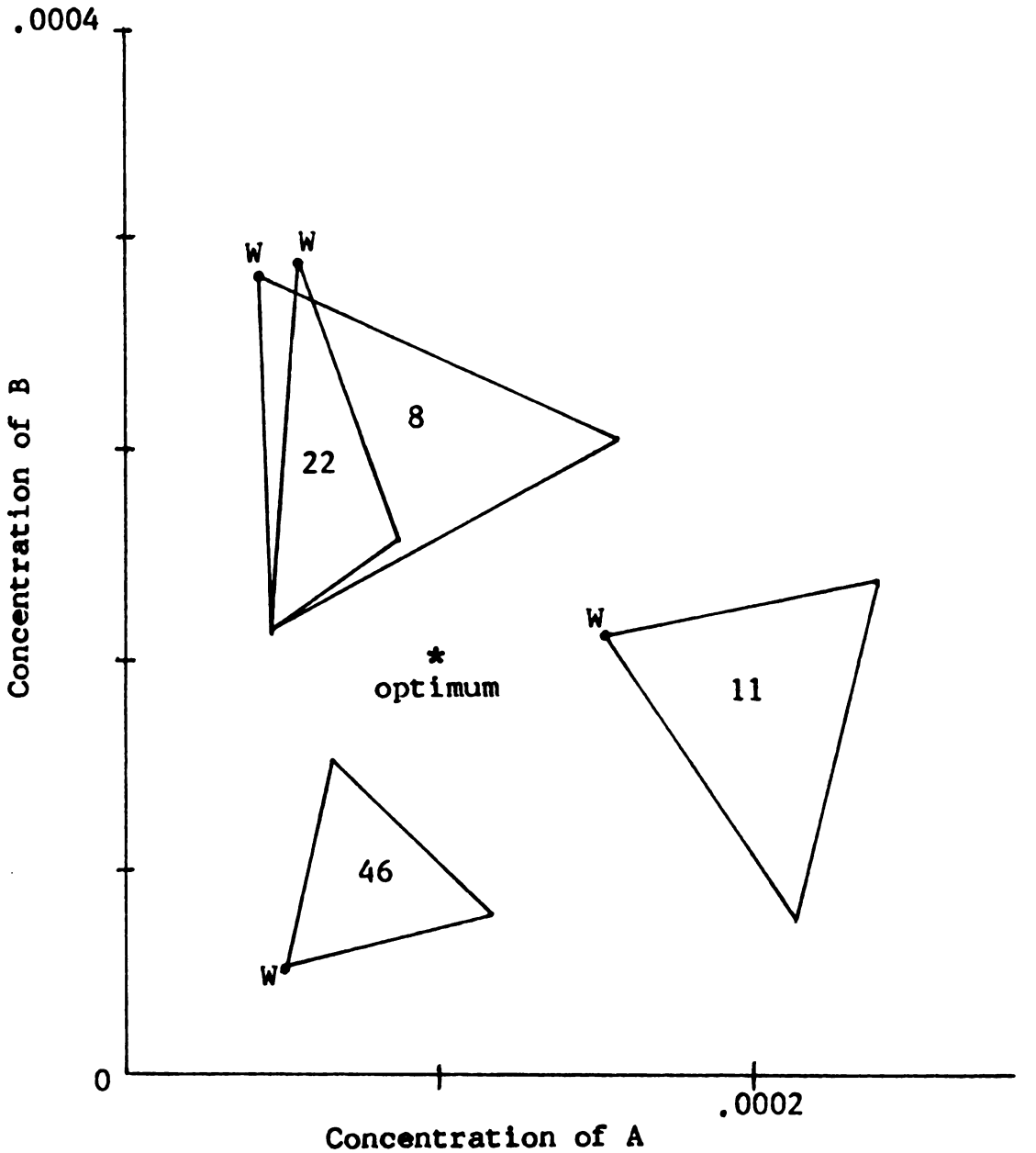


Figure A-7. Effect of initial simplex size on efficiency.

needed 46 iterations to attain the optimum, while the largest needed only 8. It is apparent therefore that a large initial simplex should be used for the SMS method.

iii) Weighting by variable data acquisition rates - Weighting of data has been done traditionally by using empirical weighting factors. Ridder and Margerum (188) attempted to account for the difference in weight of the data in terms of the magnitude of the signal and to offset the overemphasis of the slower reacting component by using variable data acquisition rates. They determined the appropriate sampling rate by the following equations:

no. of data point taken at x^{th} rate

$$n_x = \frac{N}{L} \sum_{i=x}^{L} \frac{I_x - I_{x-1}}{I_i}$$

and, rate of data acquisition for the x^{th} component

$$r_x = \frac{N}{I} \sum_{i=x}^{L} \frac{1}{I_i}$$

where N = total no. of data points;

L = total no. of reacting components;

x = no. of the component as listed in order from largest to smallest rate constant;

I = interval of time defined by 4 half-lives
of the designated components; and
= 4(0.693)/k.

According to their calculation, one should obtain two sets of equations that gives the rates of data acquisition and the number of data points taken for both the faster and the slower reacting components. These equations are used to calculate the optimum sampling rates with the input data shown in Table A-3.

The calculated optimum sampling rate is 51 points taken in 5.54 s initially. We investigated this calculation using simulated data with the simplex method, taking 50 data points for the fast reaction initially and 50 data points for the slower reaction. We varied the initial sampling periods and obtained the total errors in estimating the initial concentrations of substrates A and B. The results obtained are listed in Table A-3. The optimum data acquisition rate found by simplex is taking 50 points for 5.7 s for our reaction, which agrees quite well with the calculated values. These results confirmed the prediction made by the method of Ridder and Magerum and strongly suggested that all differential rate analysis of multicomponent mixtures should be performed in a variable data acquisition rate mode.

Table A-3. Weighing by Variable Data Acquisition Rates.

Initial Sampling Period[a]	Total Error
46.8 sec	0.46%
25.2	0.32
17.6	0.30
10.6	0.32
7.8	0.32
6.7	0.17
6.2	0.12
5.7	0.10[b]
4.6	0.13
4.4	0.13

[a]Total sampling period = 270 s; total number of data points = 100; number of data points taken in the initial period = 50.

[b]Input parameters are k_A=0.5, k_B=0.01, [A]=0.0001, [B]=0.0002, ε=2000; theoretical optimum sampling rate is taking 51 points in 5.54 s.

iv) Effect of length of sampling time on error - Should we take data for the entire duration of the slower reaction then? Ridder and Margerum suggested the time of sampling should be four times the half-life of the slower reaction. We checked this with the simplex method, and expected to observe dramatic increase in error when the total sampling time is reduced.

For the reaction we are studying, four half-lives of the faster reactant equaled 1.38 s while that of the slower reactant, four half-lives equaled 277.2 s. We proceeded to reduce the total sampling time to find its effect on error. The results are presented in Table A-4. The experiments were performed with simulated data with 1% random noise added. It is surprising to find that with a sampling time as short as 35 s (approximately one half-life of the slower reactant), the error obtained was not much higher than that of 280 s (four half-lives). As the sampling time is reduced further, the error increased dramatically. When the total sampling time is smaller than one half-life of A, the error becomes unacceptable. Our results indicate that for very slow reactions, the total sampling time may be reduced to about one half-life of the slower reactant in order to increase the sample throughput rate as well as to decrease interferences that may be caused by the products.

Table A4. Effect of Length of Sampling Time on Error.

Total Sampling Period	Total Error
280 sec	0.1%
210	0.1
140	0.15
70[a]	0.10
35	0.16
20	0.67
10	1.50
5	3.55
1	16.30

[a] τ_A=1.38 s

τ_B=69.3 s

$4\tau_B$=277.2s

E. Conclusions

We have shown that simplex is a useful tool for regression analysis. Although we did not make any actual applications with it, our studies with computer simulated data have given us several insights in the application of the super-modified simplex in numerical analysis.

We found that the super-modified version is superior to the other versions in terms of speed and efficiency in finding the true optimum. We have applied it in both linear and non-linear least-square fits of simulated data, and it worked better than at least one conventional method on a small computer. The simplex method is less prone to noise problems. We also found that one should start the SMS with a large initial simplex and weight the data by means of variable data acquisition rates. For very slow reactions, the total sampling time may be reduced to one half-life of the slower reacting species to increase the sample throughput rate as well as to decrease any interferences caused by the products.

Actual experiments should be performed in order to prove the validity of the simplex method in regression analysis and differential rate analysis.

APPENDIX B

PROGRAM PFIT06.F4

This is a curve-fitting program written in the fortran IV language.

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MSU, E. LANSING, MICH. 48824

PURPOSE--This program uses the super-modified simplex procedure to make a least-square fit to a linear or non-linear function.

USAGE --To load and run PFIT06 in fortran IV mode;

```
.R LOAD
*PFIT06<PFIT06.F4
*<FUNC06,PARMIN,LIST,INPUT,SMSMPX,SPLOT,VERTEX(ALT MODE)
.R FRTS
*PFIT06.LD
.R PFIT06
```

SUBROUTINES AND FUNCTIONS REQUIRED --

FUNC06--evaluates function given
PARMIN--inputs parameters for the main program
LIST --lists 1)initial, 2)curret, or 3)final values of parameters and variables
INPUT --input variables and initial simplex vertex value
SMSMPX--the super-modified simplex subroutine
VERTEX--(optional) inputs step sizes and initial vertex estimates, will calculate(N+1) sets of initial vertices by the method of Long.
SPLOT --(optional) plots simplex vertices on the graphics terminal after each iteration.

DESCRIPTION OF TERMS:-

IDIM=NO. OF FACTORS
V(I,J)=VALUE OF FACTOR J AT VERTEX I
ALIM(3,J)=LOWER, UPPER LIMITS, PRECISION IN EACH FACTOR J
R(I)=RESPONSE AT VERTEX I
IB=INDEX OF THE BEST POINT
IW=INDEX OF THE WORST POINT
INW=INDEX OF THE NEXT TO WORST POINT
DEL(I)=DISTANCE BETWEEN THE WORST POINT AND THE CENTROID
VTEM(1,I)=TEMPERARY NEW VERTEX FOR CENTROID
VTEM(2,I)=TEMPERARY NEW VERTEX FOR THE REFLECTED POINT
EVR=POINT VR EXTENDED 20% , RVR IS THE RESPONSE
EVW=POINT VW EXTENDED 20% , RVW IS THE RESPONSE
ALMIT=HIGHER LIMIT IN THE SIMPLIFIED COORDINATE
BLMIT=LOWER LIMIT IN THE SIMPLIFIED COORDINATE
BINT,TINT=LOWER AND UPPER SAFTY INTERVAL FOR THE CENTROID
REP=RESPONSE OF THE POINT EP

MODIFICATION OF THE PROGRAM FOR GENERAL USES--

The subroutine SMSMPX can be adapted to other optimization procedures with minor or no modification. The main program and other subroutines can be changed according to one's need in one of the following ways:

1) Input all parameters by using DATA statements in the beginning of the program PFITS6. Subroutines PARMIN and INPUT can be omitted. Change FUNC06 and LIST according to one's need.
2) Rewrite FUNC06,INPUT,PARMIN, AND LIST.

```
      SUBROUTINE SMPLX(IB)

C
C       FILENAME: SMPLEX.F4
C
C       DATE: JAN 7, 1977
C
C       VERSION: E - GENERAL PURPOSE SIMPLEX
C
C       DESCRIPTION OF PARAMETERS:
C
C        IDIM           DIMENSIONALITY OF THE SPACE (NO. OF FACTORS)
C
C        V(I,J)         VALUE OF FACTOR J AT VERTEX I
C
C        R(I)           RESPONSE AT VERTEX I
C
C        ALIM(3,J)      LOWER, UPPER LIMITS, PRECISION IN EACH FACTOR J
C
C        IRET(I)                NO. OF MOVES VERTEX I RETAINED
C
C        IMV            TYPE OF MOVE:
C                        1. BNR REFLECTION      5. BPTR TY 1 T.C.
C                        2. BNE EXPANSION       6. BPTW TY 2 T.C.
C                        3. BNCR CONTR TY 1     7. BN'CR N-REFLCT
C                        4. BNCW CONTR TY 2     8. BN'CW N-REFLCT
C
C        IWRST          INDEX OF WORST POINT
C
C        IALGO          SIMPLEX ALGORITHM TO BE USED:
C                        'SQ'   SEQUENTIAL (NO SIZE CHANGES)
C                        'NM'   NELDER AND MEAD (KEEP FAILED CONTRACTIONS)
C                        'TC'   TOTAL CONTRACTION ON FAILED CONTRACTION
C                        'NR'   N-REFLECTION ON FAILED CONTRACTION
C
C       THESE PARAMETERS MUST BE AVAILABLE TO THE SUBROUTINE.  AS WRITTEN,
C   ALL ARE STORED IN BLOCK COMMON; THEY MAY, HOWEVER, BE PASSED AS
C   ARGUMENTS IN THE CALL WITH APPROPRIATE CHANGES IN THE PROGRAM.
C
C       SUBROUTINE CALLED: FUNC (V,R,I)
C               WITH    V(I,J) AS ABOVE
C                       R(I)   AS ABOVE
C                       I      INDEX OF VERTEX BEING EVALUATED
C
C       SUBROUTINE FUNC CALCULATES THE RESPONSE R FOR THE VERTEX I.
C   V AND R MUST BE DIMENSIONED IN THE SUBROUTINE.
C



      COMMON /BLK1/V(10,9),VR(10,9)/BLK2/R(10)/BLK3/ALIM(3,9),IDIM
      COMMON /BLK4/IRET(10)/BLK5/IMV,IWRST,IALGO,DEV,IFMOD,ITER
      DIMENSION DEL(9), SUM(9), VTEM(10,9), RTEM(10)
C
C             DETERMINE BEST, WORST, NEXT-TO-WORST POINTS
C
      DO 20 I=1,IDIM+1
      DO 10 J=1,IDIM+1
      IF (I .EQ. J) GO TO 10
      IF (R(I) .LT. R(J)) GO TO 15
10    CONTINUE
      IB=I
```

```
15      DO 19 K=1,IDIM+1
        IF (I .EQ. K) GO TO 19
        IF (R(I) .GT. R(K)) GO TO 20
19      CONTINUE
        IWRST=I
        IW=I
20      CONTINUE
        DO 25 I=1,IDIM+1
        IF (I .EQ. IW) GO TO 25
        DO 24 J=1,IDIM+1
        IF (I .EQ. J) GO TO 24
        IF (J .EQ. IW) GO TO 24
        IF (R(I) .GT. R(J)) GO TO 25
24      CONTINUE
        INW=I
25      CONTINUE
C
C               DETERMINE MOVEMENT IN EACH VARIABLE TO CENTROID
C
        DO 26 I=1,IDIM
26      SUM(I)=0
        DO 30 I=1,IDIM+1
        IF (I .EQ. IW) GO TO 30
        DO 29 J=1,IDIM
29      SUM(J)=SUM(J)+V(I,J)
30      CONTINUE
        DO 35 I=1,IDIM
35      DEL(I)=SUM(I)/IDIM-V(IW,I)
C
C               DETERMINE REFLECTION R, RESPONSE
C
        DO 40 I=1,IDIM
40      VTEM(1,I)=V(IW,I)+2*DEL(I)
        CALL FUNC (1,VTEM, RTEM, 1)
C
        IF (IALGO .EQ. 2HSQ) GO TO 46
        IF (RTEM(1) .LT. R(IB)) GO TO 100



C
C               TRY EXPANSION HERE
C
      DO 45 I=1,IDIM
45    VTEM(2,I)=V(IW,I)+2.5*DEL(I)
      CALL FUNC (1,VTEM, RTEM, 2)
C
      IF (RTEM(2) .GE. R(IB)) GO TO 60
C
C               EXPANSION FAILED - RETURN BNR
C
46    DO 50 I=1,IDIM
50    V(IW,I)=VTEM(1,I)
      R(IW)=RTEM(1)
      IMV=1
      DO 51 I=1,IDIM+1
51    IRET(I)=IRET(I)+1
      IRET(IW)=1
      RETURN
C
C               RETURN EXPANSION
C
60    DO 65 I=1,IDIM
65    V(IW,I)=VTEM(2,I)
      R(IW)=RTEM(2)
      IMV=2
      DO 70 I=1,IDIM+1
70    IRET(I)=IRET(I)+1
      IRET(IW)=1
      RETURN
C
C               EXPANSION NOT ATTEMPTED
C
100   IF (RTEM(1) .GE. R(INW)) GO TO 125
      IF (RTEM(1) .GE. R(IW)) GO TO 150
```

```
C
C                TYPE 2 CONTRACTION
C
      DO 105 I=1,IDIM
105   VTEM(2,I)=V(IW,I)+DEL(I)/2
      CALL FUNC (1,VTEM, RTEM, 2)
      IF (RTEM(2) .GE. R(IW) .OR. IALGO .NE. 2HTC) GO TO 110


C
C                TOTAL CONTRACTION (TYPE 2)
C
      DO 788 I=1,IDIM
788   V(IW,I)=VTEM(2,I)
      DO 107 I=1,IDIM+1
      DO 106 J=1,IDIM
106   V(I,J)=(V(I,J)+V(IB,J))/2
107   CONTINUE
      IMV=6
      DO 108 I=1,IDIM+1
      IF (I .EQ. IB) GO TO 108
      CALL FUNC (1,V, R, I)
      IRET(I)=1
108   CONTINUE
      IRET(IB)=IRET(IB)+1
      RETURN
C
C                RETURN TYPE 2 CONTRACTION
C
110   DO 111 I=1,IDIM+1
111   IRET(I)=IRET(I)+1
      DO 115 I=1,IDIM
115   V(IW,I)=VTEM(2,I)
      R(IW)=RTEM(2)
      IRET(IW)=1
      IMV=4
      IF (RTEM(2) .GE. R(IW) .OR. IALGO .NE. 2HNR) RETURN
C
C                N-REFLECTION (TYPE 2)
C
      DO 205 I=1,IDIM
205   SUM(I)=0
      DO 210 I=1,IDIM+1
      IF (I .EQ. INW) GO TO 210
      DO 209 J=1,IDIM
209   SUM(J)=SUM(J)+V(I,J)
210   CONTINUE
      DO 215 I=1,IDIM
215   DEL(I)=SUM(I)/IDIM-V(INW,I)
      DO 220 I=1,IDIM
220   V(INW,I)=V(INW,I)+2*DEL(I)
      IMV=8
      IRET(INW)=1
      RETURN
C
C                REFLECTION
C
125   DO 127 I=1,IDIM
127   V(IW,I)=VTEM(1,I)
      R(IW)=RTEM(1)
      IMV=1
      DO 129 I=1,IDIM+1
129   IRET(I)=IRET(I)+1
      IRET(IW)=1
      RETURN
```

```
C
C                TRY TYPE 1 CONTRACTION
C
150     DO 155 I=1,IDIM
155     VTEM(2,I)=V(IW,I)+1.5*DEL(I)
        CALL FUNC (1,VTEM, RTEM, 2)
        IF (RTEM(2) .GE. RTEM(1) .OR. IALGO .NE. 2HTC) GO TO 175


C
C                TOTAL CONTRACTION (TYPE 1)
C
        DO 789 I=1,IDIM
789     V(IW,I)=VTEM(2,I)
        DO 157 I=1,IDIM+1
        DO 156 J=1,IDIM
156     V(I,J)=(V(I,J)+V(IB,J))/2
157     CONTINUE
        IMV=5
        DO 158 I=1,IDIM+1
        IF (I .EQ. IB) GO TO 158
        CALL FUNC (1,V, R, I)
        IRET(I)=1
158     CONTINUE
        IRET(IB)=IRET(IB)+1
        RETURN
C
C                RETURN TYPE 1 CONTRACTION
C
175     DO 176 I=1,IDIM+1
176     IRET(I)=IRET(I)+1
        DO 705 I=1,IDIM
705     V(IW,I)=VTEM(2,I)
        R(IW)=RTEM(2)
        IRET(IW)=1
        IMV=3
        IF (RTEM(2) .GE. RTEM(1) .OR. IALGO .NE. 2HNR) RETURN
C
C                N-REFLECTION (TYPE 1)
C
        DO 305 I=1,IDIM
305     SUM(I)=0
        DO 310 I=1,IDIM+1
        IF (I .EQ. INW) GO TO 310
        DO 309 J=1,IDIM
309     SUM(J)=SUM(J)+V(I,J)
310     CONTINUE
        DO 315 I=1,IDIM
315     DEL(I)=SUM(I)/IDIM-V(INW,I)
        DO 320 I=1,IDIM
320     V(INW,I)=V(INW,I)+2*DEL(I)
        IMV=7
        IRET(INW)=1
        RETURN
        END
```

```
C       PFIT06.F4
C
C       A PROGRAM TO SOLVE FOR THE PARAMETERS OF AN INTEGRATED
C       RATE EQUATION INVOLVING TWO CONCENTRATION TERMS
C       AND EXERCISE THE ALGORITHM FOR A SERIES OF PROBLEMS.
C       THIS PROGRAM IS DESIGNED TO FIND THE OPTIMUN SAMPLING RATE
C       WHEN KA AND KB ARE KNOWN.  THE USER CAN CHOOSE UP TO
C       TWENTY SAMPLING RATES, AND THE RATE THAT GIVES THE MINIMUM
C       % ERROR WILL BE FOUND.
C
C
C       DATE  9 MAY 1977
C
C       DESCRIPTION OF TERMS:
C
C       IALGO      SIMPLEX ALGORITHM TO BE USED
C                    'SQ'    SEQUENTIAL (NO SIZE CHANGE)
C                    'NM'    NELDER AND MEAD (KEEP FAILED CONTRACTION)
C                    'TC'    TOTAL CONTRACTION ON FAILED CONTRACTION
C                    'NR'    N-REFLECTION ON FAILED CONTRACTION
C       IDIM       NUMBER OF FACTORS
C       VINIT      INITIAL ESTIMATES OF VERTEXES
C       V(I,J)     ONE OF THE VERTEXE
C       ALIM       BOUNDARY PARAMETERS
C       SWITCH(6)  ITERATION NO. WILL BE SHOWN ON KB
C       SWITCH(7)  END THE PRESENT ITERATION
C       SWITCH(8)  RESTART
C
        COMMON /BLK1/V(10,9),VR(10,9)/BLK2/R(10)
        COMMON /BLK3/ALIM(3,9),IDIM,TOTIME,NUM,FRAC
        COMMON /BLK4/IRET(10)
        COMMON /BLK5/IMV,IWRST,IALGO,DEV,IFMOD,ITER
        COMMON /BLK6/IPT,IDB,IDIMO,VINIT(10,9),EA,AK,BK
        COMMON /BLK7/ACONC,BCONC,IP1,IP2
        DIMENSION ITIM(20),ESUM(100)
        DATA QUIT/1HQ/, NQUIT/1HG/, NO/1HN/, YES/1HY/
C
C  INITIALIZE
C
        CALL RANG(-1,0.,0.,1,0.)
        WRITE(0,220)
220     FORMAT(' ENTER ANY INTEGER SMALLER THAN 500')
        READ(4,230)INT
230     FORMAT(I3)
        DO 240 I=1,INT
240     CALL RANG(0,0.,0.,5,0.)
        IOP=1
1       IFMOD=1
        IDIM=2
        ERSUM=0.
        WRITE(0,80)
80      FORMAT(' CALL SUBROUTINE INPUT TO CHANGE NUM,ACONC,ETC.?'/)
        READ(4,110)JANS
110     FORMAT(A1)
        IF(JANS.NE.YES)GO TO 1080
        CALL INPUT
1080    WRITE(0,1190)
1190    FORMAT(' WANT TO LIST DETAIL OF EACH ITERATION?'/)
        READ(4,110)LI
1200    WRITE(0,3500)
3500    FORMAT(' WANT TO SPECIFY THE VALUE OF A FRACTION?'/)
        READ(0,110)SPEC
        IF(SPEC.EQ.NO)GO TO 3800
```

```
C
C       SPECIFYING SPECIAL FRACTION
C
        WRITE(0,4500)
4500    FORMAT(' FRACTION=?'/)
        READ(0,102)FRAC
102     FORMAT(E13.6)
103     FORMAT(I3)
        IVAR=1
        ESUM(IVAR)=0.
        CALL PARIN
        GO TO 5001
3800    WRITE(0,4000)
4000    FORMAT(' WHAT FRACTION TO START? DEFAULT=1'/)
        READ(0,103)N
        WRITE(0,5000)
5000    FORMAT(' HOW MANY INTERVALS BETWEEN FRACTIONS? DEFAULT=1'/)
        READ(0,103)INT
        IF(INT.NE.1)GO TO 5002
        M=NUM+N
        GO TO 120
5002    M=NUM*INT+N-INT
C
C
C
120     CALL PARIN
        DO 350 IVAR=N,M,INT
        ESUM(IVAR)=0.
        FRAC=1./IVAR
5001    CALL FUNC(0,0.,0.,0)
        IF (SWITCH(8.) .EQ. 1) GO TO 380
        IF(IVAR.NE.1)GO TO 703
        IF(IDB.EQ.YES)CALL LIST(1)
705     DO 329 I=1,3
        DO 328 J=1,2
        V(I,J)=VINIT(I,J)
        CALL FUNC(1,V,R,I)
328     CONTINUE
329     CONTINUE


        ITER=0
C
C ITERATE
C
        CALL PLOT(V,IDIM,ALIM,0)
100     ITER=ITER+1
        IF (SWITCH(6.) .EQ. 1) WRITE (0,735) ITER
735     FORMAT (' ITERATION NO. '13)
        IF (SWITCH(7.) .EQ. 1) GO TO 198
        IF (ITER .GT. 250)GO TO 198
        CALL PLOT(V,IDIM,ALIM,1)
        CALL SMPLX
C
C CHECK FOR CONVERGENCE
C
        DO 40 I=1,IDIM
        VMIN=V(1,I)
        VMAX=VMIN
        DO 39 J=2,IDIM+1
        IF(V(J,I).GT.VMAX)VMAX=V(J,I)
        IF(V(J,I).LT.VMIN)VMIN=V(J,I)
39      CONTINUE
        VDEL=ABS(VMAX-VMIN)
        RESID=.0001
        IF(VDEL.GT.ALIM(3,I).OR.R(I).GT.RESID)GO TO 49
40      CONTINUE
        GO TO 50
49      IF(LI.EQ.YES)CALL LIST(2)
        GO TO 100
```

```
50      WRITE(3,2000) IVAR,DEV
2000    FORMAT(' RUN NUMBER= 'I3/' NOISE= 'E13.2' % '/)
198     AVGA=(V(1,1)+V(2,1)+V(3,1))/3.
        RELACA=(ACONC-AVGA)*100./ACONC
        AVGB=(V(1,2)+V(2,2)+V(3,2))/3.
        RELACB=(BCONC-AVGB)*100./BCONC
        ESUM(IVAR)=ESUM(IVAR)+ABS(RELACA)+ABS(RELACB)
        WRITE(3,1000) FRAC,RELACA,RELACB,ESUM(IVAR)
1000    FORMAT(' FRAC= 'E13.6/' % ERROR OF A,B='2E13.6/' TOT ERR='E13.6/)
        IF(IDB.EQ.YES)CALL LIST(3)
        NM=N+1
        IF(IVAR.EQ.NM)ERSUM=ESUM(NM)
        IF(ESUM(IVAR).LT.ERSUM)GO TO 340
        GO TO 350
340     ERSUM=ESUM(IVAR)
        IOP=IVAR
350     CONTINUE
        WRITE(3,750) IOP
750     FORMAT(' OPTIMUM SAMPLING RATE IS RUN NUMBER 'I3/)
380     WRITE (0,730)
730     FORMAT (' RESTART ?'$)
        READ (0,731) IANS
731     FORMAT (A1)
        IF (IANS .EQ. 1HY) GO TO 1
        END
```

```
      SUBROUTINE VERTEX
C   THIS PROGRAM WILL ASK FOR 1)STEP SIZES, 2) INITIAL VERTEX
C   ESTIMATION, AND WILL CALCULATE (N+1) SETS OF INITIAL
C   VERTICES BY THE METHOD OF LONG FOR THE SIMPLEX OPERATION
C   UP TO TEN PARAMETERS CAN BE SPECIFIED.
C
C
      COMMON /BLK3/ ALIM(3,9),IDIM,TOTIME, NUM, FRAC
      COMMON /BLK6/ IPT,IDB,IDIMO, VINIT(10,9),EA,AK,BK
      DIMENSION SSIZE(10),P(10),Q(10)
C
C   SPECIFY STEP SIZES
C
      DO 100 I=1,IDIM
      WRITE(0,20) I
20    FORMAT(' STEP SIZE FOR PARAMETER'I2'='$)
      READ(4,40)SSIZE(I)
40    FORMAT(E13.6)
100   CONTINUE
C
C   INPUT INITIAL VERTEX
C
      WRITE(0,200)
200   FORMAT(' ENTER INITIAL VERTEX')
      DO 280 J=1,IDIM
      I=1
      WRITE(0,220) I,J
220   FORMAT(' COOR('I1','I1')='$)
      READ(4,40)VINIT(I,J)
280   CONTINUE
C
C   CALCULATING INITIAL SIMPLEX COORDINATES
C
      DO 300 I=1,IDIM
      P(I)=SSIZE(I)/IDIM/1.1416*(SQRT(IDIM+1)+IDIM-1)
      Q(I)=SSIZE(I)/IDIM/1.1416*(SQRT(IDIM+1)-1)
300   CONTINUE
      DO 500 J=1,IDIM
      DO 400 I=2,IDIM+1
      IF(I.EQ.J+1)GO TO 400
      VINIT(I,J)=VINIT(1,J)+Q(J)
400   CONTINUE
500   CONTINUE
      DO 600 I=2,IDIM+1
      J=I-1
      VINIT(I,J)=VINIT(1,J)+P(J)
600   CONTINUE
      RETURN
      END
```

```
      SUBROUTINE FUNC (MODE,V,R,I)
      DIMENSION V(10,9), R(10), A(100), TIME(100)
      COMMON /BLK3/ALIM(3,9),IDIM,TOTIME,NUM,FRAC
      COMMON /BLK5/IMV,IWRST,IALGO,DEV,IFMOD,ITER
      COMMON /BLK6/IPT,ID3,IDIMO,VINIT(10,9),EA,AK,BK
      COMMON /BLK7/ACONC,BCONC,IP1,IP2
      IF (MODE .EQ. 0) GO TO 200
      SUM=0.
      DO 5 J=1,IDIM
      IF (V(I,J) .GT. ALIM(2,J) .OR. V(I,J) .LT. ALIM(1,J)) GO TO 100
5     CONTINUE
      DO 10 K=1,100
      ACALC=EA*(V(I,1)*(1.-EXP(-AK*TIME(K)))+
     2  V(I,2)*(1.-EXP(-BK*TIME(K))))
10    SUM=SUM+(ACALC-A(K))**2
      R(I)=-SUM
      RETURN
C
C
100   R(I)=-10.E20
      RETURN
C
C  INITIALIZE - CALCULATE THE ABSORBANCE VS TIME DATA
C
200   T=0.
      TIMEIN=TOTIME*FRAC
      T1=TIMEIN/IP1
      T2=(TOTIME-TIMEIN)/IP2
      DO 210 IVAR=1,100
      IF(T.GT.TIMEIN)T=T+T2
      IF(T.LE.TIMEIN)T=T+T1
270   TIME(IVAR)=T
      SUM2=0.
      DO 300 JPTS=1,10
      APT=EA*(ACONC*(1.-EXP(-AK*TIME(IVAR)))+
     2  BCONC*(1.-EXP(-BK*TIME(IVAR))))
C
C  ADD ON NOISE
C
      IF (IFMOD .EQ. 1) SDEV=ABS(APT*DEV/100.)
      IF (IFMOD .EQ. 2) SDEV=SQRT(APT)*DEV/100.
      IF (IFMOD .EQ. 3) SDEV=DEV
      AVG=0.
      NUM=5
      CALL RANG (0, SDEV, AVG, NUM, RANDG)
      APT=APT+RANDG
300   SUM2=SUM2+APT
210   A(IVAR)=SUM2/10.
      RETURN
      END
```

```
      SUBROUTINE PARIN
C
C  INPUT PARAMETERS FOR MAIN (PARFIT.F4)
C
      COMMON /BLK3/ALIM(3,9),IDIM/BLK5/IMV,IWRST,IALGO,DEV,IFMOD,ITER
      COMMON /BLK6/IPT,IDB,IDIMO,VINIT(10,9),EA,AK,BK
      DATA NO/1HN/, YES/1HY/
1     WRITE (0,900)
900   FORMAT (10X'SIMPLEX CURVEFIT ROUTINE')
      IDIM=2
2     WRITE (0,800) EA,AK,BK
800   FORMAT (' MOLAR ABS = 'G11.4/' RATE CONST A = 'G11.4/
     2  ' RATE CONST B = 'G11.4)
      WRITE (0,805)
805   FORMAT (' CHANGES ?'$)
      READ (4,940) IANS
940   FORMAT (A1)
      IF (IANS .EQ. NO) GO TO 5
      WRITE (0,810)
810   FORMAT (' MOLAR ABS = '$)
      READ (4,970) EA
970   FORMAT (G11.4)
      WRITE (0,830)
830   FORMAT (' RATE CONSTANT A = '$)
      READ (4,970) AK
      WRITE (0,840)
840   FORMAT (' RATE CONSTANT B = '$)
      READ (4,970) BK
      GO TO 2
5     WRITE (0,910)
910   FORMAT (' ALGORITHM (SQ,NM,TC,NR) ?'$)
      READ (4,920) IALGO
920   FORMAT (A2)
      WRITE (0,1200)
1200  FORMAT (' LPT LIST EACH ITERATION ?'$)
      READ (4,940) IDB
39    DO 40 I=1,IDIM
      WRITE (0,700) I,ALIM(1,I)
700   FORMAT (' LOWER LIMIT ('I1') = 'G11.4)
      WRITE (0,710) I,ALIM(2,I)
710   FORMAT (' UPPER LIMIT ('I1') = 'G11.4)
40    WRITE (0,720) I,ALIM(3,I)
720   FORMAT (' PRECISION  ('I1') = 'G11.4)
      WRITE (0,805)
      READ (4,940) IANS
      IF (IANS .EQ. NO) GO TO 70
      DO 60 I=1,IDIM
      WRITE (0,960) I
960   FORMAT (' LOWER LIMIT ('I1') = '$)
      READ (4,970) ALIM(1,I)
      WRITE (0,980) I
980   FORMAT (' UPPER LIMIT ('I1') = '$)
      READ (4,970) ALIM(2,I)
      WRITE (0,990) I
990   FORMAT (' PRECISION ('I1') = '$)
60    READ (4,970) ALIM(3,I)
C
C  INITIAL SIMPLEX
C
70    IF (IDIMO .NE. IDIM) GO TO 850
      WRITE (0,1000)
1000  FORMAT (' USE SAME INITIAL SIMPLEX ?'$)
      READ (4,940) IANS
      IF (IANS .NE. NO) GO TO 100
850   WRITE(0,2000)
2000  FORMAT(' USE SUBROUTINE VERTEX TO CALC. INITIAL SIMPLEX?')
      READ(4,940)IANS
      IF(IANS.EQ.NO)GO TO 80
      CALL VERTEX
```

```
      GO TO 100
80    WRITE (0,1010)
1010  FORMAT (' ENTER COORDINATES OF INITIAL SIMPLEX')
      DO 95 I=1,IDIM+1
      WRITE (0,1020)
1020  FORMAT (1H0)
      DO 90 J=1,IDIM
      WRITE (0,1030) I,J
1030  FORMAT (' COOR ('I1','I1') = '$)
90    READ (4,970) VINIT(I,J)
95    CONTINUE
100   RETURN
      END
```

```
      SUBROUTINE PLOT (V,IDIM,ALIM,MODE)
      COMMON/BLK7/ACONC,BCONC,IP1,IP2
      DIMENSION V(10,9),ALIM(3,9)

      IF (MODE .NE. 0) GO TO 100
      CALL TPLINT (ALIM(1,1), ALIM(2,1), ALIM(1,2), ALIM(2,2), 2, 1)
      CALL TPLT(-1,ACONC,BCONC)
      RETURN

100   CALL TPLT (0, V(3,1), V(3,2))
      DO 110 I=1,3
110   CALL TPLT (1, V(I,1), V(I,2))
      RETURN
      END
```

```
      SUBROUTINE LIST(MODE)
C
C
C     MODE 1 INITIALIZE
C          2 CURRENT VALUES
C          3 CLOSE - FINAL VALUES
C
      COMMON /BLK1/V(10,9),VR(10,9)/BLK2/R(10)/BLK3/ALIM(3,9),IDIM
      COMMON /BLK5/IMV,IWRST,IALGO,DEV,IFMOD,ITER
      COMMON /BLK6/IPT,IDB,IDIMO,VINIT(10,9),EA,AK,BK
      GO TO (10,100,200),MODE
C
C  INITIALIZE
C
10    WRITE(3,900)IALGO
900   FORMAT(' SIMPLEX OPTIMIZATION RESULTS',5X,A2//)
      WRITE(3,910)EA,AK,BK
910   FORMAT(' INPUT CONSTANTS'//' MOLAR ABS = 'G11.4/
     2  ' RATE CONSTANT A = 'G11.4/
     3  ' RATE CONSTANT B = 'G11.4)
      WRITE(3,920)
920   FORMAT(1HO'LIMITS, DESIRED PRECISION'//' UPPER LIM'2X9)
      DO 12 I=1,IDIM
12    WRITE(3,930)ALIM(2,I)
930   FORMAT(1H+G11.4,2X9)
      WRITE(3,940)
940   FORMAT(1H+)
      WRITE(3,950)
950   FORMAT(' LOWER LIM'2X9)
      DO 14 I=1,IDIM
14    WRITE(3,930)ALIM(1,I)
      WRITE(3,940)
      WRITE(3,960)
960   FORMAT(' PRECISION'2X9)
      DO 16 I=1,IDIM
16    WRITE(3,930)ALIM(3,I)
      WRITE(3,940)
      WRITE(3,970)
970   FORMAT(1H0'INPUT INITIAL SIMPLEX  PAR(1)...PAR(K)'/)
      DO 20 I=1,IDIM+1
      WRITE(3,990)I
      DO 18 J=1,IDIM
18    WRITE(3,930)VINIT(I,J)
20    WRITE(3,940)
      WRITE(3,975)
975   FORMAT(1H 'V'8X'PAR(1)...PAR(K)...RESID**2'/)
      RETURN
C
C  CURRENT VALUES
C
100   WRITE(3,980)ITER
980   FORMAT(1H0'ITERATION 'I3)
      DO 110 I=1,IDIM+1
      WRITE(3,990)I
990   FORMAT(1H I3,2X9)
      DO 105 J=1,IDIM
105   WRITE(3,930)V(I,J)
      RI=-R(I)
      WRITE(3,1000)RI
1000  FORMAT(1H+G11.49)
110   WRITE(3,940)
      RETURN
C
C  FINAL VALUES
C
200   WRITE(3,1010)ITER
1010  FORMAT(1H0'*********'/' CONVERGENCE OBTAINED
     2  IN 'I3' ITERATIONS'/'*********'/
     3  ' FINAL RESULTS'/)
```

```
      DO 210 I=1,IDIM+1
      WRITE(3,990) I
      DO 203 J=1,IDIM
203   WRITE(3,930) V(I,J)
      RJ=-R(I)
      WRITE(3,1000) RJ
210   WRITE(3,940)
      WRITE(3,1020)
1020  FORMAT(1H1)
      RETURN
      END
```

```
      SUBROUTINE INPUT
C     THIS SUBROUTINE WILL INPUT CONTROL PARAMETERS FOR PFIT06.
C
C     DATE: 5/24/1977
      COMMON/BLK5/IMV,IWRST,IALGO,DEV,IFMOD,ITER
      COMMON/BLK3/ALIM(3,9),IDIM,TOTIME,NUM,FRAC
      COMMON/BLK7/ACONC,BCONC,IP1,IP2
      DATA NO/1HN/,YES/1HY/
      WRITE(0,91)
91    FORMAT(' TOTAL LENGTH OF SAMPLING TIME IN SEC=?')
      READ(4,102)TOTIME
102   FORMAT(E13.6)
      WRITE(0,105)
105   FORMAT(' NUMBER OF SAMPLING RATES TO BE TRIED=?')
      READ(4,103)NUM
103   FORMAT(I3)
      WRITE(0,107)
107   FORMAT(' ACTUAL CONC OF A=?')
      READ(4,102)ACONC
      WRITE(0,108)
108   FORMAT(' ACTUAL CONC OF B =?')
      READ(4,102)BCONC
      WRITE(0,200)
200   FORMAT(' CHANGE NO. OF DATA POINTS IN INITIAL SAMPLING
     2 PERIOD? DEFAULT=50'/)
      READ(4,110)JANS
110   FORMAT(A1)
      IF(JANS.EQ.NO)GO TO 300
      WRITE(0,210)
210   FORMAT(' HOW MANY DATA POINTS IN INITIAL SAMPLING PERIOD?'/)
      READ(4,103)IP1
      GO TO 3000
300   IP1=50
3000  IP2=100-IP1
      WRITE(0,310)
310   FORMAT(' ADD NOISE INTO DATA?'/)
      READ(4,110)KANS
      IF(KANS.EQ.NO)GO TO 400
      WRITE(0,340)
340   FORMAT(' SPECIFY % NOISE'/)
      READ(4,102)DEV
      GO TO 500
400   DEV=0.
500   CONTINUE
      RETURN
      END
```

```
C       SUBROUTINE SMSMPX

        SUBROUTINE SMPLX
C   THIS IS THE SUPER MODIFIED VERSION FOR SIMPLEX, FOR
C   DEFINITION OF TERMS AND DESCRIPTION OF THIS PROGRAM, CALL
C   PROGRAM SMSMPX.IN.
        COMMON/BLK1/V(10,9),VR(10,9)/BLK2/R(10)/BLK3/ALIM(3,9),IDIM
        COMMON/BLK4/IRET(10)
        DIMENSION DEL(9),SUM(9),VTEM(10,9),RTEM(10)
        DATA BINT/-.2/,TINT/.2/
C   DETERMINE BEST, WORST, NEXT-TO-WORST POINTS
        II=0
        JJ=0
        DO 20 I=1,IDIM+1
        DO 10 J=1,IDIM
        IF(I.EQ.J)GO TO 10
        IF(R(I).LT.R(J))GO TO 15
10      CONTINUE
        IB=1
15      DO 19 K=1,IDIM+1
        IF(I.EQ.K)GO TO 19
        IF(R(I).GT.R(K))GO TO 20
19      CONTINUE
        IW=I
20      CONTINUE
        DO 25 I=1,IDIM+1
        IF (I.EQ.IW)GO TO 25
        DO 24 J=1,IDIM+1
        IF(I.EQ.J)GO TO 24
        IF(J.EQ.IW)GO TO 24
        IF(R(I).GT.R(J))GO TO 25
24      CONTINUE
        INW=I
25      CONTINUE
C   DETERMINE MOVEMENT FROM THE WORST POINT TO CENTROID FOR
C   EACH VARIABLE.  (NW+B)/IDIM=COORDINATE OF THE CENTROID.
        DO 26 I=1,IDIM
        SUM(I)=0
26      CONTINUE
        DO 30 I=1,IDIM+1
        IF(I.EQ.IW)GO TO 30
        DO 29 J=1,IDIM
29      SUM(J)=SUM(J)+V(I,J)
30      CONTINUE
        DO 35 I=1,IDIM
        DEL(I)=SUM(I)/IDIM-V(IW,I)
35      VTEM(1,I)=V(IW,I)+DEL(I)
        CALL FUNC(1,VTEM,RTEM,1)
C   DETERMINE THE REFLECTION OF VW, CALL IT VTEM(2,I) AND FIND
C   ITS RESPONSE.
        DO 40 I=1,IDIM
40      VTEM(2,I)=V(IW,I)+2*DEL(I)
        CALL FUNC(1,VTEM,RTEM,2)
C   FIT A SECOND ORDER POLYNOMIAL VURVE THROUGH VW,C, AND VR.
C   USING ONE OF THE FACTORS AND THE RESPONSE AS THE COORDINATES.
C   DETERMINE A AND B FOR THE CURVE Y=A*X**2+B*X+C BY USING THE
C   MATRIX METHOD.  SETTING X(1)=-1, X(2)=0,AND X(3)=1, THE
C   COEFF. CAN BE SIMPLIFIED AS A=0.5*(RR+W-2*C) AND B=0.5*(RR-W).
        RR=RTEM(2)
        W=R(IW)
        C=RTEM(1)
        A=0.5*(RR+W-2*C)
        B=0.5*(RR-W)
        P=-B/(2*A)
```

```
C  P IS THE DERIVATIVE OF THE EQUATION Y=A*X**2+B*X+C AND IT
C  SHOWS THE LOCATION OF THE MAXIMUM.  GET THE MAX. BETWEEN
C  ALIM(1,1) AND ALIM(2,1) BUT NOT AT CENTROID.  FIRST OF
C  ALL CHANGE ALIM(I,1) INTO THE SIMPLIFIED COORDINATES.
        CF=1.0/ABS(VTEM(2,1)-VTEM(1,1))
        ALMIT=(ALIM(2,1)-VTEM(1,1))*CF
        BLMIT=(ALIM(1,1)-VTEM(1,1))*CF
C  IF A<0, THERE IS A MAXIMUM AND THE CURVE IS CONCAVE DOWN.
        IF(A.GE.0.)GO TO 300
        IF(P.GT.ALMIT)GO TO 210
        IF(P.LT.BLMIT)GO TO 220
        IF((P.GT.BINT).AND.(P.LT.0.))P=TINT
        IF((P.GE.0.).AND.(P.LT.TINT))P=TINT
        GO TO 500
210     P=ALMIT
        GO TO 500
220     P=BLMIT
        GO TO 500
C  CURVE IS CONCAVE UP.  EXTEND VW AND VR BY 20%*(VW-P) AND
C  GET THE THEORECTICAL RESPONSE.
300     EVR=1.20
        EVW=-1.20
        RVR=A*EVR**2+B*EVR+C
        RVW=A*EVW**2+B*EVW+C
C  DETERMINE THE NEW/OLD RESPONSE RATIO, PICK THE HIGHER
C  RATIO AS THE DIRECTION OF FUTHER EXPANSION.  EXPAND THE CURVE
C  BY Y=-2.75*X+6.75, WHERE X=RATIO OF RESPONSES AND Y=EXPANSION COEFF.
C  EP IS THE LOCATION OF THE REW THEORETICAL EXPANDED VALUE.
        RATIO1=RVR/RR
        RATIO2=RVW/W
        IF(RATIO1.GT.RATIO2)GO TO 310
        IF(RATIO2.GT.RATIO1)GO TO 320
310     Y=-2.75*RATIO1+6.75
        EP=Y*(1.0)
        GO TO 350
320     Y=-2.75*RATIO2+6.75
        EP=Y*(-1.0)
350     REP=A*EP**2+B*EP+C
C   CHECK IF THE NEW RESPONSE REP IS IN BOUNCE, THEN CHECK
C  IF IT IS BETTER THAN THE OLD ONE.
        IF(EP.GT.ALMIT)GO TO 210
        IF(EP.LT.BLMIT)GO TO 220
        IF((REP.GT.R).AND.(REP.GT.W))GO TO 400
        P=1.0
        GO TO 500
400     P=EP
C  CHANGE P BACK TO THE TRUE COORDINATE AND SAMPLE ITS
C  RESPONSE.  SET P NOW AS THE NEW VERTEX THAT REPLACES
C  THE WORST POINT VW.
500     SLOPE=DEL(2)/DEL(1)
        V(IW,1)=P*(1./CF)+VTEM(1,1)
510     V(IW,2)=SLOPE*(V(IW,1)-VTEM(1,1))+VTEM(1,2)
550     CALL FUNC(1,V,R,IW)
        IF(R(IW).LE.RR)GO TO 520
        GO TO 560
520     V(IW,1)=VTEM(2,1)
        V(IW,2)=VTEM(2,2)
        R(IW)=RR
560     CONTINUE
        RETURN
        END
```

APPENDIX C

ELECTROPHORESIS OF SERUM LIPOPROTEINS ON CELLULOSE ACETATE STRIPS

Equipment and Supplies:

1) Shandon "Celagram" cellulose acetate strips, 25 x 180 mm (Shandon Scientific Co., 515 Broad St., Sewickley, PA 15143).
2) Shandon electrophoresis apparatus, Model U77; power supply, Model SAI 2761.
3) Whatman No. 3MM filter paper wicks.
4) Glasswares.
5) Sample applicators.

Reagents:

1) Barbital buffer, pH 8.8, 0.1M
   1.756 g barbital acid + 20.6 g sodium barbital + 0.37 g EDTA disodium salt in one liter of water.
2) Oil Red O stain
   0.4 g Oil Red O dye (Sigma Co., St. Louis, MO) + 380 mL acetone, add 300 mL water slowly, stir at 45°C for 1 hour till 1 hour before use.
3) 1.0 N NaOH solution.

Procedures:

1) Fill the buffer reservoirs of the electrophoresis cells with 0.1M barbital buffer.
2) Load Whatman 3 MM paper wicks (1 inch wide) onto support racks after soaking them in the barbital buffer.
3) Carefully float the strip of cellulose acetate with dull side up on top of a dishful of barbital buffer. After it is thoroughly wetted, submerge it into the buffer for several minutes.
4) Remove the strip and blot dry with filter paper. Apply the sample on the dull side, then position the dull side up on the wicks across a 10 cm bridge gap inside the electrophoresis cells.
5) Set a constant current of 5 mA and allow the electrophoresis to run for 45 minutes.
6) Mix 35 mL Oil Red O stock solution with 10 mL 1.0 N NaOH solution, stain the cellulose acetate strip in this bath for one hour. Wash with water and apply a few drops of glycerol onto the surface for storage.
7) Obtain a densitometric tracing of the slide when an instrument is available.

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