droxy ester.[33] Although 70% of the theoretical AgBr was recovered, elimination and aromatization had taken place. Displacement reactions with **37** appeared even less hopeful owing to the possibility of 1,2-epoxide formation. In fact, the methyl ester **17** was first prepared by refluxing silver dibenzyl phosphate with **37** in dry acetonitrile.[32] Extensive aromatization occurred in the reaction of dibromoshikimic acid **13** with silver acetate in moist acetic acid,[9] unlike the formation of *cis*-acetoxycyclohexanol from *trans*-dibromocyclohexane under these conditions,

(33) C. B. Anderson, E. C. Friedrich, and S. Winstein, *Tetrahedron Lett.*, 2037 (1963).

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Kinetics of Carbonyl Addition Reactions. I. A Temperature-Jump Study of Carbinolamine Formation between Piperazine and Pyridine-4-aldehyde

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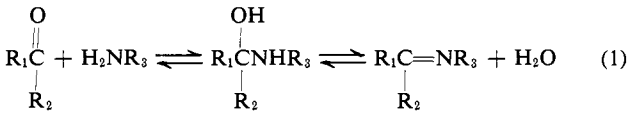
Abstract: Equilibrium and rate constants have been determined for the reactions of piperazine and piperazine monocation with pyridine-4-aldehyde to give the corresponding carbinolamines. The uncatalyzed addition rates for piperazine (pK = 9.97), k_1 = 2–3 × 10^5 M^{-1} sec^{-1}, and for piperazine monocation (pK = 5.80), k_2 = 65 M^{-1} sec^{-1}, reflect the sensitivity of the rates to the basicity of the attacking amine. The equilibrium constants for carbinolamine formation are much less sensitive to amine basicity. The reaction of piperazine has been shown to be subject to general base catalysis and the reaction of piperazine monocation has been shown to be subject to general acid catalysis. The results are discussed in terms of the detailed mechanism of carbonyl addition reactions.

The reaction of a primary amine with a carbonyl compound to give a Schiff base (eq 1) is now gen-



$$R_1C(=O)R_2 + H_2NR_3 \rightleftharpoons R_1C(OH)(NHR_3)R_2 \rightleftharpoons R_1C(=NR_3)R_2 + H_2O \quad (1)$$

erally accepted as proceeding through a tetrahedral intermediate.[1] Kinetic evidence for the existence of a carbinolamine intermediate was obtained by French and Bruice[2] in the reaction of pyridine-4-aldehyde with various amino acids. However, they could only infer its presence from evidence of a rapid preequilibrium prior to a rate determining dehydration step to give the Schiff base. Jencks[3] was able to measure the rate of carbinolamine formation in the acid pH range in the reaction of acetone with hydroxylamine. A change in rate limiting step from carbinolamine formation to dehydration was proposed to account for the pH–rate profile. More recently Sander and Jencks[4] have spectrophotometrically confirmed the existence of carbinolamine intermediates using the stopped-flow technique and have measured equilibrium constants for their formation for a series of primary and secondary amines with pyridine-4-aldehyde, formaldehyde, and *p*-chlorobenzaldehyde. However, the rates of carbinolamine formation were too fast to be measured even with the stopped-flow technique. This was also the situation in the recent study by Hine and Via[5] on the reaction of isobutyraldehyde with a series of primary amines.

The work presented in this paper is the first of a series of carbonyl addition reactions involving amines which we have studied by the temperature-jump technique in order to obtain information on those factors which affect the kinetics of these fast addition reactions. Our kinetic program is complementary to that of Jencks on relative stabilities and combined with the kinetic studies of Schuster, *et al.*,[6] on thiol addition reactions will provide a better understanding of the detailed mechanism of these biologically important reactions. The role of acid–base catalysis in these reactions is of particular interest since it may indicate the type of catalysis and the nature of the groups involved when these reactions occur enzymatically.

In this paper details are presented of the kinetics of the reaction of pyridine-4-aldehyde with piperazine in aqueous perchlorate medium. Pyridine-4-aldehyde was chosen as the carbonyl compound for the following reasons: (1) equilibrium constants for carbinolamine formation had been measured for a selection of amines by Sander and Jencks;[4] (2) a strong absorption band in the uv providing a convenient way for spectrophotometric observation of the reaction; (3) related to pyridoxal phosphate (vitamin B_6), an important cofactor in a number of enzyme systems whose mode of action in many instances is thought to involve carbinolamine and Schiff base formation.

(1) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969.
(2) T. C. French and T. C. Bruice, *Biochemistry*, **3**, 1589 (1964).
(3) W. P. Jencks, *J. Amer. Chem. Soc.*, **81**, 475 (1959).
(4) E. G. Sander and W. P. Jencks, *ibid.*, **90**, 6154 (1968).
(5) J. Hine and F. A. Via, *ibid.*, **94**, 190 (1972).
(6) P. Schuster, personal communication.

A secondary amine (like piperazine) has the advantage that the reaction sequence of eq 1 stops at the carbinolamine stage, thus leading to a simple one-step equilibrium (dehydroxylation of the secondary amine carbinolamine would result in an unstable Schiff base with a positive charge on the nitrogen). Studies of the reactions of primary amines require the use of combined stopped-flow temperature-jump techniques such that the temperature jump can be applied during the short time (*ca.* 1 sec) immediately after mixing the amine and aldehyde in which the carbinolamine is present in high concentration, before dehydration to the Schiff base occurs. Details of this technique applied to the reactions of pyridine-4-aldehyde with ethylamine, glycine, and β-alanine will be reported in a separate publication.[7]

Piperazine as a diamine allows the study of the reactions of both the neutral molecule and the monocation by extending the experiments over a wide pH range. This comparison is of particular interest since the two pK values are very different but the steric environment of the attacking nitrogen is very similar.

Experimental Section

Materials. Pyridine-4-aldehyde (Fluka) was redistilled under a reduced pressure of nitrogen (bp 80–81° (14 mm)) and stored under nitrogen at 0°. Stock solutions of pyridine-4-aldehyde (0.1 M) were stored at 0° and were stable by uv spectral criteria (λ_{max} 285 nm (ϵ_{max} 1500), for equilibrated aldehyde + hydrate, pH 6–11, μ = 1.0 M) over a period of weeks. Piperazine hexahydrate (Merck, B.P.C.), sodium perchlorate (Merck, p.a.), and perchloric acid (Merck, p.a.) were used without further purification.

Measurement of pH, Spectra, and Relaxation Times. All measurements were done at 25° and at an ionic strength μ = 1.0 M, adjusted with sodium perchlorate ($NaClO_4$ was used since it is intended to investigate the effect of metal ions on similar reactions). Reaction solutions were made up in volumetric flasks by the appropriate combination of standard stock solutions of pyridine-4-aldehyde, piperazine, perchloric acid, and sodium perchlorate. The pH was measured using a Polymetron Type 42-D pH meter with a Metrohm EA 120X combined electrode. The saturated potassium chloride solution of the reference electrode had to be replaced by a sodium chloride solution (4.6 M) in order to prevent precipitation of potassium perchlorate in the glass sinter diaphragm. The pH meter was calibrated with standard buffers (Merck) at integral pH values over the range pH 4–11. The concentrations of piperazine and piperazine monocation were calculated from the known amounts of perchloric acid added to standard piperazine solutions. These concentrations agreed with those calculated from the measured pH and pK values determined by half-neutralization.

Spectra and fixed wavelength time scans for the determination of the hydration constant were recorded on a Beckman DK-2A spectrophotometer. In the kinetic studies the temperature-jump relaxation technique[8] was applied. The apparatus used was of the double-beam type, basic design by L. De Maeyer, improved version by C. Rabl. The combined heating and rise time of the apparatus under the conditions of high ionic strength (1.0 M) was about 1 μsec. A discharge of 33 kV was used to raise the temperature from 21.0 to 25.0°. The rapid changes in absorbancy were followed with a Tektronix 549 oscilloscope at 280 nm (emission line of Hg–Xe high-pressure lamp) since this wavelength gave maximum light intensity and hence high signal-to-noise ratios close to the absorption maximum of pyridine-4-aldehyde at 285 nm.

Results

Equilibria. The following values for the protolytic equilibrium constants of the amine were determined by potentiometric measurements under the conditions of the present studies (25°, ionic strength, μ = 1.0 M),[9]

$$K_{AH} = \frac{[H^+][A]}{[AH^+]} = 1.1 \times 10^{-10}\ M \qquad (pK_{AH} = 9.97 \pm 0.02)$$

$$K_{AH_2} = \frac{[H^+][AH^+]}{[AH_2^{2+}]} = 1.6 \times 10^{-6}\ M \qquad (pK_{AH_2} = 5.80 \pm 0.02)$$

where A = the neutral piperazine molecule.

Pyridine-4-aldehyde (abbreviated to P) may also exist in a protonated form.[10]

$$K_{PH} = \frac{[H^+][P]}{[PH^+]} = 1.7 \times 10^{-5}\ M \qquad (pK_{PH} = 4.77)$$

In order to compare the kinetic data for the reaction of pyridine-4-aldehyde with piperazine with the equilibrium data obtained by spectrophotometric means it is necessary to know precisely the degree of hydration of the aldehyde under the conditions of the present studies. There are no values for the hydration constant $K_{H_2O} = [P \cdot H_2O]/[P]$ in the literature referring to these conditions, and those values which are available show a considerable variation.[4,10,11] Therefore, the hydration constant was determined by quickly adding and mixing small amounts of pure pyridine-4-aldehyde with an aqueous solution of 1.0 M $NaClO_4$ (pH 6.5) in a spectrophotometer cell and recording the change in absorbancy at 280 nm due to the hydration reaction (half-life *ca.* 10 sec) as a function of time. Since the extinction of the hydrated aldehyde at this wavelength is practically zero,[10] it follows that $K_{H_2O} = (E_0 - E_\infty)/E_\infty$, where E_∞ is the final value of the absorbancy. The initial value E_0 was obtained by extrapolating the absorbancy back to zero time. The results of three determinations gave

$$K_{H_2O} = [P \cdot H_2O]/[P] = 0.67 \pm 0.03$$
$$(25°,\ \mu = 1.0\ M\ NaClO_4)$$

The hydration constant is essentially independent of pH over the range used in these studies.[11]

The extent of carbinolamine formation between pyridine-4-aldehyde and piperazine (the neutral molecule as well as the monocation) was studied spectrophotometrically. At a given pH, solutions of 4×10^{-4} M total aldehyde and varying amounts of excess amine, 4×10^{-3} to 1×10^{-1} M of A or AH^+, respectively, were prepared and the optical density at 280 nm (absorption band of free aldehyde) was measured as function of the amine concentration. These measurements were done against reference solutions of the same pH and amine concentration (but without aldehyde) in order to compensate for a small contribution of the piperazine to the total extinction. Well-defined isosbestic points were observed at 267 and 239 nm in the complete spectra. At "low" pH values, between 5.8 and 7.7, where the concentration of unprotonated piperazine is negligible, an apparent carbinolamine formation constant

$$K_{app} = \frac{[\text{carbinolamine}]}{([P \cdot H_2O] + [P])[AH^+]}$$

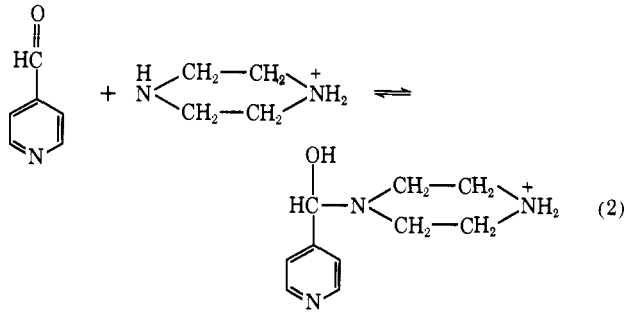
(7) H. Diebler and R. N. F. Thorneley, to be published.
(8) M. Eigen and L. De Mayer, *Tech. Org. Chem.*, **8**, 895 (1963).
(9) In this paper $[H^+]$ denotes the apparent hydrogen-ion activity as measured by the procedure outlined in the Experimental Section.
(10) K. Nakamoto and A. E. Martell, *J. Amer. Chem. Soc.*, **81**, 5857 (1959).
(11) Y. Pocker, J. E. Meany, and B. J. Nist, *J. Phys. Chem.*, 71, 4509 (1967).

was obtained from plots of $(E_0 - E)^{-1}$ *vs.* $[AH^+]^{-1}$, according to

$$\frac{1}{E_0 - E} = \frac{1}{K_{app}[AH^+]} \frac{1}{E_0 - E_\infty} + \frac{1}{E_0 - E_\infty}$$

where E = absorbancy at concentration $[AH^+]$, E_0 = initial absorbancy ($[AH^+] = 0$), and E_∞ = limiting value of the absorbancy at very high $[AH^+]$.

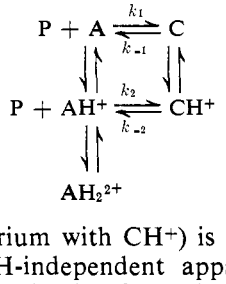
The values for K_{app} found in the pH range under consideration were essentially pH independent, indicating the reaction to be



(2)

or, abbreviated: $P + AH^+ \rightleftharpoons CH^+$ (C = neutral carbinolamine). Multiplying K_{app} by $(1 + K_{H_2O})$ yields the corresponding equilibrium constant $K_2 = [CH^+]/[P][AH^+]$. The values obtained for K_2 lie between 12.5 and 16 M^{-1}. At higher pH values, around 9–10.8, the apparent carbinolamine formation constant is pH dependent, due to contributions from both $P + AH^+ \rightleftharpoons CH^+$ and $P + A \rightleftharpoons C$. Evaluation of the experimental data from this pH range gave $K_1 = [C]/[P][A] \approx 50\ M^{-1}$ and $K_2 = [CH^+]/[P][AH^+] \approx 13\ M^{-1}$ (25°, μ = 1.0 M). Combining K_1 and K_2 with K_{AH} leads to the acid dissociation constant of the protonated carbinolamine: $K_{CH} = [C][H^+]/[CH^+] \approx 4 \times 10^{-10}\ M$.

Kinetics. Like the equilibrium measurements, the kinetic studies were done under pseudo-first-order conditions, with an initial pyridine-4-aldehyde concentration of $4 \times 10^{-4}\ M$ and a large excess of amine. The reaction mechanism for the carbinolamine formation can be represented by the following scheme (eq 3). In principle, a diprotonated carbinolamine



(3)

CH_2^{2+} (in equilibrium with CH^+) is also possible, but the essentially pH-independent apparent equilibrium constant for carbinolamine formation at the lower pH values clearly indicates that its concentration is negligible under our conditions. There was also no evidence for the formation of a carbinolamine anion up to pH 10.8. The protolytic reactions in eq 3 equilibrate rapidly because of the relatively high concentrations of A, AH^+, and AH_2^{2+}, and can be considered as preequilibria compared to the carbinolamine formation and dissociation steps. Thus only one time constant for the latter reactions is to be expected. Not included in eq 3 is the equilibrium between the free aldehyde (P) and its hydrate ($P \cdot H_2O$). This equilibrium gives rise to another relaxation effect which has a much longer time constant and which is also observed in the absence of amine. Because of the (relative) slowness of this process ($\tau \approx 3$ msec at pH 10.8, $\tau \sim 10$ sec at pH 5.8) the small shift in the concentration of $P \cdot H_2O$ which occurs during the equilibration of the reaction steps represented in eq 3 was neglected. The amplitude of this slow reaction actually changes sign as the amine concentration is varied, being rather small for most of the range studied. The reason for this is that the change in free aldehyde concentration due to the perturbation of the aldehyde–carbinolamine equilibrium almost provides the shift of the aldehyde–hydrate equilibrium required by the temperature change and at high amine concentration even exceeds it. Similar effects with coupled equilibria have been observed before.[12]

Table I. Experimental Data for the Carbinolamine Formation–Dissociation Reaction between pH 5.8 and 8.0[a]

pH	[Piperazine·H⁺], *M*	τ, msec Found	τ, msec Calcd
5.80	0.10	0.65	0.65
	0.08	0.88	0.90
	0.06	1.36	1.34
	0.04	2.30	2.27
	0.02	5.14	5.16
	0.004	26.0	25.8
6.10	0.10	1.08	1.09
	0.08	1.47	1.48
	0.06	2.28	2.18
	0.04	3.65	3.60
	0.02	7.75	7.81
	0.004	32.3	32.2
6.70	0.10	2.17	2.21
	0.08	2.95	2.97
	0.06	4.18	4.25
	0.04	6.75	6.70
	0.02	12.5	12.7
	0.01	21.2	20.5
	0.004	30.5	30.4
7.30	0.10	2.16	2.16
	0.08	2.86	2.83
	0.06	3.90	3.89
	0.04	5.80	5.72
	0.02	9.02	9.37
	0.01	12.7	12.9
	0.004	16.4	16.2
7.60	0.10	1.63	1.62
	0.08	2.20	2.13
	0.06	2.80	2.89
	0.04	4.00	4.10
	0.02	6.17	6.10
	0.004	8.50	8.55
8.00	0.10	0.89	0.87
	0.08	1.07	1.09
	0.06	1.37	1.39
	0.04	1.87	1.82
	0.02	2.35	2.43
	0.01	2.85	2.82
	0.004	3.10	3.10

[a] 25°, μ = 1.0 M; initial concentration of pyridine-4-aldehyde, 0.0004 M.

The reciprocal relaxation time (expressed in terms of $[AH^+]$) of a system like that in eq 3 under pseudo-first-order conditions, at constant pH (buffered by

(12) H. Diebler, M. Eigen, and P. Matthies, *Z. Naturforsch. B*, **16**, 629 (1961).

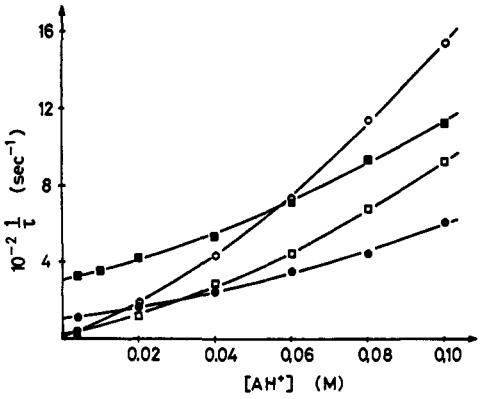


Figure 1. Plots of $1/\tau$ *vs.* the concentration of piperazine monocation at pH 5.8 (○), 6.1 (□), 7.6 (●), and 8.0 (■).

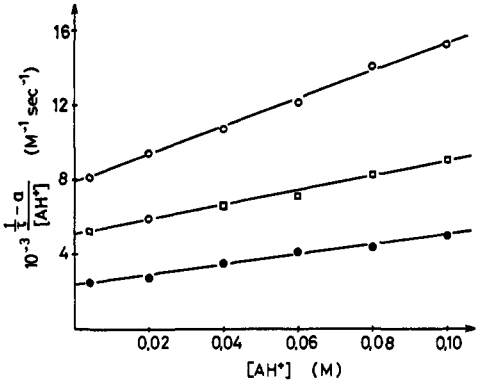


Figure 2. Plots of $(1/\tau - a)/[AH^+]$ *vs.* $[AH^+]$ at pH 5.8 (○), 6.1 (□), and 7.6 (●).

the amine species), and with fast protolytic equilibrations should be of the form

$$\frac{1}{\tau} = a + b[AH^+] \qquad (4)$$

The experimental data obtained in the pH range 5.8–8.0 are summarized in Table I. Each time constant given is the mean of four or five individual determinations. The deviations of the individual time constants from the mean were in most cases within ±4%. However, at constant pH plots of $1/\tau$ *vs.* $[AH^+]$, covering a wide concentration range from $[AH^+] = 4 \times 10^{-3}$ to 0.1 *M*, clearly deviated from linearity. This is demonstrated in Figure 1 for some of the data. Instead of fitting to eq 4, the observed relaxation times could be fitted to a relationship of the form

$$\frac{1}{\tau} = a + b[AH^+] + c[AH^+]^2 \qquad (5)$$

as shown in Figure 2, where $[(1/\tau) - a]/[AH^+]$ has been plotted *vs.* $[AH^+]$ for the data at three pH values. The quantity *a* is given by the intercepts of the curves of Figure 1 with the $1/\tau$ axis and was slightly varied—if necessary—until a good straight line was obtained. The values of *a*, *b*, and *c* which have been determined from plots similar to those in Figure 2 are presented in Table II. The values for τ which are calculated

Table II. Numerical Values of *a*, *b*, and *c* as Defined by Eq 5[a]

pH	*a*, sec^{-1}	$10^{-3}b$, M^{-1} sec^{-1}	$10^{-4}c$, M^{-2} sec^{-1}
5.80	6 (±2)	7.9 (±0.6)	7.4 (±0.6)
6.10	10 (±2)	5.1 (±0.4)	4.0 (±0.5)
6.70	23 (±3)	2.4 (±0.4)	1.9 (±0.5)
7.30	52 (±3)	2.4 (±0.4)	1.7 (±0.5)
7.60	107 (±4)	2.3 (±0.4)	2.8 (±0.5)
8.00	304 (±6)	4.6 (±0.5)	3.8 (±0.5)

[a] 25°, μ = 1.0 *M*; estimated errors.

with the figures of Table II *via* eq 5 are given in the last column of Table I for comparison.

Analogous studies at higher pH values, between pH 9.27 and 10.78, revealed the same type of behavior. Since under these conditions the predominant amine species is usually the neutral piperazine molecule, the results obtained have been expressed in

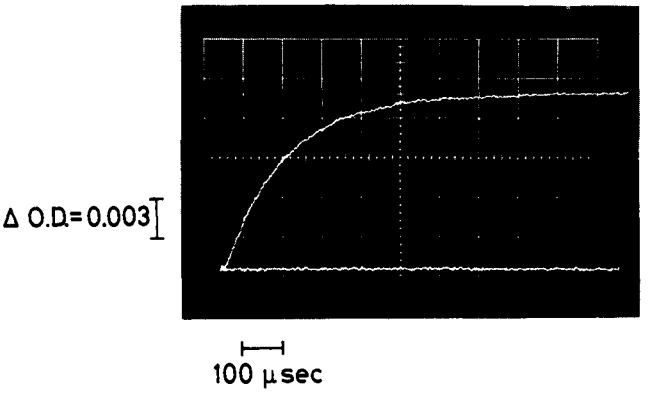


Figure 3. Chemical relaxation effect at pH 9.97 ([piperazine] = 0.004 *M*; sweep = 100 μsec/division; sensitivity = 20 mV/division).

Table III. Experimental Data for the Carbinolamine Formation–Dissociation Reaction between pH 9.28 and 10.78[a]

pH	[Piperazine], *M*	τ, μsec Found	τ, μsec Calcd
9.28	0.085	8.80	8.83
	0.06	15.2	15.3
	0.04	29.9	27.7
	0.02	65.6	66.2
	0.01	132.5	131.0
	0.004	241	243
9.97	0.09	9.66	9.56
	0.06	16.0	16.2
	0.04	25.8	26.1
	0.02	53.7	52.5
	0.01	89.9	90.7
	0.004	150	150
10.45	0.09	7.92	7.60
	0.06	12.7	13.2
	0.04	21.2	21.2
	0.02	40.7	39.2
	0.01	53.5	58.3
	0.004	81.8	77.2
10.78	0.09	6.5	6.35
	0.06	10.3	10.6
	0.04	16.6	16.3
	0.02	27.6	28.1
	0.01	40.1	39.4
	0.004	49.5	49.7

[a] 25°, μ = 1.0 *M*; initial concentration of pyridine-4-aldehyde, 0.0004 *M*.

terms of [A]. Table III gives a summary of the experimental data; the time constants are again the mean of four or five measurements. A typical oscillogram is shown in Figure 3. The relaxation times are much

shorter (microsecond range) than at the lower pH values. Their reciprocals again show a nonlinear dependence on the amine concentration. As before, the relationship can be represented by an expression

$$\frac{1}{\tau} = a' + b'[A] + c'[A]^2 \quad (6)$$

Table IV gives the corresponding values for a', b', and c'. The agreement between the measured time constants τ and those obtained from the data of Table IV *via* eq 6 is very good (last column of Table III); since errors in b' and c' may compensate for each other, the uncertainty in these two quantities is nevertheless fairly high.

Table IV. Numerical Values of a', b', and c' at High pH[a]

pH	$10^{-4}a'$, sec^{-1}	$10^{-5}b'$, M^{-1} sec^{-1}	$10^{-6}c'$, M^{-2} sec^{-1}
9.28	0.22 (±0.03)	4.4 (±1)	10.2 (±1)
9.97	0.44 (±0.05)	6.5 (±1)	5.2 (±1)
10.45	1.05 (±0.1)	5.8 (±1)	8.5 (±1)
10.78	1.70 (±0.1)	7.5 (±1)	9.0 (±1)

[a] 25°; μ = 1.0 M; estimated errors.

According to the equilibrium studies, carbinolamine formation is a 1:1 reaction of pyridine-4-aldehyde with piperazine. Therefore, the terms in the expression for $1/\tau$ which are second power in amine concentration can only be accounted for by pathways which include a second amine species acting as catalyst. The experimental findings are therefore in no way surprising, since general acid or base catalysis is a well-known feature in addition reactions to the carbonyl group.[1] The actual reaction mechanism thus becomes rather complicated. Catalysis may taken place in each of the two reaction pathways illustrated in eq 3, with A, AH^+, or OH^- possibly acting as base catalysts and AH_2^{2+}, AH^+, or H^+ as acid catalysts.

Considering all these possible pathways, a general expression for the (reciprocal) relaxation time was derived from the complete rate law. It has the form given by eq 5 or 6 with the coefficients a, b, and c (or a', b', and c') consisting of a series of terms of differing $[H^+]$ dependences, *e.g.*

$$a = a_1[H^+]^{-2} + a_2[H^+]^{-1} + a_3 + a_4[H^+] \quad (7)$$

Comparison of these expressions with the experimentally observed $[H^+]$ dependences of the coefficients as given in Tables II and IV thus allows conclusions to be drawn with regard to those terms which actually contribute to the reaction under our conditions. In this way the reasonable result was obtained that catalysis by the hydrogen ion does not contribute measurably to the overall reaction within our pH range, *i.e.*, down to pH 5.8. At high pH values, however, there is definite evidence for the catalysis of reaction 1 (P + A = C) by OH^-. Whether reaction 2 (P + AH^+ = CH^+) is also subject to OH^- catalysis cannot be decided since the corresponding rate term is kinetically indistinguishable from that of the uncatalyzed reaction 1; however, according to the interpretation of the experimental data as given in the Discussion, such a catalytic rate term must be very small compared to that for the uncatalyzed reaction 1.

With these simplifications, the mechanism will be discussed now in some more detail. If, for instance, the rate term due to OH^- catalysis of the forward reaction 1 is defined by

$$R = k_1^{OH}[P][A][OH^-] \quad (8)$$

with k_1^{OH} as the formal corresponding rate constant, then the overall reaction rate is given by[13] eq 9, where

$$\frac{d([C] + [CH])}{dt} = k_1[P][A] - k_{-1}[C] + k_1^{OH}[P][A][OH] - k_{-1}^{OH}[C][OH] + \sum_i k_1^i[P][A][i] - \sum_i k_{-1}^i[C][i] + k_2[P][AH] - k_{-2}[CH] + \sum_i k_2^i[P][AH][i] - \sum_i k_{-2}^i[CH][i] \quad (9)$$

i = A, AH, and AH_2. Starting from this rate equation, the relaxation time of the system was calculated in the usual way,[8] making use of the laws of mass conservation; the protolytic equilibria were assumed to be rapidly equilibrating preequilibria and the hydrogen-ion concentration was assumed to be constant (buffered by the amine species). The resulting expression was simplified by introducing the pseudo-first-order condition [A] + [AH] ≫ [P], [C] + [CH]. For the experiments at low pH values (5.8–8.0), where $[H^+] \gg K_{CH}$ the expression for $1/\tau$ (expressed in terms of [AH]) finally reads

$$\frac{1}{\tau} = k_{-1}^{OH}K_W K_{CH}\frac{1}{[H]^2} + k_{-1}K_{CH}\frac{1}{[H]} + k_{-2} + \left[(k_1^{OH}K_W K_{AH} + k_{-1}^{A}K_{CH}K_{AH})\frac{1}{[H^2]} + (k_1 K_{AH} + k_{-1}^{AH}K_{CH} + k_{-2}^{A}K_{AH})\frac{1}{[H]} + \left(k_2 + k_{-1}^{AH_2}\frac{K_{CH}}{K_{AH_2}} + k_{-2}^{AH}\right) + k_{-2}^{AH_2}\frac{1}{K_{AH_2}}[H]\right] \times [AH] + \left[k_1^{A}(K_{AH})^2\frac{1}{[H]^2} + (k_1^{AH}K_{AH} + k_2^{A}K_{AH})\frac{1}{[H]} + \left(k_1^{AH_2}\frac{K_{AH}}{K_{AH_2}} + k_2^{AH}\right) + k_2^{AH_2}\frac{1}{K_{AH_2}}[H]\right][AH]^2 \quad (10)$$

where $K_W = \gamma_H c_H c_{OH} = 1.3 \times 10^{-4}$ M^2, assuming $\gamma_H = \gamma_{OH}$ and $c_H c_{OH} = 1.6 \times 10^{-14}$ M^2 at 25°, 1.0 M $NaClO_4$,[14] in accordance with the experimentally obtained relationship eq 5. This expression (eq 10) reveals the following general features: the [AH]-independent part of $1/\tau$ includes the rate constants of all those *back* reaction terms which are *not* catalyzed by amine; that part which is second power in [AH] contains the rate constants of all those *forward* reaction terms which *are* catalyzed by amine; finally, the part linear in [AH] includes the rate constants of all those *forward* reaction terms which are *not* catalyzed by amine and all those *back* reaction terms which *are* catalyzed by amine.

Evaluation of Experimental Data *via* Eq 10. The experimentally determined pH dependence of a (given in Table II) is illustrated in Figure 4. The plot indicates a relationship of the form (within the pH range under consideration): $a = a_1 + a_2/[H]$. Comparison

(13) Charges have been omitted here and often further on for simplicity.

(14) R. Fischer and J. Byé, *Bull. Soc. Chim. Fr.*, 2920 (1964).

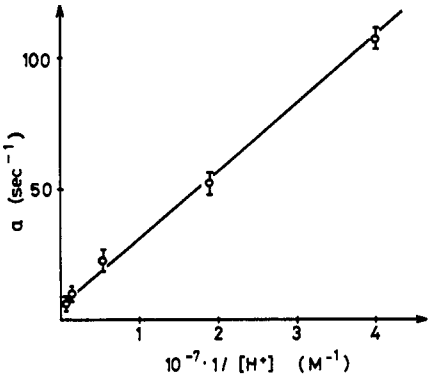


Figure 4. Plot of a (eq 5) *vs.* $1/[H^+]$.

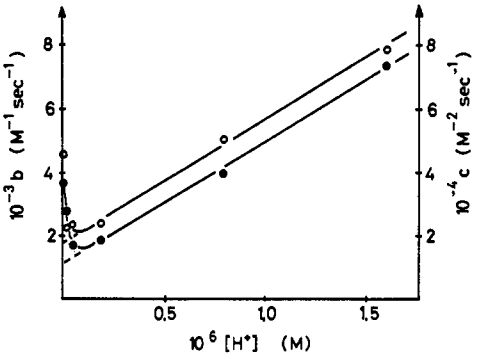


Figure 5. Plots of values for b (○, left-hand scale) and c (●; right-hand scale) *vs.* $[H^+]$.

with the [AH]-independent part of eq 10 yields $a_1 = k_{-2}$ and $a_2 = k_{-1}K_{CH}$, whereas the term with k_{-1}^{OH} does not contribute measurably to a. From intercept and slope one obtains $k_{-2} \approx 5$ sec^{-1} and $k_{-1} = 6.4 \times 10^3$ sec^{-1}. Using the equilibrium constants K_1 and K_2, it follows that $k_2 \approx 65\ M^{-1}$ sec^{-1} and $k_1 = 3.2 \times 10^5\ M^{-1}$ sec^{-1}. The $[H^+]$ dependence of b and c (data in Table II) is shown in Figure 5. Both quantities are characterized by a linear increase with $[H^+]$, *i.e.* $b = b_1 + b_2[H^+]$ and $c = c_1 + c_2[H^+]$, except at very low hydrogen-ion concentrations. Comparison with the [AH] term of eq 10 (for b) and with the $[AH]^2$ term (for c) yields $b_2 = k_{-2}^{AH_2}(1/K_{AH_2}) \approx 3.9 \times 10^9$; thus $k_{-2}^{AH_2} \approx 6.3 \times 10^3\ M^{-1}$ sec^{-1}. $b_1 = k_2 + k_{-1}^{AH_2}(K_{CH}/K_{AH_2}) + k_{-2}^{AH} \approx 1.8 \times 10^3\ M^{-1}$ sec^{-1}; since $k_2 \approx 65\ M^{-1}$ sec^{-1}, then $k_{-1}^{AH_2}(K_{CH}/K_{AH_2}) + k_{-2}^{AH} \approx 1.8 \times 10^3\ M^{-1}$ sec^{-1}. $c_2 = k_2^{AH_2}(1/K_{AH_2}) \approx 4 \times 10^{10}$, thus $k_2^{AH_2} \approx 6.4 \times 10^4\ M^{-2}$ sec^{-1}. $c_1 = k_1^{AH_2}(K_{AH}/K_{AH_2}) + k_2^{AH} \approx 1 \times 10^4\ M^{-2}$ sec^{-1}. The ratio of the slopes of Figure 5 gives $(c_2/b_2) = (k_2^{AH_2}/k_{-2}^{AH_2}) = K_2 \approx 10\ M^{-1}$, as compared to $K_2 \approx 13\ M^{-1}$ from the equilibrium studies. The increase of b and of c at very low hydrogen-ion concentrations is due to the terms with inverse $[H^+]$ dependences in the coefficients of [AH] and $[AH]^2$ (eq 10).

The calculated expression for $1/\tau$ at high pH values (*i.e.*, without the condition $[H^+] \gg K_{CH}$) and written in terms of [A] has the form given by eq 6 with the following coefficients

$$a' = \frac{K_{CH}}{K_{CH} + [H]}\left(k_{-1}^{OH}K_W\frac{1}{[H]} + k_{-1} + k_{-2}\frac{1}{K_{CH}}[H]\right)$$

$$b' = k_1^{OH}K_W\frac{1}{[H]} + k_1 + k_{-1}^{A}\frac{K_{CH}}{K_{CH} + [H]} + \left(k_2\frac{1}{K_{AH}} + k_{-1}^{AH}\frac{K_{CH}}{K_{CH} + [H]}\frac{1}{K_{AH}} + k_{-2}^{A}\frac{1}{K_{CH} + [H]}\right) \times [H] + \left(k_{-1}^{AH_2}\frac{K_{CH}}{K_{CH} + [H]}\frac{1}{K_{AH_2}K_{AH}} + k_{-2}^{AH}\frac{1}{K_{CH} + [H]}\frac{1}{K_{AH}}\right)[H]^2 + k_{-2}^{AH_2} \times \frac{1}{K_{CH} + [H]}\frac{1}{K_{AH}K_{AH_2}}[H]^3$$

$$c' = k_1^{A} + \left(k_1^{AH}\frac{1}{K_{AH}} + k_2^{A}\frac{1}{K_{AH}}\right)[H] + \left(k_1^{AH_2}\frac{1}{K_{AH}K_{AH_2}} + k_2^{AH}\frac{1}{(K_{AH})^2}\right)[H]^2 + k_2^{AH_2}\frac{1}{(K_{AH})^2K_{AH_2}}[H]^3$$

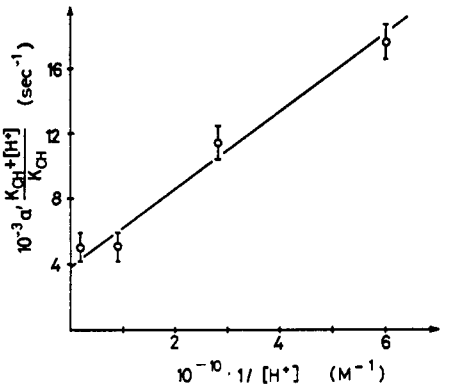


Figure 6. Plot of values for $a'(K_{CH} + [H^+])/K_{CH}$ *vs.* $1/[H^+]$.

The experimentally observed pH dependence of a' (given in Table IV) is illustrated in Figure 6 in a plot of $a'(K_{CH} + [H])/K_{CH}$ *vs.* $1/[H]$. Intercept and slope of the straight line yield $k_{-1} \approx 4 \times 10^3$ sec^{-1} and $k_{-1}^{OH} = 1.8 \times 10^7\ M^{-1}$ sec^{-1}; therefore (with K_1), $k_1^{OH} = 9.0 \times 10^8\ M^{-2}$ sec^{-1}. According to Figure 6, the term with k_{-2} does not contribute measurably to a' between pH 9.3 and 10.8, in agreement with the earlier evaluation of k_{-2}. The value of 4×10^3 for k_{-1} is somewhat lower than the one obtained from the data for low pH. It might be that this difference reflects an error in the value of K_{CH} which had been used in the former determination of k_{-1}.

The data given in Table IV for b' and for c' do not show a characteristic variation with $[H^+]$. The appreciable scatter of the values apparently indicates that these experimental figures for high pH are less accurate than those obtained in the lower pH range. It is obvious, however, that b' and c' are dominated by the $[H^+]$-independent terms. Therefore, some approximate figures can be given: $c' \approx k_1^A \approx (8 \pm 2) \times 10^6\ M^{-2}$ sec^{-1}. That the contributions of the $[H]^2$ and $[H]^3$ terms to b' and c' are negligible in this pH range agrees with the expectations based upon the results which have been obtained at low pH.

The values for b'—on the whole—show a tendency to increase slightly with increasing pH. Again this is as expected—because of the term $k_1^{OH}K_W/[H]$—although the variation is smaller than calculated with $k_1^{OH} = 9.0 \times 10^8$. The (approximately) constant contribution to b' is given by k_1 plus the term with k_{-1}^A and amounts to about $4 \times 10^5\ M^{-1}$ sec^{-1}. This

Table V. Summary of Equilibrium and Rate Constants for the Formation of Carbinolamine (C) from Pyridine-4-aldehyde (P) and Piperazine (A) (25°, μ = 1.0 M)

Equilibrium	Constant	
$[P\cdot H_2O]/[P]$	$K_{H_2O} = 0.67 \pm 0.03$	
$[A][H]/[AH]$	$K_{AH} = 1.1 \times 10^{-10}$ M	
$[AH][H]/[AH_2]$	$K_{AH_2} = 1.6 \times 10^{-6}$ M	
$[C][H]/[CH]$	$K_{CH} \approx 4 \times 10^{-10}$ M	
$[C]/[P][A]$	$K_1 \approx 50$ M^{-1}	
$[CH]/[P][AH]$	$K_2 \approx 13$ M^{-1}	
Rate term	**Constant**	**Remarks**
$k_{-1}[C]$	$k_{-1} = 6.4 \times 10^8$ sec^{-1};	From low pH data
	$k_{-1} \approx 4 \times 10^3$ sec^{-1}	From high pH data
$k_1[P][A]$	$k_1 = (2\text{-}3) \times 10^5$ M^{-1} sec^{-1}	From k_{-1} and K_1
$k_{-2}[CH]$	$k_{-2} \approx 5$ sec^{-1}	
$k_2[P][AH]$	$k_2 \approx 65$ M^{-1} sec^{-1}	From k_{-2} and K_2
$k_{-1}^{OH}[C][OH]$	$k_{-1}^{OH} = 1.8 \times 10^7$ M^{-1} sec^{-1}	
$k_1^{OH}[P][A][OH]$	$k_1^{OH} = 9.0 \times 10^5$ M^{-2} sec^{-1}	From k_{-1}^{OH} and K_1
$k_1^{A}[P][A][A]$	$k_1^{A} = (8 \pm 2) \times 10^6$ M^{-2} sec^{-1}	
$k_{-1}^{A}[C][A]$	$k_{-1}^{A} = (1.6 \pm 0.4) \times 10^5$ M^{-1} sec^{-1}	From k_1^{A} and K_1
$k_2^{AH_2}[P][AH][AH_2]$	$k_2^{AH_2} \approx 6.4 \times 10^4$ M^{-2} sec^{-1}	
$k_{-2}^{AH_2}[CH][AH_2]$	$k_{-2}^{AH_2} \approx 6.3 \times 10^3$ M^{-1} sec^{-1}	
	$k_{-1}^{AH_2}(K_{CH}/K_{AH_2}) + k_{-2}^{AH} \approx 1.8 \times 10^3$ M^{-1} sec^{-1}	
	$k_1^{AH_2}(K_{AH}/K_{AH_2}) + k_2^{AH} \approx 1 \times 10^4$ M^{-2} sec^{-1}	

value is in good agreement with the sum of these two rate constants as calculated from K_1 and the reverse rate constants k_{-1} and k_1^{A} and thus confirms the interpretation.

A summary of the results obtained in this study is given in Table V (rate constants as defined by eq 3 and eq 8.

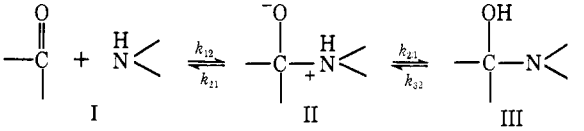
Discussion

The apparent equilibrium constants determined by spectrophotometric titration for carbinolamine formation from piperazine and piperazine monocation are in good agreement with those reported by Sander and Jencks.[4] However, since the hydration constant (K_{H_2O} = 0.67, 1.0 M $NaClO_4$) which we have determined is considerably lower than that used by Sander and Jencks (K_{H_2O} = 1.28, 1.0 M KCl), the true equilibrium constants are somewhat different. The difference in hydration constant may be due to the different media used. The kinetic data require equilibrium constants close to our determined values in order to be internally consistent. No statistical correction has been made to either the kinetic or equilibrium data for piperazine which has two nitrogen atoms capable of attacking the carbonyl group.

The reciprocal plots used for the spectrophotometric determination of the equilibrium constants indicate that at infinite amine concentrations, *i.e.* complete conversion of aldehyde to carbinolamine, little absorbancy remains at 280 nm. This means that no detectable dehydroxylation of the carbinolamine to give a positively charged Schiff base occurs, since this compound would be expected to absorb appreciably at 280 nm; the Schiff bases of primary amines exhibit enhanced absorbancy relative to pyridine-4-aldehyde at this wavelength. No spectrophotometric evidence was obtained for the formation of a *gem*-diamine from the carbinolamine. In addition, this reaction would not be expected to give changes in absorbancy at 280 nm. Thus there is no doubt that the faster of the two relaxation times observed is due to the equilibrium between pyridine-4-aldehyde, piperazine, and carbinolamine and that the slower time, which is observed also in the absence of piperazine, is due to the hydration equilibrium of pyridine-4-aldehyde, the kinetics of which have been studied previously.[15]

The forward uncatalyzed rate for piperazine (k_1 = 2-3 × 10^5 M^{-1} sec^{-1}) is more than three orders of magnitude faster than the reaction of piperazine monocation (k_2 = 65 M^{-1} sec^{-1}), yet the equilibrium constants for carbinolamine formation differ only by a factor of four. Thus, although there is little sensitivity of the equilibrium constant to the basicity of the nucleophile, as has previously been rationalized by Hine and Weimar[16] and by Sander and Jencks,[4] the addition rate increases markedly with increasing basicity. The two points from a plot of log k against pK for the uncatalyzed forward reactions of piperazine and piperazine monocation give a slope β = 0.85. This value is very similar to that obtained for the aminolysis of esters, $\beta \approx 0.8$.[17,18] A β value close to unity is indicative of a large degree of bond formation in the transition state.[17a]

The addition reaction can be formulated as a two-step process in which the equilibrium between the



I II III

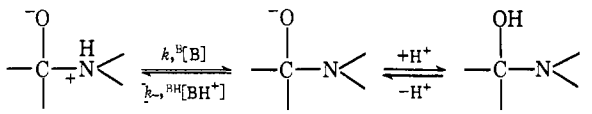
dipolar intermediate II and the neutral carbinolamine III is assumed to be far to the right,[19] even in the case of strongly basic amines.[20] The observed general base catalysis can hardly be rationalized in terms of

(15) M.-L. Ahrens, G. Maass, P. Schuster, and H. Winkler, *J. Amer. Chem. Soc.*, **92**, 6134 (1970).
(16) J. Hine and R. D. Weimar, *ibid.*, **87**, 3387 (1965).
(17) (a) W. P. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968); (b) T. C. Bruice and R. Lapinski, *ibid.*, **80**, 2265 (1958).
(18) W. P. Jencks and J. Carriulo, *ibid.*, **82**, 675 (1960).
(19) J. E. Reimann and W. P. Jencks, *ibid.*, **88**, 3973 (1966).
(20) J. Hine and F. C. Kokesh, *ibid.*, **92**, 4383 (1970); J. Hine, J. C. Craig, Jr., J. G. Underwood, II, and F. A. Via, *ibid.*, **92**, 5194 (1970).

catalysis of the bond formation step;[21] thus one is led to the conclusion that proton transfer is the rate-determining process (*i.e.*, $k_{21} \gg k_{23}$), as has been reported in some cases for similar reactions.[22,23]

The apparent forward rate constant is then given by $k_{23}k_{12}/k_{21}$ and the reverse rate constant by k_{32}. Because of the lower basicity of the nitrogen atom in the case of piperazine monocation as compared to piperazine, the value of the preequilibrium constant k_{12}/k_{21} as well as the value of k_{32} are correspondingly lower, thus accounting for the differences between k_1 and k_2 and between k_{-1} and k_{-2} (Table V).

The observed general base catalysis of reaction 1 (in the forward direction) is thought to occur by rate-determining proton transfer from the zwitterion intermediate to the general base B[23]



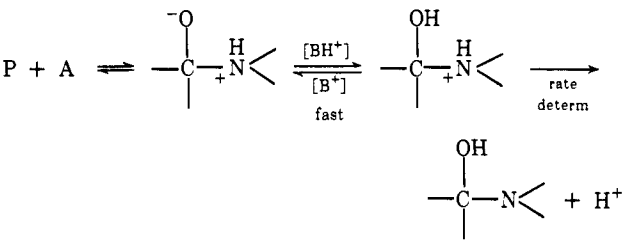
The apparent forward rate constant for the catalyzed pathway is then equal to $k_1^B = k_t^B k_{12}/k_{21}$. For addition reactions to benzaldehyde the pK_a value of the zwitterion is estimated to be 2–3 units higher than the pK_a of the protonated parent amine.[24] Therefore, diffusion controlled proton transfer may be assumed for B = OH^- in our system, *i.e.*, $k_t^{OH} \approx 5 \times 10^9\ M^{-1}\ sec^{-1}$ (including a factor ~0.5 for steric shielding). Since $k_1^{OH} = 9 \times 10^8\ M^{-2}\ sec^{-1}$, an approximate stability constant of $k_{12}/k_{21} \approx 0.2\ M^{-1}$ is calculated for the equilibrium between the zwitterion carbinolamine II and the reactants I. With $K_1 \approx 50\ M^{-1}$, the equilibrium constant for interconversion of the two forms of the carbinolamine, k_{23}/k_{32}, is then ≈250. According to this mechanism, the rate of dissociation of the zwitterion must be very high, $k_{21} \geqslant 10^7\ sec^{-1}$, as has been reported previously for other systems.[19,23]

Evaluation of the rate constant for proton transfer to piperazine as catalyzing base (B = A) yields $k_t^A = k_1^A k_{21}/k_{12} \approx 4 \times 10^7\ M^{-1}\ sec^{-1}$. The general rules governing proton transfer[25] applied to reactions of nitrogen donors with nitrogen acceptors[26] clearly indicate that the rate constant for the reverse reaction step must be approximately 10–30-fold larger than k_t^A and thus is not far below the diffusion-controlled limit. This leads to estimates of about 11–11.5 for the pK_a of the zwitterion and (with $k_{23}/k_{32} \approx 250$) of 13.4–13.9 for the pK_a of the neutral carbinolamine in the system under consideration.

Furthermore, from the expression for the rate constant of the uncatalyzed reaction 1, $k_1 = k_{23}k_{12}/k_{21} = (2\text{–}3) \times 10^5\ M^{-1}\ sec^{-1}$, a value of $k_{23} \approx 1 \times 10^6\ sec^{-1}$ is obtained. Uncatalyzed intramolecular proton transfer in this system therefore is only moderately fast; experimental studies of intramolecular proton transfer reactions in systems of similar geometry gave rate constants around $10^8\ sec^{-1}$ in the thermodynamically favored direction.[27]

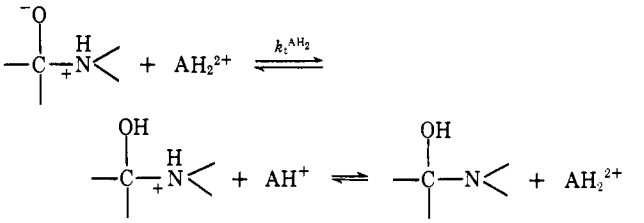
(21) W. P. Jencks, *J. Amer. Chem. Soc.*, **94**, 4731 (1972).
(22) R. E. Barnett and W. P. Jencks, *ibid.*, **91**, 2358 (1969), and references quoted therein.
(23) J. M. Sayer and W. P. Jencks, *ibid.*, **94**, 3262 (1972).
(24) J. M. Sayer, private communication.
(25) M. Eigen, *Angew. Chem., Int. Ed. Engl.*, 3, 1 (1964).
(26) M.-L. Ahrens and G. Maass, *Angew. Chem.*, 80, 848 (1968).
(27) Z. Luz and S. Meiboom, *J. Amer. Chem. Soc.*, **85**, 3923 (1963); G. Maass and F. Peters, *Angew. Chem.*, **84**, 430 (1972).

The condition $k_{21} \gg k_{23}$ (*i.e.*, proton transfer being rate determining) should also hold for reaction path 2, which has been assigned to carbinolamine formation with piperazine monocation. This interpretation is not unambiguous, however. Although N-protonated pyridine-4-aldehyde need not be considered as a reactant—this species is completely hydrated[10]—there remains some uncertainty with respect to the position of protons in the transition state, since non-rate-determining proton transfer processes may occur (after N–C bond formation) prior to the rate determining proton transfer step. For instance, the "uncatalyzed reaction 2" ($R = k_2[P][AH]$) could also be accounted for by the sequence



where the rate determining step represents uncatalyzed dissociation of a proton ($R = k[P][A][H]$). Similar considerations apply to the acid-catalyzed pathways. However, for simplicity and since there is no reason for which the piperazine monocation should not be able to form carbinolamines, we will base our discussion on the assumption that the rate terms with an $[H^+]$ dependence (rate constants with index "2," Table V) are due to reactions with the monoprotonated amine. The corresponding rate constants therefore represent—strictly speaking—only the upper limits of the "true" rate constants for the AH^+ reactions.

The observed general acid catalysis of reaction 2 by AH_2^{2+} may again be accounted for by a stepwise proton transfer process



(AH^+ is the most likely acceptor in the second proton transfer step.) The pK for protonation of the $-O^-$ of the zwitterion is certainly much higher than the pK_a of AH_2^{2+} (5.8). Therefore, proton transfer from AH_2^{2+} to the zwitterion is diffusion controlled, $k_t^{AH_2} \approx 1 \times 10^9\ M^{-1}\ sec^{-1}$ (again including a factor of 0.5 for steric shielding) whereas the rate constant for the reverse reaction is by several orders of magnitude smaller. Certainly, this reverse reaction is also much slower than the forward reaction of the second proton transfer step; thus there can be no doubt that the first proton transfer is rate determining. Then $k_2^{AH_2} = k_t^{AH_2}k_{12}/k_{21}$, and (with $k_2^{AH_2} = 6.4 \times 10^4\ M^{-2}\ sec^{-1}$) $k_{12}/k_{21} \approx 6 \times 10^{-5}\ M^{-1}$. This approximate value for carbinolamine zwitterion formation with piperazine monocation is smaller by a factor of ~3 × 10^3 than the one for reaction with unprotonated piperazine. For the equilibrium between neutral and zwitterion carbinolamine one obtains $k_{23}/k_{32} = K_2k_{12}/k_{21} \approx 2 \times 10^5$ (with piperazine monocation). Finally, the rate

constant of the uncatalyzed reaction, $k_2 = k_{23}k_{12}/k_{21} \approx 65\ M^{-1}\ sec^{-1}$, yields $k_{23} \approx 1 \times 10^6\ sec^{-1}$. Thus, there is (for the two systems under consideration) no appreciable dependence of the rate of (uncatalyzed) intramolecular proton transfer on the free-energy change of the reaction—analogous to intermolecular proton transfer reactions[25]—as long as the transfer occurs in the thermodynamically favored direction. As indicated by a molecular model of the zwitterion species, the relatively low value of k_{23} could possibly be due to a slight barrier to rotation around the C–N bond which might affect the efficiency of direct N–O proton transfer or of H_2O-mediated concerted proton transfer, respectively.

The analysis of the kinetic data obtained at low pH brought evidence for an additional catalytic rate term, which is to be interpreted either as general acid catalysis of the reaction with free piperazine by piperazine dication or as the reaction with piperazine monocation catalyzed by piperazine monocation. A distinction between the two possibilities cannot be made.

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Calorimetric Determination of Azide-Ion Binding by Ferrihemoglobin A in Water and in 5% *tert*-Butyl Alcohol[1,2]

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Abstract: Ligand binding data for the ferrihemoglobins obtained by Beetlestone and coworkers demonstrate a linear relationship between standard enthalpy and entropy changes for variation of pH, salt concentration, and species. The slope is 285 ± 15°, the same range found in small solute processes in water. Since confirmation of these results at the highest possible resolution is required, the binding of a typical ligand, azide ion, to human ferrihemoglobin A has been carried out calorimetrically using highly purified protein preparations. The standard free energies and enthalpies of binding obtained in the absence of cosolvents conform within small errors to the previous van't Hoff results and thus confirm the existence of the pattern. The so-called "turn around" or characteristic pH is the same and the slope of the ΔS *vs.* ΔH plot is 280° within error. As a first check on the possibility that water participates directly in these reactions the experiments were repeated with 5% *tert*-butyl alcohol added as cosolvent. Available evidence indicates no effect of the alcohol on the protein through direct binding. The slope of the $\Delta H°$ *vs.* $\Delta S°$ line is the same but the intercept is 0.8 kcal/mol less negative showing that the alcohol cosolvent alters the binding process quantitatively but not qualitatively. The results appear to be attributable to alteration in water mediated through the protein.

The binding of azide to ferrihemoglobin has been studied by many workers[3–6] as a function of pH, species, and temperature and it has been concluded that the thermodynamic parameters resemble those of other ligands such as SCN^-, F^-, etc., *viz.* the standard free energy of binding is generally insensitive to pH variation but the standard enthalpies and entropies of binding are pH dependent and in such a way that $\Delta H°$ and $T\Delta S°$ tend to cancel each other (compensation).[7] Values of $\Delta H°$ as a function of pH show a maximum which correlates roughly with the isoelectric point of the species. In all previous studies $\Delta H°$ has been obtained by the van't Hoff method using spectral changes of the heme group. The high degree of linearity of the van't Hoff plots implied a negligible heat-capacity change. Confirmation of these interesting results is especially important because the same pattern of enthalpy–entropy compensation is being demonstrated in more and more protein systems. These demonstrations may be accidental and it is important to establish their validity in all cases at the highest possible level of precision. The calorimetric method is distinctly better than the van't Hoff method and particularly so when heat-capacity effects may be large. We have repeated the N_3^- binding studies of Beetlestone and coworkers with human ferrihemoglobin A at 25.9° using a flow microcalorimeter.

Interpretation of the pH dependence of $\Delta H°$ and $\Delta S°$ for the ferrihemoglobin ligand binding reactions has been based on changes in the hydration sheath provoked by tautomeric shifts of the imidazole proton.[6] As an initial test of the role of water in the hydration sheath and in the bulk water phase we have studied the reaction in the presence of *tert*-butyl alcohol and observed changes in $\Delta H°$ and $\Delta S°$ especially at the more acid pH values. The significant effects we observed even at low alcohol concentration provide some support for the idea that water plays a direct role in the reaction of ferrihemoglobins.

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