Catalytic Reactions Involving Azomethines. IV.* Rates and Equilibria of Imine Formation with Pyridine-4-aldehyde and Various Amino Acids

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The reaction of pyridine-4-aldehyde with neutral, acidic, basic, and aromatic amino acids proceeds by acid-catalyzed and uncatalyzed dehydration of carbinolamine intermediates with the formation of the corresponding imines. Second-order rate constants for acid-catalyzed and uncatalyzed imine formation, equilibrium constants for imine formation and for the formation of carbinolamine intermediates, and acid- and uncatalyzed rate constants for dehydration of carbinolamines and hydrolysis of imines are reported for ten amino acids. In the pH range $pK_a \pm 0.6$, acid- and uncatalyzed rates of imine formation contribute nearly equally, and the pH-rate profile is almost flat. Rate constants for acid-catalyzed dehydration of carbinolamine range from 0.4×10^{10} to 5×10^{10} M^{-1} min^{-1}, values that are higher than those reported for overall formation of the first intermediate in enzymatic transamination of glutamate and aspartate (G. G. Hammes and P. Fasella [1963], *J. Am. Chem. Soc. 85*, 3029). A cell for the simultaneous measurement of absorbance and pH is described.

Schiff bases are of particular interest in the study of the large number of enzymatic reactions that involve pyridoxal phosphate as a cofactor. Although many studies of nitrogen-derivative formation with aldehydes have been reported, the information concerning imine formation with pyridoxal phosphate and its analogs is incomplete. The first paper in this series deals with equilibria and rates of imine formation from pyridine-4-aldehyde and various amino acids. Subsequent papers will describe the pH dependence of equilibrium constants and rate constants for imine formation from amino acids and 3-hydroxypyridine-4-aldehyde.

Elucidation of mechanisms of imine formation has depended on progress in the more general problem of mechanisms of reaction of nitrogen derivatives with carbonyl compounds. The very early work (Acree and Johnson, 1907; Barrett and Lapworth, 1908) on acid and base catalysis of oxime formation included a discussion of intermediates of the type $\diagdown \overset{+}{C}(OH)NH_2OH$.

Both the detection of such intermediates and measurement of the activity of hydrogen ion in aqueous solutions were difficult at that time. Bodforss (1924) was the first to provide clear experimental evidence for an intermediate in phenylhydrazone formation. He showed that the first-order rate of formation of the phenylhydrazone of m-nitrobenzaldehyde was slower than the rate of disappearance of phenylhydrazine; hence the addition compound must have been formed rapidly and in relatively high concentration. In a later quantitative study of acetoxime formation, in which pH was accurately measured with the hydrogen electrode, Ölander (1927) concluded from kinetic evidence alone that "acetonehydroxylamine," $(CH_3)_2C(OH)NHOH$, was an intermediate. Ölander also described maxima in pH-rate profiles for oxime formation (pH 4.5) and oxime cleavage (pH 2.3). In a related quantitative

study of semicarbazone formation Conant and Bartlett (1932) discovered sharp maxima in the pH-rate profile at slightly acid pH. They obtained both the overall equilibrium constants and rate constants for formation and hydrolysis of a variety of aldehydes and ketones. (More recently, Jencks [1959] has shown that the decrease in rate that Conant and Bartlett observed for semicarbazone formation in alkaline solution was the result of rate-limiting dehydration of the addition compound, rather than rate-limiting general acid catalysis of its formation.)

This classical work formed the basis for the technically more difficult investigations of imine formation. Since the formation equilibrium constants for imines in aqueous solution are much less favorable than for semicarbazones, oximes, and hydrazones, quantitative studies on imines have more often been concerned with hydrolysis. Initial reports of isolation of carbinolamines (Hantzch and Kraft, 1891) were soon criticized (Dimroth and Zoeppritz, 1902), and further progress on the mechanism of imine formation has come chiefly in recent years.

Zuman (1950) measured polarographically the equilibrium constants for imine formation with a variety of carbonyl compounds and amines, including amino acids. Willi and Robertson (1953) were the first to undertake a detailed kinetic investigation. From their careful measurements of the rates of hydrolysis of substituted benzylideneanilines in 50% aqueous methanol, these authors could calculate the rate constants for the specific and general acid-catalyzed reactions in buffered solutions near neutrality. By taking advantage of the fact that imine and aldehyde concentrations may be measured independently and simultaneously by polarographic methods, Kastening et al. (1956) were able to establish the presence of an intermediate during the course of hydrolysis of benzylideneaniline. They also observed base-catalyzed hydrolysis above pH 12. Willi (1956) independently deduced the presence of such an intermediate on a kinetic basis, explained the anomalies reported previously, extended his pH-rate profile to acidic and basic solutions, and set forth the general kinetic equations for imine hydrolysis. The rate-limiting step in neutral and alkaline solutions was found to be a combination of the "uncatalyzed" addition of water to imine to form carbinolamine ($\rho = -1.45$) and the acid-catalyzed addition of water to
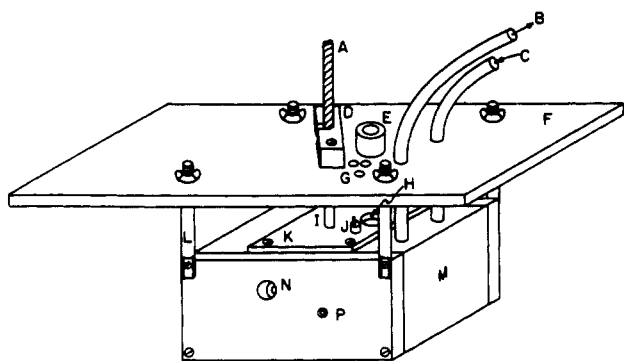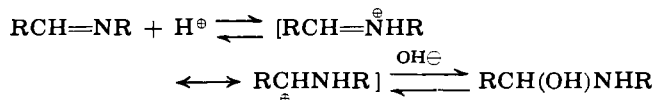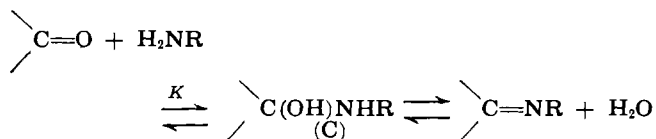
FIG. 1.—Spectrophotometric-titration cell. Key: A, flexible shaft; B, water outlet; C, water inlet; D, shaft holder; E, polypropylene guard for electrode, 0.405 i.d.; F, brass support, 21.6 × 8.9 × 0.6 cm; G, holes for tubing (nitrogen, standard base, and sample); H, hole in lid for glass electrode; I, impeller shaft, 4.8 mm diameter; J, inlet ports for nitrogen and standard base; K, polypropylene lid, 5.1 × 4.5 × 0.6 cm; L, threaded brass posts, 6.4 mm diameter; M, brass thermostat block; N, optical exit port; P, sample inlet/outlet leading to bottom of polypropylene cell. The optimal position of the cell in the compartment of the spectrophotometer is maintained by a bar on the right underside of the support and by lock nuts on the posts (not shown).

imine. The apparent rate constants for the latter reaction, which did not follow Hammett's rule, were seen to be composites of rate constants. The following mechanism for the "uncatalyzed" reaction was proposed on the basis of values for activation energies:

$$\text{RCH=NR} + \text{H}^\oplus \rightleftharpoons \text{[RCH=}\overset{\oplus}{\text{N}}\text{HR}$$

$$\xrightarrow{\hspace{1cm}} \underset{\oplus}{\text{RCHNHR}}\text{]} \overset{\text{OH}\ominus}{\rightleftharpoons} \text{RCH(OH)NHR}$$

More recently, Cordes and Jencks (1962) have measured both the rates of formation of *p*-chlorobenzylidenaniline (with semicarbazide as a trap) and rates of hydrolysis as a function of *p*H in aqueous solutions. In addition to confirming the general observations of Willi and of Kastening *et al.*, they showed that the *p*H-rate maximum in imine formation represents a transition from rate-limiting dehydration of a carbinolamine intermediate (C) in neutral and alkaline solutions to rate-limiting attack of free nitrogen base on the carbonyl compound at acid *p*H. In the acid region hydronium ion appears to act as a general acid catalyst. C accumulates rapidly at neutral *p*H, with a concomitant de-

$$\overset{\diagdown}{\underset{\diagup}{\text{C}}}\text{=O} + \text{H}_2\text{NR}$$

$$\overset{K}{\rightleftharpoons} \underset{(\text{C})}{\overset{\diagdown}{\underset{\diagup}{\text{C}}}\text{(OH)NHR}} \rightleftharpoons \overset{\diagdown}{\underset{\diagup}{\text{C}}}\text{=NR} + \text{H}_2\text{O}$$

crease in absorbance in the ultraviolet, and its dehydration is facilitated by general and specific acid catalysis. Cordes and Jencks (1963) have extended their investigations to the mechanism of hydrolysis of Schiff bases derived from aliphatic amines and substituted benzaldehydes, and have summarized rules for mechanisms of nitrogen derivative formation.

EXPERIMENTAL PROCEDURE

Materials.—The deionized distilled water that was used throughout was not further degassed. However, all solutions above *p*H 7 were either stored in screw-cap tubes or kept under nitrogen until measurements were completed.

Pyridine-4-aldehyde hydrate (CalBiochem) was used without further purification. Stock solutions, 0.01 M, were stored for no longer than 1 week at 4°. DL-α-Phenylalanine (Eastman White Label); glycine (Fisher Reagent); L-asparagine hydrate (CalBiochem A grade); and DL-aspartic acid, DL-valine, DL-leucine, DL-serine, and L-arginine hydrochloride (CalBiochem NRC grade) were used without further purification. It was necessary to reduce the absorbance at 270 mμ of Fisher reagent grade L-glutamic acid and CalBiochem NRC grade DL-alanine by recrystallization. N-Ethylmorpholine from K and K Laboratories, Inc., was distilled from sodium. Purified triethylammonium acetate was prepared according to Dawid *et al.* (1963).

Apparatus.—Spectrophotometric measurements were made with a Zeiss PMQII spectrophotometer, equipped with a thermostated cuvet holder. Readings were generally taken at 270 mμ (or 280 mμ for aromatic amino acids) in the optical density range 0.2–0.7. A stopped-flow apparatus (T. C. French, S. J. Benkovic and T. C. Bruice, paper submitted for publication) was used for the reactions with glycine at high concentration. A Radiometer *p*H-Meter 22 with scale expander was used for *p*H measurements. Spectra were recorded with a Perkin-Elmer Model 350 recording spectrometer.

Optical density and *p*H were measured simultaneously in a rectangular polypropylene cell (Fig. 1) of inner dimensions 2.5 × 2 × 3.8 cm and outer dimensions 3.5 × 4.5 × 5 cm. Cylindrical quartz windows (Pyrocell Manufacturing Co.), 7.6 mm diameter × 2.5 mm, were mounted in each face 16 mm diameter × 2.5 mm, were mounted in each face 16 mm from the bottom and 16 mm from the end of the cell. A polypropylene impeller (I), an "O" ring, 9.7 mm i.d. × 1.5 mm (located in hole H), and inlets for nitrogen and base (J) were mounted in the lid, which was in turn fixed on the rectangular cell with nylon screws and a Teflon gasket. The positions of the impeller and the combined glass electrode (Radiometer GK2021C), which was held in place by the "O" ring, were arranged so that they did not interfere with the light path through the quartz windows. The bottom and sides of the cell were surrounded by a thermostat block (M), which was suspended by posts (L) from a support (F). To provide complete electrical insulation of the glass electrode from the support it was necessary to fasten the posts to the thermostat with nylon screws and insulating washers. The impeller was connected to a 72-rpm induction motor by way of a flexible shaft (A). The entire assembly, which will hereafter be called a "spectrophotometric titration cell," could then be positioned correctly in the cell compartment of a Zeiss PMQII spectrophotometer. Solutions were pre-equilibrated at the desired temperature before addition to the thermostated cell.

Dissociation Constants of Amino Acids.—The pK_a' values of the eleven amino acids that were investigated were determined by half-neutralization of known quantities of dry amino acid with standard base. Stock solutions of half-neutralized amino acids were diluted with KCl solution to yield amino acid concentrations ranging from 0.01 to 0.50 M.

Equilibrium Constants in Buffered Solutions.—Ultraviolet spectra of buffered solutions of aldehyde, amino acid, imine, and mixtures thereof were recorded. The true equilibrium constant, K_o, was calculated from equation (1) at the higher *p*H values, and by the graphical methods of Lucas *et al* (1962) at the four lower *p*H values. Here *D* is the optical density at 270 mμ of a mixture of imine and aldehyde in the ratio $[I]/[P] =$

$$K_o = \frac{1}{[A]}\left(\frac{D - D_P}{D_I - D}\right) \qquad (1)$$

0.4–2.5; this value is corrected for absorbancy caused by impurities in the amino acid alone. Similarly, D_I is the corrected optical density of a solution of imine in which $[I]/([I] + [P]) > 0.99$. Here D_P is the optical density of aldehyde in the same concentration that is used in the measurement of D_I.

The Henderson-Hasselbach equation and the pK_a' values of Table I were used to calculate $[A]$, the concentration of unprotonated amino acid. At each pH

TABLE I
pK_a' VALUES FOR AMINO ACIDS AT 30° AND $\mu = 1$

Amino Acid	pH for Half-neutralized Amino Acid at Molarity:				
	0.01	0.02	0.05	0.10	0.50
Leucine	9.54	9.57	9.58	9.58	
Valine	9.47	9.48	9.50	9.51	9.55
Arginine	9.12	9.14	9.16	9.16	9.17
Glycine	9.51	9.54	9.56	9.58	9.63
Phenylalanine	9.01	9.01	9.02	9.03	
Serine	8.96	8.97	8.98	8.99	9.04
Aspartate	9.45	9.47	9.48	9.49	9.57
Glutamate	9.33	9.37	9.39	9.40	9.50
Asparagine	8.68	8.69	8.71	8.71	
Alanine	9.62	9.65	9.67	9.68	9.73
Phenylglycine	8.82	8.83			

five values of D in the range $0.4 < [I]/[P] < 2.5$ were measured, and the five values of K_0 that resulted were averaged.

Equilibrium Constants Measured in the Spectrophotometric-Titration Cell.—After the glass electrode had been placed in the cell and standardized, the cell was filled with 1 M KCl and positioned in the cell compartment of the spectrophotometer. The spectrophotometer was adjusted to zero optical density at 270 mμ (slit 0.1–0.15 mm). The scale of the indicating instrument was set at 0.500 and the spectrophotometric-titration cell was removed. The optical density that was recorded was used to reset the spectrophotometer to an equivalent of zero optical density over the course of a run. Repositioning of the cell in the compartment was reproducible to within 0.002 OD units. When the aperture of the quartz window in the incident beam was 4.1 mm in diameter and the exit aperture was 4.8 mm in diameter, the equivalent of zero OD lay between 0.000 and 0.100. The zero transmission setting could be obtained during the course of a run simply by rotating the filter holder to an intermediate position.

The spectrophotometric-titration cell was flushed with 8×10^{-5} M pyridine-4-aldehyde in 1 M KCl, filled with 14 ml of the same solution, and adjusted to pH 7–10 with 2.3 M KOH contained in a micrometer buret. The D_P was recorded. At the start of each series of runs, the cell was flushed with amino acid solution and filled, and the optical density of the amino acid blank was recorded as a function of pH over the range 7–9. The concentrations of total amino acid that were used to form imine were generally 0.100 M, except for phenylalanine (0.05 M), leucine (0.05 M), and phenylglycine (0.01 M). Imine formation with aromatic amino acids was measured at 280 mμ. The cell was flushed with appropriate mixtures of neutral amino acid and 8×10^{-5} M aldehyde (or 6×10^{-5} M for leucine) and filled. In each run the pH was raised in seven to twelve steps of *ca.* 0.1 unit by the addition of KOH, and pH and optical density readings were taken after equilibrium had been reached (7–15 minutes). To obtain D_I, $1/(D - D_P)$ was plotted against $1/[A]$; the intercept on the ordi-
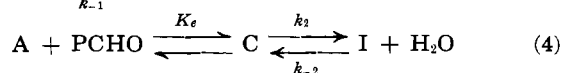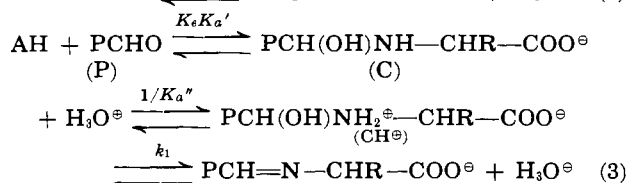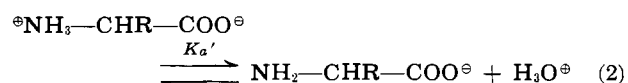
nate equals $1/(D_I - D_P)$ (Lucas *et al.*, 1962). The K_o (equation 1) was calculated for each set of readings, and the results were averaged.

Kinetics.—All kinetic experiments reported in this paper were carried out at $30.0 \pm 0.1°$ in deionized distilled water at a calculated ionic strength of 1.0 M, adjusted with KCl. Imine formation was initiated by the addition of 2.34 ml of amino acid solution ($[A]/[A_T] = 0.2$–0.8; $[A_T]$ = total concentration of amino acid $= 0.01$, 0.02, 0.03, 0.04, or 0.05 M) from a thermostated syringe to 0.06 ml of 0.01 M pyridine-4-aldehyde in a thermostated cuvet. The solutions were further mixed by inverting the stoppered cuvet three times, and readings were taken at 270 mμ (or 280 mμ for aromatic amino acids) every 10, 15, 20, or 30 seconds. Pseudo-first-order rate constants were calculated from eleven points on graphs of log $(D_\infty - D_t)$ versus time. The plots were strictly first-order within the first one to two half-lives that were studied.

In the stopped-flow experiments, the sample syringes were filled with aldehyde and amino acid solutions of nearly equal densities in that order, and the observation chamber was flushed with amino acid solution. The oscillograph pen was set at zero transmission when the slit of the monochromator was fully closed, and at 100% transmission when the galvanometer read 100% transmission (slit = 0.3 mm). Imine formation was initiated by pushing the plungers of the sample syringes down one stop (*ca.* 0.1 ml); the effluent was collected in a screw-cap tube. The plungers were advanced to the next stop after equilibrium was reached. A total of seven recordings were made with each sample. The pH values of the effluents were taken for each sample at the end of a series of runs. In each run, pseudo-first-order rate constants were calculated for two superimposable traces that were representative of the seven recordings that were measured, and the average was recorded. The plots were strictly first-order for the first one to two half-lives that were studied.

RESULTS

The following reactions of pyridine-4-aldehyde with various amino acids were considered:

$$^{\ominus}NH_3\text{—CHR—COO}^{\ominus}$$

$$\xrightleftharpoons[K_eK_a']{K_a'} NH_2\text{—CHR—COO}^{\ominus} + H_3O^{\oplus} \qquad (2)$$

$$AH + PCHO \xrightleftharpoons{K_eK_a'} PCH(OH)NH\text{—CHR—COO}^{\ominus}$$
$$(P) \qquad\qquad\qquad (C)$$

$$+ H_3O^{\oplus} \xrightleftharpoons{1/K_a''} PCH(OH)NH_2{}^{\oplus}\text{—CHR—COO}^{\ominus}$$
$$(CH^{\ominus})$$

$$\xrightleftharpoons[k_{-1}]{k_1} PCH{=}N\text{—CHR—COO}^{\ominus} + H_3O^{\ominus} \qquad (3)$$

$$A + PCHO \xrightleftharpoons{K_e} C \xrightleftharpoons[k_{-2}]{k_2} I + H_2O \qquad (4)$$

where K_a' and K_a'' are dissociation constants for AH and CH$^+$, respectively. The equilibrium constants for imine formation that are associated with equations (3) and (4) are:

$$K_H = K_oK_a' = K_eK_a'k_1/k_{-1}K_a''$$
$$= [I][H_3O]/[AH][P] \qquad (3a)$$

$$K_o = K_ek_2/k_{-2} = [I]/[A][P] \qquad (4a)$$

At low concentrations of A_T and P_o where the concentrations of C and CH$^{\ominus}$ are in low steady-state, the second-order rate constants for imine formation by the two pathways are given by:

$$k_H' = k_1K_a'K_e/K_a'' \text{ (i.e., } d[I]/dt = k_H'[AH][P] \qquad (3b)$$
$$k_0 = k_2K_e \text{ (i.e., } d[I]/dt = k_0[A][P]) \qquad (4b)$$
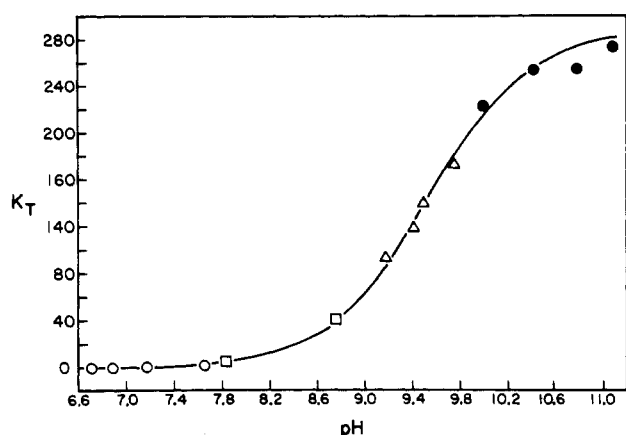
FIG. 2.—Apparent equilibrium constants, K_T (M^{-1}), for the formation of $PCH{=}NCH_2COO^-$ versus pH at $30°$ and $\mu = 1$. The solid line is a titration curve with its point of inflection at the pK_a' value observed for glycine. Buffers: —O—, potassium phosphate, 0.017 M; —□—, N-ethylmorpholine hydrochloride, 0.05 M; —△—, potassium borate, 0.05 M; —●—, triethylammonium acetate, 0.02 M.

Under these conditions the total second-order rate constant for imine formation is

$$k_f = k_0 \left(\frac{K_a'}{K_a' + a_H}\right) + k_H' \left(\frac{a_H}{K_a' + a_H}\right)$$
$$\text{(i.e., } d[I]/dt = k_f[A_T][P]) \quad (5)$$

and the total first-order rate constant for the reverse direction, hydrolysis of imine, is

$$k_r = k_{-1}(H_3O^+) + k_{-2} \quad (6)$$

Dissociation Constants of Amino Acids.—The values of pK_a' decreased by about 0.1 pH unit as the concentration of amino acid was lowered from 0.50 to 0.01 M (Table I).

Equilibrium Constants in Buffered Solutions.—The average value of K_0 for glycine at fourteen pH values in the range pH 6.7–11.1 was found to be 302 ± 20 M^{-1}. In all cases a tight isosbestic point was present at 296 mμ. By spectrophotometric titration the pK_a' values for the dissociation of the pyridinium and hydrated aldehyde groups of pyridine-4-aldehyde were found to be 4.63 (in citrate-phosphate buffers) and 12.4 (in KOH solutions), respectively; these results are consistent with those obtained at 20° by Nakamoto and Martell (1959). The absorbance of pyridine-4-aldehyde at 270 mμ over the pH range 6.7–11.1 was nearly constant. When K_T, the apparent equilibrium constant, based on $[A_T]$ rather than $[A]$, was plotted against pH, a titration curve for the amino group of glycine, $pK_a' = 9.54$, was the result (Fig. 2). This was anticipated from the derived relationship, $(K_0/K_H = 1/K_a')$. The best fit was obtained for $K_0 = 290$ M^{-1}. Since equations (1) and (4a) refer to the reactive species, unhydrated aldehyde, the slight increase in hydration that occurs at the higher pH values (Nakomoto and Martell, 1959) did not influence the values of K_0 and K_T.

There was no significant change in K_0 when K_2HPO_4 was added to imine in potassium borate buffer, pH 9.50, or when the concentration of triethylammonium acetate buffer was raised from 0.02 to 0.16 M at pH 11.08. Spectra of pyridine-4-aldehyde in borate buffers did not appear to be significantly different from spectra in other buffers at similar pH values.

The K_0 for glutamate and alanine were found to be 225 M^{-1} at pH 11.1 (triethylammonium acetate buffer).

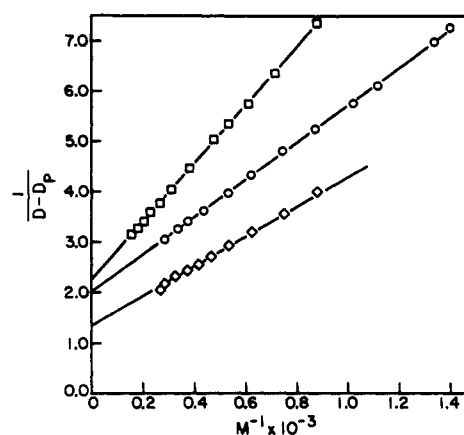*Equilibrium Constants Measured in the Spectrophoto-*

FIG. 3.—Double-reciprocal plots for the determination of D_I, the optical density of pure imine. Representative runs for glycine (—□—) and valine (—◇—), at 8 × 10^{-5} M aldehyde, and for leucine (—O—) at 6 × 10^{-5} © aldehyde are shown.

metric-Titration Cell.—Equilibrium constants for imine formation with pyridine-4-aldehyde and eleven amino acids and the average deviation of triplicate or duplicate runs are listed in Table II. In all cases there was no deviation from a straight line in the double reciprocal plot shown in Figure 3, which indicates that only the free amino group is essential to the overall equilibrium. The average of the average deviations in each run was 1.2%, whereas the corresponding value in the measurement with buffered solution was 3%. This high internal precision was partly the result of the electronic stability of the instruments used. The average of the average deviations of K_0 among different runs was (excluding phenylglycine) 2.4%. The more precise values of K_0 that were obtained with the spectrophotometric-titration cell were preferred to those obtained in buffered solutions in the calculation of second-order rate constants for imine formation.

Apparent Second-Order Rate Constants.—The following deviation for the second-order rate constant for imine formation assumes the simplest case, i.e., that no detectable carbinolamine intermediates are present. In equation (7) k_f' is the experimental apparent second-order rate constant for imine formation at constant pH and any concentration of total amino acid $[A_T]$ in both anionic $[A]$ and zwitterionic $[AH]$ forms, and k_r' is the apparent first-order rate constant for imine hydrolysis.

$$P + A_T \underset{k_r'}{\overset{k_f'}{\rightleftharpoons}} I \quad (7)$$
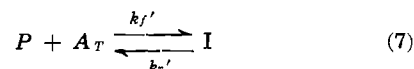
These rate constants are related to K_0, the true equilibrium constants for the reaction, by equation (8).

$$k_f'/k_r' = K_0[A]/[A_T] \quad (8)$$

Under the conditions employed, $[A_T]$ was much greater than the initial concentration of aldehyde, $[P_0]$. If the relative concentrations of intermediates in the reaction are negligible, then the concentration of aldehyde at any time is:

$$[P] = [P_0] - [I] \quad (9)$$

The rate of imine formation under these conditions is:

$$d[I]/dt = k_f'[P][A_T] - k_r'[I]$$
$$= k_f'[A_T]\left([P_0] - \frac{K_0[A] + 1}{K_0[A]}[I]\right) \quad (10)$$

Substitution of equation (11) into (10) and integration yields an expression for the observed pseudo-first-

TABLE II
EQUILIBRIUM AND RATE CONSTANTS FOR IMINE FORMATION

Amino Acid	Side Chain	K_o (M^{-1})	k_H' (M^{-1} min^{-1})	k_o (M^{-1} min^{-1})	K_e (M^{-1})	k_1/K_a'' (M^{-1} min^{-1}) × 10^{-10}	k_2 (min^{-1})	k_{-1} (M^{-1} min^{-1}) × 10^{-7}	k_{-2} (min^{-1})
Leucine	CH$_2$CH(CH$_3$)$_2$	531 ± 9	5.82	17.9	1.1	2.0	16	4.1	0.034
Valine	CH(CH$_3$)$_2$	459 ± 5	3.06	14.4	0.7	1.4	21	2.0	0.031
Arginine·HCl	(CH$_2$)$_3$NHC=NH	403 ± 8	7.08	9.5	2.2	0.44	4	2.4	0.023
	Cl$^-$ $^+$NH$_3$								
Glycine	H	377 ± 8	9.18	28.4	3.8	0.84	7	8.4	0.072
Phenylalanine	CH$_2\phi$	376 ± 17	8.28	9.9	2.7	0.66	4	4.7	0.026
Serine	CH$_2$OH	319 ± 4	4.86	16.0	0.87	0.5	19	1.5	0.050
Aspartate	CH$_2$COO$^-$	306 ± 3	5.10	8.6	0.3	5.0	30	4.8	0.03
Glutamate	CH$_2$CH$_2$COO$^-$	306 ± 21	6.30	12.2	0.56	2.5	28	4.6	0.040
Asparagine	CH$_2$CONH$_2$	300 ± 5	11.1	2.3	0.37	1.4	7	1.8	0.008
Alanine	CH$_3$	270 ± 14	2.94	19.7	0.86	1.5	23	4.6	0.072
Phenylglycine	ϕ	177 ± 18	5.76	4.4					

order rate constant for imine formation:

$$f = [I_e]/[P_o] = K_o[A]/(K_o[A] + 1) \qquad (11)$$

$$k_{obs} = k_f'[A_T]/f = (-2.303/t) \log ([P_o] - [I]/f) \qquad (12)$$

where $[I_e]$ is the concentration of imine at equilibrium and f is the mole fraction of completion of imine formation at equilibrium. The left-hand side of equation (12) is solved for k_f':

$$k_f' = f(k_{obs}/[A_T]) \qquad (13)$$

Most measurements to be reported here were made in the range $0.3 < f < 0.9$.

Second-Order Rate Constants for Imine Formation and Equilibrium Constants for Carbinolamine Formation.— In Figure 4 k_f', the second-order rate constant for imine formation with glycine at a series of nearly constant values of pH, is plotted against the total concentration of glycine. The fan of curves that is obtained indicates that: (a) k_f' decreases as the extent of initial formation of intermediate carbinolamine increases; and (b) k_f', the corrected apparent second-order rate constant at constant pH, is composed of acid- and uncatalyzed rate constants that contribute nearly equally. Hence there is a modest increase in k_f' with pH at low $[A_T]$.

The equation describing k_f is derived as follows:

$$P + A_T \underset{\overrightarrow{}}{\overset{K_e'}{\rightleftarrows}} C_T \underset{k_{-3}'}{\overset{k_3'}{\rightleftarrows}} I \qquad (14)$$
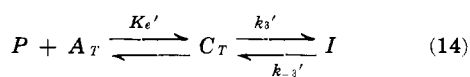
$$K_e' = [C_T]/[P][A_T] \qquad (15)$$

$$d[I]/dt = k_3'[C_T] - k_{-3}'[I]$$
$$= k_3'K_e'[P][A_T] - k_{-3}'[I] \qquad (16)$$

Under the experimental conditions $[A_T]$ is constant during the reaction. Material balance requires:

$$[P] = [P_o] - [C_T] - [I] \qquad (17)$$

Then

$$d[I]/dt = \bar{K}[P_o] - (\bar{K} + k_{-3}')[I] \qquad (18)$$

$$\bar{K} = k_3'K_e'[A_T]/(1 + K_e'[A_T]) \qquad (19)$$

At equilibrium:

$$\bar{K}[P_o] = (\bar{K} + k_{-3}')[I_e] \qquad (20)$$

Equation (11) is substituted in (20), and the result is solved for k_{-3}'.

$$k_{-3}' = \bar{K}/(1/f - 1) \qquad (21)$$

The integrated equation derived from (18) and (21) is:

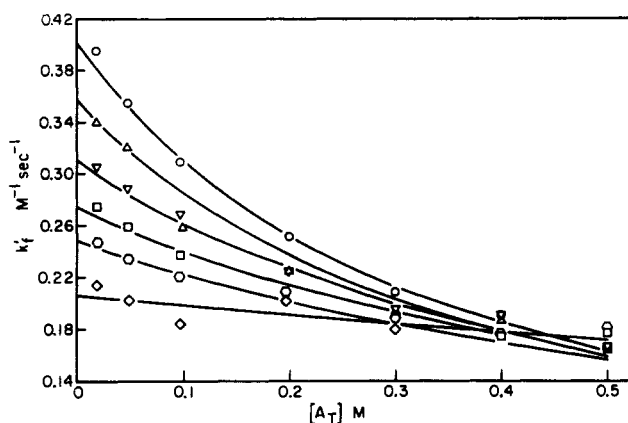$$k_{obs} = \bar{K}/f = (-2.303/t) \log ([P_o] - [I]/f) \qquad (22)$$

FIG. 4.—Plot of apparent second-order rate constants for the formation of 4-pyridylidenecarboxymethyl-amine (PCH=NCH$_2$COO$\ominus$) at 30° versus total glycine concentration. Key: —O—, pH 10.07—10.24; —△—, pH 9.67—9.73; —▽—, pH 9.49—9,56; —◇—, pH 9.30—9.41; —( )—, pH 9.15—9.21; —◇—, pH 8.91—8.98. The solid lines are calculated from equation (25); $K_e = 3.78$; $[A]$ is calculated from the Henderson-Hasselbach equation, where pH is taken as the average for a series of seven concentrations of A_T.

Equations (12) and (22) are set equal and solved for k_f'.

$$k_f' = k_3'K_e'/(1 + K_e'[A_T]) \qquad (23)$$

The true forward rate constant at constant pH, k_f, is equivalent to $k_3'K_e'$, and may be obtained graphically from equation (24):

$$1/k_f' = 1/k_f + (K_e'/k_f)[A_T] \qquad (24)$$

When plotted against pH, the values of K_e' calculated from equation (24) with the experimental points of Figure 4 fitted a titration curve for the dissociation of the amino group of glycine. Therefore equation (25) applies over the pH range that was investigated:

$$1/k_f' = 1/k_f + (K_e/k_f)[A] \qquad (25)$$

where K_e is the true equilibrium constant for the formation of neutral carbinolamine. Here K_e is 3.78 ± 0.17 (standard deviation) for glycine. Only approximate values of K_e (Table II) were obtained with other amino acids since rates were not studied beyond 0.05 M. Examination of oscillograph traces for the first few seconds of reaction of aldehyde with 0.5 M glycine revealed values of optical density that were lower than those ex-
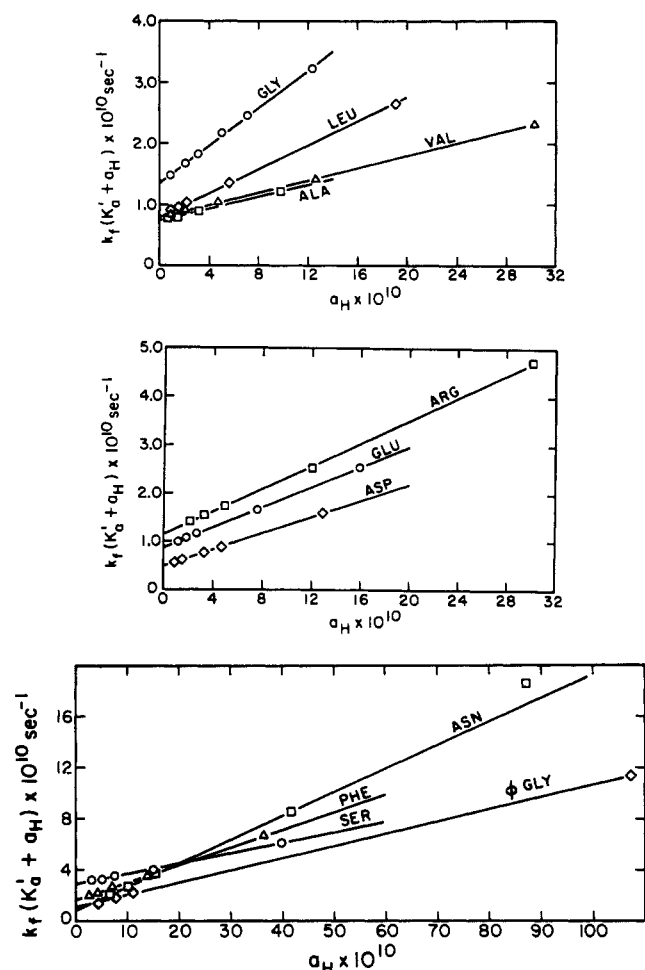
FIG. 5.—Determination of second-order rate constants for the formation of imines with pyridine-4-aldehyde and various amino acids. k_H' = slope and k_0 = intercept$/k_a'$.

pected for a mixture of imine and aldehyde alone. Therefore appreciable carbinolamine, which has a lower extinction than the more highly conjugated aldehyde and imine, must have been formed rapidly, in agreement with equation (14).

The average difference between k_f calculated from equation (25) and k_f' at 0.2 M $[A_T]$ was found to be less than 1% for the amino acid with the highest K_e, namely, glycine. It was therefore possible to use reliable measured values of pH and pK_a' for 0.02 M $[A_T]$ in the calculation of the second-order rate constants $(k_H'$ and $k_0)$ for reaction of protonated (AH) and unprotonated (A) amino acid:

$$k_f \cong k_f' \text{ at } 0.02 \text{ M } [A_T]$$
$$= k_H'[a_H/(K_a' + a_H)] + k_0[K_a'/(K_a' + a_H)] \quad (26)$$

where a_H is the hydrogen-ion activity. Plots of k_f-$(K_a' + a_H)$ versus a_H yield straight lines characteristic of each amino acid (Fig. 5) with slope equal to k_H' and intercept equal to $k_0 K_a'$. The values of k_H' and k_0 that are recorded in Table II were calculated by the method of least squares and the Student t distribution $(n = 5)$. The standard error generally lay between 3 and 8% at the 99% confidence level.

Rate Constants for Dehydration and Formation of Carbinolamine.—Values for K_0, K_e, k_H', and k_0 (Table II), and K_a' (Table I) were used to calculate k_1/K_a'', k_2, k_{-1}, and k_{-2} from equations (3b), (4b), and (6) (Table II). $k_1/K_a'' = k_H'/K_eK_a'$; $k_2 = k_0/K_e$;
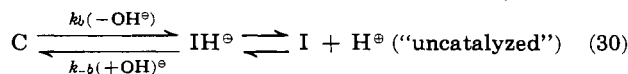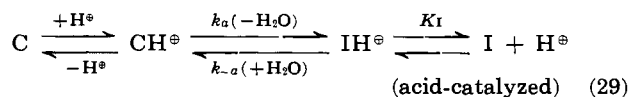
$$k_{-1} = K_ek_1/K_0K_a''; \quad k_{-2} = K_ek_2/K_0 \quad (27)$$

DISCUSSION

Mechanism of Imine Formation.—In the pH range $pK_a' \pm 0.6$, amino acids react with pyridine-4-aldehyde with pre-equilibrium formation of carbinolamine intermediate followed by rate-limiting dehydration. The acid-catalyzed and uncatalyzed terms in the dehydration step are of nearly equal importance. Between the highest and lowest second-order rate constants (Table

$$d[I]/dt = (k_H a_H + k_0) \text{ [NH}_2\text{-CHR-COO}^\ominus] \text{ [PCHO]};$$
$$k_H' = k_H a_H \cong k_0 \text{ at } pK_a' \pm 0.6 \quad (28)$$

II) there is only a 4-fold difference in k_H' and a 12-fold difference in k_0. For most amino acids, $1 < k_0/k_H' < 3$. Phenylalanine closely approaches the conditions for complete insensitivity of k_f to pH, since $k_H' \cong k_0$. Asparagine and phenylglycine are the only amino acids for which $k_H'/k_0 < 1$. Acid-catalyzed imine formation from carbinolamine is equivalent to the acid-catalyzed addition of water to imine in the reverse direction and uncatalyzed imine formation corresponds to the "uncatalyzed" step in imine hydrolysis (Willi, 1956). The

$$C \underset{-H^\ominus}{\overset{+H^\ominus}{\rightleftharpoons}} CH^\ominus \underset{k_{-a}(+H_2O)}{\overset{k_a(-H_2O)}{\rightleftharpoons}} IH^\ominus \overset{K_I}{\rightleftharpoons} I + H^\ominus$$
$$\text{(acid-catalyzed)} \quad (29)$$

$$C \underset{k_{-b}(+OH)^\ominus}{\overset{k_b(-OH^\ominus)}{\rightleftharpoons}} IH^\ominus \rightleftharpoons I + H^\oplus \text{ ("uncatalyzed")} \quad (30)$$

pH range investigated was too high to observe the change to rate-limiting amine attack that generally occurs in slightly acid solution (Cordes and Jencks, 1962, 1963) and it was too low to observe base-catalyzed dehydration (Kastening et al., 1956). No general acid or general base catalysis of carbinolamine dehydration by A or AH was detected at the relatively high concentrations of these species that were used in the glycine studies.

Figure 6, a Brönsted plot with an expanded pK_a' scale, shows that both the forward and reverse rate constants for both acid-catalyzed and uncatalyzed pathways increase moderately with the pK_a' of the amino acid. The relatively low correlation coefficients, 0.4–0.6, are to be expected for bases that differ in steric and electrostatic contributions of the side chains. These results may be rationalized as follows. Let the pK_a' values of carbinolamine or imine increase with the pK_a' of the amino acid used to form the carbinolamine or imine. In the case of uncatalyzed imine formation, the higher the pK_a' of AH, the higher will be the pK_a' of $CH^\oplus$, and the more rapid the expulsion of hydroxide ion from C. The higher the pK_a' of $CH^\oplus$, the higher will be the acid-catalyzed rates of dehydration at alkaline pH. Finally, the rates of hydrolysis of imine at alkaline pH are proportional to $[IH^\oplus]$, which will increase with the pK_a' of the imine. The slightly enhanced rates of imine formation with aspartate (Fig. 6, top) may possibly involve an electrostatic effect of the charged carboxyl of the side chain, or intramolecular general base catalysis of dehydration.

p-Nitrobenzylidenethylamine (Cordes and Jencks, 1963) is the previously investigated compound that is most closely analogous to pyridylidenecarboxymethylamine (PCH=NCH$_2$CO$^-$). The electronic effects of the substituent groups, 4-pyridyl and p-nitrophenyl, are similar (Mosher, 1950). A steady-state treatment similar to that of the former authors yields:

$$k_{obs} = \frac{k_P(k_{-1}a_H + k_{-2})}{(k_1 a_H/K_a'' + k_2 + k_P)},$$
$$a_H << K_i \cong 10^{-6} \text{ to } 10^{-7} \quad (31)$$

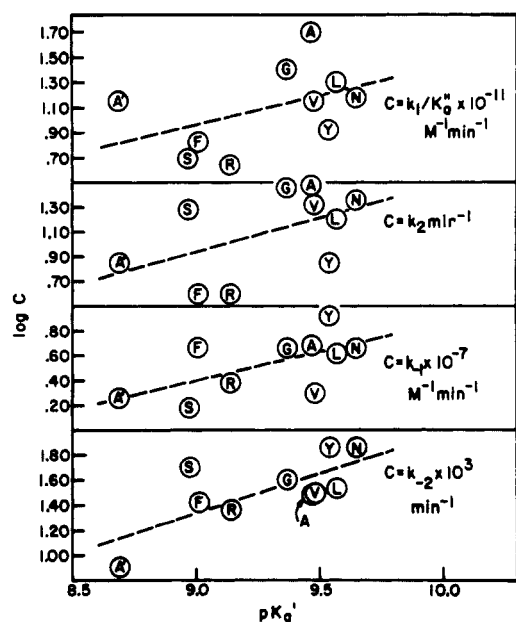where k_P is the rate constant for formation of aldehyde

Fig. 6—Relationship of rate constants for imine formation and hydrolysis to pK_a' of amino acid. The dashed lines, which were calculated by the method of least squares, have slopes of 0.5—0.6. The letters within the circles represent initials of the amino acids studied, except: A' = asparagine, N = alanine, Y = glycine, F = phenylalanine, R = arginine.

and amino acid from carbinolamine. Since C is at low steady state at low $[A_T]$ and dehydration is rate limiting, $k_P \gg k_2$. It can also be shown that $k_P \gg k_1 a_H/K_a''$ at alkaline pH, and therefore equation (31) is simplified:

$$k_{obs}(\text{min}^{-1}) = k_{-1}a_H + k_{-2}$$
$$= 8.4 \times 10^7 a_H + 0.072 \ (30°) \quad (32)$$

The corresponding equation for p-nitrobenzylidenethyl amine is approximately (Cordes and Jencks, 1963):

$$k_{obs}(\text{min}^{-1})$$
$$= 2 \times 10^7 a_H + 0.28 \ (a_H \ll K_I) \quad (25°) \quad (33)$$

The two curves associated with equations (32) and (33) are shown in Figure 7. The 4-fold increase in k_{-1} and decrease in k_{-2} that is apparent in comparison of equation (32) with (33) may represent an electrostatic effect of the ionized carboxyl of $PCH=NCH_2CO^\ominus$. The anion may stabilize the transition state for attack of water on protonated imine, and hinder the attack of hydroxide ion on protonated imine.

Velick and Vavra (1962) have shown that the maximal velocity of glutamic-aspartic transaminase is independent of pH over the range 6–9. The first step in the enzymatic reaction is postulated to involve the formation of an imine from free amino acid ($RCH(NH_2)\-COO^\ominus$) and enzyme-bound cofactor. (If free amino acid were first bound noncovalently to enzyme before imine formation without acid catalysis, then the maximal velocity would increase markedly from pH 6 to 9, as the fraction of bound $RCH(NH_2)COO^\ominus$ increased.) This observed pH insensitivity of velocity of enzymatic transamination under mildly alkaline conditions may indicate the presence of acid-catalyzed and uncatalyzed pathways for enzymatic imine formation that are of nearly equal importance. However, the fact that the acid-catalyzed rates of dehydration of carbinolamine ($0.4–5 \times 10^{10}$ M^{-1} min^{-1}) are faster than the apparent over-all enzymatic rate for imine formation with glutamate (2×10^9 M^{-1} min^{-1}, Hammes and Fasella, 1963
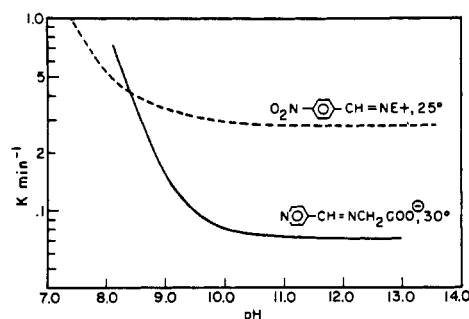

Fig. 7.—Comparison of first-order rate constants for the hydrolysis of pyridylidenecarboxymethylamine (—) at 30° and p-nitrobenzylidenethylamine (- - -) at 25° (Cordes and Jencks, 1963) in the region of alkaline pH.

suggests that transaminases might dehydrate carbinolamine intermediates by acid catalysis.

Although no general acid term was observed for imine formation in the presence of high concentrations of AH, intramolecular general acid catalysis may be favored over intermolecular specific acid catalysis. The rate constant for acid-catalyzed dehydration of carbinolamine is represented formally as k_1/K_a'', where K_a'' is presumed to be the acid-dissociation constant for carbinolamine that is protonated on nitrogen. If protonation of carbinolamine were rate limiting ($ca. 2 \times 10^{12}$ M^{-1} min^{-1}, see Emerson et al., 1960), then the maximum velocity for acid-catalyzed dehydration of carbinolamine would be $ca.$ 2000 min^{-1} at pH 9. The values of apparent pK_a'' for the carbinolamines that were studied would then be 6.5–7.5, a result that supports the assumptions of equation (3), protonation of the amino nitrogen of carbinolamine, followed by dehydration. Clearly, acid-catalyzed dehydration cannot proceed by decomposition of carbinolamine that is protonated on hydroxyl ($CH^{\oplus\prime}$), $pK_a = ca. -2$ (Arnett and Anderson, 1963), because the unfavorable equilibrium constant for the proton shift from N to O would require an impossibly large value, $ca. 10^{12}$ min^{-1}, for the rate constant for decomposition of $CH^{\oplus\prime}$. Therefore, dehydration probably occurs by an intramolecular general-acid-catalyzed reaction.
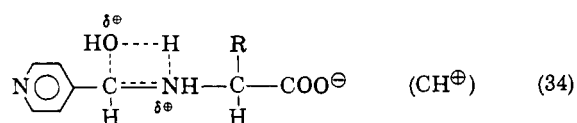
It is possible that one of the two, the lysine residue (Fischer et al., 1963) that is present in the vicinity of the carbonyl of the coenzyme may function as an intramolecular general-acid catalyst, both in amine attack and in imine formation from an intermediate.

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Carbohydrates in Protein. VIII. The Isolation of 2-Acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose from Hen's Egg Albumin*

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2-Acetamido-3,4,6-tri-O-acetyl-1-(α-benzyl N-benzyloxycarbonyl-L-β-aspartamido)-1,2-dideoxy-β-D-glucose (II) was synthesized by the condensation of 2-acetamido-3,4,6-tri-O-acetyl-1-amino-1,2-dideoxy-β-D-glucose with α-benzyl N-benzyloxycarbonyl-L-aspartate. The latter was isolated by countercurrent distribution from the products obtained by the condensation of benzyl alcohol with N-benzyloxycarbonyl-L-aspartic anhydride. Compound II was converted to pure 2-acetamido-1-[L-β-aspartamido)-1,2-dideoxy-β-D-glucose (I) by a catalytic hydrogenation followed by treatment of the compound thus obtained with 0.6 N LiOH at room temperature. In addition a compound (Ia) was isolated by chromatographic and electrophoretic techniques from a partial acid hydrolysate of a glycopeptide from hen's egg albumin, and this was shown to yield aspartic acid, glucosamine, and ammonia in molar amounts when it was hydrolyzed by acid. Some properties of compounds I and Ia were compared and it was shown that they were identical. The nature of the carbohydrate-peptide bond in this protein is thus demonstrated.

A glycopeptide was isolated by a nonchromatographic method from a tryptic hydrolysate of egg albumin by one of us in 1938 (Neuberger, 1938). This material yielded approximately 1 mole of ammonia when it was hydrolyzed with acid (5 N HCl, 3 hours, 100°). As a result of observations on the effect of alkali on this substance it was suggested that part of the glucosamine in the glycopeptide was bound to a nitrogen-containing substance, which was much later identified by a number of workers as aspartic acid (Cunningham *et al.*, 1957; Jevons, 1958; Johansen *et al.*, 1958; Kaverzneva and Bogdanov, 1960). From a study of the properties of similar glycopeptides Johansen *et al.* (1961) and Nuenke and Cunningham (1961) independently suggested that the linkage between aspartic acid and the carbohydrate moiety was formed by the condensation of a β-aspartyl residue with a 1-amino sugar, but the question of whether the latter was derived from mannose or N-acetylglucosamine was left open. Direct evidence was adduced for the participation of the β- rather than the α-carboxyl group of aspartic acid in the linkage by the conversion of a glycopeptide containing aspartic acid as the only amino acid residue to the corresponding derivative in which the aspartic acid appeared as the phenylthiohydantoin, and this was achieved without

splitting the carbohydrate-peptide bond (Fletcher *et al.*, 1963a).

That the sugar involved in the linkage was indeed N-acetylglucosamine was shown by the isolation from partial acid hydrolysates of glycopeptides of substances which yielded aspartic acid and glucosamine but no mannose on acid hydrolysis (Yamashina and Makino, 1962; Bogdanov *et al.*, 1962; Marks *et al.*, 1962, 1963). The production of approximately 1 mole of ammonia also from these fragments under similar conditions (Yamashina and Makino, 1962; Bogdanov *et al.* 1962) was in accord with its probable identification as 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose (I).

Two groups of workers (Marks *et al.*, 1963; Bolton and Jeanloz, 1963) have described the synthesis and characterization of 2-acetamido-3,4,6-tri-O-acetyl-1-(α-benzyl N-benzyloxycarbonyl-L-β-aspartamido)-1,2-dideoxy-β-D-glucose (II). One set of conditions used for removal of the protecting groups was shown to be associated with some side reactions (Marks *et al.*, 1963). It is the purpose of the present communication to describe first a procedure whereby these protecting groups may be removed to yield the desired model compound in almost quantitative yield without the occurrence of undesirable rearrangements. Second, the preparation from egg albumin of crystalline material which yields glucosamine, ammonia, and aspartic acid on acid