A New "Kinetic" Method for Enzyme Analysis
Suitable for Automation

Kenneth A. Trayser and David Seligson

A method is proposed for automatic instrumentation of enzyme activity determinations which involve spectrophotometric measurements. The procedure utilizes a kinetic method to produce one instrumental reading that is proportional to enzyme concentration. The single spectrophotometric reading can be processed by analog or digital computation, using the known absorptivity constant of the reactant or product measured, to provide a value for enzyme activity expressed in micromoles of substrate consumed or product formed per unit time per unit volume under the specified conditions. Using the spectrophotometric measurement, one can establish enzyme concentrations based on absolute constants yielding results similar to those obtained by conventional kinetic measurements of zero-order reaction rates which are proportional to enzyme concentration.

Three stages for automation in the determination of enzyme activity have been described by Schwartz and Bodansky (1). The first stage involves the use of instruments to record the results of enzyme action. In the second, the preparation of the reaction mixture is performed by machine, while in the third (1) the enzyme activities would be transformed into numerical values and (2) feedback devices would be used to control and modify the actions of various instrumental components in producing a final result. Procedures providing the first two stages of automation are known and impose no technical difficulties. We propose in this paper a method that can incorporate Part (1) of the third stage and which has the additional advantage of utilizing a kinetic method for enzyme determination which produces one instrumental reading that is proportional to enzyme concentration. This allows for economy in processing the reading by analog or digital means. Further, the method could be adopted to provide Part (2) of Stage 3.

Assays for numerous enzymes involve the measurement of a change in the absorption of radiant energy in the visible or the ultraviolet

From the Section of Clinical Pathology, Yale University School of Medicine, New Haven, Conn 06510.
Received for publication July 18, 1968; accepted for publication Oct 11, 1968.
ranges. In such procedures the substrate used or a product of the reaction differ in their absorption spectrums (eg, p-nitrophenyl phosphate/p-nitrophenate, or NAD/NADH); or the reaction of interest can be coupled with a reaction involving such a change (eg, glutamic-oxalacetic acid transaminase* coupled to the malic dehydrogenase reaction†). During that phase of the enzyme-catalyzed reaction, which is zero order with respect to substrate concentration, the reaction rate is proportional to enzyme concentration. Theoretically, therefore, the zero-order reaction rate is the one of choice for determining enzyme concentration. The frequently employed single determination of substrate consumed or product formed at a fixed elapsed time is not the most desirable measure of the rate of a reaction. Peculiarities in kinetics at initiation of the reaction and after the reaction deviates from zero-order kinetics account for the inadequacy. In practice, the measurement of changes in absorbance with time does not always provide a perfectly straight line of constant slope due to various errors inherent in the measurement. Ideally, the zero-order portion of a reaction could be represented by the output from a spectrophotometer as a perfectly linear plot of absorbance versus time. The rate of the reaction (the derivative of absorbance with respect to time) could be determined as a single reading with an analog computer (2). Practically, minute changes in absorbance (± 0.001) with time accentuate fluctuations of the derivative (ΔA/Δt) during the course of a reaction. Possible solutions to this problem are to take multiple absorbance readings at various times or to record continuously the change in absorbance with time. The best straight-line fit can then be used to calculate the desired rate of reaction. In the case of multiple readings, the method of least squares can be used to calculate an equation for the line. When continuous recording is employed, the best straight line can be drawn through the curve. In both cases, manual effort and time are required. With a computer several points could be logged and the least-squares fit calculated, but this requires a more complex computer program and use of the computer for a relatively extended time (3). Since the area under the experimental curve of absorbance versus time bears a direct mathematic relationship to the slope of the best fitting straight line, another possible procedure is to integrate the output signal. Such integration could be carried out readily with an analog computer to provide a single value.

Far more desirable would be a kinetic determination which would involve only one reading to capture the critical measurement. Because

† L-Malate; NAD oxidoreductase, I.U.B. 1.1.1.37.
serums differ (in turbidity, ultraviolet-absorbing compounds, etc.) each should serve as its own control. Since kinetics at zero time may vary because of mixing, lag phase, etc., the reading should be made after the reaction has become linear with respect to time. We propose the following procedure, which is amenable to automation.

Principle

The principle is illustrated in Fig 1. Two identical samples are prepared. The reaction in the first sample is initiated at time $t_1$ by adding substrate, and in the second at time $t_2$ by adding substrate. The time interval for the reaction is $(t_2 - t_1) = \Delta t$. The change in absorbance, $\Delta A$, can be measured at any convenient time, $t_r$, after the reaction has become linear in the second sample and before the rate deviates from zero-order kinetics in Sample 1, since $\Delta A$ is essentially constant during this time period. A double-beam spectrophotometer is employed to measure transmittance with one sample in the reference beam and the other in the sample beam. The value $\Delta A$ is the value for $\Delta t$, and $\Delta A/\Delta t$ measured at $t_r$ is then a measure of the rate of reaction and, hence, of enzyme concentration.

The difference $(A_i - A_s)$ between the absorbance by the solution in the sample beam and the reference beam is desired. For a fixed $\Delta t$ this difference is proportional to enzyme concentration. That a double-beam spectrophotometer carries out the measurement $(A_i - A_s)$ by a single measurement can be demonstrated as follows. By definition: $A_i = -\log T_i$ and $A_s = -\log T_s$, where $T$ stands for transmittance. The difference

![Fig 1](image_url)  

**Fig 1.** Upper line represents reaction started at time $t_i$, and the lower line the reaction started at a later time, $t_s$.

between these two values is $-\log T_i - (-\log T_s)$ or $\log (T_s/T_i)$. $(T_i/T_s)$ is presented by the double-beam spectrophotometer as a single reading. By the definition of absorbance, the reading $-\log (T_i/T_s)$ (or $\log (T_s/T_i)$) is the desired value, $(A_i - A_s)$. The general procedure
for detecting a difference between two such samples in a single measurement could be carried out by other means. What is required is some physical measure of substrate consumed or product formed during the course of a reaction and some instrumental means of comparing or subtracting the two readings. For example, suppose a hydrogen ion were liberated as a product during the course of the reaction. Hydrogen ion activity can be measured with a glass electrode. A difficulty becomes apparent when two readings are to be compared—i.e., when the values from two glass electrodes are to be subtracted. The two electrode systems must be matched perfectly throughout their entire ranges so that the difference between their readings is due only to a difference in the hydrogen-ion activities in the two solutions. With a double-beam spectrophotometer the matching is accomplished by the design of the instrument, and the single value presented is the measurement desired.

In the proposed method the time interval \((t_s - t_i)\) can be selected and set by the analyst, as can the time for measurement \((t_i)\). Several conflicting factors enter into the selection of the time interval for any one enzyme assay. Since absolute errors of the order of \(\pm 0.001-0.002\) absorbance units occur in the spectrophotometric measurement, making \(\Delta A\) larger by increasing \(\Delta t\) will decrease the relative error in the \(\Delta A\) measurement. If the clinically critical values for distinguishing between normal and abnormal conditions lie in the very low range of enzyme activities, then a long \(\Delta t\) is desirable. If the critical values lie in a higher range, a somewhat shorter \(\Delta t\) may be used. Prolonged incubation times do not slow the frequency of reading sample pairs. For example, if it is desired to make \(\Delta t\) 15 min, samples set up at 30-sec intervals can be read at the rate of 120/hr. There is no major loss in time or machine costs if very small enzyme samples requiring an extended reaction time are used. Finally, in order to handle a maximum number of the samples submitted without dilution for high values, \(\Delta t\) should be short enough so that the reading of high enzyme activities can be made before the reaction in Sample 1 deviates from zero order. Obviously the selection of \(\Delta t\) usually will involve some evaluation of the kinetics for each enzyme procedure and of the range desired for clinical use.

For alkaline phosphatase* a time interval of 4 min provides a \(\Delta A\) of 0.165 for 75 units (1 unit equals 1 \(\mu\) mole of product per minute per milliliter of serum), our upper limit for normal persons. This time interval is sufficiently short to measure up to 440 units, thus including 98% of the samples tested. For lactic dehydrogenase† the corresponding

---

*Orthophosphoric monoester phosphohydrolase, I.U.B. 3.1.3.1.
†L-Lactate: NAD oxidoreductase, I.U.B. 1.1.1.27.
Δt is 2½ min, which provides an absorbance difference of 0.110 for 420 units (1 unit equals 0.001 absorbance change per minute per milliliter of serum), and an upper limit of 3200 units (which includes 99% of the samples submitted). For glutamicoxalacetic acid transaminase, a 10-min time interval was selected to provide an absorbance difference of 0.100 for 40 units (1 unit equals 0.001 absorbance change per minute per milliliter of serum), and an upper limit of 335 (which includes 92% of sample values).

While the above procedure requires particularly careful work in manual operation, it lends itself readily to automation. Machine use can assure: (1) the accurate pipetting required for the preparation of two samples as nearly identical as possible, and (2) the accurate reproduction of Δt needed for successive samples. A double-beam spectrophotometer can provide an accurate measurement of ΔA. The single reading obtained can be recorded or put into a computer for subsequent processing by analog or digital computations to provide a numerical value for enzyme activity. Further, the absolute value of the single reading can be standardized with respect to absorbance units so that by incorporating in the computation the absorptivity of the substance measured, the value of enzyme activity can be reported directly in terms of micromoles of substrate consumed or product formed per unit time per unit volume. If a single spectrophotometric reading is used for the analysis, a sample can be identified more easily for machine use than if numerous readings per sample must be taken.

Some monitoring procedure to detect samples with very high values is necessary, since a single reading cannot reveal whether ΔA is constant with time or changing owing to a deviation from linearity in one or both reactions. The ΔA value may be falsely small depending on the relative stage of the reaction, so that the reading must be monitored for any consistent change in ΔA during some brief time period about time

Fig 2. Changes in ΔA as reactions in Samples 1 and 2 deviate from linearity.
This factor is illustrated in Fig 2. $\Delta A$, is the desired measure of enzyme activity. Readings taken after the first reaction deviates from zero-order kinetics are obviously inaccurate. The reaction could be monitored simply by recording $\Delta A$ versus time on a strip chart for a brief period before and after the single reading is made at $t_s$. The analyst would then survey the strip chart for tests in which $\Delta A$ changed consistently with time and repeat such samples at appropriate dilutions. The change in $\Delta A$ with time could be followed with a simple electronic circuit that would be part of a feedback circuit to detect and perhaps to repeat automatically at a greater dilution samples with a high enzyme activity, thus fulfilling Part (2) of Stage 3 stated above.

**Experimental**

Comparisons were made between the kinetic method for the determination of enzyme concentration (the rate of reaction was determined by measuring the slope of an absorbance-time plot on a recorder) and the proposed time-interval kinetic method (the reaction rate is determined by measuring $\Delta A/\Delta t$). In the time-interval method, readings were made approximately 15 sec following the initiation of the reaction in sample $t_s$.

A Perkin-Elmer Model 450 spectrophotometer, a double-beam recording instrument, was used for all assays (other comparable double-beam instruments are suitable). Results are summarized in Fig 3-5. For ease in comparison, in each case the rate for the kinetic method is expressed in terms of the change in absorbance for the time interval selected for that determination. Note that in each case the relationship

![Image](image-url)
is linear within the selected ranges of enzyme activities. Duplicate determinations agreed within ± 1%.

A typical experiment was carried out as follows. All components of the reaction mixture in one test tube, except substrate, were equilibrated at 30° in a constant temperature block (if preincubation was required, the mixture was incubated for the desired time at 30°). Aliquots of this mixture were pipetted into cuvets and placed into sample and reference beams. The absorbance difference was recorded. If ΔA differed from zero, it reflected cuvet differences. Substrate was added to one cuvet at t₁ minus 5 sec; contents of the cuvet were mixed by inversion starting at t₂. Changes in absorbance with time were recorded until t₃ minus 20 sec. (For assays in which an absorbing compound was formed, Sample t₁ was placed in the sample compartment and t₂ in the reference compartment. When an absorbing compound was consumed, Sample t₁ was placed in the reference compartment.*) Sample 2 was removed, substrate added at t₄ minus 5 sec, and mixed at t₅. The time-interval reading was made approximately 15 sec after t₅ and followed for at least 2 min thereafter. Since the cell compartments were not thermostatically controlled, the maximum temperature variation during

*Slit-width changes were compensated automatically in the double-beam spectrophotometer.
an entire series of experiments is noted for each experiment (Fig 3–5).

In summary, the method proposed for an enzyme assay provides a single, accurate, spectrophotometric reading which, with an accurate time interval, is proportional to enzyme concentration over a wide range of enzyme activities. The procedure was designed for automatic instrumentation to assure accurate sample preparation and accurate, reproducible time intervals for a large number of samples. Both the recording of the single reading and subsequent calculations can be carried out readily by an analog or digital computer. The time interval can be selected by the analyst—an extended time interval could provide greater accuracy for samples with low activity, while a shortened time interval allows the determination of samples with high activity. The spectrophotometer can be standardized with respect to absorbance so that, by using known absorptivity constants of the substances measured, results can be reported in micromoles of substrate per unit time per unit volume as recommended by the Commission on Enzymes of the International Union of Biochemistry. The system can be monitored readily for a sample with very high activity which needs to be repeated at a greater dilution.

References