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BIOSYNTHESIS OF THE PIPERIDINE NUCLEUS: THE OCCURRENCE OF TWO PATHWAYS FROM LYSINE

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Abstract—2-^{3}H,6-^{14}C-DL-Lysine was administered to the intact rat, to intact bean plants (*Phaseolus vulgaris*) and to excised shoots of *Sedum acre*. Pipecolic acid (IV), which was isolated from each of these tissues, contained ^{14}C but was free of tritium. Since incorporation of label from 6-^{3}H,6-^{14}C-DL-lysine into pipecolic acid had previously been shown[1] to take place specifically and to occur without change in ^{3}H:^{14}C ratio in each of these systems, the evidence is now complete that conversion of lysine (I) into pipecolic acid (IV) proceeds via ε-amino-α-ketocaproic acid (II) and Δ^{1}-piperideine-2-carboxylic acid (III) and not via α-aminoadipic-δ-semialdehyde (VI) and Δ^{1}-piperideine-6-carboxylic acid (V). In *S. acre*, 2-^{3}H,6-^{14}C-DL-lysine was incorporated into sedamine (X) without change in ^{3}H:^{14}C ratio. It follows that ε-amino-α-ketocaproic acid (II) cannot be an intermediate in the biosynthesis of sedamine from lysine, as had been previously suggested. Specific conversion of 6-^{3}H,6-^{14}C-DL-lysine into sedamine without change in ^{3}H:^{14}C ratio had been demonstrated earlier. This and other evidence had been interpreted to show that α-aminoadipic-δ-semialdehyde (VI) is not an intermediate on the route from lysine to sedamine and other piperidine alkaloids. An alternative pathway, which invokes neither ε-amino-α-ketocaproic acid nor α-aminoadipic-δ-semialdehyde as intermediate, is now suggested.

INTRODUCTION

IN A RECENT study[1] of the mode of incorporation of lysine into pipecolic acid it was shown that when 6-^{3}H,6-^{14}C-DL-lysine served as the precursor of pipecolic acid (IV) in the rat, in *Neurospora crassa*, and in two higher plant species, the ^{3}H:^{14}C ratio of the starting material was maintained. From these and earlier observations it was inferred that the nitrogen atom of pipecolic acid is supplied by the ε-amino group of lysine and that the conversion of lysine into pipecolic acid proceeds via ε-amino-α-ketocaproic acid (II) in each of these tissues.

Since the labelled lysine used as the tracer in our earlier study of pipecolic acid biosynthesis was an intermolecularly doubly labelled sample of 6-^{3}H,6-^{14}C-lysine, containing the two species, 6,6-^{1}H$_{2}$,6-^{14}C-lysine and 6-^{1}H,6-^{3}H,6-^{12}C-lysine, and possibly also a third species, 6,6-^{3}H$_{2}$,6-^{12}C-lysine, an alternative interpretation of the experimental data, though regarded as improbable, could not be conclusively discounted. The possibility remained that the observed similarity of the ^{3}H:^{14}C ratios of precursor and product was not the result of continuous accumulation of pipecolic acid formed from the doubly labelled lysine via ε-amino-α-ketocaproic acid (II), as inferred, but that it was the coincidental consequence of two primary hydrogen–tritium isotope effects acting in opposing directions: an isotope effect in the synthesis of product via α-aminoadipic-δ-semialdehyde (VI), yielding pipecolic acid with a ^{3}H:^{14}C ratio lower than that of the precursor, balanced exactly by an isotope effect in the partial degradation of this newly formed pipecolic acid to Δ^{1}-piperideine-6-carboxylic acid (V) (cf. Ref. 1, footnote 4). The experiments now described eliminate this remaining uncertainty in the interpretation of the earlier experimental data.

[1] R. N. GUPTA and I. D. SPENSER, *J. Biol. Chem.* **244**, 88 (1969).

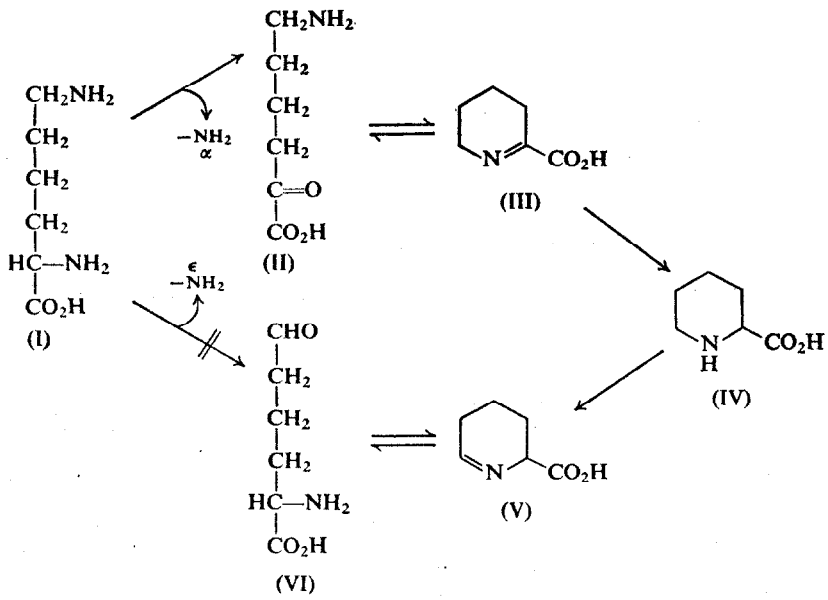

FIG. 1. TWO POSSIBLE PATHWAYS FROM LYSINE TO PIPECOLIC ACID.

The piperidine nucleus of a number of alkaloids is generated from lysine.[1-6] Since several lines of evidence[1,3-6] lead to the inference that α-aminoadipic-δ-semialdehyde cannot be an intermediate in this pathway, it has been assumed, without direct evidence, but by analogy with the origin of pipecolic acid, that ε-amino-α-ketocaproic acid lies on the route of biosynthesis of these alkaloids. This hypothesis has now been tested experimentally. On the basis of the results obtained, the view that ε-amino-α-ketocaproic acid is an intermediate in the biosynthesis of one of these alkaloids, sedamine, is no longer tenable.

RESULTS

The samples of pipecolic acid obtained from rat urine, from bean plants, and from *Sedum acre* contained radiocarbon but little, if any, tritium. The sample of sedamine, obtained from the experiment with *S. acre*, contained ^{3}H as well as ^{14}C. The ^{3}H:^{14}C ratios of the isolated products and of the administered lysine are shown in Table 1.

TABLE 1. INCORPORATION OF 2-^{3}H,6-^{14}C-DL-LYSINE INTO PIPECOLIC ACID AND SEDAMINE

	^{3}H:^{14}C ratio
Precursor: 2-^{3}H,6-^{14}C-DL-lysine	13·2 ± 0·4
Products:	
Pipecolic acid isolated from experiment with:	
Rat	0·10 ± 0·005
Phaseolus vulgaris	0·67 ± 0·06
Sedum acre	0·58 ± 0·01
Sedamine from *Sedum acre*	12·6 ± 0·1

[2] E. LEETE, *J. Am. Chem. Soc.* **78**, 3520 (1956).
[3] E. LEETE, E. G. GROS and T. J. GILBERTSON, *J. Am. Chem. Soc.* **86**, 3907 (1964).
[4] E. LEETE, *J. Am. Chem. Soc.* **91**, 1697 (1969).
[5] R. N. GUPTA and I. D. SPENSER, *Can. J. Chem.* **45**, 1275 (1967).
[6] R. N. GUPTA and I. D. SPENSER, *Phytochem.* **8**, 1937 (1969).

DISCUSSION

The doubly labelled tracer used in this investigation was an intermolecular mixture of 2-^{3}H-DL-lysine and 6-^{14}C-DL-lysine. If conversion of this 2-^{3}H,6-^{14}C-lysine into pipecolic acid (IV) occurred via α-aminoadipic-δ-semialdehyde (VI), the ^{3}H:^{14}C ratios of starting material and endproduct should be identical. Conversion by way of ε-amino-α-ketocaproic acid (II), on the other hand, should lead to a product labelled with ^{14}C but free of ^{3}H, regardless of whether or not loss of the α-amino group, by deamination or transamination, is accompanied by a hydrogen–tritium isotope effect.

Isolation of ^{14}C-labelled pipecolic acid free of tritium, from the tracer experiments with 2-^{3}H,6-^{14}C-lysine, would confirm our earlier conclusion that the conversion of lysine into pipecolic acid takes place via ε-amino-α-ketocaproic acid, and would conclusively invalidate alternative interpretations of the earlier results with 6-^{3}H,6-^{14}C-lysine.[1] Isolation of doubly labelled pipecolic acid, whose ^{3}H:^{14}C ratio corresponds to that of the administered 2-^{3}H,6-^{14}C-lysine, would, on the other hand, force us to revise the interpretation of the results of our earlier experiments.

It is evident from the data presented in Table 1 that a revision of our earlier conclusions is unnecessary. As predicted, the pipecolic acid isolated from rat urine, from bean plants, and from *Sedum acre*, after administration of 2-^{3}H,6-^{14}C-lysine (^{3}H:^{14}C ratio 13·2 ± 0·4), had lost essentially all tritium, relative to ^{14}C. It follows that in each of these tissues pipecolic acid is indeed derived from lysine by way of ε-amino-α-ketocaproic acid and Δ^1-piperideine-2-carboxylic acid. Conversion of D- and L-lysine into L-pipecolic acid by this route in the rat has recently been independently confirmed.[7]

Lysine has been implicated also in the biosynthesis of several naturally occurring piperidine derivatives other than pipecolic acid (anabasine (IX),[2,3] sedamine (X)[1,5] and *N*-methylpelletierine (VIII)).[6] The evidence is strong that the piperidine nucleus of each of these compounds originates from a C_5N unit derived from lysine: Label from 6-^{14}C-lysine has been shown to enter C-6 of the piperidine nucleus of sedamine[5] and of *N*-methylpelletierine,[6] label from 2-^{14}C-lysine enters C-2 of that of anabasine[2,3] and of sedamine.[5] Samples of sedamine[1] and of *N*-methylpelletierine,[6] obtained from feeding experiments with 4,5-$^3H_2$,6-^{14}C-lysine and with 6-^{3}H,6-^{14}C-lysine, each showed a ^{3}H:^{14}C ratio identical with that of the doubly labelled precursor. Furthermore, ε-^{15}N,2-^{14}C-lysine entered the piperidine ring of anabasine without change in ^{15}N:^{14}C ratio, whereas α-^{15}N,2-^{14}C-lysine gave anabasine which contained ^{14}C at the predicted site but was free of ^{15}N.[3]

It is evident from these results that lysine is incorporated into the piperidine nucleus in a non-symmetrical manner, and that a symmetrical compound, such as cadaverine (1,5-diaminopentane), is excluded as an intermediate.[4] The required C_5N unit, related to lysine, is present in Δ^1-piperideine (VII), a compound derivable from lysine by loss of the carboxyl group and one of the amino groups.[7] Indeed, activity from 6-^{14}C-Δ^1-piperideine is specifically incorporated into C-6 of the piperidine nucleus of anabasine (IX).[4] The position of the double bond of Δ^1-piperideine is fixed.[4,8] If this compound (or its *N*-methyl derivative) were the lysine-derived moiety which combines with the side-chain precursor in the final biosynthetic step of the pathways leading to anabasine, sedamine and *N*-methylpelletierine, as seems likely, attachment of the side-chain must, on mechanistic grounds, occur at C-2, the electrophilic center.[6] Δ^1-Piperideine is derivable by decarboxylation of either Δ^1-piperideine-2-

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[8] R. N. GUPTA and I. D. SPENSER, *Can. J. Chem.* **47**, 445 (1969).

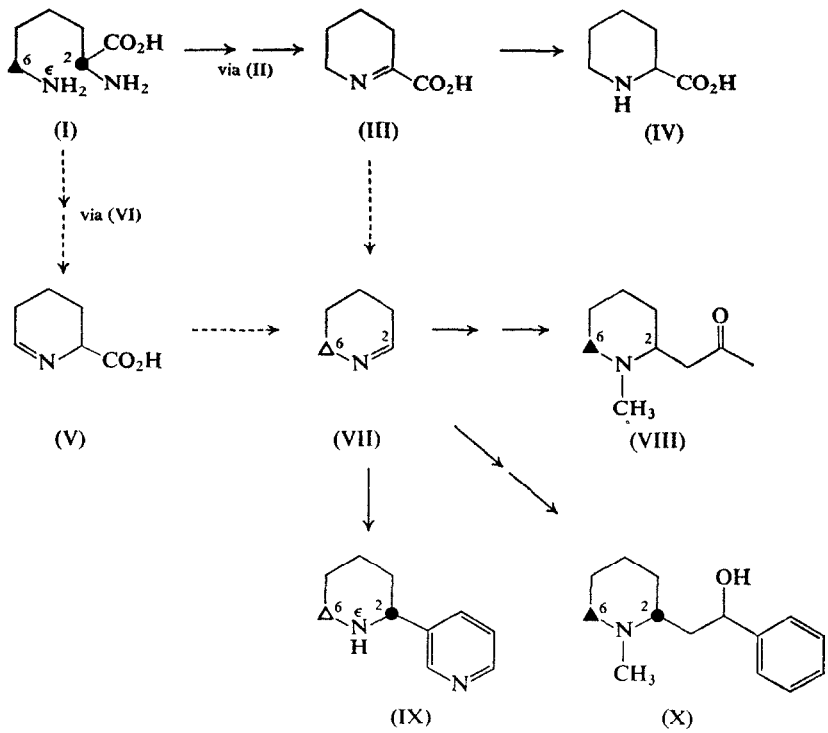

FIG. 2. INCORPORATION OF ^{14}C-LYSINE INTO PIPERIDINE BASES, AND PROPOSED INTERMEDIATES.

carboxylic acid (III) or Δ^1-piperideine-6-carboxylic acid (V). The pattern of incorporation of label from 2-^{14}C- and 6-^{14}C-lysine into the alkaloids is consistent with the intermediacy of Δ^1-piperideine-2-carboxylic acid (III), but inconsistent with intermediacy of the isomeric acid (V).[6] This inference is reinforced by the finding that the piperidine nitrogen of anabasine is derived solely from the ϵ-amino group of lysine,[3] and by the observation that the ^{3}H:^{14}C ratio of 6-^{3}H,6-^{14}C-lysine is retained in sedamine (VIII)[1] and N-methylpelletierine (IX).[6]

Retention of this ratio implies that α-aminoadipic-δ-semialdehyde (VI) and Δ^1-piperideine-6-carboxylic acid (V) can be intermediates in the conversion of 6-^{3}H,6-^{14}C- lysine to the piperidine bases only if the labelled starting material did not contain the species 6,6-$^3H_2$,6-^{12}C-lysine, and then only in the unlikely eventuality that conversion were quantitative and that ϵ-deamination of lysine, yielding the intermediate, were accompanied by a primary hydrogen–tritium isotope effect of infinite magnitude.[1] On the basis of these observations, which parallel some of the findings on the incorporation of labelled lysine into pipecolic acid,[1] and which rule out Δ^1-piperideine-6-carboxylic acid (V) as well as α-aminoadipic-δ-semialdehyde (VI) as intermediates, and by analogy with the route of lysine into pipecolic acid, it was inferred,[1,3–6] without direct experimental evidence, that the pathway of lysine into the piperidine nucleus of the piperidine bases leads through ϵ-amino-α-ketocaproic acid (II) and Δ^1-piperideine-2-carboxylic acid (III). Other possibilities had not been considered.

The present experiment on the incorporation of 2-^{3}H,6-^{14}C-lysine into sedamine in *S. acre* serves as a critical test for the involvement of ϵ-amino-α-ketocaproic acid and Δ^1-piperideine-2-carboxylic acid in the biosynthesis of this alkaloid. It was found that, whereas the pipecolic acid, isolated from *S. acre* which had been kept in contact with 2-^{3}H,6-^{14}C-lysine,

contained ^{14}C but was free of tritium, sedamine obtained from the same plants maintained, within experimental error, the $^{3}H:^{14}C$ ratio of the precursor. The assumption that pipecolic acid and sedamine originate from a common intermediate, Δ^1-piperideine-2-carboxylic acid (III), must now be abandoned.

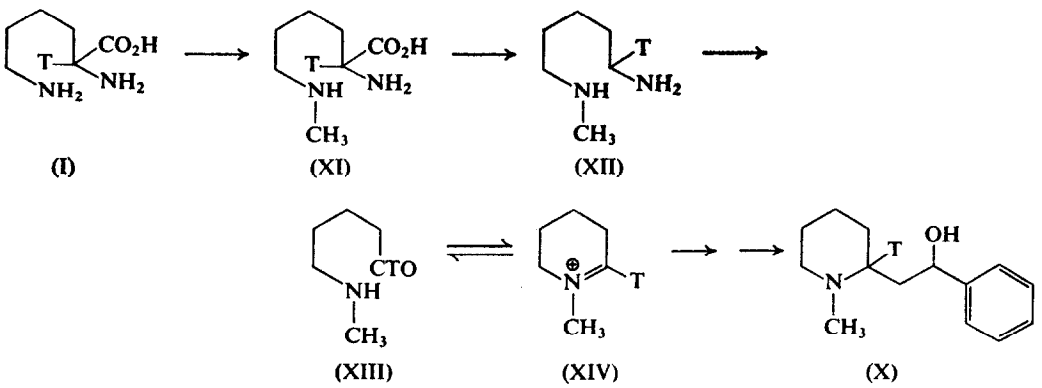

FIG. 3. A POSTULATED PATHWAY OF SEDAMINE BIOSYNTHESIS.

A pathway from lysine to the piperidine nucleus of sedamine, consistent with the experimental data, is shown in Fig. 3. It involves protection of the ϵ-amino group of L-lysine (e.g. by a methyl or acetyl substituent), followed by decarboxylation to yield a mono-*N*-substituted, and therefore non-symmetrical, derivative of cadaverine (XII). Removal of the primary amino group, i.e. of the α-amino group of the original lysine, then leads to an aminoaldehyde (XIII) whose cyclic derivative, an *N*-substituted Δ^1-piperideinium ion (XIV), serves as the immediate precursor of the piperidine nucleus of the piperidine bases. The experimental data demand that only a small amount of tritium is lost during removal of the primary amino group of the mono-*N*-substituted cadaverine derivative (XII $\rightarrow$ XIII), when this intermediate is derived from 2-^{3}H-lysine. This retention of tritium may be due to a large primary hydrogen–tritium isotope effect, or due to stereospecificity in the deamination reaction. Experimental evidence in support of this hypothesis is being sought.

EXPERIMENTAL

Labelled Compounds

2-^{3}H,6-^{14}C-DL-*lysine*. This intermolecularly doubly labelled lysine was a mixture of 6-^{14}C-DL-lysine (nominal total act. 0·3 mc, nominal spec. act. 48 mc/mmole, Commissariat á l'Energie Atomique, France) and of 2-^{3}H-DL-lysine (nominal total act. 3·9 mc, nominal spec. act. 15 mc/mmole, Commissariat á l'Energie Atomique, France). The ^{3}H-labelled compound was prepared by acid hydrolysis in tritiated water of ethyl 2-cyano-2-acetamido-6-phthalimidohexanoate,[9] followed by removal of all exchangeable tritium.[10] The distribution of tritium within this material was established by chemical degradation. A small sample of the tritiated lysine was mixed with carrier and then purified to constant activity. Oxidation[11] yielded δ-aminovaleric acid which contained negligible activity. The tritium within the original lysine is thus confined to carbon-2.

Glass-distilled water (5 ml) was added to each of the two labelled samples of lysine, the solutions were mixed and the total volume was adjusted to 20 ml. The $^{3}H:^{14}C$ ratio of this solution of "doubly labelled" lysine, determined by liquid scintillation counting, was found to be $13{\cdot}2 \pm 0{\cdot}4$. Portions of this stock solution (5 ml each) were used in each of the three tracer experiments, with intact rats, with intact bean plants, and with cuttings of *Sedum acre*.

[9] M. FIELDS, D. E. WALZ and S. ROTHCHILD, *J. Am. Chem. Soc.* **73**, 1000 (1951).
[10] L. PICHAT, personal communication, Commissariat á l'Energie Atomique, France.
[11] M. STRASSMAN and S. WEINHOUSE, *J. Am. Chem. Soc.* **75**, 1680 (1953).

Experiments with 2-^3H,6-^{14}C-DL-Lysine

Pipecolic acid from rat urine. DL-Pipecolic acid (750 mg) was dissolved in 5 ml stock solution of the doubly labelled lysine; the resulting solution was divided into two portions, and administered by intraperitoneal injection into two male rats (*ca.* wt. 180 g) which had been starved for 24 hr. Urine was collected for 24 hr. Pipecolic acid (spec. act. (^{14}C): $(5{\cdot}16 \pm 0{\cdot}06) \times 10^6$ dis/min/mmole) was isolated and purified by the published method (Ref. 1, Experiment 1). This method avoids procedures which might lead to isotope fractionation.[12]

Pipecolic acid from bean plants (Phaseolus vulgaris). Stock solution (5 ml) was administered to twenty 4-week-old bean plants by infusion into the stems through cotton wicks. Isolation of pipecolic acid (spec. act. (^{14}C): $(4{\cdot}80 \pm 0{\cdot}03) \times 10^5$ dis/min/mmole) was carried out as described in Ref. 1, Experiment 4.

Sedamine and pipecolic acid from Sedum acre. Excised shoots (~100 g fr. wt.) of *S. acre* were kept 2 days in contact with stock solution (5 ml) of the doubly labelled lysine, in which DL-pipecolic acid (500 mg) had been dissolved. Sedamine (spec. act. (^{14}C): $(1{\cdot}11 \pm 0{\cdot}01) \times 10^6$ dis/min/mmole) and pipecolic acid (spec. act. (^{14}C): $(1{\cdot}21 \pm 0{\cdot}02) \times 10^6$ dis/min/mmole) were isolated as described in Ref. 1, Experiment 6.

Determination of radioactivity. The radioactivity of all samples was determined by liquid scintillation counting.[1] Confidence limits shown in the results are standard deviation of the mean.

Acknowledgement—This work was supported by a grant from the National Research Council of Canada.

[12] L. FRANK, *Anal. Biochem.* **17**, 423 (1966).