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IN VITRO SYNTHESIS OF L-PIPECOLATE FROM L-LYSINE: INCONSISTENT WITH ε-N-ACETYL-L-LYSINE AS AN OBLIGATORY INTERMEDIATE

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Summary: In vitro synthesis of ^{14}C-L-pipecolic acid from L-[U-^{14}C]lysine was demonstrated for the first time in the rat tissues. Acetyl donor was not required for this synthesis. No labeling of ε-N-acetyl-L-lysine was detected during ^{14}C-pipecolate synthesis. When ε-N-acetyl-L-[U-^{14}C]lysine was substrate, large quantities of ^{14}C-lysine but only very small quantities of ^{14}C-pipecolate were detected, indicating the synthesis of L-pipecolate from ε-N-acetyl-L-lysine to be by way of L-lysine. Although the present data are inconsistent with the hypothesis that removal of α-NH_2 group from L-lysine in preparation for L-pipecolate formation requires ε-N-substitution, the involvement of an enzyme-bound ε-N-substituted L-lysine in this pathway cannot be ruled out.

INTRODUCTION

An outstanding feature of mammalian L-lysine metabolism is its inertness towards the attack of L-amino acid oxidase (1) and its inactivity in transamination reactions (2) which are normally known to occur in other amino acids. It has been observed, however, that even though D-amino acid oxidase and L-amino acid oxidase are not reactive with the free D- or L-lysine they are reactive with their respective ε-N-acetylated lysine isomers (3). It has also been observed that ε-N-substituted DL-lysine will support growth of rats when incorporated into their lysine-deficient diets (4-6). Observations such as these have been the basis for the suggestion of Neuberger and Sanger (3) that acetylation of the ε-NH_2 group of L-lysine to facilitate the removal of α-NH_2 group may be the first step in the utilization of L-lysine in the mammals.

Using a cerebrointraventricular injection technique we have reported earlier (7,8) that L-lysine is metabolized in the rat brain mainly to L-pipecolic acid, a reaction presumably involving the removal of the α-NH_2 group of L-lysine and the subsequent cyclization of the resultant product. Our recent success in demonstrating L-pipecolic acid formation from L-lysine

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in vitro provides a system to test the hypothesis of Neuberger and Sanger. We report here the first demonstration of this *in vitro* reaction in the rat tissues,[1] and the evidence which indicates that the metabolic pathway of L-lysine to L-pipecolic acid in the rat may not require ε-N-acetyl-L-lysine as an obligatory intermediate.

MATERIALS AND METHODS

Preparation of Tissue Samples - Male Sprague-Dawley rats (ARS/Sprague-Dawley, Madison, Wis.) weighing 350-400 g were used. The rearing conditions of the animal were same as those reported previously (8). In each experiment 3 rats were killed by decapitation. The brains and kidneys were dabbed dry, weighed and minced. Whole tissue homogenate was prepared by homogenizing 1/3 of the minced brain (approx. 1.5 g) and kidney (approx. 2.3 g) in 3 ml of 0.1 M glycyl-glycine buffer, pH 8.6 (tissue to buffer ratio 1:2, w/v) (in other experiments 0.1 M potassium phosphate buffer, pH 7 was used) in a glass tissue grinder equipped with a Teflon pestle driven by a motor at 650-750 rpm for 10 strokes.

For the preparation of the soluble and mitochondrial fractions, the rest of the minced brain and kidney were homogenized in 6 ml of 0.32 M sucrose containing 0.1 mM EDTA. One half of the two sucrose homogenates were centrifuged at 10,000 x g for 30 min. The supernatant was recentrifuged at 17,000 x g for 30 min. The supernatant fluid of the second centrifugation was designated the soluble fraction. The mitochondrial fraction was prepared from the rest of the sucrose homogenates as follows. After centrifugation at 1,000 x g for 10 min, the supernatant was subjected to another centrifugation at 14,500 x g for 10 min. The centrifugation was repeated once more at 15,000 x g for 10 min by suspending the pellets in 10 ml of 0.32 M sucrose containing 0.1 mM EDTA. The precipitate after suspending in 3 ml of the glycyl-glycine or potassium phosphate buffer was the mitochondrial fraction.

Incubation Conditions - Formation of L-pipecolic acid from L-lysine was studied by incubating 3 ml of each tissue preparation with the following ingredients (final volume 3.5 ml): 1 μCi L-[U-^{14}C]lysine (300 μCi/μmole, Schwarz/Mann Lab.), 2 μmoles acetyl phosphate (or acetyl CoA), 10 μmoles α-ketoglutarate (a possible NH_2-group acceptor), 5 μmoles pyridoxal phosphate, 300 μmoles glycyl-glycine buffer, pH 8.6 (or potassium phosphate buffer, pH 7) and 100 μmoles ε-N-acetyl-L-lysine (Calbiochem.) (as a trapping agent for labeled ε-N-acetyl-L-lysine). In separate experiments the acetyl donor and ε-N-acetyl-L-lysine was omitted from the incubation mixture. The reaction mixtures were incubated at 37°C in a water bath shaker with gentle shaking. The reaction was terminated at 0 and 120 min by adding equal volume of acetone. After storing at 4°C for 2 hr, the precipitate was separated by centrifugation and extracted with 2 ml 50% acetone. After centrifugation the two supernatants were combined and dried by blowing with a stream of N_2 gas. The extracted reaction products were stored frozen until they were analyzed.

The study of L-pipecolic acid formation from ε-N-acetyl-L-lysine was carried out under the conditions similar to those described above for labeled L-pipecolic acid formation from L-[U-^{14}C]lysine except that ε-N-acetyl-L-

[1] During our work of this *in vitro* reaction, a similar work on embryos and brains of chicks and mice was reported (9).

[U-^{14}C]lysine (300 μCi/μmole) was used in place of L-[U-^{14}C]lysine, and that L-pipecolate was used as a trapping agent instead of ε-N-acetyl-L-lysine.

Analysis of Reaction Products - Reaction products extracted from the incubation mixtures were analyzed by the Technicon amino acid analyzer equipped with a Durrum DC-1A column, and by paper chromatography. The amino acid analysis was performed similar to that described previously (7) by elution with Pico-Buffer II (Pierce Chemical Co.) programmed for buffers A, B and C for 75, 35, and 75 min, respectively. The radioactive samples collected by an automatic fraction collector were counted in a Mark I liquid scintillation spectrometer in Hydromix (Yorktown Research). Quenching was corrected by automatic external standardization.

Ascending paper chromatography was carried out on Whatman 3 MM filter paper in iso-propanol:H_2O:NH_4OH (8:1:1, v/v). Reference standards were visualized by spraying with 0.2% ninhydrin in ethanol and heating. The radioactive compounds were located by scanning with a Packard radiochromatogram scanner.

Preparation of ε-N-Acetyl-L-[U-^{14}C]Lysine - ε-N-Acetyl-L-[U-^{14}C]lysine was prepared according to the procedure of Neuberger and Sanger (6) for the unlabeled compound. One hundred μCi of L-[U-^{14}C]lysine (0.333 μmole) was used for this synthesis. The recovered compound was purified through the amino acid analyzer as described above. The purified compound was free of contamination as judged by amino acid analysis and paper chromatography described above. The overall recovery was 18%.

RESULTS

Labeling of L-Pipecolate from L-[U-^{14}C]Lysine - Two hr incubation of L-[U-^{14}C]lysine with each of the three tissue preparations of the rat brain or kidney resulted in labeling of L-pipecolic acid [the labeled pipecolate was characterized as the L-form previously (7,8)] (Table 1). Labeling of pipecolate in the 0-hr controls was negligible. In general, the kidney tissue preparations were relatively more active in the production of ^{14}C-L-pipecolate from L-[U-^{14}C]lysine than those of the brain. Whole tissue homogenate from either the brain or kidney appears to have the highest level of this activity. This activity was very much equally distributed between the soluble and the particulate (mitochondrial) preparations from either the brain or kidney.

Elimination from the incubation mixture of the acetyl donor and the unlabeled ε-N-acetyl-L-lysine as a trapping agent for the labeled intermediate did not appear to lower the labeling of L-pipecolic acid when compared to the result obtained from the samples containing these two ingredients (Table 1).

Table 1. In Vitro Formation of ^{14}C-Pipecolate from L-[U-^{14}C]Lysine by Rat Brain and Kidney Tissue Preparations.

Tissue Preparation	Labeled Metabolites Recovered		Pipecolate / Lysine
	Lysine	Pipecolate	
	dpm/g tissue		x 10^{-2}
Brain			
Whole Homogenate	484,279	11,493	2.37
Whole Homogenate*	470,840	11,786	2.50
Whole Homogenate†	266,907	12,539	4.70
Soluble Fraction	455,221	6,090	1.34
Mitochondria Fraction	929,727	15,628	1.68
Kidney			
Whole Homogenate	421,478	21,259	5.04
Soluble Fraction	480,371	12,243	2.55
Mitochondria Fraction	446,051	15,280	3.43

*Acetyl donor and unlabeled ε-N-acetyl-L-lysine omitted.

†Incubation in potassium phosphate buffer, pH 7; unlabeled ε-N-acetyl-L-lysine omitted.

Brain and kidney tissue preparations were incubated with L-[U-^{14}C]lysine in glycyl-glycine buffer, pH 8.6 (unless otherwised noted) and other ingredients as described in the text. After 2 hr incubation at 37°C, the reaction product were extracted and analyzed by the amino acid analyzer as described. The elution times of L-lysine, L-pipecolate and ε-N-acetyl-L-lysine in this system was 162,119 and 95 min, respectively.

After 2 hr incubation no labeled ε-N-acetyl-L-lysine was detected in any of the samples even though most of the unlabeled ε-N-acetyl-L-lysine was recovered as judged from amino acid analysis. It was realized that the inclusion of large quantities (150 μmoles) of unlabeled L-pipecolate in the incubation mixture did not affect the labeling of this compound (results not shown). Therefore, it was not necessary to include this unlabeled compound in order to label pipecolate. It was noted that the replacement of glycylglycine buffer with phosphate buffer slightly increased the labeling of L-pipecolate (i.e., an increase of ^{14}C-L-pipecolate to ^{14}C-L-lysine ratio) (Table 1). This result was reproducible as observed in later experiments.

The above results were confirmed by paper chromatography and radiochromatogram scanning. These experiments also showed the formation of ^{14}C-pipecolate (R_f 0.34) from L-[U-^{14}C]lysine (R_f 0.16) without detection of ^{14}C-ε-N-acetyl-lysine (R_f 0.27) (results not shown). In this instance only a very small radioactive peak on the chromatogram corresponding to L-pipecolate was visible beside the very prominent ^{14}C-L-lysine peak and no ^{14}C-ε-N-acetyl-

Table 2. In Vitro Labeling of L-Pipecolate and L-Lysine from ε-N-Acetyl-L-[U-^{14}C]Lysine by Rat Brain and Kidney Homogenates.

Tissue Preparation	Labeled Metabolites Recovered		
	ε-N-Acetyl-Lysine	Lysine	Pipecolate
		dpm/g tissue	
Brain Whole Homogenate			
No L-Lysine	18,898	157,494	726
No L-Lysine*	129,767	9,735	-
Plus L-Lysine	10,965	82,915	113
Kidney Whole Homogenate			
No L-Lysine	2,632	63,362	2,431
No L-Lysine*	75,159	6,184	-
Plus L-Lysine	5,320	47,220	862

*The reaction was stopped at 0 min by acidifying with H_2SO_4 to pH 1.

Incubation conditions were similar to those in Table 1 except that 1 μCi of ε-N-acetyl-L-[U-^{14}C]lysine was used in place of L-[U-^{14}C]lysine, and the trapping agent, if used, was 100 μmole unlabeled L-lysine. Reaction product extraction and analysis were same as those in Table 1; other details in text.

lysine was detectable. Since this technique was much less sensitive than that utilizing the combination of an amino acid analyzer and a liquid scintillation spectrometer, its usage in this work was only supplementary.

The other radioactive products in the reaction mixture detectable by the amino acid analyzer were compounds eluting at positions corresponding to saccharopine (elution time 112 min), α-aminoadipate (103 min) and Δ'-piperideine-2-carboxylate (40 min). However, no attempts were made for their definitive characterization.

Labeling of L-Pipecolate from ε-N-Acetyl-L-[U-^{14}C]Lysine - When ε-N-acetyl-L-[U-^{14}C]lysine was incubated with the whole tissue homogenate of brain or kidney for 2 hr, most of the radioactivity was recovered as labeled L-lysine (Table 2). Only very small amounts of labeled L-pipecolate was detected in the above incubation mixtures; this labeling was reduced substantially when large quantities of unlabeled L-lysine was included in the incubation mixtures (unlabeled L-lysine recovered at the end of the incubation) (Table 2).

The deacetylation of ε-N-acetyl-L-[U-^{14}C]lysine as shown above was most likely an enzymatic reaction, since similar experiments carried out with the sulfuric acid-denatured tissue preparations formed only about 7% of free ^{14}C-

L-lysine, but no ^{14}C-pipecolate, from ε-N-acetyl-L-[U-^{14}C]lysine (Table 2). Similar results could also be obtained from incubation with boiled tissue preparations.

Results obtained from analysis of reaction products by paper chromatography agree very much with those from amino acid analysis. Beside ε-N-acetyl-L-[U-^{14}C]lysine, the only labeled compound detectable was ^{14}C-lysine (results not shown). Because of the lower sensitivity of this technique, no ^{14}C-pipecolate peak was detectable.

DISCUSSION

Even though L-lysine metabolism in the mammals is in general believed to be via saccharopine (ε-N-[glutaryl-2]-L-lysine) (10,11), we have recently demonstrated its degradation to L-pipecolate to be a viable pathway (7,8). The metabolism of L-lysine to L-pipecolic acid, unlike that via saccharopine, requires the removal of the α-NH_2 group of L-lysine and the cyclization of the resultant intermediate. Elimination of the α- but not the ε-NH_2 group of L-lysine in the process of L-pipecolate formation in the intact rat has been previously shown (12). A plausible mechanism to facilitate α-deamination of L-lysine would be the substitution of its ε-NH_2 group as proposed by Neuberger and Sanger (3).

Based on the observation that ε-N-acetyl-L-lysine can be synthesized enzymatically (13), and that ε-lysine acylase from rat and hog kidney was able to catalyze the deacetylation of both ε-N-acetyl-L-lysine and α-keto-ε-acetamidocaproic acid (14,15), Paik (16) has proposed the following metabolic pathway in support of the Neuberger-Sanger hypothesis (3): L-lysine → ε-N-acetyl-L-lysine → α-keto-ε-acetamidocaproate → α-keto-ε-aminocaproate ⇄ Δ'-piperideine-2-carboxylate.

A review of the literature revealed that the experimental evidence supporting the above pathway was mainly derived from enzymatic study of the individual reactions. It was also noted that the enzyme which catalyzed the

synthesis of ε-N-acetyl-L-lysine from L-lysine was indistinguishable from ornithine transcarbamylase (13).

Our present work which demonstrated the *in vitro* formation of L-pipecolate from L-lysine does not support the contention that ε-N-acetyl-L-lysine is an obligatory intermediate in this pathway. Two pieces of evidence were presented. First, labeled ε-N-acetyl-L-lysine was not trapped by the presence of excess amounts of unlabeled compound in the incubation mixture. Secondly, when ε-N-acetyl-L-[U-^{14}C]lysine was substrate, the labeling of L-lysine was much more active than the labeling of L-pipecolate. And labeling of L-pipecolate was reduced if unlabeled L-lysine was included in the incubation. These data support the view that L-pipecolate can be derived from ε-N-acetyl-L-lysine but only by virtue of L-lysine, i.e., ε-N-acetyl-L-lysine → L-lysine → L-pipecolate.

A noteworthy point of the L-pipecolate-forming system is that it was not inhibited by phosphate buffer at 0.1 mM. Whereas the synthesis of ε-N-acetyl-L-lysine by beef liver enzyme preparation reported by Paik and Kim (13) was inhibited 75% at this phosphate concentration. Furthermore, the labeling of pipecolate from L-[U-^{14}C]lysine in our system did not require externally added acetyl donor.

It should be stressed, however, that even though our present work does not appear to support the view of Neuberger and Sanger (3), we cannot rule out the possibility of the involvement of an ε-N-substituted intermediate in the L-lysine → L-pipecolate pathway. It is known that some metabolic intermediates are enzyme bound and do not exchange with the externally added intermediates. If this is the case the addition of high quantities of unlabeled ε-N-acetyl-L-lysine would, of course, not result in the trapping of labeled ε-N-acetyl-lysine. This mechanism would predict the labeling of pipecolate from ε-N-acetyl-L-[U-^{14}C]lysine but at a much reduced level (as shown in Table 2), since the labeled substrate needs be deacetylated to free L-[U-^{14}C]-lysine first which then becomes the enzyme-bound ε-N-acetyl-L-[U-^{14}C]lysine before it can be metabolized to ^{14}C-pipecolate. In this respect, it should

be noted that although ε-N-acetyl-L-lysine has not been found in nature under normal conditions, it was found in the urine of hyperlysinemic children and normal subjects under high lysine load (17,18), suggesting its possible involvement in L-lysine metabolism. However, it is not clear whether this compound actually serves as a precursor for L-pipecolate.

The exact mechanism of the metabolic reactions leading to L-pipecolate synthesis from L-lysine is, therefore, still open to speculation. An NADH-dependent reductase capable of reducing Δ'-piperideine-2-carboxylate to L-pipecolate has been reported previously (19) and observed by us (unpublished results). It is uncertain at the present whether this reaction constitutes the final step of the metabolic pathway of L-lysine → L-pipecolate. In view of the neurological significance of this pathway (7,8,20-23), the need to elucidate the exact pathway mechanism seems imperative.

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