Catalytic Reactions Involving Azomethines. X.[1] Transamination of 1-Methyl-4-formylpyridinium Iodide

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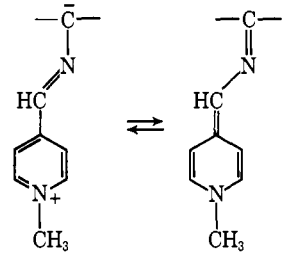
Abstract: The reactions (H_2O solvent, 30°, in the absence of metal ions) of 1-methyl-4-formylpyridinium iodide (MPCHO) with glycine, alanine, glycine ethyl ester, 2-methylalanine, and β-alanine have been investigated. All the amino acids exhibit a rapid initial reaction; a second, slower reaction is then observed with glycine, alanine, and glycine ethyl ester but not with 2-methylalanine or β-alanine. The following characteristics were established for the second reaction: (a) a deuterium isotope effect of 6.1 (comparing alanine and alanine-d_4), (b) catalysis by alanine, and (c) formation of pyruvate as the product. These results show the initial reaction to be aldimine formation and the second reaction to be a general base catalyzed conversion of aldimine to ketimine. MPCHO is thus capable of entering into a transamination reaction with α-amino acids. The second reaction (prototropy) produces a species absorbing in the visible region of the spectra (glycine 500 mμ and alanine 600 mμ), suggesting the formation of metastable dihydropyridine tautomeric structures of the intermediate carbanion zwitterion. The ability of MPCHO to enter into a transamination—whereas pyridine-4-aldehyde (PCHO) does not—is attributed to the greater electron withdrawal of the quaternized pyridine nitrogen. Though the nitrogen of PCHO may be protonated, it exists as the free base in the pH range where relatively strong general bases may be obtained at appreciable concentrations. The above results show that the catalytic effect of the 3-hydroxyl group of 3-hydroxypyridine-4-aldehyde is not essential to prototropy if the pyridine nitrogen carries a formal positive charge.

In 1954, Metzler, Ikawa, and Snell[4] summarized the minimum structural requirements for the pyridoxal-catalyzed transamination of amino acids to be the 4-formyl group and the 3-hydroxyl group substituted on the pyridine ring. The pyridine ring serves as an electron sink to assist removal of the α-hydrogen. The necessity of the formyl function requires little comment since it is involved directly in the formation of aldimine previous to the prototropic shift leading to ketimine. In this laboratory it has been established,[1] in nonmetal-ion-mediated reactions, that the 3-hydroxyl group acts as a catalyst for aldimine formation and assists the prototropic shift leading from aldimine to ketimine.[1] Also, the presence of the 3-hydroxyl group favors aldimine formation thermodynamically at acidic and neutral pH values. In addition, we have conclusively established that the prototropic shift is a general base catalyzed abstraction of the hydrogen α to the carboxyl group of the aldimine.[1]

In the absence of the 3-hydroxyl group (*i.e.*, pyridine-4-aldehyde) aldimine formation occurs but the prototropic shift leading to ketimine is immeasurably slow. It occurred to us that it would be of value to investigate the reaction of amino acids with N-methyl-4-formylpyridinium iodide (MPCHO). The derived aldimines would not possess the 3-hydroxyl group but would be characterized by a very electron-deficient quaternized pyridine ring. As a result, a positive charge could be maintained on the ring nitrogen at pH values above the pK_a' of the pyridine nitrogen of 3-hydroxypyridine-4-aldehyde. One would thus obtain an electron-deficient aldimine at pH values at which stronger bases, such as the amino group of amino acids, might act as catalysts for proton abstraction. In these higher pH ranges, existing general acids would be poorer catalysts for proton donation so that the carbanion would be longer lived. Were



(1)

this so, one might then be able to observe directly the dihydropyridine (DHP) tautomer of the carbanion. Schirch and Jenkins[5] suggested that the DHP structure is responsible for the absorbance maximum at 505 mμ observed in the ES complex of alanine with serine transhydroxymethylase.

In this paper the reaction of 1-methyl-4-formylpyridinium iodide (MPCHO) with alanine in aqueous solution at 30°, in the absence of metal ions, is described. Analysis of the reaction products has shown that pyruvate, the transamination product of alanine, is formed. In addition, the reaction has been shown to be catalyzed by alanine. The kinetic data are compared with that for the reaction of alanine with pyridine-4-aldehyde and 3-hydroxypyridine-4-aldehyde, and the deuterium kinetic isotope effect has been determined with alanine-d_4.

(1) For parts I, II, and III of this study see: (a) T. C. Bruice and R. M. Topping, *J. Am. Chem. Soc.*, **85**, 1480 (1963); (b) *ibid.*, **85**, 1488 (1963); (c) *ibid.*, **85**, 1493 (1963); for part IV: (d) T. C. French and T. C. Bruice, *Biochemistry*, **3**, 1589 (1964); part V: (e) T. C. French, D. S. Auld, and T. C. Bruice, *ibid.*, **4**, 77 (1965); part VI: (f) J. W. Thanassi, A. R. Butler, and T. C. Bruice, *ibid.*, **4**, 1463 (1965); parts VII, VIII, and IX: (g) D. S. Auld and T. C. Bruice, *J. Am. Chem. Soc.*, **89**, 2083 (1967); (h) *ibid.*, **89**, 2090 (1967); (i) *ibid.*, **89**, 2098 (1967).

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(4) D. E. Metzler, M. Ikawa, and E. E. Snell, *J. Am. Chem. Soc.*, **76**, 648 (1954).

(5) L. V. Schirch and W. T. Jenkins, *J. Biol. Chem.*, **239**, 3801 (1964).

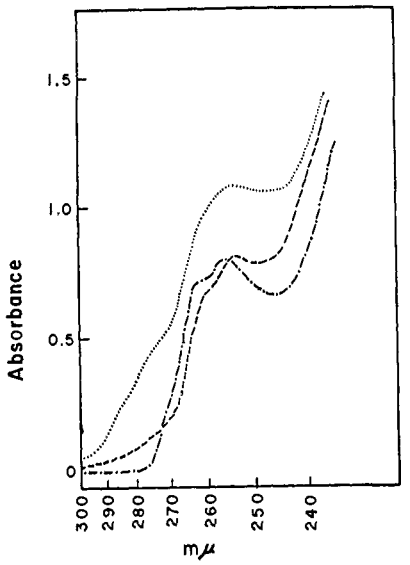


Figure 1. Reaction of MPCHO with amino acid. Spectral-time study on reaction of 0.25 M glycine with 1×10^{-4} M MPCHO at pH 9.6 and room temperature: ·–·–, MPCHO (time zero); ·····, 15 min; – – –, 2 hr.

Experimental Section

Materials. All kinetic solutions were prepared from water twice distilled from glass and purged with nitrogen. The sources and purities of chemicals used were as follows: DL-alanine, Calbiochem A grade; DL-alanine-d_4, Merck Sharp and Dohme (>95% D);[1i] DL-alanine-carboxyl-^{14}C, Calbiochem (27.6 mCi/mmol); 2-methylalanine, J. T. Baker; (ethylenedinitrilo)tetraacetic acid disodium salt, Eastman; glycine, Fisher; glycine ethyl ester hydrochloride, Aldrich (mp 145–146°); methyl iodide, Matheson Coleman and Bell; pyridine-4-aldehyde, Aldrich (n^{20}D 1.5430–1.5433); sodium pyruvate, Calbiochem A grade. Potassium chloride and hydroxide were reagent grade chemicals.

1-Methyl-4-formylpyridinium iodide was prepared by the addition of methyl iodide to pyridine-4-aldehyde in dry benzene, evaporation of solvent and excess reagent at reduced pressure, and hydration in air: mp 107–108° (lit.[6] 114–116°).

Anal. Calcd for $3(C_6H_8INO)\cdot H_2O$: C, 32.96; H, 3.42; N, 5.49; I, 49.76. Found: C, 32.70; H, 3.53; N, 5.55; I, 49.06.

Apparatus. The spectrophotometric titrations were carried out using apparatus described by French and Bruice.[1d] Ultraviolet and visible spectra were determined on a Perkin-Elmer Model 350 double-beam recording spectrophotometer. Spectral-time studies were carried out either on a Zeiss M4Q III monochromator or Beckman DU monochromator combined with a Gilford multiple-sample absorbance recorder. pH measurements were on a Radiometer Model 22 pH meter equipped with a Model PHA 620 Pa scale expander and a combined glass–calomel electrode. Polarographic product analyses were carried out on a Sargent XV polarograph with a dropping mercury electrode. Both kinetic and polarographic measurements employed cells thermostated at 30° with water jackets. Radioactivity determinations were performed at room temperature on a 720 Series Nuclear Chicago liquid scintillation system.

Kinetics. Kinetic solutions contained 1.0 M amino acid and 5×10^{-3} M ethylenediaminetetraacetic acid. The ionic strength was maintained at a calculated value of 1.0 with KCl and the pH adjusted with 2.5 M KOH. Buffer dilutions were performed with 1 M KCl. A stock solution of 2×10^{-4} M MPCHO was prepared in 1 M KCl. All solutions were preequilibrated in a water bath at 30°. Equal portions of the aldehyde and amino acid solutions were combined in a quartz cuvette and shaken and the reactions followed at 256.5 mμ. Reactions were followed to at least three half-lives and yielded linear pseudo-first-order plots. A difference of nearly a factor of 10 between rates of appearance and disappearance of imine allowed Guggenheim calculations to be made for the first reaction. Because OD_∞ values were not satisfactorily stable, Guggenheim calculations were employed to determine the rates of disappearance of imine.

Product Analysis. Chromatography. One milliliter each of the following five solutions was prepared: (a) 0.029 g of alanine, 0.017 g of MPCHO, 0.25 ml of 0.1 N KOH; (b) 0.030 g of alanine, 0.073 g of sodium pyruvate, 0.25 ml of 0.1 N KOH; (c) strongly alkaline solution of MPCHO; (d) aqueous solution of alanine; and (e) aqueous solution of MPCHO. After several hours, each solution was spotted on Whatman No. 1 filter paper and the chromatogram developed with 4:3:3 ethanol–28% NH_4OH–water. The spots were located with brom cresol green spray.

Polarography. Polarograms of reaction mixtures identical with those described in the kinetic section of this paper were obtained at 30° *vs.* a saturated calomel electrode. Samples (5 ml) of the reaction mixtures were adjusted to pH 2.73 ± 0.03, and the diffusion current was measured at the $E_{1/2}$ for pyruvate which was −0.78 V.

Isotopic Dilution. A 10-ml reaction mixture, 0.50 M in DL-alanine (7.77 μCi/mM alanine-carboxyl-^{14}C) at pH 9.75 and μ = 1.0 with KCl, containing 5×10^{-3} M MPCHO and 5×10^{-3} EDTA, was prepared and allowed to equilibrate for 5 hr at 30°. A second reaction mixture identical with the first, with the exception that the alanine contained 6.37 μCi/mM of radioactive alanine and the aldehyde was omitted, was used as a control and treated as was the aldehyde-containing mixture. After equilibration, 110 mg (1.0 mM) of sodium pyruvate was added to the reaction mixture, which was then concentrated at reduced pressure at less than 55° to dryness (less than 30 min). To the solid was then added 5 ml of methanol and 0.50 ml of concentrated HCl, the solid crushed and dispersed, and the slurry filtered through sintered glass by suction. The solid was washed with 3 ml of methanol, the filtrate (with the washings) was heated to boiling, and 0.10 ml of phenylhydrazine (bp 83–4° (1.65 mm)) was added. After boiling 10 min, the solution was diluted to ~25 ml with water and allowed to cool. The yellow crystals of pyruvic acid phenylhydrazone were then collected by suction filtration, washed with 2 ml of 1 M HCl, and dried. They were then dissolved in a minimum of hot chloroform, decolorized with Norit A, precipitated with carbon tetrachloride, and recrystallized from methanol–water to constant activity.

The activity of the phenylhydrazone was determined by counting samples of ~1 mg in 0.5 ml of methanol and 10 ml of scintillator solution (0.50 PPO, 0.03 POPOP w/v in toluene). The specific activity of the DL-alanine was determined by dissolving the radioactive alanine sample in 1.00 ml of water, extracting 10 μl by Microcap micropipet (the remainder was added to the reaction mixture), and diluting the 10-μl sample to 100 ml with methanol. A 0.50-ml sample of the methanolic solution was counted in 10 ml of scintillator solution.

The per cent transamination was determined by subtracting the counts of the product from the control solution from the counts of the product from the MPCHO-containing solution (correcting the former for the difference in initial alanine activity) and dividing by the theoretical count for 100% conversion.

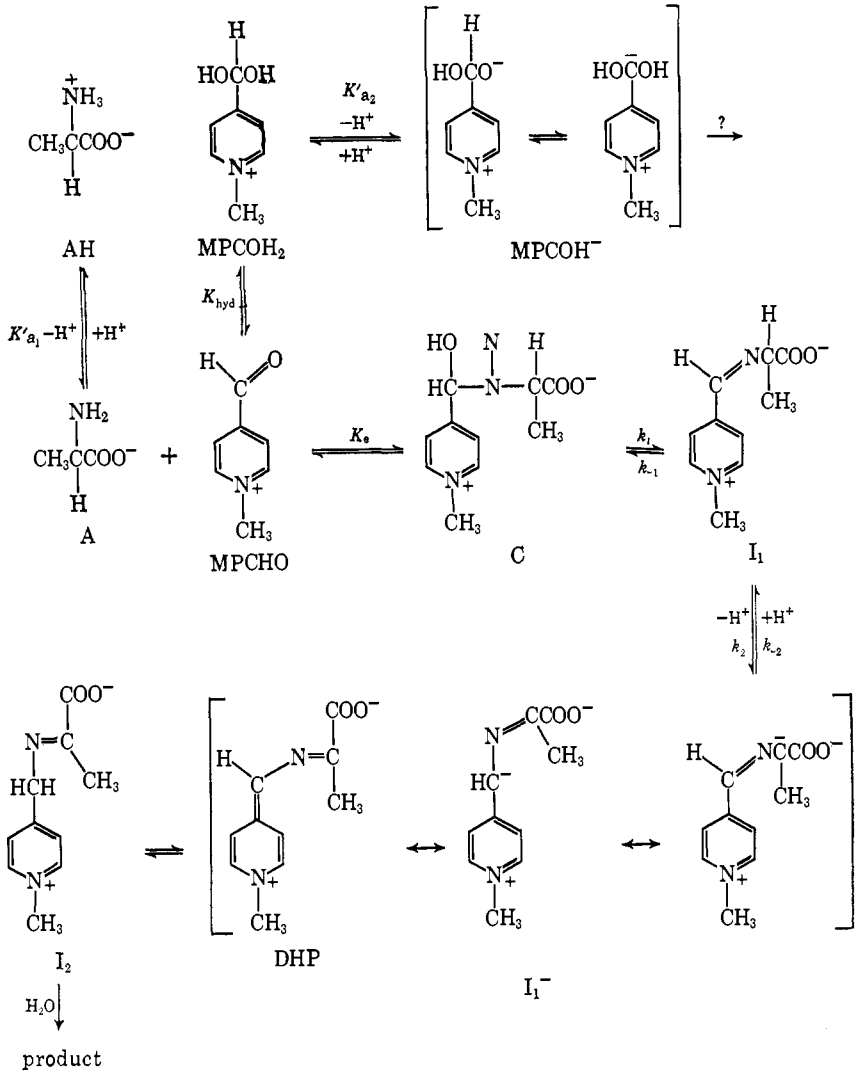
Results

A spectrophotometric titration curve (at 256.5 mμ) for MPCHO obtained by base addition fits a theoretical titration curve for $pK_a' = 10.7$–10.8. The curve for back-titration with HCl parted from the original titration curve, giving higher OD readings at low pH. Titrating successively with base and acid produced a series of curves, each with successively higher OD values and lower pK_a''s. These results establish both a dissociable proton and the instability of MPCHO in basic solution. Steinberg, *et al.*,[6] observed a pK_a' for 1-methyl-2-formylpyridinium iodide of 9.8–10.0 titrimetrically with no decomposition at high pH. They assigned this to a dissociation similar to pK_{a_2} in Chart I.

Figure 1 is representative of the changes in uv absorbance of the glycine–MPCHO or alanine–MPCHO systems in the pH range 8.4–10.5. Initially the spectrum is that of MPCHO. A rapid increase in absorbance (imine formation) occurs producing a shoulder at 276 mμ, where Schiff bases are known to absorb,[7]

(6) G. M. Steinberg, E. J. Poziomek, and B. E. Hackley, Jr., *J. Org. Chem.*, 26, 368 (1961).

Chart I



$$[A_T] = [A] + [AH]$$

$$[MPCHO_T] = [MPCHO] + [MPCOH_2] + [MPCOH^-]$$

as well as a general increase in OD from 300 to 240 mμ. A slower reaction (prototropic shift) then reduces the absorbance.

The formation and disappearance of imine were followed spectrophotometrically at 256.5 mμ, an absorbance maximum for both aldehyde and imine (Figure 1). The first reaction is (see Chart I)

$$A + MPCHO \overset{K_e}{\rightleftharpoons} (C) \xrightarrow{k_1} I_1 \qquad (2)$$

and the second reaction is

$$I_1 \xrightarrow{k_2} I_2 \qquad (3)$$

That the second and slower reaction involves the prototropic shift is established by the observation that half-neutralized (*ca.* pH 10) 0.5 *M* solutions of glycine, alanine, glycine ethyl ester, 2-methylalanine, and β-alanine exhibit the first reaction, but only glycine, alanine, and glycine ethyl ester undergo the second reaction. The 2-methylalanine significantly lacks the α proton necessary for the second reaction, and β-alanine is a simple aliphatic primary amine without an activated α hydrogen.

It is impossible to determine K_e due to the previously mentioned instability of MPCHO in base. However, under the pseudo-first-order conditions employed ($[A_T] \gg [MPCHO]$ at 0.50 *M* A_T), the rate of imine formation can be determined as $k_{obsd} = K_e k_1$. This rate with alanine was found to be essentially constant at 30° and $\mu = 1.0$ at 0.121 ± 0.027 min^{-1} from pH 8.4 to 10.5. This is ascribed to essentially complete carbinolamine formation at high $[A_T]$. A buffer dilution from $[A_T]$ 0.5 to 0.0125 at pH 10.32 ± 0.07 is shown in Figure 2. A similar upward curvature at low $[A_T]$ in a plot of the calculated second-order rate constant for imine formation *vs.* $[A_T]$ was observed by French and Bruice[1d] with PCHO and glycine and attributed to a change of rate-limiting step from carbinolamine formation at low $[A_T]$ to carbinolamine dehydration at high $[A_T]$. The over-all second-order rate constants for imine formation ($k_{obsd}/[A_T]$) at high $[A_T]$ with alanine at pH 9.73, 30°, are 11.3 and 0.242 M^{-1} min^{-1} for PCHO and MPCHO, respectively. The slower rate of forma-

(7) D. Heinert and A. E. Martell, *J. Am. Chem. Soc.*, **85**, 188 (1963).

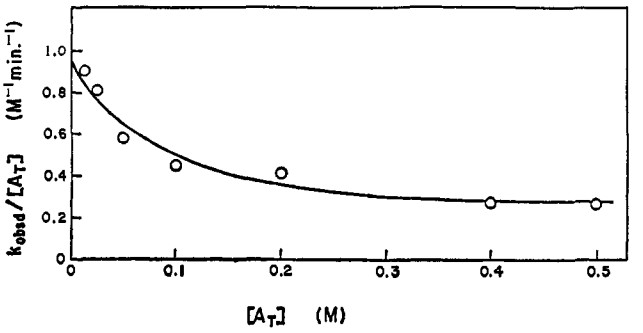


Figure 2. Dependence of $k_{obsd}/[A_T]$, apparent second-order rate constants of imine formation, on $[A_T]$ at pH 10.32 ± 0.07. Points are experimental values obtained at 30°.

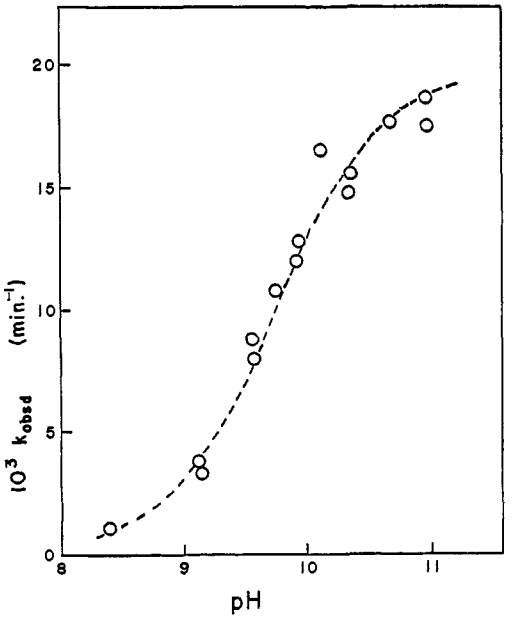


Figure 3. Dependence of k_{obsd} on pH for aldimine–ketimine interconversion: O, experimental points for transamination of MPCHO by 0.5 *M* alanine at 30°, μ = 1.0; - - - is the theoretical titration curve, pK_a = 9.73.

tion with MPCHO may be attributed to a greater degree of hydration of MPCHO than PCHO (K_{hyd}, Chart I).

The pH–rate constant profile for the aldimine–ketimine interconversion (eq 2 and k_2 on Chart I) is shown in Figure 3. The points are the pseudo-first-order rate constants determined experimentally at 256.5 mμ at 30° and $[A_T]$ = 0.50 *M*. The line is a theoretical titration curve for a dissociation constant of pK_a = 9.73, the pK_a of the amino group of alanine.[1d] This indicates a general base catalysis of the prototropic shift by alanine. A buffer dilution of k_{obsd} *vs.* $[A_T]$ from 0.8 to 0.1 $[A_T]$ at pH 10.32 ± 0.07 is a straight line with least-squares slope and intercept 2.79 × 10^{-2} M^{-1} min^{-1} and 5 × 10^{-4} min^{-1}, respectively. The slope ($k_{obsd}/[A_T]$) is the apparent second-order rate constant for general base catalysis ($k_{gb}K_a/(K_a + a_H)$) and the intercept the combined apparent first-order rate constants for lyate species catalyzed proton abstraction.

$$k_{obsd} = k_{gb}[A_T]\frac{K_a}{K_a + a_H} + k_{OH}\left(\frac{K_w}{a_H}\right) + k_0 \quad (4)$$

The rates of transamination of MPCHO by DL-alanine and DL-alanine-d_4 were determined at 30°,

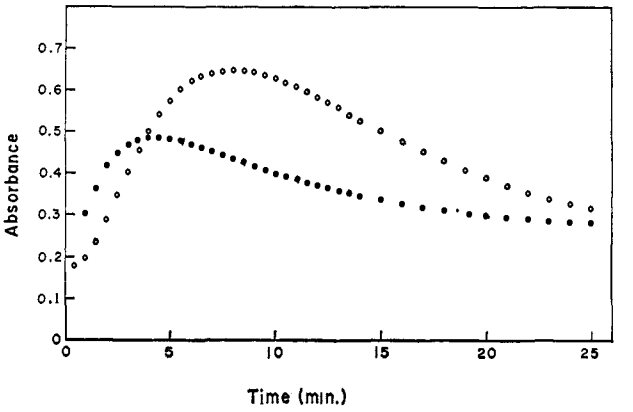


Figure 4. Formation and disappearance of DHP and I_1. An absorbance *vs.* time plot for the reaction of 1.0 *M* glycine at pH 9.89 with 5 × 10^{-3} *M* MPCHO at 500 mμ (O) and with ~1 × 10^{-4} *M* MPCHO at 256.5 mμ (●). Reactions were initiated by the addition of one drop of 0.1 or 0.02 *M* MPCHO to 2 ml of 1 *M* glycine solution at 30°. Note that the induction period in formation of DHP (500 mμ) corresponds to formation of I_1 (256.5 mμ).

pH 9.75, and $[A_T]$ = 0.50 *M*. The pseudo-first-order rates of imine formation were 0.121 min^{-1} for the nondeuterated and 0.119 min^{-1} for the deuterated alanine. The kinetic deuterium isotope effect (k^H_{obsd}/k^D_{obsd} = 0.0109/0.00178) for the prototropic shift was 6.1 ± 0.2. This value is comparable to the values of 6.06 and 6.93 observed by Auld and Bruice[1i] as kinetic isotope effects for the transamination of 3-hydroxypyridine-4-aldehyde by alanine with acetate and imidazole catalysis, respectively.

Glycine reacts with MPCHO to produce the uv spectral changes shown in Figure 1. With 1.0 *M* glycine and MPCHO approximately 5 × 10^{-3} *M* a transient visible color is observed with an absorbance maximum at 500 mμ. This is believed to be the DHP structure in Chart I. Figure 4 is a plot of the absorbance at 256.5 and 500 mμ *vs.* time for MPCHO and 1.0 *M* glycine at pH 9.89 and 30°. The induction period for the species absorbing at 500 mμ coincides with the formation of imine (rise at 256.5 mμ), indicating that the former is formed from the latter. Both species are then removed from solution by the aldimine–ketimine interconversion. The visible absorbance of the intermediate produced with alanine is at 600 mμ. These results apparently mark the first successful demonstration of the dihydropyridine structure as an intermediate in the transamination reaction with a simple amino acid in aqueous solution. The importance of the DHP structure in enzymatic reactions has been alluded to previously,[5] and Schirch and Slotter[8] have reported an intermediate species absorbing at 480 mμ in the alkoxide- or imidazole-catalyzed reaction of pyridoxal N-methochloride with diethyl aminomalonate.

Taft and McKeever[9] have shown by nmr fluorine shielding that the positive charge of the triphenylmethylcarbonium ion may be localized either on the methyl carbon or on a *para* ring substituent, depending on the nature of the substituent. A gradual change from resonance delocalization to formation of

(8) L. Schirch and R. A. Slotter, *Biochemistry*, **5**, 3175 (1966).
(9) R. W. Taft and L. D. McKeever, *J. Am. Chem. Soc.*, **87**, 2489 (1965).

a stable tautomeric structure therefore occurs. McKeever and Taft[10] have obtained similar results with carbanion structures. A plausible parallel exists in the DHP structure of Chart I in which complete localization of the electron pair of the incipient carbanion to the quaternized nitrogen may occur.

To demonstrate quantitatively and conclusively that the reaction of MPCHO with alanine is transamination, reaction mixtures were analyzed for pyruvate, the transamination product of alanine. Three methods were employed: paper chromatography, polarography, and isotopic dilution.

Paper chromatograms were identical for alanine–MPCHO and alanine–sodium pyruvate solutions (R_f alanine ~0.79, R_f pyruvate ~0.51). A basic solution of MPCHO produced a spot with R_f ~0.47. The evidence was therefore indicative of pyruvate formation but inconclusive.

Polarograms were determined for solutions identical (see Experimental Section) with those used in kinetic analysis. The results are shown in Table I. The

Table I. Polarographic Analysis for Pyruvate

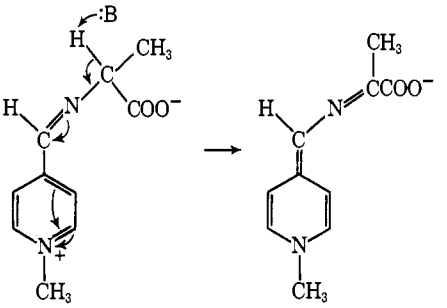
pH	i_d[a]	% transamination[b]	pH	i_d[a]	% transamination[b]
9.15	0.215	76	10.47	0.30	106
9.60	0.22	78	10.65	0.265	94
9.75	0.19	68	11.05	0.38	134
10.10	0.21	75			

[a] i_d is the diffusion current at the $E_{1/2}$ for pyruvic acid, −0.78 V. [b] Per cent transamination is determined from a calibration curve with sodium pyruvate.

results were not exactly reproducible and were quantitatively unsatisfactory. However, the data do give indication of increasing conversion of alanine to pyruvate with increasing pH.

An isotopic dilution technique (described in the Experimental Section) was employed to determine if a reaction mixture of MPCHO with DL-alanine, doped with DL-alanine-carboxyl-^{14}C, produced radioactive pyruvate. Half-neutralized, 0.5 *M* alanine produced 78% of the theoretical yield of pyruvate after 5 hr at 30°. This compares favorably with the values (68–78%) obtained from the polarographic determination at the same pH and concentration.

The kinetic deuterium isotope effect, product analysis, and dependence of the aldimine–ketimine interconversion on alanine free-base concentration establish the reaction of MPCHO with alanine to be a general base catalyzed transamination (5). The absorbance



(5)

maximum at 500 mμ in the transamination of MPCHO by glycine lends evidence to the importance of the DHP structure in the transamination of pyridinealdehydes.

It would be of interest to investigate the role of the DHP structure with a compound less labile than MPCHO. To this end we have synthesized 1-methyl-3-hydroxy-4-formylpyridinium iodide. The crude product appears stable in alkaline solution, exhibiting no change in OD with time above pH 10. The investigation of the reactions and equilibria of this aldehyde with amino acids, and those of the related 1-methyl-3-methoxy-4-formylpyridinium iodide, would help to elucidate the structural and electronic requirements of the transamination reaction.

Acknowledgments. We wish to acknowledge the aid of Dr. E. L. Triplett in the radiation determinations. This work was supported by a grant from the National Science Foundation.

(10) L. D. McKeever and R. W. Taft, *J. Am. Chem. Soc.*, **88**, 4544 (1966).