Conversion of D-Lysine to L-Lysine *via* L-Pipecolic Acid in *Neurospora crassa*

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(Z. Naturforsch. **30 c**, 253–262 [1975]; received October 30, 1974)

Neurospora crassa, Biosynthesis, D-Lysine, L-Lysine, Role of L-Pipecolic Acid

Neurospora crassa converts D-lysine to L-pipecolic acid and the latter to L-lysine by a pathway outlined herein and different from mechanisms hitherto known to be involved in interconversions of enantiomers of amino acids.

Introduction

Pipecolic acid (**4**) (Fig. 6), an imino acid occurring in plants [1], animals [2, 3], and microorganisms [4], is known to be a product of lysine catabolism. Tracer studies have shown that lysine labelled in different positions is incorporated into pipecolic acid in such diverse organisms as *Phaseolus sp.* [5, 6], *Triticum sp.* [7], *Zea mays* [8], *Acacia homalophylla* [9], *Leucaena glauca* [10], *Mimosa pudica* [11], *Rattus sp.* [12–15], *Neurospora crassa* [16, 17], *Rhizoctonia leguminicola* [18], *Pseudomonas putida* [19–21], and *Achromobacter sp.* [22].

The reactions leading to pipecolic acid are outlined in Fig. 6. The pathway involves α-deamination of lysine and formation of an intramolecular Schiff's base (**3**), Δ^1-piperideine-2-carboxylic acid (**3**), whose stereospecific reduction at C_2 yields L-pipecolic acid (**4**) [16]. Whereas the origin of pipecolic acid from lysine is well documented, there are conflicting reports whether the D or L isomer of lysine gives rise to pipecolic acid. Before 1967 it was accepted that pipecolic acid was derived from L-lysine [2, 6, 9, 13, 17, 22]. Since 1968 evidence has accumulated which leads to the conclusion – with one exception [18] – that pipecolic acid is derived from D-lysine [8, 14, 15, 19–21, 23].

In many cases inferences were based on a comparison of incorporation efficiences of radioactively labelled samples of D or L-lysine in separate experiments. Shortcomings of such an approach have been recently pointed out [23].

Pipecolic acid (**4**) is not inert. It is degraded by a bacterium *(Pseudomonas putida) via* Δ^1-piperideine-6-carboxylic acid (**5**) and α-aminoadipic acid to glutamic acid [24, 25]. Oxidation of pipecolic acid (**4**) at C_6 to give Δ^1-piperideine-6-carboxylic acid (**5**) (Fig. 6) also seems to occur in a fungus *(Neurospora)* [16]. Δ^1-Piperideine-6- carboxylic acid (**5**) is known to equilibrate with α-aminoadipic-δ-semialdehyde (**6**) [26]. The latter is reported to be a precursor of L-lysine in yeast [27]. Since naturally occurring pipecolic acid has been shown to have the L-configuration in most cases [23] and since oxidation of pipecolic acid at C_6 does not affect the chiral center at C_2, L-pipecolic acid might be a precursor of L-lysine in organisms other than bacteria. Indeed Fowden [9], and Rothstein and Saffran [28], obtained results indicating that pipecolic acid is converted to lysine in *Acacia homalophylla* and *Euglena gracilis*. The configuration of precursor and product, however, was not established in these cases.

The present investigation was designed to investigate the relationship between pipecolic acid and lysine in *Neurospora*. Contrary to a previous claim [17], it is found that, in this organism it is D-lysine which yields L-pipecolic acid, and that degradation of L-pipecolic acid yields L-lysine. Thus pipecolic acid is an intermediate in the conversion of D-lysine to L-lysine.

Materials

Neurospora crassa

Neurospora crassa Shear and Dodge wild-type strain SY 7 A No 622 was obtained from the Fungal Genetics Stock Center, Hanover, New Hampshire, USA. This strain is identical with strain No 24 740 of the American Type Culture Collection.

Chemicals, enzymes and radiochemicals

Chemicals, enzymes and radiochemicals were obtained from the following sources: DL-lysine, D-

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lysine, and L-lysine from Fluka, DL-pipecolic acid from Ega-Chemie, D-pipecolic acid, and L-pipecolic acid from Calbiochem. D-Aminoacid oxidase (DAOD) (hog kidney, E.C. 1.4.3.3.) from Boehringer and L-lysine decarboxylase (*Bac. cadaveris*, E.C. 4.1.1.18) from Sigma. DL-[4,5-T_2]lysine and L-[4,5-T_2]lysine from New England Nuclear or Amersham Buchler, DL-[1-^{14}C]lysine and L--[U-^{14}C]lysine from Amersham Buchler, DL-[6-^{14}C]lysine from Commissariat à l'Energie Atomique and DL-[6-T]lysine from New England Nuclear. Before use radioactively labelled materials were purified by chromatography in system C (vide infra). The enantiomeric purity of the commercial samples of L-lysine obtained from suppliers listed above was checked using L-lysine decarboxylase (vide infra).

Methods

Determination of radioactivity

The radioactivity of all samples was determined by liquid scintillation counting (Berthold Frieseke Liquid Scintillation Counter, Betaszint Model 5000). Activity due to ^{3}H and ^{14}C was determined simultaneously, by external standardization counting. Duplicate samples of each compound were dissolved in water and counted in Bray's solution. The standard deviation given in the tables was calculated by the scintillation computer such that 68.3% (1 σ factor) of all activity determinations of a given sample would fall within the limits given.

$T/^{14}$C-ratios listed in the tables are normalized.

Growth of Neurospora

For production of conidia *Neurospora* was propagated on agar according to Frost[29]. The conidia were used to inoculate culture flasks containing one litre of liquid medium as described by Horowitz[30]. The medium was supplemented with either 5 μg or 0.25 μg biotin per litre.

Radioactively labelled materials were sterilized by filtration and applied to shake cultures of *Neurospora* during the logarithmic phase or stationary phase of mycelial growth (*cf.* Results). Whenever radioactively labelled lysine was fed, pipecolic acid (5 mg) was applied in addition as a physiological trap. Incubations were carried out at 24 °C on a shaker. Samples of the mycelial suspension were taken out at random and checked microscopically for contaminating microorganisms.

Growth curves were obtained after simultaneous inoculation of culture flasks (100 ml medium). Each day after inoculation the mycelium of one culture flask was filtered off, washed, dried in an oven at 80 °C and weighed (Fig. 1).

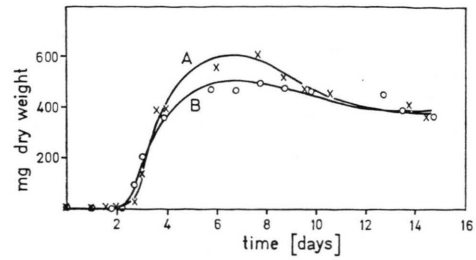

Fig. 1. Growth curves of *Neurospora* obtained with 0.5 μg (A) or 0.025 μg (B) biotin per 100 ml medium.

Chromatographic systems

TLC on silica gel G plates (0.25 mm)

System A: Chloroform, methanol, ammonia (17%) 2:2:1 (lysine $R_f = 0.3$, pipecolic acid $R_f = 0.61$).

System B: *n*-Propanol, ammonia (28%) 7:4 (lysine $R_f = 0.17$, pipecolic acid $R_f = 0.59$).

Paper chromatography on Whatman No. 1 or No. 3

System C: *n*-Butanol, acetic acid, water 2:1:1 (lysine $R_f = 0.27$, pipecolic acid $R_f = 0.61$).

System D: Pyridine, acetic acid, water 10:7:3 (lysine $R_f = 0.28$, pipecolic acid $R_f = 0.67$).

System E: Ethylacetate, formic acid, water 7:2:1 (lysine $R_f = 0.10$, pipecolic acid $R_f = 0.65$).

System F: Isopropanol, ammonia (28%), water 8:1:1 (lysine $R_f = 0.14$, pipecolic acid $R_f = 0.32$).

Two dimensional TLC on precoated plates of silica gel G.

First solvent: Butanol-2, formic acid, water 70:21:9; second solvent: Butanol-1, pyridine, acetic acid, water 50:30:20:10.

Isolation and purification of lysine and pipecolic acid from the culture filtrate of Neurospora

A portion (25 ml) of the medium (1 l) was withdrawn under sterile conditions from the mycelial suspension. The aqueous solution was passed through a column of Dowex H^+ (50 W X 4, 1.5 × 15 cm) and the column washed until the effluent was neutral. The amino acid fraction was eluted with molar ammonia and evaporated. Lysine and pipecolic acid were separated using system C. Lysine was further purified in systems A, D and B. The $T/^{14}$C ratio was constant after chromatography in system D. Pipecolic acid was further purified in system A, E and B. The $T/^{14}$C ratio of pipecolic acid was constant after chromatography in system E.

Extraction and purification of lysine and pipecolic acid from the mycelium

The mycelium was separated from the medium by filtration and washed. The mycelium (ca. 25 g fresh weight) was refluxed in water (500 ml) for 60 min and extraction repeated for 30 min. Pipecolic acid and lysine were isolated from the combined aqueous extracts as described above.

Hydrolysis of mycelium and isolation of lysine from the hydrolysate

The mycelium, which had been extracted as described above, was dried in a desiccator and a portion (1 g) was hydrolyzed in HCl (5 ml, 6 N) under nitrogen at 110 °C in a sealed tube. After 24 hours the contents of the tube were filtered, washed, inactive DL-lysine (50 mg) was added and the filtrate evaporated. The residue was repeatedly redissolved in water and evaporated to remove HCl. Lysine was isolated and purified as described above.

Degradation of lysine with L-lysine decarboxylase

Solutions of lysine and of lysine decarboxylase in phosphate buffer (1 ml, pH 6.0, 0.2 M) were combined and heated at 37 °C in a flask which was connected to a centrifuge tube attached to a Vigreux column. The centrifuge tube contained a mixture of ethanolamine (1.2 ml) and methanol (8.8 ml). After 20 min L-lysine (2 mg) dissolved in buffer (1 ml) was added to the reaction mixture and incubation continued for 40 min. The reaction was terminated when HCl (2 ml, 1 N) was added to the reaction mixture and the CO_2 formed (from C-1 of lysine) was slowly flushed with nitrogen into the tube containing the mixture of methanol-ethanolamine. The precipitated protein was centrifuged off and the supernatant evaporated. Lysine (*i.e.* D-lysine) and cadaverine (*i.e.* 1,5-diaminopentane, C-atoms 2 to 6 of L-lysine) were separated chromatographically using system F. The radioactivity of the methanol-ethanolamine mixture (containing $^{14}CO_2$ from C-1 of L-lysine) as well as of the cadaverine and the D-lysine were determined.

Assay of the enantiomeric purity of L-lysine and preparation of radioactively labelled D-lysine

Preparation of D-[^{14}C]lysine and assay of the enantiomeric purity of a given sample of lysine was carried out as described previously [23].

Synthesis of pipecolic acid labelled in different positions

The synthesis of labelled samples of pipecolic acid was carried out by the method of Rodwell [31], using appropriately labelled samples of lysine (DL-[1-^{14}C]lysine, DL-[6-^{14}C]lysine, DL-[2-T]lysine, DL-[4,5-T_2]lysine, DL-[6-T]lysine). The reaction involves replacement of the α-amino group of lysine by Cl to give 6-amino-2-chlorocaproic acid, which is then cyclized in alkaline solution. In these reactions no tritium from any of the positions of lysine should be exchanged with hydrogen from the medium. Thus, when a doubly labelled sample of lysine (DL-4,5-T_2, 6-^{14}C]lysine) was converted to pipecolic acid by this procedure no change in the T/^{14}C ratio was observed. Synthesis of [2-T] and [6-T]pipecolic acid from [2-T] and [6-T]lysine gave radiochemical yields identical with those obtained in the case of [^{14}C]pipecolic acid, suggesting that tritium exchange does not occur in these cases.

A solution of DL-lysine monohydrochloride (10 μmol) in conc. HCl (1.5 ml) was stirred and refluxed. A solution of $NaNO_2$ (30 μmol) in water (0.5 ml) was added dropwise to the mixture. The solution was refluxed for 2.5 hours, cooled to room temperature and evaporated under reduced pressure. The residue was dissolved in NaOH (5 ml, 1 N) and kept overnight at room temperature. The solution was acidified to pH 1 with conc. HCl and evaporated.

The residue was dissolved in water and passed through a column of Dowex H^+ (50 WX 4, 1.5 × 15 cm). The column was washed until the effluent was neutral and the pipecolic acid was eluted with ammonia (1 M). The eluate was evaporated and the pipecolic acid purified using system F. The purity of the samples of pipecolic acid so obtained (Fig. 2) (DL-[2-^{14}C]pipecolic acid, DL-[6-^{14}C]pipecolic acid, DL-[2-T]pipecolic acid, DL-[4,5-T_2]pipecolic acid and DL-[6-T]pipecolic acid) was checked using system A and E. Yield 35 to 50%.

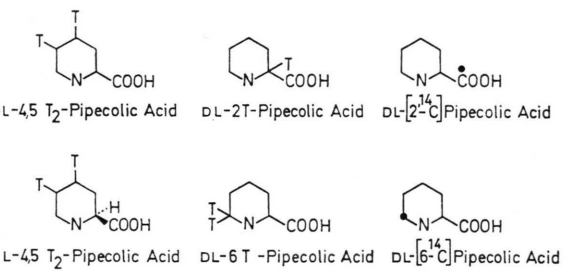

Fig. 2. Synthetic pipecolic acids labelled at different sites with Tritium (T) or ^{14}C (●).

Preparation of radioactively labelled L-pipecolic acid

DL-pipecolic acid (3.3 μmol) was dissolved in sodium diphosphate buffer (3 ml, pH 8.2, 0.1 M) and 0.02 ml of a suspension of D-amino acid oxidase

(2 units) added. Incubation was carried out at 25 °C. The reaction mixture was repeatedly flushed with oxygen. After 2.5 hours a sample of inactive D-pipecolic acid (0.8 mg) was added to ensure complete oxidation of labelled D-pipecolic acid. After 21 hours the reaction was terminated by adding HCl (2 N) and heating the sample on a steam bath. The precipitated protein was centrifuged off and the supernatant passed through a column of Dowex H^+ (50 WX 4 1×10 cm). The column was washed with water and L-pipecolic acid eluted with ammonia. The ammoniacal solution was evaporated, and the residue purified using system C. Yield = 38% of DL-pipecolic acid. The enantiomeric purity of L-pipecolic acid was checked using the following procedure.

Determination of configuration of pipecolic acid

The procedure used was essentially that described above, except that, after termination of the reaction the supernatant was evaporated and chromatographed using system A. A single peak appearing on radioscanning and corresponding in R_f value to pipecolic acid would indicate the presence of L-pipecolic acid only. More than one peak would indicate that the pipecolic acid prepared as described above or isolated from the culture filtrate of *Neurospora* after [^{14}C]lysine feeding, consisted of a mixture of L- *and* D-pipecolic acid.

Results

A. Conversion of D-lysine to pipecolic acid

L-[4,5-T_2]lysine (190 μCi, 20 Ci/mmol) and D-[6-^{14}C]lysine (12.5 μCi, 48 mCi/mmol) were simultaneously added to the mycelial suspension at the beginning of the logarithmic phase of growth (day 2 after inoculation). Reisolation and purification of [T/^{14}C]lysine from portions (25 ml) of the medium taken at intervals as indicated in Fig. 3 showed that L-lysine completely disappeared from the medium within one hour whereas D-lysine uptake was much slower. Radioactive pipecolic acid appeared in the medium 4 hours after application of labelled lysines. Pipecolic acid was almost exclusively labelled with ^{14}C (from D-[6-^{14}C]lysine). The amount of tritiated pipecolic acid (from L-[4,5-T_2]lysine) did not exceed 2 to 3% of [^{14}C]pipecolic acid (Fig. 3).

The experiment was repeated in the stationary phase of mycelial growth (day 14 after inoculation). The medium contained 0.25 μg biotin per litre medium. Uptake of amino acids proceeded much faster than in the logarithmic phase of growth. Pipecolic acid was again almost exclusively labelled from D-[6-^{14}C]lysine rather than L-[4,5-T_2]lysine (Fig. 4).

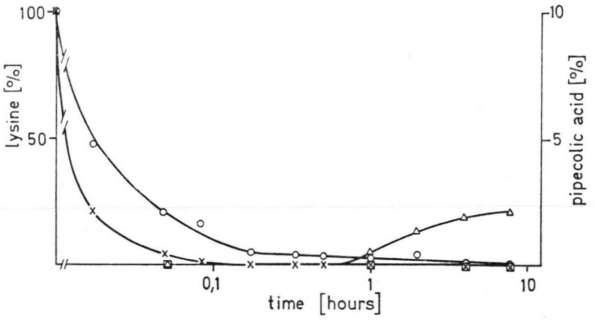

Fig. 4. Disappearance of simultaneously applied D-[6-^{14}C]-lysine (–○–○–) and L-[4,5-T_2]lysine (–×–×–) from the culture medium of *Neurospora* and incorporation efficiency (%) of labelled D-[6-^{14}C]lysine (–△–△–) and L-[4,5-T_2]lysine (–□–□–) into pipecolic acid. Incorporation efficiency refers to activity in pipecolic acid in one litre medium. The experiment was carried out on *the 14th day* after inoculation for eight hours (stationary phase of growth).

DL-[4,5-T_2]lysine (240 μCi, 20 Ci/mmol) and DL-[6-^{14}C]lysine (15.3 μCi, 48 mCi/mmol) were simultaneously applied to the mycelial suspension two days after inoculation. Pipecolic acid was iso-

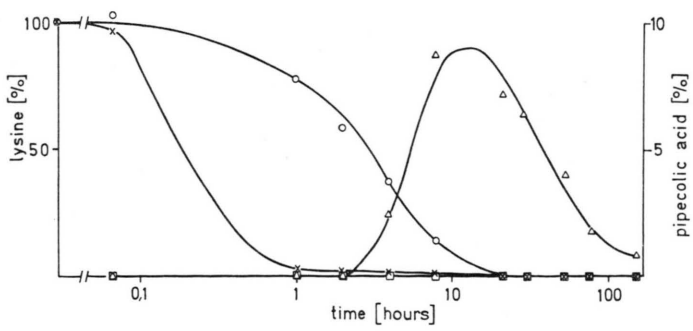

Fig. 3. Disappearance of simultaneously applied D-[6-^{14}C]lysine (–○–○–) and L-[4,5-T_2]lysine (–×–×–) from the culture medium of *Neurospora* and incorporation efficiency (%)* of labelled D-[6-^{14}C]lysine (–△–△–) and L-[4,5-T_2]lysine (–□–□–) into pipecolic acid. Incorporation efficiency refers to activity in pipecolic acid in one litre medium. The experiment was carried out between *day 3 to 9* after inoculation (logarithmic phase of growth).

* Incorporation or efficiency of incorporation (%) means: Total activity in product (pipecolic acid) divided by total activity fed (lysine), × 100.

lated from the medium at intervals as indicated in Table I. Over a period of 100 hours the pipecolic acid maintained the T/^{14}C-ratio of the lysine fed. Highest incorporation of lysine into pipecolic acid was 9.8%.

Table I. Simultaneous application of DL-[4,5-T_2]lysine and DL-[6-^{14}C]lysine (T/^{14}C-ratio 10.0±0.2) to *Neurospora* during the logarithmic phase of growth (day two to seven after inoculation).

	Pipecolic acid from culture filtrate		
Time of incubation [hours]	T/^{14}C-ratio	Incorporation * of Tritium [% of D-[T]lysine]	Incorporation * of ^{14}C [% of D-[^{14}C]lysine]
3	10.2±0.3	3.3±0.1	3.3±0.1
22	10.9±0.2	9.8±0.2	9.0±0.1
52	10.9±0.2	5.1±0.0	4.7±0.1
100	12.2±0.6	3.1±0.1	2.5±0.1

* Incorporation or efficiency of incorporation (%) means: total activity in product (pipecolic acid) divided by total activity fed (lysine), × 100.

D-[6-^{14}C]Lysine and L-[U-^{14}C]lysine were applied to the fungus in two separate experiments (Table II). The incorporation efficiency of D-lysine into pipecolic acid was 7.7% whereas the incorporation efficiency of L-lysine was 0.24% only.

Table II. Comparison of efficiencies of incorporation of labelled D- and L-lysine into pipecolic acid in two separate experiments. Start of incubation: 3 days after inoculation. Time of incubation: 16 hours.

	Substrate			Pipecolic acid From culture medium
	Activity [μCi]	Amount [μmol]	Specific activity [μCi/μmol]	incorporation [%]
D-[6-^{14}C]lysine	8.7±0.4	0.20	43.5	7.7 ±0.2
L-[U-^{14}C]lysine	8.4±0.1	0.20	42.0	0.24±0.01

The medium of *Neurospora* growing logarithmically was separated from the mycelium by filtration. L-[4,5-T_2]lysine (6.15 μCi, 20 Ci/mmol) and D-[6-^{14}C]lysine (0.22 μCi, 48 mCi/mmol) were simultaneously added to the filtrate. Chromatoscanning of the crude medium after 24 hours revealed that labelled lysine was the only radioactive compound. Samples of the medium were withdrawn after 5 min, 3 and 24 hours. Lysine was reisolated and purified. The T/^{14}C-ratio of lysine remained unchanged (10.0±0.3; 10.4±0.5; 10.3±0.3; 9.8±0.6).

B. Determination of the configuration of pipecolic acid isolated from the culture medium of Neurospora

An authentic sample of DL-[6-^{14}C]pipecolic acid was incubated with D-aminoacid oxidase. The reaction mixture was chromatographed. On radioscanning three peaks appeared, one of which corresponded in R_f value to that of an authentic sample of pipecolic acid. The other two peaks (R_f 0.8 and 0.96) were probably due to Δ^1-piperideine-2-carboxylic acid (**3**) and α-keto-ε-aminocaproic acid (**2**) (Fig. 6). The peak-area of pipecolic acid was equal to the sum of the other two peak-areas indicating complete destruction of D-pipecolic acid. When radioactive pipecolic acid obtained from the culture filtrate of *Neurospora* was chromatographed after treatment with D-aminoacid oxidase, only a single peak corresponding to L-pipecolic acid was observed (Fig. 5).

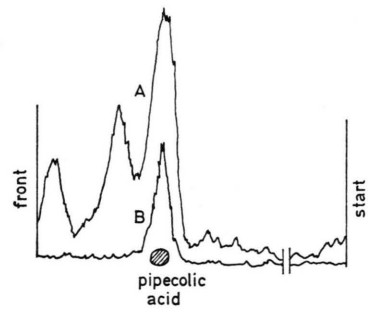

Fig. 5. Determination of configuration of pipecolic acid with D-aminoacidoxidase (DAOD) from hog kidney (E.C. 1.4.3.3.). Radioscan of a chromatogram of synthetic DL-[6-^{14}C]pipecolic acid treated with DAOD (A) and radioscan of a chromatogram with DAOD treated [^{14}C]pipecolic acid isolated from the culture filtrate of *Neurospora* (B). Solvent system *cf.* Materials and Methods.

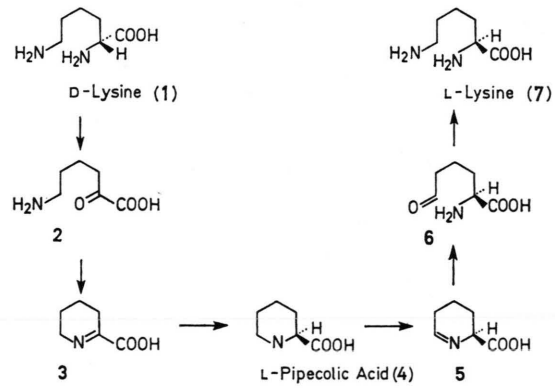

Fig. 6. Conversion of D-lysine to L-lysine *via* L-pipecolic acid.

Table III. Conversion of doubly labelled pipecolic acids into lysine. Time of incubation: 22 hours during logarithmic phase of growth.

Experiment No.	Pipecolic acid fed Configuration and position of label	T/^{14}C-ratio	Lysine isolated from aqueous extract of mycelium T/^{14}C-ratio	Lysine isolated from extracted mycelium after hydrolysis T/^{14}C-ratio
1	DL-[4,5-T_2]pipecolic acid DL-[6-^{14}C]pipecolic acid	10.0 ± 0.2	8.2 ± 0.5	8.7 ± 0.2
2	DL[6-T]pipecolic acid DL-[6-^{14}C]pipecolic acid	10.0 ± 0.1	5.6 ± 0.9	4.0 ± 0.2
3	DL-[2-T]pipecolic acid DL-[6-^{14}C]pipecolic acid	10.0 ± 0.1	8.5 ± 0.7	8.3 ± 0.2
4	L-[4,5-T_2]pipecolic acid DL-[2′-^{14}C]pipecolic acid	10.0 ± 0.1	20.2 ± 1.3	20.0 ± 1.6

C. Conversion of L-*pipecolic acid to* L-*lysine*

Application of a mixture of DL-[4,5-T_2]pipecolic acid (38.5 μCi, 9.5 mCi/mmol) and DL-[6-^{14}C]pipecolic acid (3.6 μCi, 3.6 mCi/mmol) to a shake culture of *Neurospora* three days after inoculation led to the formation of radioactive lysine (Table III, Exp. 1). The efficiency of incorporation into lysine was 0.59% ± 0.03% (water soluble fraction) and 6.6% ± 0.2% (water insoluble fraction *i. e.* proteins and cell walls), respectively. The T/^{14}C-ratio of the lysine was somewhat lower than that of the administered pipecolic acid (Table III, Exp. 1). Incorporation of pipecolic acid into lysine was also demonstrated in Exp. 2, 3 and 4 (Table III). When DL-[6-T]pipecolic acid (50 μCi, 16.5 mCi/mmol) in admixture with DL-[6-^{14}C]pipecolic acid (5.0 μCi, 36 mCi/mmol) was applied to a shake culture of *Neurospora* the T/^{14}C-ratio of lysine dropped to 5.6 and 4.0, respectively (Table III, Exp. 2). Maintainance of the T/^{14}C-ratio of lysine is expected after DL-[2-T]pipecolic acid (33.7 μCi, 11.2 mCi/mmol) and DL-[6-^{14}C]pipecolic acid (2.4 μCi, 3.6 mCi/mmol) feeding. Again a slight drop of the T/^{14}C ratio was observed (Table III, Exp. 3).

A mixture of L-[4,5-T_2]pipecolic and (22.0 μCi, 22 mCi/mmol) and DL-[2′-^{14}C]pipecolic acid (1.5 μCi, 3.6 mCi/mmol) was applied to a shake culture of *Neurospora* three days after inoculation. Uptake of L- and DL-pipecolic acid was monitored after reisolation from the medium and purification. From these data it can be concluded that the fungus does not differentiate between D- and L-pipecolic acid as far as transport is concerned. Both enantiomers of pipecolic acid are taken up at an equal rate. After 10 hours approx. 30% of pipecolic acid is still in the medium. Lysine isolated from the mycelium has a T/^{14}C-ratio twice as high as the T/^{14}C-ratio of the L-[4,5-T_2], DL-[2′-^{14}C]pipecolic acid applied (Exp. 4, Table III). Decarboxylation with L-lysine decarboxylase of lysine so obtained and analysis of the cadaverine (*i. e.* 1.5-diaminopentane) and CO_2 formed revealed that all ^{14}C was located in the carboxyl-group of lysine, whereas all tritium was recovered in the cadaverine, *i. e.* it has been located at C-atoms 2 to 6 of lysine (Table IV). Of the radioactive lysine 95% was converted to cadaverine and CO_2 indicating that at least 95% of radioactive lysine isolated from *Neurospora* after feeding of L-[4,5-T_2], DL-[2′-

Exp.	Substrate	T/^{14}C-ratio of the isolated lysine (from hydrolysate)	T/^{14}C-ratio of cadaverine (after decarboxylation of the isolated lysine with L-lysine decarboxylase)	%$^{14}CO_2$ from C_1 of L-lysine (L-lysine = 100%)
1	DL-[2-T]-/DL-[6-^{14}C]pipecolic acid	8.3 ± 0.2	8.3 ± 0.5	0
2	L-[4,5-T_2]-/DL-[2′-^{14}C]pipecolic acid	20.0 ± 1.6	∞	103.4 ± 3.0
3	DL-[6-T]-/DL-[6-^{14}C]pipecolic acid	4.0 ± 0.2	4.6 ± 0.1	0

Table IV. Degradation of [T/^{14}C]lysine isolated from *Neurospora* after administration of T/^{14}C-pipecolic acids. The degradation was carried out with L-lysine decarboxylase (*cf.* Methods).

^{14}C]pipecolic acid had the L-configuration. Two-dimensional chromatography and autoradiography of a portion of the crude extract of the mycelium after feeding of L-[4,5-T_2]pipecolic acid and DL-[6-^{14}C]pipecolic acid showed that only two spots of almost equal intensity appeared. One spot corresponded to that of an authentic sample of lysine, the other one to that of an authentic sample of pipecolic acid.

A portion of the crude hydrolysate of the extracted mycelial pad was chromatographed two-dimensionally and an autoradiogram was taken. Two major spots appeared, one corresponding to lysine. A third spot of minor intensity was also noted. None of the spots observed corresponded to α-aminoadipic acid or glutamic acid.

Degradation of lysine after administration of DL-[2-T]pipecolic acid (33.7 μCi, 11.2 mCi/mmol) and DL-[6-^{14}C]pipecolic acid (3.4 μCi, 3.6 mCi/mmol) showed (*cf.* Table III, Exp. 3; Table IV, Exp. 1) that the T/^{14}C-ratio in lysine and its products of degradation (*i. e.* cadaverine) was slightly lower than the T/^{14}C-ratio of the fed pipecolic acid.

Specific incorporation of DL-[6-T, 6-^{14}C]pipecolic acid into L-lysine was also demonstrated (Table IV, Exp. 3).

D. Overall conversion of D-*lysine to* L-*lysine*

D-[6-^{14}C]lysine (8.69 μCi, 43.5 mCi/mmol) was applied to a shake culture of *Neurospora* three days after inoculation. Incubation was terminated after 16 hours when the mycelium was separated from the suspension by filtration and washed. The mycelium was dried, hydrolyzed and lysine was isolated. The configuration of lysine was determined using L-lysine decarboxylase. Of the radioactivity of the D-lysine applied 28.5% was recovered in cadaverine (*i. e.* from L-lysine), whereas 6.1% of radioactivity of the lysine was not converted to cadaverine and thus was due to D-lysine.

Discussion

The present experiments were designed to investigate the stereospecifity of the lysine-pipecolic acid relationship in *Neurospora*. The experiments fall into four sections. In section "A" experiments were reported which show that, contrary to a previous view [17], it is D-lysine rather than L-lysine which gives rise to pipecolic acid in *Neurospora*. Section "B" contains results indicating that pipecolic acid produced by *Neurospora* has the L-configuration. This finding confirms a previous report [17]. Section "C" shows that it is L-rather than D-pipecolic acid which is specifically converted to lysine and that the lysine formed has the L-configuration. Finally it is demonstrated in section "D" that D-lysine is efficiently converted to L-lysine. Simultaneous application of tritium- and ^{14}C-labelled enantiomers was the method of choice used to establish precursor configuration in these precursor-product relationships [23]. Two prerequisites have to be fulfilled, however, if the evidence obtained by such an approach is to be interpretable in an unequivocal manner [23]. Firstly, it is essential to demonstrate that a racemic mixture of the precursor is incorporated specifically and nonrandomly into the product. Secondly it must be shown that, when differently labelled enantiomers are applied to an organism, both are taken up.

Data listed in Table I show that over a period ranging from 3 to 100 hours, a mixture of DL-[4,5-T_2]lysine and DL-[6-^{14}C]lysine is incorporated into pipecolic acid without change of the T/^{14}C-ratio. Pipecolic acid is therefore derived from the intact carbon skeleton of lysine, and thus derivation is independent of the time of incubation. Although a slight increase in the tritium content of pipecolic acid is observed after four days of incubation the specific incorporation of lysine into pipecolic acid is undebatable and in agreement with numerous tracer studies carried out previously in other organisms (vide supra).

When a mixture of L-[4,5-T_2]lysine and D-[6-^{14}C]lysine is applied to the fungus (Fig. 3), both isomers disappear from the medium and are therefore taken up by the mycelium. It is well known [32, 33] that uptake of L-lysine proceeds at a much faster rate than D-lysine-uptake. Although D-lysine enters the mycelium at a much slower rate than L-lysine, pipecolic acid isolated from the medium at intervals as indicated in Fig. 3 is almost exclusively labelled with ^{14}C (derived from D-[6-^{14}C]-lysine). In this experiment disappearance of lysine, and the amount of pipecolic acid in the medium was monitored. It was later found that ca. 95% of pipecolic acid can be recovered from the medium whereas ca. 5% is contained in the mycelium. From this observation it might be concluded that pipecolic acid is formed in the medium by exoenzymes. This is not the case, however: A mixture of DL-[4,5-T_2]-

lysine and DL-[6-^{14}C]lysine can be quantitatively reisolated from the culture filtrate of *Neurospora* without loss of activity or change in the T/^{14}C-ratio even after 24 hours incubation (*cf.* Results). Thus, lysine is taken up by the fungus and is then converted to pipecolic acid.

The differential uptake of D-lysine and L-lysine is not due to the inhibition of uptake of D-lysine by L-lysine:

When D-[6-^{14}C]lysine and L-[U-^{14}C]lysine were fed to *Neurospora* in two separate experiments, only 0.24% of the radioactivity of L-lysine but 7.7% of the radioactivity of D-lysine fed is recovered in pipecolic acid (Table II).

As pointed out in the Introduction and outlined in Fig. 6 the first step in the biosynthesis of pipecolic acid is α-deamination of lysine. This reaction is likely to be catalyzed by D-aminoacid oxidase (DAOD). This enzyme has been shown to occur in the mycelium of *Neurospora* [34, 35]. L-Aminoacid oxidase (LAOD), however. is also produced by the fungus and has been detected in the mycelium and in the culture medium [34, 35]. The same product, α-keto-ε-aminocaproic acid (**2**), would result by the action of LAOD on L-lysine and of DAOD on D-lysine. If both these reactions took place in *Neurospora*, incorporation of both D- and L-lysine into pipecolic would be expected. Formation of both enzymes, however, is known to depend on the developmental stage of the fungus and on the biotin concentration of the medium. While maximum activity of DAOD is observed one to seven days after inoculation of the medium [34, 36], maximum activity of LAOD is observed in the stationary phase of the development of the fungus between the twelvth to fourteenth day after inoculation [36]. High biotin concentration (5 μg/l) is known to inhibit LAOD activity [36]. It is therefore possible that pipecolic acid might originate from either D-lysine or from L-lysine, depending on the age and growth conditions of the fungus. To test this possibility the experiment depicted in Fig. 3 was therefore repeated (Fig. 4) under conditions (day 14 after inoculation, 0.25 μg biotin per litre) when LAOD is reported to show maximum activity. Again both isomers of lysine were taken up by the fungus, but, at a rate much faster than during logarithmic growth. This can be explained by a lack of transinhibition [37, 38]: Nutrients in the medium are exhausted and the internal pool of free amino acids is likely to be very low. Pipecolic acid isolated from the medium at this stage of growth was predominantly labelled with ^{14}C (from D-[^{14}C]lysine) and had only residual tritium activity (from L-[4,5-T_2]lysine) (Fig. 4). These data show that pipecolic acid is derived mainly if not exclusively from D-lysine and not from L-lysine, at all stages of growth of the fungus. These results are at variance with those of Schweet *et al.* [17] who reported that pipecolic acid is derived from L-lysine in *Neurospora*.

Pipecolic acid disappears from the mycelium 11 hours after application of radioactive lysine (Fig. 3). This observation suggests that pipecolic acid is degraded by *Neurospora*.

The hypothesis to be tested predicts that in the course of this degradation the configuration at C-2 of pipecolic acid is maintained during conversion to lysine. It had been shown [17], and we have confirmed, that pipecolic acid has L-configuration in *Neurospora* (Fig. 5). Thus, the product of L-pipecolic acid should be L-lysine. It was shown by two-dimensional chromatography and autoradiography of a crude extract of the mycelium and of the hydrolysate of the mycelium, that radioactive lysine was formed when *Neurospora* was incubated with radioactive pipecolic acid. No radioactive α-aminoadipic acid or glutamic acid were detectable (*cf.* Results). Degradation of pipecolic acid in *Pseudomonas* in which α-aminoadipic acid and glutamic acid are major degradation products of pipecolic acid [24, 25], and in *Neurospora* are therefore different. Pipecolic acid labelled in different positions (Fig. 2) was synthesized to obtain further information on the mechanism of conversion of pipecolic acid to lysine. When a mixture of DL-[4,5-T_2]-pipecolic acid and DL-[6-^{14}C]pipecolic acid were simultaneously applied radioactive lysine was isolated (Table III, Exp. 1).

According to the hypothesis depicted in Fig. 6 the T/^{14}C ratio of lysine (Table III, Exp. 1) should not differ from the T/^{14}C ratio of DL-[4,5-T_2, 6-^{14}C]pipecolic acid administered to the fungus. The T/^{14}C-ratio of lysine dropped slightly, however, for unexplained reasons. It might be that the commercial DL-[4,5-T_2]lysine carried some of the tritium in 6-position (the position of label is given as *nominal* [4,5-T_2]lysine). If so the pipecolic acid derived from DL-[4,5-T_2]lysine by chemical synthesis should also be labelled in 6-position to a certain extent and loss of a fraction of tritium would

occur during conversion of pipecolic acid to lysine by the fungus (vide infra, Fig. 6). The specific incorporation of pipecolic acid into lysine is ensured as indicated by degradations of lysines isolated from *Neurospora* after administration of labelled pipecolic acids (vide infra).

If the hypothesis outlined in Fig. 6 is correct, loss of tritium (50%) relative to ^{14}C after DL-[6-T]pipecolic acid and DL-[6-^{14}C]pipecolic acid feeding was expected. Lysine isolated from the hydrolysate had a T/^{14}C-ratio slightly lower than predicted (Table III, Exp. 2: 4.0 ± 0.2; theory: 5.0 ± 0.0). This result confirms the observation of Gupta and Spenser [16] who assumed that a primary isotope effect would occur during oxidation of pipecolic acid at C_6: the T/^{14}C-ratio of lysine is lower than expected due to a gain of ^{14}C relative to tritium because C – T-cleavage during oxidation at C_6 of pipecolic acid is rate limiting. The T/^{14}C-ratio of pipecolic acid reisolated from the mycelium should therefore increase. This prediction has been confirmed (Table V, Exp. 1). Increase of the T/^{14}C-ratio in pipecolic acid would in turn yield lysine with an increasing T/^{14}C-ratio as incubation proceeds. In fact free lysine (which is derived from pipecolic acid *later* during incubation) was found to have a T/^{14}C-ratio higher than that isolated from the hydrolysate (Table III, Exp. 2). These data show that pipecolic acid is converted to lysine and that oxidation of pipecolic acid precedes lysine formation (Fig. 6).

Table V. T/^{14}C-ratios of pipecolic acids reisolated from the culture filtrate or extracted from the washed mycelium.

Experiment	Pipecolic acid fed configuration and position of label	T/^{14}C-ratio	Pipecolic acid reisolated from medium after 22 hours T/^{14}C-ratio	Pipecolic acid reisolated from mycelium after 22 hours T/^{14}C-ratio
1	DL-[6-T]-DL-[6-^{14}C]-	10.0 ± 0.1	10.3 ± 0.1	31.0 ± 0.8
2	DL-[2-T]-DL-[6-^{14}C]-	10.0 ± 0.1	10.2 ± 0.2	16.4 ± 0.4
3	DL-[4,5-T_2]-DL-[6-^{14}C]-	10.0 ± 0.2	9.4 ± 0.1	9.4 ± 0.4

Oxidation at C-2 of pipecolic acid does not seem to occur, because almost all tritium is retained in lysine after DL-[2-T, 6-^{14}C]pipecolic acid feeding (Table III, Exp. 3). The slight drop in the T/^{14}C-ratio of lysine (Table III, Exp. 3) as compared to the T/^{14}C-ratio in pipecolic acid can be attributed to a secondary isotope effect. If [2-T]pipecolic acid is not metabolized at the same rate as is [6-^{14}C]pipecolic acid the T/^{14}C-ratio in the lysine which forms will decrease but the T/^{14}C-ratio of the pipecolic acid reisolated from the mycelium after incubation will increase. This was in fact observed (Table V, Exp. 2). In contrast, [4,5-T_2]pipecolic acid and [6-^{14}C]pipecolic acid are metabolized at an equal rate (Table V, Exp. 3). Treatment with L-lysine decarboxylase of lysine obtained from Exp. 3 (Table III) showed that the lysine is converted to cadaverine and hence has L-configuration (*cf.* Results). The T/^{14}C-ratio of cadaverine (*i. e.* 1,5-diaminopentane) so formed is identical with the T/^{14}C-ratio of lysine (Table IV, Exp. 1). These experiments showed that pipecolic acid is specifically converted to L-lysine.

It was also necessary to demonstrate that it is L-pipecolic acid which is converted to L-lysine. Simultaneous application of L-[4,5-T_2]pipecolic acid and DL-[2′-^{14}C]pipecolic acid to the mycelial suspension showed (*cf.* Results) that both isomers of pipecolic acid were taken up by the fungus. If L-lysine is derived from L-pipecolic acid, only L-[4,5-T_2]pipecolic acid and L-[2′-^{14}C]pipecolic acid, but not D-[2′-^{14}C]pipecolic acid should be incorporated into lysine and the T/^{14}C-ratio in the product should increase to 200% of the T/^{14}C-ratio of the substrate (= 100%). If, however, D-pipecolic acid is the precursor only ^{14}C would be expected in L-lysine. As can be seen from Table III (Exp. 4) L-lysine is derived from L-pipecolic acid.

To obtain further corroboration for the specific conversion of pipecolic acid to L-lysine, the latter (obtained from Exp. 4, Table III) was again degraded by treatment with L-lysine decarboxylase (Table IV, Exp. 2).

The CO_2 from C_1 of L-lysine contained all ^{14}C of the lysine whereas the cadaverine (C-atoms 2 to 6 of L-lysine) contained tritium only. Thus, the carboxyl group of L-pipecolic acid is converted to the carboxyl group of L-lysine, and radioactivity in C-atoms 2 to 6 of pipecolic acid is recovered in C-atoms 2 to 6 of lysine. The same conclusion can be drawn from Exp. 3 (Table IV).

All data here reported are consistent with the view that D-lysine is converted to L-lysine and that L-pipecolic acid functions as an intermediate in this conversion.

Conversion of one isomer of an amino acid to the other usually takes place by virtue of a racemase or by reversible transamination [39, 40]. The reaction sequence depicted in Fig. 6 represents a third mechanism. Unlike racemization and reversible transamination, however, the steps outlined in Fig. 6 are not reversible: L-pipecolic acid gives rise to L-lysine only and D-lysine but not L-lysine serves as the precursor of L-pipecolic acid. The irreversibility of the conversion is also evident from the observation that a mixture of DL-[6-T]lysine and DL-[6-^{14}C]lysine is incorporated into pipecolic acid without loss of tritium [16].

The conversion of D-lysine to L-lysine *via* L-pipecolic acid may also occur in higher plants. It has been shown that L-pipecolic acid is derived from D-lysine in *Sedum* and *Nicotiana* [23].

Pipecolic acid is known to have L-configuration in several higher plants [23] and Fowden has shown that pipecolic acid is degraded to lysine in *Acacia homalophylla* [9].

A mechanism similar to that outlined in Fig. 6 may also apply to the biosynthetic ornithine-proline relationship.

The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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