The free acids both distilled at 85–88° at 10 mm. The recrystallized sodium salts gave the same color yield in the keto acid procedure with 2,4-dinitrophenylhydrazine. Samples of the 2,4-dinitrophenylhydrazones were prepared in aqueous hydrochloric acid solution and tested without recrystallization. Each melted at 142° with immediate resolidification and final melting at 158–159°. No depression of melting point resulted from mixing the two samples, and the R_f values of the dinitrophenylhydrazones on paper chromatograms were identical.

AUSTIN, TEXAS

[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

A General Mechanism for Vitamin B_6-catalyzed Reactions[1]

BY DAVID E. METZLER, MIYOSHI IKAWA AND ESMOND E. SNELL

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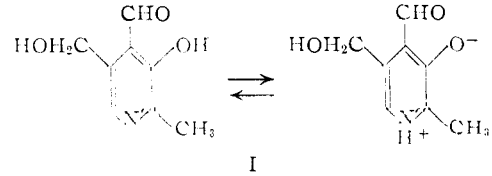
The similarity between reactions catalyzed by pyridoxal phosphate enzymes and those catalyzed by pyridoxal and metal salts is stressed. The structural features of the pyridoxal molecule essential for catalysis of these non-enzymatic reactions are the formyl group, the phenolic group and the heterocyclic ring nitrogen arranged in the 4,3- and 1-positions, respectively, as in pyridoxal, or in the electronically equivalent structure, 2-formyl-3-hydroxypyridine. The formyl group functions in formation of a Schiff base with the amino acid, the latter intermediate being stabilized to the necessary degree by chelation with the catalytic metal ion *via* the nitrogen of the resulting azomethine linkage, the phenolic group, and probably the carboxyl group of the amino acid residue. The resulting planar system of conjugated double bonds provides a mechanism for the displacement of an electron pair from any of the bonds of the α-carbon atom of the amino acid toward the strongly electrophilic heterocyclic nitrogen, a displacement that is intensified by attraction toward the strongly electronegative chelated metal ion. It is shown how such displacement can result in the observed reactions catalyzed by such systems. These include racemization, decarboxylation, transamination and elimination of an α-hydrogen together with a β-substituent of the amino acid. The reverse of the latter process also can occur, as shown by the catalysis of tryptophan formation from serine and indole.

Many different reactions of amino acids are catalyzed by pyridoxal phosphate-containing enzymes.[1a] These include transamination,[2] racemization,[3] decarboxylation,[4] elimination of the α-hydrogen together with either a β-substituent (*e.g.*, serine and threonine dehydration,[5,6] cysteine desulfhydration,[7] the tryptophanase reaction[8]) or a γ-substituent (*e.g.*, homocysteine desulfhydrase[7]), reactions that can be formulated[6] as an α,β-desaturation followed by an addition to the double bond (*e.g.*, formation of tryptophan from serine and indole[9]), and probably the reversible cleavage of β-hydroxyamino acids to glycine and carbonyl compounds.[10,11]

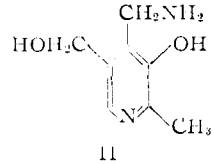
Most of these enzymatic reactions have been duplicated by non-enzymatic model reactions in which pyridoxal[11–18] or other appropriate aldehydes[18,19] and a suitable metal salt serve as catalysts. There is little doubt, therefore, that the catalytic potentialities of pyridoxal phosphate enzymes are, to an exaggerated degree, those of their prosthetic group, and that the non-enzymatic and enzymatic reactions proceed by closely similar mechanisms.

Each of these varied reactions can be visualized as resulting from the intermediate removal of an electron pair from one of the bonds to the α-carbon of the amino acid. The necessary structural features of pyridoxal that permit such reactions, together with a suggested mechanism for them, are considered herein.

In pyridoxal I there are five groups to be considered both as to presence in the molecule and relative position on the ring: (1) heterocyclic nitrogen atom, (2) methyl group, (3) phenolic hydroxyl group, (4) formyl group and (5) the hydroxymethyl group.



I



II

The primary importance of the formyl group is known from the role of pyridoxal in transamination whereby it is converted to pyridoxamine (II),[12,13]

(1) Supported in part by a grant from Sharp and Dohme, Inc.

(1a) E. E. Snell, *Physiol. Revs.*, **33**, 509 (1953).

(2) P. P. Cohen in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. 1, Part 2, Academic Press, Inc., New York, N. Y., 1951, p. 1040.

(3) W. A. Wood and I. C. Gunsalus, *J. Biol. Chem.*, **190**, 403 (1951).

(4) E. F. Gale, *Advances in Enzymol.*, **6**, 1 (1946).

(5) C. Yanofsky and J. L. Riessig, *J. Biol. Chem.*, **202**, 567 (1953).

(6) D. E. Metzler and E. E. Snell, *ibid.*, **198**, 363 (1952).

(7) R. E. Kallio, *ibid.*, **192**, 371 (1951).

(8) W. A. Wood, I. C. Gunsalus and W. W. Umbreit, *ibid.*, **170**, 313 (1947).

(9) W. W. Umbreit, W. A. Wood and I. C. Gunsalus, *ibid.*, **165**, 731 (1946).

(10) G. Ya. Vilenkina, *Doklady Akad. Nauk S. S. S. R.*, **84**, 559 (1952) [*C. A.*, **46**, 10227 (1952)].

(11) D. E. Metzler, J. B. Longenecker and E. E. Snell, THIS JOURNAL, **75**, 2787 (1953).

(12) E. E. Snell, *ibid.*, **67**, 194 (1945).

(13) D. E. Metzler and E. E. Snell, *ibid.*, **74**, 979 (1952).

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

(15) J. Olivard, D. E. Metzler and E. E. Snell, *ibid.*, **199**, 669 (1952).

(16) D. E. Metzler, J. B. Longenecker and E. E. Snell, THIS JOURNAL, **76**, 639 (1954).

(17) E. Werle and W. Koch, *Biochem. Z.*, **319**, 305 (1949).

(18) D. E. Metzler, J. Olivard and E. E. Snell, THIS JOURNAL, **76**, 644 (1954).

(19) M. Ikawa and E. E. Snell, *ibid.*, **76**, 653 (1954).

and from the inhibition of many pyridoxal phosphate enzymes by carbonyl reagents.[4,5,20]

3-O-Methylpyridoxal, in which the phenolic hydroxyl group has been converted to a methyl ether, does not exhibit non-enzymatic transamination or dehydrate serine (Table I), thus showing the importance of this group in pyridoxal function. Similarly, whereas 4-nitrosalicylaldehyde simulates the reactions of pyridoxal, *p*-nitrobenzaldehyde does not.[19]

Replacement of the 5-hydroxymethyl group by methyl as in 5-desoxypyridoxal or esterification with phosphoric acid as in pyridoxal phosphate does not result in loss of the ability to undergo non-enzymatic transamination or to catalyze serine dehydration (Table I).[13,14] Similarly, 4-nitrosalicylaldehyde, which lacks a group corresponding to the 5-hydroxymethyl group of pyridoxal, nonetheless catalyzes many of the same reactions.[19] Thus the hydroxymethyl group, although of great importance physiologically as the point of attachment of phosphate in pyridoxal phosphate, is not essential for these chemical reactions.

TABLE I

REACTIONS OF 5-DESOXYPYRIDOXAL, 3-O-METHYLPYRIDOXAL AND 2-FORMYL-3-HYDROXYPYRIDINE WITH GLUTAMIC ACID OR SERINE

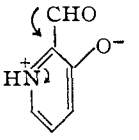
Reaction mixtures 0.01 *M* in glutamic acid, serine or α-ketoglutarate, 0.01 *M* in substituted pyridine,[a] 0.001 *M* in alum and 0.1 *M* in acetate buffer, *p*H 5.0, were heated 1 hr. at 100°.

Reactants		Products, mmoles/l.			
Pyridine cpd.	Other	α-Keto-glutarate	Pyruvate	Pyridine aldehyde	Ammonia
5-Desoxypyridoxal	.....	0.00	0.00	8.40	0.00
5-Desoxypyridoxal	Glutamate	6.76	..	2.80	..
5-Desoxypyridoxal	Serine	..	5.00	5.95	8.45
5-Desoxypyridoxamine	α-Ketoglutarate	7.02	..	2.77	..
3-O-Methylpyridoxal	Glutamate	0.03	..	..	..
3-O-Methylpyridoxal	Serine	..	0.00	..	0.00
2-Formyl-3-hydroxypyridine[a]	Glutamate	3.45	..	2.9	0.31
2-Formyl-3-hydroxypyridine[a]	Serine	..	2.09	6.4	2.75

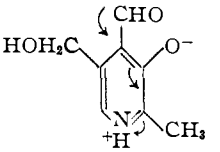
[a] Reaction mixtures were 0.0068 *M* in 2-formyl-3-hydroxypyridine; separate tests showed that both the transamination reaction of this compound with glutamate, and the dehydration reaction with serine were aluminum catalyzed, like the corresponding reactions of pyridoxal.

For non-enzymatic transamination only the formyl and phenolic groups in the proper orientation on the pyridine nucleus are required since 2-formyl-3-hydroxypyridine (III) undergoes this reaction and also catalyzes serine dehydration (Table I). In this compound the formyl group is in an electronically equivalent position on the pyridine nucleus as in pyridoxal and also in a sterically equivalent position with respect to the phenolic group.

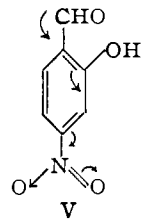
The function of the heterocyclic nitrogen atom was elucidated by studies in the benzene series where of all the compounds tested only 4-nitro- and 6-nitrosalicylaldehyde (V and VI) were found to undergo reactions similar to pyridoxal.[19] Of the aliphatic aldehydes tested glyoxylic acid was active in transamination but had very little catalytic activity toward serine dehydration or threonine cleavage.[16,18] Each of these active compounds can undergo similar and pronounced electronic displacements (III to VI).[21]



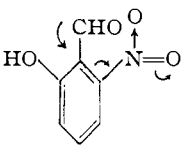
III



IV



V



VI

We propose that the strong electron attraction of these compounds is responsible for the electron-displacements from the bonds in the amino acids and that the mechanism of transmission of this electron-attraction to the α-carbon atom is provided by formation of a Schiff base[22] thus giving a conjugated system of double bonds extending from the electron-attracting group to the site of the reaction VII. The catalytic metal functions in promoting the formation of the Schiff base and maintaining planarity of the conjugated system through chelate ring formation,[23] which requires

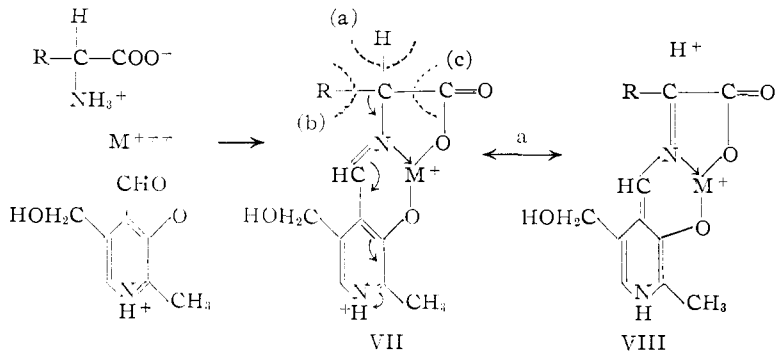
(20) E. Roberts, *J. Biol. Chem.*, **198**, 495 (1952).

(21) Glyoxylic acid can undergo a similar displacement. Under suitable conditions other aldehydes or ketones may have catalytic activity. Thus in alkaline solutions, pyruvate, benzaldehyde and salicylaldehyde catalyze the desulfhydration of cysteine (H. T. Clarke and J. M. Inouye, *J. Biol. Chem.*, **89**, 399 (1930)). These compounds can all undergo similar, though much less pronounced, electronic displacements but formaldehyde, which under the same conditions is a poor catalyst, cannot. Salicylaldehyde is a better catalyst than either pyruvate or benzaldehyde under these conditions.

(22) The Schiff bases of pyridoxal and alanine (D. Heyl, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3429 (1948)), glutamic acid (H. Brandenberger and P. P. Cohen, *Helv. Chim. Acta*, **36**, 549 (1953)), and many amines (D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **74**, 414 (1952)), form readily in high yield in alcohol. Various amines react with salicylaldehyde and copper, nickel or zinc salts in aqueous solutions to form Schiff base chelates some of which (those with amino acid esters) undergo racemization and other reactions (A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952, pp. 397–401). Blocking or loss of the amino group prevents the decarboxylation-transamination reaction of Herbst (*Advances in Enzymol.*, **4**, 75 (1944)); and no transamination between proline and pyridoxal has been observed.[12] These and additional observations[25,24] are in accord with the supposition that Schiff base formation must be intermediate to the reactions of pyridoxal considered here. Recently, G. L. Eichhorn and J. W. Dawes (Abstracts, Chicago Meeting, American Chemical Society, 63C (1953)) have provided direct spectrophotometric evidence for the formation in aqueous solutions of the Schiff base-metal chelate compounds assumed here and elsewhere[14] to be intermediates in the pyridoxal-catalyzed reactions of amino acids.

The substrates of all known vitamin B_6 enzymes contain a free primary amino group, and formation of Schiff base intermediates in enzymatic catalysis also has been assumed by A. E. Braunshtein and M. M. Shemyakin (*Doklady Akad. Nauk. S. S. S. R.*, **85**, 1115 (1952) [*C. A.*, **47**, 626 (1953)].

(23) The exact structures of the intermediate chelate compounds are not known, but are unessential to the argument presented. They may well vary from one amino acid to another, and with the *p*H of the reaction mixture. In particular, whether or not the carboxyl group is coördinated, as shown, would depend upon *p*H and other circumstances. Two such chelates have been isolated[17] and correspond approximately to VII in structure. Under the reaction conditions, unoccupied coördination positions could be filled by water or by another mole of Schiff base, yielding in the latter case chelates similar to those

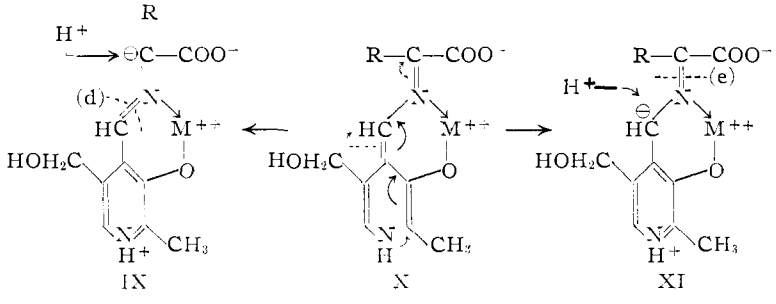


VII VIII

presence of the phenolic group. This chelated metal ion also provides an additional electron-attracting group that operates in the same direction as the heterocyclic nitrogen atom, thus increasing the electron displacements from the α-carbon atom.[24]

An electromeric displacement of electrons from bonds a, b, or c of VII would result in the release of a cation (H^+, R^+ or $COOH^+$)[25] and subsequently lead to the variety of reactions observed with pyridoxal. The extent to which one of these displacements predominates over others depends on the structure of the amino acid and the environment (pH, solvent, catalysts). We will consider the various vitamin B_6-catalyzed reactions in more detail on the basis of these proposals.

Case (a): Release of a Proton.—After the release of a proton the creation of a nucleophilic center at the α-carbon atom of the amino acid followed by the addition of a proton (VII → X → IX), and subsequent hydrolysis at d would lead to *racemization of the amino acid.*[3,15]

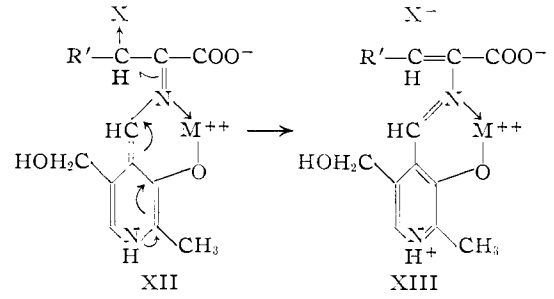


IX X XI

Creation of a nucleophilic center at the formyl carbon atom of pyridoxal would result in a net prototropic shift (VII → X → XI). Subsequent hydrolysis at e would then effect *transamination* between the amino acid and pyridoxal,[2,12,13] thus yielding pyridoxamine and an α-keto acid.[12,13]

Experimentally amino acids transaminate rapidly, but are not racemized by pyridoxal and metal salts at pH 5 except by a reversal of the transamination reaction.[15] Like the base-catalyzed prototropic rearrangements of Schiff bases studied by Ingold,[26] actual ionization does not occur but removal of a proton from the α-carbon and its addition at the formyl carbon must occur simultaneously. At pH 10 the rate of racemization exceeds that of transamination[15] suggesting an actual ionization of the α-hydrogen atom.

Instead of the creation of a nucleophilic center on the complex, the presence of an electron attracting group X at the β-carbon of the amino acid can result in the release of the extra electron pair in an anion (VII → XII → XIII).



XII XIII

Thus –OH (of serine and threonine), –SH (of cysteine) and –SR (of cystathionine) are eliminated in non-enzymatic[14,16] (see Experimental section also) and enzymatic[5,6,27,28] reactions, and indole is split from tryptophan[8] by a vitamin B_6-containing enzyme. Cleavage of the amino acid alliin by a vitamin B_6-containing enzyme[29] can be formulated in a similar fashion as the β-elimination of the elements of allylsulfenic acid. Likewise tyrosine is reported to give phenol by the action of an enzyme which is inhibited by carbonyl reagents.[30,31] In each case the resulting Schiff base of aminoacrylic acid (XIII) hydrolyzes to pyruvic acid (or ketobutyric acid from threonine), ammonia,[14] and pyridoxal or pyridoxal phosphate.

This intermediate Schiff base can however react in other ways. It can apparently be reduced by cysteine to alanine[31] and we have suggested that some enzymes permit the addition of another compound to form a new β-substituted amino acid.[6] Thus serine splits out water and adds indole to form

formed from salicylaldehyde, metal ions and amino compounds, discussed by Martell and Calvin (*cf.* footnote 22). Formation of metal chelates of this latter type in aqueous solutions of pyridoxal, metal ions and amino acids has been observed spectrophotometrically by Eichhorn and Dawes.[22] J. Baddiley (*Nature*, **170**, 711 (1952)) has isolated representatives of this type of chelate, containing, however, ethylenediamine in place of amino acids. Analysis of Baddiley's copper-pyridoxal-tyrosine chelate fits structure VII equally as well as that assumed for it by him.

(24) The catalysis of decarboxylation of β-keto acids by heavy metal ions is attributed by R. Steinberger and F. H. Westheimer (THIS JOURNAL, **73**, 429 (1951)) to a similar effect of the chelated metal ion. Martell and Calvin (ref. 22) also have emphasized this effect of chelated metal ions in explaining several reactions of bisalicylaldimine chelates, a concept extended to the analogous chelates of pyridoxal by Baddiley.[23]

(25) Single-stage (Lowry) mechanisms may be involved but for simplicity the reactions are all formulated as involving the loss of a cation.

(26) S. K. Hsü, C. K. Ingold and C. L. Wilson, *J. Chem. Soc.*, 1778 (1935); R. P. Ossario and E. D. Hughes, *ibid.*, 426 (1952).

(27) C. Yanofsky, *J. Biol. Chem.*, **198**, 343 (1952).

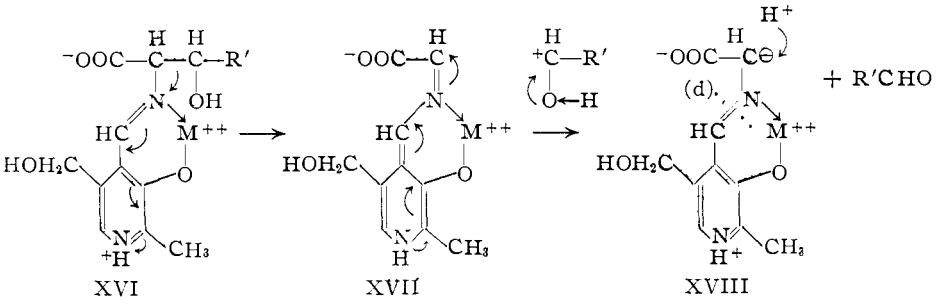
(28) F. Binkley and A. Hudgins, *Federation Proc.*, **12**, 178 (1953).

(29) E. V. Goryachenkova, *Doklady Akad. Nauk. S. S. S. R.*, **87**, 456 (1952) [*C. A.*, **47**, 4928 (1953)].

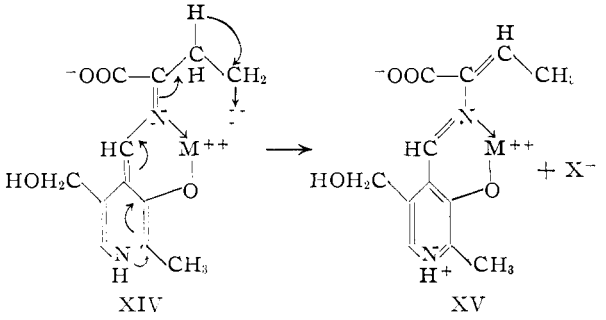
(30) A. Oshima, *J. Osaka Med. Assoc.*, **39**, 1523 (1940).

(31) K. Ohigashi, A. Tsunetoshi, M. Uchida and I. Ichihara, *J. Biochem.* (*Japan*), **39**, 211 (1952).

tryptophan in the presence of a pyridoxal phosphate activated enzyme.[9,32] Cystathionine is formed enzymatically from serine and homocysteine by a similar reaction stated to require pyridoxal phosphate.[28,33,34] The formation of tryptophan in low yield by the non-enzymatic reaction of serine and indole with pyridoxal and metal salt catalysis has also been demonstrated (Experimental section).



In contrast to these β-elimination reactions, which proceed readily both enzymatically and non-enzymatically, γ-elimination reactions analogous to the conversion of homocysteine[7] and homoserine[35] to ketobutyrate and ammonia by pyridoxal phosphate enzymes do not proceed rapidly in our non-enzymatic systems, and their observation is complicated by the occurrence of more rapid reactions (*e.g.*, transamination, β-elimination, see Experimental). The reactions may, however, be visualized in terms of the mechanism presented here as proceeding by (1) ionization of the α-hydrogen atom followed by a Wagner–Meerwein type of rearrangement (VII → XIV → XV), or (2) β–γ-desaturation followed by isomerization catalyzed

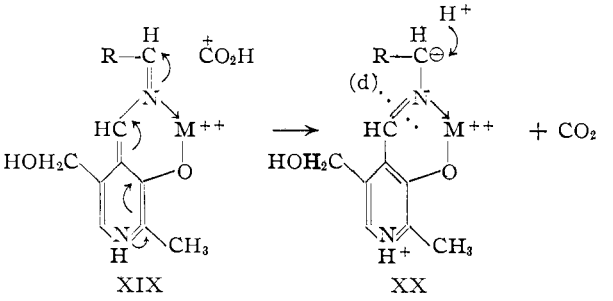


by pyridoxal through removal of a proton at the α-position and addition to the γ-carbon. In the latter case, pyridoxal may promote principally removal of the α-hydrogen but not that of the γ-substituent without the assistance of another catalyst, *e.g.*, another portion of the enzyme.

Case (b): Release of R^+.—After release of a cation (R^+) of suitable structure, such as R'–C^+HOH (XVI → XVII), the subsequent addition of a proton to the α-carbon atom of the amino acid (XVII → XVIII) followed by hydrolysis at bond (d) would result in the formation of glycine. The splitting of threonine and allothreonine (R' = CH_3) to glycine and acetaldehyde and of serine (R' = H) to glycine and formaldehyde is strongly catalyzed by pyridoxal and metal salts.[11,16] The reverse of these reactions leading to the formation of threonine from acetaldehyde and glycine and of serine from formaldehyde and glycine also occurs.[11,16] The evidence that vitamin B_6 is involved in corresponding enzymatic reactions has been summarized previously.[11]

The compound corresponding to XVI in which M = aluminum or iron and R' = (2-methyl-3-hydroxy-5-hydroxymethyl-pyridyl-4) has been isolated as a product of the reaction of glycine, pyridoxal and aluminum[16] and its subsequent hydrolysis has led to β-(2-methyl-3-hydroxy-5-hydroxymethyl-pyridyl-4)-serine.[16,36]

Case (c): Release of $COOH^+$.—The third case involving the release of $COOH^+$ would lead to decarboxylation (VII → XIX → XX) which



followed by hydrolysis at bond (d) would lead to the formation of the corresponding amine.[37]

All amino acid decarboxylases so far examined require pyridoxal phosphate for activity[4,38] and Werle and Koch[17] have reported without details that pyridoxal and pyridoxal phosphate catalyze the non-enzymatic decarboxylation of histidine to histamine.

Biochemical Significance.—These non-enzymatic reactions correspond very closely to the enzymatic

(32) C. Yanofsky, *J. Biol. Chem.*, **194**, 279 (1952).

(33) F. Binkley, G. M. Christensen and W. N. Jensen, *ibid.*, **194**, 109 (1952).

(34) A related reaction, the addition of homocysteine to the double bond of α-acetamidoacrylic acid, has been used for the preparation of cystathionine (A. Schoberl and A. Wagner, *Naturwissenshaften*, **37**, 113 (1950)). Likewise hydrogen sulfide, cysteine and benzyl mercaptan add to serine or cysteine residues of wool following their dehydration or desulfhydration by alkali (D. M. Greenberg, "Amino Acid and Proteins," 1st ed., Springfield, 1951, p. 569).

(35) F. Binkley and C. K. Olson, *J. Biol. Chem.*, **185**, 881 (1950).

(36) The condensations of aromatic aldehydes with glycine when catalyzed by aqueous alkalies, and with glycine esters in absolute ether when catalyzed by sodium, similarly yield Schiff base intermediates (E. Erlenmyer, Jr., and E. Früstück, *Ann.*, **284**, 36 (1895); C. E. Dalgliesh and F. G. Mann, *J. Chem. Soc.*, 685 (1947).

(37) A somewhat similar reaction in which an amino acid is simultaneously decarboxylated and undergoes transamination with a keto acid is formulated by R. M. Herbst (*Advances in Enzymol.*, **4**, 75 (1944)) as proceeding through an intermediate Schiff base. The reaction is not prevented by replacing the α-hydrogen of the amino acid by an alkyl group. When the α-hydrogen is present it does not exchange with the deuterium of heavy water and in this respect resembles enzymatic decarboxylation (R. Koppleman, S. Mandeles and M. E. Hanke, *Federation Proc.*, **11**, 242 (1952)). We find these reactions are not metal-salt catalyzed and are very slow compared to transamination between pyridoxal and amino acids.

(38) E. Werle, *Angew. Chem.*, **63**, 550 (1951).

reactions catalyzed by vitamin B_6 proteins, and it may be assumed that the latter proceed by a similar mechanism. The requirement for metal ions for the non-enzymatic reactions strongly suggests that such ions may likewise be essential for the enzymatic reactions, but only limited indications of this have been obtained to date,[27] and it is possible that a portion of the enzyme protein itself could fill the role played by metal ions in the non-enzymatic reactions. If metal ions are involved in the enzymatic reactions, the unoccupied coördination positions of the metal chelates formulated herein could be occupied by groups from the apoenzyme, thus furnishing a common tie between apoenzyme, coenzyme and substrate.[1a] The universally experienced difficulty (*e.g.*, ref. 3–6) in resolving these enzymes into apoenzyme and coenzyme suggests the presence of some type of non-ionic linkage. The requirement for relatively prolonged preincubation periods between coenzyme and apoenzyme for activation of various vitamin B_6 enzymes[38a] also is consistent with formation of non-ionic linkages of the type postulated here.

Experimental

2-Formyl-3-hydroxypyridine and Derivatives.—3-Hydroxypyridine and formaldehyde were condensed to yield 2-hydroxymethyl-3-hydroxypyridine,[39] isolated as its hydrochloride. To 4.0 g. of the latter crude product in 90 ml. of 0.55 *N* hydrochloric acid was added 40 g. of powdered manganese dioxide. The mixture was swirled occasionally during three days at room temperature, filtered, and the manganese dioxide washed with water. The combined filtrates were concentrated in vacuum, the resulting sirup dissolved in a small amount of water, and applied to a column (36 × 520 mm.) of Dowex 50 (200–400 mesh) in the acid form. The column was washed with 1.6 l. of water, then developed with 1 *N* hydrochloric acid. By analogy with the properties of pyridoxal, the desired product should form an insoluble dinitrophenylhydrazone, give a positive phenol test with ferric chloride, and a strongly yellow color upon treatment with sodium carbonate. The major fraction (fraction E) with these properties followed closely a band containing unreacted 2-hydroxymethyl-3-hydroxypyridine and eluted between 3500 and 4300 ml. of the acid effluent. Concentration in vacuum yielded 0.85 g. of sirup. In 25% ethanolamine[13] it gave λ_{max} 257 mμ (a_M 6.2 × 10^3) and λ_{max} 359 mμ (a_M 4.65 × 10^3). However, considering the similarity of the 359 mμ peak to that of pyridoxal at 365 mμ (a_M 6.8 × 10^3) the fraction may be estimated at approximately 68% purity. Addition of 0.1 g. of 2,4-dinitrophenylhydrazine in 30 ml. of 2 *N* hydrochloric acid to 51 mg. of fraction E in 15 ml. of water yielded 79 mg. (72%) of the insoluble dinitrophenylhydrazone. Recrystallization from 50% ethanol (100 ml.) yielded 47 mg. of fine silky needles, m.p. 219–221° dec. (cor.).

Anal. Calcd. for $C_{12}H_9O_5N_5 \cdot HCl$ (339.7): N, 20.6. Found: N, 19.7.

The aldehyde was further characterized by oxidation to the known, corresponding acid. A slurry of freshly prepared silver oxide (from 2.0 g. of silver nitrate) was added to 94 mg. of fraction E in 10 ml. of water. The mixture was shaken in the dark for 18 hours, treated with an excess of hydrogen sulfide, filtered, and the filtrate concentrated to dryness. The resulting solid (31.8 mg.) was dissolved in water (1.5 ml.), neutralized with sodium hydroxide, acidified with 0.1 ml. of glacial acetic acid, and treated dropwise with 3 ml. of 0.1 *M* cupric acetate. The green precipitate was collected, washed with water, resuspended in 5 ml. of water and decomposed with hydrogen sulfide. After coagulation with a minimum of sodium chloride, the cupric sulfide was centrifuged out, the supernate evaporated to dryness, and extracted with hot absolute ethanol. Concentration of the extract to small volume yielded glistening needles; recrystallization from ethanol yielded crystals (16.0 mg.), m.p. 205° dec. (cor.). 3-Hydroxy-2-picolinic acid obtained by permanganate oxidation of 2-hydroxymethyl-3-hydroxypyridine melts at 205°[39]; isomeric 3-hydroxypicolinic acids melt much higher.

Other Chemicals and Analytical Determinations.—5-Desoxypyridoxal,[40] 5-desoxypyridoxamine[40] and 2-methyl-3-methoxy-4-formyl-5-hydroxymethylpyridine (3-O-methylpyridoxal)[41] were gifts from Dr. Karl Folkers. Formation and disappearance of 5-desoxypyridoxal in reaction mixtures was measured spectrophotometrically at 383 mμ in 0.1 *M* phosphate buffer, *p*H 6.8, where this compound shows a pronounced absorption maximum (a_M 4.26 × 10^3) not shared by 5-desoxypyridoxamine. 2-Formyl-3-hydroxypyridine was determined by the ethanolamine procedure used for pyridoxal.[13] Keto acids and ammonia were determined as described elsewhere.[13,14,18] Tryptophan was determined microbiologically[42] with *Streptococcus faecalis* 8043, which cannot use indole in place of tryptophan.[43]

Formation of Tryptophan from Serine and Indole.—Solutions containing serine (0.05 *M*), indole (0.05 *M*), pyridoxal (0.02 *M*) and alum (0.002 *M*) at various *p*H values were heated 30 minutes at 100°. After these treatments samples of *p*H 5–6 contained small amounts of ninhydrin-positive material of R_f value identical with tryptophan when chromatographed on paper in water-saturated phenol or 77% ethanol. Microbiological assay of the sample heated at *p*H 5.0 showed the presence of 0.3–0.4 m*M* tryptophan (0.6–0.8% yield). Longer heating decreased the yield. Control samples with pyridoxal omitted as well as unheated samples assayed immediately after preparation produced no tryptophan. Samples heated at *p*H 6.0 or allowed to stand at room temperature for ten days contained 0.1–0.2 m*M* tryptophan. No tryptophan was formed at *p*H 10.0.

The low yield of tryptophan is not surprising in view of the rapid and irreversible deamination of serine under these conditions,[14] the inactivation of pyridoxal by heating with tryptophan or with indole[12] (presumably by the well-known tryptophan–aldehyde reaction), and the probability that tryptophan formed would undergo other pyridoxal-catalyzed reactions, such as degradation to indole, pyruvate and ammonia, and transamination.

Reactions of Pyridoxal with Cystine, Homocystine, Homoserine and Cystathionine.—Reaction mixtures similar to those used in studying deamination of serine and cysteine[14] were prepared, heated at *p*H 5.0, and tested qualitatively for hydrogen sulfide and keto acid production. Cystine gave a rapid production of large amounts of pyruvate, hydrogen sulfide and some precipitated sulfur. In addition to that of pyruvic acid, dinitrophenylhydrazones of other keto acids, probably transamination products, were obtained in small amounts. Homocystine gave comparatively only a small amount of hydrogen sulfide and of keto acids similar to the minor zones obtained from cystine, but little or no ketobutyrate. Homoserine gave only a very small amount of keto acid corresponding in R_f value of its dinitrophenylhydrazone to ketobutyrate, but a larger quantity of a new keto acid, probably the transamination product. Cystathionine gave a rapid production of pyruvate, but only a small amount of hydrogen sulfide, in accordance with the view that cleavage occurred by β-elimination to yield principally homocysteine, pyruvate and ammonia. Minor unidentified keto acids also were produced.

These qualitative results show that the elimination of a γ-substituent from these amino acids proceeds slowly under these conditions, but at a much slower rate than that of a β-substituent, and is difficult to observe due to the occurrence of other, more rapid reactions.

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