D-3-Hydroxybutyrate Dehydrogenase from *Rhodopseudomonas spheroides*

KINETIC MECHANISM FROM STEADY-STATE KINETICS OF THE REACTION CATALYSED BY THE ENZYME IN SOLUTION AND COVALENTLY ATTACHED TO DIETHYLAMINOETHYLCELLULOSE

By M. J. PREUVENEERS, D. PEACOCK,* E. M. CROOK,
J. B. CLARK and K. BROCKLEHURST†

Department of Biochemistry and Chemistry, St. Bartholomew's Hospital Medical College,
University of London, Charterhouse Square, London EC1M 6BQ, U.K.

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1. The reversible NAD^+ -linked oxidation of D-3-hydroxybutyrate to acetoacetate in 0.1 M-sodium pyrophosphate buffer, pH 8.5, at 25.0°C, catalysed by D-3-hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate- NAD^+ oxidoreductase, EC 1.1.1.30), was studied by initial-velocity, dead-end inhibition and product-inhibition analysis. 2. The reactions were carried out on (a) the soluble enzyme from *Rhodopseudomonas spheroides* and (b) an insoluble derivative of this enzyme prepared by its covalent attachment to DEAE-cellulose by using 2-amino-4,6-dichloro-s-triazine as coupling agent. 3. The insolubilized enzyme preparation contained 5 mg of protein/g wet wt. of total material, and when freshly prepared its specific activity was 1.2 $\mu\text{mol/min}$ per mg of protein, which is 67% of that of the soluble dialysed enzyme. 4. The reactions catalysed by both the enzyme in solution and the insolubilized enzyme were shown to follow sequential pathways in which the nicotinamide nucleotides bind obligatorily first to the enzyme. Evidence is presented for kinetically significant ternary complexes and that the rate-limiting step(s) of both catalyses probably involves isomerization of the enzyme-nicotinamide nucleotide complexes and/or dissociation of the nicotinamide nucleotides from the enzyme. Both catalyses therefore are probably best described as ordered Bi Bi mechanisms, possibly with multiple enzyme-nicotinamide nucleotide complexes. 5. The kinetic parameters and the calculable rate constants for the catalysis by the soluble enzyme are similar to the corresponding parameters and rate constants for the catalysis by the insolubilized enzyme.

D-3-Hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate- NAD^+ oxidoreductase, EC 1.1.1.30) is widely distributed in both eukaryotic and prokaryotic cells and catalyses the reversible NAD^+ -linked oxidation of D-3-hydroxybutyrate to acetoacetate:

1972). It is tightly bound to the inner membrane of the mitochondrion and closely associated with the NAD -linked electron-transport chain. The enzyme may be solubilized by removal from its associated mitochondrial membrane but in this state it is inactive. Full activity may be regained, however, by preincu-

In mammalian systems this oxidation constitutes the first step in the utilization of D-3-hydroxybutyrate for provision of cellular energy, and certain tissues, e.g. myocardium, use this ketone body as their preferred substrate for energy provision (see Wieland, 1968).

The mammalian enzyme has been studied by a number of workers (e.g. Lehninger *et al.*, 1960; Krebs *et al.*, 1962; Gotterer, 1967, 1969; Nielsen *et al.*,

bation of the enzyme in solution with phosphatidylcholine (Sekuzu *et al.*, 1963; Jurtshuk *et al.*, 1963; Gotterer, 1967).

In marked contrast with the enzyme from mammalian sources, D-3-hydroxybutyrate dehydrogenase from certain species of micro-organisms can be easily solubilized and exhibits no dependence upon phospholipid for activity (Shuster & Doudoroff, 1962; Williamson *et al.*, 1962). The enzyme from *Rhodopseudomonas spheroides* has been crystallized and its molecular weight (85000 ± 6800) determined by a sedimentation-equilibrium method (Bergmeyer *et al.*, 1967).

* Present address: Department of Botany, University of Durham, Durham City, U.K.

† To whom reprint requests should be addressed.

The present paper reports a steady-state kinetic analysis of the reaction catalysed by D-3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* at pH 8.5 to establish its kinetic mechanism. At this pH both the hydroxybutyrate oxidation and the acetoacetate reduction are relatively rapid (Bergmeyer *et al.*, 1967). The analysis was carried out on the reaction catalysed by the enzyme (a) in solution and (b) covalently attached to an insoluble polymeric matrix (DEAE-cellulose). The comparative kinetic study of the soluble and insolubilized forms of the enzyme was carried out to ascertain whether non-specific insolubilization of this oxidoreductase on an 'inert' supporting matrix necessarily gives rise to changes in the kinetic mechanism or in the parameters that characterize the catalysis. This is of interest in connexion with studies on the membrane-bound enzyme of mammalian systems. It seems possible that in these systems the phospholipid may provide a supporting matrix, which is essential for the enzyme's activity. In addition, insolubilized D-3-hydroxybutyrate dehydrogenase could find important application in clinical analysis of ketone bodies. The use of the soluble enzyme for this purpose has been described (Williamson *et al.*, 1962).

in loss of 30% of the enzymic activity. The dialysis step was therefore not carried out on stock solutions of the enzyme used for kinetic studies in solution. It was carried out, however, in the preparation of enzyme solutions for attachment to DEAE-cellulose because the $(\text{NH}_4)_2\text{SO}_4$ would have interfered with the attachment procedure (see below).

The concentration of the enzyme in solution was determined initially by the method of Lowry *et al.* (1951) with bovine serum albumin as protein standard. The protein concentration thus determined, together with a molecular weight of 85000 (Bergmeyer *et al.*, 1967) and u.v.-absorption values, was used to calculate a value of $\epsilon_{280} = 6.15 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. A spectrophotometric assay was then used as a routine to determine protein concentration.

Insolubilized enzyme. The insolubilized enzyme (DEAE-cellulose - amino-s-triazinyl - D-hydroxybutyrate dehydrogenase) was prepared by a development of the coupling method first outlined by Kay & Crook (1967) (see also Kay & Lilly, 1970). The method involves reaction of a nucleophilic centre in the enzyme (probably the ϵ -amino group of a lysine residue) with DEAE-cellulose-aminochloro-s-triazine, i.e.:

Materials and Methods

Enzyme

Soluble enzyme. D-3-Hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* was the grade II product of Boehringer Corp. (London) Ltd., London W.5, U.K. Boehringer's specification is that this product contains less than 0.2% lactate dehydrogenase and less than 5% L-malate dehydrogenase. No evidence for the presence of proteins other than D-3-hydroxybutyrate dehydrogenase was provided by analytical gel filtration. The enzyme was supplied as a suspension in $2.4 \text{ M} \cdot (\text{NH}_4)_2\text{SO}_4$. The suspended protein (1 mg in 0.2 ml) was brought into solution by addition of 0.1 M-sodium phosphate buffer, pH 6.5 (3.8 ml), at 4°C . Such a stock solution was prepared freshly each day and was sufficient for about 40 kinetic runs. In the assay procedure 0.1 ml of the stock solution of enzyme was diluted to 10 ml in the reaction cell, giving a solution containing $1.2 \text{ mM} \cdot (\text{NH}_4)_2\text{SO}_4$. In preliminary experiments the $(\text{NH}_4)_2\text{SO}_4$ was removed from the stock solution by dialysis against 10 mM-sodium phosphate buffer, pH 6.5 ($2 \times 500 \text{ ml}$), at 4°C for 12 h. This procedure resulted

The latter was prepared from DEAE-cellulose and 2-amino-4,6-dichloro-s-triazine (see below).

The protein content of the insolubilized enzyme was determined by the mass balance method (see Crook *et al.*, 1970).

Preparations

Preparation of 2-amino-4,6-dichloro-s-triazine. This was prepared from cyanuric chloride and NH_3 gas by a modification of the method of Thurston *et al.* (1951). Preliminary experiments on the attachment of yeast alcohol dehydrogenase to DEAE-cellulose by using 2-amino-4,6-dichloro-s-triazine prepared by the method of Kay & Lilly (1970) had yielded insolubilized enzyme preparations of variable activity. To ensure that the preparations of 2-amino-4,6-dichloro-s-triazine used to couple D-3-hydroxybutyrate dehydrogenase to DEAE-cellulose did not contain unchanged cyanuric chloride (which might cause cross-linking to occur) the formation of Cl^- was monitored and the reaction was allowed to continue until 1 g-ion of Cl^- /mol of cyanuric chloride had been liberated.

A solution of cyanuric chloride (92 g) in dry dioxan (1 litre) and toluene (200 ml) was cooled externally in ice and stirred vigorously. Ammonia gas, dried by passage through KOH pellets, was passed slowly through the reaction mixture at such a rate that the temperature did not rise above 6°C. A white solid precipitated as the reaction proceeded. At intervals of approx. 0.5 h, samples of the stirred suspension were removed and the solid was separated by vacuum filtration on a sintered-glass filter. The filter cake was washed thoroughly with acetone to remove organic material and the residual NH_4Cl was dissolved in water and Cl^- determined by titration with 50 mM- AgNO_3 . After approx. 4 h 1.0 g-ion of Cl^-/mol of cyanuric chloride had been liberated and the reaction was terminated by replacing the slow stream of NH_3 gas by a rapid stream of N_2 . Passage of N_2 through the reaction mixture was continued until NH_3 was no longer detected (litmus paper) in the effluent gas (approx. 1 h). The solid material in the reaction mixture was then separated by vacuum filtration, re-suspended in dioxan (1 litre), stirred in the liquid and removed again by vacuum filtration. The combined filtrates were then evaporated to dryness by rotary evaporation under reduced pressure. The residue was dissolved in acetone (1 litre) and to this solution was added water (1 litre). The volume of the mixture was halved by rotary evaporation under reduced pressure and the crystalline material that resulted was removed by vacuum filtration. The recrystallization procedure was repeated three times. The product (yield 75%) was shown to be pure by t.l.c. and elemental analysis.

Preparation of DEAE-cellulose-aminochloro-s-triazine. This was prepared from DEAE-cellulose and 2-amino-4,6-dichloro-s-triazine by the method of Kay & Lilly (1970). It was stored in 100 mM-sodium phosphate buffer, pH 7.0, at 4°C and pre-equilibrated to the required ionic strength and pH immediately before use.

Preparation of DEAE-cellulose-amino-s-triazinyl-D-3-hydroxybutyrate dehydrogenase. The attachment of the enzyme to the activated DEAE-cellulose is markedly affected by both pH and the buffer concentration. Maximal attachment at 4°C was found to occur in 10 mM-sodium phosphate buffer, pH 8.0 (D. Peacock, E. M. Crook & K. Brocklehurst, unpublished work).

The enzyme, suspended in $(\text{NH}_4)_2\text{SO}_4$ solution (see above) (10 mg in 2 ml), was dialysed against 10 mM-sodium phosphate buffer, pH 8.0 (2×500 ml), at 4°C for 12 h. The resulting enzyme solution (4 ml, containing 10 mg of protein) was added to a stirred suspension of the DEAE-cellulose-aminochloro-s-triazine (1 g wet wt.) pre-equilibrated in 10 ml of 10 mM-sodium phosphate buffer, pH 8.0, at 4°C and the mixture was stirred at this temperature for 24 h. The suspension was then centrifuged (4000 g, 10 min) at room temperature (19–22°C) and the

supernatant was removed for protein and enzymic activity determinations. The insoluble material was suspended in 1 M-NaCl (10 ml) at 4°C, stirred for 10 min and then separated by centrifugation as described above until the supernatant contained less than $0.1 \mu\text{M}$ -protein. Removal of adsorbed protein usually required three washings. The insolubilized enzyme (1 g of total material containing 5 mg of protein) was then equilibrated with 0.1 M-sodium pyrophosphate buffer, pH 8.5 (40 ml), centrifuged and resuspended in the buffer (10 ml). The specific activity of the insolubilized enzyme in 0.1 M-sodium pyrophosphate buffer, pH 8.5, at 25.0°C was $1.2 \mu\text{mol}/\text{min}$ per mg of protein, which was 67% of that of the soluble dialysed enzyme. When the suspension was stored at 4°C it gradually lost 50% of this activity during a period of 3 weeks. After each kinetic run the insolubilized enzyme was recovered by centrifugation (4000 g, 10 min) at room temperature (19–22°C) and stored at 4°C in the centrifuge tube. The same centrifuge tube was used repeatedly to collect insolubilized enzyme from a series of reaction mixtures. The insolubilized enzyme, which accumulated in the centrifuge tube, was washed with 0.1 M-sodium pyrophosphate buffer, pH 8.5 (approx. 10 ml), collected by centrifugation as described above and resuspended in fresh buffer (approx. 1 g/40 ml). This suspension was stored at 4°C. Each time insolubilized enzyme was recovered as described above, the new suspension possessed about 95% of its previous activity.

Chemicals

Substrates. DL-3-Hydroxybutyric acid [Sigma (London) Chemical Co., London S.W.6, U.K.] was resolved into its optical isomers essentially as described by Clarke (1959) but by employing the modifications of McCann & Greville (1962). The optical rotations of the individual isomers, determined with a Bendix Polaromatic 62 spectropolarimeter (for the D-isomer, $[\alpha]_D^{25} = -24.4 \pm 0.2^\circ$ and for the L-isomer, $[\alpha]_D^{25} = 24.5 \pm 0.2^\circ$; $c = 1$, water), are in good agreement with literature values (Clarke, 1959; McCann & Greville, 1962). Enzymic estimation of the purity of the D-isomer by using the equilibrium constant reported by Krebs *et al.* (1962) gave a value of 98%.

Lithium acetoacetate was prepared by the method of Hall (1962) and shown to be 95% pure by enzymic assay.

NAD^+ [Sigma (London) Chemical Co.] was purified by the method of Dalziel & Dickinson (1966), stored frozen and never kept longer than 4 days after purification. Its concentration was measured spectrophotometrically by using $\epsilon_{260} = 1.76 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Dalziel & Dickinson, 1966).

NADH (grade III) was obtained in pre-weighed vials from Sigma (London) Chemical Co. and was

shown to be 98% pure by enzymic assay (reduction of pyruvate catalysed by lactate dehydrogenase; Hakala *et al.*, 1956). The concentration of NADH in solution was calculated from $\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Horecker & Kornberg, 1948) in the concentration range over which it obeyed Beer's Law. In reaction mixtures containing NADH in concentrations such that Beer's Law was not obeyed ($>350 \mu\text{M}$) for reactions in solution and for reactions in suspension, NADH concentrations were calculated by using calibration curves.

Other chemicals. ADP-ribose was obtained from Sigma (London) Chemical Co. as the sodium salt. DL-Lactic acid (AnalaR grade) and cyanuric chloride were the products of BDH Chemicals Ltd., Poole, Dorset, U.K. Sodium pyrophosphate-phosphate buffer (0.1M-sodium pyrophosphate, pH8.5) was prepared from $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ and H_3PO_4 (AnalaR-grade products of BDH) and deionized water.

Initial-rate measurements

The reactions catalysed by both soluble and insolubilized forms of the enzyme were studied in 0.1M-sodium pyrophosphate buffer, pH8.5, at 25.0°C . The enzyme concentrations employed were (a), for the soluble enzyme, $1 \mu\text{g/ml}$ (23.5 nM) and (b), for the insolubilized enzyme, 10–50mg of total material containing 4–20 μg of protein suspended in 10ml of solution containing the substrates. The appearance or disappearance of NADH was followed by recording the u.v. absorbance at 340nm. The reaction mixture (whether solution or suspension) was stirred magnetically at constant speed in the thermostatically controlled cell compartment of a Zeiss PMQ11 spectrophotometer, which was coupled to a Smith's Servoscribe recorder.

In the reactions catalysed by the insolubilized enzyme the presence of the insoluble material in the stirred suspension affected the spectrophotometric analysis of product formation in two ways. It decreased the upper limit of the concentration range over which the u.v. absorption by NADH at 340nm obeyed Beer's Law, from 350 to $250 \mu\text{M}$ and also gave rise to 'noise' in the recorder trace with an amplitude of up to $\pm 0.002 E_{340}$ unit. These effects, however, did not interfere with the accurate measurement of the concentration of NADH in the reaction mixture nor thus of the reaction rate.

Further support for the reliability of measurement of u.v. absorption in stirred suspensions (at least at wavelengths $\geq 300 \text{ nm}$) with the Zeiss PMQ11 spectrophotometer is provided by the observation that the spectrum of sodium salicylate ($90 \mu\text{M}$, in sodium phosphate buffer, pH7.0, $I = 0.1$; λ_{max} , 300nm) is not seriously perturbed by the presence of suspended *O*-carboxymethylcellulose (at least up to

10mg/ml; 2cm cell) (K. Brocklehurst, E. M. Crook & P. L. Russell, unpublished work).

Data processing

The primary data obtained in this study consist of values of the steady-state rate (v), each one corresponding to a different concentration of one of the substrates (S), obtained while all other variables were maintained constant. Each set of primary data was plotted according to one of the linear transforms of the Michaelis–Menten equation (eqn. 1) to check that the data did appear to fit eqn. (1):

$$v = \frac{V[S_0]}{K_m + [S_0]} \quad (1)$$

The primary data ($[S_0]$, v pairs), which appeared to fit eqn. (1), were used to determine the characterizing parameters of this equation (V and K_m) by using the computer program described by Cleland (1963a). This program uses the procedure described by Wilkinson (1961). Provisional estimates of the parameters are obtained by weighted regression of the linear (reciprocal) form of eqn. (1). These are then refined by iterative fitting of the data to eqn. (1) until the value of K_m becomes constant.

Decisions about the nature of the initial-velocity patterns and the various inhibition patterns were made by inspection of the plotted data.

The constituent data of the secondary plots in the initial-velocity analysis (slopes, i.e. K_m/V , and ordinate intercepts, i.e. $1/V$, against the concentration of the fixed substrate) were analysed by weighted linear regression to provide provisional estimates of V_r , K_m^A , K_m^B , K_s^A , V_r , K_m^P , K_m^Q and K_s^Q (see eqn. 5). These estimates were subsequently refined by fitting the complete set of data from the initial-velocity analysis to eqns. (6) and (7) as described by Cleland (1963a).

In the inhibition analyses the characterizing parameters were evaluated by fitting the data to eqn. (2) for linear competitive inhibition, eqn. (3) for linear uncompetitive inhibition and eqn. (4) for linear non-competitive inhibition:

$$v = \frac{V[S_0]}{K_m(1 + [I]/K_{is}) + [S_0]} \quad (2)$$

$$v = \frac{V[S_0]}{K_m + [S_0](1 + [I]/K_{ii})} \quad (3)$$

$$v = \frac{V[S_0]}{K_m(1 + [I]/K_{is}) + [S_0](1 + [I]/K_{ii})} \quad (4)$$

In eqns. (2)–(4), $[I]$ is the inhibitor concentration and K_{is} and K_{ii} are the operationally defined inhibition constants relating to the secondary plots of slope and intercept respectively (see Cleland, 1963a).

Standard errors on the kinetic parameters obtained from apparent inhibition constants (see the Results and Discussion section) were calculated as described by Morrison & Uhr (1966).

Results and Discussion

Determination of the general form of the mechanism: initial-velocity analysis in both directions

The initial-velocity patterns (double-reciprocal plots) for the forward reaction (formation of aceto-

acetate) and for the reverse reaction catalysed by the enzyme in solution are presented in Fig. 1 and by the insolubilized enzyme in Fig. 2. When NAD^+ was the variable substrate at different concentrations of the fixed substrate, D-3-hydroxybutyrate, intersecting patterns (Figs. 1a and 2a) were obtained for both

Fig. 1. Initial-velocity patterns for (a) and (b), the forward reaction, and for (c) and (d), the reverse reaction, catalysed by the enzyme in solution

$[\text{E}_\text{T}]$: $5.87 \times 10^{-2} \mu\text{M}$. (a) $[\text{D-3-Hydroxybutyrate}]$ (μM): $\blacktriangle$, 2522; $\triangle$, 631; $\bullet$, 315; $\circ$, 189; $\blacksquare$, 126. (b) $[\text{NAD}^+]$ (μM): $\blacktriangle$, 161; $\triangle$, 80.5; $\bullet$, 40.3; $\circ$, 32; $\blacksquare$, 16. (c) $[\text{Acetoacetate}]$ (μM): $\blacktriangle$, 1000; $\triangle$, 500; $\bullet$, 250; $\circ$, 125. (d) $[\text{NADH}]$ (μM): $\blacktriangle$, 70.8; $\triangle$, 35.4; $\bullet$, 17.7; $\circ$, 8.8; $\blacksquare$, 7.1. Other conditions were as given in the Materials and Methods section.

Fig. 2. Initial-velocity patterns for (a) and (b), the forward reaction, and for (c) and (d), the reverse reaction, catalysed by the insolubilized enzyme

$[E_T]$: $3.05 \times 10^{-1} \mu\text{M}$ (a and b) or $7.05 \times 10^{-1} \mu\text{M}$ (c and d). (a) [D-3-Hydroxybutyrate] (μM): $\blacktriangle$, 2522; $\triangle$, 1261; $\bullet$, 631; $\circ$, 315; $\blacksquare$, 252. (b) $[\text{NAD}^+]$ (μM): $\blacktriangle$, 292; $\triangle$, 146; $\bullet$, 73; $\circ$, 29; $\blacksquare$, 15. (c) [Acetoacetate] (μM): $\blacktriangle$, 1000; $\triangle$, 500; $\bullet$, 250; $\circ$, 125. (d) $[\text{NADH}]$ (μM): $\blacktriangle$, 144; $\triangle$, 35; $\bullet$, 14.1; $\circ$, 7.1. Other conditions were as given in the Materials and Methods section.

catalyses. When D-3-hydroxybutyrate was the variable substrate at different concentrations of the fixed substrate, NAD^+ , intersecting patterns (Figs. 1b and 2b) were again obtained for both catalyses. The occurrence of these intersecting patterns demonstrates that both catalyses are of the sequential type involving ternary complexes (not necessarily kinetically significant) and not of the Ping Pong type involving substituted enzymes. The secondary plots of the data in the primary plots (Figs. 1 and 2), i.e. slopes and ordinate intercepts against the reciprocal of the concentration of the fixed substrate, are given in Fig. 3. The linearity of the plots in Fig. 3 suggests that both the forward and reverse reactions catalysed by both the enzyme in solution and by the insolubilized enzyme may probably be represented adequately by mechanisms in which only one molecule of each substrate binds to a particular enzyme form.

Evidence from product-inhibition and dead-end-

to a steady-state velocity that is half the maximum velocity when the complementary substrate is at saturating concentration; K_1^P and K_1^Q are operationally defined (see Cleland, 1963c) inhibition constants that characterize respectively the inhibition of the reverse reaction by B and the inhibition of the forward reaction by P and are without obvious physical significance. The result of the kinetic analysis of these catalyses (i.e. Scheme 1) is presented at this point to allow discussion of the experimental results in terms of eqn. (5) and the various equations derived from it.

The steady-state rate of the forward reaction in the absence of the products [P] and [Q] is given by eqn. (6), which is obtained from eqn. (5) by setting [P] and [Q] equal to zero. Similarly the steady-state rate of the reverse reaction in the absence of the products A and B is given by eqn. (7), which is obtained from eqn. (5) by setting [A] and [B] equal to zero and changing the sign:

$$\frac{d[Q]}{dt} = v = \frac{V_f[A][B]}{K_s^A K_m^B + K_m^A[B] + K_m^B[A] + [A][B]} \quad (6)$$

$$-\frac{d[Q]}{dt} = \frac{d[A]}{dt} = v = \frac{V_r[P][Q]}{K_s^Q K_m^P + K_m^Q[P] + K_m^P[Q] + [P][Q]} \quad (7)$$

inhibition analyses presented below strongly suggests that the predominant reaction pathway in the catalyses follows a mechanism of the ordered Bi Bi type (see Cleland, 1963b) in which the nicotinamide nucleotides bind obligatorily first to the enzyme. The basic form (i.e. without elaborations such as isomerizations) of this type of mechanism is depicted in Scheme 1. The steady-state rate (v) of the catalysis in terms of the model depicted in Scheme 1 is given by eqn. (5) (see Cleland, 1963b):

The intersecting patterns of the initial-velocity data in Figs. 1 and 2 demonstrate the presence of the $K_s^A K_m^B$ and $K_s^Q K_m^P$ terms in eqns. (6) and (7) for catalysis by both the free and the immobilized enzyme. The mechanisms of these catalyses are therefore of the sequential type. Rate equations for mechanisms which involve double displacement (Ping Pong mechanisms) do not contain the $K_s K_m$ term (see Cleland, 1963b) and are therefore not considered further.

$$\begin{aligned} \frac{d[Q]}{dt} = v = & V_f \{ [A][B] - [(V_r K_m^B K_s^A [P][Q]) / (V_f K_m^P K_s^Q)] \} / \left\{ K_m^B[A] + K_m^A[B] + [A][B] + \left(\frac{K_m^B K_m^Q K_s^A [P]}{K_m^P K_s^Q} \right) + \right. \\ & \left(\frac{K_m^B K_s^A [Q]}{K_s^Q} \right) + \left(\frac{K_m^B K_s^A [P][Q]}{K_m^P K_s^Q} \right) + K_m^B K_s^A + \left(\frac{K_m^A [B][Q]}{K_s^Q} \right) + \left(\frac{K_m^B K_s^Q [A][P]}{K_m^P K_s^Q} \right) + \\ & \left. \left(\frac{[A][B][P]}{K_1^P} \right) + \left(\frac{K_m^B K_s^A [B][P][Q]}{K_m^P K_1^B K_1^Q} \right) \right\} \quad (5) \end{aligned}$$

In eqn. (5), V_f and V_r are the maximum velocities of the forward and reverse reactions respectively; K_s^A and K_s^Q are the dissociation constants of the EA and EQ complexes respectively; K_m^A , K_m^B , K_m^P and K_m^Q are the Michaelis constants which characterize the interaction of A, B, P and Q respectively with the appropriate enzyme form and have no obvious significance, except for the operational one of the magnitude of the particular substrate concentration corresponding

Dead-end inhibition of the forward reaction: demonstration that the forward reaction is ordered and that NAD^+ binds to the free enzyme

One way to decide whether a sequential kinetic mechanism approximates to an ordered mechanism or to a random mechanism and, if to the former, to determine the order of binding, is to carry out a dead-end-inhibition analysis. One rational choice of a dead-end inhibitor is a molecule which is a close structural

analogue of one of the substrates but not of the other (for the reaction in a given direction). In a random sequential mechanism a dead-end inhibitor which is a close structural analogue of substrate A (but not of substrate B) will give rise to a competitive inhibition pattern when [A] is varied and to a non-competitive inhibition pattern when [B] is varied. Similarly, a close structural analogue of substrate B will give rise to a competitive inhibition pattern when [B] is varied and a non-competitive inhibition pattern when [A] is varied. In an ordered sequential mechanism a close

structural analogue of the substrate which binds first (substrate A) will give rise to a competitive inhibition pattern when the concentration of that substrate is varied and a non-competitive inhibition pattern when the concentration of the other substrate (substrate B) is varied. Thus a structural analogue of substrate A is of no value in distinguishing random and ordered mechanisms since the same types of inhibition patterns are obtained in each case. When a close structural analogue of substrate B, the substrate that binds obligatorily second (i.e. to the EA complex) is used as

Fig. 3. Secondary plots of the slopes and intercepts of the initial-velocity plots for (a) and (b), the forward reaction, and for (c) and (d), the reverse reaction

Plots are for reactions catalysed by ●, the enzyme in solution or ○, the insolubilized enzyme.

an inhibitor, however, an uncompetitive inhibition pattern will be obtained when the concentration of the substrate that binds first is varied. Demonstration of an uncompetitive inhibition pattern therefore allows the detection of an ordered mechanism and the delineation of the order of binding. When the concentration of the substrate that binds second is

varied a competitive inhibition pattern will be obtained.

In the present work, ADP-ribose was used as a structural analogue of NAD⁺ and DL-lactate as a structural analogue of D-3-hydroxybutyrate. Fig. 4 demonstrates that, for both catalyses, ADP-ribose gives rise to a competitive inhibition pattern when

Scheme 1. Simplest representation of an ordered Bi Bi mechanism

A, NAD⁺; B, D-3-hydroxybutyrate; P, acetoacetate; Q, NADH; E, enzyme

Fig. 4. Dead-end-inhibition patterns with ADP-ribose as dead-end inhibitor for the forward reaction catalysed by the enzyme in solution and by the insolubilized enzyme

Enzyme in solution ($\blacktriangle$, $\blacksquare$, $\bullet$): $[E_T]: 5.87 \times 10^{-2} \mu\text{M}$. Insolubilized enzyme ($\triangle$, $\square$, $\circ$): $[E_T]: 11.74 \times 10^{-2} \mu\text{M}$. (a) [D-3-Hydroxybutyrate]: $31.5 \mu\text{M}$. [ADP-ribose] (μM): $\blacktriangle$, 0.0; $\blacksquare$, 290; $\bullet$, 580; $\triangle$, 0.0; $\square$, 290; $\circ$, 740. (b) [NAD⁺]: $1.85 \mu\text{M}$. [ADP-ribose] (μM): $\bullet$, 0.0; $\blacktriangle$, 550; $\circ$, 0.0; $\triangle$, 740. Other conditions were as given in the Materials and Methods section.

NAD⁺ is the variable substrate and to a non-competitive inhibition pattern when D-3-hydroxybutyrate is the variable substrate. Fig. 5 demonstrates that, for both catalyses, when NAD⁺ is the variable substrate the secondary plots of the inhibition data (slopes of the primary plots in Fig. 4 against inhibitor concentration) for ADP-ribose are linear. Fig. 6 demonstrates that, for both catalyses, D-lactate gives rise to a competitive inhibition pattern when D-3-hydroxybutyrate is the variable substrate and to an uncompetitive inhibition pattern when NAD⁺ is the variable substrate. Fig. 7 demonstrates that for both catalyses the secondary plots of the inhibition data (slopes and ordinate intercepts of the primary plots in Fig. 6 against inhibitor concentration) for D-lactate are linear.

The dead-end-inhibition patterns presented in Figs. 4 and 6 demonstrate that catalysis by both the free and the insolubilized enzyme in the forward direction approximate closely to an ordered kinetic mechanism, in which NAD⁺ binds first (i.e. to the uncombined enzyme E) and D-3-hydroxybutyrate binds second (i.e. to the EA complex). The probable linearity of the secondary plots (Figs. 5 and 7) indicates that binding of only 1 mol of either ADP-ribose or D-lactate (see below) to each mol of the appropriate enzyme form is mainly responsible for the observed dead-end inhibitions.

The apparent inhibition constants (K_{i1} or K_{i2}) that characterize the various dead-end inhibitions were determined by fitting the data to the appropriate equation by using the computer programs described

Fig. 5. Secondary plots of the slopes of the dead-end-inhibition plots with ADP-ribose as dead-end inhibitor and NAD^+ as variable substrate for the forward reaction

Plots are for the reaction catalysed by ●, the enzyme in solution or ○, the insolubilized enzyme.

by Cleland (1963a). For the ADP-ribose inhibition when NAD^+ was the variable substrate and for the DL-lactate inhibition when D-3-hydroxybutyrate was the variable substrate, eqn. (2) was used to provide values of K_{is} . For the DL-lactate inhibition when NAD^+ was the variable substrate eqn. (3) was used to provide values of K_{il} (see the Materials and Methods section). For the ADP-ribose inhibition when D-3-hydroxybutyrate was the variable substrate the data collected were insufficient to permit reliable computation of K_{il} and K_{is} by use of eqn. (4).

The significance of each of the apparent inhibition constants was deduced by parametric comparison of the reciprocal form of one of eqns. (2)–(4) with the analogous equation of eqns. (8)–(11). The latter equations are expressions for the reciprocal of the steady-state rate v of a catalysis following an ordered sequential mechanism in the presence of particular types of dead-end inhibitor and are analogous to the reciprocal of eqn. (6), which describes such a catalysis in the absence of dead-end inhibitor:

Eqns. (8)–(11) are obtained from eqn. (6) by multiplying the appropriate term in the denominator by $(1 + [I]/K_s^I)$ (see Cleland, 1963c). In eqns. (8) and (11) $I = \text{ADP-ribose}$ and $K_s^I = \text{dissociation constant of the E-ADP-ribose complex}$; in eqns. (9) and (10) $I = \text{D-lactate}$ and $K_s^I = \text{dissociation constant of the E-D-lactate complex}$. Because, at the concentrations used in this study, L-lactate does not act effectively either as a substrate or as an inhibitor of this enzyme (Bergmeyer *et al.*, 1967), the experimental value of K_{is} for the inhibition by DL-lactate when D-3-hydroxybutyrate was the variable substrate was divided by 2 to provide the corresponding inhibition constant for D-lactate. The values of the apparent inhibition constants and the kinetic parameters calculated from them by the parametric comparison described above are collected in Table 1. The values of the kinetic parameters obtained from initial velocity, dead-end inhibition and product inhibition (see later) analyses are collected in Table 2. Calculation of the kinetic parameters from dead-end and product-inhibition data involves the combination of several parameters, each of which will be subject to error. The calculated parameters will therefore be subject to rather large errors. The most reliable values of the parameters should be those obtained from the initial-velocity analysis. When account is taken of this, the good agreement of the kinetic parameters obtained by the different kinetic analyses provides confirmatory evidence for the postulated ordered Bi Bi mechanism.

Product inhibition of the forward reaction: demonstration that the reverse reaction also is ordered and that NADH binds to the free enzyme; evidence for kinetically significant ternary complexes, for the absence of kinetically significant free-enzyme isomers and for the absence of the most common types of dead-end complexes

The results of the initial-velocity and dead-end-inhibition analyses discussed above indicate that the catalysis by the enzyme both in solution and in the insolubilized state is sequential and that in the forward reaction NAD^+ binds obligatorily first (i.e. to the free enzyme). Thus of the kinetic mechanisms that are commonly encountered, only the

$$\frac{1}{v} = \frac{1}{V_f} \left(\frac{K_s^A K_m^B}{[B]} + K_m^A \right) \left(1 + \frac{[I]}{K_s^I} \right) \frac{1}{[A]} + \frac{K_m^B}{V_f [B]} + \frac{1}{V_f} \quad (8)$$

$$\frac{1}{v} = \frac{1}{V_f} \left(\frac{K_s^A K_m^B}{[A]} + K_m^B \right) \left(1 + \frac{[I]}{K_s^I} \right) \frac{1}{[B]} + \frac{K_m^A}{V_f [A]} + \frac{1}{V_f} \quad (9)$$

$$\frac{1}{v} = \frac{1}{V_f} \left(\frac{K_s^A K_m^B}{[B]} + K_m^A \right) \frac{1}{[A]} + \frac{K_m^B (1 + [I]/K_s^I)}{V_f [B]} + \frac{1}{V_f} \quad (10)$$

$$\frac{1}{v} = \frac{1}{V_f} \left(\frac{K_s^A K_m^B}{[A]} + K_m^B \right) \left(1 + \frac{[I]}{K_s^I} \right) \frac{1}{[B]} + \frac{K_m^A (1 + [I]/K_s^I)}{V_f [A]} + \frac{1}{V_f} \quad (11)$$

Fig. 6. Dead-end-inhibition patterns with D-lactate as dead-end inhibitor for the forward reaction catalysed by (a) and (b), the enzyme in solution, and (c) and (d), the insolubilized enzyme

(a) [D-Lactate] (μM): $\blacktriangle$, 460; $\triangle$, 920; $\bullet$, 1835; $\circ$, 2750. $[NAD^+]$: $152.3 \mu M$. $[E_T]$: $2.76 \times 10^{-2} \mu M$. (b) [D-Lactate] (μM): $\blacktriangle$, 0.0; $\triangle$, 1835; $\bullet$, 3675; $\circ$, 5550; $\blacksquare$, 7350. [D-3-Hydroxybutyrate]: $1261 \mu M$. $[E_T]$: $5.87 \times 10^{-2} \mu M$. (c) [D-Lactate] (μM): $\blacktriangle$, 0.0; $\triangle$, 1835; $\bullet$, 3675; $\circ$, 5550. $[NAD^+]$: $292.2 \mu M$. $[E_T]$: $0.305 \mu M$. (d) [D-Lactate] (μM): $\blacktriangle$, 0.0; $\triangle$, 1835; $\bullet$, 3675; $\circ$, 5550. [D-3-Hydroxybutyrate]: $31.5 \mu M$. $[E_T]$: $0.305 \mu M$. Other conditions were as given in the Materials and Methods section.

ordered Bi Bi mechanism (Scheme 1) or the Theorell-Chance mechanism, i.e. an ordered Bi Bi mechanism without kinetically significant ternary complexes, could describe these catalyses. Either of these basic

mechanisms could be complicated by kinetically significant isomerizations of the free enzyme ($E \rightleftharpoons F$) and/or of the transitory complexes and/or the formation to an appreciable extent of dead-end ternary com-

Fig. 7. Secondary plots of (a) the slopes and (b) the intercepts of the dead-end-inhibition plots with D-lactate as dead-end inhibitor for the forward reaction

Plots are for the reaction catalysed by ●, the enzyme in solution, or ○, the insolubilized enzyme.

plexes. Appreciable formation of dead-end ternary complexes of the EAA and EQQ type is considered unlikely on the basis of the initial-velocity analysis (see above). The intersecting patterns of the initial-velocity data in Figs. 1 and 2 suggest the absence of EQB and EAQ complexes (see Cleland, 1970). The results presented so far, however, do not permit the elimination of dead-end ternary complexes of the EAP type. In addition to this possibility, another mechanism that has not been eliminated is one in which the reverse reaction is random even though the forward reaction is ordered, i.e. an ordered-random Bi Bi mechanism. This type of mechanism, to our knowledge has never been found to describe an enzyme catalysis.

Table 1. Apparent inhibition constants from the dead-end-inhibition analysis and kinetic parameters derived therefrom

Experimental conditions are described in the Materials and Methods section.

Variable substrate	Dead-end inhibitor	Fixed substrate	Apparent K_i (μM)				Value of kinetic parameter (μM)	
			K_{is}		K_{ii}		Soluble enzyme	Insolubilized enzyme
			Soluble enzyme	Insolubilized enzyme	Soluble enzyme	Insolubilized enzyme		
NAD ⁺	ADP-Ribose	D-3-Hydroxy-butyrate	181 ± 25	469 ± 53	—	—	$K_s^1 = 181 \pm 25$	$K_s^1 = 469 \pm 53$
D-3-Hydroxy-butyrate	D-Lactate	NAD ⁺	704 ± 158	880 ± 41	—	—	$K_s^1 = 704 \pm 158$	$K_s^1 = 880 \pm 41$
NAD ⁺	D-Lactate	D-3-Hydroxy-butyrate	—	—	2924 ± 98	2680 ± 210	$K_m^B = 443 \pm 97$	$K_m^B = 604 \pm 140$
Dissociation constant of the E-NAD ⁺ -D-lactate complex (see above)								

Table 2. Kinetic parameters obtained from the initial-velocity, dead-end-inhibition and product-inhibition analysis

Analysis	Experimental conditions are described in the Materials and Methods section.					
	Catalytic constants ($V/[E_T]$) (s^{-1})		Michaelis constants (μM)		Inhibition constants (μM)	
Initial velocity	Soluble enzyme	Insolubilized enzyme	Soluble enzyme	Insolubilized enzyme	Soluble enzyme	Insolubilized enzyme
	$k_{cat,f} = 5.25$	$k_{cat,f} = 4.03$	$K_m^A 63 \pm 12$	110 ± 20	$K_m^A 69 \pm 22$	—
Dead-end inhibition (see also Table 1)	$k_{cat,r} = 1.78$	$k_{cat,r} = 1.31$	$K_m^B 364 \pm 83$	800 ± 127	$K_m^B 21 \pm 7$	—
			$K_m^C 800 \pm 200$	417 ± 180		
			$K_m^Q 26 \pm 10$	21 ± 9		
			$K_m^B 443 \pm 97$	604 ± 140		
Product inhibition* (see also Table 3)					Dissociation constant of E-ADP-ribose complex 181 ± 25	469 ± 53
					Dissociation constant of E-NAD ⁺ -D-lactate complex 704 ± 158	880 ± 41
					$K_i^P 1046 \pm 232$	1610 ± 505
					$K_i^Q 30 \pm 4$	68 ± 6
					17 ± 5	22 ± 9
					34 ± 8	27 ± 5

* The three sets of values for K_m^P and for K_i^Q arise from a competitive inhibition pattern (one value) and a non-competitive inhibition (two values) (see Table 3).

The product-inhibition analysis of the forward reaction was carried out in an attempt to resolve at least some of these ambiguities. The product-inhibition patterns of the forward reaction catalysed by the enzyme in solution are presented in Fig. 8 and of that catalysed by the insolubilized enzyme in Fig. 9. The results of the product-inhibition analysis, which are the same for both catalyses, are summarized in Table 3. It is convenient in discussing the results of the product-inhibition analysis to make use of Table 4, which presents five types of kinetic mechanism for two-substrate reversible reactions. For each of the four types of sequential mechanism the five types of variation that seem most likely to occur are considered in addition to the basic mechanism. Table 4 demonstrates how, for these catalyses, various types of steady-state kinetic analysis have allowed the probable elimination of all except one of the kinetic mechanisms that were considered.

It shows also which mechanisms remained as possibilities when the product-inhibition study of the forward reaction was begun.

Inhibition by NADH (Q) with NAD⁺ (A) as variable substrate at non-saturating fixed concentrations of D-3-hydroxybutyrate (B)

Figs. 8(a) and 9(a) show that for both catalyses this inhibition is competitive, i.e. both A and Q bind to the same enzyme form, E. This result eliminates the possibility of free-enzyme isomerization, i.e. that when the EQ complex dissociates it produces the enzyme in a form (F) which must isomerize (to E) before it can bind A. It eliminates also the possibility of the formation of EAQ dead-end complexes. This is because saturating the system with A would not eliminate the formation of EAQ complexes that would give rise to ordinate-intercept effects. Similarly, the competitive patterns in Figs. 8(a) and 9(a) suggest that the catalysis is not of the ordered-random type. Binding of Q to EP would not be abolished by saturating the system with A. This inhibition reported in Figs. 8(a) and 9(a), however, may not be a very sensitive test for an ordered-random mechanism. In the presence of relatively high concentrations of Q and very low concentrations of P (and therefore presumably of EP) such a mechanism might approximate sufficiently closely to an ordered Bi Bi mechanism that the inhibition pattern may approximate to competitive.

Inhibition by acetoacetate (P) with NAD⁺(A) as variable substrate at fixed, non-saturating concentrations of D-3-hydroxybutyrate (B)

The postulate that this catalysis does not proceed by an ordered-random mechanism, however, is supported by the finding that the inhibition by aceto-

acetate (P) with NAD⁺ (A) as variable substrate at non-saturating fixed concentrations of D-3-hydroxybutyrate (B) is non-competitive (see Figs. 8c and 9c). Since at relatively high concentrations of P and very low concentrations of Q, the predominant route of an ordered-random mechanism might be expected to be:

the A versus P inhibition might be expected to be competitive. Strictly the possibility does still remain that the very low concentration of Q is compensated for by very low dissociation constants for EQ and/or $\text{EPQ} \rightleftharpoons \text{EQ} + \text{P}$. This could result in the observed non-competitive pattern of Figs. 8(c) and 9(c). Figs. 8(c) and 9(c) do not eliminate the possibility of an ordered-random mechanism with an EAP complex that would give the observed non-competitive pattern. That this mechanism does not describe the catalysis, however, is suggested by the competitive patterns in Figs. 8(a) and 9(a) (see above) and the linearity of some of the secondary product-inhibition plots (see below).

Inhibition by acetoacetate (P) with D-3-hydroxybutyrate (B) as variable substrate at fixed, non-saturating concentrations of NAD⁺ (A)

Figs. 8(b) and 9(b) show that for both catalyses this inhibition is non-competitive. This finding eliminates the possibility of a Theorell-Chance mechanism with or without EAP or EBQ dead-end ternary complexes. This is because in such a mechanism B and P would both effectively bind to the same enzyme form and thus the inhibition would be competitive. Any effect of P would be abolished at saturating concentrations of B and thus no ordinate-intercept effect would be observed. Because the Theorell-Chance mechanism is probably best regarded as a special case of an ordered Bi Bi mechanism in which the steady-state concentration of the EAB-EPQ central complexes is finite, although near zero, theoretically a small intercept effect would be predicted if sufficiently high concentrations of both B and P could be used. In practice, however, intercept effects would not generally be expected for a Theorell-Chance mechanism. If they were observed the mechanism would be regarded as ordered Bi Bi and not Theorell-Chance.

Probable kinetic mechanism

Of the kinetic mechanisms considered (Table 4) the number of probable, permissible mechanisms has been decreased by the A-versus-Q, A-versus-P and B-versus-P inhibition studies (Figs. 8a-c and 9a-c) to two, i.e. ordered Bi Bi with or without an EAP dead-end complex. As discussed above, the ordered-

Fig. 8. Product-inhibition patterns for the forward reaction catalysed by the enzyme in solution with (a) and (d), NADH as product inhibitor, and (b) and (c), acetoacetate as product inhibitor

(a) and (d) $[E_T]$: $5.87 \times 10^{-2} \mu M$. (b) and (c) $[E_T]$: $3.76 \times 10^{-2} \mu M$. (a) [NADH] (μM): $\blacktriangle$, 0.0; $\triangle$, 117; $\bullet$, 176; $\circ$, 235. [D-3-Hydroxybutyrate]: 1261 μM . (b) [Acetoacetate] (μM): $\blacktriangle$, 500; $\triangle$, 1250; $\bullet$, 2500; $\circ$, 5000; $\blacksquare$, 6250. [NAD $^+$]: 177 μM . (c) [Acetoacetate] (μM): $\blacktriangle$, 500; $\triangle$, 1250; $\bullet$, 1250; $\circ$, 5000; $\blacksquare$, 6250. [D-3-Hydroxybutyrate]: 1261 μM . (d) [NADH] (μM): $\blacktriangle$, 0.0; $\triangle$, 59; $\bullet$, 117; $\circ$, 176; $\blacksquare$, 235. [NAD $^+$]: 161 μM . Other conditions were as given in the Materials and Methods section.

Fig. 9. Product-inhibition patterns for the forward reaction catalysed by the insolubilized enzyme with (a) and (d), NADH as product inhibitor, and (b) and (c), acetoacetate as product inhibitor

(a) and (d) $[E_T]$: $1.76 \times 10^{-1} \mu\text{M}$. (b) and (c) $[E_T]$: $1.99 \times 10^{-1} \mu\text{M}$. (a) [NADH] (μM): $\blacktriangle$, 0.0; $\triangle$, 71; $\bullet$, 141; $\circ$, 282. [D-3-Hydroxybutyrate]: $2522 \mu\text{M}$. (b) [Acetoacetate] (μM): $\blacktriangle$, 0.0; $\triangle$, 1250; $\bullet$, 2500; $\circ$, 5000. [NAD $^+$]: $205 \mu\text{M}$. (c) [Acetoacetate] (μM): $\blacktriangle$, 0.0; $\triangle$, 1250; $\bullet$, 2500; $\circ$, 5000. [D-3-Hydroxybutyrate]: $2522 \mu\text{M}$. (d) [NADH] (μM): $\blacktriangle$, 0.0; $\triangle$, 71; $\bullet$, 141; $\circ$, 211; $\blacksquare$, 282. [NAD $^+$]: $205 \mu\text{M}$. Other conditions were as given in the Materials and Methods section.

random mechanism with or without certain dead-end complexes strictly cannot be entirely ruled out on the basis of these patterns. The other product-inhibition pattern, i.e. B versus Q, will not permit these possi-

bilities to be distinguished. Both remaining mechanisms predict that this pattern should be non-competitive. Figs. 8(d) and 9(d) demonstrate the non-competitive nature of the B-versus-Q inhibition for

both the catalyses. The inhibition patterns found in the dead-end-inhibition and product-inhibition analyses are summarized in Table 5. Similarly, these mechanisms cannot be distinguished by analysis of the product-inhibition patterns for the reverse reaction. They may be distinguished, however, at least theoretically, by consideration of some of the secondary plots of the product-inhibition data (see Cleland, 1970). This type of analysis requires decisions to be made about the linearity of the secondary plots. This criterion is often more difficult to apply than the one used in deciding whether a particular product-inhibition pattern is of the competitive or non-competitive type. The secondary plots of the product-inhibition data are presented in Fig. 10. It is considered that these data do not constitute compelling evidence that any of the secondary plots is parabolic or hyperbolic in nature. Thus the simplest kinetic mechanism for this catalysis that is consistent with the initial-velocity, dead-end-inhibition and product-inhibition analyses is the ordered Bi Bi mechanism (Scheme 1). Of the permissible mechanisms remaining from those considered in Table 4, only this one would be predicted to give rise to the linear secondary plots of all the characterizing parameters of the primary

data. Predictions of the nature of secondary plots are made on the basis of whether multiple-binding modes of a given product would affect slopes or ordinate intercepts or both of the primary plots (see Cleland, 1970). The kinetic parameters that characterize the catalysis are collected in Table 2. To calculate some of these parameters, the kinetic mechanism must be known. In these calculations it has been assumed that the catalysis follows an uncomplicated ordered Bi Bi mechanism.

Although the product-inhibition analysis has shown that the data are not consistent with the Theorell-Chance mechanisms given in Table 4, there is one type of Theorell-Chance mechanism that cannot be distinguished from the ordered Bi Bi mechanism by steady-state kinetics. This is a Theorell-Chance mechanism with EAB and EPQ dead-end ternary complexes. This situation has been discussed by Wratten & Cleland (1963).

The apparent inhibition constants that characterize the various product inhibitions discussed above were obtained by fitting the data to eqn. (2) when the inhibition was competitive and to eqn. (4) when it was non-competitive. As in the dead-end-inhibition analysis, the significance of the apparent inhibition constants in terms of the kinetic parameters that characterize the catalysis was obtained by parametric comparison with the appropriate product-inhibition-rate equation. These product-inhibition equations

for an ordered Bi Bi mechanism when the concentration of one or the other product is set to zero have been presented by Cleland (1963c). The apparent product-inhibition constants and the kinetic parameters derived therefrom are given in Table 3 (see also Table 2).

The types of possible variation in kinetic mechanism not so far considered are isomerization of the central complexes EAB and EPQ and of the non-central complexes EA and EQ. The former cannot be detected by steady-state kinetics. The latter, however, may be detected by consideration of the quantitative relationships between the kinetic parameters that characterize the catalysis (Cleland, 1970).

As discussed above, the simplest kinetic mechanism consistent with the data is the ordered Bi Bi mechanism. In an attempt to detect isomerizations of non-central ternary complexes, the first Haldane relationship and the eight rate constants of Scheme 1 were calculated on the basis of the ordered Bi Bi model.

Haldane relationship

For an ordered Bi Bi mechanism the equilibrium constant at a given pH for the reversible reaction:

is given by the Haldane relationship, eqn. (12) (see Cleland, 1963b):

$$\frac{K_{eq.}'}{[H^+]} = K_{eq.} = \frac{V_f K_m^P K_s^Q}{V_r K_m^B K_A^A} \quad (12)$$

Calculation of $K_{eq.}[H^+]$ for the equilibrium at pH 8.5 by using eqn. (12) and the kinetic parameters given in Table 2 gave a value of $1.44 \pm 0.33 \text{ nM}$ for the system catalysed by the soluble enzyme and $0.95 \pm 0.70 \text{ nM}$ for the system catalysed by the insolubilized enzyme. These values are in good agreement with the values of $K_{eq.}[H^+]$ determined directly both in the present work ($1.23 \pm 0.29 \text{ nM}$ for the soluble system and $2.33 \pm 0.61 \text{ nM}$ for the insolubilized system) and previously by Krebs *et al.* (1962) (1.18 nM ; soluble enzyme; pH 8.55) bearing in mind the large standard errors on the values determined with the insolubilized enzyme.

Thus the Haldane relationship for the ordered Bi Bi mechanism appears to be obeyed and provides no evidence for any deviation from this mechanism.

Calculation of the rate constants

By assuming that the catalysis may be described by Scheme 1 the rate constants of this model may be calculated from the kinetic parameters given in Table 2 (see Cleland, 1963b). These rate constants (with the

Table 3. Apparent inhibition constants from the product-inhibition analysis and kinetic parameters derived therefrom

Experimental conditions are described in the Materials and Methods section.

Variable substrate	Product inhibitor	Fixed substrate	Apparent K_i (μM)				Significance of apparent K_i	Value of kinetic parameter (μM)	
			K_{is}		K_{ii}			Soluble enzyme	Insolubilized enzyme
			Soluble enzyme	Insolubilized enzyme	Soluble enzyme	Insolubilized enzyme			
NAD ⁺	NADH	D-3-Hydroxy-butyrate	30 $\pm$ 4	68 $\pm$ 6	—	—	$K_{is} = K_i^Q$	$K_i^Q = 30 \pm 4$	68 $\pm$ 6
D-3-Hydroxy-butyrate	NADH	NAD ⁺	60 $\pm$ 11	91 $\pm$ 21	122 $\pm$ 21	76 $\pm$ 11	$K_{is} = K_i^Q(1 + [A]/K_m^A)$ $K_{ii} = K_i^Q(1 + [A]/K_m^B)$	$K_i^Q = 17 \pm 5$ $K_i^B = 34 \pm 8$	22 $\pm$ 9 27 $\pm$ 5
D-3-Hydroxy-butyrate	Acetoacetate	NAD ⁺	1610 $\pm$ 450	1190 $\pm$ 250	1423 $\pm$ 350	2770 $\pm$ 540	$K_{is} = K_m^P K_i^Q / K_m^Q$ $K_{ii} = K_i^Q(1 + K_m^A/[A])$	$K_m^P = 1928 \pm 739$ $K_i^A = 1046 \pm 232$	700 $\pm$ 552 1610 $\pm$ 505
NAD ⁺	Acetoacetate	D-3-Hydroxy-butyrate	3480 $\pm$ 1260	2080 $\pm$ 1700	3470 $\pm$ 670	3520 $\pm$ 560	$K_{is} = (K_m^P K_i^Q / K_m^Q) \times \left(\frac{1 + K_m^A[B]}{K_i^A K_m^B} \right)$ $K_{ii} = ([B] + K_m^B) / \left(\frac{[B]}{K_i^A} + (K_m^B K_m^P / K_m^Q K_i^Q) \right)$	$K_m^P = 946 \pm 414$ $K_m^B = 593 \pm 217$	351 $\pm$ 217 207 $\pm$ 169

exception of k_{+2} , k_{+3} and k_{-3} , see below) are presented in Table 6.

The two striking features of the data in Table 6 are first that k_{+4} (1.49 s^{-1}) is smaller than

$$k_{\text{cat},f} (= V_f/[E_T] = 5.25\text{ s}^{-1})$$

and, secondly, that k_{+3} is calculated to have a negative value. That a rate constant for a unimolecular step in a kinetic mechanism is calculated to have a value which is less than the catalytic rate constant ($V/[E_T]$) for the reaction is indicative of an inadequate kinetic model (see Cleland, 1963b). The inadequacy seems to be reflected in the value of K_m^Q/K_i^Q , which is too large to be accommodated by the simple ordered Bi Bi model (see the relationships in Table 6). Expansion of the ordered Bi Bi model to include either isomerization of the EQ complex or wrong-way binding of Q to give a dead-end QE complex could account for the low value of k_{+4} (see Cleland, 1963b) and the negative value of k_{+3} . Cleland (1963b) has pointed out that if an EQ complex isomerization results in a rate constant for one of the steps of the forward reaction that is smaller than $k_{\text{cat},f}$ the possibility then exists that an EA complex isomerization also occurs but this will not give rise to a rate constant for one of the steps of the reverse reaction which is smaller than $k_{\text{cat},r}$.

Evidence from radioisotopic redistribution at chemical equilibrium (Preuveneers *et al.*, 1973) suggests that the interconversion of the central ternary complexes is not rate-limiting in this catalysis. This evidence, together with the rate constants given in Table 6, suggests that in common with many other NAD-linked dehydrogenases the rate-limiting step(s) may be concerned with the isomerization of the enzyme-nicotinamide nucleotide complexes and/or the dissociation of the nicotinamide nucleotides from the enzyme.

Comparison of the reactions catalysed by the soluble and the insolubilized enzymes

The data presented above demonstrate that insolubilization of D-3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* by covalent attachment to DEAE-cellulose by using 2-amino-4,6-dichloro-s-triazine as coupling agent has not altered in any detectable way the permissible complexes formed between the enzyme and its four substrates or their possible pathways of interconversion at pH 8.5. To our knowledge, this study is the first determination of a kinetic mechanism for an insolubilized enzyme. It would have been difficult to undertake the large number of kinetic measurements needed in such a study without being able to use the continuous method of rate assay, which use of the

Table 4. Summary of the evidence that led to the assignment of an ordered Bi Bi mechanism to the D-3-hydroxybutyrate dehydrogenase catalysis

Mechanisms that are not in accord with a particular experimental finding are marked **X**. In general these are not then further considered. In some cases, however, a mechanism is shown to be incompatible with more than one type of experimental result. This is shown mainly in cases where the assignment of incompatibility relies on judgements of linearity in kinetic plots.

Experimental finding

Ping Pong mechanism

Basic mechanism:

Basic mechanism

Linear intersecting initial-velocity patterns for the forward reaction
 Linear intersecting initial-velocity patterns for the reverse reaction
 Dead-end inhibition A versus D-lactate, uncompetitive

Random mechanism

Basic mechanism:

Enzyme isomerization $E \rightleftharpoons F$

Basic mechanism

+	+	+	+	+	+	+	+	+	+
X	X	X	X	X	X	X	X	X	X

Dead-end complexes

EPB or EQB

EAP or EQA

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

Ordered random mechanism

Basic mechanism:

Enzyme isomerization $E \rightleftharpoons F$

Basic mechanism

Linear intersecting initial-velocity patterns for the forward reaction
 Linear intersecting initial-velocity patterns for the reverse reaction
 Product inhibition A versus Q, competitive

EAA

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

EAB or EQB

[illegible]

Table 5. Dead-end and product inhibition patterns for the reaction catalysed both by the soluble and the insolubilized enzyme

Experimental conditions are described in the Materials and Methods section. A = NAD⁺; B = D-3-hydroxybutyrate.

Variable substrate	Inhibitor	Fixed substrate (at non-saturating concentration)	Inhibition pattern
A	ADP-ribose	B	Competitive
B	ADP-ribose	A	Non-competitive
B	D-Lactate	A	Competitive
A	D-Lactate	B	Uncompetitive
A	NADH	B	Competitive
B	NADH	A	Non-competitive
A	Acetoacetate	B	Non-competitive
B	Acetoacetate	A	Non-competitive

Fig. 10. Secondary plots of slopes or intercepts, or of both, of the product-inhibition plots of the forward reaction

Plots are for the reaction catalysed by ●, the enzyme in solution, or ○, the insolubilized enzyme with (a) and (d), NADH as product inhibitor, and (b) and (c), acetoacetate as product inhibitor, and with (a) and (c), NAD^+ as variable substrate, and (b) and (d) D-3-hydroxybutyrate as variable substrate.

Zeiss PMQ11 spectrophotometer and the stirred reaction cell has permitted.

Tables 1, 2, 3 and 6 demonstrate that the kinetic parameters and the calculable rate constants that characterize the catalysis by the insolubilized enzyme do not differ dramatically from those that characterize the catalysis by the soluble enzyme. Most of them indeed are closely similar. It is possible that K_m^A , K_m^B

and K_m^P may be different by about a factor of 2 for the system involving the insolubilized enzyme compared with the values for the system involving the soluble enzyme. The large standard errors on these parameters, particularly on K_m^P , however, make the existence of a differential effect highly problematical. The markedly lower values of K_m and K_s for this insolubilized system comprising negatively charged

Table 6. *Rate constants*
Experimental conditions are as described in the Materials and Methods section.

Rate constant	Relationship to kinetic parameters	For the reaction catalysed by the soluble enzyme (assuming that $[E_T]$ = measured protein concentration)	For the reaction catalysed by the insolubilized enzyme (assuming that $[E_T]$ = protein content of the preparation uniformly distributed throughout the reaction volume)	For the reaction catalysed by the insolubilized enzyme (assuming that the catalytic constants are equal to those of the soluble enzyme catalysis)
$k_{cat,f}$	$V_f/[E_T]$	5.25 s ⁻¹	4.03 s ⁻¹	5.25 s ⁻¹
$k_{cat,r}$	$V_r/[E_T]$	1.78 s ⁻¹	1.31 s ⁻¹	1.78 s ⁻¹
k_{+1}	$V_f/K_m^f [E_T]$	$8.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$	$3.7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$	$4.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$
k_{+2}	$V_f(1 + k_{+3}/k_{+4})/K_m^f [E_T]$	—	—	—
k_{+3}^*	$1/([E_T]/V_f - \{[E_T]K_m^f/[V_f K_4^f]\})$	-0.48 s ⁻¹	-0.22 s ⁻¹	-0.15 s ⁻¹
k_{+4}^*	$V_f K_4^f/K_m^f [E_T]$	1.5 s ⁻¹	2.2 s ⁻¹	2.9 s ⁻¹
k_{-1}	$V_f K_4^f/K_m^f [E_T]$	5.8 s ⁻¹	2.4 s ⁻¹	3.2 s ⁻¹
k_{-2}	$1/([E_T]/V_f - ([E_T]K_m^f/[V_f K_4^f]))$	2.6 s ⁻¹	4.25 s ⁻¹	2.4 s ⁻¹
k_{-3}	$V_f(1 + k_{+3}/k_{+4})/K_m^f [E_T]$	—	—	—
k_{-4}	$V_f/K_m^f [E_T]$	$7.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$	$6.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$	$8.6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$

* The values of k_{+4} and k_{-3} are those calculated with $K_4^f = 21 \mu\text{M}$, which is regarded as the most reliable value. Even when the largest value of K_4^f (42 μM , see Table 2) is used, k_{+3} is still calculated to be negative and $k_{+4} < k_{cat,f}$.

substrates and positively charged supporting matrix, which would be predicted at low ionic strength (see Wharton *et al.*, 1968; Goldstein *et al.*, 1970), would not be expected to obtain at the ionic strength (1.3) employed in this study.

Comparison of the kinetic mechanisms for the reaction catalysed by D-3-hydroxybutyrate dehydrogenases from Rhodopseudomonas spheroides and from bovine heart mitochondria

When the work described in this paper had been completed, Nielsen *et al.* (1972) reported in a preliminary communication that the reaction catalysed by D-3-hydroxybutyrate dehydrogenase from bovine heart mitochondria also appears to follow an ordered Bi Bi mechanism, in which the nicotinamide nucleotides bind obligatorily first to the enzyme. This was inferred from initial-velocity and product-inhibition studies.

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