BIOORGANIC CHEMISTRY 17, 400–409 (1989)

Lactate Dehydrogenase-Catalyzed Regeneration of NAD from NADH for Use in Enzyme-Catalyzed Synthesis

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Received October 24, 1988

Lactate dehydrogenase (LDH, rabbit muscle, EC 1.1.1.27), coupled with pyruvic and glyoxylic acids as oxidants, regenerates NAD *in situ* for enzyme-catalyzed oxidations. The use of LDH has advantages of convenience, stability of the regenerating system, and economy. Pyruvate and glyoxylate differ in their advantages and disadvantages as oxidants. Pyruvate analogs, monomethyl oxalate and *S*-methyl thiooxalate, were examined as possible oxidants but were not substrates of lactate dehydrogenase.

INTRODUCTION

We herein describe the use of L-lactate dehydrogenase (LDH,[1] rabbit muscle, EC 1.1.1.27), with pyruvic and glyoxylic acids as oxidants, to regenerate NAD for enzyme-catalyzed, preparative oxidations. NAD regeneration using LDH has advantages of convenience, stability of the regenerating enzyme and reagents, and economy. In systems not sensitive to deactivation by glyoxylate, the high redox potential of glyoxylate is an advantage.

Enzymes are useful as catalysts for organic synthesis (*1*), and methods for cofactor regeneration have made synthetic applications of even cofactor-requiring enzymes practical (*2*). Although several good to excellent systems regenerate the reduced nicotinamide cofactors, NAD(P)H (*2, 3*), methods for regenerating the oxidized cofactors, NAD(P), are less developed and are currently being investigated (*4–7*). A frequent problem of enzyme-catalyzed oxidations is not the cofactor-regenerating step but the oxidation of substrate in the synthetic step. Biochemical oxidations may suffer from unfavorable thermodynamics and noncompetitive and uncompetitive product inhibition (*4, 8*).

LDH catalyzes the completely stereoselective reduction of pyruvic acid (with concomitant oxidation of NADH) to L-lactic acid, Eq. [1], R = CH_3 (*9, 10*). LDH also accepts as substrates a variety of other α-oxo acids (*11, 12*), including glyoxylic acid, which is reduced in the presence of NADH to glycolic acid, Eq. [1], R

[1] Abbreviations used: FMN, flavin mononucleotide; G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GluDH, glutamate dehydrogenase; Gly–Gly, glycylglycine; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; HLADH, horse liver alcohol dehydrogenase; αKG, ammonium α-ketoglutarate; LDH, L-lactate dehydrogenase; Mops, 4-morpholinepropanesulfonic acid; TEA, triethanolamine.

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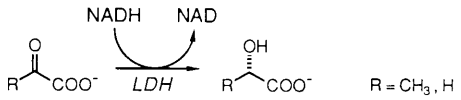
= H (*13–15*). The utility of LDH in preparative reductions, its ease of immobilization and manipulation, and its stability when protected from autoxidation have been demonstrated (*12*, *16*).

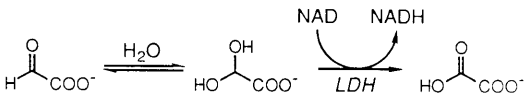


[1]

We have examined LDH as a catalyst for NAD regeneration. The two most commonly reported methods for regenerating NAD(P) use ammonium α-ketoglutarate (αKG) and glutamate dehydrogenase (GluDH, EC 1.4.1.3) (*4*, *8*, *17*) or FMN/O_2, with (*6*) or without (*4*, *18*, *19*) FMN reductase (EC 1.6.8.1) as a catalyst. LDH is less expensive and higher in specific activity than both GluDH and FMN reductase.[2] Unlike FMN/O_2, LDH permits NAD regeneration under anaerobic conditions (many enzymes are deactivated by dioxygen, superoxide, and peroxide). Pyruvic and glyoxylic acids are less expensive than α-ketoglutaric acid and FMN,[3] and glyoxylate ($E_0' = -0.90$ V[4]) is more strongly oxidizing than α-KG ($E_0' = -0.121$ V for reductive amination).

Potential obstacles to the use of pyruvate as the oxidant were the nonproductive binding of pyruvate to LDH (*20*) and the condensations of pyruvate with itself and NAD (*10*). Both Mg^{2+} and LDH catalyze the enolization of pyruvate and thus the nucleophilic addition of enol pyruvate to C-4 of NAD (*21*). Because glyoxylate cannot enolize, it should not undergo condensations as pyruvate does. Also, since glyoxylate exists in aqueous solution as the hydrate, it should be less soluble in organic solvents than pyruvate and thus more easily separated from products.

A potential problem with the use of glyoxylate, however, was the NAD-linked oxidation of glyoxylate by LDH (*14*, *22*). At high pH (pH 9), LDH oxidizes the hydrate of glyoxylate as an analog of lactate, Eq. [2]. This reaction not only consumes NAD but also generates oxalate, which is a noncompetitive inhibitor of LDH with respect to pyruvate (and presumably the oxo form of glyoxylate) (*23*).



[2]

RESULTS AND DISCUSSION

Our work began with a search for substrates of LDH other than pyruvate which would be good oxidizing agents for NAD regeneration. We measured the kinetic

[2] Cost and specific activity of LDH vs GluDH are \$0.71/1000 U and 1000 U/mg vs \$4.7/1000 U and 40 U/mg, respectively. FMN reductase (50 U/mg) requires isolation (Ref. (*9*)). Prices (Sigma, 1988) are for enzymes as research biochemicals and therefore represent upper limits. One thousand units (1 U = 1 μmol/min) is approximately the amount of enzymatic activity required to generate 1 mol of product/day.

[3] Prices of pyruvic (95%), glyoxylic (50% w/w aqueous solution), and α-ketoglutaric (95%) acids are 7.6, 3.5, and 15 \$/mol, respectively (Aldrich and ICN Biochemicals, 1988).

[4] Reduction potentials are relative to the half-reaction $2\,H^+ + 2\,e^- \rightarrow H_2$, for which $E_0' = -0.42$ V at pH 7.

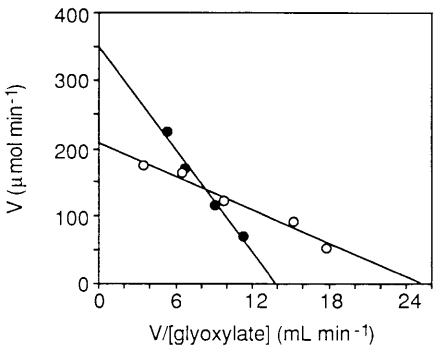


FIG. 1. Eadie–Hofstee plot used to determine K_m(app) of LDH for glyoxylate in the absence (●) and presence (○) of 1 mM sodium vanadate.

constants of LDH with glyoxylate (pH 7.0, 25°C) and found the apparent Michaelis constant,[5] K_m(app), to be 25 mM (Fig. 1). This value was in reasonable agreement with previous determinations (*13–15*) and was significantly higher than that for pyruvate (K_m = 0.15–0.33 mM) (*13, 15, 25*). The maximal velocity (V_{max}) for the reduction of glyoxylate was approximately the same as that with pyruvate and was at least 10^4 times faster than the oxidation of glyoxylate (we detected no oxidation with 50 mM glyoxylate and 2.8 mM NAD). At concentrations above 50 mM, glyoxylate inhibited its own reduction by LDH.

Since glyoxylate exists in aqueous solution as the hydrate and since vanadate has been shown to catalyze the hydration/dehydration of glyoxylate (*25*), we also measured the kinetic constants of LDH with glyoxylate in the presence of 1 mM vanadate (Fig. 1). The K_m(app) was reduced to 8 mM, but V_{max} also dropped by nearly half. In the presence of 10 mM vanadate, LDH lost all activity. We did not pursue the mechanism of this inhibition nor the further use of vanadate as a catalyst in NAD-regenerating systems.

Monomethyl oxalate (**1**) and *S*-methyl thiooxalate (**2**) were tested as pyruvate analogs, but neither was a substrate of LDH ($V < 0.005\%$ of that for pyruvate).

1 2

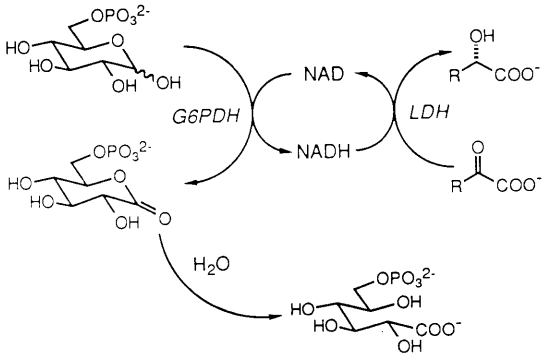
SCHEME 1

To measure the stability of the pyruvate (glyoxylate)/LDH system, we incubated 0.1 mM NAD and 50 mM pyruvate (glyoxylate) with and without LDH. The activities of the species in solution were determined periodically by the enzymatic assay. In all tests, the organic acids and enzyme remained fully active after 120 h.

[5] The K_m measured is an apparent value because it is based on the total concentration of glyoxylate, regardless of the form(s) it exists in and the species bound by the enzyme. The actual K_m is probably much smaller since glyoxylate exists in solution almost completely as the hydrate and yet LDH binds the free aldehydic species as the substrate for reduction.

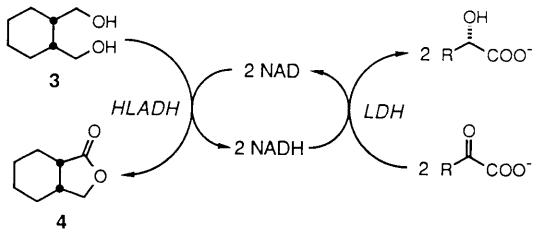
In the presence of pyruvate, active NAD decreased somewhat: After 120 h, in the presence and absence of LDH, 45 and 60%, respectively, of the original active NAD remained. With glyoxylate, in the presence and absence of LDH, 82 and 89%, respectively, of the original active NAD remained after 120 h.

LDH, coupled with pyruvic and glyoxylic acids as oxidants, successfully regenerated NAD in oxidations of 4 mmol of glucose 6-phosphate (G6P) to 6-phosphogluconate catalyzed by glucose-6-phosphate dehydrogenase (G6PDH) Eq. [3]. The G6P/G6PDH system was chosen for initial demonstrations because the oxidation of G6P is thermodynamically favorable (spontaneous hydrolysis of 6-phosphogluconolactone renders it irreversible) and not subject to product inhibition by 6-phosphogluconate. 6-Phosphogluconate was isolated as its barium salt (*26*) in 70–80% yield. Total turnover numbers (TTN)[6] (*3*) for NAD(H) were 930–1070. At the end of the reactions (4–6 h), both enzymes remained fully active, and the nicotinamide cofactor retained 76–99% of its original activity. In separate stability studies, soluble G6PDH incubated in the presence of 50 mM pyruvic or glyoxylic acid (25°C, ambient atmosphere) remained 86 and 70% active, respectively, after 95 h. When 50 mM G6P was included in the incubation mixture, G6PDH lost no activity after 95 h.



[3]

To compare the present method of NAD regeneration to methods using αKG/GluDH and FMN/O_2, we used pyruvate/LDH to regenerate NAD for the HLADH-catalyzed oxidation of *cis*-1,2-bis(hydroxymethyl)cyclohexane (**3**) to (+)-(1*R*,6*S*)-*cis*-8-oxabicyclo[4.3.0]nonan-7-one (**4**), Eq. [4] (Table 1) (*4*, *27*). Both PAN-immobilized (*28*) and membrane-enclosed (*29*) LDH regenerated NAD efficiently and economically. Particularly striking was the stability of LDH, which



[4]

[6] TTN = mol of isolated product/mol of catalytic species (enzyme or cofactor) in reaction.

TABLE 1

Comparison of Methods of Regenerating NAD from NADH for the Oxidation of **3** to **4** Catalyzed by HLADH

	Pyruvate/LDH	Glyoxylate/LDH	αKG/GluDH[a]	FMN/O_2[b]
Regenerating system				
Oxidant (mmol)	Pyruvic acid (172)	Glyoxylic acid (170 or added continuously)[e]	α-Ketoglutaric acid (153)	FMN (20.3)
Enzyme (units)	LDH (410)		GluDH (230)	—
Millimoles of NAD	0.12		0.27	1.2
Synthetic system				
Millimoles of **3**	71		69	14
Units of HLADH	520		250	80
Reaction time (days)	1.5		2.5	2.5
Product yield (%)[c]	85		85	80
TTN[c]				
NAD	1000		430	20
Regenerating enzyme	3.9×10^6			—
HLADH	1.7×10^4		1.9×10^4	2.3×10^4
Recovered activity (%)				
NAD	77		60	
Regenerating enzyme	150[f]		20	—
HLADH	110[g]		24	
$/mol NAD regenerated[d]	14		31	250

[a] Data taken from Ref. (4).
[b] Data taken from Ref. (26).
[c] Based on isolated, distilled product.
[d] Based on the total cost of NAD, oxidant, and regenerating enzyme (one-time use).
[e] Glyoxylate deactivated HLADH.
[f] Grinding of the immobilization gel into smaller particles by the stir bar increased the surface area of the gel and thus the apparent activity of the immobilized LDH.
[g] This activation of HLADH (purchased as lyophilized powder) was reproducible (three trials). Its origin is unknown.

was greater than that of GluDH (*4*). That LDH and GluDH are cytoplasmic and mitochondrial enzymes, respectively, may account for this difference in stability. Mitochondrial enzymes tend to require association with mitochondrial membranes for stability and are less stable in solution than cytoplasmic enzymes.

When glyoxylate (initial concentration = 0.23–0.25 M) served as the oxidant in Reaction [4], however, no product formed. Recovered enzymes showed nearly full LDH activity but no HLADH activity. In subsequent stability studies, glyoxylate irreversibly deactivated HLADH by a process that was neither first- nor second-order[7] with respect to the enzyme (Fig. 2). Glyoxylate probably caused its deactivation by condensing with the arginine in the binding domain of HLADH, in analogy to the deactivation of HLADH by 2,3-butadione and phenylglyoxal (*30*).

[7] HLADH exists as a dimer.

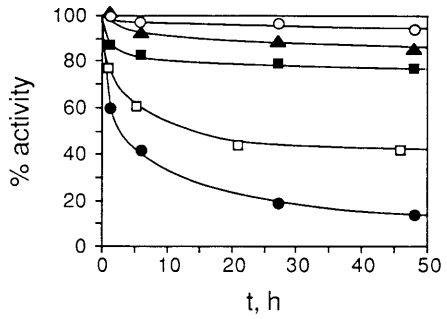


FIG. 2. Effects of 0 (○), 0.5 (▲), 5.0 (■), and 50 mM (●) glyoxylate and 50 mM glyoxylate plus 50 mM cyclohexanol (□) on HLADH.

Cyclohexanol (50 mM) slightly improved the stability of HLADH in the presence of 50 mM glyoxylate. A final attempt at using glyoxylate in Reaction [4] in which glyoxylate was added to the reactor (2.5 mmol h^{-1}) also failed. After 4 h, HLADH retained only 40% of its activity.

The advantages and disadvantages of using pyruvate and glyoxylate with LDH to regenerate NAD are summarized in Table 2. Pyruvate/LDH is perhaps the simplest, least expensive, and most stable system for regenerating NAD for reactions in which the oxidation step is thermodynamically favorable (or can be made so by removing or reacting further the product). With enzymes that are not susceptible to deactivation by glyoxylate, glyoxylate offers advantages of a high reduction potential and an even lower cost than pyruvate. A disadvantage of LDH is its inability to oxidize NADPH, and αKG/GluDH remains the only enzymatic method capable of direct regeneration of NADP. αKG has a good reduction potential. In some reactions, the zwitterionic product, L-glutamate, may be easier to separate from products than lactic or glycolic acids. Methods based on LDH and GluDH are superior to those based on the reduction of FMN.

TABLE 2

Advantages and Disadvantages of Using Pyruvic and Glyoxylic Acids with LDH to Regenerate NAD from NADH

Method	Advantages	Disadvantages
Pyruvate/LDH	Inexpensive oxidant	Moderate oxidizing potential
	High specific activity of enzyme	Mild deactivation of NAD by pyruvate
	Inexpensive enzyme	Inability to oxidize NADPH
	Small K_m for pyruvate	
	Compatibility with other enzymes	
Glyoxylate/LDH	Very inexpensive oxidant	Large K_m for glyoxylate
	High specific activity of enzyme	Deactivation of HLADH
	Inexpensive enzyme	(and other enzymes?)
	Strong oxidizing potential	Inability to oxidize NADPH
	Compatibility with NAD	

EXPERIMENTAL

Materials and methods. LDH (rabbit muscle, ammonium sulfate suspension), G6PDH (*Leuconostoc mesenteroides*, ammonium sulfate suspension), HLADH (lyophilized powder), and biochemicals from Sigma were used without further purification. *cis*-1,2-Bis(hydroxymethyl)cyclohexane (98%), pyruvic acid (98%), and glyoxylic acid (50% w/w aqueous solution) from Aldrich were used without further purification. Water was doubly distilled, the second time through a Corning AG-1b glass still. The autotitrator assembly consisted of a Radiometer PHM82 pH meter, TTT80 titrator, TTA80 titration assembly, ABU80 autoburette, REA160 titrograph, and REA270 pH stat unit. ^1H and ^{13}C NMR spectra were taken on Bruker AM-300 and AM-250 spectrometers, using $CHCl_3$ (^1H δ 7.26, ^{13}C δ 77.0 in $CDCl_3$) or sodium 3-(trimethylsilyl)-1-propanesulfonate (methyl ^1H δ 0.00, ^{13}C δ 0.00 in D_2O) as internal references. Infrared spectra were taken on a Perkin–Elmer 598 spectrophotometer. Elemental analyses were performed by Spang Microanalytical Laboratory.

Enzymatic assays. All assays were based on the spectrophotometric observation of the appearance or disappearance of NADH at 334 nm (ε = 6.18 mM^{-1} cm^{-1}). For LDH, the assay was initiated by adding 0.01 ml of a solution containing 0.01–0.1 U of LDH to 3.00 ml of 0.1 M triethanolamine (TEA) buffer, pH 7.6, containing 2.3 mM sodium pyruvate and 0.23 mM NADH. Assay of LDH with glyoxylate as substrate was performed similarly except in 0.1 M Hepes or Mops/NaOH buffer, pH 7.0, with 50 mM glyoxylate. For HLADH, 0.01 ml containing 0.01–0.1 U of HLADH was added to 1.10 ml of 0.1 M Gly–Gly buffer, pH 8.0, containing 9.9 mM cyclohexanol and 0.50 mM NAD. Units were expressed as activity with **3,** which has a maximal velocity that is 80% of that of cyclohexanol (*27*). For G6PDH, 0.02 ml containing 0.01–0.10 U of G6PDH was added to 2.98 ml 0.1 M potassium phosphate buffer, pH 7.0 or 7.6, containing 9.8 mM G6P, 6.9 mM NAD, and 3 mM $MgCl_2$. Pyruvate was assayed by adding 0.10 ml containing up to 210 nmol of pyruvate to 0.96 ml of 0.1 M TEA, pH 7.6, containing 0.33 mM NADH and 2 U of LDH. Glyoxylate was assayed by the method of Bergmeyer and Lang (*31*), substituting LDH and NADH for glyoxylate reductase and NADPH, respectively. Total NAD(H) was assayed by the method of Bernofsky and Swan (*32*), and oxalate was assayed using a diagnostic kit from Sigma.

Monomethyl oxalate. Reaction of oxalic acid (10 g) and methanol in carbon tetrachloride (*33*) gave, after distillation, 26% dimethyl oxalate [subl. 40°C (0.1 Torr); ^1H NMR ($CDCl_3$) δ 3.89 (s); ^{13}C NMR ($CDCl_3$) δ 53.5, 157.8] and 27% monomethyl oxalate: bp 50–55°C (0.1 Torr) [lit. (*33*) bp 50–52°C (0.2 Torr)]; ^1H NMR ($CDCl_3$) δ 3.94 (s, 3 H), 10.57 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 54.2, 158.2, 195.5.

Potassium S-methyl thiooxalate. Oxalyl chloride (10 mmol) was converted in four steps (*34*) to potassium *S*-methyl thiooxalate (30%): ^1H NMR (D_2O) δ 2.33 (s).

Enzyme stabilities. Pyruvic or glyoxylic acid (50 mM) and NAD (0.1 mM) were incubated with and without LDH (1 U) in 0.10 M TEA buffer, pH 7.6, 25°C. Periodic aliquots were assayed for activities of the organic acid, NAD, and LDH.

G6PDH (2 U) was incubated in 50 mM Tris/HCl buffer, pH 7.5, 6.3 mM $MgCl_2$,

25°C, in the presence of 0, 5, 15, and 50 mM pyruvate or glyoxylic acid. G6PDH was similarly incubated with 50 mM organic acid plus 50 mM G6P. Periodically, aliquots were assayed for enzymatic activity.

HLADH (0.5 mg) was incubated in 0.1 M Mops/NaOH buffer, pH 7.0, 25°C in the presence of 0.0, 0.5, 5.0, or 50 mM glyoxylate, with and without 50 mM cyclohexanol. HLADH was similarly incubated with 50 mM pyruvate, with and without cyclohexanol. Periodically, aliquots were assayed for enzymatic activity.

Oxidation of G6P using LDH to regenerate NAD. Each reaction contained in 23 ml of water: 4 mmol of G6P (disodium salt), 4 mmol of pyruvate or glyoxylate, 3 μmol of NAD, 75 μmol of DTT, and 75 μmol of $MgCl_2$. Soluble LDH (125 U) and G6PDH (200 U) were added to initiate the reaction. Reaction progress was monitored by recording the volume of 1.000 N NaOH delivered by an autotitrator assembly to maintain constant pH (pH 7.6 with pyruvate, pH 7.0 with glyoxylate). Upon completion of the reaction, the mixture was assayed for G6PDH, LDH, and NAD and oxalate if glyoxylate had been used as the oxidant. $BaCl_2 \cdot 2H_2O$ (6 mmol) and then 8 ml of ethanol were added to the stirred solution. The precipitate was filtered, dried *in vacuo*, and assayed for 6-phosphogluconate as described previously (*25*).

Oxidation of cis-1,2-bis(hydroxymethyl)cyclohexane (**3**) *using LDH to regenerate NAD.* A 3-liter, three-necked round-bottomed flask with magnetic stirring bar was charged with **3** (10.5 g, 71 mmol), pyruvic acid (12.2 ml, 172 mmol) dissolved in 150 ml of water plus 45 ml of 4 N NaOH, 37.5 ml of 0.1 M Gly–Gly buffer, pH 8.0, 520 ml of nitrogen-sparged water, HLADH (290 mg, 520 U), and PAN-immobilized (*27*) LDH (410 U, 100 ml of gel). A little 4 N NaOH was added to adjust the pH to 8.1, and the mixture was sparged with nitrogen for 30 min to remove dioxygen. A 50-μl aliquot was assayed for initial pyruvate concentration. NAD (0.12 mmol) was added to the mixture, and 50 μl was removed to assay for initial NAD(H) concentration. Hexane (1 liter) was layered over the aqueous solution, and the reaction was stirred gently. The progress of the reaction was monitored by assaying for pyruvate. After 40 h, the reaction was complete. Enzymatic assay showed 110 and 77% of the original HLADH and NAD(H) activities, respectively, remaining. The hexane was removed by steel cannula and concentrated to a yellow oil. The gel was allowed to settle and most of the aqueous layer removed by cannula. The gel was centrifuged and the supernatant decanted. The gel was then washed twice by resuspension in water, centrifugation, and decanting. All aqueous layers were combined and extracted with ether. Ethereal portions were combined with the concentrated hexane solution, dried over $MgSO_4$, and evaporated under reduced pressure. Distillation of the crude product gave 8.5 g (85%) of (+)-(1*R*, 6*S*)-*cis*-8-oxabicyclo[4.3.0]nonan-7-one (**4**) as a colorless oil: bp 65–75°C (0.3 Torr); α_D^{23} + 43.8° (neat); ^1H NMR ($CDCl_3$) δ 1.1–2.6 (m, 10 H), 3.90 and 4.15 (2 dd, J_{vic} = 1.2 and 5.0 Hz, respectively, J_{gem} = 8.8 Hz, 1 H each); ir (neat) 1770 cm^{-1}. Analytical data were in agreement with the literature (*4, 27*). The LDH gel assayed for 150% of its original activity.

A similar reaction was run using HLADH and LDH enclosed in dialysis membrane tubing (*29*). Yield of distilled product was 88%.

Attempted oxidations of **3** using glyoxylate and PAN-immobilized or mem-

brane-enclosed LDH were identical to the reactions described above except that glyoxylate replaced pyruvate. In a final attempt, a neutral solution of sodium glyoxylate (1.14 M) was added at a rate of 2.2 ml h^{-1} to a biphasic system containing 10 g of **3**, 20 mM triethanolamine, and 0.12 mmol of NAD in 800 ml of degassed water. HLADH (500 U) and LDH (1500 U) were enclosed in dialysis tubing (8 ml total volume) and submerged in the aqueous layer, which was then overlayered with hexane.

ACKNOWLEDGMENTS

This work has been supported in part by NIH Grant GM 30367 and by a grant from Firmenich, SA. H.K.C. gratefully acknowledges support as an NIH Training Grant Trainee, 1984-85 (Grant 5-T32-GM-07598).

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