amino acids (in all of our simulations, the waters show a greater tendency to bind at the carboxy groups); this serves to weaken the bonds even further. As the breathing arrives to a point wherein the hydrogen-bonding distance is very large, one of the attached water molecules realigns itself with the hydrogen so as to form a bridge between the carboxy and amino group of one hydrogen bond. This three-centered hydrogen bond serves as an intermediate which then causes the α-helix to break at that position and begin to fold up. This intermediate structure can then move in either direction: either back to the intact helix or toward a further separation. As it continues to separate, a local perturbation will occur such that the bent structure will be better accommodated; this seems to be a tendency toward a 3_{10} helix.

Alanine and glycine provide examples of two extremes that are involved in the α-helical microfolding process, and we have been able to explain the role that water plays in their helical bending. Other sequences will want to bend due to a combination of these two extremes, and so the role played by the water will demonstrate a corresponding dependence: Water will act in a passive fashion, providing a frictional force that dampens the motions of the helix (as in the case of polyglycine); it will also serve a more active role as a tool for guiding the overall bending pathway (as in the case of polyalanine). How much either of these two extremes contribute to the overall role of the bending will be dependent on the sequence of the helix and on its local environment.

It is worthwhile to mention the fact that proteins do not fold by forming one long chain that later goes on to bend at certain areas; rather, they will form some type of intermediate that consists of some initial secondary and tertiary structure. The role that will be played by the solvent must be balanced with the accessibility of the helix within this intermediate form as well as the local environment that is bestowed upon the helix by the surrounding transitionary structure. This environment will further weaken or strengthen various points along the helix and affect the role of the water within the binding process. Thus, while the internal geometry will play a crucial role in the microfolding process, so to must the local nonbonded environment that is projected by the surrounding protein, water, and any other constituents of the system.

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Registry No. Gly30, 134110-05-7; Ala30, 109376-45-6; H_2O, 7732-18-5.

The Dehydration of Glyoxylate Hydrate: General-Acid, General-Base, Metal Ion, and Enzymatic Catalysis[1a]

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Abstract: The lactate dehydrogenase catalyzed reduction of glyoxylate by NADH was studied at 25.0 °C and an ionic strength of 0.15 by using a spectrophotometric method. We have demonstrated that this reaction is a sequential one that requires prior dehydration of glyoxylate hydrate. Accordingly, reaction rates extrapolated to infinite LDH concentrations were used to determine the rate of hydration of glyoxylate hydrate. The dehydration reaction was shown to be susceptible to general-acid and general-base catalysis in phosphate buffers and to catalysis by transition-metal ions and by the high-activity zinc metalloenzyme carbonic anhydrase (CA II) from bovine erythrocytes. The Brønsted constant, β, was estimated from the catalytic rate coefficients of H_2O, HPO_4^{2-}, and OH^- and compared with those for the reversible hydration reactions of other substrates of varying electrophilicity. The magnitude and relative order of catalytic efficiency of divalent transition-metal ions, $Zn^{2+} > Cu^{2+} > Co^{2+} > Ni^{2+} > Cd^{2+} > Mn^{2+}$, were compared with other reactions in which the substrate has the capacity to bind these cations. For the dehydration of glyoxylate a mechanism involving the metal ion in the direct transfer of a water molecule and at the same time acting as a general acid is proposed. The CA II catalyzed dehydration of glyoxylate hydrate was determined as a function of pH. The data show that the dependency of enzymatic rate on pH is similar to that for the dehydration of bicarbonate, and accordingly suggest certain parallels in mechanisms for their bovine carbonic anhydrase catalyzed dehydrations.

Introduction

Glyoxylate is an anion of biochemical significance in both plants and animals. In plants and in bacteria the glyoxylate cycle provides a means of utilizing acetyl coenzyme A for the ultimate production of carbohydrates and amino acids. In mammalian brains, glyoxylate is produced from glycine by D-amino acid oxidase.[2] It has been proposed that glyoxylate may have a controlling effect on various metabolic processes in mammals.[3] It is known to inhibit oxidative metabolism, and adducts of glyoxylate with various nucleophiles appear to serve as physiological substrates for a number of oxidases. For example, thioacetates serve as substrates for L-hydroxy acid oxidase to form oxalyl thioester compounds, which may serve as intracellular messengers for insulin and other hormones.[3a-c]

In aqueous solution, glyoxylate exits almost entirely as its *gem*-diol, glyoxylate hydrate:[4]

$$\begin{matrix}\mathrm{CHO}\\ |\\ \mathrm{CO_2^-}\end{matrix} + \mathrm{H_2O} \rightleftharpoons \begin{matrix}\mathrm{CH(OH)_2}\\ |\\ \mathrm{CO_2^-}\end{matrix} \qquad K_{eq} = 163 \qquad (1)$$

It has been shown that lactate dehydrogenase (EC 1.1.1.27, L-lactate; NAD oxidoreductase) catalyzes the oxidation of glyoxylate to oxalic acid:

(1) (a) Support of this research by grants from the National Science Foundation and the Muscular Dystrophy Association is gratefully acknowledged. (b) Visiting scientist under the National Science Foundation Research Opportunity Award Program, Summer 1988. Permanent address: Department of Chemistry, Seattle University, Seattle, WA 98122.

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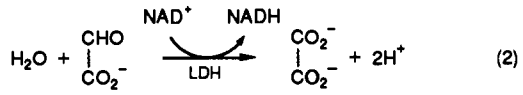
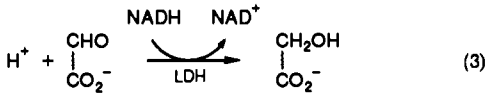


(2)

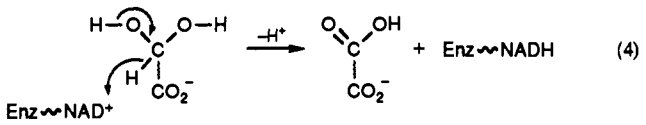
as well as its reduction to glycolate.[5]



(3)

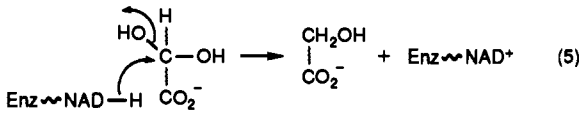
On the basis of the results of oxalate- and oxamate-inhibition studies, Warren[5c] has suggested mechanistic parallels between the oxidation of glyoxylate and lactate as well as between the reduction of glyoxylate and pyruvate. He also proposes ordered bi bi mechanisms for the glyoxylate reactions in which the coenzyme must be bound to the enzyme before glyoxylate can react.

It has been suggested that for the oxidation of glyoxylate its hydrate serves as substrate:[5a]



(4)

This mechanism parallels the oxidation of acetaldehyde by yeast aldehyde dehydrogenase.[6] Rendina et al.,[4b] as well as Everse and Kaplan,[7] have assumed that for the LDH-catalyzed reduction of glyoxylate, unhydrated glyoxylate serves as substrate by undergoing the nucleophilic acyl addition of hydride to the carbonyl. Warren,[5c] however, has suggested that it is the hydrate that undergoes reduction via nucleophilic displacement of hydroxide (activated as a leaving group by enzymatic protonation).



(5)

Studies by Rendina et al.[4b] and Craig et al.[4c] show that at high concentrations of LDH the rates of reduction of glyoxylate by NADH are no longer linear with enzyme but begin to level off. While this finding is consistent with the formation of unhydrated glyoxylate in the rate-determining step, such deviations from first-order dependency on enzyme concentration are not uncommon at relatively high enzyme concentrations. Such deviations occur, for example, when enzyme concentration limits are reached beyond which enzyme molecules fail to act completely independently or, in the extreme, begin to precipitate out of solution.

Both glyoxylate and glyoxylate hydrate may be involved in a variety of enzymatically catalyzed reactions. One would anticipate that when glyoxylate reacts with nucleophiles (for example, a thiol) or nucleophilic sources (for example, NADH), unhydrated glyoxylate would serve as the preferential substrate since the nucleophilic acyl addition reaction is generally favored over the nucleophilic displacement of hydroxide. Thus, prior to the interpretation of kinetic data, the position of equilibrium between hydrated and unhydrated forms and the rate of conversion of hydrated glyoxylate to glyoxylate (which can become rate-limiting) must be quantified.

In the present paper we demonstrate that the LDH-catalyzed reduction of glyoxylate by NADH requires the prior dehydration of glyoxylate hydrate. We show how the proper identification of unhydrated glyoxylate as the preferential substrate in its enzymatic reduction is necessary for the meaningful interpretation of the Michaelis constant in comparison to other LDH-catalyzed reactions.

Since the rates of formation of NADH at high concentrations of LDH become identical with the rate-limiting dehydration of hydrated glyoxylate, we were able to determine dehydration rates in the presence of phosphate and diethyl malonate buffers, transition-metal ions, and the zinc metalloenzyme carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1). Since carbonic anhydrase makes use of both metal ion and general-acid and -base catalytic groups, the nonenzymatic catalysis of the dehydration reaction was studied separately for comparison with the corresponding enzymatic reaction. Finally, we compare the various catalytic parameters determined for the dehydration of glyoxylate hydrate to those observed for the reversible hydration reactions of substrates studied in our earlier work.[8-10]

Experimental Section

Crystalline suspensions of rabbit muscle LDH (type V) and lyophilized high-activity CA II were products of Sigma as were NADH (grade III), the reduced form of nicotinamide adenine dinucleotide, and glyoxylic acid. Buffer components were commercially available in analytical or reagent grade.

All solutions were prepared in distilled, deionized water and kinetic runs were carried out at an ionic strength of 0.15, which was established by the addition of appropriate quantities of sodium sulfate to the buffered solutions. Values of pH were determined before and after each kinetic run.

The method of half-neutralization was used to obtain the pK_a value of the conjugate acid of monohydrogen phosphate dianion in the present work. The mean pH value for the set of buffers (pH 6.78) with a buffer ratio of unity was taken to be pK_a. Activity coefficients, $f_\pm$, were calculated from

$$\log f_\pm = \frac{-0.49Z^2\mu^{0.5}}{1 + 1.5\mu^{0.5}} \quad (6)$$

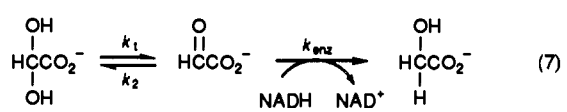
where μ and Z stand for the ionic strength of the solution and the charge of the ion under consideration, respectively.

Stock solutions of NADH were prepared in dilute buffers (ca. pH 8) immediately prior to use. The concentrations of NADH solutions were checked spectrophotometrically ($\epsilon_{340}^{mM} = 6.2$). Solutions of LDH were prepared in 0.01 M aqueous sodium chloride. All stock solutions were chilled in ice during the course of each series of kinetic runs and never used for periods exceeding ca. 3 h. The methods of standardization of the CA II used are described elsewhere.[11]

The reactions were followed on a Gilford Model 2000 high-speed recording spectrophotometer as well as on a Hewlett-Packard Model 8452A diode array spectrophotometer. The kinetic results obtained by using the two instruments were identical. The thermostated cell compartments were maintained at 25.0 ± 0.1 °C. Values of pH were determined with a Beckman 101900 research pH meter.

The LDH-catalyzed oxidation of NADH in the presence of glyoxylate was monitored at 340 nm by observing the diminution of absorbance with time. Typical concentrations of reagents used were as follows:[12] glyoxylate = 1.25×10^{-4} M; NADH = 9.5×10^{-5} M; LDH = 40–200 units/mL. In all kinetic runs reported, the stock LDH solutions were added last to initiate the chemical reactions although identical reaction rates were obtained regardless of the order of addition of the reaction components.

First-order rate constants, k_{obsd}, were determined from plots of the logarithms of absorbance vs time. In agreement with Rendina et al.[4b] and Craig et al.,[4c] it was observed that plots of k_{obsd} vs LDH concentrations were hyperbolic. If Rendina's assumption that the reaction rates achieved at high LDH concentrations are limited by the rate of dehydration of glyoxylate hydrate is valid, then the maximum value of the first-order rate constant $k_{obsd}^{max} = k_1$ for the consecutive reaction sequence below:[12]



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(5) (a) Duncan, R. J. S.; Tipton, K. F. *Eur. J. Biochem.* **1969**, *11*, 58. (b) Romano, M.; Cerra, M. *Biochim. Biophys. Acta* **1969**, *177*, 421. (c) Warren, W. A. *J. Biol. Chem.* **1970**, *245*, 1675.

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(12) The concentration of glyoxylate used was much lower than the K_m for glyoxylate, and the concentration of NADH was much larger than its K_m (see ref 5c).

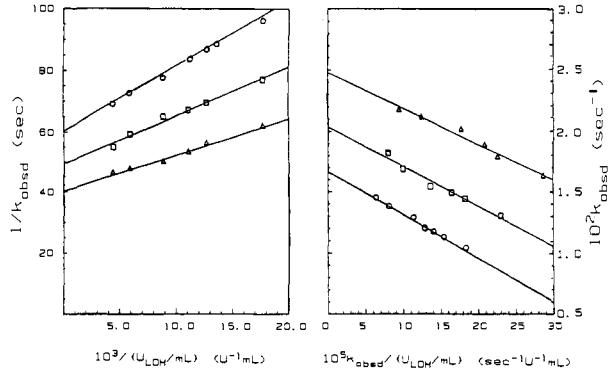


Figure 1. Graphical methods for determining rate constants for the dehydration of glyoxylate hydrate: left, method I (see text); right, method 2 (see text). All runs carried out at 25.0 °C, μ = 0.15: △, 0.050 M phosphate; □, 0.0333 M phosphate; ○, 0.0167 M phosphate. LDH concentration units are given in terms of IUB units, μ, per milliliter in the reaction mixture.

Table I. Comparison of Kinetic Data Using Graphical Methods (Phosphate Buffers, r = 0.926 at 25.0 °C, μ = 0.15)

	k_{obsd} (s^{-1})	
M phosphate (total)	method I	method II
0.0500	0.0249 ± 0.0004	0.0248 ± 0.0004
0.0333	0.0203 ± 0.0004	0.0204 ± 0.0004
0.0167	0.0167 ± 0.0005	0.0166 ± 0.0004

Under the conditions of reagent concentrations used, it may be shown for this process that

$$\frac{1}{k_{obsd}} = \frac{k_2 k_1}{k_{enz}} \frac{1}{[LDH]} + \frac{1}{k_1} \tag{8}$$

where $k_{enz} = k_{cat}/K_m$. We found that plots of $1/k_{obsd}$ vs $1/[LDH]$ were indeed linear (Figure 1A) and tentatively assigned the intercept value of $1/k_{obsd}^{max} = 1/k_1$.

Alternatively, one may express the relationship in eq 8 as follows:

$$k_{obsd} = k_1 - \frac{k_2 k_1^2}{k_{enz}} \frac{k_{obsd}}{[LDH]} \tag{9}$$

Figure 1B shows that plots of k_{obsd} vs $k_{obsd}/[LDH]$ are linear and, again, we tentatively identify $k_{obsd}^{max} = k_1$. A comparison of the data obtained from these graphical methods is shown in Table I and indicates that for the concentrations of LDH chosen for the kinetic runs, the two evaluative methods give essentially identical values of k_1 and similar limits of uncertainty.

Results and Discussion

The extrapolated reaction rates of the reduction of the glyoxylate system by NADH to infinite concentrations of LDH, k_{obsd}^{max}, were determined as a function of buffer concentration in both phosphate and diethyl malonate buffers. Typically, values of k_{obsd}^{max} were determined in reaction series in which buffer concentrations were varied while a constant acid/base ratio and consequently a constant pH were maintained. If k_{obsd}^{max} is indeed identical to the rate constant for dehydration, k_1, and furthermore if the rate of dehydration of glyoxylate is sensitive toward general-base and/or general-acid catalysis (as is the case of the reversible hydrations of other carbonyls[11]), then we would expect that k_{obsd}^{max} would include kinetic terms for each basic and possibly acidic catalytic component present in solution:

$$k_{obsd}^{max} = k_{cat}[cat] = k_0 + k_{OH^-}[OH^-] + k_{H_2PO_4^-}[H_2PO_4^-] + k_{HPO_4^{2-}}[HPO_4^{2-}] \tag{10}$$

where it is assumed that $k_0 = k_{H_2O}[H_2O]$. If the buffer ratio is defined as $r = [H_2PO_4^-]/[HPO_4^{2-}]$, then eq 9 may be rearranged:

$$k_{obsd}^{max} = k_0 + k_{OH^-}[OH^-] + [H_2PO_4^-](k_{H_2PO_4^-} + k_{HPO_4^{2-}}/r) \tag{11}$$

Figure 2A illustrates the dependency of k_{obsd}^{max} on increasing buffer concentration at constant pH and shows that k_{obsd}^{max} indeed increases linearly with increasing buffer concentration. In fact, the linearity of the plots in Figure 2A is consistent with the mechanistic implications of eqs 10 and 11 and precludes any significant catalytic contribution by a third-order kinetic term involving substrate and the simultaneous catalytic action of general acid and general base. Similar kinetic determinations were made at several other buffer ratios. According to eq 11, the slopes and intercepts of plots such as the one shown in Figure 2A for general-base- or general-acid-catalyzed reactions would have the values:

$$S = k_{H_2PO_4^-} + k_{HPO_4^{2-}}/r \tag{12}$$

$$I = k_0 + k_{OH^-}[OH^-] \tag{13}$$

Table II. Catalytic Rate Coefficients for Monohydrogen and Dihydrogen Phosphate Anions at 25.0 °C, μ = 0.15 M

pH	$1/r$	$S = k_{H_2PO_4^-} + k_{HPO_4^{2-}}/r$ (s^{-1} M^{-1})	$I = k_0 + k_{OH^-}[OH^-]$ (s^{-1})
6.23	0.280	0.197	0.0115
6.70	0.926	0.493	0.0124
6.87	1.23	0.554	0.0132
7.19	2.56	1.07	0.0158

Table III. Catalytic Rate Coefficients for the Dehydration of Glyoxylate Hydrate at 25.0 °C, μ = 0.15 M

species	buffer[d]	pH	k_{cat} (M^{-1} s^{-1})
H_2O	DEM[a]	*b*	(0.0108 ± 0.00046)/55.5
OH^-	DEM[a]	variable	2.46 × 10^4 ± 0.11 × 10^4
HPO_4^{2-}	PHOS	variable	0.378 ± 0.0081
$H_2PO_4^-$	PHOS	variable	0.106 ± 0.0026
Zn^{2+}	DEM	7.0	174 ± 1.6
Cu^{2+}	DEM	7.0	136 ± 2.5
Co^{2+}	DEM	7.0	56.0 ± 1.1
Ni^{2+}	DEM	7.0	50.2 ± 0.4
Cd^{2+}	DEM	7.0	34.2 ± 0.6
Mn^{2+}	DEM	7.0	13.3 ± 0.17
BCA	PHOS	6.8	116 ± 1.6

[a] Catalytic values determined by extrapolation to zero buffer concentration. [b] Catalytic value determined by extrapolating to zero hydroxide ion concentration. [c] $k_0 = k_{H_2O}[H_2O] = k_{H_2O}[55.5]$. [d] DEM, diethyl malonate; PHOS, phosphate.

Table II shows the values of the slope and intercepts at various phosphate buffer ratios.

Figure 2B illustrates that the process, spectrophotometrically monitored, is subject to both general-acid and general-base catalysis in phosphate buffers and allows the calculation of $k_{H_2PO_4^-}$ (intercept) and $k_{HPO_4^{2-}}$ (slope) (see Table III). Parallel to the reversible hydrations of aliphatic aldehydes,[11b,c] catalysis by HPO_4^{2-} is considerably greater than that due to its conjugate acid. Similar studies carried out in diethyl malonate buffers yielded only approximate results due to the small magnitude of catalysis. However, it was estimated that catalytic components of diethyl malonate were ca. one-twentieth those of phosphate buffers. Again, this parallels the corresponding result previously determined for the reversible hydration of other aldehydes.[11b,c]

Combined intercept values (eq 12) from phosphate and diethyl malonate studies such as those in Figure 2A plotted against hydroxide activities in Figure 2C demonstrate the sensitivity of the reaction toward hydroxide ion and water. The catalytic coefficient for hydroxide was evaluated from k_{OH^-} = slope × $f_\pm$ from the plot in Figure 2C and appears along with the value of $k_0 = k_{H_2O}[H_2O]$ in Table III.

We have shown that values of k_{obsd}^{max} are elevated by the same types of catalysts as those that enhance hydration and dehydration rates of pyruvate and other carbonyl compounds. In the paragraphs that follow, we show additional parallels to other reversible hydration reactions of carbonyls with respect to their sensitivity toward catalysis by divalent transition-metal ions and by bovine carbonic anhydrase (BCA). Furthermore, it has been shown in a separate study[13a] that the same extrapolated rates, which we

Table IV. General-Acid and General-Base Catalysis of Reversible Hydrations of Carbonyl Compounds

	CH_3CHO^a	$CH_3CH_2CHO^a$	$(CH_3)_2CHCHO^a$	glyoxylate	$2PA^{a,d}$	$4PA^{a,d}$	$MeP^{b,d}$
$k_{HPO_4^{2-}}/k_{H_2PO_4^-}$	2.25	2.13	1.4	3.6	v high	v high	v high
k_{OH^-}/k_0	4.0×10^8	1.4×10^8	2×10^8	1.3×10^8	4.0×10^8	5.2×10^8	1.2×10^9
$k_{HPO_4^{2-}}/k_{H_2O}$	2.2×10^4	1.9×10^4	2.0×10^4	2.0×10^4	5.3×10^3	5.8×10^3	1.8×10^3
$\log (k_{HPO_4^{2-}}/k_{H_2O})/(pK_a'(HPO_4^{2-}) - pK_a(H_2O))^c$	0.49	0.48	0.48	0.50	0.42	0.42	0.39
β^b	0.47	0.45	0.46				0.39

[a] Studies conducted at 0.0 °C, μ = 0.1 (see refs 11b, 11c, and 16a). [b] Studies conducted at 5.0 °C, μ = 1.0 (see ref 11a). [c] Values of pK_a for phosphate vary depending on experimental conditions of temperature and ionic strengths. The pK_a for water was taken to be −1.74. The pK_a values used for the studies on the aliphatic aldehydes, pyridinecarboxaldehydes, and methyl pyruvate were taken from references 11c, 16a, and 11a, respectively. [d] 2PA, 2-pyridinecarboxaldehyde; 4PA, 4-pyridinecarboxaldehyde; MeP, methyl pyruvate.

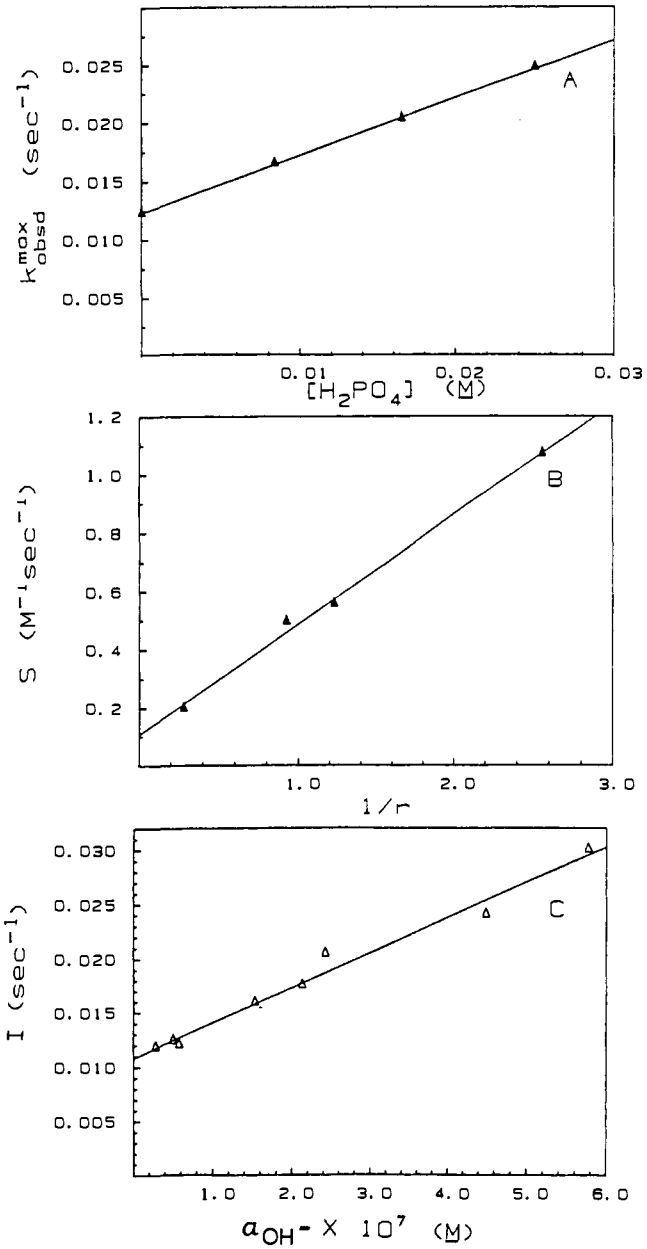


Figure 2. Determination of catalytic rate coefficients for phosphate buffer components: 2A, determination of $k_{H_2PO_4^-}$ and $k_{HPO_4^{2-}}/r$ at r = 1.08; 2B, determination of $k_{H_2PO_4^-}$ and $k_{HPO_4^{2-}}$; 2C, determination of k_0 and k_{OH^-}.

have defined as k^{max}_{obsd}, are obtained with use of glyoxylate reductase in place of LDH. Thus, we can safely conclude that what we have defined as k^{max}_{obsd} is indeed identical with the rate of dehydration of glyoxylate hydrate, and consequently that unhydrated glyoxylate is the preferential substrate of lactate dehydrogenase as it undergoes reduction by NADH.

The proper identification of the preferential substrate of LDH is necessary for the interpretation of the relative magnitude of the Michaelis constants for the reduction and oxidation of the glyoxylate system. Warren[5c] has determined that the Michaelis constant for the enzymatic reduction of glyoxylate by pig heart LDH ($K^{red}_{glyoxylate} = 6.85 \times 10^{-3}$ M) is similar to, but somewhat greater than, that for the oxidation of glyoxylate ($K^{ox}_{glyoxylate} = 2.46 \times 10^{-3}$ M). Duncan and Tipton[5a] also found similar K_m ratios for the rabbit muscle LDH-catalyzed reduction and oxidation of glyoxylate. Upon first consideration, these values, relative to each other, appear inconsistent with Warren's proposed mechanistic parallel between the reduction of glyoxylate and pyruvate and between the oxidation of glyoxylate and lactate. Since unhydrated glyoxylate structurally resembles unhydrated pyruvate (known to serve as the preferential substrate of LDH)[13b,14] and since hydrated glyoxylate structurally resembles lactate,[13c] one would expect some agreement in the ratios of Michaelis constants: $K^{ox}_{lactate}/K^{red}_{pyruvate}$ and $K^{ox}_{glyoxylate}/K^{red}_{glyoxylate}$. However, it is known that for LDH from various animal species, K_m for lactate exceeds that for pyruvate by 35–125 times.[7]

It should be noted that Warren's value of $K^{red}_{glyoxylate}$ for the reduction process was based on the total glyoxylate concentration in the reaction solution. Since it is now clear that unhydrated glyoxylate is the preferential substrate in its LDH-catalyzed reduction, the value of $K^{red}_{glyoxylate}$ must be corrected to correspond to the fraction of unhydrated glyoxylate. For the hydration of glyoxylate, the equilibrium constant has been reported:[4b] K = [glyoxylate hydrate]/[glyoxylate] = 163. Thus, the fraction of unhydrated glyoxylate is 0.0038 and $K^{corrected}_{glyoxylate} = 0.0061 \times 6.85 \times 10^{-3}$ M = 4.18×10^{-5} M for the enzymatic reduction. Accordingly, the ratio $K^{ox}_{glyoxylate}/K^{red}_{glyoxylate}(corr) = 2.46 \times 10^{-3}$ M/4.18×10^{-5} M = 59, which indeed is reasonable for the expected Michaelis constant ratio for lactate to pyruvate.

The identification of unhydrated glyoxylate as the preferential substrate for reduction by LDH is not only important for a meaningful analysis of Michaelis constants of LDH-catalyzed processes but also leads to a kinetic method for determining the sensitivity of the dehydration of glyoxylate hydrate toward catalysis by general acids, general bases, transition-metal ions, and carbonic anhydrase. We noted earlier that the sensitivity of the dehydration of glyoxylate hydrate toward both general-acid and general-base catalysis (Tables III and IV) is similar to that found for aliphatic aldehydes.[11b,c] This result is reasonable since it might be anticipated that given the similar inductive effects of the groups $-CO_2^-$ and R−, the acidic and basic catalytic requirements for glyoxylate hydrate dehydration and the hydration of aliphatic aldehydes should be similar. We see that for the more highly electrophilic substrates, methyl pyruvate and the pyridinecarboxaldehydes (Table IV), general-acid catalysis for dihydrogen phosphate is not observed.

In earlier work it was shown that the general-base-catalyzed reversible hydrations of several carbonyl compounds are good models for the application of the Brønsted catalysis law.[11] For aliphatic aldehyde hydrations,[11b] it was found that the data points for H_2O and HPO_4^{2-} were essentially on the Brønsted line (Table IV). It will be noted that for glyoxylate hydrate dehydration the

(13) (a) Lonie, N. H.; Meany, J. E. 45th Northwest Regional Meeting of the American Chemical Society, 1990, Abstract No. 256. (b) Tienharra, R.; Meany. J. E. *Biochemistry* **1973**, *12*, 2067. (c) Although it has not been demonstrated, glyoxylate hydrate is most likely the substrate of oxidation by LDH (see ref 5a).

(14) (a) Loewus, F. A.; Chen, T. T.; Vennesland, B. J. *J. Biol. Chem.* **1955**, *212*, 787. (b) Fromm, H. J. *Biochim. Biophys. Acta* **1965**, *99*, 540.

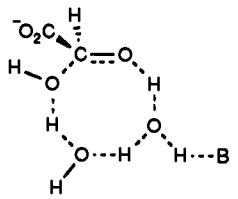
Table V. Catalysis by Divalent Transition-Metal Ions

metal ion	$k_{M^{2+}}/k_{H_2O}$ dehydration of glyoxylate	dehydration of pyruvate	hydration of 2-pyridinecarboxaldehyde	tautomerization of acetylacetone enol[a]
Zn^{2+}	8.94×10^5	3.05×10^6	6.22×10^6	1.80×10
Cu^{2+}	6.99×10^5	1.04×10^7	2.44×10^8	
Co^{2+}	2.88×10^5	1.06×10^6	7.33×10^6	4.26×10
Ni^{2+}	2.58×10^5	1.59×10^6	6.88×10^6	3.28×10
Cd^{2+}	1.77×10^5	1.86×10^5	1.11×10^6	3.27
Mn^{2+}	6.83×10^4	1.51×10^5	1.02×10^3	8.52

[a] The order of catalytic effectiveness of these ions to catalyze the enolization of 2-carbethoxycyclopentanone is identical with that observed for the tautomerization of acetylacetone enol (ref 24).

ratio of HPO_4^{2-}/k_0 is essentially identical with that for the reversible hydration of CH_3CHO, CH_3CH_2CHO, and $(CH_3)_2CHCHO$. Given the constancy of this ratio of catalytic rate coefficients, taken together with the similarity of catalytic requirements for the reactions of glyoxylate hydrate and the aliphatic aldehydes, one would predict comparable values of β (see Table IV). We note that β values for the more highly electrophilic substrates, methyl pyruvate and the pyridinecarboxaldehydes, are lower due to the greater reactivity of these substrates brought about by the electron-withdrawing effects of the carbomethoxy group (in methyl pyruvate) and the sp^2 hybridized ring nitrogen atom (in the pyridinecarboxaldehydes).

It is interesting to note that for glyoxylate dehydration the catalytic effectiveness of monophosphate is not reduced by the electrostatic repulsion of a dianionic general base and an anionic substrate in the transition state. This suggests the possibility of intervention by one or more water molecules between the substrate and the approaching general base. Cyclic transition states similar to those proposed by Eigen et al.[15] and by us[11c] would allow the necessary spatial separation of substrate and anionic catalyst:



A β value of ca. 0.50 can be interpreted to mean that approximately 50% of the bond-breaking and -making processes have occurred at the same time the transition state described above is reached. The ratio k_{OH^-}/k_0 for the dehydration of glyoxylate is also similar to those for the reversible hydrations of the aliphatic aldehydes, indicating a relative lack of electrostatic repulsion between anionic substrate and the hydroxide anion. This again may indicate a catalytic mechanism involving bridging by one or more molecules of water.

The dehydration of glyoxylate was found to be very sensitive toward divalent transition-metal ions. The overall rate constants were determined in 0.05 M diethyl malonate buffers at pH 6.7 where catalysis by the buffer components was negligible:

$$k_{obsd}^{max} = k_0 + k_{OH^-}[OH^-] + k_{M^{2+}}[M^{2+}] \qquad (14)$$

Catalytic coefficients for the metal ions were determined from a series of runs in which only the metal ion concentration was varied (see Figure 3A,B). The rates of dehydration of glyoxylate were found to be linear in metal ion concentration, indicating that one metal ion interacts with one glyoxylate hydrate molecule in the transition state. As shown in Figure 3A,B and in Table III, the order of catalytic effectiveness of the ions tested is $Zn^{2+} > Cu^{2+} > Co^{2+} > Ni^{2+} > Cd^{2+} > Mn^{2+}$. The relative catalytic effectiveness of this same group of metal ions was determined for other reactions in which the substrates have the capacity to serve as chelating agents.[9,16a,17] Table V allows a comparison of catalytic

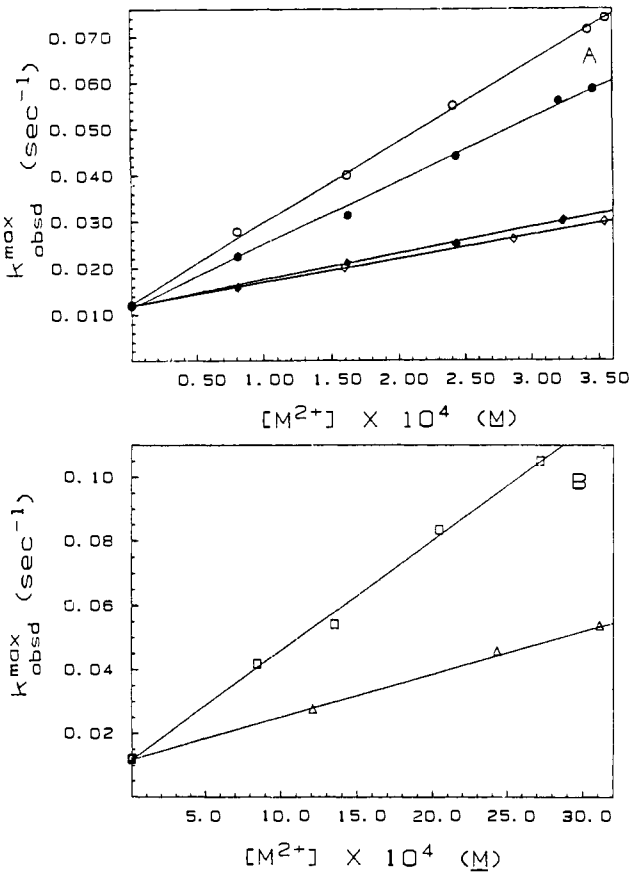


Figure 3. Metal ion catalysis of the dehydration of glyoxylate hydrate at 25.0 °C in DEM buffers, μ = 0.15: 3A, catalysis by Zn^{2+} (○), Cu^{2+} (●), Co^{2+} (◆), and Ni^{2+} (◇); 3B, catalysis by Cd^{2+} (□), and Mn^{2+} (△).

data among several reactions. Values of $k_{M^{2+}}$ are given in the table relative to k_{H_2O} to provide a common point of reference. It will be noted that in previous studies catalysis by divalent copper is greatest, catalysis by zinc, nickel, and cobalt is intermediate, and catalysis by cadmium and manganese is lowest.[9,16a,17] This general order of catalytic effectiveness parallels the predicted order of stabilities of complexes involving these divalent metal ions.[16b] We find for the dehydration of glyoxylate that catalysis by cadmium and manganese is relatively low, catalysis by zinc, nickel, and cobalt is higher and comparable, but catalysis by divalent copper is of average catalytic effectiveness. We note, however, differences in experimental conditions between the former work (μ = 1, 5.0 °C, 0.1 M diethyl malonate buffers) and the present work (μ = 0.15, 25.0 °C, 0.05 M diethyl malonate buffer). In addition, the relative abilities of the glyoxylate vs pyruvate molecules to accommodate the coordination geometries associated with the various metal ions may also be of catalytic importance.

Included in Table V are kinetic data from the metal ion catalyzed tautomerization between acetylacetone and acetylacetone enol. For this reversible reaction it has been suggested that metal

(15) Eigen, M.; Kustin, K.; Strehlow, H. *Z. Phys. Chem. (Munich)* **1962**, *31*, 140.

(16) (a) Pocker, Y.; Meany, J. E. *J. Phys. Chem.* **1968**, *72*, 655. (b) Eplattenier, F. L.; Murase, I.; Martell, A. E. *J. Am. Chem. Soc.* **1967**, *89*, 837.

(17) Meany, J. E. *J. Phys. Chem.* **1969**, *73*, 3421.

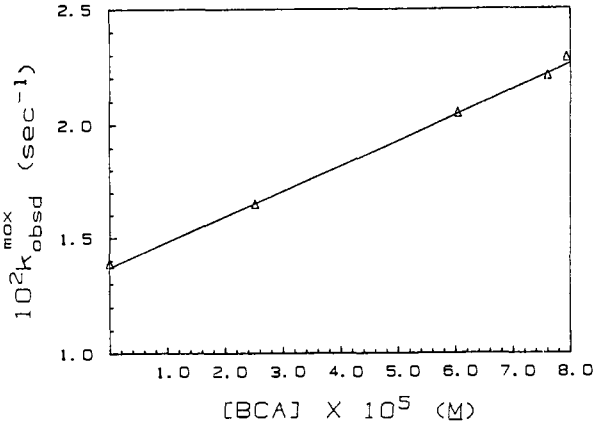
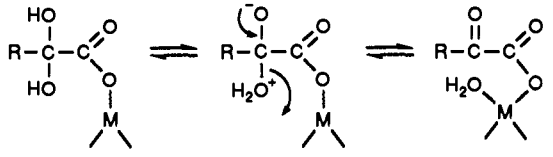


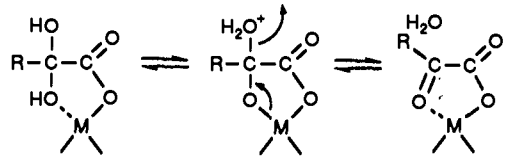
Figure 4. CA II catalysis of the dehydration of glyoxylate hydrate in 0.01 M phosphate buffer, pH 6.8, μ = 0.020, and 25.0 °C.

ions are bound by the acetylacetone/acetylacetone enol system, and thus serve in the capacity of general-acid catalysts.[17]

For the metal ion catalysis of the reversible dehydration of glyoxylate hydrate and pyruvate hydrate, two limiting mechanistic possibilities exist: (i) one in which the metal ion serves in the direct transfer of water;



(ii) one in which the metal ion serves as a general acid.



Both mechanisms are consistent with the proposal of Craig et al.[4c] regarding the intermediacy of vanadate–glyoxylate hydrate complexes in the vanadate-catalyzed dehydration of glyoxylate hydrate. Similar cyclic mechanisms were suggested earlier for the reversible hydrations of pyruvate[9] and for 2-pyridinecarboxaldehyde.[16a] While it is impossible to distinguish between mechanisms i and ii from the existing kinetic data, we note that the reversible hydration reactions, involving transfer of a water molecule, are more highly sensitive toward divalent metal ion catalysis than tautomerization reactions, which involve only proton transfers. Accordingly, for the reversible hydration reactions, the added role of the metal ion in the direct intramolecular transfer of water between the metal ion and the carbonyl carbon (mechanism i) is eminently reasonable.

The zinc metalloenzyme bovine carbonic anhydrase catalyzes not only the reversible hydration of CO_2 but also powerfully catalyzes the reversible hydration of aldehydes and pyruvate, as well as the hydrolysis of certain esters.[18] We have observed from our earlier studies that the best substrates of this enzyme prove to be compounds, the reactions of which are catalyzed by general bases and acids as well as by transition-metal ions or their complexes. Thus we anticipated that glyoxylate hydrate would serve as a particularly interesting substrate of CA II. Clearly, the enzyme reversibly hydrates glyoxylate ion, thereby providing a specific example of the hydration of an aldehyde. At the same time, the anionic glyoxylate hydrate is structurally similar to the bicarbonate anion, a natural substrate of carbonic anhydrase. Figure 4 shows the results of typical enzyme assay data in which k_{obsd}^{max} is shown to be linear with enzyme concentration. Table III shows that CA II in phosphate buffer is about one-half as catalytically active as zinc ions in solution for the dehydration of glyoxylate hydrate. A similar ratio (one-third) of catalytic activities was observed earlier for the dehydration of pyruvate hydrate.[9] Using the catalytic rate coefficient of water as the point of reference, we may also compare the enzymatic efficiency of CA II for the dehydration of glyoxylate hydrate (k_{enz}/k_{H_2O} = 5.8 × 10^5) with the corresponding calculation for the dehydration of pyruvate hydrate (k_{enz}/k_{H_2O} = 10.5 × 10^5) under similar experimental conditions. Although the values for k_{cat} and K_m were not determined in the present work, it is reasonable to assume that the low concentration of glyoxylate used in the present experiments is much lower than K_m and therefore $k_{enz} = k_{cat}/K_m$. In earlier studies of the CA II catalyzed hydration of aliphatic aldehydes,[8,19a] it was found that increasing the number of carbons on the substrate resulted in decreasing values of K_m. Thus, it is reasonable that the somewhat greater efficiency of CA II to catalyze the dehydration of pyruvate hydrate over glyoxylate hydrate is due to a smaller value of K_m for pyruvate hydrate.

Earlier work has shown that for the hydration of CO_2,[19b] the hydration of aldehydes,[8,19a] and the hydrolysis of esters,[20] the pH–rate profile for CA II catalysis is sigmoidally shaped having a point of inflection of about pH 7, with low activity on the acidic side and maximum activity on the basic side. The inference from these pH–rate profiles is that a basic group with a pK_a around neutrality is catalytically operative. In contrast, the enzymatic dehydration of bicarbonate ion produces a sigmoid curve, but one of opposite shape showing maximum activity at low pH, again with the point of inflection at around neutrality and minimum activity at high pH.[21] For this process, then, it is concluded that the acidic form of a catalytic group with a pK_a ca. 7 is catalytically active. Table VI summarizes the results of a study designed to show the variation of enzymatic activity by CA II for the dehydration of glyoxylate hydrate as a function of pH. Similar to the CA II catalyzed dehydration of bicarbonate, it is noted that enzymatic activity *decreases* in the region of pH around neutrality.

In the dehydration of bicarbonate one can envisage that an acid, HB^+, with a pK_a around neutrality (for example, the imidazolium group of protonated histidine) serves as a general acid to promote the dehydration of the zinc-bound substrate (mechanism iii). Due to the similarity of pH–rate dependencies for the dehydration of bicarbonate ion and glyoxylate hydrate, the mechanism represented by iv for the enzymatic dehydration of the latter is reasonable.[22,23]

Table VI. Dehydration of Glyoxylate Hydrate Catalyzed by Bovine Carbonic Anhydrase as a Function of pH at 25.0 °C

pH	k_{obsd}^{max} (s^{-1})	k_{buffer} (s^{-1})	[BCA] × 10^4	$k_{enz}^{BCA\,a}$ (M^{-1} s^{-1})
6.197	0.0196	0.0113	1.07	78 ± 1.9
6.375	0.0186	0.0116	1.09	64 ± 0.5
6.532	0.0181	0.0119	1.10	56 ± 1.2
6.931	0.0198	0.0136	1.08	57 ± 2.5
7.284	0.0229	0.0170	1.14	52 ± 2.6
7.653	0.0293	0.0254	1.04	38 ± 7.2

[a] Although the value of K_m for the BCA-catalyzed reaction was not determined, it can be assumed, in consideration of K_m values of other carbonyl substrates, that the concentration of glyoxylate used in these studies was much lower than K_m. Accordingly, $k_{enz}^{BCA} \simeq k_{cat}^{BCA}/K_m$.

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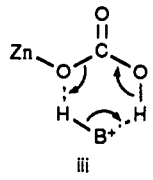
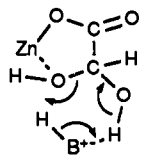
(20) (a) Pocker, Y.; Storm, D. R. *Biochemistry* **1968**, *7*, 1201. (b) Pocker, Y.; Stone, J. T. *Biochemistry* **1967**, *6*, 668.

(21) Kernohan, J. C. *Biochim. Biophys. Acta* **1965**, *96*, 304. Eigen, M.; Kustin, K.; Strehlow, H. *Z. Phys. Chem. (Munich)* **1962**, *31*, 140.

(22) The role of histidine-64 as a mediator of protons between the zinc-coordinated water or hydroxide and external buffer has been described in a previous paper.[23]



iii



iv

Thus, the present pH-rate studies suggest that the enzymatically catalyzed dehydration of glyoxylate hydrate is mechanistically similar to the dehydration of bicarbonate ion, and given the structural similarities between the two anionic substrates, glyoxylate hydrate may serve as a useful mechanistic probe for CA II.

In summary, we have demonstrated the validity of the assumption that unhydrated glyoxylate is the preferential substrate for its reduction to glycolate. This information has allowed a meaningful interpretation of the Michaelis constants for the LDH-catalyzed reduction of glyoxylate and the corresponding oxidation of glyoxylate hydrate. At the same time, the rate-limiting dehydration of glyoxylate hydrate at high concentrations of LDH allowed us to determine the catalytic rate coefficients for general acids, general bases, transition-metal ions, and carbonic anhydrase, providing an extension of our earlier work on the catalysis of the reversible hydration reactions of other carbonyl compounds. Aided by comparisons of the kinetic parameters between chemical catalysis and catalysis by CA II, we have shown that glyoxylate and its conjugate hydrate serve as a novel substrate pair for carbonic anhydrase. The glyoxylate substrate has provided a natural and important continuation of our earlier work involving acetaldehyde,[8] pyruvate,[9] and bicarbonate[10] as substrates of CA II, demonstrating the catalytic versatility of this enzyme.

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Registry No. Zn^{2+}, 23713-49-7; Cu^{2+}, 15158-11-9; Co^{2+}, 22541-53-3; Ni^{2+}, 14701-22-5; Cd^{2+}, 22537-48-0; Mn^{2+}, 16397-91-4; NADH, 58-68-4; LDH, 9001-60-9; BCA, 9001-03-0; glyoxylate, 298-12-4; glyoxylate hydrate, 563-96-2.

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Unified Strategy for Synthesis of Indole and 2-Oxindole Alkaloids

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Abstract: A concise and general entry to representative indole alkaloids of the yohimboid, heteroyohimboid, corynantheoid, and 2-oxindole classes has been developed exploiting a strategy that features intramolecular Diels-Alder reactions for the facile construction of the D/E ring subunits of the target alkaloids. The efficacy of the approach is first illustrated by a two-step total synthesis of the yohimboid alkaloid oxogambirtannine (**2**) from **22**. Thus, the Diels-Alder substrate **25**, which was prepared by nucleophilic addition of vinyl ketene acetal **24** to the intermediate *N*-acyliminium salt formed in situ upon reaction of **22** with **23**, was heated in the presence of benzoquinone to give a mixture of diastereoisomeric cycloadducts **26** and **27**; these adducts underwent spontaneous oxidation to furnish **2**. In another application of the strategy, the [4+2] heterocyclization of **34a**, which was formed upon nucleophilic addition of 1-[(trimethylsilyl)oxy]butadiene to the *N*-acyliminium salt generated in situ upon treatment of **22** with crotonyl chloride, afforded a mixture (ca. 9:1) of cycloadducts **35a** and **36a**. The major adduct **35a** was converted to **42a** using a general procedure for effecting β-carbomethoxylation of enol ethers to give vinylogous carbonates. Subsequent reduction of **42a** to the heteroyohimboid alkaloids (±)-tetrahydroalstonine (**3**) and (±)-cathenamine (**4**) was achieved by selective delivery of 2 or 1 equiv of hydride, respectively. When **42a** was treated with sodium amide, stereoselective β-elimination ensued to give **49**, which was converted by chemoselective hydride reduction into the corynantheoid alkaloid (±)-geissoschizine (**5**). Facile access to alkaloids of the 2-oxindole family was realized by using a new protocol for achieving stereoselective, oxidative rearrangements of β-carboline N_b lactams into 3,3-disubstituted 2-oxindoles. Thus, exposure of **42a** to *tert*-butyl hypochlorite followed by acid and silver ion induced rearrangement of the intermediate 3-chloroindolenine gave **50**, with only traces of the C(7) epimer being detected. Hydride reduction of **50** gave (±)-isopteropodine (**6**), acid-catalyzed isomerization of which furnished an equilibrium mixture (1:3) of **6** and (±)-pteropodine (**51**). The stereochemical course of the intramolecular hetero-Diels-Alder reaction of **34a** to give **35a** and **36a** as the only isolable cycloadducts was examined by computational analysis. The geometry of the six-atom transition state was established by semiempirical methods by using the standard closed-shell, restricted Hartree-Fock (RHF) version of the AM1 method. With use of this constrained geometry for the six-membered pericyclic array, the overall conformational energies for the four possible transition states **52-55** were minimized by MM2 calculations (MacroModel). The calculated relative energies of these transition states were in the order **52** < **53** < **54** < **55**. Since the cyclization of **34a** produced only **35a** and **36a** in an approximately 9:1 ratio via the respective transition states **52** and **53**, these calculations correlated qualitatively with the experimental results.

Introduction

Members of the indole family of alkaloids have long been subjects of scientific investigations.[1] These inquiries have been stimulated not only by the structural diversity of this family of alkaloids but also because the physiological and biological properties of some are legendary. The yohimboid, heteroyohimboid, and corynantheoid alkaloids, which are biosynthetically derived from the union of tryptophan and an unrearranged secologanin skeleton, constitute three major subgroups of the indole class. Typical of the yohimboid alkaloids is the presence of either a *cis*- or *trans*-hydroisoquinoline ring system, and in no known alkaloid is this fragment endowed with greater complexity than in reserpine (**1**).[2] At the other end of the spectrum lies oxogambirtannine

(1) For reviews of the monoterpenoid, indole alkaloids, see: (a) Cordell, G. A. *Introduction to Alkaloids, A Biogenetic Approach*; Wiley-Interscience: New York, 1981; pp 574-832. (b) Brown, R. T. In *Indoles. The Monoterpene Indole Alkaloids*; Saxton, J. E., Ed.; Wiley: New York, 1983; Chapter 4. (c) Saxton, J. E. *Specialist Periodical Reports, The Alkaloids*; The Royal Society of Chemistry, Burlington House: London, 1983; Vol. 13, pp 221-237; see also Vol. 1-12. (d) Szántay, C.; Blaskó, C.; Honty, K.; Dörnyei, G. In *The Alkaloids*; Brossi, A., Ed.; Academic Press: New York, 1986; Vol. 27, p 131.

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