Lactic Acid Racemization in *Clostridium butylicum*. Evidence for a Direct Internal Hydride Shift*

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ABSTRACT: Kinetic and isotopic studies of the enzymatic interconversion of D- and L-lactic acids by a sonicate of *Clostridium butylicum* have led to the suggestion that a single enzyme is involved in the catalytic event and that racemization proceeds *via* a direct internal hydride shift. D- and L-α-deuteriolactic acids were enzymatically prepared. The rates of racemization of the deuterated compounds compared to the undeuterated compounds showed a marked rate effect. Evidence is also presented to substantiate the fact that the deuterium does not exchange with the medium during the course of racemization.

Dennis and Kaplan (1963) reported that the racemization of D- or L-lactic acid by a soluble sonicate from *Clostridium butylicum* did not involve two enzymes acting on a dissociable symmetrical α-carbon intermediate. Methylglyoxal or acrylate added to the reaction mixture in substrate amounts did not yield lactic acid as product. [^{14}C]Pyruvic acid did not yield [^{14}C]-lactate. The rates of racemization for the two isomers were identical, and there was no net loss of lactic acid during the reaction.

Several possible mechanisms would be consistent with the previously reported data. The "hydroxyl ion displacement" and the "direct internal hydride shift" can both account for the hypothesis that a single enzyme conducts the racemization event and no dissociable symmetrical α-carbon products of lactic acid are reaction intermediates. The "hydroxyl ion displacement" assumes that the substrate when bound to the enzyme is subjected to a back-side hydroxyl attack with inversion at the α-carbon. The enzyme presumedly facilitates an hydroxyl attack.

The second proposal involves a direct internal hydride shift from the α-carbon atom to the carboxyl carbon forming an intermediate in which the α-carbon is no longer asymmetric. Hydride return back to the α-carbon atom effects racemization.

The purpose of this paper is to show that optically pure α-deuteriolactic acids will exhibit a deuterium rate effect, indicating the breaking of a C–H bond in the rate-determining step of the racemization event. The rupture of a C–H bond during racemization is consistent with the proposal of a direct internal hydride shift.

Experimental Procedure

Materials. Nicotinamide–adenine dinucleotide (NAD[1]) sodium pyruvate, L-(+)-calcium lactate, D-(−)-calcium lactate, and glutathione (GSH) were obtained from the Sigma Chemical Co. Beef heart lactic acid dehydrogenase (BH–LDH) and yeast alcohol dehydrogenase (Y-ADH) were obtained from Worthington Biochemical Corp. CH_3CD_2OH was obtained from Merck Sharp and Dohme. 3-Acetylpyridine NAD (3-APDPN) was obtained from Pabst Labs. LiOH, Tris, and EDTA were obtained from Fisher Scientific Co. AG 50W-X8 cation exchange resin and Dowex 1-X10 anion exchange resin were obtained from Bio-Rad Labs. Bacteriological nutrients were obtained from Fisher Scientific Co.; *Clostridium butylicum* No. 860 from ATCC.

Preparation of Lactic Acid Racemase. The conditions of growth were modified from those originally reported by Dennis and Kaplan (1963). The media contained 0.5% monobasic potassium phosphate, 2% glucose, 1% tryptone, 1% yeast extract, and 2% inorganic salts (Dennis and Kaplan, 1963). The bacteria were grown for 12 hr at 37° and harvested in an RC-2 continuous flow centrifuge operating at 15,000 rpm at 0°. The cells were washed three times with acetate buffer, pH 5.0, containing 0.001 M EDTA and 0.001 M GSH. The cells were suspended in acetate buffer in a ratio of 1:4 (w/v) and sonicated with a Branson Sonifier operating at full power for 15 min. The rossette containing the cell suspension was kept in an ice bath during sonication. Cell debris was removed by centrifugation in an RC-2 centrifuge at 15,000 for 30 min at 0°. This supernatant solution contained 20 mg/ml of protein as determined

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[1] Abbreviations used in this text: NAD, nicotinamide–adenine dinucleotide; $NADH_2$, dihydronicotinamide–adenine dinucleotide; $NADH_2(D)$ 4A-deuteriodihydronicotinamide–adenine dinucleotide; BH–LDH, beef heart L-(+)-lactic acid dehydrogenase; D-LDH, D-(−)-lactic acid dehydrogenase, from *Leuconostoc mesenteroides*; Y-ADH, alcohol dehydrogenase from yeast; GSH, glutathione; 3-APDPN, 3-acetylpyridine analog of NAD.

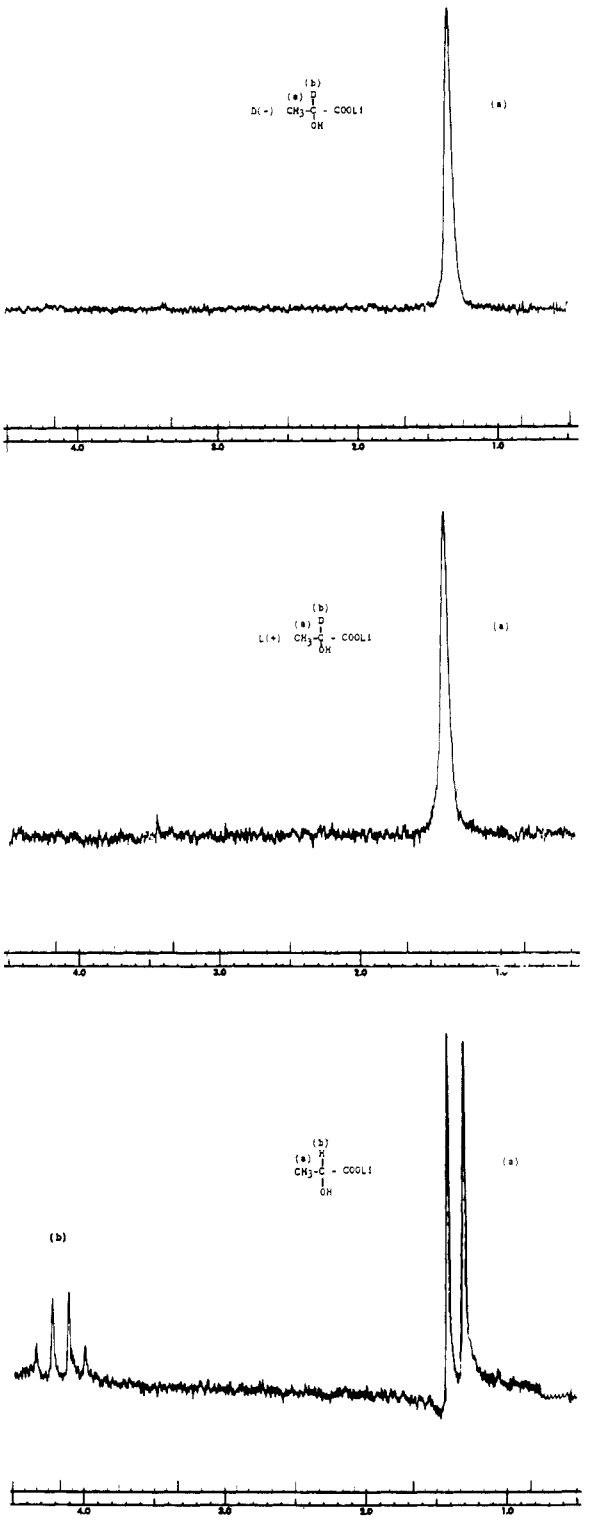


FIGURE 1: Nuclear magnetic resonance spectra of lactates. (a) D-(−)-α-Deuteriolactate, lithium salt. Concentration of lactate, 40 mg/ml in D_2O. (b) L-(+)-α-Deuteriolactate, lithium salt. Concentration of lactate, 40 mg/ml in D_2O. (c) L-(+)-Lactate, lithium salt. Concentration of lactate, 40 mg/ml in D_2O. An identical spectrum was observed with D-(−)-lactate, lithium salt.

by the method of Warburg (Warburg and Christian, 1941). The sonicate was stored at −20°.

*Preparation of α-Deuterio-*D-(−)-*lithium Lactate.* Deuterated $NADH_2$ was enzymatically prepared by the method of Rafter and Colowick (1957) employing 1 g of NAD and 0.48 g of CH_3CD_2OH. A 60% yield of $NADH_2(D)$ was obtained as the Ba^{2+} salt. The Ba^{2+} salt was converted to the Na^+ salt just prior to use. The concentration of $NADH_2(D)$ was determined by measurements at 340 mμ in pH 7 solution, using $A = 6.22 \times 10^3$. An equimolar concentration of sodium pyruvate was added to $NADH_2(D)$ in 0.002 M phosphate buffer, pH 7.5, and D-LDH from *Leuconostic mesenteroides* containing 7 mg of protein. The reaction was followed by noting the decrease in adsorbance at 340 mμ. At completion, the reaction was evaporated down to 5 ml in a Rinco evaporator and applied to an AG 50 W-X8 cation exchanger column (30 × 2.5 cm). Elution was carried out with distilled water. The eluate was then taken to near dryness and methanol was added. The precipitated nucleotides were removed by centrifugation in an RC-2 centrifuge at 10,000 rpm at 0° for 10 min. The methanol was removed in a Rinco evaporator and the oily residue was taken up in 5 ml of H_2O. The sample contained free lactic acid and trace amounts of both free pyruvic acid and nucleotides. The sample was applied to a Dowex 1-×10 formate column (32 × 2.5 cm) and eluted with a linear gradient of formic acid. The reservoir contained 400 ml of 3.6 M formic acid and the mixing chamber 400 ml of H_2O (Busch *et al.*, 1952). The tubes containing lactic acid free of nucleotides and pyruvic acid were pooled. The pooled samples were placed in a Rinco evaporator to remove formic acid and H_2O. The residual lactic acid was brought up in 10 ml of H_2O and assayed with D-LDH. The D-lactic acid was titrated potentiometrically with standard LiOH. The number of milliequivalents of LiOH used was equal to the measured concentration of D-lactic acid by enzymatic assay. The lithium lactate was taken to dryness and recrystallized two times from methanol and ethyl ether.

*Preparation of α-Deuterio-*L-(+)-*lactic Acid.* The compound was made by coupling the Y-ADH, BH-LDH reactions.[2] The reaction mixture contained 6 mmoles of CH_3CD_2OH, 12 mmoles of sodium pyruvate, 0.03 mmole of NAD, 1.8 mg of Y-ADH, and 3.7 mg of BH-LDH. The reaction was stopped by heating the reaction mixture in boiling H_2O for 3 min. The precipitate was removed by centrifugation in an RC-2 centrifuge at 15,000 rpm at 0° for 15 min. The L-(+) isomer was purified by the same procedure as used for the D-(−) isomer.

Racemase Reaction. Sonicate (0.3 ml), 0.5 ml of 0.1 M acetate buffer, pH 5.0, 0.06 ml of 0.1 M GSH, 40 μmoles of lactate, and H_2O were added to a total volume of 2.0 ml. The reaction was started by addition of enzyme and maintained at 37°. Kinetic studies were ac-

[2] The authors wish to thank Dr. R. Abeles of Brandeis University for suggesting the coupling of the two enzymatic reactions.

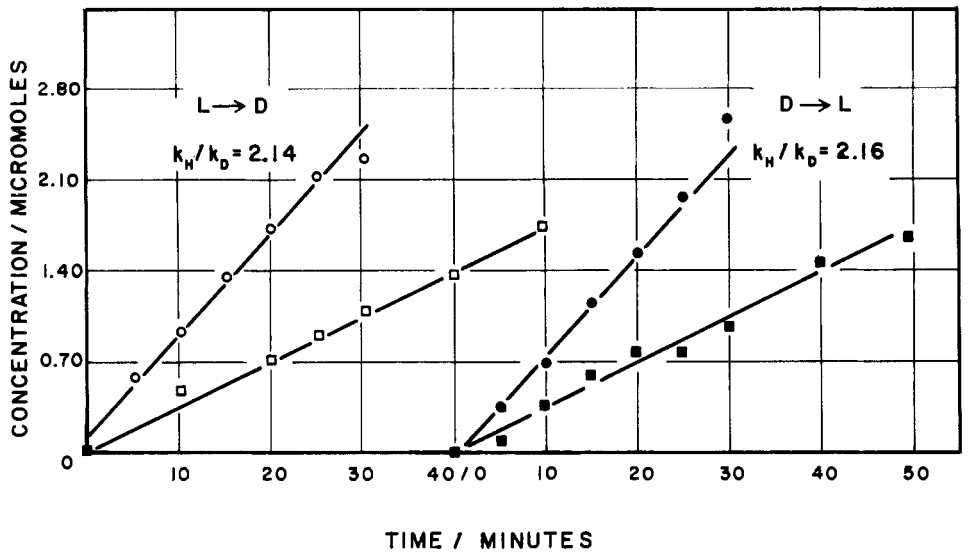


FIGURE 2: Deuterium isotope effect exhibited by lactic acid racemase. (a) Racemization of L-(+)-lactate: ○, production of D-(−)-lactate employing L-(+)-lactate as substrate; □, production of D-(−)-lactate employing α-deuterio-L-(+)-lactate as substrate. (b) Racemization of D-(−)-lactate: ●, production of L-(+)-lactate employing D-(−)-lactate as substrate; ■, production of L-(+)-lactate employing α-deuterio-D-(−)-lactate as substrate. Concentration = number of micromoles formed in reaction mixture. Components of racemase reactions are given in Experimental Procedure.

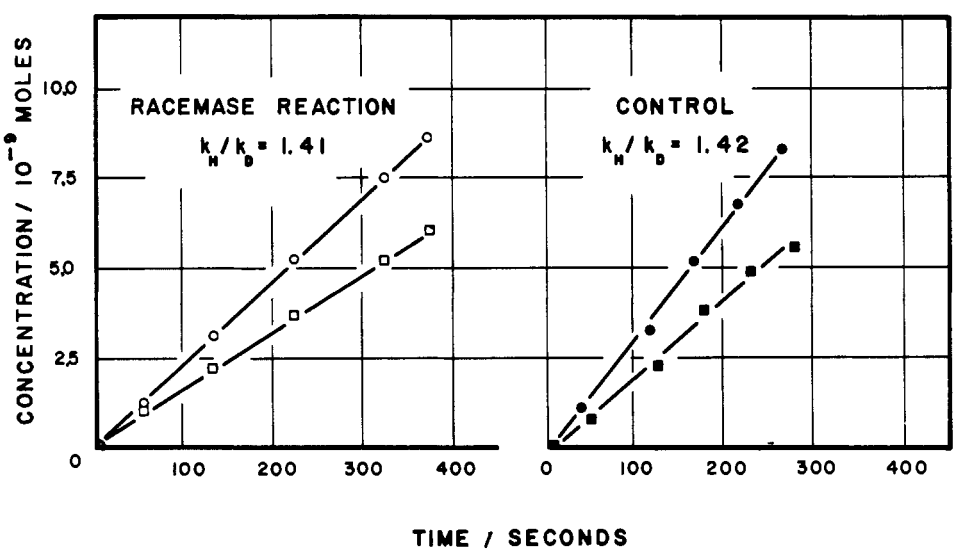


FIGURE 3: Utilization of BHLDH to show nonexchange of hydrogen atom during racemization event. (a) Rates of L-(+)-lactate oxidation with BH–LDH: ○, unsubstituted L-(+)-lactate; □, α-deuterio-L-(+)-lactate produced by the racemization of α-deuterio-D-(−)-lactate. (b) ●, unsubstituted L-(+)-lactate; ■, enzymatically prepared α-deuterio-L-(+)-lactate. Concentration = number of moles of L-(+) oxidized in the assay system. Assay system of (a) and (b) were identical except for the concentration of BH–LDH, 0.1 ml, 0.015 M APDPN, 0.1 ml, 0.1 M Tris, pH 8.6, 0.05 μmoles of lactate, BH–LDH, H_2O for total volume of 1 ml. (a) contained 0.002 ml of BH–LDH; (b) contained 0.025 ml of BH–LDH. BH–LDH concentration was 7.5 mg/ml.

complished by withdrawing 0.2-ml aliquots and heating for 3 min in boiling H_2O. The protein was centrifuged in an RC-2 centrifuge at 10,000 rpm at 0°. Aliquots of 0.02 ml were used for assay of D- or L-lactic acid.

Assay for Isomeric Lactic Acids. The assay conditions were identical with those originally described by Dennis (1959). Essentially this method involves the use of BH–LDH for L-lactate and D-LDH from *Leuconostoc*

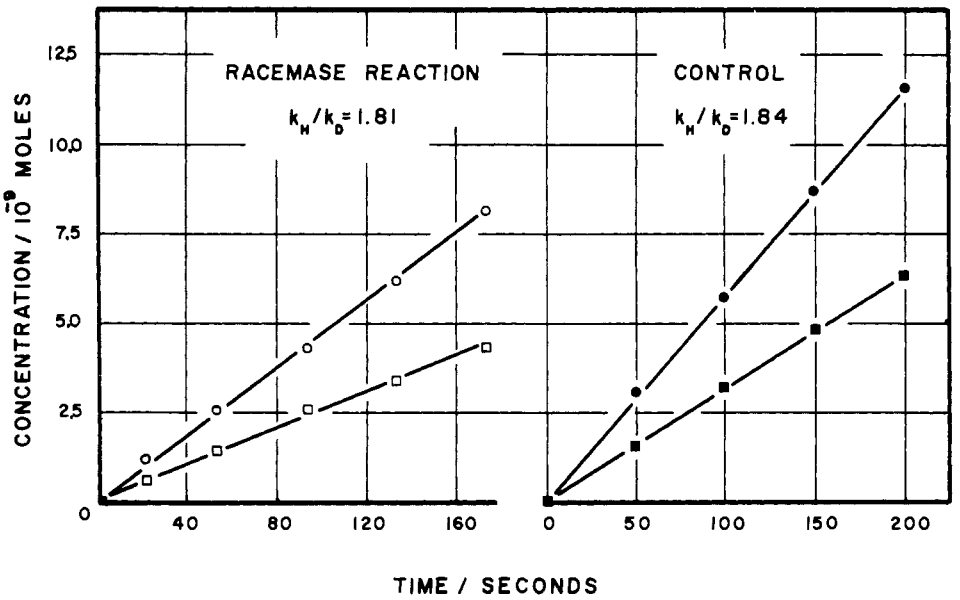


FIGURE 4: Utilization of D-LDH to show nonexchange of hydrogen atom during racemization event. (a) Rates of D-(−)-lactate oxidation with D-LDH: ○, unsubstituted D-(−)-lactate; □, deuterio-D-(−)-lactate produced by the racemization of α-deuterio-L-(+)-lactate. (b) ●, unsubstituted D-(−)-lactate; ■, enzymatically prepared α-deuterio-D-(−)-lactate. Assay system is described under Figure 3a,b. (a) and (b) contained a different number of units of D-LDH.

mesenteroides for the D-lactate. In both systems APDPN was employed rather than NAD because of the more favorable oxidation–reduction potential. Readings were taken at 363 mμ and referred to a standard curve which was linear over the range of concentrations of lactate from 0.01 to 0.10 μmole.

All spectrophotometric data were obtained with a Zeiss Model PMQ II spectrophotometer. Cells of 1-cm light path were used. pH measurements and titrations were carried out on a radiometer Model 25 pH meter.

D-LDH from *Leuconostoc mesenteroides* was prepared according to the method of Dennis (1959) without any modifications.

Results

The enzymatically prepared α-deuteriolactic acids were examined by nuclear magnetic resonance spectroscopy (see Figure 1a,b). The Li salts were dissolved in D_2O at a concentration of 40 mg/ml. The spectra for the unsubstituted compound shows the expected splitting into four peaks for the α-hydrogen, the methyl splitting into a doublet. In the case of the substituted compounds where there are no interactions between the methyl hydrogens and the α-deuterium, we observe only a single methyl hydrogen peak (see Figure 1c).

The rates of the racemase reaction were compared using four different substrates, (1) L-(+)-lithium lactate, (2) α-deuterio-L-(+)-lithium lactate, (3) D-(−)-lithium lactate, and (4) α-deuterio-D-(−)-lithium lactate. The reaction was followed for 30 min and was 14% complete in the case of the unsubstituted substrate (see Figure 2a,b). The rates of racemization for D- and L-lactate were identical. The rates of racemization for the isomeric α-deuteriolactate were essentially identical. The ratio of the rates k_H/k_D was calculated to be 2.14 for the L isomer. The ratio of the rates k_H/k_D was calculated to be 2.16 for the D isomer. The ratio is assumed to be identical, with no significance being placed on the less than 1% difference.

The BH–LDH and D-LDH enzymes used routinely for assaying either L- or D-lactic acid were also used to establish the fact that deuterium remained bound to the α-carbon atom after the racemization event. Both enzymes will catalyze the oxidation of α-deuteriolactic acid more slowly than the corresponding α-hydrogen analog. Under the assay conditions, the ratio of the rates k_H/k_D for lactate oxidation by BH–LDH is 1.42 (see Figure 3a). The ratio of the rates k_H/k_D with D-LDH is 1.86 (see Figure 4a). When a sample of L-(+)-lactate produced by the racemization reaction [which originally contained only α-deuterio-D-(−)-lactic acid] was analyzed under assay conditions, a rate effect was observed. When the rate was compared to the rate for undeuterated lithium lactate a k_H/k_D equal to 1.41 was observed (see Figure 3b), thus establishing that the deuterium was not exchanged. The same technique was used employing D-LDH and D-lactate produced from the racemization of α-deuterio-L-lactate. The k_H/k_D was equal to 1.81, again showing that there was no exchange (see Figure 4b).

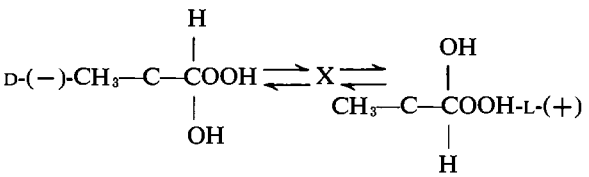
The concentration of the substituted and unsub-

stituted lactic acids were adjusted to be the same in the control assay and the assay of the products of racemization.

Discussion

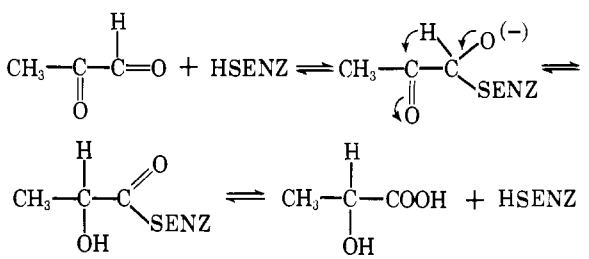
The results presented indicate that a C–H bond is ruptured during the course of racemization, and that this H is not exchanged with the medium. These observations eliminate all previously proposed mechanisms (Dennis and Kaplan, 1963) except the direct internal hydride shift.

The mechanisms which follow the general scheme:



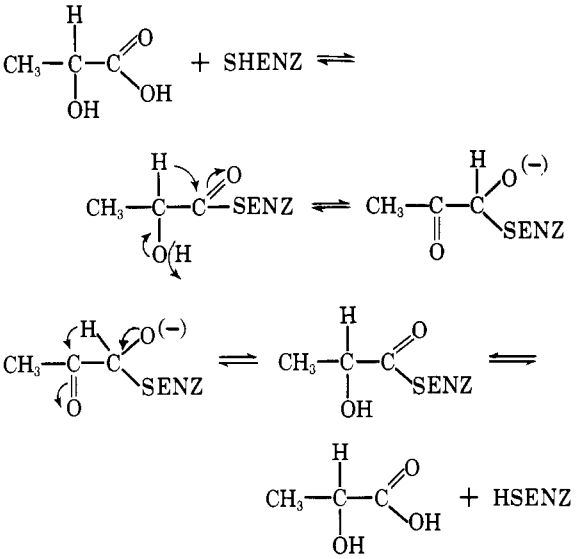
(where X = pyruvate, methylglyoxal, or acrylate) can be discarded due to the absence of hydrogen exchange. Such a system was reported in *Lactobacillus plantarum*, where X = pyruvate (Dennis and Kaplan, 1960). Any dissociable intermediate with a symmetrical α-carbon would have to exchange hydrogen with the medium. Thus the symmetrical intermediate must remain bound to the enzyme during the course of the reaction. The hydroxyl SN2 attack may also be eliminated as a possible mechanism due to the magnitude of the deuterium isotope rate effect. The possibility exists that the internal hydride shift mechanism of the racemase may be closely related to that of the glyoxylase system.

The direct internal hydride shift in the glyoxalase model system proceeds *via* a thioester intermediate (Franzen, 1955, 1956).

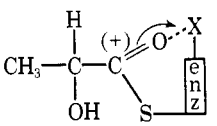


In this case a direct internal hydride transfer from a thiohemiacetal carbon to a carbonyl carbon has been demonstrated. If in the racemase system the enzyme increases the positive character on the carboxyl carbon by the formation of a thioester, we would have an analogous reaction.

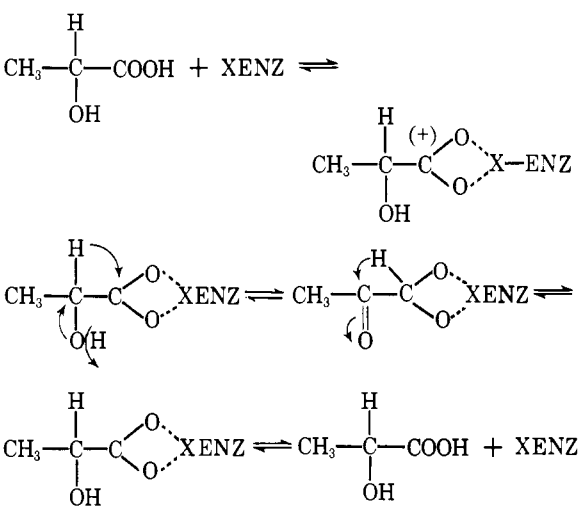
Alternatively one could consider a group on the enzyme surface that would coordinate or bind with oxygen and increase the electrophilicity of the carboxyl carbon atom. The possibility exists that the hydride shift is not dependent on a thioester intermediate, but simply involves the formation of a chelate complex.



X may be a single group or more than one functional group. At this time, the exact nature of the internal hydride shift is still speculative.



It is concluded that the enzyme does increase the electrophilicity of the carboxyl carbon atom, and a direct internal hydride transfer from the α-carbon atom to the carboxyl carbon atom is executed. The bound symmetrical intermediate possessing a carbonyl α-



carbon is now reduced by a direct hydride transfer from the reduced carboxyl atom. This event regenerates the carboxyl carbon and produces an asymmetric center at the α-carbon atom. The reduction of the symmetrical intermediate will produce either D- or L-lactic acid with equal probability. This is evident from the fact that the rates of racemization of D- or L-lactic acid are identical.

An alternate modification of our mechanistic proposal would be the involvement of a bound pyruvate–

thioester and an associate oxidation–reduction cofactor (*e.g.*, NAD), neither of which is dissociable from the enzyme surface. This proposal would require either two different reversible, direct, stereospecific hydride transfers or one reversible, direct, nonspecific hydride transfer. The direct internal hydride shift proposal is considered most likely at this time since it is analogous with the glyoxalase model system.

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The Role of the Tyrosyl Groups on the Mechanism of Action of Chicken Heart Lactic Dehydrogenase*

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ABSTRACT: About 10 of the 30 tyrosyl residues of chicken heart lactic dehydrogenase have been found free to titrat between pH 8 and pH about 11, with an average pK_{app} of 10.25. Addition of reduced diphosphopyridine nucleotide or its acetylpyridine analog shifts the pK_{app} of the free tyrosines to 10.70 and 10.90, respectively. Iodination of chicken heart lactic dehydrogenase causes a loss of enzymatic activity which is practically complete when about 4 moles of diiodotyrosine and 1–2 moles of monoiodotyrosine have been formed per mole of chicken heart lactic dehydrogenase. Reduced diphosphopyridine nucleotide seems to protect chicken heart lactic dehydrogenase from loss of activity due to the addition of iodine. Reduced diphosphopyridine nucleotide does not protect the iodinated enzyme from dissociation into subunits by sodium dodecyl sulfate. The iodinated enzyme is not denatured, as shown by its normal sedimentation coefficient, specific optical rotation, and rate of binding of *p*-mercuribenzoate. Moreover tyrosine is the only amino acid residue of chicken heart lactic dehydrogenase which reacts in substantial amounts with iodine. The evidence points to the tyrosyl residues of the enzyme as forming a part of the site of binding of the coenzymes.

Evidence has been provided (Di Sabato and Kaplan, 1963) for the involvement of four sulfhydryl residues in the binding of pyridine nucleotide coenzymes to lactic dehydrogenases. In an attempt to investigate if other amino acid residues participate in this binding, a study of the free and "buried" groups of chicken heart lactic dehydrogenase (CHLDH)[1] and their relationships with the pyridine nucleotide coenzymes has been undertaken. The present paper deals with the role played by the tyrosyl residues in the mechanism of action of CHLDH.

Iodination of lactic dehydrogenase from beef heart has been attempted by Neilands (1954) and by Nygaard (1956). These authors obtained some evidence for the binding of iodine to the sulfhydryl groups of the enzyme. More recently, Caplow (1961) studied the effect of iodination of beef heart lactic dehydrogenase on the fluorescence of the enzyme and found a decreased interaction between the iodinated enzyme and acetylpyridine analog of reduced diphosphopyridine nucleotide (AcPyDPNH). Unlike the above mentioned authors, who carried out the iodination in phosphate buffer near neutrality, we carried out the iodination of CHLDH at pH 9.5 according to the general procedure outlined by Hughes and Straessle (1950) by which maximal specificity in the iodination of the tyrosyl residues should be obtained.

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[1] The following abbreviations have been used: CHLDH; chicken heart lactic dehydrogenase; DPN^+, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; AcPyDPNH, 3-acetylpyridine analog of DPNH; SDS; sodium dodecyl sulfate; PCMB, *p*-mercuribenzoate.