Enzymatic Assay for Spermidine

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The polyamine spermidine (NH₂(CH₂)₃NH(CH₂)₃NH₂) is widespread in nature. It is present in mammalian tissues (1, 2), plants (3), bacteria (4–7), ribosomes (6, 8), and bacteriophages (9–11). There are various methods for its identification, including steam distillation (12), paper chromatography (13–18), paper electrophoresis (14, 16, 17, 19), ion exchange chromatography (2, 5), gas chromatography (31), and bioassay (20). Paper chromatography, electrophoresis, and steam distillation are not precise methods. Ion exchange chromatography, on the other hand, is accurate but too elaborate for routine use.

This paper deals with a specific and sensitive enzymatic method for the determination of spermidine. The method is based on the formation of Δ¹-pyrroline from spermidine, by freeze-dried spermidine-adapted Serratia marcescens cells. The enzymatic oxidation of spermidine proceeds according to Reaction 1 (21–23).

\[
\text{Spermidine} + \frac{1}{2} \text{O}_2 \rightarrow \text{Propane-1,3-diamine Δ¹-Pyrroline}
\]

Reaction 1

The Δ¹-pyrroline formed is determined colorimetrically with o-aminobenzaldehyde by a nonenzymatic reaction according to Reaction 2 (23–25).

\[
\text{Δ¹-Pyrroline} + \text{o-Aminobenzaldehyde} \rightarrow \text{2,3-Trimethylene-1,2-dihydroquinazolinium}
\]

Reaction 2

The final product, 2,3-trimethylene-1,2-dihydroquinazolinium, is yellow with an adsorption maximum at 435 nm and a molar absorbancy index of 1.86 × 10⁴ liter mole⁻¹ cm⁻¹ (23).

**Experimental Procedure**

* Bacteria—For the preparation of the spermidine oxidase, Serratia marcescens J (15, 23) and Serratia marcescens ATCC 8195 were used. Unless otherwise stated, the following spermidine-yeast extract medium was used: yeast extract (Difco), 0.5 g; KH₂PO₄·3H₂O, 2.0 g; KH₂PO₄, 1.0 g; glucose, 1.0 g; MgSO₄·7H₂O, 0.2 g; and spermidine·3HCl, 0.1 g, per 1000 ml of water. The pH of the medium was adjusted to 7.0 with NaOH. The medium was sterilized by autoclaving.

Bacteria were grown by shaking in a gyrotory shaker (New Brunswick Scientific Company) in 2000-ml flasks containing 1000 ml of medium. After incubation at 30° for approximately 20 hours, cells were harvested by centrifugation at 10,000 × g for 15 minutes at 4°. The cells were then washed three times with 0.15 M sodium chloride solution and freeze-dried over phosphorus pentoxide in a centrifugal freeze-drier (W. Edwards and Company, Ltd., Crowley, Sussex, England). The yield was 0.4 g of dried cells per 1000 ml of medium. The freeze-dried cells may be kept at −20° or under vacuum at room temperature for at least 6 weeks without significant loss of activity.

**Materials**—Spermidine trihydrochloride was obtained from California Corporation for Biochemical Research. Spermine tetrahydrochloride was the product of Mann Research Laboratories. 3,3'-Diaminodipropylamine was bought from Eastman Kodak Company. The diamines used were purchased from Aldrich Chemical Company. o-Aminobenzaldehyde was the product of K and K Laboratories. Yeast extract was obtained from Difco Laboratories and desiccated normal human plasma was obtained from Hyland Laboratory, Los Angeles, California. All other chemicals were commercial products.

**Assay of Spermidine by Ion Exchange Chromatography**—The method of Tabor et al. (5) was used as follows. The sample to be assayed was applied to a Dowex 50-H⁺ (2% cross-linked, 200 to 400 mesh) column (6 × 70 mm). The amines were eluted by an HCl gradient (5) and assayed calorimetrically by the 2,4-dinitro-1-fluorobenzene method (2).

**Standard Enzymatic Assay for Spermidine**—The sample to be assayed (0.1 to 0.3 ml) was added to 50 μmoles of sodium phosphate buffer, pH 6.0 (0.5 ml), and 0.1 ml of freeze-dried, spermidine-adapted S. marcescens cell suspension (5 mg of dry weight of cells freshly suspended in 1.0 ml of 0.15 M sodium chloride). Sodium chloride solution (0.15 M) was then added to a final volume of 0.9 ml. This suspension was incubated at 37° for 30 minutes, and 0.1 ml of 0.1% o-aminobenzaldehyde solution was then added. (The o-aminobenzaldehyde solution was stored in a dark bottle at −20° and used within 2 weeks.) The reaction mixture was incubated at 37° for another 30 minutes. The intensity of the yellow color formed was then determined at 435 nm in a cuvette of 1-cm light path with a Beckman model DU spectrophotometer. A reaction mixture containing 0.15 M sodium chloride solution instead of the sample to be tested served as a blank.

The molar absorbancy index for Δ¹-pyrroline-o-aminobenzaldehyde complex is 1.86 × 10⁴ liter mole⁻¹ cm⁻¹ (23).
RESULTS

Preliminary experiments showed that the highest spermidine oxidase activity of the freeze-dried cells was obtained when S. marcescens J was grown with shaking at 30° in the spermidine-yeast extract medium (0.86 μmole of Δ1-pyrroline was formed by 1 mg of dried cells per hour under standard conditions). When cells were grown at 37° with shaking or at 30° without shaking the activities were 20 and 65% lower. Cells grown in nutrient broth (Difco) or in brain heart infusion broth (Difco) had approximately one-fifth of the activity of the standard preparation.

When S. marcescens ATCC 8195 was grown in the spermidine-yeast extract medium a preparation was obtained which had approximately half the activity of that of S. marcescens J.

The optimal pH for spermidine oxidation by freeze-dried cells of S. marcescens J was 6.7 with sodium phosphate buffer. The optimal temperature for the assay was 37°.

Freeze-dried cells suspended in 0.15 M sodium chloride solution deteriorate rapidly. After storage at -20° for 48 hours, only 30% of the original activity was retained; therefore, for use in these experiments, the cells were suspended in 0.15 M sodium chloride solution immediately before use.

Oxidation of Various Substrates—It was hoped that spermidine-adapted cells of S. marcescens might serve as a specific analytical tool for the quantitative estimation of spermidine by the formation of Δ1-pyrroline, which may be assayed by o-aminobenzaldehyde. To test this assumption, various compounds were incubated with spermidine-adapted S. marcescens cells, sodium phosphate buffer, and o-aminobenzaldehyde. Table I shows that of all of the diamines and polyamines tested, only spermidine produced a compound reacting with o-aminobenzaldehyde significantly. These results agree with our previous findings that various diamines are not oxidized by the partially purified S. marcescens spermidine oxidase (23).

Stoichiometry of Spermidine Oxidation—When small amounts of spermidine were incubated with excess of enzyme, all the polyamine was oxidized within 60 minutes (Fig. 1A). During the oxidation of spermidine, Δ1-pyrroline was formed in stoichiometric amounts (Fig. 1B). The formation of Δ1-pyrroline was linear when 0.05 to 0.4 μmole of spermidine was used. The amount of Δ1-pyrroline formed was 100% of the theoretical, assuming that the molar absorbancy index for Δ1-pyrroline-o-aminobenzaldehyde complex is 1.86 X 10^3 liter mole^-1 cm^-1 (23).

Recovery of Spermidine—Spermidine may be found in biological materials along with butane-1,4-diamine (putrescine) and spermine. Since these compounds might compete with spermidine, in the enzymatic reaction, it was thought worthwhile to test the effect of butane-1,4-diamine and spermine on the oxidation of spermidine by spermidine-adapted S. marcescens cells. Butane-1,4-diamine, 0.1 to 2.0 μmoles, did not affect the oxidation rate of 0.45 μmole of spermidine when tested under standard conditions (Table II). Spermine, 2.0 and 1.0 μmoles, decreased the rate of spermidine oxidation (0.45 μmole) by 31 and 21%, respectively, when tested under standard assay conditions. However, all the spermidine was oxidized, and the expected amount of Δ1-pyrroline was produced when incubation was continued for another 60 minutes (Table II). Similar results were obtained when spermidine was incubated with the Serratia cells in the presence of 3,3'-diaminodipropylamine; the expected amount of Δ1-pyrroline was produced after incubation for 120 minutes. Table II also shows that human plasma and yeast extract did not interfere with the assay of spermidine by S. marcescens cells. The recovery of spermidine was between 88 and 103%.

In the aforementioned experiments, a known amount of spermidine was assayed under various conditions, but no comparison between the enzymatic method and other known methods such as the ion exchange chromatographic assay (5) was given. Such a comparison is given in the following experiment.

Rat liver (6.7 g, wet weight) was suspended in 130 ml of 0.4 N trichloroacetic acid and homogenized in a Waring Blender for 5 minutes. The homogenate was then heated at 100° for 5 minutes and filtered. The filtrate was extracted four times with ether (total volume, 800 ml) to remove the trichloroacetic acid. The aqueous phase was then evaporated on a steam bath and dissolved in 20 ml of distilled water. Aliquots of this solution (0.2 and 0.4 ml) were then tested for spermidine with S. marcescens cells under standard conditions. The level of spermidine was found to be 1.1 and 1.3 μmoles of spermidine per g of rat liver, wet weight. Another 2.0-ml aliquot of the liver extract was applied to a Dowex 50-H+ column (Method B of Tabor et al. (5)) and assayed with dinitrofluorobenzene (2). This assay gave a level of 1.1 μmoles of spermidine per g of rat liver, wet weight. Results obtained with the enzymatic method thus agree with the results

| Table 1 |

| Formation of Δ1-pyrroline from various amines by freeze-dried cells of Serratia marcescens J |

Freeze-dried cells, 0.5 mg, were incubated with 50 μmoles of sodium phosphate buffer, pH 6.6, and 0.1 ml of the amine solution. Total volume was 0.9 ml. After 30 minutes of incubation at 37°, 0.1 ml of 0.1% o-aminobenzaldehyde solution was added. Absorbance was measured at 435 mp after incubation for another 30 minutes at 37°.

<table>
<thead>
<tr>
<th>Diamine</th>
<th>Amount</th>
<th>Emax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermidine</td>
<td>0.4</td>
<td>0.69</td>
</tr>
<tr>
<td>Spermine</td>
<td>2.0</td>
<td>0.09</td>
</tr>
<tr>
<td>3,3'-Diaminodipropylamine</td>
<td>2.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Propane-1,3-diamine</td>
<td>2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Butane-1,4-diamine</td>
<td>2.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Hexane-1,6-diamine</td>
<td>2.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Heptane-1,7-diamine</td>
<td>2.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Octane-1,8-diamine</td>
<td>2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Nonane-1,9-diamine</td>
<td>2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Decane-1,10-diamine</td>
<td>2.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

When commercial spermine was used, as in this experiment, small amounts of Δ1-pyrroline were produced during the oxidation. This is probably due to spermidine, which is a contaminant. Spermine, purified by ion exchange chromatography, did not give rise to Δ1-pyrroline after incubation with S. marcescens cells.
435 μl. B Freeze-dried cells (0.5 mg) were incubated with 50 pmoles of sodium phosphate buffer, pH 6.6, and 0.1 ml of spermidine solution. Total volume was 1.0 ml. Incubation temperature was 37°. After 30 minutes of incubation at 37°, 0.1 ml of 0.1% o-aminobenzaldehyde solution was added. Absorbancy at 435 mN was measured after incubation for another 30 minutes.

On the other hand, the polyamine must be extracted from tissues containing higher concentrations of spermidine should be diluted before being assayed. Spermidine and 3,3'-diaminodipropylamine may affect the rate of spermidine oxidation by freeze-dried cells of Serratia marcescens. If the sample to be tested contains spermidine and one of the polyamines, it is desirable to continue to incubate the reaction mixture for another 60 minutes, to ensure the completeness of the reaction (cf. rat liver). The values obtained for rat liver with the proposed method are in good agreement with the values obtained with the ion exchange chromatographic method (6). The range of the assay is 0.05 to 0.4 μmole of spermidine. Solution containing higher concentrations of spermidine should be diluted before being assayed.

Spermidine and 3,3'-diaminodipropylamine may affect the rate of spermidine oxidation by freeze-dried cells of Serratia marcescens. If the sample to be tested contains spermidine and one of the polyamines, it is desirable to continue to incubate the reaction mixture for another 60 minutes, to ensure the completeness of the reaction (cf. rat liver). If the sample to be tested contains spermidine and one of the polyamines, it is desirable to continue to incubate the reaction mixture for another 60 minutes, to ensure the completeness of the reaction (cf. rat liver). If the sample to be tested contains spermidine and one of the polyamines, it is desirable to continue to incubate the reaction mixture for another 60 minutes, to ensure the completeness of the reaction (cf. rat liver).

The use of the partially purified spermidine oxidase from S. marcescens for the enzymatic assay of spermidine has been suggested by us earlier (23). That preparation, however, is labile and is inactivated even when stored at -20°. The use of a freeze-dried preparation of whole cells for the determination of spermidine, as proposed in this paper, has the advantage of being more stable. It can be stored at least for 6 weeks at -20° or even at room temperature under vacuum without significant deterioration. Data presented in this paper suggest that spermidine may be assayed directly, without extraction, from plasma. On the other hand, the polyamine must be extracted from tissues containing higher concentrations of spermidine should be diluted before being assayed.
negative results. Human plasma and yeast extract do not interfere with the assay. The method may be applied also to tissue extracts.

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REFERENCES
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