A Study on the Metabolism of Spermidine in Mammals: Purification and Identification of a Newly Identified Metabolite, 2-Oxo-1-Pyrrolidinepropionic Acid, in Rat Urine

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In order to study the metabolism of spermidine in mammals, radioactive spermidine was injected intraperitoneally into a rat and urine was collected for analysis. Incorporation of radioactivity into putreanine, isoputreanine, spermidic acid, and N-aminopropylpyrrolidin-2-one was confirmed by ion-exchange chromatography, thin layer chromatography, and paper electrophoresis, the highest radioactivity being observed in the non-polar and acidic fraction of the collected urine. A radioactive compound was purified from the non-polar and acidic fraction, and identified as 2-oxo-1-pyrrolidinepropionic acid by comparison of its behavior on ion-exchange chromatography and thin layer chromatography with that of authentic 2-oxo-1-pyrrolidinepropionic acid, and recrystallization with the authentic compound. Acid hydrolysis of the radioactive compound produced radioactive spermidic acid, confirming the identification. To examine the interconversion between isoputreanine and N-aminopropylpyrrolidin-2-one, these compounds were deuterated and then intraperitoneally injected into a rat. Analysis of 24-h urine by gas-chromatography–mass-spectrometry indicated no interconversion between the two metabolites of spermidine under these conditions. An intracerebroventricular injection of radioactive spermidine into a rat showed that radioactivity was also incorporated into the metabolites of spermidine in the brain, and oxidative deamination of the aminopropyl moiety of spermidine was thought to be dominant in the central nervous system and vice versa in peripheral organs.

Key words: N-aminopropylpyrrolidin-2-one, oxidative deamination, 2-oxo-1-pyrrolidinepropionic acid, spermidic acid, spermidine.

The polyamines, spermidine, and spermine, and their precursor, putrescine, have been proven to exist in a wide variety of life forms, including mammals, and may be involved in tissue growth and development. The details of their physiological actions and metabolism remain to be clarified.

Nakajima (1) and Noto et al. (2) identified N-(4-aminobutyl)-4-aminopropionic acid (putreanine) in rat brain and liver, and N-(3-aminopropyl)-4-aminobutyric acid (isoputreanine) in rat urine, respectively, as spermidine metabolites. In 1982, Seiler et al. (3) identified N-aminopropylpyrrolidin-2-one as a spermidine metabolite in mouse liver and considered this compound as a precursor amine of isoputreanine. In 1990, Kamimura et al. (4) discovered that 14C-labeled N-aminopropylpyrrolidin-2-one is produced in the brain and urine, as well as the liver, of rats treated with [14C]spermidine. Van den Berg et al. (5) found that in rats, putreanine or isoputreanine further undergoes oxidative deamination at the 8- and 1-positions, respectively, and is metabolized into N-(2-carboxyethyl)-4-aminobutyric acid (spermidic acid). Fussi et al. (6) reported the existence of spermidic acid in bovine brain and in human cerebrospinal fluid. These findings have aroused our interest in the significance of spermidine metabolism in mammals, for particularly as to what is the major metabolic pathway for spermidine and whether or not its metabolic pathway varies in different organs.

In the present study, using rats treated with [14C]spermidine, firstly an unidentified metabolite of spermidine was discovered, which was produced possibly through oxidative deamination. Secondly, in vivo interconversion between spermidine metabolites in rats was investigated. Finally, the difference in spermidine metabolism in the brain and peripheral organs was studied.

MATERIALS AND METHODS

Reagents—1,4-[14C]Spermidine trihydrochloride (CFA 512, 4.1 GBq/mmol) was purchased from Amersham International plc (Buckinghamshire, England). [D]Methanol and [D]acetic acid were purchased from CEA (Commissariat a l'Énergie Atomique, France). N-Aminopropylpyrrolidin-2-one was purchased from Aldrich Chemical Company (Wisconsin, U.S.A.). Other reagents used in this study were commercially available.

Synthesis of N-Propionitritepyrrolidin-2-One, a Synthe-
tic Intermediate—According to the method described by Kamimura et al. (4), 5 ml of acrylonitrile and 5 ml of 2-pyrrolidinone were dissolved in 10 ml of dried toluene, supplemented with 1 g of anhydrous potassium carbonate and a small quantity of dicyclohexyl-18-crown-6, stirred at room temperature for 1 h, and then filtered to remove potassium carbonate. The filtrate was applied to a 20 ml column of alumina, and the column was eluted with 200 ml of ethyl acetate to obtain N-propionitrilepyrrolidin-2-one. The eluate was evaporated to dryness under reduced pressure to obtain coarse N-propionitrilepyrrolidin-2-one. The yield was approximately 65%.

Synthesis of Isoputreanine—one hundred milligrams of N-aminopropylpyrrolidin-2-one was dissolved in 1.5 ml of 6 N hydrochloric acid and then heated in a sealed glass tube at 140°C for 10 h. The acid hydrolysate was evaporated to dryness to remove hydrochloric acid. The dried residue was dissolved in a small volume of water, and then applied to a 1.1 × 21 cm column of Amberlite CG 100 (100-200 mesh, NH₄⁺ form) and developed with 200 ml of 0.2 M ammonium hydroxide. The effluent was sequentially fractionated. Fractions containing a ninhydrin-positive compound were pooled and evaporated to dryness to obtain isoputreanine. The yield was approximately 70%. The synthesized compound was identified by high voltage paper electrophoresis in pyridine : acetic acid : water (5 : 50 : 945, v/v; pH 3.4) in a voltage gradient of 16.7 V/cm for 1 h, and visualized with a 0.2% ninhydrin-acetone solution.

In addition, an aliquot of the product was boiled in methanol saturated with hydrochloric acid under reflux for 10 min to obtain the methylated product (7). The resulting substance was then treated with pentafuoropropionic anhydride (PFPA) to produce a PFPA derivative (8). GC-MS analysis of this derivative revealed a retention time of 2.3 min and a major fragment of /m/z/ 485.

Synthesis of [D₂]N-Aminopropylpyrrolidin-2-One—Three hundred milligrams of N-propionitrilepyrrolidin-2-one was dissolved in 5 ml of [D₂]methanol, supplemented with 300 µl of [D₂]acetic acid and 25 mg of platinum(IV) oxide, and then allowed to react under high pressure (1.1 kg/cm²) with deuterium gas for 3 h. The platinum(IV) oxide was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in water and then applied to a 1.1 × 21 cm column of Amberlite CG 120 (100-200 mesh, H⁺ form). After washing the column with 200 ml each of water and 1 M pyridine, a basic fraction purified on a column of Dowex 50-2 (100-200 mesh, H⁺ form), [D₂]N-aminopropylpyrrolidin-2-one and [D₂]isoputreanine were dissolved in physiological saline, and then injected intraperitoneally into rats. After injection, urine was collected in polyethylene bottles containing 2 ml of 3N hydrochloric acid, and stored at -20°C until analysis. In addition, [14C]spermidine was injected into the cerebral ventricle, and spermidine metabolism in the brain was investigated.

Analysis of Rat Urine—[14C]Spermidine (0.5 MBq) was intraperitoneally injected into rats, and urine was collected for 24 h. Basic metabolites in the urine were isolated and purified according to the methods described by Nakajima et al. (10) and Noto et al. (2). A one-third volume of the urine collected over 24 h was filtered, and then applied to a 1.1 × 10.5 cm column of Dowex 50 × 2 (100-200 mesh, H⁺ form). The non-polar and acidic fraction, acidic and neutral ampholyte fraction, basic ampholyte fraction, and polyamine fraction were eluted with 100 ml each of the following solutions, respectively: water, 1 M pyridine, 2 M ammonium hydroxide, and 3 M ammonium hydroxide in 50% ethanol.

The non-polar and acidic fraction was evaporated to dryness, and the residue dissolved in water. The resulting solution was neutralized with 3 M ammonium hydroxide, and then passed through a 1.1 × 5.3 cm column of Dowex 1 × 4 (100-200 mesh, OH⁻ form). After washing the column with 50 ml of water, an acidic fraction was eluted with 50 ml of 1 M acetic acid. This fraction was then evaporated to dryness, and the residue dissolved in...
methanol, followed by purification by thin layer chromatography (Fig. 1). Portions in which radioactivity was distributed were scraped off, extracted with methanol, and then subjected to identification experiments.

The acidic and neutral ampholyte fraction was supplemented with 50 mg of synthesized spermidic acid, and then evaporated to dryness under reduced pressure. The residue was applied to a 1.1 × 21 cm column of Amberlite CG 120 (100–200 mesh), which had previously been equilibrated with pyridine: acetic acid: water (25:150:825, v/v; pH 3.7), followed by elution with the buffer. The eluates, 3 ml each, were collected, and an aliquot of 300 µl of each was used for measurement of radioactivity. Another aliquot of 50 µl was evaporated to dryness, subjected to color development by Dubin’s method (9), and then examined as to the distribution of radioactivity incorporated into spermidic acid.

The basic ampholyte fraction was evaporated to dryness under reduced pressure. The residue was applied to a 1.1 × 21 cm column of Amberlite CG 120 (100–200 mesh, NH₄⁺ form), and then developed with 200 ml 0.2 M ammonium hydroxide, 150 ml of 1 M ammonium hydroxide, and 180 ml of 2 M ammonium hydroxide. The eluates, 5 ml each, were collected, and an aliquot of 500 µl of each was used for measurement of radioactivity to investigate the distribution of radioactivity incorporated into isotputrene, putreanine, and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Packard Instrument). For measurement of radioactivity, 10 ml of the above mixture was used.

**RESULTS**

*Identification of 2-Oxo-1-Pyrrolidinepropionic Acid—*

Figure 1 shows a silica gel thin layer chromatogram (TLC) of the acidic fraction purified from the 24-h urine of a rat subjected to intraperitoneal injection of [14C]spermidine. The developing solution used was dichloromethane-methanol-acetic acid (8:2:0.02, v/v). A peak of a radioactive component was found at a location coincident with the authentic 2-oxo-1-pyrrolidinepropionic acid (Rf value, 0.6). This unidentified radioactive compound was purified from the acidic fraction by TLC, and then subjected to the following identification experiments.

The purified radioactive compound was supplemented with 113 mg of authentic 2-oxo-1-pyrrolidinepropionic acid, dissolved in hot isopropyl alcohol, and then crystallized adding dropwise of isopropyl ether. This procedure was repeated several times, and the radioactivity of crystals obtained each time was measured. From the second recrystallization onward, the specific radioactivity was constant (Table I).

Next, the purified radioactive substance and 85 mg of authentic 2-oxo-1-pyrrolidinepropionic acid were mixed and dissolved in 1.5 ml of 6 N hydrochloric acid, and then hydrolyzed in a sealed glass tube at 140°C for 14 h. The reaction product was chromatographed on a 1.1 × 21 cm column of Dowex 50 × 2 (100–200 mesh, H⁺ form). Basic ampholyte and polyamine fractions were purified by the same procedures as in the case of urine, and the incorporation of radioactivity into metabolites was investigated.

**Measurement of Radioactivity—** The radioactivity of β-rays was counted with a liquid scintillation counter (TRICARB 460; Packard Instrument, U.S.A.). The scintillation liquid was composed of 1 liter of toluene-Triton X-100 (2:1, v/v), 4 g of 2,5-diphenyloxazole (Wako Pure Chemical Industries), and 0.2 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Packard Instrument). For measurement of the radioactivity of each sample, 10 ml of the above mixture was used.

**Gas Chromatography-Mass Spectrometry—** A GCMS 6020 (Shimadzu, Kyoto) was used. The analysis conditions were as follows: ionization methods, EI and NH₃-CI; ionization voltage, 3.5 kV; electron acceleration voltage, 20 V; trap current, 60 µA; temperature of ion source, 280°C; column, CBP-1 (25 m × 0.25 mm); temperature of sample loading port, 300°C; temperature of column, 210–240°C (programming rate, 8°C/min); temperature of transfer line, 280°C; carrier gas, helium (1 ml/min); and analysis form of N-aminopropilpyrrolidin-2-one and isotputrene, PFPA derivative (8) (to produce the PFPA derivative, a sample was supplemented with 100 µl of each of acetonitrile and pentafluoropropionic anhydride, and then allowed to stand for 30 min at room temperature, followed by removal of the solvent). Isotputrene was previously dissolved in methanol saturated with hydrochloric acid, and then boiled under reflux for 10 min for methylation (7).
column of Amberlite CG 120 with pyridine : acetic acid : water (pH 3.7). The radioactivity emerged at the position corresponding to spermidic acid (Fig. 2), which is the acid hydrolysate of 2-oxo-1-pyrrolidinepropionic acid.

On the basis of the above results, the radioactive compound purified from the acidic fraction of urine of rats injected intraperitoneally with [14C] spermidine was identified as 2-oxo-1-pyrrolidinepropionic acid, and 0.840% of the administered radioactivity was recovered in this metabolite in the 24-h urine (Table II).

Analysis of Radioactive Metabolites in Acidic and Neutral Ampholyte Fractions of Rat Urine—The major metabolites of [14C] spermidine found in the acidic and neutral ampholyte fraction of rat urine were \( \gamma \)-aminobutyric acid and spermidic acid. The proportions of radioactivity incorporated into these compounds were 3.080 and 0.159%, respectively (Table II).

Analysis of Radioactive Metabolites in Basic Ampholyte Fraction of Rat Urine—The [14C] spermidine metabolites found in the basic ampholyte fraction of rat urine were putreanine, isoputreanine, and N-aminopropylpyrrolidin-2-one. The proportions of radioactivity incorporated into these compounds were 0.187, 0.094, and 0.571%, respectively (Table II).

Analysis of 36-h Rat Urine after an Intraperitoneal Injection of [14C] Spermidine and of Rat Brain 36 h after an Intracerebroventricular Injection of [14C] Spermidine—To determine whether or not spermidine metabolism differs between the brain and peripheral organs, analyses were performed on 36-h rat urine after an intraperitoneal injection of [14C] spermidine and on rat brain removed 36 h after an intracerebroventricular injection of [14C] spermidine. The distributions of radioactivity of spermidine and its metabolites in the urine and brain are shown in Table III. The ratio of radioactivity incorporated into putreanine and isoputreanine + N-aminopropylpyrrolidin-2-one was calculated to be 1 : 3.4 in urine and 15 : 1 in brain, suggesting a difference in spermidine metabolism between them, because the former metabolite is the product of oxidative deamination of the aminopropyl moiety of spermidine, and the latter are the products of that of the aminobutyl moiety of the polyamine.

Study of Interconversion between \([D_2] \gamma\)-Aminopropylpyrrolidin-2-One and \([D_2]\) Isoputreanine—Excretion of \([D_2]\) isoputreanine into 24-h rat urine was studied after an intraperitoneal injection of 200 mg/kg of \([D_2]\) N-aminopropylpyrrolidin-2-one, and excretion of \([D_2]\) N-aminopropylpyrrolidin-2-one into 24-h rat urine after the administration of 250 mg/kg of \([D_2]\) isoputreanine. According to the procedures described above, the urine collected was applied to a column of Dowex 50 \( \times \) 2 (100-200 mesh, H\(^+\)).

**TABLE I.** Recrystallization of radioactive 2-oxo-1-pyrrolidinepropionic acid purified from the urine of a rat injected intraperitoneally with radioactive spermidine.

<table>
<thead>
<tr>
<th>Crystallization</th>
<th>Recovery (mg)</th>
<th>Specific radioactivity (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69.4</td>
<td>605</td>
</tr>
<tr>
<td>2</td>
<td>53.1</td>
<td>430</td>
</tr>
<tr>
<td>3</td>
<td>38.4</td>
<td>445</td>
</tr>
<tr>
<td>4</td>
<td>27.3</td>
<td>425</td>
</tr>
</tbody>
</table>

Fig. 2. Ion-exchange chromatography of the acid hydrolysates of the radioactive compound purified from the "non-polar and acidic fraction" of rat urine and authentic 2-oxo-1-pyrrolidinepropionic acid. The acid hydrolysates were applied to a 1.1 \( \times \) 21 cm column of Amberlite CG 120 (100-200 mesh), which had previously been equilibrated with pyridine:acetic acid:water (25:150:825, v/v; pH 3.7). Ion-exchange chromatography was carried out with the same buffer. Three hundred microliter aliquots of 3 ml fractions of the effluent were used for measurement of radioactivity, and 50 u1 aliquots for measurement of the absorbance at 400 nm after reaction with the dinitrophenyl reagent (9).

**TABLE II.** Radioactivity of spermidine metabolites in 24-h urine of a rat injected intraperitoneally with \([14C] \gamma\)-spermidine \(3 \times 10^7\) dpm (0.5 MBq). Spermidine metabolites were purified by ion-exchange chromatography on Dowex 50 \( \times \) 2 (100-200 mesh, H\(^+\) form), Dowex 1 \( \times \) 4 (100-200 mesh, OH\(^-\) form), and Amberlite CG 120 (100-200 mesh, NH\(^+\) form). Radioactivity was counted with a liquid scintillation counter.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>dpm</th>
</tr>
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<tbody>
<tr>
<td>Putreanine</td>
<td>56,210</td>
</tr>
<tr>
<td>Isoputreanine</td>
<td>28,392</td>
</tr>
<tr>
<td>Spermidic acid</td>
<td>47,645</td>
</tr>
<tr>
<td>N′-Aminopropylpyrrolidin-2-one</td>
<td>171,295</td>
</tr>
<tr>
<td>2-Oxo-1-pyrrolidinepropionic acid</td>
<td>252,125</td>
</tr>
<tr>
<td>( \gamma )-Aminobutyric acid</td>
<td>924,030</td>
</tr>
</tbody>
</table>

**TABLE III.** Radioactivity of polyamines and spermidine metabolites in 36-h urine of a rat injected intraperitoneally with \([14C] \gamma\)-spermidine \(1.55 \times 10^7\) dpm (259 kBq) and in the brain of a rat injected intracerebroventricularly with \([14C] \gamma\)-spermidine \(1.55 \times 10^7\) dpm (259 kBq). Spermidine metabolites were purified by ion-exchange chromatography on Dowex 50 \( \times \) 2 (100-200 mesh, H\(^+\) form) and Amberlite CG 120 (100-200 mesh, NH\(^+\) form). Radioactivity was counted with a liquid scintillation counter.

<table>
<thead>
<tr>
<th></th>
<th>Urine (dpm)</th>
<th>Brain (dpm/g (wet weight))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>16,333</td>
<td>141,844</td>
</tr>
<tr>
<td>Spermidine</td>
<td>112,200</td>
<td>1,770,322</td>
</tr>
<tr>
<td>Spermine</td>
<td>5,067</td>
<td>172,166</td>
</tr>
<tr>
<td>Putreanine</td>
<td>34,955</td>
<td>7,871</td>
</tr>
<tr>
<td>Isoputreanine</td>
<td>227</td>
<td>129</td>
</tr>
<tr>
<td>N′-Aminopropylpyrrolidin-2-one</td>
<td>117,360</td>
<td>403</td>
</tr>
<tr>
<td>N′-Monoacetylspermidine</td>
<td>55,767</td>
<td>2,746</td>
</tr>
</tbody>
</table>
form) and the basic ampholyte fraction was prepared. This fraction was evaporated to dryness under reduced pressure, and the residue dissolved in a small quantity of water, which was then passed through a Polyprop chromatography column (Bio-Rad, South Richmond, U.S.A.) filled with 2 ml of Amberlite CG 120 (100-200 mesh, NH₄⁺ form). After washing the column with water, the fraction containing isoputreanine was eluted with 20 ml of 0.2 M ammonium hydroxide, and that containing N-aminopropylpyrrolidin-2-one with 20 ml of 2 M ammonium hydroxide. These two fractions were evaporated to dryness under reduced pressure and then subjected to GC-MS analysis. The 24-h urine in the both studies was proven not to contain [D₂]-isoputreanine, a ring cleavage product of the pyrrolidone derivative, or [D₂]N-aminopropylpyrrolidin-2-one, a closed ring product of the amino acid.

**DISCUSSION**

Polyamines were previously considered to be hardly metabolized in mammals, but it has become evident, through tracer experiments, that they do undergo a certain degree of metabolism. Putreanine, isoputreanine, spermidic acid, and N-aminopropylpyrrolidin-2-one have been identified as metabolites produced on oxidative deamination of spermidine. In the present study, [¹⁴C]spermidine was administered to rats, and from the acidic fraction purified from the rat urine, 2-oxo-1-pyrrolidinepropionic acid was isolated and identified. Succinic acid, which has been identified as an acidic metabolite of polyamines, exhibited almost the same behavior on TLC as that of 2-oxo-1-pyrrolidinepropionic acid. Nevertheless, succinic acid is a dicarboxylic acid and is easily separated from 2-oxo-1-pyrrolidinepropionic acid by anion exchange chromatography. The formation of spermidic acid, a ring cleavage product of the pyrrolidone derivative, by acid hydrolysis is considered to support this identification.

Seiler et al. (3, 11) described that N-aminopropylpyrrolidin-2-one is first formed and converted into isoputreanine. However, since the present study indicated no interconversion between [D₂]N-aminopropylpyrrolidin-2-one and [D₂]isoputreanine, a metabolic pathway of N-aminopropylpyrrolidin-2-one to isoputreanine is unlikely in rats, and thus these compounds may be metabolized through different courses. As illustrated in Fig. 3, it is postulated that N-aminopropylpyrrolidin-2-one may undergo oxidative deamination and be metabolized into 2-oxo-1-pyrrolidinepropionic acid, a newly identified metabolite of spermidine in this study, and isoputreanine, in the same manner, into spermidic acid (putreanine also may be converted into this acid). It is also presumed that 2-oxo-1-pyrrolidinepropionic acid and spermidic acid are the end metabolites of the oxidative deamination system of spermidine, on the basis of their chemical structures and the distribution of radioactivity incorporated into these metabolites in the rat urine collected after injection of [¹⁴C]-spermidine (Table II).

The present study demonstrated that after the administration of [¹⁴C]spermidine, the distribution of radioactivity incorporated into the spermidine metabolites in urine differed from that in brain (Table III). This suggests that oxidative deamination of the aminopropyl moiety of spermidine is predominant in the brain, and that of the aminobutyl moiety is predominant in the peripheral organs. Therefore, enzymes involved in oxidative deamination of spermidine or polyamines in the brain may be different from those in the peripheral organs.

N-Aminopropylpyrrolidin-2-one identified by Seiler et al. (3) and 2-oxo-1-pyrrolidinepropionic acid newly identified in this study are derivatives of 2-oxo-1-pyrrolidine(2-pyrrolidone), a cyclic compound of γ-aminobutyric acid. The pharmacological actions of these compounds arouse our interest because many of the drugs under clinical application or development as nootropic drugs (12) are 2-oxo-1-pyrrolidinone derivatives. We would like to further investigate whether or not 2-oxo-1-pyrrolidinepropionic acid is

![Fig. 3. Postulated pathway for the oxidative deamination of spermidine.](image-url)
produced in the brain and what pharmacological effects it exerts on the central nervous system.

REFERENCES