Biosynthesis of Slaframine, (1*S*,6*S*,8a*S*)-1-Acetoxy-6-aminooctahydroindolizine, a Parasympathomimetic Alkaloid of Fungal Origin. II. The Origin of Pipecolic Acid†

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ABSTRACT: Pipecolic acid is efficiently utilized in *Rhizoctonia leguminicola* for the synthesis of two piperidine alkaloids: 1-acetoxy-6-aminooctahydroindolizine and 3,4,5-trihydroxyoctahydro-1-pyrindine. This paper is concerned with the stereospecific synthesis of pipecolic acid and N^6-acetyllysine from lysine in this fungus. Evidence is presented that a lysine racemase is operative in this mold. By blocking the action of the racemase with hydroxylamine, it was shown that L-lysine is preferentially used to form L-pipecolic acid while D-lysine forms D-N^6-acetyllysine. Evidence from enzyme and isotopic labeling experiments establishes that pipecolic acid is formed in *R. leguminicola* as follows: L-lysine → [2-keto-6-aminohexanoic acid ⇄ Δ^1-piperideine-2-carboxylic acid] → L-pipecolic acid.

Pipecolic acid is utilized in the biosynthesis of the piperidine alkaloids 1-acetoxy-6-aminooctahydroindolizine (Guengerich *et al.*, 1973a) and 3,4,5-trihydroxyoctahydro-1-pyrindine (Guengerich *et al.*, 1973b) in *Rhizoctonia leguminicola*. Certain of the latter steps of slaframine biosynthesis were discussed in the previous paper (Guengerich *et al.*, 1973a). Pipecolic acid is known to arise from lysine catabolism in various species, and the details of the lysine → pipecolate transformations in *R. leguminicola* are reported herein. In the course of these studies evidence for a lysine racemase was found which appears to dictate stereospecific events of lysine metabolism. Thus in *R. leguminicola* L-lysine gives rise to L-pipecolate and D-lysine is converted to D-N^6-acetyllysine prior to further catabolism. The relation of these events to present knowledge of lysine catabolism in various species will be briefly discussed.

Materials and Methods

Rhizoctonia leguminicola cultures were grown in stationary culture for 10–12 days in 250 ml of hay infusion medium as previously described (Guengerich *et al.*, 1973a). Such cultures were then drained of spent medium; test compounds dissolved in 50 ml of sterile water were injected under the mold mycelium and incubated for 24 hr at room temperature. The mycelium was then rinsed with distilled water,[1] homogenized in a Waring Blendor with 95% ethanol, and filtered through a Soxhlet thimble. The ethanolic filtrate was then used to continuously extract the mycelium in a Soxhlet extractor for 24 hr. The ethanolic extract was then taken to dryness *in vacuo* and brought up in an appropriate volume of water for subsequent chromatography. Ion-exchange chromatography was employed to separate lysine and its metabolites in *R. leguminicola* extracts using a procedure modified after that of Piez *et al.* (1956). The column size was 1.2 × 50 cm; 500 ml each of sodium citrate buffer (pH 3.1) and 0.25 N NaOH were used in the gradient system (citrate to NaOH). Pipecolic acid was estimated in column eluents by a colorimetric ninhydrin method described by Piez *et al.* (1956). D-Amino acid oxidase and L-lysine decarboxylase assays were carried out by procedures described by Soda and Osumi (1971). Preparations of these enzymes were obtained from Nutritional Biochemicals Corp. and Worthington Biochemical Corp., respectively.

Radioactive lysines were purchased from New England Nuclear Corp.; DL-[*R*-^{3}H]pipecolic acid was prepared as previously described (Guengerich *et al.*, 1973a). Δ^1-Piperideine-2-carboxylic acid was synthesized according to Meister (1954). Δ^1-Piperideine-6-carboxylic acid was prepared by mild chromium trioxide oxidation of DL-2-amino-6-hydroxyhexanoic acid (Cyclo Chemical Corp.) according to Jones (1964). D-[1-^{14}C]Lysine was prepared by the action of L-lysine decarboxylase on DL-[1-^{14}C]lysine by the method of Miller and Rodwell (1971); the product was determined to be 99.7% optically pure, using the described criterion. L-[*R*-^{3}H]-Δ^1-Piperideine-2-carboxylic acid was prepared by the action of D-amino acid oxidase on DL-[*R*-^{3}H]pipecolic acid and was purified by ion-exchange chromatography (Cooksey and Greenberg, 1965). After removal of HCl by lyophilization, the material was used without further purification.

DL-[$^{15}N^6$]Lysine was prepared by a procedure adapted from Fink *et al.* (1944). Potassium [^{15}N]phthalimide (95.1% excess ^{15}N, Nuclear Equipment Chemical Corp., Farmingdale, N. Y.) was condensed with diethyl 2-(*n*-bromobutyl)-1,3-2-isoindolemalonate (prepared from diethyl phthalimidomalonate and 1,4-dibromobutane). The product (2-carbethoxy-2,6-*N*,^{15}*N*-diphthaloyllysine ethyl ester) was hydrolyzed to form DL-[$^{15}N^6$]lysine in 10% overall yield (from diethyl phthalimidomalonate). The product was pure as judged by thin-layer chromatography ($CHCl_3$–CH_3OH–14% aqueous NH_4OH, 40:40:20) of the free acid and gas chromatography (3% OV-17, 180°) of the ethyl ester. The lysine contained 89.7% excess ^{15}N.[2]

DL-[$^{15}N^2$]Lysine was prepared by a procedure adapted from Neuberger and Sanger (1944). DL-2-Bromo-6-benzamido-

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[1] The metabolites of interest, pipecolic acid and N^6-acetyllysine, accumulate in the mycelia and are apparently not excreted into the media.

hexanoic acid (mp 162–166°) was formed from DL-N^6-benzoyllysine by treatment with sodium nitrite and potassium bromide. Treatment of the bromo compound with [^{15}N]ammonium nitrate (95.5% excess ^{15}N, Mallinckrodt) gave DL-N^6-benzoyl[$^{15}N^2$]lysine (46% yield, mp 255–260°), which was hydrolyzed to DL-[$^{15}N^2$]lysine (63% yield). The product was pure as judged by thin-layer and gas chromatographies (*vide infra*) and contained 60.7% excess ^{15}N.[3]

Combined gas–liquid chromatography–mass spectrometry (LKB 9000) was used to assay ^{15}N. In all cases, the column packing was 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs.). All compounds were assayed as their ethyl esters, which were prepared according to Biemann *et al.* (1961). The following column temperatures were used for the ethyl esters: pipecolic acid, 130°; lysine, 180°; N^6-acetyllysine, 215°. In each case the M − 73 peak (loss of CO_2Et; Biemann *et al.* (1961)) was used for the determination of the ^{15}N content. The atomic per cent excess is expressed as the ratio: $(m + 1)/[m + (M + 1)]$, after correction for natural abundance ^{13}C (Biemann, 1962).

Radioactivity was measured either by counting 0.1-ml aqueous samples in 10 ml of 20% ethanol in toluene (containing 0.3% 2,5-diphenyloxazole) or by counting 1.0-ml aqueous samples in 15 ml of 33% Triton X-100 in toluene (0.3% 2,5-diphenyloxazole and 0.0033% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene]). Counting was done on Packard 3320 and Nuclear-Chicago Mark I liquid scintillation spectrometers; corrections were based on channel ratios using external standards.

Results

Catabolism of Lysine. Twelve-day old cultures of *Rhizoctonia leguminicola* were incubated with DL-[4,5-^{3}H]lysine as described in Materials and Methods and in the legend of Figure 1. Upon chromatography of the mycelial extract, two large peaks (B and C) were observed in the region of the neutral amino acids (Figure 1). Peaks B and C were also observed when similar incubations were performed with L-[U-^{14}C]-, DL-[1-^{14}C]-, DL-[2-^{14}C]-, or DL-[6-^{14}C]lysine; in each case, peaks B and C accounted for *ca.* 0.5% of the proffered label.

Peak C has been shown to correspond to pipecolic acid (Guengerich *et al.*, 1973a). Peak B elutes from the column in the region of 2-aminoadipic acid, but paper electrophoresis showed that the metabolite was a neutral amino acid. Upon subsequent analysis, essentially all the radioactivity of this peak was shown to chromatograph with N^6-acetylysine, but not with 2-aminoadipic acid or N^2-acetyllysine, on paper (Whatman No. 1, descending: 1-butanol–acetic acid–H_2O 18:1:1) and thin-layer chromatography (silica gel G: chloroform–methanol–14% aqueous NH_4OH, 2:2:1). After acid hydrolysis (6 N HCl, 12 hr, 110°), all radioactivity of the sample chromatographed with lysine in the above systems. (Further evidence for the characterization of this peak and its biosynthesis from lysine is presented below under ^{15}N Experiments.)

When pipecolic acid, isolated from *R. leguminicola* cells following incubation with DL-[2-^{14}C]lysine, was treated with D-amino acid oxidase 101.8% of the radioactivity was recovered as pipecolic acid following ion-exchange chromatography (Cooksey and Greenberg, 1965). In a control experiment when authentic DL-[*R*-^{3}H]pipecolic acid was incubated under these conditions with D-amino acid oxidase, 50.5% of the label was recovered as pipecolic acid. Thus it is concluded that only L-pipecolic acid is formed from DL-lysine, which is consistent with the results presented in the preceding paper (Guengerich *et al.* 1973a), where radioactivity derived from L-[2-^{3}H]pipecolic acid was shown to be incorporated into slaframine.

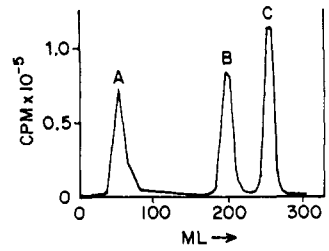

FIGURE 1: Chromatography of lysine metabolites of *Rhizoctonia leguminicola*. A 10-day old *R. leguminicola* culture was incubated for 24 hr with 70 μCi of DL-[4,5-^{3}H]lysine (200 Ci/mol). An extract of the mycelium was then prepared and applied to a cation exchange column and the column eluates (5 ml) were monitored for radioactivity as described in Materials and Methods. Peak A in each case consists of acidic and neutral compounds that are not adsorbed to the column; peak B in each case is N^6-acetyllysine; peak C is pipecolic acid (see text).

When [^{14}C]-N^6-acetyllysine isolated from an incubation with L-[U-^{14}C]lysine was treated with D-amino acid oxidase, only 5.6% of the radioactivity was recovered as N^6-acetyllysine following ion-exchange chromatography (Piez *et al.*, 1956). In a control experiment the D-amino acid oxidase preparation did not attack synthetic L-N^6-acetyllysine (98.2% of the substrate was recovered). The data thus indicate that biosynthesized N^6-acetyllysine is of the D configuration and suggest that a lysine racemase may be operative in *R. leguminicola*. Further evidence for the D configuration of N^6-acetyllysine was provided from the following experiments. (a) The product of the action of D-amino acid oxidase on biosynthesized N^6-acetyllysine was 2-keto-6-acetamidohexanoic acid, as the enzymatic product formed a 2,4-dinitrophenylhydrazone which on catalytic reduction (Meister and Abendschein, 1956) gave N^6-acetyllysine. (b) When biosynthesized N^6-acetyllysine was hydrolyzed (*vide infra*) and treated with L-lysine decarboxylase in the presence of carrier L-lysine (Miller and Rodwell, 1971) only 2.6% of the radioactivity derived from the initial N^6-acetyllysine could be found in the cadaverine isolated; control assays confirmed the stereospecific activity of the decarboxylase.

Stereospecificity of Lysine Catabolism. From the results just discussed, it seemed logical that D- and L-lysine are utilized for the syntheses of D-N^6-acetyllysine and L-pipecolic acid, respectively, and that a lysine racemase is operative as well since D-N^6-acetyllysine was formed following incubation of L-lysine in *R. leguminicola* cells. Further evidence for a lysine racemase was sought by investigating the stereospecific requirements for lysine in pipecolic acid biosynthesis as described in the experiments of Table I. It were found that the incorporation of radioactivity from lysine and the efficiency of the conversion of lysine into pipecolic acid were about the same whether L- or DL-lysine was used as the substrate (columns 2 and 3, Table I). Moreover, a high level of D-lysine had an inductive effect on pipecolate synthesis and competed

[2] The values given for per cent excess ^{15}N for lysine are expressed as the per cent ^{15}N enrichment at the nitrogen atom of interest in each case; the same convention is used with the N^6-acetyllysine in the biosynthetic experiments (Table IV).

[3] The reason for the low per cent excess ^{15}N is unclear. We did not check the [^{15}N]ammonium nitrate obtained from the manufacturer for its ^{15}N content, but assumed the stated value.

TABLE I: Observations on the Stereospecific Requirements for Lysine in Pipecolic Acid Biosynthesis.[a]

	L-[U-^{14}C]Lys, 1.94 Ci/mol	DL-[1-^{14}C]Lys, 1.96 Ci/mol		
		Unlabeled Lys Additions		
		None	500 μmol of D-Lys	500 μmol of L-Lys
Yield of pipecolate, μmol	0.14	0.12	1.35	1.17
% Incorp. [^{14}C]Lys into pipecolate	0.52	0.43	2.83	1.18
(Sp act. of pipecolate/sp act. of Lys) × 10^{-1}	1.20	1.10	0.66	0.32
% dilution radioactivity			41	71

[a] Four 10-day old *R. leguminicola* cultures were obtained as described in Materials and Methods and served for four incubation experiments in which a mycelial mat was incubated with 3.15 μmol of the particular [^{14}C]lysine isomer indicated and D- or L-lysine where stated. Following 48-hr incubation, pipecolic acid was isolated from the mycelium and its concentration and radioactivity were determined as described in Materials and Methods.

significantly with DL-[1-^{14}C]lysine for pipecolate synthesis (column 4, Table I). These latter effects were also true when a high level of L-lysine was added in the incubation mixture (last column, Table I).

Hydroxylamine has been used effectively to demonstrate racemase activity in certain microbial transformations (*e.g.*, *cf.* Miller and Rodwell, 1971), presumably by blocking the action of pyridoxal phosphate required in the reaction. Advantage was taken of this fact by incubating *R. leguminicola* cells with either D- or L-[^{14}C]lysine or the racemic mixture in the presence of hydroxylamine and then determining the extent of incorporation of the particular substrate into N^6-acetyllysine and pipecolate following separation of these amino acids by ion-exchange chromatography as described in Table II. Five hundred micromoles of carrier DL-lysine was included in each incubation to favor induction of enzymes concerned in the disposition of lysine in these experiments and to assure constancy of yield of acetyllysine and pipecolate (as shown in other experiments). The data illustrate that when racemic lysine is the substrate the ratio of acetyllysine/pipecolate is about 4:1. Hydroxylamine likely not only reduces lysine racemase activity, but likewise also reduces further catabolism of 2-keto-6-acetamidohexanoate (Guengerich, 1973) which in turn would affect the magnitude of the ratios reported in Table II. However, the data clearly show that the biosynthesis of N^6-acetyllysine predominates following incubation with D-lysine whereas pipecolate synthesis is favored on incubation with L-lysine.

The foregoing experiments (Tables I and II) thus constitute suggestive evidence for a lysine racemase in *R. leguminicola* cells and preliminary work with cell-free extracts of this mold provides direct evidence for this supposition (Guengerich, 1973). Thus when D- or L-lysine was incubated under appropriate conditions with cell-free extracts of *R. leguminicola*, the respective formation of L- or D-lysine could be demonstrated. Such epimerizations were completely abolished by hydroxylamine or by boiling the extracts.

^{15}N Experiments. In an effort to obtain more chemical and biochemical information relative to the catabolism of lysine in *R. leguminicola*, the mold was grown with either DL-[$^{15}N^2$]-lysine or DL-[$^{15}N^6$]lysine as described in Table IV. The extent of incorporation of ^{15}N into pipecolic acid and N^6-acetyllysine was determined by gas chromatography–mass spectrometry following separation of these metabolites by ion-exchange chromatography (Table III). The incorporation of N^6, but not the N^2 atom of lysine into pipecolate (Table III), suggests that pipecolic acid arises from lysine catabolism *via* Δ^1-piperideine-2-carboxylic acid. As expected, both the N^6 and N^2 atoms of lysine were incorporated into N^6-acetyl-

TABLE II: Stereospecificity of Lysine Catabolism.[a]

Radiotracer	% Incorporation		N^6-Acetyllysine/ Pipecolic Acid
	N^6-Acetyllysine	Pipecolic Acid	
DL-[2-^{14}C]Lysine	10.64	2.88	3.7
D-[1-^{14}C]Lysine	10.54	0.96	11
L-[U-^{14}C]Lysine	2.88	3.73	0.8

[a] In each case, a 10-day old *R. leguminicola* culture was drained and suspended on 50 ml of 1.2 mM hydroxylamine hydrochloride for 10 hr. The appropriate radiotracer, along with 500 μmol of carrier DL-lysine, was then added in 10 ml of water. After 24-hr incubation, N^6-acetyllysine and pipecolic acid were isolated from the mycelium by ion-exchange chromatography and the radioactivity of these metabolites was determined as described in Materials and Methods.

TABLE III: Incorporation of the Nitrogen Atoms of Lysine into Metabolites of *Rhizoctonia leguminicola*.[a]

Added Lysine	% Excess ^{15}N	
	N^6-Acetyllysine	Pipecolic Acid
DL-[$^{15}N^2$], 60.7% excess	62.8	3.0
DL-[$^{15}N^6$], 89.7% excess	76.1	40.3

[a] In each case, one 12-day old *R. leguminicola* culture was drained and suspended on 50 ml of 10 mM [^{15}N]lysine containing 1.2 mM hydroxylamine hydrochloride and 0.37 μmol of DL-[4,5-^{3}H]lysine (sp act. 200 Ci/mol) for 24 hr at room temperature. The metabolites were separated by ion-exchange chromatography. After desalting (1.2 × 50 cm Dowex 50-X8, 200–400 mesh, H^+ form, elution with 2 N NH_4OH after washing with distilled water), the ethyl esters were prepared in each case according to Biemann *et al.* (1961) and assayed for ^{15}N by combined gas chromatography–mass spectrometry as described under Materials and Methods.

TABLE IV: Δ^1-Piperideine-2-carboxylic Acid Reductase.

Description of System[a]	μmol of Pipecolic Acid
Crude homogenate, complete system	2.38
37,000*g* supernatant, complete system	1.54
Sephadexed fraction	
Complete system	2.14
−NADPH system	1.14
−NADH	2.31
−NADH, −NADPH system	0.87
Boiled enzyme	0.21
−substrate	0.08

[a] Two 10-day old *R. leguminicola* cultures were incubated with 500 μmol of DL-lysine for 24 hr, then washed, and fractionated. The preparation of a whole cell homogenate, cell-free extract, Sephadex treatment, and determination of protein is described in Materials and Methods of Guengerich *et al.* (1973a). The complete incubation system consisted of an NADPH generating system, 0.2 ml of 50 mM NADH, 0.1 ml of 100 mM Δ^1-piperideine-2-carboxylic acid (monohydrobromide monohydrate), 3.0 ml of enzyme preparation (supernatant: 4.05 mg of protein/ml; Sephadexed fraction: 3.2 mg of protein/ml), and pH 7.5 (1 mM β-mercaptoethanol, 0.2 M potassium phosphate) buffer to 4.75 ml total volume. The NADPH generating system consisted of 0.1 ml of 20 mM $NADP^+$, 0.3 ml of 40 mM glucose 6-phosphate, 0.05 ml of 20 mM $MgSO_4$, and 0.01 ml of glucose 6-phosphate dehydrogenase (625 units/ml). All incubations were for 3 hr at room temperature. Aliquots of 0.5 ml were each added to 7.0 ml of freshly prepared 0.3% ninhydrin in glacial acetic acid in duplicate. After heating for 30 min at 100°, pipecolic acid was determined at 565 nm, using a standard curve (Piez *et al.*, 1956). The value given above for the boiled enzyme incubate is due largely to the absorbance of the yellow ninhydrin adduct of residual Δ^1-piperideine-2-carboxylate at 565 nm.

lysine. In this latter experiment the retention time and mass spectrum of the test sample were identical with authentic N^6-acetyllysine ethyl ester, thus providing supplementary chemical evidence for the identity of N^6-acetyllysine as a lysine catabolite (peak B, Figure 1).

Δ^1-Piperideine-2-carboxylic Acid Reductase. As discussed in the previous section, the ^{15}N study of Table III is consistent with the view that Δ^1-piperideine-2-carboxylic acid is a precursor of pipecolic acid; hence an effort was made to demonstrate the enzymatic reduction of this compound to pipecolate in extracts of *R. leguminicola.*

[*R*-^{3}H]-Δ^1-Piperideine-2-carboxylic acid (*ca.* 0.01 μCi, sp act. 250 Ci/mol) was incubated with 4.5 mg of NADH, 2.5 mg of NADPH, and 2.0 ml of *R. leguminicola* crude homogenate in 1.0 ml of pH 7.5 (0.2 M potassium phosphate) buffer for 3 hr. The incubation was stopped with trichloroacetic acid; protein was removed by centrifugation. The neutralized supernatant was applied to a 0.6 × 60 cm Dowex 50-X8 column (H^+ form, 200–400 mesh). The radioactivity elution profile (using 1 N HCl) showed two major peaks of nearly equal magnitude. The first corresponded to authentic Δ^1-piperideine-2-carboxylic acid and the second to pipecolic acid (Cooksey and Greenberg, 1965). The second peak was verified as pipecolic acid by thin-layer chromatography (silica gel G: chloroform–methanol–14% aqueous NH_4OH, 2:2:1) with authentic material.

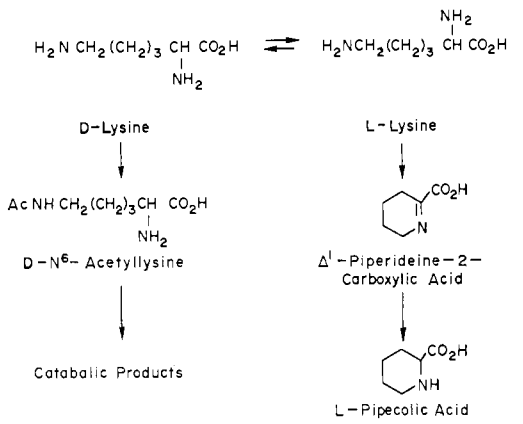

FIGURE 2: Catabolism of lysine in *Rhizoctonia leguminicola.*

The reductase was investigated further, using a colorimetric assay (Table IV). The enzymic reduction was found to proceed readily; desalted extracts appear to prefer NADPH to NADH. Δ^1-Piperideine-6-carboxylic acid was synthesized and used in similar incubations to those with Δ^1-piperideine-2-carboxylic acid; however, virtually all absorbance at 565 nm could be attributed to the small amount of absorbance of the yellow ninhydrin adduct of the substrate at that wavelength. When DL-[*R*-^{3}H]pipecolic acid was incubated with crude homogenates, oxidized pyridine nucleotides, and sodium bisulfite as a trapping agent (Cooksey and Greenberg, 1965), no evidence for the accumulation of either Δ^1-piperideine-2-carboxylic or Δ^1-piperideine-6-carboxylic acid was found.

Discussion

Evidence has been presented herein for the occurrence of the events shown in Figure 2. The finding that two separate and apparently isomer-specific pathways exist for the degradation of lysine in *R. leguminicola* is not unique. In mammals L-lysine is degraded *via* saccharopine in a reversal of the latter steps of the homocitric–aminoadipic biosynthetic pathway (Higashino *et al.*, 1965; Higashino and Lieberman, 1965), and D-lysine *via* pipecolic acid (Grove *et al.*, 1969a,b); racemase activity does not appear to be very significant. Certain *Pseudomonads* contain active racemases (Soda and Osumi, 1969) and degrade D-lysine *via* pipecolic acid (Miller and Rodwell, 1971; Chang and Adams, 1971); L-lysine is degraded *via* a pathway involving δ-aminovaleramide (Rodwell, 1969).

The efficient incorporation of the ε-nitrogen, but not the α-nitrogen, of lysine into pipecolic acid (Table III) is consistent with Δ^1-piperideine-2-carboxylic acid as an intermediate in the transformation of L-lysine to L-pipecolic acid in *R. leguminicoli.* Moreover, the cell-free reduction of Δ^1-piperideine-2-carboxylic acid, but not Δ^1-piperideine-6-carboxylic acid, to pipecolic acid was also demonstrated in this organism (Table IV). No evidence was found for the catabolism of L-pipecolic acid to aminoadipic acid or other acidic compounds (Guengerich, 1973) as in the catabolism of pipecolic acid in *Pseudomonas* (Miller and Rodwell, 1971). Experiments with resolved [^{14}C]lysines and with [^{15}N]lysine in *Neurospora crassa* gave results similar to those presented in Tables II and III for *R. leguminicola* (Guengerich, 1973). Meister *et al.* (1957) have described a Δ^1-piperideine-2-carboxylic acid reductase in *N. crassa.* Thus, the pathway described here for the biosynthesis

of pipecolic acid may be generalized among other fungi as well as *R. leguminicola*.

Figure 2 visualizes that the initial steps of lysine catabolism in *R. leguminicola* are *via* D-lysine and D-N^6-acetyllysine, whereas L-lysine is utilized for the synthesis of L-pipecolate, which in turn serves as a precursor for the synthesis of piperidine alkaloids in this mold. Purification of the lysine racemase should permit a careful study of its properties and permit a clearer view of its physiological role in determining the flow of lysine metabolites relative to the pathways of Figure 2.

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Heterogeneity of the Outer Membrane of Mitochondria†

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ABSTRACT: The arrangement of proteins in the outer membrane of rat liver mitochondria was explored. Exterior membrane proteins of the mitochondrion were labeled with ^{125}I using lactoperoxidase-catalyzed iodination. The iodine label thus served as a marker for outer membrane proteins. The specific activity of the outer membrane was 12-fold greater than that of the mitochondria from which it was isolated by digitonin treatment. Adenylate kinase, a soluble enzyme located between the outer and inner membranes, was assayed at various stages in the experiments to determine whether or not the mitochondria were intact. After iodination of the mitochondria the polypeptides of the organelles were separated into molecular weight classes by electrophoresis in a sodium dodecyl sulfate–acrylamide gel system. When intact mitochondria were iodinated, the label was distributed among polypeptides in 12 molecular weight classes. In microsomes the label was found in polypeptides of nine molecular weight classes. Although the iodinated polypeptides of both mitochondria and microsomes ranged from about 10,000 to 100,000 daltons, the radioactive gel patterns of the two organelles were quite different. About 70% of the label in the outer membrane isolated from the iodinated mitochondria was in polypeptides of the 14,000 molecular weight class. The distribution of the labeled polypeptides in the various molecular weight classes was different for proteins in isolated outer membrane fragments as compared to proteins remaining with the inner membrane matrix particles after the digitonin fractionation. This selective fractionation of outer membrane proteins suggested that the proteins were heterogeneously distributed in the plane of the membrane.

One of the most commonly used procedures for identification and determination of purity of subcellular components in cytochemical studies is the use of marker enzymes. Problems arise if the marker enzyme activity is found in more than one location. An example of this is the rotenone-insensitive NADH–cytochrome *c* reductase found in the outer membrane of mitochondria which is quite similar to the NADH–cytochrome *c* reductase system of microsomes (Raw and Mahler, 1959; Raw *et al.*, 1960; Sottocasa *et al.*, 1967a,b). Several other examples of enzymes in mitochondria with dual locations have been reported (Ernster and Kuylen-

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