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KINETIC STUDIES OF BEEF LIVER DIACETYL REDUCTASE

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SUMMARY

1. The dependence of the beef liver diacetyl reductase (acetoin:NAD oxidoreductase, EC 1.1.1.5) reaction rate on the concentration of substrates and the product inhibition pattern are investigated at optimum pH. The Michaelis and dissociation constants for the reaction in the direction diacetyl→acetoin are evaluated.

2. The results are consistent with the features of both a Theorell–Chance and a rapid equilibrium random Bi-Bi mechanism with two dead-end ternary complexes. However, it is concluded that the Theorell–Chance mechanism is the more probable one.

3. K_m values for diacetyl and NADH seem to indicate that the enzyme is operative under normal physiological conditions.

INTRODUCTION

The enzymatic reduction of diacetyl by animal tissues is now a well stablished fact[1–3], but, although some suggestions have been made[3], the physiological role of this reaction is still unknown. Although its elucidation will require an extensive study concerning the level reached by diacetyl in animal tissues and its origin, kinetic studies of the reaction allow some inferences to be made about the mechanism of enzyme action and the estimation of its biological significance. Studies of this type have not been published yet, and the only kinetic coefficient reported so far for the reaction is the apparent K_m^{diacetyl} value given for the rat liver enzyme[2]. The value is so high that it would hinder the activity under normal conditions.

The present work is an attempt to obtain reliable values for the kinetic coefficients in the initial rate-equation of the reaction catalyzed by the beef liver diacetyl reductase (acetoin:NAD oxidoreductase, EC 1.1.1.5) at its optimum pH and to obtain some knowledge about the reaction mechanism.

MATERIALS AND METHODS

Reagent solutions and buffers were prepared in deionized glass-distilled water. The diacetyl reductase preparations used were acetone (1–1.3 vol.) precipitates

obtained from beef liver as described by the authors in a previous paper[3].

NAD^+ and NADH were obtained from Boehringer. Acetoin and diacetyl were supplied by B.D.H. Acetoin was purified by washing it with peroxide-free ether to remove diacetyl traces. This procedure, recommended by Westerfeld[4], did not remove all the diacetyl impurities; its efficiency was improved by performing all operations under nitrogen but, even after this procedure, some diacetyl was still present in the acetoin samples.

Diacetyl levels in acetoin were colorimetrically determined by the method of Owades and Jakovac[5], modified by doubling the concentrations of all the components of the buffered hydroxylamine solution (in the normal procedure, concentrations of acetoin higher than 20 mg in the reaction mixture interfere with color development and prevent the detection of diacetyl up to 100 ppm; by the modified procedure 5 ppm can be measured).

Washing of acetoin with peroxide-free ether was repeated until the diacetyl content was less than 0.05%. After washing, acetoin was stored under nitrogen at −15 °C. for not more than 3 days.

Enzyme activities were spectrophotometrically determined, at optimum pH (6.1; see ref. 3) in 0.05 M sodium–potassium phosphate buffer, as already described[1].

RESULTS

Initial-rate measurements in the diacetyl→acetoin direction were made with several concentrations of each substrate in the ranges 0.05–3.9 mM diacetyl and 0.025–0.4 mM NADH. In these ranges all primary and secondary plots are linear within experimental error.

Primary double-reciprocal plots of $1/v$ *versus* 1/[diacetyl] at several NADH

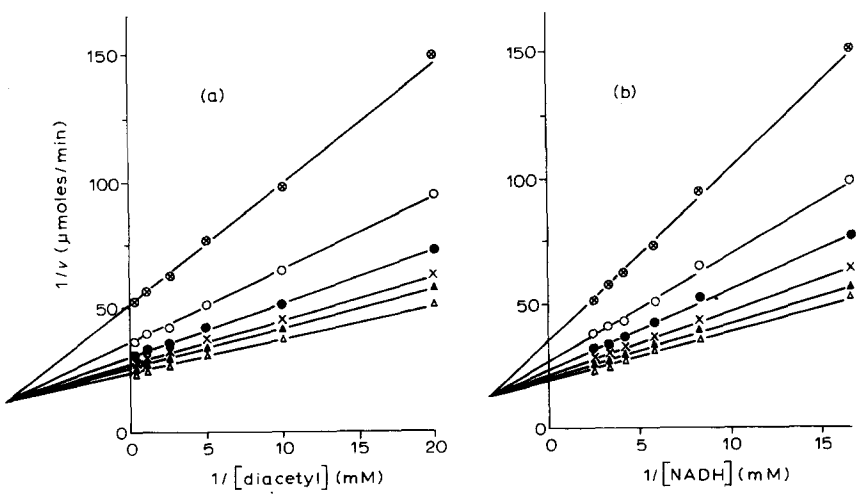


Fig. 1. Primary plots: (a) Double reciprocal plots of $1/v$ *versus* 1/[diacetyl] at the fixed NADH concentrations indicated (mM): (⊗—⊗), 0.058; (○—○), 0.117; (●—●), 0.174; (×—×), 0.233; (▲—▲), 0.3 and (△—△), 0.4. (b) Double reciprocal plots of $1/v$ *versus* 1/[NADH] at the fixed diacetyl concentrations indicated (mM): (⊗—⊗), 0.05; (○—○), 0.1; (●—●), 0.2; (×—×), 0.4; (▲—▲), 1 and (△—△), 3.9.

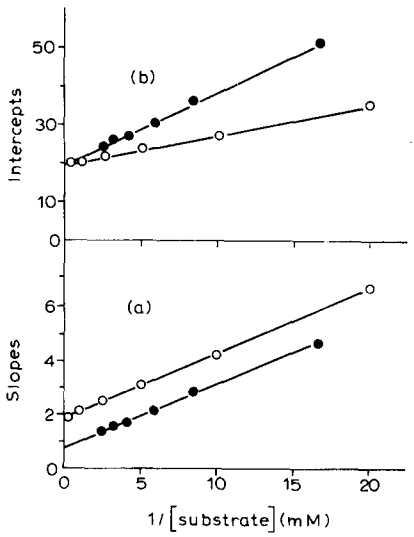


Fig. 2. Secondary plots of slopes (a) and intercepts (b) from Fig. 1 against the reciprocal of the substrates concentration: (○—○), diacetyl and (●—●), NADH.

concentrations and $1/v$ *versus* 1/[NADH] at fixed concentrations of diacetyl, give a family of straight lines converging in the second quadrant (Fig. 1).

Secondary plots of the slopes against 1/[substrate] are parallel lines; those of intercepts cut the vertical axis at the same point (Fig. 2).

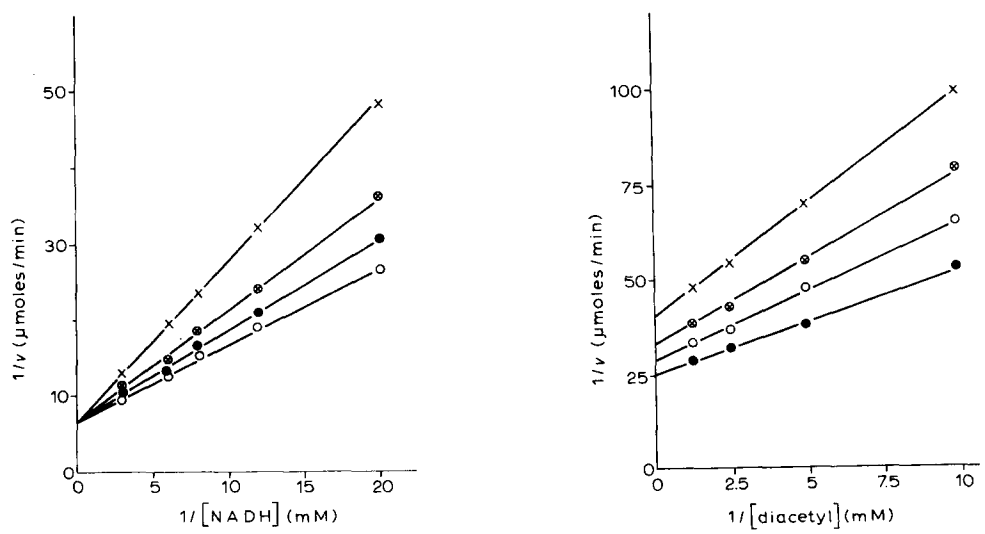


Fig. 3. Inhibition by NAD^+. Reciprocal plots of $1/v$ *versus* 1/[NADH] with constant diacetyl (0.1 mM) at different NAD^+ concentrations (mM): (×—×), 3.32; (⊗—⊗), 1.66; (●—●), 0.66 and (○—○), 0.

Fig. 4. Inhibition by NAD^+. Reciprocal plots of $1/v$ *versus* 1/[diacetyl] with constant NADH (0.2 mM) at the NAD^+ concentrations indicated (mM): (×—×), 5; (⊗—⊗), 2.5; (○—○), 1.25 and (●—●), 0.

K_s values (obtained from the intercept point of the primary reciprocal plots) and K_m (calculated from the secondary plots of the intercepts) are: $K_m^{\text{diacetyl}} = 39.8\ \mu M$; $K_m^{\text{NADH}} = 100\ \mu M$; $K_s^{\text{diacetyl}} = 122\ \mu M$; $K_s^{\text{NADH}} = 312\ \mu M$.

It has been reported that, under the standard assay conditions[3], it is not possible to measure any acetoin oxidation with diacetyl reductase from beef liver. Since the result could be due to inhibition of the reaction by diacetyl traces, the activity in the direction acetoin→diacetyl was checked with the more purified acetoin, prepared as described in Materials and Methods. No activity could be detected.

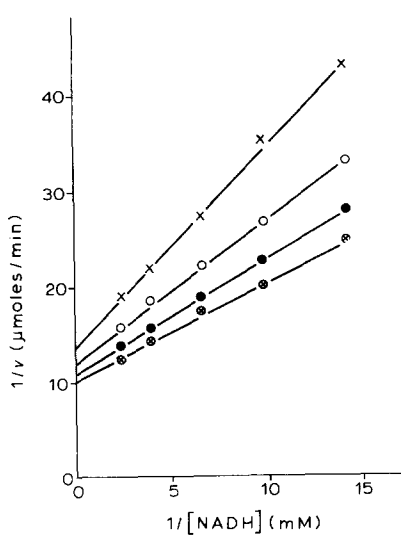


Fig. 5. Inhibition by acetoin. Reciprocal plots of $1/v$ *versus* 1/[NADH] with constant diacetyl (0.1 mM) at different acetoin concentrations (mM): (×—×), 16; (○—○), 8; (●—●), 4 and (⊗—⊗), 0.

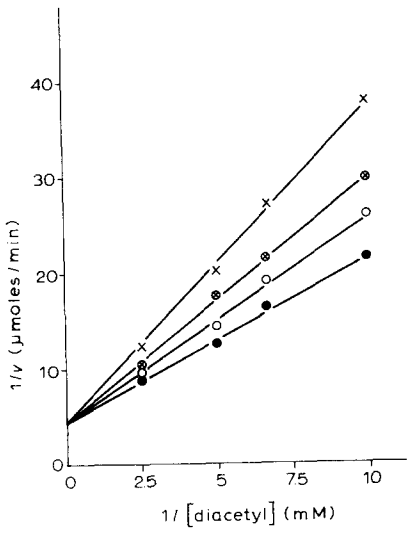


Fig. 6. Inhibition by acetoin. Reciprocal plots of $1/v$ *versus* 1/[diacetyl] with constant NADH (0.2 mM) at different acetoin concentrations (mM): (×—×), 16; (⊗—⊗), 8; (○—○), 4 and (●—●), 0.

Product inhibition pattern

In the presence of variable amounts of NAD^+, plots of $1/v$ against 1/[NADH] at a constant diacetyl concentration (Fig. 3) and $1/v$ *versus* 1/[diacetyl] at a fixed concentration of NADH (Fig. 4), demonstrate that NAD^+ inhibition is competitive with NADH and noncompetitive with diacetyl.

When diacetyl is not saturating in the system, acetoin inhibits the reaction noncompetitively for NADH (Fig. 5). Fig. 6 shows the type of inhibition of acetoin for diacetyl: the plots converge at a point on the vertical axis, proving that the inhibition is competitive. Furthermore, acetion, at concentrations up to 80 mM, does not inhibit the reaction when diacetyl is saturating.

DISCUSSION

The convergent primary double-reciprocal plots obtained demonstrate that the

diacetyl reductase reaction catalyzed by the beef liver enzyme follows a sequential mechanism whose rate law may be written as:

$$v = \frac{V}{1 + (K_m^a/[a]) + (K_m^b/[b]) + (K_m^a \cdot K_s^b/[a] \cdot [b])} \quad (1)$$

Three general mechanisms conform to this equation: ordered Bi-Bi, Theorell–Chance and rapid equilibrium random Bi-Bi (for details of mechanisms see Cleland[6]). Cleland's rules[7] predict the inhibition patterns shown in Table I for these mechanisms.

TABLE I

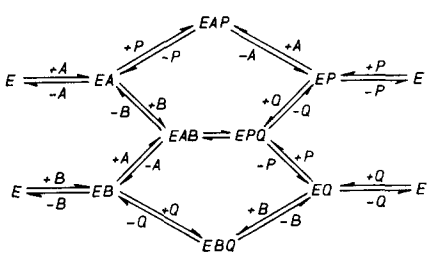
PRODUCT INHIBITION PATTERNS FOR BI-BI MECHANISMS WHOSE RATE LAW OBEYS Eqn 1

Abbreviations: C, competitive; NC, noncompetitive; UC, uncompetitive; O, no inhibition;

Mechanism[c]	*Inhibitory product*[b]	*Variable substrate*[a]			
		A		*B*	
		Unsatd with B	*Satd with B*	*Unsatd with A*	*Satd with A*
Ordered Bi-Bi	*P*	NC	UC	NC	NC
	Q	C	C	NC	O
Iso ordered Bi-Bi	*P*	NC	UC	NC	NC
	Q	NC	NC	NC	UC
Theorell–Chance	*P*	NC	O	C	C
	Q	C	C	NC	O
Iso Theorell–Chance	*P*	NC	O	C	C
	Q	NC	NC	NC	UC
Rapid equilibrium random Bi-Bi	*P* or *Q*	C	O	C	O

[a] Substrates are designated A and B according to the order of addition to the enzyme (if there is a compulsory binding order).
[b] *Q* and *P* are the products from *A* and *B*, respectively.
[c] For details of these mechanisms, see Cleland[6,7].

The inhibition pattern obtained is only consistent with one of the enzyme mechanisms in Table I; that of Theorell–Chance. There is, however, another mechanism not included in Table I whose inhibition pattern would also agree with our experimental results, namely a rapid equilibrium random Bi-Bi in which two dead-end ternary complexes, enzyme–acetoin–NADH and enzyme–diacetyl–NAD^+, are formed:



If neither P (product from B) nor Q (product from A) are present, no dead-end ternary complex is formed and the initial rate will be in accordance with Eqn 1. Cleland's rules[7] predict the same inhibitioin pattern for this mechanism as that of Theorell–Chance.

Therefore, it is not possible to distinguish between this mechanism and that of Theorell–Chance from initial-rate studies or product inhibition-pattern inspection. Nevertheless, the requirements for the rapid equilibrium random Bi-Bi mechanism discussed here are very restrictive (random order of substrate binding, two dead-end ternary complexes, all steps very rapid except the interconversion of the non-abortive ternary complexes). Thus the mechanism of Theorell–Chance seems to be the more likely reaction scheme.

Gabriel *et al.*[2] have given an apparent K_m^{diacetyl} value = 48 mM for rat liver diacetyl reductase from Lineweaver–Burk plots at a constant concentration of NADH. They assumed that the expected physiological concentrations of the substrate are probably two or three orders of magnitude lower and suggested that the enzyme must have very little activity under normal conditions and that this enzyme could be primarily specific for other substrates.

Beef liver diacetyl reductase, on the contrary, seems to have a high specificity for diacetyl[3]. Its K_m^{diacetyl} value (39.8 μM) is 10^3 times lower than the value reported for the rat liver enzyme; adequate, therefore, for using diacetyl as the primary substrate if the assumption of Gabriel *et al.*[2] about diacetyl concentration is accepted. The K_m^{NADH} value falls in the same order of magnitude as the NADH concentration expected to be found in animal tissues[8]. Thus, the beef liver enzyme could be operative under normal physiological conditions.

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