THE COMPLETE CONVERSION OF SPERMIDINE TO A PEPTIDE DERIVATIVE IN *ESCHERICHIA COLI*

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Received September 2, 1970

When full-grown cultures of *Escherichia coli* were incubated anaerobically, all of the intracellular spermidine was converted to a derivative that has been identified tentatively as glutathionylspermidine. This is in contrast to *E. coli* growing logarithmically, in which little, if any, of this derivative was present. This spermidine derivative was very rapidly converted to spermidine when cells containing the derivative were transferred to fresh medium, and subjected to vigorous aeration. An enzyme has been demonstrated in *E. coli* sonicates which carries out this cleavage.

We have recently reported that spermidine is not converted to any other derivative by *Escherichia coli* during exponential growth (1). This finding was in apparent contradiction to previous work in this laboratory (2, 3, 4), showing that *E. coli* contained a spermidine derivative, which appeared to be glutathionylspermidine. [1/] We have now found that this derivative is synthesized only under certain cultural conditions, and that under these conditions all of the spermidine in *E. coli* is present in this form.

We have previously reported (3, 6) that a spermidine-glutathione derivative can be synthesized *in vitro* by an enzyme partially purified from *E. coli* in a

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[1/] The postulation of the structure of the spermidine derivative as glutathionylspermidine was based on the following experiments: [1] Upon hydrolysis of the performic acid-treated derivative by 6 N HCl at 108° equimolar amounts of spermidine, glutamic acid, cysteic acid, and glycine were formed [2/]. [2] Hydrolysis of the performic acid-treated material in water at 105° for 18 hours resulted in the formation of a peptide containing equal quantities of cysteic acid, glycine, and spermidine. It is well-known that γ-glutamyl-linkages, as in glutathione (5), are rather labile under these conditions, and thus these results indicated that the glutamic acid is probably linked in a γ-substitution at the N-terminal end of the peptide. [3] The spermidine-glutathione derivative synthesized enzymatically had a chromatographic behavior comparable to the isolated derivative.

[2/] We wish to thank Dr. Erhard Gross and Mr. John L. Morell for these analyses.

system containing ATP, spermidine, and glutathione. We have now found that *E. coli* also contains a very active system for degrading glutathionylspermidine with the formation of free spermidine.

MATERIALS AND METHODS

To follow the metabolism of spermidine, the spermidine pool was first labeled by the addition of a small amount of ^{14}C-spermidine to the growing culture at a low optical density. In a typical experiment, *E. coli* B was grown, with good aeration at 37°, in 200 ml of a minimal salts medium with 0.4% glucose (7). After the cell density reached 1 x 10^8 per ml (A_{540} = 0.07), five aliquots of ^{14}C-spermidine were added to the culture at 15 minute intervals [3] (total spermidine added: 0.065 µmole, 325,000 cpm). The 15 minute intervals between additions were sufficient to permit the uptake of essentially all of the added ^{14}C-spermidine (8).

The culture was then allowed to grow further with shaking aerobically. Periodically bacterial samples were collected by Millipore filtration, and trichloracetic acid extracts made as previously described (1). The extracts were then chromatographed on paper in butanol-1, glacial acetic acid, pyridine, and water (4 : 2 : 1 : 2) and the fraction migrating as spermidine (R_F = 0.34) or as a derivative (R_F = 0.05 or 0.23) was determined by following the radioactivity. Alternatively, the extracts were tested by electrophoresis for 1 hour at 1,000 volts/45 cm in 8% formic acid. The spermidine derivative migrated 12.5 cm toward the cathode, while free spermidine migrated 23 cm.

RESULTS

Effect of Growth Conditions on the Metabolism of Spermidine -- In extracts from logarithmically growing cells, all of the radioactivity cochromatographed with carrier spermidine (R_F = 0.34) as previously reported (1). As the culture

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[3] The ^{14}C-spermidine was added in several portions to avoid sudden increases in the total internal concentration of spermidine within the cell. Each addition represented < 10% of the estimated internal concentration of spermidine.

approached full growth, the percentage of the radioactivity present as spermidine decreased; the remaining radioactivity was all found in a spermidine derivative (Table 1) which could be converted to free spermidine by 6 N HCl at 105° for 18 hours. If shaking was discontinued when the A_{540} was 1.1, but incubation at 37° was continued for 2 hours, as much as 84-100% of the radioactivity was found in the derivative. These findings were not just the result of cessation of growth since an *E. coli* W leucine⁻ strain, incubated with shaking, did not accumulate any spermidine derivative if growth was limited at a

TABLE 1

Formation and Utilization of Spermidine Derivative

Time [1]		A_{540}	Percent of Total Radioactivity in Spermidine Derivative
25	shaking	0.3	2
55	shaking	0.6	1
88	shaking	0.9	4
115 [2]	shaking	1.1	9
255	shaking	1.75	55
255	no shaking	1.2	84 [3]
After 4-fold dilution [4] and vigorous aeration -			
0 [5]		0.3	84
5		0.3	5

[1] Minutes after last ^{14}C-spermidine addition.

[2] The culture was divided into two portions at 115 minutes. As indicated, shaking was continued in one portion and discontinued in the second part. The latter portion was placed in a narrow cylinder to decrease the exposure to air.

[3] In other comparable experiments 100% of the radioactivity was present as the derivative.

[4] The culture which had been incubated without shaking was diluted with fresh medium and aerated vigorously.

[5] Minutes after dilution and aeration.

bacterial density of 3 x 10^8 cells by limiting the leucine concentration. Similarly, if less glucose (0.2%) was used in the medium, no accumulation of the derivative was observed in wild type *E. coli* B after the cells reached the stationary phase.

Effect of Anaerobiosis and pH on the Metabolism of Spermidine -- A large number of factors are changing in cultures that are approaching stationary phase. These are difficult to study since many of these factors are unknown, and many are closely interrelated. However, an attempt was made to study two of the most striking changes in the culture as it approached stationary phase: *i.e.*, a decrease in pH and anaerobiosis resulting from the high bacterial density.

In the first experiment, we tested the effect of varying the pH in anaerobic cultures on the accumulation of the spermidine derivative. *E. coli* was labeled with ^{14}C-spermidine in the standard medium with 0.4% glucose at an A_{540} of 0.5. Portions of this culture were adjusted to pH 7.1, 6.4, and 5.9 by the addition of HCl and placed in a N_2 - CO_2 atmosphere. The pH was maintained at these values by the frequent addition of NH_4OH. Samples were assayed at 90 minutes; no spermidine was converted to the derivative at pH 7.0, 46% at pH 6.4, and 89% at pH 5.9. Thus, an acid pH was necessary for the formation of the derivative, even under anaerobic conditions.

The following experiment, however, showed that an acid pH was not sufficient by itself to induce extensive formation of the spermidine derivative, if the culture was growing logarithmically in air. The culture was labeled with ^{14}C-spermidine at an A_{540} of 0.05 - 0.10, adjusted to pH 6.0, and allowed to grow in air with good shaking from an A_{540} of 0.10 to 0.80; only 5% of the spermidine was found as the derivative.

In Vivo Metabolism of the Spermidine Derivative -- Since this derivative accumulated best in cells that were both anaerobic and in a slightly acid medium, it was of interest to determine its fate when the cells were diluted and allowed to grow in fresh medium at neutral pH and with aeration.

As shown in Table 1 (last line), when cells containing the derivative were diluted into fresh medium (pH 7.0) and aerated vigorously, essentially all of the derivative was very rapidly converted to free spermidine.

In Vitro Degradation of Spermidine Derivative -- ^{14}C-labeled spermidine derivative, partially purified from *E. coli* as described below, was incubated with sonicated extracts of *E. coli*. The incubation mixtures contained ^{14}C-labeled spermidine derivative (6,000 cpm), 0.2 M Tris-Cl buffer, pH 8.4, and *E. coli* sonicate (100 µg of protein) in a final volume of 15 µl. After a 2 hour incubation at 37°, 70-80% of the radioactive material was found in the spermidine area. In a control incubation without enzyme, no hydrolysis of the spermidine derivative was observed.

Isolation and Preliminary Characterization of the Spermidine Derivative -- *E. coli* B was labeled with ^{14}C-spermidine as described above; when fully grown the culture was stored at 37° without shaking to allow complete conversion of spermidine to the spermidine derivative (*cf.* Table 1). The cells were then extracted with 4 volumes of 10% trichloracetic acid. After removal of the trichloracetic acid by ether extraction, and neutralization of the solution to pH 7 - 8 with NH_4OH, 35 ml of the extract were placed on an XE-64 pyridine column (2.2 x 20 cm), and eluted with an exponential gradient (500 ml water in the mixing flask; 2 N acetic acid in the reservoir). Two major peaks [4] of radioactivity were obtained (at approximately 240 and 560 ml of eluant). The material in both peaks had comparable mobility on electrophoresis, and was degraded to free spermidine by the *E. coli* sonicate described above.

The second peak was used for analytical studies, since it had little or

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[4] We tentatively consider these two peaks as the reduced and oxidized forms of the sulfhydryl moiety of the derivative. After performic acid treatment the radioactivity from both peaks had identical chromatographic and electrophoretic behavior.

When the bacterial extract was first treated overnight with 0.004 M dithiothreitol and H_2S at pH 8.4, and chromatographed as described above, but with 0.001 M dithiothreitol in the mixing chamber, only one peak of radioactivity at approximately 280 ml of eluant was found.

no non-radioactive ninhydrin-reactive material. After lyophilization, this material was rechromatographed on XE-64 pyridine and then treated with performic acid. The final product migrated as a single peak on electrophoresis in 8% formic acid. The performic acid oxidation product was hydrolyzed in 6 N HCl *in vacuo* for 20 hours and assayed for spermidine with spermidine dehydrogenase (9, 10) and for amino acids in an amino acid analyzer (11). 5/ The ratios found for glutamic acid : cysteic acid : glycine : spermidine were 1.12 : 1.14 : 1.26 : 1.0. No other amino acids were found by either the amino acid analysis or paper electrophoresis in 8% formic acid.

DISCUSSION

Even though the physiological role of the glutathionylspermidine derivative is not known, the ability of the cell to convert *all* of its spermidine to this derivative, and the presence of an active system for its hydrolysis suggest the possibility that there is normally a rapid turnover of this material in the cell that may be of physiological significance. The existence of a derivative with such a rapid turnover would result in a change in our concept of spermidine as a metabolically inert polyamine. These findings require a reevaluation of the metabolic behavior of spermidine in various physiological studies of *E. coli*. In view of the recent studies of Inoue and Pardee (12) and of Hirshfield *et al.* (13) implicating spermidine and 1,4-diaminobutane in the control of cell division, the possible role of this derivative will be of particular interest.

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