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D-3-Hydroxybutyrate Dehydrogenase from *Rhodopseudomonas spheroides*

KINETICS OF RADIOISOTOPE REDISTRIBUTION AT CHEMICAL EQUILIBRIUM CATALYSED BY THE ENZYME IN SOLUTION

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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 kinetically at chemical equilibrium by monitoring radioisotope redistribution with sodium DL-hydroxy[3-^{14}C]butyrate and [4-^{3}H]NAD^+(labelled in the nicotinamide ring). 2. When all substrates are maintained at concentrations approaching saturation (approx. 3–50 times the K_m values) the first-order rate constant for the enzyme-catalysed interconversion of NAD^+ and NADH is much smaller than that for the enzyme-catalysed interconversion of D-3-hydroxybutyrate and acetoacetate. 3. The rate of interconversion of NAD^+ and NADH increases initially with increasing concentrations of D-3-hydroxybutyrate and acetoacetate (ratio of concentrations maintained constant), passes through a maximum and approaches closely to zero at saturating concentrations of the latter substrates. 4. The rates of interconversion of NAD^+ and NADH and of D-3-hydroxybutyrate and acetoacetate increase with increasing concentration of NAD^+ (up to 66 times its K_m value) and NADH (up to 180 times its K_m value) (ratio of the concentrations of the nicotinamide nucleotides maintained constant). 5. These findings support the description of this catalysis as an ordered Bi Bi mechanism with no detectable alternative pathway, in which the interconversion of the central ternary complexes is not rate-limiting, and provide no evidence for the formation of dead-end complexes. 6. The solubility of 2,4-dinitrophenylhydrazine in HCl exhibits an acidity optimum, the maximum solubility at 25.0°C (3.8 mg/ml, 19 mM) occurring at 2.29 M-HCl; in solutions of this acidity acetone 2,4-dinitrophenylhydrazone is relatively insoluble (0.098 mg/ml, 0.413 mM).

The study of the kinetics of enzyme-catalysed radioisotope redistribution at chemical equilibrium can be useful in the delineation of certain aspects of kinetic mechanism (see, e.g., Boyer, 1959; Boyer & Silverstein, 1963; Silverstein & Boyer, 1964; Silverstein & Sulebele, 1969; Balinsky *et al.*, 1971). It may be employed to confirm results obtained by conventional steady-state kinetics and sometimes also to obtain information not accessible by this technique.

The preceding paper (Preuveneers *et al.*, 1973) reports a steady-state kinetic analysis of the NAD^+-linked reversible oxidation of D-3-hydroxybutyrate to acetoacetate catalysed by D-3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* at pH 8.5. This analysis provides evidence that the reactions catalysed by the enzyme both in solution and covalently attached to DEAE-cellulose are sequential and that the nicotinamide nucleotides bind obligatorily first to the enzyme. It suggests also that the rate-limiting step(s) of the forward reaction is probably the isomerization of the enzyme–NADH complex or the dissociation of NADH from the enzyme or both.

The results of the radioisotope-exchange study reported in the present paper support these findings. Thus evidence is presented that at pH 8.5 NAD^+ or NADH or both bind obligatorily first to the enzyme with no detectable alternative pathway and that the interconversion of the central ternary complexes is not very much slower than the other steps in the catalysis.

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Materials and Methods

Many of these are described in the preceding paper (Preuveneers *et al.*, 1973).

Sodium DL-hydroxy[3-^{14}C]butyrate and [4-^{3}H]-NAD^+ (n: labelled in the nicotinamide ring) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Control experiments with mixtures of non-radioactive D-3-hydroxybutyrate established that in the range of concentration used in this study the L-3-hydroxybutyrate present in the radioactive racemate had no effect on the kinetics of enzyme-catalysed oxidation of the D-isomer. Concentrations of 3-hydroxybutyrate given in the present paper refer to the D-isomer only. Ion-exchange cellulose paper (DE81) was obtained from Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, U.K. Silver nitrate, 2,4-dinitrophenylhydrazine, 1,4-dioxan (scintillation grade), 2,5-diphenyloxazole (scintillation grade), 1,4-bis-(5-phenyloxazol-2-yl)benzene (scintillation grade) and naphthalene (scintillation grade) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Radioisotopic redistribution techniques

Measurement of the rate of enzyme-catalysed radioisotopic redistribution at chemical equilibrium. Equilibrium mixtures of the four substrates were incubated with the enzyme in 0.1M-sodium pyrophosphate buffer, pH8.5 (total volume 1.0ml), at 25.0°C for 20min. The composition of these mixtures was calculated by using the value of $K_{equil.}$ $[H^+] = 1.23$nM reported in the preceding paper (Preuveneers *et al.*, 1973). That the mixture was at chemical equilibrium was checked by measurement of E_{340}, by using $\epsilon_{340}^{NADH} = 6.22 \times 10^3 M^{-1} \cdot cm^{-1}$ (Horecker & Kornberg, 1948) and assuming that $\epsilon_{340}^{NAD^+} = 0$. The radioisotope redistribution was then initiated by addition of a small volume (50μl) of a stock solution of either DL-hydroxy[3-^{14}C]butyrate or [4-^{3}H]-NAD^+(n). The concentration (625μM) of [4-^{3}H]-NAD^+(n) added was sufficiently high to necessitate the simultaneous addition of NADH (625μM) to maintain chemical equilibrium. Samples (0.1ml) were removed from the reaction mixture at various time-intervals, the reaction was terminated and the reaction mixture was analysed as described below.

Sets of such reaction mixtures were made up such that the concentrations of one substrate and one product (of the reaction in a given direction) were varied while being maintained in a constant ratio to each other and the concentrations of the other substrate and product were maintained constant. This resulted in the maintenance of chemical equilibrium. Reaction rates were calculated from equilibrium concentration of substrate, reaction time and fraction of radioisotope incorporated into the product (see Boyer, 1959).

Termination of the radioisotopic redistributions. This was done by inactivation of the enzyme, by addition of $AgNO_3$ to give a final concentration of 20mM for the interchange of labelled NAD^+ and NADH and by addition of HCl to give a final concentration of 2.3M for the interchange of labelled D-3-hydroxybutyrate and acetoacetate. Both of these methods of enzyme inactivation were shown to result in instantaneous cessation of the radioisotopic redistribution by spectrophotometric analysis at 340nm. Silver nitrate was used to terminate the interchange when labelled NAD^+ and NADH were being measured to keep the ionic strength as low as possible for the chromatographic separation and to avoid acid decomposition of the nicotinamide nucleotides (see Silverstein & Boyer, 1964).

Separation of the conjugate substrates

NAD^+ and NADH. These were separated by chromatography on Whatman DE 81 cellulose ion-exchange paper essentially by the method of Silverstein (1970) but with ascending chromatography. This was more convenient for the processing of large numbers of paper strips. Samples (25μl) of the reaction mixtures were applied to paper strips (20cm × 1.5cm).

DL-3-*Hydroxybutyrate and acetoacetate.* The acetoacetate was allowed to undergo decarboxylation and the resulting acetone was separated from DL-3-hydroxybutyrate as the insoluble 2,4-dinitrophenylhydrazone.

(*a*) Decarboxylation and formation of [2-^{14}C]-acetone 2,4-dinitrophenylhydrazone. Decarboxylation was carried out essentially by the method of Britton (1966). The reaction mixture (0.1ml) was added to 4.6M-HCl (0.1ml) in a test tube (5cm × 0.5cm). The test tube was sealed with a tightly fitting rubber bung and placed in a boiling-water bath for 10min. The tube was then cooled in ice for 30min, the bung carefully removed and a saturated solution of 2,4-dinitrophenylhydrazine in 2.29M-HCl (1ml) was added.

(*b*) Precipitation of [2-^{14}C]acetone 2,4-dinitrophenylhydrazone. To aid the precipitation of the very small amount of [2-^{14}C]acetone 2,4-dinitrophenylhydrazone, acetone (0.1ml, i.e. 13.1mM) was added as a carrier. 2,4-Dinitrophenylhydrazine was always in excess over total acetone. The maximum solubility of 2,4-dinitrophenylhydrazine (3.8mg/ml, 19mM) at 25.0°C in HCl occurred at 2.29M-HCl and, in solutions of this acidity, acetone 2,4-dinitrophenylhydrazone was relatively insoluble (0.098mg/ml, 0.41mM) (see Fig. 1). The results in Fig. 1 demonstrate the importance of carrying out the precipitation in solutions of the correct acidity to ensure essentially quantitative precipitation of the 2,4-dinitrophenylhydrazone while maintaining the 2,4-dinitrophenylhydrazine in solution.

After the acetone and 2,4-dinitrophenylhydrazine had been mixed, the suspension was left at 4°C for 3h. That precipitation was complete after this time was shown by the fact that essentially the same yield of acetone 2,4-dinitrophenylhydrazone was collected after 24h as after 3h (±6%, 10 samples). There was no detectable difference in yield (±6%) when precipitation was allowed to proceed for 3h at room temperature (19–22°C) instead of at 4°C.

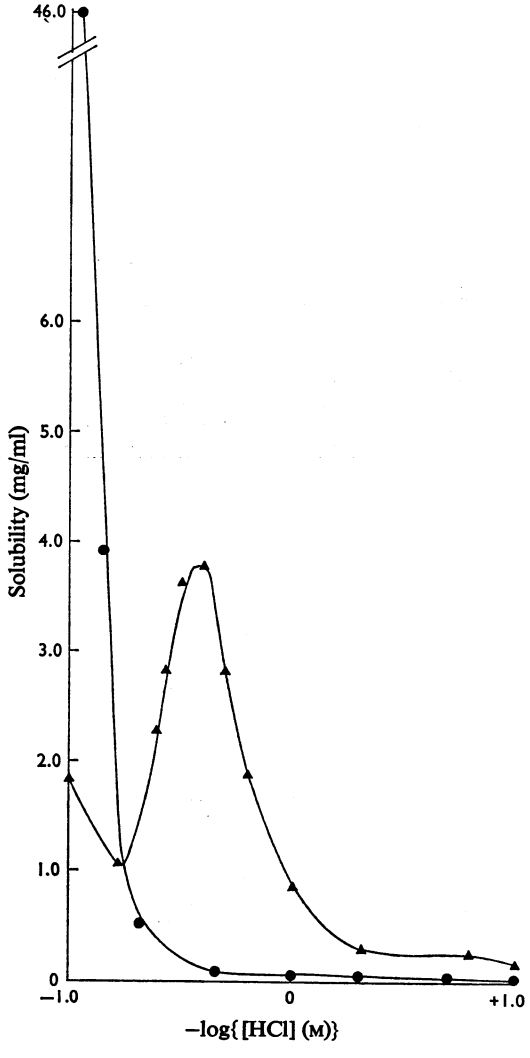


Fig. 1. *Solubilities at 25.0°C of 2,4-dinitrophenylhydrazine and acetone 2,4-dinitrophenylhydrazone*

▲, 2,4-Dinitrophenylhydrazine; ●, acetone 2,4-dinitrophenylhydrazone.

Measurement of radioactivity

[2-^{14}C]*Acetone 2,4-dinitrophenylhydrazone.* Precipitated acetone 2,4-dinitrophenylhydrazone was collected by vacuum filtration on Millipore filters and dried under an i.r. lamp. The Millipore filter discs impregnated with the precipitates were attached to cardboard discs by using rubber adhesive solution and their radioactivity was counted on planchets with a Nuclear–Chicago gas-flow counter.

4-^{3}*H-labelled NAD^{+} and NADH.* Spots of these nucleotides on the dried paper strips were located under u.v. light, cut out, placed in scintillant [10ml of a mixture of dioxan (500ml), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g), 2,5-diphenyloxazole (3.0g), naphthalene (20.0g) and methanol (50ml)] and the radioactivity was counted for 10min at 4°C in a Packard Tri-Carb liquid-scintillation counter. The total counts of the separated [4-^{3}H]NAD^{+} and [4-^{3}H]NADH were the same (±5%) as the original counts of [4-^{3}H]NAD^{+} added to initiate the radioisotopic redistribution.

Control experiments showed that when the spot size was increased from 0.5cm×0.5cm to 1.5cm×1.0cm a 5% fall in counts was observed. Spot sizes used in all kinetic experiments were kept within

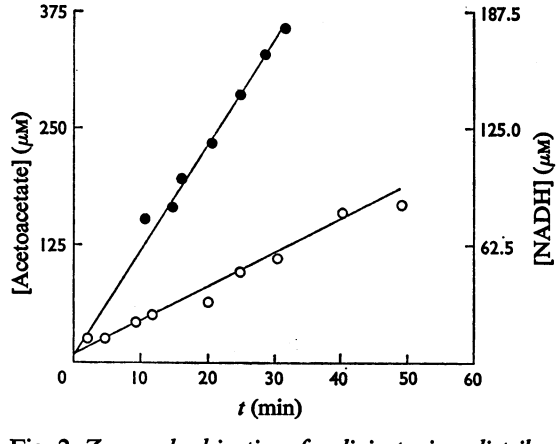


Fig. 2. *Zero-order kinetics of radioisotopic redistribution at equilibrium*

The reaction mixture (1.0ml) contained sodium pyrophosphate buffer, pH8.5 (0.1M), D-3-hydroxybutyrate (1mM), acetoacetate (2mM), NAD^{+} (1mM), NADH (1.17mM) and enzyme (12.5μg of protein/ml). The redistribution was started ●, by addition of DL-hydroxy[3-^{14}C]butyrate (1.25μCi in 50μl, to give 0.1mM) or ○, by addition of [4-^{3}H]NAD^{+}(n) (6μCi in 50μl, to give 625μM). In the latter case an equivalent amount of NADH was added to maintain chemical equilibrium (see the text).

these limits of size and correction for paper quench was not made.

Results and Discussion

Zero-order kinetics of the radioisotope redistributions

Effect of increasing the concentration of the D-3-*hydroxybutyrate–acetoacetate pair on the rate of interconversion of NAD⁺ and NADH at equilibrium: evidence that NAD⁺ or NADH or both bind obligatorily first.* Under the conditions of concentration of the enzyme and the various substrates used in this study, it was possible to ensure that the initial phases of the net formation of radioactive acetoacetate and of radioactive NADH at chemical equilibrium followed zero-order kinetics. Typical progress curves are shown in Fig. 2.

The preceding paper (Preuveneers *et al.*, 1973) reports evidence from steady-state kinetic studies strongly suggesting that the basic kinetic mechanism that describes the reactions catalysed by D-3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* at pH 8.5 is of the ordered Bi Bi type shown in Scheme 1. To obtain supporting evidence for this basic mechanism and to ascertain whether significant alternative pathways exist, the effect on the equilibrium rate of interchange of NAD⁺ and NADH of increasing the concentrations of D-3-hydroxybutyrate and acetoacetate while maintaining the ratio of the concentrations of these substrates constant was examined. The results are shown in Fig. 3. Initially there is an increase in the equilibrium rate of interchange of NAD⁺ and NADH with increasing concentration of D-3-hydroxybutyrate and acetoacetate. The rate passes through a maximum and at high concentrations of the substrates (25 times the K_m value for D-3-hydroxybutyrate and approx. 50 times

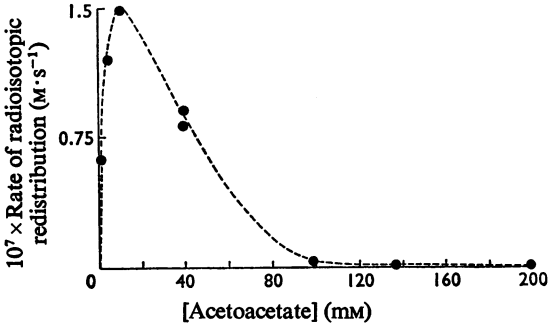
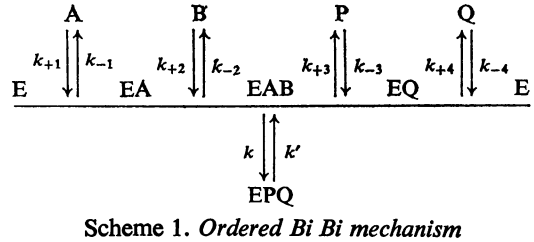


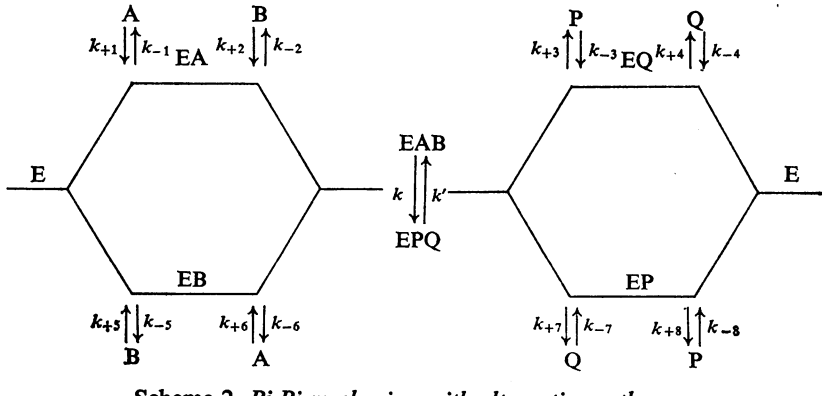
Fig. 3. *Effect on the enzyme-catalysed equilibrium rate of interchange of NAD⁺ and NADH of increasing the concentrations of* D-3-*hydroxybutyrate and acetoacetate while maintaining the ratio of their concentrations constant*

The composition of the reaction mixture with respect to the non-varying components was as given in Fig. 2. The constant ratio of [acetoacetate]/[D-3-hydroxybutyrate] = 2.



Scheme 1. *Ordered Bi Bi mechanism*

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



Scheme 2. *Bi Bi mechanism with alternative pathways*

The explanation of the symbols used is as for Scheme 1.

the K_m value for acetoacetate) approaches closely to zero. The essentially total abolition of the equilibrium rate of interchange of NAD^+ and NADH by saturating concentrations of the other two substrates provides the evidence that is usually taken to imply that the reaction proceeds essentially entirely through an ordered pathway in which the nicotinamide nucleotides bind obligatorily first (see Cleland, 1970).

The total inhibition of the A–Q interchange by saturating concentrations of B and P observed in the reactions catalysed by malate dehydrogenase at pH8.0 (Silverstein & Sulebele, 1969), and by lactate dehydrogenase at pH8.0 (Silverstein & Boyer, 1964), was regarded as evidence that these catalyses proceed by totally ordered mechanisms. Similarly, the fact that only partial substrate inhibition of the A–Q interchange at saturating concentrations of B and P is observed in the reactions catalysed by liver and yeast alcohol dehydrogenases (Silverstein & Boyer, 1964), and by malate and lactate dehydrogenases at high pH (Silverstein & Sulebele, 1969; Silverstein & Boyer, 1964), is taken as evidence of alternative pathways in these catalyses.

It is important to point out that whereas observation of a finite rate of interchange of A and Q at saturating concentrations of B and P does provide evidence of an alternative pathway, total abolition of the exchange rate does not necessarily imply a totally ordered mechanism, i.e. totally ordered in both directions. Strictly, it is necessary only for one of the substrates A or Q to bind obligatorily first to observe the total inhibition caused by saturating concentrations of B and P. This becomes apparent when the equation describing the rate of interchange of A and Q at saturating concentrations of B and P for the general model (Scheme 2), which includes the alternative pathways, is considered. The relevant equation, previously presented by Boyer & Silverstein (1963), is eqn. (1), in which R is the equilibrium rate of interchange of A and Q, $[E_T]$ is the total enzyme concentration, [A] and [Q] are the equilibrium concentrations of A and Q respectively, k, k', k_{-6} and k_{+7} are rate constants of Scheme 2, and K_6 and K_7 are dissociation constants defined by eqns. (2) and (3):

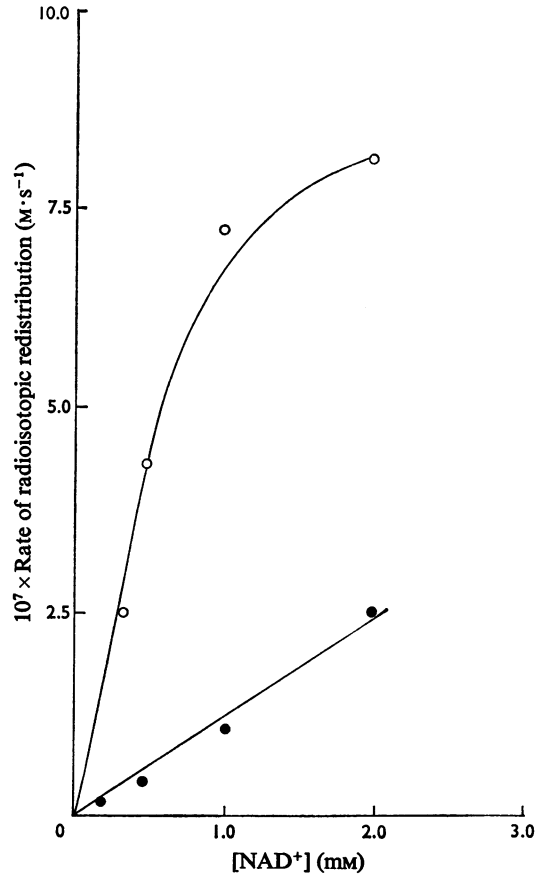


Fig. 4. *Effect on the enzyme-catalysed equilibrium rate of interchange of NAD^+ and NADH and of* D-3-*hydroxybutyrate and acetoacetate of increasing the concentrations of NAD^+ and NADH while maintaining the ratio of their concentrations constant*

The composition of the reaction mixtures with respect to the non-varying components was as given in Fig. 2. ●, NAD^+ and NADH; ○, D-3-hydroxybutyrate and acetoacetate. The constant ratio of $[NADH]/[NAD^+] = 1.15$.

$$R = [E_T]/(1 + k'/k_{+7} + k/k_{-6})\{1/k'(1 + K_7/[Q]) + (1/k(1 + K_6/[A])\} \quad (1)$$

$$K_6 = [A][EB]/[EAB] \quad (2)$$

$$K_7 = [Q][EP]/[EPQ] \quad (3)$$

It is apparent from eqn. (1) that it is necessary only that either k_{-6} (and therefore K_6) or k_{+7} (and therefore K_7) be zero to make R equal to zero; it is not necessary that both sets of rate and equilibrium constants be zero.

The results presented in Fig. 3 therefore are entirely consistent with the kinetic mechanism deduced in the preceding paper from steady-state kinetic studies, i.e. a Bi Bi mechanism in which NAD^+ binds obligatorily first and in which NADH also probably binds obligatorily first. The residual doubts about the possibility of an alternative pathway for the reverse reaction (with NADH and acetoacetate as substrates)

are discussed in the preceding paper. The above discussion demonstrates that the radioisotope-interchange studies presented here cannot resolve this ambiguity. In addition to this ambiguity there exists, of course, the usual ambiguity that arises when the formation of dead-end complexes cannot be entirely ruled out (see Cleland, 1970).

Effect of increasing the concentration of the NAD^+–NADH pair on the equilibrium rates of interconversion of NAD^+ and NADH and of D-*3-hydroxybutyrate and acetoacetate.* The effect on the equilibrium rates of interchange of NAD^+ and NADH and of D-3-hydroxybutyrate and acetoacetate of increasing the concentrations of NAD^+ (up to 66 times its K_m value) and NADH (up to 180 times its K_m value), while maintaining the ratio of the concentrations of the nicotinamide nucleotides constant, is shown in Fig. 4. Both equilibrium rates of interchange increase with increasing NAD^++NADH concentrations over the range of concentrations studied. For the D-3-hydroxybutyrate–acetoacetate interchange the onset of saturation is clearly evident. These findings, and those reported in Fig. 3 and discussed above, are in agreement with the ordered Bi Bi mechanisms proposed in the preceding paper (Preuveneers *et al.*, 1973). The results in Fig. 4 provide no evidence for the formation of dead-end complexes in the catalysis.

Comparison of the rate of interconversion of D-*3-hydroxybutyrate and acetoacetate with that of NAD^+ and NADH*

The rates of both interconversions were determined with [NAD^+] approx. 16 times its K_m value, [NADH] approx. 47 times its K_m value and [D-3-hydroxybutyrate] and [acetoacetate] approx. three times their respective K_m values. The rate of the D-3-hydroxybutyrate–acetoacetate interconversion was determined at each of five enzyme concentrations in the range 1–12 μg/ml, and the rate of the NAD^+–NADH interconversion was determined at each of five enzyme concentrations in the range 2.5–27 μg/ml. Under these conditions of concentration the rates of both interconversions were linear in enzyme concentration and the slopes of the rate–[enzyme] plots gave the apparent first-order rate constants as: 5.1 s^{-1} for the D-3-hydroxybutyrate–acetoacetate interconversion and 0.5 s^{-1} for the NAD^+–NADH interconversion.

That the apparent first-order rate constant for the interchange of D-3-hydroxybutyrate and acetoacetate is tenfold that of the corresponding parameter for the interchange of NAD^+ and NADH under the conditions of concentration of the various substrates given above constitutes evidence that the interconversion of the central (ternary) complexes is not extremely slow in relation to the other steps in this catalysis (see Boyer & Silverstein, 1963; Cleland, 1970).

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