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KINETIC MECHANISM OF THE HYDROXYPYRUVATE-LACTATE DEHYDROGENASE-NADH SYSTEM

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Summary

Chicken liver lactate dehydrogenase L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) reversibly catalyses the conversion of hydroxypyruvate to L-glycerate. The variation of the initial reaction rate with the substrate or coenzyme (NADH) concentration together with the inhibition caused by the reaction products and excess substrates, reveal that the kinetic mechanism of the reaction, with hydroxypyruvate as substrate, is of the rapid-equilibrium, ordered-ternary-complex type; NADH is the first substrate in the reaction sequence.

Rate equations have been developed for the hydroxypyruvate · E · NADH system without inhibitors, with excess substrates, and with reaction products. Comparison of the rate equations obtained with those calculated theoretically from an ordered-ternary-complex mechanism reveals the existence of E · NAD · NADH, E · NAD-hydroxypyruvate and E · hydroxypyruvate complexes.

Introduction

Lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) reduces hydroxpyruvate to L-glycerate with NADH as coenzyme and this reaction is of some physiological interest. It has been postulated [1,2] that the reduction is coupled with the oxidation of glyoxylate to oxalate, catalysed by the same enzyme the participation of lactate dehydrogenase in certain reactions in amino acid metabolism may explain how L-glycerate, D-glycerate and hydroxypyruvate are incorporated, through the same metabolic pathway, into the glycogen intermediate pool [3].

Although the physiological role of the hydroxypyruvate-lactate dehydrogenase-NADH system has been previously studied, its kinetic characteristics have not yet been established.

It has been recognised that lactate dehydrogenase kinetics at non-inhibitory levels of pyruvate and lactate are characteristic of bi-bi-sequential mechanism

when lactate dehydrogenase acts with its preferred substrate (pyruvate). The compulsory order of binding, coenzyme followed by substrate, was established as a result of equilibrium-binding experiments. Recent kinetic studies of lactate dehydrogenase have been reported by Holbrook et al. [4] and it was shown that the enzyme mechanism is of an ordered-bi-bi-ternary-complexes type.

Comparisons between kinetic studies with preferred and alternative substrates could give some information about reaction pathways, as well as indications of the molecular basis of specifity. Unfortunately, lactate dehydrogenase, as with other oxidoreductases which have been studied in this way, most of the results have been reported only as apparent maximum rates and apparent K_m values for the alternative substrates, which restricts the amount of information than can be derived.

Lactate dehydrogenase can be assayed using several 2-hydroxy and 2-keto acids as substrate [5]. With the rabbit muscle enzyme, 2-ketobutyrate and 2-keto-4-hydroxyglutarate give smaller maximum rates than pyruvate but no kinetic mechanism has been published with these alternative substrates.

Glyoxylate acts as substrate for both oxidation and reduction. Kinetic studies with this substrate are at variance with the classical ordered-ternary-complexes type [6].

Hydroxypyruvate is a less effective substrate than pyruvate and gives a low rate of catalysis suggesting a change from steady-state to rapid equilibrium, ordered kinetics; however this theoretical prediction has not been confirmed with lactate dehydrogenase. The purpose of this paper is to formulate the kinetic mechanism of lactate dehydrogenase with hydroxypyruvate as substrate and develop the rate equation of the system, which may also allow the action of lactate dehydrogenase to be compared with respect to its diverse substrates.

Materials and Methods

Freshly prepared solutions in the appropriate buffer of the following substances, after pH adjustment, were used as substrates or inhibitors: 98% lithium hydroxypyruvate and 95% oxamic acid (Sigma); 65% DL-glyceric acid aqueous solution (Merck); 78% NADH and 89% NAD (Boehringer); oxalic acid dihydrate (Union Chimie Belgium).

The enzyme activity was determined in 50 mM sodium phosphate (pH 7.4) at 340 nm (30 ± 0.1°C) in a PYE Unicam 1700 recording spectrophotometer. The reaction was started by the addition of 2.08 μg/ml of purified enzyme.

The protein content of the solutions was determined by the Warburg and Christian method [7,8]. The variations of the initial reaction rates with the substrate concentration were expressed according to the methods of Lineweaver and Burk [9] and Eadie and Hoffstee [10,11]. The true kinetic constants and the kinetic parameters were calculated according to that of Vestling and Florini [12].

The chicken liver lactate dehydrogenase used was prepared as follows: the tissue was homogenized in a Potter-Elvejehm homogenizer in 8 vols. (w/v) 0.25 M sucrose solution at 4°C and centrifuged at 12 000 × g (r_{av} = 8.7 cm) at 4°C for 30 min. The homogenate was heated and held at 56°C for 25 min, whereafter it was rapidly cooled to 4°C. The suspension was centrifuged as

above. The supernatant obtained was fractionated with $(NH_4)_2SO_4$; the precipitate having lactate dehydrogenase activity appeared between 30% and 60% saturation and was separated by centrifugation. The preparation was dissolved in 5 mM sodium phosphate buffer (pH 7.0, 1 : 2, w/v) and dialysed exhaustively against the same buffer. The dialysate was centrifuged at 18 000 × *g* at 4°C for 30 min and the supernatant chromatographed on a DEAE-Sephadex column (25 × 2 cm). The eluant was 5 mM sodium phosphate (pH 7.0) and a NaCl 0—0.3 M gradient was established. 10-ml fractions were collected, the lactate dehydrogenase activity appearing in fractions 11—16. The fractions were combined, precipitated out with 60% saturation of $(NH_4)_2SO_4$ and the precipitate stored at 4°C.

Results and Discussion

Rate equation for the hydroxypyruvate-E-NADH system. Variation of the initial reaction rates with the substrate concentration

The double reciprocal plot of v against [S] (Fig. 1) is linear if the concentrations of the variable substrate are equal to or less than the respective saturating concentration.

The true kinetic parameters calculated for the secondary plots derived from Fig. 1 (Vestling-Florini method) are:

$$v = 11.4 \frac{\mu\text{mol NADH}}{\text{min} \cdot \mu\text{g of purified prep.}}; \; K_{M(\text{hydroxypyruvate})} = 0.166 \text{ mM};$$

$\overline{K}_{(\text{hydroxypyruvate})} = \infty$ and $\overline{K}_{(\text{NADH})} = 0.023$ M.

It is observed (Fig. 1a) that the values of $1/v$ vary linearly with respect to $1/$

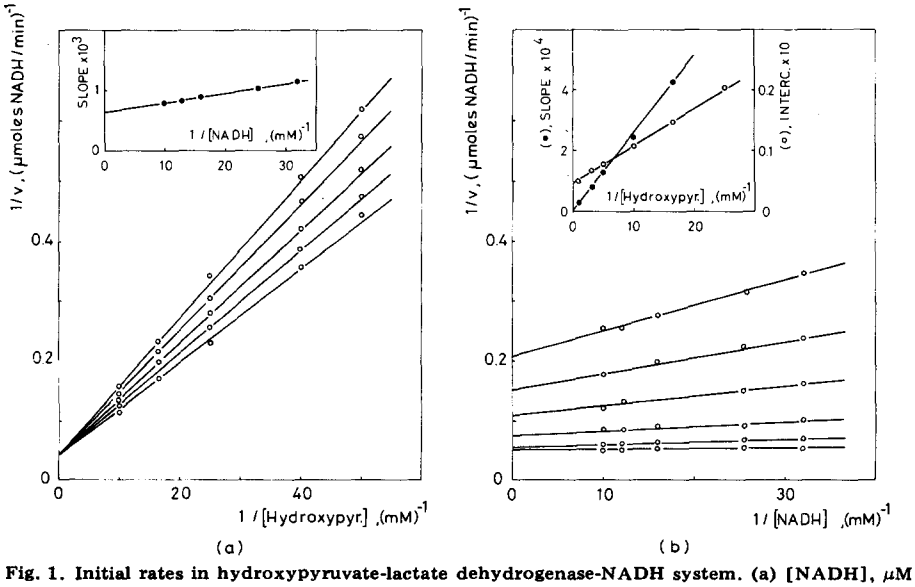

Fig. 1. Initial rates in hydroxypyruvate-lactate dehydrogenase-NADH system. (a) [NADH], μM (bottom to top): 93.6, 78, 62.4, 39, 31.2 (b) [hydroxypyruvate], mM (bottom to top): 1, 0.6, 0.2, 0.1, 0.06, 0.04.

[hydroxypyruvate], irrespective of the NADH concentrations in the reaction medium. All the straight lines in Fig. 1a meet at a common point on the ordinate axis, and the slopes derived from Fig. 1a vary linearly with 1/[NADH]. An analysis of Fig. 1b shows that $1/v$ is, likewise, a linear function of 1/[NADH]. When plotting the slopes and the intercepts of Fig. 1b in terms of 1/[hydroxypyruvate], straight lines are obtained. This suggests that the system equation in the absence of inhibitors is

$$\frac{1}{v} = \frac{a}{[\text{NADH}]\,[\text{hydroxypyruvate}]} + \frac{b}{[\text{hydroxypyruvate}]} + c\,. \qquad (1)$$

The above, experimentally-obtained rate equation is characteristic of a rapid-equilibrium, ordered-bireactant system [13], wherein the two substrates taking part in the reaction must bind to the enzyme in an ordered fashion. The plots (Fig. 1) show that NADH is the first substrate added to the reaction sequence. The mechanism suggested differs from the ordered-ternary-complex mechanism in that the rate equation solved for $1/v$ does not contain terms in 1/[NADH]. In the partial equilibrium

$$\text{E} + \text{NADH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E-NADH}\,,$$

the value of the constant k_1 is $k_1 > K_{\text{cat}}$.

Substrate inhibition

Hydroxypyruvate causes substrate inhibition (Fig. 2a); the values of $1/v$ vary linearly with the inhibition producing substrate concentrations (Fig. 2b); the inhibition is non-competitive with respect to the NADH (Fig. 2c); and the secondary plots of the slopes and intercepts are linear.

Excess NADH inhibits the system (Fig. 3a) and the plots of $1/v$ against [NADH] (Fig. 3b) and of $1/v$ against 1/[hydroxypyruvate] for various inhibitory concentrations of NADH are not linear (Fig. 3c). Under these experimental conditions, the rate equation will contain a polynomial term, of an order higher than unity, affecting the NADH concentration. In Fig. 3a and b, the deviation from linearity is more obvious with greater concentrations of NADH and hydroxypyruvate. The simplest term, whose introduction allows the above deviations to be explained, will be of the $[\text{NADH}]^2$ [hydroxypyruvate] type, but, since this term is the simplest one possible justifying the experimental results, it may be a polynomial function of a higher order.

Eqn. 2 is the simplest representative equation of the system when there is substrate inhibition but no reaction product inhibition.

$$\frac{1}{v} = \frac{a}{[\text{NADH}]\,[\text{hydroxypyruvate}]} + \frac{b}{[\text{hydroxypyruvate}]} + c + \left(\frac{d}{[\text{NADH}]} + e\right) \times [\text{hydroxypyruvate}] + (f[\text{NADH}]\,[\text{hydroxypyruvate}] + g)[\text{NADH}]\,. \qquad (2)$$

Product inhibition

NAD is a competitive and linear inhibitor of the hydroxypyruvate-E-NADH system with respect to NADH, irrespective of whether the hydroxypyruvate concentration is saturating or not (Fig. 4a), whereas it non-competitively inhibits with respect to hydroxypyruvate (Fig. 4b). Under these conditions, the rate equation should be analogous to Eqn. 1, but terms relating to the NAD

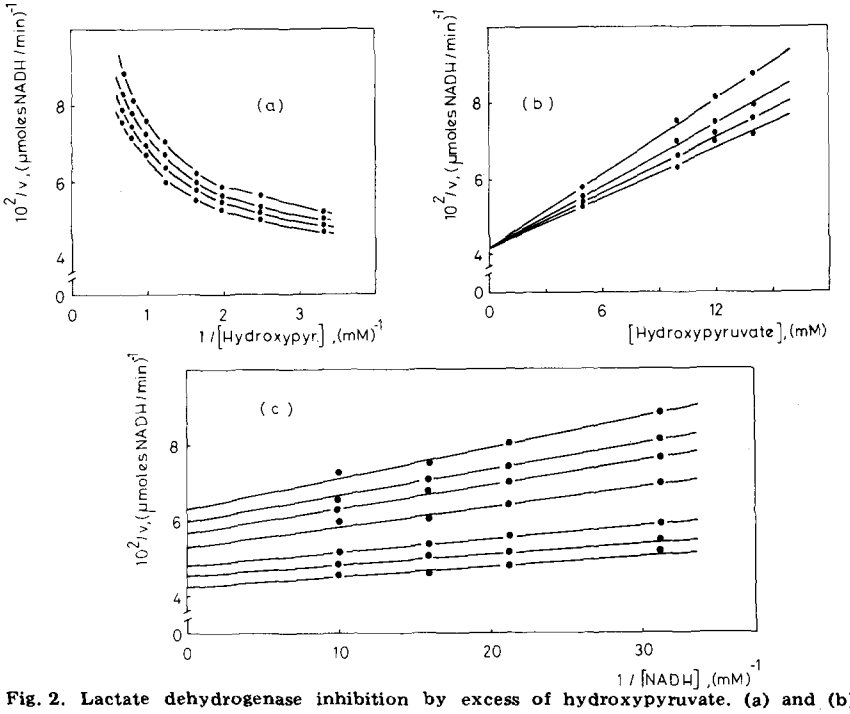

Fig. 2. Lactate dehydrogenase inhibition by excess of hydroxypyruvate. (a) and (b) [NADH], μM (bottom to top): 78, 62.4, 46, 31.2 (c) [hydroxypyruvate], mM (bottom to top) 1, 3, 5, 8, 10,12, 14.

concentration and explaining the nature of the inhibition must be introduced. Straight lines having a shallower slope (if the NADH concentration is saturating) are obtained in the secondary plots, derived from the NAD inhibi-

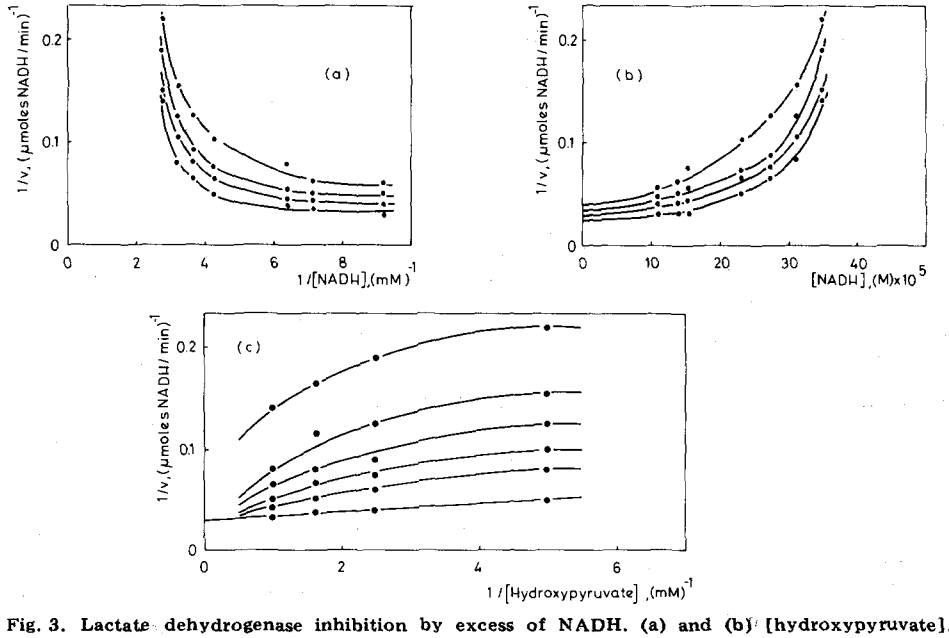

Fig. 3. Lactate dehydrogenase inhibition by excess of NADH. (a) and (b) [hydroxypyruvate], mM (bottom to top): 1, 0.6, 0.4, 0.2 (c) [NADH], mM (bottom to top): 0.078, 0.15, 0.23, 0.27, 0.31, 0.35.

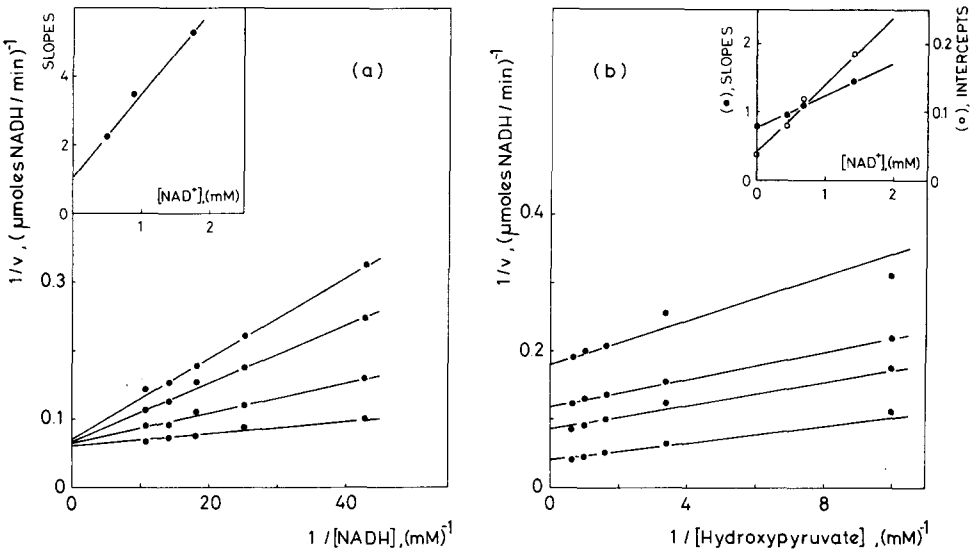

Fig. 4. Inhibition by NAD. (a) [hydroxypyruvate] = 0.2 mM; [NAD], mM (bottom to top): 0, 0.53, 0.89, 1.78 (b) [NADH] = 0.039 mM, [NAD], mM (bottom to top): 0, 0.44, 0.71, 1.42.

tion with respect to the hydroxypyruvate. The intercept with the ordinate axis, of the straight line obtained in the secondary plot of the intercepts vs. [NAD], is independent of the NADH concentration; however, the intercept with the ordinate axis, of the straight line derived from the secondary plot of the slopes vs. [NAD], is lower when the NADH is saturating. Consequently, Eqn. 3 is obtained:

$$\frac{1}{v} = \frac{a}{[\text{hydroxypyruvate}][\text{NADH}]} + \frac{b}{[\text{hydroxypyruvate}]} + \frac{c[\text{NAD}]}{[\text{hydroxypyruvate}][\text{NADH}]} + d\frac{[\text{NAD}]}{[\text{hydroxypyruvate}]} + \frac{e[\text{NAD}]}{[\text{NADH}]} + g[\text{NAD}] + h \quad (3)$$

On expressing the equation in terms of $1/v$ and $1/[\text{NADH}]$, the low mathematical significance of the terms $d[\text{NAD}]/[\text{hydroxypyruvate}]$ and $g[\text{NAD}]$ is shown, since the NAD product inhibition with respect to NADH is practically competitive; nevertheless, this may explain why the straight lines intercept each other very close to the ordinate axis, but not on the axis itself, in the double-reciprocal plot of this inhibition.

L-Glycerate is a non-competitive, linear inhibitor with respect to NADH, whether hydroxypyruvate is saturating or not (Fig. 5a) and inhibits non-competitively with respect to hydroxypyruvate, independently of NADH (Fig. 5b).

As in the previous case, L-glycerate inhibition kinetics with respect to the NADH give linear secondary plots. The secondary plots of the slopes, or of the intercepts derived from this inhibition, are straight lines in which the slope, and the intercept with the abcissa axis, is lower when hydroxypyruvate concentration is saturating.

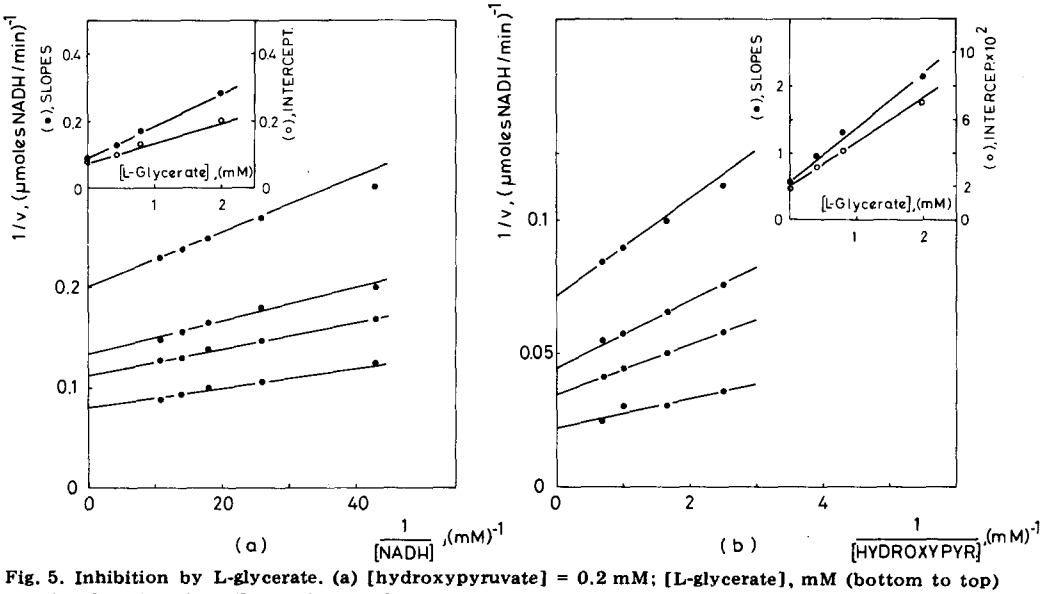

Fig. 5. Inhibition by L-glycerate. (a) [hydroxypyruvate] = 0.2 mM; [L-glycerate], mM (bottom to top) 0, 0.4, 0.8, 2 (b) [NADH] = 0.039 mM, [L-glycerate], mM (bottom to top) 0, 0.4, 0.8, 2.

In the case of inhibition induced by L-glycerate with respect to hydroxypyruvate, the secondary plots of the intercepts give straight lines which coincide, both when the NADH concentration is saturating and when it is not. Eqn. 4 satisfies the experimental results:

$$\frac{1}{v} = \frac{a}{[\text{hydroxypyruvate}][\text{NADH}]} + b\,\frac{[\text{L-glycerate}]}{[\text{hydroxypyruvate}][\text{NADH}]} + \frac{c}{[\text{hydroxypyruvate}]} + \frac{d[\text{L-glycerate}]}{[\text{hydroxypyruvate}]} + f + g[\text{L-glycerate}]\ .$$

Reaction mechanism

The data presented here are in agreement with the equations and the results confirm a change from steady-state to rapid equilibrium, ordered kinetics, when lactate dehydrogenase acts with hydroxypyruvate as substrate. Rapid equilibrium, ordered kinetics had not been evident with this enzyme using alternative substrates, but the kinetic mechanism here formulated confirms the theoretical predictions that can be formulated if one uses a poor substrate like hydroxypyruvate (which displays a lower rate of catalysis than the preferred substrate, pyruvate).

To reveal which are the abortive complexes justifying the inhibitions above mentioned, all the rate equations obtained experimentally have been compared with those described by King and Altman [14], taking, as a base, an ordered, ternary-complex mechanism (Fig. 6) in which all the possible abortive ternary complexes have been formulated. When comparing the equations, we have always borne in mind that the mechanism under study has a rapid equilibrium and that, therefore, terms in 1/[NADH] must not appear in the rate equations.

The theoretical equations of the mechanism formulated in Fig. 6 have been developed (unpublished results). Table I gives those corresponding to the

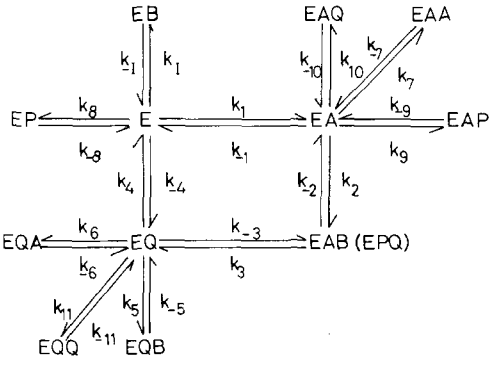

Fig. 6. Theoretical kinetic mechanism formulated for rate equations design. E: free enzyme; B: hydroxypyruvate; A: NADH; P: L-glycerate; Q: NAD.

hydroxypyruvate · E · NADH system to which the present paper relates. Table II gives the meaning of the constants appearing in Table I, in terms of the formation and dissociation constants of the complexes (Fig. 6).

The comparison of the equations shown in Table I with the experimentally deduced equations under the same conditions reveals that the terms affected only by $1/k_1$ are nil, in accordance with the suggestion that the proposed mechanism rapid equilibrium. The value of the constant, K_I, is small and the terms affected by it only have a mathematical significance if they are multiplied by the hydroxypyruvate concentration; thus, the LDH · hydroxypyruvate complex is only formed when this substrate causes inhibition as a result of an excess amount being present. The terms affected by K_7, K_8, K_9, K_{11} have no kinetic significance and, therefore, it is considered that the formation

TABLE I

THEORETICAL RATE EQUATIONS USING THE KING AND ALTMAN METHOD

A: NADH; B: Hydroxypyruvate; Q: NAD; P: L-glycerate.

Rate equation under substrate inhibition conditions:

$$\frac{1}{\tilde{v}} = \frac{k_a}{K_1K_e}\cdot\frac{1}{AB} + \frac{K_I}{k_1}\cdot\frac{B}{A} + \left(\frac{1}{k_1} + \frac{K_IK_a}{K_1K_e}\right)\cdot\frac{1}{A} + \frac{1}{K_e} + \frac{K_a}{K_e}\cdot\frac{1}{B} + \frac{K_dK_5}{K_e}\cdot B + \frac{K_dK_6}{K_e}\cdot A + \frac{K_aK_7}{K_e}\cdot\frac{A}{B}$$

Rate equation under NAD inhibition conditions:

$$\frac{1}{\tilde{v}} = \frac{K_a}{K_1K_e}\cdot\frac{1}{AB} + \frac{K_f}{K_1K_e}\cdot\left(\frac{k_3}{k_2} + \frac{1}{K_2}\right)\cdot\frac{Q}{AB} + \left(\frac{1}{k_1} + \frac{K_IK_a}{K_1K_e}\right)\frac{1}{A} + \frac{K_f}{K_e}\cdot\left(\frac{k_3}{k_1} + \frac{K_5}{K_1K_2} + \frac{k_3K_5}{K_1k_2}\right)\cdot\frac{Q}{A} + \frac{k_3K_{11}K_f}{k_1K_e}$$
$$\cdot\frac{Q^2}{A} + \left(\frac{k_3}{k_2} + \frac{1}{K_2}\right)\frac{k_fK_{11}}{K_1K_e}\frac{Q^2}{AB} + \frac{K_a}{K_e}\frac{1}{B} + \frac{1}{K_e}\left(\frac{K_6K_f(1/K_2 + k_3/k_2)}{K_1} + K_{10}K_a\right)\frac{Q}{B} + \frac{1}{K_e} +$$
$$+ \frac{1}{K_e}\left(\frac{k_3K_6K_f}{k_1} + K_{11}K_d\right)Q$$

Rate equation under L-glycerate inhibition conditions:

$$\frac{1}{\tilde{v}} = \frac{K_a}{K_1K_e}\frac{1}{AB} + \frac{1}{K_1K_e}\left(\frac{K_b}{K_2} + K_aK_8\right)\frac{P}{AB} + \left(\frac{1}{k_1} + \frac{K_IK_a}{K_1K_e}\right)\frac{1}{A} + \left(\frac{K_IK_b}{K_1K_2K_e} + \frac{K_8}{k_1}\right)\frac{P}{A} + \frac{K_a}{K_e}\frac{1}{B} + \frac{K_bK_8}{K_1K_2K_e}\frac{P^2}{AB}$$
$$+ \frac{1}{K_e}\left(\frac{K_b}{K_2} + K_aK_9\right)\frac{P}{B} + \frac{K_b}{K_e}P + \frac{1}{K_e} + \frac{K_bK_9}{K_2K_e}\frac{P^2}{B}$$

TABLE II

CONSTANTS OF THEORETICAL RATE EQUATIONS

$$K_a = \frac{k_4(k_3 + k_{-2})}{k_2(k_3 + k_4)}$$

$$K_b = \frac{k_{-3}}{k_3 + k_4}$$

$$K_d = \frac{k_3}{k_3 + k_4}$$

$$K_e = \frac{k_3 k_4}{k_3 + k_4}$$

$$K_f = \frac{k_{-4}}{k_3 + k_4}$$

$$K_I = \frac{k_I}{k_{-I}}$$

$$K_i = \frac{k_i}{k_{-i}} \quad \text{(in general)}$$

of the complexes that they regulate is nil under these experimental conditions. This is not the case with the complexes regulated by K_5 and K_6. The term, $[NAD]^2 \times$ [hydroxypyruvate] appearing experimentally, has no justification in the theoretical mechanism proposed. The formulation of the complex that would justify it has not been carried out, since although this term is the simplest one possible justifying the experimental results, it may be a polynomial function of a higher order.

The existence of the complex regulated by K_{10} is doubtful and no conclusion may be reached with respect to it.

The abortive complex, E · NAD · hydroxypyruvate, is similar to the classical one, E · NAD · pyruvate; the former complex has been postulated by other authors using kinetic, fluorimetric and spectrophotometric techniques [4,15]. The formation of the LDH · NAD · pyruvate complex is the cause of inhibition by high concentrations of pyruvate [16]. In this sense, one could expect analogous interpretations for E · NAD · hydroxypyruvate complex formation.

The kinetic mechanism suggested, as well as the rate equations derived from it, point out the existence of the E · hydroxypyruvate complex, with kinetic significance at high hydroxypyruvate concentrations. E · substrate complexes have been detected in other dehydrogenases [17,18], whilst other authors refute their existence [4]. The formation of an E · substrate complex has been demonstrated with the rabbit skeletal muscle enzyme, with high concentrations of pyruvate (unpublished results).

NAD product inhibition results give a equation that seems to suggest the kinetic significance of an E · NAD · NADH complex which could be correlated with the similar results obtained using glyoxylate as substrate [6].

In summary, it has been possible to show the existence of the E · hydroxypyruvate E · NAD · NADH and E · NAD · hydroxypyruvate complexes (Fig. 7)

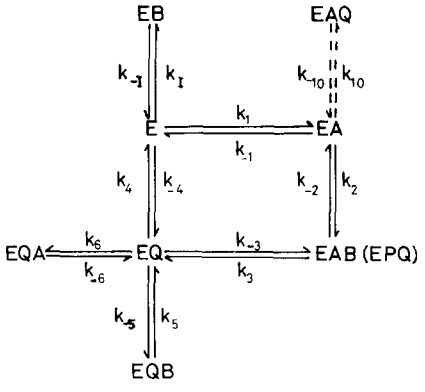

Fig. 7. Kinetic mechanism for hydroxypyruvate-lactate dehydrogenase-NADH system. E: free enzyme; B: hydroxypyruvate; A: NADH; P: L-glycerate; Q: NAD.

in the hydroxypyruvate-LDH-NADH system. Probably these are not the only complexes formed, but these have kinetic meaning under the experimental conditions described.

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