Inhibition of Ribonucleic Acid Degradation in Bacteria by Spermine*

Edward J. Herbst and B. P. Doctor†

From the Department of Biochemistry, School of Medicine, University of Maryland, Baltimore, Maryland

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A release of acid soluble constituents that absorb at 260 μm occurs during the incubation of suspensions of Escherichia coli H (1), Pseudomonas aeruginosa (2), or Lactobacillus arabinosus (3) in buffers. Lysogenic E. coli strain K12 as well as nonlysogenic E. coli strains B and B/r excrete ultraviolet-absorbing substances during growth in media deficient in glucose, and a methionine-requiring auxotroph of lysogenic E. coli (strain K12W-6) excretes 260 μm-absorbing materials when incubated in methionine-free growth medium (4). During methionine starvation occurs during the incubation of suspensions of Escherichia coli and Pseudomonas aeruginosa (2)) the number of viable cells. Holme and Palmstierna (5) incubated E. coli B in nitrogen-deficient media and observed the accumulation in the culture medium of substances causing an absorption in the ultraviolet region. Cells of Rickettsia mooseri lost 260 μm-absorbing materials in isotonic media incubated at 36° and there was a complementary decrease in the nucleic acid fraction of rickettsial suspensions (6).

We have reported (7) the release of acid soluble constituents that absorb at 260 μm during the incubation of washed cells of Hemophilus parainfluenzae in water or buffers. The 260 μm-absorbing “leakage” was derived from the ribonucleic acid fraction of the cell suspension and the temperature and pH characteristics of the reaction suggested that it is an enzymatic process. In the presence of the polyamine, spermine, which is an essential growth factor for H. parainfluenzae (8), the leakage and the associated nucleic acid breakdown is reduced or eliminated. The inhibition of nucleic acid degradation in the cell suspensions was deemed a consequence of nucleic acid-spermine complex formation (9, 10).

The experiments described in this paper more fully characterize the leakage of 260 μm-absorbing materials from suspensions of H. parainfluenzae in buffers or growth media as an autolytic degradation of cellular ribonucleic acid by a very active ribonuclease. The enzymatic degradation of bacterial ribonucleic acid can be reduced or completely inhibited by the addition of spermine to the bacterial suspensions.

**EXPERIMENTAL**

The bacteria were grown in the defined medium of Herbet et al. (11) in 1 l Erlenmeyer flasks containing 300 ml of medium. Incubation was for 16 to 24 hours at 34° with continuous shaking.

The cells were harvested by centrifugation at 2° and were washed with cold 0.9 per cent sodium chloride. A suspension containing approximately 10 mg. dry weight of cells per ml. was prepared in cold 0.1 M Tris† buffer, pH 8.1, and the weight of cells per ml. of suspension was determined turbidimetrically by reference to a standard curve relating turbidity and cell dry weights. In most experiments, an aliquot containing 3 mg. of cells was pipetted into each of the experimental tubes (13 X 100-mm. Pyrex test tubes) containing the chilled suspending medium. The final volume was 3 ml. and the tubes were incubated in a constant temperature bath at 37° without shaking. (Deviations from these conditions are indicated in the appropriate tables or figures.) At the end of the incubation period the tubes were centrifuged at 2° and the supernatants were treated with cold perchloric acid to a final concentration of 6 per cent. The very slight precipitate was removed by centrifugation and the supernatants were analyzed for 260 μm absorption with the Beckman model DU spectrophotometer and for orcinol reactive material by the procedure of Dische (12).

The cells obtained by centrifugation of the incubation mixtures were suspended in cold 6 per cent perchloric acid and extracted three times; the perchloric acid insoluble fraction was washed once with cold 0.5 per cent ethanol and resuspended in 6 per cent perchloric acid. The suspension was heated in a water bath at 90° for 15 minutes according to the modified procedure of Schneider (13) and centrifuged in the cold. The residue was re-extracted at 90° for 15 minutes and the supernatant obtained after centrifugation was combined with the first hot perchloric acid extract. The absorbance of the extract at 260 μm was determined, RNA was estimated by the orcinol procedure (12), and DNA was determined by the diphenylamine method (14). The absorbance at 260 μm of either cell supernatants (“leakage”) or cell extracts is expressed as A_{260}. Analyses of orcinol reactive material are expressed as the absorbance at 660 μm (A_{660}) and the diphenylamine analyses are expressed as the absorbance at 595 μm (A_{595}). All data have been calculated as absorbance per mg. dry weight of cells per ml.

**RESULTS**

Leakage from Washed Cells of Material Absorbing at 260 μm—Examination of the ultraviolet absorption spectra of the supernatants from suspensions of cells in distilled water, 1 per cent sodium chloride, and distilled water plus spermine or putrescine (final concentration 0.5 mM) shows a maximum near 260 μm in each series (Fig. 1). The leakage from cells in dis-
Inhibition of Nucleic Acid Degradation

Fig. 1. The absorption spectra of supernatant fluids from suspensions of *H. parainfluenzae*. Temperature 34°C; time of incubation, 4 hours; 1 mg. dry weight of cells per ml. Curve 1, distilled water; Curve 2, 1 per cent sodium chloride; Curve 3, 0.5 mM putrescine·2 HCl; Curve 4, 0.5 mM spermine·4 HCl.

Fig. 2. The effect of pH on the 260 m\(\mu\) leakage from *H. parainfluenzae*. Temperature, 37°C; time of incubation, 1 hour; 1 mg. dry weight of cells per ml. (■—■), suspensions in 0.1 M Tris buffer; (○—○), suspensions in 0.1 M Tris buffer + 0.5 mM spermine.

Fig. 3. The effect of temperature on the 260 m\(\mu\) leakage from *H. parainfluenzae*. Time of incubation, 1 hour; 1 mg. dry weight of cells per ml. (■—■), cells suspended in 0.1 M Tris buffer, pH 8.1; (○—○), suspensions in Tris buffer + 0.5 mM spermine.

Fig. 4. The release of 260 m\(\mu\) absorbing material from *H. parainfluenzae* during 3 hours of incubation at 0° and 37°. 1 mg. dry weight of cells per ml. (■—■), 0.1 M Tris buffer, pH 8.1, at 37°; (○—○), Tris buffer + 0.5 mM spermine at 37°; (●—●), Tris buffer at 0°.

tilled water is decreased somewhat by 1 per cent salt but the effect of the salt is not so pronounced as in the *P. aeruginosa* experiments where salt blocks essentially all leakage (2). The growth factors, spermine and putrescine, are more effective than salt in preventing leakage of the 260 m\(\mu\)-absorbing materials with spermine possessing the greater activity.

Effect of pH on Leakage—The pH of the suspending medium markedly influences the amount of 260 m\(\mu\) leakage from washed
cells. In Fig. 2, leakage over the pH range 7.3 to 8.45 and the effect of spermine in reducing the level of 260 m\(\mu\) leakage are illustrated. Whereas Newton (2) observed a rather sharp pH optimum at 5.3 for maximal leakage from Ps. aeruginosa, the H. parainfluenzae leakage occurs in the alkaline range with an optimal pH of 8.1. In view of these results the test system adopted for further study consisted of washed cell suspensions in 0.1 m Tris buffer, pH 8.1, and spermine as the antagonist of the leakage phenomenon.

**Effect of Temperature on Leakage**—The effect of temperature on the rate of the leakage reaction was studied over the range 1 to 55\(^\circ\). Leakage at 1 and 22\(^\circ\) is negligible while at physiological temperatures it is very great with the most leakage occurring at 37\(^\circ\) (Fig. 3); at 55\(^\circ\) the rate of leakage is reduced considerably. The protective effect of spermine is apparent over the entire range of temperatures and 0.5 m\(\mu\) growth factor provides essentially complete protection against the extensive leakage which occurs in its absence at 37\(^\circ\).

**Rate of Leakage in Tris Buffer at 0 and 37\(^\circ\)**—The rate of the leakage reaction was studied at 0 and 37\(^\circ\) to further characterize the 260 m\(\mu\) release and to establish the time required to carry the reaction to completion. In Fig. 4 the rate of 260 m\(\mu\) leakage at the two temperatures is illustrated and the protection afforded by spermine is again demonstrated at each experimental point of the 37\(^\circ\) time curve. The leakage at 0\(^\circ\) is comparable to that observed in spermine-protected cells at 37\(^\circ\).

**Origin of Leakage**—The data summarized in Table I support the concept that cellular RNA is the origin of the 260 m\(\mu\) leakage. After the incubation of the bacteria in Tris buffer, the 260 m\(\mu\) absorption of the cell extracts is lowered, RNA is lost from the cells, and the 260 m\(\mu\) leakage rises sharply; analysis of the incubation medium (“leakage”) by the orcinol method reveals the presence of pentose in support of the data on RNA degradation in the cells. The DNA content of the cells is unchanged signifying the inactivity of the DNA-specific enzymes under these conditions of incubation. In the presence of spermine the 260 m\(\mu\) leakage and the loss of RNA from the cells is substantially reduced.

In Table II the relative effectiveness of the principal H. parainfluenzae growth factors as inhibitors of the leakage reaction is compared. Spermine is the most effective inhibitor of 260 m\(\mu\) leakage from cells that have been harvested from cultures containing either spermine or putrescine. In general, neither the leakage reaction nor the protection against leakage afforded by the various growth factors is altered by growing the bacteria on spermine-containing media rather than the putrescine-supplemented medium used in all other experiments. The inhibition of leakage by the growth factors does appear, however, to be slightly less in suspensions of spermine-grown cells.

### Table I

**Ribonucleic acid as source of 260 m\(\mu\) leakage**

The bacterial suspensions were prepared and incubated in 0.1 m Tris buffer, pH 8.1, as described under “Experimental.” Spermine, when added, was present at 0.5 m\(\mu\).

<table>
<thead>
<tr>
<th>Leakage</th>
<th>Cells</th>
<th>A(_{260})</th>
<th>A(_{260})</th>
<th>A(_{260}) (RNA)</th>
<th>A(_{260}) (DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>0.300</td>
<td>0.060</td>
<td>5.340</td>
<td>0.515</td>
<td>0.058</td>
</tr>
<tr>
<td>1 hour incubation</td>
<td>1.670</td>
<td>0.255</td>
<td>3.800</td>
<td>0.345</td>
<td>0.058</td>
</tr>
<tr>
<td>1 hour incubation + spermine</td>
<td>0.630</td>
<td>0.120</td>
<td>5.060</td>
<td>0.440</td>
<td>0.058</td>
</tr>
</tbody>
</table>

* All data are expressed as absorbance per mg, dry weight of cells per ml.

† Acid soluble orcinol reactive material.

### Table II

**Relative effectiveness of amine growth factors as inhibitors of leakage**

Cells harvested from cultures containing either putrescine or spermine as the amine growth factor were tested for leakage in Tris buffer suspensions as described in Table I. The inhibition of the leakage of 260 m\(\mu\)-absorbing materials by the addition of 0.5 m\(\mu\) of the amine to these suspensions is expressed as the per cent decrease in absorbance at 260 m\(\mu\) of the cell supernatants.

<table>
<thead>
<tr>
<th>Cells grown on</th>
<th>Inhibition of leakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermine</td>
<td>Putrescine</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Putrescine</td>
<td>100</td>
</tr>
<tr>
<td>Spermine</td>
<td>92</td>
</tr>
</tbody>
</table>

### Table III

**Degradation of RNA in cells incubated in growth media**

Cells harvested from 18-hour cultures in chemically-defined medium (11) were suspended at a concentration of 1 mg, per ml, in 5 ml. of fresh media and incubated at 37\(^\circ\). Spermine, when added, was present at 0.5 m\(\mu\). Zero time and incubated cell suspensions were centrifuged and the cells were extracted and analyzed as described under “Experimental.”

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(_{260})</td>
<td>A(_{400}) (RNA)</td>
</tr>
<tr>
<td>Zero time</td>
<td>4.680</td>
</tr>
<tr>
<td>2 hours’ incubation</td>
<td>2.025</td>
</tr>
<tr>
<td>2 hours’ incubation + spermine</td>
<td>3.450</td>
</tr>
<tr>
<td>4 hours’ incubation</td>
<td>1.530</td>
</tr>
<tr>
<td>4 hours’ incubation + spermine</td>
<td>2.971</td>
</tr>
</tbody>
</table>

* Absorbance data are expressed as in Table I.
**Table IV**

*Amine and nucleic acid content of *H. parainfluenzae* at various stages of growth*

Five volumes of 300-ml. of defined growth medium were inoculated with an equal amount of logarithmic phase inoculum and a single volume of 300 ml. was centrifuged after 8, 14, 25, 36, and 50 hours' incubation at 34° C. Total growth factor was determined by microbiological analysis (11) of HCl-hydrolyzed cells while RNA and DNA were determined on cell extracts by the procedures described under "Experimental."

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>RNA (μg/mg)</th>
<th>DNA (μg/mg)</th>
<th>Growth factor†</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1.151</td>
<td>0.152</td>
<td>0.0317</td>
</tr>
<tr>
<td>14</td>
<td>1.095</td>
<td>0.111</td>
<td>0.0230</td>
</tr>
<tr>
<td>25</td>
<td>0.870</td>
<td>0.113</td>
<td>0.0143</td>
</tr>
<tr>
<td>36</td>
<td>0.712</td>
<td>0.102</td>
<td>0.0134</td>
</tr>
<tr>
<td>50</td>
<td>0.519</td>
<td>0.098</td>
<td>0.0083</td>
</tr>
</tbody>
</table>

* Absorbance data are expressed as in Table I.
† Cells grown under these conditions contain putrescine (15).

DISCUSSION

These experiments illustrate a pronounced effect of *H. parainfluenzae* growth factors, and especially spermine, in stabilizing the RNA of buffered cell suspensions or of bacteria suspended in the normal growth medium. The significance of this polyamine inhibition of the degradation of RNA by the bacterial RNase remains to be explored and defined. A related investigation in our laboratory has established that all of the growth factor in *H. parainfluenzae* cells can be precipitated as a nucleic acid-amine complex (9, 10). This observation and the effect of polyamine as an inhibitor of RNA breakdown seem indicative of an essential role of the growth factors in nucleic acid metabolism. If the role of the growth factors is that of a protector or stabilizer of RNA during the lag or early log phase of growth, then the cells incubated in buffer or growth medium without the growth factor might be damaged or degraded by the extensive RNA degradation.

**SUMMARY**

Washed cells of *Hemophilus parainfluenzae* lose acid soluble materials that absorb at 260 μm and react with orcinol during incubation in water, tris(hydroxymethyl)aminomethane buffer, or growth medium deficient in diamine or polyamine growth factor. The greatest loss occurs at 37° and at pH 8.1 while little or no loss is observed at a low temperature or low pH. The phenomenon is a consequence of the enzymatic breakdown of cellular ribonucleic acid. It is prevented by the growth factor, spermine.

REFERENCES