1968

Isolation, Characterization and Distribution of Ribosomes and Polyribosomes in Azotobacter vinelandii

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ISOLATION, CHARACTERIZATION AND DISTRIBUTION OF RIBOSOMES AND POLYRIBOSOMES IN *AZOTOBACTER VINELANDII*

by

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A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

January

1968

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INTRODUCTION

Polyribosomes, the intracellular ribosomal aggregates which compose the structural machinery upon which protein synthesis occurs, have been studied in a wide variety of biological systems, including bacteria (10, 33), yeast (21), slime molds (32), protozoa (19), plant tissues (42), and a variety of mammalian tissues, both normal (9, 25, 43, 45) and abnormal (12, 31). A large number of investigations have confirmed the occurrence of these structures in all activly metabolizing living cells; and their function in protein synthesis has now been well established (13, 25, 43-45). An interesting and important problem currently receiving intense study concerns the nature, state and distribution of polyribosomes, ribosomes and subunits in growing cells. A direct approach to this problem has not been a simple one, mainly due to polyribosomal breakdown during cell harvest and polyribosomal fragmentation during cell disruption.

One of the most striking characteristics of polyribosomes is their extreme fragility. This fragility appears to be due to the fact that polyribosomal components are held together solely by a strand of mRNA (44). Therefore, extremely gentle methods of cell disruption are required in order to isolate quantities of intact polyribosomes, or to establish with any degree of certainty the exact state and relative quantities of the various size classes of polyribosomes, ribosomes, and subunits in actively growing cells (20, 23).

In the past a wide variety of techniques have been used to disrupt

bacteria to obtain ribosomes. These include such harsh methods of cell disruption as ultrasonic oscillation, the use of the French pressure cell (36), grinding with glass beads (4), and freeze-thaw treatment (35). While these methods are effective for cell breakage, and procedures permit continued vigorous action upon disrupted cells as well as their cytoplasmic contents. The more recent and desirable method for obtaining ribosomal extracts involves the lysis of cells by osmotic shock, in which cells are subjected only to a single instantaneous trauma. This procedure was originally utilized in the rabbit reticulocyte system (44) since these cells have no cell wall but only a cell membrane thus making them extremely susceptible to osmotic lysis. Since bacteria in their normal state possess a rather rigid cell wall structure as well as cell membranes, they are not susceptible to such lysis. Thus most bacteria must first be treated either to weaken or remove the cell wall before osmotic lysis. This usually has been accomplished by incubation of cells with cell-wall lytic enzymes, EDTA, deoxycholate, antibiotics, or various combinations of these to produce spheroplasts or protoplasts which are subsequently lysed by osmotic shock (6, 8, 11, 18, 20, 37). Since in all of the previously mentioned methods for isolation of polyribosomes cells are subjected either to rigorous physical treatment or are placed under sub-optimal growth conditions to ensure lysis, polyribosome profiles obtained are not representative of the true ribosome and polyribosome content of growing cells. These profiles, rather, represent ribosomal populations which have been degraded or obtained from cells in a somewhat modified growth state.

In this study Azotobacter vinelandii a nonpathogenic, nitrogen

fixing soil bacterium, was used in order to obtain a more precise picture of the natural cellular content of polyribosomes and the mechanism of protein synthesis for two main reasons. First and most importantly is the ease with which these organisms can be disrupted. The Azotobacter are readily disrupted by direct osmotic shock (34) without prior treatment, due to the unique cell wall strcture of this bacterium. This obviates either the harsh methods of cell disruption or incubation under suboptimal growth conditions necessary in all of the previously mentioned bacterial systems. Thus, in this system it is possible to examine the cytoplasmic contents of exponentially growing cells producing a full complement of cellular components.

A second reason for studying Azotobacter is that this bacterium has been shown to possess an extensive internal membranous network (30) which is most likely part of the plasma membrane. This membrane structure may be analogous to the endoplasmic reticulum of animal cells. The attachment of ribosomes and polyribosomes to cell membrane surfaces has been known for some time in animal cells (29) and their importance in protein synthesis has been well established (15). In bacterial systems, however, while membrane-bound polysomes have been described by Schlessinger (38) and Aronson (1), it has not been not been definitely established that the association exists in vivo. Azotobacter, with its vast membranous system should offer an ideal system for the further study of membrane-bound polyribosomes in bacteria.

In this paper the polyribosome, ribosome and ribosomal subunit content of the bacterial cell under varied conditions was studied. A procedure was

developed for cell lysis that is gentle and direct and, when certain parameters are followed yields cell extracts containing a significantly larger percentage of polyribosomes than have been previously reported in other bacteria. The physical and chemical properties of these ribosomes and polyribosomes were then studied. Finally a brief consideration was given to the possible interaction between polyribosomes and bacterial membranes.

MATERIALS AND METHODS

Organism, media and cultivation. Azotobacter vinelandii strain OP, a slime-free mutant, obtained from Professor P. W. Wilson of the University of Wisconsin, was used in this study. One liter cultures were grown in a modified Burk's nitrogen-free medium, containing per liter: KH_2PO_4, 0.2 g; K_2HPO_4, 0.8 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; $CaCl_2 \cdot 2\ H_2O$, 0.04 g; $Fe_3(SO_4)_2$, 1 mg; $NaMoO_4$, 0.1 mg; and sucrose, 20 g; on a rotary shaker at 32 C in 2 liter flasks fitted with stainless steel baffles to increase aeration. Under these conditions, the Azotobacter grew with a generation time of 100 min in the exponential phase.

Harvest and lysis. Exponentially growing cultures containing 1×10^9 cells/ml were harvested by rapidly pouring the flask contents onto finely crushed ice, which lowers the temperature to 0 C within 10-15 sec. All subsequent procedures were performed at or near 0 C. The cells were then sedimented in a refrigerated centrifuge, resuspended in cold AVO buffer (Tris 0.05 M, pH 7.2; magnesium acetate 0.004 M; KCl 0.1 M; and spermidine 3 HCl 0.0002 M), centrifuged again and finally resuspended in a minimal quantity of AVO buffer containing sufficient glycerol to establish a final internal cellular concentration of 1.5 M glycerol. Cells were then lysed by abruptly adding a minimal quantity of the AVO buffer to the glycerol laden cells (1 ml of buffer per 1 ml of glycerolized cell suspension). This method of cell disruption is a slight modification of the procedure developed by Robrish and Marr (34). The lysate was freed of hulls and intact cells by centrifugation at 10,000 x g for 10 min, immediately after which the super-

natant fluid was carefully decanted.

Other methods of cell breakage.

(a) Ballistic disintegration was performed in a cooled Mickle disintegrator in which a suspension of 1 g of cells and 1 g of acid washed glass beads (120 μ diam) in 2 ml of AVO buffer was treated intermittently for a total of 90 sec in 10 sec intervals with cooling in between. The degree of breakage was followed by observation with a light microscope. After treatment the cell debris and glass beads were removed by centrifugation at 10,000 x g for 10 min and the supernatant fraction saved.

(b) Disruption with glass beads was performed by grinding 1 g of cells mixed with 2 g of acid washed glass beads (120 μ diam) for 2.5 min in a mortar packed in an ice bath. The mixture was extracted with a minimal quantity of cold AVO buffer and centrifuged at 10,000 x g for 10 min to remove debris.

(c) Sonic disruption of cells was performed in a Branson Sonifier using the large probe. A suspension of 1 g of cells in 2 ml of AVO buffer was subjected to ten 5 sec oscillations at 50 KC interspersed with 30 sec cooling intervals. Disruption was followed by microscopic observation. Intact cells and debris were removed by centrifugation at 10,000 x g for 10 min.

Sucrose density gradient centrifugation. Ten to thirty-seven percent exponential sucrose density gradients were prepared according to the method of Bock and Ling (3). Concentrated sucrose, in AVO buffer, was pretreated

with colloidal macaloid to remove traces of RNAse present in the disaccharide. Lysates were layered onto freshly prepared gradients with a wide bore pipet. The gradients were then centrifuged at 2 C in an IEC B-35 centrifuge in an SB 110 swinging bucket rotor at 105,000 x g for 4 h (unless stated otherwise).

After centrifugation, the polyallomer tubes were punctured at the bottom with a device of our design (28) and the contents of the tubes drained through a specially constructed flow cell (5 mm path length) flowing from top to bottom (22). Absorption of the effluent from the gradient was monitored continuously at 260 mu during flow through a Beckman DB spectrophotometer connected to a Sargent SRL recorder for display.

When the radioactive content of gradient samples was to be measured, 0.4 ml fractions were collected directly into vials; Bray's solution for aqeous samples (2) was added and the radioactivity was assayed in a three channel Nuclear Chicago liquid scintillation spectrometer.

Electron microscopy. Fractions for electron microscopic analysis were collected from the gradients in 0.5 ml aliquots directly into small tubes placed in an ice bath. One drop samples were placed on formvar-carbon coated grids. The polyribosomes were allowed to settle onto the grid for one min and then the grid was floated on 10% formalin (pH 7.0) for 20 sec; rinsed by immersion in distilled water for 30 sec; inverted in absolute alcohol for 60 sec and then finally placed in amyl acetate for 1 min (24). The grids were air dried and subsequently shadowed with a thin carbon-platinum coating.

The grids were examined with a Siemens Elmiskop Ia equipped with a double condenser fitted with a 50 μ objective aperture, operated at 60 KV.

Samples of whole and lysed cells were fixed with 1% osmium tetroxide buffered at pH 6.1 in veronal acetate buffer, as described by Kellenberger (17). The fixed preparations were then dehydrated and subsequently embedded in Epon 812. Thin sections of the embedded samples were cut on a Porter-Blum microtome and placed on formvar-coated grids. After staining with uranyl acetate the samples were examined with an RCA model EMU-3 electron microscope.

Analytical centrifugation. Ribosomes, suspended in AVO buffer to concentrations of 2-16 mg/ml were examined by analytical ultracentrifugation. (Ribosome concentrations were estimated by using the equivalence of A_{260} of 20 = 1 mg/ml RNA). Sedimentation analyses were performed in a Spinco model E ultracentrifuge at temperatures ranging between 4-10 C using Schlieren optics; the final speed of centrifugation was 21,740 rev/min (unless otherwise noted). After 20 min after the desired speed had been reached photographs were taken at 2 min intervals with 2 sec exposure times on Kodak plates. The rates of sedimentation of the ribosomes were calculated from the distance that the boundary moved with time. For that purpose the Schlieren photographic plates were analyzed on a Gaertner comparator. The observed experimental sedimentation coefficients (Sc) were corrected for viscosity and density to water at 20 C according to the Svedberg equation (40). These values were then extrapolated to zero concentration by plotting

sedimentation rates at 4 concentrations of the ribosome suspensions. Sedimentation coefficients using these corrections are designated $S^{o}_{20, w}$ values.

Reagents. The UL-^{14}C amino acid mixture, (specific activity, 40 mC/milliatom) was purchased from New England Nuclear, Boston, Massachusetts. RNAse and DNAse free of RNAse were purchased from the Worthington Biochemical Co., Worthington, New Jersey. Macaloid was obtained from the Inerto Co., Las Vegas, Nevada. Chloromphenicol was a gift from Parke, Davis and Co., Detroit, Michigan. Sucrose and all other chemicals were of reagent grade.

Biochemical determinations.

(a) Ribonuclease assay: RNAse levels were determined by the method described by Kalnitsky (16) wherein the hydrolysis of yeast RNA at pH 5.0 is determined by measuring spectrophotometrically the amount of acid soluble nucleotide bases liberated under specified conditions.

(b) Effect of RNAse on polyribosomes: Freshly prepared pancreatic ribonuclease (5 μg/ml) was incubated with Azotobacter cellular lysate for 10 min at 0 C, prior to layering the lysate on a sucrose gradient.

(c) Effect of DNAse on polyribosomes: Freshly prepared desoxyribonuclease (10 μg/ml) was incubated with Azotobacter cellular lysate for 15 min at 0 C, prior to layering the lysate on a sucrose gradient.

RESULTS

Resolution of polyribosomes size classes with sucrose density gradients. We are able to demonstrate the occurrence and to isolate a number of polyribosome size classes directly from cell lysates, without intervening concentration procedures. However, exponential rather than linear sucrose density gradients were necessary to achieve this resolution (23). A typical absorbancy trace is shown in Fig. 1. Seven to eight polyribosomal classes in addition to the single ribosomes and ribosomal subunits are resolved routinely. A large region of heavy unresolved polyribosomes sediments ahead of the fastest resolved peak. The polyribosomal fraction contains 80% of the total ribosomal population of which approximately 75% sediments more rapidly than the tetramers. The single ribosomes compose 10% of the ribosomal population, while the remaining 10% is made up of the ribosomal subunits.

To verify our interpretation of the absorbancy traces, fractions were collected and electron micrographs of these samples were examined. Plate 1 shows electron micrographs of samples taken from peaks 1-6 of Fig. 1. Plate 2 shows electron micrographs of peaks 7-11 and from a sample in the lightest portion of the unresolved area. Plate 3 shows electron micrographs of samples from the heavier portion of the gradient. Peak 3, contains single ribosomes, peak 4 mainly dimers, peak 5 trimers, peak 6 tetramers, etc. With the simple exponential gradients employed here, resolution decreases toward the heavy side of the octomers. While polyribosome size classes are not isolated as distinct peaks past the octomers, from our

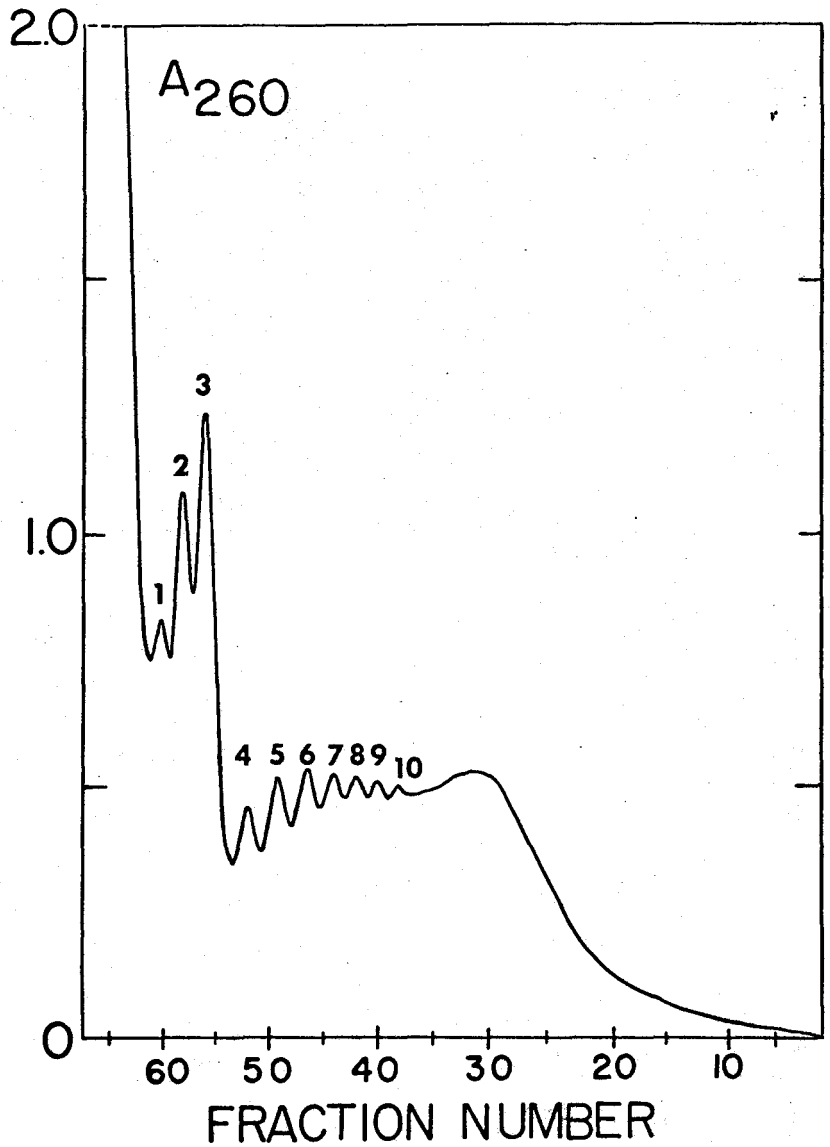


Fig. 1 Sucrose density gradient analysis of polyribosomes from cytoplasmic extracts of Azotobacter vinelandii. An exponentially growing culture was harvested and lysed by osmotic shock as described in methods and materials. An aliquot of the lysate containing 1.5 mg RNA was layered onto a 10-37% (w/v) exponential sucrose density gradient. Centrifugation was performed in an IEC B-35 centrifuge in an SB-110 swinging bucket rotor at 105,000 x g for 4 hr. After centrifugation the polyallomer tube was punctured at the bottom and the contents of the gradient was continuously monitored at 260 mμ in a double beam spectrophotometer fitted with a specially designed flow cell. One half ml fractions were collected for electron microscopic analysis.

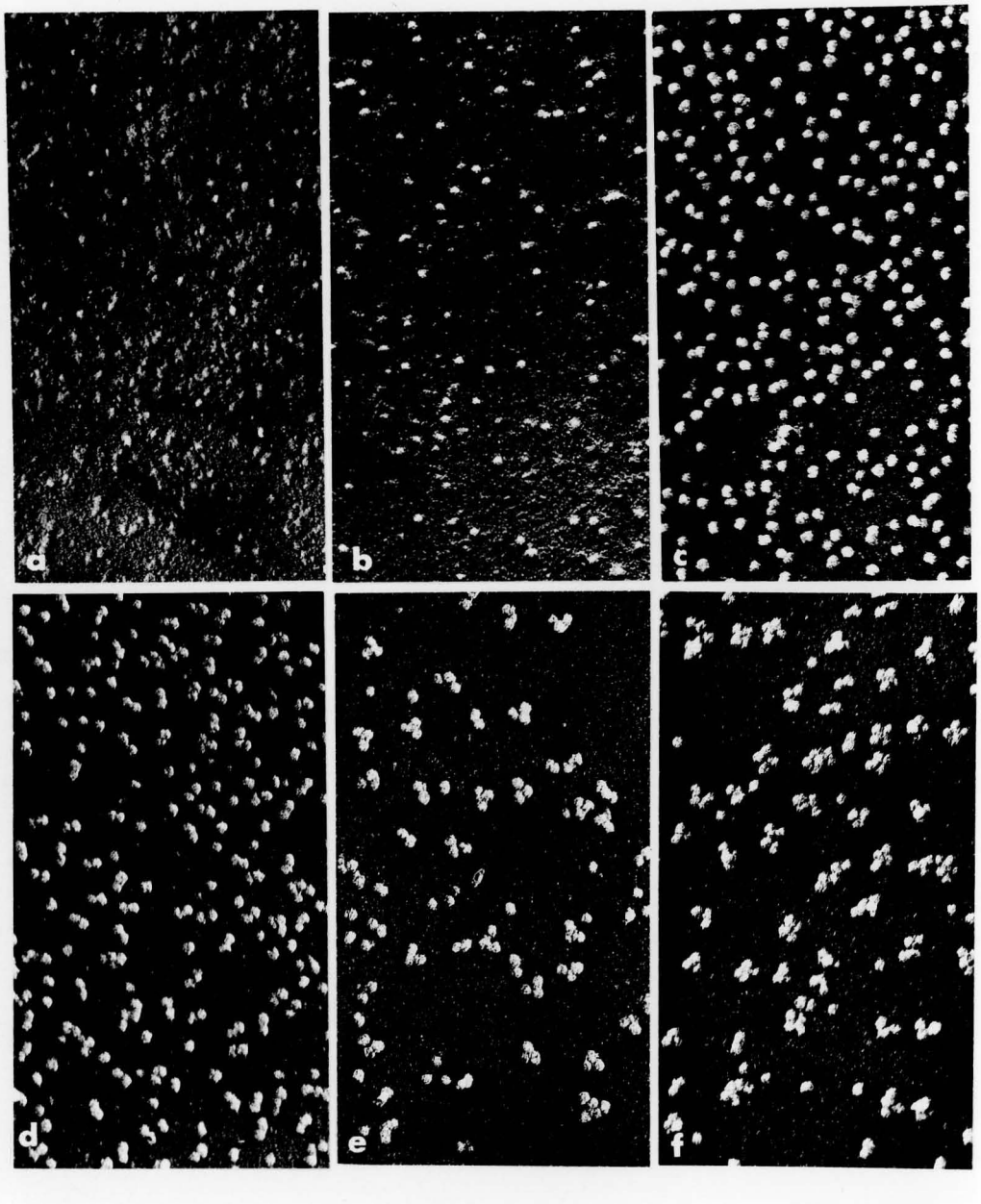


Plate 1 Electron micrographs of ribosomes, polyribosomes and subunits from Azotobacter vinelandii. (a) sample from fraction 60 of Fig. 1 showing the 36 S subunit, (b) sample from fraction 57 showing the 56 S subunit, (c) sample from fraction 55 showing the intact 72 S ribosome, (d) sample from fraction 53 showing the dimer, (e) sample from fraction 48 showing the trimer structure, (f) sample from fraction 46 showing mainly tetramers. X 60,000.

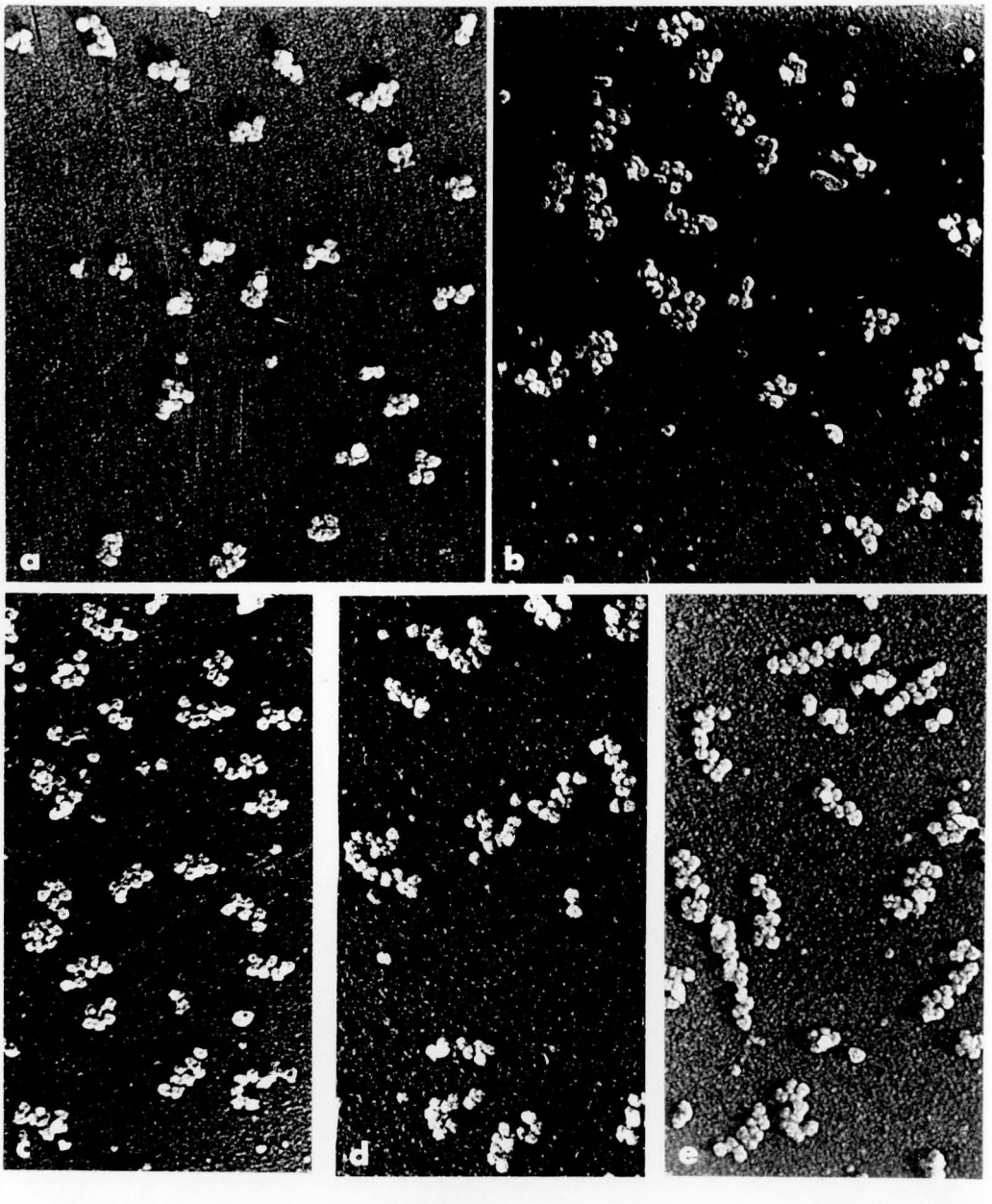


Plate 2 Electron micrographs of polyribosomes from Azotobacter vinelandii. (a) sample from fraction 44 showing pentamers, (b) sample from fraction 42 of Fig. 1 showing the hexamers, (c) sample from fraction 40 showing heptamers, (d) sample from fraction 38 showing octamers, (e) sample from fractions 34-36 showing nonomers and decamers. X 60,000.

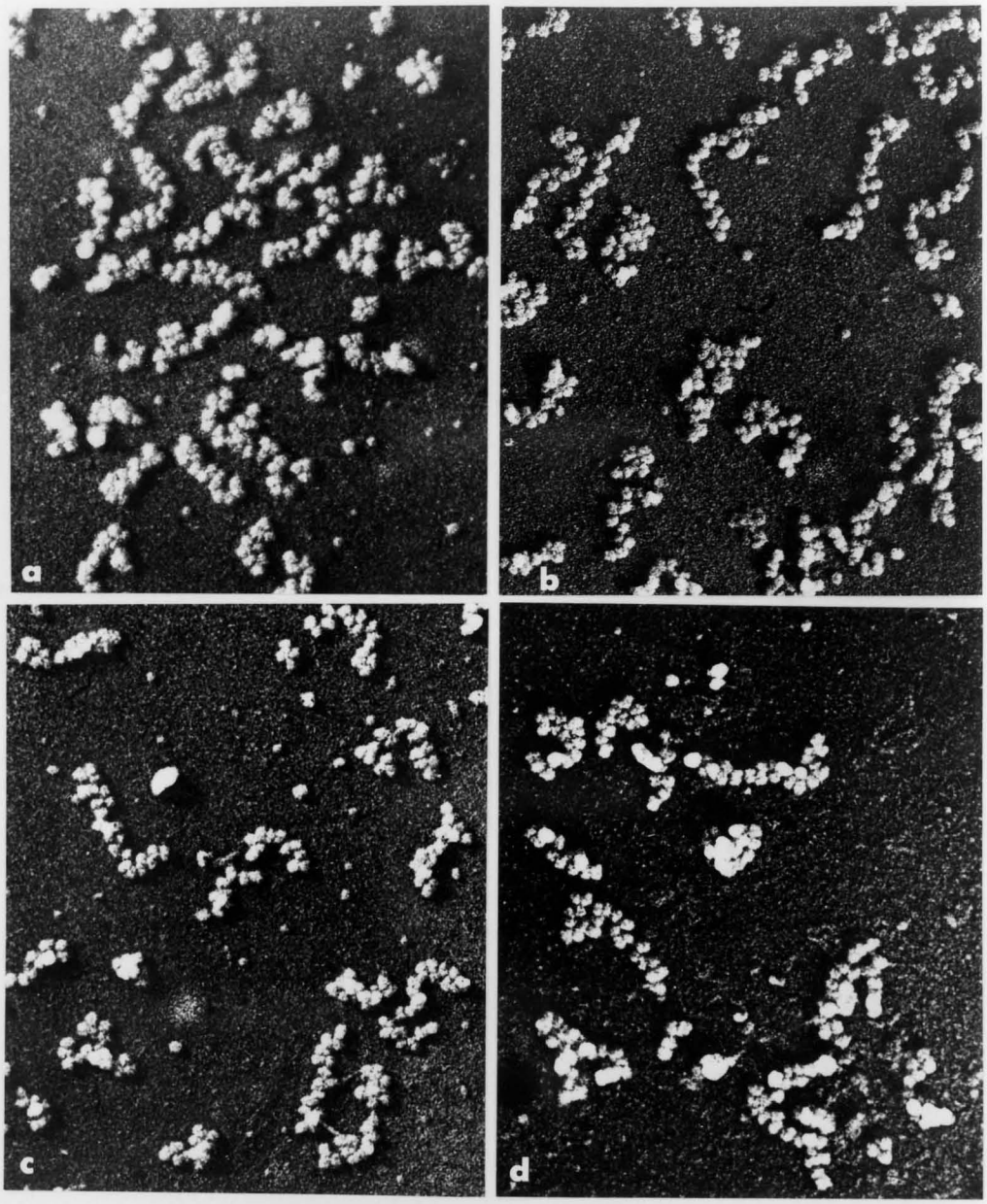


Plate 3 Electron micrographs of polyribosomes from Azotobacter vinelandii. (a) sample from fractions 27-35 of Fig. 1 showing predominantly polyribosomes containing eleven to fourteen ribosomes, (b) sample from fractions 24-26 showing polyribosome aggregates of fourteen to sixteen ribosomes, (c) sample from fractions 15-23 showing polyribosomes composed of sixteen to twenty ribosomes, (d) sample from fractions 5-15 showing large polyribosomes containing twenty to twenty-five ribosomes. X 60,000.

electron micrographic studies it is evident that there is a continued polyribosome class separation throughout the rest of the gradient. Plate 2e, drawn from a sample in the lightest portion of the unresolved area, contains nonamers and decamers. Plate 3a shows material from fractions 27-35 in the large "heavy" area, which was found to contain polyribosomes composed predominantly of 11 to 14 ribosomes. Plates 3b and 3c show material from the heaviest portions of the unresolved toe (fractions 15-26) and contains groupings of larger polyribosomes, ranging in size from 14 to 20 ribosomes. Plate 3d is from fractions 5-15 and contains polyribosomes of up to 30 ribosomes. The subunits of the single ribosomes sediment more slowly than the single ribosomes, and are shown in Plates 1a and 1b. The larger subunit is clearly resolved with the electron microscope while the smaller subunit appears as indistinct spheres.

The sedimentation values of Azotobacter ribosomes, polyribosomes and ribosomal subunits were computed from analytical ultracentrifugation studies and are shown in Table I. Plates 4 and 5 show the Schlieren diagrams obtained from a series of concentrated Azotobacter ribosomal preparations, from which the $S^{o}_{20,w}$ values were calculated. The predominant single peak is the 72.0 $S^{o}_{20,w}$ unit, which was shown by electron microscopy to result from the monomeric ribosome. The fastest moving measurable peaks had $S^{o}_{20,w}$ values greater than 400 and appeared in electron micrographs (Plate 4d) to be polyribosomes composed of more than 20 ribosomes.

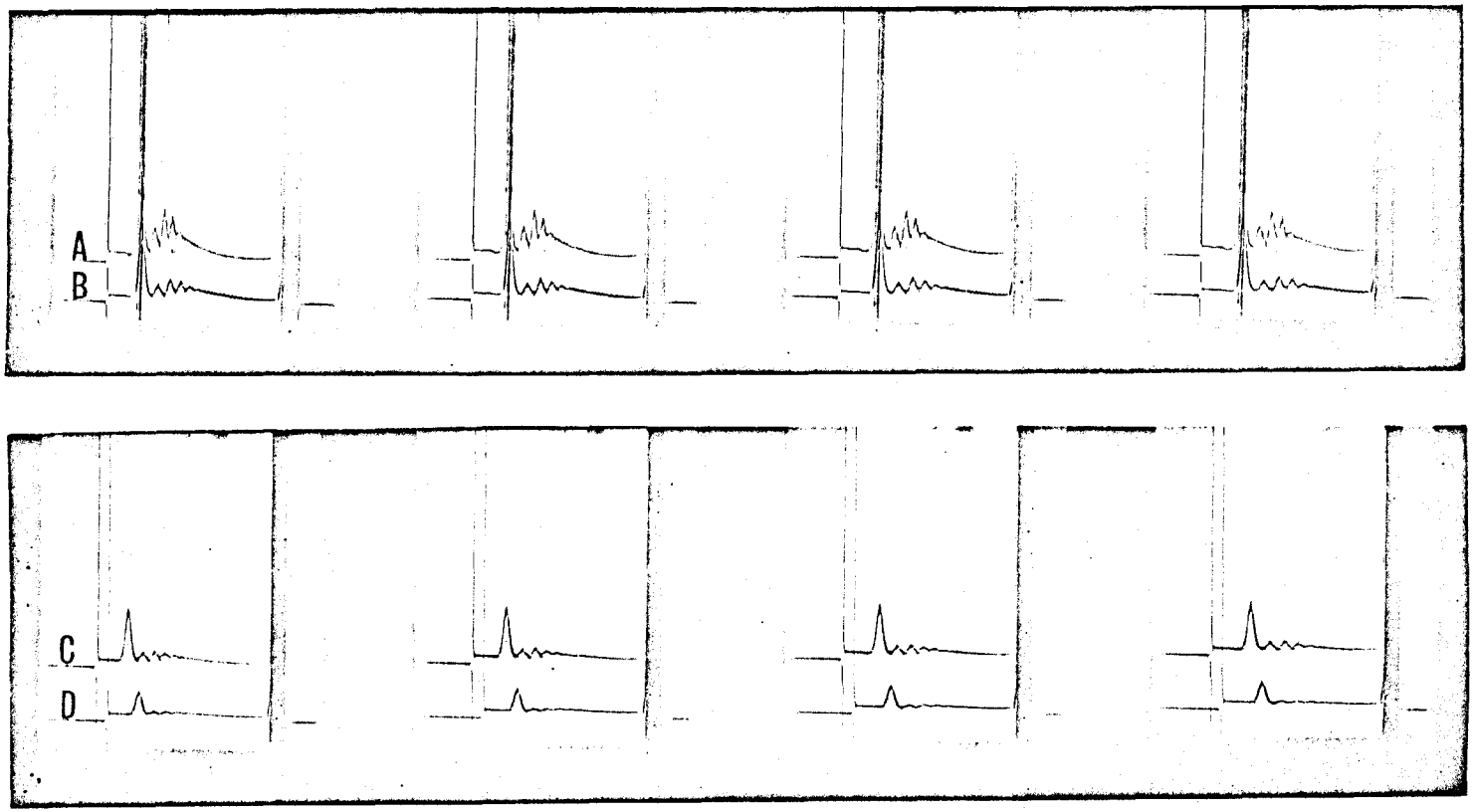
To determine precise values for the ribosomal subunits, concentrated ribosomal preparations were dialyzed in AVO buffer containing drastically

TABLE I

Corrected Sedimentation Coefficients of Azotobacter Ribosomes, Polyribosomes and Ribosome Subunits

small subunit	36.1 ± 1.4
large subunit	55.6 ± 0.3
monosome	72.0 ± 1
dimer	107.8 ± 3
trimer	133.7 ± 2
tetramer	153.1 ± 4.5
pentamer	174.5 ± 1.5
hexamer	191.8 ± 3.8
heptamer	215 ± 4
octomer	(234)*
monomer	(255.5)*
decamer	(276)*
20 25	400

* Values in parentheses are from polyribosomes present in small amounts only in the highest concentration of the ribosomal preparation used. Approximation of sedimentation values of these large aggregates were made from S_c values according to DeLey (5).



Plates 4 and 5 Analytical ultracentrifugation sedimentation patterns of *Azotobacter* polyribosomal preparations in AVO buffer. Polyribosomal concentrations of (A) 16 mg/ml, (B) 8 mg/ml, (C) 4 mg/ml and (D) 2 mg/ml were analyzed. Analyses were performed in a Spinco Model E ultracentrifuge. Pictures were taken with Schlieren optics at 2 min intervals about 20 min after the desired speed (21,740 rev per min) and temperature (4 C) were reached in each run.

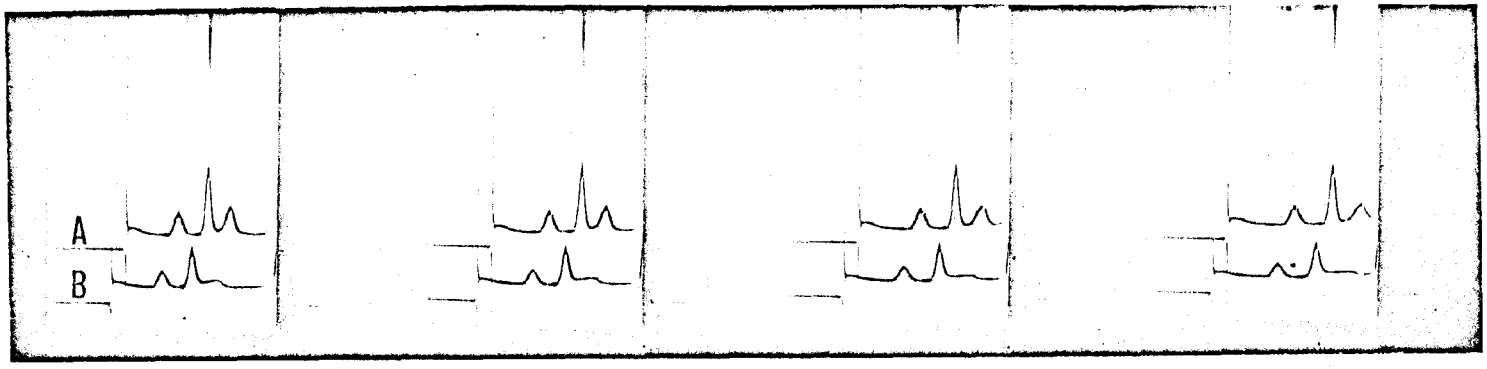


Plate 6 Analytical ultracentrifugation sedimentation patterns of dialyzed Azotobacter polyribosomal preparations. Samples with polyribosomal concentrations of (A) 10 mg/ml and (B) 5 mg/ml were dialyzed for 8 hr with 3 changes of 600-fold volumes in AVO buffer containing reduced Mg^{++} (10^{-4}M) before analysis. Pictures were taken using Schlieren optics at 2 min intervals about 40 min after the desired speed (39,460 rev per min) and temperature (7 C) were reached.

reduced Mg^{++} (10^{-4} M). At this Mg^{++} concentration most of the single ribosomes and ribosomal aggregates dissociate into ribosomal subunits. These dialyzed preparations were then examined in the analytical ultracentrifuge. Plate 6 shows the Schlieren patterns obtained using two different concentrations of the same preparation. The predominant peak contained the larger subunit (56 S), while in the trailing peak was found the smaller subunit (36 S); the fastest moving peak contains intact monosomes (72 S).

Requirements for isolation of the normal complement of cellular polyribosomes. In order to obtain consistent and reproducible polyribosome profiles for a meaningful analysis of the data, a number of parameters must be rigidly controlled. These include attention to the phase of growth of the culture, the rapid halting of cell metabolism, a gentle mode of cell breakage, avoidance of shear, use of exponential sucrose density gradients, specialized flow cells, minimizing remixing during flow through the flow cell, care in fraction collection and maintenance of low temperatures during the various steps of the procedure just described.

(a) Growth phase. The first absolute requirement for the isolation of polyribosomes involves the use of exponentially growing cells. When the growth rate diminishes as the culture enters the stationary phase a continual breakdown of polyribosomal aggregates into smaller polyribosomes occurs. Cells in late stationary phase evidence a complete lack of polyribosomes. Fig. 2 contains a series of polyribosome profiles from cultures as they progress into the stationary phase. It is interesting to note that

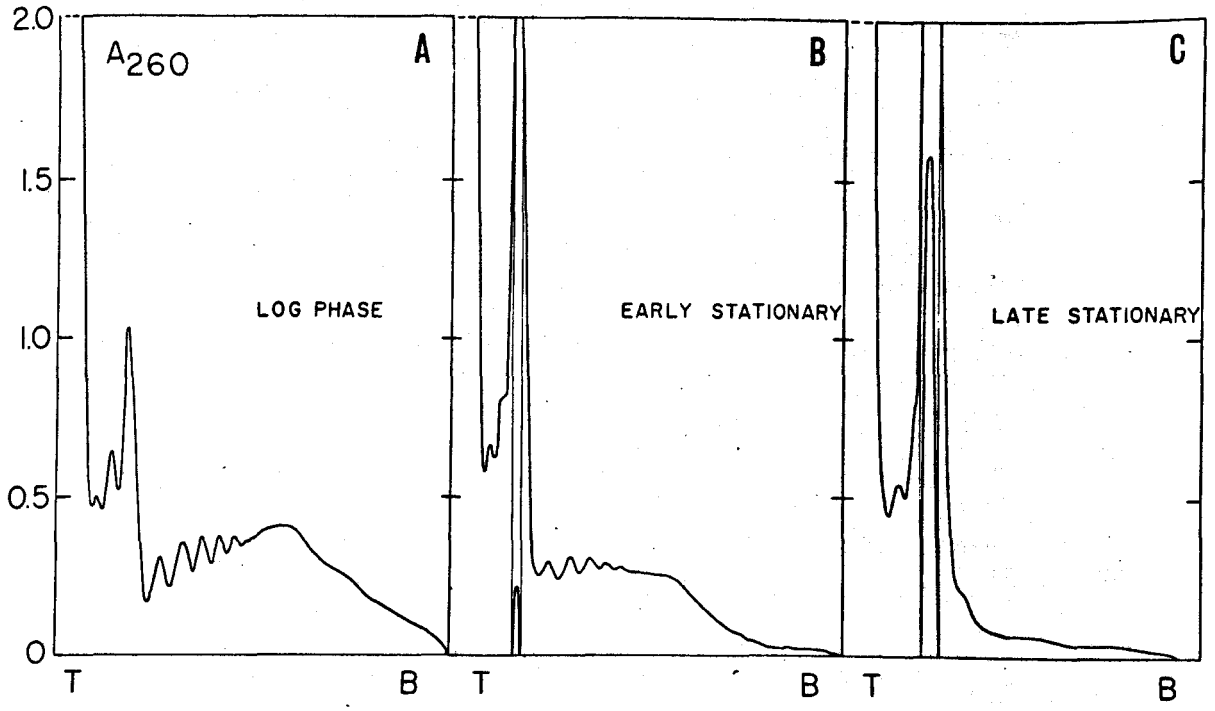


Fig. 2 Sucrose density gradient profiles of lysates from cultures in various stages of growth. Lysates were obtained from (A) an exponentially growing culture, (B) a culture grown 1 hr into the stationary phase and (C) a culture grown 10 hr into the stationary phase. Aliquots of each lysate containing 1.5 mg of RNA were layered onto 10-37% exponential sucrose density gradients. Centrifugation and A_{260} analyses were performed as described in Fig. 1.

in the stationary phase extracts, the predominant ribosomal species is the 72 S monomer while there is little, if any, increase in the ribosomal subunit pool as compared to log phase cells.

(b) Necessity for rapid cessation of metabolism. When the culture is harvested by pouring over shaved ice and is rapidly cooled to 0° (within 10-15 sec), cell extracts are obtained in which polyribosomes predominate (Fig. 3A). If, however, cultures are cooled more slowly, by simply harvesting into a refrigerated container or by swirling the culture flask in a salt-ice bath, cytoplasmic extracts are obtained which show a marked decrease of polyribosomes with a concommitant increase in monosomes (Fig. 3B and 3C). In a manner analogous to stationary cultures, slow cooled cells, depending on the rate of chilling, show increasing amounts of 72 S ribosomes at the expense of polyribosomes (Tables 2 & 3).

(c) Mode of cell breakage. The method by which the bacteria are disrupted strongly affects the polyribosomal content of the cell extracts obtained. Fig. 4 compares absorbancy profiles of cytoplasmic extracts obtained by four modes of breakage: 1) Mickle disintegration, 2) grinding with glass beads, 3) sonic oscillation, and 4) osmotic shock. Subjecting cells to ballistic disintegration or grinding cells with glass beads are rather harsh methods of cell disruption and cause an appreciable breakdown of polyribosomes into single ribosomes. Sonic oscillation, while not showing the same drastic effect, causes considerable degradation of the largest polyribosomes. Osmotic shock is the gentlest method of cell disruption,

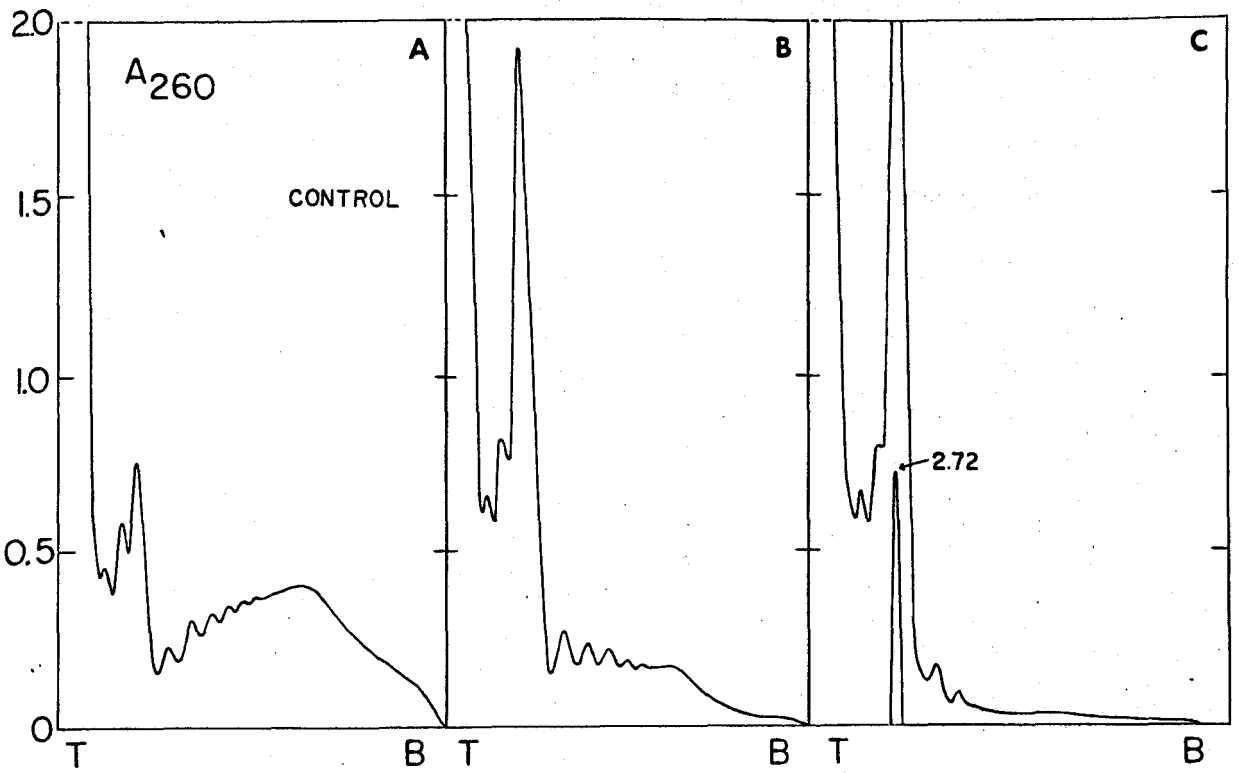


Fig. 3 Sucrose density gradient analysis of lysates from cultures in which growth was halted at different rates. Lysates were obtained from exponentially growing cultures which were (A) harvested by pouring over shaved ice and cooled to 0° in 15 sec, (B) harvested by placing the culture flask in an ice bath and swirling gently, cooling to 0 C in 15 min and (C) harvested in a refrigerated container, cooled to 0 C within 120 min. Aliquots of each lysate containing 1.25 mg RNA were layered onto 10-37% exponential sucrose gradients. Centrifugation and A_{260} analyses were performed as described in Fig. 1.

TABLE II

Ribosomal and Polyribosomal distribution as a function of the phase of growth in Azotobacter.

Phase of Growth	% 72 S Ribosomes	% Polyribosomes
Log	16	84
Early stationary	45	55
Late stationary	82	18

TABLE III

Ribosomal and Polyribosomal distribution as a function of the time allowed for the cessation of metabolism.

Time for cessation of metabolism	% 72 S Ribosomes	% Polyribosomes
Immediately (0 C within 15 sec)	16	84
15 min	57.5	37.5
60 min	82.5	17.5

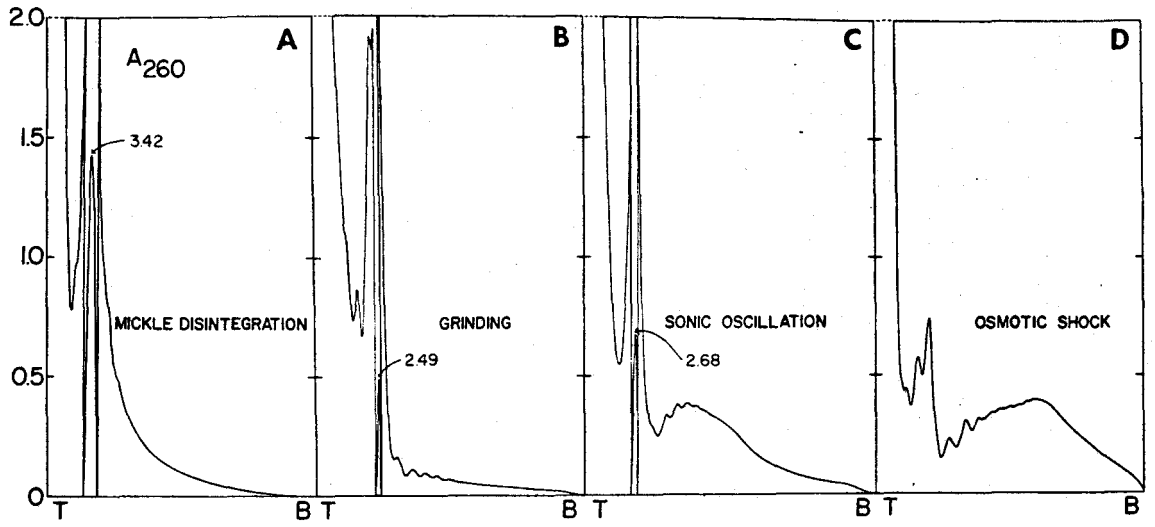


Fig. 4 The effect of the mode of cell breakage on the polyribosomal content of cell extracts. Lysates were obtained from exponentially growing cultures disrupted by (A) Mickle disintegration for 90 sec, (B) grinding with glass beads in a chilled mortar for 2.5 min, (C) sonic oscillation for 50 sec and (D) osmotic shock. (See Methods and Materials) Aliquots of each lysate containing 1.5 mg RNA were layered onto separate sucrose density gradients. Centrifugation and absorption analyses were performed as in Fig. 1.

since cells are subjected only to a single instantaneous trauma while the other methods permit continuing action upon disrupted cells and their cytoplasmic contents as well as intact cells.

(d) Avoidance of shear. Obviously it is of utmost importance to treat lysates as gently as possible, since polyribosomes are extremely susceptible to shear (36). Figure 5 shows the effect of ten pipettings on a lysate before it was layered onto a gradient. The larger polyribosome aggregates are broken down to smaller polyribosome classes. In our procedure extracts are pipetted only when being layered onto gradients; wide bore pipets are used with the sample being drawn and layered slowly. Thus, while shearing can appreciably break down polyribosomes, our procedure minimizes this degradation.

Effect of RNAse. When lysates from Azotobacter are treated with very small amounts of RNAse (Fig. 6), most of the polyribosomal material moves quantitatively to the monomer position, with only a small quantity of dimers remaining and no measurable amount appearing as additional subunits.

Effect of DNAse. DNA does not appear to be involved in the aggregation of ribosomes into polyribosomes. DNAse treated extracts do not differ in their distribution of polyribosomes as compared to untreated extracts (6, 20). Resolution of polyribosomes, in fact, may be enhanced by this treatment since the viscosity of the extracts is greatly reduced.

Effect of Mg^{++} on ribosome and polyribosome structure. In all systems examined thus far, ribosomes have been found to be very sensitive to the ionic environment, in particular, to the Mg^{++} concentration

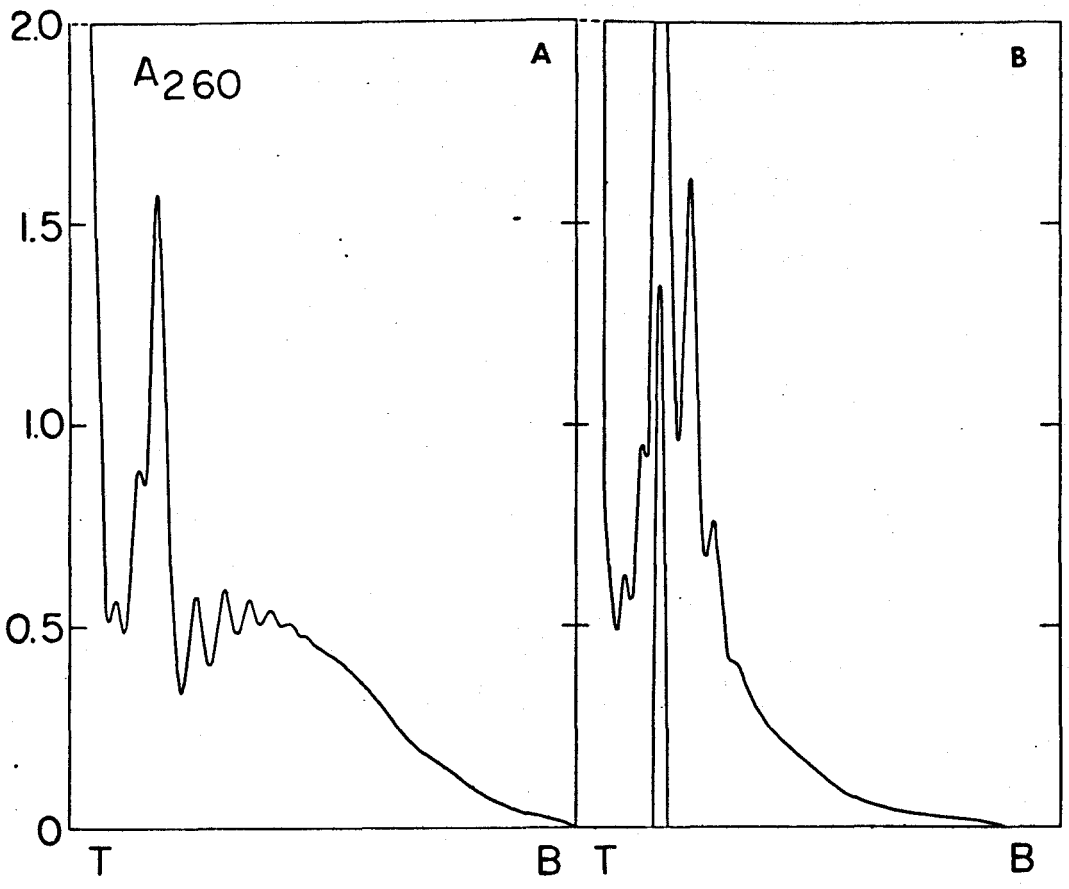


Fig. 5 The effect of shearing on polyribosome structure. Aliquot (A) from an untreated cytoplasmic extract was layered directly onto an exponential sucrose density gradient. Aliquot (B) was pipetted 10 times with a small bore 1 ml pipette before it was layered. Both gradients were centrifuged and analyzed as described in Fig. 1.

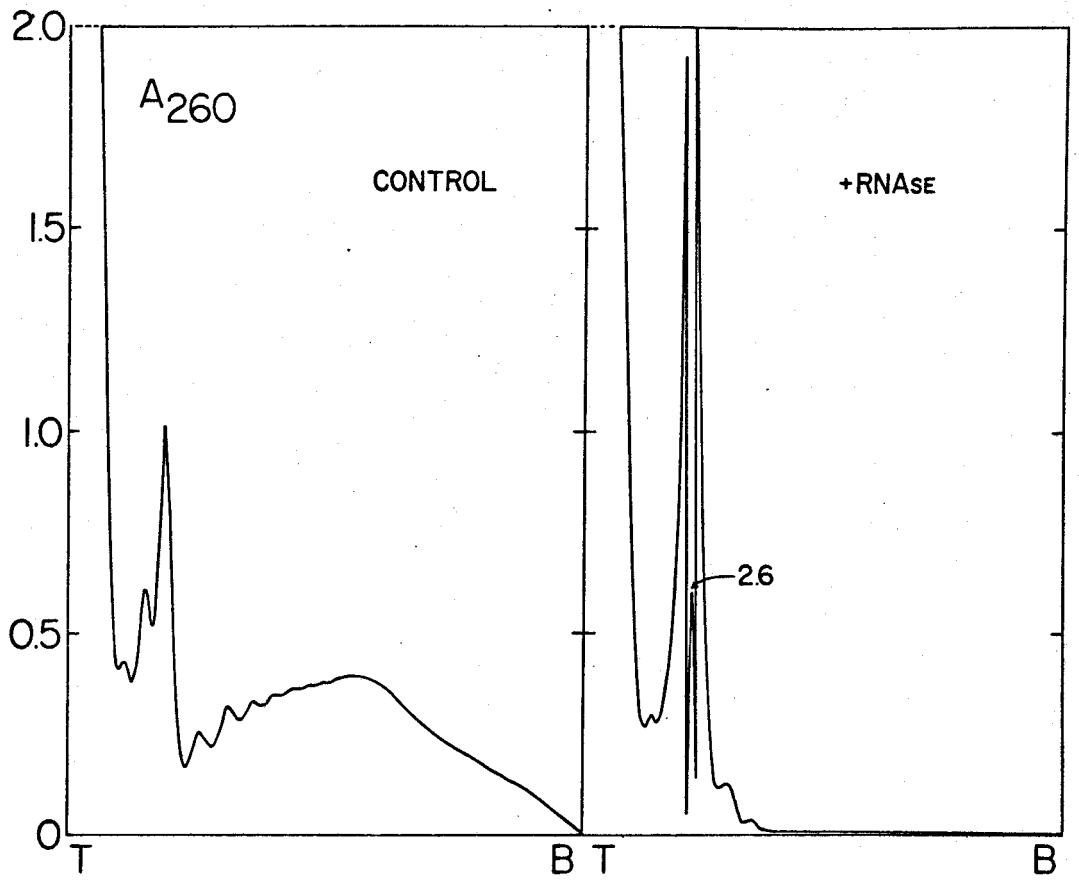


Fig. 6 The effect of RNAse on polyribosomes. One aliquot of a lysate from an exponentially growing culture obtained as described in the text was treated with ribonuclease (5 μg/ml) for 10 min at 0 C. An untreated aliquot served as control. The lysates were layered on separate exponential sucrose gradients. Centrifugation and adsorption analyses were performed as in Fig. 1.

(7, 14, 41, 42). In order to test ribosomal and polyribosomal stability, lysates were prepared in and dialyzed against a series of buffers for 8 hr with 3 changes of 600-fold vol of buffers containing 10 mM to 0.01 mM Mg^{++}. At no time were extracts exposed to a concentration of Mg^{++} other than that in which they had been initially obtained, thus avoiding artificial aggregations due to changes of Mg^{++} concentrations. The lysates were then centrifuged through exponential sucrose density gradients and their absorbancy profiles analyzed (Fig. 7). A Mg^{++} concentration of at least 4 mM is required to maintain the integrity of ribosomes and ribosomal complexes. A Mg^{++} concentration of 1 mM dissociates "free" 72 S ribosomes into 36 S and 56 S subunits, but does not affect ribosomes associated with polyribosomes. Therefore the polyribosome structure appears to protect the individual ribosomes associated with it against this lowered Mg^{++} concentration. An almost complete dissociation of all ribosomes into subunits occurs in Mg^{++} concentrations less than 1 mM.

Distribution of a pulse of ^{14}C amino acids. In order to determine what ribosome size classes in Azotobacter are functional in protein synthesis in vivo a culture of Azotobacter was given a 5 min pulse (1/24 of its generation time) of ^{14}C amino acids, and the lysate examined for polyribosomes and radioactivity as shown in Fig. 8. The bulk of the pulse-labelled polypeptide appears in the polyribosome region, especially in the larger classes. The radioactivity in the monosome paek is in part due to the diffusion of ^{14}C contaminants of low molecular weight into the 72 S region.

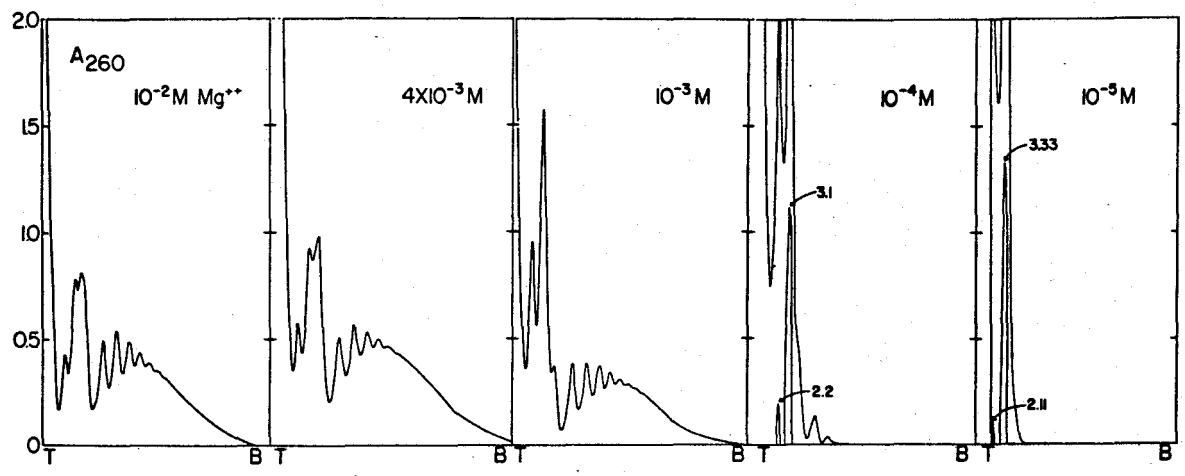


Fig. 7 The effect of Mg^{++} concentration on ribosomes and polyribosomes. Aliquots from a cytoplasmic extract were dialyzed against a series of AVO buffers containing different Mg^{++} concentrations (20 mM, 4 mM, 1 mM, 0.1 mM and 0.01 mM). Dialysis was continued for 8 hr with 3 changes of 600-fold volumes of the buffers. Each dialyzed aliquot was then examined by exponential sucrose density centrifugation as described in Fig. 1.

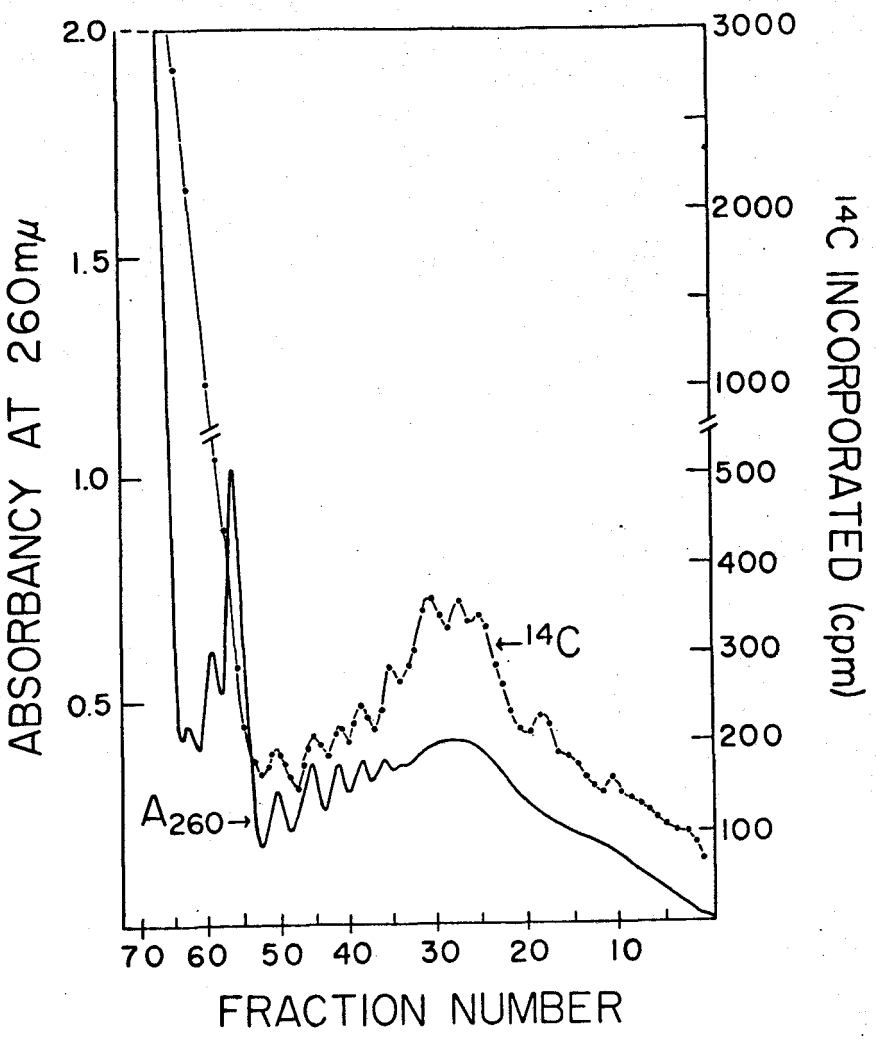


Fig. 8 The distribution of nascent protein on polyribosomes. An exponentially growing culture of Azotobacter was given a 5 min pulse with 0.1 mC of UL^{14}C amino acids. The culture was harvested and the cytoplasmic extract obtained according to our usual procedure. The lysate was centrifuged through an exponential sucrose gradient for 4-1/2 hr at 105,000 x g. After centrifugation absorbance at 260 mμ (solid line) was measured as previously described and radioactivity determined (dotted line) by collecting continuous 0.4 ml samples from the gradient and then analyzed in a scintillation spectrometer.

Since we did not precipitate the fractions these contaminants probably represent polypeptides which have been already synthesized and released into the supernatant phase of the lysate during the pulse. Upon treatment of the lysate with dilute RNAse the labelled nascent polypeptide on the polyribosomes moved quantitatively to the 72 S position.

Polyribosomal content of Azotobacter as a function of the nitrogen source in the growth medium. When the Azotobacter are grown in an enriched medium containing added amino acids a different polyribosome pattern is obtained (Fig. 9B), as compared with cells grown using gaseous nitrogen from the air as the sole nitrogen source (Fig. 9A). There is an increased quantity of heavy polyribosomes obtained when the Azotobacter are grown under the latter conditions and especially in one particular region of the profile. Since the Azotobacter is a nitrogen fixer (that is, it can utilize gaseous nitrogen for all of its nitrogen biosynthetic needs) and since this process is initiated by a series of inducible enzymes (the nitrogenases) then the appearance and absence of a distinct area in the polyribosomal distribution, depending on growth conditions, probably reflects the induction and repression of the nitrogen fixation apparatus.

Electron microscopic thin section analysis. Azotobacter possesses an extensive internal membranous system as was shown by Pangborn, Marr and Robrish (30). Electron micrographs which we have taken of thin sectioned Azotobacter illustrate this phenomenon. Plate 7 shows thin sections of both intact and osmotically broken cells. We have observed the attachment of

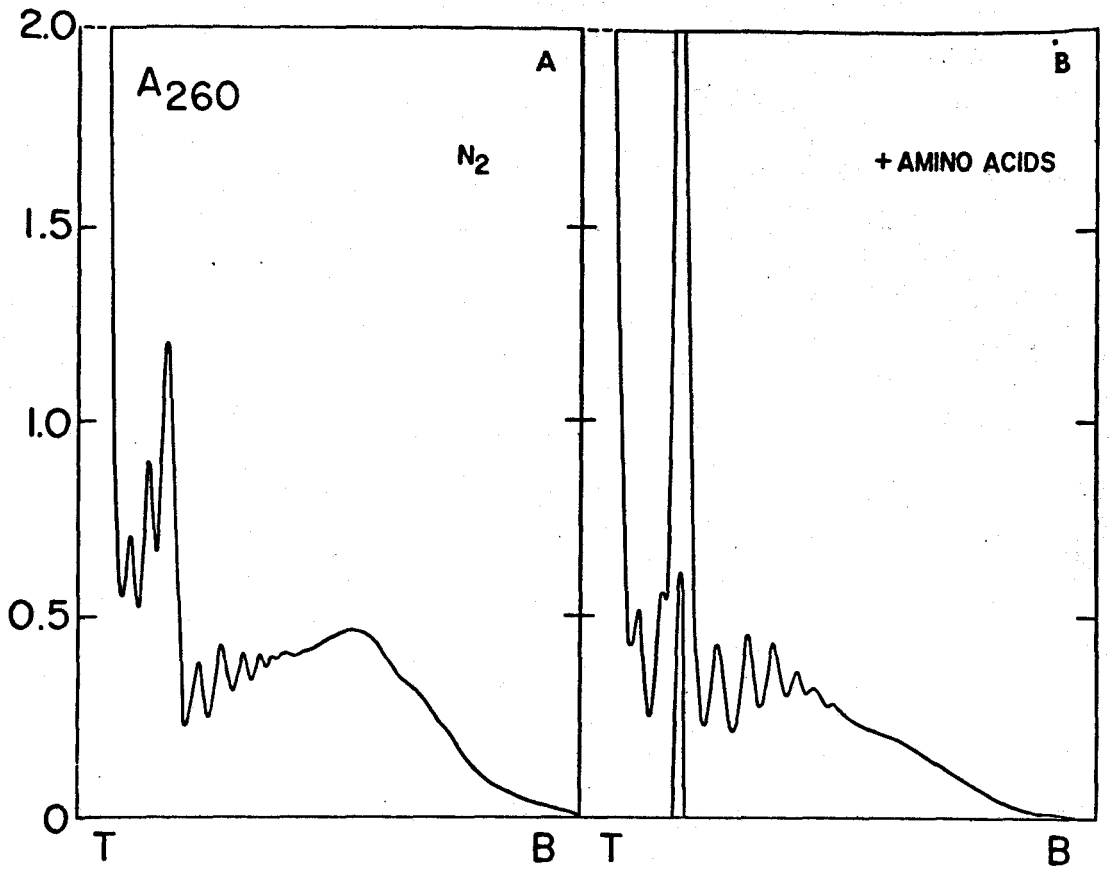


Fig. 9 Polyribosomal distribution as a function of the nitrogen source for growth. Lysates were obtained from (A) an exponential culture of the Azotobacter grown in nitrogen free medium, and (B) an exponential culture of the Azotobacter grown in an enriched medium containing 0.25% casein hydrolysate. Aliquots of each lysate containing 1.5 mg of RNA were layered onto separate 10-37% exponential sucrose density gradients. Centrifugation and A_{260} analyses were performed as described in Fig. 1.

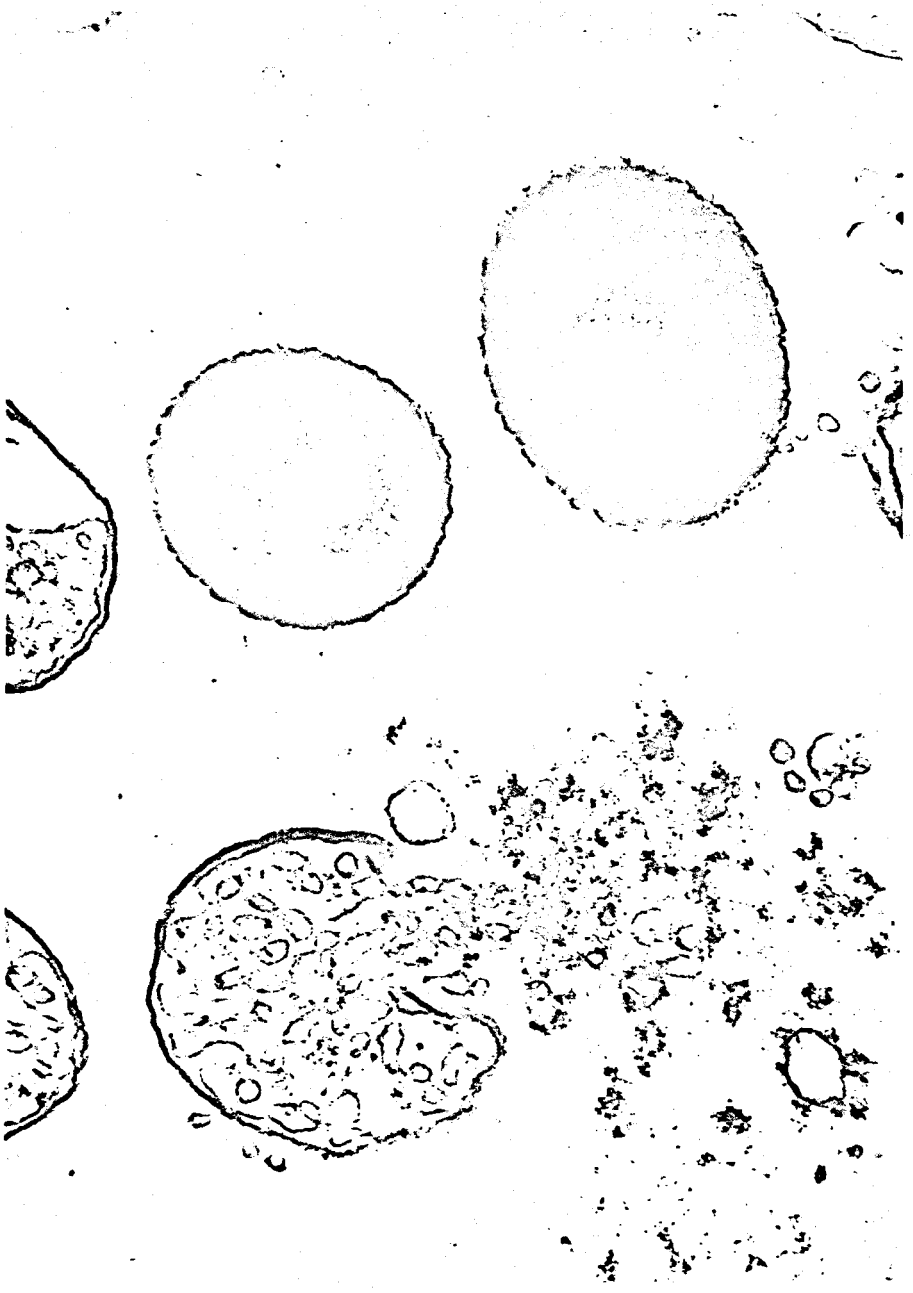


Plate 7 Electron micrograph of thin sectioned Azotobacter. The micrograph shows both intact and osmotically lysed cells. In the lysed cells the extensive internal membranous system of the bacteria is observed. The thin section was prepared as described in Materials and Methods. X 30,000.

ribosomal aggregates on the membranes in the lysed cells. To date, however, there is no direct evidence that polyribosomes exist attached to membranes in the bacterial cell. Therefore, it is not excluded that the binding of polyribosomes to membranes reported here is an artefact of adsorption or inclusion occuring when cells are broken open.

DISCUSSION

Isolation. Artificial aggregation of ribosomes or fragmentation of polyribosomes was minimized in this study, since the Azotobacter were cultured under optimal conditions, their metabolism was halted rapidly, they were disrupted gently by osmotic shock, and lysates were placed directly onto exponential sucrose density gradients. Thus it seems likely that the sucrose gradient profiles of ribosomes and polyribosomes obtained from Azotobacter extracts accurately reflect the normal cytoplasmic content of polyribosomes in the intact growing cell.

Characterization. Azotobacter ribosomes and polyribosomes possess chemical and physical properties associated with those found in other bacteria. They are sensitive to RNAse (43, 45), insensitive to DNAse (6, 20), exhibit Mg^{++} dependency for structural integrity (7, 14, 41, 42), are extremely fragile (36) and have sedimentation coefficients similar to those reported in other bacteria (5). However, unlike that observed in other bacteria, from electron microscopic analyses and preliminary experiments it appears that some portion of the polyribosomal population is intimately associated with an internal membranous network. These membrane bound polyribosomes can be released by using a lipid solubilizing agent such as Na deoxycholate (28).

Distribution. In more than 50 separate sucrose density gradient experiemnts with extracts from exponentially growing cultures, the isolated polyribosomes consistently contain between 80% and 90% of the total ribosome population. Approximately 4/5 of the polyribosomes are larger than tetramers.

The polyribosome contents of the extracts is significantly higher than previously reported in other bacterial systems. With the use of exponential sucrose density gradient centrifugation we can resolve polyribosome size classes well into the octomer class while still obtaining distinct separation between ribosomal subunits and single ribosomes. Larger size classes of polyribosomes are partially resolved. The separation and resolution of individual size classes of polyribosomes, and polyribosomes from monosomes and ribosomal subunits achieved in the Azotobacter system surpasses the results reported in any previous ribosome-polyribosome system.

In exponentially growing cells the quantity of monosomes remains fairly constant. It was observed, however, that in extracts from cultures which are progressing into the stationary phase or extracts taken from cultures in which the time for cessation of metabolism was extended, the numbers of monosomes progressively increased concomittant with a decrease in the numbers of the larger polyribosomes (Tables 2 & 3). This quantitative increase of monosomes from polyribosomes observed in these two different experiemental conditions appears to be due to the same phenomenon.

The above observed increase in monosomes does not result from the preparative procedures per se. One possible reason for the increased monosome numbers could be due to increased cellular content or activity of RNAse under the suboptimal growth conditions of cells entering stationary phase or during slow cooling. We examined this possibility and found that cell sap from extracts of the stationary phase cultures do not cause an

increase in the quantity of monosomes when added to control extracts from exponential cells, and that the levels of RNAse in the cellsaps, measured directly, was in the 0.002 - 0.004 μg/ml range. This is a level too low to affect the polyribosomes during the extraction process and an order of magnitude lower than that found in control extracts. Thus, it does not appear that the monomeric ribosomes are the degradative products of endogenous nuclease action in these experiments. A more likely explanation for the increase in monosomes is that slow cooling may stop or significantly reduce chain initiation but affect chain completion to a lesser extent which would lead to a ribosomal "runoff" (i.e., the final reading of the message). An analogous situation probably accounts for the increase in monosome numbers in stationary phase cultures. As cells go into the stationary phase, message reading goes to completion, and single ribosomes accumulate. Since few if any new messages are synthesized, and since the half-life of mRNA is short, there is little regeneration of polyribosomes. Similar observations were reported by Goldstein et.al. (12). In the stationary phase all message synthesis has stopped and all reading has been completed, thereby yielding extracts containing only single ribosomes and the normal complement of subunits.

The proportion of the ribosomal subunits to ribosomes and polyribosomes remains constant during exponential growth. Furthermore there seems to be little increase in the quantity of subunits as the culture enters and remains in the stationary phase. Thus, the subunit pool remains

constant even though the number of single ribosomes increase.

While the advantages of our methods for studying polyribosome distribution are obvious, the procedures described in this paper are most readily applied to those bacterial species which can be lysed directly by osmotic shock. (Among these are included the Azotobacter, Rhodospirillum rubrum and Serratia plymouthicum.) The precautions for cell harvest, treatment and extract handling, however, are general for all cells.

We routinely disrupt 2 to 5 g of cells (wet wt) with very little dilution. Quantities of cells can readily be increased for large scale polyribosome separation with the new large volume zonal centrifuge rotors. Thus, large quantities of individual size classes of polyribosomes can be isolated.

SUMMARY

This thesis details the procedural requirements for preparing cell-free extracts rich in polyribosomes from Azotobacter vinelandii. This enables us to demonstrate the occurence of polyribosomes in Azotobacter and to devise methods for their resolution and isolation. Azotobacter was used in this study since it is readily disrupted by osmotic shock; the gentlest mode of cell breakage available. When certain parameters are followed (the use of log phase cells, rapidly halting cell metabolism, gentle handling of lysates, sedimentation through exponential sucrose density gradients, the use of flow cells in which remixing is minimized and the maintenance of low temperatures through all procedures) cell extracts containing up to 80-90% polyribosomes of the total ribosomal population result. Individual polyribosome size classes ranging up to the octamers can be fractionated and separated from their nearest neighbors. Larger size classes are resolved partially among themselves. This was confirmed by extensive electron microscopic studies of material from the various fractions obtained upon density gradient centrifugation of Azotobacter extracts.

The physical and chemical properties of these isolated ribosomes and polyribosomes were then studied. The polyribosomes are RNAse sensitive, DNAse insensitive, exhibit Mg^{++} dependency for structural integrity, are extremely fragile and have sedimentation coefficients similar to other bacteria. It has also been observed that the polyribosomal distribution within actively metabolizing cells is dependent upon the growth medium. At least some of the polyribosomal population appears to be intimately associated with internal membranous material.

ACKNOWLEDGEMENTS

The author is greatful to Dr. Claude Biava, of the Dept. of Pathology, for his excellent electron microscopy. The author also wishes to thank Mr. Ben Massey, of the Dept. of Biochemistry of the University of Illinois Medical Center, for the operation of the analytical ultracentrifuge and for information regarding the computation of S values. Sincere appreciation is extended to Mrs. Rose Torrence for her patience in typing this thesis.

Finally I would like to extend my fullest appreciation to my advisor, Dr. Leon Marcus, for his guidance and tolerance during the course of this study and to my accomplice Julian Scheinbuks who helped me keep my sanity throughout the long days and nights.

LITERATURE CITED

1. Aronson, A. 1966. Adsorption of polysomes to bacterial membranes. J. Mol. Biol. 15: 505-514.

2. Bray, G.A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279-285.

3. Bock, R.M., and N.S. Ling. 1954. Devices for gradient elution in chromatography. Anal. Biochem. 26: 1543-1546.

4. Bretthauer, R.K., L. Marcus, J. Chaloupka, H.O. Halvorson, and R.M. Bock. 1963. Amino acid incorporation into protein by cell-free extracts of yeast. Biochemistry 2: 1079-1084.

5. DeLey, J. 1964. On the unity of bacterial ribosomes. J. Gen. Microbiol. 34: 219-229.

6. Dresden, M., and M.B. Hoagland. 1965. Polyribosomes from Escherichi coli: Enzymatic method for isolation. Science 149: 647-649.

7. Edelman, I.S., P.O.P. Ts'o, and J. Vinograd. 1960. The binding of magnesium to microsomal nucleoprotein and ribonucleic acid. Biochim. Biophys. Acta 43: 393-403.

8. Flessel, C.P., P. Ralph, and A. Rich. 1967. Polyribosomes of growing bacteria. Science 158: 658-660.

9. Gierer, A. 1963. Function of aggregated reticulocyte ribosomes in protein synthesis. J. Mol. Biol. 6: 148-157.

10. Gilbert, A. 1963. Polypeptide synthesis in Escherichia coli. I. Ribosomes and the active complex. J. Mol. Biol. 6: 374-388.

11. Grodson, G.N., and R.L. Sinsheimer. 1967. The replication of bacteriophage MS 2. VI. Interaction between bacteriophage RNA and cellular components in MS 2-infected Escherichia coli. J. Mol. Biol. 23: 495-521.

12. Goldstein, A., D.B. Goldstein, and L.I. Lowney. 1964. Protein synthesis at 0°C in Escherichi coli. J. Mol. Biol. 9: 213-235.

13. Goodman, H.M., and A. Rich. 1963. The mechanism of polyribosome action during protein synthesis. Nature 199: 318-322.

14. Hall, B.D., and P. Doty. 1959. The preparation and physical chemical properties of ribonucleic acid from microsomal particles. J. Mol. Biol. 1: 111-126.

15. Henshaw, E.C., T.B. Bojarski, and H.H. Hiatt. 1963. Protein synthesis by free and bound rat liver ribosomes in vivo and in vitro. J. Mol. Biol. 7: 122-129.

16. Kalnitsky, G., J.P. Hummel, and C. Dierks. 1959. Some factors which affect the enzymatic digestion of ribonucleic acid. J. Biol. Chem. 234: 1512.

17. Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron microscopic study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4: 671-678.

18. Kiho, Y., and A. Rich. 1965. A polycistronic messenger RNA associated with B-galactosidase induction. Proc. Natl. Acad. Sci. 54: 1751-1758.

19. Lyttleton, J.W. 1963. A simple method of isolating ribosomes from Tetrahymena pyriformis. Exp. Cell Res. 31: 385-389.

20. Mangiarotti, G., and D. Schlessinger. 1966. Polyribosome metabolism in Escherichia coli. I. Extraction of polyribosomes and ribosomal subunits from fragile, growing Escherichia coli. J. Mol. Biol. 20: 123-143.

21. Marcus, L., R.K. Bretthauer, R.M. Bock, and H.O. Halvorson. 1963. The effect of poly U size on the incorporation of phenylalanine in the cell-free yeast system. Proc. Natl. Acad. Sci. 50: 782-789.

22. Marcus, L., and G. Breuning. 1967. (in manuscript)

23. Marcus, L., and H.O. Halvorson in L. Grossman and K. Moldave. Nucleic Acids, Methods in Enzymology, Vol. 12, Academic Press, New York, N.Y., (1968), Art. 59.

24. Marcus, L., H. Ris., H.O. Halvorson, R.K. Bretthauer and R.M. Bock. 1967. Occurrence, isolation and characterization of polyribosomes in yeast. J. Cell Biol. 34: 505-512.

25. Marks, P.A., E.R. Burka, and D. Schlessinger. 1962. Protein synthesis in erythroid cells. I. Reticulocyte ribosomes active in stimulating amino acid incorporation. Proc. Natl. Acad. Sci. U.S. 48: 2163.

26. Oppenheim, J. and L. Marcus. 1967. Resolution of polysomes in Azotobacter vinelandii strain O. Bacteriol. Proc. 1967, 120.

27. Oppenheim, J., L. Marcus, and J. Scheinbuks. 1968. (in manuscript)

28. Oppenheim, J., and L. Marcus. 1968. (in manuscript)

29. Palade, G.E., and P. Siekevitz. 1956. Liver microsomes: An integrated morphological and biochemical study. J. Biophys. Biochem. Cytol. 2: 171-200.

30. Pangborn, J., A.G. Marr, and S.A. Robrish. 1962. Localization of respiratory enzymes in intracytoplasmic membranes of Azotobacter agilis. J. Bacteriol. 84: 669-678.

31. Penman, S., K. Sherrer, Y. Becker, and J. Darnell. 1963. Polyribosomes in normal and poliovirus infected HeLa cells and their relationship to messenger-RNA. Proc. Natl. Acad. Sci. 49: 654-662.

32. Phillips, W.D., A. Rich, and R.R. Sussman. 1964. The isolation and identification of polyribosomes from cellular slime molds. Biochim. Biophys. Acta 80: 508-510.

33. Risebrough, R.W., A. Tissieres, and J.D. Watson. 1962. Messenger-ribonucleic acid (mRNA) attachment to active ribosomes. Proc. Natl. Acad. Sci. 48: 430-436.

34. Robrish, S.A., and A.G. Marr. 1961. Location of enzymes in Azotobacter agilis. J. Bacteriol. 83: 158-168.

35. Ron, E., R. Kohler, and B. Davis. 1966. Polysomes extracted from Escherichia coli by freeze-thaw lysozyme lysis. Science 153: 1119-1120.

36. Schaechter, M. 1963. Bacterial polyribosomes and their participation in protein synthesis in vivo. J. Mol. Biol. 7: 561-568.

37. Schaechter, M., and K. McQuillen. 1966. Synthesis of messenger ribonucleic acid and the assembly of polysomes in Bacillus megaterium

infected with bacteriophage. J. Mol. Biol. 22: 223-234.

38. Schlessinger, D. 1963. Protein synthesis by polyribosomes in protoplast membranes of B. megaterium. J. Mol. Biol. 7: 569-582.

39. Schlessinger, D., G. Mangiorotti, and D. Apirion. 1967. The formation and stabilization of 30 S and 50 S ribosome couples in Escherichia coli. Proc. Natl. Acad. Sci. 58: 1782-1789.

40. Svedberg, T., and K.O. Pedersen. 1940. The Ultracentrifuge. Clarendon Press, Oxford.

41. Tissieres, A., J.D. Watson, D. Schlessinger, and B.R. Hollingworth. 1959. Ribonucleo-protein particles from Escherichia coli. J. Mol. Biol. 1: 221-233.

42. T'so, P.O.P., J. Bonner, and J. Vinograd. 1958. Structure and properties of microsomal nucleoprotein particles from pea seedlings. Biochim. Piophys. Acta 30: 570-582.

43. Warner, J.R., O.M. Knopf, and A. Rich. 1963. A multiple ribosomal structure in protein synthesis. Proc. Natl. Acad. Sci. 49: 122-129.

44. Warner, J.R., A. Rich, and C.E. Hall. 1962. Electron microscope studies of ribosomal clusters synthesizing hemoglobin. Science 138: 1399-1403.

45. Wettstein, F.O., T. Staehelin, and H. Noll. 1963. Ribosomal aggregate engaged in protein synthesis. Nature 197: 430-435.