Biosynthesis of the Piperidine Nucleus

THE MODE OF INCORPORATION OF LYSINE INTO PIPECOLIC ACID AND INTO PIPERIDINE ALKALOIDS

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SUMMARY

6-3H,6-14C-DL-lysine was administered in “metabolite overloading” experiments to the intact rat, to a lysine-less mutant of Neurospora crassa, to intact bean plants (Phaseolus vulgaris), and to excised shoots of Sedum acre. Pипеolic acid was then isolated from the tissues. In each case the pипeolic acid showed a 3H:14C ratio which was similar to that of the administered precursor. It follows that, in each case, the conversion of lysine into pипeolic acid proceeds via ε-amino-α-ketoacaproic acid. α-Aminoadipic-δ-semialdehyde cannot be an intermediate. These results complement earlier findings that, in the rat, the nitrogen atom of pипeolic acid is supplied by the ε-amino group of lysine; they provide evidence for a similar route in N. crassa and in higher plants. They conflict with reports that, in higher plants, it is the α-amino group of lysine which supplies the nitrogen atom of pипeolic acid.

In S. acre, the doubly labeled lysine was incorporated into sedamine without change in 3H:14C ratio.

The evidence is strong that the first of these routes (1 → 2 → 3 → 4) to pипeolic acid is operative in vertebrates. Labeled Δ1-piperideine-2-carboxylic acid (3) was formed when U-14C-lysine was incubated with a homogenate from turkey liver (13). Enzyme preparations from a variety of rat tissues catalyze the reduction of Δ1-piperideine-2-carboxylic acid (3) to pипeolic acid in vitro. Activity from 1-14C-L-lysine enters pипeolic acid in a lysine-less mutant of Neurospora crassa (5).

Labeled pипeolic acid was formed in bean plants (Phaseolus vulgaris) which had been treated with U-14C-L-lysine (4), L-14C-L-lysine (8), 2-14C-lysine (7), or 6-14C-lysine (8), in wheat (9) and in excised phyllodes of Acacia homalophylla (10) which had been exposed to U-14C-L-lysine, in Leucaena glauca plants exposed to 2-14C-lysine (11), and in Mimosa pudica plants, grown in contact with 6-14C-lysine (12).

Even though distribution of label within the 14C-pипeolic acid has not been established by chemical degradation, and conclusive evidence for the specificity of incorporation of activity from lysine into the product is thus lacking, the accumulated tracer studies make it likely that the carbon skeleton of pипeolic acid is derived from the intact carbon chain of the precursor, and that the nitrogen atom of pипeolic acid represents either the α- or the ε-nitrogen of lysine.

Two pathways for the conversion of L-lysine into L-pипeolic acid are thus particularly plausible.

One of these (1 → 2 → 3 → 4) (Fig. 1) is a route via ε-amino-α-ketoacaproic acid (2) and Δ1-piperideine-2-carboxylic acid (3), which involves loss of the α-amino nitrogen of lysine and would lead to incorporation of the ε-nitrogen into pипeolic acid. The other (1 → 6 → 5 → 4) is a pathway through α-aminoadipic-δ-semialdehyde (6) and Δ1-piperideine-6-carboxylic acid (5), leading to loss of the ε-amino group of lysine and entry of the α-nitrogen into pипeolic acid.

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According to a recent paper (7) pипeolic acid is formed from lysine in higher plants by the alternative route 1 → 6 → 5 → 4.

Numerous tracer studies indicate that L-pипeolic acid (4) is a product of lysine (1) catabolism in animals, microorganisms, and plants.

In the intact rat label from 6-14C-L-lysine (1, 2) and from U-14C-L-lysine (3) is incorporated into pипeolic acid. Liver homogenates of guinea pig (4), rat (3), and turkey (3) have been shown to convert U-14C-L-lysine into pипeolic acid in vitro. Activity from 1-14C-L-lysine enters pипeolic acid in a lysine-less mutant of Neurospora crassa (5).

Labeled pипeolic acid was formed in bean plants (Phaseolus vulgaris) which had been treated with U-14C-L-lysine (4), L-14C-L-lysine (8), 2-14C-lysine (7), or 6-14C-lysine (8), in wheat (9) and in excised phyllodes of Acacia homalophylla (10) which had been exposed to U-14C-L-lysine, in Leucaena glauca plants exposed to 2-14C-lysine (11), and in Mimosa pudica plants, grown in contact with 6-14C-lysine (12).

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It is not established which of the two routes operates in N. crassa. The findings that enzyme preparations obtained from this source catalyze the conversion of L-lysine into Δ1-piperideine-2-carboxylic acid (16) as well as the reduction of the latter to pипeolic acid (14) are compatible with the pathway 1 → 2 → 3 → 4. Tracer experiments with 14N-lysine have not been carried out, however.

According to a recent paper (7) pипeolic acid is formed from lysine in higher plants by the alternative route 1 → 6 → 5 → 4.
It is reported (7) that in P. vulgaris 2-14C, ω-15N-lysine (ωN : 14C = 1.4 × 10^-7) was incorporated into pipecolic acid (ωN : 14C = 1 × 10^-7) with a loss of less than 30% 15N, relative to 14C, whereas incorporation of 2-14C, ω-15N-lysine (ωN : 14C = 1.4 × 10^-7) into pipecolic acid (ωN : 14C = 0.3 × 10^-7) was accompanied by a loss of approximately 80% 15N, relative to 14C. This result appears to confirm earlier kinetic data on the incorporation of activity from U-14C-lysine into pipecolic acid in A. homalophylla (10) which were interpreted to show that in this plant the pathway proceeded mainly by the steps 1 → 6 + 5 + 4, i.e. via Δ'-piperideine-6-carboxylic acid (5). Also consistent with this route is the conversion of lysine into Δ'-piperideine-6-carboxylic acid, catalyzed by an enzyme preparation from seedlings of Pisum sativum (17; cf. Reference 18). On the other hand, both Phaseolus radiatus and P. sativum contain enzymes which mediate the conversion of Δ'-piperideine-2-carboxylic acid (3) into pipecolic acid (14).

Various plants contain piperidine derivatives other than pipecolic acid. The piperidine nucleus of several of these, e.g. anabasine in Nicotiana glauca (19, 20) and sedamine in Sedum acre (21), is generated from a C6N chain which is derived from lysine by loss of the carboxyl group and of one of the nitrogens. 15N from the ω-amino group, but not from the α-amino group, of lysine is incorporated into the piperidine nucleus of anabasine (20). This finding is in sharp contrast to the report on the retention of 15N from α-15N lysine in the biosynthesis of pipecolic acid in P. vulgaris.

We have investigated the mode of incorporation of 6-3H,6-14C-lysine into pipecolic acid in the rat, in N. crassa, and in P. vulgaris and S. acre. Our results lead to the conclusion that the route via ω-amino-α-ketocaproic acid (2) and Δ'-piperideine-2-carboxylic acid (3) is operable in each case.

MATERIALS AND METHODS

Labeled Compounds

6-3H,6-14C-ω-L-Lysine

This intermolecularly doubly labeled lysine was a mixture of 6-14C-ω-L-lysine (nominal total activity 0.2 mCi, nominal specific activity 9.2 mCi per mmole, Commissariat à l’Energie Atomique, France) and 6-3H-ω-L-lysine (nominal total activity 2 mCi, nominal specific activity 5.5 C per mmole, New England Nuclear). The 3H-labeled compound was prepared from DL-2-amino-5-oxo-pentanoic acid by catalytic reduction with carrier-free tritium gas.2 Since the specific activity of the 6-3H-ω-L-lysine, obtained without dilution with unlabeled carrier, was only about one-tenth of that of carrier-free tritium gas, it must be inferred that extensive exchange (1H+1H → 1H+1H) with hydrogen, presumably from the amino group of the cyanoaminopentanoic acid or from the solvent, had taken place in the course of the catalytic reduction, and that the labeled product was a mixture of singly and doubly tritiated molecules, i.e. of 6-3H,6-14C-ω-L-lysine and 6-3H,6-14C-ω-L-lysine. The distribution of tritium within this mixture has been established by chemical degradation (22). Only 75% of the tritium was found at C-6. The remaining 25% of label was located at C-5 or C-4, or both. None of the tritium resides at C-2. The absence of tritium from C-2 is crucial for the interpretation of the present results.

The radiochemical purity of each of the two labeled lysine samples was established by paper chromatography (1-butanol-acetic acid-water, 2:1:1) and radiochromatography (model 7201, Radiochromatogram scanner, Packard). Each of the samples showed a single radioactive peak (Rf 0.29).

The two labeled samples of lysine were dissolved in distilled water, the solutions were mixed, and the total volume was adjusted to 25 ml. The 3H:14C ratio of this stock solution of "doubly labeled" lysine, determined by liquid scintillation counting, was found to be 15.8 ± 0.3. This stock solution was used in Experiments 1 to 6 (see below).

4,5-3H2,6-14C-ω-L-Lysine

This intermolecularly doubly labeled lysine was a mixture of 6-14C-ω-L-lysine (nominal total activity 0.1 mCi, nominal specific activity 9.2 mCi per mmole, Commissariat à l’Energie Atomique, France) and of 4,5-3H2-ω-L-lysine (nominal total activity 1 mCi, nominal specific activity 3.0 C per mmole, New England Nuclear). The tritiated lysine, prepared by catalytic tritiation of ethyl 2-acetamido-2-carboxethoxy-6-aminohex-4-enoate,3 contained all tritium at C-4 and C-5 (23). Radiochemical purity of each of the two samples was confirmed by radiochromatography, as described above. The two samples were dissolved in glass-distilled water, the solutions were mixed, and the total volume was adjusted to 10 ml. The 3H:14C ratio of this stock solution of doubly labeled lysine was found to be 9.4 ± 0.3. It was used in Experiment 7 (see below).

FIG. 1. Two possible pathways from lysine to pipecolic acid

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Pipecolic Acid from N. crassa (Experiments 2 and 3) (cf. Reference 2)

DL-Pipecolic acid (750 mg) was dissolved in 4 ml of the stock solution of the doubly labeled lysine. Two male rats, each weighing approximately 250 g, were fasted for 24 hours. Each was then injected intraperitoneally with 2 ml of the solution containing pipecolic acid and labeled lysine. Urine was collected for 24 hours. The urine was applied to a column (25 x 4 cm) of Dowex 50 X4 (H+ form). The column was washed with water and eluted with ammonium. Eluate was collected until no further radioactivity emerged. The eluate was evaporated to dryness and the residue was dissolved in water and applied to a column of Dowex 50 X4 (H+ form). The column was washed with water until the eluate was neutral. Pipecolic acid (250 mg) was isolated as described above.

Experiment 3—The mycelium was filtered off and washed with methanol. The washings were added to the medium and the solution evaporated to dryness. Pipecolic acid (150 mg) was isolated from the residue as in Experiment 2.

Partial Degradation of Pipecolic Acid (Experiment 2) (cf. Fig. 2)

The pipecolic acid (100 mg) was dissolved in water (2 ml) and silver oxide (250 mg) was added. The mixture was refluxed for 4 hours and filtered. Concentrated sulfuric acid (1 ml) and chromic acid (100 mg) were added to the filtrate and the mixture was heated on the steam bath for 4 hours and was then diluted with water (50 ml). The solution was neutralized by the addition of saturated aqueous barium hydroxide. The mixture was filtered and the precipitate of barium sulfate was washed with water. The combined filtrate and washings were concentrated and applied to a small column of Dowex 50 X4. The column was washed with water and eluted with ammonia, and the eluate was concentrated.

Thin layer chromatography of the residue on silica gel showed β-alanine and γ-aminobutyric acid to be the major components and glyoxyl to be a minor component. No attempt was made to separate this mixture.

Pipecolic Acid from Bean Plants (P. vulgaris) (Experiments 4 and 5)

The tracer solution was administered to 4-week-old bean plants by infusion into the stems through cotton wicks (26). Two experiments were carried out. In each of these 20 intact plants were used. In one experiment 4 ml of stock solution of the doubly labeled lysine, diluted to 10 ml with glass-distilled water, was administered to the plants (Experiment 4). In the other experiment the plants were allowed to absorb 4 ml of stock solution of doubly labeled lysine, diluted to 10 ml, in which DL-pipecolic acid (350 mg) had been dissolved (Experiment 5). The plants were left to grow in contact with the tracer for 2 days, and pipecolic acid was then isolated.

Experiment 4—The aerial parts of the plants were dried at 45-50° for 24 hours and were then ground. The coarse powder (10 g) was extracted with water for 6 hours in a Soxhlet extractor. The extract was evaporated, carrier DL-pipecolic acid (100 mg) was added to the residue, and the mixture was extracted with warm methanol. The methanol extract was evaporated and the residue, dissolved in a little water, applied to a column of Dowex 50 X4 (H+ form). The column was washed with water and pipecolic acid (20 mg) was then obtained as described above.
Experiment 6—The tops and washed roots of the plants were homogenized and the homogenate was transferred to a column and percolated with water (3 liters). The eluate was evaporated and the residue was extracted with methanol. The methanol extract was evaporated and picpeolic acid (30 mg) was isolated from the residue as in Experiment 4.

Sedamine and Picpeolic Acid from S. acre (Experiment 6) (cf. Reference 21).

Excised shoots of S. acre, 2 to 3 inches in length, were cut and packed, with cut surfaces downward, into seven 100-ml beakers, each beaker containing 10 to 12 g of fresh plant material. Stock solution of the doubly labeled lysine (4 ml, diluted to a volume of 10 ml) was evenly distributed among the beakers. Distilled water (1 ml) was then added to each beaker, and again after 24 hours, when most of the original solution had been absorbed. The experiment was continued for a further 24 hours. After 48 hours in contact with tracer solution, the Sedum shoots were homogenized and the pH was adjusted to 11 by the addition of aqueous ammonia. The homogenate was transferred to a glass column (24 x 3 inches) and percolated in turn with ether, with ether containing carrier sedamine (250 mg), and again with ether until 1 liter of eluate had been collected. The ether layer of the eluate was separated from the aqueous layer and washed with water. Sedamine was isolated from the ether layer (see below). The column was further percolated with 1 liter of water containing carrier ml-pipcacheolic acid (100 mg). The combined aqueous eluates and washings were evaporated to dryness. The residue was extracted with methanol, the methanol extract was evaporated, and the residue, dissolved in water, applied to a column of Dowex 50 X4 (H+ form), which was washed with water. Picpeolic acid (25 mg) was then isolated as described above.

The ether eluate (see above) was extracted with hydrochloric acid (5%, 4 x 20 ml). The acid extract was washed with ether until colorless and was basified by the addition of ammonium hydroxide and extracted with petroleum ether (b.p. 40-60°). The nonaqueous extract was dried (NaSO4) and evaporated to dryness. The residue was mixed with carrier sedamine and dissolved in hexane. On concentration sedamine (130 mg), m.p. 80-81°, crystalized. It was sublimed under reduced pressure and resublimed to constant activity.

Experiment with 4,5-3H2,6-14C-oL-Lysine (Experiment 7)

Sedamine from S. acre (cf. Reference 21).

Stock solution of 4,5-3H2,6-14C-oL-Lysine (5 ml, diluted to a volume of 10 ml) was administered to excised shoots of S. acre, and sedamine (33 mg) isolated from the plants, after carrier dilution with cold sedamine (130 mg), in the manner outlined above.

Determination of Radioactivity

The radioactivity of all samples was determined by liquid scintillation counting (Mark 1, liquid scintillation computer, model 6860, Nuclear-Chicago). Activity due to 3H and 14C was determined simultaneously, by external standardization counting, with 133Ba. Samples, dissolved in water (lysine, picpeolic acid), were dispersed with the aid of methanol in a solution of Liquifluor (Nuclear-Chicago) diluted 25 times with toluene. Sedamine was dissolved in the diluted Liquifluor solution. Duplicate samples of each compound were counted under comparable conditions of quenching. Confidence limits shown in the results are the standard deviation of the mean. For highly quenched samples the confidence limits of the quench correction curves were ±5%.

TABLE I

Incorporation of 6-3H,6-14C-oL-Lysine into picpeolic acid

<table>
<thead>
<tr>
<th>Product</th>
<th>3H:14C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>14.5 ± 0.1</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>15.2 ± 0.1</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>17.5 ± 0.2</td>
</tr>
</tbody>
</table>

a Lysine administered in absence of unlabeled picpeolic acid. * Lysine administered in presence of unlabeled picpeolic acid.

TABLE II

Incorporation of labeled lysines into sedamine and picpeolic acid in Sedum acre

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Precursor</th>
<th>3H:14C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Picpeolic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6-3H,6-14C-oL-Lysine</td>
<td>15.6 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>4,5-3H2,6-14C-oL-Lysine</td>
<td>9.4 ± 0.3</td>
</tr>
</tbody>
</table>

comparable conditions of quenching. Confidence limits shown in the results are the standard deviation of the mean. For highly quenched samples the confidence limits of the quench correction curves were ±5%.

RESULTS

Each of the samples of picpeolic acid, isolated from Experiments 1 to 6, and of sedamine, obtained from Experiments 6 and 7, contained tritium and 14C. The 3H:14C ratio of these products is given in Tables I and II, which also show the 3H:14C ratio of the administered lysines.

Since carrier dilution was used in the isolation of the products from Experiments 2, 4, 6, and 7, and unlabeled picpeolic acid along with labeled substrate was administered in Experiments 1, 3, and 5, 3H:14C ratios, rather than specific activities, serve as a measure of conversion of precursor into products. Furthermore, 3H:14C ratios provide the only reliable basis for a comparison of results obtained in the experiments with rats, Neurospora, and higher plants.

DISCUSSION

In the course of its metabolic conversion into picpeolic acid (4), the intact carbon chain of lysine (1) is maintained whichever of the two pathways, via 2 and 3 or via 6 and 5, is operative. Experiments with 14C-lysine cannot therefore serve to distinguish between them. Two other isotopes, 15N and 3H, are suitable for diagnostic tracer experiments.

Incorporation into picpeolic acid of 15N, either exclusively from α-15N-lysine or exclusively from ε-15N-lysine, would provide evidence for one or the other of the two routes. If incorporation from both were observed, an unequivocal interpretation would not be possible, since such a result might be due to nonspecific
transamination or due to the operation of both routes simultaneously. It was inferred, from an experiment of this type, that in the intact rat pipecolic acid arises from lysine via α-amino-α-ketocaproic acid (2) (2, 15).

The pathway via α-amino-α-ketocaproic acid requires loss of the proton from C-2 of lysine. The route via α-aminoacidip-δ-semialdehyde (6), on the other hand, demands removal of 1 of the protons from C-6. In principle, the mechanism of pipecolic acid biosynthesis may thus be studied with the aid of lysine, suitably labeled with tritium.

For several practical reasons the additional presence of a 14C reference marker is desirable in such a study. First, since conversion of precursor into a single metabolic product is rarely quantitative in experiments with intact systems, an internal standard is required for a meaningful evaluation of the tritium content of the product. Retention or loss of tritium is readily apparent from a comparison of 3H:14C ratios of precursor and product, but not from a comparison of their specific activities with respect to 3H. Second, if the product occurs in quantities too small for direct isolation, “metabolic overloading” (2, 27) or carrier dilution techniques must be used. The 3H:14C ratio of the product is unaffected by these manipulations. Third, if the 3H:14C ratios of precursor and product are identical, tedious and often difficult chemical degradation to locate the sites of activity within the product may be unnecessary.

6-3H-DL-Lysine, mixed with 6-14C-DL-lysine as a reference standard, was to be used in this investigation of pipecolic acid biosynthesis. If conversion of lysine into pipecolic acid occurred via α-amino-α-ketocaproic acid (2), the 3H:14C ratios of precursor and product should be identical. Conversion by way of α-aminoacidip-δ-semialdehyde (6), on the other hand, should lead to a product whose 3H:14C ratio should be half that of the precursor if ε-transamination or ε-deamination occurred without isotope effect. If, in this conversion, C—H bond cleavage were a rate-limiting step and therefore accompanied by a primary isotopic isotope effect, the value of the 3H:14C ratio of the product would depend on the magnitude of this isotope effect, on the relative abundance, within the precursor, of the three labeled species—6,6-3H,6-14C-lysine, 6-14C,6-3H-lysine, and 6,6-14H,6-3H-lysine—and on the extent of reaction. If only a fraction of precursor is converted into product in the course of the experiment, the 3H:14C ratio of product will, in every case, be one half that of the precursor, or lower. If the labeled starting material does not contain the species 6,6-3H,6-14C-lysine, and then only in the unlikely eventuality that the reaction is accompanied by a primary hydrogen-tritium isotope effect of infinite magnitude and conversion of precursor into product is quantitative, the 3H:14C ratio of lysine would be preserved in the pipecolic acid, even if this conversion took place via α-aminoacidip-δ-semialdehyde.

Finally, a product might be obtained with a 3H:14C ratio higher than that of the precursor. Interpretations of such a result are discussed below.

The precision of the experimental approach was tested in the intact rat under experimental conditions which had earlier (2, 15) led to incorporation into pipecolic acid of 15N from ε-15N, but not from α-15N-lysine. The pipecolic acid which was isolated from rat urine, after intraperitoneal injection of a mixture of 6-14H,6-14C-DL-lysine (3H:14C = 15.6 ± 0.3) and unlabeled pipecolic acid (Experiment 1), showed a 3H:14C ratio (14.5 ± 0.1), which was, as expected, essentially identical with that of the precursor. Comparison of these two ratios provides a measure of the limits of accuracy of the method.

Pipecolic acid, obtained from a lysine-less mutant of N. crassa, grown in the presence of the doubly labeled lysine and unlabeled pipecolic acid (Experiment 2), had a 3H:14C ratio (15.3 ± 0.1), identical with that of the precursor. Conversion in N. crassa, as in the rat, thus takes place by way of α-amino-α-ketocaproic acid.

When N. crassa was grown with doubly labeled lysine in the absence of unlabeled pipecolic acid (Experiment 2) labeled pipecolic acid was obtained with a 3H:14C ratio which was 25.4 ± 0.7. This ratio indicates either gain of 3H relative to 14C or loss of 14C relative to 3H in the course of conversion of lysine into pipecolic acid.

Gain of tritium, relative to 14C, in Experiment 2, could be the consequence of nonspecific exchange or of a specific metabolic reduction step. Loss of 14C relative to 3H could be due to an isotope effect, but cannot result from a normal metabolic reaction because 14C and 3H are present at the same carbon site of the labeled precursor.

Nonspecific tritium exchange cannot be the explanation of the phenomenon since there is no reason why it should take place in the absence (Experiment 2) but not in the presence (Experiment 3) of unlabeled pipecolic acid.

A rational model can be constructed for the metabolic introduction of additional tritium into pipecolic acid and its suppression by unlabeled pipecolic acid. If pipecolic acid did not accumulate but were rapidly and irreversibly (cf. References 2, 9, and 15) degraded to α-aminoacidip-δ-semialdehyde (7) (cf. References 28 to 30) via Δ1-piperideine-6-carboxylic acid (5) and α-aminoacidip-δ-semialdehyde (6) (31), tritium from C-6 of the doubly labeled pipecolic acid would be transferred to an acceptor. If this tritiated acceptor then served as the proton donor in the reductive step (3 → 4) of the biosynthetic route, pipecolic acid would subsequently be formed from the doubly labeled lysine which would carry tritium not only at C-6 but also at C-2. This transfer of tritium would predominate only when the endogenous pool of pipecolic acid is small. In the presence of excess unlabeled pipecolic acid within the tissue, proton rather than tritium would be transferred.

This model demands that the additional tritium present in the pipecolic acid (3H:14C = 25.4 ± 0.7) should be confined to C-2 of the product. To test this prediction a partial degradation of pipecolic acid was devised. The pipecolic acid from Experiment 2 was oxidized to a mixture of γ-aminobutyric acid, β-alanine, and glycine (Fig. 2). The 3H:14C ratio of this mixture (24.3 ± 0.4) was found to be identical, within experimental error, with that of the original pipecolic acid, indicating that none of its tritium had been located at C-2. The high 3H:14C ratio of the pipecolic acid from Experiment 2 was therefore not due to a specific metabolic reduction step.

It is likely that a hydrogen-tritium isotope effect is responsible for this high 3H:14C ratio. It would appear that C—H cleavage in the degradation of pipecolic acid to Δ1-piperideine-6-carboxylic acid (5) is rate-limiting. As a consequence, the rate of degradation of 6-14H,6-14C-pipecolic acid exceeds that of 6-3H,6-14C-pipecolic acid. It can be assumed that a 14C:13C isotope effect of the reaction is insignificant in comparison to a hydrogen-tritium isotope effect. The doubly labeled pipecolic acid, generated from the intermolecularly doubly labeled 6-14H,6-14C lysine,
is a mixture of the $^3$H-$^14$C and the $^6$H-$^14$C species. The normal steady state concentration of piceclic acid in the tissue is low. In the absence of added endogenous unlabeled piceclic acid (Experiment 2) a significant fraction of the newly synthesized labeled piceclic acid undergoes further degradation. It is the $^6$H-$^14$C species which is degraded preferentially. The remaining piceclic acid which is subsequently isolated is then enriched in $^1$H, relative to $^14$C. Such an enrichment is not observed when excess unlabeled piceclic acid is introduced into the tissue (Experiment 3), since degradation of the newly synthesized labeled material, which now constitutes a quantitatively insignificant fraction of the available piceclic acid, is largely suppressed.

The results of Experiments 2 and 3 show that in Neurospora, as in the rat, conversion of lysine into piceclic acid proceeds by way of $\epsilon$-amino-$\alpha$-ketocaproic acid (2). The alternative pathway is inconsistent with maintenance or increase in the $^3$H-$^14$C ratio.

The results of the experiments with P. vulgaris lead to a similar conclusion. Piceclic acid, isolated after administration of doubly labeled lysine alone (Experiment 4), showed a $^3$H-$^14$C ratio of 22.0 ± 0.1, significantly in excess of that of the precursor. When unlabeled piceclic acid was administered along with the tracer (Experiment 5), the $^3$H-$^14$C ratio of the product dropped to 17.5 ± 0.2, somewhat larger than that of the precursor. Evidently, the amount of unlabeled piceclic acid which had been fed was insufficient to lead to complete suppression of the degradation of newly synthesized material and of the resulting hydrogen-tritium isotope effect. Even so, the results parallel those obtained in the experiments with Neurospora. They are consistent with a pathway from lysine to piceclic acid via $\epsilon$-amino-$\alpha$-ketocaproic acid, but inconsistent with a route via $\alpha$-aminoadipic-$\delta$-semialdehyde.

Our results are at variance with those of Schütte and Seelig (7) (see above), who reported incorporation of $^{15}$N from $\alpha$-$^{15}$N-lysine, but not from $\epsilon$-$^{15}$N-lysine into piceclic acid in P. vulgaris. It is likely that the $^{15}$N-enrichment found by these authors in piceclic acid (0.06 and 0.04%, respectively) was a measure of the $^{15}$N-enrichment in the nitrogen pool of the experimental plants, rather than an index of the direct conversion of lysine into the product. It may be significant that the $^{15}$N-enrichment in glutamic acid isolated from the two experiments (0.11 and 0.04%, respectively) exceeded enrichment in piceclic acid in one case and equaled it in the other. Pitfalls in the interpretation of results of tracer experiments with $^{15}$N in intact plants have been discussed (32).

Conversion of doubly labeled lysine into piceclic acid was investigated in another plant species, S. acre. The $^3$H-$^14$C ratio of the product was identical with that of the precursor (Experiment 6, Table II), a result which is again consistent with a pathway through $\epsilon$-amino-$\alpha$-ketocaproic acid. It is noteworthy that the ratio was maintained, even though tracer was administered in the absence of cold piceclic acid. It may thus be inferred that, under the conditions of this experiment, piceclic acid is not further degraded in excised shoots of S. acre.

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