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Lactate Racemase. Direct Evidence for an α-Carbonyl Intermediate†

Allan Cantwell and Don Dennis*

ABSTRACT: Evidence in support of a direct internal hydride transfer mechanism for lactic acid racemization is presented. The proposed symmetrical α-carbonyl intermediate has been trapped as an enzyme-bound oxime in the presence of hydroxylamine and as a dissociable reduced derivative lactaldehyde when the reaction is conducted in the presence of sodium borohydride.

Studies in our laboratory have presented evidence in support of the hypothesis that a direct internal hydride shift is involved in the racemization of lactic acid as catalyzed by the enzyme lactate racemase (EC 5.1.2.1.) derived from *Clostridium butylicium* (Dennis and Kaplan, 1963; Shapiro and Dennis, 1965, 1966). This report describes the effect of hydroxylamine as a potent inhibitor of the racemase reaction resulting in the trapping of bound [1-^{14}C]lactate on the enzyme as an oxime. The inhibition is reversed and the [^{14}C]lactate is released upon the addition of a competing carbonyl compound, namely pyruvic acid.

The reaction intermediate involving an α-carbonyl of lactic acid can be reduced, dissociated, and isolated when borohydride reduction is performed during the reaction in the presence of lactic acid. This dissociable reduction product has been identified as lactaldehyde derived from lactic acid.

This evidence strongly supports the hypothesis that a direct internal hydride shift of the α-hydrogen of the substrate occurs with the resultant formation of a symmetrical α-carbonyl intermediate which can undergo reduction with borohydride or oxime formation upon the addition of hydroxylamine.

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Experimental Procedure

Materials. D(−)- and L(+)-lithium lactate salts (grade A) were obtained from either Calbiochem or Miles laboratories. Sodium pyruvate was the product of Sigma Chemical Co. A crystalline ammonium sulfate suspension of beef heart lactate dehydrogenase type III was obtained from Sigma Chemical Co. Tritiated sodium borohydride, D,L-sodium [1-^{14}C]lactate, sodium [2-^{14}C]pyruvate, [1-^{3}H]ethanol, and [U-^{14}C]benzoic acid were purchased from the New England Nuclear Corporation. Tritiated H_2O was the product of Packard. Aquasol liquid scintillation counting solution and low potassium content vials were obtained from the New England Nuclear Corporation. Thin layer plates (type K301 R silica gel with fluorescent indicator) were purchased from Eastman Chemical Corp. Methylglyoxal and 1,2-propanediol were purchased from Aldrich Chemical Co. and were redistilled upon receipt. Sodium borohydride was the product of Metal Hydrides Inc. Egg white lysozyme was purchased from Worthington Biochemical Corporation. Bio-Gel 150 and Sephadex G-100 resins were obtained from Bio-Rad Laboratories and Pharmacia Fine Chemicals, respectively. Acrylamide bisacrylamide, ammonium persulfate, and Coomassie Blue were the products of Canalco. Lactaldehyde was made by the method of Abeles (1959).

Methods. STANDARD RACEMASE REACTION ASSAY SYSTEM. The reaction contained 1.0 ml of 0.1 M sodium acetate buffer

TABLE I: Purification of Lactate Racemase.[a]

Preparation	Vol (ml)	Protein (mg/ml)	Act. (units/ml)	Sp Act. (units/mg)	Total Act. Recovered (%)
Lysozyme lysate	100	10.4	12.0	1.15	
pH fractionation	110	7.2	15.0	2.1	100
Bio-Gel eluent	250	0.4	6.1	15.3	91
DEAE eluent	30	0.36	31.0	86.0	57
Heat treatment	30	0.30	30.6	102.0	56
Volume reduction	2	4.0	440	110	53
Acrylamide gel electrophoresis	12.6	0.5	63	125	48

[a] One unit of enzyme catalyzes the racemization of 1.0 μmol/30 min.

(pH 5.5), 0.1 ml of 0.2 M lithium D(−)- or L(+)-lactate and 0.9 ml of distilled water containing 0.05–2.0 mg of total protein. The reaction was initiated by the addition of the racemase enzyme and was incubated at 37°. The reaction was followed by removing aliquots at various times, heat killing for 3 min at 100°, and introducing an aliquot into a standard assay system for L(+)-lactic acid. The L(+)-lactate assay system contained 0.9 ml of 0.1 M glycine buffer (pH 10.0), 0.01 ml of sample, 0.005 ml of L(+)-lactate dehydrogenase (10 mg/ml), and 0.10 ml of NAD^+ (30 mg/ml). The reaction was initiated by the addition of L(+)-lactate dehydrogenase to the 1.0-ml cuvet. The absorbance at 340 nm after 5 min was recorded and referred to a standard curve relating absorbance to L(+)-lactate concentration.

POLYACRYLAMIDE GEL ELECTROPHORESIS. Polyacrylamide gel electrophoresis was carried out using a Canalco Model 66 disc electrophoresis. The 12.5% acrylamide gel was run at pH 4.7 with β-alanine–acetic acid buffer (0.05 M) and 10 mM lactate at both electrodes. Lactate racemase was polymerized in a sample gel layered above a stacking gel. Electrophoresis was run at a constant current of 3 mA/tube for 1.0 hr prior to sample application and then developed at the same current for 3 hr. Preparative samples were recovered after electrophoresis by slicing the gels into 1-mm bands, macerating, and eluting for 1 hr with 0.1 M acetate buffer (pH 5.5) and 1.0 mM D,L-lactate. For identification of bands the gels were fixed overnight in 12% Cl_3CCOOH rinsed with water, and stained for 1 hr with 0.1% Coomassie Blue protein stain. Excess stain was removed by washing in 7% acetic acid.

THIN LAYER CHROMATOGRAPHY. The apparatus was an Eastman chromogram chamber plate. The silica gel thin layer plates were activated by preheating at 100° for 10 min. Samples of approximately 5 μl each were spotted, dried, and partitioned in a 70:20:10 or 80:10:10 benzene–chloroform–methanol solvent system. The 2,4-dinitrophenol derivatives were directly visualized by their characteristic bright yellow orange color; other compounds were developed by exposure to iodine vapors for 2 hr.

LACTATE RACEMASE PREPARATIONS. *Clostridium butylicum* (ATCC No. 14823) was supplied in 5-kg quantities as a wet cell paste by the New England Enzyme Center where it was grown under conditions prescribed by our laboratory (Dennis and Kaplan, 1963).

A suspension of these cells was subjected to lysozyme lysis, a freeze–thaw treatment, a pH fractionation, Bio-Gel P150 gel chromatography, DEAE-cellulose ion exchange chromatography, a heat treatment, and finally a polyacrylamide gel electrophoresis resulting in 48% recovery of enzyme activity, representing a 60-fold increase in specific activity. A brief summary of the purification is presented in Table I and details will be published elsewhere.

The racemase enzyme has the following physical properties: mol wt = 82,400 daltons, $pI = 5.2 \pm 0.2$, and an operative pH range of 4.5–8.5 with a peak at 5.5.

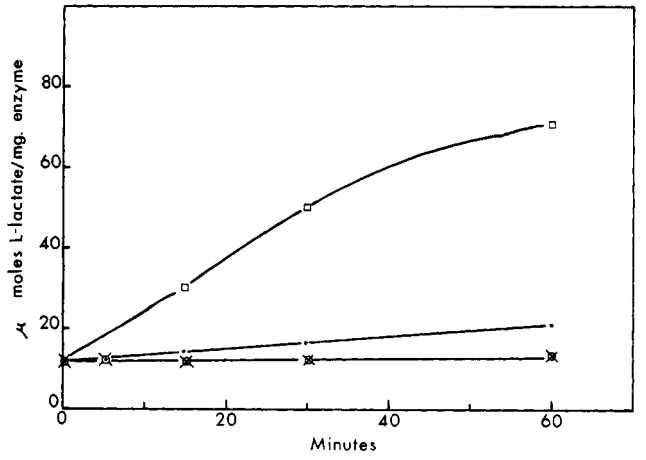


FIGURE 1: The effect of hydroxylamine on the time course of lactate racemization. Hydroxylamine at the various concentrations was added to the standard racemase reaction. The reaction was: 10 mM in D-lactate; 50 mM in acetate buffer (pH 5.4) and (□) 0 mM, (●) 0.25 mM, (○) 1 mM, (×) 2 mM in hydroxylamine. Approximately 0.2 mg of racemase was added to initiate the 1.0-ml reaction.

Results

Hydroxylamine Inhibition. The velocity of the standard racemase system was studied in the presence of several concentrations of hydroxylamine as shown in Figure 1. The racemase reaction is completely inhibited at a concentration of hydroxylamine greater than 1 mM. No detectable loss of total lactate was observed; therefore, no dissociable product was accumulated in the presence of hydroxylamine. The standard L-lactate curve was not affected by the presence of the small amounts of hydroxylamine added to the beef heart lactate dehydrogenase assay system from the racemase reaction samples.

Hydroxylamine Trapping of the Racemase-[1-^{14}C]Lactate Complex. Lactate racemase (0.2 mg) was equilibrated in 0.2 ml of 10 mM D,L-[1-^{14}C]lactate for 60 min at 37°. The mole ratio for "hot lactate" to enzyme was 100:1 and the total counts in the mixture was 2×10^8 cpm. This solution was mixed with an equal volume of 40% sucrose (to stabilize the enzyme) and layered on a column (1.0 × 6.5 cm) containing 5.0 ml of Sephadex G-25 grade fine previously equilibrated with 50 mM acetate buffer (pH 5.4). The eluent was assayed for protein and

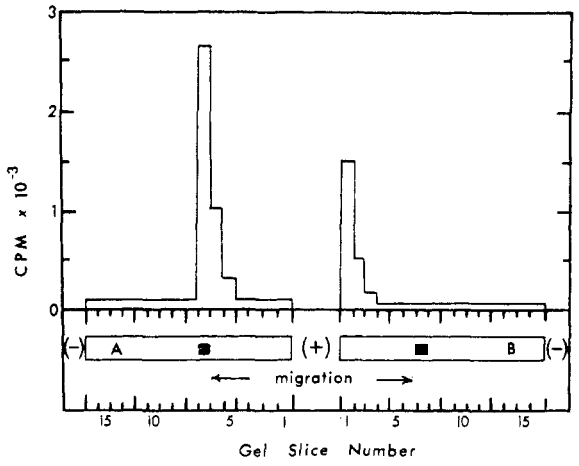


FIGURE 2: Radioactivity profile of polyacrylamide gel electrophoresis of [^{14}C]lactate-equilibrated lactate racemase. Gel A was equilibrated with 1.0 mM hydroxylamine; gel B was untreated. Gels were fixed and stained for protein revealing identical migration in both cases. Duplicate gels were sliced, hydrolyzed, and counted for ^{14}C incorporation. The results are plotted as a bar graph.

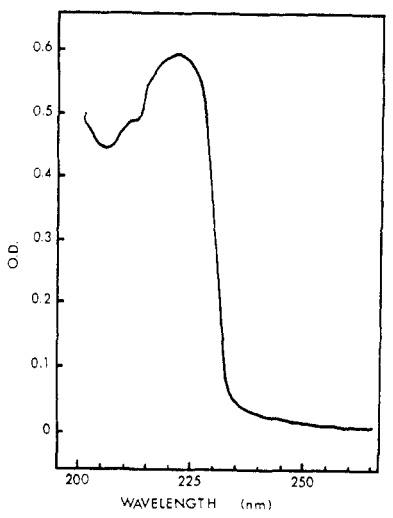


FIGURE 3: The ultraviolet difference spectrum of a hydroxylamine-treated lactate racemase–lactate sample *vs.* an untreated sample. Protein concentrations were matched at 3.8 mg/ml. The solvent was 0.01 M sodium acetate (pH 5.6).

radioactivity and a mole ratio of "hot lactate"/enzyme = 1.79 was calculated. The "desalting step" clearly did not remove the bound lactate completely, although the residual lactate present was less than that detectable in the lactate dehydrogenase assay. Half the total eluted sample (78 μg) was then cast directly into a polyacrylamide sample gel, whereas the remaining half was equilibrated in 1.0 mM hydroxylamine for 10 min prior to being cast in a similar sample gel. Both samples were then subjected to electrophoresis as described under methods for the purification of the enzyme. After the electrophoresis the gels were fixed overnight and stained. The gels were then frozen by immersion in an ethanol–Dry Ice bath and sliced into 2-mm segments along the longitudinal axis. Each segment was placed in a counting vial containing 0.5 ml of 30% H_2O_2 and incubated at 70° for 12 hr to digest the gel. Excess H_2O_2 was decomposed with 50% NaOH and the sample was neutralized with HCl. The samples were then diluted with 10 ml of aquasol counting solution and counted in a Beckmann LS-100 scintillation spectrometer using the ^{14}C channel. The results are depicted in Figure 2. In the enzyme sample treated with hydroxylamine (A) the radioactivity remained essentially associated with the racemase protein band whereas in the control (B) no radioactivity was associated with the racemase band but rather it dissociated and remained at the cathode and diffused into the buffer chamber.

Since 78 μg of protein had been applied to each gel an estimation of the moles of lactate remaining bound per mole of enzyme was made from the known specific activities of the [^{14}C]lactate (1.11×10^7 cpm/mmol) and the mol wt of the racemase (82,400 daltons). The calculated value for the mole ratio of lactate/enzyme = 0.23.

Borohydride Trapping of the Racemase–[1-^{14}C]Lactate Complex. The above procedure was repeated using tritiated-sodium borohydride as a reducing agent for the suspected carbonyl intermediate. No tritium incorporation into the racemase was observed in the electrophoresed sample. The specific activity of the borotritide was 100 mCi/g-atom of hydrogen; thus a level of incorporation of 1 g-atom of hydrogen/mol of racemase would have resulted in 1250 cpm/400-μg sample employed in the electrophoresis. The enzyme contained no detectable counts above background.

Spectral Characterization of the Hydroxylamine Trapped Intermediate. Racemase was incubated in 1.0 mM hydroxylamine at 4° for 12 hr. The reaction was desalted by passage through a Sephadex G-25 column eluted with 10 mM acetate buffer (pH 5.6). A double difference spectra of the eluted sample containing 3.8 mg of protein/ml was then taken, using as a reference the untreated racemase control at an equivalent concentration. The spectrum shown in Figure 3 exhibited an ultraviolet (uv) peak absorption at λ 223 nm. The spectrum of an authentic sample of the oxime of pyruvate was similar but not identical having a peak at λ 218 nm.

Reversal of Hydroxylamine Inhibition by Pyruvate. The time course of a standard racemase reaction which was made 1.0 mM with respect to hydroxylamine was followed for 30 min at which time a twofold molar excess of sodium pyruvate was added to the reaction mixture. The inhibition due to hydroxylamine was quickly reversed as indicated in Figure 4. A control standard racemase reaction which was made 5 mM with respect to pyruvate was also run to determine the effect of pyruvate on the racemase reaction and the beef heart lactate dehydrogenase

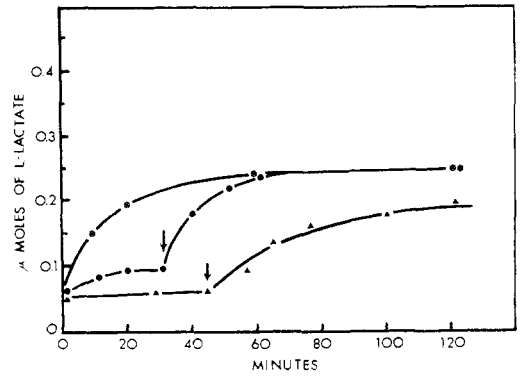


FIGURE 4: The reversal of hydroxylamine inhibition by pyruvate. The reaction system consisted of: 0.335 ml of acetate buffer (0.1 M, pH 5.4), 0.05 ml of purified racemase (100 U/mg), 0.1 ml of D-lactate (0.2 M), and 5 μmol of either 1.0 M (▲) or 0.1 M (●) hydroxylamine. The control reaction is indicated by (○). The reaction in the presence of 5 mM sodium pyruvate corrected for the effect on the lactate dehydrogenase end point standard curve is indicated by (X). The arrows indicate the value for L-lactate immediately prior to the addition of a twofold molar excess of sodium pyruvate to hydroxylamine.

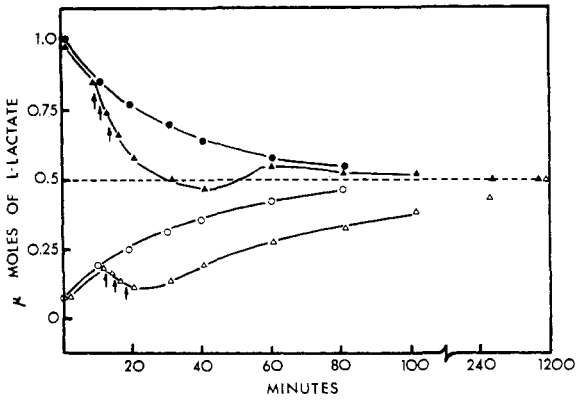


FIGURE 5: A comparison of the time course of lactate racemization in the presence and absence of sodium borohydride: (●) control reaction in the L- to D,L-lactate direction; (▲) borohydride additions in the L- to D,L-lactate direction; (○) control reaction in the D- to D,L-lactate direction; (△) borohydride additions in the D- to D,L-lactate direction. The reaction system consisted of: 0.02 ml of racemase (200 U/ml), 0.32 ml of Tris-acetate buffer (0.1 M, pH 7.2), 0.02 ml of D- or L-lactate (0.2 M). The experimental runs were initiated with racemase addition at room temperature. Sodium borohydride (0.2 M) in pH 10 NaOH was added in three 6-μl injections at the points indicated by arrows on the plot. The theoretical end point is indicated by the dashed line.

assay system. When the lactate dehydrogenase assay system standard curve for lactate was corrected for the effect of pyruvate on the equilibrium end point, the control racemase reaction was observed to be unaffected by the presence of pyruvate.

Borohydride Inhibition of Lactate Racemase. Sodium borohydride was investigated as a potential trapping agent for the transient carbonyl formed during the racemase reaction. When the time course of a racemase reaction was followed at pH 7.2 and at a low ratio of substrate to enzyme an anomalous loss of L(+)-lactate concentration was noted after the addition of the inhibitor, sodium borohydride. In Figure 5 the results are shown for a racemization proceeding in the direction D → D,L where either the appearance or disappearance of L(+)-lactate was monitored. After the interruption in the rate profile caused by the addition of 1 mol of borohydride/mol of substrate there was a slow recovery of total lactate such that a 10-hr sample had the expected end point and D/L ratio.

Isolation of the Borohydride-Reduced Racemase Intermediate Derived from [^{14}C]Lactate. The standard racemase reaction was conducted at pH 7.2 in the presence of D,L-[1-^{14}C]lactate and sodium borohydride. The reaction was terminated by the addition of 12% $ZnSO_4$ after 35 min at which time the total lactate concentration was at a minimum. The mixture was centrifuged and the supernatent extracted into ethyl ether and chromatographed on thin layer chromatography (tlc). Similar samples derived from reaction controls (*e.g.*, no borohydride, heat-killed racemase), authentic samples of lactaldehyde, 1,2-propanediol, and methylglyoxal were also examined in the thin layer chromatography (tlc) system. The results shown in Table II clearly identify the reduced transient intermediate as lactaldehyde. The complete reaction also contained a faint radioactive spot with an R_F corresponding to methylglyoxal. The reaction mixture spot with an R_F corresponding to lactaldehyde derived from [^{14}C]lactate was recovered in an H_2O wash of the spot scraped from the tlc plate. The control reactions showed only a radioactive spot near the origin corresponding to [^{14}C]lactate.

TABLE II: Identification of Borohydride-Reduced Intermediate by Tlc on Silica Gel.

Sample	R_F of ^{14}C
Racemase reaction[a]	0
Racemase reaction + BH_4^- [a]	(0.90) (0.68 faint)
Heat-killed racemase reaction + BH_4[a]	0
Lactaldehyde	(0.90)
Propanediol	(0.50)
Methylglyoxal	(0.68)
Lactate	0

[a] The substrate was D,L-[1-^{14}C]lactate.

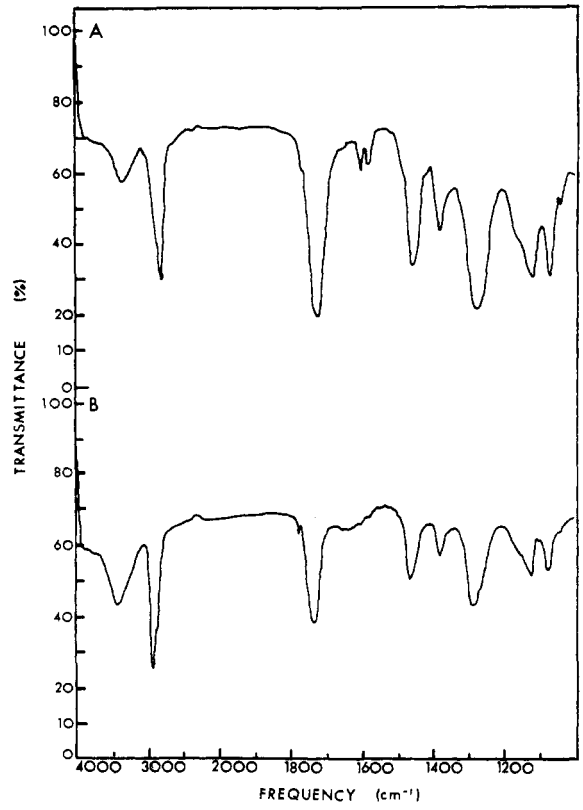


FIGURE 6: (A) The infrared spectra of lactaldehyde synthesized by the method of Abeles (1959). (B) The infrared spectrum of the product isolated from a borohydride-reduced lactate racemase reaction. The samples were run "neat," as a 0.001-cm layer between NaCl plates.

Identification of Lactaldehyde as the Borohydride-Reduced Racemase Intermediate. The 2,4-dinitrophenol derivative of the ether extract was made and compared to an authentic sample of the 2,4-dinitrophenol derivative of lactaldehyde. The 2,4-dinitrophenol derivative of the ether extract had a mp of 155.2–157.5°, whereas the authentic 2,4-dinitrophenol derivative of lactaldehyde had a melting point of 156.6–157°.

A portion of the ether extract dried over P_2O_5 and paraffin shavings along with an ether extract of authentic lactaldehyde treated in a similar manner were compared "neat" in NaCl blocks scanned on an infrared (ir) spectrophotometer. The ir spectra are shown in Figure 6. The spectra are essentially identical at all absorption wavelengths examined.

Quantitative Analysis of Lactaldehyde after Reduction to Propanediol. The total volume of ether extract was added to 0.2 ml of 0.1 M sodium borohydride (pH 10.0) and incubated for 1.0 hr at room temperature and then assayed for 1,2-propane-

diol in a spectrophotometric assay at λ 595 nm. Absorbance was referred to a standard curve (linear for 5–50 μg) and a yield of 465 μg of 1,2-propanediol was determined. This corresponded to 6.2 μmol of lactaldehyde/ml of reaction mixture assuming that complete reduction to the diol had occurred upon borohydride reduction. The total D-lactate produced from L-lactate in 35 min in the control racemase reaction was 8.2 μmol/ml; therefore, the rate of lactaldehyde formation was comparable to the rate of racemization.

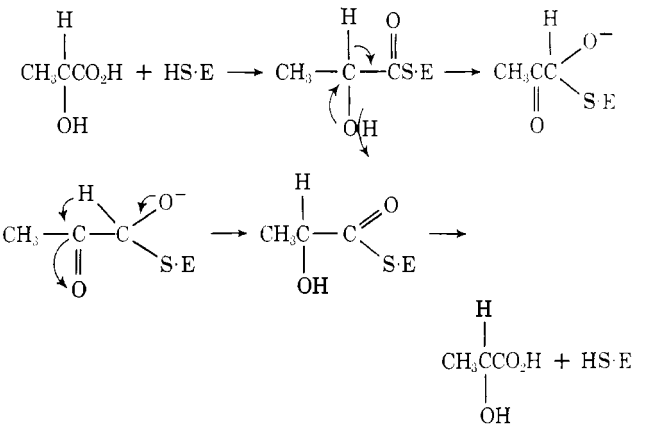
Discussion

The mechanism of lactate racemase is suggested to involve an enzyme-bound symmetrical α-carbonyl as an obligatory intermediate. Previous information in support of this suggestion may be summarized as follows. The breaking of the α-hydrogen C_2 bound occurs in the rate-determining step (k_H/k_D = 2.14) and this hydrogen does not exchange with the media during the course of the reaction (Shapiro and Dennis, 1965). The α oxygen does not exchange with the media during the course of the reaction (Shapiro and Dennis, 1966). The velocities of the reaction D → D,L and L → D,L are identical (Dennis and Kaplan, 1963). Neither pyruvic acid, methylglyoxal, or acrylic acid is converted to lactic acid by the enzyme (Dennis and Kaplan, 1963). The inhibition of racemase by hydroxylamine and the trapping of ^{14}C-labeled lactate on the enzyme by this carbonyl reagent constitutes strong evidence for the existence of a carbonyl as an obligatory intermediate during the course of the reaction. The accumulation of lactaldehyde as a dissociable borohydride reduction product of the lactate–enzyme complex confirms the existence of the carbonyl as well as establishing the location at the α position of the lactate. Scheme I is our proposal for the racemization event, where E is the enzyme.

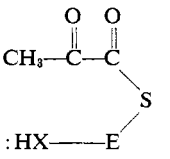
It is suggested that the enzyme increases the electrophilicity of the carboxyl carbon in order to facilitate a direct internal hydride shift; however the role of the sulfhydryl in this event is speculative.

A previous alternative involving a bound pyruvyl thio ester

SCHEME I



resulting from a hydride transfer from the α hydrogen to an enzyme-bound acceptor (*e.g.*, NAD^+) was considered.



This "caged hydride transfer mechanism" cannot be ruled out since the borohydride reduction product lactaldehyde could have been formed from this intermediate, if one considers that the intermediate (:HX—E–) could reduce the thio ester concomitant with the $NaBH_4$ reduction of the α-carbonyl.

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