Polyribosomes of Growing Bacteria

Abstract. *A method of extracting polyribosomes has been developed which uses lysozyme on bacteria in the exponential phase of growth. This procedure recovers more than 80 percent of the total ribonucleic acid and nearly all of the soluble protein. Analysis of the lysate on sucrose gradients shows that 80 to 90 percent of the ribosomes sediment with the polysome fractions, indicating little or no degradation. The method has been applied to both a Gram-positive and a Gram-negative organism under various growth conditions and has proved uniformly successful.*

Studies of protein synthesis in bacteria are frequently performed by analysis of the polyribosomes (polysomes) on which the synthesis takes place. However, the yield and size distribution of polysomes obtained from the organism depends critically upon the method used in lysing the cells. Here we present the results obtained by the use of low-temperature lysis with lysozyme and EDTA (ethylenediaminetetraacetate), supplemented with chloramphenicol to inhibit protein synthesis. This method has been applied successfully to a Gram-positive and a Gram-negative bacterium and yields a significantly larger percentage of polysomes than previously reported. Thus, this percentage may more closely represent the ribosomal distribution in growing cells.

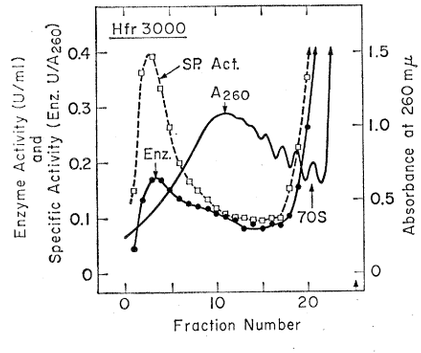

Fig. 1. Sucrose gradients of polyribosomes from *E. coli* K12, strain Hfr 3000. The culture, at a density of 3×10^8 cells per milliliter, received a final concentration of $10^{-3}M$ methyl-β-thiogalactopyranoside (TMG) for 6 minutes to induce the formation of β-galactosidase. Then 50-ml portions were harvested, and the polysomes were extracted as described in the text. Portions (0.8 ml) of clarified lysate were layered on cold, 16-ml, linear sucrose gradients (15 to 30 percent) in the standard buffer (0.01*M* tris *p*H 7.4, 0.01*M* $MgSO_4$, 0.05*M* NH_4Cl) and spun for 130 minutes at 25,000 rev/min in a Spinco SW 25.3 rotor at 4°C. At the end of the ultracentrifugation, the bottom of the density-gradient tube was punctured with a needle, and the contents were pumped through a Gilford recording spectrophotometer to monitor the absorption at 260 mμ continuously. The enzymatic activity was measured as previously described (*12*). The arrow at the lower right indicates the top of the gradient.

Various techniques have been used to open bacteria, including vigorous methods such as ultrasonic oscillation or use of the French press. However, the more usual methods for the study of polysomes involve conversion of cells to spheroplasts or protoplasts (*1*) and incubation of these altered cells to effect incorporation of radioactive materials or induction of an enzyme, after which the cells are lysed osmotically in the presence of detergent. Other procedures have utilized lysozyme treatment alone or with EDTA either at room temperature or at 0°C (*2*). In addition, mutants with weak cell walls have been used (*3*) as well as freezing and thawing methods (*4*). In our method, protoplasts are formed at 0°C. It offers several advantages, permitting enzyme induction or radioactive labeling of bacterial cells under normal conditions of exponential growth on various media. Chloramphenicol is used to rapidly stop protein synthesis, and the cells are immediately chilled to 0°C; metabolic processes are thus rapidly arrested. Since all subsequent operations are carried out at 0°C, this procedure permits rapid kinetic studies of normal cell polysomes. It is a modification of the procedure for preparing *Escherichia coli* protoplasts (*5*).

Strains of *E. coli* were grown exponentially at 37°C with a doubling time of 45 to 50 minutes (*6*). In a typical experiment, 25 ml of culture were grown to 3×10^8 to 4×10^8 cells per milliliter. Chloramphenicol (Parke Davis) was then added to a final concentration of 100 μg/ml, and the cells were immediately poured over an equal volume of crushed ice. All subsequent operations were performed at 0°C. The cells were harvested by centrifugation at 5000*g* for 5 minutes. After the supernatant was decanted, 0.30 ml of a solution containing 2.0 mg of chloramphenicol per milliliter was added. The pellet was then resuspended in the same tube at 0°C with 4.5 ml of sucrose-salt solution containing sucrose (0.5 mole/liter), tris(hydroxymethyl)aminomethane buffer (0.1 mole/liter, *p*H 8.0) and NaCl (0.1 mole/liter). Then 0.6 ml of freshly dissolved lysozyme (Mann, 1.0 mg/ml in sucrose-salt solution) and 0.4 ml of 0.14*M* EDTA (*p*H 8.0) was added. Approximate final concentrations were 0.4 mole of sucrose per liter, 100 μg of lysozyme per milliliter, 10^{-2} mole of EDTA per liter, at *p*H 8.

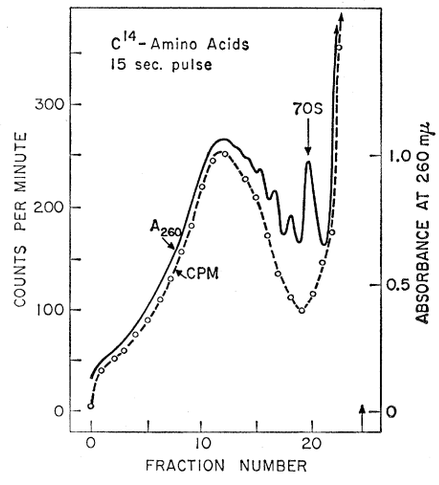

Fig. 2. A 100-ml portion of a culture of *E. coli* Hfr 3000 growing exponentially at 37°C was exposed for 15 seconds to 20 μc of a mixture of amino acids labeled with C^{14}. Then a lysate was made as described in the text. The lysate was centrifuged on a 15 to 30 percent sucrose gradient for 3 hours in an SW 25.3 Spinco rotor. The absorbance and radioactivity were measured.

Protoplasts were formed after 2 minutes at 0°C (with occasional gentle swirling of the tube), followed by addition of 0.12 ml of 1*M* $MgSO_4$ to restore the magnesium concentration to $10^{-2}M$. The protoplasts were centrifuged for 5 minutes at 5000*g*, the supernatant was decanted, and the inside of the centrifuge tube was wiped dry. To the pellet was added 0.5 to 1.0 ml of lysing medium (*7*). We then disrupted the protoplasts by dispersing them uniformly in the lysis medium. The crude lysate was centrifuged for 10 minutes at 10,000*g* to sediment cell debris; the supernatant was carefully removed and analyzed by sucrose density-gradient centrifugation.

Figure 1 shows the polysomal pattern obtained from strain Hfr 3000. The absorbance profile characteristically shows few single ribosomes, with 80 to 90 percent of the ribosomes sedimenting as polysomes. The polysome peak in Fig. 1 occurs in the region

of the ribosomal decamers, with approximately half of the polysomal material sedimenting more rapidly. The polysomes prepared by this method are completely sensitive to ribonuclease, which quantitatively degrades them to single 70*S* ribosomes.

Two tests have been carried out to assay these preparations for possible degradation. Polysomal-bound β-galactosidase was measured on the gradient fractions. Polysomes have been prepared previously from spheroplasts formed by the action of penicillin (*1*) without the use of chloramphenicol or deoxyribonuclease. It has been found that the polysomes involved in the synthesis of β-galactosidase are very large, containing 40 to 50 ribosomes; hence they can be used to provide a sensitive index of breakdown. With our method we have also observed a peak of polysomal β-galactosidase activity at the same position near the bottom of the gradient (Fig. 1). The fact that there are low amounts of enzyme between this peak and the supernatant at the top of the gradient suggests that degradation is minimal in this procedure.

Proteins are synthesized largely on polysomes rather than on isolated ribosomes. Hence another sensitive index of polysomal breakdown is the presence of radioactivity in nascent polypeptide chains on single ribosomes. We tested this preparative method by exposing *E. coli* to radioactive amino acids for 15 seconds before adding chloramphenicol and pouring on crushed ice. Sucrose gradient analysis of this preparation indicates that there is a minimum of radioactivity near the single 70*S* ribosomes, with a peak associated with polysomes on one side, and soluble protein at the top of the gradient on the other side (Fig. 2). If the preparation resulted in a substantial breakdown of polysomes, single ribosomes would accumulate in the 70*S* peak together with the attached nascent polypeptide chain. The absence of radioactivity in this region is an additional indication that there has been little degradation during polysome preparation.

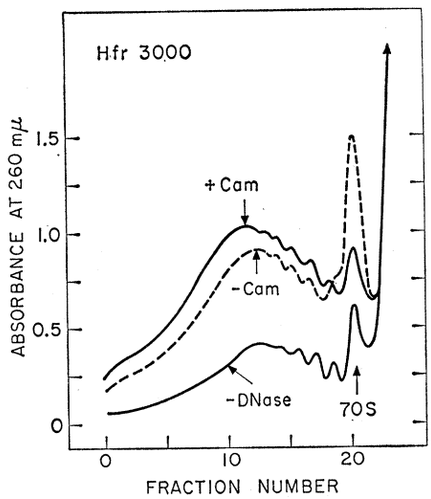

Fig. 3. Effect of chloramphenicol and deoxyribonuclease on polysomes of *E. coli* strain Hfr 3000. The upper solid curve shows the polysome profile obtained in a sucrose gradient when both deoxyribonuclease and chloramphenicol are used in preparing the cell lysate. The dashed curve shows the effect of omitting chloramphenicol, while the lower solid curve shows the effect of omitting deoxyribonuclease. The absence of this enzyme substantially decreases polysome yield.

Table 1. Release of macromolecules from protoplasts of *E. coli* strain γ-13. Cells were labeled for two generations with 0.01 μc of C^{14}-uracil per milliliter, and β-galactosidase was induced in $5 \times 10^{-4}M$ TMG for 20 minutes before harvest. Protoplasts, prepared as described in the text, were disrupted in complete lysing medium or in lysing medium minus sodium deoxycholate (DOC). We determined the release into the clarified lysate of β-galactosidase, material that absorbs ultraviolet light, and radioactive material insoluble in 5 percent trichloroacetic acid. The results shown are the average of three experiments and agree to ± 5 percent.

Lysing medium	Absorbance at 260 mμ recovered per 10^{10} cells	Release of β-galactosidase (%)	Release of RNA labeled with C^{14}-uracil (%)
Complete	27.2	92	83
Complete, minus DOC	14.2	91	36

We have determined the yield of RNA and of soluble protein obtained by this procedure (Table 1). Over 80 percent of the cellular RNA and over 90 percent of a soluble protein (β-galactosidase) is released, which is comparable to that obtained by other methods (*2–4*). Although sodium deoxycholate (DOC) generally increases polysome yields, it is not necessary in the lysing medium with Hfr 3000; the omission of DOC from the lysing medium has no effect on the results in Fig. 1.

If deoxyribonuclease is omitted from the preparation, uniform dispersion of the protoplasts in the lysing medium is made more difficult, and much lower yields of ribosomal material are obtained (Fig. 3). There also appears to be a preferential loss of the larger polysomes. When polysomes are extracted in the absence of chloramphenicol, relatively more single ribosomes are observed, the number of 70*S* ribosomes increasing from 10 to 30 percent (Fig. 3). It should be emphasized, however, that this is due to changes which occur after the cells have been chilled on ice and harvested. The polysome patterns obtained from cells chilled on ice in the absence of chloramphenicol, harvested, and then resuspended in medium containing the drug are comparable to those obtained by the standard method, as described above, in which chloramphenicol is added immediately before the cells are poured on ice.

We have also applied the method to *Micrococcus lysodeikticus* (*8*), a Gram-positive organism, with only minor alterations. Cells were grown at 32°C to a cell density of approximately 5×10^8 bacteria per milliliter, with a doubling time of 80 to 100 minutes, and the suspension was then poured over an equal volume of ice and harvested in the cold. Chloramphenicol

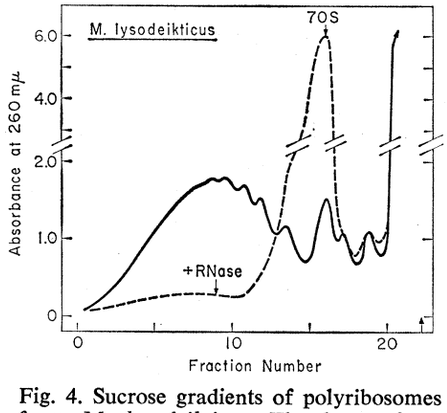

Fig. 4. Sucrose gradients of polyribosomes from *M. lysodeikticus*. The lysate from approximately 4×10^9 cells obtained as described in the text was divided in half, and one portion was treated with ribonuclease (Worthington) at a concentration of 5 μg/ml for 15 minutes at 4°C. The lysates were then layered on separate 27-ml, 15 to 45 percent sucrose gradients and centrifuged for 6 hours at 25,000 rev/min in an SW 25.1 rotor. Adsorption was monitored as in Fig. 1. Solid curve: untreated lysate: dashed curve: effects of treatment with ribonuclease.

was not used in these experiments. For formation of protoplasts, cells were resuspended in ice-cold medium containing 1.0 mole of NaCl (*9*), instead of the 0.4 mole of sucrose (which proved less effective in stabilizing these protoplasts), 0.02 mole of sodium phosphate at *p*H 6.8, and 0.01 mole of $MgSO_4$ per liter. Lysozyme was added to a final concentration of 1.2 mg/ml. After protoplast formation, as judged by microscopic examination (usually about 5 minutes), the action of lysozyme was arrested by the addition of 10 volumes of the aforementioned medium. The protoplasts were centrifuged and lysed as before, and they were analyzed on a sucrose density gradient (Fig. 4). The polysomal pattern showed less than 10 percent single 70*S* ribosomes, and the polysomes were sensitive to small amounts of ribonuclease.

In all the strains of bacteria examined, we find that the quantity of polyribosomal material is considerably larger than that found by previous methods. In the strains of *E. coli* approximately 85 to 90 percent of the ribosomal material sediments as polysomes, suggesting that there may be few free single ribosomes in rapidly proliferating cells. This view has been supported by Mangiarotti and Schlessinger (*3*), who have also used chloramphenicol in the preparation of bacterial polysomes. However, their results are qualitatively different from ours. We find 85 to 90 percent of the ribosomal material on polyribosomes and only 10 to 15 percent as single ribosomes and ribosomal subunits; Mangiarotti and Schlessinger find only about half of the ribosomes on polysomes and the remainder as 50*S* and 30*S* ribosomal subunits.

The question of what chloramphenicol does to the equilibrium of ribosomes and polysomes in the cell must be taken into account in evaluating our results. Chloramphenicol apparently blocks protein synthesis by preventing the movement of the ribosome along the messenger (*8, 10*). It might therefore tend to freeze the polysomal distribution and preserve it during the lysis and extraction procedure. However, since chloramphenicol apparently does not block attachment of the ribosome to messenger RNA, it is possible that the dearth of free ribosomes which we observed may be caused by an attachment, at 0°C, of the free ribosomes normally found in cells to existing polysomes. Alternatively, the polysome profile obtained in the absence of the drug may reflect the differential release of ribosomes due to residual protein synthesis at 0°C (*11*).

The advantages of our method are that it is gentle, easy to apply, and apparently of wide application. The cells are maintained metabolically inactive during preparation of the polysomes; thus this method is likely to yield a better representation of the true intracellular state of bacterial ribosomes. It does permit the extraction of essentially all the polysomes in largely undegraded state from exponentially growing bacteria and thus makes possible rapid kinetic studies of normal cell polysomes.

Note added in proof: Recently Hotham-Iglewski and Franklin have described a method for preparing polysomes from *Escherichia coli* which is very similar to that described above. Their polysomal distributions are close to those reported here, with more than 80 percent of the ribosomes found in polysomes.

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6. Strain Hfr 3000 B_1 (obtained from B. Magasanik) and strain γ-13 B^- pro$^-$ (obtained from J. Beckwith) were grown on a medium containing in final concentration 0.02*M* NH_4Cl, 0.01*M* $MgSO_4$, 0.06*M* sodium phosphate at *p*H 7.0, 0.5 percent glycerol, 0.25 percent casamino acids, 4 μg of vitamin B_1 per milliliter and 100 μg of proline per milliliter. Similar results have been obtained with the K12 strain T3 B_1^- try$^-$ and *E. coli* B.
7. The lysing medium contains in final concentration 0.5 percent Brij 58 (Atlas Chem. Co.), 0.5 percent sodium deoxycholate, 4 μg of deoxyribonuclease per milliliter (Mann Research-DNase I "DPFF"), 100 μg of chloramphenicol per milliliter, 0.05*M* NH_4Cl, 0.01*M* $MgSO_4$, 0.01*M* Tris-HCl, *p*H 7.4. Deoxycholate is added just before use.
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14. We thank Dr. R. Rolfe for assistance in the initial stage of this investigation. This research was supported by NIH and NSF grants. C.P.F. and P.R. are supported by an NIH predoctoral fellowship and training grant, respectively.

16 August 1967

Daily Rhythm in Tyrosine Concentration in Human Plasma: Persistence on Low-Protein Diets

Abstract. *The concentration of tyrosine in the plasma of normal males varies diurnally. It is lowest (9.5 ± 0.35 micrograms per milliliter) between 0130 and 0230 hours and rises to a peak of 16.2 ± 0.82 micrograms per milliliter by 1030 hours. This rhythm persists when subjects are maintained for 2 weeks on a diet that is very low in protein.*

Circulating tyrosine can undergo at least three kinds of metabolic transformation (*1*): the amino acid can be taken up in the tissues and incorporated into peptides and proteins; small amounts can be converted to low-molecular-weight compounds such as thyroxine, melanin, and the catecholamines; or it can be deaminated to form *p*-hydroxy phenylpyruvic acid, which is a substrate for gluconeogenesis.

Recently (*2*) it was demonstrated that the activity of tyrosine transaminase, the enzyme that catalyzes this last transformation, shows marked diurnal fluctuation in the livers of untreated rats. When animals are kept under light for 12 hours each day, enzyme activity is greatest several hours after the onset of darkness and falls to basal levels by the beginning of the light period. Although tyrosine transaminase activity is known to be induced by certain adrenocortical steroids (*3*), this enzyme rhythm is not generated by the adrenal secretory cycle inasmuch as it persists in the adrenalectomized or hypophysectomized rat (*2*).

Since transamination may account for a major fraction of the tyrosine that leaves the blood stream, we have examined the concentration of this amino acid in plasma, from normal