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Pyridoxal-Catalyzed Decarboxylation of Amino Acids*

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When α-amino acids (*e.g.*, α-aminoisobutyric acid, α-methylserine, α-phenylglycine) are heated with pyridoxal in dilute aqueous solutions in the absence of metal ions, two closely related but independent reactions occur as follows:

$$RR'CNH_2COOH \xrightarrow{\text{Pyridoxal}} RR'CHNH_2 + CO_2 \quad (1)$$

$$RR'CNH_2COOH + \text{Pyridoxal} \longrightarrow RR'C{=}O + CO_2 + \text{Pyridoxamine} \quad (2)$$

Reaction (1) is analogous to decarboxylation of amino acids by pyridoxal phosphate enzymes. Reaction (2) is a decarboxylation-dependent transamination reaction for which no enzymatic analogy is known. Reactions (1) and (2) are both partially inhibited by those metal ions that catalyze previously studied reactions between pyridoxal and amino acids. These observations are explained in terms of the general mechanism for pyridoxal-catalyzed reactions presented previously (Metzler *et al.*, 1954; Snell, 1958).

Most reactions of amino acids that are catalyzed by pyridoxal phosphate enzymes are also catalyzed at slower rates by pyridoxal in dilute aqueous solution (Metzler *et al.*, 1954; Snell, 1958). Study of such nonenzymatic reactions has provided a sound experimental basis for current concepts of the catalytic role of the coenzyme in pyridoxal phosphate–dependent enzymes. Nonenzymatic decarboxylation of amino acids is known to occur in such systems from the study of Werle and Koch (1949), who used pharmacologic techniques to detect histamine formation from histidine in the presence of pyridoxal. However, no detailed study of this reaction has appeared. Results presented herein show that such nonenzymatic decarboxylation reactions are readily observable. They are of a special interest since, unlike other model reactions catalyzed by pyridoxal, they are independent of or actually inhibited by metal ions. A novel decarboxylation-dependent transmination reaction can be observed under these conditions when amino acids lacking an α-hydrogen atom are employed.

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Experimental Procedures

To minimize metal ion contamination, all stock solutions were prepared with distilled water that was passed through a mixed bed deionizing resin, and buffer salts were recrystallized from dilute solutions of disodium ethylenediaminetetraacetate. Procedures were similar to those described by Metzler and Snell (1952a,b). Reaction mixtures (1–10 ml) were heated for the desired time in sealed soft-glass tubes or in flasks equipped with reflux condensers. The contents were cooled and the products formed were identified and analyzed by the following procedures.

Pyridoxal was estimated by the ethanolamine procedure, and *pyridoxal plus pyridoxamine* by the absorbancy at 323.5 mμ and *p*H 6.7 (Metzler and Snell, 1952a). *Pyridoxamine* was separated from pyridoxal by chromatography on paper

located by examination under ultraviolet light,[1] eluted with water, and estimated spectrophotometrically at 323.5 mμ and *p*H 6.7.

Amino acids and *benzylamine* were separated on chromatograms and estimated by the procedure of Giri *et al.* (1952). Heating of the mixtures following their application to the paper was avoided to eliminate occurrence of the reactions between keto acids and amino acids observed on heated chromatograms (Giri and Kalyankar, 1953; Giri *et al.*, 1954). The upper layer of *n*-butanol–acetic acid–water (40:10:50) was the developing solvent for reaction mixtures containing α-phenylglycine or α-amino*iso*butyric acid; pyridine–water (80:20) was used for mixtures containing α-methylserine or α-hydroxymethylserine. *Isopropylamine* was distilled from alkalinized reaction mixtures and identified by chromatography against appropriate standards. *Benzylamine* was identified by extraction into ether from the alkalinized reaction mixture. Addition of a saturated ethanolic solution of picric acid to the concentrated ethereal extract yielded crystalline benzylamine picrate. On recrystallization from ethanol, it melted at 196°, showed no depression of melting point on admixture with authentic benzylamine picrate, and analyzed satisfactorily for nitrogen (found, 16.49; calculated, 16.65%). *Acetone* was identified by distillation from acidified reaction mixtures and isolation as its 2,4-dinitrophenylhydrazone (m.p. 125–126°, no depression with an authentic sample; N, found, 23.68%; calculated, 23.51%). It was estimated routinely by the method of Behre (1940).

Acetol was identified by isolation as its 2,4-dinitrophenylosazone, which, after recrystallization from dioxane, melted at 298° (with decomposition). No change in melting point occurred on admixture with an authentic sample of acetol 2,4-dinitrophenylosazone. Acetol was estimated by conversion to lactic acid by copper-lime treatment and analysis of the lactic acid with *p*-hydroxydiphenyl (Huggins and Miller, 1956). In the presence of high concentrations of α-methylserine, slightly high values were obtained. This difficulty was overcome by use of standards containing appropriate amounts of α-methylserine. Formaldehyde, if present, was quantitatively removed by the addition of dimedon (Brin and Olson, 1952), holding overnight, and centrifuging out the precipitated formaldemethone.

Dihydroxyacetone was isolated from reaction mixtures as its 2,4-dinitrophenylosazone (m.p. 278°; mixed m.p. with the authentic compound, 278°). It was estimated routinely by the phosphomolybdate method of Campbell (1926). None of the reactants or reaction products interferes except pyruvate at high concentrations.

Ammonia was determined by aeration from alkalinized samples into acid and Nesslerization (Metzler and Snell, 1952b).

Pyruvate was estimated colorimetrically as its 2,4-dinitrophenylhydrazone after the removal of the 2,4-dinitrophenylhydrazone of pyridoxal (Metzler and Snell, 1952a).

Formaldehyde was estimated by the chromotropic acid procedure of Frisell *et al.* (1954). Interference due to pyridoxal was minimized by addition of an equal volume of saturated thiourea in water, which reduces the color intensity of the pyridoxal complex (λ_{max} 615 mμ)[2] without affecting that of the formaldehyde complex (λ_{max} 575 mμ). Experiments in which *carbon dioxide* was determined were carried out in a small flask equipped with a short reflux condenser fitted with an inlet tube through which a slow stream of nitrogen was passed and an outlet connected to an absorption train. Carbon dioxide in the acid-washed effluent gas was trapped in barium hydroxide solution; the barium carbonate was centrifuged out, washed with distilled water, dissolved in standard HCl, and titrated.

Results

Observation of nonenzymatic decarboxylation is complicated by the occurrence of a variety of competing pyridoxal-catalyzed reactions, *e.g.* transamination, many of which are dependent upon the presence of an α-hydrogen atom (Snell, 1958). To minimize such reactions, decarboxylation of α-substituted amino acids was investigated.

Reactions of α-Aminoisobutyric Acid.—When α-amino*iso*butyric acid and pyridoxal are heated together in dilute aqueous solution, CO_2, isopropylamine, acetone, and pyridoxamine are formed (Table I), together with at least two unidentified products which fluoresced on chromatograms under ultraviolet light. Control experiments revealed no detectable transamination between isopropylamine and pyridoxal, or between acetone and pyridoxamine under these conditions; furthermore, both CO_2 and pyridoxamine production was *inhibited* by addition of Cu^{++} or Al^{+++}, but not by EDTA. In the absence of added metal ions, the CO_2 formed is equivalent to the acetone *plus* pyridoxamine produced, and approximately equals α-amino*iso*butyrate disappearance if the latter is corrected for destruction of the amino acid in the absence of pyridoxal. Acetone and pyridoxamine are formed in equimolar amounts. These findings suggest occurrence of two independent reactions, (1*a*) and (2*a*), of approximately equal quantitative significance. Neither of these reactions is metal ion–dependent, but they are instead somewhat

[1] The characteristic orange color formed with ninhydrin on chromatograms served to confirm the position of pyridoxamine.

[2] This color reaction provides a means for estimation of pyridoxal which is somewhat more sensitive than the ethanolamine procedure of Metzler and Snell (1952a) but less sensitive than the phenylhydrazine procedure of Wada and Snell (1961). Pyridoxine and pyridoxamine do not interfere.

TABLE I

REACTIONS OF α-AMINO*iso*BUTYRIC ACID IN THE PRESENCE OF PYRIDOXAL AT 100°

The complete reaction mixture contained 40 μmoles of pyridoxal and 40 μmoles of α-amino*iso*butyrate in 10.0 ml of 0.1 N sodium acetate buffer, *p*H 5.0, and was heated at 100° for 4 hours.

	Reactants Disappearing (μmoles)		Products Formed (μmoles)			
Additions	Pyridoxal	α-Amino-*iso*-butyrate	Isopropyl-amine	Pyridox-amine	Acetone	CO_2
None[a]	6.32	4.8	+	1.69	1.67	3.53
EDTA (0.01 M)	5.80	5.0	+	1.72	1.68	3.58
Al^{+++} (0.01 M)	5.20	3.9	+	1.2	1.2	3.34
Fe^{+++} (0.01 M)	4.02				1.40	3.14
Cu^{++} (0.01 M)	4.17				0.97	3.06

[a] When pyridoxal was omitted, no isopropylamine was formed but 1.22 μmoles of α-amino*iso*butyrate disappeared during this 4-hour heating period. When α-amino*iso*butyrate was omitted, 1.02 μmoles of pyridoxal disappeared.

TABLE II

COMPARISON OF PRODUCTS FORMED ON HEATING PYRIDOXAL WITH α-METHYLSERINE IN THE PRESENCE AND ABSENCE OF Cu^{++}

Reaction mixtures contained 10 μmoles of α-methyl-DL-serine and 2 μmoles of pyridoxal, with and without Cu^{++} (1 μmole) as indicated, in 3.0-ml volumes. The *p*H was adjusted to the indicated value with dilute NaOH. The mixtures were heated for 2 hours at 100°.

	Residual Reactants		Products					
*p*H	α-Methyl-serine	Pyri-doxal	Pyridox-amine	Acetol	Pyru-vate	Alanine	HCHO	NH_3
				(μmoles per 3 ml)				
4.9	8.90	1.66	0.35	0.32	0.0	0.0	0.0	0.0
6.7	7.60	1.60	0.30	0.28	0.0	0.0	0.0	0.0
6.8 (+ Cu^{++})	4.8	1.14	Traces	0.12	1.32	0.92	2.65	0.56

inhibited by addition of metal ions. The reac-

α-Aminoisobutyric acid $\xrightarrow{\text{Pyridoxal}}$ Isopropylamine + CO_2 (1a)

α-Aminoisobutyric acid + Pyridoxal ⟶ Pyridoxamine + Acetone + CO_2 (2a)

tions are slow, and their stoichiometry could not be established more fully for lack of a convenient method for determining the highly volatile isopropylamine, and because of the destruction of pyridoxal *via* other reactions during the long heating period. The optimal *p*H, as measured by formation of either CO_2 or acetone, was 3.5 to 6.0, but some reaction occurs from *p*H 2.0 to *p*H 9.0.

Reactions of α-Methylserine and α-Hydroxymethylserine.—With α-methylserine as substrate, reaction (1) should lead to 2-aminopropanol and CO_2, and reaction (2) to acetol, pyridoxamine, and CO_2 as products. In addition, as shown by Longenecker *et al.* (1957), α-methylserine undergoes reaction (3) in the presence of pyridoxal and metal ions, which may be followed by reaction (4) (Metzler and Snell, 1952a,b). In the presence of Cu^{++} a pyridoxal-catalyzed oxidation of amines [*e.g.* reaction (5)] also occurs to yield ammonia (Ikawa and Snell, 1954a).

$$HOCH_2C(CH_3)(NH_2)COOH \xrightarrow{\text{Pyridoxal}} HCHO + CH_3CHNH_2COOH \quad (3)$$

$$CH_3CHNH_2COOH + \text{Pyridoxal} \xrightarrow{Cu^{++}} CH_3COCOOH + \text{Pyridoxamine} \quad (4)$$

$$\text{Pyridoxamine} \xrightarrow[\text{Pyridoxal}]{Cu^{++},O_2} \text{Pyridoxal} + NH_3 \quad (5)$$

The products formed from this amino acid in the presence of pyridoxal are shown in Table II. In the absence of added metal ions, acetol and pyridoxamine are formed in equimolar amounts in accordance with equation (2); CO_2 and 2-aminopropanol were not determined. No products corresponding to reactions (3) to (5) are observed when cupric ion is omitted; when Cu^{++} is added, these latter reactions predominate.

Entirely similar results were obtained when α-hydroxymethylserine was the amino acid. With no added metal ions, dihydroxyacetone and pyridoxamine were formed in equimolar amounts; no pyruvate, serine, glycine, formaldehyde, or NH_3 appeared. On addition of Cu^{++}, each of

TABLE III

PYRIDOXAL-CATALYZED FORMATION OF BENZYLAMINE FROM α-PHENYLGLYCINE UNDER VARIOUS CONDITIONS

The reaction mixtures contained 10 μmoles of α-phenylglycine, 10 μmoles of pyridoxal, and (where indicated in B) 2 μmoles of metal ion in 3.0 ml of 0.1 M acetate buffer, *p*H 4.00. They were heated 30 minutes at 100°. All analytical values are in terms of μmoles per 3.0 ml of reaction mixture.

A. *Stoichiometry*

Gain or Loss of Reactants and Products					
α-Phenylglycine	Pyridoxal	Pyridoxamine	Benzylamine	Benzoylformate	Benzaldehyde
−5.02	−2.87	+2.84	+2.19	0.00	+++

B. *Effect of Metal Ions and EDTA*

	Changes in		Additions (μmoles)		Changes in	
Addition	Pyridoxal	Benzylamine	Al^{+++}	EDTA	Pyridoxal	Benzylamine
None	−2.86	+2.19	None	None	−2.86	+2.17
EDTA[a]	−2.65	+2.18	2.0	None	−4.32	+1.14
Al^{+++}	−4.32	+1.65	2.0	1.0	−3.54	+1.36
Ca^{++}	−2.86	+2.27	2.0	2.0	−2.65	+1.84
Cu^{++}	−3.05	+1.12	2.0	10.0	−2.65	+2.05
Fe^{+++}	−3.20	+1.48				
Mg^{++}	−2.81	+2.18				

[a] 2 μmoles per 3.0 ml.

the latter products was formed (*cf.* Longenecker *et al.*, 1957) and only traces of dihydroxyacetone appeared.

Reactions of α-Phenylglycine.—Decarboxylation of α-phenylglycine could be followed readily by appearance of benzylamine on chromatograms of reaction mixtures. With short heating periods in the absence of metal ions, the phenylglycine destroyed approximately equaled the sum of the benzylamine and pyridoxamine produced (Table III); benzaldehyde was detected by its characteristic odor but was not determined quantitatively. The reaction is most rapid at *p*H 4.0 and is wholly dependent on pyridoxal (Fig. 1). Again, the decarboxylation reaction, as measured by benzylamine formation, is inhibited by the same metal ions (Al^{+++}, Cu^{++}, Fe^{+++}) that promote nonenzymatic transamination, but is unaffected by those ions that do not (Table III, Fig. 2). Control experiments showed there was no transamination between pyridoxal and benzylamine or between pyridoxamine and benzaldehyde under these conditions of temperature and *p*H. These latter reactions occurred when the *p*H was raised to 8.5 or above, but only to a slight extent. These results thus indicate that benzylamine arises by reaction (1b). Benzaldehyde can arise by the decarboxylation-dependent transamination reaction (2b), but since α-phenylglycine possesses an α-hydrogen, benzaldehyde may also arise by a metal ion–catalyzed transamination reaction, (6), similar to those studied previously (Metzler and Snell, 1952a), followed by decomposition of the benzoylformate formed [reaction (7)]. The increase in pyridoxal disappearance (principally pyridoxamine formation) that occurs when Al^{+++} is added (Fig. 2, curve C) provides evidence that

$$C_6H_5CHNH_2COOH \xrightarrow{\text{Pyridoxal}} C_6H_5CH_2NH_2 + CO_2 \quad (1b)$$

$$C_6H_5CHNH_2COOH + \text{Pyridoxal} \longrightarrow \text{Pyridoxamine} + CO_2 + C_6H_5CHO \quad (2b)$$

$$C_6H_5CHNH_2COOH + \text{Pyridoxal} \longrightarrow \text{Pyridoxamine} + C_6H_5COCOOH \quad (6)$$

$$C_6H_5COCOOH \longrightarrow CO_2 + C_6H_5CHO \quad (7)$$

reactions (6) and (7) contribute to the over-all reaction, especially in the presence of metal ions. The coincident decrease in benzylamine formation (Fig. 2, curve B) confirms previous data showing that the rate of the decarboxylation reaction is slowed by metal ions.

No decarboxylation (or transamination) of α-phenylglycine occurred when pyridoxal was replaced in reaction mixtures by salicylaldehyde or by 2 - methyl - 3 - methoxy - 4 - formyl - 5 - hydroxymethylpyridine,[3] in which the phenolic hydrogen of pyridoxal is replaced by a methyl group. The structural requirements for catalysis of the decarboxylation reaction thus resemble those found for the transamination reaction (Ikawa and Snell, 1954b; Snell and Jenkins, 1959).

DISCUSSION

Three points of principal interest emerge from these studies: (1) occurrence of a readily observed pyridoxal-catalyzed nonenzymatic decarboxylation of α-amino acids in dilute aqueous solutions that serves as a model for decarboxylation of

[3] We are indebted to Dr. Karl Folkers of Merck, Sharpe and Dohme for a small sample of this compound.

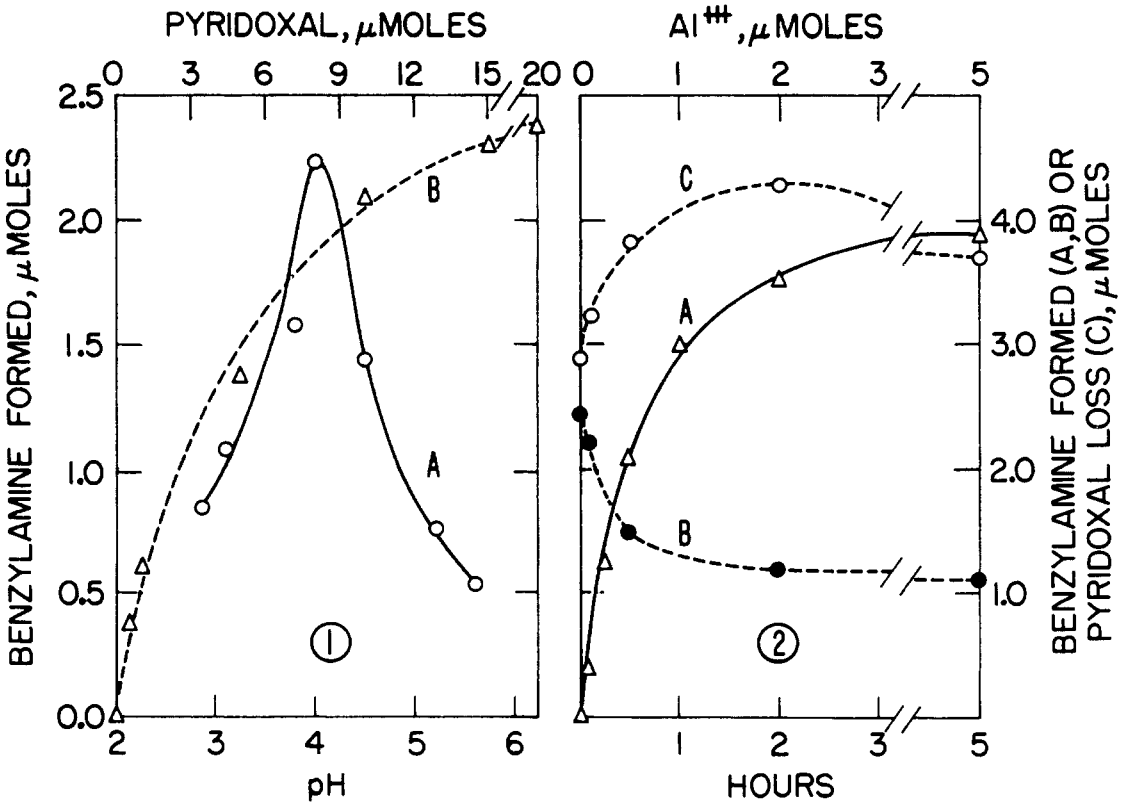

FIG. 1.—*Curve A* (bottom abscissa): *p*H optimum for benzylamine formation from α-phenylglycine in the presence of pyridoxal. The reaction mixture contained 10 μmoles each of α-phenylglycine and pyridoxal in 3.0 ml 0.1 M acetate at the indicated initial *p*H. The mixtures were heated for 30 minutes at 100°. *Curve B* (top abscissa): Relation of benzylamine formation to pyridoxal concentration. Indicated amounts of pyridoxal were heated for 30 minutes at 100° with 10 μmoles of α-phenylglycine in 3.0 ml of 0.1 M acetate buffer, *p*H 4.0.

FIG. 2.—*Curve A* (bottom abscissa): Relation of benzylamine formation to time during reaction between α-phenylglycine and pyridoxal. Ten μmoles of each reactant per 3.0 ml of 0.1 M acetate buffer, *p*H 4.0, 100°. *Curve B* (top abscissa): Effect of concentration of Al^{+++} on benzylamine formation or (*Curve C*) pyridoxal loss during reaction of α-phenylglycine with pyridoxal. Reaction mixtures as in *A* with added Al^{+++} as indicated were heated for 30 minutes at 100°.

amino acids by pyridoxal phosphate enzymes; (2) the coincident occurrence of a previously unobserved decarboxylation-dependent transamination reaction between pyridoxal and amino acids; and (3) the observation that these reactions are inhibited by those same metal ions that catalyze all previously studied model reactions between pyridoxal and amino acids. Each of these observations can be visualized in terms of the general mechanism for pyridoxal-catalyzed reactions developed previously (Metzler *et al.*, 1954; Snell, 1958). Reaction (1) proceeds (Fig. 3) by Schiff's base formation between pyridoxal and amino acid followed by an electromeric shift of electrons from the bond to the carboxyl group in I to yield carbon dioxide and a transition form, II, which stabilizes as the Schiff's base, III, of pyridoxal and an amine, which exists in aqueous solution in equilibrium with the latter compounds. However, II can also stabilize as IV, the Schiff's base of pyridoxamine with an aldehyde or ketone, thus giving the decarboxylation-dependent transamination reaction, (2). No enzymatic analogy for the latter reaction is known.[4] The two reactions are analogous to those between α-keto acids and α-amino acids discussed by Herbst (1944), but occur at substantially higher rates than the latter and in the physiologic *p*H range.

When an appropriate metal ion replaces the phenolic hydrogen of these chelate structures, two types of compounds, V and VI (Fig. 3), become possible, with a pronounced tendency toward

[4] The pyridoxal phosphate–dependent, enzymatic synthesis of sphingosine (Brady *et al.*, 1958) is a closely related reaction. It can be formulated as an addition of palmitaldehyde to the complex II or III (Fig. 3) derived from serine by decarboxylation, followed by hydrolysis. Dr. A. Neuberger (unpublished; see Snell, 1961) has observed a similar nonenzymatic reaction in which aminomalonic acid and formaldehyde interact in the presence of pyridoxal to form serine and carbon dioxide. The reaction is not catalyzed by metal salts and is accompanied by decarboxylation of aminomalonic acid to glycine.

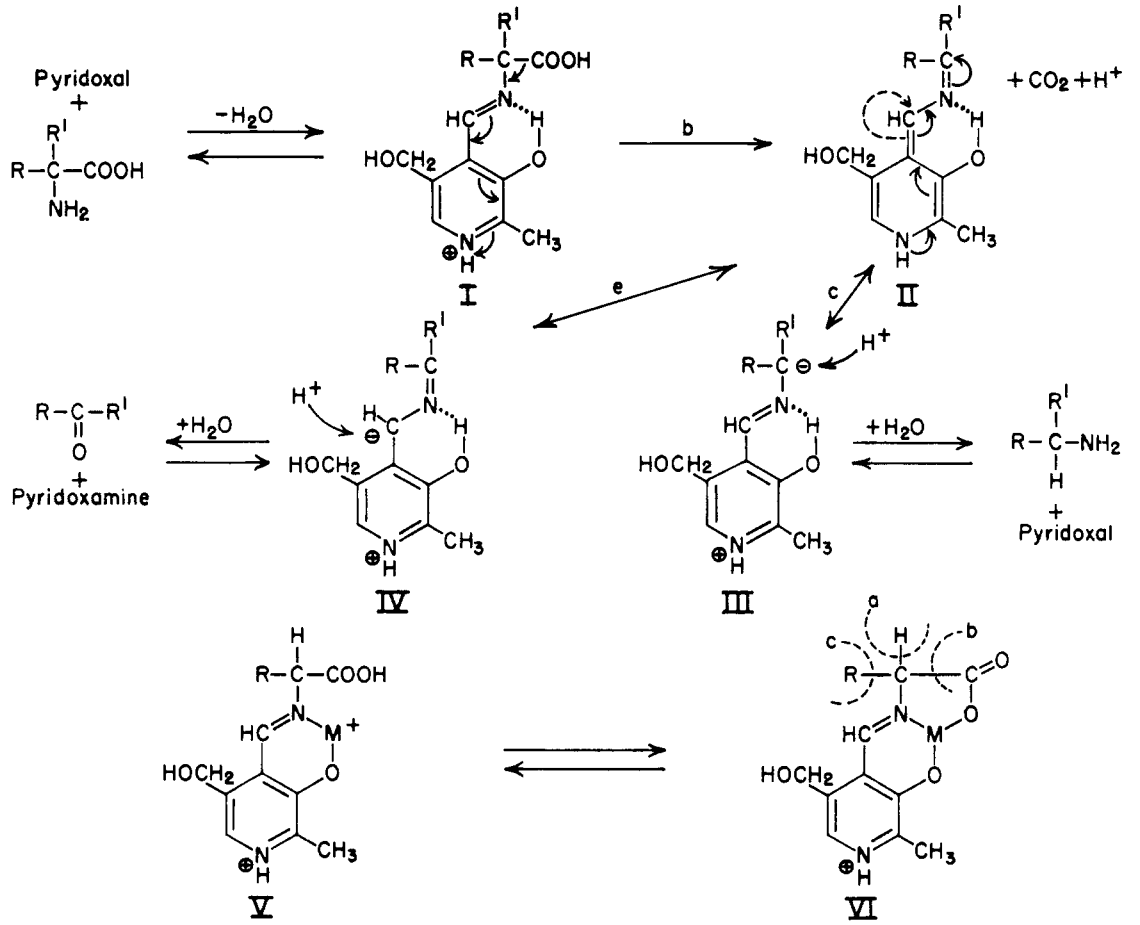

FIG. 3.—Diagrammatic representation of the decarboxylation and decarboxylation-dependent transamination of amino acids in the presence of pyridoxal (R or R′ represent hydrogen or organic radicals, M an appropriate di- or trivalent metal ion).

formation of the latter because of charge neutralization and the added stability conferred by the additional chelate ring. To the extent that the latter is formed, both the decarboxylation and the decarboxylation-transamination reaction will be partially inhibited, since the escaping tendency of the carboxyl group due to labilization of bond *b* (VI) is reduced because of its spatial orientation and bonding to the metal ion. In addition, the electrophilic character of the C=O is enhanced by chelate formation, thus increasing the tendency toward labilization of bonds *a* and *c*, and promoting the tendency toward reaction at these sites. It is thus clear why the same metal ions reduce the rate of the decarboxylation reaction (and the decarboxylation-dependent transamination reaction) and enhance the rate of other pyridoxal-dependent reactions of amino acids. These combined effects appear largely to explain the pronounced shift in nature of the products formed from α-methylserine (Table II) depending upon whether or not metal ions are added to the reaction mixture.

In contrast to the nonenzymatic systems, where a single amino acid may undergo several competing pyridoxal-catalyzed reactions, pyridoxal-phosphate enzymes are reaction specific. The observations and interpretations reported here suggest one basis for this specificity. Each of the pyridoxal-catalyzed reactions is a consequence of labilization of the bonds (*a*, *b*, or *c* in VI, Fig. 3) that hold the groups surrounding the α-carbon atom of the amino acid. If one or more such groups is bound to the enzyme in such a manner as to maintain the normal conformation of the amino acid substrate, labilization of the bond to that group will be nonproductive. Conversely, productive bond labilizations would be those to groupings not bound to the protein, or, more effectively, to groups bound in such a way as to enhance the lability of the bond induced by pyridoxal phosphate.

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The Preparation and Some Properties of α-Aminoadipic-δ-Semialdehyde (Δ¹-Piperideine-6-carboxylic Acid)*

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α-Aminoadipic-δ-semialdehyde (Δ¹-piperideine-6-carboxylic acid), which has been suggested as an intermediate in the degradative metabolism of lysine and in its biosynthesis from α-aminoadipic acid, has been prepared by ozonolysis of 2-amino-6-heptenoic acid. 2-Amino-6-heptenoic acid was prepared by alkylation of ethylacetamidocyanoacetate with 5-bromo-1-pentene, followed by alkaline hydrolysis of the condensation product. *N*-Acetyl-α-aminoadipic-δ-semialdehyde was also prepared, and preparation of α-ketoadipic-δ-semialdehyde was attempted. Nonenzymatic transamination of lysine with glyoxylate at *p*H 5 and 100° gave α-keto-ε-aminocaproic acid rather than α-aminoadipic-δ-semialdehyde; under these conditions, ε-*N*-acetyllysine (but not α-*N*-acetyllysine) transaminated with glyoxylate. Evidence for the enzymatic reduction by liver and kidney preparations of *N*-acetyl-α-aminoadipic-δ-semialdehyde to the corresponding ω-alcohol in the presence of reduced pyridine nucleotides has been obtained. Diphosphopyridine nucleotide–dependent enzymatic oxidation of α-aminoadipic-δ-semialdehyde and of its *N*-acetyl derivative to the corresponding dicarboxylic acids was observed.

The enzymatic steps in the degradative metabolism of lysine and in its biosynthesis by certain yeasts and fungi from α-aminoadipic acid have not been fully elucidated. Although it has been suggested that α-aminoadipic-δ-semialdehyde may be formed as an intermediate in both pathways, this has not yet been experimentally demonstrated. The present work was initiated in an attempt to achieve a synthetic preparation of this compound that would be useful for enzymatic studies. In analogy with glutamic-γ-semialdehyde (Vogel and Davis, 1952; Strecker, 1960) and α-keto-ε-aminocaproic acid (Meister, 1954), α-aminoadipic-δ-semialdehyde would be expected to exist in solution in equilibrium with the corresponding cyclic form Δ¹-piperideine-6-carboxylic acid, as shown in reaction (1). Reduction of the cyclic form would give pipecolic acid, while oxidation of the open-chain form would yield α-aminoadipic acid. Reductive amination or trans-

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