Conversion of D-Lysine into L-Lysine *via* L-Pipecolic Acid in *Nicotiana glauca* L. Plants and Cell-suspension Cultures

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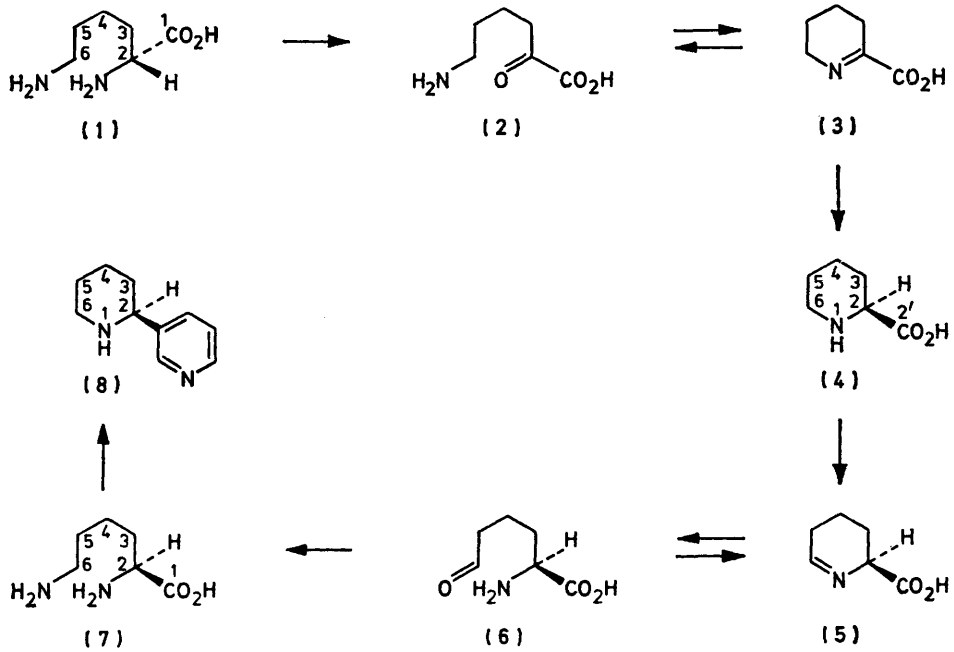
It is demonstrated by labelling experiments that D-lysine (1) is specifically converted into L-pipecolic acid (4) in *Nicotiana glauca* and that L-pipecolic acid (4) is specifically converted into L-lysine (7). Thus, pipecolic acid (4), which is widely distributed in micro-organisms, animals, and plants, is an intermediate in the conversion of D-lysine (1) into L-lysine (7). This conversion occurs not only in intact *N. glauca* plants, but also under sterile conditions in a cell-suspension culture of *N. glauca*.

D-AMINO-ACIDS are widely distributed in nature and enzymes which catalyse different reactions for their metabolism, *e.g.* racemization, reversible transamination, and oxidation, are available.[1] Among mosses and fungi, *N*-acetylation of D-amino-acids is a major pathway [2] whereas *N*-malonylation occurs in the higher plants.[3] The latter reaction is not encountered in D-lysine (1) metabolism.[1] This amino-acid, when administered to bacteria,[4-6] fungi,[7] mammals,[8,9] and higher plants,[10-13] is converted into pipecolic acid (4) with high efficiency (Scheme 1). The first step in this conversion is either a transamination or an oxidation. These reactions are common to L- and D-amino-acids.[14] The resulting product, α-keto-ε-aminocaproic acid (2), undergoes ring-closure to give Δ^1-piperidine-2-carboxylic acid (3). An enzyme system catalysing the stereospecific reduction of this compound into L-pipecolic acid (4) has been isolated from various sources.[15] Another enzyme which oxidizes pipecolic acid (4) at C-6 to give Δ^1-piperidine-6-carboxylic acid (5) is known to occur in *Pseudomonas putida* [16,17] and *Rhodotorula glutinis*.[18] One of the products of this reaction, *viz.* L-α-aminoadipic acid ε-semialdehyde (2-aminoadipaldehydic acid) (6), is a substrate for a bacterial transaminase generating L-lysine.[19] The reaction sequence from pipecolic acid (4) into L-lysine (7) appears also to occur in higher plants; the specific conversion of compound (4) into compound (7) has been shown to occur in *Acacia* plants.[20] Whereas individual steps shown in Scheme 1 have been well documented, the overall process, *i.e.* the conversion of D-lysine into L-lysine *via* L-pipecolic acid, has not been extensively studied and little attention has been paid to the stereochemistry of the conversion of the precursor *via* the intermediates into the product. Scheme 1 shows a reaction sequence in which one enantiomer (D-lysine) of an amino-acid is converted into the other enantiomer (L-lysine) by a process other than racemization or reversible transamination. This Scheme also sheds light on the role of pipecolic acid, an imino-acid which is very widely distributed in nature. We have demonstrated previously that this pathway



SCHEME 1 Conversion of D-lysine (1) into L-lysine (7) and anabasine (8) *via* L-pipecolic acid (4)

operates in *Neurospora crassa*.[7,21] We now show that this reaction sequence also occurs in a higher plant, *Nicotiana glauca*.

RESULTS AND DISCUSSION

Malonylation is a major pathway for the metabolism of D-amino-acids in higher plants.[3,22] It is now confirmed that this pathway occurs also in *N. glauca*. Administration to the plant of D-[3-^{14}C]phenylalanine resulted in the formation of radioactive malonyl-D-[^{14}C]phenylalanine (26.5% incorporation) which was isolated by ion-exchange and paper chromatography. The malonyl-D-[^{14}C]phenylalanine was identified [22] by degradation and co-chromatography. Whereas D-phenylalanine is converted into the malonyl derivative in *N. glauca*, a similar experiment with D-[6-^{14}C]lysine did not yield radioactive malonyl derivatives of lysine. Thus, although malonylation occurs in *N. glauca*, D-lysine does not undergo this reaction.

Experimental results listed in Tables 1 and 2 show,

TABLE 1

Incorporation of radioactivity from a mixture of L-[4,5-^{3}H]- (12.5 μCi) and D-[6-^{14}C]- (1.2 μCi) lysine (^{3}H/^{14}C ratio 10.2) into pipecolic acid (4), L-lysine (7) (isolated from protein), and anabasine (8) from *N. glauca*. Incorporation = (total radioactivity in product/total radioactivity applied) × 100

Product	Incorporation (%)	^{3}H/^{14}C Ratio	Retention of ^{3}H relative to ^{14}C (%)
(4)	1.8 (^{14}C)	1.3	12.4
(7)	0.6 (^{3}H)	80.6	790.2
(8)	0.9 (^{3}H)	200.0	1 960.8

TABLE 2

Incorporation of radioactivity from a mixture of D-[6-^{3}H]- (7.64 μCi) and D-[1-^{14}C]- (2.0 μCi) lysine (^{3}H/^{14}C ratio 3.82) into pipecolic acid (4), L-lysine (7) (isolated from protein), and anabasine (8) from *N. glauca*. Incorporation = (total radioactivity in product/total radioactivity applied) × 100

Product	Incorporation (%)	^{3}H/^{14}C Ratio	Retention of ^{3}H relative to ^{14}C (%)
(4)	11.80(^{14}C)	3.70	96.8
(7)	0.37(^{14}C)	1.88	49.2
(8)	0.10(^{3}H)	(no ^{14}C)	(no ^{14}C)

however, that D-lysine (1) is metabolized to give L-pipecolic acid (4). This observation is consistent with previous data [12] which show that L-lysine is the precursor of the piperidine ring of anabasine (8) whereas pipecolic acid (4) is derived from D-lysine. We have repeated our previous experiments and confirmed the results. When a mixture of D-[6-^{14}C]lysine and L-[4,5-^{3}H]lysine was applied to the plant and pipecolic acid (4) and anabasine (8) then isolated, the imino-acid (4) contained ^{14}C (from D-[6-^{14}C]lysine), but little ^{3}H, where anabasine (8) and the L-lysine isolated from the protein of the plant contained ^{3}H (from L-[4,5-^{3}H]lysine), but little ^{14}C. The data also show that a racemase, if present, is rather slow with respect to the incorporation of D-lysine into the piperidine ring of pipecolic acid and of L-lysine into that of anabasine. It has been shown in earlier publications [7,12,21,23] that the conversion of D-lysine (1) into L-pipecolic acid (4) and of the atoms C-2 to C-6 of L-lysine into the piperidine ring of anabasine occurs specifically and non-randomly. Consideration has also been given [7,12,21] to the individual steps involved in the conversion of D-lysine into L-pipecolic acid.

The hypothesis to be tested (Scheme 1) predicts that pipecolic acid (4) is converted into L-lysine (7). During oxidation of pipecolic acid (4) at C-6 (Scheme 1) the chiral centre at C-2 is not affected. It follows that the products of degradation [(5), (6), and (7)] should have the same configuration at C-2 as the pipecolic acid from which they are derived. Both enantiomers of pipecolic acid (4) are found in nature. The D-enantiomer was isolated from beech nuts,[24] while the L-enantiomer was isolated from various other sources.[1,7] It was therefore essential to determine the configuration of pipecolic acid isolated from *N. glauca*. This was done [7] using D-amino-acid oxidase (E.C. 1.4.3.3). Under identical conditions the enzyme attacked a synthetic sample of D-[6-^{14}C]pipecolic acid, but no pipecolic acid isolated from *N. glauca*. Therefore this latter sample had the L-configuration.

Degradation of L-pipecolic acid by the steps shown in Scheme 1 should therefore give L-lysine. Data listed in Tables 1 and 2 show that this is the case. When D-[6-^{3}H]lysine mixed with D-[1-^{14}C]lysine was administered to the plant no loss of tritium occurred during the course of its conversion into pipecolic acid (Scheme 1 and Table 2). When the [6-^{3}H, *carboxy*-^{14}C]pipecolic acid which is formed in the plant is further metabolized into L-lysine a 50% loss of tritium relative to ^{14}C is predicted [(4)→(5), Scheme 1]. The L-lysine so formed should be incorporated into anabasine (8) with loss of the carboxy-group, the C-2 to C-6 atoms of L-lysine giving rise to the piperidine ring of anabasine.[12,21] The alkaloid should then contain tritium, but no ^{14}C because the L-lysine formed from the L-[6-^{3}H,*carboxy*-^{14}C]pipecolic acid should be L-[6-^{3}H,1-^{14}C]lysine, whose ^{14}C-labelled carboxy-group would be lost *en route* to anabasine. The data listed in Table 2 clearly show that these predictions are correct. The fact that the L-lysine (isolated from the plant protein) contains ^{3}H and ^{14}C, whereas anabasine (8) contains ^{3}H only (Table 2) confirms that the radioactive labels in pipecolic acid (4) and L-lysine (7) are located non-randomly within the molecules.

The specific conversion of L-pipecolic acid into L-lysine is also demonstrated by the data listed in Tables 3, 4, and 5. When pipecolic acid, labelled either in the carboxy-group (Table 3) or in the piperidine ring (Table 4), was administered to the plant, specifically labelled L-lysine was isolated from the plant. The specific labelling pattern in L-lysine was demonstrated by degradation using L-lysine decarboxylase (E.C. 4.1.1.18) from *Bacillus*

TABLE 3

Incorporation of radioactivity from L-[*carboxy*-^{14}C]pipecolic acid (4) (18.0 μCi) into L-lysine (7) (isolated from protein) and the distribution of radioactivity in the degradation products of L-lysine

Product	C-*n* of lysine	Total activity (disint. min^{-1})	Specific activity (disint. min^{-1} μmol^{-1})
(7)	all	1.62×10^5	0.69×10^5
CO_2	1	1.45×10^5	0.62×10^5
(9)	2—6	Inactive	Inactive

TABLE 4

Incorporation of radioactivity from L-[4,5-$^3H_2$]pipecolic acid (4) (4.5 μCi) into L-lysine (7) (isolated from protein) and the distribution of radioactivity in the degradation products of L-lysine

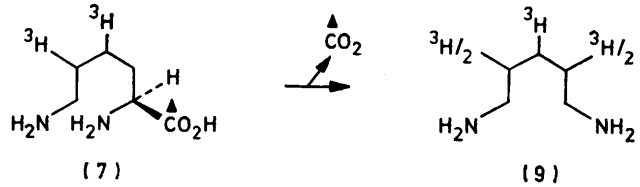
Product	C-*n* of lysine	Total activity (disint. min^{-1})	Specific activity (disint. min^{-1} μmol^{-1})
(7)	all	3.20×10^5	1.36×10^5
CO_2	1	Inactive	Inactive
(9)	2—6	3.04×10^5	1.30×10^5

TABLE 5

Incorporation of D-[4,5-^{3}H]lysine (37.0 μCi) into L-pipecolic acid (4) and L-lysine (7) (expt. A) and L-[4,5-^{3}H]pipecolic acid (4) (6.7 μCi) into L-lysine (7) (expt. B) in a cell-suspension culture of *Nicotiana glauca*

Expt.	Product	Total activity (disint. min^{-1})	Incorporation (%)
A	(4)	4.15×10^4	0.051
	(7)	3.26×10^6	4.0
B	(7)	0.85×10^5	0.57

cadaveris (Scheme 2). The carbon dioxide liberated in this reaction was trapped and the cadaverine (9) formed (Scheme 2) was isolated. The specific activity of both degradation products was determined. After application to the plant of carboxy-labelled pipecolic acid all the activity of L-lysine was located in the carbon dioxide



SCHEME 2 Degradation of labelled L-lysine with L-lysine decarboxylase (E.C. 4.1.1.18) from *Bacillus cadaveris*

(*i.e.* C-1 of L-lysine) and none in the cadaverine (C-2 to C-6 of L-lysine) (Scheme 2, Table 3). In contrast, after application of pipecolic acid labelled in the piperidine ring all the activity was located in the cadaverine (9) (Scheme 2, Table 4).

All the data reported here are consistent with the hypothesis outlined in Scheme 1. It has been demonstrated that D-lysine is specifically converted into L-lysine *via* L-pipecolic acid. Previous data show that D-lysine is the precursor of pipecolic acid in *Sedum* and *Nicotiana* plants,[12] but also that pipecolic acid is converted into lysine in *Acacia* plants.[20] The stereochemistry of the latter conversion had not been determined. The conversion of D-lysine into L-lysine *via* L-pipecolic acid had also been observed in *Neurospora crassa*.[7,21] The possibility therefore exists that the reactions reported here might be due to microbial contamination of the *N. glauca* plants. This was ruled out by the following experiment. A sterile cell-suspension culture of *N. glauca* was established and grown in a medium which had been used for anthraquinone-producing cell cultures.[25,26] The medium contained 2,4-dichlorophenoxyacetic acid, kinetin, indolylacetic acid, and naphthylacetic acid as hormones. The culture did not contain detectable amounts of anabasine. The data shown in Table 5 indicate that the conversion of D- into L-lysine *via* L-pipecolic acid takes place under sterile conditions, as well as in the intact plants (Tables 1 to 4).

EXPERIMENTAL

Plant Material and Feeding Techniques.—*Nicotiana glauca* plants were grown from seed in the greenhouse of this institute. Four-month old plants were fed by the wick technique for 70 h. In order to establish a cell-suspension culture of *N. glauca* the stem of a plant was sterilized twice (10 min each) in NaOCl (10%) and ethanol, respectively, cut into segments and transferred to agar which contained the components which had previously been used.[26] The callus, which developed within 2 to 3 weeks, was transferred into a liquid medium (50 ml) containing the same components as the agar medium, except the agar, and shaken at 60 revolutions per min at 27 °C. The suspension culture was fed for 24 h before the stationary growth phase was reached.

Radioactive Compounds.—The sources of labelled lysines and pipecolic acids has been described previously.[7] Radioactively labelled D-[3-^{14}C]phenylalanine was obtained from a racemic mixture which had been treated with L-amino-acid oxidase (Sigma) (LAO, E.C. 1.4.3.2). DL-[3-^{14}C]-Phenylalanine (1 mg) was dissolved in sodium phosphate buffer (0.2M, 2 ml, pH 6.5). LAO (5 mg) was added and the incubation carried out at 25 °C. After 2 h, L-phenylalanine (inactive, 1 mg) was added and incubation was continued for 20 h. The mixture was repeatedly flushed with oxygen. The incubation was terminated by adding methanolic HCl and heating. Protein was centrifuged off, the supernatant liquid removed, the pellet re-suspended in water, and centrifugation repeated. L-[^{14}C]Phenylalanine was isolated from the combined supernatant liquids by ion-exchange and paper chromatography (yield, 35% relative to the DL-mixture).

Isolation of Pipecolic Acid, Anabasine, and Lysine.—Pipecolic acid and anabasine were isolated from the intact plant and from the cells of the suspension culture as described previously.[12] The extracted plant material was stirred in NaOH (1M) for 24 h. The suspension was centrifuged and the supernatant liquid acidified with trichloroacetic acid (3M). Protein was centrifuged off and the pellet re-suspended in water and the centrifugation was repeated. The pellet was suspended in HCl (6M) and the suspension sealed under nitrogen in a glass tube. The tube was kept at 110 °C for 24 h. The contents of the tube were diluted (H_2O) and filtered, and from the filtrate amino-acids were isolated after treatment with Dowex H^+ and paper chromato-

graphy.[7] L-Lysine, anabasine, and L-pipecolic acid were isolated carrier-free. Yield (per plant) was *ca.* 90—100 μM of compound (8), *ca.* 2.5 μM of (7), or *ca.* 5.0 μM of (4). Each compound was purified until a constant $^{3}H/^{14}C$ ratio or a constant specific activity was reached.

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