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Analysis of enzyme kinetics by using integrated rate equations

Arginine decarboxylase

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We have used an integrated rate equation to analyse the reaction catalysed by the inducible arginine decarboxylase from *Escherichia coli* B. The stoichiometry

$$\text{Arginine} \rightarrow \text{agmatine} + CO_2$$

is the simplest of the multiple-substrate/multiple-product cases. Twenty-one time courses were carried out at various initial concentrations of arginine and agmatine, and were then fitted to the integrated equation by using appropriate analytical procedures. Values were obtained for six of the seven possible kinetic constants, corresponding to $k_{cat.}$, K_{Arg}, the terms for competitive inhibition by agmatine, by CO_2 and by agmatine and CO_2 together, and the term for uncompetitive inhibition by agmatine. The uncompetitive constant for CO_2 was indeterminate. Our results indicate that it is both practical and experimentally economical to obtain kinetic constants from full time courses.

INTRODUCTION

We have used the inducible arginine decarboxylase of *Escherichia coli* B (Blethen *et al.*, 1968; Boeker & Snell, 1968; Boeker *et al.*, 1969; Nowak & Boeker, 1981) to test the feasibility of determining enzyme kinetic parameters by fitting entire progress curves to the integrated rate equation (Boeker, 1984*a*,*b*, 1985). Arginine decarboxylase is a pyridoxal 5′-phosphate-dependent enzyme that cleaves arginine to CO_2 and agmatine. The pH optimum is 5.2. Under normal atmospheric conditions the CO_2 is immediately evolved; the reaction is essentially irreversible.

Arginine decarboxylase was chosen for this study because its stoichiometry, $A \rightarrow P + Q$, irreversible, is the simplest of the multiple-substrate/multiple-product cases. Also, the standard assay is a discontinuous radioactive measurement of CO_2. Assays of this type are inherently tedious and subject to experimental error, factors that discourage kinetic analysis by initial rates.

The general derivative rate equation for this stoichiometry (Wong & Hanes, 1962; Boeker, 1984*b*) is:

$$\frac{dP}{dt} = \frac{e_0 k_{cat.} J_A A}{J_0 + J_A A + J_P P + J_Q Q + J_{PQ} PQ + J_{AP} AP + J_{AQ} AQ} \quad (1)$$

In this notation, the coefficients J (Boeker, 1984*b*, 1985) are collections of microscopic rate constants that, for a particular mechanism, result directly from a King & Altman (1956) derivation. J_P, J_Q and J_{PQ} correspond to the competitive product inhibition constants; J_{AP} and J_{AQ} correspond to the uncompetitive constants.

Eqn. (1) can be transformed into the standard Michaelis–Menten form by dividing numerator and denominator by $J_A A$. J_0/J_A is K_A, J_P/J_A is normally identified as K_A/K_P, etc. Unfortunately, this particular transformation obscures both the fundamental experimental measurements of kinetics and the decisions that must be made from them. The numbers that are measured directly, before any transformations, are the Dalziel (1957) constants. These appear when eqn. (1) is transformed by dividing the numerator and denominator by $J_A A k_{cat.}$. In other words, the Dalziel constants are $1/k_{cat.}$, $J_0/J_A k_{cat.}$ ($= K_A/k_{cat.}$), $J_P/J_A k_{cat.}$ (the competitive constant for P) etc. We will continue to write the Dalziel constants in terms of the coefficients J in order to make clear the identity and origin of each product inhibition term.

Eqn. (1) is general for this stoichiometry in that it contains essentially any term that can arise for any mechanism (with one exception, described in the Discussion section). The goal of kinetic analysis is to determine the empirical rate equation for a particular enzyme, and to use it to make statements about the mechanism. In particular, this means determining which of the Dalziel terms are significantly different from 0. For example, if $J_P/J_A k_{cat.}$ is finite but $J_{AP}/J_A k_{cat.}$ is 0, the product inhibition is competitive; if both are finite, it is mixed, etc. The nature of the product inhibition can then be related to particular mechanisms (Cleland, 1963*b*). In

Notation used: A, P and Q and A_0, P_0 and Q_0 are respectively the instantaneous and initial concentrations of substrates and products. ΔP is $P - P_0$, the net change in product concentration at time t. $k_{cat.}$ is the catalytic constant or turnover number, e_0 is the enzyme concentration and K_A is the Michaelis constant for the substrate. K_P, K_Q and K_{PQ} are the competitive product inhibition constants for P, for Q, and for P and Q together; K_{iP} and K_{iQ} are the uncompetitive constants. The coefficients J and C are defined in the text.

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order to decide whether a Dalziel term differs from 0, it is not sufficient simply to measure its value; it is necessary to apply a quantitative statistical test, either by measuring the standard deviation of the term or by assessing the need for it in the empirical rate equation.

In the present paper we report values for six of the seven possible Dalziel terms for arginine decarboxylase. One of these six, the competitive term for CO_2, may not be significant. The seventh, the uncompetitive constant for CO_2, was indeterminate because we were not able to measure one of the terms in the progress-curve equation with sufficient accuracy.

MATERIALS AND METHODS

L-Arginine hydrochloride, pyridoxal 5'-phosphate and bovine serum albumin were obtained from Sigma Chemical Co. Agmatine sulphate (99%) was from Aldrich Chemical Co. Omnifluor and L-[U-^{14}C]arginine were from New England Nuclear. All other chemicals were reagent grade. Arginine decarboxylase was prepared according to the method of Blethen *et al.* (1968) and Boeker *et al.* (1969). The concentration of the purified protein was determined spectrophotometrically at 280 nm by using an absorption coefficient of 1.57 for a 1 mg/ml solution (Blethen *et al.*, 1968).

The enzyme assay measured the evolution of $^{14}CO_2$ from L-[U-^{14}C]arginine (Morris & Pardee, 1965). The enzyme was diluted in 0.2 M-sodium acetate buffer, pH 5.2, containing bovine serum albumin (1 mg/ml), and added to a solution containing substrate at 37 °C. The final composition of the assay solution was: 0.2 M-sodium acetate, pyridoxal 5'-phosphate (0.2 mg/ml), bovine serum albumin (0.02 mg/ml), 0.5–30 mM-L-arginine hydrochloride, 0–80 mM-agmatine sulphate and L-[U-^{14}C]arginine (0.2 μCi/ml), all at pH 5.2. The $^{14}CO_2$ was trapped on filter paper soaked with ethanolamine/2-methoxyethanol (1:2, v/v). The reaction was stopped by adding 0.1 vol. of 100% (w/v) trichloroacetic acid, and the CO_2 was allowed to absorb for at least 20 min. The filter paper was then transferred to a scintillation vial containing 10 ml of counting solution (10 g of Omnifluor, 2 litres of toluene and 500 ml of 2-methoxyethanol) and its radioactivity counted to at least 1% precision in a Beckman LS-100 scintillation counter.

For each progress curve, arginine decarboxylase was added to a series of assay tubes and the reaction was stopped at intervals corresponding to more-or-less equal increments of product. The time courses were designed so that the third point was taken at approximately 15% reaction. Blank values were determined by adding the trichloroacetic acid to the substrate solution before the enzyme. Every curve had at least ten points, each measured in triplicate, and was complete in 60 min or less. Twenty-one curves were carried out at various initial concentrations of arginine and agmatine, and 15 of these were carried out at least twice, at two enzyme concentrations. Results for different enzyme concentrations were combined by plotting $\Delta P/A_0$ against e_0t in order to evaluate possible enzyme inactivation (Selwyn, 1965; see the Results section). A sample progress curve is shown in Fig. 1.

The direct measurements of radioactivity (c.p.m.) were converted into a reaction fraction ($\Delta P/A_0$) by determining, in triplicate, a substrate-exhaustion value for each progress curve. A 50-fold excess of enzyme was added to an otherwise normal assay; the resulting values of radioactivity (c.p.m.) gave an effective specific radioactivity for the particular initial substrate concentration. This procedure automatically corrects for any losses of CO_2, and for any quenching during scintillation counting, and ensures an accurate end point for the reaction.

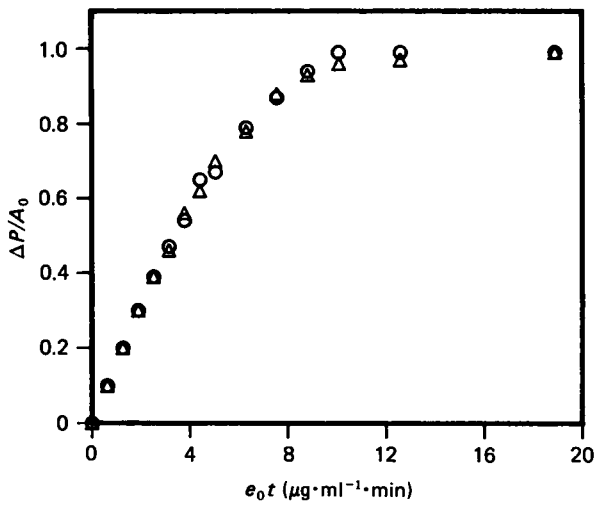

Fig. 1. Arginine decarboxylase progress curves

The initial arginine concentration was 2 mM. The enzyme concentrations were 0.28 μg/ml (○) and 0.42 μg/ml (△). Each point is the average for three determinations.

The arginine decarboxylase reaction results in the uptake of a proton from the medium; the reaction is heavily buffered for this reason. In dummy progress curves, carried out in large volumes with unlabelled arginine and monitored on a pH-meter, we observed that arginine concentrations of 40 mM or greater led to a significant increase in pH, 0.1 unit or more. Initial arginine concentrations greater than 30 mM were therefore not used.

In the experiments presented in this paper, only one of the products, agmatine, was added to the time courses. For notation purposes only, agmatine has been designated Q. The integrated equation for this stoichiometry is then, in the absence of added P:

$$e_0t = -C_f \cdot \ln\left(1-\frac{\Delta P}{A_0}\right) + C_1 \cdot \Delta P + \tfrac{1}{2}C_2(\Delta P)^2 \quad (2)$$

$$C_f = \frac{J_0}{J_A k_{cat.}} + \frac{J_P + J_Q}{J_A k_{cat.}} A_0 + \frac{J_Q}{J_A k_{cat.}} Q_0 + \frac{J_{PQ}}{J_A k_{cat.}} A_0(A_0+Q_0) \quad (3)$$

$$C_1 = \frac{1}{k_{cat.}} - \frac{J_P + J_Q}{J_A k_{cat.}} + \frac{J_{AQ}}{J_A k_{cat.}} Q_0 - \frac{J_{PQ}}{J_A k_{cat.}}(A_0+Q_0) \quad (4)$$

$$C_2 = \frac{J_{AP} + J_{AQ} - J_{PQ}}{J_A k_{cat.}} \quad (5)$$

The progress curves were fitted to these equations by using the analytical procedures described in the accompanying paper (Boeker, 1987). Briefly, the coefficients C were estimated for each time course by a non-linear regression, and their variances were estimated with the

jackknife technique. C_f, C_1 and C_2 were then weighted according to variance, and values for the kinetic constants calculated by the multiple regressions suggested in eqns. (3)–(5).

The time courses were also analysed by initial-rate methods. Initial rates were obtained by the method of Boeker (1982) and fitted to eqn. (1) by non-linear regression. Standard errors were obtained by matrix inversion (Matyska & Kovář, 1985).

RESULTS

A potential source of error in using progress curves is the possibility of enzyme inactivation. Arginine decarboxylase is a stable enzyme (Blethen *et al.*, 1968), but it undergoes dissociation to a form that is either inactive or much less active (Boeker & Snell, 1968; Nowak & Boeker, 1981). We have employed a simple test, known to early enzymologists [see Cornish-Bowden (1979) for a discussion of this] and described by Selwyn (1965), to detect inactivation. Two progress curves, done at different enzyme concentrations but otherwise identical initial conditions, should superimpose if ΔP is plotted against e_0t (see eqn. 2). If enzyme inactivation occurs, the curve at the lower enzyme concentration will be lower on the graph, since it requires longer times to reach a given value of e_0t, allowing more inactivation to occur.

Most of the time courses used in this analysis were tested in this way for enzyme inactivation. Curve separation was observed regularly in the absence of bovine serum albumin. The problem was eliminated with serum albumin except at initial arginine concentrations of 60 mM or more. It seems likely that the albumin prevents adsorption of the enzyme on the glass of the assay tube. The separation at high arginine concentrations is probably due to consumption of protons, beyond the capacity of the buffer, by the decarboxylation reaction. The analysis was therefore limited to arginine concentrations of 30 mM or less.

An effort was made to establish the sensitivity of this test by simulating progress curves over a 20 min time course and assuming a first-order inactivation process. The simulations suggested that 10% inactivation could be detected with a 3-fold difference in enzyme concentrations. When the inactivation was 25%, the reactions did not even reach completion in 20 min.

The experimental values of C_2 are expected to be constant, independent of the initial concentrations of substrate and product (see eqn. 5). Values and standard errors for C_2 are shown in Table 1. The fifth column of this table gives the results of a t test on C_2. C_2 is significantly different from 0, at the 95% or better confidence level, in only two of the progress curves. We conclude that, under these conditions, the contribution of C_2 to the arginine decarboxylase reaction is too small to measure.

The values of C_f and C_1 obtained from the 21 progress curves are displayed in Figs. 2–5. For graphing purposes, the curves displaying C_f and C_1 have been sorted into four experimental designs (see eqns. 3 and 4): (1) and (2), initial arginine concentration varied (1) in the absence of added product and (2) in the presence of 40 mM added agmatine (C_f is shown in Fig. 2 and C_1 in Fig. 3); (3) and (4), initial agmatine concentration varied (3) at 0.5 mM initial arginine and (4) at 30 mM initial arginine (C_f is shown in Fig. 4 and C_1 in Fig. 5).

Table 1. Data for C_2

The units of C_2 and its standard error are $\mu g \cdot ml^{-1} \cdot min \cdot mM^{-2}$. The value of t is C_2 divided by its standard error.

Initial concentration (mM)		Value of C_2	Standard error of C_2	Value of t
Arginine	Agmatine			
0.05	–	340	580	0.59
0.2	–	36	35	1.03
0.5	–	0.99	7.3	0.14
1.0	–	−0.21	1.5	0.15
2.0	–	0.095	0.34	0.28
5.0	–	0.029	0.23	0.13
10	–	−0.012	0.038	0.32
20	–	−0.098	0.080	1.23
30	–	−0.022	0.043	0.51
0.5	10	3.0	13	0.23
0.5	20	3.5	6.9	0.51
0.5	40	−8.8	28	0.32
0.5	80	−9.5	45	0.21
5.0	40	−0.53	0.16	3.31*
10	40	−0.035	0.066	0.53
20	40	−0.16	0.035	4.57*
30	40	−0.062	0.050	1.24
30	10	−0.022	0.019	1.16
30	20	−0.024	0.028	0.86
30	60	−0.049	0.053	0.92
30	80	−0.092	0.050	1.84†

* Significant at the 95% level.
† Significant at the 90% level.

The values of C_f and C_1 were fitted to eqns. (3) and (4) by weighted multiple regression, and the resulting Dalziel constants are shown in Table 2, along with their uncertainties. In Table 2, $J_{AP}/J_A k_{cat.}$ is indeterminate

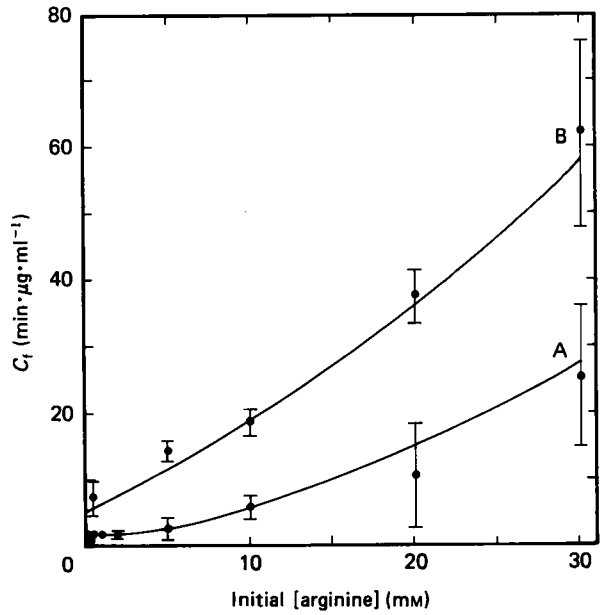

Fig. 2. Dependence of C_f on the initial arginine concentration

The fits, calculated from the results shown in column 3 of Table 2, are: curve A (initial [agmatine] = 0), $C_f = 0.93+0.28[Arg]_0+0.020[Arg]_0^2$; curve B (initial [agmatine] = 40 mM), $C_f = 5.56+1.12[Arg]_0+0.020[Arg]_0^2$. In terms of the C_f axis, the error bars on the fitted curves are, at 0 and 30 mM-arginine respectively: curve A, ±0.3 and ±4.5; curve B, ±1.0 and ±5.6.

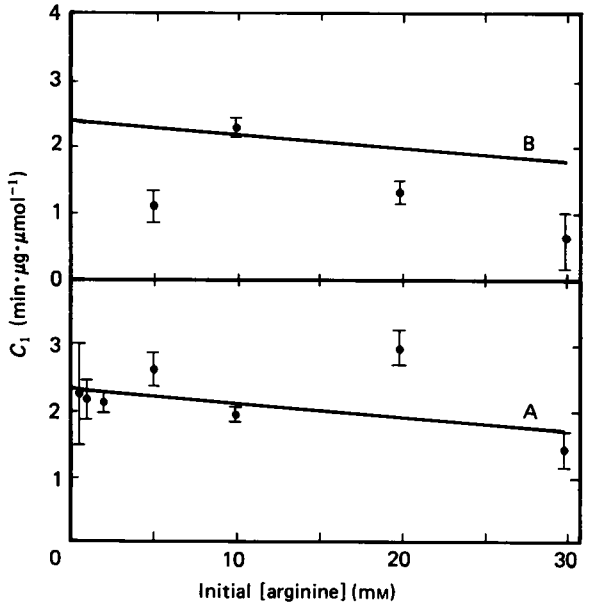

Fig. 3. Dependence of C_1 on the initial arginine concentration

The fits are: curve A (initial [agmatine] = 0), $C_1 = 2.28-0.020[Arg]_0$; curve B (initial [agmatine] = 40 mM), $C_1 = 2.70+0.020[Arg]_0$. The error bars on the fitted curves are, regardless of the arginine concentration: curve A, ±0.2; curve B, ±0.5.

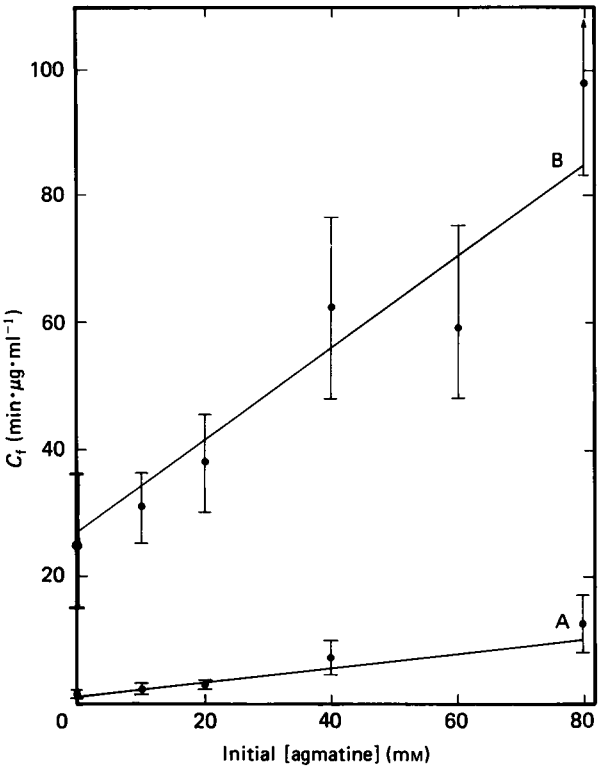

Fig. 4. Dependence of C_f on the initial agmatine concentration

The fits are: curve A (initial [arginine] = 0.5 mM), $C_f = 1.07+0.12[Agm]_0$; curve B (initial [arginine] = 30 mM), $C_f = 26.5+0.60[Agm]_0$. The error bars on the fitted curves are, at 0 and 80 mM-agmatine respectively: curve A, ±0.3 and ±1.9; curve B, ±4.5 and ±10.7.

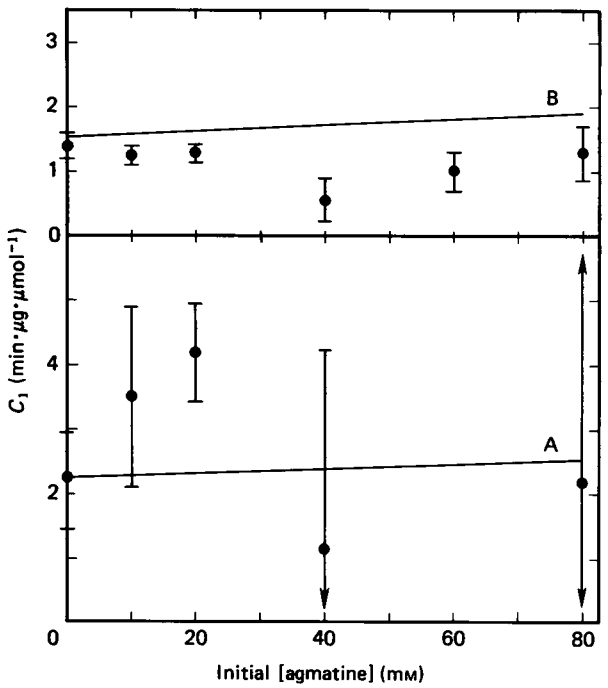

Fig. 5. Dependence of C_1 on the initial agmatine concentration

The fits are: curve A (initial [arginine] = 0.5 mM), $C_1 = 2.27+0.023[Agm]_0$; curve B (initial [arginine] = 30 mM), $C_1 = 1.68+0.023[Agm]_0$. The error bars are, at 0 and 80 mM-agmatine respectively: curve A, ±0.2 and ±1.0; curve B, ±0.2 and ±1.0.

Table 2. Values and standard deviations of the Dalziel constants

Product P corresponds to CO_2, product Q to agmatine.

Constant	Units	All data	No C_2	Three curves	Initial rates
$1/k_{cat.}$	$(\mu mol/min\ per\ \mu g)^{-1}$	2.56 ± 0.20	2.44 ± 0.18	2.87	2.37 ± 0.13
$J_0/J_Ak_{cat.}$	$mM\cdot(\mu mol/min\ per\ \mu g)^{-1}$	0.93 ± 0.27	1.30 ± 0.20	0.86	1.46 ± 0.37
$J_P/J_Ak_{cat.}$	$(\mu mol/min\ per\ \mu g)^{-1}$	0.17 ± 0.12	0.12 ± 0.12	0.46	Indeterminate
$J_Q/J_Ak_{cat.}$	$(\mu mol/min\ per\ \mu g)^{-1}$	0.12 ± 0.02	0.10 ± 0.02	0.25	0.12 ± 0.06
$J_{AP}/J_Ak_{cat.}$	$mM^{-1}\cdot(\mu mol/min\ per\ \mu g)^{-1}$	Indeterminate	Indeterminate	Indeterminate	Indeterminate
$J_{AQ}/J_Ak_{cat.}$	$mM^{-1}\cdot(\mu mol/min\ per\ \mu g)^{-1}$	0.023 ± 0.012	0.028 ± 0.011	0.007	0.009 ± 0.005
$J_{PQ}/J_Ak_{cat.}$	$mM^{-1}\cdot(\mu mol/min\ per\ \mu g)^{-1}$	0.020 ± 0.003	0.016 ± 0.003	0.009	Indeterminate

because C_2 could not be extracted from the progress curves. The lines shown in Figs. 2–5 are based on these Dalziel values, and represent a best fit to all of the data at once. The plots themselves were not used to fit the data.

The Dalziel term $J_{PQ}/J_Ak_{cat.}$ appears in both eqns. (3) and (4). Thus fitted values for this term arise from both C_f and C_1. The observed values were 0.019 ± 0.003 and 0.023 ± 0.012; the weighted average of these values is given in Table 2 and was used to calculate the lines shown in Figs. 2–5. The differences between the observed value where C_1 is fitted and the weighted average from the two fits account for the differences between the lines and the data shown in Figs. 3 and 5.

In assembling the results in column 3 of Table 2, we have used values and uncertainties for C_f and C_1 that were calculated when C_2 was being fitted as well. Since a term in C_2 is present in eqn. (2), and must contribute to the rate equation, we believe that this is the proper procedure. Nevertheless, as C_2 does not appear to differ significantly from 0, we have also fitted the data to a reduced form of eqn. (2), omitting the term in C_2. The immediate effect of this is to reduce the uncertainties in C_f and C_1. The Dalziel values resulting from this calculation are shown in column 4 of Table 2. The values themselves are not much changed. However, the competitive constant for CO_2 ($J_P/J_Ak_{cat.}$) is now completely indeterminate, although the uncompetitive constant for agmatine ($J_{AQ}/J_Ak_{cat.}$) appears to be somewhat more certain.

In principle, for this stoichiometry, estimates of the kinetic constants can be obtained from just three progress curves, including at least two initial substrate concentrations and two initial concentrations of one of the products. As a test of this possibility, time courses at 1 mM and 30 mM initial arginine, with no added agmatine, and at 30 mM arginine with 60 mM added agmatine were used to calculate the kinetic constants. These curves were chosen only so as to have a good spread in arginine concentration and a high added concentration of agmatine. The results of this calculation are shown in column 5 of Table 2. Uncertainties are not shown because the solution to the fitting problem is in this case exact. Obviously, using so few data may give a less than reliable answer, but the correspondence with the Dalziel values from the complete data set is quite reasonable, and suggests that complete kinetic analysis can be obtained from relatively few time courses.

As a reference for the results from the integrated equation, the data were also analysed by initial-rate methods. A Hanes plot of the initial rates is presented in Fig. 6. Agmatine shows a mixed product-inhibition pattern: the competitive component changes the intercept in Fig. 6, and the uncompetitive component changes the slope. Dalziel constants are shown in column 6 of Table 2. As with the integrated equation, the initial rates for the 21 curves were all fitted to eqn. (1) simultaneously; the lines shown in Fig. 6 represent this best fit. The only apparent difference between columns 3 and 6 in Table 2 is the value for $J_{AQ}/J_Ak_{cat.}$, the uncompetitive constant for agmatine. A t test on the difference between the two values gives $t = 0.93$, suggesting that the two numbers are not in fact significantly different.

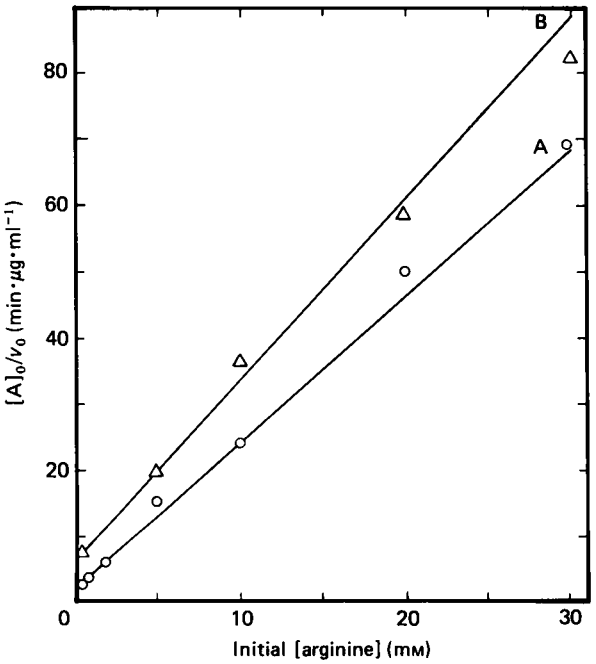

Fig. 6. Hanes plot of initial-rate data

The fits, calculated from the results shown in column 6 of Table 2, are: curve A (initial [agmatine] = 0), $[Arg]_0/v_0 = 1.46+2.37[Arg]_0$; curve B (initial [agmatine] = 40 mM), $[Arg]_0/v_0 = 6.26+2.73[Arg]_0$.

DISCUSSION

A potential source of error in this analysis is the use of statistical tests that rely on distributional assumptions. First, it is important to realize that the methods used to calculate standard errors (the jackknife for coefficients

C_f, C_1 and C_2, and matrix inversion for the Dalziel constants) do not themselves depend on the assumption of normality. However, tests for the significance of these terms do rely on distributional assumptions. A t test has already been used to test for the significance of values of C_2; additional t tests will be used for the Dalziel terms. These tests are appropriate only if the underlying residuals, which are $\Delta P_i - \Delta\hat{P}_i$ for C_2 and either $C_{fi} - \hat{C}_{fi}$ or $C_{1i} - \hat{C}_{1i}$ for the Dalziel terms, are normally distributed. This appears to be the case for both sets of residuals, as is shown in the following paper (Boeker, 1987).

Twenty-one time courses were used in this analysis. With the clear vision of hindsight, it is apparent that many of them contribute little to the final result. We began by choosing substrate concentrations centred around K_A, i.e. arginine concentrations of 0.05 to 2 mM. This is the wrong approach, for two reasons.

(1) The range of values in C_f and C_1 can be large only if there are large changes in the substrate concentration. In the absence of product, C_f is a quadratic in A_0, and C_1 is a linear function. Even with an arginine concentration range of 30 mM, C_1 does not change very much (Fig. 3). The change in C_f with substrate concentration can be clearly seen in Fig. 2. Unlike initial-rate analysis, where the object is only to measure as wide a range of initial velocities as possible, the object here is to measure as wide a range of curve shapes as possible. This implies a good spread in the initial substrate concentrations, including many where the initial velocity is near maximal. A qualitative way of looking at this is that at low initial substrate concentrations the products can never build up very much; product inhibition can never become much of a factor. At high initial substrate concentrations, on the other hand, product inhibition will become very important toward the end of the reaction.

(2) C_2 is not measurable in most of the progress curves, basically because it contributes so little to the overall equation. The t test employed here [an alternative possibility is described in the following paper (Boeker, 1987)] tests the null hypothesis that $C_2 = 0$ and, in general, shows that this hypothesis cannot be rejected for these data. With the experimental conditions used, two of the three terms in C_2 also appear in C_f and C_1. From eqn. (5) and Table 2, $C_2 = J_{AP}/J_A k_{cat.} + 0.023(\pm 0.012) - 0.20(\pm 0.003) = J_{AP}/J_A k_{cat.} + 0.003$ (± 0.012). In other words, the value of C_2 is approximately the value of $J_{AP}/J_A k_{cat.}$. If $J_{AP}/J_A k_{cat.}$ is about the same size as $J_{AQ}/J_A k_{cat.}$ and $J_{PQ}/J_A k_{cat.}$, the possible contribution of the term in C_2 can be assessed. At 10 mM-arginine and 50% reaction, $C_2 = 0.20$ gives a term in eqn. (2) that is less than 4% of the sum of the terms of C_f and C_1; at 30 mM-arginine and 50% reaction, the term in C_2 is still less than 10% of the other terms. The only possibility of measuring C_2 is at high substrate concentrations, where ΔP can become large, increasing the relative importance of the term in $(\Delta P)^2$.

Eqns. (1)–(5) are not completely general for this stoichiometry, on two counts. (1) A fourth term, $\frac{1}{3}C_3(\Delta P)^3$, where $C_3 = J_{APQ}/J_A k_{cat.}$, should be added to eqn. (2) (Boeker, 1984*b*, 1985). Terms in J_{APQ} appear to arise only if a stable enzyme form (i.e. the free enzyme) isomerizes between catalytic events (Cleland, 1963*a*). As we are unable to detect terms in $(\Delta P)^2$ with any certainty, we cannot expect to detect terms in $(\Delta P)^3$. (2) Eqn. (1) does not include all possible steady-state terms; there are, for example, no terms to account for substrate inhibition. The Dalziel constants shown in Table 2 represent the best fit between these equations and the data; the remaining question is whether this fit is, in some absolute sense, very good, i.e. whether eqns. (2)–(5) are in fact sufficient to explain the data.

Because we have carried out most of the progress curves at two enzyme concentrations, a test of this question is possible: the pairs of curves can be used to estimate the experimental ('pure') error, and the difference between each measured curve and the corresponding fitted curve (calculated from the initial conditions and the Dalziel values in column 3 of Table 2) can be used to estimate the fitting error. The average absolute value of the experimental error is 5.8%, and that of the fitting error is 7.5%. Both sets of residuals appear to be normally distributed, and a t test on the difference between the two values indicates that they are not significantly different.

The constants shown in column 3 of Table 2 have, in Table 3, been transformed into more usual Michaelis constants. Previously reported values for $k_{cat.}$ are 0.52 μmol/min per μg (Blethen *et al.*, 1968) and 0.56 μmol/min per μg (O'Leary & Piazza, 1978). Blethen *et al.* (1968) and Boeker *et al.* (1969) report specific activities of 0.41–0.42 μmol/min per μg for the pure enzyme, using an assay where the arginine concentration was 25 mM. This number is in good agreement with the $k_{cat.}$ value of 0.39 ± 0.03 μmol/min per μg found here. It seems possible that the initial-rate methods, relying as they do on a slope extrapolation, overestimate $k_{cat.}$ somewhat.

K_A was found to be 0.56 mM by Gale (1940), 0.65 mM by Blethen *et al.* (1968) and 1.0 ± 0.5 mM by O'Leary & Piazza (1978). All of these are in reasonable agreement with the value of 0.36 ± 0.11 mM in Table 3. Blethen *et al.* (1968) also report a value of 1.5 mM for K_Q, which is somewhat different from 7.8 ± 2.6 mM. This is probably a consequence of the relatively insensitive manometric technique used in that study.

Our intention was not to investigate the mechanism of arginine decarboxylase action. Nevertheless, our results

Table 3. Derived macroscopic constants

Product P corresponds to CO_2, product Q to agmatine. K_P, K_Q and K_{PQ} are competitive product-inhibition constants, K_{iQ} is an uncompetitive constant.

Constant	Derived from	Value
$k_{cat.}$	$(1/k_{cat.})^{-1}$	0.39 ± 0.03 μmol/min per μg
K_A	$\frac{J_0}{J_A k_{cat.}}\left(\frac{1}{k_{cat.}}\right)^{-1}$	0.36 ± 0.11 mM
K_P	$\frac{J_0}{J_A k_{cat.}}\left(\frac{J_P}{J_A k_{cat.}}\right)^{-1}$	5.5 ± 4.2 mM
K_Q	$\frac{J_0}{J_A k_{cat.}}\left(\frac{J_Q}{J_A k_{cat.}}\right)^{-1}$	7.8 ± 2.6 mM
K_{PQ}	$\frac{J_0}{J_A k_{cat.}}\left(\frac{J_{PQ}}{J_A k_{cat.}}\right)^{-1}$	46 ± 15 mM^2
K_{iQ}	$\left(\frac{J_{AQ}}{J_A k_{cat.}}\right)^{-1}$	43 ± 23 mM

bear on this question. A rate equation in which the release of (either) product is strictly irreversible will not have a term in J_{PQ}. However, our results are quite clear. A t test on $J_{PQ}/J_A k_{cat.}$ shows that it is significantly different from 0 at well above the 99% confidence level. Thus CO_2 release must be kinetically reversible even though it is thermodynamically irreversible.

Secondly, if the product release mechanism is strictly ordered, CO_2 first, as suggested by Metzler *et al.* (1954), agmatine should behave as a pure competitive inhibitor and the term in J_{AQ} should equal 0. Again, by use of a t test, $J_{AQ}/J_A k_{cat.}$ is significantly different from 0 at just about the 95% confidence level. The reverse possibility, in which agmatine is released first, cannot be ruled out, because $J_{AP}/J_A k_{cat.}$ is indeterminate. However, this possibility seems unlikely on chemical grounds.

The remaining possibility is that product release can occur by either pathway. The simplest random-release mechanism is one in which the rapid-equilibrium assumption is justified; this requires that the terms in both J_{AP} and J_{AQ} be 0. If equilibrium is not established before release of product, all the terms in Table 3 do appear in the rate equation. In principle, four additional terms, in P^2, Q^2, P^2Q and PQ^2, also appear. However, such terms can be expected to be very small and may not be experimentally detectable (Gulbinsky & Cleland, 1968).

On balance, we think it is likely that arginine decarboxylase catalyses product release by either pathway, probably with a preference for the CO_2-first path. It is possible that the agmatine-first path occurs when the product is displaced by transaldimination with an incoming substrate molecule. Such a mechanism has been suggested for the pyruvate-containing histidine decarboxylase from *Lactobacillus* 30a (Recsei & Snell, 1984).

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