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Pyridoxal- and Metal Ion-catalyzed Oxidative Deamination of Alpha Amino Acids

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Abstract

This paper describes the pyridoxal- and metal ion-catalyzed oxidation of alanine and phenylalanine to pyruvic acid and phenylpyruvic acid, respectively. The reactions were found to occur at moderate, easily measurable rates at 25 °C in aqueous and methanol solutions. Conversions of amino acid to keto acid of up to 89% were observed in the presence of relatively low concentrations of catalysts. The relative catalytic activities of the metal ions employed, as measured by initial rate constants and product yields are Mn(II) > Co(II) > Cu(II) ≫ Ni(II) ~ 0. Of special interest is the fact that little or no ammonia was evolved from the reaction mixture and no hydrogen peroxide was formed. However a considerable amount of the ammonia was accounted for by the formation of pyridoxal oxime, and the trapping of some hydroxylamine from the volatile products. The results are discussed in terms of two possible intermediates involving different reaction mechanisms, one with dioxygen coordinated to the metal ion, originally suggested by Hamilton, and a mechanism by which the oxygen attacks the carbanionic form of the delocalized α-deprotonated intermediate to give a hydroperoxide, which then undergoes O–O fission to form the keto acid and coordinated pyridoxal oxime. The relative merits of these reaction mechanisms are discussed.

Introduction

The transformation of primary amines to carbonyl compounds and alkenes has received considerable attention, and a variety of metal oxidizing reagents have been used for this purpose with varying success. The deamination reaction has been intensively investigated recently and most alpha amino acids undergo slow irreversible decarboxylation during the oxidation reaction [1–3]. Of the twenty amino acids found in proteins, only aspartic acid [4] and asparagine [5] are known to deaminate nonenzymatically. Vitamin B_6 (pyridoxal)-catalyzed conversion of amino acids to keto acids has been suggested to involve transamination rather than direct amine oxidation [6–8]. Earlier work has shown that some amine oxidases [9, 10] require pyridoxal-5′-phosphate and Cu(II) as cofactors [11–15]. Catalysis of the nonenzymatic deamination of α-alanine by molecular oxygen with Mn(II) and pyridoxal as catalysts has been described [14].

In this report, a study of the catalysis of amino acid oxidation by molecular oxygen in the presence of pyridoxal and transition metal ions is described. Methanol was employed as solvent in place of water because there are previous indications [7] that pyridoxal Schiff bases are formed much more completely and exist as relatively simple molecular species in this medium. This investigation has resulted in the extension of catalysis of nonenzymatic pyridoxal-catalyzed deamination of α-amino acids to additional transition metal ions and the identification of an oxygenated intermediate.

Experimental

Materials

Pyridoxal hydrochloride, DL-alanine, DL-α-phenylalanine and sodium pyruvate were obtained from Sigma Chemical Co. All organic and inorganic compounds were obtained from commercial sources, were certified reagent grade chemicals, and were used without further purification. The methanol employed as solvent was of spectroscopic grade. Phenylpyruvic acid was synthesized by an appropriate literature procedure [16] and was purified by recrystallization. Nitrogen was passed through a basic pyrogallol solution, a sulfuric acid column, and basic drying tube, and saturated with methanol vapor. Oxygen was purified with a Matheson Model 460 Gas Purifier and was also saturated with methanol vapor.

Preparation of Solutions

Alanine, phenylalanine and pyridoxal hydrochloride were dissolved with equimolar sodium hydroxide. Pyridoxal solutions were prepared for each experiment and used immediately to avoid possible oxidative decomposition. Copper(II) chloride,

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manganese(II) acetate, cobalt(II) acetate and nickel(II) acetate were employed for metal chelate formation with pyridoxal and amino acid in the form of their solutions in methanol.

Oxygen Uptake

The reactions were initiated by mixing the high level concentration of Schiff base metal chelate solutions with solutions containing the appropriate concentration of the sodium salt of the amino acid. The rates of oxygen uptake were measured by the manometric technique at 25.0 °C. Corrections were made for changes in atmospheric pressure.

Determination of Keto Acids

Pyruvic and phenylpyruvic acid, the transaminated and oxidized products of alanine and phenylalanine respectively, were determined colorimetrically as the 2,4-DNP hydrazones with 2,4-dinitrophenylhydrazine (2,4-DNP) in basic solution. The concentrations of products were determined by comparison with a linear calibration curve [17]. The 2,4-DNP hydrazone of pyruvic acid was isolated from 10% sodium carbonate solution by acidification with concentrated hydrochloric acid and extraction with organic solvents such as toluene and ethylacetate. The yellow crystalline hydrazone melted at 224 °C, and there was no lowering of the melting point for a mixture of this hydrazone with an authentic sample. The yellow crystalline hydrazones were recrystallized from 95% ethanol. The formaldehyde formed by the reactions run in methanol was isolated as the hydrazone from the organic layer after extracting the keto acid with 10% sodium carbonate. Identification was made by paper chromatography by comparing its R_f value with that of the authentic material. The yellow needles which crystallized from 95% ethanol melted at 164 °C, and there was no melting point depression of a mixture with the authentic hydrazone.

For the reaction of the copper(II) chelate system under oxygen, a pale blue precipitate appeared after 20 min. The precipitate was separated by filtration, washed with ethanol, and dried at 56 °C under reduced pressure for a few hours. Pyridoxal was identified by paper chromatography of the solution obtained by dissolving the precipitate in dilute hydrochloric acid. Tests for pyruvic acid, alanine, and pyridoxamine were negative.

The elemental analysis of 1:1 copper(II) chelate of pyridoxal oxime (Galbraith Laboratories, Knoxville, Tenn.) is in good agreement with the calculated values. *Anal.* Calc: C, 39.20; H, 4.39; N, 10.16; Cu, 23.04. Found: C, 38.84; H, 4.24; N, 9.93; Cu, 23.71%.

Volatile Materials

While the reaction was taking place, the volatile materials, which were expected to be ammonia and some type of amine, were absorbed in dilute sulfuric acid. The ammonia formed was determined by Nessler color development [18] and paper chromatography after the sample solutions had been concentrated.

Hydrogen Peroxide

Hydrogen peroxide [19], one of the products expected in this reaction [14], was analyzed by formation of the vanadate complex. The sample solution was acidified with sulfuric acid and mixed with ammonium metavanadate–perchloric acid solution. A spectrophotometer was used to determine the peroxide complex concentration from its absorbance at 460 nm.

Paper Chromatography

The reaction products were identified by paper chromatography by the one-dimensional descending method, with Whatman No. 1 chromatography paper. The solvents used were (proportions by volume): (1) n-butanol:acetic acid:water (4:1:5); (2) n-butanol: water:0.5 M ammonium hydroxide (85:10:5); (3) n-butanol:95% ethanol:0.5 M ammonium hydroxide (7:10:2).

Spots were developed as follows: amino acids and amines, blue–violet color on spraying with 0.2% ninhydrin solution; pyridoxal, yellow color on spraying with 10% ethanolamine solutions; and 2,4-DNP hydrazones, yellow spots without spraying, brown spots on spraying with 20% aqueous sodium hydroxide.

Absorbance Measurements

The electronic absorption spectra were recorded with a Cary Model 14 Spectrophotometer with matched 1.000 cm cells at 25 °C. For the spectral studies, the sample solutions of Schiff base-metal chelates were prepared under a nitrogen stream to avoid reaction with oxygen. Precipitation occurred with only the copper(II) ion in the presence of air, and these precipitates were removed by filtration. A Cary Model 14 spectrophotometer was employed for the colorimetric determination of the keto acids produced. The copper(II) chelate precipitate obtained from the reaction mixture (which turned out to be the Cu(II) chelate of pyridoxal oxime) was studied in KBr disks with a Beckman IR-8 Infrared Spectrophotometer.

Results and Discussion

Oxygen Uptake and Formation of Keto Acids

The reactions were studied in basic solution to avoid transamination which occurs optimally at pH 4–5. Some inconveniences under highly basic conditions would be expected, such as the length of time

TABLE I. Initial Rates of Oxygen Uptake of Metal–Schiff Base in Methanol Solution

Metal	Alanine Schiff base (μl/s)	Phenylalanine Schiff base (μl/s)
Cu(II)	0.30	0.25
Mn(II)	1.22	0.98
Co(II)	0.44	0.40
Ni(II)	0.15	0.026

TABLE II. Initial Rate of Oxygen Uptake of Metal–Schiff Base in Aqueous Solution

Metal	Alanine Schiff base (μl/s) × 10	Phenylalanine Schiff base (μl/s) × 10
Cu(II)	1.15	0.71
Mn(II)	0.91	0.79
Co(II)	0.76	0.30
Ni(II)	0.003	

required to complete Schiff base formation, the greater ease of oxidation of metal ion, and the fact that certain amino acids are oxidized at higher pH [20]. Unlike aqueous solutions, in methanol metal hydroxide precipitates were not observed under basic conditions during the experimental runs. However, when oxygen was passed through the copper(II) chelate solution, a pale blue precipitate was found for both the alanine and phenylalanine systems, as discussed in the 'Formation of Oxime' section.

The initial rates of oxygen uptake in methanol are shown in Table I. Alanine Schiff bases are more reactive than phenylalanine Schiff bases and the rates of oxygen uptake are very dependent on the metal ions present. Of all the metals studied, the manganese-(II) chelate showed the most rapid oxygen uptake. The results of oxygen uptake in aqueous solution are shown in Table II. The initial rates of oxygen uptake in aqueous solution were much slower than in methanol and gave oxygen absorption curves different from those measured in methanol. For example, in the Co(II)–chelate system an initial induction period was observed.

Comparisons between the volumes of oxygen taken up and the quantities of products formed are presented in Table III. Oxygen uptake in each run was determined by subtracting the value measured in the absence of amino acid under the same conditions. The formation of keto acid was measured under purified nitrogen and oxygen streams. Zero time of reaction was taken as the time of addition of metal.

Reaction Products

The reaction products identified by paper chromatography are summarized in Table IV. Unreacted amino acids were identified and determined by comparisons with the R_f values of authentic samples of the amino acids. Pyridoxal, which was essentially the catalyst for the oxidative deamination reaction, was identified in the acetal form by paper chromatography.

Pyruvic acid, the main reaction product, was obtained from the reaction mixture by extraction with sodium carbonate solution. It was identified as the

TABLE III. The Stoichiometry of the Amino Acid Oxidation by Air at 25.0 °C

	Oxygen taken up (μmol)	Keto acid produced (μmol)	Conversion (%)
In methanol			
Cu(II)–alanine[a]	9.2	4.4	88.9
Cu(II)–phenylalanine[a]	9.8	3.1	61.5
Mn(II)–alanine	8.6	8.1	81.5
Mn(II)–phenylalanine	15.7	7.0	70.4
Co(II)–alanine	7.8	7.5	75.9
Co(II)–phenylalanine	12.7	5.5	55.6
In aqueous solution			
Cu(II)–alanine	5.6	3.2	32.2
Cu(II)–phenylalanine	9.9	2.0	17.9
Mn(II)–alanine	1.5	1.1	10.7
Mn(II)–phenylalanine	7.2	5.1	51.4
Co(II)–alanine	2.6	1.7	16.7
Co(II)–phenylalanine	4.3	4.6	4.57
Ni(II)–alanine	0.1	0.1	0.2
Ni(II)–phenylalanine	1.4	1.5	14.9

[a]Concentration of metal chelate is 1/2 of the other runs.

TABLE IV. R_f Values of Reagents and Products on Whatman No. 1 Paper at 23.5 °C

	Solvent[a] (1)	Solvent (2)	Solvent (3)
Amino acids			
Alanine	0.27		
Phenylalanine	0.61		
Pyridoxal (aldehyde)	0.32–0.34		
Pyridoxal (acetal)	0.41–0.54		
Hydrazones			
Pyruvic acid (*trans*)		0.30–0.31	0.43–0.45
Pyruvic acid (*cis*)		0.49–0.50	0.65–0.67
Pyridoxal			0.41–0.43
Formaldehyde		0.80–0.81	0.83
Reference compounds			
Methylamine	0.23–0.25		
Pyridoxamine	0.16–0.18		
Acetaldehyde (hydrazone)		0.91–0.92	0.86
2,4-DNP			0.75

[a]Solvents employed (*v/v*): (1) n-butanol:acetic acid:water (4:1:5); (2) n-butanol:water:0.5 ammonium hydroxide (85:10:5); (3) n-butanol:95% ethanol:0.5 M ammonium hydroxide (7:10:2).

hydrazone of 2,4-DNP, which was separated from the acidified carbonate solution by extraction with ethyl acetate. Its identity was confirmed by the mixed melting point of a mixture with an authentic sample.

The formaldehyde formed by the reactions run in methanol was isolated as the hydrazone and was identified by paper chromatography, by melting point, and by mixed melting point.

Formation of Oxime

The precipitate obtained from the copper chelate systems under oxygen was identified as the neutral 1:1 Cu(II) chelate of pyridoxal oxime (which yields pyridoxal in the acetal form by hydrolysis in dilute hydrochloric acid) on the basis of its elemental analysis and a strong characteristic IR absorption band at 1650 cm^{-1}, assigned to the C=N stretching vibration of the oxime group.

The reaction products obtained from the alanine Schiff base and from the phenylalanine Schiff base were found by infrared (KBr disk) to be identical, based on the IR spectra in the range 4000–650 cm^{-1}. The reaction solution, which was concentrated by removing the methanol solvent, was examined for the presence of pyridoxamine by paper chromatography. There was no color spot at R_f 0.16–0.18, which would be expected if transamination had occurred.

Schiff Base Equilibria

Metal-free systems

The assignments of the electronic absorption bands for the Schiff base systems are well established [21, 22]. The formation of Schiff base from pyridoxal and alanine was brought to completion by the use of a ten-fold excess of amino acid over pyridoxal in basic methanol solution. Complete formation of Schiff base was demonstrated by the fact that there was no change in the ultraviolet and visible absorption spectra at higher alanine concentrations, and clear isosbestic points resulted when the alkali hydroxide (KOH) concentration was increased. In alkaline solution, the spectra underwent gradual changes involving both a lowering of intensity and a blue shift of the 370 nm band over a long period of time.

From changes in absorbance at 370 nm which occurred with increases of concentration of excess amino acid, the log of the equilibrium constant K for formation of the Schiff base was found to be 3.80,

$$K = \frac{[\text{pyridoxylidenealanine}]}{[\text{pyridoxal}]\,[\text{alanine}]}$$

where [pyridoxal] includes both the hemiacetal and the aldehyde forms of pyridoxal.

Oxidation with Metal Schiff Base Complexes as Catalysts

The Schiff base systems obtained from equimolar quantities of alanine and pyridoxal were first equilibrated and metal solutions were then added to give varying concentrations of the catalysts. In the Schiff base chelate systems for most of the metal ions in neutral methanol, spectral changes did not occur up to 24 h, indicating that the keto acid was not formed. The manganese–Schiff base chelate, on the other hand, underwent gradual changes involving both a lowering of intensity and a red shift of the 385 nm band, and an increase in intensity of the 278

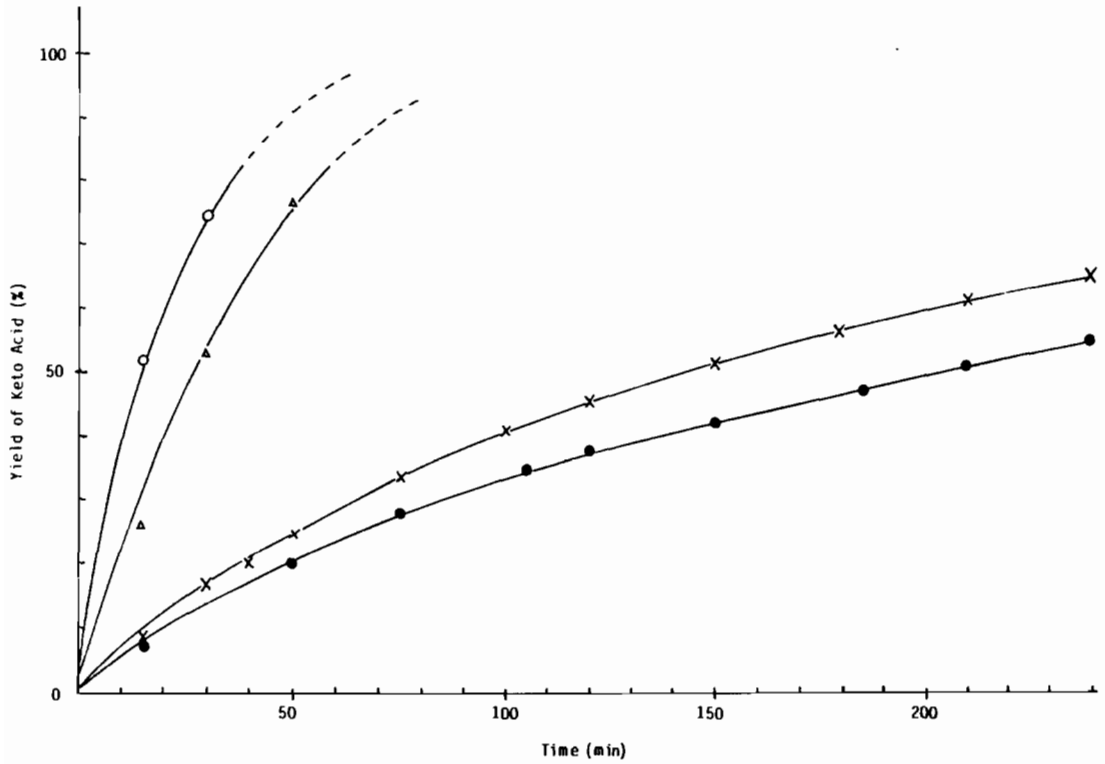


Fig. 1. Effect of chelate concentration on reaction: $\frac{1}{2}$ mol, ○; $\frac{1}{4}$ mol, △; $\frac{1}{8}$ mol, ×; $\frac{1}{10}$ mol, ● (*vs.* amino acid concentration).

nm band. The absorption band at 229 nm disappeared concurrently with the increasing absorbance at 278 nm, indicating partial conversion to the keto acid.

In basic solutions, the spectra of the metal–Schiff base chelates underwent gradual lowering of intensities of the absorption bands near 390 nm and 280 nm, and increasing intensity of a new band around 350–360 nm. For these solutions the growth of the new absorption band correlated with the detection of keto acid by the methods described above. The systems containing chelates of cobalt(II) and manganese(II) showed rapid spectral changes, with the absorbances of the absorption bands at 390 nm and 395 nm rapidly decreasing, concurrent with the appearance of new absorption bands at 345 and 349 nm.

For the Cu(II)–alanine Schiff base system, the time dependence of yield of keto acid at different chelate concentrations at p[OH] = 1.58 is shown in Fig. 1. Also for a 1:10 molar ratio of chelate to amino acid, the observed first order rate constant was found to be 4.5×10^{-5} s^{-1} for an extended period of time (up to 350 min). This system produced 60% conversion to reaction product.

For high ratios of the copper(II) alanine Schiff base chelate to alanine, the rates of formation of pyruvic acid are shown in Fig. 2. The observed first order rate constants calculated over the initial 30 min of the reaction are reported in Table V.

TABLE V. Effect of Base Concentration on Observed Rate Constant

$-\log [OH^-]$	$k_{obs} \times 10^3$ (s^{-1})
2.48	10.32
2.23	9.14
1.72	7.92
1.58	3.02
1.27	2.81

Methanol Oxidation

The initial rates of oxygen uptake in methanol solution are much faster than in aqueous solution. According to Table III, the molar quantity of oxygen taken up is much greater in methanol than in aqueous solution. Also, the quantities of formaldehyde found in the paper chromatographic studies indicate that the unequal values of oxygen taken up and keto acid formed were due to the formation of formaldehyde. The stoichiometric relationship between yields of keto acid and formaldehyde cannot be explained simply by a secondary oxidation of the keto acid, although some of the formaldehyde may have been formed in that manner. The remainder of the formaldehyde appears to be the oxidation product of the methanol solvent itself. Decarboxylation is considered to be the first step in the formation of

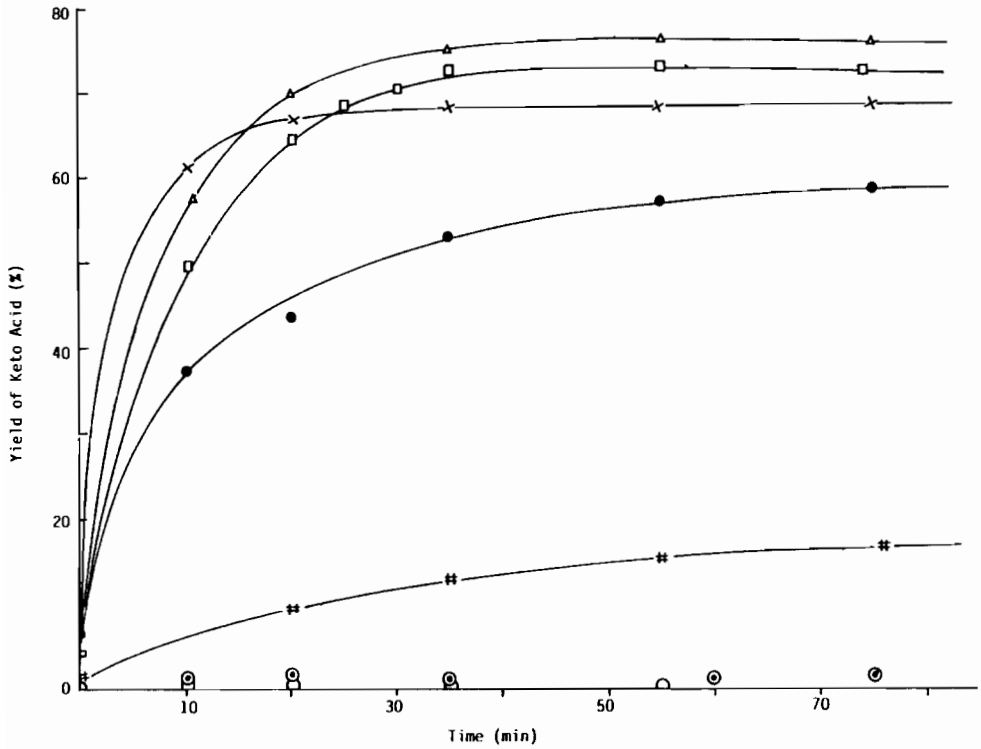


Fig. 2. Effect of base concentration of reaction: p(KOH) = 3.27, ○; p(KOH) = 2.48, #; p(KOH) = 2.23, ●; p(KOH) = 1.72, □; p(KOH) = 1.58, △; p(KOH) = 1.27, X ; neutral, ○.

formaldehyde from pyruvic acid, but it is not clear whether decarboxylation takes place in the highly basic reaction medium or during conversion of the keto acid to the hydrazone in the highly acidic medium employed for its formation and isolation.

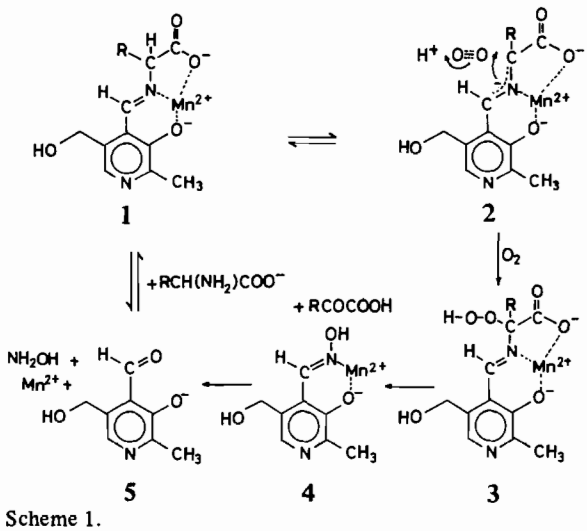
Volatile Materials

The tests for volatile material, carried out as described in the experimental part, showed that very little volatile compounds were liberated in the course of the reaction runs of the type illustrated in Figs. 1 and 2. Ammonia was not evolved in any detectable amount. The Nessler tests for ammonia did not show interference from methanol or by any impurities that may be present. The slow development of pale yellow color in the Nessler solutions is not due to the presence of ammonia, which would give a much different color, and is assigned to the presence of a small amount of hydroxylamine. Thus the results of the present investigation indicate a new reaction pathway which had not been previously suggested for these reaction systems.

Reaction Pathway

Acetaldehyde was also detected in the reaction systems for the higher catalyst concentrations, apparently as the result of the decarboxylation of pyruvic acid. The formation of aldehydes in reactions of amino acids with carbonyl compounds is well known [23]. Also, Ikawa and Snell [24] found that the quantity of keto acid formed in pyridoxal-catalyzed oxidative deamination was invariably less than the ammonia formed at pH 9.6, as a consequence of the instability of α-keto acids. On the other hand Hamilton and Revesz [14] suggested that the low yield of pyruvate was due to the fact that pyruvate rapidly reacts with the hydrogen peroxide formed in the reaction. In both cases, however, no further experimental evidence was available to support these suggestions.

For alanine, the extent of formation of pyruvic acid with Mn(II) as catalyst was more than 70% under nitrogen and 80% under oxygen at zero time while for phenylalanine the conversions were 35% and 70%, respectively. The initial rate of formation of keto acid in the presence of Mn(II) was the largest of all the metal ions studied. In the presence of oxygen and Mn(II), the quantity of phenylpyruvic acid formed decreased as the reaction progressed, indicating decomposition of the α-keto acid, which is unstable in solution in the presence of metal ions. The hydrazone of phenylpyruvic acid was also found through colorimetric measurements to be unstable in solution. Parallel results were obtained by Metzler and Snell [20] in their study of transamination reactions catalyzed by aluminum(III) under oxygen at pH 5.

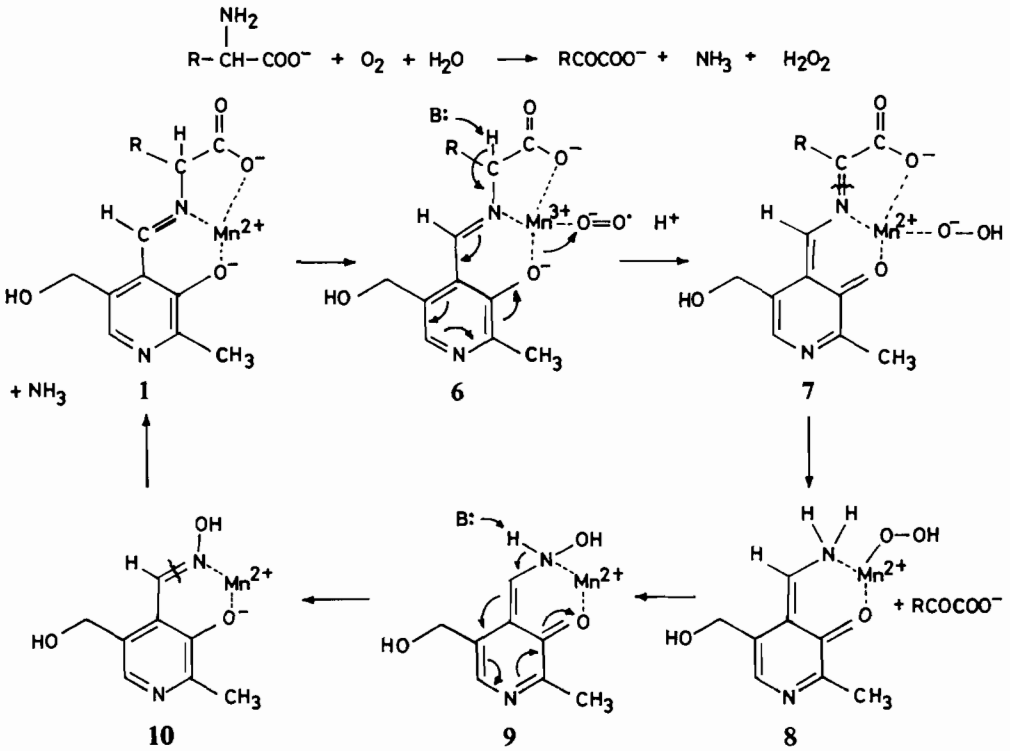


Scheme 1.

In the case of the Cu(II) chelate systems the formation of keto acid increases gradually with time, while the nickel(II) chelate systems do not show an increase. These results parallel the rates of oxygen uptake. The increase of concentration of keto acid in the presence of nitrogen in the case of Mn(II) and Co(II) during the initial 10 min was probably caused by the presence of dissolved oxygen in the metal solutions. It was found on the other hand that in the absence of oxygen more than 70% of the Mn(II) aldimine chelate rapidly forms the ketimine chelate through transamination.

The spectral changes that occurred during these reactions, consisting of decreasing intensity of the aldimine absorption bands, accompanied by the development of new bands around 350 nm for each metal chelate system, are considered due to the formation of pyridoxal oxime complexes, with dissociation of the keto acid from the Schiff base complex, as indicated by Schemes 1 and 2. The very rapid catalytic reactions observed in the case of Mn(II) and Co(II) can be considered due to at least partial oxidation of these ions to the trivalent form by molecular oxygen. This may happen when the metal ions are already complexed in the form of their Schiff base chelates. The more rapid reactions catalyzed by vitamin B_6 such as transamination, β-elimination, decarboxylation, etc., have been shown by many observers to be catalyzed much more effectively by trivalent ions. Even in neutral methanol solution in the absence of complexing agents Mn(II) and Co(II) ions are observed to be rapidly (*i.e.*, in a few minutes) oxidized to the trivalent form by oxygen. Such oxidation could occur more rapidly in the form of the Schiff base chelates, because of the well known effect of coordination on decreasing reduction potentials of complexed metal ions.

Hydrogen peroxide has been suggested as the product of oxygen reduction for oxidative deamina-



Scheme 2.

tion reactions in enzyme systems [14], but it has not been detected in nonenzymatic oxidative deamination reactions. In the present investigation no hydrogen peroxide was detected in the reaction mixture by the method described in the Experimental. This is an important observation and it is of interest to consider the possible reasons for the absence of H_2O_2 formation. The absence of hydrogen peroxide as a reaction product was explained by Hamilton and Revesz [14] as possibly due to its reaction with components of the system, and correlated with the observed low yield of pyruvate and the decomposition of pyridoxal. Such reactions, however, generally occur in organic low-polarity solvents [25, 26] to give hydroperoxides. In alkaline solution these compounds decompose to the corresponding alcohols with the release of oxygen. The possibility that hydrogen peroxide reacts with the methanol present can be ruled out because such reactions take place only in acid solutions [27]. Because of the lack of a satisfactory explanation for the loss of H_2O_2, it is appropriate to consider the reaction mechanism illustrated in Scheme 1 which does not involve the formation of hydrogen peroxide under the reaction conditions employed. The suggested formation of intermediates **1** and **2** is similar to earlier proposals advanced to explain other pyridoxal-catalyzed reactions of amino acids. Oxygen attacks the α-carbon of the amino acid moiety of the Schiff base, **1**, which is activated by metal ion-catalyzed formation of the delocalized carbanion **2**, to form the hydroperoxide **3**, which then collapses to the keto acid and the metal chelate of pyridoxal oxime. The bidentate oxime ligand forms a less stable metal chelate than does the Schiff base, and in the presence of an excess of the latter is displaced and hydrolyzes, liberating hydroxylamine.

In order to investigate the possibility of oxidation of the amino acid via a free radical mechanism, 2-propanol was added to the alanine–Cu(II)–Schiff base dioxygen reaction system. This resulted in an approximately two-fold increase in the rate of pyruvic acid formation. Since 2-propanol has been reported to be an efficient scavenger for OH radicals [28], the addition of organic free radical scavenger should have decreased the reaction rate. This result would seem to eliminate the possibility of a free-radical reaction mechanism.

The reasons for the difference in reactivity in water and methanol solution are not fully understood. Also, it is of interest to consider the reaction mechanism proposed by Hamilton [29, 30] involving formation of a metal-Schiff base intermediate with oxygen coordinated to the metal ion as in mononuclear oxygenated metal complexes (superoxide type, **6**). Subsequent steps **7**–**9** in Scheme 2 constitute a modification of the originally suggested two-electron transfer process [29], with reduction of dioxygen to peroxide. This is followed by direct oxidation of the potential imino group in **9** by the adjacent coordinated peroxide to form the oxime chelate **10**. Although there are as yet insufficient data to distinguish between the mechanisms in Schemes 1 and 2, the Hamilton mechanism is attractive in that it assigns a greater role to the metal ion in the activation of oxygen.

Additional work in progress in this laboratory on pyridoxal-catalyzed oxidative deamination involves the use of derivatives of pyridoxal and unnatural amino acids, both of which are designed to answer some of the questions brought up in this paper.

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