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Kinetic analysis of lactate dehydrogenase using integrated rate equations

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Abstract. The reaction catalyzed by lactate dehydrogenase was analyzed under fully second-order conditions using integrated rate equations. A two-step regression analysis was utilized to fit twenty-one progress curves repeated in sextuplicate to the general mechanism second-order integrated rate equation[1] with additional terms for substrate inhibition. The fitting error was less than one percent. The resulting kinetic constants support a ternary complex mechanism; in no case were constants supporting another mechanism predicted. The inhibition constant for oxamate was also determined.

Key words. Integrated rate equation; non-linear regression; progress curve; ternary complex.

We have used the M_4 isoenzyme of lactate dehydrogenase to determine the kinetic parameters of an irreversible second-order reaction using integrated rate equations. The stoichiometry of the reaction is: $A + B \rightarrow P + Q$, and the reaction is:

$$NADH + H^+ + Pyruvate \rightarrow L\text{-}Lactate + NAD^+$$

This stoichiometry is described by the general derivative rate equation (1)[1].

The corresponding but vastly simpler problem for a one-substrate reaction has been examined numerous times, including work from our laboratory[2,3]. Because of its complexity, the two-substrate problem has previously been studied only under special conditions. Schwert[4,5] used a special solution to the integration problem to study lactate dehydrogenase, Bates and Frieden[6] studied glutamate dehydrogenase using numerical integration, and Duggleby and Morrison[7,8] studied aspartate aminotransferase under pseudo-first-order conditions. We have applied analytical solutions[1] to the integration problem to determine the kinetic parameters of lactate dehydrogenase under fully second-order conditions.

The complexities of the second-order problem are most easily understood by considering the progress curve equations for one-substrate and two-substrate reactions, respectively:

$$e_0 t = -C_f \ln(1 - \Delta P/A_0) + C_1 \Delta P + 0.5C_2(\Delta P)^2 \quad (2)$$

$$e_0 t = -C_f \ln(1 - \Delta P/A_0) - C_s \ln(1 - \Delta P/B_0) + C_1 \Delta P + 0.5C_2(\Delta P)^2 + 0.33C_3(\Delta P)^3 \quad (3)$$

The coefficients C are linear or quadratic functions of the kinetic constants and the initial substrate and product concentrations, A_0, B_0, P_0, and Q_0, as follows:

$$C_f k_{cat} = \frac{J_B}{J_{AB}} + \frac{J_0}{J_{AB}}\frac{1}{B_0 - A_0} + \frac{J_P}{J_{AB}}\frac{A_0 + P_0}{B_0 - A_0} + \frac{J_Q}{J_{AB}}\frac{A_0 + Q_0}{B_0 - A_0} + \frac{J_{BP}}{J_{AB}}(A_0 + P_0) + \frac{J_{BQ}}{J_{AB}}(A_0 + Q_0) + \frac{J_{PQ}}{J_{AB}}\frac{(A_0 + P_0)(A_0 + Q_0)}{B_0 - A_0} + \frac{J_{BPQ}}{J_{AB}}(A_0 + P_0) \times (A_0 + Q_0) \quad (4)$$

$$C_s k_{cat} = \frac{J_A}{J_{AB}} + \frac{J_0}{J_{AB}}\frac{1}{A_0 - B_0} + \frac{J_P}{J_{AB}}\frac{B_0 + P_0}{A_0 - B_0} + \frac{J_Q}{J_{AB}}\frac{B_0 + Q_0}{A_0 - B_0} + \frac{J_{AP}}{J_{AB}}(B_0 + P_0) + \frac{J_{AQ}}{J_{AB}}(B_0 + Q_0) + \frac{J_{PQ}}{J_{AB}}\frac{(B_0 + P_0)(B_0 + Q_0)}{A_0 - B_0} + \frac{J_{APQ}}{J_{AB}}(B_0 + P_0) \times (B_0 + Q_0) \quad (5)$$

$$C_1 k_{cat} = 1 + \frac{J_{PQ} - J_{AP} - J_{AQ} - J_{BP} - J_{BQ}}{J_{AB}} + \frac{J_{ABP}}{J_{AB}}P_0 + \frac{J_{ABQ}}{J_{AB}}Q_0 - \frac{J_{APQ}}{J_{AB}}(B_0 + P_0 + Q_0) - \frac{J_{BPQ}}{J_{AB}} \times (A_0 + P_0 + Q_0) \quad (6)$$

$$\frac{dP}{dt} = \frac{e_0 k_{cat} AB}{\frac{J_0}{J_{AB}} + \frac{J_A}{J_{AB}}A + \frac{J_B}{J_{AB}}B + \frac{J_P}{J_{AB}}P + \frac{J_Q}{J_{AB}}Q + \frac{J_{AB}}{J_{AB}}AB + \frac{J_{AP}}{J_{AB}}AP + \frac{J_{AQ}}{J_{AB}}AQ + \frac{J_{BP}}{J_{AB}}BP + \frac{J_{BQ}}{J_{AB}}BQ + \frac{J_{PQ}}{J_{AB}}PQ + \frac{J_{ABP}}{J_{AB}}ABP + \frac{J_{ABQ}}{J_{AB}}ABQ + \frac{J_{APQ}}{J_{AB}}APQ + \frac{J_{BPQ}}{J_{AB}}BPQ + \frac{J_{ABPQ}}{J_{AB}}ABPQ} \quad (1)$$

$$C_2 k_{cat} = \frac{J_{ABP} + J_{ABQ} - J_{APQ} - J_{BPQ}}{J_{AB}} + \frac{J_{ABPQ}}{J_{AB}} \times (P_0 + Q_0) \quad (7)$$

In general, for the one-substrate two-product case (eq. 2), C_f depends on $J_A/J_{AB}k_{cat}(K_A/k_{cat})$ plus 3 product inhibition terms, C_1 depends on $1/k_{cat}$ plus 3 product inhibition terms, and C_2 depends on product inhibition terms alone. For the two-substrate two-product case (eq. 3), C_f and C_s depend respectively, on $J_B/J_{AB}k_{cat}(K_A/k_{cat})$ or $J_A/J_{AB}k_{cat}(K_B/k_{cat})$ plus $J_0/J_{AB}k_{cat}(K_{ia}K_B/k_{cat})$ and 5 product inhibition terms, C_1 depends on $1/k_{cat}$ plus 11 product inhibition terms, and C_2 depends on 5 product inhibition terms. (The terms in C_3 can ordinarily be ignored since they arise only if a free enzyme form isomerizes.) Measurements of terms in C_2 do not appear to be statistically significant, as shown below and by Cox and Boeker[2]. There is one substrate constant and 6 possible product inhibition constants for the one-substrate two-product case, and 3 substrate constants and 12 possible product inhibition constants for the two-substrate case. As will be shown, the logarithmic term in $1\text{-}\Delta P/B_0$ is perfectly correlated with the logarithmic term in $1 - \Delta P/A_0$, and when written as in equation 2, the progress curve equation does not have a unique solution (ill-conditioned). This is not a fundamental property of the integrated equation, but is a result of the way that it is factored here and nevertheless must be taken into account computationally. Although initially somewhat daunting, this complexity is what gives this technique its power; each progress curve is affected by the product inhibition and contains a corresponding amount of information.

Two problems must be dealt with in order to apply integrated rate equations to two-substrate reactions. The first is to find a suitable method for fitting the data despite the correlation between the two logarithmic terms. The second problem is to make use of the various product inhibition constants to elucidate the mechanism. Combinations of inhibition constants are mechanism dependent and therefore define the mechanism. A ternary complex mechanism, for example, contains 7 of the 12 possible inhibition constants, while a ping-pong mechanism contains a different set of 5. Duggleby[9] has recently presented a method which will fit data with a particular combination of product inhibition terms. This has been applied to the first-order case. This approach requires that the correct combination be known in advance or that the entire procedure be repeated each time a new combination of terms is to be fitted.

In this paper we present analytical methods that initially incorporate all the possible product inhibition terms, but leave the final choice of terms open. The computer programs utilize the standard statistical techniques of forward and backward step regression for choosing which terms give the best fit.

The method presented here for fitting the data is a substantial modification of that used by Cox and Boeker[2] for one-substrate reactions; a non-linear regression is first used to fit equation 2 to obtain the C coefficients. A linear regression is then applied to the equations for the coefficients C (eqs 4–7). The non-linear regression is modified to take into account the linear dependence between the two logarithmic terms, and the linear regression is modified so that all coefficients C are fitted simultaneously rather than individually. Rate equations are fitted and discussed using 'J' notation.

The coefficient notation 'J' is used to represent the parameters because it gives the most straight-forward description of the complex two-substrate equations. J_0 is, for example, the King-Altman[10] term which, in the denominator of the derivative equation (eq. 1), has no dependence on substrate or product concentration; J_A is the term that depends on [A], etc. Consider, as an example, the Michaelis-Menten equation as being written:

$$v_0 = e_0 k_{cat} J_A A/(J_0 + J_A A) \quad (8)$$

When divided by $J_A A$, equation 8 becomes $v_0 = e_0 k_{cat}/(1 + J_0/J_A A)$. Here, J_0/J_A is the term in $1/A$ (i.e., K_A). For two-substrate reactions, K_A is J_B/J_{AB}, K_B is J_A/J_{AB}, etc.[1]

Materials and methods

Notation. A, B, P and Q, and A_0, B_0, P_0 and Q_0 are, respectively, the instantaneous and initial concentrations of substrates and products. A is NADH, B is pyruvate, P is NAD^+ and Q is lactate. The terms that appear in a ternary-complex or ping-pong mechanism assume only that A and P, and B and Q, correspond in structure. 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 total enzyme concentration, and K_A, K_B are typical kinetic parameters as defined by the Nomenclature Committee of the International Union of Biochemistry[15]. The subscript i indicates a particular measurement, a superscribed ^ (hat) indicates a best fit (rather than measured) value, and f indicates a fractional reaction. Matrices are represented in bold type (i.e. **P**, **D**). The parameters C and J are defined in the text.

Experimental methods. Sodium pyruvate, l(+) lactic acid, NADH, NAD^+, bovine serum albumin, sodium oxamate, lactic acid standard solution, and the M_4 isoenzyme of rabbit muscle lactate dehydrogenase were obtained from Sigma. All other chemicals were reagent grade.

Sodium pyruvate and NADH were prepared fresh daily in 0.1 M sodium phosphate buffer, pH 7.0. Lactate and NAD^+ were prepared as stock solutions at pH 7.0 and kept frozen at −20 °C. An extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ at 340 nm was used for NADH[11].

Table 1. Experimental conditions and average values of the coefficients C[a]

NADH	Pyr	Lac	NAD	C_f	sd	C_s	sd	C_1	sd	C_2	sd
0.035	0.280	3.00	0.00	0.083	0.007	0.001	0.001	0.49	0.30	35	77
0.034	0.183	0.00	23.60	0.017	0.003	0.010	0.001	1.68	0.07	10	11
0.035	0.235	0.00	35.70	0.018	0.003	0.008	0.001	1.61	0.05	10	11
0.137	0.236	5.07	0.01	0.103	0.005	0.049	0.004	0.63	0.06	0.9	0.4
0.024	0.039	0.00	0.02	0.056	0.006	0.027	0.009	1.27	0.25	45	23
0.028	0.040	0.01	2.00	0.068	0.008	0.037	0.009	0.72	0.18	41	31
0.054	0.100	4.86	0.00	0.129	0.007	0.017	0.006	0.33	0.08	7.9	3.2
0.058	0.097	3.48	0.00	0.124	0.006	0.021	0.005	0.42	0.05	3.5	2.4
0.057	0.096	1.52	0.02	0.066	0.005	0.034	0.005	0.88	0.07	4.3	4.0
1.715	0.298	7.04	0.13	0.032	0.013	0.123	0.078	0.46	0.17	3.6	3.5
1.353	0.146	0.22	0.00	0.006	0.004	0.424	0.068	0.89	0.17	32	8
1.071	0.052	0.11	29.60	0.004	0.002	0.213	0.098	1.59	0.69	−16	123
0.841	0.064	0.06	22.20	0.002	0.004	0.251	0.075	0.70	0.63	−18	101
0.177	0.041	1.01	0.02	−0.020	0.031	0.227	0.116	−0.88	0.87	−117	251
0.150	0.020	2.67	0.01	0.003	0.005	0.123	0.042	1.30	1.54	−72	431
1.011	0.104	0.00	40.04	0.001	0.007	0.518	0.112	1.19	0.10	−93	66
0.104	0.056	5.07	0.02	0.003	0.013	0.208	0.015	−0.03	0.01	10	10
0.192	0.119	0.02	10.00	0.052	0.015	0.156	0.012	0.43	0.08	3.1	2.4
1.307	0.222	1.94	20.40	0.033	0.007	0.190	0.021	0.90	0.06	1.8	1.0
0.026	0.016	0.00	0.00	−0.003	0.011	0.173	0.010	−1.12	1.26	−17	122
0.982	0.167	0.10	35.40	0.024	0.004	0.244	0.033	0.87	0.09	2.5	1.9

[a]Each progress curve was repeated at least 5 times, ordinarily 6. All concentrations are mM. The dimensions of C_f and C_s are units ml^{-1} min; C_1, units ml^{-1} min mM^{-1}; C_2, units ml^{-1} min mM^{-2}. A unit of enzyme has a specific activity of 330 μmoles/min/mg.

The concentration of NAD^+ stock solutions was determined at 259 nm using an extinction coefficient of 18,000 M^{-1} cm^{-1} (ref. 11). The concentrations of lactate and pyruvate in stock solutions were determined spectrophotometrically by measuring the change in NADH concentration in the lactate dehydrogenase reaction under conditions where the lactate or pyruvate was the limiting substrate.

Lactate dehydrogenase was purchased and stored as an $(NH_4)_2SO_4$ suspension. Before use, it was dialyzed overnight against 0.1 M sodium phosphate buffer, pH 7.0. The concentration of solubilized protein was determined at 280 nm using an extinction coefficient of 1.13 for a 1 mg/ml solution[12]. The enzyme was assayed by following the disappearance of NADH at 340 nm, based on the procedure of Kornberg[13]. The final composition of the assay solution: 0.1 M sodium phosphate, pH 7.0, 0.93 mM sodium pyruvate, 0.22 mM NADH, 0.1 mg/ml bovine serum albumin, and 0.01 to 0.02 units/ml of lactate dehydrogenase.

Time courses were carried out in sextuplicate at 25 °C in water-jacketed cell holders in a Cary 219 spectrophotometer by following the disappearance of NADH at 340 nm. The initial substrate and product concentrations for each progress curve are shown in table 1.

The integrated rate equations (eqs 2 and 3) assume that the reaction is irreversible. In the direction of NADH oxidation, the equilibrium constant for this reaction is on the order of 10^4 at pH 7.0 and 25 °C[14]. The progress curve conditions were chosen such that each reaction would proceed to at least 99% of completion. No experiments were conducted with both products at high levels. The initial conditions in table 1 reflect these limits.

For each time course a record of absorbance versus time was collected by an Apple IIe computer interfaced directly to the spectrophotometer, and transferred to the Utah State University Vax. Progress curves were corrected for enzyme addition and mixing time, as determined with a stopwatch. The end point of each curve was determined by adding a large excess of enzyme after the reaction appeared to have reached completion. Evenly spaced data points, at intervals of 1% of the total product formed, were selected so that all portions of the curve were equally weighted.

Non-linear regression for the coefficients C. Equation 2 can be written in matrix form as $\mathbf{t} = \mathbf{Pc}$, where $\mathbf{t}$ is the vector of $e_0 t_i$ observations, $\mathbf{P}$ is the matrix formed with $-\ln(1 - \Delta P_i/A_0)$, $-\ln(1 - \Delta P_i/B_0)$, ΔP_i, and $0.5(\Delta P_i)^2$ values as the columns, and $\mathbf{c}$ is the unknown vector of the best-fit coefficients C. If the experimental error were primarily in $\mathbf{t}$, finding $\mathbf{c}$ would be an ordinary multiple regression problem, minimizing $\Sigma(t_i - \hat{t}_i)^2$; $\mathbf{c}$ would then be the solution to $\mathbf{P'Pc} = \mathbf{P't}$. However, inspection of equation 3 shows that it is 'backwards' with respect to the dependent and independent variables. The experimental error lies in the measurement of product, not of time; $\Sigma(\Delta P_i - \Delta \hat{P}_i)^2$ must be minimized. This requires a non-linear regression in which the coefficients are first approximated and then refined until the $\Sigma(\Delta P_i - \Delta \hat{P}_i)^2$ reaches a minimum value. The approximations are obtained by solving the linear regression problem, and the corrections, by successively solving $\mathbf{e} = \mathbf{Dx}$ for $\mathbf{x}$. $\mathbf{e}$ is the vector of the fitting error, $\Delta P_i - \Delta \hat{P}_i$; $\mathbf{D}$ is the matrix formed from the partial derivatives of ΔP, at each point i, with respect to each coefficient C, and $\mathbf{x}$ is the unknown vector of the corrections. (This is the Gauss-

Table 2. Characteristics of matrices needed for the non-linear regression problem[a]

Problem	Matrix	Eigenvalue for $1-\frac{\Delta P}{A_0}$	Eigenvalue for $1-\frac{\Delta P}{B_0}$	ΔP	$\frac{1}{2}(\Delta P)^2$	Least diagonal element	Tau
Two substrate, full	**P'P**	82.3	0.00000	0.517	0.0091	0.54×10^{-4}	91×10^{-4}
	D'D	1.09	0.00000	0.107	0.0036	0.72×10^{-4}	99×10^{-4}
Two substrate, reduced	**P'P**	81.7	-	0.504	0.0091	0.0994	0.0090
	D'D	1.59	-	0.0143	0.00033	0.0191	0.0010
Two substrate, reduced	**P'P**	80.0	0.00000	0.484	-	2.3×10^{-3}	8.9×10^{-3}
	D'D	1.56	0.00000	0.0136	-	0.44×10^{-3}	1.0×10^{-3}
One substrate	**P'P**	18.3	none	0.0904	0.0013	0.0387	0.0043
	D'D	0.45	none	0.0043	0.00012	0.0134	0.00067

[a]The data in the first 3 rows is abstracted from the fitting problem for a lactate dehydrogenase progress curve; initial conditions, 0.054 mM NADH (A_0), 0.10 mM pyruvate (B_0), 4.9 mM lactate, no NAD^+. The last row is from an arginine decarboxylase progress curve reported in Cox and Boeker[2]; initial conditions, 1 mM arginine, no added product.

Newton method for doing non-linear regression.) The corrections **x** are the solution to the least-squares problem $\mathbf{D'Dx} = \mathbf{D'e}$.

For one-substrate reactions, C_s does not occur in the progress curve equation (eq. 2); **P'P** and **D'D** are 3×3. For arginine decarboxylase, a one-substrate reaction, the non-linear regression problem converged in nearly all cases[3]. However, for lactate dehydrogenase, **P'P** and **D'D** are 4×4; initially, this non-linear regression converged only rarely, or gave answers that were physically impossible.

The reason for this is made clear by the data in table 2. Matrices which contain both logarithmic terms are singular: one of their eigenvalues is zero. This regression is rank-deficient. It is clear that, for these data, it is the column corresponding to the second logarithmic term that causes the problem; not only does the zero eigenvalue disappear when this column is removed, it remains when one of the other columns is removed. This is true of both the matrix needed for the approximations (**P'P**) and the matrix needed for the corrections (**D'D**). Note that the initial concentration of pyruvate (B_0) is greater than that of NADH (A_0) resulting in an eigenvalue of zero for the $1 - \Delta P/B_0$ term; when this condition is reversed an eigenvalue of zero is associated with the logarithmic term in $1 - \Delta P/A_0$.

Regression problems that are rank-deficient or near rank-deficient are fairly common, and computational procedures for dealing with them are well-known to numerical analysts. We implemented Lawson and Hanson's algorithm HFTI, a procedure that does not rely on a predetermination of the linearly dependent column and incorporates a test for rank-deficiency[16]. In this approach, a matrix **P** is factored into matrices **HRK'** by a QR decomposition with accompanying column interchanges. **H** is an orthogonal matrix resulting from a series of Householder transformations; **K'** is an orthogonal matrix that accounts for the column interchanges. Matrix **R** is right triangular, with the columns ordered such that the fourth column corresponds to the dependent column and contains numbers that approach zero. After **P** has been decomposed into **HRK'**, its rank can be determined by comparing the size of each of the diagonal elements of **R** to a number tau. Lawson and Hanson[16] suggest that the value of tau should be taken as 10^{-3} times the norm of **P**; the norm of **P** is the square root of the maximum eigenvalue of **P'P**. This comparison is shown in table 3, and the results are exactly what is expected from the eigenvalues: when the second logarithmic term is included in the problem, the smallest diagonal element is less than tau. There are two additional noteworthy features of this analysis: 1) when the second logarithmic term is omitted from the fitting problem, the smallest diagonal element corresponds to the column arising from $0.5(\Delta P)^2$ and is only 10–20 × tau. This condition undoubtedly causes the large observed variance in C_2. 2) The initial approximation regression and the final non-linear regression have the same characteristics with respect to rank-deficiency. This is computationally desirable.

The solution vector for this least squares problem is $\mathbf{c} = \mathbf{Ky_1} + \mathbf{Ky_2}$, where $\mathbf{y_1}$ is the solution vector obtained for a reduced problem of full rank and $\mathbf{y_2}$ is arbitrary. In other words, this rank-deficient problem has an infinite number of solutions, depending on the choice of $\mathbf{y_2}$. The standard choice, which we have implemented, is to let $\mathbf{y_2} = \mathbf{0}$, minimizing the size of the solution vector.

This non-linear regression requires that the root of equation 3 be found iteratively; this problem was solved by Boeker[3]. She has also described the remaining conditions and convergence criteria for the non-linear regression.

Multiple regression for the kinetic parameters. The equations for the coefficients C for the two-substrate two-product general case are displayed in table 3. Previously, values of the kinetic constants were obtained by performing *separate* multiple regressions for each coefficient C (ref. 3); this would correspond, for example, to a multiple regression of the value of C_f obtained in each progress curve against that curve's experimental

Table 3. Expressions for the coefficients C[a]

Parameter	C_f	C_s	C_1	C_2
1 $\frac{1}{k_{cat}}$	0	0	1	0
2 $\frac{J_A}{J_{AB}k_{cat}}$	0	1	0	0
3 $\frac{J_B}{J_{AB}k_{cat}}$	1	0	0	0
4 $\frac{J_P}{J_{AB}k_{cat}}$	$\frac{A_0+P_0}{B_0-A_0}$	$\frac{B_0+P_0}{A_0-B_0}$	0	0
5 $\frac{J_Q}{J_{AB}k_{cat}}$	$\frac{A_0+Q_0}{B_0-A_0}$	$\frac{B_0+Q_0}{A_0-B_0}$	0	0
6 $\frac{J_0}{J_{AB}k_{cat}}$	$\frac{1}{B_0-A_0}$	$\frac{1}{A_0-B_0}$	0	0
7 $\frac{J_{AP}}{J_{AB}k_{cat}}$	0	B_0+P_0	-1	0
8 $\frac{J_{AQ}}{J_{AB}k_{cat}}$	0	B_0+Q_0	-1	0
9 $\frac{J_{BP}}{J_{AB}k_{cat}}$	A_0+P_0	0	-1	0
10 $\frac{J_{BQ}}{J_{AB}k_{cat}}$	A_0+Q_0	0	-1	0
11 $\frac{J_{PQ}}{J_{AB}k_{cat}}$	$\frac{(A_0+P_0)(A_0+Q_0)}{B_0-A_0}$	$\frac{(B_0+P_0)(B_0+Q_0)}{A_0-B_0}$	1	0
12 $\frac{J_{ABP}}{J_{AB}k_{cat}}$	0	0	P_0	1
13 $\frac{J_{ABQ}}{J_{AB}k_{cat}}$	0	0	Q_0	1
14 $\frac{J_{APQ}}{J_{AB}k_{cat}}$	0	$(B_0+P_0)(B_0+Q_0)$	$B_0+P_0+Q_0$	-1
15 $\frac{J_{BPQ}}{J_{AB}k_{cat}}$	$(A_0+P_0)(A_0+Q_0)$	0	$A_0+P_0+Q_0$	-1

[a]Specific expressions can be obtained by multiplying the parameter in column 1 by the corresponding predictor in column 2, 3, 4 or 5. The results are independent of the assignment of A, B, P and Q. Using only the convention that A and P, and B and Q, correspond in structure, terms 1–5 plus 6, 8, 9, 11, 13 and 15 are expected for a ternary complex mechanism, and terms 1–5 plus 7, 10 and 11 for ping-pong.

predictors listed in column 2 of table 3. With an appropriate choice of experimental conditions, eight parameters ($J_B/J_{AB}k_{cat}$, $J_P/J_{AB}k_{cat}$, etc.) are obtained. The difficulty with this approach is that many of these same parameters occur in the expressions for C_s (column 3) and C_1 (column 4) as well; best-fit parameters from C_f will not be optimized for C_s and/or C_1.

To overcome this problem, we have performed a single regression that fits all of the coefficients C simultaneously. The main idea lies in the fact that equations 4 thru 7 include the *same* predictors (the terms in X_i) even though they may not be explicitly written. For example, equation 4 as written does not contain the term J_{AP}/J_{AB}, which is explicit in the equation for C_s (eq. 5). However, in fact, this term *is* in equation 5; the predictor is equal to zero. This idea is illustrated below for two general linear equations:

$$Y_1 = X_1b_1 + X_2b_2 + X_3b_3 + (0)b_4 + (0)b_5 + (0)b_6 \quad (9)$$

$$Y_2 = (0)b_1 + (0)b_2 + (0)b_3 + X_4b_4 + X_5b_5 + X_6b_6 \quad (10)$$

Note that all possible predictors are included in both equations. Writing the equations in this manner permits them to be solved simultaneously. In these equations, $\mathbf{Y_i}$ is a vector of dependent variables, $\mathbf{B_i}$ is a vector of parameters to be estimated, and $\mathbf{X_i}$ is the matrix of independent variables. Thus the solution to the matrix equation $\mathbf{Y = XB + e}$ can be obtained by ordinary multiple regression.

When, as here, C_2 does not differ significantly from zero (see 'Results'), columns 2, 3 and 4 become the row of the regression, and each progress curve contributes 3 rows. Since the dimensions of C_1 differ from those of C_f and C_s (see table 1), all entries in the C_1 row were multiplied by the limiting substrate concentration. The term in C_1 in equation 3 is now expressed as a fractional reaction, $C_1S_0(\Delta P/S_0)$, corresponding to the terms in C_f and C_s. The parameters ($1/k_{cat}$, etc.) were then obtained by a standard multiple regression technique, and their standard deviations were estimated from the jack-knife; both computations have been previously described[3].

Estimation of experimental and fitting errors. Each progress curve was repeated 5 or 6 times, and the experimental error was approximated as follows: For each set of curves, an average curve was generated by finding the average fractional reaction, f_i, for a given value of $e_0 t_i$. This average curve was used to determine the values of $e_0 t_i$ corresponding to $f = 0.1, 0.2, \ldots, 0.8$; the values of $e_0 t_i$ were then used to determine the corresponding values of f_i on each actual progress curve. The experimental error was then taken to be the square root of $\{[\Sigma(f_i - 0.1)^2]/n + [\Sigma(f_i - 0.2)^2]/n + \cdots + [\Sigma(f_i - 0.8)^2]/n\}/8$, where n is the number of progress curves in the group. The fitting error of the non-linear regression for the coefficients C is simply the square root of $[\Sigma(f_i - f_i)^2]/(n - m + 1)$, where n is the number of observations on the progress curve and m is the number of coefficients fit.

The fitting error for the linear regression for the kinetic parameters may be calculated as the residuals between the actual and the fitted values of C_f, C_s, C_1 and C_2. However, this residual is indirect and yields no simple measurement for how well the calculated parameters fit the data. Alternatively, we have calculated a fitting error similar to the experimental error above. For each progress curve, values of $e_0 t_i$ corresponding to fractional reactions of 0.1 through 0.8 were calculated. These values of $e_0 t_i$ were then used to calculate the corresponding fitted values of f_i. The fitting error was then taken as the square root of $\{[\Sigma(f_i - 0.1)^2]/n + [\Sigma(f_i - 0.2)^2]/n + \cdots + [\Sigma(f_i - 0.8)^2]/n\}/8$, where n is the *total* number of progress curves. Thus, the error is expressed as a fractional reaction.

Results

Enzyme inactivation. Enzyme inactivation must be avoided if entire progress curves are to be analyzed successfully. In order to avoid adsorption of lactate dehydrogenase on cuvette walls, bovine serum albumin was added to each time course. More fundamentally, however, lactate dehydrogenase is known to form a stable adduct between NAD^+ and pyruvate at high pyruvate concentrations (17 thru 21). This adduct binds very tightly to the active site and essentially reduces the concentration of enzyme.

Enzyme inactivation can be detected by comparing progress curves that have identical initial conditions except for the concentration of the enzyme itself[22,23]. Plotting product formed against $e_0 t_i$ should, in the absence of inactivation, give superimposing curves. In the presence of inactivation, the progress curve at the lower enzyme concentration will appear to be slower, as the enzyme has a longer time to be inactivated. Results for lactate dehydrogenase at 2 concentrations of pyruvate are shown in figure 1; it is apparent that inactivation occurs at high concentrations of pyruvate. The concentration of pyruvate was therefore kept below 0.3 mM in all experiments (see table 1).

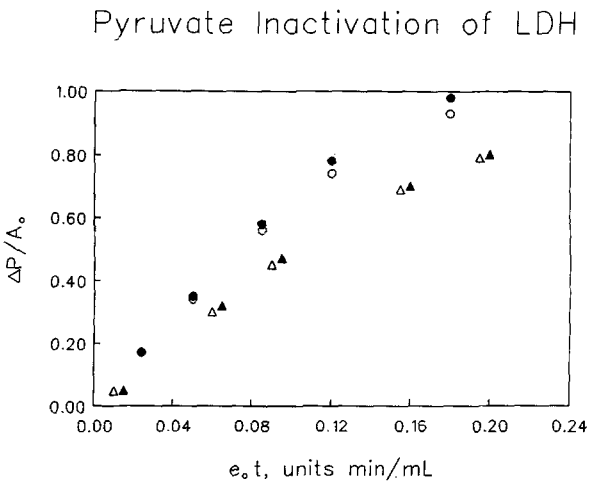

Figure 1. Pyruvate dependence of the inactivation of lactate dehydrogenase. Two initial enzyme concentrations were used: 0.021 units/ml (closed symbols) and 0.004 units/ml (open symbols). The initial NADH concentration was held constant at 0.170 mM, and the initial pyruvate concentrations were: 0.700 mM (●, ○), and 0.280 mM (▲, △).

Progress curves. The fitted values for C_f, C_s, C_1 and C_2 obtained for 21 sets of initial conditions are shown in table 1. These data represent average values from a total of 122 progress curves fitted with the non-linear regression program described in 'Materials and methods'. The fitted curves match the experimental curves very well; the average value of the fitting error for all 122 curves was 0.74% of the total reaction.

It is clear from table 1 that the relative sizes of C_f and C_s depend in all cases on which substrate is present in excess. C_f is greater than C_s when [pyruvate] is greater than [NADH], and vice-versa. It is clear from table 3 why this is so: When [pyruvate] is greater than [NADH], B_0 is greater than A_0 and the denominators of the terms in C_f are positive while those in C_s are negative. The reverse is true when [NADH] is greater than [pyruvate].

The fact that denominator terms in table 3 are negative under some conditions and positive under others emphasizes the fact that these denominators are zero when the two substrate concentrations are equal; the limiting expression for the integrated rate equation under these conditions is given in Boeker[1]. We have taken care to avoid this situation experimentally by always maintaining one substrate in at least a one and a half-fold excess over the other (see table 1).

For each of the coefficients in table 1, the average value of the t-score, the ratio between the coefficient's value and its standard deviation, is: C_f, 6.6; C_s, 7.2; C_1, 7.7; and C_2, 1.2. The small size of t for C_2 suggests that it does not differ significantly from 0; i.e., that the contribution of the term in C_2 to the overall progress curve is too small to measure. Cox and Boeker[2] obtained a similar result for arginine decarboxylase. The 21 lactate

dehydrogenase progress curves were then fitted a second time while holding C_2 zero. The resulting values for C_f, C_s and C_1, which differ slightly from the averaged values in table 1, were used in all subsequent calculations.

Data fitting. Once C_f, C_s and C_1 have been extracted from a set of progress curves, the best-fit kinetic parameters may be determined. Two problems must be addressed: 1) which of the 15 possible parameters in table 3 actually influence the progress curves, and 2) how should the coefficients C be weighted, if at all, in order to obtain the best fit?

Our experimental design was chosen with question 2) in mind. Each progress curve was repeated 6 times in order to obtain, experimentally, reliable estimates of the standard deviations of the coefficients. We have examined 5 weighting schemes: 1, no weights; 2, weights are 1/(standard deviation); 3, weights are 1/(standard deviation)^2; 4, weights are t; and 5, weights are t^2. The usual weighting method is number 3. We tried schemes 2, 4 and 5 in the hope that they might prove somewhat less dependent upon individual measurements.

For each weighting scheme, a forward step regression was done by first fitting the data only with $1/k_{cat}$, $J_B/J_{AB}k_{cat}(K_{NADH}/k_{cat})$ and $J_A/J_{AB}k_{cat}(K_{pyr}/k_{cat})$. Each of the remaining 12 terms were then sequentially added to determine which improved the fit the most. The term which improved the fitting error the most was added or, if there was a tie, the term which also had the greater t value was added. Terms which gave negative parameters were not included. Once the fourth term was established, each of the remaining 11 terms was added one at a time in order to establish the fifth term, and so on. The procedure was terminated when adding a term no longer improved the fitting error or when terms no longer had a significant value of t. For each weighting method, this procedure appeared to produce the best fit. A backward-step regression gave similar results.

The results of the step-regression are shown in table 4. The results when the weights were 1/(standard deviation) and t^2 were extremely similar to those shown for 1/(standard deviation)^2 and t, respectively, except that the fitting error was slightly larger. All of the terms obtained are those expected for a ternary complex mechanism. The fitting procedure selected only terms expected for this mechanism (see table 3).

The results in table 4 demonstrate the importance of choosing the correct weighting method. For this set of data, the standard weighting procedure, 1/(sd)^2, produces the fit with the smallest error. Different weighting methods obtain different parameters in the final fits. The importance of a particular progress curve in the overall fit is determined by the size of its predictors and the size of its weights.

Of the 11 parameters in table 4 expected for a ternary-complex mechanism, 8 are mathematically independent.

Table 4. Best-fit kinetic parameters[a]

Parameter	Weights none	1/(sd)^2	t	8 independent 1/(sd)^2
1 $\frac{1}{k_{cat}}$	0.53 ±0.07	0.79 ±0.06	0.88 ±0.09	0.77 ±0.06
2 $\frac{J_A}{J_{AB}k_{cat}}$	0.112 ±0.004	0.109 ±0.017	0.115 ±0.002	0.113 ±0.016
3 $\frac{J_B}{J_{AB}k_{cat}}$	0.027 ±0.003	0.010 ±0.008	0.024 ±0.003	0.010 ±0.007
4 $\frac{J_P}{J_{AB}k_{cat}}$	0.00067 ±0.00005	-	0.00065 ±0.00005	-
5 $\frac{J_Q}{J_{AB}k_{cat}}$	-	-	-	-
6 $\frac{J_0}{J_{AB}k_{cat}}$	0.00067 ±0.00008	-	0.00068 ±0.00032	0.0011 ±0.0002
8 $\frac{J_{AQ}}{J_{AB}k_{cat}}$	0.0010 ±0.0003	0.0037 ±0.0009	0.00063 ±0.00032	0.0015 ±0.0006
9 $\frac{J_{BP}}{J_{AB}k_{cat}}$	0.0037 ±0.0013	0.0167 ±0.0021	0.0065 ±0.0015	0.0156 ±0.0018
11 $\frac{J_{PQ}}{J_{AB}k_{cat}}$	-	0.0057 ±0.0011	-	0.0038 ±0.008
13 $\frac{J_{ABQ}}{J_{AB}k_{cat}}$	0.0257 ±0.0030	-	0.0083 ±0.0037	-
15 $\frac{J_{BPQ}}{J_{AB}k_{cat}}$		0.0051 ±0.0012		0.0033 ±0.0009
Fitting error	0.087	0.078	0.088	0.081

[a]The terms shown are those expected for a ternary complex mechanism; none of the omitted terms were predicted by any of the fits. A is NADH, B is pyruvate, P is NAD^+ and Q is lactate.

There is a very complex relationship between parameters 11, 13, 15, and the remaining two relationships are $2 \times 5 = 6 \times 8$ and $3 \times 4 = 6 \times 9$. A possible way of fitting the data is then to find the fit which gives the 8 independent parameters, with the best fitting error. We examined this by beginning with the best fits from the 5 original weighting methods and adding and/or subtracting single parameters. The result is shown in the fifth column of table 4. This fit is very similar to the best fit, both in terms of parameter values and the fitting error. Figure 2 illustrates the success of the fitting technique. Eighteen of the 21 experiments had fitting errors comparable to this one. The 3 remaining curves with poor fits had high lactate concentrations.

Competitive inhibition. Oxamic acid (H_2NCH_2COOH) inhibits lactate dehydrogenase competitively with respect to pyruvic acid[24]. In order to demonstrate the

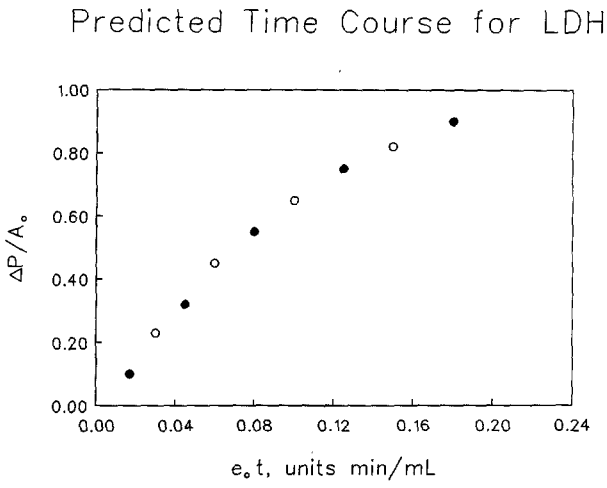

Figure 2. Observed (●) and predicted (○) time courses. The initial concentrations were: NADH, 0.0243 mM; pyruvate, 0.0391 mM; lactate, 0.016 mM; NAD^+, 0. The lactate dehydrogenase concentration was 0.0435 units/ml.

utility of progress curves in measuring inhibition, we have measured the oxamate inhibition constant. The effect of a competitive inhibitor on a rate equation is to multiply by $1 + [I]/K_I$ the King-Altman[10] terms for the enzyme species that combine with the inhibitor[23]. For oxamate and lactate dehydrogenase, those terms are $J_A/J_{AB}k_{cat}$, $J_{AQ}/J_{AB}k_{cat}$ and $J_{PQ}/J_{AB}k_{cat}$. From table 3 it is clear that the principal effect of oxamate inhibition would be on C_s, which contains all 3 of these terms. Consequently, we carried out inhibition experiments under conditions that maximized the effect of C_s on equation 5; i.e., where [NADH] > [pyruvate]. By further keeping the absolute pyruvate concentration low, and by not adding either product initially, the equation for C_s (table 3) should reduce simply to the term in $J_A/J_{AB}k_{cat}$; i.e., to the intercept.

The results for 7 sets of conditions are shown in table 5. A regression of C_s against the predictors in table 3 showed that indeed only the intercept contributed to the fit. The average value of C_s in table 5 is 0.54; this is $J_A(1 + I/K_I)/J_{AB}k_{cat}$. Using a value of 0.11 from table 4 for $J_A/J_{AB}k_{cat}$ gives an oxamate K_I of 0.026 mM, in agreement with the value of 0.026 reported by Nova et al.[24].

Table 5. Determination of oxamate inhibition

[NADH] mM	[pyruvate] mM	C_s units ml^{-1} min
0.225	0.023	0.63
0.201	0.026	0.42
0.208	0.037	0.44
0.898	0.103	0.44
0.495	0.069	0.53
0.071	0.011	0.55
0.092	0.013	0.62

[1]The oxamate concentration was 0.1 mM in all experiments.

Discussion

The application of progress curve analysis to the second-order lactate dehydrogenase reaction has required a more demanding approach than simpler first-order enzymatic reactions. Thus the analysis of the first-order arginine decarboxylase reaction studied in this laboratory[2] was improved with several standard statistical procedures. The nonlinear regression of equation 2 using the code of Cox and Boeker[2] results in what is termed an 'ill-conditioned' matrix primarily due to correlation between the 2 logarithmic terms. The HFTI algorithm described in 'Materials and methods' has successfully resolved the linear dependence between columns of this matrix as demonstrated in table 2. Also in the arginine decarboxylase analysis, each of 3 C coefficients obtained from the nonlinear regression of equation 1 were fit in separate multiple regressions yielding multiple predictions of some kinetic parameters. This procedure was not a problem in the first-order analysis because multiple predictions were not significantly different; the weighted average was reported. In the second-order analysis, separate regressions of the C coefficients are troublesome because most kinetic constants are predicted in more than one equation as shown in table 3. This problem is avoided by fitting all C coefficients simultaneously.

Various step-regression data weighting schemes of C coefficients were investigated (see table 4). Regardless of the weighting method, kinetic parameters were estimated consistently. Although weighting has only minor effects on the size of predicted constants, it changes which of the 11 possible ternary-complex constants are predicted. Kinetic parameters that are inconsistently predicted using different weighting schemes have minor effects on the overall fitting error. Inconsistent predictions of a kinetic constant using different weighting schemes creates a degree of uncertainty for this value. The questionable prediction of some constants in models containing many predictors is not unique to the integrated rate analysis or these data, but is a common regression problem.

Two of the kinetic parameters inconsistently predicted using different weighting schemes, constants 13 and 15 in table 4, are the constants which distinguish between Theorell-Chance and ternary-complex mechanisms. The significance of these constants is not consistent in the literature. While Nygaard[25], Takenaka and Schewert[26], and Thompson and Darling[27] predict these constants thus supporting a ternary-complex mechanism, Zewe and Fromm[28] have not. The fact that these constants appear, depending upon the weighting method, suggests a ternary-complex mechanism. In table 4 the $1/k_{cat}$ value would be 1 if the specific activity and V_{max} were equivalent. In initial rate kinetics this is the extrapolation of infinite substrate concentration in order to find V_{max}.

This investigation is a continued effort to exploit the potential value of progress curve kinetics. Investigation of a second-order reaction here demonstrates the power of this technique. For lactate dehydrogenase all macroscopic constants were extracted from 21 progress curves repeated in sextuplicate. A comparable initial-rate analysis would involve substantially more measurements. Less data are required in an integrated rate analysis for two reasons: 1) Each point on a progress curve is a reaction rate for a particular substrate and product concentration. One point contains the same information as one initial rate measurement. 2) All data from every progress curve is used to determine each constant. For an initial rate analysis, constants (e.g. K_m) are determined from a subset of the data. The integrated rate analysis is also easily adapted to include inhibition studies as demonstrated here using oxamate (see table 5).

The development of integrated rate analysis requires further investigation in several areas. As the fitting of the C coefficients in the step-regression are condensed to fit C_f, C_s, C_1, and C_2 simultaneously, the nonlinear and step-regressions can be condensed in the same manner. This has been suggested by Duggleby[9] and will involve substantial revision of the present code.

Acknowledgment. We wish to dedicate this paper to Dr. Elizabeth A. Boeker who recently passed away.

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