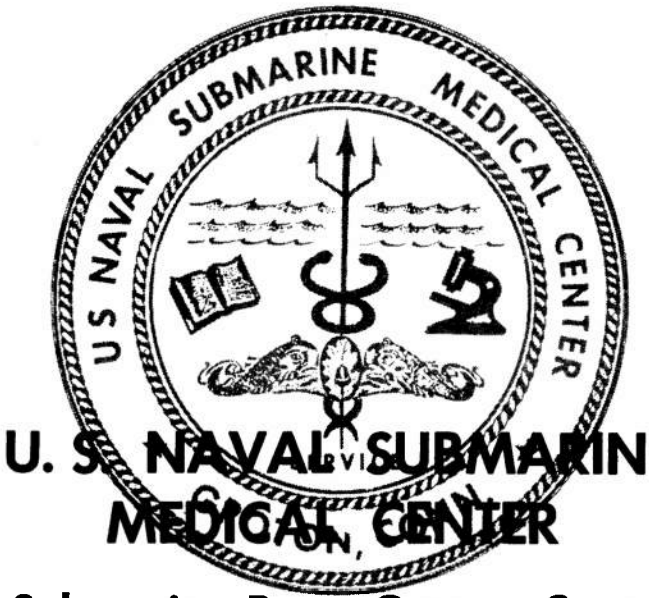

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ENZYME CONCENTRATION INFLUENCES ON ACTIVITY MEASUREMENTS

by

Donald V. Tappan

Bureau of Medicine and Surgery, Navy Department
Research Work Unit MR005.04-0053.04

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Naval Submarine Medical Center

14 May 1968

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SUBMARINE MEDICAL RESEARCH LABORATORY
NAVAL SUBMARINE MEDICAL CENTER REPORT NUMBER 522

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SUMMARY PAGE

THE PROBLEM

To develop an approach to the evaluation of enzyme assay results which adequately allows for the lack of proportionality commonly observed between reaction velocity and enzyme concentration in such measurements.

FINDINGS

Enzyme activity-concentration relationships fit a linear or simple curvilinear pattern either of which may be described quantitatively by constants derived from reciprocal plots of the data. A wide variety of results from many other kinds of experiments may be accurately quantitated by application of this principle.

APPLICATIONS

The method derived from handling data is useful in most experimental situations in which measurements of a dependent variable must be related to an independent variable. The procedure may be applied to linear and non-linear data and is applicable to manual or automated computational techniques.

ADMINISTRATIVE INFORMATION

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ABSTRACT

Most plots of enzyme concentration versus catalytic activity are linear, or fall on a single arm of an hyperbola, allowing treatment in the double reciprocal manner of "maximum rate of product formation" kinetics. Since curves of similar, if undefined, shape arise from many experimental measurements, the double reciprocal approach with its characterizing constants, can be employed to give an approximate description to many kinds of data.

ENZYME CONCENTRATION INFLUENCES ON ACTIVITY MEASUREMENTS

A recently proposed generalization by Srere (1) states that many if not most enzymes are present **in vivo** in concentrations far higher than those employed for **in vitro** measurements of enzyme activity. Tissue levels are in the order of 10^{-6} to 10^{-5} Molar(M), whereas assay procedures generally utilize 10^{-10} to 10^{-7} M, enzyme concentrations (1). To arrive at reasonably valid interpretations concerning the operation of enzymes within living tissues, methods must be devised for estimating their activities at physiological concentations.

As a partial answer to the problem of evaluation of concentration effects outside the ranges convenient for enzymic analysis, the author proposes the application of a system justifiable empirically as shown here and, in some situations, theoretically on the basis of the **maximum rate** enzyme kinetics developed by Darvey, Prokhovink and Williams (2). By employment of a series solution analysis to the rate equations for the generalized simple enzyme reaction,

$$E + S = X = E + P \qquad (1),$$

a relationship between reaction velocity, v, and enzyme concentration, E, at a constant substrate level was derived:

$$v = V_e E / (K_e + E) \qquad (2),$$

with V_e, maximum velocity, and K_e, a complex reaction constant (2).

The reciprocal of Equation 2,

$$1/v = 1/V_e + K_e/V_e\ (1/E) \qquad (3),$$

provides a linear plot of 1/v vs. 1/E in a similar manner to the double reciprocal procedure of Lineweaver and Burk (3) for treating substrate dependent kinetics. K_e/V_e is the slope of the resulting line with $1/V_e$ the intercept on the 1/v axis. Expressions analogous to the variations of the Michaelis equation may also be derived from Equation 2 which predict the linearity of v/E vs. v and of E/v vs. E (4). Statistical procedures are available for evaluating the reliability of estimates of the reaction constants calculated from such treatments (5).

To illustrate the employment of Equation 3, several sets of data have been taken from recent biochemical literature reporting effects of enzyme concentration on reaction velocity. From these data, summarized in Table I, the constants V_e and K_e were calculated and are shown with an estimation of the success achieved in each case in fitting the data to the linear model. The data were calculated according to the least squares procedure of Smith and Mathews (6) which minimizes the percentage error rather than absolute error in the squares of the deviations from the calculated line (7).

TABLE I.

MAXIMUM RATE CONSTANTS FOR DATA PUBLISHED FOR SEVERAL ENZYMIC REACTIONS

Reference	System	N*	K_e§	V_e§	S.D.**
8a	Thymidylate Synthetase	3	.3266	30.57	.1043
8b	Cellulase	9	.3725	111.7	.0215
8c	Rubredoxin (cytochrome c reductase)	14	1.869	5.275	.0507
8d	Bacterial reductase	9	298.8	10.43	.0311
8e	NAD+ kinase	5	.4754	.7145	.0253
9a	Protein synthesis by cytoplasmic S 150 (phenylalanine incorporation)	3	2.854	79.75	.0298
9b	" (valina incorporation)	3	89.27	10450	.0932
9c	" (leucine incorporation)	3	78.08	13250	.0362
9d	DPN-synthetase	8	533.2	1223	.0167
9e	PPi-glucose phophotransferase	7	240.7	.5146	.0348
10a	Carbamyl-P synthetase (+ ammonia)	8	8.167	5.701	.0931
10b	Methionyl-S RNA transformylase	4	36.70	41.63	.1478
10c	Intestinal enzyme converting β-carotene to retinal	6	23.53	110.5	.1183
10d	Elastase (trypsin activated)	3	.9131	.5781	.0132
10e	pH 5 enzymes. Leucine incorporation into polyribosomes	6	4.415	81.14	.2055
10f	Ibid corrected for "blank," see text	5	314.8	145.5	.0880

* Number of data points available for calculations.

§ K_e and V_e values in each case expressed in units employed on x and y axes of plots indicated in references.

** Relative standard deviation (7). All calculations include an error component introduced by uncertainties in reading of graphic data.

Data reported in the table were chosen because of their widely varying abilities to conform to the relationship,

$$v = kE \quad (4),$$

which is the basis on which most enzymes are quantitatively estimated (11). The calculated standard deviations indicate that all of the data may be described by Equation 3 in a general way; while with notable exceptions, those sets composed of larger numbers of points define their curves in a more accurate manner. The overall standard deviation for the procedure is 7.5% with errors of only 2-3% for some of the curves.

The plots shown in Figure 1 generalize the various sets of data represented in the table. Since scatter of the data about the calculated curves accounts for much of the variability reported, smooth curves have been drawn to better demonstrate the logic for forcing all of the tabulated data into the reciprocal model, Eqn. 3. Figure 2 shows graphically the results of expressing the information of Figure 1 in such a manner (14).

Plots of many reciprocal curves as in the second figure and calculations of the constants for describing such curves have led to the empirical conclusion that most smooth curves which do not approach a point of inflection may be approximated with at least fair accuracy by Equation 3. For results to be meaningful in terms of the enzyme concentration - velocity relationship, however, curves should be shifted to pass through the origin of the plots as illustrated by nos. 10e and 10f in Table I. In this case the value at zero enzyme concentration in the original data, #10e, was subtracted from each succeeding value thus effecting a "blank" correction and markedly improving the relative error of the estimation, 10f.

The data of curves C of the figures represent measurements of the activity of an electrophoretically purified bovine carbonic anhydrase B (12) eluted from a 2.5 cm polyacrylamide gel column (13). An analytical system was employed in which substrate was provided by a carefully regulated

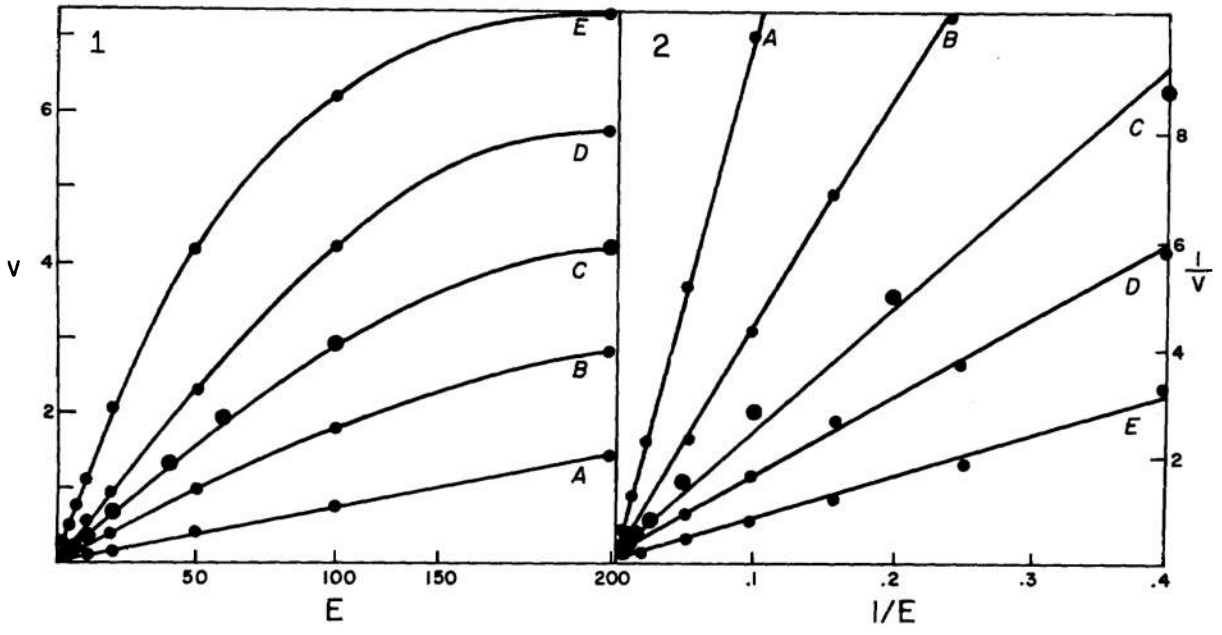

Fig. 1. Generalized Curves Representing Data of Table I Projected onto Plot of Bovine Carbonic Anhydrase, Curve C, for which v = moles x 10^3/l-sec. and E = µl enzyme solution equivalent in activity to 1/48 dilution of whole blood (17).

Fig. 2. Double Reciprocal Plots of Data of Fig. 1. For C, $K_e = 212.85$, $V_e = .008687$ (6).

stream of CO_2 bubbling into the reaction vessel. The course of the reaction was monitored by recording continuously the pH of a tris-acetate (0.15 M) buffered enzyme mixture between 7.68 and 7.48 at 30°. Velocities were corrected for the non-enzymic component of the reaction occurring simultaneously. Carbonic anhydrase is an example of an enzyme system for which investigators over a period of many years (14) and employing widely different reaction conditions have often not obtained activity values proportional to concentration of enzyme.

The advantage of describing non-linear data as in curves B to D of Figure 1, by an expression characterized by two readily obtainable constants is quite clear, especially when electronic or other automated means are employed to assist with the handling and reduction of data. On the other hand, the relative merits of treating "linear" data as in curves A by the reciprocal routine, I, must be considered as an important exceptional situation before a general evaluation of the procedure can be reached since many data conform, approximately, to this pattern. The reciprocal of Equation 4, $1/v = 1/k\ (1/E)$, is linear and predicts the relationship between the curves A in the figures. Equation 3 reduces to this form when V_e is large or Equation 2 reduces to Equation 4 when E is small with respect to K_e. Each of these conditions is met as v versus E approaches perfect linearity.

To study the foregoing problem further with experimental data, constants were calculated according to Equation 4 or more generally, $y = a + bx$, **II** (15), for those reaction curves of Table I most nearly approaching the linear form, #'s 8d, 8e, 9b, 9c and 9e, with relative S.D.'s for the resulting errors in calculated v or y values obtained. Mean S.D. for the five data sets was .0650 by **II** compared to .0587 by the procedure described for the table, **I**. These findings substantiate correlated studies which indicate that with any slight systematic curvature, either concave or convex, the application of **I** leads to superior results. Only when arcs of more than one curve are involved which are situated symmetrically around the calculated line or when points are distributed in an absolutely random manner about the line does **II** prove advantageous. If perfect linear data are employed, only **II** gives useful information, but if one or more points deviate from the perfect line by any slight amount, the x vs y plot becomes effectively curved and, except under the very restricted conditions described, calculation **I** becomes most accurate. Experimental measurements, of course, seldom if ever furnish perfect data.

In view of the superiority of **I** over approach **II**, especially when constants characterizing the respective lines are calculated according to the latest squares principle employed here (6), I suggest that enzyme activity versus concentration data be plotted routinely or calculated in the reciprocal manner. Particularly when calculations are made without the aid of computers, it is usually more satisfactory to calculate E for assay results at unknown enzyme concentrations from $1/E = V_e/K_e(1/v) - 1/K_e$ than from $E = vK_e/(V_e - v)$ since K_e is large in situations approximating the A curves of Figures 1 and 2 allowing $1/K_e$ to be neglected for many calculations; whereas V_e/K_e is readily available, being, in fact, the slope constant of Equation 4. Small inaccuracies in the individual values of V_e of K_e produce relatively serious errors in calculated values of E by the alternative method.

For those situations in which experimental data are satisfactorily described by Equations 2 or 3, the calculation of enzyme activities at tissue concentrations by these expressions may reasonably be considered. Very different information from simple extrapolations of activity data acquired at low concentrations, however, is obtained from these calculations. Certainly the physiological significance of such estimates varies in proportion to the nearness of the **in vitro** assay conditions to the situation within the tissues. The carbonic anhydrase data of Figure 2 will illustrate the possibilities and pitfalls of calculation of activities at high enzyme levels.

Following the leadership of Roughton (16), it has frequently been pointed out that the activity of erythrocyte carbonic anhydrase

utilized as the blood passes through the lungs is a very small fraction of the potential activity measured in blood hemolysates. Although the data described by curves C of the figures represent a bovine enzyme preparation, similar information is forthcoming from the more active forms of the human enzyme. Calculation of activity at the concentration within whole blood from the constants presented shows only 4.1% of the activity expected from multiplication of the activity at 100 μl of diluted enzyme preparation by the total dilution of the sample (17). The activity predicted within erythrocytes is thus about 2% of the apparent potential activity.

In contrast to such calculated data, however, Kernohan, **et al.** (18) have recently reported reaction rates, obtained by rapid measuring techniques, proportional to enzyme levels at concentrations approaching those in intact erythrocytes. The statement remains valid, however, that the enzyme does not operate physiologically at the level predicted directly from measurements made at low enzyme concentrations. Many other questions concerning transport of substrates, localization of enzymes within the cell and duplication of the milieu of the cell must be dealt with before the discrepancies in these data can be finally resolved.

Since many processes in chemistry, biology and other experimental disciplines may be described by the equation,

$$y = Y_x x/(K_x + x) \qquad (5),$$

and even more by the relationships depicted generally by the curves of Figure 1, reciprocal treatment of various data other than those discussed here may prove useful. Spectrophotometric data that do not follow Beer's law, for example, may usually be given a quantitative status by application of the reciprocal form of Equation 5. Although the following applications are not more important than many others, time vs. extent of reaction plots, activation or inhibition plots and plots describing binding reactions occur frequently in biochemical literature. The constants Y_x and K_x not only may define these curves which are frequently hyperbolic or nearly hyperbolic, but also may themselves have useful meaning. In the first case, for example, Y_t defines maximum extent of reaction and K_t describes reaction half time, T/2.

While the reciprocal plotting of curvilinear data cannot be used without further considerations to predict or confirm reaction mechanisms or other descriptive models, it would seem to have wider application than presently realized. Numerous results that have hitherto been presented qualitatively may be given a more quantitative form providing the generality and limitations of the technique are not overlooked.

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13. ABSTRACT

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Enzyme activity measurement						
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