Excretion and Degradation of Ribonucleic Acid by *Bacillus subtilis*

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Received for publication 9 October 1964

**ABSTRACT**

DEMAIN, A. L. (Merck & Co., Inc., Rahway, N.J.), R. W. BURG, AND D. HENDLIN. Excretion and degradation of ribonucleic acid by *Bacillus subtilis*. J. Bacteriol. 89:640-645. 1965.—*Bacillus subtilis* MB-1480 was found to produce several 5'-ribonucleotides in the extracellular medium, in addition to the previously found 5'-guanosine monophosphate and guanosine diphosphate. The nucleotides accumulated after excretion of high-molecular-weight ribonucleic acid (RNA) and subsequent extracellular breakdown. Excretion of RNA paralleled growth closely, and was accompanied by deoxyribonucleic acid (DNA) and protein excretion. The process occurred in the absence of visible lysis. Whereas extracellular DNA was stable, the other macromolecules were degraded after excretion. With extracellular DNA as a measure of maximal lysis, it was calculated that the major part of the extracellular RNA must have been excreted by cells which had not undergone lysis.

The cross-feeding of purineless mutants by wild-type *Bacillus subtilis* cultures has been reported (Demain et al., 1964a; Demain, Miller, and Hendlin, 1964b). With a guanineless *B. subtilis* mutant as assay organism, it was shown that wild-type *B. subtilis* MB-1480 accumulates a high concentration of extracellular guanine-containing compounds during growth in a chemically defined medium. From such a broth, 5'-guanosine monophosphate (GMP) and a smaller amount of guanosine diphosphate (GDP) were isolated (Demain et al., 1964c).

The mechanism of accumulation has been investigated in the present work. The possibilities considered were: (i) *de novo* synthesis and excretion of guanine nucleotides alone; (ii) *de novo* synthesis and excretion of nucleotides of several bases; (iii) intracellular breakdown of nucleic acids and excretion of constituent nucleotides; and (iv) excretion of nucleic acids and extracellular breakdown into constituent nucleotides. The results to be described indicate the last-mentioned possibility to be the true mechanism.

**MATERIALS AND METHODS**

_Growth and production_. The growth of wild-type _B. subtilis_ MB-1480 in flasks was carried out in a chemically defined medium, as previously described (Demain et al., 1964b).

_Fractionation of cells for kinetic study_. At each sampling period, several flasks were removed from the shaker and their contents were pooled. To correct for evaporation, water was added to the original volume of broth. After growth was determined spectrophotometrically, 25 ml of whole broth were centrifuged at 25,000 X g for 10 min. The cell pellet was washed once with 25 ml of a mineral salts mixture [0.3% KHPO₄, 0.1% KH₂PO₄, 0.05% NH₄Cl, 0.1% NH₃NO₃, 0.01% Na₂SO₄, 0.001% MgSO₄·7H₂O, 0.0001% MnSO₄·4H₂O, 0.0001% FeSO₄·7H₂O, 0.00005% CaCl₂ (pH 7.0)], centrifuged, suspended in 25 ml of salts, homogenized in a Dounce homogenizer, mixed with an equal volume of cold 1.0 M perchloric acid, and centrifuged to separate the cell acid-soluble fraction from the cell acid-insoluble fraction. The centrifuged broth was also treated with perchloric acid to yield the broth acid-soluble and acid-insoluble fractions.

The acid-insoluble fractions of both broth and cells were treated as follows for protein and nucleic acid assays. The pellets were washed with 0.5 M perchloric acid, neutralized with dilute sodium hydroxide, and homogenized. Samples were assayed for protein. The remaining portions were mixed with an equal volume of 1.0 M perchloric acid, heated at 70°C for 30 min, and filtered. The filtrates were assayed for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

_Assays_. Growth was estimated by absorbancy at 660 μm in a Bausch & Lomb Spectronic-20 colorimeter in tubes with an outside diameter of 0.5 in. The absorbancy was converted to dry weight by means of a previously constructed calibration curve.

Quantitative estimation of guanine-containing compounds was accomplished with a tube dilution assay with the guanineless _B. subtilis_ auxotroph, MB-1517 (Demain et al., 1964b). The assay stand-
ard was disodium GMP dihydrate, and the results were calculated as micromoles of guanines per milliliter. The term "guanines" is used to designate guanine-containing compounds utilizable by the assay organism. These include guanine, guanosine, guanine nucleotides, and partially degraded RNA; highly polymerized nucleic acid was not used by the organism.

RNA was determined by the orcinol reaction with yeast RNA as standard (Mejbbaum, 1929). DNA was assayed by the diphenylamine reaction. The Dische (1930) method with salmon sperm DNA as standard was used for the initial work on the 5-liter fermentor broth. The Burton (1956) modification was employed for the kinetic studies; calf thymus DNA was the standard. Hydrolysis, chromatography, and base analysis of nucleic acids were done according to Bendich (1957). The method of Chen, Toribara, and Warner (1956) was used to determine inorganic phosphate. Protein was estimated according to Lowry et al. (1951) with bovine serum albumin as standard.

Ultraviolet (UV) absorbancy measurements were done in a Beckman model DB spectrophotometer with 1-cm light path.

Values for all assays are presented on the basis of their original concentration in broth.

Chromatography. Circular paper chromatography was done in the following four systems: (A) n-propanol-NH₄OH-water (6:3:1, v/v/v); (B) isobutyrlic acid-NH₄OH-water (66:1:33, v/v/v); (C) isobutyrlic acid-NH₄OH-water (57:43:9, v/v/v); (D) (NH₄)₂SO₄-0.1 M sodium phosphate buffer (pH 7.0)-n-propanol (30:50:1, w/v/v).

Results

Presence of extracellular nucleic acid. The possibilities considered as likely mechanisms for accumulation of extracellular GMP (and GDP) are listed in the introduction. The first mentioned (de novo synthesis and excretion of guanine nucleotides alone) was unlikely, because of the following observations: (i) the UV absorbancy of the broth supernatant fluid was several times greater than could be accounted for by its content of guanines; (ii) the broth supernatant fluid was capable of feeding not only guanineless and purineless mutants of B. subtilis, but also a pyrimidineless auxotroph; (iii) paper chromatography of the supernatant fluid showed UV-absorbing bands in addition to GMP; these had the mobilities of the 5'-monophosphates of adenosine, inosine, cytidine, and uridine.

Of the three remaining possibilities, the one involving excretion of intact nucleic acids was suggested by the observation that broths became highly viscous during growth. This was especially noticeable when the cells were grown in 5-liter fermentors. To determine whether intact nucleic acid was excreted, cells were removed from broth by centrifugation at 20,000 × g for 30 min after 4 days of growth in a fermentor. The resulting viscous broth had a UV spectrum typical of nucleic acid. Its absorbancy value indicated a nucleic acid content equivalent to about 4 mg/ml. Samples of the broth were subjected to the following three treatments, each designed to separate nucleic acids from low molecular weight materials: precipitation with perchloric acid, precipitation with protamine sulfate, and dialysis. Table 1 shows the results. All three treatments removed about half of the UV absorbancy and bioactivity, indicating the presence of a large amount of high molecular weight and UV-absorbing and guanine-containing material in the broth. Nucleic acid assays of the perchloric acid precipitate and the non-dialyzable material showed approximately 2 mg/ml of RNA and 0.5 mg/ml of DNA to be present. Further proof of the presence of nucleic acid was obtained by hydrolysis of the acid-insoluble fraction with 11 M perchloric acid and estimation of the phosphate and the bases released (Table 2). The total concentration of released bases agreed well with the amount of phosphate liberated. An additional, but minor, UV-absorbing component (not included in the table) was also seen on the chromatograms. It had the mobility of thymine, but there was not sufficient material for its characterization. The microbiological assay figures for guanine in Table 1 indicate that the nucleic acid was highly polymerized, since the guanine was relatively unavailable before hydrolysis.

The above data supported the mechanism involving the excretion of highly polymerized

<table>
<thead>
<tr>
<th>TABLE 1. Effect of various treatments on ultraviolet absorbancy and on concentration of guanines in broth*</th>
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<tbody>
<tr>
<td>Medium</td>
</tr>
<tr>
<td>----------------------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Broth......................... 257</td>
</tr>
<tr>
<td>Acid-soluble broth fraction.......... 257</td>
</tr>
<tr>
<td>Protamine-soluble broth fraction.... 257</td>
</tr>
<tr>
<td>Dialyzable broth fraction........... 256</td>
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</tbody>
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* Cells were removed from 5-liter fermentor broth by centrifugation. Acid precipitation was done with 0.1 M perchloric acid; protamine precipitation with 0.2% protamine sulfate. Dialysis was done on 20 ml of broth against 1 liter of distilled water for 16 hr in the cold.
The concentration of perchloric acid used was 2N.

Table 2: Acid hydrolysis of acid-insoluble fraction of broth

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bases (μmoles/ml)</th>
<th>Inorganic phosphate</th>
<th>Guanines by microbial assay</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Guanine</td>
<td>Adenine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>Before hydrolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After hydrolysis</td>
<td>1.03</td>
<td>0.88</td>
<td>0.61</td>
</tr>
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</table>

* The perchloric acid precipitate (see footnote of Table 1) was dried, hydrolyzed with 2N perchloric acid, chromatographed, and assayed (see Materials and Methods). Assays were conducted in triplicate with two levels of hydrolysate. Agreement was within 2%.

Table 3: Release of nucleotides by incubation

<table>
<thead>
<tr>
<th>Medium</th>
<th>Incubation</th>
<th>Nucleotide bands on paper chromatograms</th>
<th>Guanines by microbial assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth</td>
<td>hr</td>
<td></td>
<td>μmoles/ml</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>Absent</td>
<td>0.1</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>Present</td>
<td>0.8</td>
</tr>
<tr>
<td>Nondialyzable frac-</td>
<td>0</td>
<td>Absent</td>
<td>0.38</td>
</tr>
<tr>
<td>tion of broth</td>
<td>18</td>
<td>Present</td>
<td>0.76</td>
</tr>
</tbody>
</table>

* Centrifuged broth and its nondialyzable fraction (see footnote of Table 1) were incubated separately at 28°C for 18 hr under toluene. The resulting mixtures were chromatographed in system A and bioassayed.

nucleic acid and its subsequent breakdown into nucleotides. To study this further, both broth and its nondialyzable fraction were incubated at 28°C for 18 hr under toluene before being paper chromatographed in system A and assayed for guanines. The data in Table 3 show that incubation brought about the release of nucleotides and increased the concentration of bioactive material. A similar increase in bioactivity was noted with the broth.

Kinetics of extracellular accumulation. The excretion of nucleic acid was followed for 7 days in 250-ml shake flasks. At the end of this period, the centrifuged broth and its acid-soluble fraction showed UV spectra typical of nucleic acid or breakdown products (Fig. 1).

Synthesis of cellular components is shown in Fig. 2A. Of interest is the linear growth and synthesis of protein, RNA, and DNA. Lysis of the culture was noted after 6 days, at which time the concentration of all three cell-bound macromolecules began to decrease. When the increases in RNA, DNA, and protein occurring before lysis were plotted against cell weight (Fig. 2B), it was seen that each component formed a constant fraction of the cells throughout growth.

The excretion of UV-absorbing (at 280 μm) materials is shown in Fig. 3. Elaboration of such material into the broth began when the cells entered their linear phase of growth and continued for the rest of the experiment. Up until about 110 hr, about half of the excreted material was acid-precipitable. At 120 hr, depolymerization of the nucleic acid was noted, and, by the end of the experiment, the excretion was almost entirely acid-insoluble.
experiment, most of the UV-absorbing material was acid-soluble. The extracellular accumulation of bioassayable guanines lagged behind. This is a reflection of the need for some depolymerization of nucleic acid before the assay organism could use it.

In Fig. 4 is shown a comparison of protein, RNA, and DNA found in the cells and in the broth during the 6 days of growth preceding lysis. It is obvious that all three types of macro-

The absorbancies of the broth and of its acid-insoluble fraction were determined after heating for 30 min at 70°C in 1.0 N perchloric acid so that the values could be directly compared with that of the low molecular weight material in the acid-soluble fraction.

The curves of Fig. 4 give some clue to the mechanism of RNA excretion. Cell lysis during growth does not appear to be solely responsible. Since broth DNA is stable, it can be used as a measure of maximal lysis. The amount of excreted DNA was equal to that present in the cells at the end of the growth phase (6 days). Thus, at most, half of the cells underwent lysis. This could account for the amount of protein and degradation products of protein in broth, since this total amount equaled the amount of cell protein. However, the RNA excretion was much in excess of cellular RNA. The maximal level of RNA which accumulated in the broth was 2.5 times as much as that of the cellular RNA, and, if one adds the amount of broth RNA degradation products to broth RNA, the total excreted RNA was over six times the amount in the cells. It thus appears that the

![Fig. 3. Excretion of nucleic acids as a function of time. The absorbances of the broth and of its acid-insoluble fraction were determined after heating for 30 min at 70°C in 1.0 N perchloric acid so that the values could be directly compared with that of the low molecular weight material in the acid-soluble fraction.](image)

![Fig. 4. Production of cellular components and extracellular materials as a function of time.](image)
major part of the RNA was excreted by cells which had not undergone lysis.

**Discussion**

The results obtained in the present investigation show that wild-type *B. subtilis* cells feed purineless mutants by excreting RNA into the medium where it is degraded into smaller utilizable derivatives. Since these derivatives were found to be 5'-nucleotides, it is concluded that phosphodiesterase, rather than ribonuclease, is involved in the degradation. Phosphodiesterase is known to be excreted by *B. subtilis* (Kakinuma et al., 1964). The isolation of GDP from broths containing 5'-GMP (Demain et al., 1964c) suggests that polynucleotide phosphorylase may also participate in the degradation; this enzyme has been found in *B. subtilis* (Steiner and Beers, 1961). The presence of both phosphodiesterase and polynucleotide phosphorylase could account for the low content of GDP, as compared with that of GMP, and for the fact that GDP was not always found in the broths. In *Escherichia coli*, both enzymes participate in the degradation of ribosomal RNA into predominantly ribonucleoside 5'-monophosphates (Wade and Lovett, 1961). It would be interesting to compare the excretion of RNA with that of the two enzymes as a function of time during growth of *B. subtilis*.

One ordinarily considers bacteria to be impermeable to substances of high molecular weight. However, the existence of such genetic phenomena as transformation, recombination, lysogeny, and release of male-specific bacteriophage without cell lysis suggests that large molecules can pass inwardly through cytoplasmic membranes without permanent damage to cells. Likewise, the outward excretion of exoenzymes and transforming DNA by microorganisms is well documented. Surprisingly, little has been published on excretion of intact RNA.

Although many reports on exoenzymes have appeared in the literature, the mechanism of protein excretion is still unclear (Pollock, 1962). Nomura, Hosoda, and Yoshikawa (1958) concluded that amylase excretion by *B. subtilis* resulted from a change in the cell surface caused by an autolytic enzyme, although visible lysis did not occur. On the other hand, it has been found that penicillinase liberation by *B. subtilis* can occur without damage to the cell envelope (Pollock, 1961a, b; Kusher and Pollock, 1961). The enzyme is membrane-bound before excretion and appears to be released enzymatically before passing through the cell wall.

Less is known about the mechanism of DNA excretion (Catlin, 1969; Takahashi and Gibbons, 1957; Takahashi, 1962). Extracellular DNA originates from intracellular DNA, is active in transformation, and is excreted under conditions in which lysis is not observed. Although it is generally considered that extracellular DNA results from cell rupture, excretion by intact cells has not been disproved (Ottolenghi and Hotchkiss, 1962). That this is possible is suggested by the observation (Campbell et al., 1961) that transfer of cells in the act of DNA excretion from growth medium to water stopped the process. When the same was done to cells which had not yet begun to excrete DNA, liberation was prevented.

The stability of DNA excreted by *B. subtilis* in the present study agrees with the recent finding (Okazaki and Kornberg, 1964) that this species is very low in nuclease content. The possibility exists, however, that the stability of DNA is due to inhibition of exoyribonuclease by RNA and other polyanions. Although we do not know whether DNA excretion is a result of lysis during growth, if we assume this to be the case, its stability allows us to calculate the maximal amount of lysis which could have occurred. This figure of 50% lysis agrees with our finding that the total amount of protein and its degradation products in the broth equaled the amount of protein in the cells. Since the total amount of excreted RNA and degradation products was over six times that of cellular RNA, it is apparent that RNA excretion from intact cells had occurred. This massive excretion of RNA is emphasized by the observation that at the point of maximal cell yield, the weight of excreted RNA and derivatives was equal to over half the weight of the cells present.

An intriguing question for future investigation is whether the excretion phenomenon involves a lytic RNA phage. It is doubtful, however, that much of the "excess" RNA formation could be phage RNA. Excretion of RNA occurs before there is a decrease in the turbidity of the culture. Not only does the turbidity increase, but so do the viable counts; these reach a peak of 4.2 × 10^9 cells per milliliter at 6 days and then decline in parallel with the drop in turbidity. Even if we were dealing with an RNA phage similar to the male-specific phage fr of *E. coli* K-12 (Hoffmann-Berling and Maze, 1964) which multiplies and is released during bacterial growth without lysis, it is doubtful that the "excess" RNA is predominantly that of the phage. Assuming a cell concentration as high as 10^10 cells per milliliter and a release of as many as 10^4 particles per cell, there would be 10^14 particles per milliliter or 1.7 × 10^-15 moles of phage RNA per ml. At a molecular
weight of $1.3 \times 10^4$ for phage RNA (Schindler, 1964), there would be 220 $\mu$g of phage RNA per milliliter of broth. In the present work, we have found 2,450 $\mu$g of extracellular RNA and degradation products and 390 $\mu$g of intracellular RNA per milliliter of culture. Assuming 50% lysis, we are left with an "excess" of over 2,000 $\mu$g of extracellular RNA and degradation products, 10 times the amount calculated above for phage RNA.

It is interesting to compare these results with those published reports of ribonucleotide excretion during normal growth (Okabayashi and Masuo, 1960; Arima et al., 1963; Sugimori, Tazuke, and Hamada, 1963; Ogata, Imada, and Nakao, 1962) and during incubation of cell suspensions in the presence of buffers and chelating agents (Higuchi, Tanaka, and Uemura, 1962; Imada, Nakao, and Ogata, 1962). Depending on the organisms, the excreted nucleotides were either ribonucleoside 2'(3')-monophosphates or mixtures of 5'-monophosphates and 5'-diphosphates. These events are thought to be due to the action of ribonuclease and polynucleotide phosphorylase, respectively, on intracellular RNA, since the concentration of this macromolecule decreased during excretion. However, since the intracellular nucleotide pool did not increase, one wonders whether intact RNA, rather than nucleotides, was excreted and rapidly broken down. In agreement with such a suggestion is the finding (Higuchi and Uemura, 1959) that over 60% of the nucleotide material excreted by yeasts is in the form of oligo- and polynucleotides. Although the mechanism of RNA excretion by *B. subtilis* may be similar to that described in these reports, it should be borne in mind that, in the present study, there was no decline in intracellular RNA during excretion.

In considering the mechanism of RNA excretion, the possibility that RNA is synthesized at the membrane is an attractive one. The cytoplasmic membrane of bacteria contains RNA (Luria, 1960) presumably because of its content of metabolically active ribosomes (Hendler and Tani, 1964). The kinetics of incorporation of exogenous nucleic acid bases by *E. coli* can be explained on the basis of RNA synthesis at the membrane (Britten, 1962). Experimental evidence for RNA synthesis by isolated membranes has been obtained with several bacteria (Spiegelman, 1958; Hill, 1962). The *E. coli* membrane system shows an absolute requirement for Mn++, which we have found to be essential for RNA excretion by *B. subtilis* (Demain et al., 1964b). In *Streptococcus faecalis* (Abrams, Nelson, and Thaemert, 1964), the most rapidly synthesized RNA fraction is that of the membrane-bound ribosomes. During nucleotide excretion by yeast (Higuchi and Uemura, 1962), the RNA content of the membrane fraction decreased markedly, but that of the free cytoplasmic ribosomes did not drop until a later autolytic phase characterized by a decrease in viability.

In conclusion, it appears that the major part of the material responsible for cross-feeding of purineless mutants by wild-type *B. subtilis* arises via excretion of high-molecular-weight RNA by intact cells, followed by extracellular degradation. As a working hypothesis, it is suggested that the RNA is synthesized at the membrane, but, for some reason, it fails to become incorporated into ribosomes and is excreted. Preliminary results indicate that this wasteful process can be inhibited by addition of amino acids to the medium. Such supplementation has been found to increase growth and decrease excretion.

**Acknowledgments**

We thank Joanne Fabian, Sara Currie, and Emily O’Leary for technical assistance.

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