resonance interactions, etc.) in the case of the reactions to which the Hammett equation has been applied, practically all of which involve ionic species, hydroxylic solvents and/or rate processes.

The inapplicability of Hammett substituent constants to our disproportionation data may stem from the fact that the nature of the atom attached to the aromatic ring is being changed. McDaniel has pointed out that linear plots of $\sigma_m$ *vs.* $\sigma_p$, which would be expected if the Hammett equation were entirely general, are usually found when the atom attached to the aromatic ring is held constant, but not otherwise.[22]

We hope to learn more about the proper method of calculating the energy of polar interaction of substituents in subsequent work.

**Acknowledgment.**—We wish to acknowledge our indebtedness to the National Science Foundation for a grant in support of this work.

(22) D. H. McDaniel, *J. Org. Chem.*, **26**, 4692 (1961).

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

# Catalytic Reactions Involving Azomethines. I. The Imidazole Catalysis of the Transamination of Pyridoxal by α-Aminophenylacetic Acid[1a]

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

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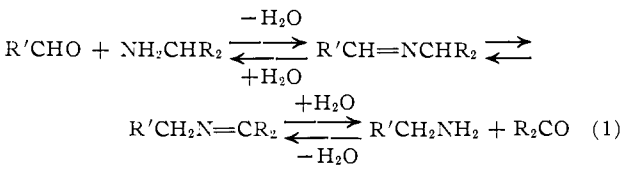
The transamination of pyridoxal by α-aminophenylacetic acid has been studied in aqueous medium at *p*H 8.6, 30° and $\mu$ = 0.05 *M*. The reaction occurred readily in the presence of imidazole buffer and in both product isolation (reactants at $5 \times 10^{-2}$ *M*) and spectrophotometric (reactants at $10^{-4}$ *M*) studies the catalyzed reactions could be followed up to 95% completion (approach to an equilibrium position) without divergence from apparent first-order kinetics. Spectrophotometrically the reaction was established to occur in two distinct phases. In the first phase equilibrium is established between the initial reactants and ketimine with aldimine at a low steady state (*i.e.*, pyridoxal + amino acid ⇄ aldimine ⇄ ketimine), while in the final phase equilibrium is established between all reactants, products and intermediates (*i.e.*, pyridoxal + amino acid ⇄ aldimine ⇄ ketimine ⇄ α-keto-acid + pyridoxamine). Employing imidazole as the buffer, the rate of completion of phase one is dependent on the square of the total imidazole concentration at low buffer concentration and independent of imidazole concentration at higher buffer concentration. The most logical mechanism involves the formation of a complex of the aldimine with two molecules of an imidazole species which then undergoes an intracomplex-catalyzed prototropic shift converting aldimine to ketimine. Support for the suggestion of a specific pre-equilibrium complex formation between imidazole catalyst and substrate is provided by the observation that certain bases (*e.g.*, morpholine and carbonate) are catalytically effective only when used at high concentrations. In experiments in which $Al^{+++}$ ions were added to the reaction mixture no effect on the rate of the imidazole-catalyzed reaction was noted.

## Introduction

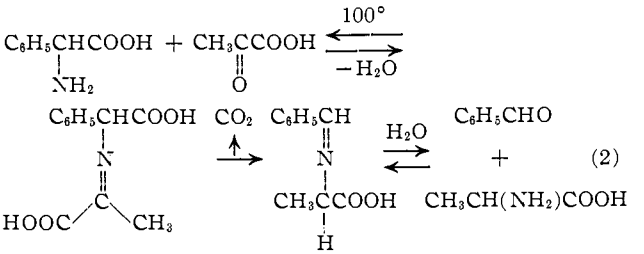
The mechanism for the catalysis of the interconversion of azomethines, as found in the transamination reaction 1, has long held the interest of organic chemists and biochemists alike.



$$R'CHO + NH_2CHR_2 \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} R'CH{=}NCHR_2 \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} R'CH_2N{=}CR_2 \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} R'CH_2NH_2 + R_2CO \quad (1)$$

This, the first paper in a series dealing with the catalysis of reactions proceeding through intermediate azomethines, pertains to the imidazole catalysis of the transamination of pyridoxal by α-aminophenylacetic acid.

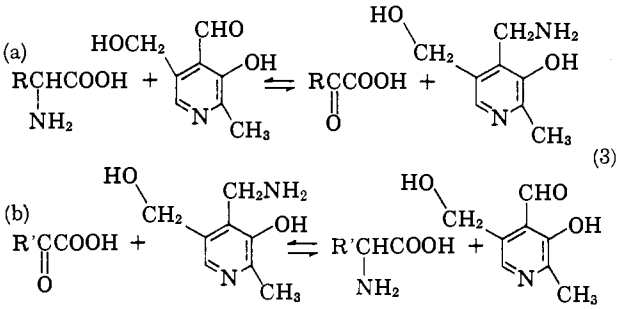
Interest in the biological transamination of amino acids led Herbst and Engell[2] to investigate the reaction of α-amino acids with α-keto acids. The latter studies were carried out in boiling aqueous solution, the products recovered indicating that the initially formed imine underwent decarboxylation.[3] Only in the case of glyoxylic acid reacting with α-amino acids could a transamination of type 1 be realized.[4] The glyoxylic acid reactions were carried out in water at 25° and the rate of product formation (followed chromatographically) was found to be increased by $OH^{\ominus}$, whereas in 2 an apparent *p*H optimum of about 3 was noted. The rate of the transamination of glyoxylic acid was found



$$C_6H_5CHCOOH + CH_3CCOOH \underset{-H_2O}{\overset{100°}{\rightleftharpoons}} C_6H_5CHCOOH\ CO_2\ C_6H_5CH \overset{H_2O}{\rightleftharpoons} C_6H_5CHO + CH_3CCOOH + CH_3CH(NH_2)COOH \quad (2)$$

to be strongly dependent on the nature of the amino acid.

Biochemical transamination reactions were shown by Braunstein[5] and Cohen[6] to be mediated by pyridoxal phosphate-requiring enzymes. Recently Snell[7] has established certain transaminases to require nonphosphorylated pyridoxamine as cofactor. The nonenzymatic transamination of pyridoxal (3a) by an α-



(a) $HOCH_2$, CHO, OH, $CH_3$, $RCHCOOH + \rightleftharpoons RCCOOH +$, HO, $CH_2$, $CH_2NH_2$, OH, $CH_3$

(b) HO, $CH_2$, $CH_2NH_2$, OH, $CH_3$, $R'CCOOH + \rightleftharpoons R'CHCOOH +$, HO, $CH_2$, CHO, OH, $CH_3$ (3)

amino acid was first shown by Snell in 1945.[8] In the experiments of Snell, which were followed by both

(1) (a) A portion of this study has appeared in preliminary form (see T. C. Bruice and R. M. Topping, *J. Am. Chem. Soc.*, **84**, 2448 (1962)). (b) Post-doctoral Fellow of the Department of Chemistry, Cornell University.

(2) R. M. Herbst and L. L. Engell, *J. Biol. Chem.*, **107**, 505 (1934); *J. Am. Chem. Soc.*, **58**, 2239 (1936).

(3) R. M. Herbst and D. Rittenberg, *J. Org. Chem.*, **8**, 380 (1943).

(4) H. I. Nakada and S. Weinhouse, *J. Biol. Chem.*, **204**, 831 (1953).

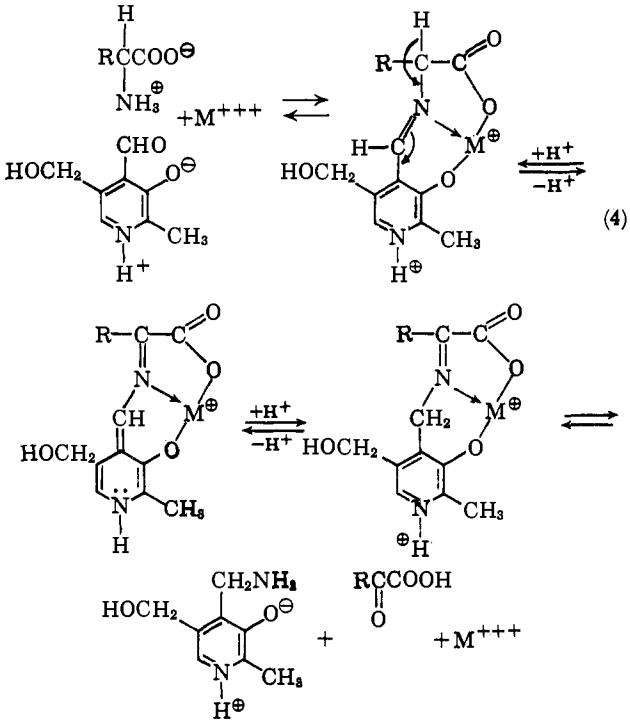
(5) A. E. Braunstein, *Enzymol.*, **7**, 25 (1934).

(6) P. P. Cohen, *Biochem. J.*, **33**, 1478 (1939).

(7) H. Wada and E. E. Snell, *J. Biol. Chem.*, **237**, 127, 133 (1962).

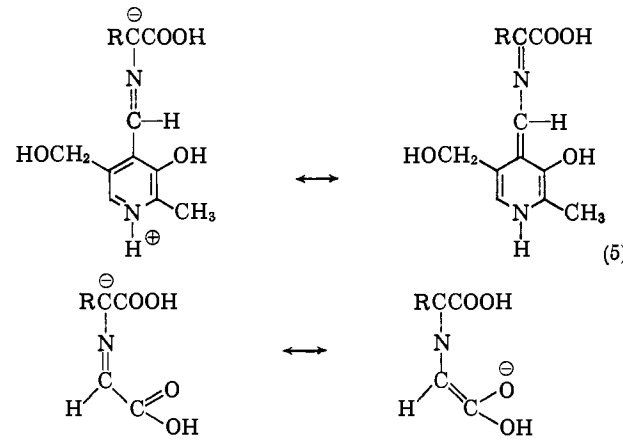
(8) E. E. Snell, *J. Am. Chem. Soc.*, **67**, 194 (1945).

chemical and biological assay procedures, autoclave temperatures and a large excess of pyridoxal were employed. The order of reactivity of various amino acids with pyridoxal was found to be that previously noted with glyoxylic acid and, as in the latter case, the transamination occurred without decarboxylation. On the basis that the addition of chelating agents, as citrate and EDTA, slowed down the reaction, the influence of metal ions on the transamination reactions of pyridoxal and amino acids was investigated.[9] These studies were generally carried out at 100° in water and the most effective metal ions for promoting the transamination of amino acids by pyridoxal were found to be $Cu^{++}$, $Fe^{+++}$ and $Al^{+++}$. The transamination of glyoxylic acid by α-amino acids (*p*H 4–10 at 100° in water) was also shown to be metal ion promoted.[10] For the pyridoxal case transamination was found to be reversible and facilitated in both directions by metal ions.[11] The rates of product appearance were found to be proportional to metal ion concentration only at low metal ion concentrations and the reactions exhibited *p*H optima. The mechanism of the transamination reactions involving pyridoxal and metal ion have been suggested by Snell and Metzler[12] to be




(4)

The metal ion, in forming a chelate with the imine, was suggested to facilitate the reaction by: (a) stabilization of the imine formed from pyridoxal and amino acid, (b) providing a planar conjugated system, and (c) increasing the inductive withdrawal of electrons away from the α-carbon and thereby increasing its acidity. The metal ion promotion of the transamination of pyridoxal by α-amino acids may or may not be of biochemical significance. It would appear as though a number of highly or partially purified pyridoxal phosphate-requiring enzymes do not require addition of metal ions for full activity.[13] In the interconversion of the α-amino acid imines of pyridoxal and glyoxylic acid, the driving force for the prototropic shift would, *a priori*, be the stabilization of the intermediate carbanion owing to a gain in resonance energy by the creation of a particularly favorable conjugated system (5). This supposition finds support in the L.C.A.O. approximations




(5)

in which the role of the metal is ignored.[14] The metal ion by chelating with the imine would provide an extra-stabilizing influence.

The important step in the transamination reaction is then the abstraction of the proton from the α-carbon of the imine.[15] In the enzymatic reaction the abstraction of the proton must be carried out by the weakly basic groups available to the protein at *p*H values near neutrality, the most effective of which would be the imidazolyl group of histidine.

In this paper we report a detailed kinetic analysis of the imidazole-catalyzed transamination of pyridoxal by α-aminophenylacetic acid in water at 30 ± 0.1° and at *p*H 8.61. In following papers this initial study will be further extended. The imidazole-catalyzed transamination of pyridoxal by α-aminophenylacetic acid has been found to be considerably more facile than reactions previously reported[2-4,8-12] and to be free of side reactions showing no change in kinetic order to as much as 80–95% completion of reaction. During the investigation leading to this paper, Banks, Diamantis and Vernon[16] reported the results of a study on the non-metal ion mediated reactions of pyridoxamine and pyruvic acid and pyridoxal and alanine. Owing to the complexities of their system and the slowness of their reactions they could only determine initial rates (maximum of 6% to completion) preventing the determination of order, etc.

## Experimental

**Apparatus.**—The majority of the spectrophotometric measurements were made with a Perkin–Elmer model 350 double beam spectrophotometer equipped with a thermostated cylindrical cell housing. Slower reactions, which provided only small absorbance changes, were followed using a single-beam Zeiss PMQII spectrophotometer, equipped with a hollow-brass cuvette holder which could be thermostated by circulation of constant temperature water. Constant temperature (30 ± 0.1°) was maintained by Haake circulating water-baths. Standard taper stoppered cuvette and cylindrical cells were used in all kinetic experiments. These were filled so as to leave no air space and thus alleviate the problem of air oxidation of pyridoxal. Measurements of *p*H were carried out with a model 22 Radiometer *p*H meter at constant temperature (30 ± 0.01°) employing a combined type GK Radiometer electrode.

**Chemicals.**—Imidazole was Eastman Kodak Co. White Label and was recrystallized prior to use and stored over $P_2O_5$ in a vacuum desiccator. Imidazole hydrochloride was prepared by

(9) D. E. Metzler and E. E. Snell, *J. Am. Chem. Soc.*, **74**, 979 (1952).
(10) D. E. Metzler, J. Olivard and E. E. Snell, *ibid.*, **76**, 644 (1954).
(11) J. B. Longenecker and E. E. Snell, *ibid.*, **79**, 142 (1957).
(12) D. E. Metzler, M. Ikawa and E. E. Snell, *ibid.*, **76**, 648 (1954).
(13) (a) W. T. Jenkins and I. W. Sizer, *ibid.*, **79**, 2655 (1957); *J. Biol. Chem.*, **234**, 51, 1179 (1959); **235**, 620 (1960); (b) Y. Matsuo and D. M. Greenberg, *ibid.*, **230**, 545, 561 (1958); (c) N. Alexander and D. M. Greenberg, *ibid.*, **220**, 775 (1956); (d) M. A. Karasek and D. M. Greenberg, *ibid.*, **227**, 191 (1957).
(14) A. M. Perault, B. Pullman and C. Valdemoro, *Biochem. et Biophys. Acta*, **46**, 555 (1961).
(15) D. E. Metzler, *J. Am. Chem. Soc.*, **79**, 485 (1957).
(16) B. E. C. Banks, A. A. Diamantis and C. A. Vernon, *J. Chem. Soc.*, 4235 (1961).

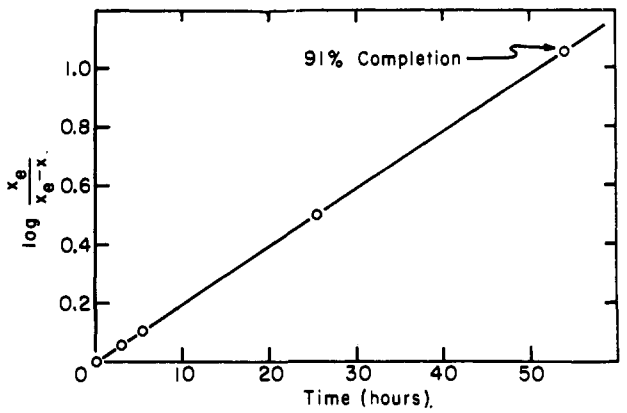



Fig. 1.—First-order plot for the formation of phenylglyoxylic acid (isolated as the 3-phenyl-2-oxyquinoxaline derivative) in the reaction of pyridoxal with α-aminophenylacetic acid in imidazole buffer (*p*H 8.61, 30°).

passing dry hydrogen chloride gas into an ethanolic solution of imidazole, followed by precipitation with ether. The product, after collecting and washing with dry ether, was desiccated over $P_2O_5$ *in vacuo*. The *dl*-α-aminophenylacetic acid, pyridoxal hydrochloride and pyridoxamine dihydrochloride were obtained from Mann Research Laboratories, Inc., and employed without recrystallization. Phenylglyoxylic acid was obtained from K and K Laboratories.

**Isolation of Phenylglyoxylic Acid (as the Derivative 3-Phenyl-2-oxyquinoxaline) from the Reaction of α-Aminophenylacetic Acid with Pyridoxal at *p*H 8.61.**—In 20 ml. of water there was dissolved 204.2 mg. (1.0 mmole) of pyridoxal hydrochloride, 3.4 g. (50 mmoles) imidazole and 105.2 mg. (1.0 mmole) imidazole hydrochloride. To the clear solution there was then added 151 mg. (1.0 mmole) of α-aminophenylacetic acid. The suspension was stirred magnetically until all amino acid had dissolved when the *p*H was adjusted to 8.6 by addition of a small volume of concentrated aqueous potassium hydroxide. Three-ml. aliquots of the resultant clear yellow solution were then distributed using a pipet into 5-ml. Erlenmeyer flasks which were tightly stoppered, sealed with Parafilm and covered with aluminum foil. At various time intervals flasks were opened and the reaction quenched by addition of 0.4 ml. of a 0.9 *M* *o*-phenylenediamine hydrochloride solution. A copious orange precipitate immediately formed. Aqueous hydrochloric acid (9 *N*) was then added with stirring until the orange precipitate redissolved. The reaction mixture was then allowed to stand for 48 hours. The resultant suspension of crystals was transferred to a centrifuge tube and the crystals spun down, the supernatant decanted and the crystals washed several times by centrifuging out of water. The crystalline derivative was then dissolved in a small aliquot of 95% ethanol and made up to a 25-ml. volume with additional 95% ethanol in a volumetric flask. An aliquot of this solution was suitably diluted with additional 95% ethanol. The concentration of the 3-phenyl-2-oxyquinoxaline derived from the phenylglyoxylic acid was then determined by comparing the absorbance at 370 mμ to a Beer's line prepared from an authentic sample of said derivative (Beer's law followed to at least $1.1 \times 10^{-4}$ *M*).

A plot of log $(a/a - x)$ *vs.* time was found to be linear when $a$ was taken as the concentration of the phenyloxyquinoxaline derivative formed at $t_\infty$ as determined from a Guggenheim[17] calculation and $x$ the determined concentration of the derivative at any time $t$. The formation of phenylglyoxylic acid could be shown by this procedure to follow first-order kinetics to 91% completion (Fig. 1). At completion, the reaction had gone to 43% conversion of α-aminophenylacetic acid to phenylglyoxylic acid (equilibrium position of the reaction).

In separate experiments, the imidazole buffer was replaced by carbonate buffer (0.3 *M*) and by morpholine buffer (0.1 *M*), all other conditions remaining identical. Owing to the insolubility of the α-aminophenylacetic acid in these media, no attempt was made to determine the kinetics of these reactions which were instead allowed to attain equilibrium (one week, at which time all amino acid had dissolved) before isolation of the phenylglyoxylic acid by the procedure outlined previously. In this way the reaction involving carbonate buffer was found to yield 34% of the keto-acid while the morpholine buffer gave 43%. When borate buffer was employed (0.4 *M*) no keto-acid was obtained.

3-Phenyl-2-oxyquinoxaline has previously been shown[18] to be the product of reaction of phenylglyoxylic acid and *o*-phenylenediamine. This substance was prepared from phenylglyoxylic acid and *o*-phenylenediamine[18] and established to be identical with the derivative isolated in the reaction of α-aminophenylacetic acid and pyridoxal (see above) by melting point (m.p. 250°, lit.[18] 247°), lack of mixture melting point depression and superimposition of infrared (Nujol) and ultraviolet (ethanol between 400 and 235 mμ) spectra.

**Spectra and extinction coefficients of initial and final products** were determined at the wave lengths employed in this study in order to calculate rate and equilibrium constants. The absorbance of all components were shown to follow Beer's law (*p*H 8.61, $\mu$ = 0.05 *M*, solvent water, $T$ = 30 ± 0.1°).

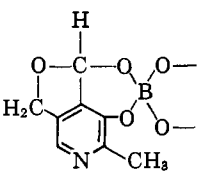
| | ε 395 mμ | ε 246 mμ |
|---|---|---|
| Pyridoxal | 1290 | 6,170 |
| Pyridoxamine | .. | 5,450 |
| Keto-acid | .. | 9,750 |
| Phenylglycine | .. | 200 |
| Ketimine | .. | 13,600 |

**Kinetics.**—All kinetic experiments reported in this paper were carried out at 30 ± 0.1° and at *p*H 8.61 ± 0.02 in de-aerated water at a calculated ionic strength of 0.05 *M* (with KCl). In the initiation of a kinetic run an aliquot of pyridoxal hydrochloride solution (prepared freshly before each kinetic experiment) was mixed with an aliquot of a stock solution of α-aminophenylacetic acid dissolved in the proper concentration of imidazole buffer. The buffer solutions were prepared by mixing solutions of imidazole and imidazole hydrochloride (at constant ionic strength (KCl)) and were invariably of *p*H 8.61 ± 0.02. The time of mixing was employed as zero time for the run. The reaction mixture was then transferred to a cuvette or cylindrical cell. The change of absorbance (read against buffer and KCl in the reference cell) at wave lengths between 235 and 340 mμ and the change in transmittance between 340 and 400 mμ were recorded at convenient time intervals. The initial base line of the recorder was set by scanning distilled water against distilled water and the setting checked and adjusted if necessary between runs.

## Results

In product isolation experiments at *p*H 8.61 using imidazole buffers a reaction occurs between pyridoxal and α-aminophenylacetic acid to yield phenylglyoxylic acid (see Experimental). The kinetics, determined by the isolation of phenylglyoxylic acid as the 3-phenyl-2-oxyquinoxaline derivative, were found to be first order in appearance of phenylglyoxylic acid (Fig. 1). Furthermore, the reaction was found, under the conditions employed, to go to only 43% completion at $t_\infty$. These results establish imidazole and/or its conjugate acid to be catalysts for the transamination of pyridoxal by α-aminophenylacetic acid (*i.e.*, reaction 1 catalyzed in both forward and reverse directions).

Similar runs in which the imidazole buffer is replaced by carbonate or morpholine buffers (all other conditions remaining unchanged), if allowed to stand for a sufficient period, also yield phenylglyoxylic acid (34% and 43% over-all yields, respectively). Thus, imidazole is not unique as a catalyst in this system and other bases will also catalyze the transamination reaction. An extended study of the susceptibility of this transamination reaction to general acid and general base catalysis is being pursued. The catalysis of the reaction by imidazole in the presence of morpholine buffers forms the subject matter for part III of this study. The use of borate buffer, under the conditions of both the isolation experiments and the spectrophotometrically investigated runs, was found to provide no catalysis for the transamination. On the grounds that the catalytic inactivity of borate buffer might arise as a result of the possible formation of a complex with the inactive internal hemiacetal form of pyridoxal (6), the effect of borate buffer on the imidazole-catalyzed reac-



(6)

(17) E. A. Guggenheim, *Phil. Mag.*, **2**, 538 (1926).

(18) J. Buraczewski and L. Marchlewski, *Ber.*, **34**, 4009 (1901).

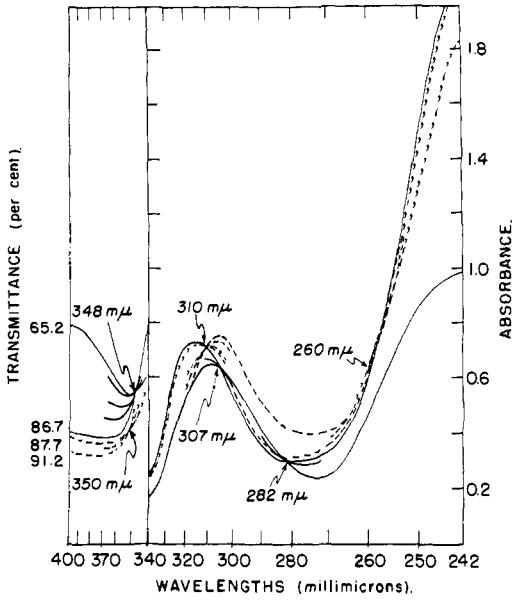



Fig. 2.—Spectral-time study for the reaction of pyridoxal ($10^{-4}$ $M$) with α-aminophenylacetic acid ($2 \times 10^{-4}$ $M$) in the presence of imidazole (1.8 $M$) in water at $p$H 8.61 and a temperature of 30°. The tracing shows the shape of the absorbance curves and isosbestic points for the first (———) and second (·· – – –) phases of the reaction.

tion was investigated. For these experiments the spectrophotometric procedure described below was employed (*i.e.*, $PCHO_0 = A_0 = 10^{-4}$ $M$, $p$H 8.6, solvent $H_2O$, $T = 30°$, $\mu = 1.0$ $M$; 0.1 $M$ borate). The catalytic rate constant for imidazole was insignificantly influenced by addition of borate (*i.e.*, $k_0 = 8.79 \times 10^{-3}$ min.$^{-1}$ *vs.* $7.23 \times 10^{-3}$ min.$^{-1}$ for approach to equilibrium of phase one as defined below) as was the position of equilibrium for the reaction. Thus, it would appear as though borate (at least to 0.1 $M$) is simply an inert reagent toward the transamination reaction.

The unique feature of the imidazole catalysts is obtained from studies carried out at low initial reactant concentrations (PCHO = A = $10^{-4}$ $M$). At these concentrations the catalysis by imidazole buffers is easily discernible while no catalysis by carbonate or morpholine can be detected even after several days. This may be attributed to the ability of imidazole buffer to form a strong complex with certain reacting species.

The remainder of this study describes a detailed kinetic investigation of the imidazole-catalyzed transamination reaction in aqueous solution under restricted conditions of acidity ($p$H 8.61 ± 0.02), temperature (30 ± 0.1°) and ionic strength (calcd. $\mu = 0.05$ $M$ with KCl). In the initial experiments to be described, pyridoxal and α-aminophenylacetic acid were at equal initial concentrations of $1 \times 10^{-4}$ $M$. The results of a typical spectral-time study are presented in Fig. 2. Inspection of Fig. 2 reveals that the over-all reaction may be divided conveniently into two phases. In the initial phase the reactants are converted into a product (S″) without the accumulation of an intermediate (intermediates would have to be present at a low steady state concentration) (7). This conclusion is reached from the observation of true isosbestic points at 348, 307 and 282 mμ. Furthermore, a linear relationship exists between the decrease in absorbance at 395 mμ and the increase in absorbance at 246 mμ (Fig. 3). The decrease in absorbance at 395 mμ may be related to the disappearance of pyridoxal or conceivably to the disappearance of the imine of pyridoxal and α-amino-

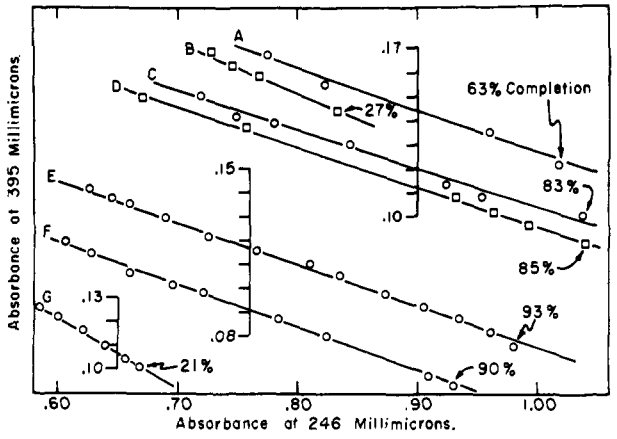



Fig. 3.—Linear relationship between the decrease in absorbance at 395 mμ and increase in absorbance at 246 mμ for phase one in the reaction of pyridoxal with α-aminophenylacetic acid.

phenylacetic acid (S′) while the increase in absorbance at 246 mμ may be related to the formation of the imine of pyridoxamine and phenylglyoxylic acid (S″) and/or phenylglyoxylic acid (PG) plus pyridoxamine ($PCH_2NH_2$) (7). That the product whose absorbance is increasing at 246 mμ is not a mixture of $PCH_2NH_2$ and PG is shown by the fact that there are no common isosbestic points between the combined spectra of $PCH_2NH_2$ + PG and the combined spectra of PCHO + A (Fig. 4). Inspection of Fig. 4 shows that PG does show absorbance at 246 mμ. It would then be expected that S″ would also exhibit absorbance at 246 mμ and that the product of the first phase is S″ (Fig. 5). The rate of disappearance of PCHO measured at 395 mμ is necessarily identical with the rate of production of S″ as measured at 246 mμ (see below). The reaction of amino acids with PCHO in water to form aldimines has been studied by Metzler[19] and found to be a rapid process. The equilibrium constant ($K_1$) for aldimine formation (8) has been determined[19] to be in the range of 1 to 100 for a number of α-amino acids. Therefore, one would anticipate that the concentration of S′

$$K_1 = S'/PCHO{\cdot}A;\ K_2 = S''/S';\ K_1K_2 = S''/PCHO{\cdot}A \quad (8)$$

(where S′ denotes total aldimine, both free and as any complex with the catalyst) would be $10^{-6}$ $M$ to $10^{-8}$ $M$, in our experiments in which PCHO = A = $10^{-4}$ $M$ at $t_0$. These results are all in agreement with our supposition that the first phase of the reaction, which is characterized by three isosbestic points at 282, 307 and 348 mμ, can be related to the conversion of PCHO + A through S′ to S″ with S′ at a low steady state concentration.

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

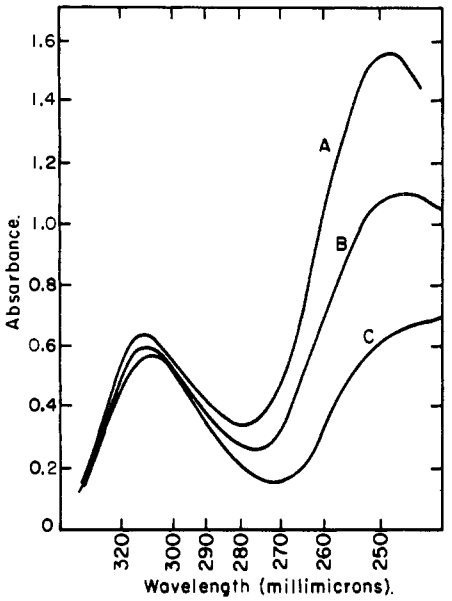



Fig. 4.—Combined spectra of: A, phenylglyoxylic acid and pyridoxamine both at $10^{-4}$ $M$; B, phenylglyoxylic acid, pyridoxamine, pyridoxal and $\alpha$-aminophenylacetic acid all at $0.5 \times 10^{-4}$ $M$; C, pyridoxal and $\alpha$-aminophenylacetic acid both at $10^{-4}$ $M$. Curves A, B and C show that there would be no isosbestic points for the direct conversion of pyridoxal plus $\alpha$-aminophenylacetic acid to phenylglyoxylic acid plus pyridoxamine. Spectra taken at $p$H 8.61, 30°, in aqueous solution, 1.8 $M$ in imidazole buffer.

The identification of the ultraviolet spectrum of the reacting system at any time with the reactants, intermediates and products producing that spectrum was unequivocally established by following the rate of attainment of equilibrium of the transamination reaction using both the spectrophotometric analytical procedure and the product isolation procedure and showing that these independent methods provide the same value for $k_{obs}$. Thus from a common reaction mixture aliquots were periodically withdrawn and divided into two parts, a and b. Part a was analyzed spectrophotometrically (after suitable dilution first in water and then in the appropriate buffer) for species on both sides of the rate-determining prototropic shift (*i.e.*, reactants at 395 m$\mu$ and products at 246 m$\mu$). Part b was treated with an excess of *o*-phenylenediamine allowing the determination of total ketimine and keto-acid through formation of the 3-phenyl-2-oxyquinoxaline derivative and thus providing an independent method for measuring the species at 246 m$\mu$. In this way it was shown that the same kinetic constants resulted whether product appearance was followed by quantitative isolation, by estimation at 246 m$\mu$ or by determining the disappearance of reactants at 395 m$\mu$.

In the second phase of the reaction absorbance at 395 m$\mu$ continues to decrease. This clearly then represents a reaction in which PCHO is still consumed. Isosbestic points are no longer encountered at 282, 307 and 348 m$\mu$, where absorbance continually increases, but are now present at 260, 310 and 350 m$\mu$ (Fig. 2). This second phase, a slower reaction, represents conversion of S'' to PG and $PCH_2NH_2$. This reaction does not go to completion as can be shown by the fact that the spectra at $t_\infty$ do not superimpose upon the combined spectra of $10^{-4}$ $M$ PG and $10^{-4}$ $M$ $PCH_2NH_2$ (Fig. 4). Also, at $t_\infty$ considerable PCHO remains, as can be seen by the residual absorbance at 395 m$\mu$. The second phase of the reaction then represents the attainment of equilibrium between all components [*i.e.*, PCHO + A $\rightleftarrows$ S' (steady state) $\rightleftarrows$ S'' $\rightleftarrows$ PG + $PCH_2NH_2$].

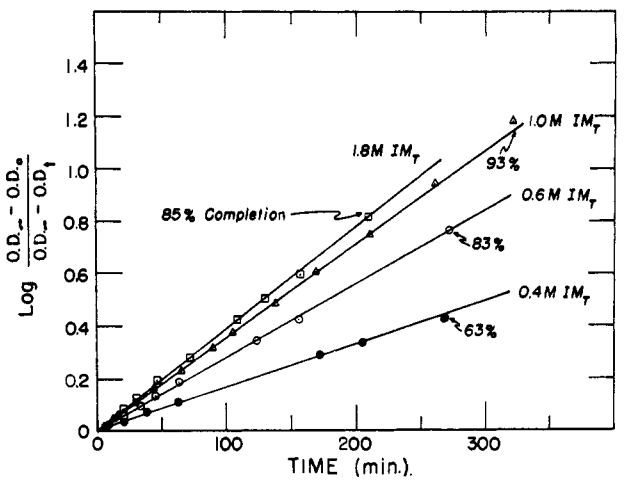



Fig. 5.—First-order plots for the appearance of S'' (as determined at 246 m$\mu$) in the presence of varying concentrations of imidazole (pyridoxal and $\alpha$-aminophenylacetic acid initially at $10^{-4}$ $M$, $p$H 8.61, $\mu$ = 0.05 $M$, 30°).

In the first phase of the reaction, the appearance of S'' as measured at 246 m$\mu$ and the disappearance of PCHO as measured at 395 m$\mu$ have been found to follow apparent first-order kinetics at all concentrations of PCHO and A investigated. Examples of the first-order plots for the appearance of S'' are provided in Fig. 5. Inspection of Fig. 5 reveals the reaction to be first order (often to 85–95% of attainment of equilibrium) and inspection of Fig. 3 reveals that the same rate law pertains to the disappearance of PCHO. In practice, the value of the experimentally obtained first-order rate constant ($k_{obs}$) was initially obtained by the method of Guggenheim.[17] From the value of $k_{obs}$ so determined the absorbance at 246 m$\mu$ at $t_\infty$ could be calculated and was found to be that obtained at the end of the first phase of reaction (within 3.0%). Employing the value of the absorbance at $t_\infty$ so calculated, the value of $k_{obs}$ was then recalculated employing the usual integrated expression of the first-order rate law (Fig. 5). The values obtained by the Guggenheim and log $(xe/xe - x)$ plots were found to agree within a few per cent. Thus, the rate of attainment of the equilibrium $K_1K_2$ (7) is strictly first order.

The use of absolute alcohol instead of water as the reaction medium renders the separate identification of the two phases of the reaction difficult at low imidazole buffer concentrations (*e.g.*, IM = 0.25 $M$, $IMH^\oplus$ = 0.005 $M$) owing to the absence of definitive isosbestic points. This appears to result from a greater rate of cleavage of ketimine in the alcohol system than in the water system. Only at high imidazole buffer concentrations (*e.g.*, IM = 1.25 $M$, $IMH^\oplus$ = 0.025 $M$) are isosbestic points again discernible as in the water systems, indicating that a high catalyst concentration has greatly enhanced the rate of formation of ketimine with relatively little effect on its cleavage. As in the water system the reaction, as measured by the increasing absorbance at 246 m$\mu$, adheres strictly to first-order kinetics. Examination of the spectrum from 400 to 235 m$\mu$ of the above system after attainment of equilibrium reveals a similar distribution of reactants and products to that found in the water system. Thus, a residual absorption at *ca.* 395 m$\mu$ indicates unchanged PCHO in agreement with the less than 100% conversion to PG and $PCH_2NH_2$ revealed by the absorption at 246 m$\mu$. In contrast, however, the reaction may be driven to 100% completion by replacing the imidazole buffer by imidazole free base. Thus, when a mixture of PCHO·HCl ($10^{-4}$ $M$), A ($10^{-4}$ $M$) and imidazole (0.5 $M$) in

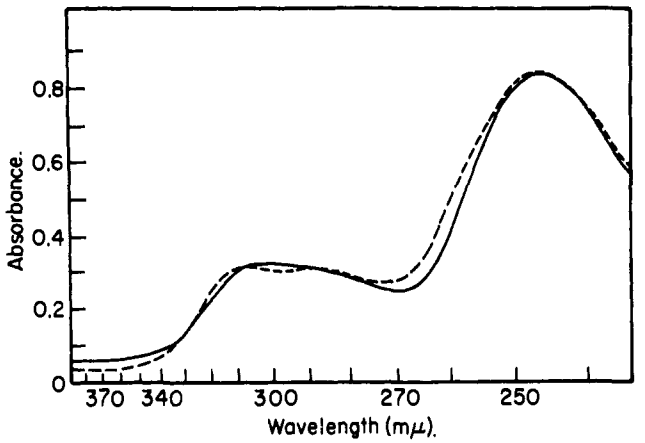



Fig. 6.—A comparison of the spectra of (a) a mixture of pyridoxal ($10^{-4}$ $M$), $\alpha$-aminophenylacetic acid ($10^{-4}$ $M$), and imidazole (0.5 $M$) in absolute alcohol at equilibrium (diluted to 50% aq. alcohol) (full line); and (b) a mixture of phenylglyoxylic acid ($10^{-4}$ $M$), pyridoxamine ($10^{-4}$ $M$) and imidazole (0.5 $M$) in absolute alcohol (diluted to 50% aqueous alcohol) (broken line).

absolute alcohol is allowed to attain equilibrium (5 days), the ultraviolet spectrum of a sample (diluted to 50% aqueous alcohol) revealed *no* absorption in the 395 m$\mu$ region indicating complete removal of PCHO from the system (full line, Fig. 6). Furthermore, the spectrum was found to closely conform to that of a mixture of PG ($10^{-4}$ $M$), $PCH_2NH_2$ ($10^{-4}$ $M$) and imidazole (0.5 $M$) in absolute alcohol after similar dilution to 50% aqueous alcohol (broken line, Fig. 6). These results suggest 100% conversion of reactants to products. This result provides striking confirmation of the stoichiometry of the system under investigation and provides further support for the proposal that interference from side reactions under the conditions used in the study is negligible.

Returning now to the experiments in aqueous solution, the values of $k_{obs}$ for phase one are sensitive to the concentration of imidazole buffer. In Fig. 7 are plotted the determined values of $k_{obs}$ *vs.* the square of the total concentration of imidazole ($IM_T$) as buffer. The points of Fig. 7 are experimental while the curve has been drawn from the theoretical equation 9

$$k_{obs} = (0.95 \times 10^{-2}(IM_T)^2)/(0.20 + (IM_T)^2) \quad (9)$$

From 9 it can be seen that the experimentally determined rate of approach to equilibrium in phase one proceeds from a dependence on the square of $IM_T$ to independence in $IM_T$ concentration. The form of eq. 9 is of the Michaelis–Menten type and represents saturation of an intermediate with two molecules of an imidazole species or alternatively like saturation of a rate-determining step.

Employing the knowledge that the attainment of equilibrium in S'' is first order, we may now calculate the numerical values for equilibrium constants (8). The low steady state in S' prevents its spectroscopic measurement. Although this condition is ideal for the application of the steady state approximations, as will be seen later, it prevents the separate determination of the constants $K_1$ and $K_2$. The product $K_1K_2$, however, may be evaluated as follows. From the Guggenheim method for determining the *pseudo*-first-order rate of the approach to equilibrium in S'' we may determine the optical density corresponding to the maximum value of S'' *prior* to its cleavage to PG + $PCH_2NH_2$. By suitable extrapolation of this value on the linear plot of $O.D._{395}$ *vs.* $O.D._{246}$ (Fig. 3), the corresponding decrease in absorbance at 395 m$\mu$ is found. As the maximum absorbance at 395 m$\mu$ is an index of the known initial concentration of PCHO (after a small correction for the

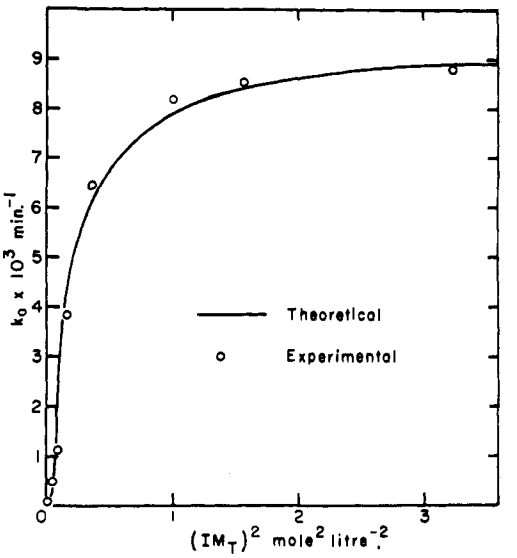



Fig. 7.—Plot of the observed first-order rate constants ($k_{obs}$) for the appearance of S'' *vs.* the square of the concentration of total imidazole. The points are experimental and the curve is that obtained from eq. 9.

absorption of other species at this wave length), it is possible to compute the PCHO remaining after establishment of the equilibrium $K_1K_2$. The essential conditions of Beer's law are followed by PCHO at 395 m$\mu$. Since stoichiometrically equivalent amounts of PCHO and A are consumed, the concentration of the latter at equilibrium is also obtained. Subtraction of the determined concentration of PCHO at equilibrium from the known initial concentration provides the equilibrium molar concentration of S'' before its cleavage to PG + $PCH_2NH_2$. Also obtained is the extinction coefficient of S'' at 246 m$\mu$ ($\epsilon$ = 13,600, *p*H 8.61). The values of PCHO, A and S'' now known, the value of the product $K_1K_2$ is obtained (8). Having determined $K_1K_2$, it is possible to trace the course of the second phase. The second phase is sufficiently slow that it is permissible to assume that the first phase maintains equilibrium. Thus, at any time subsequent to departure from the isosbestic points, signifying establishment of equilibrium in the first phase, it is possible to determine the absorbance at 395 m$\mu$ and hence evaluate the concentration of PCHO and A in the system at that time. The use of 8 then permits the determination of the concentration of S'' and from a material balance PG and $PCH_2NH_2$. The relative slowness of phase two, however, prevents the precise determination of final equilibrium owing to complicating side reactions. The validity of the above method of calculation (for phase 2) as a semiquantitative tool and justification for the premises on which it is based is provided by the close agreement between the calculated and experimental values for the distribution of products at any given stage of phase 2. Two examples, the first at low and the second at high buffer concentration follow. In run Sp-53 (Table I) the product distribution after 19 hours was estimated to be: PCHO ($0.39 \times 10^{-4}$ $M$), A ($0.39 \times 10^{-4}$ $M$), S'' ($0.32 \times 10^{-4}$ $M$), $PCH_2NH_2$ ($0.29 \times 10^{-4}$ $M$) and PG ($0.29 \times 10^{-4}$ $M$). Knowing the extinction coefficients of each component at 246 m$\mu$ a value of 1.126 for the absorbance of a mixture of the above composition at 246 m$\mu$ was obtained, in good agreement with the experimental value of 1.155. In a run in which the initial concentrations were $IM_T$ = 2.5 $M$, PCHO = A = $10^{-4}$ $M$, the product distribution after 2 days resulted in a calculated value of $O.D._{246\ m\mu}$ of 1.121 and an experimental value of 1.170.

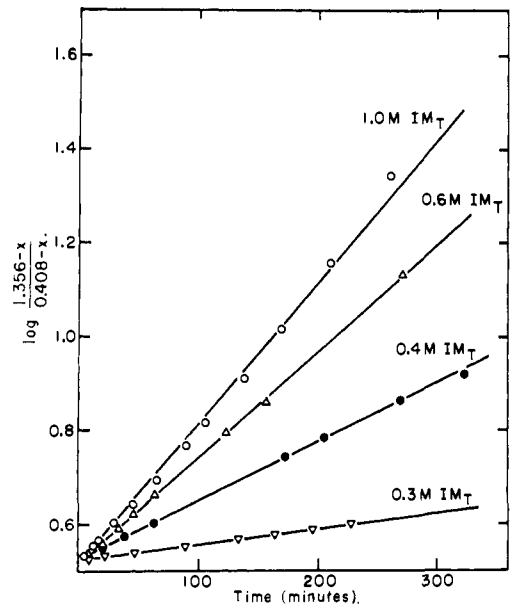



Fig. 8.—Plots of the experimentally determined rates of formation of S'' (determined at 246 mμ) employing the derived theoretical rate expression for approach to equilibrium in phase one (15).

The expected side reaction between PCHO and the $PCH_2NH_2$ produced in phase 2 is only of possible significance in the slowest reactions studied (below 0.3 *M* imidazole buffer concentration). This conclusion is based upon a separate determination of the rate constant for the reaction PCHO + $PCH_2NH_2$ → PCH = N—$CH_2$P. Thus at initial concentrations of reactants of $10^{-3}$ *M* (*i.e.*, ten times greater than the initial PCHO concentration in the kinetic runs and many hundred times the concentration of $PCH_2NH_2$ before completion of phase one) in the presence of 1.0 *M* imidazole buffer (*p*H 8.6, $T$ = 30°, $\mu$ = 0.05 *M*), the observed pseudo-first-order rate constant for approach to equilibrium was only $1.78 \times 10^{-3}$ min.$^{-1}$.

TABLE I

THE DETERMINED RATE CONSTANTS FOR THE IMIDAZOLE-CATALYZED TRANSAMINATION OF PYRIDOXAL ($10^{-4}$ *M*) BY α-AMINOPHENYLACETIC ACID ($10^{-4}$ *M*) AT *p*H 8.6, $T$ = 30° $\mu$ = 0.05 *M*

| Run | $IM_T$, *M* | $k_{obs} \times 10^3$, min.$^{-1}$ | $k_{obs} \times 1.15/c$, l. mole$^{-1}$ min.$^{-1}$ | $k_2K_1$ |
|---|---|---|---|---|
| 60 | 0.000[a] | Negl. | ... | ... |
| 4 | .102 | 0.05 | 0.9 | 0.7 |
| 3 | .204 | 0.49 | 8.9 | 6.2 |
| 55 | .306 | 1.12 | 20.2 | 14.1 |
| 54 | .408 | 3.84 | 69.1 | 51.8 |
| 53 | .612 | 6.47 | 116.3 | 94.7 |
| 1 | 1.02 | 8.20 | 147.6 | 131.3 |
| 2 | 1.275 | 8.54 | 153.6 | 136.3 |
| 56 | 1.836 | 8.79 | 158.2 | 186.3 |

[a] *p*H 8.6 maintained by 0.4 *M* borate buffer.

The question arises as to whether the approach to the equilibrium conditions given by $K_1K_2$ in phase one should follow first-order kinetics.

$$\text{PCHO} + \text{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{S}' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{S}'' \qquad (10)$$

At $t_0$, PCHO = $a$, A = $b$ and at any time ($t$), S'' = $x$ and PCHO = $(a - x)$, A = $(b - x)$, since S' is at a low steady state. Then

$$dS''/dt = dx/dt = k_2S' - k_{-2}S'' \qquad (11)$$

Since we suppose a rapid establishment of steady state in aldimine

$$S' = K_1 \cdot \text{PCHO} \cdot \text{A} \qquad (12)$$

$$dx/dt = k_2K_1 \cdot \text{PCHO} \cdot \text{A} - k_{-2}S'' \qquad (13)$$

and from 8

$$dx/dt = k_2K_1\ \text{PCHO} \cdot \text{A} - (k_2S'')/K_2 \qquad (14)$$
$$= k_2K_1\ [(a - x)(b - x) - (x/K_1K_2)]$$

Integration of 14 provides 15

$$k_2K_1t = \frac{1}{2c} \ln\left(\frac{c - \mu}{c + \mu}\right) + B \qquad (15)$$

where

$$c = \left[\frac{a^2 + b^2 + 2\left(\frac{[a + b]}{K_1K_2} - ab\right) + \frac{1}{(K_1K_2)^2}}{4}\right]^{1/2} \qquad (16)$$

$$\mu = x - \left[\frac{a + b + 1/(K_1K_2)}{2}\right]$$

$$B = \frac{-1}{2c} \ln\left(\frac{c - \mu + x}{c + \mu - x}\right)$$

In 16, $(c - \mu)$ changes by only ~20% while $(c + \mu)$ goes to zero at $t_\infty$ so that the term ln $(c - \mu)/(c + \mu)$ approaches very closely the general form ln $(ya - x)/(a - x)$ where $ya >> x$ resulting in a close approximation to first-order kinetics. The observed first-order rate constant for attainment of equilibrium is then

$$k_{obs} \cong k_2K_1 \times \frac{2c}{2.303} = \frac{1}{t} \log\left(\frac{c - \mu}{c + \mu}\right) + B \qquad (17)$$

and the second-order rate constant ($k_{II}$) for the reaction of PCHO and A to equilibrium in S'' is

$$k_{II} \cong k_2K_1 = \frac{1.15}{ct} \log\left(\frac{c - \mu}{c + \mu}\right) + B \qquad (18)$$

The value of $k_{II}$ is then obtained from plots of log $[(c - \mu)/(c + \mu)]$ *vs.* $t$ (Fig. 8). Inspection of Fig. 8 reveals that the derived equation is as satisfactory in correlating the experimental data as is the conventional first-order rate equation (Fig. 5). It should be noted (Table I) that $1.15k_{obs}/c$ and $k_2K_1$ are quite comparable. For the evaluation of $c$ and $\mu$ the product $K_1K_2$ had to be known with certainty. The calculated values of $K_1K_2$ from runs in which the initial concentration of PCHO and A were equal were found to be independent of the total imidazole concentration. However, when the initial concentrations of PCHO and A are other than equal (*e.g.*, 0.5:1, 2:1, 3:1, etc.) then the reactant at lower concentration may be all but consumed at equilibrium, rendering the computed value of $K_1K_2$ unduly sensitive to the experimental error in assessing equilibrium concentrations. As a result the value of $K_1K_2$ used in eq. 15 to 18 to evaluate $k_{II}$ was an average, calculated from runs in which the initial concentrations of reactants were equal.

An interesting confirmation of eq. 15–18 is provided by a study of the influence of the variation of initial concentration of reactants upon the observed first-order rate constant. It may be seen from Table II that at a constant concentration of $IM_T$ of 1.8 *M*, the value of $k_{obs}$ increases by only 40% on doubling the initial concentration of either PCHO or A, while halving the initial concentration of A has little effect on the rate constant. This influence of initial concentration on $k_{obs}$ is predicted by eq. 15. Upon converting the first-order constants to the corresponding second-order constants by dividing by the appropriate value of $0.87c$ (see 18), the same second-order constant of 156.8 ± 5.1 l. mole$^{-1}$ min.$^{-1}$ is obtained. For these experiments a constant imidazole-buffer concentration of 1.8 *M* was chosen owing to its favorable position well along the plateau of the Michaelis–Menten curve (Fig. 7) allowing initial reactant concentrations to be varied without affecting any critical catalyst/substrate ratio (*i.e.*, $(IM_T)^2/S'$).

TABLE II

THE INFLUENCE OF VARIATION OF REACTANT CONCENTRATION UPON THE DETERMINED RATE CONSTANTS FOR THE IMIDAZOLE-CATALYZED TRANSAMINATION OF PYRIDOXAL BY α-AMINOPHENYLACETIC ACID

($IM_T$ = 1.8 $M$, $p$H 8.6, $T$ = 30°, $\mu$ = 0.05 $M$)

| Run | Pyridoxal, $M \times 10^4$ | α-Aminophenylacetic acid, $M \times 10^4$ | $k_{obs} \times 10^2$, min.$^{-1}$ | $c$, $M \times 10^4$ | $k_{obs} \times 1.15/c$, l. mole$^{-1}$ min.$^{-1}$ |
|---|---|---|---|---|---|
| 56 | 1 | 1 | 8.79 | 0.64 | 158.2 |
| 59 | 2 | 1 | 12.09 | .92 | 151.3 |
| 57 | 1 | 2 | 12.17 | .92 | 152.2 |
| 58 | 1 | 0.5 | 8.92 | .62 | 165.6 |

## Discussion

The reaction of pyridoxal (PCHO) with α-aminophenylacetic acid (A) at 30° and $p$H 8.6 in water at a constant ionic strength of 0.05$M$ has been established to be catalyzed by imidazole buffer ($IM_T$). This has been demonstrated both by spectrophotometric analysis of the reacting system and also by the isolation and identification of phenylglyoxylic acid (PG), the product of transamination of the amino acid. The reaction proceeds in two distinct phases (7); the first phase provides an equilibrium mixture of pyridoxal (PCHO), amino acid (A), aldimine (S′) (at a low steady state concentration) and ketimine (S″), while the second phase involves cleavage of S″ to provide a final equilibrium mixture of A, PCHO, S′, S″, PG and pyridoxamine ($PCH_2NH_2$). A plot of the decreasing concentration of PCHO (395 mμ) *vs.* the increasing concentration of S″ (246 mμ) for runs at various imidazole buffer concentrations results in a series of lines (see Fig. 3) which are parallel above 0.3 $M$ $IM_T$ but which show increasing slope below 0.3 $M$ $IM_T$. The linearity of these plots indicates that the assumption of a low steady state in S′ is justified as was anticipated from the close conformity to the isosbestic points. The parallelism of the lines indicates that for rates greater than those catalyzed by 0.3 $M$ imidazole buffer, interference from side reactions during the first phase is negligible, and only below this buffer concentration do side reactions cause a greater consumption of PCHO than is reflected by an increase in S″. One of the principal sources of interference is possibly the condensation reaction between the $PCH_2NH_2$ which results from cleavage of the S″ and the unreacted PCHO and this process has been shown to be negligible above 0.3 $M$ imidazole buffer concentration (see Results section).

The absence of any metal ion catalysis on the system under the conditions of our experiments (*e.g.*, PCHO = A = $10^{-4}$ $M$, solvent $H_2O$, $p$H 8.6, $T$ = 30°, $\mu$ = 1.0 $M$, $IM_T$ = 1.8 $M$) was shown by running parallel experiments which differed only in the content of $Al^{+++}$ ion. Thus addition of $Al^{+++}$ ions ($10^{-3}$ $M$) as $AlNH_4(SO_4)_2 \cdot 12H_2O$ to the above system resulted in no appreciable change in either the equilibrium position of phase one or the rate of approach to equilibrium.

The first phase of the reaction, which results in a 54% conversion to S″ at equilibrium, has been established to follow the kinetic expressions of 9 and 17 which provide an expression 19 for the second-order rate constant ($k_{II}$), in terms of the concentration of imidazole buffer ($IM_T = IM + IMH^{\oplus}$).

$$k_{II} = k_2K_1 = \frac{1.09 \times 10^{-2}(IM_T)^2}{[0.20 + (IM_T)^2]c} \qquad (19)$$

in which $c$, as previously defined, is a function of the concentration of initial reactants and the product of the equilibrium constants for formation of S′ and S″ ($K_1K_2$). Under the simplest conditions of equal initial reactant concentrations ($a = b = 10^{-4}$ $M$), eq. 19 reduces to eq. 20.

$$k_{II} = k_2K_1 = \frac{2.18 \times 10^{-2}K_1K_2(IM_T)^2}{[0.20 + (IM_T)^2][4 \times 10^{-4}K_1K_2 + 1]^{1/2}} \qquad (20)$$

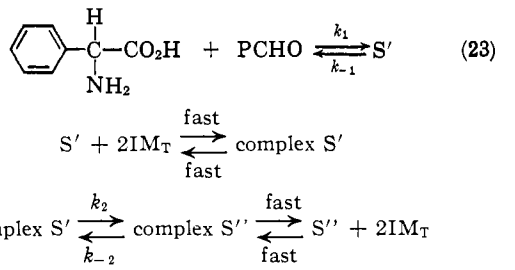
At low concentrations of imidazole buffer the second-order rate of attainment of equilibrium in phase 1 is second order in total imidazole concentration (21), while at high concentrations of imidazole buffer the

$$k_{II} = \frac{10.90 \times 10^{-2}K_1K_2(IM_T)^2}{[4 \times 10^{-4}K_1K_2 + 1]^{1/2}} \qquad (21)$$

rate becomes independent of imidazole concentration (22).

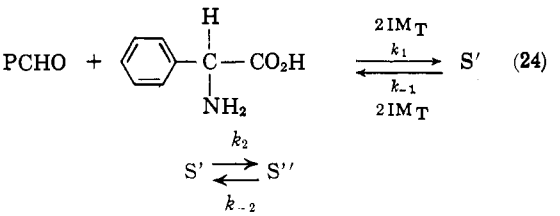
$$k_{II} = \frac{2.18 \times 10^{-2}K_1K_2}{[4 \times 10^{-4}K_1K_2 + 1]^{1/2}} \qquad (22)$$

Three mechanisms may be invoked to explain the Michaelis–Menten type kinetics, but only the first, as will be shown, is tenable. The first mechanism requires saturation of S′ by two molecules of an imidazole species followed by a rate-determining intracomplex general base and/or general acid conversion of S′ to S″ as depicted in 23



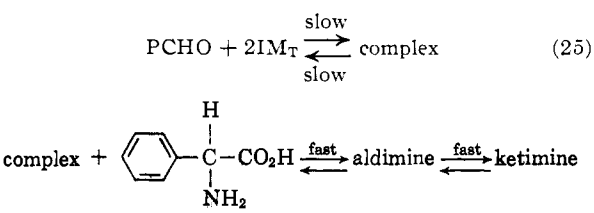
(23)

The second mechanism (24) involves the catalysis of a rate-determining formation of S′ by two imidazole species. At high imidazole buffer concentrations this step would no longer be rate-controlling



(24)

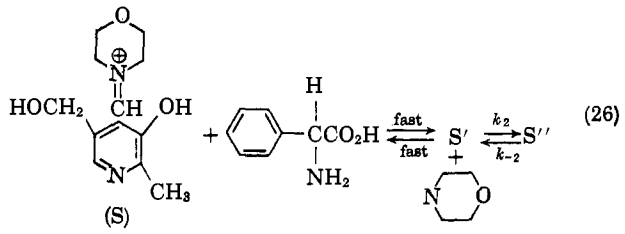
The third mechanism (25) involves a rate-controlling complex formation between PCHO and two imidazole species which at high imidazole buffer concentrations becomes zero order in imidazole as a result of saturation of the PCHO



(25)

Thus, imidazole buffer is acting as a catalyst for the prototropic shift in 23 and for the formation of Schiff base in 24 and 25. Although these mechanisms explain the observed rate dependence on imidazole buffer concentration (9) equally well, compelling evidence points to mechanism 23 as being the correct one. Thus, if mechanism 25 were operative then the observed overall rate should be independent of amino acid concentration. The observed rate, however, is found to show exactly the same dependence upon amino acid concentration as it does upon PCHO concentration. Furthermore, all previous work on this reaction[15] suggests that

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




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.

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(20) Details of this study are reported separately in part III, *J. Am. Chem. Soc.*, **85**, 1493 (1963).
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# Catalytic Reactions Involving Azomethines. II. The pH Dependence of the Imidazole Catalysis of the Transamination of Pyridoxal by α-Aminophenylacetic Acid

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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 $\mu$ = 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]

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

Phase one — Phase two

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(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 $IM_T = IM + IMH^{\oplus}$.
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