Enzymatic Degradation of Ribosomes During Endogenous Respiration of Pseudomonas aeruginosa

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ABSTRACT

GRONLUND, AUDREY F. (University of British Columbia, Vancouver, B.C., Canada), AND J. J. R. CAMPBELL. Enzymatic degradation of ribosomes during endogenous respiration of Pseudomonas aeruginosa. J. Bacteriol. 90:1-7, 1965.—From sedimentation analyses it was found that the ribosomal content of Pseudomonas aeruginosa decreased during endogenous respiration. A greater degree of degradation of 50S than 30S ribosomes occurred during the 3-hr starvation period. The enzyme responsible for the initiation of ribosome degradation and present in the ribosome fraction was identified as polynucleotide phosphorylase. The enzyme was inactive in intact 70S ribosomes, but was active in low magnesium ion concentrations which allowed the 70S ribosome to dissociate. Polynucleotide phosphorylase was not solubilized after dissociation of the 70S particle, but remained firmly attached to the 50S and 30S ribosomes, the ribonucleic acid of which served as substrate.

The release of ultraviolet (UV)-absorbing material from microorganisms which have been subjected to various conditions of starvation has been noted by many workers. More specifically, the degradation of ribonucleic acid (RNA) by cells respiring endogenously has been reported to occur with Sarcina lutea (Burleigh, Dawes, and Ribbons, 1963) and Escherichia coli (Dawes and Ribbons, 1962), and a net loss of RNA has been found during endogenous respiration of Aerobacter aerogenes (Strange, Dark, and Ness, 1961) and Pseudomonas aeruginosa (Gronlund and Campbell, 1963).

Ribosomal RNA has been implicated as an endogenous substrate of P. aeruginosa (Gronlund and Campbell, 1963), and the work presented here describes the influence of endogenous respiration on the ribosomal components of the cell and the identification and localization of an enzyme responsible for ribosome degradation.

MATERIALS AND METHODS

Resting-cell suspensions. P. aeruginosa (ATCC 9027) was grown in a glucose-ammonium phosphate-salts medium: 20% glucose, 2 ml; 20% yeast extract, 2 ml; and uracil-β-C14 (specific activity, 32 μc/mg), 30 μc. A 90-min period of incubation in this medium resulted in a suitable uptake of radioactivity into cold trichloroacetic acid-insoluble material before a significant increase in cell numbers occurred.

Preparation of cell-free extracts. At zero time and after a 3-hr period of starvation on the Warburg respirometer, 2.8-ml samples of C14-labeled and nonlabeled cells were removed from Warburg vessels and were centrifuged. The resulting cell pellets were resuspended in 1.4 ml of 0.01 M Tris buffer [pH 7.4; 20 mg (dry weight) of cells per ml], and cell-free extracts were prepared with a French pressure cell (Milner, Lawrence, and French, 1950). The Tris buffer contained 10-2 M, 10-4 M, or no magnesium ions. Viscosity of the cell extracts was reduced by the addition of deoxyribonuclease, and unbroken cells were removed by centrifugation at 5,000 X g for 10 min.

Sucrose gradients. Linear sucrose gradients from 5 to 20% were prepared in 8-ml Lusteroid centrifuge tubes by a method analogous to that of Britten and Roberts (1960). An amount (0.2 ml) of cell-free extract [4 mg (dry weight)] was applied to the top of the gradients by means of a tuberculin syringe fitted with a no. 18 hypodermic needle. The samples were centrifuged at 37,500 rev/min in a SW 39 rotor in a Spinco model L preparative ultracentrifuge. The length of centrifugation time was dependent on the magnesium ion concentration of the sucrose solutions and cell-free extracts, and was as follows: 10-2 M Mg++, 90
min; $10^{-4}$ M Mg++, 150 min; and 0.5% sodium monolauryl sulfate (Duponol), no magnesium, 360 min. After centrifugation, the bottom of each Lusteroid tube was punctured with a no. 22 hypodermic needle, and the tube contents were dispensed dropwise into 1-ml centrifuge tubes. Approximately 25 equal fractions were collected from each gradient in this manner.

The distribution of ribosomal material in the collected fractions was determined by diluting the samples 1:9 with distilled water and measuring the optical density (OD) at 260 m$\mu$ with a Beckman model DU spectrophotometer. The distribution of radioactivity in the various fractions was measured as follows: 0.2 ml of 1.4 N perchloric acid (PCA) was added to each fraction, the tube contents were mixed, and the tubes were held in ice for 20 min. After centrifugation, the supernatant fluid was removed from each tube and discarded; 0.05 ml of 0.2 N NaOH was added to the cold PCA-insoluble material. The tube contents were mixed by means of a Vortex mixer and a Pasteur pipette. The resulting solutions were plated directly onto stainless steel planchets, the centrifuge tubes were rinsed twice with distilled water, and the washings were added to the planchets. Radioactivity was measured with a model 151 Nuclear-Chicago scaler with an automatic gasflow counter which had a thin end-window Geiger tube.

Identification and localization of the degradative enzyme. Ribosome fractions were obtained by the differential centrifugation procedure of Campbell, Hogg, and Strasdine (1960), and an assay for polynucleotide phosphorylase activity, similar to that described by Littauer and Kornberg (1957) which involved the measurement of charcoal-absorbable radioactive phosphorus, was carried out.

To isolate 70S, 50S, and 30S ribosomes, ribosome pellets were resuspended in 0.05 M Tris buffer (pH 7.4), containing either $10^{-2}$ M or $10^{-3}$ M Mg++, at a concentration approximately equivalent to the ribosome content of 100 mg (dry weight) of cells per ml. An amount (0.30 ml) [3 mg (dry weight)] of the appropriate ribosome suspension was layered on top of each of three sucrose gradients containing 4.7 ml of 5 to 20% sucrose in 0.01 M Tris buffer (pH 7.4) with either $10^{-2}$ M or $10^{-3}$ M MgCl$_2$. The tubes were centrifuged at 37,500 rev/min for 90 or 150 min, fractions were collected, and the OD at 260 m$\mu$ was determined on 1:99 dilutions of each fraction. The two fractions containing the greatest OD from 70S, 50S, or 30S peaks, from each of three appropriate gradients, were pooled, and samples of each ribosome were dialyzed, with stirring, against 0.05 M Tris buffer (pH 7.4) containing $10^{-2}$ M, $10^{-4}$ M, or no magnesium ions for 90 min at 6 C. Polynucleotide phosphorylase assays were carried out in 1-ml volumes containing ribosomes, $6 \times 10^{-2}$ M inorganic phosphate, 0.05 M Tris buffer, and $5 \times 10^{-2}$ M, $10^{-2}$ M, $10^{-3}$ M, or no added magnesium ions. Reactions were stopped at zero time or after a 60-min incubation at 30 C by the addition of 1 ml of 1.4 N PCA. After centrifugation, the increase in cold PCA-soluble UV-absorbing material was measured with a Bausch and Lomb double-beam recording spectrophotometer.

**Results and Discussion**

Changes in the ribosomal components of *P. aeruginosa* during endogenous respiration. Sedimentation analyses of cell-free extracts of starved and nonstarved cells in $10^{-2}$ M Mg++ demonstrated a decrease in both OD and radioactivity from the 70S ribosomes and an increase in the 50S and 30S ribosomes (Fig. 1 and 2). The changes in the 70S and 50S particles are further exemplified in Table 1, in which the total OD and total radioactivity have been calculated for each of these ribosomal peaks.

As the conditions for preparing sedimentation analyses of starved and nonstarved cells were identical, and as these conditions promote the aggregation of 50S and 30S particles, the observed decrease in the 70S fraction must be considered to be a result of endogenous respiration and to be a reflection of an alteration in the 50S and 30S components such that aggregation is less favored.

Only a small percentage of the total ribosomal RNA in the cells was labeled under the experimental conditions employed; therefore, if newly formed ribosomes were selectively degraded during endogenous respiration, then the ratio of
the radioactivity in the 3-hr 70S component relative to the 0-hr 70S component would be considerably lower than the same ratio using OD measurements at 260 m´. However, from Table 1 it is obvious that there was no substantial difference in the two ratios, thus indicating that there was a random degradation of 70S ribosomes during starvation.

The change in 50S ribosomes relative to 30S ribosomes was demonstrated by preparing sucrose gradients in $10^{-4}$ M Mg++, thereby allowing the dissociation of the 70S components. From OD measurements, it is apparent that both 50S and 30S ribosomes decreased during starvation (Fig. 3). The changes observed for these two ribosomal units after the 3-hr period of starvation revealed a more pronounced degradation of the 50S than of the 30S component (Table 2). The radioactivity in both 23S and 16S RNA decreased during starvation, and 23S RNA, comparable with 50S ribosomes, decreased to a greater extent than did 16S RNA (Table 3).

The relative instability of ribosomes during endogenous respiration is, no doubt, partially due to the conditions of magnesium starvation imposed upon the organism. When cells were allowed to respire endogenously for 3 hr in the presence of $3.33 \times 10^{-3}$ M Mg++, less UV-absorbing material was excreted into the suspending fluid (Fig. 4), but only a 12% reduction in endogenous C\(^{14}\)O\(_2\) release from uracil-2-\(^{14}\)C-labeled cells was observed (Gronlund and Campbell, 1963). Therefore, magnesium starvation is
fluids during peak per TABLE 4.

0.2

8.88

23S 16S

Hr    RNA

<table>
<thead>
<tr>
<th>Hr</th>
<th>RNA</th>
<th>23S 16S 23S/16S</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40.40</td>
<td>21.60 1.87</td>
</tr>
<tr>
<td>3</td>
<td>29.70</td>
<td>19.20 1.55</td>
</tr>
<tr>
<td>3/0</td>
<td>0.695</td>
<td>0.890</td>
</tr>
</tbody>
</table>

* Values given are for total counts per minute per peak × 10⁻⁴.

 responsible for a small but significant role in the dissociation of ribosomes under the conditions employed.

These data support the previous evidence which suggested that ribosomes are degraded during the endogenous respiration of P. aeruginosa (Campbell, Gronlund, and Duncan, 1963). Further, the data demonstrate that 50S ribosomes are more susceptible to degradation than are the 30S ribosomes, and that there is no obvious preferential usage of either newly formed or "old" ribosomal material as the endogenous substrate.

Identification of the degradative enzyme. Wade and Robinson (1963) compared the autodegradation of Pseudomonas fluorescens ribosomes with that of E. coli ribosomes, and demonstrated that the degradative processes of the two microorganisms were considerably different, thus reflecting a variance in their ribosomal nucleic content. Ribonuclease activity in E. coli ribosomes was stimulated markedly by ethylenediamine tetraacetate (EDTA), but was not detectable in P. fluorescens ribosomes; it therefore was concluded to be absent from the ribosomes of the pseudomonad. Orthophosphate enhanced the breakdown of P. fluorescens ribosomes, via polynucleotide phosphorylase, to a considerably greater extent than that observed with E. coli, thus suggesting that P. fluorescens has a higher content of polynucleotide phosphorylase in the ribosome fraction.

The influence of EDTA and inorganic phosphate on the degradation of P. aeruginosa ribosomes was followed by incubating ribosome suspensions in Tris buffer (pH 7.4) for 1 hr at 30°C in the presence and absence of inorganic phosphate (60 μmoles/ml) or EDTA (2.7 μmoles/ml). The increase in cold PCA-soluble UV-absorbing material released during the incubation period was measured (Table 4).

The results are analogous to those found with P. fluorescens in that EDTA, which stimulates ribonuclease activity (Wade, 1961), completely inhibited ribosome degradation, whereas inorganic phosphate promoted a 16-fold increase in degradative activity. These data suggested that, like P. fluorescens, P. aeruginosa ribosomes do not contain ribonuclease.

Ribosome suspensions were examined for nonspecific phosphodiesterase, ribonuclease, and polynucleotide phosphorylase activity. No increase in OD at 440 μm was observed when concentrations up to 5 mg (dry weight) of ribosomes were incubated at 30°C in Tris buffer (pH 7.4 and 8.5) with 1 μmole of bis (p-nitrophenyl) phosphate in presence and absence of magnesium ions. As bis (p-nitrophenyl) phosphate serves as a substrate for phosphodiesterases (Koerner and Sinsheimer, 1957), the absence of measurable activity, under the conditions which promote the release of cold PCA-soluble UV-absorbing material from ribosomes, demonstrates that a nonspecific phosphodiesterase is not responsible for the observed ribosome degradation.

<table>
<thead>
<tr>
<th>Suspension</th>
<th>ΔOD at 260 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomes</td>
<td>0.141</td>
</tr>
<tr>
<td>Ribosomes plus EDTA</td>
<td>0.000</td>
</tr>
<tr>
<td>Ribosomes plus PO₄³⁻</td>
<td>2.22</td>
</tr>
</tbody>
</table>
To detect ribonuclease activity, ribosomes were incubated at 30°C in Tris buffer (pH 7.4) with the propyl ester of uridine monophosphate (Tener and Khorana, 1955). As a control, pancreatic ribonuclease was incubated with the substrate under identical conditions. Samples of the ribosomal reaction mixture were removed at zero time and at 15-min intervals for a period of 1 hr, spotted on Whatman no. 1 paper, and chromatographed with iso-propyl alcohol:ammonia:water (7:1:2) as the solvent system. After 1 hr of incubation, a sample of the pancreatic ribonuclease reaction mixture was treated in a like manner. In the presence of the commercial ribonuclease, all of the propyl ester of uridine monophosphate (UMP) was converted to 2'- or 3'-UMP. There was no appearance of either 2'- or 3'-UMP or 2',3'-cyclic UMP in the reaction mixture containing ribosomes. The propyl ester of UMP, in the chromatographed test samples, was eluted from the paper, and the OD at 260 μm was recorded. There was no measurable decrease in substrate concentration from the zero-time to the 1-hr test sample. From these results, then, it may be concluded that the enzyme responsible for the degradation of ribosomes in P. aeruginosa is not ribonuclease.

By the procedure of Littauer and Kornberg (1957) for measuring polynucleotide phosphorylase, ribosome suspensions were incubated with radioactive inorganic phosphate. The cold PCA-soluble UV-absorbing material released during the incubation period was examined for charcoal-absorbable P₃² which would be present through the conversion of inorganic phosphate to organic phosphate if polynucleotide phosphorylase were the enzyme involved in ribosome degradation. A comparison of the amount of radioactivity and 260 μm-absorbing material recovered from the zero-time control and the test-reaction mixture charcoal eluates is shown in Table 5. As P₃² was incorporated into the UV-absorbing material enzymatically released from ribosomal RNA, it is clear that the enzyme associated with the ribosomes and responsible for their degradation during endogenous respiration is polynucleotide phosphorylase. Previous work in this laboratory (Strasdin, Hogg, and Campbell, 1962) conclusively established the presence of polynucleotide phosphorylase in the ribosome fraction of P. aeruginosa by demonstrating the rapid polymerization of adenosine diphosphate to form polyadenylic acid, with concurrent release of inorganic phosphate.

**Ribosomal location of polynucleotide phosphorylase.** The 70S, 50S, and 30S ribosomes were isolated and examined for enzyme activity to determine whether or not polynucleotide phosphorylase was associated with a particular ribosomal component. The last or top three fractions from sucrose gradients were also examined under conditions in which 70S ribosomes were isolated and under conditions in which 50S and 30S ribosomes were isolated, to detect a possible solubilization of the enzyme during the dissociation of 70S particles.

The release of cold PCA-soluble UV-absorbing material by 70S ribosomes in the presence of various magnesium ion concentrations is shown in Table 6. Enzyme activity was completely inhibited by magnesium ion concentrations of 5 × 10⁻² and 1 × 10⁻² M, which do not allow the dissociation of the 70S particles. Significant activity was evident, however, in 10⁻⁴ M Mg⁺⁺, which does allow the dissociation of the 70S ribosomes into the 30S and 50S subunits. Enzyme activity was maximal with no added magnesium.

The influence of magnesium ion concentration on the polynucleotide phosphorylase activity of 50S and 30S ribosomes was determined (Table 7). The 50S ribosome showed no enzyme activity with 5 × 10⁻⁴ M Mg⁺⁺, very slight activity

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### Table 5. Radioactivity and UV-absorbing material in charcoal eluates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts per min per ml</th>
<th>OD/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time control</td>
<td>300</td>
<td>1.0</td>
</tr>
<tr>
<td>After 1 hr of incubation</td>
<td>800</td>
<td>9.45</td>
</tr>
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</table>

### Table 6. Influence of magnesium ion concentration on the polynucleotide phosphorylase activity of 70S ribosomes

<table>
<thead>
<tr>
<th>Mg⁺⁺ conc</th>
<th>ΔOD at 260 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td></td>
</tr>
<tr>
<td>5 × 10⁻²</td>
<td>0.0</td>
</tr>
<tr>
<td>1 × 10⁻²</td>
<td>0.0</td>
</tr>
<tr>
<td>1 × 10⁻⁴</td>
<td>0.334</td>
</tr>
<tr>
<td>No added Mg⁺⁺</td>
<td>0.300</td>
</tr>
</tbody>
</table>

### Table 7. Polynucleotide phosphorylase activity of 50S and 30S ribosomes in various magnesium ion concentrations

<table>
<thead>
<tr>
<th>Mg⁺⁺ conc</th>
<th>ΔOD at 260 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>50S Ribosomes</td>
</tr>
<tr>
<td>5 × 10⁻²</td>
<td>0.0</td>
</tr>
<tr>
<td>1 × 10⁻²</td>
<td>0.041</td>
</tr>
<tr>
<td>1 × 10⁻⁴</td>
<td>0.560</td>
</tr>
<tr>
<td>No added Mg⁺⁺</td>
<td>0.552</td>
</tr>
</tbody>
</table>

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with $10^{-3}$ M Mg$^{++}$, and maximal activity at $10^{-4}$ M Mg$^{++}$. The results with no added Mg$^{++}$ are within experimental error of those obtained in the presence of $10^{-4}$ M Mg$^{++}$. The 30S ribosomes demonstrated maximal activity in $10^{-4}$ M Mg$^{++}$, 20% of maximum in $5 \times 10^{-2}$ M, 50% of maximum in $10^{-2}$ M, and 80% of maximum with no added magnesium ions.

No polynucleotide phosphorylase activity was detected in the low-density fractions from sucrose gradients under conditions in which 70S ribosomes were isolated intact or in which they were allowed to dissociate into 50S and 30S particles. It is apparent, then, that the enzyme is attached to the 70S intact unit in an inactive form, and, on dissociation of this unit, the enzyme is not solubilized but remains firmly bound to the 50S and 30S subunits, the RNA of which serves as substrate.

As polynucleotide phosphorylase requires Mg$^{++}$ for activity (Grunberg-Manago and Ochoa, 1955), the low activity obtained with 30S ribosomes in the absence of added Mg$^{++}$ indicates that the Mg$^{++}$ concentration of the 30S ribosome suspension was below that required for optimal enzyme activity. The low polynucleotide phosphorylase activity observed with isolated 50S and 30S ribosomes in high magnesium ion concentrations, in all probability, may be attributed to the aggregation of these particles. The dimerization of purified 50S ribosomes of E. coli in high magnesium ion concentrations has been shown to take place, and the aggregation of the particles occurred at the same "active site" as that involved in the aggregation of 50S particles with 30S particles (Huxley and Zubay, 1960). This suggests either that the enzyme is bound at this "active site" after the aggregation of the ribosomal particles, or that the substrate is bound, thus being unavailable to enzyme attack.

Measurements of the amount of enzyme activity associated with 50S and 30S ribosomes, when expressed as the increase in PCA-soluble UV-absorbing material released per hour per ribosome suspension containing an OD of 5.0 at 260 mµ, demonstrated 28% more activity in the 30S than in the 50S ribosomes (Fig. 5). However, when enzyme activity is expressed per ribosomal unit, then the 50S ribosome exhibits 30% more activity than the 30S component. When considering the total activity associated with 70S particles, then 62% may be attributed to the 50S ribosomes and 38% to the 30S ribosomes. These data are consistent with the results obtained from sedimentation analyses of cell-free extracts of starved and nonstarved cells, which showed a greater degradation of the 50S component than of the 30S component during endogenous respiration.

The presence of polynucleotide phosphorylase firmly bound to 50S and 30S ribosomes and inactive in the intact 70S ribosome is, no doubt, a control mechanism. Under conditions in which the need for structural ribosomes is at a minimum, and the cells do not have an adequate energy source, the 70S particles disassociate and consequently activate the degradation of ribosomal material which subsequently provides either energy or required nitrogenous intermediates.

As microorganisms are generally considered to utilize carbohydrate, lipid, or volutin as normal endogenous reserves, the degradation of ribosomal material, at first, appears to represent an abnormal situation. It would appear to be uneconomical from the point of view that the energy to be gained from the oxidation of RNA and protein is relatively less than that to be gained from the oxidation of carbonaceous reserves. Also, it would appear to be physiologically unsound in that it suggests that a loss of viability would be encouraged by the degradation of the "essential" cellular constituents. As ribosomal degradation is initiated by polynucleotide phosphorylase in this

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**Fig. 5. Relative polynucleotide phosphorylase activity associated with 50S and 30S ribosomes.**

Enzyme activity was followed by measuring the increase in cold PCA-soluble UV-absorbing material released. Symbols: ∆, 30S ribosomes and □, 50S ribosomes expressed for the same number of physical units; ○, 50S ribosomes with an amount of RNA equivalent to that of the 50S ribosomes.
organism, the immediate products of the degradation are nucleoside diphosphates. *P. aeruginosa* contains an active adenylate kinase (Campbell et al., 1962) which effectively removes adenine diphosphate (ADP) by the following reaction:

\[ 2 \text{ADP} \rightarrow \text{AMP} + \text{ATP} \]

thus supplying the organism with an available high-energy compound. The nonspecific interconversion of nucleotides by transphosphorylation reactions is known to occur in microorganisms. By this mechanism, the purine and pyrimidine nucleoside diphosphates may be converted to nucleoside monophosphates and nucleoside triphosphates. The sequential activity of nucleotidases and nucleosidases would yield free bases, ribose, and inorganic phosphate. *P. aeruginosa* contains a ribokinase which allows the pentose to enter the pentose phosphate cycle (Gronlund, 1961) and enzymes for nucleoside degradation, and purine and pyrimidine oxidation have been shown to be constitutive in the organism.

Under conditions of starvation, then, the ribosome concentration in microorganisms is far in excess of that required for protein synthesis. Therefore, ribosomes may function adequately in the role of an endogenous substrate, provided that the enzymes concerned with ribosome degradation are part of the enzyme complement of the cell.

Acknowledgments

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Literature Cited


