Isolation, Characterization, and Turnover of Glutathionylspermidine from *Escherichia coli*  

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**SUMMARY**

Most of the spermidine in *Escherichia coli* is converted to glutathionylspermidine at the end of logarithmic growth. Methods are presented for the determination and isolation of glutathionylspermidine and for its characterization as γ-glutamylcysteinylglycylspermidine. Isotopic experiments demonstrate that the spermidine of glutathionylspermidine is in equilibrium with free intracellular spermidine.

*Escherichia coli* contains 3 to 4 μmol of spermidine per g, wet weight, when grown in minimal medium (2-8); in logarithmically growing cells this amine is present in an unsubstituted form (8, 9) and there is no apparent metabolism (9). During stationary phase (10), however, the spermidine is largely converted to a compound which, in preliminary studies from this laboratory, was partially identified as a glutathione derivative of spermidine (11-13). The present paper describes the isolation of this derivative, its characterization as glutathionylspermidine (Fig. 1), and studies on its turnover in *E. coli*.

**EXPERIMENTAL PROCEDURES**

Amino acids were determined in the amino acid analyzer (14) after performic acid oxidation (15), followed by hydrolysis in 6 N HCl for 18 hours at 108° in vacuo. Spermidine was determined with the gradient described previously≥ (8). Usually 5 to 20 nmol of spermidine were present in the sample placed on the column.

Glutathionylspermidine was assayed on the amino acid analyzer with the same elution system as that used for spermidine. This assay permitted the determination of both the reduced (monosulfide) and the disulfide forms of glutathionylspermidine. Elution patterns for each form are given in Fig. 2, A and B. The validity of this assay for glutathionylspermidine is shown by the following observations: (a) no other naturally occurring amines are eluted at these positions (8); (b) in the absence of glutathionylspermidine (i.e., in extracts obtained from logarithmically growing *E. coli*), no ninhydrin-positive compounds are eluted at these positions; and (c) when *E. coli* extracts containing glutathionylspermidine are oxidized by performic acid, or treated with N-ethylmaleimide after reduction with dithioerythritol, or hydrolyzed by 6 N HCl at 108°, there is no longer any ninhydrin-positive material eluted at these positions, and new peaks appear, which correspond to the expected products (8).

For the assay of glutathionylspermidine in *E. coli*, the cells from 25 ml of culture were collected on a Millipore filter and extracted rapidly with 2.5 ml of cold 10% trichloroacetic acid. After centrifugation, 0.01 volume of 1 N HCl was added, and the trichloroacetic acid was removed by ether extraction. The added HCl ensured an acid pH at the end of the extraction. If the assay was not carried out immediately, the solution was stored at −20° in a stoppered tube. Prior to assay, portions of the extract (0.15 ml) were treated with dithioerythritol (or dithiothreitol) as previously described (8). The value for reduced glutathionylspermidine thus obtained represents the total glutathionylspermidine. If the dithioerythritol were omitted, the assay could be used to determine the proportion of glutathionylspermidine present in the reduced and disulfide forms. However, in the absence of dithioerythritol, any mixed disulfides between glutathionylspermidine and other sulfhydryl compounds would migrate elsewhere, and would not be included in this calculation.

Glutathionylspermidine, as well as spermidine (9), adsorbs easily to glass. Therefore, solutions of glutathionylspermidine were usually made in 0.01 N HCl or 0.2 N acetic acid, and plastic containers were used wherever possible.

14C and 3H were counted in a scintillation counter. To avoid loss of counts due to adsorption, plastic scintillation vials were...

1 We have found it convenient to use an LKB Ultrograd, fitted with two valves, to construct the gradient. Since magnetic stirring resulted in the formation of air bubbles, we eliminated the LKB mixing chamber and stirrer. Adequate mixing of the buffers was accomplished by passing the fluid into the upper end of a vertical glass tube, 5 X 0.6 cm, filled with glass beads, 3 mm in diameter. Prior to each run, any accumulated air bubbles were removed through a 3-way stopcock.

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*COOHCHCH₂CH₂CONHCHCONHCH₂CONH(CH₂)₄NH(CH₂)₄NH₂*  
| NH₂ | CH₂ | SH |

**FIG. 1.** Glutathionylspermidine.
N<sup>1</sup>-Acetylspermidine was synthesized by treatment of N-monoacetyl-1,3-diaminopropane with 4-bromobutyronitrile, followed by catalytic reduction. To 1.45 g of N-acetyl-1,3-diaminopropane hydrochloride, prepared essentially as described (17) for the synthesis of N-acetyl-1,4-diaminobutane, were added 45 ml of absolute ethanol and 3.5 g of K<sub>2</sub>CO<sub>3</sub>. A solution of 1.47 g of 4-bromobutyronitrile in 50 ml of absolute ethanol was added dropwise with magnetic stirring. After 1 hour at room temperature, the mixture was refluxed for 12 hours. After the solution was cooled, 100 ml of absolute ethanol, 0.7 ml of concentrated H<sub>2</sub>SO<sub>4</sub>, and 300 mg of PtO<sub>2</sub> were added, and hydrogenation was carried out for 2.5 hours at room temperature at atmospheric pressure. The catalyst was removed by filtration; the solution was diluted with water and passed through a Dowex 50-X2 column (H<sup>+</sup> form; 2 × 20 cm). The column was washed with water and 250 ml of 0.5 N HCl. The acetyl spermidine was then eluted with 500 ml of 1 N HCl; the solution was evaporated to dryness <i>in vacuo</i>, and acetyl spermidine dihydrochloride was crystallized from pro panol-2 (yield 590 mg). This method of synthesis of N<sup>1</sup>-acetylspermidine is superior to the less specific procedure that we described previously (17).

N<sup>2</sup>-Acetylspermidine was synthesized by condensation of acrylonitrile with N-monoacetyl-1,4-diaminobutane and catalytic reduction of the resultant nitrile, as previously described (17).

Each isomer was checked for purity by elemental analysis and by analysis on the amino acid analyzer (8). No contaminating ninhydrin-reacting materials were present in either preparation.

Materials—[5,8-<sup>14</sup>C]spermidine trihydrochloride<sup>2</sup> with a specific activity of 2.74 × 10<sup>6</sup> cpm per pmol (2.2 μCi per pmol) and H<sub>2</sub>SO<sub>4</sub> (carrier-free) were obtained commercially. [N-<sup>14</sup>]<sup>H</sup>spermidine trihydrochloride was prepared commercially by the procedure of Wilzbach and, after the addition of carrier spermidine, was purified by chromatography on Dowex 50 (2); the final specific activity was 1.1 × 10<sup>7</sup> cpm per pmol (32 μCi per pmol).

Amberlite CG-50 (XE-64) carboxylate resin was used in the pyridine form for preparative chromatography of glutathionylspermidine. The resin was suspended in 5 volumes of 10% pyridine, packed in a column (2 cm diameter, 24 cm height) and washed with 200 ml of 1% pyridine just before use. All chromatographic procedures were carried out at 20-25°.

**RESULTS**

**Isolation of Glutathionylspermidine**

**Growth of Cells and Preparation of Extract**—Preliminary studies (10) had indicated that, even though logarithmically growing <i>E. coli</i> had no glutathionylspermidine, >85% of the spermidine in the organism was converted to this derivative during stationary phase, particularly if the culture was anoxic during this period. In order to obtain the maximal conversion, sufficient glucose had to be present to permit the pH to fall, usually to pH 5.5 to 6.0.

On the basis of these findings a 320-liter culture of <i>E. coli</i> B was grown at 37° with aeration in a minimal medium (19), containing 0.5% glucose.<sup>4</sup> At the end of logarithmic phase, 640 g of glucose were added, and the temperature was maintained at 37°, but the aeration was discontinued. After 2 hours the cells (yield, 1980 g) were collected by centrifugation and were stored at −20°. For each isolation, 25-g portions were thawed and extracted with 4 volumes of 5% trichloroacetic acid.

**Fig. 2.** Elution patterns of oxidized glutathionylspermidine (disulfide), reduced glutathionylspermidine, 1,4-diaminobutane, and spermidine in the amino acid analyzer, with the buffer system described under "Experimental Procedures." A, glutathionylspermidine (disulfide form): 11.5 nmol (containing 23 nmol of spermidine after hydrolysis) dissolved in 1 ml of citrate-NaCl buffer (pH 5.81, 1.35 M Na<sup>+</sup>). The color yield was 110% of that obtained from 23 nmol of spermidine. B, reduced glutathionylspermidine (monosulfide form): 23 nmol, prepared by reducing 11.5 nmol of the disulfide for 2 hours with di thioerythritol as previously described (8), were mixed with sufficient citrate-NaCl buffer to give a final concentration of 1.35 M Na<sup>+</sup> and a total volume of 1 ml. To avoid contamination of the analyzer coil with di thioerythritol, the elution was diverted to drain for the first 10 min; the recorder was not started until 20 min after the sample was injected. The color yield was 80% of that obtained from 23 nmol of spermidine. C, 1,4-diaminobutane (20 nmol) and spermidine (20 nmol) dissolved in 1 ml of the same buffer as in A.

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<sup>2</sup>The abbreviation used is: dansyl, 5-dimethylaminonaphthalene-1-sulfonyle.

<sup>3</sup>The numbering system used in this paper is the same as that which we have used previously (17), and was suggested by Drs. R. S. Tipson and W. E. Cohn (personal communication) on the basis of a modified azo nomenclature (IUPAC Rule 814.6).

<sup>4</sup>We wish to thank Dr. Herbert A. Sober and Mr. David L. Rogerson, Jr., for the preparation of the <i>E. coli</i>.
To facilitate isolation and characterization of glutathionyl spermidine, isotopically labeled material was prepared. The conditions used were the same as above, except that the culture volume was small (50 to 1000 ml). 35S-Labeled cells were grown in 50 ml of medium (19) containing 0.4 mCi of 35SO4 and 35 μmol of 35SO4. Alternatively, 14C or 3H labeling was carried out by adding [3H]spermidine or [3H]spermidine when the cell density was 7 x 10^6 cells per ml; 3 aliquots of 3 to 15 μCi of the desired isotope were added per liter of culture at 10- to 15-min intervals. When the culture reached stationary phase, the incubation was continued as described above. The labeled cells were collected by Millipore filtration and extracted with 5% trichloroacetic acid.

The labeled extracts were then added to the trichloroacetic acid extract from 25 g of unlabeled cells; the trichloroacetic acid was removed either by extraction with ether (three times, each with 2 volumes of diethyl ether) or by passage through a column of Amberlite CG-45 (column dimensions: 3.1-cm diameter, 4.2-cm height, for 110 ml of a 5% trichloroacetic acid extract).

**Chromatography of Glutathionylspermidine on Amberlite CG-50**

The extract was reduced with 0.01 M dithiothreitol, adjusted to pH 7 with 0.1 N NH4OH, and stored in a stoppered vessel for 24 hours at 0°C. If this reduction step was omitted, the chromatography was poor, presumably because of the formation of mixed disulfides of glutathionylspermidine with glutathione and other sulfhydryl compounds during the isolation procedure. The reduced extract (110 ml) was passed through a column of Amberlite CG-50 pyridine prepared as described under "Experimental Procedures." The column was washed with 150 ml of 0.001 M dithiothreitol and 1000 ml of water, in order to remove most of the sulfhydryl compounds other than glutathionylspermidine. The column was then washed with 1000 ml of 1% pyridine over a 48-hour period. Under these conditions glutathionylspermidine was converted to the disulfide form, presumably by dissolved oxygen. This step was introduced since the disulfide form of glutathionylspermidine was adsorbed more tightly to the resin than the reduced form, and hence was purified more readily from contaminants.

The column was then washed successively with: (a) 750 ml of H2O; (b) 1000 ml of 0.2 N acetic acid; (c) 1000 ml of 0.4 N acetic acid; (d) 1000 ml of 0.5 N acetic acid; and (e) 2000 ml of 1 N acetic acid. Most of the glutathionylspermidine was eluted by 1 N acetic acid. This fraction was evaporated to dryness in vacuo and stored in 5 to 10 ml of 0.2 N acetic acid. The yield of glutathionylspermidine disulfide was 13.2 μmol from 25 g wet weight of packed E. coli.

**Characterization of Glutathionylspermidine**

The isolated glutathionylspermidine was eluted as a single peak on the short column of the amino acid analyzer (Fig. 2A). After reduction with dithioerythritol, the material was also eluted as a single peak, but at an earlier time (Fig. 2B). Thus these two elution patterns represent the oxidized (disulfide) and reduced (monosulfide) forms, respectively.

Upon electrophoresis on Whatman No. 3MM paper for 1 hour at 1000 volts/45 cm in 8% formic acid, glutathionylspermidine (detected by radioactivity) migrated as a broad peak 12.5 cm toward the cathode, whereas free spermidine migrated 23 cm. Glutathionylspermidine was further characterized by analysis for amino acids and spermidine after hydrolysis (6 N HCl in vacuo for 18 hours at 108°C) of the performic acid-treated material. In a typical example, the following results were obtained, expressed as nanomoles per sample: glutamic acid, 17.1; cysteic acid, 17.6; glycine, 19.4; spermidine, 17.4 (ratio 1.0:1.0:1.1:1.0). No other amino acids (<5%) were present in the hydrolyzed material. Similarly, no free amino acids or free spermidine (<5%) were present before hydrolysis.

**Sequence Studies**

**Determination of the NH2-Terminal Amino Acid by Dansylation**—When performic acid-oxidized glutathionylspermidine was treated with dansyl chloride and hydrolyzed, only dansylglutamic acid and bisdansylspermidine were found (Table I), indicating that glutamic acid was in the NH2-terminal position. The NH2-terminal position of glutamic acid was confirmed by the marked decrease in glutamic acid observed upon amni acid analysis of the dansylated sample after hydrolysis. All of the cysteic acid (23.6 nmol) and glycine (24.6 nmol) was recovered; only 3.8 nmol of glutamic acid and 1.5 nmol of spermidine were found.

**Partial Hydrolysis**—Further evidence for the sequence of the components of glutathionylspermidine was obtained by analyses of the fragments obtained by partial hydrolysis of the performic acid-oxidized material. [35S]Glutathionyl-[3H]spermidine was treated with performic acid, partially hydrolyzed with 5 N HCl.

**Table I**

<table>
<thead>
<tr>
<th>Solvent I</th>
<th>Solvent II</th>
<th>Solvent III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dansylated products (after hydrolysis)</td>
<td>0.92, 0.53</td>
<td>0.25, 0.07</td>
</tr>
<tr>
<td>Standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dansylglutamic acid</td>
<td>0.53</td>
<td>0.07</td>
</tr>
<tr>
<td>N1, N4-Bisdansylspermidine*</td>
<td>0.92</td>
<td>0.25</td>
</tr>
<tr>
<td>N1, N4-Bisdansylspermidine**</td>
<td>0.92</td>
<td>0.25</td>
</tr>
<tr>
<td>Dansylglycine</td>
<td>0.60</td>
<td>0.17</td>
</tr>
<tr>
<td>Dansyleysite acid</td>
<td>0.30</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Procedure of Hartley (18) in which chromatography on polyamide thin layer plates (Gallard-Schlesinger Chemical Mfg. Corp.) is carried out first in Solvent I in one direction, followed by Solvent II in a perpendicular direction on the same plate. Solvent I, 1.5% (v/v) formic acid; Solvent II, benzene-glacial acetic acid, 9:1 (v/v).

† Carboxymethylcellulose plates (Brinkmann MN-Polygram CEL 300 CM) were used. Solvent III was 0.3 m sodium acetate, pH 5.5, with 5% by volume of 33% formaldehyde.

‡ A blue fluorescent spot due to dansyl hydroxide was also seen.

§ The spot at 0.20 was about 3 to 4 times as intense as the one at 0.08.

¶ This dansylated standard was obtained by dansylation and hydrolysis of N1-acetylspermidine.

‖ This dansylated standard was obtained by the dansylation and hydrolysis of N4-acetylspermidine.
Chromatography of partial hydrolysate of performic acid-treated glutathionylspermidine on Amberlite CG-50

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Elution volume</th>
<th>Per cent of total radioactivity</th>
<th>Nanomoles</th>
<th>Ratio of amino acids to spermidine (after hydrolysis)</th>
<th>Composition of fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unhydrolyzed compound</td>
<td>ml</td>
<td>% H</td>
<td>% S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Unadsorbed</td>
<td>4</td>
<td>70</td>
<td>830</td>
<td>1.0</td>
</tr>
<tr>
<td>B</td>
<td>76-127</td>
<td>30</td>
<td>30</td>
<td>250</td>
<td>0.8</td>
</tr>
<tr>
<td>C</td>
<td>319-415</td>
<td>27</td>
<td>20</td>
<td>220</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>D</td>
<td>482-662</td>
<td>41</td>
<td>0</td>
<td>340</td>
<td>0</td>
</tr>
</tbody>
</table>

* The figures were calculated on the basis of the total radioactivity placed on the column.
* The number of nanomoles of spermidine-containing material in each fraction was calculated from the counts per min of H and the specific activity of the spermidine moiety of the starting glutathionylspermidine.
* Trace amounts of contaminating amino acids (<5%) were present in some of these fractions both before and after hydrolysis. No corrections for these trace contaminants have been made. No free spermidine was found in Fractions B or C before hydrolysis.

Glutathionylspermidine oxidized with performic acid at 37°C, and chromatographed on Amberlite CG-50, as described in Table II. Almost all of the material containing H was adsorbed to the CG-50 resin and was eluted in three fractions. Each fraction was analyzed for its amino acid content before and after hydrolysis; the amount of spermidine was calculated from the H content. On the basis of these analyses, Fraction B contained the starting material, Fraction C contained cysteyleyllyspermidine, and Fraction D contained both glycyspermidine and spermidine. The material which was not adsorbed to the CG-50 column (Fraction A) was predominantly labeled with 35S, with little H. This fraction was chromatographed on Dowex 1-C1 as described in Table III. Four fractions were obtained which, on the basis of amino acid analyses before and after hydrolysis, appeared to be (E) cysteyleylglutamate, (F) cysteyleylglycine, (G) glutamycysteyleylglycine, and (H) glutamyllyspermidine. These results indicate that the sequence is glutamycysteyleyllyspermidine.

Characterization of Glutamycysteyleyllyspermidine

To determine whether the material in Fraction H (Table III) was glutamyllyspermidine or glutamycysteyleyllyspermidine, this material was compared with authentic samples by chromatography on Whatman No. 1 paper (ascending) in n-butyl alcohol:glacial acetic acid:water, and pyridine (20:10:10:5). The glutamyl cysteyleyllyspermidine acid was prepared by performic acid treatment of synthetic glutamyllyspermidine, which was kindly supplied by Dr. E. P. Abraham (20). The glutamyllyspermidine acid was prepared by performic acid treatment, partial hydrolysis, and Dowex 1 chromatography of commercial 35S-glutathione, essentially as described above for glutathionylspermidine. The Rf of α-glutamylcysteyleyllyspermidine acid was 0.24, while the Rf of both the authentic glutamyllyspermidine acid and the isolated material in Fraction H was 0.18.

Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Elution volume</th>
<th>Per cent of total radioactivity</th>
<th>Nanomoles</th>
<th>Ratio of amino acids to spermidine (after hydrolysis)</th>
<th>Composition of fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>Glutamic acid</td>
<td>Cysteine acid</td>
<td>Glycine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>145-166</td>
<td>13</td>
<td>0.04</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>F</td>
<td>320-355</td>
<td>9</td>
<td>0.05</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>G</td>
<td>460-490</td>
<td>17</td>
<td>0.55</td>
<td>1</td>
<td>1.05</td>
</tr>
<tr>
<td>H</td>
<td>520-570</td>
<td>40</td>
<td>0.94</td>
<td>1</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* The amount of 35S placed on the Dowex 1-C1 column represented 67% of the 35S placed on the CG-50 column (Table II). No 3H was detected in any of the fractions listed.

Table III

Dowex 1-C1 chromatography of unadsorbed fraction from CG-50 chromatography.

The material that was not adsorbed to the CG-50 column (Fraction A, Table II) was placed on a Dowex 1-C1 column (0.8 cm diameter, 13 cm high; 200 to 400 mesh; 8% cross-linkage). Elution was carried out by an exponential gradient, consisting of 250 ml of water in the mixing flask and 250 ml of 0.1 M HCl in the reservoir; this was followed by a gradient consisting of 250 ml of 0.01 M HCl in the mixing flask, and 0.1 M HCl in the reservoir flask. Portions of the fractions containing 35S were concentrated, hydrolyzed, and analyzed for amino acids.
Characterization of Glycylspermidine Linkage

The above analyses do not indicate which end of the spermidine is linked to the carboxyl group of glycine, i.e. the N1- or N4-nitrogen. This question was investigated by two methods and the results indicated that at least 85% is linked at the N1 position.

As discussed earlier, dansylation of glutathionylspermidine, followed by hydrolysis in 6 N HCl, yielded bisdansylspermidine. Thin layer chromatography (Solvent III, Table I) showed that this derivative was mainly N4-N3-bisdansylspermidine, indicating that the glutathionyl substitution was predominantly on the N1 position of the spermidine. The presence of some N1-N3-bisdansylspermidine could not be excluded, however, since double spots were obtained even when authentic N1-N3-bisdansylspermidine was chromatographed in Solvent III.

Further evidence for the N1 substitution was obtained by cleaving glutathionylspermidine with spermidine dehydrogenase from Serratia marcescens (21, 22), which has been shown to oxidize and cleave spermidine and its acetyl derivatives at the secondary nitrogen. Thus, oxidation of glutathionylspermidine would result in different products, depending on the position of the glutathionyl substitution.

Therefore, glutathionylspermidine was oxidized with excess spermidine dehydrogenase as described in the legend to Table IV. Sodium borohydride was then added to reduce the aldehydes formed. One aliquot was treated with dithioerythritol to reduce any residual disulfides, and was analyzed for amines; another aliquot was hydrolyzed in 6 N HCl overnight before analysis. The major product of the enzymatic oxidation of glutathionylspermidine was a derivative of diaminopropane, presumably N-glutathionyl 1,3-diaminopropane, which was converted to diaminopropane by acid hydrolysis. A bound form of diaminopropane could only have arisen if the original glutathionyl substitution was at the N1 position. The absence of diaminopropane before hydrolysis or of any 1,4-diaminobutane after hydrolysis, indicated that little or none of the N2 substitution existed. Although an N2 substitution is not strictly excluded by these experiments, it seems unlikely that such a compound would be oxidized by spermidine dehydrogenase.

The position of cleavage when spermidine dehydrogenase oxidizes spermidine and acetyl spermidine is indicated by the dotted lines:

Spermidine

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH2CH2CH2CH2NH2CH2CH2CH2NH2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N1-Acetylspermidine

CH3CONHCH2CH2CH2NH2CH2CH2CH2NH2 |

N8-Acetylspermidine

NH2CH2CONHCH2CH2CH2NH2CH2CH2CH2NH2 |

or

NH2CH2CH2CH2NHCCH2CH2CH2NHOCOC3

The position of the dotted line is based on the following observations:

1. Spermidine is converted stoichiometrically to diaminopropane and 4-aminobutan-1, which is converted spontaneously to Δ1-pyrroline (21, 23).

2. N1-Acetylspermidine is converted stoichiometrically to N-acetyl-1,3-diaminopropane (unpublished data).

3. N4-Acetylspermidine is oxidized very slowly, i.e. at less than 2% of the rate with spermidine at a concentration of 5 X 10^-4 M. Thus, even though at first it appeared not to be a substrate for this enzyme (22), we have recently found that with excess enzyme it is degraded with the formation of both N-acetyl-1,4-diaminobutane and 1,3-diaminopropane, i.e. the oxidation can split N4-acetylspermidine on either side of the secondary nitrogen (unpublished data).

### Table IV

Products formed by action of spermidine dehydrogenase on glutathionylspermidine

<table>
<thead>
<tr>
<th>Glutathionyl-spermidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaminopropane</td>
</tr>
<tr>
<td>Before hydrolysis</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Untreated glutathionyl-</td>
</tr>
<tr>
<td>spermidine.........</td>
</tr>
</tbody>
</table>

* The glutathionylspermidine was added as the disulfide, and contained 20 nmol of acid-hydrolyzable spermidine.

* No residual glutathionylspermidine, in either the reduced or disulfide form, was detected by the automated column assay. Upon hydrolysis, however, 1.45 nmol of spermidine, equivalent to 0.72 nmol (7%) of the original glutathionylspermidine, were found.

* A peak was noted at 44 min (in the 0.35 M NaCl buffer); this peak disappeared after hydrolysis and a new peak appeared in the diaminopropane position. The peak at 44 min was presumably glutathionyl-1,3-diaminopropane. The other oxidation product expected would be 4-aminobutan-1. After reduction, a small amount of 4-aminobutan-1-1 was found, i.e. elution time 60 min in the 0.35 M NaCl buffer. The remaining material presumably was cyclized to Δ1-pyrroline, which, after reduction to pyrrolidine, would not be detected by the ninhydrin reagent.

Turnover of Glutathionylspermidine in Escherichia coli

It was not possible to carry out turnover experiments in either logarithmically growing cultures, or in the usual stationary cultures, since the former cells do not have glutathionylspermidine, and the latter organisms have very little spermidine. Conditions for a turnover experiment could be obtained, however, if the pH of a postlogarithmic culture was maintained at 6.0 to 6.1, and aeration was limited by controlled shaking. Under these conditions the ratio of spermidine to glutathionylspermidine was approximately 0.6.

In a typical experiment, E. coli B was grown in 300 ml of minimal medium (19) containing 0.6% glucose in a 1-liter Erlenmeyer flask with shaking at 250 rpm in a New Brunswick rotary shaker at 37°C. When the A_{540 nm} was 0.6 (9 X 10^8 bacteria per ml), 43.5 nmol of [3H]spermidine containing 1.2 X 10^6 cpm were added. Four hours later the A_{540 nm} was 1.38 and the pH was 6.04. At this point 242 nmol of [3H]spermidine, containing 2.7 X
were collected and the cells were extracted with trichloroacetic acid. These extracts were assayed for glutathionylspermidine, spermidine, $^{3}H$, and $^{14}C$, as described in the text.

Table V

<table>
<thead>
<tr>
<th>Time after addition of $[^{3}H]$spermidine</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{3}H]$Spermidine uptake (%)$^{a}$</td>
<td>44</td>
<td>73</td>
<td>90</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Glutathionylspermidine in cells (mol)</td>
<td>2.8</td>
<td>2.7</td>
<td>2.9</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>$^{3}H$:14C in Spermidine$^{c}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{3}H$:14C in Glutathionylspermidine$^{d}$</td>
<td>0</td>
<td>5.0</td>
<td>15.0</td>
<td>10.6</td>
<td>22.7</td>
</tr>
<tr>
<td>$^{3}H$:14C in Spermidine$^{e}$</td>
<td></td>
<td>17.2</td>
<td>23.8</td>
<td>25.8</td>
<td>24.3</td>
</tr>
</tbody>
</table>

$^{a}$ Immediately before $[^{3}H]$spermidine was added to the culture.

$^{b}$ The amount of $H$ found in the trichloroacetic acid extract, expressed as per cent of the total amount of spermidine added to the medium.

$^{c}$ Chromatography on the Beckman PA-35 column was carried out in duplicate. In the first chromatography the amount of glutathionylspermidine and of spermidine was determined with ninhydrin in the amino acid analyzer, after reduction with dithioerythritol as described under "Experimental Procedures," and the results are expressed in the table per 300 ml of culture. In the second chromatography the eluates were collected from a fraction collector, and the $^{14}C$ and $^{3}H$ content of the spermidine and glutathionylspermidine areas were determined. $^{3}H$ assays showed no change in the $^{14}C$ content of these compounds during the experimental period. The $^{3}H$:14C ratio, however, increased in both compounds (data not shown), essentially as reported in the table.

$^{d}$ These data were obtained from fractions eluted from paper after electrophoresis.

10$^{6}$ cpm, were added. Shaking was continued, and the pH was maintained for 20 min at 6.04 to 6.09 by the repeated additions of 50-ml portions of 5 N KOH. During this period there was no increase in the A$_{140}$ nm reading. Periodically 25-ml samples were collected rapidly by a Millipore filter. The cells were extracted with 2.5 ml of 10% trichloroacetic acid. After removal of the trichloroacetic acid, portions of each extract were analyzed for amines as described under "Experimental Procedures." Another aliquot of each extract was subjected to electrophoresis for 75 min at 100 volts/45 cm in 8% formic acid. The area corresponding to glutathionylspermidine (10 to 15 cm from the origin) and to spermidine (20 to 25 cm from the origin) were eluted with 0.2% acetic acid, and the $^{3}H$:14C ratio was obtained by scintillation counting.

As shown in Table V, the $[^{3}H]$spermidine was taken up relatively rapidly by the bacteria, i.e. 73% in 10 min and 95% in 20 min. During this period the $^{3}H$:14C ratio increased in both spermidine (0 to 24.3) and glutathionylspermidine (0 to 22.7). No net change occurred in the amount of either glutathionylspermidine or spermidine. Therefore, the increase in $^{3}H$:14C ratio in glutathionylspermidine shows that the spermidine in glutathionylspermidine is continually exchanging with free spermidine.

**DISCUSSION**

The data presented in this paper characterize glutathionylspermidine as $\gamma$-glutaamylcysteinylglycylspermidine (Fig. 1). Essentially all of the intracellular spermidine and a large part of the intracellular glutathione (24) are converted to glutathionylspermidine during stationary phase (10, 25). The data in Table V show that glutathionylspermidine is in equilibrium with intracellular spermidine. These experiments are consistent with our earlier findings (10, 25) that glutathionylspermidine is rapidly converted to spermidine when a stationary culture is diluted into fresh medium. These results suggest the possibility that glutathionylspermidine is being formed at all stages of growth, but that it has a rapid turnover rate and a very low steady state level in logarithmically growing cells. Consistent with this postulation are our findings that extracts of E. coli from any stage of growth contain an enzyme which synthesizes a glutathione derivative of spermidine in the presence of glutathione, AT$^{1}$, and Mg$^{2+}$ (13), and another enzyme that degrades the disulfide form of glutathionylspermidine to free spermidine (10). The accumulation of glutathionylspermidine in stationary cells might, in part, be the result of changes in enzyme activity resulting from changes in the intracellular pH or of the fraction of glutathione present in the reduced or in the disulfide form.

Both glutathione (24, 26, 27) and spermidine (7) are widely distributed in biological materials, and have been implicated in various theories of control of growth and nucleic acid metabolism. It is possible that glutathionylspermidine, which contains both compounds in a covalent linkage, plays an integral role in these processes and in their regulation.

**REFERENCES**

Isolation, characterization, and turnover of glutathionylspermidine from Escherichia coli.
H Tabor and C W Tabor


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