The Intracellular Distribution of Polynucleotide Phosphorylase in *Escherichia coli* Cells*

Y. Kimhi and U. Z. Littauer

ABSTRACT: The intracellular distribution of polynucleotide phosphorylase in *Escherichia coli* B cells has been studied. The major part of the enzyme activity (80%) is found in the soluble fraction; 10% of the total activity is bound to the cell "membrane" and cell "debris"; and about 10% remains bound to washed ribosomes. The specific enzyme activity is similar in all three fractions. Examination of the ribosome-bound enzyme revealed that the enzyme is found in all types of "native" ribosomes, namely, particles of 70, 50, and 30 S. The specific activity in these ribosomal fractions increases toward the lighter particles and reaches a maximum in the 30S region. Most of the enzymatic activity is released by lowering the Mg^{2+} concentration or by ribonuclease (RNase) action. Enzyme activity was also detected in polyribosomes. The specific activity was found to be low in the heavy-polysome region and to increase toward the smaller polysomes. By analytical ultracentrifuge and sucrose gradient analysis, 50S "native" particles were found to be susceptible to RNase action under conditions in which the "native" 70S and 30S particles are left intact.

It was suggested by Sekiguchi and Cohen (1963) and by Andoh *et al.* (1963) that a possible role of polynucleotide phosphorylase in *Escherichia coli* cells is to degrade mRNA. This hypothesis was based on the analysis of the *in vitro* degradation products of phage-induced mRNA and normal *E. coli* mRNA associated with ribosomal particles. The existence of substantial amounts of polynucleotide phosphorylase in ribosomes of *E. coli* (Wade and Lovett, 1961; Kimhi and Littauer, 1962; Grunberg-Manago, 1963) and other microorganisms (Strasdine *et al.*, 1962) was compatible with this assumption. Little is known about the distribution of the enzyme among various ribosomal subparticles and polyribosomes. This distribution is the subject of this communication.

Materials and Methods

Growth of Cells. E. coli strain B cells were grown at 37° with aeration, in a yeast extract medium (Littauer and Kornberg, 1957) to the middle of the exponential growth phase. At this stage, the temperature of the medium was lowered to 10° by the addition of crushed ice, and the cells were harvested in a Sharples centrifuge. The cells were then washed twice with cold Tris-HCl buffer (0.01 M, pH 7.4) containing magnesium acetate (0.01 M) and stored at −20°.

Preparation of Cell Extract. Cells (20 g) were suspended, while thawing in 50 ml of Tris-HCl buffer (0.01 M, pH 8.0) containing magnesium acetate (0.01 M) and DNase to 5 μg/ml. Cells were broken in a French pressure cell, precooled to 0°, at a pressure of 4000 psi. The crude homogenate was stirred for an additional 10 min at 4° to complete the degradation of DNA by the DNase. Unbroken cells and large cell debris were spun down by centrifuging at 4500*g* for 10 min. The supernatant was further centrifuged at 30,000*g* for 10 min to sediment membranes and small cell particles. The 30,000*g* supernatant was designated as the "soluble" fraction. The remaining pellet was resuspended in 10 ml of Tris-Mg buffer; this suspension was designated as the "cell membrane" fraction.

Preparation of "Native" Ribosomes. The soluble fraction was centrifuged for 4 hr at 150,000*g* (Spinco rotor no. 50). The supernatant was decanted and the ribosome pellet was resuspended in Tris-Mg buffer. This procedure of precipitation and resuspension was repeated four times. Undissolved material, consisting of various ribosomal aggregates and denatured protein, was removed after the last resuspension by centrifuging for 10 min at 10,000*g*. The clear solution, containing native ribosomes of 70, 50, and 30 S in Tris-HCl buffer (0.01 M, pH 8.0) and magnesium acetate (0.01 M), was designated as "washed ribosomes."

Dissociation into Subunits. Dissociation of 70S ribosomes to 30S and 50S subunits was achieved by dialyzing washed ribosomes for 12–15 hr against Tris-HCl buffer (0.01 M, pH 8.0) containing magnesium acetate (0.001 M) and NaCl (0.05 M) (Tal and Elson, 1963).

Sucrose Gradient. For the isolation of native ribosomes, 1.0 ml of the suspension was layered directly onto a 30-ml linear gradient of 5–20% sucrose solution containing Tris-HCl buffer (pH 8.0, 0.01 M), magnesium acetate (0.01 or 0.001 M, as indicated), and ammonium acetate (0.086 M). (Ammonium acetate was added to prevent association of 70S ribosomes to 100S particles; Gilbert, 1963.) The gradients were prepared at least 12 hr before use and equilibrated at 4°. After centrifugation

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at 25,000 rpm (Spinco rotor no. SW-25) at 3–5°, the bottom of the tube was pierced, and 10-drop fractions were collected.

Preparation of Polysomes. Polysomes were prepared according to Dresden and Hoagland (1965). The cells were grown in 400 ml of yeast extract medium at 37° up to the middle of the logarithmic growth phase, harvested, and suspended in 4 ml of Tris-HCl buffer (0.01 M, pH 7.4) containing magnesium acetate (0.01 M). A 2-ml aliquot of this suspension was layered onto 26 ml of a discontinuous gradient containing the following layers from the top down: (1) 5 ml of sucrose solution (5%) buffered with Tris-HCl (0.005 M, pH 7.9), (2) 7 ml of sucrose solution (10%) containing EDTA (0.05 M), (3) 7 ml of sucrose solution (15%) containing Tris-HCl buffer (0.03 M, pH 8.2) and lysozyme (1 mg/ml), and (4) 7 ml of sucrose solution (25%) containing Tris-HCl buffer (0.01 M, pH 7.4) and magnesium acetate (0.01 M).

The tubes were placed in rotor SW-25 of the Spinco centrifuge and accelerated to 20,000*g*. After 2 min at that speed, the centrifugation was stopped with the aid of the brakes. All the work up to this stage was carried out at room temperature. The following steps were performed at 0–4°. The sucrose solution was decanted, and the cell pellet was resuspended in a small volume of Tris-HCl buffer (0.01 M, pH 7.4) containing magnesium acetate (0.01 M). Sodium deoxycholate solution (10%) was added to a final concentration of 0.25% and the suspension was left for 5 min in ice. The total lysate was centrifuged for 10 min at 20,000*g*. The clear supernatant (1 ml) was carefully removed and placed directly onto a 30-ml linear sucrose density gradient (10–30%). The sucrose solution contained Tris-HCl buffer (0.01 M, pH 7.4), magnesium acetate (0.01 M), and ammonium acetate (0.086 M). Centrifugation was carried out in the Spinco rotor no. SW-25 for 3 hr at 25,000 rpm. After centrifugation, 15-drop fractions were collected.

Enzyme Assay. The activity of polynucleotide phosphorylase was measured in two ways: (a) the phosphorolysis of poly A and (b) the exchange of $^{32}P_i$ with the terminal phosphate of ADP[1] (Littauer and Kornberg, 1957).

Phosphorolysis of Poly A. The reaction mixture (0.1 ml) contained in micromoles: Tris-HCl buffer (pH 8.0), 10; $MgCl_2$, 0.05; EDTA (pH 8.0), 0.005 [^{32}P]potassium phosphate buffer (pH 8.0), 1 (about 10^6 cpm/μmole of PO_4); polyadenylic acid, 0.1; and enzyme, 0.005–0.5 unit. Incubation at 37° was carried out for 15 min, after which the tubes were cooled and 0.9 ml of perchloric acid (1%) was added. Extraction of inorganic phosphate was carried out as follows (Avron, 1960). To the reaction mixture, water saturated with isobutyl alcohol–benzene (1:1, v/v) was added to give a final volume of 2.5 ml, and 0.8 ml of ammonium molybdate reagent (5% in 4 N HCl) was added. The phosphomolybdate complex was extracted with 7 ml of isobutyl alcohol–benzene mixture (1:1, v/v). The upper organic layer was removed by suction and the extraction was repeated (with 7 ml of isobutyl alcohol–benzene). Special care was taken to remove traces of the organic solvent. Aliquots of 1 ml (out of 3.3-ml total volume) were dried and counted in a gas-flow counter. One unit of enzyme is defined as the amount which liberates 1 μmole of ADP in 15 min.

Exchange of $^{32}P_i$ with ADP. The reaction mixture (0.5 ml) contained in micromoles: Tris-HCl buffer (pH 8.3), 100; ADP, 0.4; $MgCl_2$, 2; [^{32}P]sodium phosphate buffer (pH 8.0), 0.35 (about 10^6 cpm/μmole); activator [(Littauer and Kornberg, 1957) ammonium sulfate fraction (40–53% saturation) chromatographed on Sephadex G-200 and boiled for 5 min at 100° (Y. Kimhi and U. Z. Littauer, in preparation)], 0.1 ml; and 0.001–0.05 unit of enzyme. After 20-min incubation at 37°, the tubes were cooled in an ice bucket and 0.5 ml of perchloric acid (3%) was added. The extraction of inorganic phosphate was carried out as described above. Aliquots of 1 ml were added to 12 ml of Bray's (1960) scintillation fluid and counted in a liquid scintillation spectrometer (Packard, Tri-Carb). One unit of enzyme is defined as the amount of enzyme which exchanges 1 μmole of ADP in 1 hr.

Enzymes. Polynucleotide phosphorylase was purified 720-fold from *E. coli* B cells. The method involved ammonium sulfate precipitation, acetone fractionation, and DEAE-Sephadex and Sephadex G-200 column chromatography (Y. Kimhi and U. Z. Littauer, in preparation). Five-times-crystallized ribonuclease was obtained from Mann Reasearch Laboratories, Inc.; crystalline DNase was a Worthington Biochemical Corp. product.

Chemicals. ADP was purchased from Sigma Chemical Inc. Polyadenylic acid was received from Miles Chemical Co. Sodium deoxycholate was obtained from the British Drug House Ltd. [^{32}P]Orthophosphate was obtained from Radiochemical Centre, Amersham, England.

Results

The Intracellular Location of the Enzyme. As shown in Table I, the enzyme is found in all cell fractions. The major part of the activity (80–90%) is in the soluble fraction which also contains ribosomes. The membrane fraction (containing also small cell fragments) does not contain more than 10–15% of the total activity. This amount may result from rupturing the cells in a French pressure cell, and perhaps is not a reflection of *in vivo* conditions. However, preparations of alumina-ground cells gave similar results.

Is the Enzyme Bound to Ribosomes? As shown in Table I, the soluble fraction contains about 80–90% of the total polynucleotide phosphorylase activity in the cell. It has been previously shown (Wade and Lovett, 1961; Kimhi and Littauer, 1962; Grunberg-Manago, 1963) that a large part of the cellular activity of this enzyme is ribosome bound. It was, therefore, of interest to examine the nature and stability of the binding of the enzyme to the ribosomes. Ribosomes were washed by repeated centrifugation in Tris-HCl buffer (0.01 M, pH 8.0) containing magnesium acetate (0.01 M). Table II

[1] Abbreviations used: ADP and UDP, adenosine and uridine diphosphates.

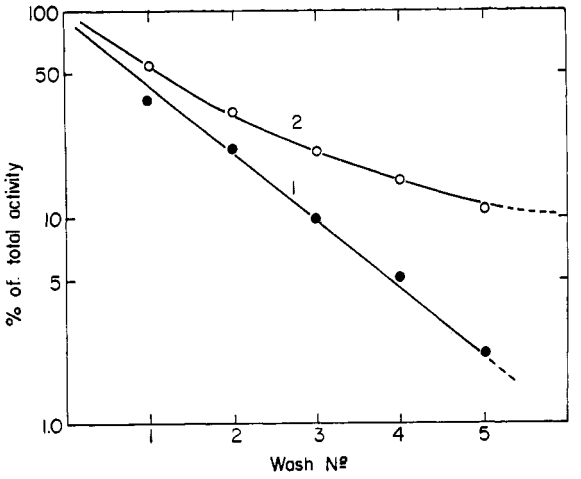

FIGURE 1: Release of enzyme from ribosomes by repeated centrifugation. Curve 1 shows the amount of polynucleotide phosphorylase liberated into the supernatant after each washing. Curve 2 shows the amount of enzyme which remains bound to the ribosomes. The values are expressed as per cent of the initial total activity. Enzymatic activity was measured by the phosphorolysis assay.

shows that ribosome-bound activity decreased after the fifth washing to 10–13% of the total cell activity. The open circles in Figure 1 show the per cent of the total enzyme present in the ribosome fraction after each wash. The enzyme content of the ribosomes starts to level off at about 10–13% of the total after the fifth wash. The solid circles show the amount of enzyme liberated (out of the initial total activity) into the supernatant after each washing. Extrapolation of these values indicates that no more than an additional 3% of the total would be released after additional washing.

TABLE I: The Intracellular Distribution of Polynucleotide Phosphorylase.[a]

Fraction	% of Total Act. in the Cell	Sp Act. (units/mg of protein)
Cell debris	9	0.32
Cell membrane and small cell fragments	7	0.35
Soluble fraction (containing ribosomes)	84	0.33
Whole cells	100	0.30

[a] The various fractions were subjected to sonication for 20 min at 4° in a Raytheon, Model DF 101, sonic oscillator. Activity was measured by the phosphorolysis assay.

TABLE II: Polynucleotide Phosphorylase Activity Bound to Washed Ribosomes.[a]

Fraction	Enzyme Activity: Total Units	Enzyme Activity: %[b]
First precipitation	504	60
Second precipitation	283	34
Third precipitation	192	23
Fourth precipitation	143	17
Fifth precipitation	109	13
Dissociation of Five-Times-Washed Ribosomes		
Supernatant after dissociation	60	7
Dissociated ribosomes	42	5

[a] The starting material for these experiments was the supernatant of the 30,000*g* centrifugation which contains 80% of the total activity in the cell. "Washing" of the ribosomes was performed by suspension in Tris-HCl buffer (0.01 M, pH 8.0) containing magnesium acetate (0.01 M). The phosphorolysis assay was used in these experiments. [b] This value is expressed as per cent of the total enzyme activity in crude extracts before centrifugation.

To What Ribosomal Fraction Is the Enzyme Bound? As noted above, 7–10% of the total activity is firmly bound to ribosomes. In order to determine the types of ribosomes to which the enzyme is attached, the ribosome suspension was assayed for enzyme activity after centrifugation on linear sucrose gradients. Enzymatic activity is found in the ribosomal region and increases toward the soluble fraction (Figure 2). The same picture was obtained when the activity of the enzyme was assayed by phosphorolysis of polyadenylic acid or by exchange of $^{32}P_i$ with ADP. Sucrose itself does not affect the assays.

In order to achieve better resolution, it was necessary to place a small amount of ribosomes on the gradient. Since the exchange assay is more sensitive than the phosphorolysis assay, the fomer was used in the following experiments.

Fractionation on a sucrose gradient in the presence of 0.01 M Mg^{2+} reveals that the enzyme is found in all types of native ribosomes, namely, particles of 70, 50, and 30 S. The specific activity in these ribosomal fractions increases toward the lighter particles and reaches its maximum in the 30S region (Figure 2). In some experiments the differences in specific activity were more pronounced. Other experiments (Kimhi and Littauer, 1962), not presented here, demonstrate that the specific activity of the 20–25S fraction may reach higher values, but this could not be shown unequivocally since the soluble enzyme, having a value of approximately 8 S (Y. Kimhi and U. Z. Littauer, in preparation), overlaps with that region.

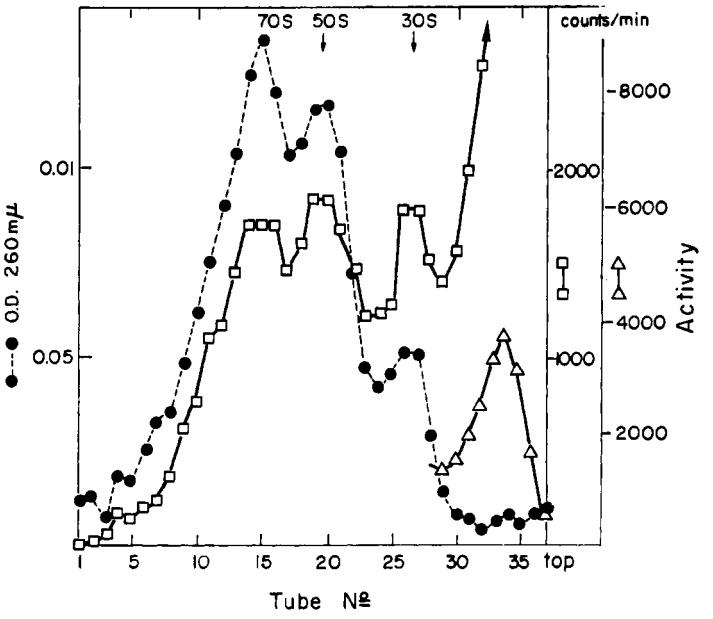

FIGURE 2: Separation of native ribosomes (washed five times) in a linear density sucrose gradient. Ribosomes [(3.3 mg) in 0.15 ml of Tris-HCl buffer (pH 8.0, 0.01 M), magnesium acetate (0.01 M), and ammonium acetate (0.086 M)] were layered directly onto a 30-ml linear density sucrose gradient (5–20%) containing the same buffer and centrifuged for 7 hr at 25,000 rpm in a SW-25 rotor at 4°. Enzymatic activity was measured by the exchange assay. The enzyme activity was corrected for a blank of 166 cpm obtained in the absence of enzyme.

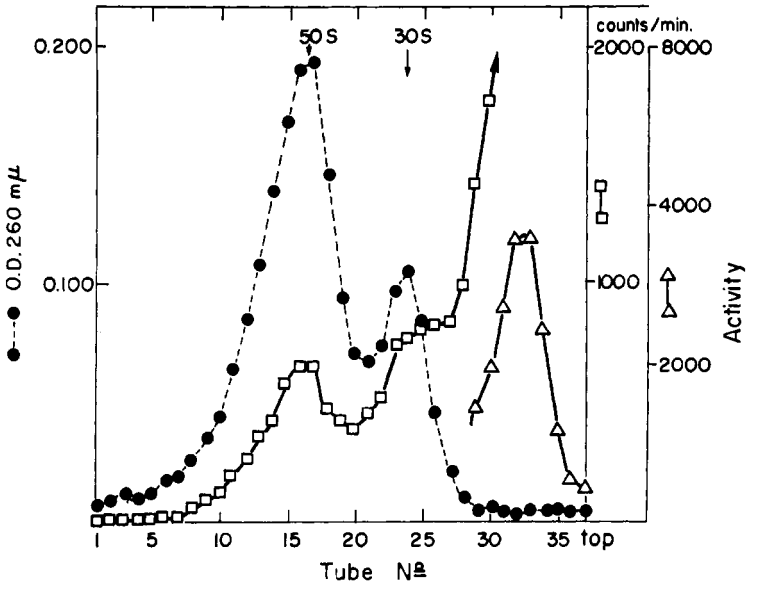

FIGURE 3: Separation of ribosomes after dissociation in 10^{-3} M Mg^{2+} in the presence of 0.05 M NaCl. Dissociated ribosomes [(3.0 mg) in 0.15 ml of Tris-HCl buffer (pH, 8.0, 0.01 M), magnesium acetate (0.001 M), and ammonium acetate (0.086 M)] were layered directly onto a 30-ml linear density sucrose gradient (5–20%) containing the same buffer and centrifuged for 9 hr at 25,000 rpm in a SW-25 rotor at 4°. Enzymatic activity was measured by the exchange assay. The enzyme activity was corrected for a blank of 145 cpm obtained in the absence of the enzyme.

The activity found in the lighter region of the sucrose gradient is one-third of the total activity contained in the five-times-washed ribosomes. This value is in agreement with the amount of enzyme which would be released from the ribosomes if washings were continued three to four times (Figure 1).

The Nature of the Enzyme–Ribosome Bond. Two methods were used to investigate the nature of the en-

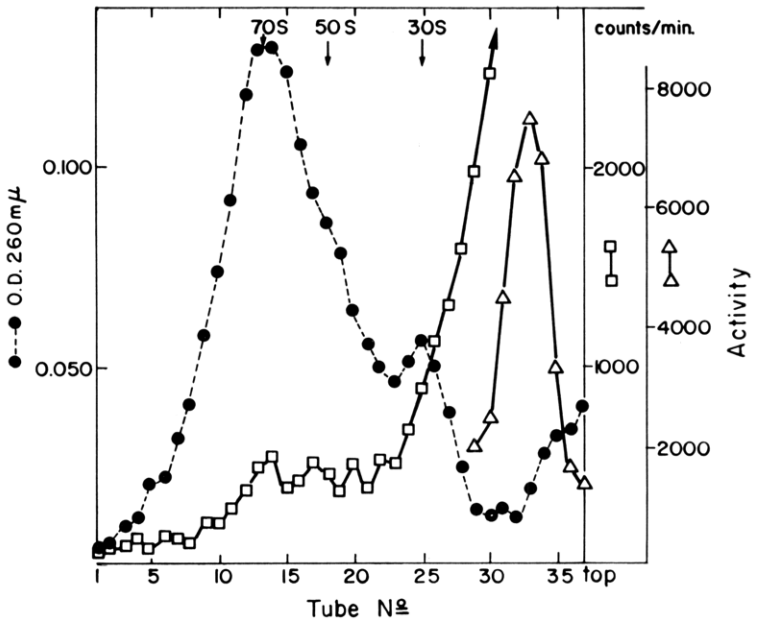

FIGURE 4: Separation of ribosomes treated with RNase (10 μg/ml) for 10 min at 4°. Amount of ribosomes and conditions are the same as in Figure 2.

zyme–ribosome bond: (a) lowering the Mg^{2+} concentration which causes the dissociation of the native 70S ribosomes into the derived 30S + 50S subunits (Tissieres *et al.*, 1959), and (b) action of RNase.

Table II and Figure 3 show that the dissociation of the 70S particle liberates the major part of the ribosomal activity. Thus, after lowering the Mg^{2+} concentration, only 2.5–5% of the total activity of the crude extract remains bound to the 30S and 50S ribosomes. The residual enzyme activity found in the 30S and 50S ribosomes (Figure 3) may be bound to the native 30S and 50S particles which are also present in the preparation. Evidence supporting this assumption comes from other experiments in which the native 70S fraction was selectively pelleted by short centrifugations thus eliminating the bulk of the lighter native 30 and 50 S. After dissociation of these enriched 70S ribosomes, 1–2% of the activity remains bound to the ribosomes.

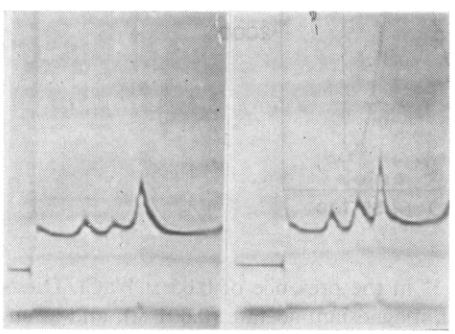

FIGURE 5: The effect of RNase treatment on native ribosomes. (a) (left) Plus RNase (10 μg/ml for 10 min at 4°); (b) (right) minus RNase. Each sample contained about 7 mg of ribosomes in 1 ml of Tris-HCl buffer (pH 8.0, 0.01 M) containing magnesium acetate (0.01 M) and ammonium acetate (0.086 M). The ultracentrifuge pictures were taken with schlieren optics at bar angle of 60° after 80 min at 50,740 rpm and 20°. Sedimentation is from left to right.

Figure 4 shows that incubation of native ribosomes with RNase (10 μg/ml, for 10 min at 0°) renders most of the enzyme soluble. These experiments indicate that polynucleotide phosphorylase is bound to the ribosomes through RNA and magnesium ions. It was also observed that when RNase was applied to native ribosomes (compare Figure 4 to Figure 2), most of the native 50S particles' peak disappeared. This finding was confirmed by examining the digest in the analytical ultracentrifuge (Figure 5) which indicated a degradation of approximately 50% of the native 50 S without a detectable change in the 70S or 30S fractions.

Presence of Polynucleotide Phosphorylase in Polysomes. The observation that polynucleotide phosphorylase activity was liberated from ribosomes by RNase treatment and by lowering the magnesium concentration suggests that the enzyme may be bound to mRNA (Brenner *et al.*, 1961; Gros *et al.*, 1961). The presence of enzyme activity in polysomes was, therefore, examined. About 1–3% of the enzyme activity was found in the polysomal region (Figure 6). The specific activity was found to be low in the heavy-polysome region and to increase toward the smaller polysomes. Figure 7 shows that incubation of the crude extract with RNase releases the enzyme from the polysome region.

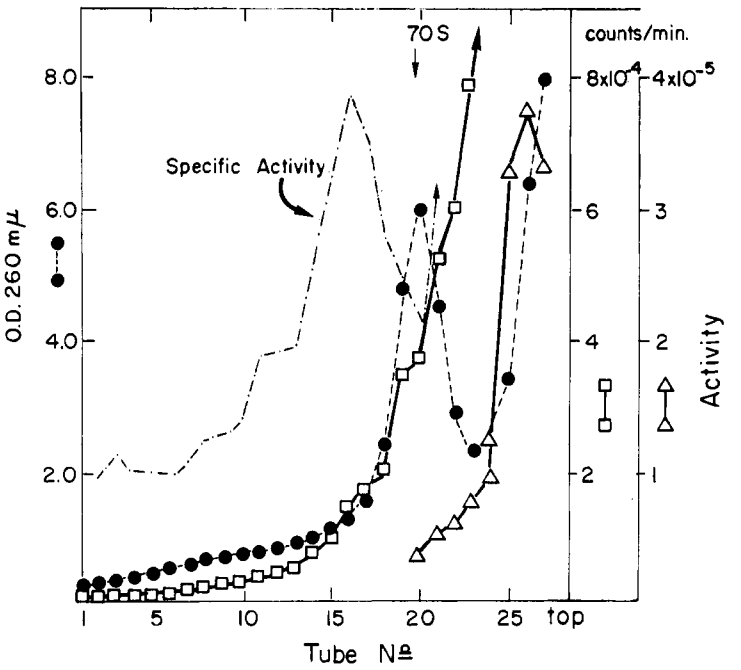

FIGURE 6: Separation of polysomes on a linear density sucrose gradient. Polysome solution (1 ml) was layered directly onto a 30-ml linear density sucrose gradient (10–30%) containing Tris-HCl buffer (pH 7.4, 0.01 M), magnesium acetate (0.01 M), and ammonium acetate (0.086 M) and centrifuged for 3 hr at 25,000 rpm in a SW-25 rotor. The enzymatic activity was measured by the exchange assay. The enzyme activity was corrected for a blank of 168 cpm obtained in the absence of the enzyme.

Discussion

Abrams and McNamara (1962) have demonstrated that polynucleotide phosphorylase is located in the membrane of *Streptococcus faecalis* and that the specific activity in this fraction is ten times higher than that of the soluble fraction. As shown in this work, *E. coli* B membrane fractions prepared by homogenization in a French pressure cell or alumina grinding do not differ markedly in their specific activity from the other fractions. Furthermore, most of the enzyme is found in the soluble fraction.

Sekiguchi and Cohen (1963) and Andoh *et al.* (1963) suggested that polynucleotide phosphorylase is involved in the degradation of mRNA. Their conclusions were based on (a) the presence of the enzyme in ribosomes and (b) isolation of nucleoside diphosphates among the degradation products of mRNA after addition of orthophosphate and Mg^{2+}. Wade and Lovett (1961) have shown by various methods, that *E. coli* ribosomes contain this enzyme. Recent studies by Hardy and Kurland (1966) indicated that washed *E. coli* ribosomes contain only a small amount of polynucleotide phosphorylase. It was also observed by these authors that crude *E. coli* extracts contain a potent inhibitor which antagonizes the polynucleotide phosphorylase assay (incorporation of labeled ADP or UDP into an acid-insoluble precipitate). Because of the presence of this inhibitor the per cent of enzyme contained in the ribosomes could not be ascertained unambiguously. On the other hand, Grunberg-Manago (1963) found that 40% of the enzyme is contained in the ribosomes. However, our findings show that this value can be further reduced to 10–13% by repeated washings (Table I). Figure 1 shows that additional washings could reduce this value to no more than 7–10%. It is possible that this amount of "ribosomal" enzyme (7–10%) can still be adequate for degrading activity. It should be indicated that the major part of this activity is released from the ribosomes by RNase treatment or by lowering the Mg^{2+} concentration to 10^{-3} M. These findings may be due to binding of the enzyme to mRNA rather than to the ribosomes. If this assumption is correct, then one molecule of enzyme should be found on each polysome since the enzyme acts as an exonuclease and attacks the 3′-OH end of the mRNA chain. One would expect that the longer the mRNA chain, the higher the number of ribosomes in the polysome, and that the specific activity of the enzyme, namely, the number of enzyme molecules per polysomes (or counts per minute per optical density at 260 mμ), would be higher in the small polysomes, and lower in the large polysomes. The results presented in Figure 6 are consistent with this assumption. (a) It can be seen that activity of the enzyme is present in the polysome region. (b) The specific activity increases from the heavy region toward the lighter, reaches a peak in the dimer–tetramer region, and then falls in the monomer (70 S) region which presumably contains less mRNA per ribosome.

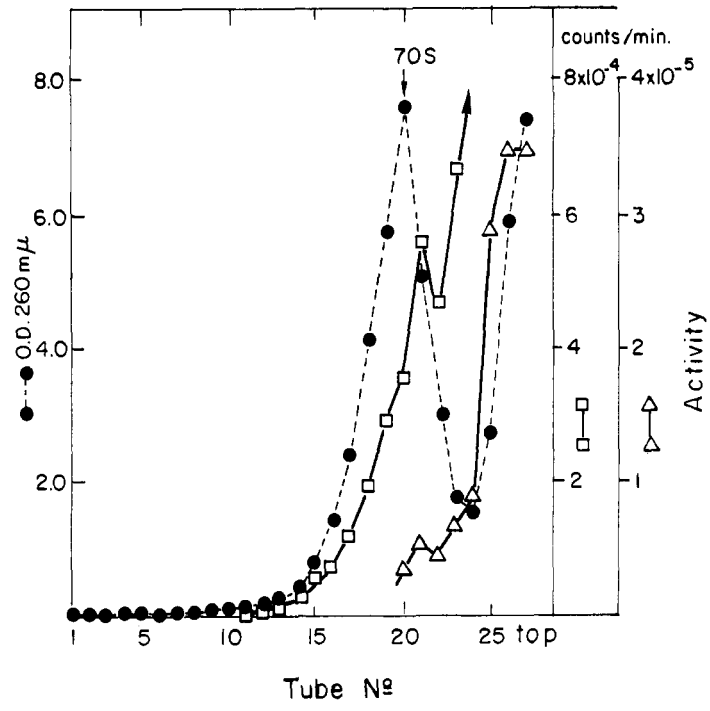

FIGURE 7: The effect of RNase (1 μg/ml for 5 min at 0°) on polysomes. The amount and conditions were the same as in Figure 6.

In addition, Figure 6 shows that the specific activity in the polysome region increases in a stepwise fashion rather than a linear fashion. (Though a stepwise increase in specific activity was consistently found in all the gradients examined, this conclusion is tentative since the values obtained are close to the experimental error.) (c) Incubation with RNase diminishes the polysome region and the enzyme activity associated with it (Figure 7).

The mode of degradation of mRNA by polynucleotide phosphorylase may explain the observation that the level of the various enzymes produced by a polycistronic messenger decreases in a single direction (modulation; Ames and Hartman, 1963; Zabin, 1963). It was shown recently that the reading of the mRNA in protein synthesis is directed from the 5′-OH end to the 3′-OH end (Salas *et al.*, 1965; Thach *et al.*, 1965). On the other hand, the degradation of polyribonucleotides by polynucleotide phosphorylase proceeds according to an exonucleolytic mechanism, starting from the 3′-OH end and moving toward the 5′-OH end. In the present work it was shown that polynucleotide phosphorylase activity is found in polyribosomes. The specific activity was shown to be low in the heavy-polysome region and to increase toward the smaller polysomes. If polynucleotide phosphorylase degrades mRNA and if one assumes that the mRNA is translated in the direction of decreasing polypeptide production, then the modulation of enzyme levels is compatible with the finding of the present communication and can be explained by postulating that the direction of the degradation is opposite to the direction of the reading of mRNA.[2] On the other hand, our observations are also consistent with the idea that the polynucleotide phosphorylase activity associated with the polysomes (1–3%) reflects the last stage of *de novo* synthesis of the enzyme. Also, the existence of microorganisms with an apparent absence of polynucleotide phosphorylase activity (Ochoa *et al.*, 1961) and the isolation of mutants which appear to be deficient in polynucleotide phosphorylase (Gesteland, 1965) indicate that the cell may have other mechanisms for degrading mRNA (*cf.* Spahr, 1964). Further experiments are, therefore, needed to substantiate or to rule out these suggestions.

The biological role of the native 30S and 50S particles is still obscure (Aronson and McCarthy, 1961; Green and Hall, 1961). It is possible that their function is somehow related to the high specific activity of polynucleotide phosphorylase found in these particles. The observation that RNase degrades selectively the native 50S particles suggests that the RNA in these particles is not fully protected as in the other ribosomal species and that the structure of 50S native ribosomes is different from that of the other particles.

Acknowledgment

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[2] The possibility that modulation is governed by exonucleolytic degradation was also discussed by Kaempfer (1965).

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