DIFFERENTIAL DISTRIBUTION OF RNA AND PROTEIN IN THE RIBOSOMAL FRACTIONS OF ESCHERICHIA COLI

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SUMMARY

The RNA to protein ratio profiles of sucrose density gradient-separated crude extracts and ribosomal preparations of E. coli were used to study the homogeneity of ribosomes, and to determine the effects of buffer salts, pH, ribonuclease treatment, and differential centrifugation on all ribosomal components. The following results were obtained:

1. Contrary to earlier findings with whole preparations that all classes of ribosomes had the same RNA to protein ratio, very different ratios were obtained for different components in the ribosomal region of the gradient. The ratios were always highest in the 70s or 100s components, and considerably lower in the polysome region.

2. The ratio profile in the ribosomal region, contrary to the constancy expected on the basis of a simple Mg-mediated aggregation of similar sub-units, showed well-defined and periodically spaced peaks, indicating the presence of protein-rich and protein-poor components within each sedimentation class of ribosomes.

3. The height of the peaks in the ratio profile as well as the difference in protein content between the polysome region and the main peak were largest in crude extracts, and became attenuated, but not obliterated, in the course of purification of the ribosomes.

4. In Tris-Mg buffers, the total amount of protein bound to sedimentable components is less at pH 7.4 than at either pH 7.1 or 8.1. The addition of K^+ or NH_4^+ to the buffers caused more protein to be bound in crude extracts, but less in purified ribosomes.

5. Treatment with small amounts of ribonuclease in the cold was effective for releasing the "extra" protein and obtaining high RNA to protein ratios in all components. Since the "extra" protein does not remain bound to smaller ribosomes, it is postulated that the binding of this protein is dependent on the presence of ribonuclease-sensitive RNA, presumably polysomal mRNA.

6. RNA to protein ratio profiles coupled with sucrose density gradient centrifugation provide a most sensitive analytical test for homogeneity of ribosomes and permit detection of very subtle changes in the minor as well as in the major components of a preparation.

INTRODUCTION

It is universally assumed that ribosomes provide the structural matrix in the cell for the synthesis of proteins, and ribosomes from many sources have been extensively studied and characterized. Yet despite this large amount of chemical and physicochemical information on ribosomes as ribonucleoprotein particles, we find that we are surprisingly ignorant when we attempt to define ribosomes as <u>biochemically</u> active structures, for the simple reason that physicochemical integrity and chemical purity are not at all correlated with biochemical, i.e., amino acid incorporating activity.

The recent recognition of polysomes as the active site of protein synthesis in mammalian (1 - 3), as well as in bacterial cells (4 - 6) is

helpful in that it shows that a biochemically active ribosome has to be bound to messenger or template RNA (mRNA). It is not sufficient, however, since only a small proportion of ribosomes as commonly separated seem to be able to form specific complexes with added mRNA (7, 8) and it is not known what enables these ribosomes to do so. (The attachment of poly u, which under some conditions occurs with more than 50 % of the ribosomes (9), is probably non-specific (10).)

Further recent findings have shown (11, 12) that isolated ribosomes have bound to them various amounts of sRNA, denoting the presence of several non-equivalent binding sites on each ribosome, and that there is additional binding of specific sRNA when polysomes are formed (13 - 15). All this makes it likely that a biochemically active ribosome has bound to it some sRNA, but this, also, is an insufficient definition, because, again, the factor or factors responsible for the binding of functional sRNA are unknown. It is also possible that in addition to transfer RNA, ribosomes contain another kind of low-molecular weight RNA (16).

There are a number of reports in the literature (17 - 19) where "active" and "inactive" ribosomes, obtained from the same source under slightly different conditions, cannot be distinguished by the usual physico-chemical criteria and do not seem to be deficient in any of the known partial reactions of amino acid incorporation, with the possible exception of the ability to bind transfer RNA (17). The differences between biochemically active and inert ribosomes are thus for the most part quite subtle and most likely involve some ribosomal property or component not yet recognized. The present series of investigations was started with the purpose of looking for any demonstrable heterogeneity in ribosomal preparations, and possibly demonstrating novel qualitative differences between different components, quite independently of any connection with their amino acid incorporating ability.

The first step consisted in an investigation of the relative distribution of RNA and protein in the different sedimentable components of crude extracts and ribosomal preparations in different stages of purification and under different conditions, since detailed data on this point are not available. (RNA to protein ratios have usually been determined on isolated gross preparations (cf. 20).) The results, reported in this paper, show that within each sedimentation class of ribosomes there exist protein-rich and protein-poor components, and that in general polysomes are richer in protein than single ribosomes.

The second part of the investigation consisted of detailed enzyme assays. The results are reported in the accompanying papers (21, 22) and show that ribosomes have bound to them specific nucleoside triphosphatases of a novel kind, and that different kinds of enzymatic activities are associated with the protein-rich and the protein-poor components.

EXPERIMENTAL

Cells:

E. coli B, supplied by Dr. G. Stent of the University of California, was grown in a rich medium according to Tissieres *et al.* (23). The cells were harvested in the middle of the exponential growth curve, corresponding, under our conditions, to an optical density at 650 mμ of 0.9, and washed twice with Tris-Mg buffer, as given under results.

Crude extracts:

For the preparation of crude extracts, washed cells were frozen and mixed with three volumes of buffer containing 10 μg DNAase per g of cells. After one hour in the cold, the partially thawed cell paste was passed through a French press, in the cold, at a pressure of 6000-7500 psi. It should be noted that Roberts and his coworkers use a pressure of about

10,000-15,000 psi (cf. 24) at which polysomes are completely disrupted (25; see also (6)). Schaechter (5) used 3000 psi and found polysomes, but had many whole cells left. Most cells were broken under our conditions.

The disrupted cells were centrifuged at 10,000 g first for 10 minutes, and after decantation for an additional 20 minutes. The crude extracts thus obtained contained between 20 and 25 mg protein per ml.

Ribosomes:

Crude ribosomes were prepared from the crude extract by centrifuging in the No. 50 Rotor of a Spinco Model L ultracentrifuge, at 145,000 g for 1 - 2 hours. They were then suspended in the appropriate buffer by gentle teasing with a glass rod, and washed by one to three cycles of differential centrifugation at alternating low and high speeds to yield purified ribosomes.

Purified ribosomes were also prepared in a one step operation by centrifuging crude extract through layers of concentrated sucrose, according to Wettstein et al. (3). Some ribosomes were also prepared by the method of Wood and Berg (26).

Supernatant:

Supernatant was prepared by spinning the first ribosomal supernatant for 4 hours at 145,000 g and taking the upper two thirds of the tube.

Sucrose density gradients:

SDG analysis was performed by layering 0.5 to 1 ml of the sample on 29 ml of a linear sucrose gradient obtained by running equal amounts of 5 and 20 % sucrose from a two-arm mixing device. (The final sucrose concentrations were determined from the refractive index of the fractions after centrifugation, and were found to be from 7 to 18 %.) The tubes were centrifuged in a SW 25.1 swinging bucket rotor of a preparative Spinco ultracentrifuge at 25,000 rpm for periods of time varying between two and

three hours. After completion of the run, the bottoms of the tubes were pierced and fractions were collected manually into chilled test tubes.

Buffers:

Standard buffers described and tested in the literature were used in order to allow as much comparison as possible with the data of other workers. All buffers contained 0.005 M Tris-HCl, at pHs of 7.1, 7.4 or 8.1, and Mg^{++}; and one series, in addition, contained monovalent salt.

Since in the presence of salt more Mg is needed in order to maintain the same effective Mg concentration, buffers containing one of the following pairs: 0.0175 M $Mg(Cl)_2$ and 0.086 M KCl, or 0.0175 M Mg acetate and 0.19 M NH_4 acetate (27) were compared with buffers containing 0.01 M Mg acetate in the case of crude extracts, and 0.005 M Mg acetate in the case of purified ribosomes.

Crude extracts were prepared directly in the buffer later used in the SDG analysis. Ribosomes were prepared in the buffers appropriate to a given procedure, and the purified suspension was adjusted to the desired salt and Mg concentration just prior to layering on the gradient.

RNA and protein determinations:

RNA was determined by reading the optical density at 260 mμ of an appropriately diluted sample, assuming an O.D. of 24 for a 0.1 % solution. In gradient analyses, the first 10 tubes were diluted 1:10 and subsequent tubes, 1:30. All dilutions were made with micro-pipets. The solutions were read in micro-cells with a Zeiss PMQ II spectrophotometer.

Protein was determined on 50 μl aliquots by a micro modification of the method of Lowry et al. (28), in which all the volumes are one fifth that of the standard method. The slope is linear from 10 to 50 μg of protein (using crystalline Armour Bovine Albumin as a standard) and in this region

corresponds to 7.5 μg per 0.1 O.D. unit at 750 mμ. Up to 0.2 O.D., the slope is 6 μg per 0.1 O.D. unit. As little as 1 - 2 μg of protein can thus be determined quite accurately. The maximum deviation from the standard curve is 5 % for a single determination, and 2 % for duplicates. On the average, however, the deviations are lower.

RNA to protein ratios were calculated for all fractions of the gradients. The maximum error of the ratios is estimated to be 10 %, and the average error of the order of 3 - 5 %.

RESULTS

Crude extracts:

The distribution of RNA-containing components varied somewhat from extract to extract, but the pattern obtained for each individual extract could be reproduced in detail in duplicate analyses. Furthermore, changes in the patterns produced by artificially manipulating the extract were always rather minor compared to the differences between extracts. Despite apparent standardization of the growth conditions, it is therefore likely that the observed differences are due to inherent differences in the state of the ribosomes of the cells at the time of harvesting.

The distribution of RNA relative to that of protein, on the other hand, was more reproducible on the whole, as can be seen from Table I where the RNA to protein ratios at different points along the gradient for a number of extracts, in four different buffers, are given. The distribution of RNA and the corresponding RNA to protein ratio profiles of two extracts of divergent types are given in Fig. 1 A - C. The pronounced 100s peak found in both of these is, however, not always observed, there frequently being only one unresolved peak like that shown in Fig. 2.

Both Fig. 1 and Table I illustrate the fact that the RNA to protein ratio in the ribosomal region of the gradient is far from constant, it being generally highest in the region of the 70s of 100s peak, and lowest in the region of ribosomal aggregates. Fig. 1, furthermore, shows that the ratio profile does not vary in a continuous manner across the gradient, but rather that it forms a series of well-defined and periodically-spaced peaks.

It is also seen from these data that the addition of salt to the buffers has a pronounced and complex effect. Besides causing the reduction in size of the 100s peak and a general sharpening of all the peaks as observed by Gilbert (27), it also renders less pronounced the peaks in the ratio profile (Fig. 1) by lowering the ratio in the heavy components (Table I).

The effect of the pH of the buffer is illustrated in Fig. 2, which shows a comparison of RNA and of RNA to protein ratio profiles of three extracts prepared from the same batch of bacteria by separate extractions at three different pHs. While the RNA profiles are identical, there are small, but significant differences in the ratio profiles. It seems that at a pH of 7.4, which is the pH most commonly used for the preparation and purification of ribosomes from E. coli, there is the smallest amount of excess protein in the heavy fractions, and at a pH of 7.1, there is the greatest.

Ribosomes:

Because of the considerable heterogeneity in the ribosomal components of crude extracts as shown by their RNA to protein ratio profiles, it was of interest to apply these criteria to ribosomes in different stages of purification. Fig. 3 A shows the RNA and the RNA to protein ratio profiles of a very crude ribosomal pellet, having an RNA to protein ratio of 0.7;

Fig. 3 B shows the same data for a more purified preparation obtained by the sucrose layering technique and having an RNA to protein ratio of 1.47. While the RNA profiles are not very different, there was a pronounced difference between the ratio profiles for the two preparations. The ratio profile for the crude ribosomes shows much greater heterogeneity, i.e. more pronounced peaks, and also the characteristic preponderance of protein in the heavy regions seen in the crude extracts, whereas the RNA to protein ratios of purified ribosomes were more or less constant all across the gradient. Intermediate patterns may be obtained with preparations of intermediate "purity" as judged by their overall RNA to protein ratios.

Constancy of the ratios was also achieved by taking the peak tubes from a SDG run of an impure preparation, pelleting the material and re-centrifuging on a SDG under the same conditions. Such an experiment is shown in Fig. 4. The RNA to protein ratios could not be determined all across the gradient because the values for the heavier fractions did not differ significantly from the blank; which shows that a given component runs true on recentrifugation, and that under our conditions aggregation and dissociation did not occur in the course of the experiment. It also shows that when material is found in the heavy fractions, its presence is not due to "tailing" or "smearing" of the components present in high concentration, but probably to heavier and discretely sedimenting components. This is born out both by enzyme assays (21) and by previously reported amino acid incorporation assays (29; see also 27) which show that the rapidly sedimenting components, small though their absolute amount may be, have properties distinctly different from the bulk of the material.

By examining a number of ribosomal preparations through several stages of differential centrifugation, it was uniformly found that the

high relative protein content of the impure preparations was only to a small degree due to contamination with soluble protein. The excess protein was found mainly in the rapidly sedimenting fractions, and was lost from these in the course of the purification. Loss of protein from the main ribosomal peak occurred to a smaller extent, as evidenced by smaller changes in the RNA to protein ratios in the corresponding fractions of the gradient as a function of overall purification.

Effect of salt and ribonuclease on ribosomes:

In the early experiments the RNA to protein ratio in the main peak seemed rather variable and not directly correlated with "purity", as measured by the relative RNA content of the preparation as a whole. Tabulation of a number of data (Table II) indicated that the values were lowest when the ribosomes had been kept in Tris-Mg buffers throughout, and were increased by exposure to salt (KCl, NH_4Ac and $(NH_4)_2SO_4$ in that order), either during their preparation or in the gradient. The only exceptions were some preparations which had been treated with ribonuclease in the cold, and which also showed an increased RNA to protein ratio, even in the absence of salt.

Somewhat surprisingly, salt and ribonuclease seemed thus to have similar actions, and the effect of these agents on a given ribosomal preparation was therefore examined in detail. A suspension of twice-washed ribosomes prepared according to Tissieres et al. (23) in 0.01 M Mg^{++}-Tris was divided into three portions: the first was adjusted to 0.005 M Mg^{++}, the second was treated with 1 μg of crystalline pancreatic ribonuclease (Armour) and also adjusted to 0.005 M Mg^{++}, and the third was adjusted to 0.0175 M Mg acetate and 0.19 M NH_4 acetate. (The latter salt was used instead of KCl to minimize the action of the K^+-activated phosphodiesterase

of E. coli (9) on the remaining mRNA in the preparation.) All three aliquots were then centrifuged on a SDG containing the corresponding buffer and analyzed for RNA and protein. The results are shown in Fig. 5. The differences in the RNA profiles (Fig. 5 A) for the three gradients were very small, but the differences in RNA to protein ratio profiles (Fig. 5 B) were quite large. Moreover the RNA profile, denoting concentration rather than total amount is dependent on fraction volume, and could be misleading if two different gradients are compared; whereas the ratios are not subject to such limitations and are more directly comparable.

The ratio profile (Fig. 5 B) again showed that salt and RNAase had a rather similar action on the larger ribosomes in that both caused a greater displacement of protein than of RNA from all fractions heavier than 70s. The effect of these two agents is, however, seen to be different in the light particles region and supernatant, where salt causes no change or a decrease in the RNA to protein ratio, whereas RNAase causes a marked increase. Ratios can, thus, give a quick, graphic picture of any relative changes and are more useful in this respect than either the RNA or the protein profiles alone.

The similarity, however, of the ratio profiles after treatment with two agents known to have opposite effects—one enhances amino acid incorporation while the other inhibits it—also emphasizes that ratios in themselves are not sufficient to describe changes completely, since they do not tell us if there is a net transfer of material from one fraction to another. For this it is necessary to calculate quantitative recoveries for each fraction, as shown in Table III, and from which it can be seen that the two agents are in fact very different. RNAase effects a redistribution of RNA as well as of protein, whereas salt changes the protein only.

Furthermore, all of the RNA, e.g. optical density, liberated enzymatically from the heavy fractions appears in the light fractions, whereas most of the protein is precipitated and appears in a pellet at the bottom of the tube. In the case of salt, on the other hand, a large portion of the stripped protein appears in the supernatant.

DISCUSSION

Discussion of the present paper might usefully be centered on two unexpected results, namely, the presence of "extra" protein in the ribosomal aggregates, and the heterogeneity shown up by the RNA to protein ratio profile.

The low RNA to protein ratios in the heavy fractions of crude extracts raises the possibility that cell-wall or membrane fragments high in protein and low in RNA might sediment in this region. Similar low ratios have, however, also been observed with washed ribosomal preparations from both pea seedlings (30) and reticulocytes (31) where contamination with cell membranes is less likely to occur than in E. coli. Furthermore, the previously reported (29) fact that practically all of the amino acid incorporating activity of the extracts is concentrated in the heavy region, and that this activity can be substantially diminished by pre-incubation or ribonuclease treatment, which also effects the release of "extra" protein (Table III), indicates that polysome-like ribosomal aggregates are responsible for the binding of this protein. The data do not exclude the presence of small amounts of membrane fragments bound to polysomes (32), but minimize their importance for the high protein content of this fraction.

The ribonuclease data also show that any aggregates of ribosomal protein, formed as a result of ribosome denaturation or destruction, pellet easily and do not appear as discretely sedimentable structures.

Any presumptive role of denaturation is further minimized by the repeated observation in this laboratory that the amount of "extra" protein is largest in the most carefully handled extracts and in ribosome preparations with high endogenous amino acid incorporating activity. The observed phenomenon, therefore, seems to depend on the presence of mRNA, although it is quantitatively influenced by other factors such as the pH and the salt concentration of the buffer.

Since polysomes are thought to be formed by several 70s ribosomes and a strand of messenger RNA (1 - 3), the low RNA to protein ratio of the aggregates compared to that of the monomeric unit is surprising, especially since the difference is much too large to be accountable by nascent protein on the polysomes.

The present data indicate that this picture of polysome structure is too simple, and that the functional unit is capable of specifically binding not only additional amounts of sRNA, as shown by several workers (13 - 15), but also relatively large amounts of protein. That this protein might have functional significance is indicated by the fact that E. coli polysomes are self-sufficient for amino acid incorporation (33) and hence possess the necessary enzymes. In addition, however, the "extra" protein comprises most of the specific nucleoside triphosphatase activity of the extracts, as detailed in the following paper (21), and therefore definitely does not represent a cross-section of all the proteins in the extract. While the significance of the triphosphatases cannot be envisaged at present, one of the general functions of the "extra" protein might be to protect labile RNA from nucleases in the cell sap.

In this connection it might be mentioned that because of the method of cell disruption used, our preparations must contain only a fraction

of the polysomes obtainable by gentler procedures (see (4, 6)). It is therefore possible that the above picture applies only or particularly to a more resistant class of polysomes.

While many a priori considerations make it reasonable to expect some inhomogeneity in ribosomal preparations, the particular kind of heterogeneity shown up by the regularly spaced peaks in the RNA to protein ratio profile is both unexpected and difficult to explain. Yet it seems to be a general phenomenon, since, again, very similar findings have been made with ribosomal preparations of both pea seedlings (30) and reticulocytes (31).

According to present concepts of ribosome structure, the larger particles are formed by a successive Mg-mediated aggregation of two fundamental sub-units, the 30s and the 50s particles. Since both of these have the same initial RNA to protein ratio (20), this ratio should remain constant throughout the gradient, regardless of the number and even the type of aggregates. The pronounced fluctuations observed are not consistent with this picture of a simple aggregation of similar sub-units, and mean either that the building blocks are not homogeneous and each type undergoes a species-specific aggregation independent of other types, or that components other than the fundamental particles and Mg are either needed for the aggregation process or added as a result of it.

Although the possibility of heterogeneity in both the 50s particles (34) and the 30s (24) has been pointed out in the literature, there is no real evidence for sub-units of a different type which could aggregate in a manner required by our results. The so-called natural 50, 30 and 20s particles (35) present in extracts of E. coli do not seem to aggregate, even in high Mg concentrations (see also Fig. 1). Other small particles

present in low concentration include ribosomal precursors deficient in protein (24), 43s particles apparently derived from 50s through loss of one molecule of sRNA, as well as protein (36, 37), and a 25s particle (38). Of all of these, only the 25s particle is rich enough in protein (75 %) to potentially influence the RNA to protein ratios in the observed manner.

Since, however, evidence for the participation of this particle in ribosomal aggregation is lacking, it is well to consider heterogeneity of a less fundamental type which could arise from the fact that different RNAs are bound to the two sub-units, sRNA to the 50s (11, 36, 37) and mRNA to the 30s (10). Cannon et al. (11) reported that the average number of sRNA molecules per ribosome in washed preparations was 5, of which 1 was bound more firmly than the rest. There could, therefore, be at least ten classes of ribosomes with different numbers of bound sRNAs, but because of the low molecular weight of transfer RNA compared to ribosomal RNA, in centrifugation experiments this heterogeneity would be manifested merely by a spreading of the peak and a gradual increase of about 10 % in the RNA to protein ratios from one side of the peak to the other. It could not, therefore, account for the observed sharp peaks in the ratio profile. Similar reasoning shows that the binding of different mRNAs to different ribosomes likewise could not per se account for the observations.

Binding of different amounts or different types of RNA could, nevertheless, account for our results if the further, entirely reasonable assumption is made that this RNA, in turn, causes a more or less specific binding of a proportionally large amount of protein. On the basis of quantitative considerations alone, peaks in the profile would correspond

to a class of components with less bound RNA, and the valleys to a class relatively richer in protein-binding RNA, and hence even richer in protein. It is equally possible, however, that different RNAs bind different amounts of protein, and that the peaks and valleys actually are indicative of qualitatively dissimilar components.

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Table I

RNA TO PROTEIN RATIOS IN CRUDE EXTRACTS OF E. COLI

AFTER SDG CENTRIFUGATION

Buffer in gradient	Approximate s values of peaks								
	> 200	180	140	100	85	70	50	30	4
0.0175 M Mg^{++},	0.1-0.6	0.6	1.2	1.4	1.1	1.0	0.7	0.2	0.2
0.086 M KCl,	0.3-0.6	0.7	0.9	1.3	-*	1.2	0.7	0.3	0.2
Tris pH 7.4	0.3-0.5	0.8	1.1	1.2	1.1	1.0	0.6	0.2	0.2
Av:	0.2-0.6	0.7	1.1	1.3	1.1	1.1	0.7	0.2	0.2
0.01 M Mg^{++},	0.6-1.4	1.6	1.2	1.8	-	0.9	0.5	-	0.3
Tris pH 7.4	0.4-0.8	0.8	1.0	1.8	1.3	1.8	0.6	0.3	0.3
	0.6-0.7	0.9	1.1	1.3	1.2	1.0	0.8	0.4	0.3
	0.8-1.0	1.0	1.3	1.5	1.4	1.2	0.7	0.3	0.3
Av:	0.6-1.0	1.1	1.2	1.6	1.3	1.3	0.6	0.3	0.3
0.01 M Mg^{++}, Tris pH 7.1	0.5-0.8	0.8	1.1	1.3	1.2	1.1	0.8	0.4	0.3
0.01 M Mg^{++}, Tris pH 8.1	0.6-0.7	0.8	1.1	1.2	1.1	1.0	0.6	0.3	0.4

* Dashes mean that no discernible peak was observed in the appropriate region in that particular run.

Table II

RNA TO PROTEIN RATIOS OF THE 70 S PEAK AFTER SDG CENTRIFUGATION OF DIFFERENT RIBOSOMAL PREPARATIONS

RNA to protein ratios		Buffer salts*		Ref. to method of preparation
Starting material	Peak tube of SDG	During preparation	In SDG	
0.70	1.20	$Mg(Ac)_2$	$Mg(Ac)_2$	(23)
1.06	1.52	$Mg(Ac)_2$	$Mg(Ac)_2$	(23)
1.21	1.55	$Mg(Ac)_2$	$Mg(Ac)_2$	(23)
1.20	1.80	$Mg(Ac)_2$	$NH_4 \cdot Ac$, Mg^{++}	(23)
1.20	1.96†	$Mg(Ac)_2$	$Mg(Ac)_2$	(23)
1.06	1.75†	$Mg(Ac)_2$	$Mg(Ac)_2$	(23)
1.49	1.60	KCl, Mg^{++}	KCl, Mg^{++}	(3)
1.47	1.65	KCl, Mg^{++}	KCl, Mg^{++}	(3)
1.49	1.85†	KCl, Mg^{++}	KCl, Mg^{++}	(3)
1.47	1.60	KCl, Mg^{++}	$Mg(Ac)_2$	(3)
1.25	1.89	$(NH_4)_2SO_4$, Mg^{++}	$Mg(Ac)_2$	(26)
1.21	1.89	$(NH_4)_2SO_4$, Mg^{++}	$Mg(Ac)_2$	(26)
1.52	2.05	$(NH_4)_2SO_4$, Mg^{++}	$Mg(Ac)_2$	(26)

* All buffers contained Tris, pH 7.4, Mg^{++} and salt as given under experimental.

† Treated with RNAase.

Table III

EFFECT OF RIBONUCLEASE AND OF SALT ON THE DISTRIBUTION OF RNA AND PROTEIN IN THREE FRACTIONS OBTAINED BY SDG CENTRIFUGATION OF A RIBOSOMAL PREPARATION OF *E. COLI*

Fraction*	Gradient	mg total**		Percent of total†		RNA to protein ratio	Percent recovery††	
		RNA	Protein	RNA	Protein		RNA	Protein
Starting material	--	6.35	5.4	54	46	1.17		
Whole gradient	A‡	5.08	4.78	50.5	49.5	1.06	80	89
	B	5.15	3.10	62.5	37.5	1.67	81	57.5
	C	5.10	3.77	57.5	42.5	1.35	80	70
Polysomes	A	0.30	0.95	6.0	20.0	0.32		
	B	0.16	0.16	3.1	5.3	1.00		
	C	0.25	0.22	5.0	5.7	1.17		
Main peak	A	4.65	3.28	92.0	69.0	1.42		
	B	4.33	2.38	84.0	77.0	1.82		
	C	4.70	2.80	92.0	74.0	1.68		
Supernatant	A	0.13	0.54	2.0	11.0	0.24		
	B	0.66	0.46	12.9	18.0	1.42		
	C	0.16	0.75	3.0	20.0	0.21		

Footnotes to Table III

* "Starting material" refers to amount of material actually put on the gradient; "whole gradient" refers to the sum of material in all the tubes collected from the gradient, but does not include the small pellets at the bottom of the tubes; "polysomes" is the pooled material from the tubes 1-26 of Fig. 5; "main peak" corresponds to tubes 27-53, and "supernatant" to tubes 54-64. (The latter also includes small particles in addition to true supernatant.)

** The amount of material in the gradient or in a given fraction was calculated by multiplying the sum of the concentrations (per ml), obtained from the analytical procedures, by the average fraction volume. The latter was calculated by dividing the total volume of the gradient (30 ml) by the number of fractions, which does not take into account the variability of individual fractions. The excellent reproducibility from one gradient to another shows, however, that neither the fraction volume nor the analyses can be in serious error.

† Percent of total in the case of "whole gradient" refers to portion of total material recovered from the gradient which is RNA or protein. In the case of the three fractions, percent of total refers to portion of total RNA or protein in the gradient which is found in that particular fraction.

†† Percent recovery refers to portion of starting material recovered in the gradient only, and does not include the pellet.

‡ The composition of the gradients is given in Fig. 5. A is untreated ribosomes; B, RNAase-treated; and C contains $NH_4 \cdot Ac$.

REFERENCES

1. J. R. Warner, P. M. Knopf and A. Rich, Proc. Natl. Acad. Sci. U.S., 49 (1963) 122.
2. A. Gierer, J. Mol. Biol., 6 (1963) 148.
3. F. O. Wettstein, T. Staehelin and H. Noll, Nature, 197 (1963) 430.
4. T. Staehelin, C. Brinton, F. Wettstein and H. Noll, Nature, 199 (1963) 865.
5. M. Schaechter, J. Mol. Biol., 7 (1963) 561.
6. Y. Kiho and A. Rich, Proc. Natl. Acad. Sci. U.S., 51 (1964) 111.
7. R. W. Risebrough, A. Tissieres and J. D. Watson, Proc. Natl. Acad. Sci. U.S., 48 (1962) 430.
8. G. J. Spyrides and F. Lipmann, Proc. Natl. Acad. Sci. U.S., 48 (1962) 1977.
9. W. Gilbert, Cold Spring Harbor Symp., 28 (1963) 287.
10. M. Takanami and T. Okamoto, J. Mol. Biol., 7 (1963) 323.
11. M. Cannon, R. Krug and W. Gilbert, J. Mol. Biol., 7 (1963) 360.
12. M. Takanami, Biochim. Biophys. Acta, 61 (1962) 432.
13. A. Kaji and H. Kaji, Biochem. Biophys. Res. Com., 13 (1963) 186.
14. T. Nakamoto, T. W. Conway, J. E. Allende, G. J. Spyrides and F. Lipmann, Cold Spring Harbor Symp., 28 (1963) 227.
15. P. Cammarano, G. Guidice and G. D. Novelli, Biochem. Biophys. Res. Com., 12 (1963) 498.
16. R. Rosset and R. Monier, Biochim. Biophys. Acta, 68 (1963) 653.
17. G. Webster and S. L. Whitman, Biochim. Biophys. Acta, 68 (1963) 653.
18. I. D. Raacke, unpublished; see also, I. D. Raacke in "Metabolic Pathways", D. M. Greenberg, ed., New York (1961), p. 263.

19. G. W. Dietz, Jr. and M. V. Simpson, Federation Proc., 23 (1964) 219.

20. A. Tissieres and J. D. Watson, Nature, 182 (1958) 778.

21. I. D. Raacke, Biochim. Biophys. Acta.

22. I. D. Raacke and J. Fiala, Biochim. Biophys. Acta.

23. A. Tissieres, D. Schlessinger and F. Gros, Proc. Natl. Acad. Sci. U.S., 46 (1960) 1450.

24. B. J. McCarthy, R. J. Britten and R. B. Roberts, Biophys. J., 2 (1962) 57.

25. K. McQuillen, R. B. Roberts and R. J. Britten, Proc. Natl. Acad. Sci. U.S., 45 (1959) 1437.

26. W. B. Wood and P. Berg, Proc. Natl. Acad. Sci. U.S., 48 (1962) 94.

27. W. Gilbert, J. Mol. Biol., 6 (1963) 374.

28. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193 (1951) 265.

29. I. D. Raacke and J. Fiala, Proc. Natl. Acad. Sci. U.S., 51 (1964) 323.

30. I. D. Raacke, in preparation.

31. J. Fiala and I. D. Raacke, in preparation.

32. D. Schlessinger, J. Mol. Biol., 7 (1963) 569.

33. I. D. Raacke, J. Fiala and T. H. Jukes, Federation Proc., 23 (1964) 218.

34. C. G. Kurland, J. Mol. Biol., 2 (1960) 83.

35. A. I. Aronson and B. J. McCarthy, Biophys. J., 1 (1961) 215.

36. D. Elson, Biochim. Biophys. Acta, 53 (1961) 232.

37. D. Elson, Biochim. Biophys. Acta, 61 (1962) 460.

38. M. Takai, Y. Oota and S. Osawa, Biochim. Biophys. Acta, 55 (1962) 202.

LEGENDS TO FIGURES

Fig. 1. Distribution of RNA and RNA to protein ratios of two different crude extracts of E. coli in Tris- Mg buffers in the presence and absence of KCl.

A and B, extract I; C and D, extract II. B and C in 0.005 M Tris, pH 7.4, 0.01 M Mg acetate; A and D in 0.005 M Tris, pH 7.4, 0.0175 M $Mg(Cl)_2$, 0.086 M KCl. Heavy solid lines denote RNA, dashed lines the RNA to protein ratios. The dashed diagonal line represents the sucrose concentrations in the gradient at the end of the run. The gradients were centrifuged at 25,000 rpm for two and a half hours.

Fig. 2. Distribution of RNA (A) and RNA to protein ratios (B) of the same crude extract as a function of pH.

All buffers contained 0.005 M Tris and 0.01 M Mg acetate. --●--● pH 7.1; —O—O— pH 7.4; ...x...x pH 8.1. The gradients were centrifuged as in Fig. 1.

Fig. 3. Distribution of RNA and RNA to protein ratios in crude (A) and in purified (B) ribosomal preparations from E. coli.

The solid lines denote RNA and the dashed ones the ratios. The crude ribosomes were prepared by the method of Tissieres et al. (23), the first ribosomal pellet being suspended in 0.005 M Tris, pH 7.4, 0.01 M Mg acetate. The RNA to protein ratio of the pellet was 0.7.

The purified ribosomes were prepared by layering 5 ml of the crude extract, prepared in 0.005 M Tris, pH 7.4, 0.0175 M $Mg(Cl_2)$, 0.086 M KCl, on 5 ml of 1.3 sucrose containing the same buffer. Centrifugation was

at 145,000 g for 5 and a half hours. The pellet was suspended in buffer, dialyzed to remove sucrose and layered in the usual manner on a SDG containing 0.005 M Tris, 0.01 M Mg acetate. The RNA to protein ratio of the starting material was 1.47.

Both gradients were centrifuged at 25,000 rpm for 3 hours.

Fig. 4. The effect of repeated SDG centrifugation on the RNA and RNA to protein ratio profiles of isolated ribosomes.

The ribosomes used for the first SDG run were isolated by the method Tissieres et al. (23) and had a RNA to protein ratio of 1.0. They were suspended in 0.005 M Tris, pH 7.4, 0.005 M Mg acetate (to minimize formation of the 100s peak) and centrifuged on gradients containing the same buffer, in three separate buckets. After the run the three buckets were pooled tube by tube, the optical density determined in the region of the main peak, and a cut taken as indicated in the figure. The pooled tubes were centrifuged at 100,000 g (40 rotor) for 4 hours, the pellets suspended in the same buffer and dialyzed to remove the sucrose. The suspension was then layered on a gradient and run under the same conditions as before, 25,000 rpm, 3 hours.

The RNA to protein ratio profile refers to the re-run.

Fig. 5. The effect of salt and of ribonuclease on the distribution of RNA (A) and the RNA to protein ratio profile (B) of a ribosomal preparation of E. Coli.

X-X- untreated preparation; --●--● treated with 1 μg RNAase (0.5 ml, 13 mg ribosomes) in the cold; ...o...o untreated preparation suspended in 0.005 M Tris, pH 7.4, 0.0175 M Mg acetate, 0.19 M NH_4·Ac, and run

on a SDG containing the same buffer. Both the untreated, and the RNAase-treated preparation were in 0.005 M Tris, pH 7.4, 0.005 M Mg acetate throughout. Centrifugation was as in Fig. 1.

The ribosomes were prepared by the method of Tissieres et al. (23), taken through three cycles of differential centrifugation. Analytical details are given in Table III.

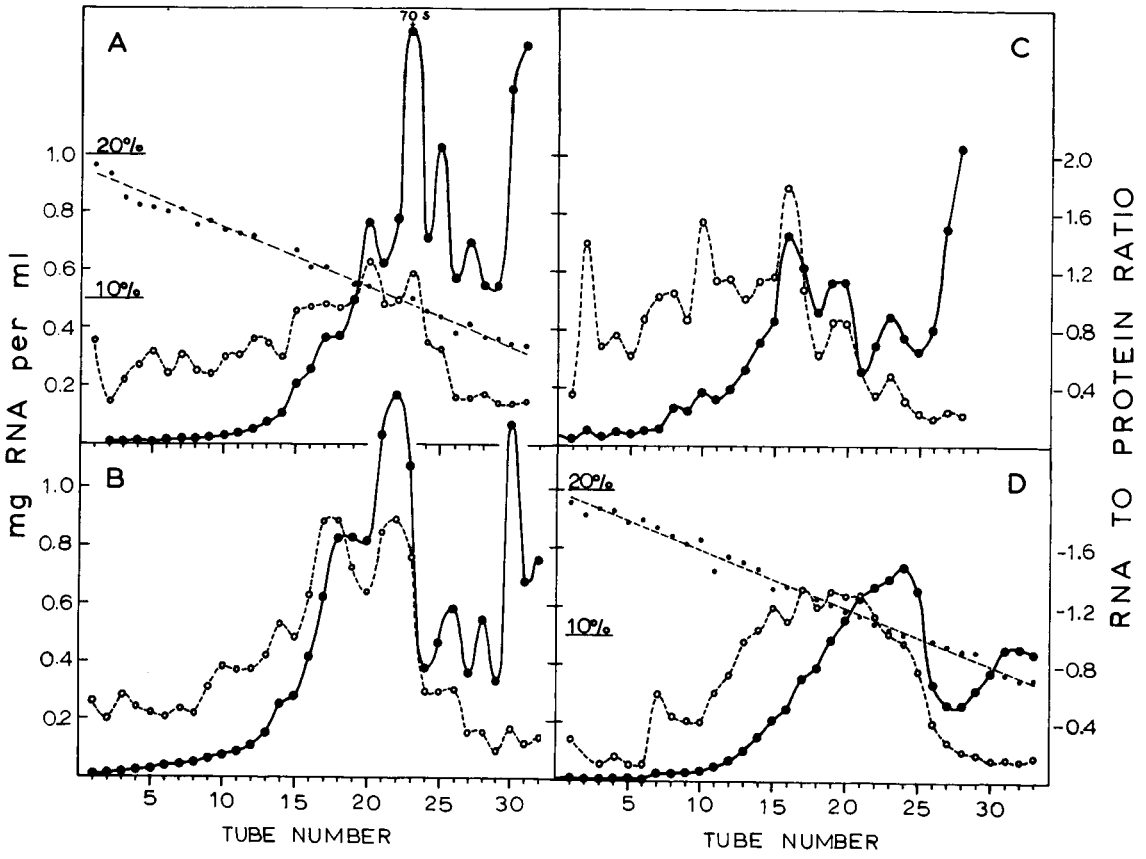

Figure 1

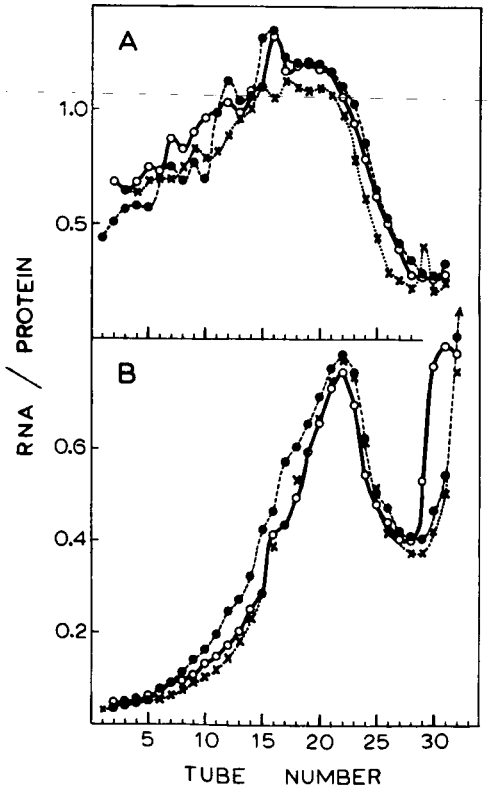

Figure 2

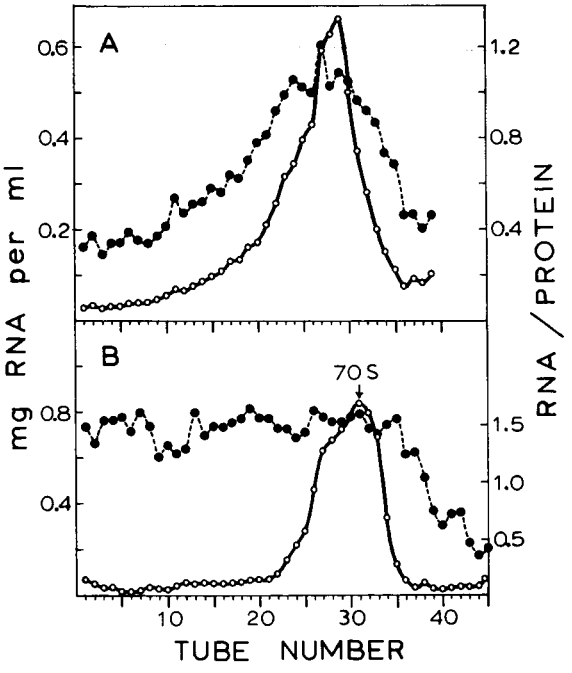

Figure 3

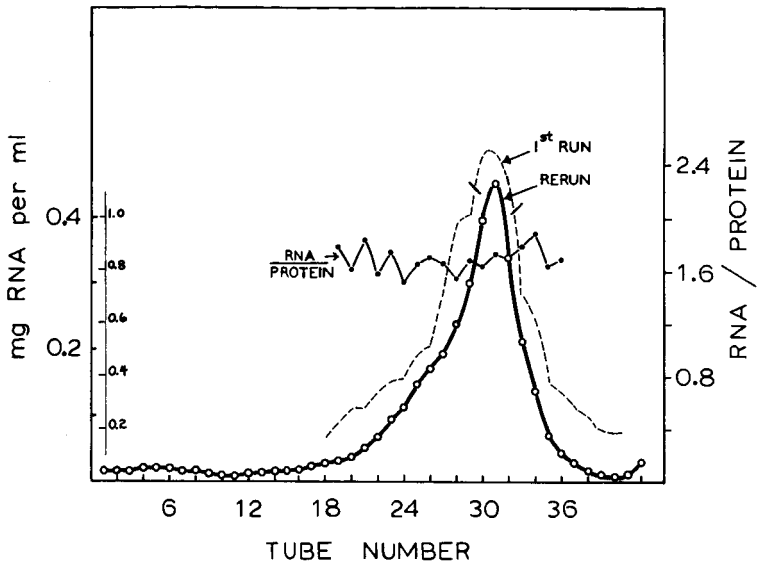

Figure 4

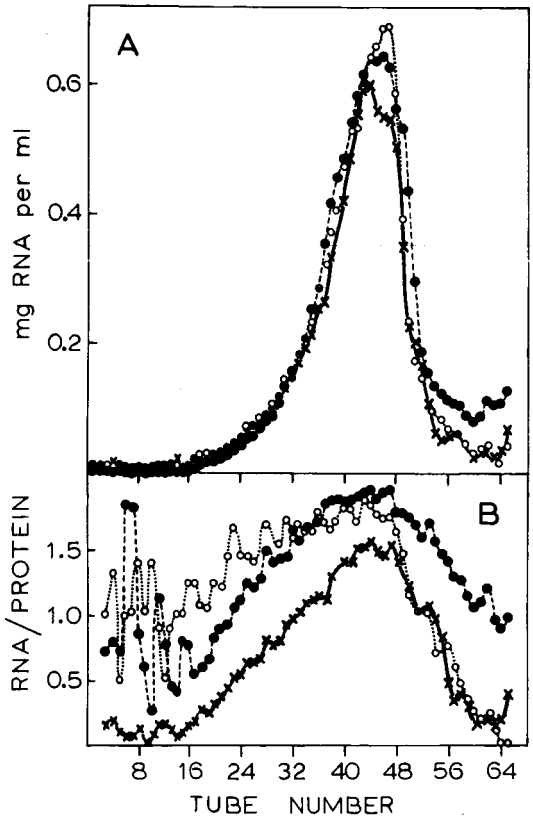

Figure 5