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MAXIMUM RATE KINETIC ANALYSIS APPLIED TO ENZYME ASSAY DATA

by

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SUMMARY PAGE

THE PROBLEM

To evaluate a new procedure for the handling of enzyme assay data for which available techniques have proven unsatisfactory.

FINDINGS

A method is described and is justified, theoretically. This procedure makes possible the description of enzyme activity curves or calculation of enzyme concentrations for most systems in which activity is directly proportional to protein concentration, as well as for cases where this simple correlation does not hold. Data from a carbonic anhydrase analysis are used to illustrate the procedure.

APPLICATION

The "maximum rate" analysis described is proposed as a general method for handling enzyme assay data. The widespread use of enzymatic methods, for example in clinical medicine, provides immediate application for improved methods of evaluation or interpretation of results.

ABSTRACT

Double reciprocal plots of enzyme concentration vs. reaction velocity provide a rational basis for determining enzyme concentrations by assay methods based on measurement of reaction rates. Maximum rate kinetics furnishes a theoretical basis for the procedure, which is not limited to specific ranges of substrate concentration. Constants somewhat analogous to those of the Michaelis expression for characterizing the enzyme reactions are useful by-products of the method. The hydration of CO_2 by bovine carbonic anhydrase illustrates the applicability of the technique.

MAXIMUM RATE KINETIC ANALYSIS APPLIED TO

ENZYME ASSAY DATA

The effect of substrate concentration on the kinetics of an enzyme reaction has been known and utilized by biochemists for many years. Recently, however, the theory of maximum rate of product formation, (or 'maximum-rate kinetics'), was proposed by Darvey, Prohovnik and Williams[1] to describe the effect of enzyme concentration on the reaction:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E+P \qquad (1)$$
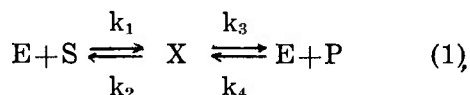
This equation describes the conversion of substrate, S, to product, P, under the influence of enzyme, E, with an intermediate complex, X, and with rate constants, k_X, for the various steps. If it can be assumed that during the time an enzyme reaction is monitored, the measured velocity, v, represents the maximum rate of product formation, not to be confused with V_m, V_s, or V_e, the theoretical maximum or limiting velocity, the new kinetic analysis provides a description of reaction (1) as valid as the equilibrium[2], or steady state analyses[3]. In addition, the present model accounts for the constant, k_4, which is considered to be negligible in some of the earlier procedures, but which may significantly influence the course of the action.[4]

Common practice in enzyme chemistry has demanded that in order to determine the quantity of an enzyme by measurement of its activity the assay must demonstrate the direct proportionality of enzyme activity to enzyme concentration.[5]

On the other hand, the "Michaelis equation," in some variation,

$$v = V_mS/K_m + S \qquad (2),$$

is usually employed to account for the influence of substrate concentration on enzyme velocities.[2] K_m, as well as K_s and K_e introduced below, are composite reaction constants.[6]

In order for the foregoing expression to be compatible with the activity-enzyme concentration proportionality, or:

$$v = k_XE \qquad (3),$$

the substrate must be adjusted so that, as nearly as possible, S is large with respect to K_m allowing v to approximate V_m, i.e., v approaches k_3E, justifying equation (3).[7]

With the proposal of the maximum rate analysis, an expression is also available for the influence of substrate on velocity that is equivalent in appearance to equation (2) although K_s, replacing K_m, is more complex than the Michaelis constant as it is usually defined.[1,2] In addition, however, to a statement of a relationship between velocity and substrate concentration (at a constant enzyme level), the maximum rate analysis also provides a relationship between velocity and enzyme concentration (at a constant substrate level):

$$v = V_eE/K_e + E \qquad (4),$$

Providing equation (4) is valid, it furnishes an explanation for the approximate proportionality which is often observed between the amount of an enzyme and its activity but which holds over a somewhat limited range of enzyme concentrations. Since V_e and K_e are constants, an expression such as that of equation (3) could replace equation (4), if E in the denominator of the latter were small with respect to K_e. For k_X to be approximately constant, E must therefore be limited to a very narrow range of values.

On the other hand, the possibility is provided by equation (4) for a much more general statement of an enzyme concentration-velocity relationship if the reciprocal of the expression is utilized:

$$1/v = K_e/V_e (1/E) + 1/V_e \qquad (5),$$

In this case a true proportionality constant, i.e., the ratio K_e/V_e relates the reciprocals of velocity and enzyme concentration,

modified only by the added constant $1/V_e$. A plot of $1/v$ vs. $1/E$ yields values for the constants in the manner of the Lineweaver-Burk[8] treatment of equation (2). K_e/V_e is the slope of the linear plot and $1/V_e$ the intercept on the $1/v$ axis.[9]

The statement for enzyme activity (equation 5) which is independent of substrate restrictions and for which enzyme activity and concentration are theoretically proportional over any range, provides a significant new tool for enzyme chemistry. If such an expression should prove to be generally applicable, several of the restrictions[5] imposed by even the simplest attempts to measure quantitatively the amount of an enyzme by determining its activity can be eliminated.

To illustrate the advantages of the employment of equation (5) in one particular situation, a series of assays was performed on an electrophoretically purified bovine carbonic anhydrase, form B.[10] An analytical system was utilized in which substrate for the reaction was provided by a carefully regulated stream of CO_2 bubbling into the reaction mixture.[11] While pCO_2 analyses indicate that the substrate concentrations can be held approximately constant during the portion of the reaction measured, it is not possible to define absolutely the substrate concentration at the initial instant of the reaction, nor is it experimentally easy to employ an absolutely defined range of substrate concentrations.

Figure 1 illustrates that under the conditions described a linear relationship between reciprocal velocity and enzyme concentration is obtained for the hydration of CO_2 by this carbonic anhydrase preparation.

From data such as those illustrated by the figure, it is possible not only to calculate the constants V_e and K_e, which characterize the reaction under the conditions employed, but also to determine an absolute or relative concentration value for an unknown quantity of enzyme.[13] Particularly when a single enzyme species is being investigated, it is simpler to calculate a value for E for the unknown amount of enzyme from the linear equation (5) than from a curved plot of v vs. E.
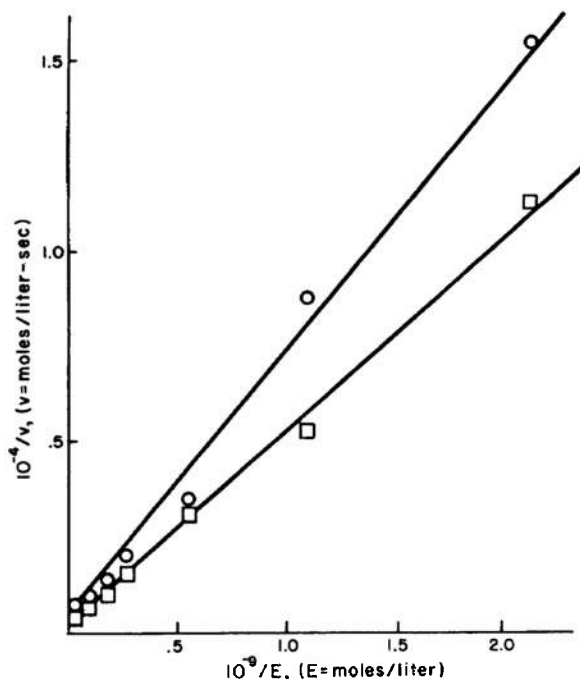
Double reciprocal velocity vs enzyme concentration plots for purified bovine carbonic anhydrase studied at two substrate, CO_2, concentrations. (Circles describe experiment employing approximately .0014 M substrate; squares .0047 M. The constants for the two sets of data calculated by a weighting technique appropriate for use with equation (5) (Johansen, Lumry, ref. 9) are $K_e = 1.54 \times 10^{-8}$ and 5.48×10^{-8} and $V_e = 2.49 \times 10^{-3}$ and 1.09×10^{-2}, with coefficients of correlation between the data and linear plots according to the "Pearson product-moment formula"[12] = .996 and .997, respectively).

It should be recognized, of course, that information about the proportional concentrations of any possible isozymes must be available if absolute enzyme levels are to be assigned. This complication arises from the very wide differences in molar activities of various members of some enzyme groups, for example, those of the human carbonic anhydrases.[14]

The use of double reciprocal plots of velocity vs. concentration should be considered

for evaluation of data from enzyme assays currently in use, since equations (5) will be valid for many cases to which equation (3) has heretofore been applicable as well as for the more general situation for which it was derived.[15]

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5. V. R. Potter, in **Advances in Enzymology,** F. F. Nord, C. H. Werkman, Eds. (Interscience, New York, 1944), vol 4; J. M. Reiner, loc. cit.

6. Since mathematical definitions of the various constants are not required for the present argument, the reader is referred to the original literature (1,2) for this information.

7. Expression 3 has sometimes been justified, e.g., see Reiner (3), on the basis of equation 2 written as: $v = (k_3 S / S + K_m) E$, which, however, presumes that E has no influence on the magnitude of K_m.

8. H. Lineweaver, D. Burk, **J. Am. Chem. Soc. 56,** 658 (1934).

9. Other methods for treatment of data conforming to equations of the type, $y = Kx/k + x$, are also available, e.g., see M. Dixon, E. C. Webb, loc. cit.; G. Johansen, R. Lumry, **Compt. rend. trav. lab. Carlsberg 32,** 185 (1961).

10. E. E. Rickli, A. S. Ghazanfar, B. H., Gibbons, J. T. Edsall. **J. Biol. Chem. 239,** 1965 (1964).

11. The course of the reaction was monitored by recording continuously the pH of a 0.15 M tris buffered enzyme mixture between 7.68 and 7.48 at 30°. Further details of the procedure will be described elsewhere.

12. F. E. Croxton, **Elementary Statistics with Applications in Medicine and the Biological Sciences** (Dover, New York, 1953).

13. $E = vK_e / (V_e - v)$.

14. B. H. Gibbons, J. T. Edsall. **J. Biol. Chem. 239,** 2539 (1964); D. V. Tappan, M. J. Jacey, H. M. Boyden. **Ann. N. Y. Acad. Sci. 121,** 589 (1964).

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