The Lactate Dehydrogenase Catalyzed Reduction of Pyruvate. Active Substrate and Substrate Inhibition†

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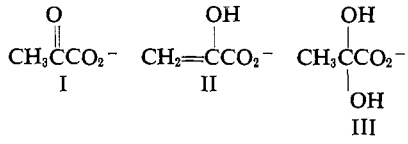
ABSTRACT: The reduction of pyruvate catalyzed by lactate dehydrogenase was studied using a spectrophotometric method in which the rate of oxidation of DPNH was monitored at 340 nm. Detailed kinetic analyses were carried out with respect to the enzymatic activity of beef heart lactate dehydrogenase as a function of pH, pyruvate concentration, and coenzyme concentration. The relative rates of the enzymatic process at various stages of pyruvate hydration, H_2O + $CH_3COCO_2^- \rightleftharpoons CH_3C(OH)_2CO_2^-$, were investigated and comparative studies involving the deuterated and the protiated pyruvate were carried out. The results obtained indicate that the true substrate involved in this enzymatic process is the unhydrated keto form rather than either a hydrated or an enolized form. Furthermore, the existence of even relatively small quantities of the hydrated pyruvate accounts for significant "substrate" inhibition.

Pyruvate undergoes both enolization and hydration; accordingly, its aqueous solutions exist as equilibrated systems consisting of pyruvate (I) as well as its enolized (II) and hydrated (III) forms



The enol form accounts for less than 1% of the total concentration of pyruvate (Eigen *et al.*, 1962; Pocker *et al.*, 1969). Detailed kinetic studies of the *enolization* of pyruvic acid have been carried out by following its rate of iodine uptake (Albery *et al.*, 1965; Meany, 1971; Hegazi and Meany, 1972). The reversible *hydration* of pyruvic acid and the pyruvate ion catalyzed by general acids and bases as well as by metal ions and bovine carbonic anhydrase have been thoroughly investigated (Pocker and Meany, 1970). From thermodynamic measurements, the distribution of hydrated *vs.* unhydrated pyruvic acid and pyruvate ion is known over wide ranges of pyruvate concentration, pH, and temperature (Eigen *et al.*, 1962; Pocker and Meany, 1970; Strehlow, 1962; Gold *et al.*, 1964; Griffiths and Socrates, 1967).

The reversible reduction of pyruvate by DPNH catalyzed by lactate dehydrogenase is an important part of the anaerobic glycolytic system in animal cells (Schwert and Winer, 1963). The enzymatically assisted hydride transfer from DPNH to pyruvate might occur *via* any of three possible routes: (1) as a nucleophilic addition of hydride to the carbonyl carbon of pyruvate, (2) as a nucleophilic addition of hydride to the sp^2 hybridized carbon of the enol form, or finally, (3) by the nucleophilic substitution of hydride for hydroxide on the hydrated pyruvate ion.

Conflicting opinions appear in the literature regarding the participation of the enolized form of pyruvate as the preferential substrate for lactate dehydrogenase (Fromm, 1965; Griffin and Criddle, 1970; Vennesland, 1956). The possible involvement of hydrated pyruvate in the enzymatic process has not been considered. It is the purpose of the present work to determine the nature of the preferential substrate for lactate dehydrogenase and also to characterize the "substrate" inhibition generally observed for this system.

Experimental Section

Reagent grade pyruvic acid was obtained from Sigma Chemical Corp. and was freshly distilled under reduced pressure in an atmosphere of nitrogen prior to each set of experiments. The concentration of the distilled material was checked prior to use by titration with a standardized sodium hydroxide solution. Sodium pyruvate was obtained from City Chemical Corp. Buffer components and all other inorganic compounds used in this work were obtained from commercial sources either in analytical or reagent grade. Buffer solutions were prepared in deionized water or in deuterium oxide (International Chemical and Nuclear Co.) and adjusted to an ionic strength of 0.1 M by the addition of the appropriate quantities of sodium chloride.

Solutions of β-DPNH (Sigma Type III) were prepared immediately before each series of kinetic runs by dissolving accurately weighed amounts of the cofactor in phosphate buffer (pH 8). The resultant concentrations of β-DPNH were checked spectrophotometrically (E_{340}^{mM} 6.2).

Most of the kinetic studies reported in the present paper involve the use of beef heart lactate dehydrogenase (Worthington Biochemical Corp.). A value of 2.1×10^5 cm^{-1} M^{-1} was used as the molar extinction coefficient for this enzyme preparation which consists predominently of the H_4 isozyme (Millar *et al.*, 1969). The molecular weight was taken to be 142,000 amu (Jaenicke and Knof, 1968). Stock solutions of the enzyme in 0.01 M sodium chloride were prepared directly before each set of kinetic runs. Kinetic measurements were also carried out on pyruvate reduction catalyzed by rabbit muscle lactate dehydrogenase (Worthington), pig heart lactate dehydrogenase (Sigma Chemical Co.), as well as the H_4 and M_4 isozymes of lactate dehydrogenase (Boehringer Mannheim Corp.).

Reaction rates were followed on a Beckman Kintrac VII recording spectrophotometer, the cell compartment of which

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was thermostated to 25 ± 0.02° by means of a Beckman Thermocirculator Accessory. The oxidation of β-DPNH was monitored by following the decrease in absorbancy at 340 nm. For kinetic studies involving the rate of equilibration between the hydrated and unhydrated forms of pyruvate, the dehydration of 2,2-dihydroxypropionate was initiated by injecting small amounts of a preacidified (pH 0.45) aqueous solution of 1.2 M pyruvic acid (consisting of *ca.* 62% of the hydrated form) (Pocker *et al.*, 1969) into the buffered reaction media. The almost instantaneous pH change brought about by the 0.01 M phosphate buffer (pH 6.8) causes the dehydration of 2,2-dihydroxypropionate to occur, since around neutral pH the fraction of hydrated pyruvate is 6% (Pocker *et al.*, 1969). The quantities of the acidified solutions introduced into the phosphate buffers were usually small enough so that no significant changes in pH occurred. When it was necessary to employ larger amounts of the acidified pyruvic acid solutions, the appropriate amount of standardized sodium hydroxide was added to counterbalance any decrease in pH. All pH measurements employed the use of a Beckman Century SS expanded scale pH meter and were carried out before and after each kinetic run.

The dehydration of 2,2-dihydroxypropionate initiated in this manner may be followed directly by monitoring the increase in absorbancy at 260 nm as the concentration of unhydrated pyruvate increases (Pocker and Meany, 1970). The pseudo-first-order rate constants for the equilibration process may be determined by plots of log $(A_\infty - A_t)$ *vs.* time: k_{obsd} = 2.3 × slope. Due to the reversibility of the reaction, the observed rate constant obtained, $k_{obsd} = 1.4\ min^{-1}$ [in 0.01 M phosphate buffer (pH 6.8) at 25.0°], is actually a sum of rate constants for the hydration and dehydration processes. The rate constant associated with the hydration process may be deduced by multiplying the observed rate constant by the fraction of hydration at equilibrium: $k_{hyd} = \chi k_{obsd} = 0.06 \times 1.4\ min^{-1} = 0.084\ min^{-1}$.

Deuterated pyruvate, $CD_3COCO_2^-$, was obtained by preparing pyruvate solutions in heavy water and allowing them to incubate for a sufficient period of time for deuterium exchange ($2CH_3COCO_2^- + 3D_2O \rightleftharpoons 2CD_3COCO_2^- + 3H_2O$) to occur as a result of enolization. Immediately after the solutions were prepared, proton magnetic resonance (pmr) scans (Varian Model HA60IL) were made. Tetramethylsilane (10.0 ppm) was used as an external reference. Two peaks were observed at 7.8 and 8.7 ppm, which correspond to the protons associated with the unhydrated, $CH_3COCO_2^-$ and hydrated, $CH_3C(OD)_2CO_2^-$ species, respectively (Pocker *et al.*, 1969). Additional pmr scans were made as the deuterium exchange progressed. Ultimately the original peaks very nearly disappeared and were replaced by a water (H_2O) peak at *ca.* 5.2 ppm. Integration of the peak areas showed that the pyruvate was at least 95% deuterated.

In the kinetic runs involving the enzymatic reduction of the deuterated pyruvate system, small quantities of pyruvate in D_2O were added as the last component to the reaction solutions (in H_2O). The rate of oxidation of DPNH was much more rapid than the enolization of the pyruvate system (Hegazi and Meany, 1972) so that the enzymatic process was complete before any significant amount of the pyruvate could undergo hydrogen exchange.

Results and Discussion

Enzymatic Oxidation of DPNH as a Function of Enzyme Concentration. Reactions were carried out at constant pyruvate (6.7×10^{-5} M) and DPNH concentrations (9.6×10^{-5} M) while varying only the enzyme concentration. It was observed that the enzymatic rate of reduction of DPNH is first order in enzyme over a relatively wide range of enzyme concentrations ($V = 8.0 \times 10^{-5}$ M min^{-1} at 5.1×10^{-6} M lactate dehydrogenase).

Activity of Lactate Dehydrogenase as a Function of pH. The pH–rate profile for the enzymatic reduction of pyruvate was deduced in 0.01 M phosphate buffer in the pH range from 5 to 8. Using 6.7×10^{-5} M pyruvate and 9.6×10^{-5} M DPNH a broad bell-shaped curve was obtained exhibiting inflections at pH 5.5 and 7.8 and a relatively flat maximum at pH 6.8. The region of pH at which maximum activity was observed under these conditions was chosen for subsequent kinetic work described in this paper.

Activity of Lactate Dehydrogenase as a Function of DPNH and Pyruvate (Pyr) Concentration. The Michaelis constants were obtained by treating the data by the method of Florini and Vestling (1957): $K_{Pyr} = 1.0 \times 10^{-4}$ M, $K_{DPNH} = 0.65 \times 10^{-5}$ M, $K_{DPNH-Pyr} = 6.5 \times 10^{-10}$ M[2]. These values are consistent with those reported by Winer and Schwert (1958) considering the differences in experimental conditions (pH, temperature, ionic strength, and choice of buffer components) under which the parameters were determined.

Variation of Enzymatic Oxidation of DPNH with the Dehydration of 2,2-Dihydroxypropionate. The rates of DPNH oxidation catalyzed by lactate dehydrogenase were followed under two sets of conditions: (i) an aqueous solution of pyruvate at neutral pH was the last component injected into the cuvette containing phosphate buffer at pH 6.8 and (ii) a preacidified pyruvic acid solution (pH 0.45) was injected to initiate the enzymatic reduction. Under these latter conditions, two processes commence at the kinetic zero; the dehydration of 2,2-dihydroxypropionate and the enzymatic reduction of the pyruvate system. Figure 1 illustrates a typical run in which the solid curve (A) demonstrates that as the dehydration of 2,2-dihydroxypropionate takes place, the rate of DPNH oxidation increases until equilibrium is reestablished between the hydrated and unhydrated forms of pyruvate. The upper dotted line (A′) is an extrapolation of the resultant linear portion of the solid line. Line A′ has a slope which was always found to be identical with that obtained from the corresponding runs in which the aqueous solution of pyruvate at neutral pH was injected as the last reaction component, ie. where the equilibrium between the hydrated and unhydrated forms at pH 6.8 had already been established. These experiments were carried out at a DPNH concentration (9.6×10^{-5} M) which was much larger than K_{DPNH} and at low enough pyruvate concentration (6.7×10^{-5} M) such that the rates were strictly first order in pyruvate.[1]

If the curvature of line A in Figure 1 directly corresponds to the formation of the preferential substrate (unhydrated pyruvate) for lactate dehydrogenase action, one would expect that its approach to linearity should follow a first-order relationship. Furthermore, the rate at which it approaches linearity should be identical with the rate at which the dehydration process approaches equilibration. This is indeed shown to be the case as illustrated in Figure 2, where the logarithms of the differences between the absorbancies associated with the two curves, log $(A' - A)_t$ are plotted against time. The slope of the resultant straight line allows the calculation of the rate

[1] It may also be calculated that under these same experimental conditions the hydration equilibration is essentially complete before more than 10% of the DPNH is oxidized.

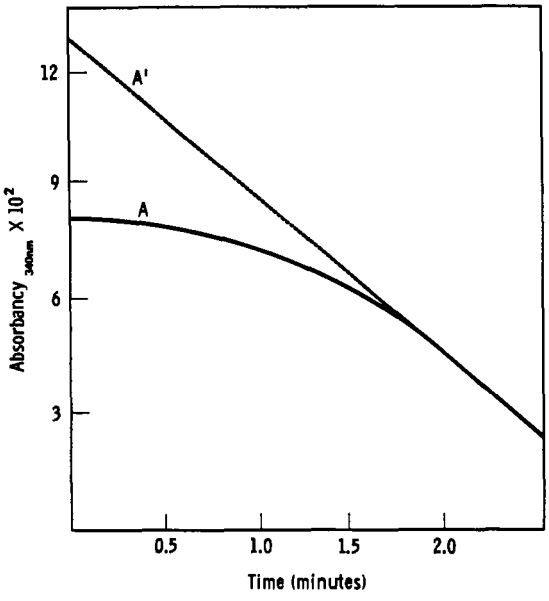

FIGURE 1: Variation in the rate of enzymatic DPNH oxidation during the progress of the dehydration of 2,2-dihydroxypropionate in 0.01 M phosphate buffer (pH 6.88) at 25.0°. Total concentration of pyruvate and hydrate was 6.7×10^{-6} M; the concentration of DPNH was 9.6×10^{-5} M.

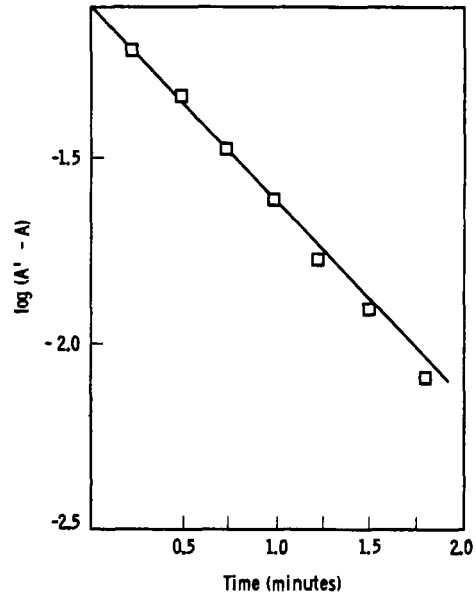

FIGURE 2: First-order rate plot of the convergence of the lower curve in 1 to linearity: $k = -2.3 \times$ slope $= 1.3$ min^{-1}.

constant for the first-order process, $k = -2.3 \times$ slope $=$ 1.3 min^{-1}. This value indeed correlates very well with the observed rate constant associated with the hydration–dehydration equilibration, $k_{obsd} = 1.4$ min^{-1} determined by direct spectrophotometric measurements as described earlier in the experimental section. Other typical results, given in Table I, show the direct correspondence between the dehydration of 2,2-dihydroxypropionate and the enzymatic oxidation of DPNH and establish that unhydrated pyruvate is the preferential substrate involved in the enzymatic process.

Although the data presented above were obtained from experiments involving beef heart lactate dehydrogenase purchased from Worthington Biochemical Corp. (see Experimental Section), similar results were observed using enzyme samples isolated from pig heart (Sigma) and rabbit muscle (Worthington) and the H_4 and M_4 isozymes (Boehringer Mannheim Corp.). Thus, for each of the lactate dehydrogenase preparations studied, unhydrated pyruvate would appear to be the preferential substrate for the enzymatically catalyzed oxidation of DPNH.

It is interesting to compare the initial and final rates of DPNH oxidation from runs such as that illustrated by the lower curve (A) in Figure 1. It should be noted that these data were obtained from runs in which the pyruvate concentration was within the range where its enzymatic reduction is first order in pyruvate. Accordingly one might expect that the ratio V_∞/V_0 would be equal to the fraction of unhydrated pyruvate after reequilibration (at pH 6.8) divided by that at the kinetic zero (corresponding to pH 0.45), 0.94/0.38 = 2.5. This is not, however, the case. Instead we observe for all such comparisons represented by Figure 1 that the ratio $V_\infty/V_0 \sim 10$, is much larger (Table I). We interpret this as an indication that the initial velocity is perhaps much slower than expected as a result of an appreciable degree of enzymatic inhibition brought about by the presence of hydrated pyruvate. Thus, this finding may suggest that for the lactate dehydrogenase catalyzed reduction of pyruvate the well-known "substrate" inhibition (Schwert and Winer, 1963) may be largely due to 2,2-dihydroxypropionate.

Finally, we compared the rate of reduction of deuterated pyruvate, $CD_3COCO_2^-$ by the enzyme to that observed for the protiated pyruvate. Using deuterated pyruvate (6.7×10^{-5} M) eight kinetic runs were made at a DPNH concentration of 9.6×10^{-5} M (pH 6.8). The results obtained were identical with those observed for the protiated pyruvate. These observations parallel the results of similar studies involving rabbit muscle lactate dehydrogenase (Fromm, 1965) and lactate dehydrogenase from *Pseudomonas* (Vennesland 1956). Thus, the conclusion of these authors that it is the keto rather than the enol form of pyruvate which acts as the preferential substrate of the enzyme can be extended to include the beef heart lactate dehydrogenase used in the present work.

Thus for the lactate dehydrogenase catalyzed reduction of the pyruvate system, it is neither a hydrated form nor an enolized form but unhydrated pyruvate which acts as the preferential substrate. Since experimental values of Michaelis constants, K_{exp}, have previously been based on the total concentration of the pyruvate–2,2-dihydroxypyruvate system, they should be corrected by multiplying by the fraction of unhydrated substrate. Although for the pyruvate system the correction is small: $K_{cor} = K_{exp}(1 - \chi) = K_{exp}$ (0.94), this type of consideration becomes important in comparative studies involving enzyme substrates whose fractions of hydra-

TABLE I: Initial and Final Velocities for the Enzymatic Reduction of Pyruvate in 0.01 M Phosphate Buffers at 25.0°.

Final pH of mixture	6.88	6.88	6.90
k (min^{-1}) (calculated from converging curves such as in Figure 3A)	1.3	1.5	1.2
V_0 (M min^{-1})	0.066	0.076	0.068
V_∞ (M min^{-1})	0.76	0.78	0.68
V_∞/V_0	12	9.8	10

tion are different (Gregory *et al.*, 1972). In this connection, one may consider the comparison of Michaelis constants for lactate dehydrogenase action on pyruvate and fluoropyruvate. Eisman *et al.* (1965) and Lee *et al.* (1965) report that the Michaelis constants for pyruvate and fluoropyruvate as substrates of lactate dehydrogenase are almost identical. It is now known through nuclear magnetic resonance measurements (J. E. Meany and J. Spaulding, unpublished results) that in contrast to pyruvate, fluoropyruvate is better than 85% hydrated at 25°. If one assumes, in analogy to pyruvate itself, that unhydrated fluoropyruvate is the preferential substrate of lactate dehydrogenase; and accordingly applies the respective correction factors $(1 - \chi)$ to the corresponding Michaelis constants, it is found that instead of being nearly identical, the complex constant for fluoropyruvate is about ten times smaller than that for pyruvate itself.

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Binding of the By-Product Analog Benzylsuccinic Acid by Carboxypeptidase A†

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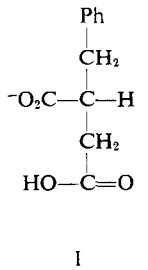
ABSTRACT: A variety of carboxylic and dicarboxylic acids were examined as inhibitors of bovine carboxypeptidase A. Of these, the most effective was 2(*R*)-benzyl-3-carboxypropionic acid (the L isomer of benzylsuccinic acid). This inhibitor appeared to be purely competitive with respect to hydrolysis of both esters and peptides, and exhibited $K_i = 4.5 (\pm 0.8) \times 10^{-7}$ M when assayed against a variety of substrates at pH 7.5 in 0.5 M NaCl at 25°. The stoichiometry, site, and pH dependence of binding were examined; the results suggest that a single molecule of a monoanionic species of L-benzylsuccinic acid is bound, and that this occurs at the active site. Binding is associated with detectable changes in ultraviolet spectra, resistance of the enzyme to various kinds of irreversible inactivation, an increase in enzyme solubility, and a change in the sites of enzyme derivatization with tetranitromethane. Kinetic analysis of enzyme inhibition by the products hippuric acid and L-phenylalanine suggests that binding of one of these products does not appreciably affect binding of the other. It is suggested that L-benzylsuccinate resembles the collected products of peptide hydrolysis, and is thus bound with an affinity resembling their combined affinity.

In a preliminary communication, we described the inhibition of bovine carboxypeptidase A by several carboxylic acids. In particular, it was noted that L-benzylsuccinic acid (I), which appears to resemble the collected substrates for the reverse reaction II, was an unusually potent inhibitor (Byers and Wolfenden, 1972).



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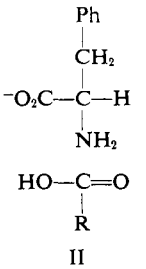

II

In this paper, the structural requirements for tight binding of carboxylic acid inhibitors of carboxypeptidase A are considered in detail. The stoichiometry, site, and pH dependence

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