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MODEL DISCRIMINATION AND NONLINEAR PARAMETER ESTIMATION IN THE ANALYSIS OF THE MECHANISM OF ACTION OF β-HYDROXYBUTYRATE DEHYDROGENASE FROM *RHODOPSEUDOMONAS SPHEROIDES*

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SUMMARY

1. β-Hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30) from *Rhodopseudomonas spheroides* was prepared and initial velocity studies in the presence and absence of product at zero time were performed.

2. The product inhibition pattern was found to be consistent with an ordered sequential mechanism.

3. Nonlinear regression analyses were applied to selected combinations of the velocity data to provide estimation of the parameters, their standard deviations, and confidence limits.

4. Analysis of variance provided a basis for determination of the acceptability of the model and for discrimination of points that showed extreme lack of fit.

5. A data-blocking procedure has been introduced that permits the estimation of parameters using combinations of data sets that were obtained with different concentrations of enzyme.

6. The lack of one-to-one correspondence between the set of kinetic constants and the set of rate constants imposed difficulties in the initial attempt to apply the nonlinear regression analysis to the full rate equation in rate constant form.

7. A reformulation of the kinetic constants based on the requirement for a non-zero Jacobian for the transformation has been developed that provides a one-to-one correspondence with the set of rate constants.

8. A method for the evaluation of the approximate 95% confidence regions of the parameters and for the assessment of the experimental design by means of a determination of the eigenvalues of the solution matrix ($X'X$) is introduced.

INTRODUCTION

Evaluation of the parameters of an enzymic reaction implies the assumption

of a mechanism for the interaction of the enzyme intermediates and the substrate components. Kinetic analysis therefore should give some indication of the acceptability of the model as well as a measure of the reliability of the estimates of the parameters. The classical method of transforming the enzymic rate equation from rate constants to kinetic constants, which are defined as maximum velocities, Michaelis constants, and inhibitor constants, and the application of an experimental design based on linearization procedures[1], are useful for obtaining preliminary information about the transformed model but do not provide a suitable basis for testing the fundamental rate constant model.

Estimation of the kinetic constants by means of separate analysis of the replot of intercepts and slopes, which are derived from the reciprocal relationship defined between velocity and variable substrate[1], is not reliable since the interdependence of the parameters has been removed as a factor in their evaluation. A consequence of this is that meaningful standard deviations for the kinetic constants cannot be obtained. Moreover, the least square lines for the replot of slopes and intercepts provide a very limited number of degrees of freedom for statistical analysis of the data. These deficiencies can be overcome to some extent by the application of nonlinear regression analysis to the full rate equation when it is modified to conform to the limiting conditions of the experimental design employed. The existence of constant variance can then be determined by examination of the distribution of the residual error and, if homogeneity of variance is not observed, a re-evaluation of the model can be performed.

This approach was tested by its application to combinations of data sets obtained from an initial velocity study of the mechanism of action of D(−)-β-hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30) from *Rhodopseudomonas spheroides*. Nonlinear regression analyses were carried out by a modification of the Gauss linearization method that made possible the simultaneous estimation of large numbers of parameters using provisional values obtained from a previous determination of the kinetic constants by the reciprocal plot procedure.

Extracts of *R. spheroides* have been shown by Carr and Lascelles[2] to reduce nicotinamide adenine dinucleotide (NAD^+) in the presence of DL-β-hydroxybutyrate as substrate but only the D(−) isomer was oxidized. The reaction was freely reversible and NADH was oxidized in the presence of free acetoacetate. Neuberger and Tait[3] obtained a soluble D(−)-β-hydroxybutyrate dehydrogenase from cell free extracts of *R. spheroides* and the Michaelis constants, determined with crude enzyme at saturating concentrations of the second substrate were found to be 0.63 mM for D(−)-β-hydroxybutyrate and 0.04 mM for NAD^+. Bergmeyer *et al.*[4] crystallized the enzyme from *R. spheroides* and the molecular weight was calculated to be 85 000 by sedimentation equilibrium. The apparent Michaelis constants were 0.41, 0.08, 0.28, and 0.054 mM for the substrates β-hydroxybutyric acid, NAD^+, acetoacetate, and NADH, respectively.

In the present study, the results of initial velocity and product inhibition experiments were found to be consistent with an ordered sequential type of mechanism. Analyses of the combination of all the data indicated that the method of transformation of the full rate equation to kinetic constant form has to be assessed carefully to ensure that the definitions of the kinetic constants for the transformed model

are in agreement with the degrees of freedom implicit in the fundamental rate constant equation.

MATERIALS AND METHODS

Preparation of the enzyme

The enzyme was prepared by the method of Williamson *et al.*[5] from the organism *R. spheroides* (NCIB 8253) grown aerobically in the light. Since the L(+) isomer of β-hydroxybutyric acid has been shown not to inhibit the enzymic reaction[6], the racemic mixture, DL-β-hydroxybutyric acid, was used as substrate. All concentrations and kinetic constants are therefore expressed in terms of the racemic mixture.

Reagents

DL-β-Hydroxybutyric acid, in the form of its sodium salt, was obtained from L. Light and Co., Ltd, Colnbrook, England.

β-NAD^+ and β-NADH were obtained as the disodium salts (Grade III) from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Trishydroxymethylaminomethane (Tris) and citrate were obtained from British Drug Houses Ltd, Toronto, Ontario, Canada.

Sodium acetoacetate was prepared by hydrolysis of ethylacetoacetate by the method of Ljunggren[7].

Assay of enzyme activity

The enzyme reaction was studied by measuring the reduction of NAD^+ or the oxidation of NADH. Enzyme assays were performed in a Unicam SP-800 spectrophotometer equipped with a variable speed slave recorder having a full scale expansion equal to the range, 0.0 to 0.1 absorbance units. Kinetic measurements were made at 340 nm in silica cells of 1 cm light path. Four reactions were followed simultaneously and the temperature during assay was maintained at 30 °C by means of a thermostatically controlled cell holder. All kinetic studies were carried out using Tris–citrate buffer, pH 8.0, final concentration 0.1 M with respect to both Tris and citrate. The major volume components of the assay mixture were kept at 30 °C prior to each group of experiments. 1 ml of buffer (0.3 M) was added initially to the cuvette. Substrate components were made up in 10 times their final concentration and 0.3 ml of the appropriate solution was then added followed by 1.7 ml of water. The enzymic reaction was initiated by the injection of 10 μl of enzyme solution and the mixture was stirred with a Nalgene stirrer. Since the enzyme was found to be more stable in cysteine, all enzyme samples were diluted with 0.2 M cysteine, pH 7.4, in a 1:1 volume ratio before each experiment and maintained at 0 °C until added to the assay mixture. Accurately standardized amounts were not employed because of the instability of the enzyme preparation but active samples were chosen to lie within the linear range of activity versus volume of enzyme as represented in Fig. 1.

Each experiment consisted of a group of 30 to 36 assays using different concentrations of the substrate components. The slopes of the resultant tracings from the spectrophotometric analysis were measured over an initial 5 min period and the velocity of the enzymic reaction was expressed as the change in absorbance at 340 nm per minute.

The velocity data were analyzed initially to determine the degree of fit to Eqn 1

$$1/v = \text{intercept} + \text{slope} \times 1/[\text{substrate}] \quad (1)$$

and secondary effects for variation in intercept or slope were assessed for the alternate fixed substrate component. Least square lines were determined by a multiple regression method[8] adapted to provide estimates of the maximum velocity (V) and the Michaelis constant (K_m) from Eqn 1. Nonlinear regression analyses of combined data sets were carried out by a modification of the Gauss linearization method (Appendix I).

The graphs of least square lines for reciprocal plotting were obtained using the IBM/360 Calcomp plotter and were computed from the parameters provided by fitting the inverse of Eqn 1 to each set of 6 data points represented in Figs 3 to 8.

RESULTS

Enzyme activity

The purified D(−)-β-hydroxybutyrate dehydrogenase preparation was found to have a trace (0.1%) of NAD^+ reducing and NADH oxidizing activity in the absence of added β-hydroxybutyric acid or acetoacetate. Although this activity could be removed by dialysis, it was expedient, because of the instability of the enzyme, to correct for it by the use of blank cells containing all the components of the assay mixture except the acid substrates.

The variation of enzyme activity relative to concentration of the dehydrogenase is shown in Fig. 1. The initial velocity was linearly dependent on the enzyme concentration over a 10-fold range for the assay conditions chosen.

The pH optimum for enzyme activity in the presence of Tris–citrate buffer was investigated (Fig. 2). In the direction for β-hydroxybutyrate oxidation the optimum was pH 9.0 and for acetoacetate reduction, pH 7.7. Because of the fact that both pH curves were relatively flat, the choice of pH 8.0 for kinetic studies of the reaction in both directions was a reasonable one.

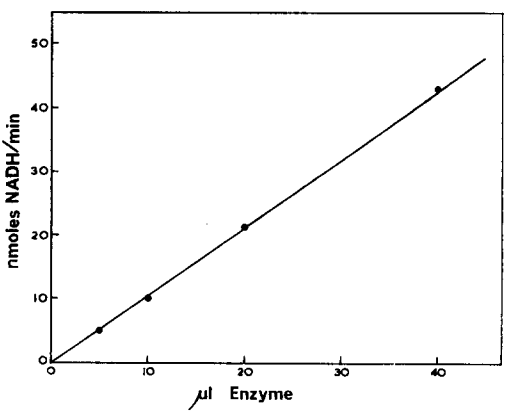


Fig. 1. Effect of enzyme concentration on initial velocity in the presence of 10 mM β-hydroxybutyrate and 0.93 mM NAD^+ in 0.1 M Tris–HCl buffer, pH 9.0.

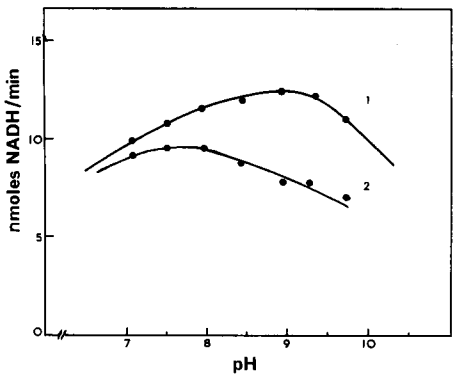


Fig. 2. Effect of pH on the activity of β-hydroxybutyrate dehydrogenase. Curve 1, activity in the direction of oxidation of β-hydroxybutyrate. Initial velocities were determined with 10 mM β-hydroxybutyrate and 0.76 mM NAD^+ in 0.1 M Tris–citrate buffer. Curve 2, activity in the direction of reduction of acetoacetate. Initial velocities were determined with 8.5 mM acetoacetate and 0.18 mM NADH in 0.1 M Tris-citrate buffer.

Initial velocity studies

When the concentration of NAD^+ was varied for different fixed concentrations of β-hydroxybutyrate, the least square lines for the reciprocal plot intersected to the left of the vertical axis (Fig. 3a). A similar pattern of intersecting lines was obtained

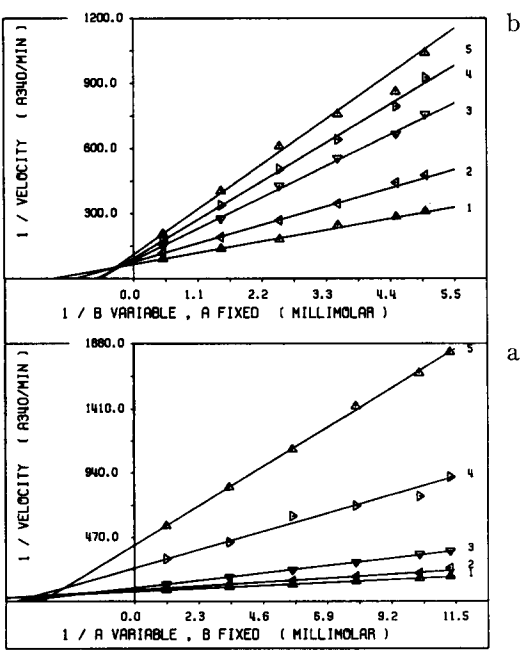


Fig. 3(a) Initial velocity analysis with NAD^+ as the variable substrate and β-hydroxybutyrate fixed at the following concentrations: 2.0 mM (1); 1.33 mM (2); 0.67 mM (3); 0.20 mN (4); 0.10 mM (5). (b) Initial velocity analysis with β-hydroxybutyrate as the variable substrate and NAD^+ fixed at the following concentrations: 0.876 mM (1); 0.292 mM (2); 0.146 mM (3); 0.110 mM (4); 0.088 mM (5).

when β-hydroxybutyrate was the variable substrate for different fixed concentrations of NAD^+ (Fig. 3b). For the reaction in the reverse direction, using NADH or acetoacetate as variable substrate respectively in the presence of fixed concentrations of the second substrate, the same type of reciprocal plot was obtained as shown in Fig. 4a and 4b. The results of the initial velocity studies indicate that the substrates add in a sequential manner to the enzyme before any products are released[1].

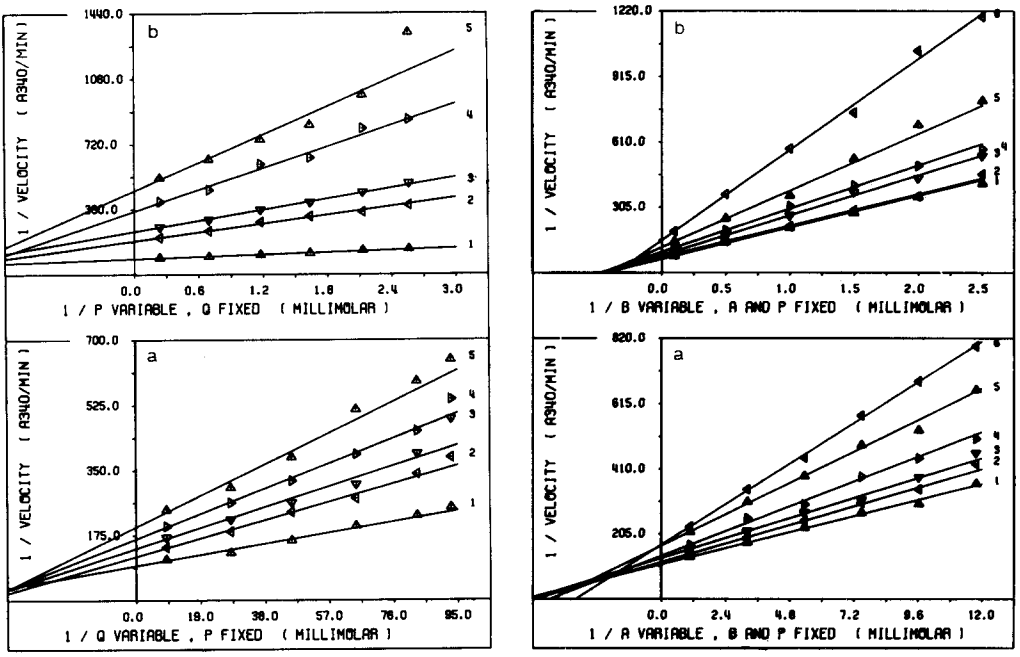
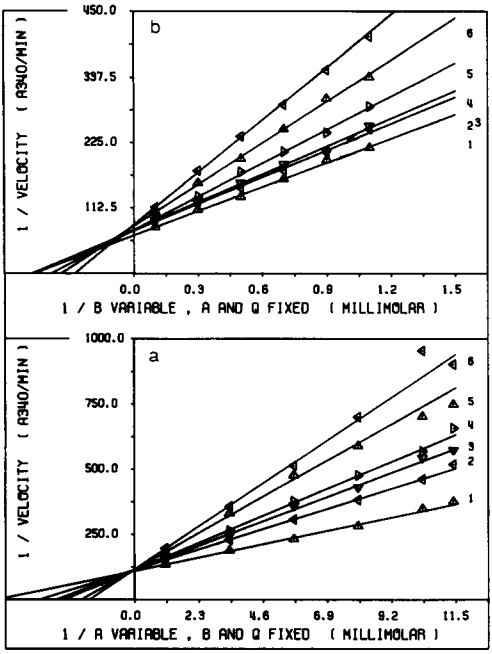


Fig. 4(a) Initial velocity analysis with NADH as the variable substrate and acetoacetate fixed at the following concentrations: 4.25 mM (1); 0.532 mM (2); 0.386 mM (3); 0.284 mM (4); 0.212 mM (5). (b) Initial velocity analysis with acetoacetate as the variable substrate and NADH fixed at the following concentrations: 0.15 mM (1); 0.015 mM (2); 0.010 mM (3); 0.005 mM (4); 0.0037 mM (5).

Fig. 5(a) Product inhibition by acetoacetate with NAD^+ as the variable substrate with β-hydroxybutyrate at 1 mM and acetoacetate fixed at the following concentrations: 0.0 (1); 0.071 mM (data rejected by multiple regression program for significant difference in standard error[8]) (2); 0.142 mM (3); 0.283 mM (4); 0.567 mM (5); 0.850 mM (6). (b) Product inhibition by acetoacetate with β-hydroxybutyrate as the variable substrate with NAD^+ at 0.296 mM and acetoacetate fixed at the following concentrations: 0.0 (1); 0.053 mM (data rejected by multiple regression program for significant difference in standard error[8]) (2); 0.213 mM (3); 0.425 mM (4); 0.850 mM (5); 1.70 mM (6).

Product inhibition studies

With NAD^+ as variable substrate, acetoacetate provided a non-competitive inhibition effect when β-hydroxybutyrate was present at non-saturating concentration (Fig. 5a). NADH was a competitive inhibitor for the same conditions (Fig. 6a). When β-hydroxybutyrate was the variable substrate for fixed concentrations of NAD^+, both acetoacetate (Fig. 5b) and NADH (Fig. 6b) caused non-competitive inhibition. The same product inhibition pattern was obtained for the reverse reaction



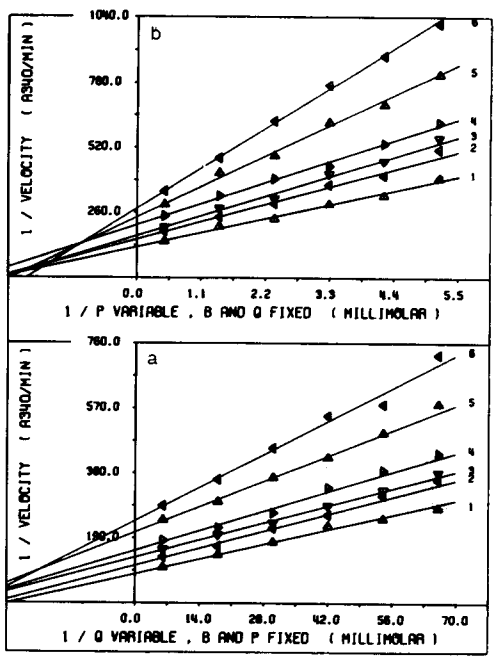


Fig. 6. (a) Product inhibition by NADH with NAD^+ as the variable substrate with β-hydroxybutyrate at 1 mM and NADH fixed at the following concentrations: 0.0 (1); 0.026 mM (2); 0.041 mM (3); 0.052 mM (4); 0.082 mM (data rejected by multiple regression program for significant difference in standard error[8]) (5); 0.103 mM (6). (b) Product inhibition by NADH with β-hydroxybutyrate as the variable substrate with NAD^+ at 0.28 mM and NADH fixed at the following concentrations: 0.0 (1); 0.0093 mM (2); 0.0185 mM (3); 0.037 mM (4); 0.074 mM (5); 0.11 mM (6).

Fig. 7. (a) Product inhibition by β-hydroxybutyrate with NADH as the variable substrate with acetoacetate at 0.68 mM and β-hydroxybutyrate fixed at the following concentrations: 0.0 (1); 0.83 mM (2); 1.65 mM (3); 3.33 mM (4); 6.67 mM (5); 10.0 mM (6). (b) Product inhibition by β-hydroxybutyrate with acetoacetate as the variable substrate with NADH at 0.044 mM and β-hydroxybutyrate fixed at the following concentrations: 0.0 (1); 0.83 mM (2); 1.65 mM (3); 3.33 mM (4); 6.67 mM (5); 10.0 mM (6).

when NADH and acetoacetate were employed as variable substrates and the products β-hydroxybutyrate (Fig. 7a, 7b) and NAD^+ (Fig. 8a, 8b) were present at fixed initial concentrations. The results of the product inhibition studies are summarized in Table I. The slope and intercept effects were analyzed separately for their degree of fit to linear, parabolic, and hyperbolic functions. No significant reduction in the error sum of squares was observed for the nonlinear functions in comparison with the linear.

DISCUSSION

The product inhibition pattern indicated in Table I is compatible with an Ordered Bi Bi mechanism[1] represented schematically by Model I where A and B are specified respectively by NAD^+ and β-hydroxybutyrate for the forward reaction and

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3 B}{\rightleftharpoons}} EAB\text{–}EPQ \underset{k_6 P}{\overset{k_5}{\rightleftharpoons}} EQ \underset{k_8 Q}{\overset{k_7}{\rightleftharpoons}} E \qquad \text{(Model I)}$$

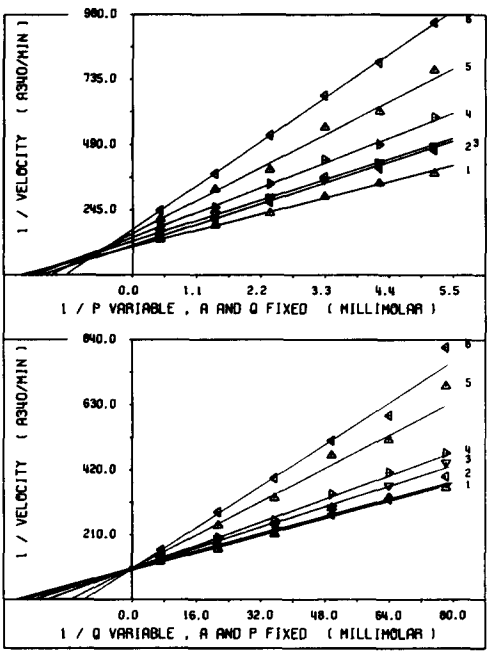


Fig. 8. (a) Product inhibition by NAD^+ with NADH as the variable substrate with acetoacetate at 0.68 mM and NAD^+ fixed at the following concentrations: 0.0 (1); 0.059 mM (2); 0.117 mM (3); 0.233 mM (4); 0.465 mM (5); 0.698 mM (6). (b) Product inhibition by NAD^+ with acetoacetate as the variable substrate with NADH at 0.0423 mM and NAD^+ fixed at the following concentrations: 0.0 (1); 0.078 mM (2); 0.156 mM (3); 0.312 mM (4); 0.624 mM (5); 0.935 mM (6).

TABLE I

PRODUCT INHIBITION PATTERN FOR D(−)-β-HHYDROXYBUTYRATE DEHYDROGENASE FROM *R. spheroides*

Variable substrate	*Fixed substrate*[b]	*Inhibition by product added at zero time*[a]			
		NAD^+	*β-Hydroxybutyrate*	*Acetoacetate*	*NADH*
NAD^+	β-Hydroxybutyrate	–	–	non	comp
β-Hydroxybutyrate	NAD^+	–	–	non	non
Acetoacetate	NADH	non	non	–	–
NADH	Acetoacetate	comp	non	–	–

[a] Comp and non signify competitive and non-competitive inhibition by product.
[b] Substrate fixed at less than half saturation.

P and *Q* are specified by acetoacetate and NADH for the reverse reaction. The full rate equation for this mechanism has been derived[1] by the method of King and Altman[9] and written in kinetic constant form by various methods of transformation[1,10–12]. The set of kinetic constants (V_f, V_r, K_A, K_B, K_P, K_Q, K_{iA}, K_{iB}, K_{iP}, K_{iQ}) used in this study have been defined previously[12].

For the initial velocity conditions illustrated in Fig. 3a and 3b the rate equation may be written in the form

$$v = \frac{V \cdot [A] \cdot [B]}{[A] \cdot [B] + K_A \cdot [B] + K_B \cdot [A] + K_{iA} \cdot K_B} \tag{2}$$

Combination of the data sets for the experiments in Fig. 3a and 3b provided a total of 60 experimental values for fitting Eqn 2. Because of the fact that the enzyme concentration was not the same for the two sets of initial velocity studies, an additional parameter was needed to provide a basis for estimation of the value of V_{AB} for A as variable substrate in Fig. 3a and the value of V_{BA} for B as variable substrate in Fig. 3b. To accomplish this an array of variables, Z_1 and Z_2, were introduced with each element in each set of Z corresponding to each element in the set of dependent variables v for each data point (Appendix I). Accordingly each element in Z_1 was set equal to 1 for the data set from Fig. 3a and to 0 for the data set from Fig. 3b; Z_2 was set equal to the converse values. The introduction of the Z array provides the increase in the degrees of freedom needed for the evaluation of the additional parameter. This procedure is equivalent to the standard method of normalization in which the values of the dependent variable are adjusted after evaluation of the enzyme concentration for a given block of data but it has the advantage that the primary data are not subjected to the possible inclusion of an extraneous error. The modified form of the equation to permit the estimation of the maximum velocity for each of the data blocks in Fig. 3a and 3b is shown in Eqn 3

$$v = \frac{(V_{AB} \cdot Z_1 + V_{BA} \cdot Z_2) \cdot [A] \cdot [B]}{[A] \cdot [B] + K_A \cdot [B] + K_B \cdot [A] + K_{iA} \cdot K_B} \tag{3}$$

The solution of Eqn 3 by nonlinear regression analysis permitted the estimation of the 5 parameters, V_{AB}, V_{BA}, K_A, K_B, and K_{iA}, and their standard deviations. Analysis of variance provided the total sum of squares, the sum of squares due to deviation from regression, and the sum of squares due to regression; significance could be estimated with $(n, nw-n)$ degrees of freedom using the F statistic (Appendix I). The results of this analysis are presented in Table II.

For the reverse reaction, under initial velocity conditions, the equation is of the same form. Since the equation is symmetrical, it does not provide a basis for defining the sequential relationship indicated in Model I and convergence to the same minimum sum of squares due to error occurs irrespectively of the definition of the substrates, A and B or P and Q. The designation of the parameters in Table II is therefore a result of an overall appraisal of the data.

Combination of the data sets from Fig. 4a and 4b permitted the estimation of the following set of parameters, V_{QP}, V_{PQ}, K_Q, K_P, and K_{iQ}, and their standard deviations (Table II), by means of an appropriately modified form of Eqn 3. The F statistic for these data was found to be very low in comparison with the data for the forward reaction and an examination of the distribution of the residual error indicated the presence of an extreme bias in the higher ranges for both NADH as variable substrate and acetoacetate. When the data points associated with 0.15 mM NADH in Fig. 4b and with 4.25 mM acetoacetate in Fig. 4a were removed, a normal distribution of the residual error was obtained. Comparison of the variance for the different treatment of the data in Table II demonstrates a highly significant reduction in the residual sum of squares. It should be noted that the use of the $F_{partial}$ for this type of comparison is not an orthodox procedure but the marked differences observed

TABLE II

KINETIC PARAMETERS, STANDARD DEVIATIONS, AND ANALYSIS OF VARIANCE FOR INITIAL VELOCITY DATA FOR D(−)-β-HYDROXYBUTYRATE DEHYDROGENASE FROM *R. spheroides*

K_M and K_{iM} defined as mM × 10^2; maximum velocity as $A_{340\ nm}$ × 10^2 per min.

	Experimental conditions		
	NAD^+ + β-hydroxybutyrate[a]	*NADH + acetoacetate*[b]	*NADH + acetoacetate*[c]
K_A	5.78 ± 0.55	–	–
K_B	52.67 ± 2.51	–	–
K_{iA}	42.65 ± 2.46	–	–
V_{AB}	1.74 ± 0.03	–	–
V_{BA}	1.61 ± 0.02	–	–
K_Q	–	2.01 ± 0.14	1.79 ± 0.21
K_P	–	22.03 ± 2.36	37.70 ± 4.94
K_{iQ}	–	4.14 ± 0.66	2.46 ± 0.28
V_{QP}	–	1.23 ± 0.04	1.41 ± 0.10
V_{PQ}	–	1.30 ± 0.02	1.21 ± 0.07
Variance × 10^8	1.3340	7.4749	1.4321
F value (df. n, m)[d]	20 880 (5, 55)	3 220 (5, 55)	6 680 (5, 43)
$F_{partial}$ (removal of 12 data points)[e]			20.4 (12, 43)

[a] Combination of 60 data points from Fig. 3a and 3b.

[b] Combination of 60 data points from Fig. 4a and 4b.

[c] Six data points for acetoacetate fixed at 4.25 mM and 6 data points for NADH fixed at 0.15 mM eliminated for combination of data from Fig. 4a and 4b.

[d] Degrees of freedom (df) for n parameters and for m equal to the number of data points *minus* the number of parameters.

[e] The $F_{partial}$ for difference in residual sum of squares (RSS) is defined by $(RSS_1 - RSS_2) \times df_2/(RSS_2 \times (df_1 - df_2))$. $F_{critical}$ (0.005:12, 40) = 2.95.

provide a reasonable basis for accepting the hypothesis that an additional effect, not explained by the structure of Eqn 3, is present in the data for the higher concentration range of NADH and acetoacetate. Elimination of additional data points did not contribute a significant change in the residual sum of squares.

The competitive and non-competitive inhibition effects for low concentrations of NADH in the presence of NAD^+ and β-hydroxybutyrate as variable substrate (Fig. 6a, 6b) can be described by Eqn 4.

$$v = \frac{(V_{ABQ}\cdot Z_1 + V_{BAQ}\cdot Z_2)\cdot[A]\cdot[B]}{[A]\cdot[B] + K_A\cdot[B] + K_B\cdot[A] + K_{iA}\cdot K_B + K_A\cdot[B]\cdot[Q]/K_{iQ} + K_{iA}\cdot K_B\cdot[Q]/K_{iQ}} \quad (4)$$

The variables, Z_1 and Z_2, in Eqn 4 are defined as before to distinguish between the value of V_{ABQ} for A as variable substrate in Fig. 6a and V_{BAQ} for B as variable substrate in Fig. 6b. The combination of 72 data points permitted the estimation of the 6 parameters, V_{ABQ}, V_{BAQ}, K_A, K_B, K_{iA}, and K_{iQ}, and their standard deviations (Table III). Eqn 4 may be modified to permit combination of data from Figs 3a, 3b, 6a, and 6b by the expansion of the numerator to provide Eqn 5

$$v = \frac{(V_{AB}\cdot Z_1 + V_{BA}\cdot Z_2 + V_{ABQ}\cdot Z_3 + V_{BAQ}\cdot Z_4)\cdot[A]\cdot[B]}{\text{Denominator 4}} \quad (5)$$

where Denominator 4 represents the denominator as shown in Eqn 4. The desig-

nations of V for each of the data blocks are defined by an expansion of the Z array for each concordant data set. The results are presented in Table III.

Combination of data from Fig. 5a and 5b for evaluation of the non-competitive inhibition effect for low concentrations of acetoacetate in the presence of variable concentrations of the substrates, NAD^+ and β-hydroxybutyrate, was tested initially by fitting Eqn 6

$$v = \frac{(V_{ABP}\cdot Z_1 + V_{BAP}\cdot Z_2)\cdot[A]\cdot[B]}{[A]\cdot[B]+K_A\cdot[B]+K_B\cdot[A]+K_{iA}\cdot K_B+K_B\cdot K_{PQ}\cdot[A]\cdot[B]\cdot[P]/K_{iB}+K_B\cdot K_{PQ}\cdot[A]\cdot[P]+K_{iA}\cdot K_B\cdot K_{PQ}\cdot[P]} \quad (6)$$

The presence of a nonlinear dependence between the parameters, K_Q, K_P, and K_{iQ}, was eliminated by the definition $K_{PQ} = K_Q/(K_P\cdot K_{iQ})$. This substitution is necessitated by a requirement of the nonlinear regression program (Appendix I) that no dependent relationships may occur between the columns of the solution matrix $(X'X)$. Such a dependence causes a condition of near-singularity and is signalled by the existence of a determinant for the $X'X$ matrix that is too small. This may also occur if the experimental design is not adequate, indicating that a dependency may exist because of the choice of experimental points. It was found that the combined data from Fig. 5a and 5b did not support the estimation of the 7 parameters defined in Eqn 6 and convergence to a satisfactory minimum could only be obtained by decreasing the number of parameters. This was accomplished by setting K_{iB} equal to K_{PQ} to yield Eqn 7

$$v = \frac{(V_{ABP}\cdot Z_1 + V_{BAP}\cdot Z_2)\cdot[A]\cdot[B]}{[A]\cdot[B]+K_A\cdot[B]+K_B\cdot[A]+K_{iA}\cdot K_B+K_B\cdot[A]\cdot[B]\cdot[P]+K_{iB}\cdot K_B\cdot[A]\cdot[P]+K_{iA}\cdot K_{iB}\cdot K_B\cdot[P]} \quad (7)$$

An additional problem in the assessment of the data from Fig. 5a and 5b was due to the anomalous effect of acetoacetate at 0.85 mM in Fig. 5a when NAD^+ was the variable substrate and at 1.70 mM in Fig. 5b when β-hydroxybutyrate was the variable substrate. Elimination of the data points associated with these concentrations of acetoacetate along with those rejected by the multiple regression program[8], permitted a satisfactory convergence for the nonlinear regression analysis and the results presented in Table III were accordingly obtained for 48 data points fitted to Eqn 7.

When the data for Figs 3a, 3b, 5a, and 5b were combined it was possible to estimate the 9 parameters, V_{AB}, V_{BA}, V_{ABP}, V_{BAP}, K_A, K_B, K_{iA}, K_{iB}, and K_{PQ}, using Eqn 8, which is an expanded form of Eqn 6

$$v = \frac{(V_{AB}\cdot Z_1 + V_{BA}\cdot Z_2 + V_{ABP}\cdot Z_3 + V_{BAP}\cdot Z_4)\cdot[A]\cdot[B]}{\text{Denominator 6}} \quad (8)$$

Examination of the estimates of K_{iB} and K_{PQ} (Table III) shows no significant difference in their values.

Combination of all of the data for the forward reaction (Figs 3a, 3b, 5a, 5b, 6a, and 6b) was assessed using Eqn 9

$$v = \frac{(V_{AB}\cdot Z_1+V_{BA}\cdot Z_2+V_{ABP}\cdot Z_3+V_{BAP}\cdot Z_4+V_{ABQ}\cdot Z_5+V_{BAQ}\cdot Z_6)\cdot[A]\cdot[B]}{[A]\ \ B]+K_A\cdot[B]+K_B\cdot[A]+K_{iA}\cdot K_B+K_A\cdot[B]\cdot[Q]/K_{iQ}+K_{iA}\cdot K_B\cdot[Q]/K_{iQ}+K_B\cdot K_{PQ}\cdot[A]\cdot[B]\cdot[P]/K_{iB}+K_B\cdot K_{PQ}\cdot[A]\cdot[P]+K_{iA}\cdot K_B\cdot K_{PQ}\ [P]} \quad (9)$$

Convergence to an acceptable minimum was achieved without difficulty using

TABLE III

KINETIC PARAMETERS, STANDARD DEVIATIONS, AND ANALYSIS OF VARIANCE FOR INITIAL VELOCITY DATA FOR THE FORWARD REACTION CATALYZED BY D(−)-β-HYDROXYBUTYRATE DEHYDROGENASE IN THE PRESENCE OF ADDED PRODUCT

K_M and K_{iM} defined as mM × 10^2; maximum velocity as $A_{340\ nm}$ × 10^2 per min. K_{PQ} equals $K_Q/(K_P \cdot K_{iQ})$.

	Experimental conditions				
	NAD⁺ + NADH + β-hydroxybutyrate[a]	*NAD⁺ + NADH + β-hydroxybutyrate*[a,b]	*NAD⁺ + acetoacetate + β-hydroxybutyrate*[c]	*NAD⁺ + acetoacetate + β-hydroxybutyrate*[b,c]	*NAD⁺ + NADH + acetoacetate + β-hydroxybutyrate*[a,b,c]
K_A	2.88 ± 0.57	1.07 ± 0.43	3.13 ± 2.65	2.53 ± 1.26	1.46 ± 0.50
K_B	91.33 ± 6.52	52.46 ± 4.00	108.30 ± 14.4	59.10 ± 6.51	63.19 ± 4.40
K_{iA}	37.29 ± 2.84	56.49 ± 4.31	37.47 ± 3.54	49.54 ± 6.02	50.04 ± 3.44
K_{iB}	–	–	116.60 ± 8.36	175.70 ± 39.7	146.80 ± 25.2
K_{iQ}	4.89 ± 0.24	4.42 ± 0.22	–	–	4.63 ± 0.24
K_{PQ}	–	–	–	190.00 ± 18.1	168.20 ± 14.1
V_{AB}	–	1.74 ± 0.04	–	1.82 ± 0.07	1.86 ± 0.05
V_{BA}	–	1.63 ± 0.04	–	1.70 ± 0.07	1.75 ± 0. 05
V_{ABQ}	1.67 ± 0.05	1.38 ± 0.03	–	–	1.47 ± 0.04
V_{BAQ}	1.58 ± 0.04	1.37 ± 0.03	–	–	1.43 ± 0.03
V_{ABP}	–	–	1.92 ± 0.16	1.48 ± 0.06	1.52 ± 0.04
V_{BAP}	–	–	1.70 ± 0.14	1.42 ± 0.06	1.43 ± 0.03
Variance × 10^8	2.3824	5.5206	2.2491	7.6256	6.0976
F value (df. n, m)[d]	14 363 (6, 66)	7 791 (8, 124)	7 373 (6, 42)[e]	3 470 (9, 99)	6 059 (12, 168)

[a] Combination of 72 data points from Fig. 6a and 6b.

[b] Combination of 60 data points from Fig. 3a and 3b.

[c] Combination of 48 data points from Fig. 5a and 5b. Twelve data points for acetoacetate at 0.071 and 0.85 mM (Fig. 5a) and 12 for acetoacetate at 0.053 and 1.7 mM (Fig. 5b) not included.

[d] See Footnote d, Table II.

[e] Number of parameters decreased by one by setting K_{iB} equal to K_{PQ}.

provisional estimates of the parameters obtained from the regression analyses resulting from the successive applications of Eqns 3, 4, 5, 7, and 8 to the data in a stepwise fashion. A comparison of the results for the different initial reactant conditions is given in Table III.

For the reverse reaction a similar procedure was followed utilizing the same equations but respecifying the parameters on the basis of the symmetry in Model I and similarly redefining the variables. The estimates of the parameters for the various combinations of the data from Figs 4a, 4b, 7a, 7b, 8a, and 8b are presented in Table IV. The experimental design for the non-competitive effect of β-hydroxybutyrate in the presence of NADH and acetoacetate as variable substrate (Fig. 7a, 7b) was also found to be unsatisfactory for the estimation of 7 parameters by Eqn 6 but this limitation was removed by the inclusion of the initial velocity data as in the case of the forward reaction.

Having obtained estimates of the parameters for both the forward and reverse reactions, it was now possible to determine the degree of fit to the full rate equation in kinetic constant form as shown in Eqn 10

TABLE IV

KINETIC PARAMETERS, STANDARD DEVIATIONS, AND ANALYSIS OF VARIANCE FOR INITIAL VELOCITY DATA FOR THE REVERSE REACTION CATALYZED BY D(−)-β-HYDROXYBUTYRATE DEHYDROGENASE IN THE PRESENCE OF ADDED PRODUCT

K_M and K_{iM} defined as mM × 10^2; maximum velocity as $A_{340\ nm}$ × 10^2 per min. K_{AB} equals $K_A/(K_B \cdot K_{iA})$.

	Experimental conditions				
	NADH + NAD⁺ + acetoacetate[a]	*NADH + NAD⁺ + Acetoacetate*[a,b]	*NADH + acetoacetate + β-hydroxybutyrate*[c]	*NADH + acetoacetate + β-hydroxybutyrate*[b,c]	*NADH + NAD⁺ + acetoacetate + β-hydroxybutyrate*[a,b,c]
K_Q	1.81 ± 0.35	3.62 ± 0.48	2.14 ± 0.32	3.00 ± 0.46	3.12 ± 0.40
K_P	30.69 ± 7.29	74.86 ± 10.2	21.81 ± 2.61	42.12 ± 7.07	52.50 ± 7.15
K_{iQ}	6.45 ± 1.54	2.07 ± 0.27	7.73 ± 0.87	2.74 ± 0.49	2.97 ± 0.41
K_{iP}	–	–	18.15 ± 2.80	46.54 ± 11.8	30.27 ± 7.07
K_{iA}	41.39 ± 1.69	38.30 ± 1.92	–	–	40.28 ± 2.24
K_{AB}	–	–	–	22.39 ± 3.39	15.65 ± 2.37
V_{QP}	–	2.26 ± 0.19	–	1.67 ± 0.14	1.93 ± 0.14
V_{PQ}	–	2.02 ± 0.18	–	1.64 ± 0.16	1.81 ± 0.14
V_{QPA}	1.46 ± 0.11	2.01 ± 0.15	–	–	1.74 ± 0.11
V_{PQA}	1.31 ± 0.09	1.79 ± 0.14	–	–	1.57 ± 0.10
V_{QPB}	–	–	1.46 ± 0.07	1.67 ± 0.13	1.86 ± 0.12
V_{PQB}	–	–	1.15 ± 0.05	1.29 ± 0.10	1.44 ± 0.10
Variance × 10^8	2.0552	3.4774	4.2427	5.2859	4.8647
F value (df. n, m)[d]	9 347 (6, 66)	5 856 (8, 112)	3 892 (6, 66)[e]	3 084 (9, 111)	4 484 (12, 180)

[a] Combination of 72 data points from Fig. 8a and 8b.
[b] Combination of 48 data points from Fig. 4a and 4b (*cf.* Footnote c, Table II).
[c] Combination of 72 data points from Fig. 7a and 7b.
[d] See Footnote d, Table II.
[e] Number of parameters decreased by one by setting K_{iP} equal to K_{AB}.

$$v = \frac{(V_{AB}\cdot Z_1 + V_{BA}\cdot Z_2 + V_{ABP}\cdot Z_3 + V_{BAP}\cdot Z_4 + V_{ABQ}\cdot Z_5 + V_{BAQ}\cdot Z_6)\cdot[A]\cdot[B]\cdot K_P\cdot K_{iQ} + (V_{QP}\cdot Z_7 + V_{PQ}\cdot Z_8 + V_{QPB}\cdot Z_9 + V_{PQB}\cdot Z_{10} + V_{QPA}\cdot Z_{11} + V_{PQA}\cdot Z_{12})\cdot[P]\cdot[Q]\cdot K_{iA}\cdot K_B}{K_P\cdot K_{iQ}\cdot[A]\cdot[B] + K_A\cdot K_P\cdot K_{iQ}\cdot[B] + K_B\cdot K_P\cdot K_{iQ}\cdot[A] + K_B\cdot K_{iA}\cdot K_P\cdot K_{iQ} + K_A\cdot K_P\cdot[B]\cdot[Q] + K_B\cdot K_{iA}\cdot K_P\cdot[Q] + K_B\cdot K_Q\cdot[A]\cdot[B]\cdot[P]/K_{iB} + K_B\cdot K_Q\cdot[A]\cdot[P] + K_{iA}\cdot K_B\cdot K_Q\cdot[P] + K_A\cdot K_P\cdot[B]\cdot[P]\cdot[Q]/K_{iP} + K_B\cdot K_{iA}\cdot[P]\cdot[Q]} \quad (10)$$

With the parameter values obtained from fitting Eqn 9 to the data for the forward reaction (Table III, Column 5) and for the reverse reaction (Table IV, Column 5) it was possible to evaluate the 20 parameters of Eqn 10 with relative ease, convergence to a satisfactory minimum being obtained in 6 iterations. The results are presented in Table V. The standard deviations for the maximum velocities are in general much lower than those for the Michaelis constants and the product inhibition constants. In Table VI the 95% confidence limits for the kinetic constants are compared for the various initial reactant conditions described in Tables II, III, and IV with those obtained for the combined data assessed by means of Eqn 10. Significant differences occur for K_A, K_B, K_{iP}, and K_{iQ} and for V_{AB}, V_{BA}, and V_{PQ}.

Examination of Eqn 10 shows that the set of kinetic constants (V_f, K_A, K_{iA}, K_B, K_{iB}, V_r, K_Q, K_{iQ}, K_P, K_{iP}) does not exist in one-to-one correspondence with the set of rate constants (k_1, k_2, k_3, k_4, k_5, k_6, k_7, k_8) and therefore the transformation from one set to the other cannot be carried out by means of unique definitions unless

TABLE V

KINETIC PARAMETERS, STANDARD DEVIATIONS, AND ANALYSIS OF VARIANCE FOR THE COMBINATION OF ALL INITIAL VELOCITY DATA FOR D(−)-β-HYDROXYBUTYRATE DEHYDROGENASE FROM *R. spheroides* TESTED FOR FITTING EQN 10

K_M and K_{iM} defined as mM × 10^2; maximum velocity as $A_{340\ nm}$ × 10^2 per min.

K_A	2.80 ± 0.43	V_{AB}	1.96 ± 0.04
K_{iA}	41.95 ± 1.83	V_{BA}	1.85 ± 0.04
K_B	75.01 ± 3.59	V_{ABP}	1.59 ± 0.04
K_{iB}	134.30 ± 17.3	V_{BAP}	1.50 ± 0.03
K_Q	2.47 ± 0.16	V_{ABQ}	1.56 ± 0.04
K_{iQ}	4.46 ± 0.20	V_{BAQ}	1.53 ± 0.03
K_P	37.90 ± 2.56	V_{QP}	1.71 ± 0.07
K_{iP}	11.00 ± 1.97	V_{PQ}	1.60 ± 0.08
		V_{QPB}	1.65 ± 0.06
		V_{PQB}	1.28 ± 0.05
		V_{QPA}	1.55 ± 0.05
		V_{PQA}	1.40 ± 0.05
Variance × 10^8	5.9437		
F value (degrees of freedom)	5 728 (20, 352)		

TABLE VI

COMPARISON OF 95% CONFIDENCE LIMITS FOR KINETIC CONSTANTS EVALUATED FOR DIFFERENT COMBINATIONS OF INITIAL VELOCITY DATA FOR D(−)-β-HYDROXYBUTYRATE DEHYDROGENASE FROM *R. spheroides*

Confidence limits are computed by multiplying the standard deviations by two. For the structure of the equations applied to the data see text.

Kinetic constant	*Equation applied*	*95% confidence limits* × 10^2	*95% confidence limits for Eqn 10* × 10^2
K_A	3	4.68– 6.88[a]	1.94– 3.66
K_{iA}	3	37.70– 47.6	38.30– 45.6
K_B	3	47.70– 57.7[a]	67.80– 82.2
K_{iB}	8	96.40–255.0	98.80–170.0
K_Q	3	1.37– 2.21[b]	2.15– 2.79
K_{iQ}	3	1.90– 3.02[a]	4.06– 4.86
K_P	3	27.80– 47.6	32.80– 43.0
K_{iP}	8	22.90– 70.2[a]	7.05– 14.9
V_{AB}	3	1.60– 1.80[a]	1.88– 2.04
V_{BA}	3	1.54– 1.65[a]	1.77– 1.93
V_{ABP}	8	1.36– 1.60	1.51– 1.67
V_{BAP}	8	1.20– 1.54[b]	1.44– 1.56
V_{ABQ}	4	1.57– 1.77[c]	1.48– 1.64
V_{BAQ}	4	1.50– 1.66	1.47– 1.59
V_{QP}	3	1.21– 1.61[d]	1.57– 1.85
V_{PQ}	3	1.07– 1.35[a]	1.44– 1.76
V_{QPB}	8	1.41– 1.93	1.53– 1.77
V_{PQB}	8	1.09– 1.49	1.18– 1.38
V_{QPA}	4	1.24– 1.68	1.45– 1.65
V_{PQA}	4	1.13– 1.49	1.30– 1.50

[a] Estimate of kinetic constant is significantly different from that calculated using Eqn 10.

[b] Significant difference between the mean values at the 0.5% level using Student's t distribution for $t = (\bar{x}_1 - \bar{x}_2)/\sqrt{(s_1)^2 + (s_2)^2}$ for m_2 degrees of freedom.

[c] Significant difference between the mean values at the 5% level.

[d] Significant difference between the mean values at the 1% level.

the values of one set are exact. Since this is not the case because of the residual error in the data, the application of the set of kinetic constants as defined in Eqn 10 introduces two additional degrees of freedom that are not represented in Model I. This criticism does not apply to Eqns 1 to 9 where the number of degrees of freedom implied by the definitions of the kinetic constants employed is less than the number of degrees of freedom for the set of rate constants under the limiting conditions chosen. This inconsistency in defining the rate equations for the various conditions of reactant concentrations, using kinetic constants formulated on the basis of Cleland's approach[1,12], was therefore considered as a possible explanation for the serious discrepancies in the 95% confidence limits of the kinetic constants (Table VI). It was accordingly necessary to determine the degree of fit using the rate equation in rate constant form in order to eliminate ambiguity about the acceptability of Model I.

The definitions of the maximum velocity for the forward reaction (V_f) and for the reverse reaction (V_r) contain the concentration of the enzyme as a factor[1,12]. In Eqn 10 the maximum velocities in the numerator terms are estimated separately for each block of data by means of the coding of the Z array, but all of the combined data contribute to the evaluation of the Michaelis and product inhibitor constants. For the enzyme equation in rate constant form, the enzyme component exists essentially as a scaling factor for the dependent variable (v) and can be set arbitrarily to unit concentration. However, because of the symmetry of the rate equation, some method of defining the relative velocities for the forward and reverse reactions must still be included in the equation since the enzyme concentrations for the various data blocks were not accurately controlled. Cleland[1] has indicated that the full rate equation can only be applied to data for which the same enzyme concentration has been employed. This difficulty has been surmonted in Eqn 10 by means of the Z array in which, for mx data blocks,

$$Z_i = 1;\ Z_j\ (j \neq i) = 0 \qquad (i, j = 1, 2, \ldots mx)$$

The additional variables introduced by the Z array do not increase the number of degrees of freedom implicit in the model but simply provide a means of evaluating the maximum velocity for each data block. The numerator of the full rate Eqn 10 was made positive because no data points for A, B, P, and Q present at initial time are included in the experimental design. In the application of the enzyme equation in rate constant form, nevertheless, the problem arises that the separation of the rate constants from the enzyme component in the definition of the maximum velocities would introduce an additional degree of freedom. To overcome this difficulty the relative velocities for the different data blocks may be expressed as ratios and the ratio for the data block from Fig. 3a may be set arbitrarily equal to 1. This would effectively eliminate the enzyme concentration from the estimation of the maximum velocities and permit V_f to be determined by the values of k_5 and k_7 and V_r to be determined by the values of k_2 and k_4 (ref. 12). The full rate equation in rate constant form, modified in this manner, is shown in Eqn 11. The E vector in the numerator provides a basis for obtaining estimates of the relative enzyme concentrations for the various data blocks studied.

$$v = \frac{k_1k_3k_5k_7[A]\cdot[B]\cdot(Z_1+E_{BA}\cdot Z_2+E_{ABP}\cdot Z_3+E_{BAP}\cdot Z_4+E_{ABQ}\cdot Z_5+E_{BAQ}\cdot Z_6)+k_2k_4k_6k_8[P]\cdot[Q]\cdot(E_{QP}\cdot Z_7+E_{PQ}\cdot Z_8+E_{QPB}\cdot Z_9+E_{PQB}\cdot Z_{10}+ +E_{QPA}\cdot Z_{11}+E_{PQA}\cdot Z_{12})}{k_1k_3(k_5+k_7)\cdot[A]\cdot[B]+k_3k_5k_7[B]+k_1k_7(k_4+k_5)\cdot[A]+k_3k_7(k_4+k_5)+k_3k_5k_8[B]\cdot[Q]+k_3k_6(k_4+k_5)\cdot[Q]+k_1k_3k_6[A]\cdot[B]\cdot[P]+k_1k_4k_8[A]\cdot[P]+ +k_2k_4k_6[P]+k_3k_6k_8[B]\cdot[P]\cdot[Q]+k_6k_8(k_2+k_4)\cdot[P]\cdot[Q]} \quad (11)$$

In order to apply Eqn 11 to the analysis of the combined data points from Figs 3a–8b, provisional estimates of the rate constants were calculated on the basis of the values of the kinetic constants for Eqn 10 that are listed in Table V. The failure of these kinetic constants to yield unique values was emphasized by the inability to achieve a solution to Eqn 11 since the nonlinear regression program diverged and could not find a satisfactory direction in which to search for a minimum. An attempt to modify Eqn 10 to provide relative enzyme concentrations in a manner comparable to Eqn 11 failed due to violation of the constraint for positive, non-zero eigenvalues of the $X'X$ matrix[13,14].

The possibility was therefore considered that the additional degrees of freedom introduced by the definitions of Michaelis constants, product inhibition constants, and maximum velocities, as described for Eqn 10, were providing an analysis of some unknown mechanism that contained additional reaction steps not indicated in Model I. In order to evaluate the acceptability of the Ordered Bi Bi mechanism, a reformulation of the kinetic constants was undertaken to produce consistent definitions of a set of parameters that would be in one-to-one correspondence with the set of rate constants and under the constraint that the absolute value of the Jacobian[15] for the transformation would be non-zero. Definitions of the set (K_1, K_2, . . . K_8) that meet these conditions are defined in Table VII*. The full rate equation for Model I using these parameters is given in Eqn 12. When the analyses of the data in Figs 3a–8b were repeated with Eqns 3 to 9 transformed using the reformulated kinetic constants, it was possible to obtain provisional estimates that permitted a solution to Eqn 12.

$$= \frac{[A]\cdot[B]\cdot(Z_1+E_{BA}\cdot Z_2+E_{ABP}\cdot Z_3+E_{BAP}\cdot Z_4+E_{ABQ}\cdot Z_5+E_{BAQ}\cdot Z_6)+[P]\cdot[Q]\cdot(K_2K_6K_8/(K_1K_4K_7))\cdot(E_{QP}\cdot Z_7+E_{PQ}\cdot Z_8+E_{QPB}\cdot Z_9+E_{PQB}\cdot Z_{10}+ +E_{QPA}\cdot Z_{11}+E_{PQA}\cdot Z_{12})}{[A]\cdot[B]/K_3+[B]/K_1+[A]/K_4+K_2/(K_1K_4)+[B]\cdot[Q]\cdot K_8/(K_1K_7)+[Q]\cdot K_2K_8/(K_1K_4K_7)+[A]\cdot[P]\cdot K_6/(K_4K_7)+[P]\cdot[Q]\cdot K_2K_6K_8/(K_1K_4K_5K_7)+ +[A]\cdot[B]\cdot[P]\cdot(K_6/K_7)\cdot(1/K_3-1/K_2+1/K_5-1/K_7)+[P]\cdot K_2K_6/(K_1K_4K_7)+[B]\cdot[P]\cdot[Q]\cdot(K_6K_8/(K_1K_7))\cdot(1/K_3-1/K_2+1/K_5-1/K_7)} \quad (12)$$

The results of the nonlinear regression analysis based on Eqn 12 are presented in Table VIII. From the values of the set of (K_1, K_2, . . . K_8) it was now possible to calculate provisional estimates of the rate constants that were consistent using the transformation described in Table VII. A satisfactory convergence to a minimum sum of squares using the rate equation for Model I in rate constant form could now

* The set of (K_1. . . K_8) is comparable to the set of (Φ_1, Φ_2, Φ_3, Φ_{12}, Φ_1', Φ_2', Φ_3', Φ_{12}') described by Dalziel[11], but represents less complex functions of the rate constants and has been chosen to provide the simplest basis for the evaluation of the differentials in the determination of the Jacobian. The set of Φ_i can be expressed as a function of the set of (K_1. . . K_8) by the relationship

$$\delta\Phi_I = [A_{IJ}]\cdot\delta K_J$$

The determinant of matrix [A] has been found to be non-zero and therefore the Jacobian for the set of Φ_i with respect to the set of rate constants will be non-zero. However the inclusion of the inhibitor constants defined by Dalziel[11] will negate the requirement for a one-to-one correspondence. The acceptability of other combinations of the kinetic constants defined by Dalziel[11] has not been evaluated on this basis.

TABLE VII

REFORMULATION OF KINETIC CONSTANTS FOR THE ORDERED BI BI MECHANISM BASED ON THE REQUIREMENT FOR ONE-TO-ONE CORRESPONDENCE WITH THE SET OF RATE CONSTANTS AND THE EXISTENCE OF A JACOBIAN WITH A NON-ZERO DETERMINANT

$K = f[k_1, k_2, \ldots k_8]$	$k = f[K_1, K_2, \ldots K_8]$	$K = f[V_f, K_A, K_{iA}, K_B, V_r, K_Q, K_{iQ}, K_P]$
$K_1 = k_1$	$k_1 = K_1$	$K_3 = V_f/EX_O$
$K_2 = k_2$	$k_2 = K_2$	$K_1 = K_3/K_A$
$K_3 = k_5k_7/(k_5 + k_7)$	$k_4 = K_2K_5/(K_2-K_5)$	$K_2 = K_1 \cdot K_{iA}$
$K_4 = k_3k_5/(k_4 + k_5)$	$k_5 = K_3K_7/(K_7-K_3)$	$K_4 = K_3/K_B$
$K_5 = k_2k_4/(k_2 + k_4)$	$k_3 = K_4(k_4 + k_5)/k_5$	$K_5 = V_r/EX_O$
$K_6 = k_4k_6/(k_4 + k_5)$	$k_6 = K_4(k_4 + k_5)/k_4$	$K_6 = K_5/K_P$
$K_7 = k_7$	$k_7 = K_7$	$K_8 = K_5/K_Q$
$K_8 = k_8$	$k_8 = K_8$	$K_7 = K_8 \cdot K_{iQ}$

Definition of non-zero elements of Jacobian for $K = f[k_1, k_2, \ldots k_8]$[a]

$J_{35} = (k_7)^2/(k_5 + k_7)^2$ $J_{37} = (k_5)^2/(k_5 + k_7)^2$ $J_{43} = k_5/(k_4 + k_5)$
$J_{44} = -(k_3k_5)/(k_4 + k_5)^2$ $J_{45} = (k_3k_4)/(k_4 + k_5)^2$ $J_{52} = (k_4)^2/(k_2 + k_4)^2$
$J_{54} = (k_2)^2/(k_2 + k_4)^2$ $J_{64} = (k_5k_6)/(k_4 + k_5)^2$ $J_{65} = -(k_4k_6)/(k_4 + k_5)^2$
$J_{66} = (k_4)/(k_4 + k_5)$ $J_{11} = J_{22} = J_{77} = J_{88} = 1$

Determinant of $J_{ij} = k_4k_5(k_2k_7)^2/[(k_2 + k_4)\cdot(k_4 + k_5)\cdot(k_5 + k_7)]^2$, [b]

$$= \frac{(K_2-K_5)^3\cdot(K_7-K_3)^3}{K_3K_5(K_2K_7)^3\cdot(1/K_3-1/K_2 + 1/K_5-1/K_7)^2}$$

[a] $J_{ij} = \partial K_i/\partial k_j$

[b] Since the determinant of the Jacobian matrix is non-zero therefore $\delta k = [J^{-1}]\cdot\delta K$ can be obtained from $\delta K = [J]\cdot\delta k$ and the definitions of the two sets of constants are consistent. For the relationships $\delta K_i = (X'X)^{-1}\cdot X'Y$ and $\delta k_i = (Z'Z)^{-1}\cdot Z'Y$ the transformation $J'(X'X)J = Z'Z$ can be considered to provide a reasonable basis for estimating the distribution of variance for the set of $[k_1, k_2, \ldots k_8]$ from $(Z'Z)^{-1}\cdot(s)^2$.

TABLE VIII

KINETIC PARAMETERS, STANDARD DEVIATIONS, AND ANALYSIS OF VARIANCE FOR THE COMBINATION OF ALL INITIAL VELOCITY DATA FOR D(−)-β-HYDROXYBUTYRATE DEHYDROGENASE FROM *R. spheroides* TESTED FOR FITTING EQN 12

K_j (j = 1, ... 8) defined as mM × 10^2 and enzyme ratios for E_{AB} equal to 100.

Reformulated kinetic constants[a]		*Ratios of enzyme concentrations*	
K_1	69.02 ± 9.96	E_{BA}	94.50 ± 1.33
K_2	28.40 ± 4.83	E_{ABP}	81.15 ± 1.39
K_3	2.03 ± 0.05	E_{BAP}	77.85 ± 1.25
K_4	2.52 ± 0.08	E_{ABQ}	79.20 ± 1.31
K_5	6.36 ± 1.51	E_{BAQ}	76.60 ± 0.99
K_6	16.97 ± 3.51	E_{QP}	26.60 ± 5.80
K_7	14.55 ± 2.87	E_{PQ}	23.75 ± 5.15
K_8	303.65 ± 63.0	E_{QPB}	25.50 ± 5.58
		E_{PQB}	19.80 ± 4.34
		E_{QPA}	23.95 ± 5.32
		E_{PQA}	21.45 ± 4.77
Variance × 10^8	6.4544		
F value (degrees of freedom)	5 747 (19, 353)		

[a] Kinetic constants are defined in Table VII.

be achieved and the values of the parameters obtained from the regression analysis for Eqn 11 are given in Table IX. A comparison of the estimates of the rate constants calculated on the basis of the definitions of the parameters in Eqns 10 and 12 are

TABLE IX

KINETIC PARAMETERS, STANDARD DEVIATIONS, AND ANALYSIS OF VARIANCE FOR THE COMBINATION OF ALL INITIAL VELOCITY DATA FOR D(−)-β-HYDROXYBUTYRATE DEHYDROGENASE FROM *R. spheroides* TESTED FOR FITTING EQN 11

k_j (j = 1, . . . 8) defined as mM × 10^2 and enzyme ratios for E_{AB} equal to 100.

Rate constants		*Ratios of enzyme concentrations*	
k_1	69.02 ± 10.1	E_{BA}	94.50 ± 1.33
k_2	28.40 ± 4.91	E_{ABP}	81.15 ± 1.39
k_3	11.30 ± 2.92	E_{BAP}	77.85 ± 1.25
k_4	8.20 ± 2.18	E_{ABQ}	79.20 ± 1.31
k_5	2.36 ± 0.12	E_{BAQ}	76.60 ± 0.99
k_6	21.85 ± 3.15	E_{QP}	26.60 ± 5.91
k_7	14.55 ± 2.92	E_{PQ}	23.75 ± 5.25
k_8	303.65 ± 64.1	E_{QPB}	25.50 ± 5.68
		E_{PQB}	19.80 ± 4.42
		E_{QPA}	23.95 ± 5.42
		E_{PQA}	21.45 ± 4.86
Variance × 10^8	6.4544		
F value (degrees of freedom)	5 747 (19, 353)		

presented in Table X along with the 95% confidence limits for the rate constants provided by means of Eqn 11.

The extent to which a proposed model provides an acceptable representation of a particular set of data may be assessed by an examination of the precision with which the parameters are estimated. In a multi-parameter case this means that joint confidence intervals for all of the parameters are required. The joint confidence regions can be determined from the sum of squares surface[16]. When the number of parameters is large, a detailed exploration of the sum of squares surface is difficult and it is therefore necessary to find an approximation to it. If it can be assumed that the linearized model is a satisfactory representation near the least squares parameter estimate, then an approximate ellipsoidal confidence region can be delineated[16]. Information about the shape of the response surface can therefore be obtained from an examination of the eigenvalues and eigenvectors of the normalized $X'X$ matrix used in the regression analysis for the estimation of the parameters (Appendix I). If the ellipsoidal confidence region is elongated, showing high correlation between the parameters, this would indicate that the data do not contain sufficient information to provide good estimates and that the experimental design does not permit an adequate definition of the model. A measure of elongation is the ratio of the maximum and minimum axes of the ellipsoidal confidence region defined by the quadratic form

$$(k-\hat{k})' X'X (k-\hat{k}) \leqslant nS(\hat{k}) F/(nw-n) \tag{13}$$

where $S(\hat{k})$ is the variance for the least squares estimate of $\hat{k}$ and F is the F statistic for n and $nw-n$ degrees of freedom at the 95% confidence level. Diagonalization of Eqn 13 yields the eigenvalues $\lambda_1, \lambda_2 \ldots \lambda_n$ and since the principal axes of an ellipsoid are proportional to the reciprocal of the square root of the eigenvalues, the ratio of the square roots of the maximum and minimum values of the eigenvalues, defined as the condition number (Table X), gives an indication of the elongation of the sum of squares surface. Values of this ratio in excess of 10 may be taken to indicate an

TABLE X

COMPARISON OF THE VALUES OF THE RATE CONSTANTS ESTIMATED BY NONLINEAR REGRESSION ANALYSES FOR MODELS I AND II

k_j (j = 1, . . . 8) defined as mM × 10^2.

	Eqn 11[a]	*Eqn 10*[b]		*Eqn 12, Model I*[c]	*Eqn 12, Model II*[d]	
		(1)[e]	*(2)*	*(3)*	*(4)*[f]	*(5)*[f]
k_1	48.78– 89.26	70.06	70.00	69.02	68.50	60.86
k_2	18.59– 38.23	29.39	29.37	28.41	28.13	24.63
k_3	5.45– 17.13	2.75	11.73	11.29	11.16	9.62
k_4	3.84– 12.56	1.24	8.23	8.20	8.12	7.28
k_5	2.12– 2.60	23.75	2.36	2.36	2.37	2.49
k_6	15.55– 28.15	63.23	22.50	21.85	21.26	19.05
k_7	8.71– 20.39	2.14	11.61	14.55	14.28	13.96
k_8	175.4 –431.9	47.96	260.20	303.65	298.84	266.33
Residual error × 10^5	2.2784	2.0922		2.2784	2.2777	2.8828
Variance × 10^8	6.4544	5.9437		6.4544	6.4707	7.4299
Condition numberg	195.1	24.5		137.3	143.3	138.5
F value (degrees of freedom)	5 747 (19, 353)	5 728(20,352)		5 747 (19, 353)	5 260 (20, 352)	5 398 (20,388)
F_{partial}	2.67 (35, 353)[h]	31.3 (1, 352)[i]		–	0.11 (1, 352)[j]	2.6 (36, 352)[k]

[a] 95% confidence limits for rate constants for Model I computed from data presented in Table IX.

[b] Estimates of rate constants for (1) computed from data in Table V from the set (V_{AB}, K_A, K_{iA}, K_B, V_{QP}, K_Q, K_{iQ}, K_P) assuming a 1:1 ratio of enzyme concentrations and similarly for (2) but assuming a 1:0.2661 ratio).

[c] Estimates of rate constants calculated from the set (K_1, K_2, . . . K_8) using data in Table VIII.

[d] Eqn 12 modified to include ($1 + K_{IP}\cdot[P]$) factor for AB, ABP, and PQ terms and tested for fit to 372 data points for (4) and for an additional 36 data points for (5).

[e] Significant differences for k_3 to k_8 in comparison with results for Eqn 11.

[f] Estimate of K_{IP} for (4) is 0.001 (not significantly different from zero) and for (5) is 0.0105.

[g] Condition number equals the square root of the ratio of maximum and minimum values of the eigenvalues of the $X'X$ matrix.

[h] F_{partial} for Eqn 11 based on comparison with residual error for Eqn 12, Model II, (5). F_{critical} (0.005:30,∞) = 1.79.

[i] F_{partial} for Eqn 10 based on comparison with residual error for Eqn 11. F_{critical} (0.005: 1,∞) = 7.88.

[j] F_{partial} for Eqn 12, Model II, (4), based on comparison with residual error for Model I, (3). F_{critical} (0.005:1,∞) = 7.88.

[k] F_{partial} for Eqn 12, Model II, (5), based on comparison with residual error for Model II (4). F_{critical} (0.005:30,∞) = 1.79

inadequate experimental design. The eigenvalues and eigenvectors of the $X'X$ matrices for all of the analyses were determined and, in all cases where a form of the full rate equation was applied to the data, a high degree of correlation between the parameters was observed as indicated by the condition number (Table X). This may be considered to be due to the fact that the experimental design, which is based on a determination of the velocity of the enzymic reaction extrapolated to zero time, fails to provide sufficient information to delineate the equilibrium relationship at infinite time. The distribution of the residual error among the parameters however indicates a remarkably good fit to the proposed model despite the restricted form of the experimental design.

Although Eqn 10 is not acceptable as a representation of Model I, it did yield a lower variance and condition number than Eqns 11 and 12, showing a highly significant reduction in the residual error with an F_{partial} value of 31.3. The probability of a reduction of this magnitude occurring if Model I were correct is less than 0.001. Since Eqn 10 represents a model with essentially 10 degrees of freedom, it was therefore decided to explore the possibility of the formation of a dead-end inhibitor complex which would introduce additional terms into the denominator of the rate equation. Model II was found to provide a reasonable explanation for the data that had been discarded due to lack of fit for Model I but it did not yield an improvement in the variance.

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} EAB\text{–}EPQ \underset{k_6P}{\overset{k_5}{\rightleftharpoons}} EQ \underset{k_8Q}{\overset{k_7}{\rightleftharpoons}} E \qquad \text{(Model II)}$$

$$EAB\text{–}EPQ \xrightarrow{K_{IP}} EPPQ$$

The possibility arises that the additional parameters, imposed by the necessity of evaluating the enzyme ratio for each block of data, are responsible for the extremely high condition numbers obtained using Eqns 11 and 12. In the initial application of Eqn 10 to the combined data, the structure of the equation implicitly fixed the enzyme ratios at one for each data set on the basis of the definitions of the Z array. Subsequently, when the ratio of V_{AB}/V_{QP} was arbitrarily fixed at 0.2661 (Table X, Footnote b), the estimates of the rate constants corresponded closely to those obtained using the set of kinetic constants (K_1, K_2, . . . K_8). Thus acceptable values of the rate constants could only be obtained from Eqn 10 by a restriction of the linearly dependent parameters that appear in the numerator. In contrast, Eqns 11 and 12 provide an unrestricted determination of both the linearly dependent enzyme ratios and the nonlinearly dependent rate constants or kinetic constants. The extremely high condition numbers obtained with the application of Eqns 11 and 12 to the data may be taken as a reflection of an additional degree of freedom. When Eqn 11 was modified to restrict E_{AB} to 1 and E_{QP} to 0.2661, a drastic reduction in the condition number was observed and a marked improvement in the standard deviations of the rate constants was achieved (Table XI). A similar result was obtained when all of the enzyme ratios were fixed at the values presented in Table IX.

The assumption was therefore made that, in the application of nonlinear regression analysis to the full rate equation, a satisfactory delineation of the confidence limits for the parameters was dependent upon additional information with respect to the interrelationship of the set of parameters defining the forward reaction as represented by (K_1, K_2, K_3, K_4) and those defining the reverse reaction (K_5, K_6, K_7, K_8) (Table VII). The interdependence of these subsets is defined by the equilibrium constant.

To test this hypothesis the equilibrium constant was set equal to the value of $k_1k_3k_5k_7/k_2k_4k_6k_8$ obtained from the estimates of the rate constants presented in Table IX and the number of nonlinearly dependent parameters in Eqn 11 was reduced by one by the substitution of the relationship ($K_{eq} \cdot k_2k_4k_6k_8/k_3k_5k_7$) for k_1. When this reduced model was tested very rapid convergence was obtained for the estimation of 7 nonlinear and 11 linear parameters. Although there was no change in the residual sum of squares, the distribution of the residual error among the parameters was modified drastically and the standard deviations of the rate constants

TABLE XI

EFFECT OF PARAMETER REDUCTION ON THE STANDARD DEVIATIONS AND THE CONDITION NUMBER IN THE ESTIMATION OF RATE CONSTANTS AND ENZYME RATIOS USING MODEL I

k_j ($j = 1, \ldots 8$) defined as mM × 10^2 and enzyme ratios for E_{AB} equal to 100.

Parameter	*Estimate*[a]	*Standard deviations*			
		1[b]	*2*[c]	*3*[d]	*4*[e]
Rate constants					
k_1	69.03	–	2.79	3.56	9.89
k_2	28.41	1.85	1.52	1.86	4.80
k_3	11.29	0.65	0.40	0.58	2.85
k_4	8.20	0.47	0.34	0.40	2.13
k_5	2.36	0.05	0.04	0.05	0.12
k_6	21.85	0.92	0.75	0.90	3.09
k_7	14.55	0.99	0.71	0.88	2.86
k_8	303.65	13.84	8.66	11.98	62.72
Enzyme ratios					
E_{BA}	94.47	1.33	–	1.33	1.33
E_{ABP}	81.15	1.38	–	1.38	1.39
E_{BAP}	77.85	1.25	–	1.25	1.25
E_{ABQ}	79.18	1.29	–	1.30	1.31
E_{BAQ}	76.63	0.98	–	0.98	0.99
E_{QP}	26.61	6.86	–	–	5.77
E_{PQ}	23.75	7.88	–	6.65	5.13
E_{QPB}	25.50	6.76	–	5.12	5.55
E_{PQB}	19.81	5.59	–	4.42	4.31
E_{QPA}	23.95	6.01	–	4.48	5.29
E_{PQA}	21.45	5.59	–	4.40	4.74
Residual error × 10^5		2.2784	2.2784	2.2784	2.2784
Variance × 10^8		6.4362	6.2594	6.4362	6.4362
Condition number[f]		18.99	20.06	25.14	190.4

[a] Estimates of parameters for fitting Eqn 11 obtained using an adaptation of nonlinear regression program written for the Burroughs 6700 by Broekhoven and Watts[17]. Identical parameter estimates were obtained for analyses 1 to 4.

[b] Model restricted by replacing k_1 by $K_{eq} \cdot k_2 k_4 k_6 k_8 / (k_3 k_5 k_7)$.

[c] Model restricted by elimination of all enzyme ratio parameters and fixing these values to those presented in Table IX by setting $Z_1 = 1.0$, $Z_2 = 0.9447$, $Z_3 = 0.8115$, $Z_4 = 0.7785$, $Z_5 = 0.7918$, $Z_6 = 0.7663$, $Z_7 = 0.2661$, $Z_8 = 0.2375$, $Z_9 = 0.2550$, $Z_{10} = 0.1981$, $Z_{11} = 0.2395$, and $Z_{12} = 0.2145$ for the current value of Z_i. Values of Z_j for j not equal to i were set to zero (see text).

[d] Model restricted by setting $Z_7 = 0.2661 = E_{QP}$.

[e] Unrestricted model as defined for Eqn 11.

[f] See Footnote g, Table X.

became comparable to those observed when the enzyme ratios were fixed as shown in Table XI. The resultant improvement in the confidence limits for the rate constants is accordingly a function of the accuracy of the value of the equilibrium constant and would therefore be dependent upon a definitive evaluation of this factor.

Comparison of the results presented in Tables X and XI demonstrates that the nonlinear regression procedure described in Appendix I makes possible a consistent evaluation of enzyme rate equations containing a large number of parameters. For any kinetic analysis it is essential to take into consideration a fundamental model which is the rate constant model in the sense that it is descriptive of each rate process in the mechanism. The models defined by Cleland[1] and others[9–12] are attempts to reduce the computational difficulties inherent in the rate constant model and this

has resulted in various definitions of other parameters. This procedure is acceptable provided that the parameters in the transformed model are in one-to-one correspondence with the parameters of the fundamental model. For this condition to be true, it is necessary that the number of parameters be the same and that the Jacobian for the transformation be non-zero. The method presented in this paper demonstrates the importance of combining all of the data and testing a model that describes the complete mechanism. Other methods, using only parts of the data, produce models that describe separate parts of the mechanism and these procedures are unsatisfactory because they give misleading parameter estimation and integrate the mechanism without ever placing the total model in jeopardy.

In the present study on the mechanism of action of β-hydroxybutyrate dehydrogenase from *R. spheroides* the experimental design, based on the reciprocal plot procedure, does not provide a suitable basis for model discrimination even though the distribution of the residual error among the parameters is reasonable. Although an accurate estimate of the rate constants for any restricted region of the joint confidence interval is not possible without additional information, the hypothesis of an ordered sequential mechanism is acceptable within the confidence limits indicated by the analysis of variance.

APPENDIX I

Nonlinear regression analysis

The quasi-steady state relationship for an enzymic reaction can be expressed by

$$V_i = f(A_{ji}; P_k; EX_0) + e_i \qquad (i = 1, nw; j = 1, m; k = 1, n)$$

where the dependent variable V is a vector of nw velocity determinations; A is an $m \times nw$ matrix of initial concentrations of m reactant components for the ith experiment; P is a vector of n parameters, defined as a set of rate constants or as a set of kinetic constants that are combinations of rate constants; e is a vector of the corresponding errors in the experimental determination of V, and EX_0 is the enzyme concentration. The residual functions e_i are assumed to be independent and the error randomly distributed with constant variance. If, on examination of the residuals, these conditions are not met, then appropriate action must be taken to resolve this problem.

The required solution is the vector P whose estimated values provide a minimum for the residual sum of squares. For most enzymic rate equations $f(V)$ is nonlinear in the parameters and the least squares estimate of the set of parameters that effectively minimizes the residual sum of squares must be achieved by iterative procedures. Convergence to acceptable approximations is not always a simple matter since the variance contour surface for a nonlinear model is frequently narrow, elongated, and non-ellipsoidal. The modified Gauss linearization method[16] with the variable incrementation procedure recommended by G. E. P. Box (personal communication) has been found to overcome some of the difficulties that may arise[13,14]. Generalization of the quasi-steady state rate equation may be achieved by considering it as a ratio of two functions. The numerator may be represented by

$$DN_i = f(A_{ji}; Z_{zi}; P_k; EX_0) \qquad (i = 1, nw; j = 1, m; z = 1, mx; k = 1, n)$$

where DN is a polynomial expression; Z is an $mx \times nw$ matrix employed for the estimation of the respective maximum velocity parameters for each of the mx blocks of data. Z_{zi} is given the value of 1 if the zth data block is associated with the current value of i, otherwise it is given the value of 0. The number of reactant components is given by m.

The denominator of the quasi-steady state equation may be represented by

$$D_i = f(A_{ji}; P_k) \qquad (i = 1, nw; j = 1, m; k = 1, n)$$

where D is a polynomial expression containing at least $n-m+1$ terms.

For provisional values (P°) of the parameters and known values for the A_{ji} reactants, estimates of the velocities can be obtained from

$$V_{esti} = DN_i/D_i$$

and the evaluation of the residuals from

$$Y_i = V_i - V_{esti}$$

The total variation is expressed by

$$\mathrm{SUM}_1 = \sum_{i=1}^{nw} V_i^2$$

and the sum of squares due to deviation from regression is given by

$$\mathrm{SUM}_2 = \sum_{i=1}^{nw} (V_i - V_{esti})^2$$

The variance E is defined by $\mathrm{SUM}_2/(nw-n)$ and the F value for n and $(nw-n)$ degrees of freedom by $(\mathrm{SUM}_1-\mathrm{SUM}_2)\cdot(nw-n)/(n\cdot\mathrm{SUM}_2)$. For the initial trial vector P° a variance estimate E° is obtained and a vector of increments δP is defined for which $P^\circ + \delta P = P^1$ yields a new variance estimate E^1.

The matrix solution for the vector of δP values is based on the following relationships

$$\boldsymbol{Y} = (Y_i) = \delta V_i$$

$$\boldsymbol{X} = (X_{ij}) = \partial V_{est_i}/\partial P_j$$

$$X'Y = X'X\cdot\delta P$$
$$\delta P = (X'X)^{-1}\cdot X'Y$$

The elements of matrix X are defined by

$$X_{ij} = [D_i\cdot(\partial DN_i/\partial P_j) - DN_i\cdot(\partial D_i/\partial P_j)]/D_i^2 \qquad (i = 1, nw; j = 1, n)$$

The definitions of DN, D, $\partial DN/\partial P$, and $\partial D/\partial P$ are determined by the structure of the rate equation being tested and must be provided as an external function.

In practice a fraction of δP is used in the incrementation of the parameters so that

$$P^1 = P^0 + \delta P\cdot\Phi$$

The variable factor Φ is defined by

$$\Phi = 1/(1+\mu)$$

The value of μ is set initially to some convenient value (usually in the range 0.1 to 10) dependent upon the accuracy of the provisional estimate of the parameters and is changed at each iteration in accordance with the nature of the change in variance given by

$$\delta E = E^0 - E^1$$

If δE is negative, the values of P^1 obtained in the incrementation step are discarded to prevent divergence and the incrementation factor is changed by the replacement of the current value of μ by the value of $\mu/0.618$ where 0.618 is the Pythagorean golden section. New values of $\delta P \cdot \Phi$ are estimated and the vector P° is retained until a positive value of δE is reached.

When δE is positive, the current value of μ is replaced by the value of $0.618 \times \mu$ and iteration is continued using the new trial vector P^1 in place of P°. This process is repeated until the convergence criteria have been satisfied. A stationary value of E is considered to have been attained when the ratios, $\delta E/E^\circ$ and $\delta P_i/P^\circ_i$, are all less than respectively assigned critical values.

A positive constraint is imposed on the parameters by a simple means of reparameterization defined by

$$\theta_i = \log_e P_i$$

Alternatively

$$P_i = e^{\theta_i}$$

The partial derivatives for the set of θ can be obtained conveniently from those defined for the set of P by the relationship

$$\partial V/\partial \theta = (\partial V/\partial P) \cdot \theta$$

Thus the procedure for estimating δP can be used for the estimation of $\delta\theta$ since

$$X_\theta = X_P \cdot \theta$$

The method for estimating $\delta\theta$ is used until the convergence criteria are satisfied and then a final estimation is obtained using the procedure for δP. In all stages of iteration the value of the variance is computed from values of $P = e^\theta$.

When the convergence criteria have been satisfied, the variance-covariance table is obtained from the $X'X$ matrix by the relationship

$$S_{ij} = (X'X_{ij})^{-1} \cdot \mathrm{SUM}_2/(nw - n) \qquad (i = 1, n; j = 1, n)$$

and the standard deviations of the parameters from

$$s_i = \sqrt{(S_{ii})} \qquad (i = 1, n)$$

The correlation matrix for the intercorrelation of the parameters is obtained by the relationship

$$U_{ij} = S_{ij}/(s_i \cdot s_j) \qquad (i = 1, n; j = 1, n)$$

The eigenvalues and eigenvectors are obtained from the normalized form of the $X'X$ matrix in order to eliminate differences due to scaling of the X vector. Conversion of $X'X$ to the normalized form is given by

$$Z'Z_{ij} = X'X_{ij}/(\sqrt{X'X_{ii}} \cdot \sqrt{X'X_{jj}})$$

Computation of the eigenvalues and eigenvectors of the $Z'Z$ matrix is accomplished by means of the EIGEN subroutine[8] modified to produce the angles of the direction vectors in degrees and the ratios of the eigenvalues and their square roots to those of the largest eigenvalue. Direction vectors close to 45° and 135° indicate high correlation for the dominant component.

Abnormal termination of the iterative procedure may occur for the following reasons:

(i) Failure to find a positive value of δE after a specified number of trials. Conclusion is that the $X'X$ matrix is extremely ill-conditioned.

(ii) Determinant of the $X'X$ matrix equals zero. The matrix is printed out to check for occurrence of a zero column or a linear dependency between columns (usually the result of an error in the construction of the equation for $f(V)$).

(iii) Determinant of $X'X$ matrix is too small. This signals a condition of near singularity. Failure may be due to insufficient data to satisfy the degrees of freedom implicit in the model. A solution may be possible by changing the scale of the dependent variable. The corresponding scale changes in the parameters will depend upon their interrelationship in the rate equation.

(iv) Estimate of parameter approaches zero. Indication is that the parameter is not required to explain the regression.

(v) Parameter increases beyond defined limit. Indication of gross discrepancy in the provisional estimate of the parameters.

A flow chart for the iterative procedure is presented in Fig. 9.

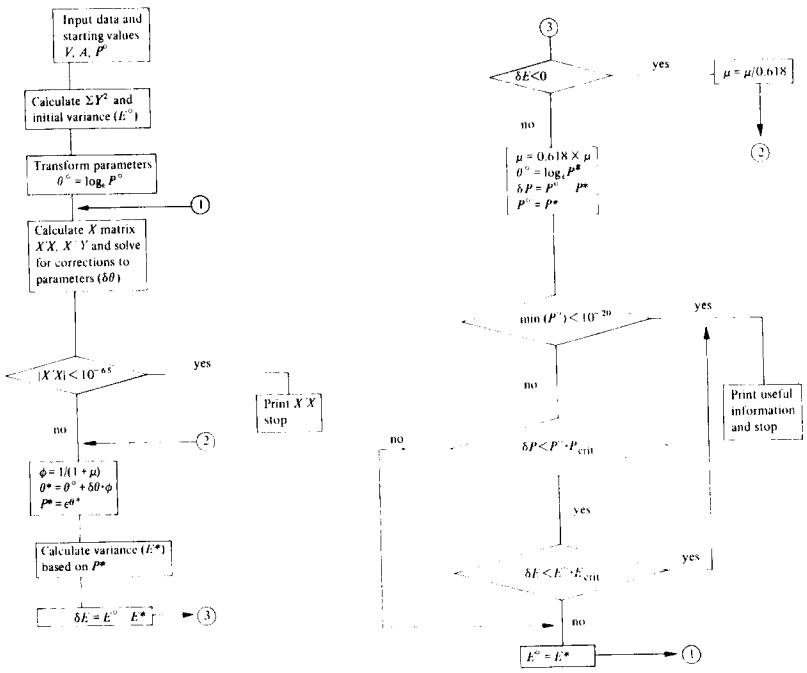


Fig. 9 Flow diagram for nonlinear regression analysis.

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