Properties and Distribution of Intracellular Putrescine in a *Pseudomonas*

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Received for publication 12 August 1965

ABSTRACT

KIM, KI-HAN (Wayne State University, Detroit, Mich.). Properties and distribution of intracellular putrescine in a *Pseudomonas*. J. Bacteriol. 91:193-197. 1966.—A *Pseudomonas* species which contains putrescine as the only intracellular polyamine was used to study the distribution of putrescine in the cells and the changes in putrescine content upon nitrogen or carbon and nitrogen starvation. In the cell-free extract, approximately 80 to 90% of the putrescine was found in the soluble fraction, and the rest was found in the ribosomal fraction; 50% of the putrescine could be removed from the cells by nitrogen starvation. Putrescine content in the ribosomes prepared from nitrogen-starved cells was about one-half of that in the unstarved cells. Putrescine was found in both 30S and 50S ribosomal particles. In the presence of 10^{-3} M Mg^{++} , the ribosomal particles did not exchange bound putrescine for free putrescine, but did incorporate free spermine from the medium. Cells grown on glucose- NH_3 medium contained large amounts of acetyl putrescine. Cells grown on putrescine contained negligible amounts of acetyl putrescine, but readily formed acetyl putrescine when subjected to starvation.

Recently, there has been an ever increasing interest in the role of polyamines in microorganisms. There have been a number of reports which suggest some relationship between nucleic acids and polyamines in vitro. However, the function(s) of these amines in vivo is not well understood. There have also been other reported effects of polyamines involving membranes (3, 8, 9), as well as isolated enzyme systems (9, 10).

We have isolated a species of *Pseudomonas* which contains only putrescine as the intracellular polyamine. This bacterium can readily be adapted to grow on putrescine as the sole carbon and nitrogen source. We have been studying the various properties of the cellular components of this organism upon nitrogen and carbon starvation. In this communication, the effects of starvation on putrescine content, as well as on the distribution of putrescine in the cell, are reported.

MATERIALS AND METHODS

Putrescine was purchased from K & K Laboratories, Jamaica, N.Y. Putrescine-1,4- C^{14} ·2HCl was purchased from New England Nuclear Corp., Boston, Mass. Deoxyribonuclease was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Other chemicals were purchased from commercial sources.

Growth of bacteria. The *Pseudomonas* sp. was

grown with aeration at room temperature in the following medium (grams per liter): Na_2HPO_4 , 16.4, KH_2PO_4 , 1.5; $MgSO_4$, 0.097; $CaCl_2$, 0.01; $FeSO_4$, 0.001; and neutralized putrescine, 1 (P medium). The cells were also grown on the same salts medium with 0.2% glucose and 0.2% $(NH_4)_2SO_4$ (GN-medium) instead of putrescine. The cells were harvested in the early log phase and washed once with basal salts medium. If the cells were to be subjected to nitrogen starvation, the washed cells were resuspended in the salt medium supplemented with 0.2% glucose.

Preparation of ribosomes. The washed cells were suspended in buffer (1:4, w/v) containing 0.01 M tris(hydroxymethyl)aminomethane (Tris)-HCl; 0.001 M magnesium acetate·4 H_2O , and 0.004 M succinate (pH 7.8; standard buffer), and were disrupted with a French pressure cell at 2,000 psi. After standing at room temperature for 5 min with 100 μ g of deoxyribonuclease per g of wet cells, the suspension was centrifuged at $31,000 \times g$ for 30 min, and the debris was discarded. The ribosomes were sedimented from cell-free extract by centrifugation at $105,000 \times g$ for 2 hr. The ribosomes were usually washed once with the Tris buffer. Further washing with Tris buffer caused no loss of polyamines. The separation of 30S and 50S ribonucleoproteins was accomplished by using a linear sucrose density gradient (5 to 20%).

Assay of putrescine. Putrescine was measured quantitatively by the method of Dubin and Rosenthal (2). The sample to be assayed was added to an equal

volume of 10% trichloroacetic acid. After removal of the precipitate by centrifugation, the supernatant fluid was extracted several times with ethyl ether to remove the trichloroacetic acid. The solution was neutralized with KOH and applied to a Dowex 50W-x4 column. Gradient elution was performed with 300 ml of water in the mixing flask and 500 ml of 2.5 N HCl in the reservoir. Fractions (3 ml) were collected. A sample of each fraction was dried over KOH in vacuo. Then, 0.1 ml of dinitrofluorobenzene (DNFB) solution (5 ml of a mixture of 0.65 ml of DNFB and 50 ml of acetate added to 100 ml of 0.066 M sodium tetraborate) was added to each dried sample. The samples were then incubated in a water bath at 65°C for 10 min and allowed to cool. A 1-ml amount of dioxane-HCl (100:1, v/v) was then added, and the absorption at 370 m μ was measured. Putrescine was also determined by paper chromatography by the methods of Kay, Harris, and Entenman (5) and Herbst, Keister, and Weaver (4).

RESULTS

The general column chromatographic pattern of the trichloroacetic acid-extractable materials from the *Pseudomonas* grown on P medium is shown in Fig. 1. The only quantitatively significant diamine or polyamine in the bacterium is putrescine. Its identity was established both by comparing its elution pattern with authentic putrescine and by paper chromatography and electrophoresis (4). Spermidine and spermine were not detected by column chromatography

FIG. 1. Putrescine content of the *Pseudomonas* cells grown in P medium. (I) Inorganic salt and nonbasic amino acids. (II) Basic amino acids. (III) Unknown material which does not react with DNFB. (IV) Putrescine. The volume of the fractions was 3 ml each. The elution pattern of putrescine was verified and identified by paper chromatography with authentic compound. The identity of the other peaks was deduced from the report of Dubin and Rosenthal (2).

where authentic samples were eluted between 210 to 240 and 285 to 330 ml, respectively. Nor were they detectable by paper chromatography or electrophoresis of the total polyamines extracted by tertiary butanol in the presence of alkali (7). When the bacteria were grown on GN medium, an additional peak, which has been assumed to be acetyl putrescine by its chromatographic behavior (2), was observed (Fig. 2). The total free putrescine content per gram of wet cells, however, was approximately 6 μ moles, regardless of the growth medium (Table 1). About 80 to 90% of this putrescine is located in the cytoplasmic fraction, and the rest is associated with the ribosomal fraction (Table 2).

When bacteria grown on P medium were subjected to nitrogen starvation in the presence of glucose, about 50% of the total putrescine was lost (Table 1). Less decrease was observed when the cells were subjected to both carbon and ni-

FIG. 2. Putrescine and acetyl putrescine content of starved (----) and unstarved *Pseudomonas* cells grown in GN medium (—) Only tube numbers from 40 to 60 are recorded. (I) Acetyl putrescine. (II) Putrescine. The identity of acetyl putrescine is deduced from the chromatographic behavior. Other conditions of the experiment are described in Materials and Methods.

TABLE 1. *Effect of starvation on putrescine content**

Original culture medium	N starvation		GN starvation at 24 hr
	0 hr	24 hr†	
P	5.6	2.43	5.33 4.76
P	6.5	3.2	
P	6.4	3.3	
GN	6.0		

* Figures represent micromoles of putrescine per gram of wet cells.

† During nitrogen starvation, OD of culture increased two to threefold, and these figures represent the total amount of putrescine in these increased populations.

TABLE 2. *Distribution of putrescine*

Original culture medium*	Ribosomal putrescine
	%
P	8.5
P	12.1
P	15.7
Pst	9.7
Pst	8.5
GN	17.3
GN	11.9
GN	12.9

* Different batches of bacterial culture were used to show the distribution of putrescine. These ribosomal pellets were washed once with the standard buffer at the original volume. The rest of the putrescine is found in soluble fraction. Abbreviations: P = cells grown in P medium, Pst = cells grown in P medium and then subjected to N starvation, GN = cells grown in GN medium.

trogen starvation. Similar loss of putrescine was also observed with the cells grown on GN medium. The changes in putrescine content under different conditions are shown in Table 1.

The kinetics of putrescine disappearance during nitrogen starvation is shown in Fig. 3. Putrescine depletion was complete after 24 hr, and a longer period of starvation had little or no additional effect. The same results were obtained if the nitrogen starvation was carried out in the presence of 10^{-2} M magnesium or 10^{-3} M calf thymus histone. Starvation in the presence of 10^{-3} M spermine led to variable results, due, probably, to complications arising from permeability barrier and bacterial adaptation to degrade spermine.

The distribution of this "starvation-resistant" putrescine again showed that about 80 to 90%

is in the cytoplasmic fraction with the rest in the ribosomal fraction (Table 2).

To determine whether the decrease in putrescine is due to depletion within the cells or due to subsequent events occurring after the breakage of cells, such as enzymatic breakdown in the starved cell extract, the following experiment was carried out. Known amounts of normal and nitrogen-starved cells were mixed and disrupted with the French pressure cell. The putrescine content in the mixture was the same as the sum of putrescine of the two kinds of cells if they were disrupted separately, indicating that the loss of putrescine resulting from starvation had occurred inside the cells during the starvation and not outside the cells after breakage (Table 3).

The ribosomes prepared from nitrogen-starved

FIG. 3. *Effect of N starvation on putrescine and acetyl putrescine. A 1-liter culture at 0.160 OD at 650 mμ was subjected to nitrogen starvation. Samples (100 ml) were taken at the indicated times and assayed for amine content as described in Materials and Methods.*

TABLE 3. *Distribution of putrescine*

Cells	Soluble putrescine	Ribosomal putrescine
	μmoles	μmoles
Unstarved	2.4	0.75
Starved	1.05	0.30
50% Unstarved + 50% starved	1.55	0.45

TABLE 4. *Putrescine content in ribosomes**

Expt	Unstarved cells	N-starved cells
1	0.012	0.005
2	0.009	0.005

* Figures represent micromoles of putrescine per 1 optical unit at 260 mμ. These ribosomes were washed once with the standard buffer at the original volume. Other experimental procedures are described in Materials and Methods.

FIG. 4. A fraction (0.5 ml) of a ribosomal suspension was dialyzed against 100 ml of standard buffer containing $10 \mu\text{C}$ (0.52 mg) of putrescine-1,4- C^{14} ·2 HCl for 12 hr. The sample was subjected to a sucrose density gradient (5 to 20% of sucrose in the standard buffer) for 2 hr at 37,000 rev/min. Ten drops per sample were collected. After adding 0.5 ml of Tris buffer to each sample, 0.2 ml was used to assay radioactivity (broken line). An 0.8-ml amount of Tris buffer was added to another 0.2-ml sample, and its absorbance at 260 m μ was measured (solid line).

FIG. 5. All the procedures were the same as those in Fig. 4 except that $5 \mu\text{C}$ (0.41 mg) of spermine- C^{14} was used instead of putrescine. Broken line, radioactivity; solid line, UV absorbance.

cells contained only one-half of the putrescine content of the normal, as shown in Table 4. However, their ability to synthesize polyphenylalanine catalyzed by polyuridylic acid is not impaired (Moller and Kim, unpublished data). Ribosomal putrescine is found in both the 50S and the 30S ribosomal particles at a concentration of 0.15 and 0.66 $\mu\text{mole/mg}$ of ribosomes, respectively. Since dialysis of the ribosomes against a buffer containing C^{14} -labeled putrescine did not result in any uptake of radioactivity by the ribosomes (Fig. 4), the ribosomal putrescine appeared not to be the result of the nonspecific absorption of cationic putrescine of polyanionic

ribosomes after the breakage of the cells. This agrees with some of the earlier experiments of Cohen and Lichtenstein (1). However, dialysis against a buffer containing C^{14} -spermine caused the ribosomes to incorporate labeled spermine (Fig. 5). Further studies on the nature of the ribosomal putrescine are in progress.

DISCUSSION

It is well known that in many bacteria, the intracellular concentration of polyamine is of the order of 0.01 M, of which only a portion is associated with the ribosomes (1, 9, 10). We have previously reported that, by starvation of *Escherichia coli* grown on putrescine as the sole source of carbon and nitrogen, one can completely remove the intracellular putrescine (6). However, as these *E. coli* cells also contain large amounts of spermidine, it was difficult to interpret the results in terms of the intracellular function and essentiality of the polyamines. The *Pseudomonas* sp. reported here has the advantage that it contains only one polyamine, namely, putrescine. The results clearly show that, even in cells grown on putrescine as the sole carbon and nitrogen, half of the intracellular putrescine cannot be removed by carbon and nitrogen starvation.

The major portion of putrescine is found in the soluble fraction after breakage of the cell. Approximately half of this amount is removed during starvation. The remainder is probably bound to some subcellular component and is metabolically nonavailable. Since this amount is found in the soluble fraction of cell-free extracts, it seems logical to conclude that it is deoxyribonucleic acid (DNA) which binds this polyamine in intact cells and renders it unavailable for metabolic transformation. This result, taken together with the protective property of polyamine against mutagenic agents (Bach, personal communication), suggests that the polyamines in bacteria are normally associated with the DNA and that this may be a major role of these compounds.

The results also show a partial depletion of ribosomal putrescine during starvation, even though no decrease in ribosomes took place. This seems to be somewhat at variance with the results of Cohen and Lichtenstein (1) which showed that *E. coli* ribosomes contain tightly bound polyamines. The exchangeability of the *Pseudomonas* ribosomal putrescine was, therefore, examined. The results show that at neutral pH and in the presence of 10^{-3} M Mg^{++} , no exchange between the ribosomal putrescine and free putrescine took place in 12 hr. This is in agreement with the report of Cohen and Lichtenstein (1) and shows that the ribosomal putrescine is tightly bound and not loosely adsorbed. However, if

putrescine in the dialysis medium were replaced by spermine, the latter was found to be associated with both the 50S and 30S components of the ribosomes when the latter were subjected to sucrose density gradient centrifugation. The exact significance of the above results is not known, but the problem is being further studied. We observed that the "exchangeability" of the ribosomal putrescine is critically dependent upon the experimental conditions employed. We also observed that the polyamine-deficient ribosomes are biologically active, but have readily detectable differences in physical chemical properties from the normal ribosomes. Some of these results have been reported elsewhere (6a; Moller, Kim, and Tchen, *Federation Proc.* **21**:654, 1965), and others will be reported later.

The results also show that the level of acetyl putrescine is greatly dependent upon the conditions of growth. Essentially no acetylated putrescine was observed when the cells were grown on putrescine medium, whereas a substantial amount of this derivative is present in glucose-ammonium grown cells. Previously it was suggested that the formation of the acetylated derivatives of polyamines was more or less the result of homeostatic control of the amine concentration at the intracellular level (2). This, however, does not explain the observed increase of this derivative upon starvation of putrescine-grown cells, particularly as the presence or absence of glucose during starvation did not effect the formation of this product (Kim, *unpublished data*). Nor can we offer any explanation for the increase in the sum of putrescine and acetyl putrescine during early stages of starvation. These problems on the metabolism and the role of acetyl putrescine are currently under investigation.

ACKNOWLEDGMENTS

The author expresses his appreciation to T. T. Tchen, Mary L. Moller, and Ruth Michaels for their helpful discussions.

This investigation was supported by Public Health Service grants GM 12648, AM 5384, and HE 4139.

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