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The Mechanism of the Bond Forming Events in Pyridine Nucleotide Linked Oxidoreductases. Studies with Epoxide Inhibitors of Lactic Dehydrogenase and β-Hydroxybutyrate Dehydrogenase†

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ABSTRACT: 2,3-Epoxybutyrate and 2,3-epoxypropionate act as effective competitive inhibitors of pig heart lactic dehydrogenase. $K_I$app for both inhibitors was pH dependent and varied according to the general equation $K_I\text{app} = K_I(1 + K_a/H^+)$ which may be predicted if the binding of the epoxide to the E-NADH complex involves a compulsory protonation step. Values of $K_{I(\text{epoxybutyrate})}$, $K_{I(\text{epoxypropionate})}$ and pK_a were estimated as 150 μM, 860 μM, and 6.8, respectively. The formation of an E-NADH epoxide inhibitor complex was followed directly by fluorescence measurements. Both epoxybutyrate and epoxypropionate enhanced fluorescence of the E-NADH complex and caused a 20-nm blue shift in the maximum emission wavelength. The dissociation constants measured by fluorescence titration for both epoxides increased as the pH was raised reflecting a decreased affinity for the E-NADH complex. 2,3-Epoxybutyrate was also shown to inhibit β-hydroxybutyrate dehydrogenase by a mechanism which is consistent with compulsory protonation prior to addition of the epoxide. These results are discussed in terms of a general mechanism for the bond forming events in pyridine nucleotide linked oxidoreductases.

Pyridine nucleotide oxidoreductases have long been the subject of extensive mechanistic studies and in particular a considerable amount of kinetic data have been obtained concerning the formation and dissociation of binary and ternary complexes (Holbrook and Gutfreund, 1973). Despite a detailed understanding of these phenomena for a number of oxidoreductases, little information is available on the precise nature of events involved in the transformation of substrate to product. Although the classical work of Westheimer and Vennesland (Fisher et al., 1953; Loewus et al., 1953) established that there was a direct hydride transfer between substrate and coenzyme, with the concomitant involvement of a proton to maintain stoichiometry, the precise mechanism of activation of the substrate in the ternary complex has not been determined. Studies on the pyridine nucleotide linked reductions of certain carbon–carbon double bonds (Wilton et al., 1966, 1968, Watkinson et al., 1971a,b; Akhtar et al., 1972) established that the orientation of addition of the hydride ion and proton was consistent with a Markovnikov mechanism where substrate activation is achieved by initial protonation to give a carbonium ion intermediate which is subsequently neutralized by hydride transfer (Scheme I). This mechanism of substrate activation by initial protonation has also been proposed in the case of pyridine nucleotide linked carbonyl oxidoreductases (Akhtar and Wilton, 1970, 1973; Akhtar et al., 1972).

In order to evaluate the role of enzyme mediated sub-

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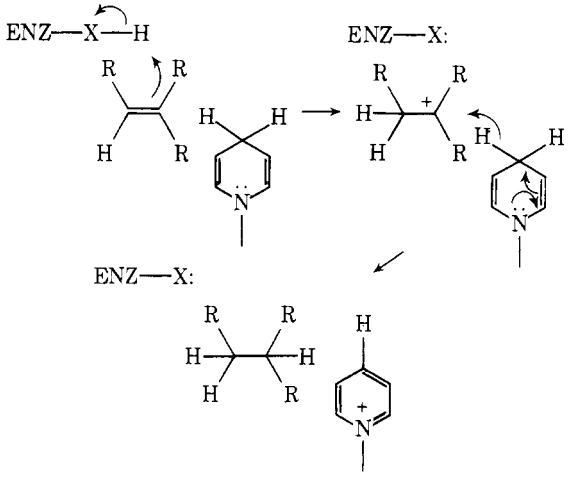
Scheme I: The Mechanism of the Reduction of Nonpolarized Carbon-Carbon Double Bonds.



strate protonation in pyridine nucleotide linked dehydrogenases we have studied the interaction of a number of substrate analogs with lactic dehydrogenase. It is concluded that the binding of the carbonyl oxygen of the substrate or inhibitor to the protonated group of the enzyme is by a hydrogen bond and the resulting partial proton transfer to substrate achieves the necessary substrate activation to allow hydride transfer from the reduced coenzyme.

Materials and Methods

2,3-Epoxybutyric acid was prepared by treating crotonic acid with H_2O_2 in the presence of sodium tungstate catalyst (Payne and Williams, 1959).

Potassium 2,3-epoxypropionate was prepared by treating β-chlorolactic acid (Sigma Chemical Co.) with alkali using a modification of the procedure described by Blau et al. (1954); 0.074 mol of KOH was dissolved in 20 ml of very dry methanol and cooled to 0°. To this was added dropwise with stirring, 0.037 mol of β-chlorolactic acid in 20 ml of very dry methanol. After the mixture was stirred for 3 hr at 0°, precipitated KCl was removed by filtration and the product was precipitated by addition of very dry ether to give the maximum yield of white granular precipitate. The product was recrystallized from very dry ether–methanol. At this point, incubation of samples of the product with lactic dehydrogenase and NADH showed that it contained a small (0.5–2%) contamination of pyruvate which arises presumably through dehydrochlorination of β-chlorolactic acid. In order to remove this contaminant, 600 mg of the product was dissolved in 10 ml of methanol and treated with 10 mg of $NaBH_4$. After 1 hr, insoluble material was removed by filtration. Addition of ether yielded 400 mg of potassium epoxypropionate which contained no detectable pyruvate when assayed with lactic dehydrogenase. Pig heart lactic dehydrogenase was purchased from Boehringer-Mannheim Co. and β-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* was obtained from The Sigma Chemical Co., St. Louis, Mo.

Lactic dehydrogenase crystals in ammonium sulfate suspension were collected by centrifugation and dissolved in 0.1 *M* potassium phosphate buffer (pH 7.0) to give a stock solution at a protein concentration of 5 mg/ml. Assays of enzyme activity were performed at 24° using 0.1 μg of protein in 0.1 *M* potassium phosphate solutions (pH adjusted as required with 1 *M* KOH) containing 0.2 m*M* NADH and variable concentrations of pyruvate and inhibitor as required. With lactate as a substrate, 0.2 m*M* NAD^+ was used as coenzyme. Initial reaction velocities were recorded by measuring the optical density change at 340 nm. When assayed at 24° in 0.1 *M* potassium phosphate (pH 7.0), the maximal reaction velocity corresponded to 370 IU/mg which agrees closely with the estimate of Stinson and Holbrook (1973) on pig heart lactic dehydrogenase. On this basis it was assumed that for the fluorescence experiments the stock solution of enzyme had an active site concentration of 140 μ*M* based on a protomer molecular weight of 36,000 (Everse and Kaplan, 1973).

β-Hydroxybutyrate dehydrogenase was assayed at 24° in 0.1 *M* Tris-HCl solutions containing 0.01 IU of enzyme, 0.2 m*M* NADH, and varying concentrations of acetoacetate. For analysis of kinetic data, an initial check was made that double reciprocal plots were linear. Then the data were fitted to the Michaelis–Menten equation using an unweighted least-squares hyperbolic regression analysis (Wilkinson, 1961). After analysis of the slopes and intercepts as a function of inhibitor concentration the data were fitted to the equations for either linear competitive inhibition or for linear noncompetitive inhibition. The data output included the $K_I$app, $K_m$app, and $V_m$app. The programs used, based on those of Cleland (1967), were written in Focal-12 and run on a Digital Equipment Corporation PDP-12 computer. For the epoxide inhibitors used in the present work the error variance was least when the experimental data were fitted to the linear competitive inhibition model.

Fluorescence titrations with lactic dehydrogenase were performed using an Aminco Bowman ratio spectrophotofluorimeter with an excitation wavelength of 340 nm and an emission wavelength of 420 nm. Titrations were performed manually by the successive addition of small volumes of a concentrated solution of the ligand to a solution of 7 μ*M* in active sites of lactic dehydrogenase and 3.5 μ*M* NADH in 1.0 ml of 0.1 *M* potassium phosphate at the appropriate pH. A correction was made for the dilution effect of the ligand during titration. Dissociation constants and the maximum fluorescence change (F_{max}) were also calculated by an unweighted least-squares hyperbolic regression analysis of the data for change in relative fluorescent intensity with ligand concentration.

Results

The lactic dehydrogenase catalyzed reaction proceeds through a sequential mechanism in which bonding of the nicotinamide adenine dinucleotide to the enzyme precedes the binding of the second substrate (Winer and Schwert, 1958; Novoa et al., 1959; Novoa and Schwert, 1961). Demonstration of the existence and the rate of formation of individual complexes in the reaction sequence has been the subject of extensive investigation by rapid reaction techniques (Holbrook and Gutfreund, 1973; Sudi, 1974). However, the rate of addition of both pyruvate and H^+ to the E-NADH complex is so fast that the order of binding of these components has not been determined. It is our opinion that mechanistic considerations, as presented in the introduction, predict that the enzyme functional group involved in binding the substrate carbonyl must always be present in the protonated form. We felt that further evidence for this concept might be obtained from the use of substrate analogs of pyruvate.

Initially, a variety of compounds were tested as inhibitors of lactate dehydrogenase. Methacrylic acid (100 m*M*), propionic acid (100 m*M*), and cyclopropanecarboxylic acid

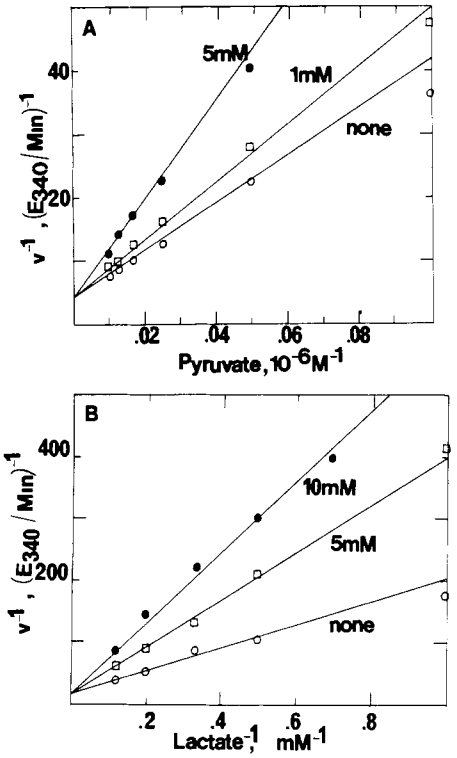


FIGURE 1: Inhibition of lactic dehydrogenase by 2,3-epoxybutyrate. Lactic dehydrogenase was assayed at pH 8.0 using either pyruvate and NADH (A) or lactate and NAD^+ (B) in the presence of the indicated concentrations of 2,3-epoxybutyrate. The lines indicate the calculated result for competitive inhibition with respect to either pyruvate ($K_{pyruvate}$app, 86 μM; $K_{I(epoxybutyrate)}$app, 4.62 mM; V_m, 370 μmol per min per mg of protein) or lactate ($K_{lactate}$app, 2.47 mM; $K_{I(epoxybutyrate)}$app, 4.97 mM; V_m, 116 μmol per min per mg of protein).

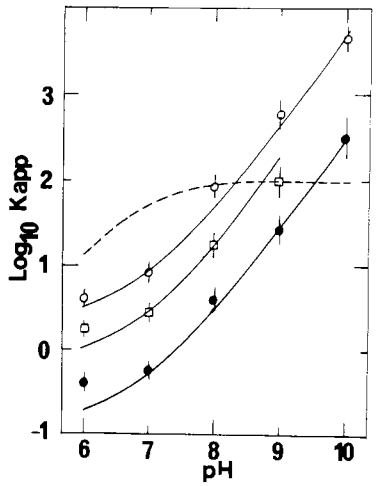


FIGURE 2: Influence of pH on the apparent dissociation constants of epoxybutyrate and epoxypropionate for lactate dehydrogenase. $K_{I(epoxybutyrate)}$app (●, mM), $K_{I(epoxypropionate)}$app (□, mM), and $K_{pyruvate}$app (○, μM) were measured at several pH values. The solid lines indicate the calculated results (eq 2) for a compulsory protonation mechanism using values of pK_a = 6.8; $K_{I(epoxybutyrate)}$ = 150 μM; $K_{I(epoxypropionate)}$ = 860 μM; $K_{m(pyruvate)}$ = 2.5 μM. The dotted line indicates the calculated result (eq 4 or 5) for the pH dependence of a dissociation constant for a compulsory substrate addition mechanism using pK_a = 6.8 and K = 100 μM (arbitrary).

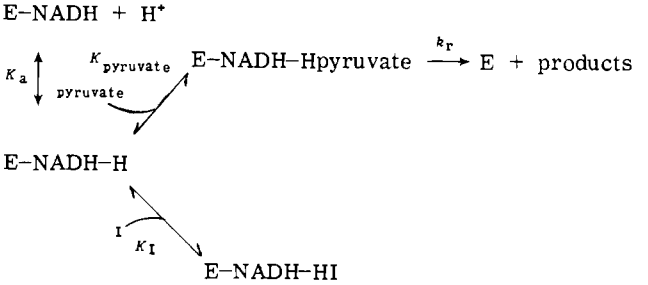
(100 mM) produced inhibition (10%) which was of the same order as acetate (100 mM). Furthermore, the small amount of inhibition observed with all these compounds was noncompetitive with respect to pyruvate. In contrast, two epoxide analogs, 2,3-epoxybutyrate and 2,3-epoxypropionate, were effective inhibitors of the enzyme. This result indicates that the ligand must contain a functional oxygen, in addition to the carboxyl, in order to bind to the E-NADH complex and strongly suggests hydrogen bond formation between the functional oxygen and an enzyme derived hydrogen from the active site histidine (Adams et al., 1970; Holbrook and Ingram, 1973). In the case that hydrogen bonding between the enzyme and functional oxygen is a vital element in binding to the E-NADH complex, then it will be expected that the epoxides should bind most effectively to the protonated E-NADH complex and that binding should show the characteristics of a compulsory protonation mechanism. Evaluating these parameters constitutes the major proportion of the subsequent experimental work.

Figure 1 shows that at pH 8.0, 2,3-epoxybutyrate acts as an apparently competitive inhibitor with respect to either pyruvate when the enzyme is assayed in the direction of oxidation of NADH or with lactate in the reverse direction. The observation that the inhibition is competitive with respect to both the inner substrate pairs of the lactic dehydrogenase reaction indicates that the epoxide binds to both the E-NADH and E-NAD^+ enzyme forms. The bonding of inhibitors to both enzyme nucleotide complexes is consistent with studies using oxamate and oxalate (Novoa et al., 1969; Winer and Schwert, 1959; Whitaker et al., 1974). The ability of these ligands to form ternary complexes with the enzyme may be determined by the protonation state of the active site histidine in addition to the nature of the coenzyme.

Epoxides have been used in a number of instances as alkylating inhibitors of enzymes (Schray et al., 1973; O'Connell and Rose, 1973). When lactic dehydrogenase was incubated in the presence or absence of NADH for periods up to 48 hr between pH 6 and 10, there was no demonstrable irreversible inhibition of the enzyme. The result is not surprising since the tertiary structure of the enzyme indicates a lack of suitable nucleophiles in the ternary complex.

It is interesting to compare the affinity of lactic dehydrogenase for both epoxybutyrate and epoxypropionate. In the α-oxo acid series, lactate dehydrogenase has a much higher affinity for pyruvate than for α-oxobutyrate (Nisselbaum et al., 1964). This result is not shared by the epoxides, where the C-4 epoxide (K_I, 150 μM; Figure 2) binds preferentially to the C-3 epoxide (K_I, 860 μM; Figure 2). We attribute this to the possibility that in the epoxide inhibitor, the entire oxiran ring acts as an analog of the carbonyl. In this case epoxybutyrate resembles pyruvate more closely than epoxypropionate. Furthermore, epoxypropionate is effectively an analog of glyoxalate which has a lower affinity for lactic dehydrogenase than pyruvate (Warren, 1970).

If the epoxides (I) bind to lactic dehydrogenase following a compulsory protonation of the enzyme, the reaction profile can be represented as



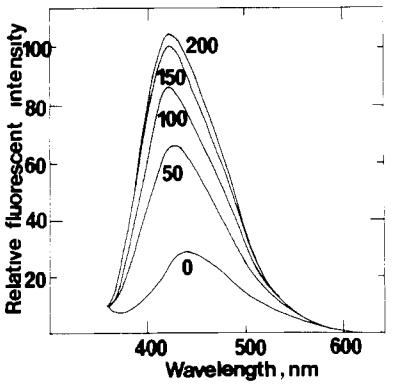


FIGURE 3: Enhancement of the lactic dehydrogenase-NADH fluorescence by 2,3-epoxybutyrate. Fluorescence emission spectra were recorded in 0.1 *M* potassium phosphate, pH 6.0, containing 7 μM active sites of lactic dehydrogenase, 3 μM NADH, and the indicated concentration (μM) of 2,3-epoxybutyrate.

The rate equation derived by steady-state kinetics will be of the form

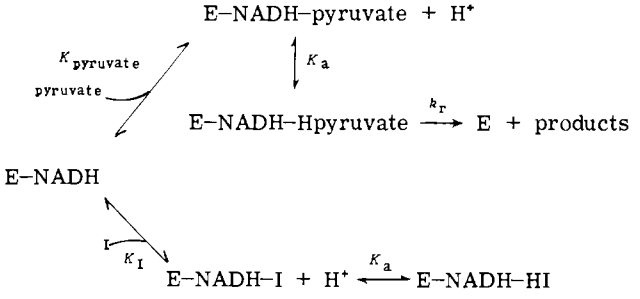
$$v = \frac{V_{m(pyruvate)}}{\text{pyruvate} + K_{pyruvate}\left(1 + \frac{K_a}{H^+} + \frac{I}{K_I}\right)} \quad (1)$$

from which it can be deduced that $K_I$app will vary as a function of pH according to

$$K_I\text{app} = K_I[1 + (K_a/H^+)] \quad (2)$$

Inspection of eq 2 shows that at a pH greater than pK_a, the $K_I$app will increase approximately tenfold for each increase of 1 pH unit. This prediction is particularly useful in establishing the role of protonation in promoting the binding of carbonyl analogs to lactic dehydrogenase. Figure 2 shows the effect of pH on a number of parameters for lactic dehydrogenase including $K_{m(pyruvate)}$app, $K_{I(epoxybutyrate)}$app, and $K_{I(epoxypropionate)}$app. It may be noted that over the range of pH studied the apparent dissociation constants for the epoxide inhibitors vary in accordance with eq 2. In both cases, pK_a may be deduced as 6.8 which agrees with previous estimates of the pK_a of the essential histidine in lactic dehydrogenase (Holbrook and Gutfreund, 1973; Holbrook and Ingram, 1973). V_m (370 μmol per min per mg of protein) was approximately constant over the pH range (Holbrook and Gutfreund, 1973).

It is worthwhile considering what the result of the pH experiment would have been if substrate or inhibitor binding to E–NADH was compulsory prior to binding of the proton. In this case the reaction profile would be of the form



If it is assumed that the protons bind to the E–NADH-pyruvate and E–NADH–I forms of the enzyme equally well, then the rate equation will be

$$v = \frac{\frac{V_{m(H^+(pyruvate))}}{H^+ + K_a}}{\text{pyruvate} + \frac{k_a k_{pyruvate}}{H^+ + K_a}\left(1 + \frac{I}{K_I}\left\{1 + \frac{H^+}{K_a}\right\}\right)} \quad (3)$$

it can be deduced that

$$K_{pyruvate}\text{app} = K_{pyruvate}\left(\frac{K_a}{K_a + H^+}\right) \quad (4)$$

$$K_I\text{app} = K_I\left(\frac{K_a}{K_a + H^+}\right) \quad (5)$$

Figure 2 shows the theoretical variation of either $K_{m(pyruvate)}$app or $K_I$app (K value set arbitrarily as 100 μM) and it is obvious that the experimental results are not consistent with these predictions. Therefore a mechanism which involves substrate binding prior to protonation is unlikely.

It is concluded that the binding of epoxide analogs to lactic dehydrogenase is consistent with compulsory protonation of the enzyme. Studies on the binding of pyruvate (Schwert et al., 1967), *o*-nitrophenylpyruvate (Holbrook and Stinson, 1973), and oxamate (Winer and Schwert, 1959; Holbrook and Stinson, 1973) also lead to the same conclusion. This binding characteristic appears to be restricted to ligands which serve as acceptors in hydrogen bond formation with the active site histidine, since the $K_{lactate}$app increases below pH 7 (Schwert et al., 1967), which is consistent with lactate binding to the deprotonated form of the enzyme.

Binding of Epoxides to the LDH–NADH Complex. The kinetic results clearly point toward the formation of an E–NADH–H epoxide complex. When NADH binds to lactic dehydrogenase there is an enhancement of the nucleotide fluorescence which is accompanied by a blue shift in the emission maximum (Winer et al., 1959). The fluorescence characteristics of this complex are modified by suitable ligands of lactic dehydrogenase and this provides a direct and convenient method of demonstrating their binding to the E–NADH complex. Figure 3 shows that when lactic dehydrogenase (7 μM in sites) is mixed with 3 μM NADH and then titrated with epoxybutyrate there is an increase in fluorescent intensity. This is accompanied by a 20-nm blue shift in the emission maximum which decreases from 439 nm in the E–NADH complex to 419 nm in the E–NADH–epoxybutyrate complex. This property is shared by epoxypropionate which increases fluorescence to approximately the same extent although it has a lower affinity for the E–NADH complex. The requirement in fluorescence enhancement for a group to enter into hydrogen bond formation is reflected by the fact that cyclopropanecarboxylic acid (50 mM) has virtually no ability to enhance fluorescence.

When the E–NADH complex of lactic dehydrogenase in 0.1 *M* potassium phosphate (pH 6.0) at 5° was titrated with 100 μM epoxybutyrate using a stopped flow spectrophotofluorimeter then the fluorescence enhancement was so fast that it was complete within the dead time of the instrument (5 msec). This would give a minimum value of 400 sec^{-1} for a pseudo-first-order rate constant for the process. A similar rate of reaction must hold in the case of oxamate binding to the E–NADH complex since this is also too fast to be followed by stopped flow kinetics (Holbrook and Gutfreund, 1973).

Although the E–NADH–epoxybutyrate complex has an enhanced fluorescence, the binding of another ligand, oxa-

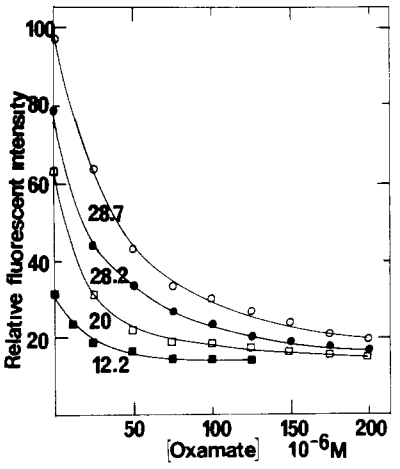


FIGURE 4: Decreased fluorescence of the E-NADH-epoxybutyrate complex in the presence of oxamate. The fluorescent complex between lactic dehydrogenase (7 μM active sites), NADH (3 μM), and epoxybutyrate was titrated with oxamate at pH 6.0. The concentrations of epoxybutyrate were: (■) none; (□) 50 μM; (●) 100 μM; (○) 200 μM. The values of $K_{oxamate}$app (μM) are shown on the curves. For each titration, the relative fluorescence of the E-NADH-oxamate complex was 10 ± 1 at infinite oxamate concentration.

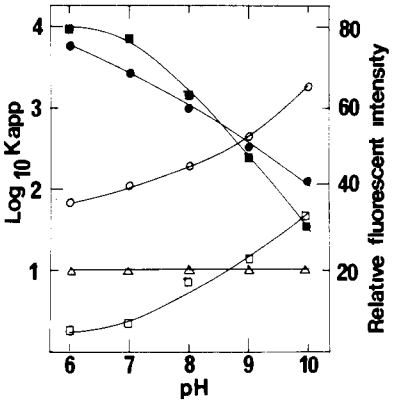


FIGURE 5: Influence of pH on the characteristics of the E_{NADH} epoxide fluorescent complex. The following parameters were measured: (○) $K_{epoxybutyrate}$app, μM; (●) F_{max} E-NADH-epoxybutyrate; (□) $K_{epoxypropionate}$app, mM; (■) F_{max} E-NADH-epoxypropionate; (△) relative fluorescent intensity of the E-NADH complex.

mate, to the E-NADH complex produces a decrease in fluorescence (Winer and Schwert, 1959). It follows that if both oxamate and epoxybutyrate bind to similar sites on the E-NADH complex, then the addition of increasing concentrations of oxamate to the E-NADH-epoxybutyrate complex should progressively displace the epoxide and result in a decrease in fluorescence. When this experiment was performed (Figure 4) it was found that oxamate did decrease the fluorescence of the E-NADH-epoxybutyrate complex and the final quenched value of fluorescence of the E-NADH complex at high oxamate concentrations was not markedly influenced by the presence of epoxybutyrate. The only effect of epoxybutyrate was to produce the expected increase in the apparent dissociation constant for oxamate. This result indicates that oxamate competitively displaces epoxybutyrate from the E-NADH complex and is consistent with the suggestion that oxamate and epoxybutyrate possess similar binding sites on the E-NADH complex. The effect of pH on $K_I$app revealed that as the pH was increased there was a marked decrease in the affinity of the epoxide inhibitors for the E-NADH complex. This can be demonstrated directly by measuring the ability of the epoxides to enhance fluorescence of the E-NADH complex over a range of pH. Figure 5 shows that the dissociation constants for both epoxybutyrate and epoxypropionate rises progressively as the pH is increased. This is accomplished by a decrease in the maximum fluorescence enhancement which is in contrast to the basal level of fluorescence which is approximately constant over the pH range studied. The variation of the apparent dissociation constant for either epoxybutyrate or epoxypropionate, as determined by fluorescence study, is only in general agreement with that predicted in eq 2 for a compulsory protonation mechanism. Thus $K_I$app does not approach ten per pH unit as predicted. A similar discrepancy has been noted for the pH dependence of the binding constant for oxamate as determined by oxamate's effect on quenching of fluorescence of the E-NADH complex (Winer and Schwert, 1959). These results presumably reflect additional complexities of the events responsible for changes in fluorescence of the E-NADH complex.

Ligands which influence the fluorescence of the E-NADH complex of lactic dehydrogenase can be divided into two classes. Thus carbonyl containing (pyruvate, *o*-nitrophenylpyruvate) or pseudocarbonyl containing (oxamate) ligands quench fluorescence (Winer and Schwert, 1959; Holbrook and Gutfreund, 1973), whereas oxalate (Winer and Schwert, 1959), epoxybutyrate, and epoxypropionate all enhance fluorescence.

It is attractive to presume that the difference in fluorescence response to the ligands is related to the mechanism of their binding to the E-NADH complex. We feel that the critical difference between the two types of ligands is that although they all have a group suitable for hydrogen bonding (giving binding to the E-NADH complex) only the carbonyl containing ligands have a polarizable group which could serve to initiate the withdrawal of electrons from the dihydronicotinamide ring which will result in the disappearance of fluorescence as the dihydronicotinamide nature of the coenzyme decreases. These concepts are represented in the general mechanism for lactic dehydrogenase presented in Scheme IV (reaction 1 and 2) of the discussion.

β-Hydroxybutyrate Dehydrogenase. The concept of compulsory protonation of the E-NADH complex has been extensively developed for lactic dehydrogenase, however, the concept of activation of the carbonyl by hydrogen bonding to a protonated group on the enzyme should be a general feature of the reaction mechanism of NADH dependent oxidoreductases. To see whether the epoxides could yield any more information on this concept, the effect of epoxybutyrate on β-hydroxybutyrate dehydrogenase was studied. Although the epoxide analogs were specifically synthesized as analogs of pyruvate, it is reasonable to suppose that epoxybutyrate should also serve as an analog of acetoacetate. Analysis showed that when β-hydroxybutyrate dehydrogenase was assayed at pH 8.0 with saturating NADH, then epoxybutyrate was competitive with respect to acetoacetate. Furthermore, both the $K_{m(acetoacetate)}$app and $K_{I(epoxybutyrate)}$app increased with raising pH (Figure 6). In both cases the changes are in general agreement with the expected values for a compulsory protonation as described by eq 2 assuming a predicted pK_a value of 8.0.

General Discussion

It has previously been established (Wilton et al., 1966, 1968; Watkinson et al., 1971a,b; Akhtar et al., 1972) that

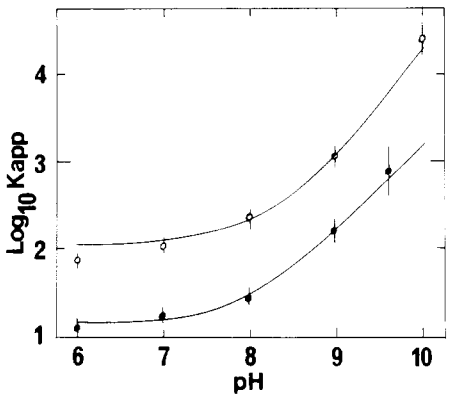
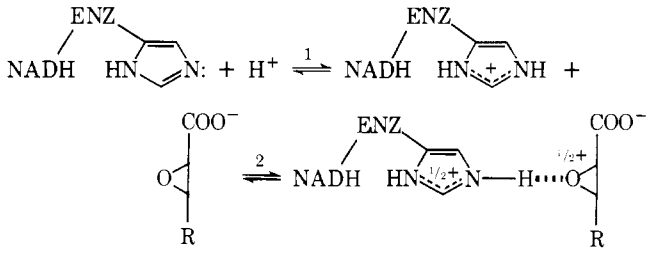


FIGURE 6: Influence of pH on $K_{I(epoxybutyrate)}$app (●, mM) and $K_{m(acetoacetate)}$app (○, μM) for β-hydroxybutyrate dehydrogenase. The solid lines indicate the calculated results (eq 2) for a compulsory protonation mechanism using values of pK_a, 8.0; $K_{I(epoxybutyrate)}$, 14.4 mM; and $K_{m(acetoacetate)}$, 110 μM.

the pyridine nucleotide linked reduction of nonpolarized carbon–carbon double bonds is achieved by a mechanism involving an initial protonation of the substrate to give a carbonium ion intermediate which is subsequently neutralized by hydride transfer from the reduced coenzyme (Scheme I). This mechanism is essentially that of Markovnikov addition. Inspection of Scheme I indicates a compulsory requirement for the protonated form of the enzyme to allow reduction to occur. It has been postulated (Akhtar and Wilton, 1971, 1973; Akhtar et al., 1972) that this mechanism may be extended to other pyridine nucleotide linked reductions including, most notably, the reduction of carbonyl groups.

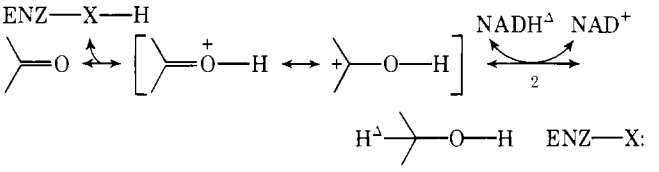
Considering the known tertiary structure of lactic dehydrogenase the most reasonable type of binding of the carbonyl oxygen to the enzyme would be to a hydrogen of the active site histidine (Adams et al., 1970; Holbrook and Ingram, 1973) via a hydrogen bond and therefore the state of protonation of this histidine should be of prime importance not only in catalysis but also in the binding of the substrate. This concept was investigated by studying the effect of pH on the affinity for lactic dehydrogenase of 2,3-epoxypropionate and 2,3-epoxybutyrate which are nonreducible analogs of pyruvate. The result clearly established a relationship between $K_I$app and pH as would be expected if the inhibitory complex (E-NADH-HI) is formed through the participation of two reactions shown by Scheme II. A decrease in pH will increase the concentration of E-NADH-H and will give a more favorable equilibrium for the binding of the inhibitor by the reaction 2 (Scheme II). At higher pH values, the concentration of the protonated enzyme will progressively decline thus requiring increased concentrations of the inhibitor to convert the enzyme into the inhibitory complex. Moreover a critical kinetic analysis of the data has established that compulsory protonation of the histidine must precede the binding of the epoxide rather than the alternative mechanism where protonation would normally follow the formation of the ternary complex.

Scheme II: Compulsory Protonation Mechanism for the Binding of Epoxide Inhibitors to Lactic Dehydrogenase.

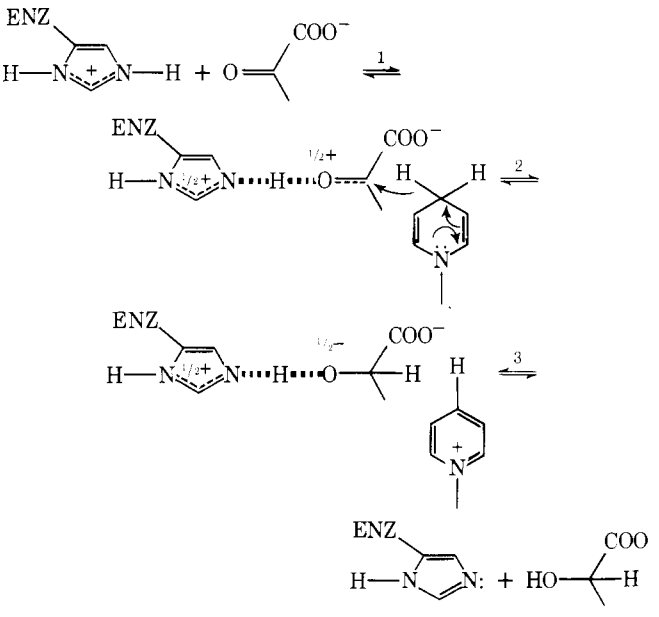


If we assume that a part of the catalytic machinery normally required for the physiological reaction is utilized in the formation of the epoxide–enzyme complex (E-NADH-HI), then it may be deduced that the binding of pyruvate to lactate dehydrogenase occurs through the formation of a hydrogen bond between the carbonyl oxygen and the protonated histidine. This will result in enhanced polarization of the carbonyl group facilitating hydride transfer from the reduced pyridine nucleotide to the now more positively charged carbon atom as shown in Scheme III. The degree of proton transfer which may be achieved in the formation of the pyruvate–lactate dehydrogenase complex raises an interesting mechanistic question. If the first step in the direction of substrate reduction involves a full proton transfer as shown in Scheme III then, in order not to violate the law of microscopic reversibility, the initial event in the back reaction must be an unfavorable abstraction of a hydride ion from a nonactivated molecule. This dilemma may be resolved by postulating that the initial step in the reduction of pyruvate involves only a partial proton transfer and that a full protonation occurs only after hydride transfer from the coenzyme, in the last step of the reaction (Scheme IV). The reversal of this mechanism will also involve a hydrogen bond between the substrate, now lactate, and the nonprotonated histidine and substrate activation is achieved by partial deprotonation of the alcohol which will facilitate hy-

Scheme III: Full Proton Transfer Mechanism for the Reduction of Carbonyl Groups.

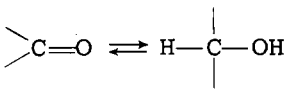


Scheme IV: Mechanism of Pyruvate Reduction by Lactic Dehydrogenase Involving Partial Proton Transfer.



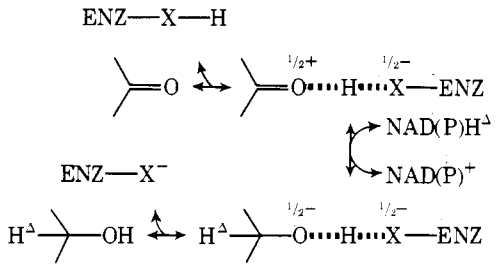
dride removal. Hence the enzyme could catalyze both forward and reverse reactions by a mechanism involving initial substrate activation by partial proton transfer due to hydrogen bond formation. It is interesting to recall that in the reduction of carbon–carbon double bonds, the first step of the proposed mechanism involved a full protonation of the substrate to give a carbonium ion (Scheme I) which is then converted into the saturated compound after hydride transfer. Since the reduction reaction (Scheme I) is irreversible, the mechanistic difficulty posed by a full proton transfer does not exist in the reduction of olefins.

The mechanism indicated in Scheme IV may have widespread relevance to the reversible interconversion



This is emphasized by a parallel study in which we have investigated the inhibitory properties of 2,3-epoxybutyrate on β-hydroxybutyrate dehydrogenase. The compound is an effective competitive inhibitor of the enzyme and there is an excellent correlation between $K_I$app and pH as would be predicted if binding required the prior compulsory protonation of the enzyme in order to allow hydrogen bond formation. The general mechanism in Scheme V previously ad-

Scheme V: General Mechanism for Pyridine Nucleotide Linked Oxidoreductases.



umbrated by us (Akhtar and Wilton, 1973) highlights the role of the enzyme in initiating the reaction so that hydride transfer occurs to or from an already activated substrate. Using other approaches, substrate activation by an enzyme mediated polarization of the carbonyl has been suggested for the reduction of acetaldehyde by yeast alcohol dehydrogenase (Klinman, 1972; Shore et al., 1974). Also in a number of studies involving the reduction of model compounds by dihydropyridine analogs hydride transfer has only been demonstrated where prior polarization of the substrate has been achieved (Pandit and Mas Cabré, 1971; Creighton and Sigman, 1971).

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