Amino Acid Synthesis by Reversal of the Amino Acid Oxidase Reaction*

A. N. Radhakrishnan† and Alton Meister

From the Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts

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The reaction catalyzed by the general L- and D-amino acid oxidases (2) has been represented as a dehydrogenation of an amino acid by a flavoenzyme to yield reduced flavoenzyme and the corresponding imino acid (where E-FAD[1] stands for enzyme-flavin adenine dinucleotide):

$$\begin{matrix}\mathrm{RCHCOOH} \\ | \\ \mathrm{NH_2}\end{matrix} + \text{E-FAD} \rightarrow \text{E-FADH}_2 + \left[\begin{matrix}\mathrm{RCCOOH} \\ \| \\ \mathrm{NH}\end{matrix}\right] \quad (1)$$

The proposed intermediate would be expected to be unstable and to hydrolyze spontaneously to the corresponding α-keto acid and ammonia:

$$\left[\begin{matrix}\mathrm{RCCOOH} \\ \| \\ \mathrm{NH}\end{matrix}\right] + \mathrm{H_2O} \rightarrow \begin{matrix}\mathrm{RCCOOH} \\ \| \\ \mathrm{O}\end{matrix} + \mathrm{NH_3} \quad (2)$$

Although the intermediate formation of an imino acid is consistent with available data, direct experimental evidence for it is still lacking. The reaction in the presence of air proceeds to completion and is essentially irreversible because of reoxidation of the reduced flavoenzyme by molecular oxygen. Previously, the amino acid oxidase reaction has been studied only in the direction of α-keto acid and ammonia formation. We have recently found that L- and D-amino acid oxidases, under anaerobic conditions, can catalyze the reverse reaction leading to the synthesis of an amino acid from the corresponding α-keto acid and ammonia (1). This paper describes studies on the formation of amino acids catalyzed by purified snake venom L-amino acid oxidase, and sheep kidney D-amino acid oxidase.

EXPERIMENTAL

Compounds—Δ^1-Pyrroline-2-carboxylic acid (3), Δ^1-piperidine-2-carboxylic acid (3), and most of the α-keto acids (4) were obtained as described. α-Ketoisocaproic acid-1-C^{14} was prepared by enzymatic oxidation of DL-leucine-1-C^{14} at pH 7.8 with a mixture of D-amino acid oxidase and L-amino acid oxidase in the presence of catalase, by a procedure similar to that described (5). DL-Proline and α-ketoglutaric acid were obtained from Nutritional Biochemicals Corporation, and DL-α-aminophenylacetic acid was purchased from Eastman Kodak Company. Sodium pyruvate was obtained by the procedure of Price and Levintow (6), and DL-pipecolic acid hydrochloride was prepared according to Stevens and Ellman (7). The D- and L-amino acids were prepared by enzymatic resolution of the corresponding racemates by the method of Greenstein (8), or were purchased from Schwarz Laboratories, Inc. The amino acid isomers were tested for optical purity by enzymatic procedures (9); all isomers thus examined contained less than 0.1 per cent of the corresponding enantiomorph.

Enzyme Preparations—D-Amino acid oxidase was prepared from sheep kidney as described by Negelein and Brömel (10); preparations of the enzyme carried to the fourth, fifth, and final steps of this procedure catalyzed reactions of the type described in this report. Material processed to Step 4 of the procedure of Negelein and Brömel was used in the experiments described here, since further purification was accompanied by considerable reduction in the yield of enzyme. The enzyme preparations, which were devoid of appreciable catalase activity, were treated with an excess of FAD (0.02 μmole per mg. of protein), and dialyzed against 0.1 M sodium pyrophosphate buffer (pH 8.3) at 5° until free of ammonia. Preparations lacking FAD lost considerable activity on dialysis. The oxidative activity values for the enzyme preparations employed in the present studies were 1060 (DOX-A) and 1880 (DOX-B); these values are expressed in terms of microliters of oxygen absorbed per mg. of protein per hour in air at 37° in a reaction mixture consisting of enzyme and 50 μmoles of DL-proline in a final volume of 1.5 ml. of 0.067 M sodium pyrophosphate buffer (pH 8.3).

The L-amino acid oxidase preparations were obtained from rattlesnake and moccasin venoms by following the first 3 steps of the procedure of Singer and Kearney (11). Activity (Q_{O_2}) values between 4000 and 16,000 were obtained. In one preparation from moccasin venom, a preparation comparable in activity to that reported by Singer and Kearney was obtained; however, although considerable purification was achieved, preparations obtained by this procedure from rattlesnake venom and other batches of moccasin venom were less active. It seems possible that this variability in activity is related to the nature of the starting materials. The oxidative activity values of the preparations employed in the present studies are given in terms of units, where one unit is defined as the quantity of enzyme that will catalyze the uptake of 1 μmole of oxygen per hour at 37° in air in a reaction mixture consisting of enzyme, 20 μmoles of L-leucine, 200 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 7.2), and 30 μmoles of potassium chloride, in a final volume of 3.0 ml. Preparations of crude venom exhibited glutamic-aspartic transaminase activity; however, this activity

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† Traveling scholar of the J. N. Tata Endowment, India.

[1] The abbreviations used are: FAD, flavin adenine dinucleotide; E, enzyme.

could not be detected after the first step of the purification procedure.

Methods—The experiments were carried out in Warburg vessels or in small test tubes with side bulbs. In general, all of the components were added to the main compartment of the vessel or tube and the amino acid was placed in the side bulb. After flushing with nitrogen (previously bubbled through 3 traps containing Fieser's solution (12)) for 15 minutes, the contents of the vessel were mixed. The reaction was stopped by addition of 0.25 volume of N hydrochloric acid followed by 3 volumes of absolute ethanol. The precipitated protein was removed by centrifugation, and an aliquot of the supernatant solution was evaporated and analyzed for amino acid or keto acid. In some experiments, deproteinization was accomplished by heating the acidified mixture at 70° for 3 minutes. The coagulated protein was removed by centrifugation. Each series of experiments included controls without enzyme and with enzyme inactivated by heating at 100° for 3 minutes; controls in which reactants were separately omitted were also employed.

In the experiments with N^{15}-ammonia, the reaction mixtures were made alkaline by addition of sodium carbonate, and the free ammonia was removed by exhaustive aeration. The mixture was then adjusted to pH 7.2 by cautious addition of 6 N hydrochloric acid, and was then treated with L-amino acid oxidase in air until there was no further uptake of oxygen. The mixtures were subsequently made alkaline by addition of sodium carbonate and the ammonia released by treatment with L-amino acid oxidase was aerated into sulfuric acid traps. After addition of carrier ammonium sulfate, and conversion to nitrogen, N^{15} analyses were carried out with a mass spectrometer.[2]

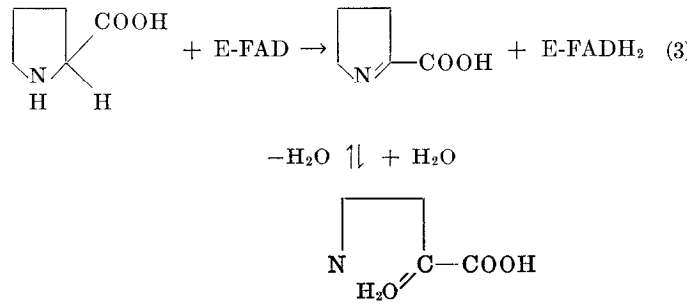
Proline was determined by the procedure of Troll and Lindsley (13). The procedure was modified as previously described (14) in order to avoid interference by Δ^1-pyrroline-2-carboxylate and Δ^1-piperidine-2-carboxylate. The other amino acids were determined after elution from paper chromatograms (with Whatman No. 3 paper) as described by Giri *et al.* (15). Radioactive leucine was separated by paper strip chromatography, and 1 cm. sections of the strip were counted with a thin, mica window tube. Ammonia was determined by nesslerization after aeration into sulfuric acid traps. Pyruvate was quantitatively determined with crystalline lactic dehydrogenase and reduced diphosphopyridine nucleotide. Phenylpyruvate (16) and pipecolic acid (17) were determined as described.

The optical configuration of the enzymatically formed amino acids was established by treating samples of the deproteinized reaction mixtures with D- or L-amino acid oxidases. The mixtures were shaken in air at 37° for 2 hours; after deproteinization, samples were chromatographed on paper, together with an untreated sample. Authentic samples of the respective isomers were included in each series. Under these conditions, complete disappearance of the susceptible isomers was observed. A similar procedure for proline was previously described (14). That the proline formed in the experiments with D-amino acid oxidase was of the D configuration was also shown by an independent procedure involving the use of a proline-requiring mutant of *Escherichia coli* (Davis, 55-1). Autoclaved reaction mixtures containing enzymatically synthesized proline did not support the growth of this mutant, which grew luxuriantly when such reaction mixtures were supplemented with microgram quantities of L-proline.

[2] The authors are indebted to Dr. S. Hartman and Dr. J. M. Buchanan for the N^{15} analysis.

RESULTS

Synthesis of D-Proline by D-Amino Acid Oxidase—The oxidation of D-proline by D-amino acid oxidase may be represented as a dehydrogenation leading initially to Δ^1-pyrroline-2-carboxylic acid, which has been shown to exist in solution in equilibrium with α-keto-δ-aminovaleric acid (3).



Thus, the oxidation of proline (as well as hydroxyproline (18) and pipecolic acid), in contrast to the oxidation of α-amino acids, yields products that may be regarded as substituted imino acids of the type postulated above in Reactions 1 and 2. Under anaerobic conditions it is possible to demonstrate the reversibility of Reaction 3. Thus, incubation of D-amino acid oxidase with Δ^1-pyrroline-2-carboxylate and a D-amino acid yielded D-proline and the corresponding α-keto acid and ammonia. In the experiment described in Table I, stoichiometric formation of D-proline, pyruvate, and ammonia was observed in a system consisting initially of D-alanine and Δ^1-pyrroline-2-carboxylate. When Δ^1-piperidine-2-carboxylate was employed instead of the corresponding pyrroline compound, D-pipecolic acid was formed; the rate of formation of D-proline was more rapid than that of D-pipecolic acid under these conditions. The optical configuration of the imino acids was established as described in the experimental section. As indicated in Table II, a number of D-amino acids were active in the reaction leading to D-proline

TABLE I

*Stoichiometric formation of pyruvate, ammonia, and D-proline from Δ^1-pyrroline-2-carboxylate and D-alanine catalyzed by D-amino acid oxidase**

Time	NH_3 formed	D-Proline formed	Pyruvate formation
hours	*μmole*	*μmole*	*μmole*
1		0.195	0.200
2		0.400	0.415
3	0.830	0.789	0.810
4†	0.405	0.355†	0.365

* The reaction mixtures consisted initially of 10 μmoles of Δ^1-pyrroline-2-carboxylate, 2 μmoles of D-alanine, 1.31 mg. of protein (DOX-B), 20 μmoles of sodium pyrophosphate (adjusted to pH 6.8 with HCl) in a final volume of 0.4 ml.; incubated under nitrogen at 37°.

† Δ^1-Piperidine-2-carboxylate added; D-pipecolic acid formed.

TABLE II

*Formation of D-proline from various D-amino acids and Δ¹-pyrroline-2-carboxylic acid catalyzed by D-amino acid oxidase**

Amino acid	D-Proline formed
	μmole
D-Methionine	0.100
D-Alanine	0.048
D-Phenylalanine	0.110
D-Valine	0.049
D-Leucine	0.025
D-Tyrosine	0.095
Allohydroxy-D-proline	0.087†
D-Isoleucine	0.100
D-Tryptophan	0.064
D-Serine	0.025
D-Threonine	0.017
DL-α-Aminophenylacetic acid	0.050
D-Lysine	0
D-Glutamic acid	0
D-Aspartic acid	0

* The reaction mixtures consisted initially of 2 μmoles of Δ¹-pyrroline-2-carboxylate, 2 μmoles of D-amino acid, 87 μg. of protein (DOX-B), and 20 μmoles of sodium pyrophosphate (adjusted to pH 6.8 with HCl) in a final volume of 0.5 ml. The mixtures were incubated for 4 hours under nitrogen at 37°.

† Formation of pyrrole-2-carboxylic acid (18) was observed by paper chromatography.

TABLE III

*Formation of various D-amino acids catalyzed by D-amino acid oxidase**

System	Amino acid formed	μmoles
D-Alanine + Δ¹-pyrroline-2-carboxylate	D-Proline	0.942
D-Alanine + α-ketoisocaproate	D-Leucine	0.032
D-Alanine + *d*-α-keto-β-methylvalerate	D-Alloisoleucine	0.025
D-Alanine + α-keto-γ-methiolbutyrate	D-Methionine	0.103
D-Alanine + α-ketoisovalerate	D-Valine	0.037
D-Alanine + phenylpyruvate	D-Phenylalanine	0.123
D-Methionine + pyruvate	D-Alanine	0.078
D-Methionine + α-ketoglutarate	None	

* The reaction mixtures consisted initially of 10 μmoles of the sodium salt of the α-keto acid, 25 μmoles of D-amino acid, 10 μmoles of NH_4Cl, 2.6 mg. of protein (DOX-A) and 20 μmoles of sodium pyrophosphate buffer (pH 8.3) in a final volume of 0.7 ml.; incubated for 3 hours at 37° under nitrogen.

formation, which may be represented as the sum of Reactions 4 and 5:

$$\text{D-Amino acid} + H_2O + \text{E-FAD} \rightleftharpoons \alpha\text{-keto acid} + NH_3 + \text{E-FADH}_2 \quad (4)$$

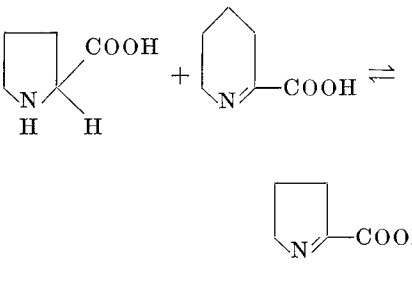
$$\text{E-FADH}_2 + \Delta^1\text{-pyrroline-2-carboxylate} \rightleftharpoons \text{D-proline} + \text{E-FAD} \quad (5)$$

$$\text{D-Amino acid} + H_2O + \Delta^1\text{-pyrroline-2-carboxylate} \rightleftharpoons \text{D-proline} + \alpha\text{-keto acid} + NH_3 \quad (4 + 5)$$

Only D-amino acids that were susceptible to aerobic oxidation by D-amino acid oxidase were active in this system; thus, no proline was formed with D-glutamic acid, D-aspartic acid, and D-lysine. L-Amino acids were not active. Enzyme preparations that did not contain FAD were not active.

The synthesis of D-proline was directly proportional to concentration of enzyme, and, with a given concentration of enzyme, it was linear with time (Fig. 1*B*). In contrast to the aerobic oxidation of D-proline (and other D-amino acids) which occurs most rapidly at values of pH between 8.2 and 8.8, the formation of D-proline exhibited a pH optimum at approximately 6.8 (Fig. 1*A*). The synthesis of proline was approximately 6 times more rapid at pH 6.8 than at pH 8.3. The effects of varying D-amino acid and Δ¹-pyrroline-2-carboxylate concentrations on the rate of D-proline formation are described in Fig. 1*C*. The concentration of Δ¹-pyrroline-2-carboxylate necessary at pH 8.3 for maximal rate of proline formation was considerably greater than that of D-phenylalanine. This result is consistent with the conclusion that Reaction 5 is rate-limiting. A similar experiment carried out at pH 6.8 revealed no essential difference between the saturation curves for D-phenylalanine and Δ¹-pyrroline-2-carboxylate, and *Curve 1* of Fig. 1*C*.

The formation of D-proline may be coupled with the dehydrogenation of imino acids such as allohydroxy-D-proline (Table II) or D-pipecolic acid. This type of reaction is unique in that ammonia does not participate:



(6)

Reaction 6 has been observed in both directions (Fig. 1*D*); the equilibrium is in favor of pipecolic acid formation at pH 6.8 and 37°; the equilibrium constant for Reaction 6 calculated from the data of Fig. 1*D* is approximately 6.

Synthesis of Other D-Amino Acids by D-Amino Acid Oxidase—The studies described above were extended to systems consisting of various α-keto acids and ammonia. It was found that D-amino acid oxidase catalyzed the formation of a number of D-amino acids from the corresponding α-keto acids in the presence of ammonia and another D-amino acid substrate (Table III). D-Aspartic acid, D-glutamic acid, D-lysine, L-amino acids, and α-ketoglutaric acid were not active in this system. The formation of D-amino acids from α-keto acids and ammonia was considerably less rapid than the formation of D-proline from Δ¹-pyrroline-2-carboxylate. In contrast to reactions leading to D-proline formation, the optimal pH was approximately 8.3 or about the same as that for the aerobic oxidation reaction (Fig. 1*A*). The general reaction may be represented as the sum of Reactions 7 and 8:

$$\text{Amino acid } (A) + H_2O + \text{E-FAD} \rightleftharpoons \text{keto acid } (A) + NH_3 + \text{E-FADH}_2 \quad (7)$$

$$\text{E-FADH}_2 + NH_3 + \text{keto acid } (B) \rightleftharpoons \text{amino acid } (B) + H_2O + \text{E-FAD} \quad (8)$$

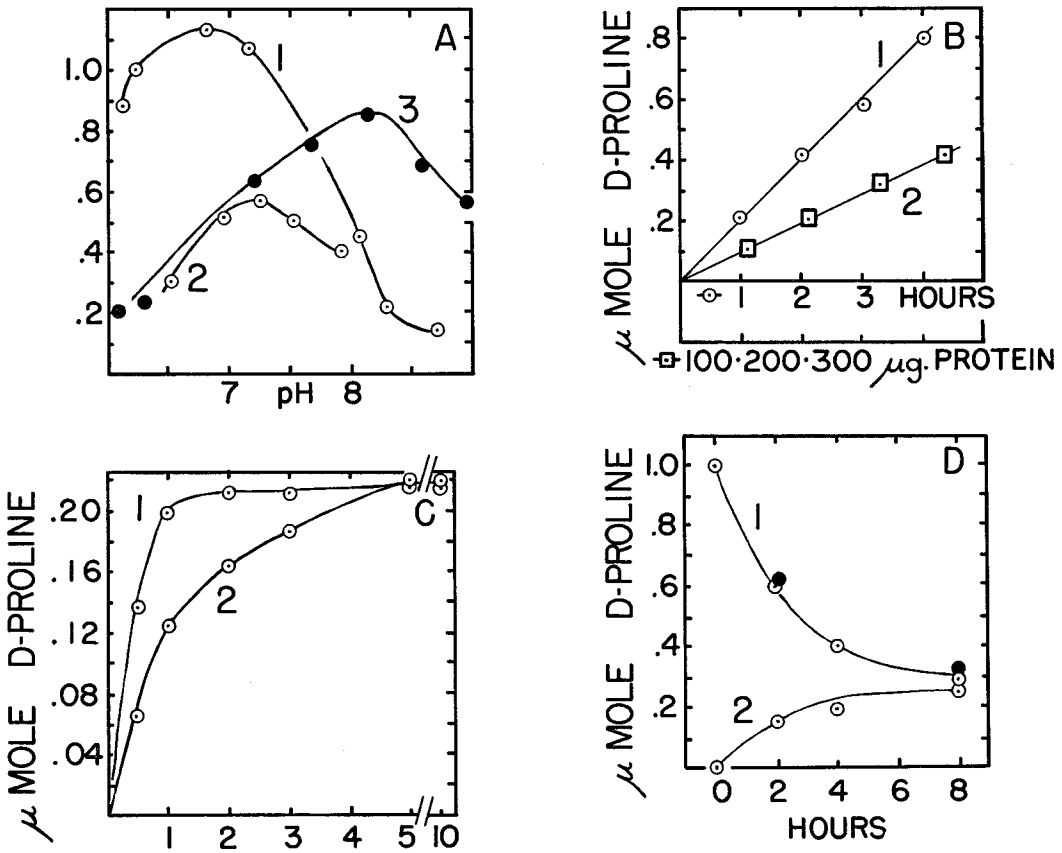


FIG. 1. *A*, pH-activity curves. *Curve 1*, formation of D-proline from Δ^1-pyrroline-2-carboxylate and D-phenylalanine catalyzed by D-amino acid oxidase. The reaction mixtures consisted initially of 25 μmoles of D-phenylalanine, 12.5 μmoles of Δ^1-pyrroline-2-carboxylate, 435 μg. of protein (preparation DOX-B), and 90 μmoles of sodium pyrophosphate in a final volume of 2 ml. The pH was adjusted to the indicated values by addition of HCl; incubated under nitrogen at 37° for 4 hours. *Ordinate* = μmole of D-proline formed. *Curve 2*, formation of L-phenylalanine from phenylpyruvate and L-tyrosine catalyzed by L-amino acid oxidase. The reaction mixtures consisted initially of 5 μmoles of L-tyrosine, 20 μmoles of sodium phenylpyruvate, 20 μmoles of NH_4Cl, 50 units of L-oxidase, and 45 μmoles of tris(hydroxymethyl)aminomethane buffer of indicated pH in a final volume of 1 ml.; incubated under nitrogen for 4 hours at 37°. *Ordinate* = μmole × 2 of L-phenylalanine formed. *Curve 3*, formation of D-phenylalanine from phenylpyruvate and D-alanine catalyzed by D-amino acid oxidase. The reaction mixtures consisted initially of 10 μmoles of D-alanine, 20 μmoles of sodium phenylpyruvate, 20 μmoles of NH_4Cl, 1.39 mg. of protein (DOX-B), and 50 μmoles of sodium pyrophosphate adjusted to the indicated values of pH, in a final volume of 1.2 ml. incubated under nitrogen for 4 hours at 37°. *Ordinate* = μmole × 10 of D-phenylalanine formed.

B, *Curve 1*, time course of the reaction between D-phenyalanine and Δ^1-pyrroline-2-carboxylate. *Curve 2*, effect of enzyme concentration. The reaction mixtures consisted initially of 10 μmoles each of D-phenylalanine and Δ^1-pyrroline-2-carboxylate, 435 μg. of protein (DOX-B) (*Curve 1*), 40 μmoles of sodium pyrophosphate (adjusted to pH 6.8 with HCl), in a final volume of 1 ml.; incubated at 37° under nitrogen for 2 hours (*Curve 2*).

C, effect of varying concentrations of D-phenylalanine and Δ^1-pyrroline-2-carboxylate on formation of D-proline. The reaction mixtures consisted initially of the reactants at the concentrations indicated, 90 μmoles of sodium pyrophosphate (pH 8.3) and 1.10 mg. of protein (DOX-B) in a final volume of 1 ml.; incubated at 37° for 2 hours under nitrogen. *Curve 1*, D-phenylalanine concentration varied (10 μmoles of Δ^1-pyrroline-2-carboxylate); *Curve 2*, Δ^1-pyrroline-2-carboxylate concentration varied (10 μmoles of phenylalanine). *Abscissa*-μmoles of reactant per ml.

D, formation of D-proline and D-pipecolic acid. *Curve 1*, the reaction mixtures consisted initially of 2 μmoles of DL-proline (or 1 μmole of D-proline), 1 μmole of Δ^1-piperidine-2-carboxylate, 10 μmoles of sodium pyrophosphate (adjusted to pH 6.8 with HCl), and 1.31 mg. of protein (DOX-B) in a final volume of 0.2 ml.; incubated under nitrogen at 37°; ● = values that were obtained with D-proline. *Curve 2*, the reactants were DL-pipecolic acid and Δ^1-pyrroline-2-carboxylate; other conditions as for *Curve 1*.

Amino acid (*A*) + keto acid (*B*) $\rightleftharpoons$
amino acid (*B*) + keto acid (*A*) (7 + 8)

The over-all reaction is identical with a transamination reaction; however, several lines of evidence indicate that amino group transfer is not involved, and that ammonia is a participant in these reactions leading to D-amino acid formation and in the analogous reactions catalyzed by L-amino acid oxidase described below. It is evident that amino group transfer is not involved in the reactions leading to D-proline formation. Furthermore, D-amino acid oxidase catalyzes exchange between the α-amino group of a D-amino acid and N^{15}-ammonia (1); a similar reaction catalyzed by L-amino acid oxidase is described below. An additional finding consistent with the participation of ammonia is the marked acceleration of the over-all reactions produced by addition of ammonia. Thus, the reaction between α-ketoiso-

TABLE IV

*Formation of various L-amino acids catalyzed by L-amino acid oxidase**

Reactants	Amino acid formed	μmole
L-Leucine + α-ketobutyrate	L-α-Aminobutyric acid	0.06
L-Leucine + α-keto-γ-methiolbutyrate	L-Methionine	0.20
L-Leucine + phenylpyruvate	L-Phenylalanine	0.58
L-Leucine + p-hydroxyphenylpyruvate	L-Tyrosine	0.25
L-Leucine + α-ketoglutarate	None	
L-Leucine + α-ketophenylacetate	L-α-Aminophenylacetic acid	0.15
L-Methionine + *d*-α-keto-β-methylvalerate	L-Isoleucine	0.12
L-Methionine + α-ketoisocaproate	L-Leucine	0.19
L-Tyrosine + α-ketoisocaproate	L-Leucine	0.05
L-Tyrosine + phenylpyruvate	L-Phenylalanine	0.40

* The reaction mixtures consisted initially of 10 μmoles of the sodium salt of the α-keto acid, 5 μmoles of L-amino acid, 10 μmoles of NH_4Cl, 47.2 units of L-oxidase, and 10 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 7.2) in a final volume of 0.25 ml.; incubated for 195 minutes at 37° under nitrogen.

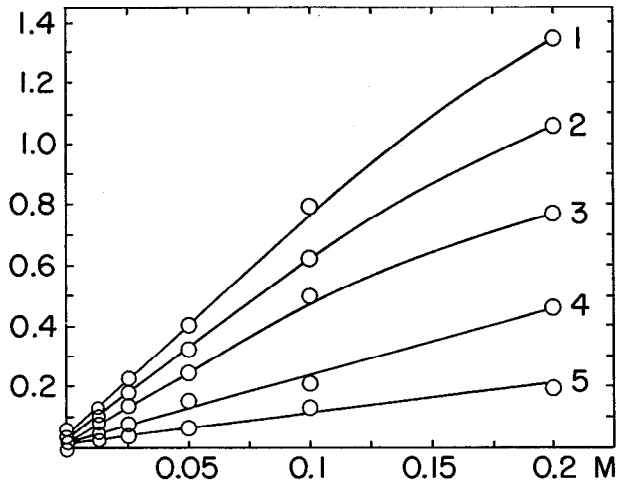


FIG. 2. Effect of varying concentrations of ammonium chloride and α-ketoisocaproate on the formation of phenylpyruvate from L-phenylalanine by L-amino acid oxidase. The reaction mixtures consisted initially of 5 μmoles of L-phenylalanine, 80 units of L-oxidase, NH_4Cl in the indicated concentrations, and sodium α-ketoisocaproate in a final volume of 0.4 ml. of 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.2); incubated for 2 hours at 37° under nitrogen. The concentration of α-ketoisocaproate was 0.2, 0.1, 0.05, and 0.025, and 0.0125 M in *Curves 1, 2, 3, 4,* and *5*, respectively. *Abscissa* = molar concentration of NH_4Cl; *ordinate* = μmoles of phenylpyruvate formed.

caproate and D-phenylalanine occurred much more rapidly in the presence of added ammonia; the rate of the reaction increased linearly with increasing concentration of added ammonium chloride (0.0125 to 0.100 M). An analogous experiment was carried out with L-amino acid oxidase (see Fig. 2 and text, below). When methylamine hydrochloride was incubated with D-amino acid oxidase, α-ketoisocaproate, and D-phenylalanine, the rate of phenylpyruvate formation was less than 10 per cent of that observed with ammonium chloride.

Synthesis of L-Amino Acids by L-Amino Acid Oxidase—Reactions analogous to those described in the previous section (Reactions 7 and 8), but leading to the synthesis of L-amino acids rather than D-amino acids, are catalyzed by L-amino acid oxidase. Several representative experiments are summarized in Table IV. The pH optimum for the reaction between L-tyrosine and phenylpyruvate was 7.2 (Fig. 1*A*), or approximately the same as that for the aerobic oxidation of amino acids by this enzyme.

The effects of varying the concentrations of ammonium chloride and α-keto acid on the rate of formation of phenylpyruvate from L-phenylalanine and α-ketoisocaproate are described in Fig. 2. In these studies the concentration of L-phenylalanine was 0.0125 M; this concentration of phenylalanine gave optimal rates of reaction with various concentrations of ammonium chloride and α-ketoisocaproate and, under these conditions, the formation of phenylpyruvate was linear with time. The rate of phenylpyruvate formation tended to reach a maximal value with increasing concentrations of α-ketosiocaproate; the concentrations of α-ketoisocaproate necessary for half maximal velocity were 0.0469, 0.0515, 0.0500, 0.0530, and 0.0628 M for ammonium chloride concentrations of 0.0125, 0.0250, 0.0500, 0.100, and 0.200 M, respectively. On the other hand, the reaction velocity did not reach a maximal value even with concentrations of ammonium chloride of 0.200 M. Acceleration of the reaction by ammonium chloride was evident at all concentrations of α-ketoisocaproate studied. These observations suggest that the formation of L-leucine from α-ketoisocaproate and ammonia is the rate-limiting step, and that the accelerating effect of ammonia is due to an increase in the rate of this reaction.

Conclusive evidence for the participation of ammonia in the reaction was obtained by incubating L-amino acid oxidase with an L-amino acid, the analogous α-keto acid, and N^{15}-ammonia. Significant incorporation of N^{15} into the amino groups of the amino acids was observed (Table V). In experiments in which α-keto acid was omitted, the incorporation of isotope was approximately 9 per cent of the values given in Table V. No incorporation was observed in experiments in which the enzyme was inactivated by heating at 100° for 3 minutes. It is of interest that the incorporation of $N^{15}H_3$ into L-phenylalanine was somewhat greater than into L-leucine or L-methionine. In the coupled reactions also (Table IV), reactions leading to L-phenylalanine appeared to proceed more rapidly than did the other reactions studied. The experiments with N^{15}-ammonia are consistent with the occurrence of the separate Reactions 7 and 8. As expected, L-amino acid oxidase also catalyzed (anaerobically) the formation of C^{14}-L-leucine in a system consisting initially of L-leucine, α-ketoisocaproate-1-C^{14}, and ammonia. Thus, incubation of L-oxidase (80 units), 25 μmoles each of L-leucine, sodium α-ketoisocaproate-1-C^{14} (85,200 c.p.m.), and ammonium nitrate, in 1.0 ml. of 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.2) at 37° for 3 hours, resulted in the formation of radioactive leucine (1730 c.p.m.). An analogous experiment with D-leucine and D-amino acid oxidase gave similar results.

Reaction 7 was also demonstrated spectrophotometrically by following the characteristic absorption band of L-amino acid oxidase at 465 mμ. Bleaching of the amino acid oxidases by amino acid substrates and by reducing agents has been reported (10, 11). Reoxidation of reduced L-amino acid oxidase by molecular oxygen is associated with restoration of the absorption band at 465 mμ (11). We have found that oxidation of L- and D-amino

acid oxidases (reduced with hydrosulfite or an amino acid substrate) may also be carried out anaerobically by addition of α-keto acid plus ammonium chloride. In the experiment described in Fig. 3, L-amino acid oxidase was reduced with hydrosulfite and reoxidized by addition of relatively large quantities of α-ketoisocaproate and ammonium chloride; addition of either α-keto acid or ammonium chloride alone did not result in restoration of the absorption band at 465 mμ. Similar results were obtained with enzyme reduced with L-leucine, and reoxidation was also achieved by adding phenylpyruvate and ammonium chloride. Reoxidation of the enzyme was somewhat greater with phenylpyruvate than with α-ketoisocaproate; this result is consistent with other experiments (Tables IV and V). The spectrophotometric studies indicate that the equilibrium of Reaction 7 favors α-keto acid and ammonia formation. Similar conclusions follow from the experiments demonstrating acceleration of the coupled reaction by ammonium chloride (Fig. 2). Preliminary data[3] on the equilibrium constant of Reaction 7 at pH 7.2 and 26° with L-leucine lead to a value of approximately 100 (concentration of water taken as unity).

DISCUSSION

The present studies demonstrate that the reaction catalyzed by the general amino acid oxidases is reversible, and therefore that these enzymes can catalyze the synthesis of α-amino acids from the corresponding α-keto acids and ammonia. It is possible that under appropriate physiological conditions, reversal of the amino acid oxidase reaction may be responsible for amino acid synthesis. Such reactions might represent pathways alternative to transamination for conversion of α-keto acids to α-amino acids, and an additional mechanism for the utilization of ammonia. Additional work is necessary to determine whether synthesis of amino acids by this mechanism is of physiological significance. Although under our conditions the rates of amino acid synthesis were much lower than the rates of amino acid oxidation in the aerobic systems usually employed, it may not be valid to base conclusions on results of experiments carried out under arbitrarily chosen conditions *in vitro*. It is conceivable that alternative pathways for the transfer of hydrogen to the amino acid oxidases exist *in vivo*, and that the isolated enzyme systems represent fragments of more complicated integrated cellular mechanisms. The present studies were carried out with the amino acid oxidases that can readily be isolated from snake venom and sheep kidney; however, it seems possible that the amino acid oxidases present in other biological materials, such as microorganisms, may also catalyze amino acid synthesis. Although we have no new suggestions to offer as to the function of mammalian D-amino acid oxidase, it may be observed that, despite the lack of evidence demonstrating the occurrence of D-amino acids in mammalian tissues, the presence of some D-amino acids in mammalian tissues has not yet been unequivocally excluded. The occurrence of D-amino acids in certain bacteria is at least compatible with the possibility that reactions of the type studied here may be involved in their formation.

The formation of an intermediate imino acid has not been experimentally demonstrated; however, evidence excluding α,β-unsaturation in the course of the amino acid oxidase reaction gives indirect support to the imino acid hypothesis. Such evidence includes the finding that the four isomers of isoleucine are enzymatically oxidized by amino acid oxidases to the corresponding optically active α-keto-β-methylvaleric acids (19), and the observation that the L-isomers of β-phenylserine are converted by L-amino acid oxidase to the respective optical isomers of mandelic acid (20). Furthermore, the L- and D-isomers of α-aminophenylacetic acid, which does not possess a β-hydrogen atom, are susceptible to the action of amino acid oxidases (21, 9). Additional evidence excluding α,β-unsaturation has arisen from studies on the oxidation of L-leucine in the presence of D_2O by L-amino acid oxidase; the isolated α-ketoisocaproate did not contain appreciable deuterium (22).

The present finding that Δ^1-pyrroline-2-carboxylate, which may be regarded as a substituted imino acid, was a much more active substrate than was a mixture of α-keto acids and ammonia, may be considered as evidence in favor of the concept that an

[3] D. Wellner, and A. Meister, unpublished data.

TABLE V

*Participation of ammonia in the synthesis of L-amino acids catalyzed by L-amino acid oxidase**

Amino acid	Atom per cent excess N^{15} in amino acid
L-Methionine	0.28
L-Phenylalanine	2.68
L-Leucine	0.37

* The reaction mixtures consisted initially of 10 μmoles of L-amino acid, 20 μmoles of the sodium salt of the α-keto acid, 20 μmoles of $N^{15}H_4NO_3$ (63.0 atom per cent excess $N^{15}H_4$), 100 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 7.2), and 157 units of L-oxidase in a final volume of 1.0 ml.; incubated for 3 hours at 37° under nitrogen.

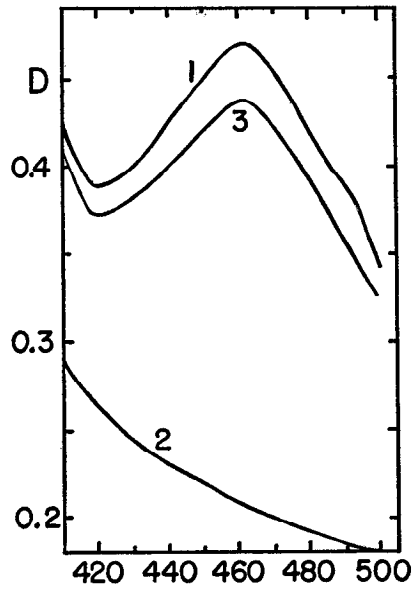


FIG. 3.—Spectrophotometric observation of reduction and reoxidation of L-amino acid oxidase. *Abscissa* = wave length (mμ); *ordinate* = optical density (1 cm. light path). *Curve 1*, absorption curve of oxidized enzyme at pH 7.2 in 0.1 M tris(hydroxymethyl)aminomethane buffer; volume = 1 ml., 26°; *Curve 2*, after addition of 0.5 μmole of sodium hydrosulfite to enzyme solution used in *Curve 1; Curve 3*, after anaerobic addition of 500 μmoles each of sodium α-ketoisocaproate and ammonium chloride to reduced enzyme; corrected to volume of 1 ml.

imino acid (formed by nonenzymatic reaction of α-keto acid and ammonia) is the actual substrate. However, it is possible that the concentration of free imino acid may be negligible and that an enzymatically-bound imino acid is formed by successive reaction of the enzyme with keto acid and ammonia or *vice versa*. The latter type of mechanism is not inconsistent with the observations made with Δ^1-pyrroline-2-carboxylate, for it is possible that the open-chain form of this compound, α-keto-δ-aminovaleric acid, reacts initially with the enzyme. The relatively rapid conversion of this compound to D-proline might then be ascribed to condensation between the α-keto and δ-amino groups to form an enzyme-Δ^1-pyrroline-2-carboxylate complex. Studies on the interconversion of Δ^1-pyrroline-2-carboxylate and α-keto-δ-aminovaleric acid may be significant in terms of the enzymatic mechanism. Another problem that remains for investigation is whether ammonia reacts in these systems in the unionized form or as ammonium ion. Further studies of these new reactions catalyzed by the amino acid oxidases may yield additional information concerning the reaction mechanisms; such work is in progress.

SUMMARY

1. The reaction catalyzed by the general amino acid oxidases, amino acid + enzyme-flavin adenine dinucleotide (FAD) + $H_2O \rightleftharpoons$ α-keto acid + NH_3 + enzyme-$FADH_2$, has been shown to be reversible. Amino acid synthesis has been observed in systems in which either L-amino acid oxidase (from snake venom) or D-amino acid oxidase (from sheep kidney) was incubated with an amino acid substrate, ammonia, and the α-keto acid analogue of another amino acid substrate; the formation of the respective amino acid isomer was observed under anaerobic conditions.

2. D-Amino acid oxidase catalyzed the formation of D-proline in reaction mixtures containing Δ^1-pyrroline-2-carboxylate and a D-amino acid substrate. This reaction proceeded more rapidly than reactions involving α-keto acids and ammonia and, in contrast to these reactions, exhibited a pH optimum at 6.8. The reversible reaction (not involving ammonia), D-proline + Δ^1-piperidine-2-carboxylate $\rightleftharpoons$ D-pipecolic acid + Δ^1-pyrroline-2-carboxylate, was observed and the equilibrium constant was determined at pH 6.8 and 37°.

3. Both L-amino acid oxidase and D-amino acid oxidase catalyzed the incorporation of $N^{15}H_3$ into L- and D-amino acids, respectively, in the presence of the α-keto acid analogue of the amino acid. The formation of radioactive L- and D-leucine in systems containing leucine, ammonia, and C^{14}-α-ketoisocaproate was also catalyzed by the respective oxidases.

4. The rates of the reactions, L- (and D)-phenylalanine + α-ketoisocaproate $\rightleftharpoons$ L- (and D)-leucine + phenylpyruvate, increased linearly with increasing concentrations of added ammonium chloride (0.0125 to 0.100 M); evidence is presented that these reactions are not transamination reactions, but involve the the participation of ammonia.

5. Reduction of L-amino acid oxidase and of D-amino acid oxidase by hydrosulfite and by L- or D-amino acid substrates respectively, was observed spectrophotometrically by following the absorption band of the oxidized flavoproteins in the region of 465 mμ. Anaerobic reoxidation of the reduced enzymes was carried out by addition of ammonia and α-keto acid.

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