A Comparative Study of the Enolization of Pyruvate and the Reversible Dehydration of Pyruvate Hydrate

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Received June 24, 1991

Abstract: The enolization of pyruvate and the reversible dehydration of pyruvate hydrate were studied at 25.0 °C using spectrophotometric methods. The enolization of pyruvate was followed at 353 nm by monitoring the rate of uptake of triiodide ion. The dehydration of pyruvate hydrate was initiated by introducing small quantities of preacidified solutions of pyruvic acid containing, at the kinetic zero, ca. 60% of the hydrate into buffer solutions. A decrease in absorbance at 325 nm took place as the reaction progressed to a final solution composition of 6% hydrate. The reactions were studied in acetate, MES, phosphate, arsenate, imidazole, 1-methylimidazole, HEPES, Tris, and borate buffers. The dehydration of pyruvate hydrate was found to be sensitive toward general-acid and general-base catalysis, while the enolization of pyruvate was catalyzed only by the basic components of the buffers studied. The corresponding rate coefficients were determined for the acidic and basic catalysts, and taking into account the appropriate statistical correction factors associated with the capacity of the catalysts to donate and accept protons, Brønsted plots were constructed. Brønsted coefficients were determined for enolization (β = 0.47) and for dehydration (α = 0.54, β = 0.52). While relatively normal catalytic behavior was observed for the enolization of pyruvate, deviations for the dehydration of hydrated pyruvate were noted. Analysis of these deviations, in light of a comparison of the relative magnitude of the catalytic rate coefficients for the reversible hydrations of other carbonyl compounds, suggests the possible contribution of a general-base catalytic path involving the intramolecular participation of the carboxylate group of hydrated pyruvate. The data are also considered in terms of the possible roles the rates of interconversion and positions of equilibria between keto, enol, and hydrated species may play in the physiological reactions of pyruvate. Finally, the Brønsted analysis provides the necessary basis for a comparison of the relative susceptibilities of the many substrates of carbonic anhydrase II including pyruvate hydrate.

Introduction

The substrates of many oxidoreductase enzymes are carbonyl compounds, most of which, in aqueous solution, exist as equilibrated mixtures of keto, enol, and *gem*-diol forms. Interconversions between these chemical species are catalyzed by acids and bases[2-15] and sometimes by trace concentrations of divalent metal ions[2,6a,d,e,7c,15,16] as well as by certain enzymes.[17,18] It has long been known that many enzymatic oxidations and reductions of carbonyl compounds are subject to what is commonly regarded as "substrate" inhibition.[19] Two questions arise regarding these enzymatic processes: (i) Which substrate form serves as the preferential substrate for the enzyme? (ii) Which substrate form is operative in enzymatic inhibition? The answers to these questions are not only required for the appropriate interpretation of kinetic data obtained in mechanistic studies but may also lead to a greater understanding of metabolic regulation.

The biochemical sources and fates of pyruvate are many. For example, pyruvate is the product of aerobic glycolysis and forms a bridge between the metabolism of carbohydrates and a number of amino acids. It is converted into acetyl coenzyme A which fuels the tricarboxylic acid cycle and is available for fatty acid synthesis. Depending on oxygen availability, it is reversibly reduced to lactate and, in the process of fermentation, forms ethyl alcohol. Each of the competing reactions involved is kinetically controlled with specific enzymes, assuming, of course, favorable free energies of reaction. Each enzyme, in turn, may depend in some way on the distribution and/or rates of interconversion between keto, enol, and hydrated pyruvate. For example, enol pyruvate is a substrate and product of the action of pyruvate kinase.[6e,17a,b,20] It also has been shown to be the substrate of transcarboxylase and pyruvate carboxylase.[17c] Keto pyruvate serves as the preferential substrate of lactate dehydrogenase catalyzed reduction by NADH,[21,22] and it has been reported that both hydrated pyruvate[21,23] and enol pyruvate[24] form ternary abortive complexes with lactate dehydrogenase and NAD^+.

Thus, the fate of pyruvate or pyruvic acid when introduced into aqueous solution is of physiological as well as of chemical interest. Since pyruvic acid ionizes and undergoes both enolization and

(1) (a) Seattle University. (b) McGoldrick Fellow, Seattle University, 1990–1991. (c) University of Washington.
(2) Craig, M. M.; Baff, M.; Gresser, M. J. *J. Am. Chem. Soc.* **1988**, *110*, 3302.
(3) Rendina, A. R.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5148.
(4) Sørensen, P. E.; Jencks, W. P. *J. Am. Chem. Soc.* **1987**, *109*, 4675.
(5) Gregory, D.; Goodman, P. A.; Meany, J. E. *Biochemistry* **1972**, *11*, 4472.
(6) (a) Miller, B. A.; Leussing, D. L. *J. Am. Chem. Soc.* **1985**, *107*, 7146. (b) Leussing, D. L. *J. Org. Chem.* **1990**, *55*, 666. (c) Emly, M.; Leussing, D. L. *J. Am. Chem. Soc.* **1981**, *103*, 628. (d) Leussing, D. L.; Emly, M. *J. Am. Chem. Soc.* **1984**, *106*, 443. (e) Covey, W. D.; Leussing, D. L. *J. Am. Chem. Soc.* **1974**, *96*, 3860.
(7) (a) Pocker, Y.; Meany, J. E. *J. Phys. Chem.* **1967**, *71*, 3113. (b) Pocker, Y.; Dickerson, D. G. *J. Phys. Chem.* **1969**, *73*, 4005. (c) Pocker, Y.; Meany, J. E. *J. Phys. Chem.* **1970**, *74*, 1486.
(8) Schellenberger, A.; Hubner, G. *Chem. Ber.* **1965**, *98*, 1938.
(9) Meany, J. E. *J. Phys. Chem.* **1969**, *73*, 3421.
(10) Hegazi, M.; Meany, J. E. *J. Phys. Chem.* **1972**, *76*, 3121.
(11) Albery, W. J.; Bell, R. P. *Trans. Faraday Soc.* **1965**, *61*, 1194.
(12) Burgner, J. W., II; Ray, W. J. *Biochemistry* **1974**, *13*, 4229.
(13) Germay, T.; Meany, J. E.; Pocker, Y. *Joint 45th Northwest/Rocky Mountain Regional Meeting, American Chemical Society* **1990**, Abstract No. 255.
(14) (a) Bruice, P. Y.; Bruice, C. *J. Am. Chem. Soc.* **1978**, *100*, 4793. (b) Bruice, P. Y. *J. Am. Chem. Soc.* **1983**, *105*, 4982.
(15) Meany, J. E.; Pocker, Y. *J. Am. Chem. Soc.* **1991**, *113*, 6155.
(16) Pocker, Y.; Meany, J. E. *J. Phys. Chem.* **1970**, *74*, 1486.
(17) (a) Kuo, D. J.; Rose, I. A. *J. Am. Chem. Soc.* **1978**, *100*, 6288. (b) Kuo, D. J.; O'Connell, L.; Rose, I. A. *J. Am. Chem. Soc.* **1979**, *101*, 5025. Kuo, D. J.; Rose, I. A. *J. Am. Chem. Soc.* **1982**, *104*, 3235.
(18) (a) Pocker, Y.; Meany, J. E. *J. Am. Chem. Soc.* **1965**, *87*, 1809. (b) Pocker, Y.; Meany, J. E. *Biochemistry* **1965**, *4*, 2535. (c) Pocker, Y.; Dickerson, D. G. *Biochemistry* **1968**, *7*, 1995.
(19) (a) Segel, I. H. *Enzyme Kinetics*; Wiley: New York, 1975. (b) Dixon, M.; Webb, E. C. *Enzymes*, 2nd ed.; Academic Press: New York, 1964.
(20) Dougherty, T. M.; Cleland, W. W. *Biochemistry* **1985**, *24*, 5875.
(21) Tienharra, R.; Meany, J. E. *Biochemistry* **1973**, *12*, 2067.
(22) Vennesland, B. J. *J. Biol. Chem.* **1955**, *212*, 787.
(23) Waddington, M. D.; Meany, J. E. *Arch. Biochem. Biophys.* **1981**, *211*, 447.
(24) Burgner, J. W., II; Ray, W. J. *Biochemistry* **1978**, *17*, 1654.

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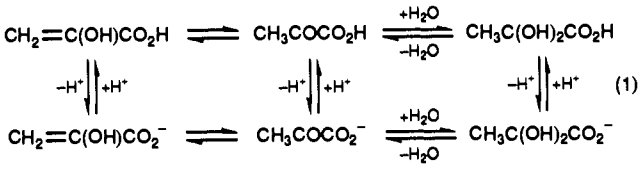
hydration, aqueous solutions contain a total of six chemical species in equilibrium:[25a-f]



(1)

The enol form accounts for a very small fraction of the total concentration of the pyruvate–pyruvic acid system in either neutral or acidic aqueous solution.[25a] Concentrations of the hydrated species, however, are much higher and vary with temperature and pH.[25a] Relatively high percentages of hydrate exist on the acidic side of the pK_a of pyruvic acid (pK_a = 2.2) while considerably lower percentages prevail on the basic side.[25a]

Thus, pyruvate is a molecule for which the rates of tautomerization and hydration can both be monitored and provides a unique opportunity to make direct kinetic and mechanistic comparisons between these reactions as they are catalyzed by acids and/or bases. The present paper describes the degree of sensitivity of the reversible hydration of pyruvate and its enolization to general-acid and -base catalysis. We studied a total of nine different buffers, most of which are, or have been, commonly used in the determination of kinetic parameters of enzymes and of related model systems. The catalytic rate coefficients of the acidic and basic components of these buffers were determined, and a comparison of Brønsted parameters is made.

Experimental Section

Kinetic studies were carried out in buffered solutions prepared from distilled, deionized water. Ionic strength was adjusted by the addition of the appropriate quantities of sodium sulfate. The buffer components, sodium pyruvate, pyruvic acid, sodium sulfate, and all other reagents, were used in their commercially available reagent grade forms. Identical kinetic results were obtained in the present work using either sodium pyruvate or pyruvic acid (with appropriate adjustments in pH). Commercially available pyruvic acid was twice distilled through a Vigreux column in an atmosphere of nitrogen gas; bp (18 Torr) 60–64 °C. All pyruvic acid solutions were prepared just prior to use.

The method of half-neutralization was used to obtain the pK_a values of the conjugate acid component of the buffers used. Measurements of pH were carried out on a Radiometer PHM84 research pH meter. The mean pK_a value was calculated for each set of buffers using the Henderson–Hasselbalch equation. Activity coefficients, $f_\pm$, were calculated from

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

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

Reaction rates were monitored on a Gilford high-speed recording spectrophotometer, Model 2000, or on a Hewlett-Packard diode array spectrophotometer, Model 8452A. The initiation of all kinetic runs was accomplished with the rapid mixing of the final reaction components by means of a small stirring paddle attached to the needle tip of the Hamilton microliter syringe.[26] Ultraviolet and visible absorption scans were carried out on a Varian Cary 210 instrument interfaced to an Apple IIe. The thermostated cell compartments of the spectrophotometers used were maintained at 25.0 ± 0.1 °C using a Forma-Temp Jr. bath and circulator.

The enolization of pyruvate was followed by its iodination as described in earlier work.[10-12] The kinetic runs were initiated by injecting 0.050 mL of a stock solution of 2.0×10^{-3} M iodine and 1.0 M potassium iodide into 3 mL of the buffer solution containing 0.10 M sodium pyruvate. The disappearance of the triiodide band was monitored at 353 nm (ϵ = 24 500 M^{-1} cm^{-1}). The pseudo-zero-order rate constants for the enolization reactions were calculated from the linear portions of the traces obtained, $k_{enol} = v/[\text{pyruvate}]$, which followed the initial "burst" in the uptake of iodine first described by Albery et al.[11] Typically, for each series of runs at a given buffer ratio, rate constants were calculated from the averages of duplicate runs carried out at five different buffer concentrations. Reproducibility was generally to within 1–2%.

Aqueous solutions of pyruvic acid at pH ≪2.2 consist predominantly of the hydrated form (e.g., 82% at 0.0 °C and 61% at 25.0 °C) while at higher values of pH the pyruvate ion is hydrated to a much lesser extent (e.g., 17% at 0.0 °C and 6% at 25.0 °C).[25a] Thus, the injection of an acidic solution of pyruvic acid into a neutral buffered solution causes the almost instantaneous deprotonation of hydrated pyruvic acid. The first-order dehydration is thereby initiated to yield a greater percentage of keto pyruvate.[7c] The shift in equilibrium results in an increase in absorbance due to the $n \rightarrow \pi^*$ transition associated with the carbonyl group. Spectrophotometric comparisons of acidic aqueous solutions of pyruvic acid (pH 1.3) with neutral solutions of pyruvate at 25.0 °C allowed the determination of the extinction coefficient for pyruvic acid (keto + hydrated), λ_{max} = 315 nm, ϵ = 7 M^{-1} cm^{-1}, and for pyruvate anion (keto + hydrated), λ_{max} = 325 nm, ϵ = 20 M^{-1} cm^{-1}.

In the present work, the dehydration of pyruvate hydrate was initiated by injecting 0.10 mL of an acidic solution of 0.227 M pyruvic acid (pH 1.3) into a reaction mixture containing the buffer to be studied along with the appropriate quantity of aqueous sodium hydroxide to neutralize the addition of acid. Measurements of pH before and after each kinetic run were shown to be virtually identical, ensuring the exact balance of added acid and base to the buffers. Following the rapid mixing of the pyruvic acid solution into the reaction mixture, the absorbance change monitored at 325 nm typically was from about 0.05 to 0.15 unit and strictly followed first-order kinetics. Rate constants k_{dehyd}, were determined from plots of ln $(A_\infty - A_0)$ vs t from data points taken over a time interval corresponding to approximately 90% of the reaction. For the dehydration studies, five buffer concentrations were studied at each buffer ratio, and runs at each concentration were run in triplicate. Reproducibility of these runs, for which the half-lives were as short as 6 s, was ±5%.

Results

For general-acid, general-base catalyzed reactions of pyruvate, the observed rate constants, k, refer to a series of terms, one for each acid and/or base present in the reaction mixture

$$k = k_0 + k_{OH^-}[OH^-] + k_{H_3O^+}[H_3O^+] + k_A[A] + k_B[B] + k_P[P] \quad (3)$$

where A and B represent the acidic and basic components of the buffer and P the pyruvate anion.

Defining the buffer ratio as $r = [A]/[B]$, eq 3 takes the form

$$k = k_0 + k_{OH^-}[OH^-] + k_{H_3O^+}[H_3O^+] + [A](k_A + k_B/r) + k_P[P] \quad (4)$$

If one carries out a series of kinetic runs in a given buffer at a fixed buffer ratio while varying simultaneously both the acidic and basic buffer components, a plot of k vs [A] is expected to be linear (assuming the absence of simultaneous catalysis by the acid and base) where the slope, S, and the intercept, I, have the following values:

$$S = k_A + k_B/r \quad (5)$$

$$I = k_0 + k_{OH^-}[OH^-] + k_{H_3O^+}[H_3O^+] + k_P[P] \quad (6)$$

Values of the catalytic rate coefficients for the acids and bases may then be determined from data collected at various buffer ratios from plots of S vs $1/r$. Values for the catalytic rate coefficients for hydroxide and hydronium ion (depending on the predominance of hydroxide or hydronium ion catalysis at the pH under investigation) and k_P are calculated from plots of I vs $[OH^-]$ or I vs $[H_3O^+]$ at a given pyruvate concentration. As in earlier work,[7a,15,27] these coefficients are calculated from the slopes of the lines obtained: k_{OH^-} = slope × $f_\pm$ or $k_{H_3O^+}$ = slope × $f_\pm$. Hydronium and hydroxide ion activities were computed from observed pH values extrapolated to zero buffer concentration for each series of runs.[7a,15,27,28a] By determining values of I at multiple

(25) (a) Pocker, Y.; Meany, J. E.; Nist, B. J.; Zadorojny, C. *J. Phys. Chem.* **1969**, *73*, 2879. (b) Strehlow, H. *Z. Elektrochem.* **1962**, *66*, 392. (c) Eigen, M.; Kustin, K.; Strehlow, H. *Z. Phys. Chem.* **1962**, *31*, 140. (d) Gold, V.; Socrates, G.; Crampton, M. R. *J. Chem. Soc.* **1964**, 5888. (e) Griffiths, V. S.; Socrates, G. *Trans. Faraday Soc.* **1967**, 673. (f) Becker, M. *Ber. Bunsenges. Phys. Chem.* **1964**, *68* (7), 669.

(26) Spyridis, G. T.; Meany, J. E. *J. Chem. Educ.* **1988**, *65*, 461.

(27) Pocker, Y.; Meany, J. E.; Jones, R. C. *J. Am. Chem. Soc.* **1982**, *104*, 4885.

(28) At a given buffer ratio small pH changes, generally on the order of 0.01–0.03 unit, occur over the range of buffer dilutions with salt solution of identical ionic strength.

Table I. General-Acid, General-Base Catalyzed Dehydration of Hydrated Pyruvate and Enolization of Pyruvate at 25.0 °C

dehydration					enolization		
base	pK_a (μ = 0.6)	k_B, M^{-1} s^{-1}	acid	k_A, M^{-1} s^{-1}	base	pK_a (μ = 0.35)	k_B, M^{-1} s^{-1}
OH^-	15.7	$(1.6 \pm 0.09) \times 10^4$	H_3O^+	$(1.1 \pm 0.09) \times 10^4$	OH^-	15.7	0.78
Tris	8.3	0.346 ± 0.019	HOAc	0.19 ± 0.07	borate	9.1	$(4.79 \pm 0.11) \times 10^{-4}$
HEPES[b]	7.6	0.0805 ± 0.005	$MESH^+$	0.08 ± 0.01	Tris	8.1	$(5.65 \pm 0.04) \times 10^{-4}$
Im	7.3	0.182 ± 0.005	$H_2PO_4^{2-}$	0.416 ± 0.012	Im	7.1	$(2.73 \pm 0.04) \times 10^{-4}$
CH_3Im	7.3	0.151 ± 0.004	$H_2AsO_4^{2-}$	0.294 ± 0.006	CH_3Im	7.0	$(1.65 \pm 0.04) \times 10^{-4}$
HPO_4^{2-}	6.6	0.214 ± 0.007	ImH^+	0.0998 ± 0.009	HPO_4^{2-}	6.6	$(2.86 \pm 0.02) \times 10^{-4}$
$HAsO_4^{2-}$	6.6	0.130 ± 0.005	CH_3ImH^+	0.0700 ± 0.006	$HAsO_4^{2-}$	6.0	$(3.61 \pm 0.01) \times 10^{-4}$
MES[b]	6.3	0.19 ± 0.02	$HEPESH^+$	0.0630 ± 0.006	OAc^-[a]	4.7	5.3×10^{-6}
OAc^-	4.7	0.06 ± 0.02	$TrisH^+$	0.0814 ± 0.011	H_2O	−1.74	$(5.4 \pm 1) \times 10^{-9}$
H_2O	−1.74	$(3.2 \pm 0.1) \times 10^{-4}$					

[a] See ref 8. [b] HEPES = *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MES = 2-*N*-morpholinoethanesulfonic acid.

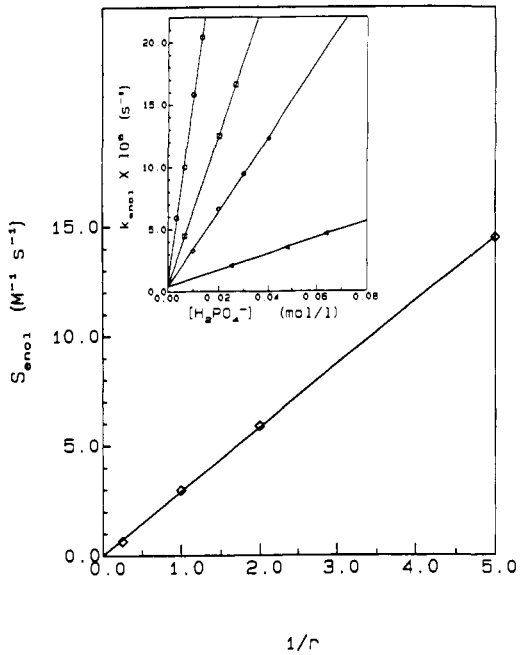


Figure 1. Phosphate-catalyzed enolization of pyruvate: plot of slope, S, vs $1/r$. Inset: Rate constants, k_{enol}, as a function of $[H_2PO_4^-]$ at various buffer ratios. Key: ○, r = 0.2; □, r = 0.5; ◇, r = 1.0; ▽, r = 4.0

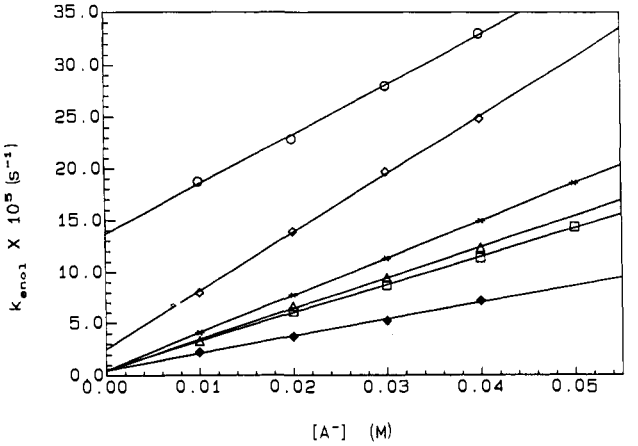


Figure 2. The general base catalyzed enolization of pyruvate. ○, borate; ◇, Tris; ✳, arsenate; △, phosphate; □, imidazole; ◆, 1-methylimidazole.

pyruvate concentrations, the kinetic term, k_0, commonly defined as $k_{H_2O}[H_2O]$, and k_P may be evaluated.

For the enolization of pyruvate in phosphate buffers, the above analysis was carried out at four different buffer ratios: r = 0.2, 0.5, 1.0, and 3.0. In the concentration ranges of the buffer components studied, plots of k_{enol} vs $[H_2PO_4^-]$ were linear (Figure 1, inset). The slope of the plot of S_{enol} vs $1/r$ was used to evaluate $k_{HPO_4^{2-}}$. It will be noted that the intercept value for the plot shown in Figure 1 is indistinguishable from zero, indicating that the reaction is relatively insensitive toward general-acid catalysis by acids in this pK_a range. Since we have considered catalysis in solutions around neutral pH, we may assume that the catalytic contributions of the acidic components of the other buffers studied are also small.

It will be noted from eq 3 that, in the absence of detectable catalysis by the general-acid component, plots of k vs [B] are expected to be linear for a series of runs at constant r as the concentrations of B and A are varied simultaneously. This relationship is shown in Figure 2 for borate, Tris, arsenate, phosphate, imidazole, and 1-methylimidazole buffers. The catalytic rate coefficients for the general bases were calculated from the slopes of the linear plots, and k_{OH^-} was determined from values of I_{enol} (eq 6) at the various hydroxide ion activities for each of the buffers used (Table I). These latter determinations were made using 0.10 M pyruvate solutions, where I_{enol} extrapolated to zero hydroxide ion concentration was evaluated: $k_0 + k_P[0.10\ M] = 4 \times 10^{-7}$ s^{-1}. When I_{enol}, extrapolated to zero hydroxide concentration, was determined using 0.01 M pyruvate, a slightly smaller value of $k_0 + k_P[0.01\ M] = 3 \times 10^{-7}$ s^{-1} was obtained. Thus, $k_0 = 3 \times 10^{-7}$ s^{-1} and $k_P = 1 \times 10^{-6}$ s^{-1}. We also list in Table I for comparison the value of k_{OAc^-} determined by Schellenberger and Hubner.[8]

The dehydration of hydrated pyruvate was studied in phosphate, arsenate, acetate, Tris, imidazole, 1-methylimidazole, HEPES, and MES buffers. For each buffer studied, sets of observed rate constants were determined at several buffer ratios and the data analyzed in terms of eq 4. The linearity of the associated plots precludes any significant contribution by a third-order kinetic term, implying substrate, general base, and general acid. The catalytic contributions of the acidic and basic components of the various buffers were determined from the plots shown in Figure 3A,B. We have grouped the data for buffers with anionic bases and those with nitrogen bases to avoid an overly complex set of plots. The intercept values are easily detectable, demonstrating the sensitivity of this reaction to catalysis by the acidic components of the buffers. The catalytic rate coefficients for the various acids and bases were calculated from the plots in Figure 3 and are listed in Table I. It will be noted that the kinetic determinations in acetic acid/acetate buffers involved greater degrees of uncertainty. In this region of lower pH the intercept values of the plots of k_{dehyd} vs [HOAc] were more scattered. Nevertheless, the approximate value for the catalytic rate coefficient for the acetate ion is included in Table I for comparison.

Intercept values (eq 6) were determined at various buffer ratios for each of the buffers studied.[28b] These values, I_{dehyd}, were plotted against hydronium ion concentration (for phosphate, arsenate and MES buffers; pH range 5.7–6.8) or hydroxide ion concentration (imidazole, 1-methylimidazole, HEPES, and Tris buffers; pH range 7.1–8.5). The catalytic rate coefficients, k_{OH^-} and $k_{H_3O^+}$ (Table I), were determined from intercept values where catalysis by only one of the species was clearly dominant. For data points close to the origin, small corrections were made to allow complete separation of the terms $k_0 + k_{H_3O^+}[H_3O^+]$ and $k_0 + k_{OH^-}[OH^-]$. Such corrections, when made, accounted for no more than 15% of the actual values of I_{dehyd}.

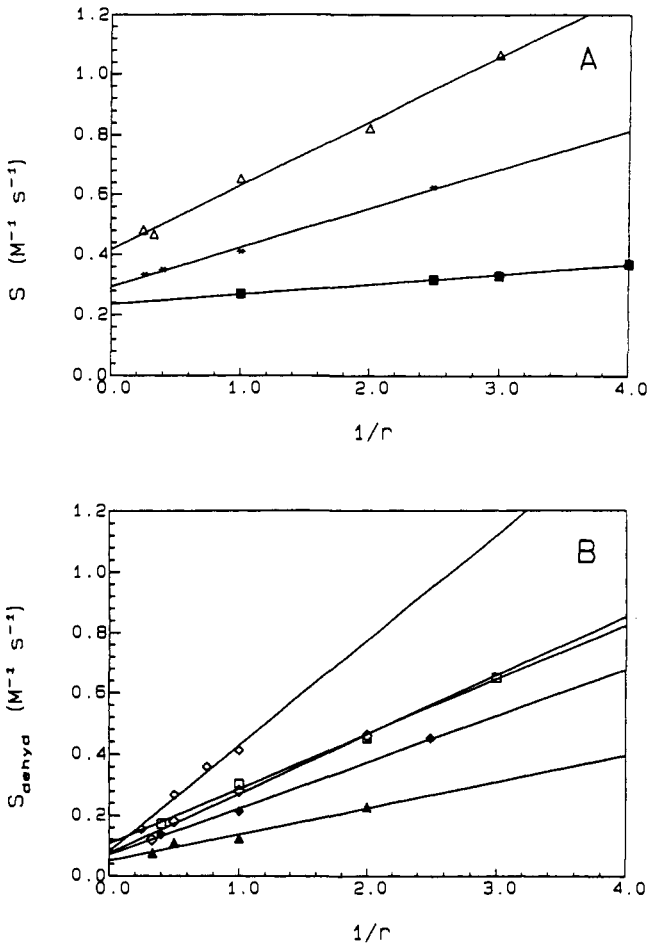


Figure 3. General-acid, general-base catalyzed dehydration of pyruvate hydrate. (A) Catalysis by anionic bases and their conjugate acids: △, phosphate; ✱, arsenate; ■, acetate. (B) Catalysis by nitrogen bases and their conjugate acids: ◇, Tris; □, imidazole; ◆, 1-methylimidazole; ▲, HEPES; ⬙, MES.

Brønsted plots were constructed for the enolization of pyruvate and the dehydration of pyruvate hydrate (Figure 4). It can be seen for the enolization reaction (Figure 4A) that the points fall reasonably well on the line. Even the data points for water, log (k_0/55.5) (not shown), and hydroxide ion were to within ca. 0.2 log unit of the line (negative deviation). Accordingly, the slope of the plot was determined by the catalytic coefficients associated with the series of general bases indicated in the figure legend. We found somewhat more scatter in the Brønsted plots for the general-acid- and general-base-catalyzed dehydration of pyruvate (Figure 4B,C). For each graph, the data point corresponding to the water term showed a high positive deviation. Following Sørensen and Jencks,[4] we calculated the slopes of these Brønsted plots for acid and base catalysis, omitting in each case the data point for "water catalysis". We excluded the data point for acetate from the least-squares analysis due to the approximate nature of the kinetic data determined in these buffers. We also note that the lower values of pH which prevailed in these latter studies were relatively close to the reported pK_a of hydrated pyruvate (3.7)[29a] and could thereby allow concurrent mechanistic channels for the dehydration reaction.

Discussion

The observed rate constants we report for the dehydration of pyruvate hydrate actually refer to those for the equilibration between keto and hydrated forms and therefore are sums of rate constants: $k_{equil,hyd} = k_{hyd} + k_{dehyd}$. The equilibrium constant for the reversible hydration of pyruvate, P, to its hydrate, H, at 25.0 °C was determined in earlier work:[25a] $K_{hyd} = [H]/[P] = k_{hyd}/k_{dehyd} = 0.064$. Accordingly, values of k_{hyd} and k_{dehyd} were calculated from k_{equil} and are listed in Table II.

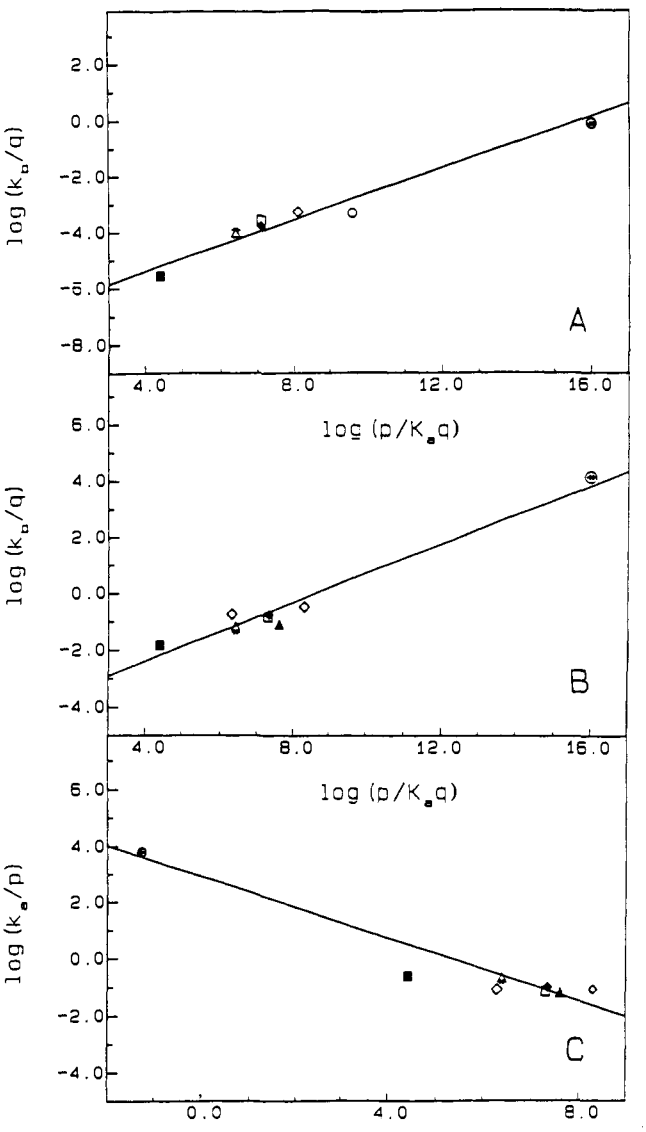


Figure 4. Brønsted catalysis plots: (A) enolization of pyruvate by general bases; (B) dehydration of pyruvate hydrate by general bases; (C) dehydration of pyruvate hydrate by general acids. Key: ■, acetate; ◇, MES; △, phosphate; ✱, arsenate; ◆, 1-methylimidazole; □, imidazole; ▲, HEPES; ◇, Tris; ○, borate; ⊛, hydroxide ion; ⊕, hydronium ion.

Since our kinetic data for the enolization reaction were determined from rates of iodination of enolpyruvate, E, formed in the rate-determining step, the observed rate constants refer to k_{enol}. The equilibrium constant for this reaction, K_{enol}, can be estimated from our data taken together with that in the literature. Kuo, O'Connell, and Rose[17b] have determined the half-life at 20 °C for the ketonization of enolpyruvate in dilute aqueous solution (in the absence of catalytically significant concentrations of acids or bases) to be approximately 30 s.[29b,c] From this measurement, $k_0 = 0.023$ s^{-1}, which when corrected to correspond to 25.0 °C becomes ca. 0.035 s^{-1}. Using our spontaneous rate constant for enolization, we calculate $K_{enol} = k_{enol}/k_{keto} = 3 \times 10^{-7}$ s^{-1}/0.035 s^{-1} = 9×10^{-6}.

Miller and Leussing[6a] determined the catalytic rate coefficient for acetate for the ketonization of enolpyruvate, $k_{keto,OAc^-} = 0.65$ s^{-1}, while Schellenberger and Hubner[8] obtained the corresponding coefficient for the iodination, $k_{enol,OAc^-} = 5 \times 10^{-6}$ s^{-1}, which

(29) (a) Fisher, M.; Warneck, P. *Ber. Bunsenges. Phys. Chem.* **1991**, *95*, 523. (b) After submission of this paper to this journal for review, a value for the half-life for the ketonization reaction was estimated (see ref 29c) to be about twice that given in ref 17b. (c) Peliska, J.; O'Leary, H. *J. Am. Chem. Soc.* **1991**, *113*, 1841. (d) Kresge, J. A. *Pure Appl. Chem.* **1991**, *63*, 213.

(30) Bell, R. P.; Rand, M. H.; Wynne-Jones, K. M. A. *Trans. Faraday Soc.* **1956**, *52*, 1093.

Table II. Comparison of Catalytic Rate Coefficients (M^{-1} s^{-1}) for the Reversible Hydration and Enolization of Pyruvate

	reversible hydration: K_{hyd} = [H]/[P] = 0.064			reversible enolization: K_{enol} = [E]/[P] = 8.2×10^{-6}		
catalyst	$k_{equil,hyd} = k_{hyd} + k_{dehyd}$	k_{hyd}	k_{dehyd}	$k_{equil,enol} = k_{enol} + k_{keto}$	k_{enol}	k_{keto}
OH^-	1.6×10^4	9.6×10^2	1.5×10^4	9.5×10^4	0.78	9.5×10^4
boratee				58	4.79×10^{-4}	58
Tris	0.346	0.021	0.33	69	5.65×10^{-4}	69
HEPES	0.0805	0.0048	0.076			
Im	0.182	0.011	0.17	43	2.73×10^{-4}	33
CH_3Im	0.151	0.0091	0.14	20	1.65×10^{-4}	20
HPO_4^{2-}	0.214	0.013	0.20	35	2.86×10^{-4}	35
$HAsO_4^{2-}$	0.130	0.0078	0.12	44	3.61×10^{-4}	44
MES	0.19	0.0114	0.18			
OAc^-	0.06	0.0036	0.056	0.65	5.3×10^{-6}	0.65
H_2O	3.2×10^{-4}	1.92×10^{-5}	3.0×10^{-4}	6.6×10^{-4}	5.4×10^{-9}	6.6×10^{-4}
H_3O^+	1.1×10^4	6.6×10^2	$1.0 \times 10^{+4}$			
HOAc	0.19	0.011	0.18			
$MESH^+$	0.08	0.0048	0.075			
$H_2PO_4^-$	0.416	0.025	0.39			
$H_2AsO_4^-$	0.294	0.018	0.28			
ImH^+	0.0998	0.0060	0.094			
CH_3ImH^+	0.0700	0.42	0.066			
$HEPESH^+$	0.0630	0.0038	0.066			
$TrisH^+$	0.0814	0.0049	0.77			

becomes 5.3×10^{-6} s^{-1} after correction for the fraction of pyruvate in the hydrated form. Thus, K_{enol} = [E]/[P] = k_{enol}/k_{keto} = 8.2×10^{-6}, which is in excellent agreement with our value calculated above. Finally, Kresge[29d] reports equilibrium constants at 25 °C which were determined using two methods: $K_{enol} = 7.6 \times 10^{-6}$ from the ratio of hydroxide ion catalytic rate coefficients for the enolization and ketonization reactions, respectively, and K_{enol} = 1.1×10^{-5} from the initial burst in the uptake of triiodide ion.

Rate constants for equilibration between keto and enol forms, $k_{equil,enol}$, and for the ketonization, k_{keto}, were calculated from our experimental data and the value of $K_{enol} = 8.2 \times 10^{-6}$ and are included in Table II.

The most obvious conclusion one draws from the data in Table II, taken together with the magnitude of the equilibrium constants for the two reactions under consideration, is that the hydration of pyruvate is much more rapid and thermodynamically much more favorable than the enolization. However, the spontaneous rates of equilibration for both reactions are relatively rapid. Extrapolated to zero buffer concentration at neutral pH and 25.0 °C, the reversible enolization is about twice as fast as the reversible hydration ($\tau_{1/2hydration}$ = 39 s and $\tau_{1/2enolization}$ = 19 s). One may predict that these approximate rates of equilibration prevail, for example, in the cytoplasm of cells although account must also be taken of catalytic components of existing general bases and divalent metal ions which also catalyze both reversible reactions[6a,16] and the fact that the reversible hydration is catalyzed by general acids as well. The data also suggest that the specific acid catalytic component for the latter reaction would not be significant under conditions of physiological pH. Finally, pyruvate concentrations would be too low under physiological conditions for the catalytic contribution by pyruvate ion to be important.

The kinetic and thermodynamic parameters associated with the reversible hydration and enolization of pyruvate may be used to access the likelihood of each of the different forms of pyruvate as potential participants either as enzyme substrates or as enzyme inhibitors. The data provide a basis to determine whether there exists a high enough concentration of a given form of pyruvate to explain a hypothetical kinetic scheme or whether a given form of pyruvate may be formed rapidly enough to be consistent with known kinetic parameters. Such data have also been used to design experiments aimed at distinguishing between keto, enol, and hydrated pyruvate as the preferential substrate[21] or "substrate" inhibitor[23] of lactate dehydrogenase.

We have demonstrated that the reversible hydration reaction is sensitive toward catalysis by the acidic components of the buffers used in this study (Figure 3; Table I). In fact, we find that the tendency toward catalysis by general acids relative to their conjugate bases is considerably greater for the reversible hydration of pyruvate than for aliphatic aldehydes.[7a,b] Catalysis of the reversible hydration of pyruvate by H_3O^+ also seems to be unusually potent. Whereas catalyses by hydronium and hydroxide ions are similar for this reaction (Table I), catalysis by H_3O^+ is only 1/60th that by OH^- for the corresponding reaction of acetaldehyde.[7b] Table III compares the "spontaneous" rate constants and the catalytic rate coefficients for the hydronium ion, hydroxide ion, and the acidic and basic components of phosphate buffers for the reversible hydrations of acetaldehyde, glyoxylate, and pyruvate. Acetaldehyde and glyoxylate were chosen as model substrates for comparison because their hydrations exhibited Brønsted β coefficients of around 0.5.

Table III. Comparison of Catalytic Rate Coefficients, $k_{equil,hyd} = k_{hyd} + k_{dehyd}$, for the Reversible Hydration of Carbonyl Compounds

	acetaldehyde[a]	glyoxylate[b]	pyruvate[c]
β	0.47[d,e]	−0.5	0.52
k_0 (s^{-1})	1.6×10^{-3}	1.8	1.8×10^{-2}
$k_{H_3O^+}$ (M^{-1} s^{-1})	186		1.1×10^4
k_{OH^-} (M^{-1} s^{-1})	1.1×10^4	4.0×10^6	1.6×10^4
$k_{H_2PO_4^-}$ (M^{-1} s^{-1})	0.27	17	0.42
$k_{HPO_4^{2-}}$ (M^{-1} s^{-1})	0.60	62	0.21
k_{OH^-}/k_0 (M^{-1})	6.9×10^6	2.2×10^6	8.9×10^5
$k_{HPO_4^{2-}}/k_0$ (M^{-1})	380	34	12

[a] See ref 7a,b. [b] See ref 15. [c] Present work. [d] See ref 30. [e] Sørensen and Jencks[4] have determined β = 0.39 for the dehydration of acetaldehyde hydrate catalyzed by carboxylate anion bases in the pK_a range 2.2–4.6.

As expected, we observe for the reversible hydration of pyruvate that the ratios of catalytic coefficients for bases to those for their conjugate acids, k_B/k_A, are dependent on the pK_a's of the acids. There also seems to be some dependency on the charge type of the catalyst as well. The data in Table I indicate that, for the anionic bases, $k_B/k_A < 1$, whereas, for the neutral zwitterionic nitrogen bases, $k_B/k_A > 1$. For the corresponding reactions of aliphatic aldehydes,[7a,b] catalysis by general bases with pK values around neutrality is consistently stronger than that by their conjugate acids regardless of the charge types of the catalysts. For example, for acetaldehyde, $k_{HPO_4^{2-}}/k_{H_2PO_4^-}$ = 2.2 (Table III), and the ratios are similar for other aliphatic aldehydes.[7b] The different catalytic behavior exhibited by the reversible hydration of pyruvate may be due in part to electrostatic repulsion which develops in the transition state when the reaction of the pyruvate anion is catalyzed by anionic bases.

From our earlier work[15] with the corresponding dehydration of glyoxylate hydrate, the diminution in the catalytic potency of anionic general bases was not observed (Table III). For this latter reaction the more favorable solvation of the transition state by water (due to the absence of the methyl group of pyruvate) may compensate for the buildup of negative charge. Alternatively, as

has been suggested earlier, several water molecules may be more specifically involved in a cyclic transition state through bridging, thereby allowing the necessary spatial separation of the carboxylate group and the anionic catalyst.[15]

We found the enolization of pyruvate to be general-base catalyzed but relatively insensitive to catalysis by the acidic components of the buffers used in the present work. This observation is consistent with the studies of Miller and Leussing[6a] who reported that the catalytic rate coefficient for acetate is 5 times greater than for its conjugate acid. Indeed, one would expect much lower catalytic contributions for the general acids studied in the present work where the pK_a values are at least 2 log units higher than that for acetic acid. The relative insensitivity of the *ketonization* of enolpyruvate in phosphate buffers was observed by Peliska and O'Leary.[29c]

Available data (Table I and ref 31) suggest that this reaction may be less sensitive to the charge type of the general bases than was observed for the reversible hydration. For example, the same order of catalytic effectiveness was observed for the enolization of pyruvate anion: arsenate > phosphate > imidazole as was observed earlier for the enolization of the electrically neutral acetone molecule.[31] One might expect the sensitivity of catalysis for the enolization of pyruvate toward bases of different charge types to be less than that for the reversible hydration. In the formation of the transition state for enolization, the attack by the general base occurs at the end of the pyruvate molecule, opposite to the carboxylate group.

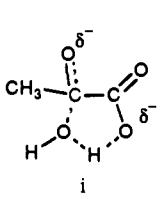
Brønsted coefficients were calculated from the data in Figure 4: enolization, $\beta = 0.47$; hydration, $\alpha = 0.54$, $\beta = 0.52$; using the assumptions made in the Results.[32a] We recognize that the strict relationship between Brønsted coefficients and the degree of proton transfer at the time of transition state development is perhaps naive. However, for the reactions in the present investigation it is not unreasonable to assume that the exchange of protons between pyruvate and the various acid and base catalysts studied is ca. half complete when the respective transition states are fully developed.

The Brønsted plot for the enolization of pyruvate shows little in the way of deviations. The data point for hydroxide ion is to within about 0.2 log unit of the line, indicating that it serves as a general base. Normal Brønsted behavior has also been observed[6c,14a,6d] for the enolization of oxaloacetate dianion catalyzed by imidazole, hindered tertiary amines, DPEA, and several oxyanions. From the published data, one can calculate $\beta = 0.38$ and ascertain that the data point for hydroxide ion conforms well to Brønsted general-base behavior just as it does in the enolization of pyruvate. Finally, the close conformity of the data point for water (deviation −0.26 log unit) indicates relatively good Brønsted behavior for the enolization of pyruvate over an extensive pK_a range.

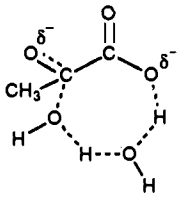
For the general-base-catalyzed reversible hydration of pyruvate deviations are observed for hydroxide ion (+0.5 log unit) and water. Catalytic rate coefficients for the hydration of the carbonyl group of oxaloacetate were also found to be large for hydroxide ion and water.[6c,13b] Large, positive deviations for catalysis by hydroxide ion have been observed in a number of related reactions, for example, the reversible hydration of acetaldehyde,[7a,30] decomposition of acetaldehyde hemiacetals,[4] and hydration and hydrolysis of methyl pyruvate ester,[27] all of which are reactions where hydroxide ion is believed to participate directly as a nucleophile rather than as a general base. In fact, for such reactions the positive deviations observed are generally in the range of 1–2 log units.[4,7a,27,30] The smaller positive deviation noted for the reversible hydration of pyruvate may be due to a decrease in stability of the transition state brought about by the close proximity of the negative charges of the hydroxide nucleophile and the carboxylate group of the substrate.

The positive deviation for the data point for water is relatively large (+1.7 log units). A related analysis would compare ratios k_B/k_0 for the reversible hydration of pyruvate with the corresponding ratios for the hydration of acetaldehyde and glyoxylate. For example, we note considerably larger values of $k_{HPO_4^{2-}}/k_0$ for the reversible hydration of acetaldehyde[7a] and other aliphatic aldehydes[7b] than for the corresponding reaction of pyruvate (Table III). Again, this reflects an unusually large "spontaneous" rate for the latter.

One explanation for the larger than expected value for k_0 for the reversible hydration of pyruvate is that there exists an intramolecular general-base-catalyzed component involving either (i) the direct interaction of carboxylate with the participating water molecule or (ii) the indirect interaction of carboxylate with the participating water through bridging by a second water molecule.



i



ii

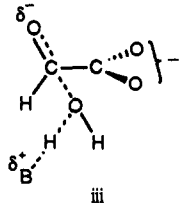
If intramolecular catalysis is indeed operative, one may represent the apparent spontaneous rate constant as the sum of terms: $k_0 = k_{H_2O}[H_2O] + k_{intra}$.

One can demonstrate the reasonableness of attributing the relatively high value of k_0 to an intramolecular general-base-catalyzed path by calculating the effective concentration of the carboxylate group at the reaction site which would be necessary to result in k_0. The pK_a of hydrated pyruvate has been reported,[29a] $pK_a = 3.7$, so that the catalytic rate coefficient for its carboxylate group may be approximated from our Brønsted plot, $k_{cat} = 0.0025$ M^{-1} s^{-1}. Since the data point originally assigned for H_2O catalysis is almost 2 log units above the Brønsted line, one may assume that k_0 represents almost exclusively the first-order rate constant associated with the intramolecular catalytic path, $k_{intra} = 0.0178$ s^{-1}. This would imply an effective catalytic concentration of the carboxylate group at the reaction site of 0.0178 s^{-1}/0.0025 M^{-1} s^{-1} = 7.1 M. This effective catalytic concentration is considerably smaller than 55.5 M, which at one time was considered to be the upper limit for intramolecular general-base catalysis.[32b] More recently in fact, Page and Jencks[32c] have suggested that maximum effective concentrations of neighboring catalytic groups in solution may be much larger. Thus, if an intramolecular catalytic path is indeed operative for the reversible hydration of pyruvate, its contribution is relatively small. This is not surprising since the reacting carbonyl group (or *gem*-diol group) and the carboxylate group can easily attain many alternative conformations in which the latter is catalytically unreactive.

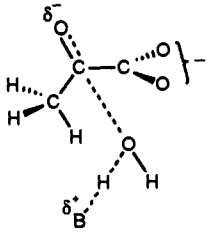
In contrast to the reversible hydration of pyruvate, the ratio $k_{HPO_4^{2-}}/k_0$ for the corresponding reaction of glyoxylate[15] is closer to those observed for the hydrations of aliphatic aldehydes (Table III). This may be an indication that the intramolecular catalyzed path we have hypothesized for the reversible hydration of pyruvate is not as important for glyoxylate. In this regard, we suggest that for the latter reaction a transition state with the required positioning of the general base is sterically unimpeded, thereby allowing the intermolecular catalyzed paths to effectively compete with the intramolecular reaction (structure iii). In contrast, the site of reaction of pyruvate is flanked on one side by methyl and on the other by the carboxylate group. These groups would be expected to retard the intermolecular reaction (by requiring the presence of an additional molecule or ion of general base in the transition state, structure iv) relative to the intramolecular process (utilizing only the carboxylate group already present and positioned appropriately, structure i or ii).

(31) Spaulding, J.; Stein, J. E.; Meany, J. E. *J. Phys. Chem.* **1977**, *81*, 1359.

(32) (a) It is realized that α for the dehydration reaction is tentative since the value may partially reflect the inherent uncertainties associated with the data point for H_3O^+. (b) Koshland, D. E., Jr. *J. Theor. Biol.* **1962**, *2*, 75. (c) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678. (d) We note that transition states v and vi could exist as seven-membered rings, similar to transition state ii, in which an additional water molecule could participate as a bridge.

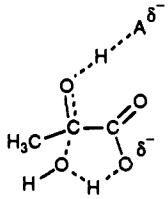


iii



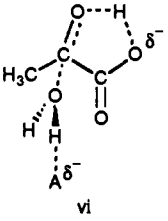
iv

The absence of a third-order kinetic term for the dehydration of hydrated pyruvate involving substrate, general base, and general acid precludes a mechanism in which the base and conjugate acid act simultaneously. However, the observed tendency for stronger catalysis by acids and the unusually large value of $k_{H_3O^+}$ for the reversible hydration of pyruvate in comparison to the corresponding reaction of aliphatic aldehydes may imply the involvement of a mechanism different from, or in addition to, the simple concerted general-acid catalytic mode. One possibility would be the participation of a mechanistic path involving a transition state in which *intramolecular* general-base catalysis by carboxylate is coupled with *intermolecular* general- (or specific-) acid catalysis (structure v).[32d]



v

An alternative would be a mechanistic parallel to that proposed by Leussing[6b] for what had appeared to be the general-acid-catalyzed enolization of oxaloacetate dianion. He reported that the actual mechanism involved preprotonation of the dianionic substrate by the acid followed by the simultaneous action of general-base catalysis and intramolecular acid catalysis by the 4-CO_2H group. The corresponding mechanism for the reversible hydration of pyruvate would involve transition state vi.[32d] The



vi

available kinetic data cannot distinguish between mechanisms proceeding through transition states v or vi. Neither can be excluded on the basis of diffusion control limitations.

For the enolization of pyruvate, Figure 2 indicates that there exists no significant simultaneous catalysis by the acids and bases studied. The close conformity of the data point for water on the Brønsted plot for this reaction suggests no detectable intramolecular catalytic component. General-base-catalyzed enolization reactions of carbonyl compounds involve the direct attack of the base on the carbon-bound hydrogen[33] as opposed to the intervention by one or more molecules of water between the substrate and the general base as is the case for reversible hydration reactions. A five-membered ring transition state would be involved if the carboxylate group of pyruvate directly attacked a carbon-bound proton from the methyl group. It would appear that the number of degrees of freedom which would be lost in order to develop such a transition state prevents this mechanistic path from contributing effectively to the overall reaction rate.

The question of intramolecular general-acid catalysis arose earlier for the enolization and hydration of pyruvic *acid* at low values of pH.[8,11,34] However, it was shown for both these reactions[9,10,35] that the spontaneous rate constants for methyl and pyruvate esters (for which the intramolecular catalyzed path is impossible) are almost identical to that for pyruvic acid. The relatively low values of $k_{H_3O^+}$ determined for these reactions and the absence of detectable catalysis by general acids[9,10,11,35] indicate a lack of susceptibility toward catalysis by acids, an observation which is not surprising for such electrophilic substrates. The relatively large spontaneous rates of enolization and hydration of pyruvic acid and the pyruvate esters may simply be attributed to the high reactivity of these substrates brought about by the strong electron-withdrawing effect of the protonated carboxyl group or alkyl carboxyl group.

We have shown in our previous work that the enzyme, carbonic anhydrase II from bovine erythrocytes (BCA II), catalyzes the reversible hydration of a wide variety of carbonyl compounds and the hydrolysis of esters.[15,16,18,36] The molecular activity of BCA II was reported earlier,[13] $k_{BCA} = 420\ M^{-1}\ s^{-1}$, for the dehydration of pyruvate hydrate in 0.05 M diethyl malonate buffers, pH 6.9 at 25.0 °C. Brønsted catalysis plots provide appropriate standards to allow valid comparisons to be made of the relative effectiveness with which this enzyme catalyzes these various reactions. For example, assuming a pK_a for BCA II around neutrality,[37] the data point for enzymatic catalysis shows a deviation of +3.5 log units from the Brønsted line, a deviation which is slightly higher than those observed for the BCA-catalyzed reversible hydrations of aliphatic aldehydes. The data point for the corresponding enzymatic reaction of a glyoxylate shows a somewhat smaller deviation of approximately +2.2 units.[15] Although the values for k_{cat} and K_m are not yet available for pyruvate or glyoxylate, it is reasonable that the concentrations of these substrates used in the enzyme assays to which we refer[13,15] were lower than K_m so that $k_{enz} = k_{cat}/K_m$. For the carbonic anhydrase catalyzed hydration of aliphatic aldehydes, it was found that K_m decreased with increasing numbers of carbon atoms on the substrate. Thus, the greater capacity of carbonic anhydrase to catalyze the reversible hydration of pyruvate over glyoxylate (relative to their corresponding buffer catalyzed reactions) may be the result of a smaller value of K_m for the former.

Acknowledgment. We thank the Muscular Dystrophy Association for their generous support of the experiments carried out at the University of Washington. One of us (J.E.M.) thanks the Sabbatical and Leave Faculty Development Committees of Seattle University for their support of this project and the National Science Foundation (Grant CSI-8650548) for providing funds for spectrophotometric instrumentation. Finally we thank Greg Spyridis for his helpful comments and expert assistance in the preparation of this paper.

Registry No. Pyruvate, 57-60-3; pyruvate hydrate, 27998-78-3.

(33) (a) Bell, R. P. *The Proton in Chemistry*, 2nd ed.; Cornell University Press: New York, 1973; pp 194–225. (b) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; p 243. (c) Pocker, Y. *Chem. Ind. (London)* **1958**, 1117; **1959**, 1383. (d) Huang, H. H.; Robinson, R. R.; Long, F. A. *J. Am. Chem. Soc.* **1966**, *88*, 1866. (e) Goodall, D. M.; Long, F. A. *J. Am. Chem. Soc.* **1968**, *90*, 238. (f) Walters, E. A.; Long, F. A. *J. Am. Chem. Soc.* **1969**, *91*, 3733.

(34) Strehlow, H. *Z. Elektrochem.* **1962**, *66*, 392. Eigen, M.; Kustin, K.; Strehlow, H. *Z. Phys. Chem.* **1962**, *31*, 140.

(35) Pocker, Y.; Zadorojny, C.; Meany, J. E. *J. Phys. Chem.* **1971**, *75*, 792.

(36) (a) Pocker, Y.; Meany, J. E.; Davis, B. C. *Biochemistry* **1974**, *13*, 1411. (b) Kaiser, E. T.; Lo, K. W. *J. Am. Chem. Soc.* **1969**, *91*, 4912. (c) Pocker, Y.; Sarkanen, S. *Biochemistry* **1978**, *17*, 1110. (d) Rickli, E. E.; Edsall, J. T. *J. Biol. Chem.* **1962**, *237*, 258. (e) Rickli, E. E.; Ghanzanfar, S. A. S.; Gibbons, B. H.; Edsall, J. T. *J. Biol. Chem.* **1964**, *239*, 1065. (f) Thorslund, A.; Lindskog, S. *Eur. J. Biochem.* **1967**, *3*, 117. (g) Verpoorte, J. A.; Mehta, S.; Edsall, J. T. *J. Biol. Chem.* **1967**, *242*, 4221. (h) Henkens, R. W.; Sturtevant, J. M. *J. Am. Chem. Soc.* **1968**, *90*, 2669.

(37) The values of pK_a for carbonic anhydrase have been determined from inflection points of pH–rate profiles. See, for example, refs 15, 16, 18, and 36.