Kinetics and Equilibria of the Reaction of Pyridoxal 5′-Phosphate with Ethylenediamine to Form Schiff Bases and Cyclic Geminal Diamines: Evidence for Kinetically Competent Geminal Diamine Intermediates in Transimination Sequences[1]

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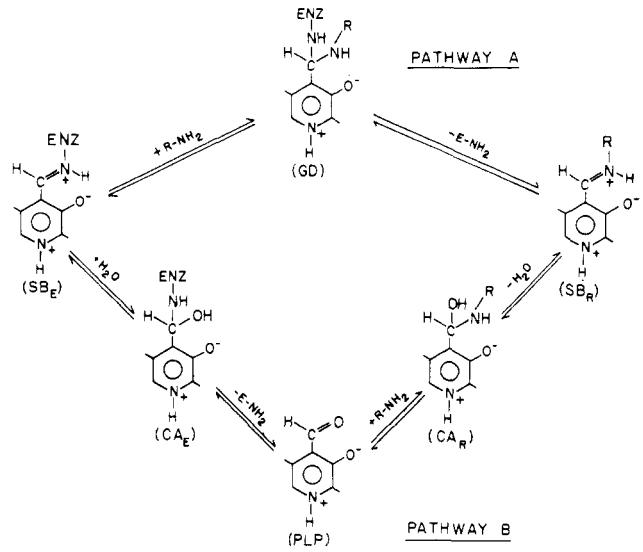
Abstract: Ethylenediamine and pyridoxal 5′-phosphate form Schiff bases and cyclic geminal diamines (imidazolidines) in aqueous solution in the pH range 7.5 to 14. The various pH-independent open chain cyclic tautomerization constants and the microscopic proton dissociation constants for the Schiff bases and cyclic geminal diamines have been determined from (1) the pH dependencies of electronic absorption spectra, (2) from the pH dependency of apparent association constants for addition of ethylenediamine to pyridoxal 5′-phosphate and (3) model compounds. The rates of the interconversion of the open chain Schiff bases and cyclic geminal diamines obtained from temperature-jump relaxation experiments appear to be hydronium ion catalyzed in the pH range 11.5 to 13 with kinetic constants reaching values $\sim 10^5$ sec^{-1} at the lower pH limit. This rate constant for a reaction which is in effect an intramolecular transimination sequence is about 10^7 larger than the rate constants for the alternative transimination route involving Schiff base hydrolysis and reformation and suggests that the transimination reactions in which the pyridoxal 5′-phosphate moiety is transferred from the ϵ-amino group of a lysine residue of an enzyme to the α-amino group of a substrate amino acid at the active sites of pyridoxal 5′-phosphate dependent enzymes almost certainly involve enzyme bound geminal diamine intermediates and *not* enzyme bound pyridoxal 5′-phosphate itself. Furthermore, from a comparison of the rates of the intramolecular transimination process and the turnover numbers of pyridoxal 5′-phosphate dependent enzymes, it appears that such enzymes may not need to catalyze the transimination process by contributions that are other than entropic in nature.

Since 1951, when the structure of codecarboxylase was first clearly established to be pyridoxal 5′-phosphate (pyridoxal-P)[3,4], it has been amply shown that pyridoxal-P is capable of catalyzing, without the apoproteins, the reactions catalyzed by the transaminases and most of the other pyridoxal-P dependent enzymes as well. However, in these studies of the pyridoxal-P dependent model reactions, elevated temperatures and polyvalent cations were generally employed, and the reactions were virtually always observed to proceed at much slower rates and to exhibit less specificity than the enzyme catalyzed reactions.[5] The catalytic cycles of these enzymes, as well as their nonenzymatic pyridoxal-P dependent models, all have as their central theme the reactivity of Schiff bases formed from pyridoxal-P and substrate amino acids; the first phase of such catalytic cycles must therefore be the formation of a Schiff base formed from pyridoxal-P and substrate. One possible pathway for the formation of such Schiff bases (pathway B, Scheme I) consists of two addition–elimination sequences with two carbinolamines and pyridoxal-P as enzyme bound intermediates.[6] However, since pyridoxal-P is stored at the active site of pyridoxal-P dependent enzymes as a Schiff base with the ϵ-amino group of a lysyl residue[5a] and the carbonyl group is not readily available as such, an alternative route is possible for the transfer of the pyridoxal-P moiety from the lysyl residue to the substrate amino acid, namely via a single addition–elimination sequence incorporating a single enzyme bound C_4' geminal diamine intermediate (Pathway A, Scheme I).[7]

Transimination reactions of pyridoxal-P have not been widely studied; there are only a few reports of intermolecular transimination involving pyridoxal-P[8a,b] and only a single communication on an intramolecular transimination which employed pyridoxal, not pyridoxal-P as the carbonyl component.[9] The latter study by Abbott and Martell led to the conclusion that intramolecular transimination must proceed via pathway B in which both nucleophilic catalysis and

Scheme I

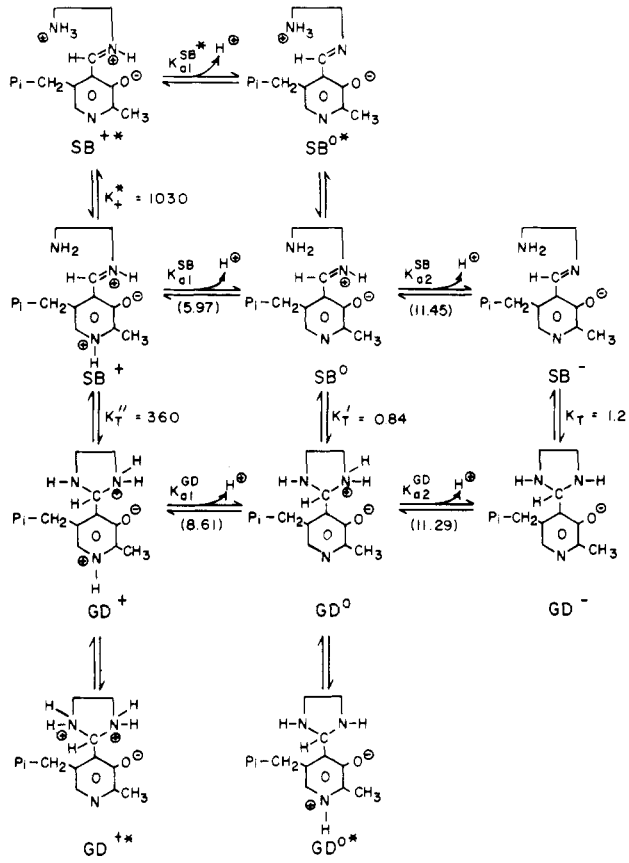


the entropic advantage of an intramolecular reaction are unavailable, while the former studies provide evidence in support of pathway A. Nucleophilic catalysis has been observed for benzaldehyde[10a] and pyridoxal-P[8a] semicarbazone formation, thiazolidine-4-carboxylic acid formation,[10b] and N^5,N^{10}-methylenetetrahydrofolic acid[11a] formation, and the reaction pathways have been described in terms comparable to pathway A. The formation of N^5,N^{10}-methylenetetrahydrofolic acid, from the Schiff base form of "active formaldehyde", to form in this case a very stable geminal diamine, may be viewed as the first half of a pathway A transimination sequence.[11a]

This report describes the thermodynamic and kinetic characterization of the elements of an intramolecular transimination reaction observable in solutions containing pyridoxal-P and ethylenediamine (EDA). At various pH

values, such solutions contain Schiff bases and geminal diamines in different states of ionization and in different proportions (See Results and Scheme II). Taken together with

Scheme II



kinetic studies of the formation of the Schiff bases and geminal diamines from EDA and pyridoxal-P, these data indicate that pathway A is the far more likely route for transimination (cf. Abbott and Martell[9]). Further, the transimination reaction occurs at rates which suggest that there is no necessity for apoenzyme catalysis of transimination by PLP dependent enzymes by contributions that are other than entropic in nature.

Results

Equilibria. Electronic absorption spectra of solutions of pyridoxal-P at high concentrations of EDA at several pH values are shown in Figures 1 and 2. The concentration of EDA in these solutions was such that more than 99% of the pyridoxal-P was complexed with EDA (Figure 3). Typically Schiff bases formed from pyridoxal-P and alkylamines have absorption maxima near 410 nm at pH values where the imine is protonated (>C=N$^+$H-, iminium) and near 340 nm at more alkaline pH values where the imine free base (>C=N-) exists.[12] That pyridoxal-P in EDA solutions is at least partly in the form of a Schiff base is shown by the 410 nm peaks at pH 7.6 and 10 and the 340 nm shoulder at pH 13.7 (see Figure 1). However, the additional absorption maxima at 340 (pH 7.6) and at 313 nm (pH 10 and 13.7) and the molar absorptivity values at 340 (pH 13.7) and 410 nm (pH 7.6 and 10) of about 50% of the expected value based upon aliphatic monoamine Schiff bases[17] suggest that the pyridoxal-P is only partly in the form of a Schiff base. Since pyridoxal-P derivatives with sp^3 hybridization of the C_4' carbon have characteristic absorption maxima near 310 (above pH 8) and near 325 nm (below pH 8),[13] we conclude that a solution containing pyridoxal-P and EDA consists of

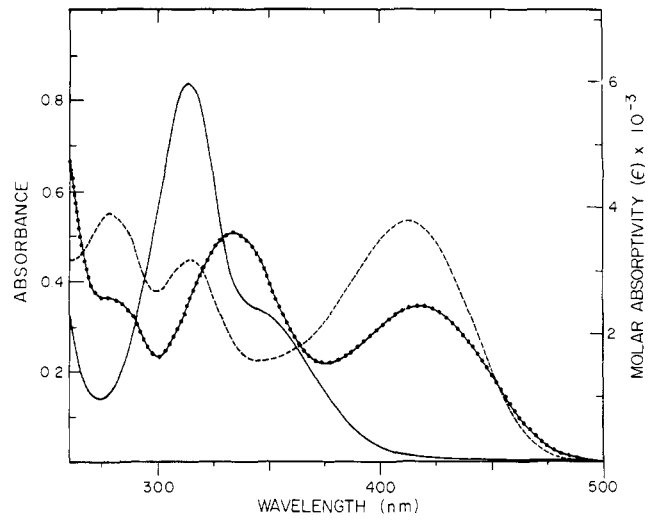


Figure 1. Electronic absorption spectra of solutions of EDA and pyridoxal-P (1.40×10^{-4} *M*), 25°, 1 *M* ionic strength. Dotted line (···), pH 7.55, [EDA] = 0.1 *M*; dashed line (----), pH 10.02 [EDA] = 0.5 *M*; solid line (—), pH 13.73, [EDA] = 0.5 *M*.

two entities and is a mixture of Schiff base and one (or more) C_4' saturated derivative. On the basis of spectra similar to those in Figure 1 for mixtures of pyridoxal-P and 1,3-diaminopropane (DAP), O'Leary concluded that such solutions contain a mixture of Schiff base and C_4' geminal diamine (GD)[14] while, from NMR observations of mixtures of pyridoxal and various diamino acids, Abbott and Martell concluded that solutions of pyridoxal and 2,3-diaminopropionic acid contained a mixture of Schiff base and C_4' carbinolamine (CA).[9]

From the following consideration of the relative magnitudes of the apparent association constants for complex formation from pyridoxal-P and EDA (K^c_{app}) and other amines, it is apparent that the C_4' sp^3 derivative of pyridoxal-P must be a geminal diamine. If there is an adduct formed from EDA and pyridoxal-P in addition to the Schiff base, then at any given pH, K^c_{app} contains additive contributions from the association constant for addition of EDA to pyridoxal-P to form a Schiff base (K^{SB}_{app}) and the association constant for addition of EDA to pyridoxal-P to form the unidentified species X (K^X_{app}), i.e., $K^c_{app} = K^{SB}_{app} + K^X_{app}$. From the data of Figure 3, in which K^c_{app} is plotted vs. pH together with association constants for Schiff base formation from pyridoxal-P and a variety of monoamines, an upper limit for the value of K^{SB}_{app} may be estimated to be 1.2×10^3 M^{-1} at pH 8.5 (i.e., twice the value of the highest K^{SB}_{app} for a monoamine since EDA contains two equivalent amino groups) and $K^X_{app} \geq 2.4 \times 10^3$ M^{-1}. If X is a carbinolamine, then this K^X_{app} value is >30- and 60-fold larger, respectively, than the association constants for carbinolamine formation from methylamine or *n*-propylamine and pyridine-4-carboxaldehyde,[15] which represent the largest alkylamine–aromatic aldehyde carbinolamine association constants of which we are aware. The largest carbinolamine formation constant from an amine and pyridoxal-P is about 13.5 M^{-1} for semicarbazide,[17] an α effect amine, at pH 8.3, and estimates[17] for serine and cysteine applicable to this pH range place the values <1 M^{-1}. We consider it therefore very unlikely that X is a carbinolamine and conversely very likely that X is a C_4' geminal diamine as depicted in Scheme II.

Several of the protonated structures shown in Scheme II may be eliminated in order to simplify the equations by means of which the significant equilibrium constants of

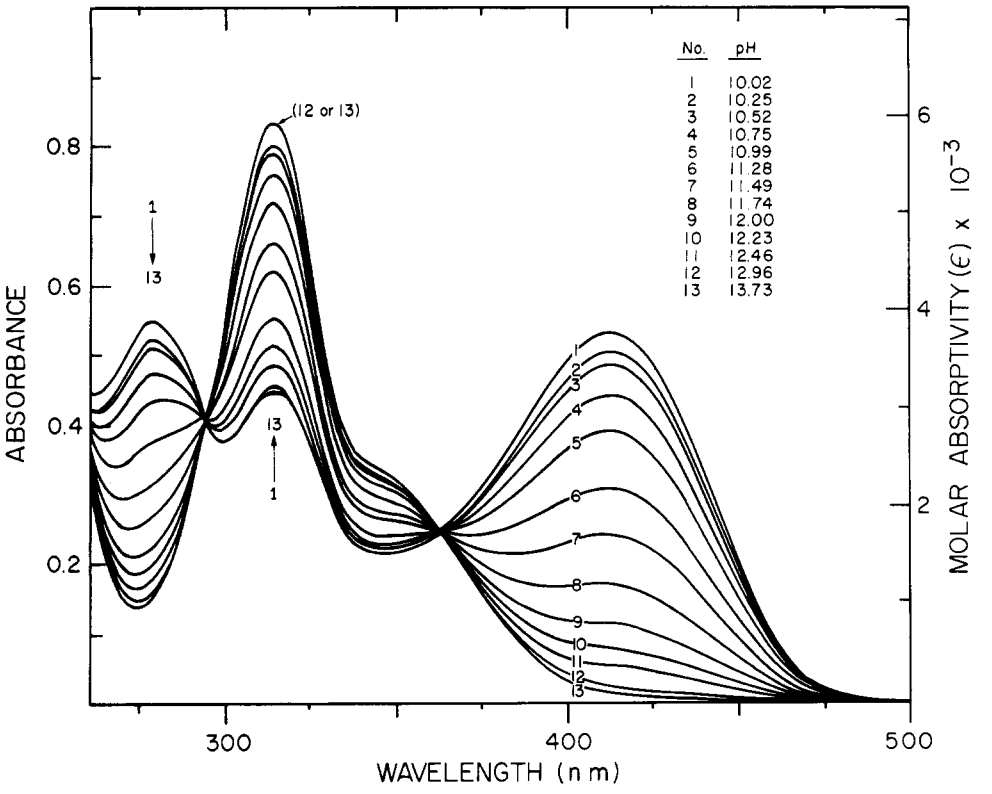


Figure 2. Electronic absorption spectra of solutions of EDA and pyridoxal-P (1.40×10^{-4} *M*), 25°, 1 *M* ionic strength; pH values as indicated, [EDA] = 0.5 *M*.

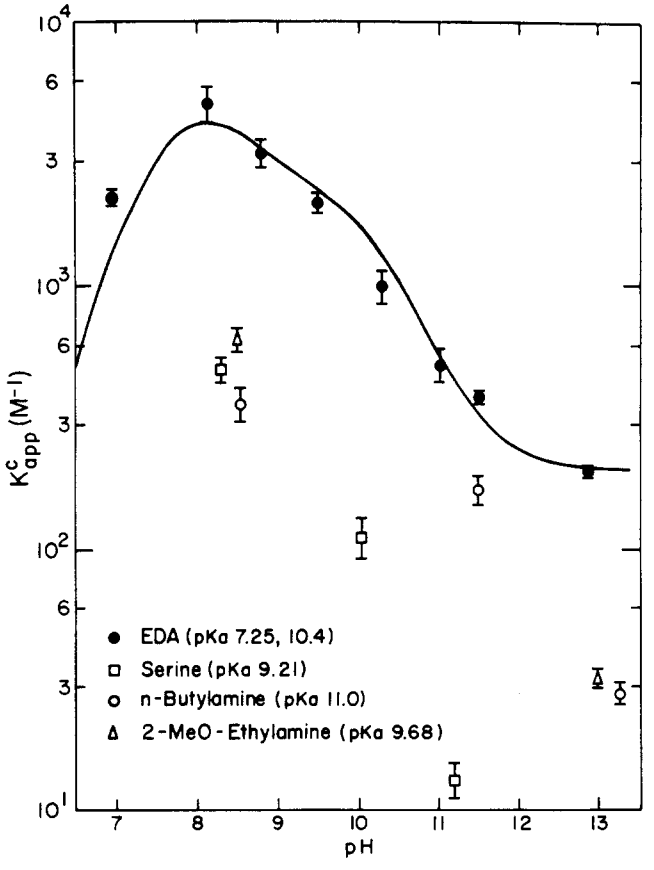


Figure 3. Association constants (K^c_{app}) for addition of EDA (●), serine (□), n-butylamine (○), and 2-methoxyethylamine (△) to pyridoxal-P, 25°, 1 *M* ionic strength. *Solid line* calculated from eq 1 and the constants contained in Table I. The pH was maintained with potassium phosphate or pyrophosphate, HEPES, and Dabco buffers and KOH.

Scheme II may be evaluated. First, GD^{0*} and GD^0 simply represent different ways of protonating GD^-. Since the basicity of the various functional groups of GD^-, as a first approximation, will be similar to those of pyridoxamine 5′-phosphate (pyridoxamine-P), the fact that the first protonation of pyridoxamine-P occurs exclusively at the C_4' amino group[16] effectively eliminates the need for consideration of GD^{0*} as a stoichiometrically significant prototropic tautomer of GD^0. Second, the less stable juxtaposition of positive charge around the C_4' carbon of GD^{+*} (cf. GD^+) makes species GD^{+*} unlikely to be of any stoichiometric significance with respect to species GD^+. Finally SB^{0*} may be eliminated from consideration since the pK_{a1}^E value (eq 1, Table I) is 10.3, while pK_{a2}^{SB} values for the Schiff bases formed from pyridoxal-P and *n*-butylamine or 2-methoxyethylamine are 12.4 and 11.4, respectively.[17] Thus the imine nitrogen of SB^- is at least tenfold more basic than the terminal amino group and protonation of SB^- will occur primarily at the imine nitrogen. Thus simplified, the microscopic equilibrium constants of Scheme II may be combined to yield the two macroscopic apparent acid dissociation constants K_{a1}^c and K_{a2}^c which appear in eq 1 (see eq 1i in Appendix) and Table I. This equation describes the pH dependence of the apparent equilibrium constant for the formation of the complex(es) between EDA and pyridoxal-P with the pH range 7–13.

$$K^c_{app} = \left\{ \frac{K_{a1}^E K_{a2}^E}{K_{a1}^c K_{a2}^c} K_{as}^c(\alpha_{E^{2+}}) + \frac{K_{a2}^E}{K_{a2}^c} K_{as}^c(\alpha_{E^+}) + K_{as}^c(\alpha_{E^0}) \right\} (\alpha_{P^-}) \quad (1)$$

At pH values of 13.4 or above, the absorption spectra of pyridoxal-P in EDA solutions become independent of pH, and only SB^- and GD^- are present in solution. From the apparent molar absorptivity value for the alkaline solutions

Table I. Equilibrium Constants, 25°, 1 *M* Ionic Strength (KCl)

Equilibrium	Symbol	K	−Log K	Source
$EDA^{2+} \rightleftarrows EDA^{+} + H^{+}$	$K_{a_1}E$	2.57×10^{-8}	7.59 ± 0.02	*a*
		3.89×10^{-8}	7.41	*b*
$EDA^{+} \rightleftarrows EDA^{0} + H^{+}$	$K_{a_2}E$	5.14×10^{-11}	10.29 ± 0.03	*a*
		6.92×10^{-11}	10.16	*b*
$PLP^{+} \rightleftarrows PLP^{0} + H^{+}$	$K_{a_1}P$	1.45×10^{-4}	3.84 ± 0.04	*a, d*
		7.25×10^{-5}	4.14	*c*
$PLP^{0} \rightleftarrows PLP^{-} + H^{+}$	$K_{a_2}P$	4.07×10^{-9}	8.39 ± 0.04	*a, d*
		2.04×10^{-9}	8.69	*c*
$\left\{\begin{matrix}SB^{+}\\+\\SB^{+}{*}\\+\\GD^{+}\end{matrix}\right\} \rightleftarrows \left\{\begin{matrix}SB^{0}\\+\\GD^{0}\end{matrix}\right\} + H^{+}$	$K_{a_1}c$	1.41×10^{-9}	8.85 ± 0.10	*a*
$\left\{\begin{matrix}SB^{0}\\+\\GD^{0}\end{matrix}\right\} \rightleftarrows \left\{\begin{matrix}SB^{-}\\+\\GD^{-}\end{matrix}\right\} + H^{+}$	$K_{a_2}c$	4.26×10^{-12}	11.37	*a*
$SB^{+} \rightleftarrows SB^{0} + H^{+}$	$K_{a_1}SB$	1.07×10^{-6}	5.97	*a, e*
$SB^{0} \rightleftarrows SB^{-} + H^{+}$	$K_{a_2}SB$	3.55×10^{-12}	11.45	*a*
$GD^{+} \rightleftarrows GD^{0} + H^{+}$	$K_{a_1}GD$	2.48×10^{-9}	8.61	*a, e*
$GD^{0} \rightleftarrows GD^{-} + H^{+}$	$K_{a_2}GD$	5.07×10^{-12}	11.29	*a*
$EDA^{0} + PLP^{-} \rightleftarrows \left\{\begin{matrix}SB^{-}\\+\\GD^{-}\end{matrix}\right\}$	$K_{as}c$	200		*a*
$SB^{-} \rightleftarrows GD^{-}$	K_T	1.2		*a*
$SB^{0} \rightleftarrows GD^{0}$	K_T'	0.84		*a*
$SB^{+} \rightleftarrows GD^{+}$	K_T''	3.6×10^{2}		*a, e*
$SB^{+} \rightleftarrows SB^{+}{*}$	$K_{+}{*}$	1.03×10^{3}		*a, e*

[a] This work. [b] Reference 29. [c] Reference 16. [d] Assigned as in ref 12. [e] See text.

Table II. Electronic Absorption Spectral Data, 25°, 1 *M* Ionic Strength (KCl)

Compd	λ_{max}, nm	$\epsilon \times 10^{-3}$, M^{-1} cm^{-1}	pH	Conditions[a]	Source
Pyridoxal-P					
n-Butylamine Schiff base	339	5.61[b]	13.7	0.50 *M* amine 0.45 *M* KOH	*c*
	409	7.37	8.94	0.20 *M* amine 0.10 *M* diazabicyclo-[2.2.2]-octane	*c*
Pyridoxamine-P	308	8.04	13.6	0.5 *M* KOH	*c*
	308	8.00	~13	0.1 *M* NaOH	*d*
	311	7.36	9.77	0.1 *M* K_2CO_3	*c*
	325	8.30	7.0	0.1 *M* sodium phosphate	*d*

[a] Chosen in this work to obtain, as nearly as possible, spectral data for a single ionic species. Buffers adjusted with HCl. [b] Corrected for lack of complete pyridoxal-P complexation using measured association constant of 26 M^{-1}. [c] This work. [d] Reference 13.

of pyridoxal-P and EDA at 340 nm, K_T may be determined from the analogous Schiff base formed from pyridoxal-P and *n*-butylamine at pH 13.7 and 339 nm,[17] Table II). Since pyridoxamine-P at pH 13.7 has no appreciable absorption at 340 nm,[13] GD^- is also expected to be transparent at this wavelength. K_T is calculated to be 1.2 from the data of Figure 1 at pH 13.7 and $K_T = ([P_{TOT}]\epsilon_{SB^-}/A_{340}) - 1$.

Above pH 10, as discussed above, one may reasonably anticipate that only the four species GD^0, GD^-, SB^0, and SB^- will be present in solution. Since SB^0 is expected to be the only species absorbing at 413 nm and K_T is known, with an estimated molar absorptivity value for SB^0 (ϵ_{SB^0} based upon the molar absorptivity of the Schiff base formed from *n*-butylamine and pyridoxal-P at 409 nm and pH 8.94,[17] Table II), the data of Figure 2 may be plotted in accordance with eq 2, where $[P_{TOT}] = [SB^0] + [SB^-] + [GD^0] + [GD^-]$, a_H is the hydronium ion activity, and A_{413} is the absorbancy of the solution at 413 nm (Figure 4), in order to determine K_T' and K_{a2}^{SB}. From the ordinate intercept of the plot in Figure 4, $K_T' = 0.84$ and, from the slope, $K_{a2}^{SB} = 3.55 \times 10^{-12}$ ($pK_{a2}^{SB} = 11.45$). Finally, from the thermodynamic relationship $K_{a2}^{GD} = K_{a2}^{SB}K_T/K_T'$, $K_{a2}^{GD} = 5.07 \times 10^{-12}$ ($pK_{a2}^{GD} = 11.29$). The near equality of K_T and K_T' or of K_{a2}^{SB} and K_{a2}^{GD} is consistent with the tight isosbestic points at 294 and 363 nm in the series of spectra shown in Figure 2. The linearity of the plot in Figure 4 appears to justify the assumption that, above pH 10, the doubly protonated species of Scheme II do not comprise any appreciable fraction of total pyridoxal-P. From these results, pK_{a2}^{c} was calculated to be 11.37 from eq 3b (see Appendix).

$$\frac{[P_{TOT}]}{A_{413}} = \frac{(1 + K_T')}{\epsilon_{SB^0}} + \frac{(1 + K_T)K_{a2}^{SB}}{\epsilon_{SB^0}} \times \frac{1}{a_H} \quad (2)$$

Between pH 7 and 10, plots of absorbancy at a given wavelegth vs. pH look very much like titration curves. However, such plots cannot be analyzed in a simple manner as has been done for the pH range above pH 10 since there is no wavelength at which only one of the five species which may exist in this pH range (GD^+, GD^0, $SB^+{*}$, SB^+, and SB^0) uniquely absorbs in the uv–vis wavelength range. However, since all the constants on the right hand side of eq 1 are now determined with the exception of K_{a1}^{c}, this last constant was obtained iteratively utilizing eq 1 and the data of Figure 3 ($pK_{a1}^{c} = 8.85 \pm 0.10$).

Finally with estimates of K_{a1}^{SB} and K_{a1}^{GD} from compounds analogous to SB^+ and GD^+, K_T'' and $K_+{*}$ were calculated. For pK_{a1}^{GD} the reported value of 8.61 for the acid dissociation constant of the pyridoxamine-P pyridinium group[16] was employed and, for pK_{a1}^{SB}, the value of 5.97 for

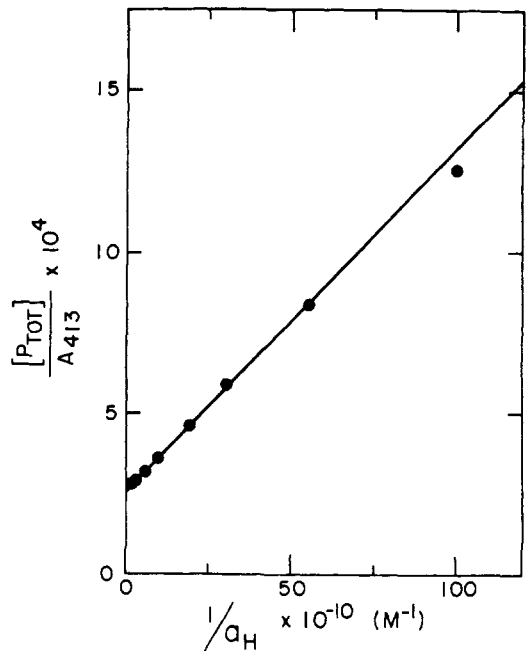


Figure 4. Plot of the data of Figure 2 according to eq 2 for the purpose of obtaining K_T' and $K_{a_2}^{SB}$ values. *Solid line* calculated from eq 2, the constants contained in Table I, and $\epsilon_{SB^0} = 7.37 \times 10^3\ M^{-1}\ cm^{-1}$ (Table II).

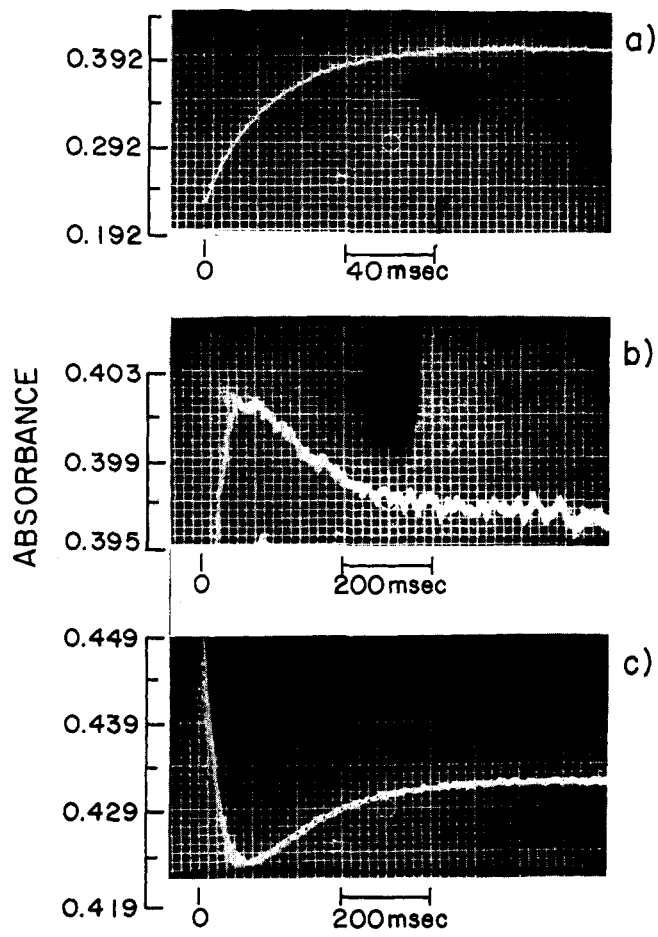


Figure 5. Oscillographic recordings of the absorbancy changes observed in the stopped-flow spectrophotometer upon mixing of EDA with pyridoxal-P at pH 10.34, 25°, 1 *M* ionic strength. After mixing, [EDA] = 0.25 *M* and [pyridoxal-P] = 2.5×10^{-4} *M*. Wavelengths: (a) and (b) at 313 nm; (c) at 410 nm.

the corresponding ionization for the Schiff base formed from pyridoxal-P and serine[17] was utilized. Thus from the relation $K_T'' = K_{a1}^{SB} \times K_T'/K_{a1}^{GD}$, K_T'' is estimated to be 3.7×10^2 and, from equation 3a, K_+^* is estimated to be 1.03×10^3. The solid line in Figure 3 is calculated from eq 1 and the constants in Table I. Equation 1 is similar to the equations utilized by Metzler[18a] or French et al.[18b] to describe similar association equilibria of monoamines and heteroaromatic aldehydes.

Kinetics. The kinetics of the reaction of pyridoxal-P with EDA were studied in part in an effort to obtain association constants for carbinolamine formation from a type of "saturation" kinetics (i.e., progression of the dependence of the pseudo-first-order rate constants for product formation from first order to zero order in respect to EDA concentration).[6,10b,11a] Such data might have lent additional direct experimental support to our previous conclusion (based upon the magnitude of the association constants for the formation of the C_4' sp^3 hybridized pyridoxal-P derivative) that the C_4' saturated product of the reaction of pyridoxal-P with EDA was a geminal diamine. However, it was not possible to accomplish this because of the fact that the rate of dehydration of pyridoxal-P hydrate became rate determining at high EDA concentrations. Thus, the reaction at pH 10.34 of pyridoxal-P with EDA at or above 0.25 *M* EDA is clearly biphasic when observed with a stopped-flow spectrophotometer at either 313 or 410 nm. At 313 nm, a large rapid increase in absorbancy was followed by a slower, small decrease (see a and b in Figure 5), while, at 410 nm, a large, rapid decrease was followed by a slower, smaller increase in absorbancy (see c in Figure 5). These traces appear to be a composite of two exponential processes (Figure 6), which we shall designate k^f_{obsd} and k^s_{obsd}, for the faster and slower exponentials, respectively. At lower concentrations of EDA, i.e., below 0.25 *M*, only a single exponential was observed at either wavelength, the absorbancy increasing at 313 nm and decreasing at 410 nm. The concentration dependencies of the pseudo-first-order rate constants (k_{obsd}) for the various exponential processes are plotted vs. EDA concentration in Figure 7. The k_{obsd} values obtained at low EDA concentration together with the k^f_{obsd} values for the fast phase describe a straight line defined by the second-order rate constant k_F (first order in both EDA and pyridoxal-P). The plot of k_{obsd} values at less than 0.05 *M* EDA and the k^s_{obsd} values for the slow phase vs. EDA concentration appears to become independent of the EDA concentration at high EDA concentration. These latter data have been fit as a rectangular hyperbola defined by eq 4 and the constants, *G*, and maximum first-order rate constant, $k^s_{obsd_{max}}$ (eq 4, Table III).

$$k_{obsd} \text{ or } k^s_{obsd} = k^s_{obsd_{max}}/\{1 + 1/(G[EDA])\} \quad (4)$$

$$K_{CA} = [>C(OH)HNR]/[RNH_2][>C{=}O];$$

$$K_d = [>C{=}O]/[>C(OH)_2]$$

We account for these results in terms of Scheme III in which k_F is equal to the second-order rate constant $(1 + K_d)\,K_{CA}k_2$ for Schiff base formation (with carbinolamine (CA) dehydration being rate limiting), and $k^s_{obsd_{max}}$ is equal to k_d, the first-order rate constant for pyridoxal-P hydrate ($PLP{\cdot}H_2O$) dehydration.[19a] An alternate scheme in which $k^s_{obsd_{max}}$ is identified with the rate constant for CA dehydration[6,10b,11a] is inconsistent with the increase in $k^s_{obsd_{max}}$ values as the pH increases from 10.3 to 14[19b] and with estimates[17] of K_{CA} (which should equal *G*) at pH values of 10.3 ($2.8\ M^{-1}$) and 8.03 ($11\ M^{-1}$). Furthermore,

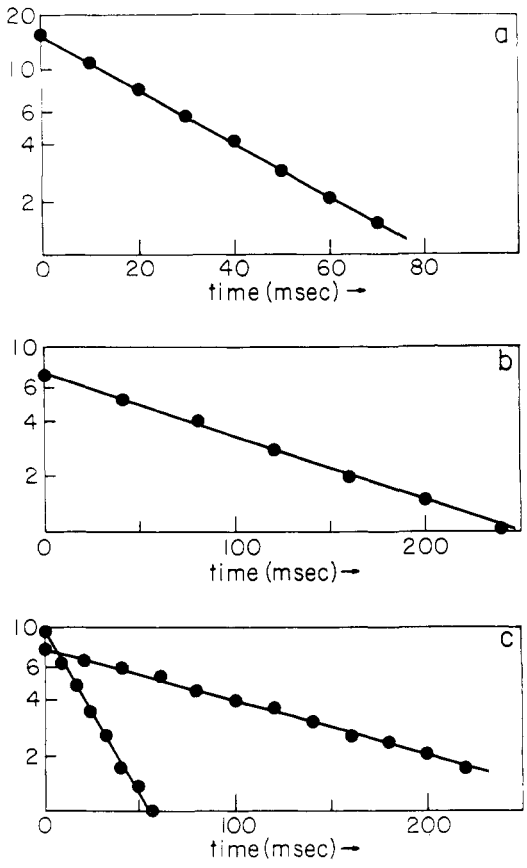


Figure 6. Semilogarithmic plots of the data in Figure 5. Ordinate scale arbitrary.

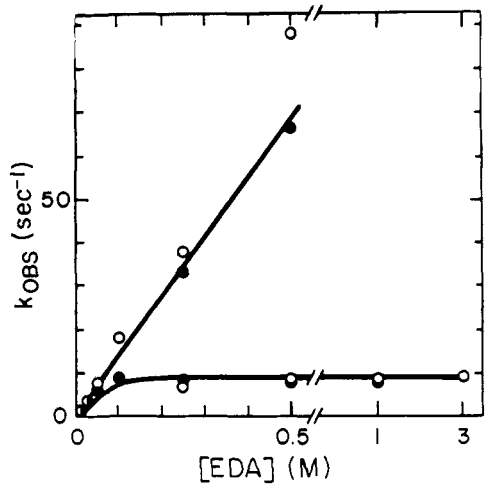


Figure 7. Pseudo-first-order rate constants (k_{obsd}) for the addition of EDA to pyridoxal-P obtained at 410 (○) or 313 nm (●), pH 10.34, 25°, 1 *M* ionic strength. *Solid line* (linear) calculated as follows: $k_{obsd} = K_{CA}(1 + K_d)k_2$[EDA], where $(1 + K_d)K_{CA}k_2$ is 142 M^{-1} sec^{-1} and *solid line* (nonlinear) calculated from eq 4 and the parameters contained in Table III.

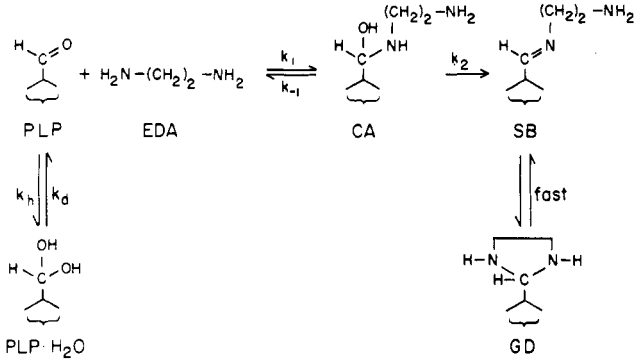
Table III. Kinetic Constants[a] for the Reaction of Pyridoxal-P with EDA, 25°, 1 *M* Ionic Strength (KCl).

pH	k_F, M^{-1} sec^{-1}	G, M^{-1}	$k^s_{obsd_{max}}$ sec^{-1}	k_d, sec^{-1}	K_{CA}^{lim}, M^{-1}
8.01	34 (±5)	4.3 (±1.7)	10.5 (±1.0)	10[b]	<0.5
10.34	134 (±10)	17.5 (±2.5)	8.7 (±0.2)	8.6[b]	<1
14.0	*c*	0.9 (±0.1)	106 (±5)	118[b]	*c*

[a] k_F obtained from linear relationship of k_{obsd} vs. [EDA] and $k^s_{obsd_{max}}$ from eq 4 in text. [b] See ref 20. [c] Not measureable.

the fact that $k^s_{obsd_{max}}$ and k_d[20] values are identical (Table III), at each pH value studied over the pH range 8–14, is unlikely to be coincidental and argues very strongly for Scheme III in which $k^s_{obsd_{max}}$ is assigned to the pyridoxal-P

Scheme III



hydrate dehydration step. Finally the fraction of total pyridoxal-P which is unhydrated (obtained from the preexponential constants calculated from traces in Figures 5 and 6) is similar to that found by other workers.[21b]

Instrumental limitations precluded further studies of the dependencies of either the absorbance change or the kinetics of the fast phase upon the concentration of EDA at EDA concentrations greater than 0.5 *M*, and thus a direct determination of K_{CA} was not possible. The fact that the dependence of the k_{obsd} values for the fast phase upon [EDA] remains linear at all of the experimental pH values studied does nevertheless appear to set an upper limit upon the value of K_{CA}, denoted K_{CA}^{lim}. The values of K_{CA}^{lim} (see Table III) are of the order of magnitude of our aforementioned estimates of the carbinolamine association constant for pyridoxal-P and EDA and are clearly much less than the magnitude required of K_{app}^{X} to account for the data for EDA in Figure 3.

The rate constant for SB → GD must be greater than the maximum observed value of $(1 + K_d)K_{CA}k_2$[EDA] since the observed rate constants for each phase are the same whether the appearance of Schiff base or C_4' saturated derivative is followed.

Kinetic observation of the geminal diamine Schiff base ring–chain tautomerization reaction was accomplished by temperature jump perturbation of solutions of pyridoxal-P and EDA at various pH values. The observed first-order rate constants (k'_{obsd}) were found to be independent of pyridoxal-P concentration over the range 2 to 10 × 10^{-4} *M* and appear to show a first-order hydronium ion dependence in the pH range 11.5 to 13.6 (Figure 8). Since the electronic time constant of the instrument used corresponds to the plateau value of the observed rate constants (1.6 × 10^5 sec^{-1}), whether or not the rate constants for the chemical reaction do plateau at 1.6 × 10^5 sec^{-1} with an apparent pK_a value of 11.3 (dashed line) or continue acid catalyzed below pH 12 (dotted line) cannot be concluded at present (Figure 8). In any event below pH 12, the plateau (dashed line, Figure 8) represents a lower bound and the dotted line of slope −1 based on the assumption of a simple first-order hydronium ion dependence, an upper bound for the rate constants for the Schiff base–geminal diamine reequilibration. Writing the ring–chain tautomerization reaction as shown in eq 5, where an asterisk is used to distinguish the nitrogen atoms, $k'_{obsd} = 2(k_{+3} + k_{-3})$ and K_T^{obsd} = [geminal diamine]/[Schiff base] = k_{+3}/k_{-3}. From the value of k'_{obsd} extrapo-

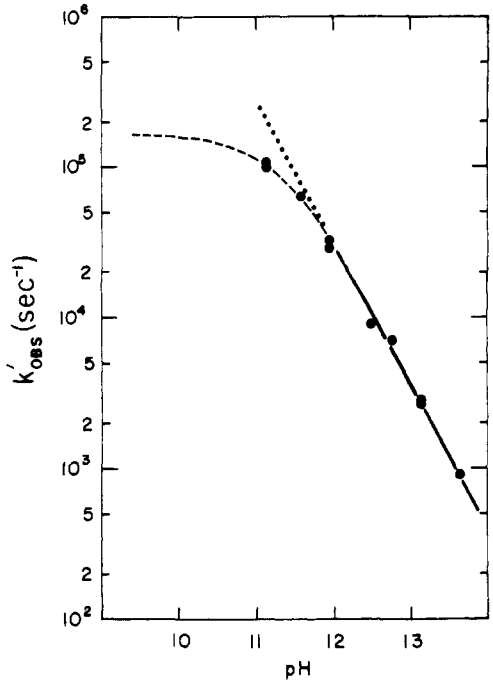
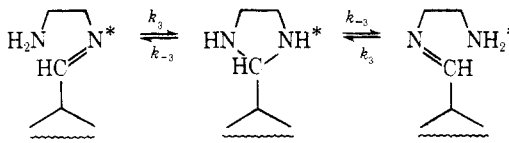


Figure 8. First-order rate constant (k'_{obsd}) obtained by temperature jump relaxation methods applied to solutions of pyridoxal-P (5.6×10^{-4} *M*) and EDA (0.5 *M*), 25°, 1*M* ionic strength. See text for details.

lated to pH 10 via the dotted line and the fact that, near pH 10, $K_T^{obsd} = K_T'$, one obtains $k_{+3}' \sim k_{-3}' \geq 4 \times 10^4$ sec^{-1}.



(5)

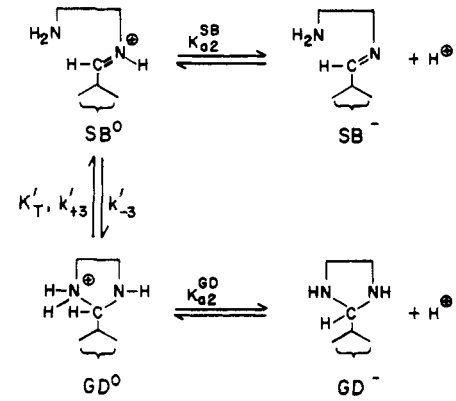
Discussion

The Mechanism and Kinetics of Transimination. The two possible pathways for transimination are shown in Scheme I. Pathway A, first proposed by Snell,[7] is a single addition elimination sequence with a geminal diamine as its only intermediate, while pathway B consists of two addition elimination sequences with two carbinolamines and free pyridoxal-P as intermediates. The relative kinetic competence of the two pathways has typically been examined in model systems by comparing the rate of transimination via pathway A with the rate of formation of the final Schiff base from free pyridoxal-P and amine.

To our knowledge, three intermolecular transimination model systems have been studied. Cordes and Jencks found that pyridoxal-P semicarbazone formation was catalyzed 4–80 fold by a variety of primary and secondary amines and concluded therefore that pathway A was kinetically favored.[8a] Mackay studied thiazolidine-4-carboxylic acid formation from pyridoxal-P and cysteine in the presence of varying amounts of glutamic acid, having previously shown that the rate-determining step for thiazolidine formation was the formation of the Schiff base formed from pyridoxal-P and cysteine. At sufficiently high concentrations of glutamic acid to complex essentially all of the pyridoxal-P as its Schiff base with glutamic acid, thiazolidine formation was some two- to threefold slower than in the absence of glutamic acid.[8b] Thus, although pathway A was available for transimination, the Schiff base was somewhat less reactive than pyridoxal-P itself under those conditions. Similar studies utilizing 6 aminocaproic acid instead of glutamic acid gave essentially the same results.[8c] Abbott and Martell reasoned that intramolecular transimination, such as might occur at the active site of an enzyme, could be much faster than intermolecular transimination and that the proposed geminal diamine might be directly observable in an intramolecular system.[9] From the line widths and time dependency of the NMR spectra of mixtures of pyridoxal (not pyridoxal-P) and various diamino acids, they came to the surprising conclusion that the intramolecular transimination sequence of pathway A was not observable at all and that transimination occurred via pathway B on a time scale of hours.[9]

In contrast to this NMR study, based upon the measured values of k_3 and k_{-3} (Scheme IV), we propose that intramolecular transimination of pyridoxal-P from one alkylamine to another proceeds via pathway A and does so at very rapid rates indeed.

Scheme IV



From studies of the reactions of imines, it is clear that the addition of basic nucleophiles such as an alkylamine occurs to the cationic iminium species and, from studies of the elimination of basic amines from carbinolamines, it appears that prior N-protonation is required for amine elimination.[6,19b] This leads to the conclusion that the pathway for transimination in the pyridoxal-P and EDA system is best described by Scheme IV, which incorporates specific acid catalysis[22] of both the ring opening and closing reactions involving EDA.

From Scheme IV it would be predicted that a graph of k'_{obsd} vs. pH should reach a plateau when both the Schiff base and geminal diamine are protonated, i.e., SB^0 and GD^0 of Scheme IV predominate in solution. For this reason, we feel that the dashed line and data points are a better description of the pH dependency of k'_{obsd} than the dotted line of slope −1. Then from $K_T' \sim 1$, one obtains the following, $k'_{+3} \simeq k'_{-3} \geq 4 \times 10^4$ sec^{-1}.

The quantitative comparison of the rates of geminal diamine formation via pathways A and B follows. The association constant for the formation of the Schiff base from pyridoxal-P and EDA near pH 10 is given approximately by $K_{app}^c/(K_T' + 1)$ and, at 10 *M* EDA, an upper limit of the rate constant for hydrolysis of the Schiff base formed from pyridoxal-P and EDA is given by $k^f_{obsd}[EDA]/\{K^c_{app}/(K'_T + 1)\} = 2.7 \times 10^{-3}$ sec^{-1}. Since the rate constant near pH 10 for transimination via pathway A is at least 4×10^4 sec^{-1}, pathway A is favored as the route for transimination by a factor of at least 1.5×10^7, a very large factor indeed.

The difference between our results and those of Abbott and Martell[9] is certainly striking. We can only speculate that the unblocked 5-hydroxymethyl group in pyridoxal and/or the high concentrations (~ 0.1 *M*) of pyridoxal and diamine necessitated by the NMR studies account for the

differences between their results and those reported herein.

It is interesting to attempt an extrapolation of our results to the active site of a pyridoxal-P dependent enzyme at pH 7 in view of prior statements to the effect that enzymatic transimination must be apoenzyme catalyzed.[8a] We limit ourselves to considerations of specific acid-base catalysis of the addition and elimination reactions, rate constants of which will be $k_{3(7)}$ and $k_{-3(7)}$ at pH 7, respectively, and assume that the effects of reactant approximation at an active site are not more significant than reactant approximation in our model system. The pyridine nitrogen of the Schiff base holoenzymes is thought to be maintained in an effective state of protonation at pH ~7,[5a,b] i.e., as SB^+, while, at pH 10 and above as in our experiments, it certainly is unprotonated. The effect of such protonation is to increase the nucleophilic susceptibility of the Schiff base formed from pyridoxal-P and serine about 40- and 600-fold toward water and hydroxide ion, respectively,[17] and we assume as a first approximation a similar effect (~100-fold) for nitrogen nucleophiles. Thus $k_{3(7)}$ is estimated to be greater than 4×10^6 sec^{-1} and, with the K_T'' value of 3.6×10^2, $k_{-3(7)} > 1 \times 10^4$ sec^{-1}.

In all of the temperature jump relaxation studies of enzyme active site transimination, a large number of relaxations are observed with disappointingly few relaxations assignable to individual chemical events.[23] Despite these limitations, the results of a study of the kinetics of the interaction of glycine with serine hydroxymethylase (EC 2.1.2.1) at pH 7.3 by Schirch[24] are summarized in eq 6, in which

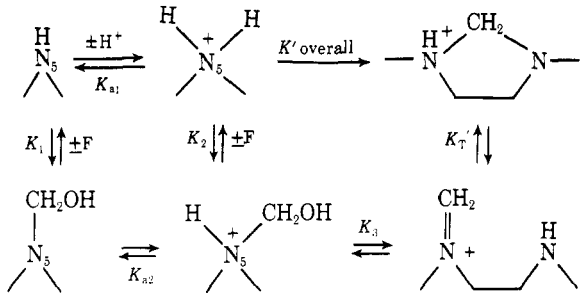
$$E + Gly \underset{1.2 \times 10^3\ sec^{-1}}{\overset{7 \times 10^4 M^{-1}\ sec^{-1}}{\rightleftharpoons}} EX \underset{1.3 \times 10^3\ sec^{-1}}{\overset{3.2 \times 10^3\ sec^{-1}}{\rightleftharpoons}} EY \qquad (6)$$

Gly is glycine, E is enzyme, EX is a geminal diamine, and EY is a noncovalent complex of enzyme and the Schiff base of glycine and pyridoxal-P. The equation is strictly analogous to pathway A of Scheme I. If the acid dissociation constant of the ϵ-ammonium group of the lysine residue in EY is the same as that for lysine in solution,[25] i.e., pK_a = 10.8, then from our lower limit of k_3, the rate constant for EY → EX (assuming that only the free base form of the lysyl residue is reactive) should be 1.3×10^3 sec^{-1}, surprisingly close to the observed value (eq 6) with the serine hydroxymethylase-glycine system. Similarly, the ratio [EX]/[EY] ~ 0.4 is very close to the value of 0.36 calculated for the analogous ratio (see below) in our model system studies.

Since the results on the model system and serine hydroxymethylase provide rate constants for transimination which are similar to each other and at the same time larger than any turnover number for a pyridoxal-P enzyme of which we are aware, there is neither a need for apoenzyme catalysis of transimination nor does there appear to be any catalysis of this process.

Acidity of SB^0 and GD^0. On the basis of a structure reactivity correlation developed[17] for the purpose of predicting iminium proton dissociation constants for pyridoxal-P Schiff bases from the acid dissociation constants of the reactant alkylammonium ions, we would expect pK_{a2}^{SB} to be 11.75 ± 0.25, in fair agreement with the experimental value of 11.45. An estimate of pK_{a2}^{GD} is based upon the pK_a value of 10.9 for the ammonium conjugate acid of pyridoxamine-P,[16] an adjustment of +0.3 for the increased basicity of secondary amines,[26] an adjustment of −0.8 based on the structure reactivity correlation of Fox and Jencks[27] employing their value of $\rho_I = -8.4$ and $\sigma^1 = 0.1$ for an amine or alkylamine substituent,[28a] an adjustment of +0.3 since the five-membered ring cyclic amine pyrrolidine is 0.3 units more basic than diethylamine,[26] and finally +0.3 for statistical factors (two sites for protonation). Thus we would expect pK_{a2}^{GD} to be 11.0 as compared with an experimental value of 11.29. Therefore, it appears reasonable that the values of pK_{a2}^{GD} and pK_{a2}^{SB} are very nearly equal, and we conclude that neither GD^0 or SB^0 possesses any markedly unusual acidic properties.

Scheme V



$K_1 = [>NCH_2OH]/[>NH][F]$

$K_2 = [>NHCH_2OH^+]/[>NH_2^+][F]$

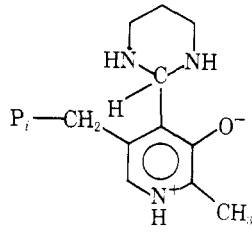
$K_{a_1} = [>NH]a_H/[>NH_2^+]$

$K_{a_2} = [>NCH_2OH]a_H/[>NHCH_2OH^+]$

$K_3 = [>N^+{=}CH_2]/[>N^+HCH_2OH]$

$K_T' = [>N^+H\text{-}CH_2\text{-}N<]/[>N^+{=}CH_2]$

$F = ([H_2C(OH)_2] + [H_2C{=}O])$



Geminal Diamine Schiff Base Ring-Chain Tautomerization Equilibria. One of the striking results of this study is the finding that the ratio of geminal diamine to Schiff base in solutions of pyridoxal-P and EDA varies by less than a factor of 4 over the pH range 7-14. At about pH 14 K_T = 1.2, at about pH 10 K_T' = 0.84, and at about pH 7 the ratio $[GD^+]/([SB^+] + [SB^{+}*]) = K_T''/(K_+* + 1) = 0.36$. The basis of this pH independency clearly derives from the value of K_T, modified at lower pH values by the coincidentally offsetting acid-base properties of the variously protonated geminal diamines and Schiff bases of Scheme II. Other stable five-membered ring cyclic geminal diamines of biochemical relevance are known, for example, N^5,N^{10}-methylenetetrahydrofolic acid[11a] and a variety of its model compounds.[11b,c] These imidazolidines involve rather different substitutions of the geminal diamine ring, secondary amines, and different aldehydes. A calculation[28b] of the pH independent equilibrium constant for the Schiff base geminal diamine tautomerization (K_T') in the N^5,N^{10}-methylenetetrahydrodolate system is 7.9×10^4 (Scheme V), and this value indicates that, for aliphatic aldehydes, the cationic iminium species is many orders of magnitude less stable than the cationic cyclic geminal diamine (imidazolidine). As far as we are aware, comparable five-membered ring cyclic geminal diamines formed from aromatic aldehydes and primary diamines, for example, from EDA and benzaldehyde, have not been observed.[29] Analogous six-membered ring geminal diamines formed for aromatic aldehydes appear to be more common, for example, from pyridoxal and 2,4-diaminobutyric acid,[9] or from 1,3-diaminopropane (DAP) and pyridoxal-P or pyridine-4-aldehyde.[14] In studies involving pyridoxal and diamines, Schiff bases were not ob-

served among the final products. In contrast to the system composed of pyridoxal and 2,4-diaminobutyric acid, pyridoxal-P reacts with DAP to form a mixture of Schiff base and geminal diamine.[14] With respect to the data on the interaction of pyridoxal-P with DAP,[14] we should like to point out the following. (1) Since spectral observations were not made above pH 11, a significant part of the pH dependency of the geminal diamine–Schiff base tautomerization and the proton dissociations of structures analogous to SB^0 and GD^0 was missed. (2) As shown below (XIII in ref 14), the site of protonation of the GD^0 analog formed from DAP and pyridoxal-P was assigned to the pyridine nitrogen and not to one of the C_4' amino groups. This is inconsistent with what is known about the relative basicity of the various functional groups of pyridoxamine-P[16] as we have discussed earlier with respect to GD^{0*} and the predicted and calculated values of pK_{a2}^{GD}. The fact that both pyridoxamine-P and pyridoxine 5′-phosphate have absorption maxima at 325 nm at pH 7,[13] at which pH the pyridine nitrogens of both are protonated whereas the solutions of DAP and pyridoxal-P at pH 10-11 clearly exhibit an absorption maximum at 310 nm, is also inconsistent with O'Leary's assignment of structure. (3) In the discussion of the pH dependency of the Schiff base–geminal diamine tautomerization between pH 7 and 11, the existence of a geminal diamine protonated at both the pyridine nitrogen and at one of the C_4' amino groups (e.g., GD^+) was not considered and species analogous to SB^- and GD^- were ignored. No evidence is presented for these omissions, although it is possible that they may be justified for that particular system. In view of these limitations, the analysis of the pH dependency for cyclization and the determination of a cyclization equilibrium constant $K_C = 25$, analogous to our K_T', may be in error. Indeed, the value of K_C is plainly inconsistent with the spectral data presented in Figure 3 of ref 14, from which one would estimate $K_C \leq 3$ at pH 10. Since six-membered rings are generally thought to be more stable than five-membered rings,[30] the small difference between our value K_T' and our estimate of K_c for DAP is somewhat surprising, although it may well be that this generalization does not hold in the comparison of imidazolidine and hexahydropyrimidine stabilities.

Summary

It has been shown that both Schiff bases and geminal diamines are formed in solutions containing pyridoxal-P and EDA over the pH range 7.5–14. Furthermore, the pathway for intramolecular transimination via a geminal diamine is extremely rapid relative to the alternative transimination pathway involving a free pyridoxal-P intermediate. These results strongly suggest that geminal diamines can serve as kinetically competent intermediates for transimination sequences at the active sites of pyridoxal-P dependent enzymes and that such enzymes may not need to catalyze the transimination process by contributions that are other than entropic in nature.[33]

Experimental Section

Materials. Liquid amines were redistilled from CaH_2 before use. Pyridoxal-P was obtained from Sigma as the monohydrate (based upon the molar absorptivity value of Peterson and Sober[13]) and was used without further purification with solution concentration determined spectrophotometrically and gravimetrically. Buffer components, inorganic salts, and disodium ethylenediaminetetraacetic acid (Na_2EDTA) were reagent grade and used without further purification. Deionized water of greater than 5×10^5 ohms cm specific resistance was used throughout with KCl added to maintain ionic strength constant at 1.0 ± 0.1 M except where noted otherwise. All solutions contained 0.005 M Na_2EDTA to chelate with any polyvalent cations present as impurities in buffer components, etc.

Methods. All experimental measurements were made on solutions maintained at $25 \pm 1°$.

Curve fitting of experimental data to theoretical equations (straight lines, rectangular hyperbolae, and the titration of EDA) was performed by linear or nonlinear[34b] least-squares computer programs made available to us by Dr. R. O. Viale.

Measurements of pH were obtained with a Radiometer Model 25SE or Model 26 pH meter equipped with GK2302B or GK2303C (Radiometer) combination electrodes calibrated with pH 7 and 10 standard buffers (Beckman) and 0.1 N NaOH.[34a]

Absorbance measurements were obtained with a Beckman DU equipped Model 2000 Gilford, a Cary Model 14 or a Cary Model 118C spectrophotometer.

Acid dissociation constants for EDA dihydrochloride and pyridoxal-P were obtained by potentiometric titration with sodium hydroxide.[34c]

Association constants for 1:1 complexes formed from pyridoxal-P and amines were determined by spectrophotometric titration of pyridoxal-P with amine at either 410–435 nm and/or at 310–335 nm.[34b]

Kinetic measurements were made either with the rapid mixing stopped-flow spectrophotometer described earlier[35] or with an electrical discharge temperature jump perturbation instrument type SPA7, Messanlagen Studien Gesellschaft mbH, Göttingen, Germany. A temperature jump of 5° was used. Rate constants which were either first order or pseudo-first order were obtained by two methods: (a) the usual semilogarithmic plots[10b] when the two reactions had sufficiently distinct rate constants that the two reactions could be observed independently or when the absorbancy change of the second reaction was negligible compared to the first; or (b) by a nonlinear least-squares program provided by Dr. R. O. Viale for the curve fitting of double exponentials when method a could not be employed.

Acknowledgments. We are grateful for the excellent technical assistance of Ms. Mary Frederick and the aid and advice of Drs. Richard O. Viale and Fred Kayne. We appreciate the communication of results prior to publication from Drs. LaVerne Schirch and Jules Shafer.

References and Notes

(1) Supported by the National Institutes of Health, United States Public Health Service, Grant No. GM-13,777 from the Institute of General Medical Sciences, and Research Career Development Award No. K4CA 70,487 from the National Cancer Institute. A portion of this work has been presented in a preliminary form at the Meeting of the American Chemical Society, Atlantic City, N.J., Sept 1974.
(2) Postdoctoral Fellow of the National Institutes of Health (GM 38549-01). Present address: Department of Obstetrics and Gynecology, The University of Chicago, Pritzker School of Medicine, Chicago, Ill. 60637.
(3) a_H, hydronium ion activity; CA, carbinolamine; DAP, 1,3-diaminopropane; EDA, ethylenediamine; GD, geminal diamine; P_i–, ester of phosphoric acid, ionization state unspecified; Na_2EDTA, disodium dihydrogen ethylenediaminetetraacetate; pyridoxal-P or PLP, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; SB, Schiff base.
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(19) (a) An exact kinetic treatment of Scheme III, omitting the carbinolamine intermediate reveals that k^s_{obsd} is not an hyperbolic function of the concentration of EDA, although k^e_{obsd}(max) should equal k_d. In addition, k^l_{obsd} does not intersect the k_{obsd} axis at $k_{obsd} = 0$, but rather at $k_{obsd} = k_d + k_h$ although, at larger values of k^l_{obsd}, the slope of this line does approach the second-order rate constant for EDA addition to unhydrated PLP. The derivation of this exact kinetic treatment of Scheme III will be included in a future publication. We make use of eq 4 in interpreting these data to show that the numerical results are inconsistent with the alternate scheme mentioned in the text following this note. (b) Base catalyzed dehydration of carbinolamines derived from aliphatic amines and aromatic aldehydes has not been observed. See ref 6, 17, and 19c. (c) E. H. Cordes and W. P. Jencks, *J. Am. Chem. Soc.*, **85**, 2843 (1963).
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(22) (a) General acid catalysis, rather than specific acid catalysis as an explanation of the pH dependency of the data in Figure 8, is unlikely for the following reasons: The only acid catalysts in the system are H_2O, H_3O^+, EDA^{2+}, and EDA^+. As general acid catalysts, H_2O would not lead to the observed pH dependency, and the required catalytic rate constant for H_3O^+ is 10^{10} M^{-1} sec^{-1}, greater than the upper limit of 10^{10}–10^{11} M^{-1} sec^{-1} for a diffusion-controlled reaction.[22b] Insofar as EDA^{2+} and EDA^+ are concerned, neither requirement of the "Libido Rule" for predicting general acid catalysis[22c] is fulfilled; i.e., there is no large difference between the pK_a values of reactants and products during the course of the reaction nor is the pK_a value of the catalyst intermediate between the initial and final pK_a values of the substrate site. (b) M. Eigen, *Angew. Chem., Int. Ed. Engl.*, **3**, 1 (1964); (c) W. P. Jencks, *J. Am. Chem. Soc.*, **94**, 4731 (1972).
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(25) (a) R. M. C. Dawson, D. C. Elliott, W. H. Elliot, and K. M. Jones, Ed., "Data for Biochemical Research"; Clarendon Press, Oxford, 1969, p 43; (b) The pK_a value for the ϵ-ammonium group of lysine may be substantially different from this value because of local effects: e.g., D. E. Schmidt, Jr., and F. H. Westheimer, *Biochemistry*, **10**, 1249 (1971).
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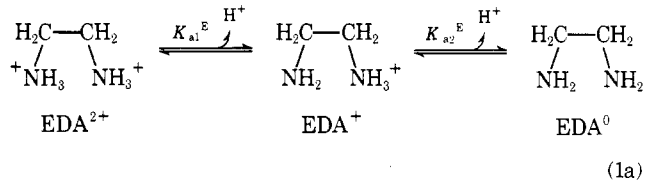
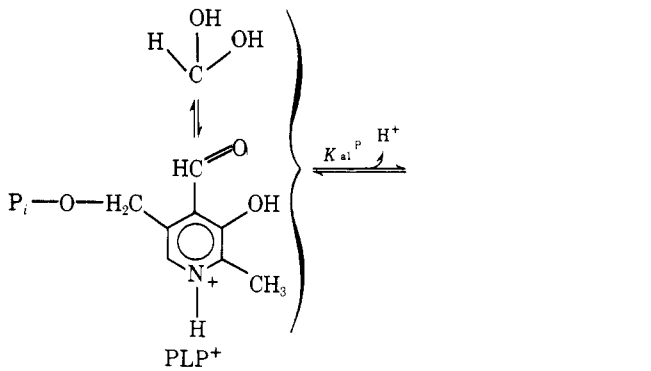
(27) J. P. Fox and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 1436 (1974).
(28) (a) S. Ehrenson, *Prog. Phys. Org. Chem.*, **2**, 195 (1964). (b) The value of K_2 is estimated from $K_2 = K_1Ka_1/Ka_2$ and values for K_1 of 32 M^{-1} (ref 11a) and $Ka_1/Ka_2 = 0.033$ (ref 28c). Values of K'(overall) of 785 M^{-1} (ref 28c) and K_3 of 10^{-2} (ref 28d and 34c) were employed to calculate $K_{T'}$ from $K_{T'} = K'$(overall) (K_2K_3). (c) R. G. Kallen, in "Methods in Enzymology: Vitamins and Coenzymes", Vol. 18, Part B, Academic Press, New York, N.Y., 1971, p 705. (d) J. De Luis "The Chemistry of Formaldehyde Amine Condensation Products", Ph.D. Thesis, Pennsylvania State University, 1964; *Chem. Abstr.*, **63**, 8184d (1965).
(29) Imidazolidine adducts of glyoxalate, isobutyraldehyde, and acetone have been studied. See A. Hilton and D. L. Leussing, *J. Am. Chem. Soc.*, **93**, 6831 (1971); J. Hine and K. W. Narducy, *ibid.*, **95**, 3362 (1973), and references therein.
(30) Thermochemical data supporting this generalization in five- and six-membered cycloalkanes, cycloalkanols, cycloalkanones, and cycloalkenes have been summarized by Brown et al.[31] The same phenomenon is observed in the formation of sugar hemiacetals, although not with sugar lactones[32] (but see also Brown et al.[31]).
(31) H. C. Brown, J. H. Brewster, and H. Shechter, *J. Am. Chem. Soc.*, **76**, 467 (1954).
(32) W. W. Pigman and R. M. Goepp, Jr., "Chemistry of the Carbohydrates", Academic Press, New York, N.Y., 1948, p 10.
(33) M. I. Page and W. P. Jencks, *Proc. Nat. Acad. Sci. U.S.A.*, **68**, 1678 (1971).
(34) (a) V. Gold, "pH Measurements", Wiley, New York, N.Y., 1956; (b) R. O. Viale and R. G. Kallen, *Arch. Biochem. Biophys.*, **146**, 271 (1971); (c) R. G. Kallen, *J. Am. Chem. Soc.*, **93**, 6227 (1971).
(35) K. L. Brown and R. G. Kallen, *J. Am. Chem. Soc.*, **94**, 1894 (1972).

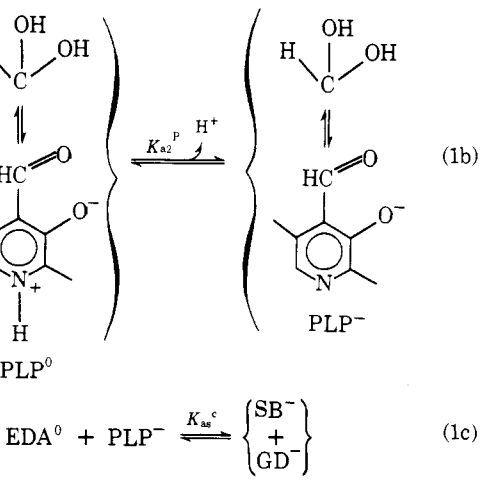
Appendix



(1a)



(1b)



(1c)

$$\alpha_{E^{2+}} = \frac{[EDA^{2+}]}{[EDA_{TOT}]} = \frac{1}{1 + (K_{a1}^E/a_H) + (K_{a1}^E K_{a2}^E/a_H^2)} \quad (1d)$$

$$\alpha_{E^{+}} = \frac{[EDA^{+}]}{[EDA_{TOT}]} = \frac{1}{1 + (K_{a2}^E/a_H) + (a_H/K_{a1}^E)} \quad (1e)$$

$$\alpha_{E^0} = 1 - \alpha_{E^{2+}} - \alpha_{E^{+}} \quad (1f)$$

$$[EDA_{TOT}] = [EDA^{2+}] + [EDA^{+}] + [EDA^0] \quad (1g)$$

$$\alpha_{P^-} = \frac{1}{1 + \frac{a_H}{K_{a2}^P} + \frac{a_H^2}{K_{a1}^P K_{a2}^P}} \quad (1h)$$

$$K^c_{app} = \left\{ \frac{K_{a1}^E K_{a2}^E}{K_{a1}^c K_{a2}^c} K_{as}^c(\alpha_{E^{2+}}) + \frac{K_{a2}^E}{K_{a2}^c} K_{as}^c(\alpha_{E^{+}}) + K_{as}^c(\alpha_{E^0}) \right\} (\alpha_{P^-}) \quad (1i)$$

$$\frac{[P_{TOT}]}{A_{413}} = \frac{(1 + K_T')}{\epsilon_{SB^0}} + \frac{(1 + K_T)K_{a2}^{SB}}{\epsilon_{SB^0}} \times \frac{1}{\alpha_H} \quad (2)$$

$$K_{a1}^c = \frac{([SB^0] + [GD^0])a_H}{([SB^{+*}] + [SB^+] + [GD^+])} = \frac{(1 + K_T')K_{a1}^{GD}K_{a1}^{SB}}{(1 + K_+^*)K_{a1}^{GD} + K_T'K_{a1}^{SB}} \quad (3a)$$

$$K_{a2}^c = \frac{([GD^-] + [SB^-])a_H}{([GD^0] + [SB^0])} = \frac{(1 + K_T)K_{a2}^{GD}K_{a2}^{SB}}{K_TK_{a2}^{SB} + K_{a2}^{GD}} \quad (3b)$$