Abstract: This paper reports kinetic parameters \((K_m, k_{cat})\) for reduction of approximately 20 \(\alpha\)-keto acids by \(t-LDH\) (EC 1.1.1.27) from 5 sources (porcine heart, rabbit muscle, chicken liver, bovine heart, lobster tail). The \(t-LDH\)-catalyzed reduction reaction of four substrates representative of the range of activities observed has been carried out on a preparative scale using rabbit muscle \(t-LDH\), and absolute configurations and values of enantiomeric excess (ee) of the products have been determined: 2-hydroxybutanoic acid, ee >99%, S; 2-hydroxypentanoic acid, ee >99%, S; cyclopropaneglycolic acid, ee >99%, S; 3-phenyllactic acid, ee >99%. This enzyme-catalyzed reduction provides a practical method for preparing 1-25-g quantities of a range of 2-hydroxy acids with high ee. To illustrate the value of these compounds as chiral synthons, (S)-2-hydroxybutanoic acid was converted to (S)-1-butenoxime having >98% ee on a 6-g scale.

Transformations based on enzymatic catalysis are providing an increasingly valuable component of the methodology of enantioselective synthesis. Some enzymes [for example, porcine pancreatic lipase (EC 3.1.1.3), porcine liver dehydrogenase (EC 1.1.1.1), and horse liver alcohol dehydrogenase (EC 1.1.1.1)] used in recent synthetic applications have broad substrate specificity but show variable enantioselectivity; others [for example, furmarase (EC 4.2.1.2), glyceraldehyde kinase (EC 2.7.1.30), and glycerol dehydrogenase (EC 1.1.1.1)] have high enantioselective but narrow substrate specificity. Only a few enzymes [for example, acylase (EC 3.5.1.1)] simultaneously possess broad substrate specificity and high enantioselectivity. This paper explores the utility in organic synthesis of an enzyme \(t-LDH\) (EC 1.1.1.27) that, we believe, belongs in this third category.

1-Lactate dehydrogenase is commercially available and inexpensive (less than $10 per 1000 units). Although this enzyme has an air-sensitive thiol group, it has satisfactory stability if immobilized and protected against autooxidation. \(t-LDH\) catalyzes the reduction of pyruvate to \(t\)-lactate by NADH in vivo with absolute enantioselectivity. It accepts 2-oxo acids other than pyruvate, but its enantioselectivity in these reactions has not been established. The utility of this enzyme as a catalyst in organic synthesis has not been systematically examined. We have previously used \(t-LDH\) in the multigram scale synthesis of \(L-\beta\)-chlorolactic acid having >97% ee. This work convinced us that \(t-LDH\) was indeed an efficient catalyst that should be useful in the enantioselective reduction of unnatural 2-oxo acids and encouraged us to examine the substrate specificity and stereoselectivity of several commercially available \(t-LDH\) dehydrogenases.

The objectives of this work were to explore the range of substrates accepted by \(t-LDH\) at a rate sufficient in organic synthesis, to establish the absolute configuration and enantioselective excess of representative products, to determine if there are any significant differences in specificity among the enzymes from different sources, and to illustrate practical synthetic procedures using \(t-LDH\) dehydrogenase.

Homochiral 2-hydroxy acids are valuable chiral synthons. Procedures currently available for their preparations include chemical methods, fermentation, and enzymatic catalysis.

(1) This work was supported by the National Institutes of Health, Grant GM 30367.
Few of the chemical methods yield products having high values of ee; fermentation methods (almost certainly utilizing lactate dehydrogenase in vivo) can give good values of ee\(^{20}\) but suffer from low concentrations of product and difficult isolation. Enzymatic methods are relatively straightforward for this application.

L-Lactate dehydrogenase is found in all higher organisms. It is a tetramer with a molecular weight of ca. 140000.\(^{22}\) The amino acid sequences\(^{23}\) and crystal structures\(^{24}\) of several lactate dehydrogenases have been partially defined.\(^{25}\) The tetrameric assembly is composed of subunits whose composition correlates with the tissue in which it is found.\(^{13}\) The homogeneous isoenzymes from animal heart have H-type subunits (H\(_+\)), whereas those from animal muscle have M-type subunits (M\(_+\)). The other, heterogeneous, isoenzymes have a mixture of both subunits (H\(_+M_+\), H\(_+M_+\), H\(_+M_+\)). The isoenzymes have been reported to show significant differences in substrate selectivity and in their sensitivity to substrate or product inhibition.\(^{13}\) H-type enzymes have higher activity toward a wider range of substrates and are more sensitive to substrate or product inhibition than M-type enzymes.\(^{13}\) Both types of homogeneous enzymes were tested in this work.

L-Lactate dehydrogenase has several characteristics useful for synthetic application. It has a high specific activity (400–1500 units/mg) and is readily immobilized. The equilibrium constant of the L-LDH-catalyzed reaction of pyruvate greatly favors the reduction;\(^{26}\) \(K_{\text{eq}} = 10^{11}\) M\(^{-1}\) (eq 1; \(K_{\text{eq}} = 10^4\)). The supporting techniques for the synthetic use of L-LDH, including immobilization\(^{27}\) and in situ regeneration of NADH,\(^{28}\) are well developed.

**Results**

**Kinetics Analysis.** Five l-lactate dehydrogenases (from rabbit muscle, porcine heart, bovine heart, chicken liver, and lobster tail) were surveyed with several 2-oxo acids in 30 mM phosphate buffer at pH 7.2 and 25 °C in the direction of reduction (Table I). Figure 1 shows representative kinetic data. The relative values of \(k_{\text{cat}}\) of bovine heart enzyme cited from the studies of others were obtained, with the exception of fluoropyruvate, by using the reactivity of the substrate measured at a single concentration (1.7 or 3.3 mM).

**Substrate Specificity.** The most important observation from Table I is that a variety of compounds are substrates for L-LDH. Because L-LDH is inexpensive, even substrates showing reactivity as low as 0.1\% of that of pyruvate can be considered for practi-

\[
\text{CH}_3\text{COOH} + \text{NADH} + H^+ \xrightarrow{L-LDH} \text{CH}_3\text{OH} + \text{COOH} + \text{NAD}^+\quad (1)
\]

![Figure 1. Eadie-Hofstee plots for reduction of several 2-oxo acids by NADH catalyzed by L-lactate dehydrogenase from porcine heart. The compounds were pyruvate (O), 2-oxopentanoate (A), fluoropyruvate (O), and chloropyruvate (O).](image)

![Figure 2. Structural characteristics of substrates that are accepted by L-lactate dehydrogenase. Values of \(k_{\text{cat}}\) are relative to that of pyruvate.](image)

![Scheme I. L-LDH-Catalyzed Synthesis of (S)-2-Hydroxy Acids](image)
usually run in concentrated (i.e., \( f_{\text{max}} \)) conditions, and rates are determined by \( k_{\text{cat}} \).

**Syntheses of (S)-2-Hydroxy Acids Using t-LDH.** As substrates for illustrative practical-scale syntheses, we selected 2-oxobutanolic acid (3), 2-oxopentanoic acid (4), cyclopropaneglyoxylic acid (7), and phenylpyruvic acid (20). Rabbit muscle t-lactate dehydrogenase was chosen as the catalyst, and formate/formate hydrogenase was chosen as the NADH-regenerating system (Scheme I). The enzymes (t-LDH, FDH) were immobilized in polyacrylamide (PAN) gel. All reactions were carried out by a similar procedure. A 1-equiv aliquot of substrate was allowed to react with 0.005–0.007 equiv of NADH in the presence of a slight excess of formate. The solution was kept in the presence of 1 mM mercaptoethanol, and air was excluded from it. The progress of reaction was followed by observing the amount of HCl solution required to keep the solution between pH 7.4 and 7.6; typical data are summarized in Figure 3. The reaction was stopped when the theoretical amount of HCl solution had been added, the enzyme-containing gels were removed, the aqueous solution was concentrated and acidified, and the products were extracted with ether. The products were almost pure on the basis of their \(^1H\) NMR spectra and did not require further purification. The enzymes recovered from the first run were reused for the next reaction. This recycling was repeated 5 times to prepare 4 different products in 2–25-g quantities. The enzymes recovered after five cycles still possessed more than half of the initial activities. The results from these syntheses are summarized in Table II. The yields ranged from 94 to 99%.

![Figure 3](image-url)  
**Figure 3.** Reaction progression for the t-LDH-catalyzed reductions of 2-oxo acids (RCOCOOH) to (S)-2-hydroxy acids (RCH(OH)COOH): (a) \( R = \mathrm{C}_2 \mathrm{H}_5 \mathrm{CH}_2 \), run 1; (b) \( R = \mathrm{CH}_3 \mathrm{CH}_2 \mathrm{CH}_2 \), run 2; (c) \( R = \mathrm{CH}_3 \mathrm{CH}_2 \mathrm{CH}_2 \), run 3; (d) \( R = \mathrm{CH}_3 \mathrm{CH}_2 \mathrm{CH}_2 \), run 4; (e) \( R = \mathrm{C}_2 \mathrm{H}_5 \mathrm{CH}_2 \), run 5.

**Determination of Absolute Configuration and Enantiomeric Purity.** We assigned the absolute configuration of the enzymatically reduced products (25–28) on the basis of the analysis of \(^1H\) NMR spectra of the (R)-(+) -MTPA derivatives. We
correlated the $^1$H NMR chemical shifts for one to three characteristic protons of each MTPA derivative with its configuration based on the correlation scheme described by Yamaguchi. These correlations suggest that all of the enzymatically reduced products have an S configuration (Table II), as expected from the specificity of t-LDH for (S)-2-lactate.

We determined the values of ee by carefully adding 1-33% of the MTPA ester of the racemic 2-hydroxy acid to the MTPA ester of the enzymatically reduced product (Figure 4). With careful calibration, we were able to detect the impurity of the minor enantiomer in quantities as low as 0.5%.

The enzymatic synthesis of (S)-1-butene oxide (32) was achieved in three steps from (S)-2-hydroxybutanoic acid (Scheme II). The overall yield was 55% based on 2-oxobutanoic acid. The optical purity of the epoxide was determined by $^1$H NMR spectroscopy in the presence of Eu(hfc)$_3$ (Figure 5). With careful calibration, we could detect 1% enantiomeric impurity. We conclude that 32 has >98% ee.

### Discussion

**Range of Substrate Useful in Organic Synthesis.** Among the kinetic parameters, the relative value of $k_{cat}$ provides the best basis for the determination of substrate utility in organic synthesis. The value of $k_{cat}$ limits the fastest possible rate of reaction with a substrate showing low activity; provided that $K_m$ is not too large, it thus limits the practical scale of the reaction. The range of values of $k_{cat}$ observed in this work is approximately $10^4$. On the basis of experience with the compounds in Table II, we found that it is practical to carry out multigram (1-5-g) syntheses in convenient laboratory-scale reactions using substrates having reactivities as low as 0.1-1% that of pyruvate (Figure 2). We believe that this scale can be increased to more than 10 g without difficulty by extending the reaction time or reusing the recovered enzymes. We conclude that the substrates having reactivity >0.1% that of pyruvate can be used for >50-g-scale preparations; the substrates having reactivity between 1 and 10% that of pyruvate can be reduced on >10-g scale; the substrates having reactivity 0.1-1% that of pyruvate can be reduced without major difficulty on a multigram scale.

**Enantioselectivity.** Although t-lactate dehydrogenase generates only t-lactate in the direction of reduction, the enantioselectivity of t-LDH toward other substrates had not been established. Only one unnatural substrate, 3-chloropyruvate, has been shown to be reduced in an t-LDH-catalyzed reaction with high enantioselectivity.
Enzymic Homochiral 2-Hydroxy Acid Synthesis

Substrates listed in Table 1 are highly enantioselective toward the whole range of unnatural compounds (>97% ee). On the basis of the results described in the literature, the progress of reaction, and related matters, the enzymes used as catalysts in this work are rabbit muscle LDL and yeast formate dehydrogenase and yeast formate dehydrogenase. All are readily immobilized in polycrylamide gel (PAN), and LDL can also be used enclosed in a membrane (membrane-enclosed enzymatic catalysis, MEEC). The MEEC technique is more convenient than covalent immobilization and is especially useful when a large quantity of enzyme is required. We believe that the MEEC technique will be the method of choice for syntheses using relatively unreactive substrates and requiring large amounts of LDL. PAN-immobilized LDL will be the better choice for large-scale syntheses using highly reactive substrates and a small amount of enzymes and requiring recycling of the enzymes.

The progress of a reaction carried out using LDL can be conveniently followed titrimetrically by observing the amount of HCl solution added to keep the pH of the reaction mixture neutral. The enzymatically reduced products are stable under the reaction conditions and can be isolated in almost pure form and in excellent yield by simple extraction with ether. Formate dehydrogenase is expensive, but the cost can be reduced by recycling the recovered enzymes. The reactant and product (CO₂) used in the NADH-regenerating system do not complicate the workup of the reaction. We believe that synthetic procedures using LDL are straightforward, practical, and economical.

Experimental Section

General Procedures. LDL-lactate dehydrogenases (porcine heart, bovine heart, chicken liver, rabbit muscle, lobster tail; EC 1.1.1.27) were obtained as crystalline suspensions in ammonium sulfate solution or as lyophilized powders from Sigma. Formate dehydrogenase (yeast; EC 1.2.1.2) was obtained in lyophilized form from Boeringer Mannheim. The enzymes in lyophilized form were used as received and those in ammonium sulfate solution centrifuged before use. All enzymes used for kinetic measurements were dissolved in 30 mM phosphate buffer solution. Enzymes used as catalysts in synthesis were immobilized in PAN-1000 as listed in Table 1 and used without further purification unless otherwise indicated. Water used for workup operations was distilled twice, the second time from glass. Chemical shifts in the (3)C NMR spectra were referenced to DSS or solvent peaks. IR spectra were recorded as KBr pellets in the range 4000-400 cm⁻¹. UV spectra were recorded in a Perkin-Elmer 242 polarimeter. 1H and (3)C NMR spectra were referenced to TMS or solvent peaks and those in the literature.

Kinetic Measurements. The activities of LDL-lactate dehydrogenases were measured in the direction of reduction in the presence of NADH and various concentration of substrates, and various amounts of enzymes. The concentration of substrates increased from 0.2 to 8 mM, and the amount of enzyme was increased by a factor of 1000 in order to get a reasonable signal to noise ratio. The kinetic parameters were obtained from Eadie-Hofstee plots. The activities of formate dehydrogenases were measured in the direction of oxidation at pH 7.5 and 25 °C by following the increase in UV absorbance at 340 nm. The assay solution contained 80 mM Tris, 150 mM formate, 0.7 mM NAD, and about 0.02 units/ml of enzyme. The immobilized enzymes were assayed under the same conditions as for the soluble enzymes.

LDL-Catalyzed Synthesis of (S)-2-Hydroxy Acids. We performed five reactions with recycled enzymes to prepare four different products on a 15-150-mmol scale.

(S)-2-Hydroxybutyric Acid (25). Run 1. A 300-mmol aqueous solution containing 2-oxobutyric acid (3, 101 mmol), sodium formate (117 mmol), mercaptoethanol (0.5 mmol), and Tris (2.34 mmol) was transferred to a 1-L, three-necked flask with magnetic stirring bar, three septa, and a Fisher penile combination electrode connected to a Chemtrix pH controller. The solution was adjusted to pH 7.5 with concentrated KOH solution. NAD (0.5 mmol) and a 100-mL aqueous suspension of immobilized LDL (400 units) and FDH (40 units) were introduced into the flask. A bubbler and a nitrogen supply were attached with needles through septa to the flask. An HCl solution (2.56 N) placed in a 5-L, 10-bottle was connected (by a silicon tube through a septum to the flask) via a peristaltic pump, which was remotely controlled by the pH controller. The flask was checked for a tight seal, and then nitrogen was bubbled through the solution for 30 min to degas it. The solution was maintained under positive nitrogen pressure. The solution was kept at pH 7.4-7.6 by the controlled addition of 2.56 N HCl. The reaction was followed by observing a change in amount of HCl solution added. When the addition of HCl solution had stopped when 39.5 mL of 2.56 N HCl had been added (48 days). The enzyme-containing gel particles were isolated by centrifugation, washed twice with degassed distilled water, resuspended in cold degassed water containing 2 mM mercaptoethanol, and assayed by the UV method. The combined washes (400 mL) were acidified to pH 2.0 with 6 N HCl and extracted continuously with diethyl ether for 4 days. The ethereal phase was dried over anhydrous MgSO₄ and concentrated by rotary evaporation to give an oily residue that solidified after further drying in vacuo to yield a white solid. The solid was dried at 0.5 Torr for 10 h to yield 10.4 g (100 mmol, 99%) of 2-hydroxybutyric acid: mp 54.5-55.5 °C; δ (3)C NMR (CDCl₃) 174.96, 173.97, 67.51, 67.46, 54.5-55.5 °C: [α]D +1.150 (c 8.1, 3% CF₃Cl): mp 97-98 °C; 1H NMR (CDCl₃) δ 5.66 (br s, 2.2 H, -l.Si-OCH₂CH₃), 2.74 (2H, -CHO), 2.97 (2H, -OCH₂CH₃), 3.79 (2H, -CHO), 4.27 (2H, -OCH₂CH₃). Run 2. The second run was performed on a 150-mmol scale with the enzyme recovered from the first run. The starting materials included 150 mmol of 2-oxobutyric acid, 170 mmol of sodium formate, 0.73 mmol of mercaptoethanol, and 2.23 mmol of Tris. The overall procedure, except for the workup, was followed as described in run 1. The reaction was stopped when 59.8 mL of 2.56 N HCl had been added (42 days). The mother liquor obtained after the removal of the enzyme-containing gel particles was concentrated by rotary evaporation in vacuo to 80 mL, acidified to pH 2 with 6 N HCl, and extracted 4 times with 170-mL portions of ether. The ethereal layer was dried over anhydrous MgSO₄ and evaporated to give the crude white product. Further drying for 24 h in vacuum provided almost pure white solids (15.02 g, 144 mmol, 96%): mp and spectroscopic data were indistinguishable from those described in run 1.

(S)-2-Hydroxypropionic Acid (26). Run 3. The synthesis of 26 was performed on a 30-mmol scale using the procedure for run 2. The starting materials comprised 30 mmol of sodium formate, 30 mmol of mercaptoethanol, and 11.4 N HCl for titration. The reaction was stopped when 26.7 mL of 1.14 N HCl had been added (13 days). The products obtained as white solids weighed 3.27 g (28 mmol, 94%): mp 57.8-58.5 °C; 1H NMR (CDCl₃) δ 6.92 (4s, 2H, OH), 4.26 (4s, 2H, -CHO), 1.78 and 1.68 (2H, 2H, CHO), 1.05 (3H, 3H, Me) 3.79 (2H, 2H, -CHO), 4.27 (2H, 2H, -OCH₂CH₃), 5.66 (br s, 2H, -CHO), 2.74 (2H, 2H, -OCH₂CH₃), 3.79 (2H, 2H, -CHO), 4.27 (2H, 2H, -OCH₂CH₃), 3.79 (2H, 2H, -CHO). (S)-Cyclopropaneglycolic Acid (27). Run 4. The synthesis of 27 was performed on a 35-mmol scale using the procedure for run 3 except that potassium cyclopropaneglycolate 7 was the starting material. The reaction was stopped when 26.6 mL of 1.14 N HCl had been added (13 days). The products obtained as white solids weighed 3.35 g (29 mmol, 97%): mp 97-98.5 °C; 1H NMR (CDCl₃) δ 5.66 (br s, 2H, OH), 3.83 (br s, 2H, OH).

(33) Lane, R. S.; Dekker, E. E. Biochemistry, 1969, 8, 2958.
allowed to stir under nitrogen at room temperature for 20 h. A water-
in the dropping funnel and added dropwise over 2 h. The mixture was
solved in dry THF (80 mL) and transferred into the flask with a long
round-bottomed flask fitted with a silicon stopper, magnetic stirring bar,
the minor enantiomer could be detected.

The mixture containing 60 μL of Eu(hfc)₃ (100 mg/mL CDCl₃), 0.5 mL of CDCl₃, and
chemical shift for methoxy protons.

Determination of Enantiomeric Excess by ¹H NMR Spectroscopy.
(S)-2-Hydroxy Acids. For calibration, five samples were prepared by
mixing 0.5 mL of CDCl₃ and 15 μL of one of the following: (1) the
MTPA derivative of (S)-1-Butene Oxide; (2) the MTPA derivative of racemic 25; (3) the
characteristic protons are summarized in Table III.

Table III. ¹H NMR Chemical Shifts (ppm) for Three Characteristic
Protons from (R)-(+) MTPA Derivatives

<table>
<thead>
<tr>
<th>Acids</th>
<th>ACa</th>
<th>CH₃OC</th>
<th>CH₂O</th>
<th>CH₂CH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂CH₂(HOH)COOH</td>
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<td>3.63</td>
<td>0.87</td>
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<tr>
<td>CH₂CH₂CH₂(HOH)COOH</td>
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<td>3.55</td>
<td>0.99</td>
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<tr>
<td>(CH₂)₂CH₂(OH)COOH</td>
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<td>3.75</td>
<td>3.57</td>
<td>0.92</td>
</tr>
<tr>
<td>C₅H₅CH(OH)COOH</td>
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<td>3.54</td>
<td>0.92</td>
</tr>
<tr>
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*AC, absolute configuration.