Interpretation of the Kinetics of Consecutive Enzyme-catalyzed Reactions

STUDIES ON THE ARGINASE-UREASE SYSTEM

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Physicochemical properties of beef liver arginase are reported, particular attention being given to its state of aggregation in the concentration range encountered in enzymic assays. It is shown that a species of molecular weight 114,000 is the operational kinetic unit. Evidence is also provided that arginase does not associate heterogeneously with urease, and therefore, in the absence of macromolecular interactions, the arginase-urease couple provides a suitable experimental system to test the applicability of theory previously developed to guide the interpretation of coupled assay results. Application of the theory led to values of the Michaelis constant and maximal velocity describing the first reaction in the sequence, catalyzed by arginase, which agreed within experimental error with the corresponding values obtained by studying the arginase-catalyzed reaction alone. Comment is also made on the product inhibition of arginase by ornithine, which must be considered in the comparison of experimental results describing the time course of a coupled assay with theoretical solutions obtained by numerical integration.

Several descriptions of the conduct of coupled assays have suggested that, in the elucidation of the kinetic parameters governing the first reaction, large excesses of the consecutive enzymes should be employed, the approach being typified by the work of McClure (1) and Easterby (2). It has been pointed out (3) that this requirement cannot be met with certain systems and places an undue restriction on the study of others. In this previous communication (3), a theory was developed which gave the concentration of final product as an explicit function of time which is valid regardless of the relative mixing concentrations of the enzymes, provided the approximation is met that all enzyme-substrate complexes are in a steady state. This suggested an analysis procedure based on determination of limiting tangents which would lead to evaluation of the kinetic parameters characterizing the first reaction in the sequence. A major purpose of this work is to show that the previously developed theoretical concepts (3, 4) apply to the interpretation of the experimental system in which arginine is converted to ornithine and urea, and the urea hydrolyzed to form ammonium and bicarbonate ions, the respective reactions being catalyzed by arginase and urease.

EXPERIMENTAL PROCEDURE

Materials—Arginase was prepared from young beef liver by a modified procedure described by Harell and Sokolovsky (5). Batches of nonpathological liver (2 kg) were treated according to the first four steps described in detail by the previous workers (6) leading to a protein concentration (7.3 g in 64 ml of 0.01 M Tris-HCl, 0.05 M MnCl₂, pH 7.5) which possessed 68% of the activity of the original extract. The next step of the purification utilized a Sephadex G-100 column, which has superior flow characteristics to the Bio-Gel P-150 stationary phase suggested earlier: the column dimensions were 4 × 100 cm and 8-ml samples were eluted with 0.01 M Tris-HCl, 0.05 M MnCl₂, pH 7.5, 4°C at a flow rate of 40 ml h⁻¹. Enzyme activity measurements of the eluted fractions (13 ml) led to the discarding of the first 80 ml of eluant which contained protein and the retention of the next 90 ml which contained 90% of the applied arginase activity (specific activity 125 to 300 units mg⁻¹). The Sephadex G-100 chromatography step was repeated on eight successive batches, the resulting 720 ml of pooled eluant being concentrated to 20 ml by ultrafiltration using a UM10 membrane, without loss of activity. The final two steps, SP-Sephadex chromatography and isoelectric focusing, were conducted as described previously (5). Removal of the ampholyte by ultrafiltration led to a solution of arginase in 0.01 M Tris-HCl, 0.05 M MnCl₂, pH 7.5 which retained its activity for several weeks on storage in the cold: the final yield was 33 mg of arginase with specific activity 720 units/mg, which represented 10% of the original total activity.

Specific activities were determined in the environment specified by Harell and Sokolovsky (5) utilizing the spectrophotometric procedure developed by Ward and Srere (6). Protein concentrations were estimated spectrophotometrically at 278 nm with ε₁ₐ₉₅₅ of 0.96 (5). Urease (jack bean) of specific activity 110,000 Summer units g⁻¹ (7) was obtained from Sigma Chemical Co. Sedimentation velocity analysis of the sample revealed three peaks of the following sedimentation coefficients and proportions, 19 S (75%), 27 S (20%), and 32 S (5%) corresponding to a mixture of noninteracting monomer, dimer, and trimer (8). Since these oligomeric forms appear to possess closely similar enzymic activities (9, 10), the sample was used in kinetic experiments without further purification.

AR reagents were used throughout, the L-arginine and L-ornithine (free base) being obtained from Calbiochem, the urea and MnCl₂·2H₂O from Mallinkrodt, and diacetylmonoxime and antipyrine from Sigma Chemical Co.

Kinetic Experiments—In experiments with arginase alone, arginine in 0.05 M Tris-HCl, 0.02 M NaCl, 0.01 M MnCl₂, pH 7.5, of concentra-
tion cited in the text was used as initial substrate and all experiments were conducted at 25°. Two methods were employed to follow the reaction. One method involved monitoring the concentration of urea produced by the diacyl monoxime method (11), after derivatization of the reaction by the addition of 2 M HCl. The assay protocol was to mix 2 ml of reaction mixture with 1 ml of Solution I (0.25 g dl⁻¹ of diacetyl monoxime in 3% (v/v) acetic acid) and 1 ml of Solution II (0.8 g dl⁻¹ of antipyrine in 80% (v/v) sulfuric acid), and to develop the chromatophore in a light-free chamber at 100° for 30 min, prior to recording the absorbance at 460 nm. The second method involved measuring the deflection of the substrate arginine, spectrophotometrically at 205.7 nm (16), using a Cary 14 spectrophotometer with a slit setting of 0.8 mm.

In experiments with urease alone, the enzyme was incubated for 90 min in the above specified pH 7.5 buffer prior to its addition to urea solutions in the same buffer. The ammonium ions produced were separated from the reaction mixture by employing the Conwy microdiffusion method (12). One milliliter of the reaction mixture was placed in the outer compartment and 1 ml of 60% (w/v) potassium acetate was added to liberate the ammonia: the inner compartment contained a solution of 2% (w/v) boric acid and 0.0001% methylene blue, 0.0004% methyl red, the absorbed ammonia being estimated by back-titrations with 0.01 M potassium biurate.

Polyacrylamide Gel Electrophoresis—Experiments were conducted according to the method of Davis (13) employing 7.5% acrylamide gels in glass tubes (0.5 x 7.5 cm) at 25° in a Buchler apparatus. The electrode buffer was 0.006 M Tris, 0.037 M glycine, pH 8.3; and 0.001% bromophenol blue was used as a migration marker.

Frontal Gel Chromatography—Sephadex G-100 columns (1.27 x 45 cm) were equilibrated with 0.05 M Tris-HCl, pH 7.5. Sufficient volumes of arginase solutions (~25 ml) were applied to the column to ensure a plateau region in the elution profile, according to the design of frontal analysis (14, 15). Weight average elution volumes were determined from the median bisectors of the advancing fronts found by assaying the collected fractions (0.5 ml) for arginase activity. The void volumes of the columns, used in the calculation of $K_v$, defined by Laurent and Killander (16), were determined with the use of blue dextran 2000.

Ultracentrifugation Studies—Sedimentation velocity experiments were performed in a Spinco model E ultracentrifuge at 20,006 rpm, the latter being of the meniscus depletion type (18). Apparent specific density meter at 20 * 0.01".

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RESULTS

Four criteria were used for homogeneity of the arginase samples used in this work. First, a single band at pH 5.9 ± 0.05 was observed in isoelectric focusing in accordance with the reported isoelectric point of the enzyme (5). Second, polyacrylamide gel electrophoresis revealed a single band with an $R_f$ of 0.28. Thirdly, a series of sedimentation velocity experiments at pH 7.5 revealed a single peak typified by the result shown in Fig. 1A and to the specific that $\bar{s} = 6.15 (1 - 0.069 c) \times 10^{-13}$ s where c is in grams per dl. Use of this relation permitted an analysis of the boundary shape (19, 20) shown in Fig. 1A. The essential analytical plot in the procedure is that of Equation 1 of Baldwin (20), which in the present instance proved to be linear, providing another index of the homogeneity of the sample with respect to hydrodynamic parameters.

Fig. 1B shows the result obtained in the meniscus-depletion experiment, the linearity of this plot and that obtained from the experiment at 6,000 rpm providing the fourth index of sample homogeneity. The slopes of these lines determined by least squares linear regression led to an estimate of the molecular weight of 114,000 ± 3,000 which may be compared with the values of 112,000 and 115,000 reported by Harell and Sokolovsky (5). In these calculations a value of 0.734 g⁻¹ ml⁻¹, obtained from density measurements, was used for the partial specific volume. From Fig. 1 it is clear that arginase exists as a single noninteracting species in the concentration range 1.0 to 0.01 g dl⁻¹. This range does not encompass that usually employed in enzymic assays (~5 x 10⁻⁹ M) and the lower value just bears on the range of concentration encountered in the cell (21). For this reason, it was decided to examine the state of aggregation of arginase at lower concentrations by the method of frontal analysis chromatography. Fig. 2A presents a typical elution profile from such an experiment illustrating the existence of a plateau region and the method used for determining the weight average elution volume: Fig. 2B summarizes the derived partition coefficients (16) obtained in a series of experiments as a function of applied (plateau) concentration. The invariance of the $K_v$ value with concentration shows that the operational unit of arginase continues to be of molecular weight 114,000 even at concentrations as low as 5 x 10⁻⁸ g dl⁻¹.

A second aspect concerning interactions which might complicate the interpretation of coupled assay results (4) is the possibility of a heterogeneous association between arginase and urease. Sedimentation velocity analysis in pH 7.5 Tris buffer on mixtures of these enzymes showed that no chemical interaction occurred between them, the sedimentation coefficients of the peaks and their relative proportions being those observed in control experiments conducted with the enzymes separately.

All kinetic studies were performed in the same pH 7.5 Tris buffer as used for the physical characterization; this medium was selected on the basis of the pH dependence of activity of both arginase and urease (22, 23). The first set of kinetic experiments were performed on the enzymes studied individually to establish their basic steady state kinetic parameters.

![Graph](http://www.jbc.org/)
For several preparations of arginase, and with each assay performed in duplicate, double reciprocal plots of initial velocity versus substrate concentration (arginine of concentrations 1 to 10 mM) proved to be linear, yielding values of $K_m = 5 \pm 1$ mM and $k_{cat} = 8.2 \times 10^4$ min$^{-1}$. In this connection it is noted that there was close agreement between results found by assaying urea formation by the diacetylmonoxime method and arginine depletion spectrophotometrically. Further kinetic studies with arginase were directed at exploring the effects of assaying urea formation by the diacetylmonoxime method and noted that there was close agreement between results found by tions 1:10 mM) proved to be linear, yielding values of $K_m = 5 \pm 1$ mM. In the set of control experiments in which urease was studied alone with urea as substrate (1 to 10 mM), Lineweaver-Burk plots were linear and values of $K_m = 3 \pm 1$ mM and $k_{cat} = 1.66 \times 10^4$ min$^{-1}$ were derived, the latter referring to the rate of depletion of urea on the molar scale. The obedience of the mechanism to the basic Michaelis-Menten format, which has been observed by several other workers (10, 24, 25), supports the view that the coexistence of various polymeric forms of urease does not complicate the kinetic interpretation within experimental error. The compounds, arginine, ornithine, urea, and ammonium ions in the concentration range (0 to 10 mM) and arginase were shown to be neither inhibitors nor activators for the catalyzed urea hydrolysis: the well known inhibition by excess substrate (25) occurs in a concentration range of urea (>0.4 mM) much higher than employed in these studies. Indeed in relation to the components of a coupled assay, the only species which complicates the urease-catalyzed hydrolysis of urea is Mn(II), which with incubated urease solutions proved to act as a noncompetitive inhibitor, the Michaelis constant determined in its presence (10 to 100 mM) remaining unchanged (4 $\pm$ 1 mM) but with apparent maximal velocity varying linearly with the MnCl$_2$ content of the solution. While the $k_{cat}$ value reported above refers to the specified buffer containing 10 mM MnCl$_2$ in relation to coupled assays, the maximal velocity pertaining to urease was determined separately for each sample of enzyme employed.

Although several coupled assay experiments were performed (with arginine concentration the variable in any given set), it is convenient first to present the results of a single study. A solution containing both enzymes was incubated at 25° for 90 min prior to the addition of 1 ml of it to 1 ml of a solution of arginine. The reaction was terminated at a specified time with 0.5 ml of 2 M HCl, the procedure being repeated to obtain results at various times. The ammonium ions produced were separated from the reaction mixture by the Conway microdiffusion method employing 1% (w/v) boric acid in the center chamber as absorbent, and estimating the ammonium ion concentration by direct Nesslerization. Fig. 3 presents a plot of the molar concentration of ammonium ions produced, $[S]$, as a function of time, $t$, obtained with a mixture containing 5.6 $\times$ 10$^{-7}$ M arginase, 1.81 $\times$ 10$^{-2}$ M urease, and an initial arginine concentration of 10 mM. The broken curve attempts to average these results and it is clear from it that the initial velocity of product formation is zero as predicted theoretically (3). This theoretical study suggested that, for a coupled system involving two enzymes, the data be replotted with time squared as

![Fig. 2. Frontal analysis chromatographic results obtained with arginase in 0.05 M Tris-HCl, 0.02 M NaCl, 0.01 M MnCl$_2$, pH 7.5 at 20°.](http://www.jbc.org/)

![Fig. 3. Results of a coupled assay in which arginase (6.6 $\times$ 10$^{-7}$ M) and urease (1.81 $\times$ 10$^{-2}$ M) catalyzed consecutive reactions in which the initial substrate arginine (10 mM) was converted ultimately to the final product, ammonium ions, the molar concentration of which is denoted by $[S]$. The experiment was conducted at 25° in the buffer 0.05 M Tris-HCl, 0.02 M NaCl, 0.01 M MnCl$_2$, pH 7.5, further details being given in the text. The solid points and broken curve refer to a plot of $[S]$ versus time $t$, while the points (x) are the same values of $[S]$ plotted versus $t^2$. The solid line was obtained by linear least square regression of the latter data.)
the abscissa which yields the solid line shown in Fig. 3. It is clear that this plot is linear over the time domain examined and that its slope may readily be determined as $2 \times 10^{-5}$ M min$^{-1}$. Since 2 mol of ammonia are formed for each mole of urea depleted, a quantity $m$ of value $1 \times 10^{-5}$ M min$^{-1}$ is defined as the equivalent slope in terms of urea depletion, consistency thereby being maintained with the reference scale used previously to define the maximal velocity, and hence $k_{cat}$ of the urease-catalyzed reaction. Corresponding values of $m$ and $[S]_0$ were obtained from a series of such experiments employing initial arginine concentrations in the range 1.33 to 10 mM. In principle those values may be employed to evaluate the steady state kinetic parameters pertaining to the first reaction in the sequence when the kinetic parameters pertaining to the second reaction have been evaluated separately (3).

It is relevant therefore to derive the magnitudes of the Michaelis constant ($K_v$) and maximal velocity ($V_i$) pertaining to arginase on this basis; for then such values may be compared with those obtained in studies with arginase alone in order to test the applicability of the coupled assay theory (3).

For a coupled assay system involving two enzymes of the Michaelis-Menten type with no heterogeneous enzyme-enzyme interaction occurring, it has been shown that

$$
\frac{1}{m} = \frac{d[S]/2}{d(t^2)} = \frac{\frac{V_i}{K_v} [S]_0}{2K_v v_0 [S]_0} = m
$$

where in the present instance, $V_i$ and $K_v$ refer to the maximal velocity and Michaelis constant for urease. A plot is therefore suggested of $1/m$ versus $1/[S]_0$, which is shown in Fig. 4. It is clear that the linearity of this experimental plot is in accord with Equation 1 which predicts that the ordinate intercept ($6.3 \pm 0.5 \times 10^4$ M$^{-1}$ min$^2$) equals $2K_v V_i$, while the intercept on the abscissa ($-1.7 \pm 0.3 \times 10^4$ M$^{-1}$) equals $1/K_v$. In this study $V_i$ equalled $3.0 \times 10^{-4}$ M min$^{-1}$ and with the use of $K_v = 4$ mM the values obtained from the coupled assay results for $K_v$ and $V_i$ are $6.0 \pm 1$ mM and $4.3 \times 10^{-4}$ M min$^{-1}$, respectively. The value of $K_v$ agrees within experimental error with that of $5 \pm 1$ mM obtained when arginase was studied alone, and likewise there is reasonable agreement between the $k_{cat}$ for arginase of $7.7 \times 10^{-4}$ M min$^{-1}$ derived from the coupled assay and the value of $8.2 \times 10^{-4}$ M min$^{-1}$ reported previously. This agreement is also illustrated in Fig. 4 by comparing the lines calculated by weighted least squares linear regression of the experimental results, with the broken lines, computed on the basis of Equation 1 with the kinetic parameters determined for the individual enzymes. The coupled assay experiment was repeated twice with similar composition of original mixed enzyme solutions and yielded $K_v$ for arginase of $6.0 \pm 1$ mM and $5.0 \pm 1$ mM. There was also agreement between the derived $V_i$ values and those obtained in separate studies on arginase alone.

Fig. 5 presents results pertaining to the time course of the sequential reactions obtained with a reaction mixture of the same composition as specified in Fig. 3. The urea concentrations shown were determined on aliquots by the diacetylmonoxime method, the ammonia ion concentration by Conway microdiffusion analysis, and the arginine concentrations by difference according to the conservation of mass requirement. The solid lines are the numerical solutions of the set of differential equations which describe the sequential reactions in terms of two Michaelis-Menten mechanisms with competitive inhibition of arginase by ornithine. The parameters used in obtaining the numerical solutions, performed with an analogue computer, are cited in the caption which provides a summary of these values derived from both coupled assay and individual kinetic studies. The close agreement in Fig. 5 between experimental and calculated values shows that the postulated mechanism (including the absence of enzyme-enzyme interaction) is valid even for long times.

**Discussion**

Three findings in this work show that the arginase-urease couple is a particularly suitable system with which to examine coupled assay theory (3). Thus, the enzymes do not associate in the pH 7.5 buffer employed; arginase does not dissociate even

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**Fig. 4.** Results pertaining to the arginase-urease couple studied in the environment reported in Fig. 5. $m$ was calculated from the slope of $[S]_0$ versus $t^2$ plots illustrated in Fig. 3, $[S]_0$, being the corresponding initial substrate (arginine) concentration. The solid points are experimental and the solid line was calculated from these by the method of weighted least squares linear regression. The broken line was calculated using Equation 1 and steady state kinetic parameters found by studying the kinetics of reactions catalyzed by arginase and urease individually.

**Fig. 5.** The time course of the consecutive reactions catalyzed by arginase and urease with an initial substrate concentration (arginine) of 10 mM. Other experimental parameters were those reported in Fig. 3. The concentrations of species are denoted as follows: ○, arginine; ▽, urea; and O, ammonium ions. In the latter case the points have been plotted as $[S]_0/2$ for consistency with Equation 1. The solid line is an analogue computer solution obtained by numerical integration of the set of differential rate equations describing the two Michaelis-Menten reactions and the competitive inhibition of arginase by ornithine $K_v = 4$ mM. These solutions were obtained with the aid of an analogue computer. The values of the steady state kinetic parameters employed in the simulation were as follows: for arginase, $K_v = 6.0$ mM, $V_i = 4.3 \times 10^{-4}$ M min$^{-1}$ ($k_{cat} = 7.7 \times 10^4$ min$^{-1}$); and for urease $K_v = 4$ mM, $V_i = 3.0 \times 10^{-4}$ M min$^{-1}$ ($k_{cat} = 1.66 \times 10^5$ min$^{-1}$).
at high dilution (Fig. 2B) and the mechanisms of both enzymes are describable in terms of a simple Michaelis-Menten model. The comparison of theoretical and experimental curves shown in Figs. 4 and 5 suffices to illustrate that the kinetic parameters pertaining to the first enzyme, derived from coupled assay results, do indeed describe the first reaction. There is however no question that it would be preferable, in a general sense, to establish kinetic parameters on any given enzyme by studying it alone, since as Equation 1 clearly illustrates the results of a coupled assay reflect also the properties of the second enzyme and additional error is therefore introduced associated with the kinetic parameters of this second enzyme. In the present instance, separate studies on the individual enzymes were possible, the system being chosen deliberately to illustrate the applicability of a theory which might be the only available basis for the interpretation of certain systems where neither the substrates nor products can be monitored readily.

The general applicability of the method depends on the ability to determine the slope m from plots such as shown in Fig. 3. The time domain over which linearity might be expected depends on the mixing ratios of the two enzymes concerned, the linear region being shortened with an excess of the second enzyme. This arises since the equation which describes the concentration of final product as a function of time is a polynomial (3); but in any event in the limit as \( t^2 \to 0 \) Equation 1 is rigorous and thus, provided \( m \) may be defined as a limiting slope, the procedure is applicable to any initial mixing composition of enzymes. Clearly in the present work there was no difficulty in defining \( m \) (Fig. 3). Another example is provided by the work of Easterby (2) where a replot of the data shown in his Fig. 2 with \( t^2 \) as the abscissa shows that \( m \) may be defined with reasonable precision, even though the concentration of the second enzyme was sufficiently large to lead to a transient time of only 40 s. It also follows from the use of a limiting slope at \( t^2 \to 0 \) that product inhibition, such as the inhibition of arginase by ornithine, is not reflected in Equation 1; determination of the basic steady state kinetic parameters therefore being possible without consideration of such complication. This may be shown readily by utilizing Equations 1 and 2 of Ref. 3, the former equation being written to include the standard term for linear competitive inhibition: it follows that \( d[S]_1/d(t^2) \) evaluated at \( t = 0 \) does not contain the inhibition constant and, therefore, that Equation 1 continues to apply. Certainly higher order terms in the Maclaurin polynomial contain \( K_i \) and it is, therefore, of interest to enquire as to the reason for the maintenance of linearity over an extended time domain in Fig. 3. The answer is to be found by inspecting Fig. 5 where it is seen that the amount of ornithine produced in this time domain is negligibly small with respect to its inhibitory action. In contrast at longer times the fit shown in Fig. 5 was only possible by considering the inhibitory effect of ornithine with \( K_i = 3 \pm 1 \) mM. This provides an illustration of the way in which solutions obtained by computer simulation, using numerical integration or analogue methods, may be utilized together with basic steady state kinetic parameters, derived by analyzing experimental results as \( t^2 \to 0 \) (\( n \) being the number of enzymes involved in the sequence), in elucidating a complete mechanism.

It will not have escaped the reader’s attention that the previous theoretical treatments (3, 4) and the present experimental study considered only sequences in which each enzyme acted on a single substrate, whereas it is well established that many enzymes act on two substrates, their detailed mechanisms being diverse (26). It is important therefore to show that the present approach directly applies to these more complicated systems when kinetic experiments are designed following usual practice to ensure saturating concentrations for all but one of the substrates for each of the enzymes. This generalization is established in the “Appendix” which follows and it is hoped that it, together with the illustration provided by the arginase-urease couple, may assist in the further analysis of coupled assays of diverse kind.

APPENDIX

It is desired to provide a general formulation of an equation analogous to Equation 1, which will permit the analysis of experimental results obtained in the form of the time dependence of final product formation from a linear sequence of reactions catalyzed by enzymes acting on more than one substrate. Consider the reaction scheme,

\[
S_1 \rightarrow E_1 \rightarrow S_2 \rightarrow E_2 \rightarrow \ldots \rightarrow S_n \rightarrow E_n \rightarrow P
\]

where there are \( n \) enzymes in the sequence each catalyzing a Bi Bi reaction. The symbols \( S \) and \( A \) are used to denote substrates forming products denoted by \( R \) and \( P \), where \( P \) is the particular product monitored by the experimenter. The appropriate set of differential equations describing this kinetic scheme is,

\[
\frac{d[P]}{dt} = H[S]_1[A]_1[P]_1[Q]_1 \ldots + \frac{d[P]}{dt} = H[S]_2[A]_2[P]_2[Q]_2 \ldots
\]

where the functions \( F, G, \) and \( H \) are written in terms of the species of variables concentrations to encompass all Bi Bi mechanisms (26) including reversible reactions. It has been shown that the concentration of final product at any time \( t \) may be expressed as the Maclaurin polynomial (3),

\[
[P] = \sum_{k=2}^{n} [P]_0 \left( \frac{a}{k} \right) k
\]

where in the general term \( \alpha > \beta > \gamma > \ldots \) is the order of the derivative and the subscript 0 to this derivative implies evaluation at \( t = 0 \). It is now noted that the first two terms of Equation 3 are zero since \([P] = 0 \) at \( t = 0 \) and for all Bi Bi mechanisms the numerator of the expression for \([P]_0 \) is the form \( \alpha_1 [S]_1 [A]_1 - \alpha_1 [R]_1 [P]_1 \) with \([S]_1 \), \([P]_1 \), and \([R]_1 \) all being zero at \( t = 0 \) and \( \alpha_1 \) and \( \alpha_2 \) being products of individual rate constants. Accordingly, Equation 3 may be rewritten as,

\[
[P] = \sum_{k=2}^{n} [P]_0 \left( \frac{a}{k} \right) k
\]

Application of the chain and product rules of differentiation shows that for \( k < n \),

\[
[P]_0 = \left| \frac{[d[S]_1/a_t]}{[d[S]_0/a_t]} \right| \left| \frac{[d[S]_2/a_t]}{[d[S]_1/a_t]} \right| \left| \frac{[d[S]_3/a_t]}{[d[S]_2/a_t]} \right| \ldots
\]

where \( s_n + 1 = P \). A general expression relevant to a system involving \( n \) enzymes is available by combining Equations 4 and 5, the use of which may be most simply illustrated by writing the combination for the case \( n = 2 \) to obtain,
Equation 6 first may be considered in relation to the arginase-urease system for which, from Equation 26, \( \frac{d[S_1]}{dt} = V_s[S_1]d(K_s + [S_1]_b) \), since all variables in Equation 2b are zero at \( t = 0 \) except for \([S_1]\) which is functionally related to the rate by the Michaelis-Menten expression. The remainder of the product in Equation 6 is seen from Equation 2c to be \( V_sK_s \), and hence it is apparent that the more general Equation 6 is consistent with Equation 1. For sequences involving Bi Bi reactions, application of Equation 6 requires explicit definition of the functions \( F, G, \) and \( H \) in Equation 2.

However two important general points emerge. First, from Equation 26 it follows that \( \frac{d[S_1]}{dt} = -\frac{d[S_1]}{dt} \) whereupon \( m \) is seen to be a product of the kinetic expression pertaining to the depletion of initial substrate by the first enzyme acting alone, and half of the first order rate constant \( V_s/K_s \) of the second reaction. The latter expression is valid only when \([A_1]\) is in excess so that it may be regarded as a constant in the differentiation of the function \( H \) with respect to \( S_2 \). Secondly, it follows that an analytical function is available relating \( m \) to \([S_1]_b\) which may be utilized to obtain steady state kinetic parameters relevant to the first enzyme, provided the second enzyme is studied alone in the presence of excess substrate \( A_2 \) to obtain \( V_{2s} \) and \( K_s \) independently. The use of this relation would also require that the coupled assay be performed in the presence of excess of \( A_1 \) (or of \( S_1 \)) so that the initial velocity expression for the first enzyme becomes a function only of \([S_1]_b\) (or \( [A_1]_b \)). The practice of utilizing an excess of one substrate in studying the kinetics of a two substrate enzyme to define the kinetic parameters pertaining to the variable substrate is familiar to enzyme kineticists.

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