Enzymatic Kinetic Rate and End-Point Analyses of Substrate, by Use of a GeMSAEC Fast Analyzer

T. O. Tiffany, J. M. Jansen, C. A. Burtis, J. B. Overton, and C. D. Scott

Enzymatic substrate analysis is an attractive means of analysis in clinical chemistry because of its sensitivity and specificity. The GeMSAEC Fast Analyzer, in conjunction with a small computer, provides a means of performing routine enzymatic substrate analysis and offers the following advantages: (a) selectivity of approaches to enzymatic analysis, i.e., end-point or kinetic; (b) essentially parallel analyses of multiple samples, yielding a unique method for performing kinetic fixed-time analysis; (c) on-line data reduction, resulting in rapid calculation and output of results and the minimization of data handling errors; and (d) a small reagent volume per test (400 μl), which reduces the cost of analysis. The analysis of substrate with enzymatic end-point and kinetic procedures is examined by use of a computer-interfaced Fast Analyzer. Computer programs were written to facilitate this study. Glucose (hexokinase/GPD), urea (urease/GMD), and uric acid (uricase) have been used as examples in evaluating both end-point and kinetic analyses. The advantages and limitations of each type of analysis are presented, with the emphasis being placed on enzymatic substrate analysis and means by which the computer-interfaced Fast Analyzer can facilitate both end-point and kinetic analyses.

Additional Keyphrases hexokinase • urease • uricase • equilibrium analysis • Focal • glucose • urea • uric acid • rate or fixed-time analyses

The use of enzyme assays for substrate analysis of single components in complex biological matrices, such as serum and urine, is of interest to clinical chemists because of the selectivity and sensitivity of these assays. However, the relatively high cost per analysis and low sample throughput have made such analyses, in general, prohibitive for routine work. The GeMSAEC Fast Analyzer, developed by Anderson (1) to perform rapid, multiple chemical analyses on small volumes of sample and reagent, has provided a means of incorporating enzymatic substrate analysis into the routine clinical chemistry work load.

Enzymatic end-point substrate analysis and enzymatic rate measurement analysis have been reviewed by Guilbault (2). Theoretical and experimental considerations of analysis by kinetic rate measurements have been discussed by Pardue (3) and by Ingle and Crouch (4), who have provided much practical experimental information concerning the accuracy of kinetic rate analysis. Their discussions show that several features of the Fast Analyzer make this instrument suitable as a kinetic substrate analyzer for routine applications. These include: (a) a relatively stable multieuvet-photometer or spectrophotometer with program- mable digital averaging, providing instrumental noise levels of less than 1% from 0.10 to 2.0 A at 340 nm; (b) centrifugal addition of discrete samples and reagent, providing rapid, simultaneous initiation of the reactions, which proceed essentially in parallel with respect to time; and (c) real-time, on-line data reduction in conjunction with a computer, which eliminates the need for an off-line calculator, thereby decreasing analysis time and eliminating an error-prone step.

There are two general approaches to enzymatic substrate analysis: (a) an end-point or equilibrium analysis, and (b) rate measurement analysis. An investigation was undertaken to demonstrate the

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1 Nonstandard abbreviations used: GeMSAEC is an acronym derived from two of the major sources of support, the National Institute of General Medical Sciences and the U.S. Atomic Energy Commission; K_{m}, Michaelis-Menten constant; PM, photomultiplier; GMD, glutamate dehydrogenase (1-glutamate:NAD(P)) oxidoreductase deaminating, EC 1.4.1.3; G6P, glucose-6-phosphate dehydrogenase (6-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49); and Tris, tris(hydroxymethyl)aminomethane.
potential that the Fast Analyzer offers, as an analytical system, in using either approach. A secondary objective was to examine the feasibility of each analytical approach for use in routine analysis, with regard to accuracy, precision, time of analysis, sample capacity per transfer disk, and general ease of analysis. To accomplish these objectives, two general programs were written in focal, one for enzymatic end-point substrate analysis and the other for kinetic rate analysis of substrate, and these are discussed in the text.

The program for enzymatic end-point analysis of substrate eliminates the requirement for sample blanks by calculating absorbance at zero time (see "Results and Discussion"); calibration reference standards are unnecessary. It has been used in the enzymatic analyses of urea nitrogen, glucose, and uric acid, as described herein.

The computer program for kinetic rate analysis allows the use of initial rate, rate at a given time, or fixed-time analysis. Fixed-time analysis has been considered as useful only with reactions obeying first-order or pseudo-first-order kinetics, because linearity of instrumental signal as a function of concentration is imposed for simple, rapid, and direct calculation of substrate concentration. A computer-interfaced analyzer, with nonlinear data-reduction capability, does not have such restrictions placed on the analysis. The kinetic program has been evaluated for use in the analysis of urea nitrogen both by rate and by linear fixed time, and in the analysis of glucose by nonlinear fixed time. Advantages of rate-measurement analysis are the elimination of sample blanks and a shorter analysis time as compared with that required for enzymatic end-point substrate analysis. However, calibration standards, which decrease the number of samples per run, are required.

A discussion of the two types of analysis and their respective programs is presented.

Materials and Methods

Reagents. Glucose and urea nitrogen were determined by using prepackaged hexokinase/glut 
(5) and urease/glut (6) "Stat-Packs" (Calbiochem, San Diego, Calif. 92112). Reagents were prepared 1.5 times more concentrated than the manufacturer's recommendation, to compensate for a 200-µl sample washout used with our automated diluter.

Uric acid was determined by using both "Determinate-U" prepackaged porcine liver uricase (7) 
(Worthington Biochemical Corp., Freehold, N.J. 07728) and bacterial uricase (8) (Novo Enzyme 
Corp., Mamaroneck, N.Y. 10543). The latter reagent was prepared by adding 200 µl of uricase (10 units/ml) to 20.0 ml of borate buffer (0.2 mol/liter, pH 9.5). This was sufficient for 30 analyses on the Fast Analyzer.

A stock uric acid solution (100 mg/dl) was prepared by dissolving 100 mg of No. 913 standard reference uric acid (9) (National Bureau of Standards, Washington, D.C. 20234) in 50 ml of distilled water containing 60 mg of Li2CO3 at 60°C. The solution was allowed to cool and then diluted to 100 ml with distilled water. Reference solutions of uric acid were prepared at the following concentrations: 3 mg/dl, 6 mg/dl, 9 mg/dl, 12 mg/dl, and 18 mg/dl.

A stock urea solution (100 mg/dl as urea nitrogen), was prepared by dissolving 2.166 g of urea (NBS reference material No. 912) in 1 liter of double-distilled water. One milliliter of chloroform was added as a preservative.

A stock glucose solution (10 mg/ml) was prepared by dissolving 1 g of reagent-grade glucose in 100 ml of filtered, saturated benzoic acid.

Procedures. A modified "Nilab" sample and reagent loader (10) was used for the automatic loading of all reagents and samples into the transfer disk. The system functions by pipetting 56 ±0.2 µl of sample from a sample ring containing 15 places for samples and standards, and dispensing the sample with 198 ±0.3 µl of distilled water as washout into outer cavities 1 through 15 of the transfer disk. A total of 300 ±0.2 µl of reagent is dispensed into each of 15 inner cavities of the transfer disk.

Serum dilutions. Serum was diluted 20-fold with saline solution (9 g/liter) before analysis, and the resulting solution was used for both glucose and urea nitrogen analyses. The final dilution in the reaction mixture was approximately 200-fold.

Serum was diluted fivefold with saline (9 g/liter) before uric acid analysis. This provided a 50-fold dilution in the final reaction mixture.

Substrate analysis. Prepackaged reagents were used, where possible, to simplify the procedure and to permit adaptability of our methods to other systems. Table 1 lists the reaction conditions and general computer parameters for the particular substrate analysis being performed. Calibration standards are not required for the end-point analysis. Calibration reference standards are required for kinetic methods, and four are generally used for the linear rate and linear fixed-time analyses. More standards can be utilized (the number is variable); five are usually used for the nonlinear fixed-time analysis.

We have found bacterial uricase (8) to have excellent properties as an analytical reagent for uric acid analysis. These properties include low absorbance contribution, rapid turnover rate, larger Ks value (1 × 10−4 mol/liter as opposed to 1 × 10−5 mol/liter for porcine liver), stability of the enzyme under refrigeration, and ease of reagent preparation.
**Table 1. Procedures and Computer Parameters Used in the Enzymatic Analysis of Substrate**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction time, min</th>
<th>Substrate factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5</td>
<td>567.9</td>
<td>(6)</td>
</tr>
<tr>
<td>Urea nitrogen</td>
<td>15</td>
<td>44.3</td>
<td>(6)</td>
</tr>
<tr>
<td>Uric acid</td>
<td>20</td>
<td>68.2</td>
<td>(7)</td>
</tr>
<tr>
<td>(porcine liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>10</td>
<td>68.2</td>
<td>(8)</td>
</tr>
<tr>
<td>(bacterial)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A. End-point method**

**B. Kinetic methods**

1. **Rate**  
   - Urea nitrogen  
     - Delay time, s: 30  
     - Rate interval, s: 60  
     - Analysis time, s²: 120  

2. **Fixed-time**  
   - Uric acid  
     - T<sub>f</sub>: 4  
     - Fixed time interval, s: 300  
     - Analysis time, s²: 330  
   - Urea nitrogen  
     - 30  
   - Glucose  
     - 4  
   - 60  
   - 90  

- Calibration reference standards are not required.  
- Calibration reference standards are required.  
- T<sub>f</sub> is the time of the first computer reading.  
- Time from the initiation of the reaction to the teletype print-out of data.

**Instrumentation**

**G-IIC-system.** The Fast Analyzer used in this work is a 15-cuvet system, and has been previously described (11-13). Several modifications such as the addition of a high-intensity gradient monochromator (Bausch & Lomb 33-86-07; 200-700 nm) and a choice of three lamp sources (tungsten-quartz iodide, xenon, or deuterium) have been made. The tungsten lamp is routinely used as the light source for operation between 340 nm and 620 nm. A change from tungsten lamp source to deuterium lamp source can be quickly made, thereby allowing conversion from a multicuvet visible spectrophotometer to a multicuvet ultraviolet spectrophotometer. Quartz windows in the rotor allow use of the system in the ultraviolet or visible spectral regions.

**Ultraviolet optical system.** The use of the Fast Analyzer for enzymatic substrate analysis at wavelengths below 340 nm has not been previously described. We determined the feasibility of this system for the analysis of uric acid at 292 nm by measuring the absorbances of a series of uric acid reference solutions, with the various lamp sources being used in conjunction with the high-intensity monochromator. A Cary 14 recording spectrophotometer was used as the reference spectrophotometer. The results (Figure 1) indicate that the deuterium lamp source in combination with the monochromator provided the best agreement (less stray light) with the Cary 14 system. The molar absorptivity of uric acid, as calculated from the absorbance data obtained on the Fast Analyzer equipped with the deuterium lamp source, was $1.21 \times 10^4$ cm<sup>2</sup>/mol (0.071 absorbance unit per microgram of uric acid per milliliter).

The linear instrumental response was demonstrated by measuring the absorbances of NADH solutions in Tris buffer (0.1 mol/liter, pH 7.4) at 340 nm (Figure 2). The light source used was a tungsten-quartz iodide lamp. A molar absorptivity of $6.20 \times 10^3$ cm<sup>2</sup>/mol was calculated from these data.

**Instrumental noise.** An experimental parameter that needs to be mentioned is the level of "instrumental noise" of the analyzer relative to the absorbance being measured. The term "instrumental noise" is used here to refer to electro-
optical and computer conversion uncertainties (14). The precision in measuring absorbance decreases owing to low light intensities at 340 nm (PM voltage = 0.7 kV) and at 292 nm (PM voltage = 0.8 kV). Because small absorbance changes are involved in the enzymatic analysis of substrate (i.e., 0.050 to 0.100 absorbance unit in the normal range), it was necessary to establish the uncertainties in measuring absorbances at all levels that could be encountered in the analyses.

Figures 3 and 4 show the standard deviation for absorbance (1 SD vs. absorbance level at 340 nm and 292 nm, respectively. Also shown in these Figures is the effect of digital averaging on the standard deviation of absorbance vs. increasing absorbance. The data were obtained by taking 10 readings for each absorbance level, in triplicate, for increasing concentrations of NADH and uric acid solutions. Digital averaging was obtained by taking 1, 4, 9, and 16 readings per cuvet. The use of digital averaging should decrease the instrumental noise by \(1/\sqrt{N}\), where \(N\) is the number of readings per cuvet per sample interval. The data in Figures 3 and 4 demonstrate that such an improvement can be obtained by using the Fast Analyzer; as a consequence, all readings for both the end-point and the kinetic rate measurement programs are made by using 10 readings per cuvet. In this manner, instrumental noise contributions were found to be 0.1 to 0.2% over a range of 0.1 to 2.0 absorbance units at 340 nm, and 0.3 to 0.5% over a range of 0.1 to 1.3 absorbance units at 292 nm. Aqueous solutions were used to make these measurements; therefore, light-scattering contributions from serum protein samples are not included in the above data. However, these data do provide a reasonable criterion for determining the effect of instrumental noise on the precision of the results when very dilute solutions are used, as required in the substrate analysis.

Computer data system

HARDWARE: The G-IIC Fast Analyzer is interfaced with a PDP 8/1 computer (Digital Equipment Corp., Maynard, Mass. 01754). This system has been described elsewhere (12, 13).

SOFTWARE: The computer programs are written in FOCAL (programming language developed by Digital Equipment Corp.). The programming concepts for the design of software for the computer-interfaced Fast Analyzer have been discussed by Kelley and Jansen (12). Basically the various programs are similar in that each of them (a) obtains preliminary information concerning the analysis such as date, procedure, technician, delay time before initial data, reaction time, number of readings, concentration of standards (if employed), etc.; (b) uses the preliminary information to set up the computer clock and data-taking functions; (c) awaits relay contact closure at the initiation of the reaction and then obtains data; and (d) calculates concentration or enzyme activity and presents the data, in a useful format, on a teletype unit or display scope. The programs for enzymatic substrate analysis are, in essence, subroutines of a basically flexible and versatile GEMSAEC programming format, and have been specifically designed for enzymatic substrate analysis in clinical chemistry.
Enzymatic substrate analysis programs. The enzymatic substrate programs have a unique element; that is, the use of a clock-reset subroutine that allows a variable rate of data acquisition. Use of this function enables several absorbance readings to be obtained in the early stages of a reaction; then, with the clock reset, considerable time can pass before the final data points are taken. This option serves to conserve care data storage by obtaining the maximum amount of useful information during the initial reaction, ignoring the intermediate data, and obtaining a final accurate absorbance reading. Figure 5 shows the reaction progress curve of a glucose determination with hexokinase, illustrating how the initial data could be used, by extrapolation to \( t = 0 \), to obtain an \( A_0 \). The clock-reset routine can also be used to obtain a series of reaction rates at various time intervals throughout a reaction.

The enzymatic end-point program establishes the necessary analysis information and asks for the substrate factor \([\text{mg dl}^{-1} \text{(absorbance unit)}]^{-1}\). If unknown, the program calculates it from parameters supplied by the technician. The initial absorbance, \( A_0 \), is computed by extrapolation (see "Results and Discussion"); then, after a time delay based on the reaction time supplied to the computer by the technician, the final absorbance is obtained. The substrate concentration is subsequently calculated and printed out, in units of mg/dl, by cuvet number. Although no calibration standards are required, controls in the normal and abnormal range are included in every run to monitor the performance of the system.

The kinetic rate program establishes the necessary analysis information, including the concentrations of four reference standards used for calibration. Either the initial rate (i.e., the velocity of the reaction as time approaches zero) or a rate at a given time \( (t) \) can be obtained by using an appropriate delay-time interval before the computer takes data. A calibration factor is calculated by dividing the rate by the concentration of the corresponding standard and obtaining a mean value of the calibration factor. The rate of the unknown times the mean value of the calibration factor is the concentration of the unknown, and this value is printed out by cuvet, in appropriate concentration units, along with the rate.

The fixed-time program also requires standard reference compounds for calibration purposes; however, the number of standards can be varied. The first several cuvets (2 to 6) are used for calibration with reference standard solutions. For linear fixed-time analysis the absorbance change for each standard over the fixed-time interval is divided by the corresponding concentration of that standard, and the mean value of \( \Delta A / \text{concentration} \) is used as a calibration factor. The substrate concentration is obtained by multiplying this factor by the absorbance change over the fixed time of each unknown time interval. Concentrations in units of mg/dl are printed out in an acceptable format. For nonlinear fixed time, a simple numerical analysis method is used to bracket the absorbance change of the unknown within the absorbance change of two standards such that the absorbance change of the unknown is greater than the absorbance change of one of the standards but less than that of the next higher concentrated standard. A linear interpolation is made between these two standards to find the concentration of the unknown. This concentration is then printed out in units of mg/dl. The calibration standards are chosen to fit a range corresponding to approximately 95% of all samples (for glucose, 0 to 400 mg/dl) to be encountered; absorbance changes for any unknowns greater than the absorbance change of the highest standard are labeled "out of range."

Results and Discussion

Enzymatic End-Point Analysis

The most common type of enzymatic substrate analysis is the end-point or equilibrium method, in which the reaction is allowed to go to completion. For spectrophotometric analysis, the initial absorbance \( (A_0) \) and the final absorbance \( (A_f) \) of a coenzyme, substrate, or product are obtained, and the initial concentration of the unknown substrate is related directly to the absorbance change \( (A_f - A_0) \) either by using a standard

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4 Computer programs described here available from T. O. T. on request.
A problem associated with conventional enzymatic end-point determinations is the requirement to analyze a separate sample without reagent to determine the initial absorbance \(A_0\). A significant feature of the computer-interfaced Fast Analyzer is that the initial absorbance readings after initiation of the reaction can be obtained in the first few seconds of the reaction and extrapolated back to zero time in order to obtain \(A_0\). This eliminates measuring a separate transfer disk containing serum without reagent to obtain \(A_0\). The enzymatic end-point analysis program written for the GEMS AEC Fast Analyzer uses a quadratic least-squares-curve-fitting routine to obtain \(A_0\). The absorbance at any given time \(t\) is assumed to fit a second-degree polynomial equation such that

\[
\text{Absorbance} \ (t) = a_0 + a_1t + a_2t^2
\]

where \(a_0\), \(a_1\), and \(a_2\) are the coefficients of the quadratic equation. Therefore, at time \(t = 0\), the initial absorbance should equal \(a_0\).

The success in using the coefficient \(a_0\) to obtain an accurate initial absorbance depends upon two factors: (a) obtaining \(A_1\), the first absorbance reading, as rapidly as possible after mixing, and (b) obtaining the successive readings \(A_1, A_2, A_3, \ldots\) etc., in equal time increments such that \(\Delta t\) is equal to the delay interval between the start of the reaction and the first reading. The Fast Analyzer used in this study obtains \(A_1\) at 3.2 s after acceleration of the rotor has been initiated. Eleven readings are then taken at 2-s intervals, with digital averaging of 10 readings per cuvet during each of the 11 time increments to increase the precision of the absorbance readings.

The ability to obtain a measurement at 3.2 s after the reaction is started does not eliminate the necessity of extrapolating back to zero time to obtain an accurate \(A_0\) value. Comparison of three end-point enzymatic substrate analyses—glucose (hexokinase), uric acid (uricase; procine liver and bacterial), and urea (urease)—showed that the glucose hexokinase reaction is sufficiently rapid to produce a significant difference between the calculated \(a_0\) value and \(A_1\), the initial absorbance reading obtained at 3.2 s. For a normal glucose sample (83 mg/dl), the difference between the absorbances of the sample at \(t = 0\) and \(t = 2\ s\) is \(5 \times 10^{-3}\) absorbance unit. This corresponds to a glucose concentration of 2.9 mg/dl, or a 3.5% error in concentration based on the use of the normal glucose sample (83 mg/dl).

The accuracy with which \(a_0\) reflects \(A_0\) was determined by comparing the extrapolated \(A_0\) with experimentally determined \(A_0\) values. The results are shown in Table 2. For the worst cases, samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc, mg/dl</th>
<th>(A_s)</th>
<th>(A_s) (extrapolated)</th>
<th>(A_s) (blank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>74.7</td>
<td>0.149</td>
<td>0.017</td>
<td>0.015</td>
</tr>
<tr>
<td>B</td>
<td>91.4</td>
<td>0.184</td>
<td>0.023</td>
<td>0.021</td>
</tr>
<tr>
<td>C</td>
<td>118.6</td>
<td>0.236</td>
<td>0.027</td>
<td>0.027</td>
</tr>
<tr>
<td>D</td>
<td>178.9</td>
<td>0.353</td>
<td>0.039</td>
<td>0.040</td>
</tr>
<tr>
<td>E</td>
<td>93.7</td>
<td>0.184</td>
<td>0.019</td>
<td>0.020</td>
</tr>
<tr>
<td>F</td>
<td>187.2</td>
<td>0.371</td>
<td>0.040</td>
<td>0.036</td>
</tr>
<tr>
<td>G</td>
<td>353.5</td>
<td>0.699</td>
<td>0.080</td>
<td>0.073</td>
</tr>
</tbody>
</table>

A, B, C, D = \(1/10, 1/50, 1/100, 1/1000\) dilutions of a serum control. E, F, G = \(1/10, 1/50, 1/1000\) dilution of a serum control. \(A_s\) is final absorbance. \(A_s\) is initial absorbance.

\(A\) and \(G\), the errors in glucose concentration resulting from the errors in \(A_0\) would be 1.5% and 1.0%, respectively. An estimation of the precision in obtaining \(a_0\) was made by analyzing 13 aliquots of a normal glucose control serum. The standard deviation was \(\pm 1.6 \times 10^{-3}\) absorbance unit, with a mean blank value of \(19.6 \times 10^{-3}\) absorbance unit.

### Calculation of Substrate Concentration

After values have been obtained for \(A_0\) and \(A_s\), the substrate concentration is determined by multiplying \(\Delta A\) by a substrate factor that is calculated by solving Beer's law for absorbance in terms of mg/dl, as shown in the following expression:

\[
F (\text{mg dl}^{-1} A^{-1}) = \left(\text{mw} \frac{V t}{V s} \right) (100) \frac{a}{(V a)} \frac{1}{(b)}
\]

where \(\text{mw} = \text{molecular weight of the substrate, } V t = \text{total reaction volume, } V s = \text{sample volume (actual volume, not diluted volume), } a = \text{molar absorptivity of the component being monitored, and } b = \text{path length, cm. The proper use of the substrate factor demands that the spectrophotometric system be properly calibrated and that volumetric devices used for measuring sample and reagent volumes also be calibrated.}

### Evaluation of the Enzymatic End-Point Program

Uric acid (uricase), glucose (hexokinase), and urea (urease) analyses were used to evaluate the performance of the Enzymatic End-Point Program. In this evaluation, the analytical methods themselves were examined with regard to their utility for routine chemical analysis. A discussion of the results is presented following the section on Kinetic Analysis.

Reference solutions of urea and glucose were prepared as previously described and these ref-
ference solutions were analyzed by using the enzymatic end-point program. The data obtained (Table 3) indicate that use of the Fast Analyzer coupled with a computer analysis of the data yields results with a relative error of 1 to 2%.

Several replicate samples of normal and supranormal control sera were analyzed to establish the in-run precision of the analyses. These results are given in Table 4. The accuracy and precision of the results shown in Tables 3 and 4 are reasonable. We are currently using the glucose procedure routinely in the ORNL Health Division and find the program simple to use in routine operations. The elimination of calibration standards allows a greater number of samples to be run per disk. (Three controls are routinely included per transfer disk.) The run-to-run variation is of the order of 2 to 4% in the normal range.

The analysis of uric acid with bacterial uricase and the end-point program produces in-run precision of 2 to 3% in the supranormal range and 3 to 4% in the normal range.

Kinetic Enzymatic Substrate Analysis

Linear fixed-time and rate analyses for the determination of initial substrate concentration. It is of some interest in certain cases (i.e., uric acid and urea nitrogen) to approach the analysis of substrate concentration by the use of rate measurements instead of by the equilibrium procedures that are commonly employed as described in the preceding section. Advantages in the rate measurement approach are a decreased overall analysis time and elimination of sample blanks. However, a disadvantage of this approach is the requirement for calibration reference solutions, which decrease the sample capacity of the rotor; in addition, the accuracy of the analysis is directly related to the quality of the standards.

The subject of enzymatic rate measurement analyses has been discussed by Guibault (2) and, to some extent, by Ingle and Crouch (4). The discussions generally focus on the Michaelis-Menten expression:

\[ V_0 = -\frac{dS}{dt} = \frac{dP}{dt} = \frac{V_{\text{max}}S_0}{K_m + S_0} \]  

where \( V_0 \) = initial velocity, \( S \) = substrate concentration, \( P \) = product concentration, \( V_{\text{max}} \) = maximum enzyme velocity, \( S_0 \) = initial substrate concentration, and \( K_m \) = Michaelis-Menten constant, which is equivalent to the substrate concentration at one-half maximum velocity. For the case when \( K_m \gg S_0 \), \( K_m + S_0 \approx K_m \), the initial velocity of the reaction is apparently first order with respect to initial substrate concentration, \( S_0 \), or

\[ V_0 = -\frac{dS}{dt} = \frac{dP}{dt} = \frac{V_{\text{max}}S_0}{K_m} = k'S_0 \]  

Table 3. Calculated Substrate Concentrations by Using Enzymatic End-Point Substrate Analysis

<table>
<thead>
<tr>
<th>A. Glucose(^a)</th>
<th>Calculated substrate concn. mg/dl</th>
<th>Relative error, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference solution concn. mg/dl</td>
<td>Calculated substrate concn. mg/dl</td>
<td>Relative error, %</td>
</tr>
<tr>
<td>50.0</td>
<td>49.5</td>
<td>1</td>
</tr>
<tr>
<td>100.0</td>
<td>99.1</td>
<td>1</td>
</tr>
<tr>
<td>150.0</td>
<td>155.3</td>
<td>4</td>
</tr>
<tr>
<td>200.0</td>
<td>201.4</td>
<td>0.7</td>
</tr>
<tr>
<td>400.0</td>
<td>390.2</td>
<td>2.4</td>
</tr>
</tbody>
</table>

where \( k' = V_{\text{max}}/K_m \). It is apparent that there must be a range of initial substrate concentrations in the reaction mixture above which the enzymatic reaction is no longer first order with respect to substrate concentration. Guibault (2) has suggested that the region in which linearity between reaction rate and substrate concentration is achieved, and in which an analytical determination of substrate concentration can be based on reaction rate, is for values below 0.2 \( K_m \). Cook and Hallett (1) have reported a rate method for the enzymatic analysis of urea (urease/GMD), which they find to be linear to 250 mg of urea per 100 ml. Under the conditions of reaction used, this corresponds to a starting reaction concentration of \( 1.4 \times 10^{-4} \) mol/liter, or an initial substrate/\( K_m \) ratio of \( \approx 0.05 \). The rate procedure discussed in the section on "Linear Rate and Fixed-Time Analysis" is linear to at least 50 mg of urea nitrogen per 100 ml, which corresponds to a reaction concentration of \( 1.6 \times 10^{-4} \) mol/liter or a \( K_m \) of approximately 0.05.

Table 4. End-Point Analysis Data

<table>
<thead>
<tr>
<th>Glucose analysis (hexokinase); reaction time, 4 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose concentration mg/dl</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>66.5</td>
</tr>
<tr>
<td>130.0</td>
</tr>
<tr>
<td>189.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Urea nitrogen analysis; reaction time 10 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea nitrogen concentration mg/dl</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>15.0</td>
</tr>
<tr>
<td>34.6</td>
</tr>
</tbody>
</table>

CLINICAL CHEMISTRY, Vol. 18, No. 8, 1972 835
Equation 2 can be integrated and rearranged to give the following equation in terms of $A_0$, the initial substrate concentration:

$$A_0 = \frac{\Delta A}{e^{-k'_{t_1}} - e^{-k't_2}}$$

(3)

A basic property of a first-order or pseudo-first-order reaction is evident in Equation 3. If $t_1$ and $t_2$ are accurately defined and all other variables such as reaction pH, temperature, etc., are held constant, then the initial substrate concentration, $A_0$, is linearly related to the change in substrate concentration, $\Delta A$, during that time period. The Fast Analyzer is ideally suited for this approach since $t_1$ and $t_2$ are accurately defined for all determinations in one rotor because of the unique parallel analyses of these samples under the same constant conditions.

From the preceding discussion it is apparent that two approaches can be made to the enzymatic rate analysis of substrate. Integration of the Michaelis–Menten single substrate–enzyme expression yields a mixed first-order–zero-order expression, and the validity of the linear rate method and fixed-time method based on expression 3 is clearly limited by the substrate-to-$K_m$ ratio. These limits, especially for expression 3, have not been well defined. The useful concentration range over which pseudo-first-order kinetics can be applied is of particular importance in clinical chemistry because of the large range of concentrations that are encountered in routine analysis. Table 5 lists the enzymatic substrate analyses commonly used in clinical laboratories, the corresponding $K_m$ values, the absorbance change due to total conversion of substrate at 0.01 $K_m$ in the assay mixture, and the resolution ($\Delta A$ mg$^{-1}$/dil$^{-1}$). It is obvious, in terms of initial reaction substrate concentration ranging from 0.01 to 0.1 $K_m$, that urea (urease/OMP) and uric acid (bacterial uricase) are the most likely to fit the pseudo-first-order approximation, and still provide sufficient resolution to assure adequate precision.

First-order enzymatic rate and fixed-time substrate analyses of urea nitrogen. The reactions catalyzed by urease and glutamate dehydrogenase are essentially irreversible, and both fit the criterion for pseudo-first-order (with respect to substrate) enzymatic reactions. The $K_m$ value for urease is of the order of $2 \times 10^{-4}$ to $3 \times 10^{-4}$ mol/liter (16). The $K_m$ value for glutamic dehydrogenase with respect to ammonia is $3.2 \times 10^{-4}$ mol/liter (17). The concentration of urea in serum samples under conditions of the analysis ranges from $0.83 \times 10^{-6}$ to $2.49 \times 10^{-6}$ mol/liter, and the conditions specifying that the substrate concentration must be equal to 0.01 to 1.0 $K_m$ for both enzymes in this coupled system is met. Therefore, in analyzing for urea by use of the coupled enzymic system for rate analysis, one is confronted with two first-order reactions occurring in series.

These equations can be most simply represented as two irreversible reactions:

$$U \rightarrow_k A$$

(4)

$$A \rightarrow_k G$$

(5)

which can be represented by the following differential equations:

$$\frac{dU}{dt} = -k_1U$$

(6)

$$\frac{dA}{dt} = k_1U - k_2A$$

(7)

$$\frac{dG}{dt} = k_2A$$

(8)

The following solutions for $U$, $A$, and $G$ can be obtained:

$$U = U_0 e^{-k_1t}$$

(9)

$$A = \frac{U_0 k_1}{k_2 - k_1} (e^{-k_1t} - e^{-k_2t})$$

(10)

$$G = U_0 [1 + \frac{1}{k_2 - k_1} (k_2 e^{-k_1t} - k_1 e^{-k_2t})]$$

(11)

Discussions of the above and of the more general
reversible series first-order reactions are presented in detail by Frost and Pearson (18).

The determination of urea by the above reaction is accomplished by monitoring the conversion of NADH to NAD, which is analogous in series first-order reactions to following the formation of product G. Thus, the rate of production of G or NAD becomes expression 12 by substituting Equation 10 into Equation 8:

\[
\frac{dG}{dt} = \frac{k_0(k_1e^{-kt} - e^{-kt})}{k_1 - k_1} U_0
\]

Expression 12 is important to the rate analysis because it shows that the rate of production of G (NAD) is directly proportional to the initial concentration \(U_0\), which is urea in this case. Figure 6 shows the rate, as \(\Delta A_{460}/\text{min}\), plotted against the urea nitrogen concentration. It is obvious that a linear relationship exists between the rate and the concentration.

A second relationship that is important for consideration in the rate analysis of urea results from considering Equation 11 at \(t = t_1\) and \(t = t_2\):

\[
U_0 = \frac{\Delta G}{(k_1 - k_2)[(k_0e^{-kt} - k_0e^{-kt}) - (k_0e^{-kt} - k_0e^{-kt})]}
\]

or, if we substitute \(K''\) for the complex rate constant,

\[
U_0 = \frac{\Delta G}{K''}
\]

The important point of Equation 14 is that, when \(t_1\) and \(t_2\) are defined for consecutive or series reaction, the change in product (\(\Delta G\)) is proportional to the initial substrate concentration, \(U_0\). A plot of \(\Delta A_{460}\) at different values of \(\Delta t\) is shown in Figure 7. Again, a linear relationship exists for the absorbance change between two fixed times, \(t_1\) and \(t_2\), as a function of urea nitrogen concentration.

Analysis of Urea Nitrogen by Use of Linear Rate and Linear Fixed-Time Programs

Urea nitrogen was determined in normal and supranormal control sera with fixed-time and rate analyses by means of the linear fixed-time and linear rate programs.

The results of the rate analysis for urea nitrogen in the normal control serum (21.7 ± 2 mg/dl) was 12.9 ± 0.7 mg/dl with a relative standard deviation of 5.1% and for the rate analysis of the supranormal control (34.3 ± 2 mg/dl) the result was 35.6 ± 1.0 mg/dl with a relative standard deviation of 2.9%. The precision of the rate procedure is somewhat better than that reported by Cook and Hallett (15); however, the fixed-time analysis gave the best overall results. The result of the fixed-time analysis of urea nitrogen for the normal control serum (12.7 ± 2 mg/dl) was 13.4 ± 0.3 mg/dl with a percent relative standard deviation of 1.8%, and the result of the analysis of the supranormal control (34.3 ± 2 mg/dl) was 34.3 ± 0.3 mg/dl with a percent relative standard deviation of 0.8%.

In Table 6 the three analytical approaches are compared: end-point, rate, and fixed time. The fixed-time analysis of urea nitrogen is seen to be the method of choice.

Linear Fixed-Time Analysis of Uric Acid

The end-point analysis of uric acid with porcine liver uricase requires 20 min; with bacterial uri-
Table 6. Three Approaches to the Analysis of Urea Nitrogen (Comparison of Results)

<table>
<thead>
<tr>
<th>Analysis time</th>
<th>End-point</th>
<th>Fixed time</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration</td>
<td>Substrate factor</td>
<td>Reference stds.</td>
<td>Reference stds.</td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>X 11.7 mg/dl</td>
<td>13.3 mg/dl</td>
<td>12.9 mg/dl</td>
</tr>
<tr>
<td></td>
<td>SD .2 mg/dl</td>
<td>.3 mg/dl</td>
<td>.7 mg/dl</td>
</tr>
<tr>
<td></td>
<td>CV 1.5%</td>
<td>2.0%</td>
<td>5.0%</td>
</tr>
<tr>
<td>Sample B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>X 34.6 mg/dl</td>
<td>34.3 mg/dl</td>
<td>35.6 mg/dl</td>
</tr>
<tr>
<td></td>
<td>SD .5 mg/dl</td>
<td>.3 mg/dl</td>
<td>1.0 mg/dl</td>
</tr>
<tr>
<td></td>
<td>CV 1.2%</td>
<td>.8%</td>
<td>2.9%</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1/20 dilution of normal control serum (12.7 ± 2 mg/dl).
<sup>b</sup> 1/20 dilution of abnormal control serum (34.3 ± 2 mg/dl).

The introduction of bacterial uricase (11) as an analytical reagent has made it possible to analyze for uric acid at 292 nm by using linear fixed-time analysis. The $K_m$ for this enzyme is $1.0 \times 10^{-4}$ mol/liter as compared with $1.7 \times 10^{-4}$ mol/liter for porcine liver uricase (20), and increases the concentration of initial substrate that can be used sixfold. Figure 8 shows the absorbance change vs. uric acid concentration at various fixed-time intervals with $t_i$ equal to 4 s. The reaction concentration of uric acid corresponding to 12 mg/dl is $1.4 \times 10^{-4}$ mol/liter, which is a molar-substrate-concentration-to-$K_m$ ratio of 0.14.

The precision of analysis for uric acid control sera is 4 to 6% relative standard deviation in the normal range and 2% relative standard deviation in the supranormal range.

Nonlinear Fixed-Time Analysis

It has previously been indicated that some limit of initial concentration of substrate exists, beyond which the pseudo-first-order kinetic approximation is not followed and the initial rate, rate at time (series first order), or absorbance change over a fixed time interval is no longer linearly dependent on initial substrate concentration. A limitation in using enzymatic fixed-time analysis or enzymatic rate analysis of substrate in a routine analytical mode is due, in part, to instrumental data output. Noncomputerized digital instrumentation, available to the laboratory, that is capable of printing out concentration based on absorbance change, does so by multiplying a stored digital constant by the absorbance change to obtain the concentration. This demands that absorbance vs. concentration be linear over the analytical range of interest. Computerized instrumentation can be programmed to perform nonlinear data reduction and broaden the usefulness of enzymatic rate or fixed-time analysis.

The hexokinase-glucose procedure has been chosen as an example of the Nonlinear Fixed-time Substrate Analysis Program. For glucose concentrations ranging from 0 to 400 mg/dl, with a sample volume of 2.8 $\mu$l in a total reaction volume of 550 $\mu$l, the actual reaction concentration is 9 to 12.5 $10^{-4}$ mol/liter; the upper limit concentration corresponds to 0.73 $K_m$. The absorbance change per fixed time interval with respect to concentration should be nonlinear, and Figure 8 shows this to be the case. Table 7 presents the concentrations of several glucose reference solutions and serum samples as a function of fixed time interval and shows that close correlation is achieved between the expected concentration and the determined concentration even after an analysis time of only 30 s ($\Delta t = 20$ s). The precision of analysis of a glucose control serum (85 mg/dl) was 84.3 ± 2 mg/dl (relative standard deviation = 2.5% at a $\Delta t$ of 20 s and $t_i = 9$ s), and 85.0 ± 1.6 mg/dl (relative standard deviation = 1.9% at a $\Delta t$ of 90 s and $t_i = 9$ s).

**Summary and Conclusion**

Enzymatic substrate analysis has been considered by evaluating both end-point and kinetic methods. Elementary computer programs have been written for both end-point and kinetic enzymatic analysis of substrate to facilitate these considerations.
Table 7. Nonlinear Enzymic Fixed-Time Analysis of Glucose by the Hexokinase/GPD Method

<table>
<thead>
<tr>
<th>Glucose (mg/dl), expected concn</th>
<th>Glucose (mg/dl), calc concn at various fixed time intervals (ΔT) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔT = 20 s</td>
</tr>
<tr>
<td>50</td>
<td>51.7</td>
</tr>
<tr>
<td>100</td>
<td>101.8</td>
</tr>
<tr>
<td>200</td>
<td>199.3</td>
</tr>
<tr>
<td>66</td>
<td>68.2</td>
</tr>
<tr>
<td>82</td>
<td>80.8</td>
</tr>
<tr>
<td>109</td>
<td>110.0</td>
</tr>
<tr>
<td>164</td>
<td>164.7</td>
</tr>
</tbody>
</table>

a T = 9 s, time of first computer reading.

End-point enzymatic analysis allows accurate, precise results but demands that the spectrophotometric system and the volume dispensing systems be properly calibrated to ensure accuracy. The need for calibration (reference) standards is eliminated if the monitored reaction specie has a determined and constant molar absorptivity.

It was demonstrated that it is feasible to obtain the initial absorbance, A₀, even for fast reactions such as hexokinase-glucose, without running separate sample blanks. This was achieved by extrapolation of the initial data points to zero time. The main disadvantage of end-point enzymatic substrate analysis is that the reaction time is, in the case of urea nitrogen and uric acid, too long (15–20 min).

Rate or fixed-time analyses, which are more rapid (1 to 2 min) are, therefore, of great interest. The problem associated with the sample blank is eliminated by using these approaches. The main disadvantages of kinetic analyses are the requirement for calibration (reference) standards and the possible nonlinearity of the relationship between rate or fixed-time absorbance change and initial substrate concentration. However, by incorporating nonlinear data reduction with a simple numerical analysis method, the linearity problem is eliminated and the fixed-time method becomes a rapid, simple, and precise means of substrate analysis.

Common enzymatic substrate analyses such as glucose (hexokinase), urea (urease/GPD), and uric acid (uricase), were used to demonstrate certain characteristics of each type of analysis. The determination of uric acid at 292 nm has not previously been demonstrated on an automated system such as the Fast Analyzer. The commercial availability of bacterial uricase, with a sixfold larger γmax than porcine liver uricase, in conjunction with a more rapid turnover rate, makes it feasible to perform linear fixed-time analysis of uric acid in 3 to 5 min, as compared with 10 to 20 min required for end-point analysis.

The procedures are not limited to those described in this paper, but include all those for which specific enzymes are commercially available to perform enzymatic substrate analysis. The application of the methods discussed here could be used in the rapid, simultaneous analysis of lactate and pyruvate or other substrates present in a variety of pathological states.

We conclude from this study that analytical systems are available for inexpensive, rapid, accurate, and precise enzymatic substrate analysis and that their use will simply depend on the availability of procedures for a specific substrate analysis.

References


