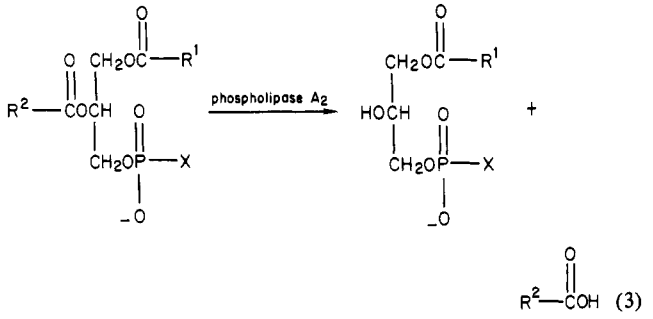
hydrolysis of esters to carboxylic acids. Indeed, the criteria for direct nucleophilic catalysis as enunciated by Jencks[4b] are eminently fulfilled in the system here presented: (1) the desolvated carboxylate ion is more nucleophilic than the final acyl acceptor (i.e., water); (2) the intermediate mixed anhydride is more reactive than the starting ester; and (3) the intermediate is thermodynamically less stable than the final product (i.e., the carboxylic acid).

The biochemical significance of the reaction between "naked" acetate and *p*-nitrophenyl *o*-toluate is in that it provides a chemical precedent for a possible route by which active-site carboxylates might participate in enzyme-catalyzed acyl transfer reactions. Mechanistic proposals implicating "buried" residues of metalloproteases acting as direct nucleophiles have been put forward in connection with carboxypeptidase A and thermolysin.[11] It appears, however, that the system here described is more closely related to the possible mechanism of action ester-hydrolyzing lipolytic enzymes such as phospholipase A_2 (eq 3). Structural analysis



(3)

has shown that this enzyme has no catalytic serine residues. X-ray crystallographic data on pancreatic porcine phospholipase have revealed that an aspartate residue might be involved in the catalytic functioning of the enzyme.[3] This has been further supported by chemical-modification studies implicating an essential carboxylate residue in the pancreatic phospholipase.[12] Thus, formation of a catalytically competent acyl-enzyme intermediate in the form of an anhydride might be a particularly attractive proposal for the first step in the enzymatic hydrolysis of the acyl linkage at the glycerol 2 position of the phospholipid substrate. In analogy with the amide to ester interconversion involved in the mechanism of serine proteases, the ester to anhydride sequence could provide a catalytically feasible path en route to the final products, including the free fatty acid and the secondary alcohol moiety. The conformational flexibility of the protein could readily provide the suitable microenvironment necessary for the formation and subsequent decomposition of the acyl-enzyme anhydride. It is important to point out that in order to fully understand the mechanistic implications of such a mixed-anhydride intermediate in enzyme-catalyzed acyl transfer reactions, further studies of the chemistry of mixed anhydrides in dipolar aprotic media must be carried out.[20]

In conclusion, it might be noted that the reaction discussed in this work demonstrates the facility with which catalytic acyl transfer reactions can occur in nonprotic media. These findings provide additional evidence in support of the view that the transition states of the corresponding enzymatic reactions are apolar in nature.[9] Indeed, as it has been pointed our recently,[2] none of the currently accepted mechanisms for enzyme-catalyzed hydrolytic reactions involve charge separation at the rate-limiting transition states. Further studies presently underway in our laboratory are aimed at the detailed mechanistic elucidation of carboxylate-dependent acyl transfer reactions. It is hoped that these efforts will lead to a better understanding of the mechanism of this fundamentally important reaction.

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Reaction Kinetics and Equilibria of β-Elimination of Some Schiff Base Complexes

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Abstract: The kinetics of β-elimination of *O*-phosphoserine, β-chloroalanine, *S*-ethylcysteine, and β-chloro-α-aminobutyric acid was investigated by NMR in deuterium oxide media at 31.5 ± 0.5 °C in the presence of pyridoxal and in the presence and the absence of Ga(III), Al(III), and Zn(II) ions. The reaction rate constants and relevant equilibrium constants are reported for these systems. The specific rate constants for the individual molecular species in solution were determined from the observed reaction rate constants and the equilibrium constants applicable to these systems. Catalysis by pyridoxal in the absence of metal ions showed an increase in rate as pD was increased, with a rate maximum in the region of pD 8–9, and a moderate decrease at higher pH. The first-order rate constants varied with the amino acid moiety in the order β-chloroalanine > *O*-phosphoserine > β-chloro-α-aminobutyric acid > *S*-ethylcysteine. The rate constants reported for pyridoxal catalysis in the presence of metal ions show rate enhancements in the order of ten times the values of metal-free systems. Possible mechanisms of these reactions are discussed.

A series of potentiometric and spectrophotometric studies of amino acid Schiff bases of pyridoxal and pyridoxal 5′-phosphate in aqueous media have been carried out. Earlier equilibrium studies on these systems consisted of potentiometric measurements of hydrogen ion concentration supplemented by spectrophotometric data.[2,3] Because of the complex pH dependence in these proton- and metal ion-coordinated Schiff base systems, only the pH-dependent conditional rate constants have been reported.[4,5] With the introduction of computer-assisted equilibrium calculations,

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a more complete elucidation of molecular species present in a number of Schiff base systems has appeared in the literature.[6,7]

The kinetics of the catalysis of elimination of electronegative substituents from the β-position of α-amino acids by pyridoxal and metal ions has recently been described.[8] The methods previously employed for determining the equilibrium distribution of ionic species are now applied to determination of the distribution of complex species in aqueous solution containing pyridoxal and one of amino acids: β-chloroalanine, *O*-phosphoserine, *S*-ethylcysteine, and β-chloro-α-aminobutyric acid, in the absence and presence of Zn(II), Al(III), and Ga(III) ions. The resulting distributions of species are employed for a detailed analysis of the kinetic data to determine the specific β-elimination rate constants for the individual ionic species of the Schiff bases and their metal complexes in these systems.

Experimental Section

Pyridoxal hydrochloride was obtained as Mann Analyzed grade and was used without further purification. The amino acids were obtained from the following companies: β-chloroalanine from Cyclo Chemical, *O*-phosphoserine from Calbiochem, *S*-ethylcysteine from United States Biochemical, and β-chloro-α-aminobutyric acid from Vega-Fox Biochemical. The NaOD, D_2O, and DCl were obtained from Diaprep Corp. The purities of these reagents were as follows: D_2O, 99.7%; DCl, 20%; NaOD, 40%. The DCl and NaOD samples were diluted to the appropriate concentrations under dry nitrogen. Stock solutions of Al(III) sulfate and Zn(II) nitrate were prepared by dissolving hydrates of $Al_2(SO_4)_3$ and $Zn(NO_3)_2$ in D_2O. After repeated evaporation to dryness and dissolution in D_2O, the solutions were diluted with D_2O to the approximate desired concentration and standardized by conventional chelatometric titration.[9] The standard gallium(III) chloride solution was prepared by dissovling a specific amount of gallium metal in DCl and diluting to the appropriate volume.

The pH values of the solutions used for kinetic studies were measured with a Corning Model 101 digital pH meter fitted with a Beckman miniature combination glass electrode. The instrument was calibrated before and after each kinetic run by standardization with dilute hydrochloric acid and NaOH at 1.00 M (KNO_3) ionic strength to read $-\log [H^+]$. In the case of D_2O solutions, the pD value was computed by adding 0.40 to the observed reading.[10] The temperature of the reaction was maintained at 31.5 ± 0.5 °C, the ambient temperature of the NMR probe.

All kinetic runs were carried out in homogeneous systems, with the analytical concentrations of amino acid and pyridoxal set at 0.10 M. The ionic strength was maintained at μ = 1.00 M with potassium nitrate. NMR spectra were obtained with a Varian HA-100 nuclear magnetic resonance spectrometer. The chemical shifts are reported in hertz with respect to the resonance of tetramethylsilane (Me_4Si) which was inserted into the reaction mixture in a coaxial tube. The fraction of Schiff base in the experimental solutions was determined by (the sum of) the resonances of 6-H, 4-CH, and 2-CH_3 groups. The concentrations of Schiff bases, amino acid, and pyridoxal employed in the equilibrium calculations were obtained from the initial NMR measurements in the kinetic runs.

A simple graphical method for determining the protonation constants by the use of UV–visible absorption spectra was employed[11,12] for the amino acid Schiff bases. The values of pK_a's were determined from the inflection points of the absorbance–pH curves.[13-16] Electronic absorption spectra were measured with a Cary Model 14 recording spectrophotometer. Matched 0.10-cm quartz cells were employed. Solutions for spectrophotometric study were 1.00×10^{-3} M in pyridoxal and 0.100 M in amino acid. Ionic strength was maintained at 0.10 M by the addition of KCl. Adjustments of pH were made by adding small volumes of NaOH from a Gilmont screw syringe.

Table I. Acid Dissociation Constants for Pyridoxal and Various Amino Acids (t = 25 °C; μ = 0.10 M (KNO_3))

compd	pK_1	pK_2	pK_3	Schiff base pK_1	Schiff base pK_2
β-chloralanine	1.95	8.18		6.40	9.75
O-phosphoserine[a]	2.07	5.62	9.71	6.43	9.80
β-chloro-α-aminobutyric acid	1.48	8.07		6.70	9.80
S-ethylcysteine	1.92	8.65		6.55	9.85
pyridoxal[b]	4.20	8.66	13.0		

[a] Reference 13. [b] Reference 15.

Table II. Equilibrium Constants for Schiff Base Formation[a] (t = 30 °C; μ = 1.00 M (KNO_3))

compd	$\log K_{f_0}$	$\log K_{f_1}$	$\log K_{f_2}$
β-chloroalanine	0.54	10.29 ± 0.04	16.69 ± 0.07
O-phosphoserine	0.52	10.32 ± 0.04	16.77 ± 0.07
β-chloro-α-aminobutyric acid	0.62	10.42 ± 0.04	17.12 ± 0.07
S-ethylcysteine	0.53	10.38 ± 0.04	16.93 ± 0.07

[a] For definitions of constants see eq 1.

Potentiometric equilibrium measurements were made by using a jacketed, air-tight, all-glass titration vessel of roughly 50-mL capacity. This cell was equipped with O-ring fittings for glass and saturated calomel electrodes and with gas inlet and outlet tubes. A microburette was connected to the cell with a Teflon adapter. Solutions were kept under an atmosphere of nitrogen which was purified with two alkaline pyrogallol scrubbers to remove O_2 and CO_2. The gas stream was raised to the proper humidity by bubbling through an 0.100 M KNO_3 solution prior to passing it through the experimental solution. The gas outlet tube was also connected to a small bubble counter to maintain a slight positive pressure within the cell. All solutions were adjusted to 0.10 M ionic strength by the addition of 1.00 M KNO_3 and were maintained at 25.00 ± 0.05 °C by circulation of thermostated water through the outer jacket of the cell. Hydrogen ion concentrations were measured with a Beckman Research Model pH meter. The apparatus was standardized with dilute hydrochloric acid at 0.10 M (KNO_3) ionic strength to read $-\log [H^+]$.

Results and Discussion

Equilibria. The proton dissociation constants, pK_a, for β-chloroalanine, *O*-phosphoserine, *S*-ethylcysteine, and β-chloro-α-aminobutyric acid, and of their pyridoxal Schiff base are listed in Table I. The conditions employed for the equilibria differ slightly from the kinetic experiments for the equilibrium data to be in par with previously reported constants.[6,13] The protonation constants of the amino acids defined by $K_H^n = [H_nL]/[H^+][H_{n-1}L]$ were determined by measuring $-\log [H^+]$ of aqueous solutions of the ligands as a function of the moles of base added per mole of ligand. A slightly modified version of an iterative computer program written by Dr. R. J. Motekaitis was used to calculate the protonation constants from potentiometric data.

The Schiff base protonation constants were determined spectrophotometrically. The notation employed for expressing Schiff base formation from amino acids and pyridoxal is the same as that employed by Leussing.[6]

$$PAL^- + AA^- + jH^+ \overset{K_{f_j}}{\rightleftharpoons} SBH_j^{j-2} \quad (1)$$

$$K_{f_j} = \frac{[SBH_j^{j-2}]}{[PAL^-][AA^-][H^+]^j} \quad (2)$$

The protonation dissociation constants correspond closely to the constants reported previously for similar pyridoxal–amino acid Schiff base systems.[14-20] The lower pK_a values for azomethine groups reported in the present study are expected since they are in general agreement with the lower pK_a values of the amino acid themselves.

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Table III. The Equilibrium Constants for Metal Ion-Pyridoxal-Amino Acid Systems (t = 25 °C; μ = 0.10 M (KNO_3))

log binary constants	Cl-Ala	Phoser	Cl-Ami	PAL
$\beta_1(Zn^{2+} + L^- \rightleftharpoons ZnL^+)$	4.63	5.88	4.75	
$\beta_2(Zn^{2+} + 2L^- \rightleftharpoons ZnL_2)$	8.65	9.72	8.67	
$\beta_3(Zn^{2+} + 3L^- \rightleftharpoons ZnL_3^-)$	~10.9			
$\beta_1(Al^{3+} + L^- \rightleftharpoons AlL^{2+})$	4.05	4.79	4.13	
$\beta_2(Al^{3+} + 2L^- \rightleftharpoons AlL_2^+)$	6.05	8.81	6.33	
$\beta_3(Al^{3+} + 3L^- \rightleftharpoons AlL_3)$		10.68		
$\beta_1(Ga^{3+} + L^- \rightleftharpoons GaL^{2+})$	4.51	6.18	4.89	
$\beta_2(Ga^{3+} + 2L^- \rightleftharpoons GaL_2^+)$	8.39	9.87	9.0	
$\beta_3(Ga^{3+} + 3L^- \rightleftharpoons GaL_3)$	10.34			
$\beta_1(Zn^{2+} + PAL^- \rightleftharpoons ZnPAL^+)$				2.32
$\beta_1(Al^{3+} + PAL^- \rightleftharpoons AlPAL^{2+})$				2.45
$\beta_1(Ga^{3+} + PAL^- \rightleftharpoons GaPAL^{2+})$				2.67
$\beta_{11}(Zn^{2+} + SB^{2-} \rightleftharpoons ZnSB)$	10.3	10.5	10.3	
$\beta_{11}(Al^{3+} + SB^{2-} \rightleftharpoons AlSB^+)$	10.5	11.2	10.5	
$\beta_{11}(Ga^{3+} + SB^{2-} \rightleftharpoons GaSB^+)$	13.8			
$\beta_{12}(Zn^{2+} + 2SB^{2-} \rightleftharpoons ZnSB_2^{2-})$	17.1			
$\beta_{12}(Al^{3+} + 2SB^{2-} \rightleftharpoons AlSB_2^-)$	18.2			
$\beta_{12}(Ga^{3+} + 2SB^{2-} \rightleftharpoons GaSB_2^-)$	~20			
$K_1(M^{n+}SB^{2-} + H^+ \rightleftharpoons MSBH^{n+})$	6.42			
$K_{11}(M^{n+}SB_2^{2-} + H^+ \rightleftharpoons MSB_2H^{n+})$	7.4[a]			
$K_{12}(MSB_2H^{n+} + H^+ \rightleftharpoons MSB_2H_2^{n+})$	5.4[a]			

[a] Reference 6.

The corresponding equilibrium constants obtained from NMR measurements in the kinetic runs are listed in Table II. The protonation equilibrium constants reflect the pK's obtained from the separate spectrophotometric experiments. These values also closely resemble the values previously reported.[8,19,21,22]

NMR Assignments. The proton chemical shifts of pyridoxal resonances have been assigned and discussed in detail elsewhere.[20,23] The resonances for the amino acids were assigned through the use of standard procedures. All resonances shift to higher field with increasing basicity of the solution. As the pD is increased above 6, Schiff base resonances became apparent.

Kinetic Studies. Rate Law. The rate measurements were based on quantitative NMR detection of appearance of the products, as well as the disappearance of the amino acids. For the metal-free systems, the rate equations are based on the reaction scheme

$$PAL + \chi\text{-AA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SB \xrightarrow{k_2} \alpha\text{-KA} + PAL + NH_3 \quad (3)$$

For the metal–Schiff base (1:1) systems

$$PAL + \chi\text{-AA} + M^{n+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SBM \xrightarrow{k_2} \alpha\text{-KA} + PAL + NH_3 + M^{n+} \quad (4)$$

where PAL = pyridoxal; χ-AA = amino acid with electronegative group χ (chloride, phosphate, or ethyl sulfide); SBM and SB = Schiff base with and without metal ion; α-KA = α-ketoacid; M^{n+} = Zn^{2+}, Al^{3+}, or Ga^{3+}. The second-order rate equation (5) is employed for the metal-free systems in the absence of an appreciable amount of Schiff base.

$$-\frac{d[\chi\text{-AA}]}{dt} = [PAL][\chi\text{-AA}]\left(\frac{k_{-1}k_1}{k_{-1} + k_2} - k_1\right) = k_{obsd}[PAL][\chi\text{-AA}] \quad (5)$$

Similarly, for the metal–Schiff base systems

$$-\frac{d[\chi\text{-AA}]}{dt} = [M^{n+}][PAL][\chi\text{-AA}]\left(\frac{k_{-1}k_1}{k_{-1} + k_2} - k_1\right) = k_{obsd}[PAL][\chi\text{-AA}][M^{n+}] \quad (6)$$

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Table IV. Observed α,β-Elimination Rate Constants for the *O*-Phosphoserine-Pyridoxal System (1:1)[a]

pD	10^4k_{obsd}, s^{-1} M^{-1}	$10^3k'_{obsd}$, s^{-1}
4.08	1.30 ± 0.03	
5.28	1.40 ± 0.03	
6.30	3.10 ± 0.06	
7.42		1.22 ± 0.03
7.97		1.55 ± 0.03
8.20		1.63 ± 0.03
8.77		1.76 ± 0.04
8.95		1.95 ± 0.04
9.12		1.64 ± 0.04
9.46		1.45 ± 0.04
9.74		1.16 ± 0.05
10.05		0.93 ± 0.07

[a] Standard deviations calculated as in ref 25.

Table V. Rate Constants Observed for the 1:1 β-Chloroalanine-Pyridoxal System[a]

pD	10^4k_{obsd}, s^{-1} M^{-1}	$10^3k'_{obsd}$, s^{-1}
4.13	1.60 ± 0.02	
4.90	2.10 ± 0.03	
6.00	2.70 ± 0.06	
6.80		1.62 ± 0.04
7.08		2.06 ± 0.03
7.33		2.38 ± 0.02
7.67		2.71 ± 0.02
8.00		2.96 ± 0.03
8.24		3.04 ± 0.05
8.69		2.82 ± 0.04
9.30		2.38 ± 0.03
9.62		2.14 ± 0.04
9.89		1.84 ± 0.03

[a] Standard deviations calculated as in ref 25.

All the Schiff base complexes studied in this research involve 1:1:1 molar ratios of metal ion, pyridoxal, and amino acid. When the steady-state assumption is not applicable to Schiff base formation (i.e., when the accumulation of Schiff base in solution is appreciable), the first-order rate equation (7) was employed.

$$-\frac{d[\chi\text{-AA}]}{dt} = k'_{obsd}[SB] \text{ or } k'_{obsd}[SBM] \quad (7)$$

Thus, when possible the reaction kinetics were related directly to the concentration of Schiff base in solution. The reaction stoichiometry, in the absence of byproducts, gives the relationship

$$[\alpha\text{-KA}] = [\chi\text{-AA}]_0 - [\chi\text{-AA}] - ([SB] \text{ or } [SBM]) \quad (8)$$

where $[\alpha\text{-AA}]_0$ is the initial concentration of amino acid. The values of the first-order rate constants k'_{obsd} is then found from the slope of the plot of $d[\alpha\text{-KA}]/dt$ vs. [SB]. Since [χ-AA] and [SB] are both found directly from the NMR integrations, the slope of [α-KA] vs. time and the corresponding k'_{obsd} values are readily obtained.

In acidic media, the pyruvic acid resonances observed as the result of β-elimination of β-chloroalanine and *O*-phosphoserine, up to 1 half-life of the reaction, were those of the keto or diol form. The pyruvic acid resonances were used as a measure of the progress of the elimination reaction since H–D exchange of pyruvic acid is relatively slow in neutral and weakly acid solution. At high pH, H–D exchange of pyruvate is too rapid, and the pyruvic acid resonance was therefore not used as a measure of the extent of the reaction under these conditions. These considerations applied to both metal–free and metal–chelate systems.

The rate constants observed for the elimination of phosphate from *O*-phosphoserine are listed in Table IV. The rate constants observed for β-chloroalanine, β-chloro-α-aminobutyric acid, and *S*-ethylcysteine are listed in Tables V, VI, and VII, respectively. The rate constants determined in the lower pD range where very little Schiff base is present are second order, as expected, in accordance with eq 5. At higher pD's the concentrations of Schiff

Scheme I

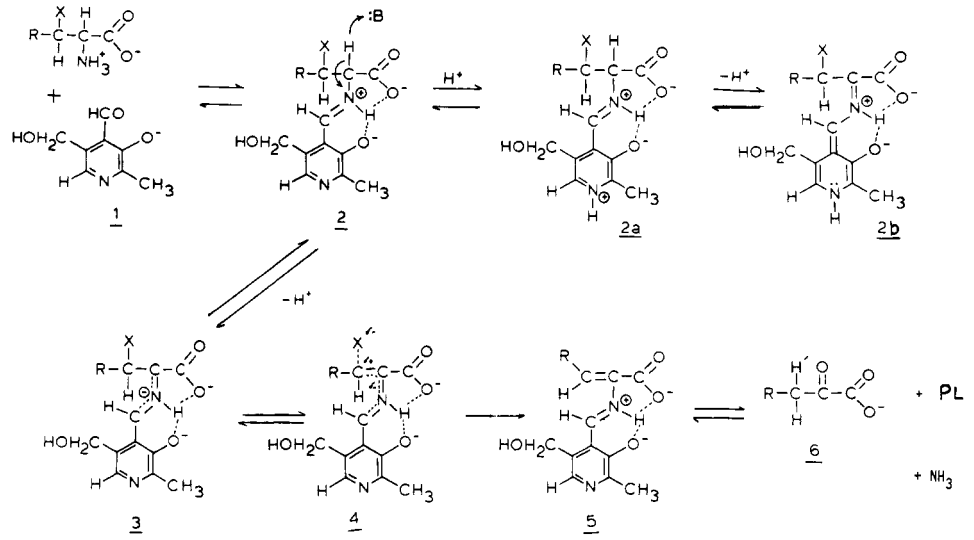

Table VI. Observed β-Elimination Rate Constants for the 1:1 β-Chloro-α-aminobutyric Acid–Pyridoxal System

pD	$10^5 k_{obsd}$, s^{-1} M^{-1}	$10^4 k'_{obsd}$, s^{-1}
4.21	0.72 ± 0.05	
4.94	1.57 ± 0.03	
5.74	2.42 ± 0.03	
6.89	2.50 ± 0.03	2.30 ± 0.07
7.25	2.83 ± 0.04	2.70 ± 0.05
7.75		4.96 ± 0.04
8.39		7.16 ± 0.03
8.80		7.64 ± 0.03
9.28		7.68 ± 0.03
9.52		7.44 ± 0.03
9.91		7.14 ± 0.04
10.27		6.71 ± 0.04
10.74		6.32 ± 0.05

[a] Standard deviations calculated as in ref 25.

Table VII. Observed Rate Constants for β-Elimination in the 1:1 S-Ethylcysteine–Pyridoxal System[a]

pD	k_{obsd}, s^{-1} M^{-1}	k'_{obsd}, s^{-1}
4.33	$(3.21 \pm 0.04) \times 10^{-6}$	
4.74	$(7.65 \pm 0.05) \times 10^{-6}$	
5.47	$(7.50 \pm 0.05) \times 10^{-6}$	
7.11		$(1.2 \pm 0.2) \times 10^{-5}$
7.42		$(1.8 \pm 0.2) \times 10^{-5}$
8.27		$(2.3 \pm 0.2) \times 10^{-5}$
8.92		$(2.8 \pm 0.2) \times 10^{-5}$
9.15		$(2.5 \pm 0.2) \times 10^{-5}$
9.56		$(2.0 \pm 0.3) \times 10^{-5}$

[a] Standard deviations calculated as in ref 25.

base for the elimination reaction were obtained. The rate constants thus determined show considerable pH dependence. Precipitation during the kinetic runs hampered kinetic measurements in the pD range 5.5–7.0, and rate constants determined in this range were therefore determined by recalculation of the concentration of initial reactants from the normalized integration of NMR spectra. The high reaction rate and the broadening of the resonances that occur at high pD made quantitative integration of the NMR resonances somewhat more difficult. In spite of these problems, however, the tolerances of the rate constants reported at high pD's are considered reasonable.

The factors that influence the rates in the absence of metal ion are (1) the amount of the Schiff base formation and the degree of protonation of the Schiff base, (2) the influence of the azomethine group and the electronegative neutral or protonated nitrogen of the pyridine ring on the lability of the α-hydrogen of the amino acid moiety, and (3) the electronegativity of the leaving group. Calculations based on equilibrium constants determined in this study demonstrate that the fraction of Schiff base in these systems never exceeds 20% of the total substrate below pH 6 but increases at higher pH. This low degree of formation of the Schiff base must be partly responsible for the observed low reaction rates observed in acidic media. Also, the low reaction rate observed at pH values below pH 6 may be related to the fact that under these conditions the pyridine ring of the Schiff base is protonated (see Scheme I, formula **2a**).

The labilization of the α-proton of the amino acid moiety is promoted by the ability of Schiff base to accommodate the liberated electron pair through the extended conjugated π-bond system (formulas **2** → **3**). In addition, this dissociation is further promoted by both the coordinate bonding and electrostatic effects on the azomethine nitrogen by the coordinated proton or metal ion and the inductive (–I) effect of the heterocyclic nitrogen atom. The latter effect is greatly amplified upon protonation of the pyridine ring. These inductive and bonding effects that favor α-proton dissociation to give the protonated pyridine intermediate **2a** would render the generated lone pair of electrons in the α-position less available for the withdrawal of the electronegative leaving group and thus tend to slow down the elimination reaction. It seems possible that these two opposing effects may very well largely cancel each other and that the low rate below pH 6 could be due primarily to the lower degree of Schiff base formation.

In the intermediate pH range, it is seen that the first-order rate constants increase with pH, probably because of a change in the nature of the reactive Schiff base species. The observed rate may be considered the summation of the rates of the individual molecular species differing in degree of protonation. Thus

$$k'_{obsd}[SB_T] = k'[H_2SB] + k''[HSB^-] + k'''[SB^{2-}] \quad (9)$$

where SB_T is total Schiff base and H_2SB, HSB^-, and SB^{2-} are its di-, mono-, and nonprotonated forms. The concentrations of the three Schiff base species vary with pH, in accordance with the protonation equilibrium constants listed in Tables I and II. As the pH increases from 6.8 to 8.0, the ratio of HSB^-/H_2SB increases, indicating that β-elimination takes place more rapidly with the monoprotonated Schiff base species than with the fully protonated Schiff base, in which the pyridine ring is protonated (i.e., $k'' > k'$). Since the latter species is a much stronger promoter of α-proton dissociation of the α-amino acid moiety of the Schiff base, the observed increase in the rate seems to support the hypothesis made in the previous paragraph concerning the rate-determining nature of removal of the electronegative group from the β-position.

The decrease in the first-order rate constant at still higher pH may be accounted for by the deprotonation of the Schiff base at the azomethine nitrogen, resulted in the formation of a higher

Table VIII. Specific Rate Constants for β-Elimination

Schiff base	pK_1	pK_2	k'	k''	k''_{OH}[a]	σ[b]
β-chloroalanine	6.40	9.75	3.18×10^{-4}	3.05×10^{-3}	1.44	9.35×10^{-5}
O-phosphoserine	6.45	9.80	2.39×10^{-4}	1.85×10^{-3}	1.14	5.49×10^{-5}
β-chloro-α-aminobutyric acid	6.70	9.80	2.44×10^{-5}	7.98×10^{-4}	5.41×10^{-1}	1.97×10^{-4}

[a] $pK_w = 13.79$. [b] $\sigma = (\Sigma_i^n(\text{observed rate} - \text{calculated rate}))/(n-1)$.

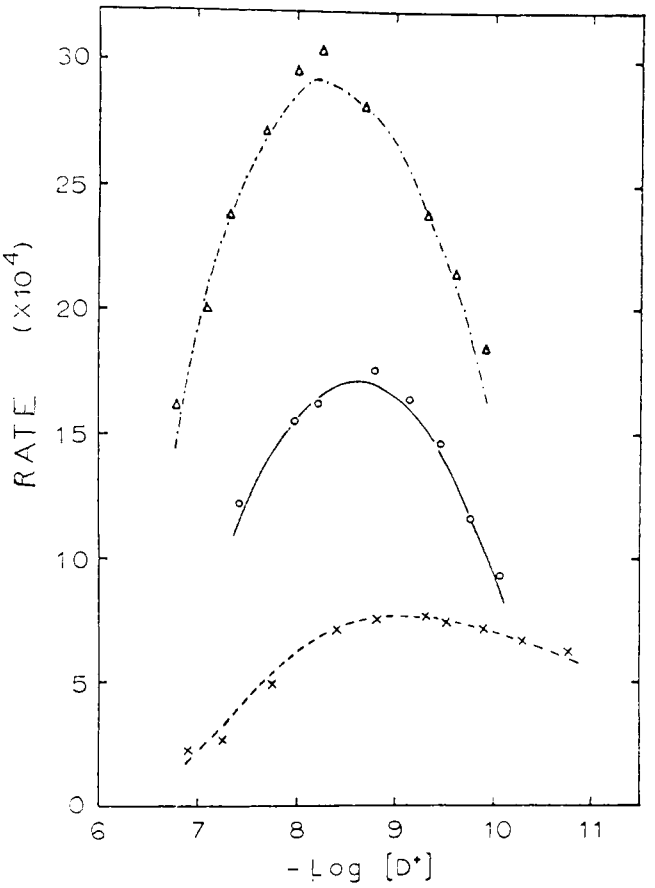

Figure 1. Rate vs. pD for the metal-free systems containing β-chloroalanine, *O*-phosphoserine, or β-chloro-α-aminobutyric acid with pyridoxal. The curves represent calculated rate and symbols represent observed rate: β-chloroalanine, Δ, ···; *O*-phosphoserine, O, —; β-chloro-α-aminobutyric acid, ×, ---

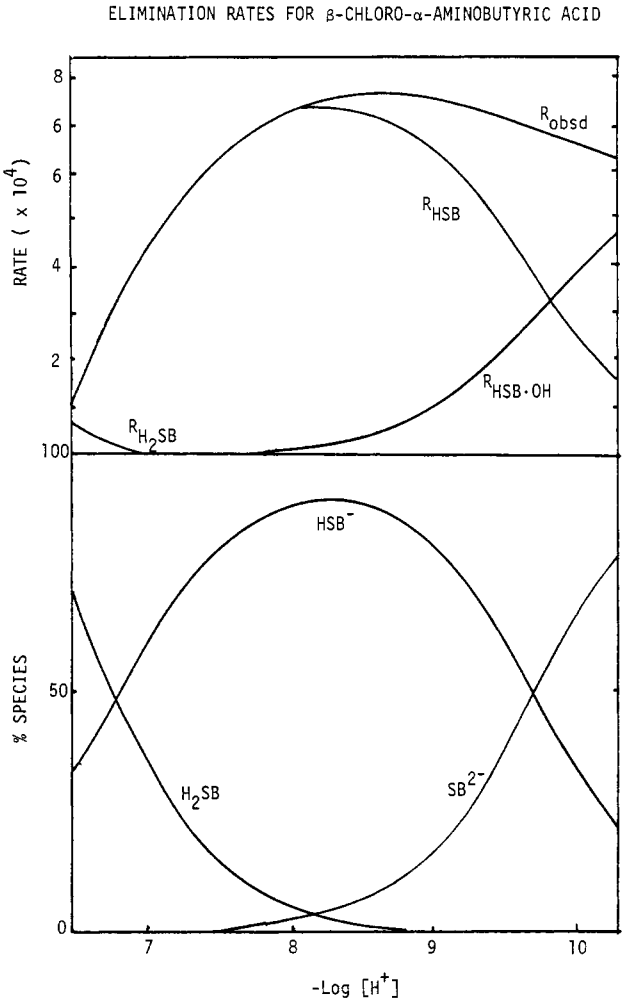

Figure 2. A. Rate vs. pH for the β-chloro-α-aminobutyric acid–pyridoxal system showing contributions of individual Schiff base species to total β-elimination rate. B. Relative concentrations of specific protonated species of Schiff base of pyridoxal and β-chloro-α-aminobutyric acid as a function of -log [H^+].

proportion of the SB^{2-} species. In the latter form, the catalytic effect of the Schiff base azomethine nitrogen and of the deprotonated pyridine ring on the α-proton dissociation of the α-amino acid moiety is minimal, since it is due only to the electronegativity of the neutral azomethine nitrogen, amplified somewhat by conjugation with the pyridine ring. This observation is in accord with the concept that the initial deprotonation step is also rate determining and is an essential step in the β-elimination reaction. Thus the model chosen for these systems is expressed by eq 10.

$$k'_{obsd}[SB_T] = k'[H_2SB] + k'[HSB^-] + K''_{OH}[HSB^-][OH^-] \quad (10)$$

Although the third specific rate constant k''_{OH} in this equation is second order and thus seems quite different from the third constant k''' in the previous equation (9), it cannot be distinguished from the other specific rate constant, k''', since k''_{OH} may be converted to k''' by the use of the protonation equilibrium constant of the most basic form of the Schiff base. Thus

$$k''_{OH} = k'''(K_{a_2}/K_w)$$

Assuming the data to be adequately represented, we calculated the values of $[H_2SB]$ and $[HSB^-]$ from the protonation constants and the appropriate mass balance equations. The values of k', k'', and k''_{OH} were obtained by a regression analysis[25] by which the sum of the squares of the errors, U, is minimized. The term U is defined as

$$U = \Sigma\{k'_{obsd}[SB_T] - k'[H_2SB] - k'[HSB^-] - k''_{OH}[HSB^-][OH]\}^2 \quad (11)$$

The values of protonation constants were varied so as to minimize the error U. The protonation constants thus obtained are remarkably close to the values obtained spectrophotometrically, as reported in Table I. The specific rate constants thus obtained for the individual Schiff base species are listed in Table VIII. Smoothed data were used in the computer calculation of these rate constants. Figure 1, containing plots of rate constants vs. pD, shows the degree of reproducibility between the observed and calculated values. The percent differences between the smoothed data and calculated values were less than 5% for all amino acids investigated. The reproducibility of each curve is remarkably good

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Table IX. Observed Rate Constants for Zinc(II)-Catalyzed β-Elimination (Pyridoxal-Amino Acid-Zinc(II) (1:1:1))[a]

amino acid	pD	$10^3 k^{M}_{obsd}$, M^{-2} s^{-1}	$10^3 k^{M'}_{obsd}$, s^{-1}
β-chloroalanine	4.20		1.2 ± 0.1
	5.00		2.0 ± 0.1
	5.40		1.4 ± 0.1
O-phosphoserine	3.44	1.1 ± 0.1	
	4.06	2.2 ± 0.1	
	4.34	4.0 ± 0.1	
	4.56	4.1 ± 0.1	
β-chloro-α-aminobutyric acid	3.88	0.64 ± 0.01	
	4.63	1.36 ± 0.01	
	5.23	2.17 ± 0.01	
S-ethylcysteine	3.57	0.34 ± 0.01	
	4.39	0.63 ± 0.01	
	5.14	0.89 ± 0.01	

[a] Standard deviations calculated as in ref 25.

Table X. Observed Rate Constants for Al(III)- and Ga(III)-Catalyzed β-Elimination (Pyridoxal-Amino Acid-Metal Ion (1:1:1))

amino acid	pD	$k^{Al(III)}_{obsd}$, M^{-2} s^{-1}	pD	$10^3 k^{Ga(III)}_{obsd}$, M^{-2} s^{-1}
β-chloroalanine	3.54	1.3×10^{-3}	3.13	3.4
	3.98	2.0×10^{-3}	3.44	4.8
	4.35	2.4×10^{-3}		
O-phosphoserine	3.12	1.0×10^{-3}	3.08	2.8
	3.67	1.8×10^{-3}	3.35	3.3
	4.13	3.6×10^{-3}	4.08	3.4
	4.52	3.9×10^{-3}		
β-chloro-α-aminobutyric acid	3.40	1.0×10^{-3}	3.09	2.7
	3.92	1.9×10^{-3}	3.44	3.5
	4.21	2.3×10^{-3}	3.82	4.1
S-ethylcysteine	3.45	8.9×10^{-4}	3.11	1.2
	4.12	9.8×10^{-4}	3.68	3.8
	4.47	1.1×10^{-3}		

considering the simple model chosen for these systems. The values obtained in these calculations indicate that the second-order rate constant of the base-catalyzed reaction makes a small but significant contribution to the observed rate constant. The graphical illustrations in Figure 2 show the contributions of the individual species to the observed rates. The decrease in k'_{obsd} above pD 8.0–8.5 is due mainly to the decrease in the concentration of the monoprotonated Schiff base. The fact that k'_{obsd} decreases in the higher pD range is due to the fact that k''_{OH} is relatively small (for a second-order rate constant) and therefore makes only a small contribution to the observed rate. This contribution, which increases linearly with $[OH^-]$, is not sufficient to compensate for the concomitant decrease in the concentration of the monoprotonated species, HL.

It is interesting to use the kinetic data on the contribution of the deprotonated species to the overall reaction rate to obtain further insight concerning the nature of the reaction mechanism and the rate-determining steps. If the deprotonation at the α-carbon of the amino acid moiety is the only rate-limiting step, the rate constants for the fully protonated Schiff base should be larger than the values for the monoprotonated Schiff base. The smaller values obtained for the fully protonated Schiff base leads to a conclusion that the loss of the electronegative group in the β-position is also rate determining. The additional positive charge of this species would be expected to slow down the removal of the electronegative substituents with its bonding electron pair in accordance with the observation that the rate constant k_{obsd} decreases with decreasing pH.

Metal Ion Catalysis. Systems containing 1:1:1 stoichiometric ratios of pyridoxal, amino acid, and metal ion were investigated under conditions similar to those described above for the metal-free Schiff bases. The β-elimination rates were calculated entirely on the basis of the disappearance of the α-amino acid moieties of the Schiff bases. It was found that under comparable conditions the observed rates of β-elimination were greater for the metal-catalyzed systems than for the metal-free systems. In measurements at low pH where either no Schiff base was observed, or where the fraction of Schiff base was less than 5%, the kinetic data were interpreted in terms of eq 6, and third-order rate constants were calculated. When the accumulation of the Schiff base was greater than 5%, the concentrations of these species were sufficiently well-known to treat the reaction kinetics on the basis of the rate of disappearance of Schiff base, the first-order rate constants were therefore reported.

The kinetic results obtained for Zn(II) and for Ga(III) and Al(III) catalysis of β-elimination for β-chloroalanine, *O*-phosphoserine, *S*-ethylcysteine, and β-chloro-α-aminobutyric acid are summarized in Tables IX and X, respectively. Formation of metal chelates of the intermediate Schiff base results in considerable rate enhancement for all the amino acids, as illustrated by the rate constants reported for metal ion catalyzed reactions. The degree to which keto acid tautomerized to the enol form of the pyruvic acid, as well as the degree of hydration of the keto form to the diol,[26,27] was greatly enhanced by the presence of the metal ion.

With *O*-phosphoserine as an example, the values of $k'_{obsd}[M]$ for Zn(II) at pH 4.06 is 2.2×10^{-4} M^{-1} s^{-1}, for Al(III) at pD 4.13 it is 3.6×10^{-4} M^{-1} s^{-1}, and for Ga(III) at pD 4.08 it is 3.4×10^{-4} M^{-1} s^{-1} compared to the rate constants k_{obsd} of 1.30×10^{-4} M^{-1} s^{-1} for pD 4.08 in the metal-free system, a twofold rate enhancement. A larger rate increase is observed for β-chloro-α-aminobutyric acid. The value of $k'_{obsd}[M]$ for Al(III) at pD 4.21 is 2.3×10^{-4} M^{-1} s^{-1} while at pD 4.21 the metal-free system k_{obsd} is 7.2×10^{-6} M^{-1} s^{-1}, a 32-fold rate enhancement. These rate enhancements are also due in part to the electron-withdrawing effect of the metal ion, and its labilizing effect on the α-proton of the amino acid moiety.

Precipitation at higher pD regions made quantitative rate measurements difficult for most of the amino acids studied. Although the precipitates redissolved as the pD was increased above 8–8.5, NMR measurements of these solutions were rendered difficult because of turbidity and/or an increase in viscosity. Although rate constants were not determined at higher pDs, β-elimination was observed to proceed faster in the presence of the metal ion above pD 8 than in the metal-free system.

For all the amino acids studied, elimination proceeded faster for the Ga(III) complexes than for either Al(III) or Zn(II) complexes under the same experimental conditions. The relative rate enhancements for metal ion catalysis of β-elimination raises some interesting questions about the nature of this reaction, since the electron shift for the breaking of the bond between the carbon atom and the electronegative leaving substituent is in opposition to the electron effect of the metal ion.

As indicated in scheme I, abstraction of the α-hydrogen from the amino acid moiety of the Schiff base complex for either metal complex or metal-free system is an essential first step. Thus, if this were the only rate-limiting step, the influence of the ionic species attached to the azomethine nitrogen, which directly affects the labilization of the H–C bond would be the main factor determining the magnitude of the observed reaction rate. The metal ion with a higher charge thus should labilize the bond more effectively and would be expected to catalyze the elimination reaction more effectively than one with lower charge, the predicted order being Ga(III), Al(III) ≫ Zn(II). If the removal of an electronegative substituent were also rate limiting, then the electronegativity of the β-substituent would affect the β-elimination. The charge of the metal ion and its affinity for the chelating donor groups (the azomethine nitrogen and adjacent carboxylate and phenolate donors) would also influence the rate, since it would control the preequilibrium that results in the formation of the α-deprotonated intermediate. The ability of the electronegative substituent to withdraw the delocalized electron

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pair from the azomethine group is illustrated by the differences in the values obtained for the rate constants of the various amino acids studied, with the most electronegative substituents having the highest rates: chloride followed by phosphate and sulfide. This result supports the idea that the β-elimination step is rate limiting. It is interesting to note that the differences in catalytic effects of di- and trivalent metal ions are quite small, whereas one would expect on the basis of first principles that their relative effects on α-hydrogen dissociation would be considerable, mainly because of differences in ionic charge. It is suggested that part of the large catalytic increase expected from an increase in the charge of the metal ion is considerably mitigated by the fact that increase of the charge would tend to slow down the removal of the electronegative β-substituent. Thus the experimental data indicate that both α-proton labilization and β-elimination steps are rate limiting.

The differences between the values of β-chloroalanine and β-chloro-β-aminobutyric acid rate constants point out the fact that the molecular groups attached to the α-carbon, other than the functional group, have considerable effect on the rate of the reaction. Electron-donating groups tend to slow down the reaction while electron-withdrawing groups aid the reaction. The differences in rate between α-proton labilization and β-elimination reaction would determine the relative importance of these individual steps in the overall elimination process.

The rate constants obtained for the metal-free β-chloroalanine and *O*-phosphoserine systems were of approximately equivalent magnitude for the β-elimination step and α-deprotonation. The rate constants of α-proton labilization of the β-chloro-α-aminobutyric acid system were 1.1 times larger than those obtained for β-elimination, and the differences are thus close to the limit of experimental error. The rates of α-proton labilization in the *S*-ethylcysteine system were less and were 1.1 times larger than those obtained for β-elimination. Because of the lack of a sufficient amount of Schiff base formation at low pDs for the metal Schiff base complexes, the relative rates of α-proton labilization and β-elimination could not be measured. The closeness of the β-elimination and α-hydrogen labilization rate constants that have been measured indicate the delicate balance between these two rate-limiting steps and the possibility that extensive constitutional changes could eliminate the second step without greatly influencing the first. The reverse influence is of course not possible.

Further experimental work is needed to elucidate the interplay between these two reaction steps in metal-free as well as in metal chelate systems and for the influence of the constitution of the amino acid on the absolute magnitudes and the relative values of the rates of these sequential steps in elimination reactions.

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Pyridoxal- and Metal-Catalyzed β-Elimination, Decarboxylation, and Dealdolation Reactions of β-Hydroxyglutamic Acid

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Abstract: Pyridoxal-catalyzed β-elimination and dealdolation reactions of β-hydroxylglutamic acid were found to occur simultaneously. The rate constants for the parallel elimination and dealdolation reactions in D_2O in the absence of metal ions were determined by proton NMR over the pD range of 4–10. The variations of the rates with pD and solution conditions were found to be similar to those previously reported for the β-phenylserine–pyridoxal system although the magnitudes of the rate constants were found to be much smaller. Sequential pyridoxal-catalyzed reactions consisting of β-elimination followed by δ-decarboxylation were observed in the pD range near the pK_a of the δ-carboxyl group. Metal ion catalysis was found to be less effective than proton catalysis in the promotion of γ-decarboxylation, and the catalytic effects of metal ions were found to decrease with increase in charge on the metal ion.

Metzler et al.[2] have described a general mechanism for the vitamin B_6-catalyzed reactions of Schiff bases of α-amino acids. The metal ion- and pyridoxal-catalyzed dealdolation reactions of β-hydroxy-α-amino acids have been described,[3-6] and probable reaction mechanisms have been proposed. Martell et al.[7,8] have described the kinetics and a proposed general mechanism for vitamin B_6-catalyzed β-elimination reactions of Schiff bases of α-amino acids having β-hydroxyl substituents. In the case of β-phenylserine β-elimination and dealdolation reactions were found to occur simultaneously.[9] For pyridoxal Schiff base model systems, decarboxylation of aspartic acid to alanine and of β-hydroxy aspartate to serine has been described.[10-12] The general mechanism proposed for δ-decarboxylation involves the ketimine Schiff base as an intermediate, thus requiring transamination of the aldimine Schiff base. The mechanisms proposed for these decarboxylation reactions involved consideration of the relative electron-withdrawing effects of the azomethine and pyridyl groups. Similarly, the capacity of the Schiff base to accommodate the electron pair shifted in the formation of an intermediate in the decarboxylation reaction was indicated as important in the proposed mechanism.[12] The present study of pyridoxal-catalyzed

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