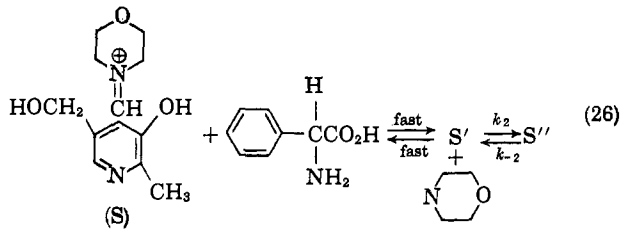
imine formation is much more rapid than prototropy. Convincing evidence that 24 does not represent the reaction path is obtained from experiments in which the morpholine imine of PCHO (S) is used in place of PCHO.[20] Thus the catalytic rate-constants for the imidazole-catalyzed formation of ketimine (S'') from the morpholine imine of PCHO (S) (26) are similar to those for the reaction involving PCHO. This result



(26)

would not be predicted on the basis of 24 since it is well established that imines react at a greater rate with general reagents of type R–NH_2 than do the corresponding aldehydes or ketones.[21–24] Recently, Cordes and Jencks[25] have shown that S reacts with semicarbazide at a greater rate than does PCHO.

It may be noted that morpholine, which is a stronger base than imidazole, does not alone catalyze the transamination reaction between PCHO and α-aminophenylacetic acid under conditions (*e.g.*, 10^{-4} *M* reactants) which provide a facile transamination using imidazole buffers. This observation lends support to the suggestion that the catalytic activity of imidazole buffer arises from its ability to form a complex with a reactant.

The catalysis would, therefore, appear to be best expressed by the path of 23 in which a complex of S' with two molecules of an imidazole species is followed by a rate-controlling intracomplex catalysis of the prototropic shift converting S' to S''. Only 23 is compatible with the determined kinetic scheme (10–18). Thus, the kinetic treatment is based upon the assumption of a rate-determining prototropic shift following a rapidly established low steady-state in S', and is found not only to provide a rate equation which accommodates the rate data to 80–90% completion but also predicts the somewhat unusual rate variation caused by variation of the initial reactant concentrations (see Table II).

In the kinetic considerations of this paper we have treated PCHO, S', and S'' as discrete chemical entities. Of course they are not. Pyridoxal can exist in several ionic forms in both the free aldehyde and internal hemiacetal forms.[26] Much the same is true of S' and S''. The consideration of these complications was unessential to the arguments presented herein since the reactions were studied under restricted conditions of acidity, ionic strength, solvent composition and temperature. The present study suffices to establish the catalysis to occur through a complex of one or more of the aldimine species formed from PCHO and A with two neutral, two acidic or one neutral and one acidic imidazole species.

Acknowledgments.—The authors express their gratitude to Mrs. Patricia Benkovic for performing many of the rate measurements necessary to the completion of this study. This work was supported by grants from the National Institutes of Health and from the National Science Foundation. For this support we are deeply grateful.

(20) Details of this study are reported separately in part III, *J. Am. Chem. Soc.*, **85**, 1493 (1963).
(21) E. H. Cordes and W. P. Jencks, *ibid.*, **84**, 826 (1962).
(22) Mme. Bruzau, *Ann. Chim.*, [11] **1**, 332 (1934).
(23) E. A. Brodhag and C. R. Hauser, *J. Am. Chem. Soc.*, **77**, 3024 (1955).
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(26) D. E. Metzler and E. E. Snell, *J. Am. Chem. Soc.*, **77**, 2431 (1955).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, N. Y.]

Catalytic Reactions Involving Azomethines. II. The pH Dependence of the Imidazole Catalysis of the Transamination of Pyridoxal by α-Aminophenylacetic Acid

BY THOMAS C. BRUICE AND RICHARD M. TOPPING[1]

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The pH dependence of the catalysis of the transamination of pyridoxal by α-aminophenylacetic acid has been investigated. The first phase of the reaction (*i.e.*, pyridoxal + amino acid ⇄ aldimine ⇄ ketimine) has been shown to occur *via* pre-equilibrium complexing of aldimine and ketimine with one molecule of imidazole free base and one ion of the conjugate acid of imidazole. The essential prototropic shift has been suggested to take place *via* an intracomplex general acid, general base mechanism in which imidazole acts as the general base and imidazolium ion as the general acid. Since the final equilibrium concentration of ketimine is not influenced by the catalyst concentration (nor the pH) it is essential for the proposed mechanism that either pyridoxal or amino acid also be complexed by imidazole. It has been established that α-aminophenylacetic acid forms a complex in aqueous solution with imidazole and imidazolium ion and that the formation constant for this complex is comparable to that determined kinetically for the imines.

Introduction[2]

In the preceding paper[3] the imidazole-catalyzed transamination of pyridoxal by α-aminophenylacetic acid at pH 8.6 (30° in water at μ = 0.05 *M*) was described. It was established that the reaction occurred in two distinct stages, the first leading to the formation of an equilibrium mixture of pyridoxal, amino acid, aldimine (at low steady state) and ketimine, and the second stage of the reaction to a final equilibrium mixture of pyridoxal, amino acid, aldimine (low steady state), ketimine, pyridoxamine and phenylglyoxylic acid.[2]

$$\text{PCHO} + \text{A} \underset{\text{fast}}{\overset{K_1}{\rightleftharpoons}} (\text{S}') \underset{\text{slow}}{\overset{K_2}{\rightleftharpoons}} \text{S}'' \rightleftharpoons \text{PCH}_2\text{NH}_2 + \text{PG} \quad (1)$$

Phase one

Phase two

(1) Post-doctoral Fellow of The Department of Chemistry, Cornell University.
(2) Abbreviations employed in this paper are: pyridoxal, PCHO; pyridoxamine, PCH_2NH_2; α-aminophenylacetic acid, A; phenylglyoxylic acid, PG; the aldimine formed between pyridoxal and α-aminophenylacetic acid, S'; the ketimine formed between pyridoxamine and phenylglyoxylic acid, S''; the complex of S' with one molecule of imidazole and one molecule of the conjugate acid of imidazole, S_c'; like complexes of S'' and A are similarly abbreviated as S_c'' and A_c, respectively; total imidazole IM_T, where $IM_T = IM + IMH^{\oplus}$.
(3) T. C. Bruice and R. M. Topping, *J. Am. Chem. Soc.*, **85**, 1-80 (1963).

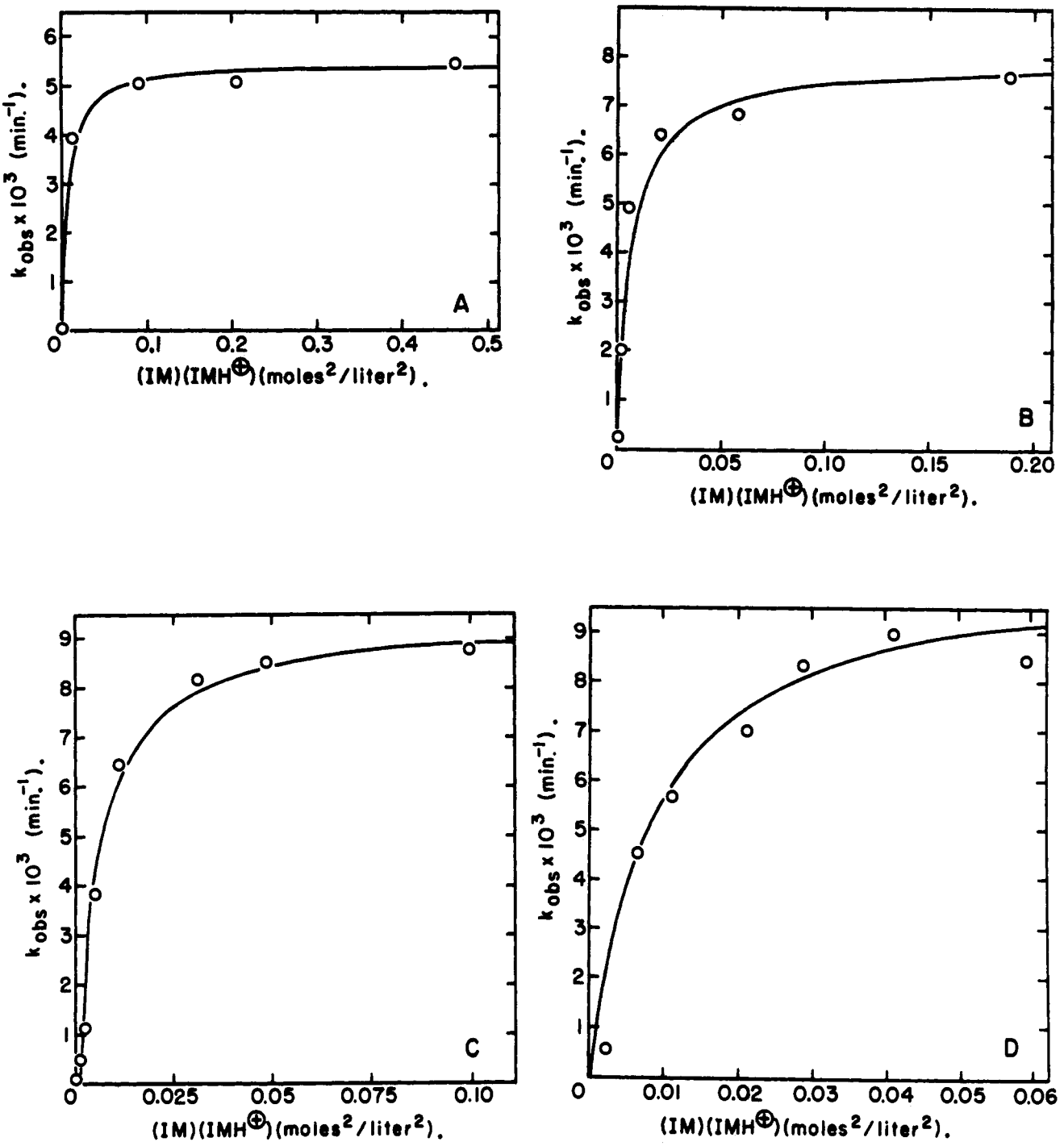


Fig. 1.—Plots of the observed first order rate constants (k_{obsd}) for the appearance of S'' *vs.* the product of the concentrations of imidazole and imidazolium ion. The points are experimental and the curves are those obtained from equation 3 employing the constants provided in Table I: (A) pH, 7.8; (B) pH, 8.3; (C) pH, 8.6; (D) pH, 9.3.

Though the reactions of (1) could be shown to occur in imidazole buffer at both very low (spectrophotometric) and high (isolation procedures) concentrations of PCHO and A, the reaction was found to occur at measurable rates in morpholine or carbonate buffers only at high reactant concentrations. The efficiency of imidazole buffer as catalyst was ascribed to its ability to form complexes with reactants and intermediates. In accord with this supposition, it was found that the rate of approach to equilibrium in phase one was dependent upon the second power of the total imidazole concentration at low concentrations of imidazole buffer and to be independent of imidazole concentration at higher concentrations of the buffer. The apparent first order rate of approach to equilibrium in phase one, therefore, allowed the well known Michaelis–Menten form

$$k_{obsd} = \frac{0.95 \times 10^{-2}\,(IM_T)^2}{0.2 + (IM_T)^2} \text{ for pH } 8.61 \qquad (2)$$

In order to determine the imidazole species involved in complex formation and catalysis and to shed further light on mechanism, we have extended our investigations to other acidities. The results of this study are reported herein.

Results

In Fig. 1 are presented plots of the observed first-order rate constants for the approach to equilibrium in phase one (1) at several pH values *vs.* the product of the concentrations of free imidazole (IM) and the

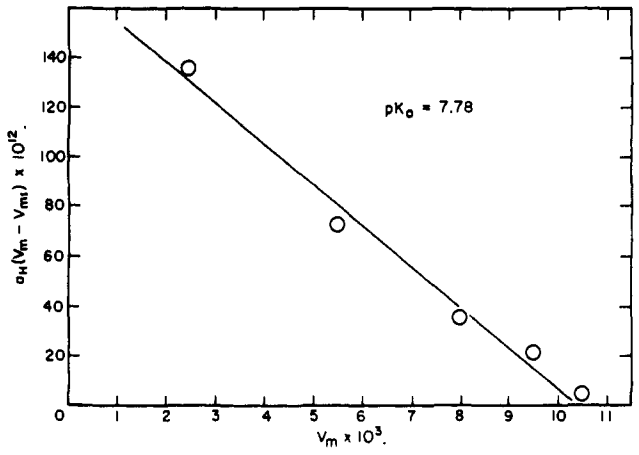


Fig. 2.—Plot of equation 6. The slope provides the value of $-K_a'$ and the intercept at $a_H(V_m - V_{m1}) = 0$ is V_{m2}.

conjugate acid of imidazole ($IMH^{\oplus}$). The points represent experimental values and the lines are theoretical having been calculated from equation 3.

$$k_{obsd} = \frac{V_m(IM)(IMH^{\oplus})}{K_m + (IM)(IMH^{\oplus})} \quad (3)$$

In Table I are recorded the values of V_m and K_m so determined for each pH. Also recorded in Table I are the final equilibrium concentrations of S'' for phase one (1) at t_∞. The latter values have been calculated in the manner previously outlined in part I of this study (see ref. 3).

TABLE I

VALUES OF K_m AND V_m CALCULATED FROM EQUATION 3 AND THE FINAL EQUILIBRIUM CONCENTRATIONS OF S'' AT t_∞ FOR THE IMIDAZOLE CATALYSIS OF PHASE ONE[a]

pH	Buffer	IM Mole fraction	$IMH^{\oplus}$ Mole fraction	$V_m \times 10^4$, min.$^{-1}$	K_m, mole^2 l.$^{-2}$	S'' at t_∞, M
7.06	Imidazole	0.466	0.534	2.46	...	...
7.80	Imidazole	.827	.173	5.5	7.15×10^{-3}	5.2×10^{-5}
8.30	Imidazole	.938	.062	8.0	7.0×10^{-3}	5.3×10^{-5}
8.60	Imidazole	.968	.032	9.5	6.2×10^{-3}	5.6×10^{-5}
9.30	Carbonate	.993	.0066	10.5	8.5×10^{-3}	5.3×10^{-5}
10.20	Carbonate			>11	...	4.8×10^{-5}

[a] Initial concentration of pyridoxal and amino acid was 10^{-4} M, temperature 30°, ionic strength 1.0 M and solvent water.

In part I[3] of this study it was established kinetically that the only satisfactory mechanism, proceeding through the formation of imines, was one in which S' was at a low steady state concentration and the rate determining step was the prototropic shift leading to the reversible conversion of S_c' to S_c''. If S' must be complexed prior to conversion to S'' then from microscopic reversibility S'' must be complexed prior to conversion to S'. The constancy of the values of K_m, calculated on the basis of the involvement of one neutral and one acidic species of imidazole (Table I), is convincing evidence that the complexes of S' and S'' which partake in the reversible prototropic shift have one of the compositions of (4). It is of course not possible to differentiate kinetically between (4a), (4b) and (4c).

$$S_c' = S', IM, IMH^{\ominus} \text{ or } S', IMH^{\oplus}, IMH^{\oplus}, OH^{\ominus} \text{ or } S', IM, IM, H^{\oplus}$$
$$S_c'' = S'', IM, IMH^{\oplus} \text{ or } S'', IMH^{\oplus}, IMH^{\oplus}, OH^{\ominus} \text{ or } S'', IM, IM, H^{\oplus} \quad (4)$$
$$\text{(a)} \qquad \text{(b)} \qquad \text{(c)}$$

The catalytic complex as represented by (4a) would appear to be the most likely, however, only three species, rather than four, as in (4b) and (4c), being involved.

Inspection of Table I shows that V_m increases with increasing pH. The values of V_m have been shown to follow equation 5.

$$V_m = \frac{V_{m1}a_H + V_{m2}K_a'}{K_a' + a_H} \quad (5)$$

where V_{m1} and V_{m2} are rate constants for approach to equilibrium of phase one at saturation by catalyst species, a_H the hydrogen ion activity as determined by the glass electrode and K_a' a kinetically apparent dissociation constant. Rearrangement of (5) provides (6).

$$a_H(V_m - V_{m1}) = K_a'(V_{m2} - V_m) \quad (6)$$

The value of V_{m1} was determined to be 9×10^{-4} min.$^{-1}$ by the fitting of a theoretical dissociation curve to a plot of V_m *vs.* pH. In Fig. 2 is plotted $a_H(V_m - V_{m1})$ *vs.* V_m and from the slope of the line the value of K_a' has been determined to be 1.64×10^{-8} ($pK'_{app} = 7.78$) while from the intercept at $a_H(V_m - V_{m1}) = 0$, the value of V_{m2} was determined to be 10.2×10^{-3} min.$^{-1}$.

Examination of the values of S'' at t_∞ (Table I), calculated from experiments carried out under saturation conditions of imidazole buffer, reveals that the over-all equilibrium of phase one of the transamination reaction is not pH dependent. In like manner the final equilibrium position of the reaction (determined by the absorbance of ketimine at 246 mμ) is not influenced unduly at constant pH by alteration in the concentration of catalyst (Table II).

TABLE II

INFLUENCE OF IMIDAZOLE ON POSITION OF EQUILIBRIUM OF PHASE ONE AT t_∞ (pH 9.3; μ = 1.0 M; T = 30°)

IMT (M)	O.d.$_{246\ m\mu}$
0.6	0.400
1.0	.386
1.3	.420
1.8	.400
2.1	.482
2.5	.477
3.0	.440
	Av. 0.430 ± 0.030

As shown in part I[3] of this study, S' is at steady state and at completion of phase one and at saturation concentrations of catalyst, considerable PCHO remains. It is required, therefore, that PCHO or A also be complexed by imidazole and imidazolium ion and that the dissociation constant for the complex with the reactant be of the same magnitude as K_m. The formation of a complex between α-aminophenylacetic acid and imidazole was suspected on the basis of the greater solubility of the amino acid in aqueous solutions containing imidazole buffers than in aqueous solutions containing borate, morpholine or carbonate buffers at the same pH (see Experimental section of part I[3]). The formation constant for complex formation between amino acid and imidazole buffer was approximately determined by means of solubility studies at pH 8.6 and pH 7.06 and calculated using equation 12. The formation constants were found to be quite similar if calculated on the basis of the formation of a complex composed of one imidazole molecule, one imidazolium ion and one amino acid molecule in the form of the zwitterion.[4]

(4) Essentially the same formation constants are obtained if it is considered that the imidazole species complex with both the zwitterion and anionic species of the amino acid but are not at all similar if it is assumed that only the anionic species of the amino acid forms the complex. Differentiation between these possibilities could not be made since at pH values of 9.0 and above the mole fraction of imidazolium ion is an exceedingly small number and the resultant solubility of the amino acid in imidazole buffers in this pH range is too small to determine with any degree of accuracy (see Experimental).

The similarity of the constant so determined to the value of $1/K_m$ derived from the Michaelis–Menten equations provides the necessary rationale to our experimental finding that the equilibrium position of phase one is independent of catalyst concentration, that measurable pyridoxal remains at t_∞ and that aldimine species are at steady state. The question as to the nature of the cohesive forces binding the complex remains at this time open. It would appear that the portion of the aldimine and ketimine which is complexed by the imidazole includes the benzene ring of the amino acid since the amino acid and the imines are complexed to about the same degree. The formation of complexes between aromatic, lyophobic compounds and heterocyclic compounds in water is a well established, if perhaps not well understood phenomenon,[5,6] though catalysis proceeding through such complexes in aqueous solution has not been observed previously.

Combining equations 3 and 5 with equation 14 of part I[3] of this series provides the experimentally derived differential equation which describes the kinetics for phase one at various acidities and catalyst concentrations (7).

$$\frac{d\,S_T''}{dt} = \frac{[K_a' V_{m2} + a_H V_{m1}](IM)(IMH^\oplus)}{[K_m + (IM)(IMH^\oplus)][K_a' + a_H]} \times \left[P_T A_T - \frac{S_T''}{K_\infty}\right] \quad (7)$$

where P_T = total concentration of all pyridoxal and aldimine species at time t; A_T = total concentration of all amino acid species at time t; S_T'' = total concentration of all ketimine species at time t; K_∞ is defined by equation 9.

From (7) the apparent first order rate constant is given by (8)

$$k_{obsd} = \frac{[K^1{}_a V_{m2} + a_H V_{m1}](IM)(IMH^\oplus)c}{[K_m + (IM)(IMH^\oplus)][K_a' + a_H]1.15} \quad (8)$$

where

$$c = \left[\frac{PCHO_0^2 + A_0^2 + 2\left(\frac{PCHO_0 + A_0}{K_1K_2} - PCHO_0A_0\right) + \frac{1}{(K_1K_2)^2}}{4}\right]^{1/2}$$

and K_1K_2 is defined by equation 1.

At t_∞, $dS_T''/dt = 0$ and K_∞ is pH and catalyst invariant

$$K_\infty = \frac{S_{T\infty}''}{P_{T\infty}A_{T\infty}} \quad (9)$$

The second-order rate constant for the forward reaction (formation of S″) is provided approximately by

$$\frac{k_{obsd} \times 1.15}{c} \cong k_2K_1 \quad (10)$$

where k_2 is the first-order rate constant for the prototropic shift and K_1 is the formation constant for aldimine. If one initial reactant concentration (*i.e.*, $PCHO_0$ or A_0) remains constant, the other initial reactant concentration may vary from zero up to the initial concentration of the reactant at constant concentration with only minor changes in c (8). However, if the initial concentration of both reactants vary, then c is markedly changed. Metzler[7] has shown that the pH profile (log K_{eq} *vs.* pH) for the formation constant of pyridoxal aldimines from α-amino acids exhibits a slope of *ca.* 1.0 in the pH range we have employed. This may be associated with the necessity to have the α-amino acid in the amino free base form for imine formation and suggests that the pK_a' of pyridoxal of 8.6 has little influence upon the extent of pyridoxal aldimine formation (*i.e.*, both the protonated and unprotonated forms must react with the amino acid to about the same extent). We would anticipate then, that the c value of (10) would remain essentially constant and the pH dependence of V_m must reflect the

TABLE III

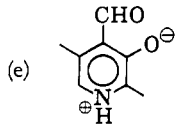
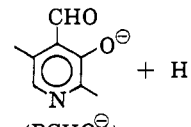
A LIST OF THE RATE AND EQUILIBRIUM STEPS REQUIRED TO PROVIDE A FULL DESCRIPTION OF PHASE ONE OF THE IMIDAZOLE CATALYSIS OF THE TRANSAMINATION OF PYRIDOXAL BY α-AMINOPHENYLACETIC ACID

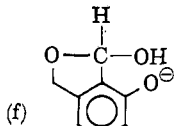
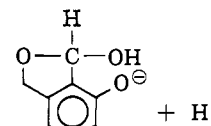
(a) $C_6H_5CH(NH_3^\oplus)COO^\ominus \underset{}{\overset{K_a^I}{\rightleftharpoons}} C_6H_5CH(NH_2)COO^\ominus + H^+$
(AH) (A⊖)

(b) $AH + IM + IMH^\oplus \overset{K_m^I}{\rightleftharpoons}$ Complex. AH

(c) $A^\ominus + IM + IMH^\oplus \overset{K_m^{II}}{\rightleftharpoons}$ Complex. $A^\ominus$

(d) Complex. AH $\overset{K_a^{II}}{\rightleftharpoons}$ Complex. $A^\ominus + H^\oplus$

(e)  $\overset{K_a^{III}}{\rightleftharpoons}$  $+ H^\oplus$
(PCHO) (PCHO⊖)

(f)  $\overset{K_a^{IV}}{\rightleftharpoons}$  $+ H^\oplus$
($PCHO_i$) ($PCHO_i^\ominus$)

(g) $PCHO_i \overset{K_h^I}{\rightleftharpoons} PCHO$

(h) $PCHO_i^\ominus \overset{K_h^{II}}{\rightleftharpoons} PCHO^\ominus$

(i) $PCHO + A \overset{K_1}{\rightleftharpoons} S'$

(j) $PCHO^\ominus + A \overset{K_1'}{\rightleftharpoons} S^{\ominus\prime}$

(k) $S' + IM + IMH^\oplus \overset{K_m^{III}}{\rightleftharpoons} S_c'$

(l) $S^{\ominus\prime} + IM + IMH^\oplus \overset{K_m^{IV}}{\rightleftharpoons} S_c^{\ominus\prime}$

(m) $S' \overset{K_a^V}{\rightleftharpoons} S^{\ominus\prime} + H^\oplus$

(n) $S_c' \overset{K_a^{VI}}{\rightleftharpoons} S_c^{\ominus\prime} + H^\oplus$

(o) $S_c' \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} S_c''$

(p) $S_c^{\ominus\prime} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} S_c^{\ominus\prime\prime}$

(q) $S'' + IM + IMH^\oplus \overset{K_m^V}{\rightleftharpoons} S_-''$

(r) $S^{\ominus\prime\prime} + IM + IMH^\oplus \overset{K_m^{VI}}{\rightleftharpoons} S_c^{\ominus\prime\prime}$

(s) $S'' \overset{K_a^{VII}}{\rightleftharpoons} S^{\ominus\prime\prime} + H^\oplus$

(t) $S_c'' \overset{K_a^{VIII}}{\rightleftharpoons} S_c^{\ominus\prime\prime} + H^\ominus$

(5) T. Higuchi and D. A. Zuck, *J. Am. Pharm. Assoc., Sci. Ed.*, **XLII**, 132 (1953); T. Higuchi and J. L. Lach, *ibid.*, XLIII, 349 (1954); E. H. Gans and T. Higuchi, *ibid.*, **XLVI**, 458 (1957); T. Higuchi and S. Bolton, *ibid.*, **XLVIII**, 557 (1959); J. W. Poole and T. Higuchi, *ibid.*, **XLVIII**, 592 (1959).

(6) H. A. Harbury and K. A. Foley, *Proc. Natl. Acad. Sci., U. S.*, **44**, 662 (1958); H. A. Harbury, K. F. LaNoue, P. A. Loach and R. M. Amick, *ibid.*, **45**, 1708 (1959).

(7) D. E. Metzler, *J. Am. Chem. Soc.*, **79**, 485 (1957).

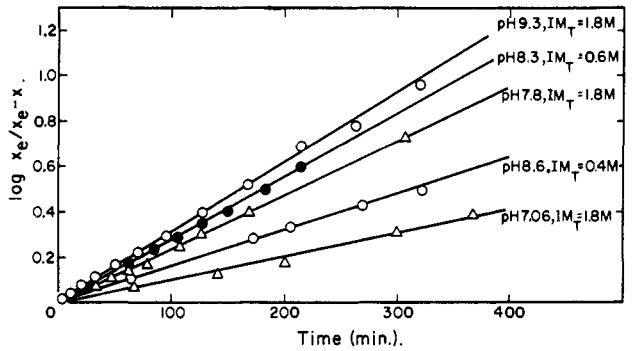


Fig. 3.—First order plots for the appearance of S″ (as determined at 246 mμ) in the presence of various concentrations of imidazole and at various pH (pyridoxal and α-aminophenylacetic acid initially at 10^{-4} *M*; μ = 1.0 *M*; T = 30°).

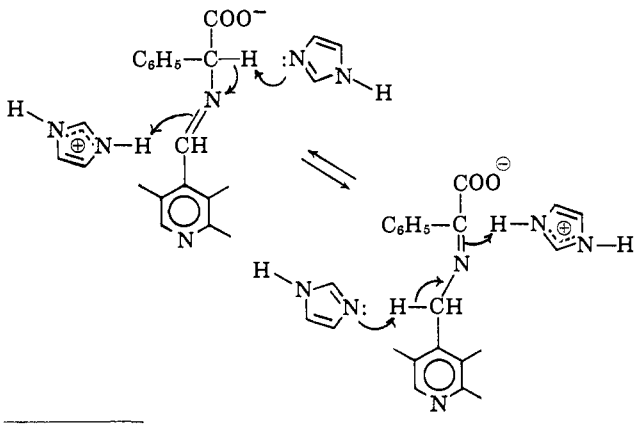
pK_a' of the α-amino acid and that of intermediates and final product.

Listed in Table III are the various equilibrium and rate steps that must be taken into account in deriving any theoretical rate equation for phase one. Of the equilibrium and rate constants of Table III, only a few can be determined separately. For this reason it is not possible to interpret meaningfully the pH dependence of V_m. Less complex systems will have to be investigated for this purpose. We are at present actively engaged in this endeavor.

Discussion

In the transamination of pyridoxal by α-aminophenylacetic acid, the final equilibrium position for phase one (1) is independent of catalyst concentration, but the rate of approach to equilibrium is catalyst dependent. This is so at all pH values investigated (Table I). We are, therefore, dealing with a true catalysis. The constancy of K_m (3), calculated on the basis of the formation of catalytic complexes involving one imidazole and one imidazolium species, suggests the possibility that the essential prototropic shift is catalyzed *via* the mechanism of Chart I. The position of the critical transition state along the reaction coördinate cannot be specified from the data of this study, though it may be noted that Banks, Diamantis and Vernon[8] have reported a feeble general acid catalysis in the transamination of pyruvic acid by pyridoxamine. The key experiments to resolve this issue will be the determination of the kinetic isotope effects when α-deuterioamino acid is employed and when the reaction is carried out with deuterioimidazolium ion in D_2O as solvent. The complete symmetry of the mechanism of Chart I is compelling and it may be noted that the mechanism is formally of the "push–pull" type as reported by Swain and Brown[9] for the catalysis of the mutarotation of tetramethylglucose by carboxylic acids and α-pyridone in benzene solution. Like the latter reaction, the imidazole catalysis of the transamination of pyridoxal by α-aminophenylacetic acid also involves a pre-equilibrium complex formation. Unlike the catalysis of Swain and Brown, however, the imidazole catalysis of the transamination reaction takes place in aqueous solution.

CHART I



The model reaction reported herein bears a close similarity to the enzymatic reaction. Hammes and Fasella have examined the kinetics of glutamic-aspartic transaminase by employing the temperature jump method.[10,11] Six relaxation times were found for the over-all reaction at high enzyme concentrations (10^{-5} *M*). The measured rate constants were assigned as

$$E_L + As \underset{>5\times10^3\ sec.^{-1}}{\overset{>10^7\ M^{-1}\ sec.^{-1}}{\rightleftharpoons}} X_1 \underset{26\ sec.^{-1}}{\overset{80\ sec.^{-1}}{\rightleftharpoons}} X_2 \underset{1.4\times10^2\ sec.^{-1}}{\overset{7\times10^7\ M^{-1}\ sec.^{-1}}{\rightleftharpoons}} E_M + Oa \quad (11)$$

$$Kg + E_M \underset{70\ sec.^{-1}}{\overset{2\times10^7\ M^{-1}\ sec.^{-1}}{\rightleftharpoons}} Y_2 \underset{61\ sec.^{-1}}{\overset{30\ sec.^{-1}}{\rightleftharpoons}} Y_1 \underset{3.3\times10^7\ M^{-1}\ sec.^{-1}}{\overset{2.8\times10^3\ sec.^{-1}}{\rightleftharpoons}} G_m + E_L$$

where E_L is the aldehyde form of the enzyme, E_M the amino form, As is aspartate, Oa is oxalacetate, Kg is ketoglutarate, Gm is glutamate and the X's and Y's are reaction intermediates. Inspection of the rate constants of (11) reveals that the rate determining steps involve the interconversion of the intermediates which were shown by their absorption spectra to be imines. The magnitude of the rate constants suggests a diffusion controlled formation of catalyst–substrate complex followed by imine formation. The rate constants for the interconversion of intermediate imines are small enough for acid–base catalysis to be involved.[12] It is important to note that the enzyme preparation employed by Hammes and Fasella was found to be devoid of all heavy metal ion and not to exhibit enhanced activity on addition of metal ions.[13]

Experimental

Kinetic procedures were those employed in part I. All reactions adhered strictly to first order kinetics as shown by the representative sample given in Fig. 3 at various pH values and various catalyst concentrations.

Determination of the Formation Constant for the α-Aminophenylacetic Acid–Imidazole Complex.—On the basis of the apparent increased solubility of α-aminophenylacetic acid in aqueous solutions containing imidazole buffers, the possible formation of a complex was investigated and an attempt was made to determine by solubility studies the size of the formation constant for such a complex.

The determinations were made at pH 8.6 and 7.06 by suspending a known amount of the amino acid (sufficient to provide an excess of undissolved solid at equilibrium) in a known volume of water containing the imidazole buffer at several concentrations and sufficient KCl calculated to maintain 1.0 *M* ionic strength. The mixtures were sealed into water-tight vials immersed in a reciprocating constant temperature water bath at 30° and vigorously and continuously agitated over a period of at least 72 hr. to ensure attainment of equilibrium. The solutions then were filtered rapidly through sintered glass crucibles, the residual amino acid washed with a minimum of ice-cold water and estimated by spectrophotometric analysis in 1% HCl solution at

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TABLE IV

THE DETERMINATION OF THE SOLUBILITY OF α-AMINOPHENYLACETIC ACID IN IMIDAZOLE BUFFERS AT pH 8.6 AND 7.06 (30°; μ = 1.0 M with KCl)

pH 8.6; T = 30°; intercept gave $A = 27.9 \times 10^{-3}$ M

$(IM)(1MH^{\oplus})$ [(moles/l.)^2 × 10^2]	Solubility of amino acid—A [moles/l. × 10^4]
4.96	6.7
4.96	6.5
3.72	2.8
2.79	2.9
1.95	2.0
1.24	0.6
0.31	1.2
0.195	0.3

pH 7.06; T = 30°; intercept gave $A = 21.6 \times 10^{-3}$ M

$(IM)(1MH^{\oplus})$ [(moles/l.)^2 × 10^2]	Solubility of amino acid—A [moles/l. × 10^4]
3.98	18.7
2.99	11.4
2.24	11.3
0.25	1.9

260 mμ; Beer's law calibration curves were prepared for the amino acid in 1% HCl solutions and were used for the analyses.

The formation constants were found to be approximately independent of pH only if calculated for a complex of amino acid containing one free imidazole species and one imidazolium ion. The formation constants thus were calculated on the basis of equation 12 in which K_m^I represents the formation constant for the complex, A_c the amino acid complex and A the free amino acid

$$K_m^I = \frac{A_c}{A(IM)(IMH^{\oplus})} \quad (12)$$

The value of K_m^I thus was determined from a plot of the solubility of the amino acid (in moles/l.) *vs.* the corresponding concentration of imidazole buffer (expressed as $(IM)(IMH^{\oplus})$) to give straight lines of slope $A_c/(IM)(IMH^{\oplus})$ and intercept A. The results are tabulated in Table IV.

The results of Table IV indicate the trend of greater solubility of amino acid with increasing imidazole buffer concentrations. As, however, under the conditions of the experiment, a solubility difference of only *ca.* 5 mg. of amino acid is involved between the media of lowest and highest imidazole buffer concentrations, a relatively large though unavoidable experimental error allows only an approximate assignment of the value of K_m^I. Thus the results at pH 8.6 give a value for K_m^I of 37 and at pH 7.06, K_m^I was evaluated to be 21. The dissociation constant of the complex, $1/K_m^I$, is thus approximately 20–50 × 10^{-3} mole^2 liter^{-2} and may be seen to be at least comparable to the value of the dissociation constant (K_m = 7.2 × 10^{-3} mole^2 liter^{-2}) of the aldimine complex derived from the Michaelis–Menten kinetics. Such agreement provides further support for the postulate of complex formation on which the kinetic scheme is founded.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, N. Y.]

Catalytic Reactions Involving Azomethines. III.[1] The Influence of Morpholine upon the Imidazole Catalysis of the Transamination of Pyridoxal by α-Aminophenylacetic Acid. The Transamination of the Morpholine Imine of Pyridoxal

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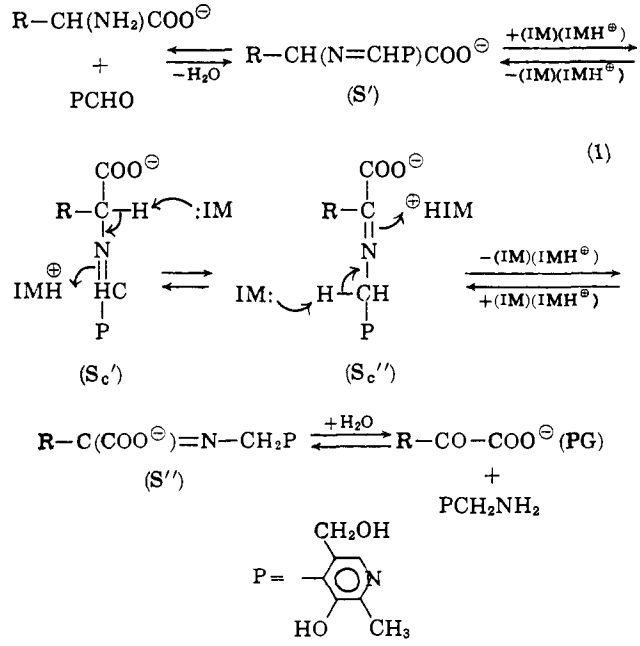
The influence of morpholine upon the rate and final equilibrium position of the imidazole-catalyzed transamination of pyridoxal (and the resultant morpholine imine of pyridoxal) by α-aminophenylacetic acid has been investigated. Morpholine is found to provide no apparent catalysis under the experimental conditions and the conversion of pyridoxal to the morpholine imine is shown to result in no significant change in the kinetic characteristics of the transamination reaction. The study is suggested to provide a model for the enzymatic process in which the pyridoxal co-enzyme is bound to the enzyme surface through an azomethine link necessitating a "transimination" step prior to transamination.

Introduction

In the preceding papers[1] it has been established that imidazole catalyzes the transamination of pyridoxal by α-aminophenylacetic acid *via* a pathway involving pre-equilibrium complex formation of the intermediate imines with one molecule of imidazole and one ion of the conjugate acid of imidazole. The prototropic shift leading to the reversible interconversion of aldimine (S′) and ketimine (S″) has been postulated to occur *via* intracomplex general acid–general base catalysis (*i.e.*, in (1) $S_c' \rightleftarrows S_c''$).

In the enzymatic catalysis of the transamination of pyridoxal phosphate by amino acids, the pyridoxal phosphate is present on the enzyme surface in combination with the ε-amino group of a lysine residue as an imine.[3–5] The formation of the imine of substrate and enzyme-bound cofactor then occurs *via* a "transimination" reaction (2). It is generally known that imines are more reactive toward "carbonyl reagents" than are



the corresponding carbonyl compounds themselves.[6] The possibility thus arises that reaction 2 may facilitate

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