Kinetic Methods That Are Independent of the Rate of Reaction

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Kinetic methods for the quantification of substrates offer a number of advantages over equilibrium methods, such as speed and economy. However, in their most commonly practiced form they are liable to error from various sources in addition to those ordinarily encountered in equilibrium methods, owing to variations in enzymic activity between test and standard assays. In certain circumstances the differences in enzymic activity may be such as to completely invalidate the method. Such difficulties may, however, be avoided if three or more measurements are made during the reaction of the sample and are evaluated by one of five means. In each case data from the early course of the reaction are used to predict the total change in sensor signal at infinite time, from which the concentration of the substrate may be calculated as for an equilibrium method.

Additional Keyphrases: enzymic assays  •  equilibrium methods

For the large class of chemical assays that depend upon the production or consumption of substrate, product, or cofactor, most can be divided into "kinetic" or "equilibrium" methods. According to the classification of Pardue (1) a kinetic method is one in which calculation of the analyte concentration is based upon data collected during the early part or kinetic phase of a reaction, whereas in an equilibrium method the data are collected after equilibrium has been reached.

Most kinetic methods used for measuring substrate concentrations involve the estimation of one or more aspects of the reaction curve, and the value derived for the unknown sample is then compared with values obtained under identical conditions with samples of known concentration. Various methods of data collection/evaluation may be used, examples of which are:

Method 1a: estimation of the initial velocity (defined as the rate of change in sensor signal per unit time) of the reaction.

Method 1b: estimation of the velocity of the reaction at a pre-specified time, such as t min after the start of the reaction.

Method 2: estimation of the change in sensor signal between two fixed time points, such as t₁ and t₂ min.

Although kinetic methods are faster than equilibrium methods, their dependence upon the use of standards, which must be run under identical conditions to those used when assaying samples, predisposes them to various additional sources of error. In the case of enzyme-catalyzed reactions, enzyme activity must be kept constant. Variations due to differences in amounts of enzyme added, pH, incubation temperature, and the relative amounts of inhibitors or activators will result in a variable degree of error in the estimates of sample concentration. In practice, the first three sources of error can easily be controlled—but not so the fourth, where samples contain variable amounts of activators or inhibitors. In this respect kinetic methods are less robust than equilibrium methods.

The ideal solution would therefore be to devise a kinetic method which enables the equilibrium point of the reaction to be predicted. Such a method would still retain the advantages of the classical kinetic method in terms of speed of assay, but would now be independent of factors affecting the rate of reaction. In the special case of a photometric assay the end point of the method would be the calculation of the absorbance at infinite time (ΔA₀), enabling the change in absorbance (ΔA₁) to be calculated. If the molar absorptivity of either the product or the substrate is known then the assay can be freed totally from the obligatory use of standards.

Methods for the prediction of the equilibrium point in a first-order reaction were in fact first described as long ago as 1926 (2, 3), and today there are at least five computational procedures available for this class of reaction. The most recent of these (4), in which a regression method is utilized to estimate the change in sensor signal at equilibrium, has been applied also to reactions with combined zero-order/first-order kinetics (5). Even the simplest method requires at least three data points, and therefore it would come under the heading of multi-point kinetic methods (1).

As far as we are aware none of the major texts on enzymic methods for the determination of substrate concentrations even consider predictive-equilibrium point methods, although nearly all describe in detail the basic theory behind conventional equilibrium and kinetic methods—this is in spite of the fact that, as noted earlier, the first two methods of calculation were described many years ago. In this article we would like briefly to outline the different procedures that may be used and to illustrate these by reference to a first-order reaction that is monitored photometrically.

Principle and Formulas

Consider the following reaction in which the substrate S is converted to the product P in the presence of enzyme E.

\[ S \xrightarrow{E} P \]

Under the conditions of assay it is arranged that this reaction proceeds exponentially such that the concentration of P at any time is given by:

\[ C_P = C_{S0} - C_{S0} \cdot \exp(-k \cdot t) \]

where \( C_P \) is the concentration of P at time \( t \), \( C_{S0} \) is the concentration of S at the start of reaction, \( k \) is the exponential rate constant, and \( t \) is the time elapsed from the start of the reaction.

Let us suppose that the progress of the reaction is monitored by measuring the increase in absorbance (Δ) due to formation of P at a wavelength of λ, a wavelength at which S is not absorbant.

Two typical traces for this reaction are shown in Figure 1. The concentration of S may be found by either allowing the reaction to run to completion and estimating \( C_{S0} \) from the total change in absorbance—i.e., the conventional equilibrium method—or by utilizing the data collected during the early part of the reaction—i.e., the kinetic method.

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Fig. 1. The increase in absorbance (A) due to the formation of product P in a first-order reaction. In this figure it has been arranged that the absorbance at the start of the reaction (after addition of enzyme) is 0.08, and that sufficient enzyme is added to drive the reaction to 95% completion within 3 min (i.e., \( k = 1.0 \text{ min}^{-1} \)). In trace 1 the total change in absorbance by infinite time \((\Delta A_i)\) is 0.10 and in trace 2, 0.20 units; i.e., the initial concentration of S giving rise to trace 2 is twice that giving rise to trace 1.

Historically, the first method proposed that enables us to calculate or predict the absorbance at infinite time \((A_\infty)\) from data collected during the early part of the reaction, was that described by Christie Smith (3). It required three absorbance measurements to be made \((A_1, A_2, \text{and } A_3)\) such that the time interval between the first pair \((t_2 - t_1)\) is equal to that between the second pair. \(A_\infty\) is then calculated from

\[
A_\infty = A_1 + (A_2 - A_1)^2/(2A_2 - A_1 - A_3)
\]

However, to calculate the change in absorbance \((\Delta A_\infty)\) an accurate measurement of the absorbance at the start of the reaction \((A_0)\) is still needed. In practice, a measurement of \(A_0\) may prove difficult to obtain, particularly with instruments that automatically inject the last component of the reaction mixture and mix the contents of the cuvette. Personal experience with a machine of this type indicated that data collected during the first few seconds was unreliable if the reagent solution contained a high concentration of protein, because of transient foaming after injection and mixing of the last component. In 1977 we proposed an alternative method of calculation (6), again based upon just three absorbance readings made during the course of reaction, which allowed \(\Delta A_\infty\) to be calculated directly. In this method, measurement of \(A_0\) is not required. Again, three measurements \((A_1, A_2, \text{and } A_3)\) are made such that:

\[
t_2 - t_1 = t_3 - t_2 = x \text{ min}
\]

\(\Delta A_\infty\) may then be calculated from:

\[
\Delta A_\infty = (A_3 - A_1)[\exp(\ln(A_3 - A_2)/(A_2 - A_1)) \cdot t_1/x] - \exp[\ln((A_3 - A_2)/(A_2 - A_1)) \cdot t_2/x]
\]

Although an improvement on the calculation of Christie Smith, the procedure is still dependent upon spot measurements of absorbance, with the result that the precision of the derived estimate of \(\Delta A_\infty\) will largely depend upon the accuracy of the photometric system being used. In the remaining three methods, which rely upon multiple measurements of absorbance during the course of reaction, this dependence is decreased.

In 1975, we had earlier shown (7) that \(A_\infty\) could be estimated from a plot of the reaction rate made at various times \(t\) — i.e., \((\text{d}A/\text{d}t)\) — vs absorbance at time \(t\) — \((A_0)\). This is illustrated in Figure 2 and shows that a straight line is obtained which intercepts the x-axis at a point equal to \(A_\infty\). Interestingly, it is the data from the earliest part of the reaction, obtained while the reaction rate is still high, that are the most useful in the calculation, and this tends to minimize the overall assay time. The method, however, again required an accurate measurement of \(A_0\) — as in the Christie Smith procedure — to calculate \(\Delta A_\infty\).

A multipoint method for the direct calculation of \(\Delta A_\infty\) had in fact been proposed in 1926 by Guggenheim (2) a few months after the first appearance of Christie Smith's paper (3). (We were, in fact, unaware of either of these two papers as we struggled between 1975 and 1977 with our own solution to the problem.) Formally stated, if \(A_1, \ldots, A_j, \ldots, A_n\) measurements are made at times \(t_1, \ldots, t_j, \ldots, t_n\) (without any restriction as to the intervals) and \(n\) more readings, \(A_1^*, \ldots, A_j^*, \ldots, A_n^*\), are made at \(t_1 + x, \ldots, t_j + x, \ldots, t_n + x\), where \(x\) is a constant time interval, then a plot of \(
\ln (A_i^* - A_2) - \ln (A_j^* - A_1))\) results in a straight line with a slope equal to \(k\), the exponential rate (Figure 3). From the estimate of \(k\), \(\Delta A_\infty\) may be calculated from

\[
\Delta A_\infty = (A_n^* - A_1)\left(\exp(-k \cdot t_1) - \exp(-k \cdot (t_n + x))\right)
\]

where \(A_1\) and \(A_n^*\) will, in practice, be the first and last estimates of absorbance at times \(t_1\) and \(t_n + x\). Guggenheim's method of calculation is, in fact, a multipoint of that later suggested by us in 1977 (6), in which \(-k\) was calculated from:

\[
-k = \ln \left(\frac{(A_3 - A_2)/(A_2 - A_1)}{x}\right)
\]

The last method of calculation was proposed in 1978 by Mieling and Pardue (4) and utilizes a multiple-linear regression program to compute estimates of the rate constant \(k\), initial absorbance \(A_0\), and final absorbance \(A_\infty\) that best fit the data to a first-order model. The program involves an iterative calculation about an expansion of the basic first-
order equation with use of a simplified Taylor series. For further details of the method of calculation the reader is referred to the paper by Mieling and Pardue (4). This method of calculation has also been applied to a reaction with combined zero-order/first-order kinetics (5).

Discussion

As a class, kinetic methods that have as their end point the prediction of sensor signal at infinite time (e.g., $A_\infty$) exhibit the speed of convention kinetic methods, but because they are largely insensitive to variations in factors that affect the rate of reaction, such as enzymic activity, it is to be expected that they will exhibit more of the robustness of an equilibrium method. The calculation procedures used are eminently suited to computerization. Kinetic methods predicting $A_\infty$ or $\Delta A_\infty$ have been used in this laboratory for the determination of fructose 1-phosphate (7) and glucose in whole blood by use of glucose dehydrogenase (6 and unpublished results), and by Mieling et al. (8) and Hamilton and Pardue (5) for the determination of glucose by use of hexokinase and glucose-6-phosphate dehydrogenase, and uric acid by use of uricase.

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References

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