A Nuclear Magnetic Resonance Investigation of the Metal Ion and Proton-Catalyzed Reaction of Some Vitamin B_6 Schiff Bases[1,2]

Edwin H. Abbott and Arthur E. Martell

Contribution from the Department of Chemistry, Texas A&M University, College Station, Texas 77843. *Received June 9, 1969*

Abstract: By means of nmr measurements in D_2O solution, a new vitamin B_6 catalyzed reaction—β-proton exchange of α-amino acids—has been studied by both proton and metal ion catalysis. β deuteration of α-aminobutyric acid and valine have been studied as a function of pH (pD) in the presence and in the absence of Zn(II) and Al(III) ions. Progress of the reactions through disappearance of reactants, appearance of Schiff bases, and formation of deuterated products was followed by nmr measurements. Similar studies are reported on the deuteration of α-ketobutyric acid. The interferences of possible side reactions, such as metal ion- and proton-catalyzed aldol condensation of the α-keto acids are measured and discussed. Mechanisms are proposed for β-proton exchange in which either Schiff bases or Schiff base metal complexes are reactive intermediates. These reactions are also considered significant since they provide the first model systems in which unsaturation is introduced into the amino acid or keto acid moiety of the Schiff base by electron withdrawal. The biological implications of the proposed new reaction mechanisms are discussed.

Vitamin B_6 is known to catalyze many reactions of α-amino and α-keto acids in living systems. In aqueous media containing pyridoxal or pyridoxamine, many of these reactions can be carried out in the absence of enzymes. Metzler, Ikawa, and Snell[3] and Braunstein[4] have suggested mechanisms by which these model reactions may proceed, and their mechanisms have been used for inferring possible mechanisms of the analogous enzyme systems.

Publications of Bender[5] and Westheimer[6] have presented strong evidence that protonated Schiff bases are the reactive intermediates in the primary amine catalyzed enolization of acetone and of acetaldehyde polymerization. Since vitamin B_6 is, in one form, the primary amine pyridoxamine, it is of interest to examine its effect on the enolization of keto acids and to explore the biological implications of the possibility that this reaction occurs through a Schiff base intermediate.

In this paper, the pyridoxamine-catalyzed β deuteration of α-ketobutyric acid is examined in the absence and presence of zinc(II) ion. The results are interpreted in terms of a mechanism in which either a Schiff base or a Schiff base–metal complex is the reactive intermediate. The reaction is also used to deuterate the β position of valine and α-aminobutyric acid in the presence of aluminum(III) ion and pyridoxal.

Experimental Section

Materials. Pyridoxal hydrochloride, pyridoxamine dihydrochloride, valine, and α-aminobutyric acid were obtained from Mann Laboratories as "Mann Analyzed" grade and were used without further purification. α-Ketobutyric acid from the same source, certified to be better than 99.5% pure, was stored in a refrigerator and used soon after receipt. Sodium α-ketobutyrate was prepared by dissolving the α-ketobutyric acid in anhydrous methanol and adding a less than stoichiometric quantity of sodium methoxide dissolved in methanol. The precipitate which formed was filtered and washed with methanol and then with ether and dried under vacuum over P_2O_5. When the resulting finely divided colorless solid was dissolved in D_2O, its nmr spectrum was identical with that of α-ketobutyric acid solutions which had been neutralized with NaOD. Pyridoxol was obtained from Matheson Coleman and Bell and was recrystallized from ethanol–acetone solution. Its melting point was 206–207° dec. Zinc(II) ion was used as a stock solution of $ZnCl_2$ obtained from Mallinckrodt. The solution was standardized by titration with ethylenediaminetetraacetic acid (EDTA) in the presence of eriochrom black T (EBT) as an indicator. The D_2O was 99.5 mol % from Matheson Coleman and Bell and 99.8 mol % from Mallinckrodt. Sodium deuteroxide was prepared by dropping D_2O onto clean bright reagent grade sodium metal in an inert atmosphere. Reagent grade sodium chloride and potassium nitrate were used to control ionic strength. Tetramethylammonium chloride was obtained from Eastman Kodak Co. It was recrystallized from ethanol–water solution and washed with chloroform.

Measurements. Nmr spectra were recorded primarily with the Varian HA 100 nuclear magnetic resonance spectrometer. A Varian A 60 nuclear magnetic resonance spectrometer was used to verify the assignments of resonances due to coupling. Solvents were D_2O or H_2O. Chemical shifts are reported in parts per million with respect to the center of the tetramethylammonium chloride resonance; positive values represent resonances at higher field than the reference. Temperature was controlled at 30° with a Varian variable-temperature controller for the more rapid reactions. For the slower reactions, samples were stored in a constant-temperature bath and spectra were taken periodically.

The pD was determined with a Beckman Model G pH meter fitted with a Beckman one-drop glass electrode system. Beckman buffers were used to calibrate the instrument. The relationship, $pD = pH + 0.40$ was used to determine pD.[7] Values of activity were converted to concentration by means of tabulated activity coefficients.[8]

Preparation of Solutions. Solutions of pyridoxamine and the keto acid were prepared in D_2O. Exchange of the β protons of the ketobutyrate is quite slow under strongly acid conditions and this stock solution therefore was, on occasion, stored for short periods of time in a refrigerator without detectable deuteration. Aliquots were pipetted into volumetric flasks. Appropriate volumes of base and other components were added and the solutions were then

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(2) This work was supported by the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, under Research Grant No. AM11694.

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

(4) A. E. Braunstein and M. M. Shemyakin, *Biokhimiya*, **18**, 393 (1953).

(5) M. L. Bender and A. Williams, *J. Amer. Chem. Soc.*, **88**, 2502 (1966).

(6) F. H. Westheimer and M. Cohen, *ibid.*, **57**, 2674 (1935).

(7) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

(8) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolyte Solutions," Reinhold Publishing Corp., New York, N. Y., 1958.

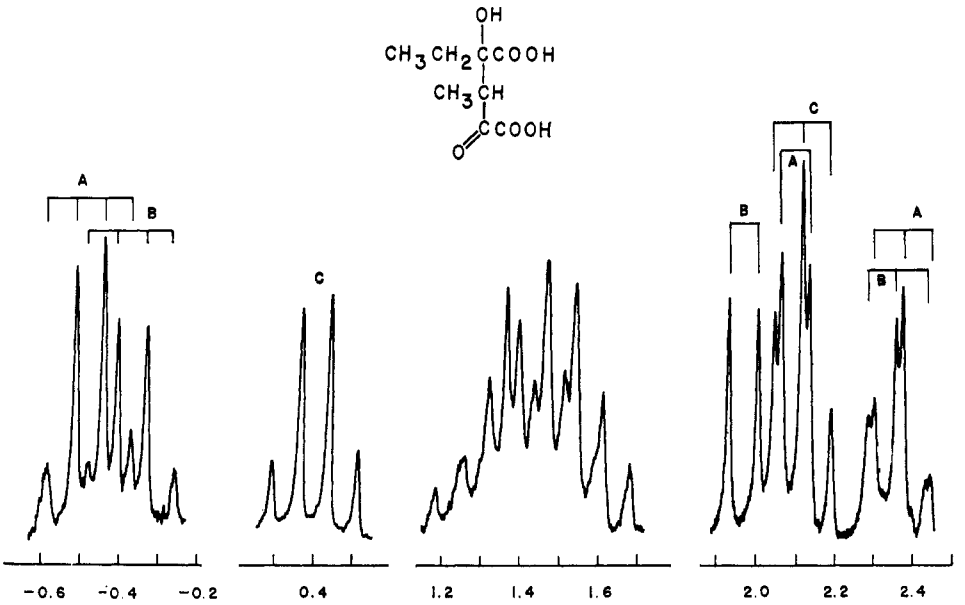


Figure 1. The 100-MHz nmr spectrum of a solution of AKBA in equilibrium with dimeric forms in H_2O.

diluted to the desired volume. In some instances base was added to known volumes of stock solution and the concentration was calculated from the resulting volume.

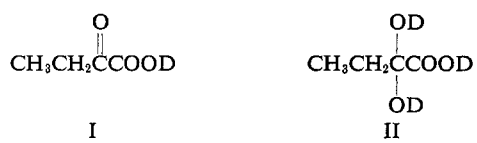
Between pD 8.0 and 10.5 pyridoxamine dihydrate precipitates from pyridoxamine solutions in the concentration range studied. However, with scrupulously clean glassware, it was possible to make kinetic runs before precipitation occurred. When zinc(II) chloride was added to pyridoxamine α-ketobutyrate solutions above pD 7, a precipitate formed and then slowly redissolved. Quantitative kinetic data were unobtainable under these conditions because the exchange reaction was faster than the dissolution of the precipitate.

Measurement and Treatment of Nmr Data. The rate of enolization was followed by measuring the decrease in the integral of the β-proton signal. When the 2-methyl peak of the pyridoxamine was close to the β-proton region of the keto form of the acid, the integral over both was taken and the integral over the γ-methyl group of the acid, multiplied by the ratio of pyridoxamine to ketobutyrate, was subtracted from it. The remainder was divided by the integral over the γ-methyl protons of the acid. Above pD 6, the integral over the β protons of the keto form was sufficiently isolated that it could be determined directly.

The logarithm of the β-proton integral was plotted *vs.* time and the slope of the best straight line through these points was used to compute a pseudo-first-order constant. Consideration of the secondary isotope effect arising from the exchange of the mono β-deuterated form of the α-ketobutyrate was avoided by using only the points comprising the initial part of the reaction. Second-order constants were computed by dividing the pseudo-first-order constant by the total pyridoxamine concentration.

Results

α-Ketobutyric Acid Spectrum. The spectrum of α-ketobutyric acid (hereafter abbreviated AKBA) at pD 1.4 consists of quartets of 0.30 and 1.35 ppm and triplets at 2.16 and 2.32 ppm. Comparison of the relative areas of the multiplets as pD is varied in the region shows that the AKBA is in two forms, and the quartet at 0.30 is consistent with a methylene group adjacent to a carbonyl group while the quartet at 1.35 indicates that the methylene group is adjacent to a saturated carbon atom. Therefore, the resonances at 0.30 and 2.16 are assigned to the keto form, I, and the resonances at 1.35 and 2.32 are assigned to the hydrate form, II. This is analogous to the results for pyruvic acid in strongly acid media.[9] In addition, the quartet at 1.35 is broader than the one at 0.30. Since this is the case even when the two forms are in approximately equal concentrations, this cannot be due to interconversion of the two species but rather must result either from exchange of the alcoholic deuterons of II with the solvent or from long-range coupling of these atoms to the methyl group. The triplet at 2.32 is not substantially broadened with respect to the one at 2.16.



I

II

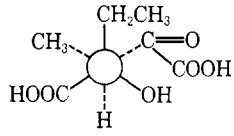
When the pD is raised, the hydrated form decreases in relative concentration until at pD 4 the spectrum is nearly completely that of the keto form. The disappearance of the hydrate is immediate within the time scale of the experiments; however, small unresolved peaks remain in the region of 1.3 and of 2.3 ppm. In the absence of catalyst and between pD 4.0 and 7.0, the exchange of β protons is very slow and is not observed after several days at 30°.

In the presence of zinc(II) ion or at high pH, a very different spectrum is obtained (Figure 1). In H_2O the spectrum has quartets at −0.475 and −0.365, doublets at 1.975 and 2.105, triplets at 2.365 and 2.380, and a number of unresolved peaks between 1.2 and 1.7. In D_2O all resonances vanish. In the methyl region, the doublets and triplets are all replaced by broadened singlets 1 Hz to higher field. This shift to higher field[10] and loss of multiplicity is consistent with the deuteration of the carbon atoms adjacent to the methyl groups.

On the basis of spin decoupling experiments and of relative areas, the quartet at −0.475 arises from a proton on a carbon atom adjacent to the methyl group that gives the doublet at 2.105. Similarly the quartet at −0.365 is associated with doublet at 1.975. The triplets at 2.365 and 2.38 are both associated with multiplets in the region 1.2 to 1.7. In addition to these peaks, the AKBA keto form quartet is observed at 0.41 and the triplet at 2.12 (C in Figure 1).

(9) D. L. Leussing and C. K. Stanfield, *J. Amer. Chem. Soc.*, **86**, 2805 (1964).
(10) H. S. Gutowsky, *J. Chem. Phys.*, **31**, 1683 (1959).

These results are consistent with the formation of an AKBA dimer by condensation of the methylene group of one molecule with the carbonyl group on another. This dimer exists in two diasteriomeric forms, III and IV, which have different nmr spectra (A and B, Figure 1) because of the different steric requirements and possibilities for hydrogen bonding. The dimerization constant (eq 1) was computed from the relative concentra-



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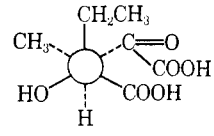


IV

tions of the respective species at known total concentrations. The constant was found to be 5.5 ± 0.5 in the range of total initial monomer concentrations of 0.2 to 1.5 *M*.

$$K_d = \frac{\text{dimer}}{(\text{monomer})^2} \qquad (1)$$

After a day at 30°, further alterations were observed to have occurred in the AKBA spectrum in the presence of zinc(II) ion. A sharp singlet grows in progressively at 1.42 ppm and a triplet grows in at 2.42. In D_2O the singlet is unaltered but a broad singlet appears in place of the triplet. This reaction is more rapid in weakly acid solution than in neutral or weakly basic solution. After many days, the dimer is converted primarily to this form.

AKBA β Deuteration. In D_2O from pD 4 to pD 8, and in the presence of pyridoxamine or of general bases such as acetate ion, the methyl triplet of AKBA undergoes the alterations shown in Figure 2. Two broadened peaks grow in with a center about 0.7 Hz to higher field than the triplet. With smaller sweep widths, these new peaks are seen to each be a 1:1:1 triplet. Comparison with spectra run at 60 MHz shows that the two new peaks are a doublet, over-all, with a coupling constant of 7.0 Hz, identical with the coupling constant for the normal AKBA triplet. Eventually, both the new doublet of triplets and the original triplet are replaced by a broad singlet at 0.5 Hz to still higher field. During the time scale of these changes, the methylene resonances broaden and decrease steadily in area. No such changes occur when H_2O is the solvent. Therefore, the reaction being observed is the deuteration of the β position of the AKBA.

The same reaction is observed at a higher rate when small amounts of zinc(II) chloride are added to solutions of pyridoxamine and AKBA in D_2O. In both the metal-free and metal-catalyzed cases, the thermodynamically more stable dimer is formed subsequently to the exchange of the β protons even with initial AKBA concentrations of 1.0 *M* or more.

Zinc(II) ion serves to catalyze the enolization of AKBA, a reaction which has been shown to be subject to general base catalysis by acetate ion.[11] However, above pD 4.5, the changes in the nmr spectrum of the methyl region of AKBA are quite different than when pyridoxamine is present. This is illustrated in Figure 3. Initially, the reaction appears as though it were being carried out in H_2O. That is, the spectrum of the dimer which is formed initially (Figure 3B) is identical

(11) A. Schellenberger and G. Huebner, *Ber.*, 98, 1938 (1965).

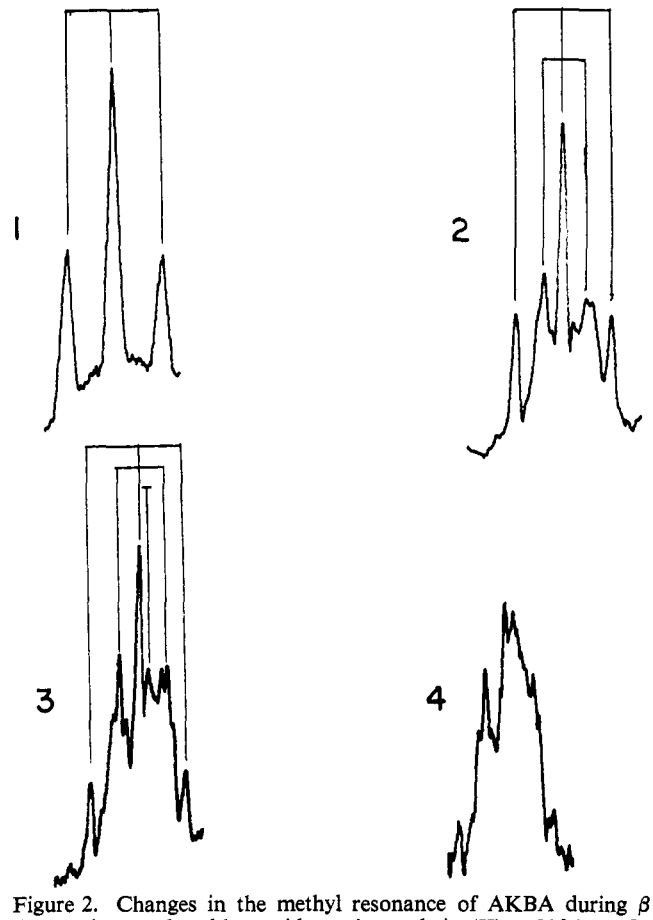


Figure 2. Changes in the methyl resonance of AKBA during β deuteration catalyzed by pyridoxamine and zinc(II) at 30° in D_2O: 1, initially; 2, 15 min.; 3, 30 min.; 4, 1 hr.

with that of the dimer formed in H_2O (Figure 1). Only later does the β-deuterated dimer V form as is indicated by the appearance of singlets shifted to higher field in place of the doublets and triplets of the protonated dimer (Figure 3C and 3D). This efficiency of dimerization of AKBA in the presence of zinc(II) ion is observed whether the reaction is slower than the pyridoxamine-catalyzed case, as in the absence of acetate ion, or more rapid as in 1 *M* acetate ion. When the AKBA concentration is high enough to conveniently observe the dimer, it is found that only at the lowest concentrations of zinc(II) and at the lower pD's does exchange precede dimerization as it does in the pyridoxamine-catalyzed case. Thus, in the region above pD 4.5, the mechanism of the pyridoxamine, zinc(II) catalysis of the deuterium exchange of AKBA is different from the zinc(II) catalysis in the absence of pyridoxamine.

Quantitative Studies. Figure 4 shows the variation of the second-order rate constant with $-\log [D^+]$ for the pyridoxamine-catalyzed deuterium exchange of AKBA. Addition of millimolar EDTA did not significantly change the rate constants nor did doubling the ionic strength from 1.0 to 2.0 have a significant effect.

The general base catalysis due to the phenolic group of pyridoxol VI was also investigated in the same way as was the pyridoxamine-catalyzed case. The second-order constant at 30° was found to be 0.9×10^{-3}.

In Figure 5, the dependence of the second-order rate constant with $-\log [D^+]$ is shown for samples initially 0.4 *M* in pyridoxamine and AKBA and 0.04 *M* in zinc(II) ion. The rate constant is everywhere greater

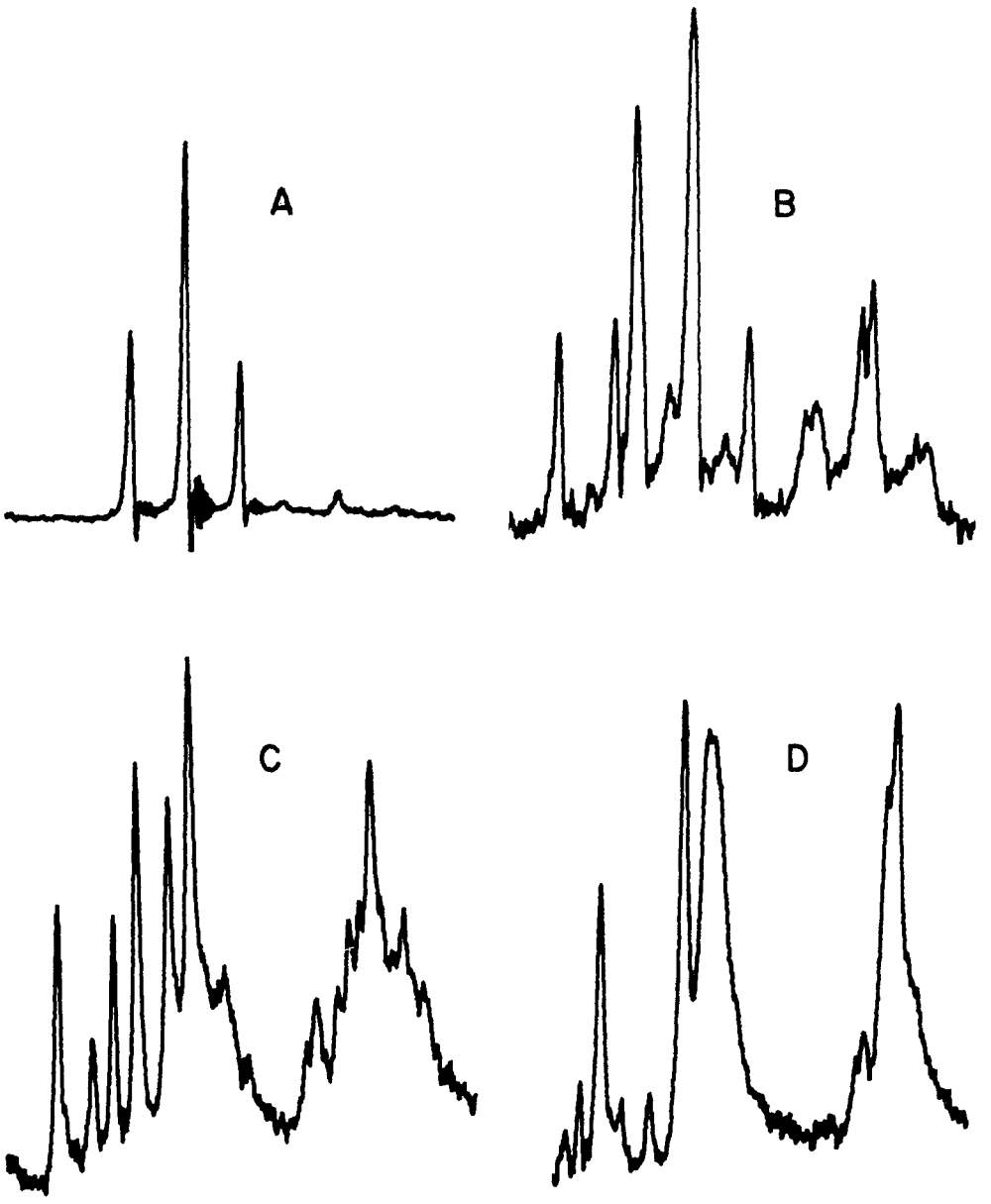


Figure 3. Changes in the methyl resonance of 1*M* AKBA during reaction with acetate ion and zinc(II) at 30° in D_2O: A, initially; B, 20 min; C, 1 hr; D, 2 hr.

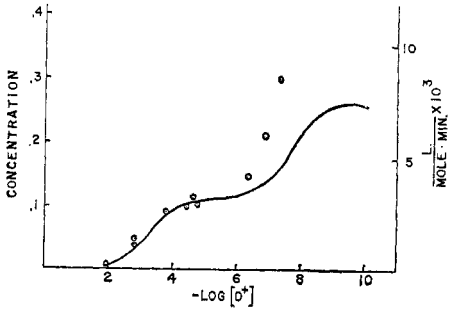
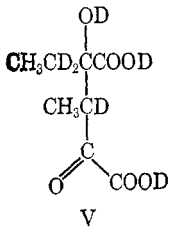


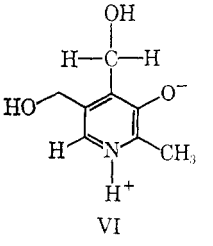
Figure 4. A comparison of rate constant for deuterium exchange with Schiff base formation for 0.40 *M* AKBA and 0.40 *M* pyridoxamine at 30° in D_2O. The points are rate constants and the line is the calculated molar concentration of Schiff base.

than in the absence of zinc(II) ion. The dependence of rate constant on zinc(II) concentration was linear for solutions initially 0.4 *M* in pyridoxamine and AKBA.

α-Deuterio-, β-Deuteriovaline. The nmr spectrum of protonated valine consists of doublets at 2.25 and 2.20, one for each of the magnetically nonequivalent methyl groups; a multiplet at 0.9, the β proton; and a



V



VI

doublet at −0.42, the α proton. When a 1.0 *M* valine solution buffered at pD 5.9 with acetate and containing 0.2 *M* pyridoxal and 0.1 *M* aluminum(III) ion, is incubated at 100° in a steam bath, the β and α resonances decrease relative to the methyl resonances while two new somewhat broadened peaks grow in with a center

shifted to slightly higher field than the center of the original four-line pattern. At 100 MHz, the distance between these new resonances is 5.0 Hz while at 60 MHz, it is 3.0 Hz. This distance between the two methyl doublets of valine is also 5.0 Hz at 100 MHz and 3.0 Hz at 60 MHz. Addition of H_2O and continued heating regenerates the four-line pattern of the protonated valine. Therefore, α and β deuteration of the amino acid are occurring. Accordingly, no such alterations of the valine spectra are observed when the solvent is H_2O, even after 14 days of incubation.

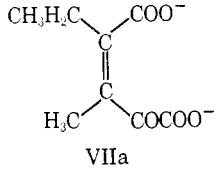
The β deuteration also occurs with copper(II) and zinc(II) as catalysts. Because of the paramagnetism of the copper(II) complexes, 0.010 *M* copper(II) was used, but with 0.10 *M* zinc(II) the reaction was slower than with 0.10 *M* aluminum(II) ion. In the absence of these metal ions, the reaction is much slower.

Experiments at other pD's showed that β deuteration is very slow below pD 4. At pD's above 7, the rate of exchange of the α proton became progressively more rapid with respect to β exchange as the pD was raised. At pD 9, the α doublet was observed to have vanished within 8 hr. Small amounts of precipitate were noticed in many of the experiments.

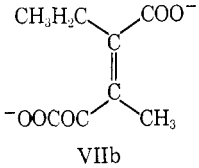
α-Deuterio-, β-Dideuterio-α-aminobutyric Acid. The nmr spectrum of α-aminobutyric acid consists of a triplet at 2.22, the methyl resonance; a multiplet at 1.305, the methylene resonance; and a triplet at −0.515, the α-proton resonance. This amino acid is also deuterated under the same conditions as those for valine except that the reaction is more rapid than with valine. The outer lines of the methyl triplet are seen to decrease steadily while the central line becomes larger and broader and shifts progressively to higher field, the transformation being complete in about 24 hr. During the same time span, the integrals over the α and β positions decrease steadily. No doublet is observed as would be expected from the mono β-deuterated, mono β-protonated form. Thus, the monodeuterio form of the amino acid is not an intermediate in the deuteration reaction.

Discussion

The Spectra of AKBA and of Pyridoxamine. The resonances at 1.42 and 2.42 which appear gradually in the dimer spectrum in the presence of zinc(II) suggest the formation of an unsaturated compound, perhaps the dehydrated dimer VIIa or VIIb. However, al-



VIIa



VIIb

ternatives cannot be ruled out, particularly since both species should be present and have different spectra. At any rate, these resonances cannot be attributed to the lactone, such as has been reported in the pyruvic acid system.[12] The results for the AKBA keto form and hydrate are similar to those of previous workers.[9,13] The pyridoxamine resonances agree with the assignments made by Korytnyk and Singh[14] and the dependence of the chemical shifts with pD agrees with that reported by Gansow and Holm.[15]

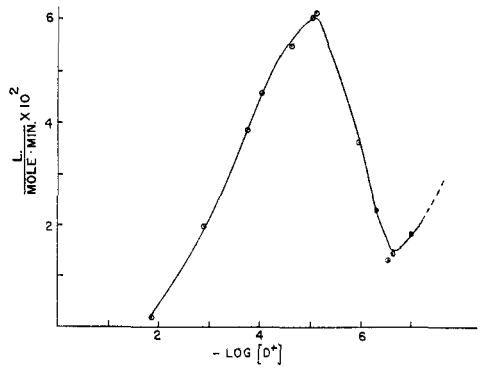


Figure 5. The dependence of rate constant for deuterium exchange of AKBA with $-\log [D^+]$ for 0.4 *M* AKBA, 0.4 *M* pyridoxamine, and 0.04 *M* zinc(II) ion at 30° in D_2O.

Pyridoxamine, AKBA Schiff Base Spectra. In the absence of zinc(II) ion, the resonances of the ketimine are clearly observed, as has recently been reported for the pyridoxamine–pyruvate system[16] and, though the resonances of the pyridoxamine portion of the Schiff base are sharp, those from the β protons of the AKBA are not. At pD 8, where the Schiff base is about half formed for initial pyridoxamine and AKBA concentrations of 0.4 *M*, a sharp quartet is observed for the free AKBA at 0.46 ppm and a broad absorption is observed at 1.18. The latter resonance is probably due to the β protons of the ketimine although it is surprising to find it shifted to so high a field since the ketimine is most likely protonated at the azomethine nitrogen in this range. In addition to this resonance, integration shows a broad absorption from 1.1 to 1.6 ppm. The hydrated form of the free keto acid absorbs in this region, but the sharpness of the quartet of the keto form precludes attributing this broad resonance to hydrate in rapid equilibrium with the keto form. Furthermore, no hydrate is observed under these conditions in the absence of pyridoxamine. This resonance can, however, be assigned to the Schiff base analog of the hydrate—the carbinolamine which is an intermediate in Schiff base formation.[17] Decreasing the pD from 7 to 6 causes the ketimine resonances to broaden and coalesce with the free pyridoxamine resonances. Since the free pyridoxamine is in excess of the Schiff base under these conditions, this is observed, in the limit, as a broadening of the base of the sharp pyridoxamine resonances. In this range and down to pD 4, integration shows that there are broad resonances in the range of 1.1 to 1.7, possibly due to the β protons of the carbinolamine. Below pD 4, the fairly sharp resonances due to the hydrated form of the keto acid are observed in this region.

In all cases, the changes in the pyridoxamine AKBA spectra due to Schiff base formation were immediate with respect to the time required to take the spectrum. This is indicative of the rapid equilibrium between the Schiff base and its components observed in a spectro-

(12) L. Wolff, *Ann. Chem.*, **305**, 154 (1899).
(13) G. Oejelund and I. Wadsoe, *Acta Chem. Scand.*, **21**, 1408 (1967).
(14) W. Korytnyk and R. P. Singh, *J. Amer. Chem. Soc.*, **85**, 2813 (1963).
(15) O. A. Gansow and R. H. Holm, *Tetrahedron*, 4477 (1968).
(16) O. A. Gansow and R. H. Holm, *J. Amer. Chem. Soc.*, **90**, 5629 (1968).
(17) E. H. Cordes and W. P. Jencks, *ibid.*, **84**, 832 (1962).

photometric study of the pyridoxamine–pyruvate system.[18]

The zinc(II) Schiff base complex was not observed as a separate entity because of its rapid equilibrium with the free metal and free Schiff base. At $-\log [D^+]$ greater than 6, considerable broadening of the pyridoxamine 2-methyl resonance was observed, apparently the result of a slowing down in the exchange rate.

Pyridoxamine-Catalyzed Deuterium Exchange of AKBA. The Brønsted relationship, eq 2, shows that the higher the pK_a the greater the general base-catalytic

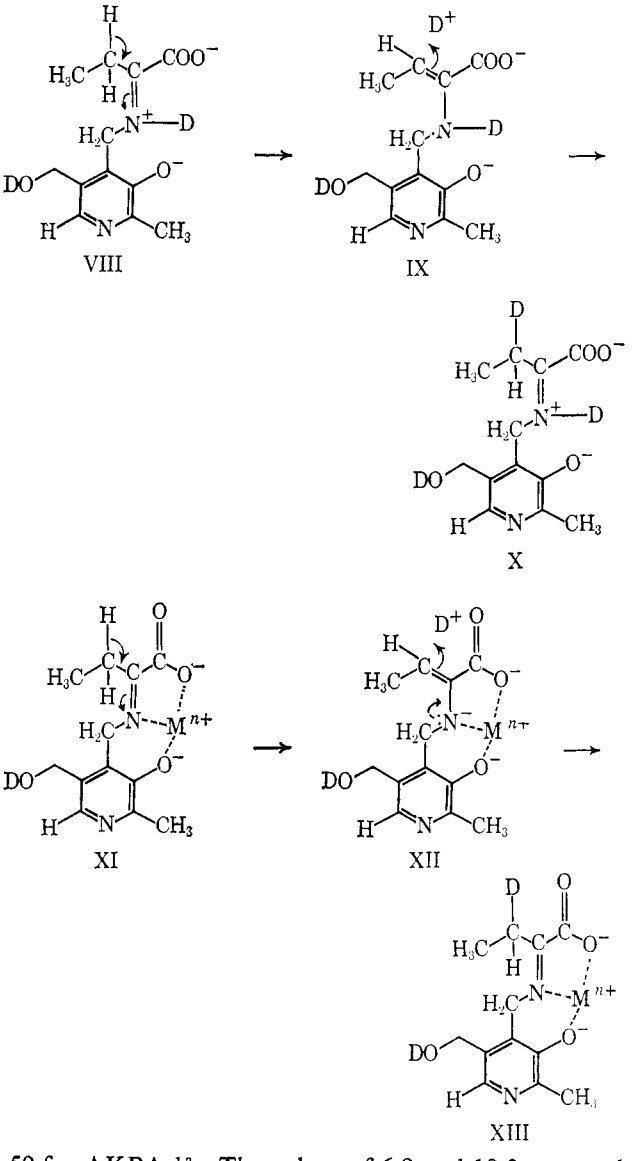
$$k_b \propto K_B^{\beta} \qquad (2)$$

constant. The rate constant for the catalysis by pyridoxamine ($pK_2 = 3.31$ in H_2O[19]) in the region of $-\log [D^+]$ from 4.5 to 6 is about 3×10^{-3} l. mol^{-1} min^{-1} while the rate constant for general base catalysis of AKBA enolization in water is about 5×10^{-4} l. mol^{-1} min^{-1}.[11] Thus, if a Brønsted plot were constructed with β equal to 0.5, the point for pyridoxamine catalysis would lie nearly two orders of magnitude from a line passing through the acetate point. This would not be surprising if the first pK of pyridoxamine were so low because the ions formed in the deprotonation were much more strongly solvated than the parent ion so that the pK observed in water was very much lower than the "intrinsic pK". Thus, the general base catalysis by phenolic group of pyridoxol ($pK_a = 4.7$[20] to 5.0[21] in H_2O) was investigated and its rate constant (0.9×10^{-3} l. mol^{-1} min^{-1}) was found to be less than a factor of 2 greater than that of the acetate ion. Pyridoxamine differs from pyridoxol in that it has an NH_3^+ group at the 4-methylene position rather than an OH group. Therefore, whatever its influence on the solvation of the ion, the NH_3^+ most probably exerts a greater electron-withdrawing effect on the phenolic oxygen than the neutral OH group does, and the rate constant for catalysis by pyridoxamine consequently should be much lower than for catalysis by pyridoxol; it is, however, observed to be greater by almost a factor of 4. Thus, an alternative mechanism for the pyridoxamine reaction must be sought.

A reasonable alternative is shown in Scheme I as VIII–X. The Schiff base that is formed between AKBA and pyridoxamine should react to exchange the β protons of the AKBA portion because the strongly electropositive deuterated azomethine nitrogen attracts the bonding electrons from the β carbon toward the α carbon according to the electron shift in VIII. Thus, the enhancement of the rate of exchange is due to the stability (*i.e.*, higher concentration) of the intermediate IX for the acid catalysis of imine–enamine tautomerization IX with respect to the intermediate for acid catalysis of the keto–enol tautomerization of the free keto acid.

A computation of the quantity of Schiff base in solution was carried out by the method used by Metzler for the Schiff base of pyridoxal and valine.[22] The pK's used were 3.31, 8.01, and 10.14 for pyridoxamine[21] and 2.50 for AKBA.[13] The values of 6.9 and 10.3 reported for the pyruvate pyridoxamine Schiff base[18] were used for the pK's of the very similar AKBA–pyridoxamine Schiff base. These pK's in H_2O were converted to pK's in D_2O by Bell's method.[23] The results of this calculation appear in Figure 4 with rate constants observed under similar conditions superimposed. Small variations of the several pK's do not change the over-all shape of the curve appreciably.

Scheme I. Metal-Free and Metal-Catalyzed Mechanisms for Schiff Base β-Proton Exchange



Above $-\log [D^+]$ of 7, the deviations of the curve reflect the increasing importance of base-catalytic factors in the exchange. This region could not be extensively investigated because of the insolubility of the neutral pyridoxamine hydrate, as mentioned in the Experimental Section; however, it was observed that the rate of exchange increases continuously instead of leveling off as does the concentration of Schiff base.

Below $-\log [D^+]$ of 6, the constancy of the second-order constant with varying concentrations indicated near first-order dependence on both keto acid and pyridoxamine. The failure to observe additional catalysis of the Schiff base reaction by the phenolic oxygen may

(18) B. E. C. Banks, A. A. Diamantis, and C. A. Vernon, *J. Chem. Soc.*, 4235 (1962).
(19) R. L. Gustafson and A. E. Martell, *Arch. Biochem. Biophys.*, **68**, 485 (1957).
(20) N. R. Williams and J. B. Neilands, *ibid.*, **53**, 56 (1954).
(21) A. K. Lunn and R. A. Morton, *Analyst* (London), **77**, 718 (1952).
(22) D. E. Metzler, *J. Amer. Chem. Soc.*, **79**, 485 (1957).
(23) R. P. Bell, "The Proton in Chemistry," Cornell Univ. Press, Ithaca, N. Y., 1959, p 189.

indicate that its base-catalytic constant is small with respect to that of the solvent or that the shielding observed for the β protons in the AKBA portion of the Schiff base is indicative of stronger bonding and lower susceptibility to base catalysis.

Catalysis in the System Pyridoxamine–AKBA–Zinc(II). In the case of the zinc(II)–pyridoxamine-catalyzed reaction, the catalysis is particularly strong in the region of $-\log [D^+]$ of 5 in comparison with the metal-free case. Therefore, the possibility must be considered that the acceleration of the rate in the region of $-\log [D^+]$ of 5 is due to the action of the phenolic oxygen of pyridoxamine in general base catalysis.

In the Results section, it was shown that the changes in the nmr spectrum of AKBA as it reacts in the presence of zinc(II) ion are consistent with very efficient dimerization followed by a slower incorporation of deuterium while, if pyridoxamine is present, the exchange is more rapid than the dimerization and so the dimer that is formed already has deuterium incorporated. This observation is in accord with a possible template mechanism involving 2:1 or higher complexes of AKBA, so that when one of them is enolized, another is close by in the coordination sphere to condense with it. An alternative explanation could be the increase in stability (*i.e.*, greater lifetime) of the enolate form of the keto acid coordinated to the metal ion. In the presence of pyridoxamine, this efficiency of dimerization is not observed and so the pyridoxamine-catalyzed mechanism must differ from that observed in the case of general base catalysis.

Furthermore, the pK_a of AKBA is reported to be 2.5[13] and the log of the stability constant of the 1:1 zinc(II) complex with pyruvate is 1.38. Thus, if the rate of the reaction were governed by the zinc(II)–AKBA complex concentration, it should show a maximum below $-\log [D^+]$ of 4 instead of near 5.

Estimates for the concentrations of the various ions in the pyridoxamine–AKBA–zinc(II) equilibrium mixture at various values of $-\log [D^+]$ were computed as indicated in Figure 6. Since the stability constants for this complex system have not been measured, constants for closely analogous complexes were used to give a reasonable distribution diagram for comparison with rate variations. In the system of 0.40 *M* AKBA, 0.40 *M* pyridoxamine, and 0.040 *M* zinc(II), the product of the concentrations of the zinc(II)–AKBA complex and the pyridoxamine deprotonated at the phenolic oxygen passes through a maximum below $-\log [D^+]$ of 4 whereas the rate data show a maximum near 5. Furthermore, at $-\log [D^+]$ of 6 to 7, the rate is still more than twice as great as the metal-free case even though the zinc(II)–AKBA complex concentration has fallen below 10^{-5} *M*. Thus, some mechanism other than general catalysis is probably acting in this pD range.

Above $-\log [D^+]$ of 6, the computations show that nearly all the zinc(II) in solution is complexed by two Schiff bases, and since the rate is everywhere higher than in the absence of zinc(II) ion, this region most probably involves a Schiff base–metal complex mechanism such as the one shown in Scheme I, (XI–XIII). This mechanism is entirely analogous to the proton-catalyzed mechanism shown as VIII–X except that here the zinc(II) ion serves to withdraw electrons.

Using the various models mentioned, the ratio of the zinc(II)-catalyzed rate to the proton-catalyzed rate for equal concentrations is about 3 at $-\log [D^+]$ 6.5. In general, the proton is expected to be a better polarizing agent at a particular site than a metal ion,[24] particularly a relatively weakly binding metal such as zinc(II). Though these Schiff bases are protonated at the azomethine nitrogen through the range of these experiments, the pK_a for the carboxyl proton is probably about 1.0. Hydrogen, though it is strongly bound to the azomethine nitrogen, is then able to hydrogen bond only weakly to the carboxyl group that is deprotonated. Furthermore, when the phenoxide oxygen is deprotonated, it represents a stronger base than the carboxyl group and so hydrogen bonding is more likely to include it, leaving the carboxyl group free. Transition metals have a number of relatively equivalent sites and so zinc(II) may then bind strongly to all the points of attachment in the ligand and, though it is substantially poorer polarizing agent at the azomethine nitrogen, it binds strongly to the carboxyl group reducing the negative charge on it and thus exerting a more powerful withdrawing effect on the binding electrons of the β hydrogens than would a metal ion bound only through the azomethine nitrogen and the pheolate oxygen. Further, the presence of the five-membered metal chelate ring containing the carboxylate group would greatly stabilize intermediates such as XI, XII, and XIII relative to VIII, IX, and X.

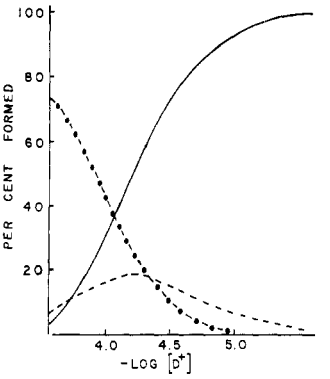


Figure 6. Species in solution for 0.4 *M* AKBA, 0.4 *M* pyridoxamine, and 0.04 *M* zinc(II): ——— 1:2 zinc(II)–Schiff base complex, - - - - 1:1 zinc(II)–Schiff base complex, – · – · – 1:1 zinc(II)–AKBA complex.

The dependence of rate constant on $-\log [D^+]$ cannot, however, be entirely explained by a single Schiff base mechanism because the concentration of the Schiff base 2:1 complex should increase monotonically with $-\log [D^+]$ under the conditions of these experiments. Thus the strong catalysis in the range of 4 to 6 is probably not entirely due to the 2:1 complex. One species that could be responsible for the rate maximum is the 1:1 Schiff base complex which, using the models assumed, passes through a region of maximum concentration near 5. It does not seem likely that the rate for the 1:1 complex can be so much greater than for the 2:1, however.

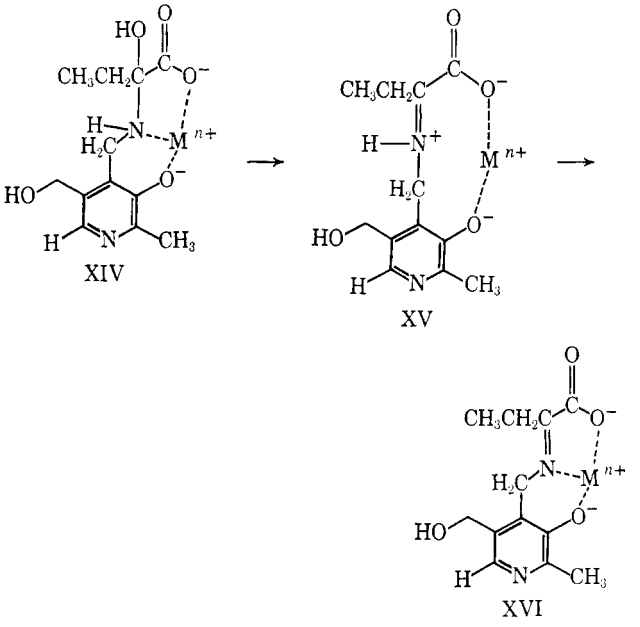
An alternative possibility could be an intermediate of the type proposed by Leussing and Stanfield[25] to explain the pH dependence of the formation of the pyr-

(24) A. E. Martell in "Chemical and Biological Aspects of Pyridoxal Catalysis," Interscience Publishers, New York, N. Y., 1963, pp 27, 28.

(25) D. L. Leussing and C. K. Stanfield, *J. Amer. Chem. Soc.*, **86**, 2805 (1964).

uvate glycine complex with zinc(II). In their study, they judged that a substantial quantity of the carbinolamine complex could be formed and could undergo acid-catalyzed dehydration, passing through a protonated Schiff base–metal complex form. In our system this would appear as the reaction shown in Scheme II. Species XV, with both the metal and the proton to

Scheme II. A Mechanism for the Generation of a Species Catalytically Active toward β-Proton Exchange *via* Carbinolamine Dehydration (after Leussing and Stanfield[25])



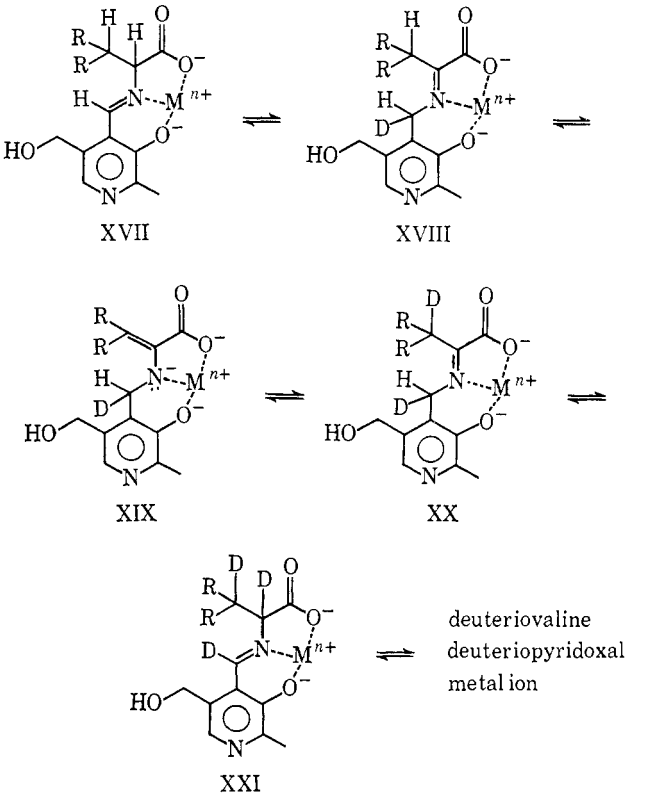
attract the electrons from the β carbon atom of the keto acid portion would not be very stable and hence would be present in relatively low concentrations, but would be expected to be a much more powerful catalytic species than either the protonated Schiff base or the Schiff base–metal complex.

Several other possible explanations for the strong catalysis in the region of $-\log [D^+]$ of 5 must also be considered. These include the mixed ligand complexes of AKBA, zinc(II), and pyridoxamine and of AKBA, zinc(II), and the Schiff base of AKBA and pyridoxamine. Since the pyridoxamine or Schiff base in the coordination sphere would probably prevent coordination of more than one AKBA, the condensation reaction would be inhibited with respect to exchange. No stability data are yet available for these species; however AKBA is completely dissociated in the region of stability of the 1:1 Schiff base–metal chelate, and most certainly combines with it to form appreciable amounts of the 1:1:1 mixed ligand chelate, Zn(II)–AKBA–Schiff base. The possibility of the contribution of this type of species to metal catalysis of the exchange reaction cannot be overlooked.

Deuteration of Amino Acids. Junk and Svec[26] have observed that β deuteration of leucine occurs at pH 5 in the presence of pyridoxal but offered no mechanism or interpretation for the reaction. Comparison of their conditions with the reactions reported here for valine and α aminobutyric acid makes it possible to propose a mechanism for their reaction. Accordingly, it is suggested that the pyridoxal-catalyzed deuteration of α-

(26) G. A. Junk and H. J. Svec, *J. Org. Chem.*, 29, 947 (1964).

Scheme III. A Mechanism for the β Deuteration of α-Amino Acids



amino acids occurs by the mechanism in Scheme III. The reaction should be general for all amino acids, and any amino acid derivative containing a free or potential basic amino group. By this mechanism, it should be possible to β deuterate terminal and branching amino acid portions of polypeptides that contain free amino groups.

The nonenzymic transamination steps are known to be slow and to require relatively high temperatures to go at convenient rates.[27] Thus, as reported in the Results section, α-aminobutyric acid is not observed to be monodeuterated because after transamination of aldimine to ketimine, the β deuteration occurs much more rapidly than the transamination back to aldimine.

Olivard, Metzler, and Snell[28] have shown that at pH 9 in the presence of pyridoxal and aluminum(III), racemization of amino acids occurs more rapidly than does transamination. The nmr results confirm this and suggest a method for preferentially deuterating either the α or β positions of amino acids. For example, as has been shown, the α position of amino acids are deuterated in a few hours at 100° in D_2O in the presence of catalysts. Conversely, the amino acid could be α and β deuterated at pD 5, evaporated to dryness, H_2O added, and the pH adjusted to about 9. After a few hours of further incubation at 100° the amino acid should be largely α protonated and β deuterated.

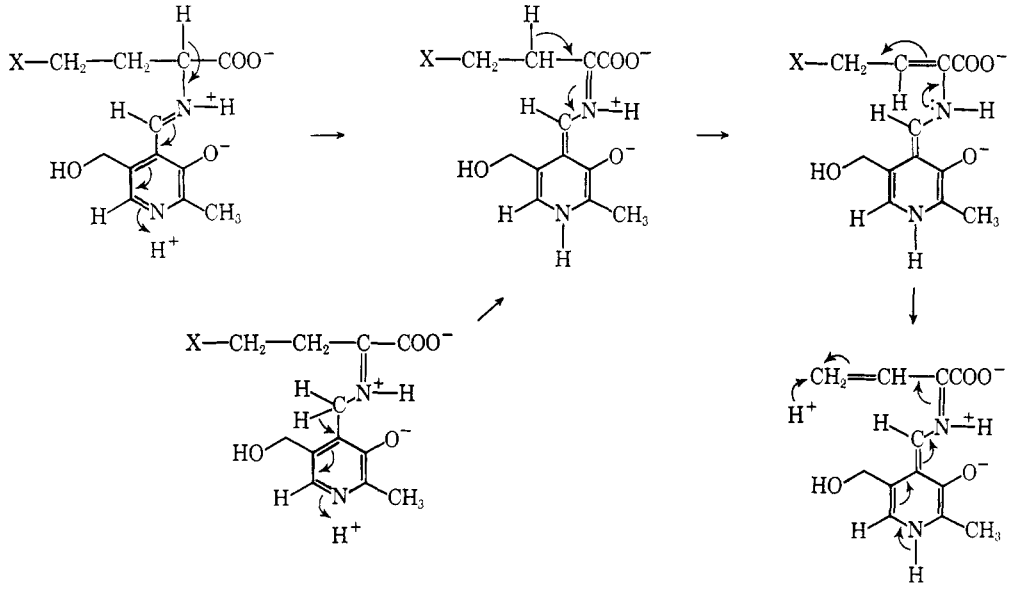
Biological Implications. Previously, it was thought that vitamin B_6 could introduce unsaturation into the complementary portion of the Schiff base only by electron donation.[3,29] Thus, the elimination of electroneg-

(27) D. E. Metzler and E. E. Snell, *J. Amer. Chem. Soc.*, 74, 979 (1952).

(28) J. Olivard, D. E. Metzler, and E. E. Snell, *J. Biol. Chem.*, 198, 353 (1952).

(29) A. E. Braunstein, "The Enzymes," P. E. Boyer, H. Lardy Vol. 2, and K. Myuback, Ed., Academic Press, New York, N. Y., 1960.

Scheme IV. A Mechanism for the Elimination of Electronegative Groups from the γ Position of Amino Acids



ative γ substituents was thought to occur by the same type of shift, shifting a negative hydride from the β position in place of the electronegative group.

A Schiff base catalyzed imine–enamine tautomerization suggests an alternative mechanism for γ elimination reactions, as shown in Scheme IV. In this mechanism the electronegative γ substituent is eliminated by donation of electrons of the β carbon to the β–γ carbon–carbon bond XX, rather than by the replacement of the negative group by a hydride ion from the β position.

In the theory of vitamin B_6 catalysis, the first step is the deprotonation of the methylene group α to the 4 position of the heterocyclic ring, if the ketimine is the reactant, or deprotonation of the carbon α to the carboxyl group, if the aldimine is the reactant.[3] This extends the resonance in the ring to the carboxyl group of the amino or keto acid portion. In the keto acid exchange reactions studied in this work, this process is much slower than the β-proton exchange and it has been found that the β position is fully deuterated before there is a perceptible exchange of the methylene protons α to the four positions. Presumably the strong catalysis observed for the vitamin B_6 enzymes is due to their ability to catalyze this deprotonation. Extension of the ring resonance to the azomethine nitrogen should serve to further accelerate imine–enamine tautomerization and γ elimination as shown in Scheme IV, because the unsaturation then connected to the azomethine nitrogen should exert a still stronger electron-withdrawing effect on the bonding electrons of the β hydrogen than does the protonated imine group alone.

In model systems, the γ elimination is very slow if it proceeds at all.[3] This may be due to the relatively high concentrations and mobilities of protons which can block the reaction by protonating the β group. In the active site of enzymes, this may be a problem. Protons may be sufficiently screened out that the kinetic pathway to γ elimination becomes predominant.

Flavin and Slaughter[30] have found evidence against hydride-ion transfer in the γ elimination reactions catalyzed by several enzymes, as discussed in the introduction, and have considered several enzymic mechanisms which are similar to the new γ elimination mechanism suggested here (Scheme IV) for model systems. In the light of their results on enzyme systems and these results on model systems, the proton-transfer mechanism should be considered a more reasonable basis for the initiation of γ elimination than the widely accepted hydride ion transfer mechanism.

Acknowledgment. The authors express their gratitude to Professors R. H. Holm and O. A. Gansow for providing preprints of related work prior to publication.

(30) M. Flavin and C. Slaughter, *J. Biol. Chem.*, **235**, 1112 (1960).