D-Lysine Catabolic Pathway in *Pseudomonas putida:*
Interrelations with L-Lysine Catabolism

YUNG-FENG CHANG and ELIJAH ADAMS

Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

Received for publication 2 November 1973

The isolation of several mutant strains blocked in L-lysine degradation has permitted an assessment of the physiological significance of enzymatic reactions related to lysine metabolism in *Pseudomonas putida.* Additional studies with intact cells involved labeling of metabolic intermediates from radioactive L- or D-lysine, and patterns of enzyme induction in both wild-type and mutant strains. These studies lead to the conclusions that from L-lysine, the obligatory pathway is via \( \delta \)-aminovaleramide, \( \delta \)-aminovalerate, glutaric semialdehyde, and glutarate, and that no alternative pathways from L-lysine exist in our strain. A distinct pathway from D-lysine proceeds via \( \Delta^1 \)-piperideine-2-carboxylate, L-pipecolate, and \( \Delta^1 \)-piperideine-6-carboxylate (\( \alpha \)-aminoacrylic semialdehyde). The two pathways are independent in the sense that certain mutants, unable to grow on L-lysine, grow at wild-type rates of D-lysine, utilizing the same intermediates as the wild type, as inferred from labeling studies. This finding implies that lysine racemase in our strain, while detectable in cell extracts, is not physiologically functional in intact cells at a rate that would permit growth of mutants blocked in the L-lysine pathway. Pipecolate oxidase, a D-lysine-related enzyme, is induced by D-lysine and less efficiently by L-lysine. Aminooxyacetate virtually abolishes the inducing activity of L-lysine for this enzyme, suggesting that lysine racemase, although functionally inactive for growth purposes, may still have regulatory significance in permitting cross-induction of D-lysine-related enzymes by L-lysine, and vice versa. This finding suggests a mechanism in bacteria for maintaining regulatory patterns in pathways that may have lost their capacity to support growth. In addition, enzymatic studies are reported which implicate \( \Delta^1 \)-piperideine-2-carboxylate reductase as an early step in the D-lysine pathway.

The oxidation of L-lysine in *Pseudomonas* is believed to involve a series of intermediates shown in the right branch of Fig. 1, sometimes referred to as the \( \delta \)-aminovaleraldehyde pathway. The physiological significance of these reactions has not been explicitly tested by the use of mutants; its role as a major pathway rests on the characterization of the pathway reactions themselves (17, 20–22, 33, 34, 37, 39) and on the induction of certain of the enzymes by exposure of cells to lysine (33, 41). Another set of reactions in *Pseudomonas,* L-pipecolate \( \rightarrow \) \( \Delta^1 \)-piperideine-6-carboxylate \( \rightarrow \) \( \alpha \)-aminoacprodate, studied most definitively by Rodwell and his associates (5–7, 9, 31), has not been integrated clearly into an overall scheme of lysine metabolism; an earlier undefined relationship to L-lysine metabolism was suggested by the finding that L-lysine, like pipecolate itself, induces pipecolate oxidase (5).

One difficulty in analyzing pathway steps in relation to L- or D-lysine, either by tracer methods or induction studies, is the existence of lysine racemase, first reported by Ichihara et al. (20). Thus, it could be inferred, from the effect of hydroxylamine in selectively inhibiting D-lysine oxidation by acetone powder preparations, that D-lysine is metabolized, after racemization, via the \( \delta \)-aminovalerate pathway.

Recent studies by Miller and Rodwell (28) based on intermediate-labeling techniques and the use of inhibitors, and a subsequent preliminary report by ourselves (10) based additionally on the use of mutants, have clarified the status of the utilization of L- and D-lysine. Both studies, each in somewhat different ways, strongly suggested the existence of two complete pathways in *Pseudomonas,* one related to...
L-lysine utilization and the other to D-lysine utilization.

This paper presents further studies of the L- and D-lysine pathways and their interrelation. In extension of the earlier report of Miller and Rodwell (28), we provide evidence for the fol-
ollowing: (i) a physiological assessment of the L-lysine pathway based on growth studies with mutants blocked at specific steps of this pathway; (ii) segregation of the two pathways both by mutant growth properties and intermediate-labeling techniques; (iii) studies of the patterns of enzyme induction under various growth conditions in wild-type and mutant strains; (iv) a consideration of the role of lysine racemase; this enzyme, although detectable in extracts, appears inadequate to support growth, on L-lysine, of mutants blocked in enzymes of the L-lysine pathway, or to prevent segregation of intermediate-labeling patterns from D- or L-lysine; (v) the recognition and partial characterization of Δ^1-piperidine-2-carboxylate reductase, previously postulated (28) as a step in the D-lysine pathway.

The two lysine pathways in *Pseudomonas*, as they are now understood both from previous publications and from the present studies, are outlined in Fig 1. The same figure also provides literature references to individually defined enzymatic steps and indicates the site of enzyme blocks in the mutant strains referred to in the present paper.

**MATERIALS AND METHODS**

**Bacterial strain and growth procedures.** *P. putida*, biotype A (ARCC 15070), has been described in detail elsewhere (1). Wild-type and mutant stocks were grown on a minimal salts medium (25) with added carbon sources as noted in individual experiments. Solutions of substrates to be added were neutralized with NaOH and were either autoclaved and added to the basic media or, where stability to autoclaving was uncertain, were sterilized by filtration through membrane filters (Millipore Corp.). Cells were grown for 18 to 20 h at room temperature in Erlenmeyer flasks with side arms (Nephelo flasks) on a rotary shaker. Cell density was measured in a Klett-Summerson colorimeter (no. 54 filter) against a water blank. After 24-h growth periods, both mutant and wild-type stocks could be maintained in the growth medium for at least several weeks at 4 C and at least 1 year at −70 C. Relations between turbidity, cell number, and dry weight were based on previous measurements with this strain (15).

**Preparation of cell extracts.** For large-scale preparations, cell pellets (freshly harvested or after storage at −15 C) were homogenized with a motor-driven Teflon pestle in 5 volumes of 5 mM potassium phosphate, pH 7.0, containing 5 mM GSH (freshly made neutral solution) and 1 mM ethylenediaminetetraacetic acid (EDTA). Volumes of 30 to 50 ml in a Rosett cell (Branson Instruments) were subjected to cell breakage with a Branson Sonifier; the temperature was kept below 10 C by cooling in a dry iced-ethanol bath.

For standardized small-volume extracts, cells harvested from 100-ml cultures were suspended in 8 ml of 0.05 M potassium phosphate, pH 6.7, and subjected to sonic oscillation in 4-ml batches with the microtip of a Branson Sonifier; cooling was carried out as above.

**Mutagenesis and mutant isolation.** It has been reported that this strain gives rise to two colony types (3) described earlier (36) for other strains. Before cells were treated with mutagens, a single colony (the translucent, faster-growing type) was isolated for further treatment. Mutagens were ethyl methane sulfonate, used as described earlier (24), and N-methyl-N′-nitro-N-nitrosoguanidine used by the method of Adelberg et al. (4). Wild-type cultures grown on L-lysine (0.2%) were harvested at an optical density of about 100 Klett units and treated with either mutagen under the general conditions recommended. Approximately 1% survival was observed after ethyl methane sulfonate treatment and less than 0.1% survival after nitrosoguanidine treatment. Mutants were isolated after serial dilution of mutagenized cultures to yield a convenient colony density on 2% agar plates. In searches for mutants blocked in a specific reaction or region of a pathway, a substrate before the desired step (e.g., lysine) was present at high concentration (0.2%), whereas an intermediate following the desired step (e.g., δ-aminovalerate, glutaric semialdehyde, or glutarate) was present at low concentration (0.01%).

After incubation of plates at 30 C for 2 to 3 days, mutant cells blocked in a given reaction were recognized as forming small colonies under the appropriate selective conditions. Such colonies were tested further for selective growth on plates containing the specific substrates before and after the presumptive blocked step, and then tested further by growth on various pathway intermediates in liquid culture. Finally, cell extracts were tested for the activity of specific enzymes. Negative growth was defined by failure to grow during 48 h.

Mutants isolated are indicated in Fig. 1. American Type Culture Collection numbers for the mutant strains cited in the present paper are as follows: A-3(ATCC 25990), I-5(ATCC 25992), I-8(ATCC 25993), M-7(ATCC 25991), and M-14(ATCC 25996).

**Enzyme assays; enzyme tests for mutants.** Cell extracts, obtained as described above, yielded L-pipepoxide oxidase in the supernatant solution from a preliminary centrifugation for 30 min at 10,000 × g. The enzyme was then obtained in the pellet after a further 2-h centrifugation at 100,000 × g. The assay procedure was essentially that of Baginsky and Rodwell (6), using 0.1 to 1.0 mg of protein (crude particulate preparation) in a final volume of 0.4 ml. The quantity of product, Δ^1-piperidine-6-carboxylate, was calculated by using a molar absorption coefficient of 164 M^-1 cm^-1 (7) at 430 nm; the rate of product formation was linear with enzyme.

Δ^1-Piperidine-2-carboxylate reductase was assayed by the rate of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at room temperature in a 1-ml incubation volume containing 0.08 M potassium phosphate (pH 6.5), 0.15 mM
NADPH, 0.85 mM substrate, and 0.01 to 0.02 ml of crude enzyme; crude enzyme was the supernatant solution recovered from the 100,000 x g centrifugation step described above. The reaction was started by adding \( \Delta^1 \)-piperideine-2-carboxylate, and the rate of decrease in optical density at 340 nm was recorded with a Gilford spectrophotometer. The reaction rate was constant for at least 2 min and was linear with enzyme. A small blank rate of NADPH oxidation in the absence of added substrate was subtracted.

Lysine racemase was assayed by measuring the formation of L-lysine from D-lysine under anaerobic conditions to prevent lysine oxygenase action on the L-lysine formed. The enzyme source was an acetone powder prepared by suspending washed cells in acetone at \(-15^\circ\)C (50 ml/g of cells). The suspension was homogenized in a Sorvall Omnimixer at full speed for 1 min with cooling (less than \(-10^\circ\)C) in an alcohol-dry ice bath. The acetone powder, obtained by filtration with suction on a Buchner funnel, was washed with cold acetone and air-dried. Suspensions were made by gently stirring the acetone powder (10 mg/ml) in 0.07 M potassium phosphate, pH 7.4. Samples of the acetone powder suspension (0.02 to 0.2 ml) were added to the main chamber of a Thunberg tube containing 0.02 M potassium phosphate, pH 7.4, and 0.2 mg of pyridoxal phosphate in a final volume of 2 ml. The side arm contained 0.1 or 0.4 ml of 0.1 M D-lysine; there was little or no dependence of the reaction rate on substrate concentration over this range. After several sequences of evacuation and nitrogen flushing, the reaction was started by mixing substrate with the other components. Tubes were incubated at 30 C for 1 h and then held in a boiling-water bath for 5 min. L-Lysine formed during the racemase incubation was assayed by conversion to cadaverine, catalyzed by L-lysine decarboxylase (EC 4.1.1.18; Worthington Biochemical Corp.); and measurement of cadaverine as the dinitrophenyl derivative as was described by Hutzler et al. (19). Controls included zero-time samples of D-lysine added to boiled incubation mixtures made as described above, as well as L-lysine standards carried through the entire procedure. The reaction rate was proportional to the quantity of acetone powder assayed.

Glutarate semialdehyde dehydrogenase was assayed by the procedure described earlier (11), by following the formation of NADPH at 340 nm. \( \delta \)-Aminovalerate transaminase was assayed by coupling with glutarate semialdehyde dehydrogenase under conditions similar to the assay for glutarate semialdehyde dehydrogenase with the following modification: 0.02 M \( \alpha \)-ketoglutaric acid (neutralized), 0.015 M \( \delta \)-aminovalerate and excess partly-purified glutarate semialdehyde dehydrogenase (11) (free of \( \delta \)-aminovalerate transaminate) were included; glutarate semialdehyde was omitted.

In all assays cited, a unit of enzyme activity means 1 amol of product formed per min under the assay conditions used.

**Preparation and assay of substrates.** \( \Delta^1 \)-Piperideine-2-carboxylic acid was prepared by the action of purified D-amino acid oxidase (EC 1.4.3.3; DAOFF, Worthington Biochemical Corp.) on D-pipecolic acid (Calbiochem). The latter compound was neutralized with NaOH and added as a 0.3 M solution, in 8 ml, to 32 ml containing 0.02 M sodium pyrophosphate, (pH 8.3) 4 mg of enzyme (2.5 U/mg, Worthington Biochemical Corp.), and 250 U of catalase (EC 1.11.1.6; Worthington Biochemical Corp.). After 8 h at room temperature, an equal volume of absolute ethanol was added to the incubation mixture, precipitated protein was removed by centrifugation, and the supernatant solution was reduced in volume to 20 ml by flash evaporation in a 45 C water bath. The solution was acidified to pH 4 with HCl and placed on a column (1 by 39 cm) of Dowex 50 (H\(^+\)) (x8; 100 to 200 mesh). The column was eluted with 0.5 M HCl at a rate of 0.5 ml/min, collecting 10-ml fractions. \( \Delta^1 \)-Piperideine-2-carboxylic acid was detected initially by a color test with \( \alpha \)-aminobenzaldehyde as described by Soda et al. (34); subsequently a more sensitive and convenient detection method was based on an acid ninhydrin method (30). The ninhydrin spectrum of \( \Delta^1 \)-piperideine-2-carboxylic acid showed peaks at 466 and 500 nm; in contrast to the 3 h required for color development with \( \alpha \)-aminobenzaldehyde, the ninhydrin method required only 30 min for full color development. The compound was collected in a volume of 160 ml, beginning at 220 ml. Pooled fractions were free from residual piceolic acid, as determined by absence of its typical purple color in the acid ninhydrin assay of Pize et al. (30). The pooled eluates were concentrated by repeated flash evaporation in a 45 C water bath to remove HCl (avoiding complete drying) and were neutralized with 5 N NaOH before use. The compound was assayed as piceolate (30) after reduction with excess NaBH\(_4\). Alternatively, \( \Delta^1 \)-piperideine-2-carboxylic acid was assayed by the stoichiometric oxidation of excess NADPH catalyzed by crude \( \Delta^1 \)-piperideine-2-carboxylic acid reductase (see above). Both assay methods gave results in good agreement. The yield of \( \Delta^1 \)-piperideine-2-carboxylic acid represented about 16% of the D-pipecolic in the initial incubation mixture.

\( \Delta^1 \)-Piperideine-6-carboxylic acid was prepared by scaling up the incubation mixture described for the assay of piceolate oxidase (see above) and prolonging the incubation time to several hours. The reaction was stopped by adding an equal volume of absolute ethanol, and the crude preparation of product was used after concentration by flash evaporation. No ninhydrin-positive compound other than \( \Delta^1 \)-piperideine-6-carboxylate and piceolate was detected by chromatography on amino acid analyzer columns (see below). The piperideine reaction product was estimated as noted for the assay of piceolate oxidase (see above). Glutaric semialdehyde was synthesized and assayed as described previously (2). \( \delta \)-Aminovaleramidate was prepared as described (32).

**Identification and isolation of metabolic intermediates by ion exchange chromatography.** A reproducible technique for the resolution of intermediates of interest utilized the Technicon amino acid analyzer system with a column (0.6 by 60 cm) of DC-1A resin (Durrum Chemical Corp.). Samples were evapo-
rated to dryness, dissolved in 0.5 ml of 0.01 N HCl, and placed on the column. The column was eluted with sodium citrate buffer (pH 6.35, 1.6 M in Na+) at a rate of 35 ml/h and at 52 C. In some experiments, approximately one-half of the column output passed through the auto analyzer-recorder system, while the remainder passed into a fraction collector; for preparative purposes, the entire column output was pumped into a fraction collector. (The elution positions of a number of relevant substrates and intermediates are shown in Fig 2.)

**Purification of [14C]lysine.** Samples of L-[U-14C]-lysine or DL-[2-14C]lysine, obtained from New England Nuclear Corp. or Calbiochem, contained significant radioactive impurities. Some of these, on ion-exchange chromatography, overlapped the elution positions of metabolic intermediates, eluting at 15, 21, 26 and 41 min in the system described above. For this reason, it was essential to purify radioactive lysine samples before use, as has been independently noted elsewhere (12). Samples of [14C]lysine were chromatographed as noted for Fig 2; fractions corresponding to the lysine peak, which emerged at 50 to 58 min, were collected and evaporated to dryness under vacuum at 45 C; the residue, redissolved in 0.01 N HCl, was desalted by applying to a column (0.9 by 20 cm) of Dowex 50 (H+) (x8; 100 to 200 mesh) and eluting with 1 N NH4OH after the column was washed with water (250 ml). The eluates were evaporated several times to remove ammonia, and the purified [14C]lysine samples were stored at 4 C. Approximately 80% of the radioactivity in the lysine fractions was recovered.

**Preparation of D-[2-14C]lysine.** D-[2-14C]lysine was prepared by exhaustive treatment with L-lysine decarboxylase of commercially obtained DL-[2-14C]lysine which had been chromatographically purified as described above. Approximately 20 piCi (6 mol) of DL-[2-14C]lysine was mixed with 4 mg of L-lysine decarboxylase (see below) in a volume of 1 ml containing 0.2 M potassium maleate (pH 6), 5 mM EDTA, and 0.6 mM pyridoxal phosphate, and incubated at 37 C for 2.5 h; another 2 mg of enzyme was added and the incubation was continued for another 2 h. The reaction mixture was made 0.1 N in HCl, precipitated protein was removed by centrifugation, and the precipitate was washed with 0.1 N HCl. The combined supernatant solutions and wash were flash-evaporated to dryness, dissolved in 5 ml of water, and subjected to ion-exchange chromatography to separate D-lysine and cadaverine by the method of Tabor and Rosenthal (38). Gradient elution of labeled material from a column of Amberlite XE-64 (K+, 0.9 by 20 cm) yielded two radioactive components: lysine, between 52 and 74 ml, and cadaverine, between 146 and 160 ml/h.

**Fig. 2.** Relative radioactivity in lysine intermediates after incubation of intact cells with L-[U-14C]lysine or D-[2-14C]lysine. Cells of mutant M-7 (see Table 1) were grown to a density of about 300 Klett units in the standard medium containing 0.4% L-glutamate and 0.4% D,L-lysine, harvested, washed with 0.033 M potassium phosphate (pH 7), and suspended in this buffer to a final density of 1000 Klett units. Samples of the suspension (0.08 ml) were incubated (final volume, 2 ml) with 1.6 piCi (1.6 mol) of L-[U-14C]lysine or D-[2-14C]lysine and 48 piCi of unlabeled L-piperidine. After 90 min of incubation at 30 C, with aeration, acetone was added to 40% to stop the reaction and freeze the cells. The mixture was centrifuged, and the supernatant solution was evaporated to dryness in a flash evaporator, taken up in 0.5 ml of 0.01 N HCl, and chromatographed on an amino acid analyzer column as described in the text. Approximately one-half the column output passed through the recorder system. The remainder was collected in a fraction collector for measurement of radioactivity. The bottom half of the figure shows the distribution of radioactivity from L-[U-14C]lysine (●) or from D-[2-14C]lysine (O). The radioactive fractions shown were identified, as indicated, by runs on standard compounds. The top half of the figure shows the ninhydrin absorbance (solid line, 570 nm; dashed line, 440 nm) of various peaks in the same experiment. The peak labeled "unknown" resembles α-aminoadipic acid in its chromatographic behavior in this system as well as with a pH 3.5 elution buffer, and on paper chromatography. Essentially identical results were obtained in similar incubations of wild-type cells, but in such experiments 48 piCi of unlabeled δ-aminovalerate was also added; cells of mutant M-7 did not require this addition because their block in δ-aminovalerate transaminase led to endogenous accumulation of this intermediate.
178 ml. The ratio of radioactivity in the cadaverine to that in the lysine was 1.04, suggesting essentially complete freedom of D-[2-14C]lysine from residual L-lysine. Pooled eluate fractions containing D-[2-14C]lysine were evaporated to dryness and desalted as described in the purification of [14C]lysine (see above). The desalted sample of D-[2-14C]lysine contained 27% of the radioactivity initially present in DL-lysine, representing a yield of approximately 54% of the D-isomer.

Commercially obtained enzymes and compounds. L-Lysine decarboxylase of Bacterium cadaveris was purchased as an acetone powder from Worthington Biochemical Corp. For use both in the assay of lysine racemase and in the preparation of D-[2-14C]lysine, the enzyme was first suspended (2 mg/ml) in 0.02 M potassium maleate (pH 6) containing 5 mM EDTA. Insoluble material which contained the enzyme was collected by centrifugation at 800 × g for 10 min. D-Amino acid oxidase (DAOFF, 2 U/mg) was purchased from Worthington Biochemical Corp. Samples of DL-pipeolic acid were purchased from Sigma Chemical Corp., and D- and L-pipeolic acids were from Calbiochem. D-Aminovaleric acid was obtained from Sigma Chemical Corp., α-aminobenzaldehyde was from K & K Laboratories, and DL-α-aminoadipate was from Eastman Kodak Co.

Transport measurements. Estimates of the initial rate of uptake by cells of L- or D-lysine were made as described previously for other substrates (16). Cells were washed and suspended in 0.033 M potassium phosphate, pH 7, to a final density of 100 Klett units. D-[2-14C]lysine or L-[U-14C]lysine, each at final concentrations of 0.125 mM or 0.5 mM and at a specific activity of 0.23 μCi/μmol, were incubated under aeration at 30°C with 4 ml of the above cell suspension. Samples (0.5 ml) of the incubation mixture were filtered through Millipore filter membranes (HAWP, average pore size 0.45 μm); the membranes were washed with 10 ml of 0.033 M potassium phosphate, pH 7, at room temperature and dissolved in scintillation fluid for counting. Uptake rates remained linear for at least 3 min after substrate addition.

Radioactivity determination. Radioactivity was measured in a Packard scintillation counter by using a modified Bray solution (8). Eluates from the amino acid analyzer showed negligible quenching by salt in the small samples counted.

Protein measurement. Protein in crude enzyme preparations was estimated by a turbidimetric method (35).

RESULTS

Growth studies of mutants. Wild-type cells grew well (optical density of about 200 Klett units in 24 h) on both L- and D-lysine, as well as on all intermediates shown in Fig. 1 except for δ-aminovaleramide and α-aminoadipate; on these compounds, growth was distinctly slower, requiring about 48 h for full turbidity. Since each of the latter substrates is utilized by cell-free extracts (18, 39) it seemed probable that slow growth reflects poor uptake of these substrates; we have observed considerably faster growth after repeated transfer of cells through media containing δ-aminovaleramide or α-aminoadipate as carbon sources.

Table 1 shows the growth characteristics of several mutants with intermediates of the sequence: Δ1-piperideine-2-carboxylate → L-pipeolate → Δ1-piperideine-6-carboxylate, as well as L- or D-lysine, as the carbon source. The physiological significance of the reactions leading from L-lysine through δ-aminovaleramide is shown by failure of mutants blocked in several regions of this pathway to grow on L-lysine. The separation of DL-lysine and L-lysine metabolism in our strain is similarly indicated by the inability of several mutants (M-7, A-3, M-14), blocked in the L-lysine pathway, to grow freely on D-lysine and its presumptive metabolic products. A puzzling exception to the above are mutants I-5 and I-8, blocked in the first reactions of L-lysine; these strains were incapable of growth on either L- or D-lysine but reverted readily to growth capacity on both isomers.

Selective labeling of intermediates from D- or L-lysine. Tabular data have been presented

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth on substrates:</th>
<th>Missing enzyme*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Lysine</td>
<td>D-Lysine</td>
</tr>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I-5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I-8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M-7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A-3, M-14</td>
<td>+</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Growth tests were carried out as described in the text. Substrates tested were added to the basal medium at 0.2% as sole carbon source. Positive growth (+) indicates growth indistinguishable from that of the wild type on a given substrate. Negative growth (0) was based on observing no significant growth for 48 h. NT, Not tested. Mutants I-5, I-8, and M-7 were isolated after treatment with ethyl methane sulfonate; A-3 and M-14 were isolated after treatment with nitrosoguanidine.

† Refers to blocked reaction as numbered in Fig. 1.

‡ M-14 is a secondary mutant of A-3 and additionally lacks the hydroxyproline-induced dehydrogenase for α-ketoglutarate semialdehyde (23).

§ Because of the existence of several isoenzymes catalyzing glutarate semialdehyde oxidation (23), these mutants are leaky in showing retarded, but not zero, growth on L-lysine.
in a brief report of this work (10), indicating markedly greater labeling of pipecolate and \( \Delta^1 \)-piperideine-6-carboxylate from \( \text{D-}[2-^{14}\text{C}] \)lysine than from equal radioactivity in \( \text{L-}[^{14}\text{C}] \)lysine. Reciprocally, radioactive \( \text{L} \)-lysine was a much better source of label in \( \delta \)-aminovalerate than was \( \text{D} \)-lysine. Comparable data were obtained for both wild-type cells and the M-7 mutant. An illustration of these findings is shown in Fig. 2 for an experiment with the M-7 mutant.

**Uptake of \( \text{D} \)- and \( \text{L} \)-lysine.** Because of striking differences in the labeling of characteristic intermediates produced by administering \( \text{L-}[^{14}\text{C}] \)lysine or \( \text{D-}[^{14}\text{C}] \)lysine, it was of interest to examine possible differences in uptake rate. It should be noted that differences in transport of the antipodes seemed an unlikely basis for the above labeling difference since each isomer showed a selective pattern, labeling certain intermediates better than did the other. Experimentally, uptake studies by wild-type or mutant (M-7) cells showed differences no greater than twofold in the rate of \( \text{L} \)-lysine uptake compared with \( \text{D} \)-lysine, whether or not cells were induced by growth in the presence of \( \text{DL} \)-lysine (Table 2). Rates were similar in magnitude to saturating rates measured for the uptake of hydroxyproline by the same strain of cells after full induction (16). In contrast to the status of hydroxyproline uptake by our strain and to lysine uptake by another strain of \( \text{P. putida} \) (27), exposure to lysine produced no stimulation of uptake rate; in contrast, growth in lysine media led to consistently lower initial uptake rates of \( \text{L} \)- or \( \text{D} \)-lysine. In magnitude, rates that we have measured for \( \text{L} \)-lysine uptake after cell growth on lysine, are comparable to those for uninduced cells of another strain of \( \text{Pseudomonas putida} \) (27).

\( \Delta^1 \)-Piperideine-2-carboxylate as a precursor of pipecolate. Although the enzymatic reduction of \( \Delta^1 \)-piperideine-2-carboxylate to \( \text{L} \)-pipecolate has been reported in animal tissues (26, 29), plant extracts, and \( \text{Neurospora} \) (26), this reaction has apparently not been studied in bacteria. Since the piperideine compound represents a logical precursor of pipecolate in \( \text{Pseudomonas} \) and might therefore be an earlier product of \( \text{D} \)-lysine metabolism, a role for \( \Delta^1 \)-piperideine-2-carboxylate was examined both in labeling studies of whole cells and in enzymatic studies of cell extracts. Efforts to demonstrate conversion of \( \text{D} \)-lysine to \( \Delta^1 \)-piperideine-2-carboxylate in radioactive experiments like those described in Fig. 2 did not yield clear results; only a suggestion of a radioactive peak in the region of this presumed intermediate was seen when \( \text{D-}[2-^{14}\text{C}] \)lysine was the substrate (Fig. 2). The addition of \( \text{NaHSO}_3 \), however, did lead to the accumulation of a small peak of radioactivity in the position of \( \Delta^1 \)-piperideine-2-carboxylate (not shown). A possible interpretation is the existence of this intermediate in the cell, primarily as the open-chain equilibrium form rather than the cyclic piperideine form; the open-chain form has been tentatively identified as the form bound to \( \Delta^1 \)-piperideine-2-reductase from animal sources (13, 29).

**Enzymatic studies of \( \Delta^1 \)-piperideine-2-carboxylate as an intermediate.** Attempts were unsuccessful to detect the formation of a piperideine (i.e., \( \alpha \)-aminobenzaldehyde-reactive) product of \( \text{D} \)-lysine (or \( \text{L} \)-lysine) by using either crude sonic extracts or a particulate fraction centrifuged from these extracts at 100,000 \( \times \) g. Such incubation mixtures (2 ml) contained 0.02 M \( \text{L} \)- or \( \text{D} \)-lysine and were incubated at pH 6.5 (potassium phosphate) at 30 C. In contrast, when \( \text{L} \)-pipecolate was the substrate incubated, an \( \alpha \)-aminobenzaldehyde-reactive product (\( \Delta^1 \)-piperideine-6-carboxylate) was readily detected. A comparable failure to detect conversion of radioactive \( \text{D} \)-lysine to pipecolate by cell-free \( \text{Pseudomonas} \) extracts has been noted by Miller and Rodwell (28). Although an enzymatic reaction responsible for \( \Delta^1 \)-piperideine-2-carboxylate formation was not detected, the reduction of this compound to \( \text{L} \)-pipecolate by cell extracts was easily demonstrated. Samples of the 100,000 \( \times \) g supernatant solution from sonic extracts of \( \text{Pseudomonas} \) were active in the oxidation of NADPH in a

### Table 2. Initial rate of uptake of \( \text{L} \)- or \( \text{D} \)-lysine

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Growth substrate</th>
<th>Uptake on substrates (µmol/g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \text{L} )-Lysine</td>
</tr>
<tr>
<td><strong>Wild type</strong></td>
<td>Glutamate</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Glutamate/lysine</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>M-7</strong></td>
<td>Glutamate/lysine</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Uptake measurements were made as described in the text. Uninduced cells were grown in basal medium containing 0.2% \( \text{L} \)-glutamate; induced cells were grown in the same medium containing glutamate plus 0.2% \( \text{DL} \)-lysine. Numerical values shown are micromoles of substrate taken up per gram of cell dry weight per minute and are averaged from at least two experiments at 0.125 m or 0.5 mM substrate concentration (see text).*
reaction requiring \( \Delta^1 \)-piperideine-2-carboxylate. Depending on induction conditions (see below), the activity of such crude extracts varied between 0.03 and 0.3 U/mg of protein. The product of the reaction was shown to be piperolic acid on the basis of similarity of its acid-ninhydrin spectrum (30) with that of authentic \( L \)- or \( D \)-piperolate or with the NaBH₄ reduction product of \( \Delta^1 \)-piperideine-2-carboxylate; additional points of identity with reference \( DL \)-piperolate included elution position from the Technicon amino acid analyzer (method as in Fig. 2), paper chromatography, and paper electrophoresis at pH 3.6.

In subsequent studies (Y. F. Chang, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 170, 1972), \( \Delta^1 \)-piperideine-2-carboxylate reductase has been partly purified from cells of the present strain of *Pseudomonas* grown on \( L \)-lysine. The reaction product was shown to be the \( L \)-isomer of piperocate by tests with \( D \)-amino acid oxidase and was shown to be formed in a 1:1 stoichiometry with NADPH oxidized.

**Induction of \( D \)-lysine related enzymes in wild-type cells.** The postulated relationship of \( D \)-lysine to the reaction sequence, \( \Delta^1 \)-piperideine-2-carboxylate \( \rightarrow \) \( L \)-piperolate \( \rightarrow \) \( \Delta^1 \)-piperideine-6-carboxylate, was examined by induction studies in wild-type cells (Table 3). \( L \)-Lysine, whether used as sole carbon source or added to glutamate, was an efficient inducer of piperocate oxidase as reported earlier (5); indeed, in our experience, it was generally more efficient as an inducer than piperolate itself (Table 3). Both with wild-type and mutant cells (Tables 3 and 4), \( D \)-lysine was consistently a somewhat better inducer of this enzyme than was \( L \)-lysine. Another intermediate of the \( L \)-lysine pathway, \( \Delta^1 \)-piperideine-2-carboxylate, was also an inducer of piperolate oxidase; in contrast, intermediates of the \( L \)-lysine pathway tested were not inducers. \( L \) and \( D \)-lysine were indistinguishable as inducers of \( \Delta^1 \)-piperideine-2-carboxylate reductase. It is notable that in this instance, the substrate of the enzyme had no inducer activity. The data of Table 3 also suggest that addition of glutamate at equal concentration with an inducing substrate had no inhibitory or repressing effect on the level of induction, nor, in independent studies by one of us (Y. F. Chang, unpublished data), has any diauxie phenomenon been suggested by growth curves on such substrate combinations. For this reason most of our data were obtained with glutamate as a carbon source for mutant strains, in which compounds to be tested as inducers could not themselves be utilized for growth; much of our comparative data with wild-type cells therefore also utilized a glutamate medium.

**Induction of piperolate oxidase in mutant strains.** Table 4 summarizes some observations on the induction of piperolate oxidase in several mutant strains. A point of interest concerning the relation of piperolate oxidase to \( D \)-lysine is the difference between \( D \) and \( L \)-lysine as an inducer of piperolate oxidase in mutant I-5.

**Experimental separation of \( L \)-lysine and \( D \)-lysine as inducers of piperolate oxidase.** Some of the findings above suggested that \( D \)-lysine might be a selective inducer of piperolate oxidase and that \( L \)-lysine might exhibit inducer activity only or, largely, by virtue of its conversion to \( D \)-lysine through the action of lysine racemase. Many attempts to isolate a mutant strain lacking lysine racemase were unsuccessful, perhaps because of difficulty in screening for such mutants in cells having independent degradative pathways for \( L \) and \( D \)-lysine. As an alternative, a likely inhibitor of lysine racemase, aminoxyacetic acid (14), was tested for

| Table 3. Induction of two \( D \)-lysine-related enzymes in wild-type cells* |
|-------------------------------|-------------------------------|-------------------------------|
| Substrates in growth medium   | Pipecolate oxidase (U/mg of protein) | \( \Delta^1 \)-Piperideine-2-carboxylate reductase (U/mg of protein) |
| L-Glutamate                   | 0.23 (9)                       | 0.04 (4)                      |
| L-Lysine                      | 7.9 (3)                        |                               |
| D-Lysine                      | 24 (1)                         |                               |
| DL-Piperolate                 | 2.0 (5)                        |                               |
| Cadaverine                    | 0.20 (1)                       |                               |
| L-Glutamate + L-lysine        | 9.4 (9)                        | 0.21 (4)                      |
| L-Glutamate + D-lysine        | 10.4 (9)                       | 0.16 (4)                      |
| L-Glutamate + \( \delta \)-aminovaleramidé  | 0.11 (1)                      |                               |
| L-Glutamate + \( \delta \)-aminovalerate   | 0.13 (3)                      |                               |
| L-Glutamate + L-piperolate    | 9.7 (1)                        | 0.03 (1)                      |
| L-Glutamate + D-piperolate    | 2.6 (1)                        | 0.04 (1)                      |
| L-Glutamate + \( \Delta^1 \)-piperideine-2-carboxylate | 6.7 (1) | 0.03 (1) |

* Growth conditions, preparation of extracts and enzyme assays were as described in the text. All compounds tested as inducers were also growth substrates. Both glutamate (where present) and the compound tested were added at 0.2%, except as noted. Values shown are mean values; the number of trials of separate cultures are noted in parentheses.
* Added at 0.1%.
* Added at 0.03%.
its effects on the induction pattern of L-lysine compared with D-lysine. At a concentration of 10^{-2} M aminooxyacetate, growth on either lysine antipode was comparable and showed only slight inhibition. In Table 5, data from a specific experiment, typical of several carried out, indicate that in the presence of aminooxyacetate, L-lysine, but not D-lysine, loses most of its ability to induce piperolate oxidase. In contrast, in the presence of this inhibitor, Δ^1-piperideine-2-carboxylate reductase appears to be induced to comparable levels both by L- and D-lysine.

**Levels of lysine racemase.** The existence of mutants having definable enzyme blocks in the L-lysine pathway, but which grow normally on D-lysine, implies that in such cells, and by extension in wild-type cells, the level of lysine racemase is insufficient to catalyze L-lysine racemization at a rate required for measurable growth. Direct measurements of lysine racemase yielded values of 0.003 U/mg of acetate powder in uninduced (glutamate grown) cells. On transfer to lysine-containing media, the racemase activity increased to a maximum of only about 0.005 U/mg of acetate powder, measured 10 h after addition of L-lysine to the growth media. A comparison of racemase activity in extracts of mutants M-7, I-5, and I-8 indicated no notable differences from the wild-type levels. In particular, the I-5 mutant, whose failure to show induction of piperolate oxidase by L-lysine might suggest an associated block in lysine racemase, had no reduction from the wild-type activity of lysine racemase.

Data provided by Ichihara et al. (20) represent the only other assay values of a *Pseudomonas* lysine racemase known to us. If we compare units of racemase activity per milligram of acetone powder, calculated from their data (20), it appears that their cells might have contained approximately five times as much enzyme as ours. The possible significance of this difference is discussed below.

### Table 4. Induction of piperolate oxidase in several lysine-pathway mutants

<table>
<thead>
<tr>
<th>Substrates in growth medium</th>
<th>Enzyme activity (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>0.23 (9)</td>
</tr>
<tr>
<td>L-Glutamate + β-aminovalerate</td>
<td>0.13 (3)</td>
</tr>
<tr>
<td>L-Glutamate + L-lysine</td>
<td>9.4 (9)</td>
</tr>
<tr>
<td>L-Glutamate + D-lysine</td>
<td>10.4 (5)</td>
</tr>
<tr>
<td>DL-Pipicolate</td>
<td>2.0 (5)</td>
</tr>
</tbody>
</table>

*See Table 3 for details. Values for wild-type cells from Table 3 are included for convenience. Numbers of separate trials are given in parentheses. See Table 1 and text for characterization of mutant strains.*

### Table 5. Effect of aminooxyacetate on enzyme induction by D- or L-lysine

<table>
<thead>
<tr>
<th>Additions to glutamate medium at 16 h</th>
<th>Pipicolate oxidase (U/mg of protein)</th>
<th>Δ^1-Piperideine-2-carboxylate reductase (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.26</td>
<td>0.033</td>
</tr>
<tr>
<td>Aminooxyacetate</td>
<td>0.22</td>
<td>0.039</td>
</tr>
<tr>
<td>D-Lysine</td>
<td>11.7</td>
<td>0.10</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>9.9</td>
<td>0.073</td>
</tr>
<tr>
<td>D-Lysine + aminooxyacetate</td>
<td>4.0</td>
<td>0.042</td>
</tr>
<tr>
<td>L-Lysine + aminooxyacetate</td>
<td>0.45</td>
<td>0.059</td>
</tr>
</tbody>
</table>

*Wild-type cells were grown on the standard medium containing 0.2% L-glutamate for 16 h; at this time D- or L-lysine was added to 0.2% and, where shown, aminooxyacetate was added to 10^{-2} M. Growth continued for another 7 h, at which time cells were harvested for enzyme assays.*

### DISCUSSION

A general scheme for the separate pathways in *Pseudomonas* that degrade L-lysine or D-lysine, and the potential connection between these pathways via lysine racemase (Fig. 1), are supported by the present data. The physiological significance of the pathway from L-lysine via β-aminovalerate is indicated directly by the isolation of mutants blocked in specific regions of this pathway which are unable to grow on L-lysine. The metabolic distinctness of the D-lysine pathway is similarly demonstrated by growth studies of the same mutants: all are capable of growth on intermediates beginning with Δ^1-piperideine-2-carboxylate. Mutants blocked in β-aminovalerate transaminase or glutaric semialdehyde dehydrogenase also grow at the wild-type rate on D-lysine. The two mutants (I-5 and I-8) blocked at an earlier point in the L-lysine pathway, however, were also
incapable of growth on D-lysine. The latter finding was unexpected and is unexplained by present knowledge of these pathways or their component reactions. That these two mutants might be double mutants lacking, additionally, a reaction between D-lysine and Δ1-piperideine-2-carboxylate or a transport system for D-lysine, is unlikely since both revert readily to prototrophy with respect to growth on both L- and D-lysine. The behavior of these mutants suggests a possible link between the early reactions of the L- and D-lysine pathways; some such relationship is also suggested by the unexplained difference between I-5 and I-8 with respect to the relative induction efficiency of piceolate oxidase by L- or D-lysine (Table 4); as noted above, this finding was not explained by a possible difference in the apparent activity of lysine racemase in the two mutants. One purely speculative possibility is the involvement of L-lysine oxygenase, in some manner, in the initial oxidation of D-lysine; this is prompted by the report that lysine oxygenase can act anaerobically to convert L-lysine to the corresponding α-keto acid (42).

The labeling pattern of metabolites from D- or L-lysine similarly relates those intermediates of the left branch of Fig. 1 to D-lysine and those of the right branch to L-lysine. Thus, Fig. 2 shows that in whole-cell experiments, δ-aminовалerate is labeled almost exclusively by L-lysine and virtually not at all from D-lysine. In contrast, piceolate, and particularly Δ1-piperideine-6-carboxylate, are labeled primarily from D-lysine. Such patterns were obtained both from wild-type and mutant (M-7) cells and with quantitatively similar utilization of the added L- or D-lysine; a gross difference in uptake rates between L- and D-lysine, which might favor alternative labeling patterns for other reasons than pathway segregation, was ruled out by direct experiments.

An experiment, with another strain of P. putida, leading to a similar conclusion was recently reported by Miller and Rodwell (28). In their case, a large added pool of unlabeled DL-piceolate trapped some 20% of counts added to whole cells as D-lysine, but only 2% of counts added as L-lysine, in the presence of hydroxylamine. Similarly, loss of the α-carboxyl of D-[1-14C]lysine (presumably as a result of lysine oxygenase action) but not that of L-[1-14C]lysine was prevented by hydroxylamine in cell extracts. These findings were interpreted as an effect of hydroxylamine in inhibiting lysine racemase and were, therefore, consistent with the conclusion that D-lysine was utilized selectively via piceolate, and L-lysine selectively via δ-aminovalerate, i.e., inhibition of lysine racemase restricted each lysine antipode to its own characteristic pathway.

Our experiments demonstrate similar relative segregation of the two lysine antipodes to the appropriate pathway without the necessity of adding inhibitors, a probable consequence of the functionally low level of lysine racemase in our strain.

Although the labeling data presented clearly support the pathway formulation shown in Fig. 1, these experiments raise further questions that are not completely clarified by present knowledge. The fact that piceolate was labeled significantly from D-lysine, trapping about 10 to 15% as much label as from D-lysine, indicates either that the lysine racemase in our cells permits this degree of mixing of the two pathways or that piceolate may also be formed directly from L-lysine. In data reported by Miller and Rodwell (28), it is notable that L-[1-14C]lysine labeled piceolate almost half as efficiently as did D-[1-14C]piceolate; the considerably greater mixing of the two pathways in their experiments than in ours may reflect a higher level of racemase activity in cells of their strain.

Further findings consistent with the formulation of Fig. 1 involve induction patterns. The L-related intermediates past L-lysine, specifically δ-aminovaleramide and δ-aminovalerate, did not induce piceolate oxidase; in contrast, as will be reported separately (Y. F. Chang and E. Adams, unpublished data) δ-aminovalerate is a good inducer of two L-lysine related enzymes—its own transaminase as well as glutarate semialdehyde dehydrogenase. An exception to this pattern is Δ1-piperideine-2-carboxylate reductase, which appears to be induced not by its substrate but by L- or D-lysine to equal extent. In contrast, piceolate oxidase appears to be induced primarily by D-lysine and less so by piceolate itself. The role of L-lysine as an inducer of piceolate oxidase is less clear. Since its induction action is largely abolished by aminooxyacetate, it may be considered that it acts primarily by conversion to D-lysine via the action of lysine racemase, as discussed below.

Δ1-Piperideine-2-carboxylate has been proposed as a reasonable hypothetical precursor of piceolate in Pseudomonas (28); the enzymatic and induction studies presented here provide evidence for this relationship.

The role of lysine racemase in the interrelation of L- and D-lysine metabolism is of particular interest. The enzyme can be detected in cell
extracts of our strain but appears unable to permit appreciable growth on L-lysine by mutants blocked in the latter pathway. This could be explained by our finding that assay values for this enzyme from our strain are considerably lower than seems compatible with observed growth rates on added N-lysine. It is difficult to determine whether, in this respect, our strain is typical of other Pseudomonas strains studied; experiments comparable to ours, that test the functional capacity of lysine racemase in whole cells, have not been reported. It is significant, however, that for comparable crude extracts a specific activity considerably higher than ours can be inferred from the data of Ichihara et al. (20), in studies of the lysine racemase of another Pseudomonas strain, as noted above.

From the data of Table 5, the interpretation seems plausible that aminooxyacetate blocks induction of pipecolate oxidase by L-lysine but not by D-lysine as a consequence of inhibiting lysine racemase. An obvious requirement for this conclusion is the demonstration that aminooxyacetate inhibits lysine racemase in our strain. Although such experiments have been repeatedly undertaken, both in whole cell preparations and in cell extracts, difficulties with a quantitatively satisfactory assay of the low level of lysine racemase in our strain have so far prevented a reliable conclusion. At this time, we propose tentatively that in our strain the lysine racemase is present but is insufficient to support growth by adequate flow from L-lysine through D-lysine pathway or vice versa. We propose also that the low level of racemase present may be responsible for cross-induction of L-specific enzymes by D-lysine and vice versa. If clearly demonstrable, this may represent an example of the preservation of regulatory patterns even when the pathways concerned are nonfunctional, by way of a weakly active enzyme which permits the formation of products at an inducing but not growth-supporting level.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM 11105 from the National Institute of General Medical Sciences.

LITERATURE CITED


