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Δ'-PYRROLINE: AN INTERMEDIARY METABOLITE IN THE CONVERSION OF PUTRESCINE TO 2-PYRROLIDONE

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SUMMARY: Δ'-Pyrroline, an oxidative product of putrescine metabolism, was chemically synthesized and incubated with a rat liver homogenate. The incubation mixture was fractionated on an amino acid analyzer before and after acid hydrolysis. The hydrolyzed sample, in contrast to the unhydrolyzed sample, contained a ninhydrin positive compound that co-chromatographed with γ-aminobutyric acid, the product of 2-pyrrolidone acid hydrolysis. Authentic 2-pyrrolidone had the same retention time as the Δ'-pyrroline metabolite when analyzed by high-pressure liquid chromatography. It is concluded that Δ'-pyrroline is an intermediary metabolite in the pathway from putrescine to 5-hydroxy-2-pyrrolidone.

INTRODUCTION

The metabolic pathway from putrescine (1,4-diaminobutane) to the polyamines spermidine and spermine has been studied in great detail and, moreover, it is generally accepted that these aliphatic amines play an important role in the early stages of growth, differentiation and transformation (1-3). Putrescine, however, is metabolized by alternate pathways but, in contrast to polyamine biosynthesis, very little is understood with respect to their precise intermediary steps and physiological functions.

Recently it was shown that rat liver slices metabolize putrescine to 2-pyrrolidone (4-aminobutyric acid lactam) which, in turn, is oxidized to 5-hydroxy-2-pyrrolidone (4,5). Although the precise step(s) leading to

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the biosynthesis of 2-pyrrolidone from putrescine were unknown the data suggested that a possible intermediate was Δ'-pyrroline (5), a well known oxidative product of putrescine metabolism (6).

In this report we provide direct evidence that rat liver metabolizes Δ'-pyrroline to 2-pyrrolidone.

MATERIALS AND METHODS

Tissue Preparation: Liver was obtained from female Sprague-Dawley rats (80 to 100 days) after decapitation. The tissue was homogenized (10 volumes/g) in sucrose (0.25 M) buffered with Bis Tris Propane (0.05 M), pH 7.4. The homogenate was centrifuged at 500 x g for 15 min at 5° and the supernatant incubated with Δ'-pyrroline as discussed below.

Δ'-Pyrroline Synthesis: Δ'-Pyrroline was synthesized from ornithine and bromosuccinimide (7) and quantitated spectrophotometrically utilizing o-aminobenzaldehyde (8). The reaction mixture contained other ninhydrin positive compounds. Therefore, an aliquot, to which [^{14}C]ornithine was added, was loaded on a double ion exchange column (0.9 x 20 cm) containing Dowex 50 Na^+ (5.0 cm) and AG 1X-2 Cl^- (5.0 cm). The column was washed consecutively with 20 ml of H_2O, 0.1 N HCl and 0.5 N HCl until the effluent was free of ^{14}C. Then Δ'-pyrroline was eluted with 20 ml of 1.0 N HCl. This procedure removed all ninhydrin positive compounds from the Δ'-pyrroline fraction.

Incubation and Sample Preparations: Δ'-Pyrroline (14 μl, 73.5 mM) in 1.0 N HCl was added to the liver homogenate (986 μl) and incubated at 37° shaking for the desired length of time. The reaction was stopped with 2 N acetic acid (1.0 ml) and centrifuged at 3000 x g for 15 min. The supernatant was extracted with 10 volumes of chloroform/methanol (2:1). Metabolites of putrescine are in the chloroform phase (5).

Hydrolysis of Δ'-Pyrroline Metabolite: A portion (2.0 ml) of the chloroform phase was taken to dryness under a stream of nitrogen. The residue was resuspended in 2.0 ml of H_2O and loaded on the double ion exchange column described above. The metabolites were eluted with H_2O (20 ml) and the water wash taken to approximately 1 ml with a rotary evaporator at 30°. The residues were resuspended in 2.0 ml H_2O and 1 ml was hydrolyzed with an equal volume of 12 N HCl for 2 h at 110°. The HCl was removed and the residue dissolved in H_2O. The hydrolyzed and non-hydrolyzed samples were analyzed on an amino acid analyzer as described below. Parallel experiments to which [^{14}C]2-pyrrolidone was added to the acetic acid treated homogenate revealed that approximately 79 percent of the carrier 2-pyrrolidone was recovered after extraction and ion exchange chromatography.

γ-Aminobutyric Acid Analysis: Analysis of γ-aminobutyric acid was carried out utilizing a Beckman 119 CL amino acid analyzer equipped with an automatic sample injector. The column (0.6 x 11.5 cm) contained Beckman W-3 resin. γ-Aminobutyric acid was eluted from the column with an 0.35 M potassium citrate buffer, pH 3.25. A buffer flow rate of 44 ml/h, a ninhydrin flow rate of 22 ml/h and a column temperature of 54° was utilized throughout each run.

2-Pyrrolidone Analysis: The identification of 2-pyrrolidone was carried out with a Waters Associates high-pressure liquid chromatography system equipped with a model 6000A pump, a U6K injector and a LKB 2138 Uvicord S UV monitor equipped with an 8 μl flow cell. The procedure employed reverse phase chromatography utilizing a Whatman Inc. Partisil-10 ODS-2 column (4.6 x 250 mm) and the

solvent was acetonitrile/water (1.5:98.5, V/V). The lower limit of reliable detection of 2-pyrrolidone was determined to be approximately 20 ng and reproducibility was estimated to ± 5 percent.

RESULTS AND DISCUSSION

A previous study (5) had shown that the metabolism of putrescine to 2-pyrrolidone by rat liver slices was significantly inhibited by agmatine and methylglyoxyl bis-(guanylhydrazone). Since agmatine (9) and methylglyoxyl bis(guanylhydrazone) (10) are known to be potent inhibitors of diamine oxidase, the enzyme which oxidizes putrescine to Δ'-pyrroline, the question arose: Is Δ'-pyrroline an intermediary metabolite in the conversion of putrescine to 2-pyrrolidone?

To answer the above question a specific and sensitive assay for 2-pyrrolidone was developed. It was appreciated that acid hydrolysis of 2-pyrrolidone yields γ-aminobutyric acid, and that unlike γ-aminobutyric acid, 2-pyrrolidone does not bind to either cationic or anionic exchange resins. Furthermore, 2-pyrrolidone can be extracted from aqueous solutions with chloroform/methanol (5). Based on these properties of 2-pyrrolidone, an indirect assay was developed to test whether Δ'-pyrroline is metabolized to 2-pyrrolidone. Δ'-Pyrroline was incubated with rat liver homogenate for 2 h and the incubation medium treated as described under "Materials and Methods". Partially purified reaction mixture was hydrolyzed with HCl and fractionated on an amino acid analyzer. Several ninhydrin positive compounds were detected (Fig. 1A), one of which eluted from the column at approximately 44 min and co-chromatographed with an authentic γ-aminobutyric acid (Fig. 1C). γ-Aminobutyric acid was not detected in incubation medium obtained at zero time (Fig. 1B), in homogenates to which Δ'-pyrroline was not added, nor in 2 h incubation medium analyzed prior to acid hydrolysis. It was concluded that the hydrolytic product of Δ'-pyrroline metabolism is extractable with chloroform/methanol; has little affinity for either cationic or anionic exchange resins; and co-chromatographs with γ-aminobutyric acid on an amino acid analyzer. A direct assay to show that Δ'-pyrroline is metabolized to 2-pyrrolidone was developed.

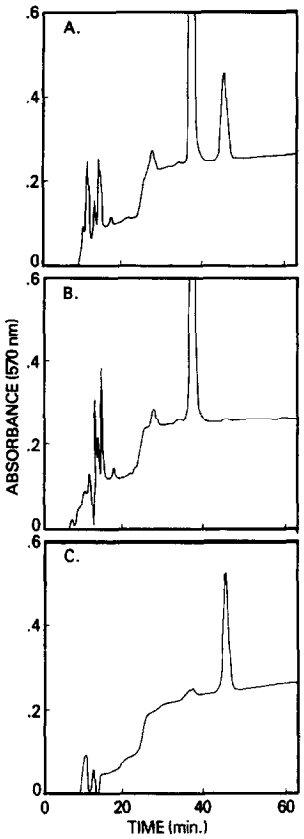
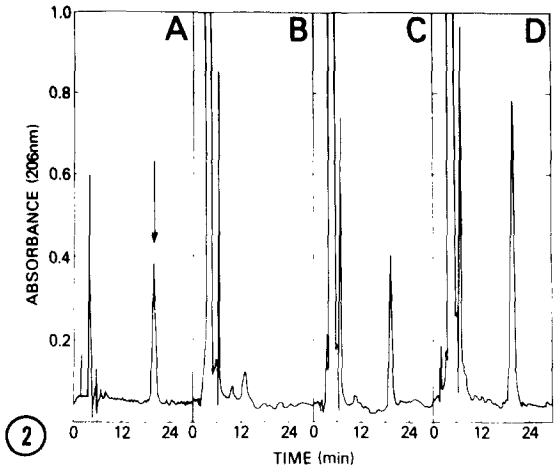


Fig. 1 Amino acid analyzer elution profiles from homogenates incubated with Δ'-pyrroline. Panel A. hydrolyzed incubation medium. Homogenized liver was incubated with Δ'-pyrroline, extracted with chloroform/methanol, run through a double ion exchange column, hydrolyzed with HCl and fractionated as described under "Materials and Methods". Panel B, same as Panel A except the sample was obtained at zero time. Panel C, 10 nmoles of γ-aminobutyric acid.

It was recently reported that caprolactam (6-aminocaproic acid lactam) can be quantitated utilizing reverse phase high-performance liquid chromatography by monitoring the column effluent at 210 nm (11). Using commercial 2-pyrrolidone (4-aminobutyric acid lactam), it was demonstrated that a similar system detected ng quantities of 2-pyrrolidone (Fig. 2A). Direct analysis of chloroform/methanol extracts of rat liver homogenates incubated for 2 h with Δ'-pyrroline revealed the presence of a compound (Fig. 2C) with the same retention time as authentic 2-pyrrolidone standard (Fig. 2A). This peak was not present in a zero time rat liver homogenate containing Δ'-pyrroline (Fig. 2B). Addition of authentic



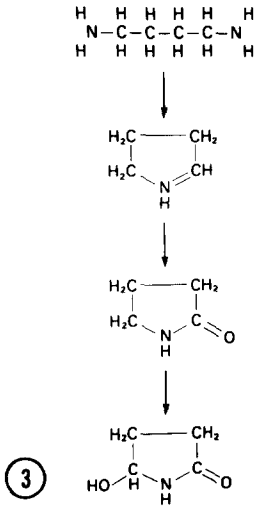


Fig. 2 High-pressure liquid chromatography retention profiles obtained from rat liver homogenates incubated with Δ'-pyrroline. Panel A, 2-pyrrolidone standard (5 µl, 125 ng). The HPLC system utilized is as described under "Materials and Methods". Panel B, extracted liver homogenate at zero time. Δ'-pyrroline was added to a rat liver homogenate containing 2 N acetic acid and extracted with chloroform/methanol as described under "Materials and Methods". An aliquot (50 µl) of the chloroform extract was injected directly into the HPLC system. Panel C, same as Panel B but the liver homogenate was incubated for 2 h with Δ'-pyrroline before the incubation medium was terminated and extracted with chloroform/methanol. Panel D, the same as Panel C except 5 µl (125 ng) of authentic 2-pyrrolidone was added and the total volume of 55 µl was injected into the HPLC system.

Fig. 3 Proposed metabolic pathway from putrescine to 5-hydroxy-2-pyrrolidone showing Δ'-pyrroline as an intermediary metabolite between putrescine and 2-pyrrolidone.

2-pyrrolidone to the 2 h chloroform extract (Fig. 2C) showed that the unknown Δ'-pyrroline metabolite has the same retention time as 2-pyrrolidone (Fig. 2D).

We conclude that Δ'-pyrroline is metabolized to 2-pyrrolidone by rat liver and suggest that the complete pathway from putrescine to 5-hydroxy-2-pyrrolidone is as shown in Figure 3.[#]

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[#]Dr. Julio J. Ludwieg, Brain Tumor Research Center, University of California, San Francisco has confirmed the original observation that putrescine is metabolized to 2-pyrrolidone and Δ'-pyrroline is an intermediary metabolite (personal communication).

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