A Systematic Study of the Isolation of Murine Plasma Cell Ribosomal Subunits, Their Sedimentation Properties and Activity in Polyphenylalanine Synthesis†

C. H. Faust, Jr.,‡ and H. Matthaei*

ABSTRACT: A detailed study is described on the preparation of ribosomal subunits from ribosomes of murine myeloma tumors. Subunits are separated by sedimentation at either 5 or 20° through a linear sucrose gradient, containing the same KCl and $MgCl_2$ concentrations as the sample overlay. The temperature of centrifugation does not affect the sedimentation profile of the subunits. However, their polyuridylic acid directed polyphenylalanine-synthesizing ability is strongly influenced: the 5° preparation is about 2.5 times more active than the 20° preparation. Comparisons of different methods of subunit preparation are made with respect to the influence of a preliminary 0.5 M KCl washing, preincubation at 37° and use of puromycin. The effects of the K^+:Mg^{2+} molar ratio upon ribosome dissociation and subunit sedimentation behavior are noted in terms of possible breakdown and/or conformational changes, as reflected through A_{260}:A_{280} ratios and $s_{20,w}$ values. The A_{260}:A_{280} ratios range from about 1.85 to 2.10 for both the small and large subunits, while the s coefficients vary from 40 to 23 S for the small subunit and 66 to 38 S for the large subunit. Agarose–acrylamide gel electrophoreses of rRNA verify the composition of the various subunit peak fractions. In some cases, the small subunit, as dimers, contaminates the large subunit fraction very much, while in others the 5S rRNA of the large subunit appears to be missing. The 60S:40S ratios ranged from 6.5:1.0 to 2.2:1.0. The functional assay of polyuridylic acid dependent polyphenylalanine synthesis demonstrates the biological integrity of these subunits. The 80S ribosomes are active, and with certain methods of preparation 80 S, reconstituted from subunits, are also active. Noteable exceptions are preparations in which the 5S rRNA appears to be missing. The contamination of the large subunit fractions by small subunit dimers is also verified by these assays. Subunit preparation is optimal in the range of 275–525 mM K^+ and 2.5–5.0 mM Mg^{2+}, with a value of as low as 1.5 mM Mg^{2+} for 275 mM K^+. The sedimentation profiles seem to suggest that the subunits undergo an almost stepwise unfolding with the salt concentrations employed here. For the small subunit, the sequence can be approximated as 40 S (40 S–38 S) → 35 S → 27 S (30 S–26 S) → 23 S, while for the large subunit it appears to be 60 S (66 S–60 S) → 56 S (58 S–53 S) → 46 S → 38 S (41 S–38 S). As the 56 S goes to the 38 S form, the 5S rRNA appears to be lost from this subunit, possibly through a 46S transition state.

Recently, there have appeared several reports on the preparation of active 40S and 60S subunits from various types of mammalian ribosomes (Martin *et al.*, 1969; Nolan and Arnstein, 1969; Falvey and Staehelin, 1970; Blobel and Sabatini, 1971). These methods may seem confusing, since they employ various different salt conditions and temperatures. Therefore, the present comprehensive study was undertaken to systematically determine the effects of a preliminary high-salt washing, centrifugation temperature, preincubation of sample, puromycin treatment, different K^+ and Mg^{2+} concentrations, and the influence of the K^+:Mg^{2+} molar ratio on the dissociation of ribosomes into 40S and 60S subunits or other subparticles. Since certain salt conditions can result in the dimerization of 40S particles, the 60S peak is sometimes contaminated. This contamination by 40S subunits was shown by an assay of the ability for polyphenylalanine synthesis and by agarose–acrylamide gel electrophoresis of the rRNA. The latter method also revealed a possible loss of 5S rRNA from the large subunit, with a concomitant loss of biological activity. No EDTA or other Mg^{2+} chelators were employed in our studies, because it was previously shown (Martin *et al.*, 1969) that this results in permanent biological inactivity. A comparison of the results of this work to results of other methods of subunit preparation suggests that significant differences may exist among the ribosomes derived from different tissues.

Experimental Section

Ribosome Preparation. Plasmacytomas, MOPC 46B and 70E, were obtained originally from Dr. Michael Potter. Both κ-light-chain-secreting tumors were serially transplanted subcutaneously in the axial region as a cell suspension in physiological saline (0.85%, w/v) into BALB/c F strain mice. This is a genetically controlled substrain bred over one and one-half years in this laboratory, originally obtained from Jackson Laboratories, Bar Harbor, Maine. After two weeks of growth, the tumors were excised (average weight 3.5–4.0 g/mouse) and placed in 2.5–3 volumes of ice-cold medium a,[1] containing 350 mM sucrose. These were then coarsely minced with a scissors and homogenized with a motor-driven Potter-Elvehjem homogenizer, using a loose-fitting pestle

† Max-Planck-Institut für experimentelle Medizin, Arbeitsgruppe Biochemie, 34 Göttingen, Germany. *Received November 1, 1971.* C. F. was a recipient of an American Cancer Society Postdoctoral Fellowship, Grant No. PF-667F, during the course of this work.

‡ Present address: Département de Pathologie, Université de Genève, 1211 Genève 4, Switzerland.

[1] Abbreviations used are: medium a, 50 mM Tris-HCl (pH 7.5)–10 mM KCl–1 mM $MgCl_2$–7 mM β-mercaptoethanol; medium b, 50 mM Tris-HCl (pH 7.5)–25 mM KCl–5 mM $MgCl_2$–21 mM β-mercaptoethanol; medium c, medium a but with 21 mM β-mercaptoethanol. S-100, the supernatant fraction obtained from the postmitochondrial supernatant, after the 2.5-hr centrifugation at 360,000g.

with six or seven up-and-down strokes. Unless otherwise stated, all procedures were carried out at 0–4°.

The homogenate was centrifuged for 20 min at 10,000*g* and the supernatant was made to contain a final concentration of 0.5% (w/v) sodium deoxycholate from an aqueous 6% (w/v) stock. The sodium deoxycholate treated supernatant was centrifuged for 2.5 hr at 360,000*g* maximum (59,000 rpm), using a Spinco type 60 Ti rotor. The yield of unwashed ribosomes and polysomes was as much as 8 mg/g of tumor tissue.[2] The pelleted particles were then resuspended in medium b plus 250 mM sucrose. The KCl concentration was adjusted to 0.5 M with a 4 M stock solution, stirred gently for 4–6 hr and repelleted as described above. The washed ribosomes were resuspended in medium c plus 250 mM sucrose. If the ribosomes did not recieve the 0.5 M KCl preliminary washing, they were resuspended directly in medium c instead of medium b. The unwashed ribosomes were consistently found to have an A_{260}:A_{280} ratio of about 1.72–1.78, while the one-time 0.5 M KCl-washed ribosomes had an A_{260}:A_{280} ratio of about 1.82–1.86. Total amounts of ribosomes ranging from 10,000 to 15,000 A_{260} units were processed at one time, so that each one of the three individual centrifugation experiments run were uniform with respect to ribosomes used. At a concentration of 250–350 A_{260} units/ml, the ribosomes were quickly frozen at −160° and stored at −50°.

Subunit Preparation. Subunits were obtained from the above-described ribosome preparation. Using the SW 25.3 or SW 27 rotors ($^5/_8$ × 4 in. tubes), 17 ml of 5–25% (w/v) linear sucrose gradients were prepared, containing 50 mM Tris-HCl (pH 7.5), 21 mM β-mercaptoethanol, and the indicated KCl and $MgCl_2$ concentrations. The 320-μl reaction mixture, containing 20 A_{260} units of ribosomes, 50 mM Tris-HCl (pH 7.5), 21 mM-β-mercaptoethanol, no sucrose, and the indicated KCl and $MgCl_2$ concentrations, was treated as described in the legends. A 300-μl sample from this mixture was then layered onto the gradient and centrifuged at 5 or 20° for the times and speeds listed. The gradients were fractionated and monitored with a flow-through cell at 260 mμ, collecting the peak fractions by taking that portion between the half-heights of the ascending and descending boundaries of the peak tracing when possible. The absorbance in all figures is corrected to 1-cm path length.

The subunits were concentrated by one of two methods. With the first method, the Mg^{2+} was raised to about 25 mM with a 1 M $MgCl_2$ stock to cause aggregation. This was followed with a 5.0-hr centrifugation at 360,000*g*, using a Spinco type 60 Ti rotor to pellet the aggregates. These were resuspended and dialyzed against medium c containing 30% (v/v) glycerol, and frozen as described above. In the other method, the Mg^{2+} concentration in the peak fractions was raised to 10 mM; the particles were precipitated with an equal volume of 95% ethanol (−35°), allowed to stand for 1 hr at −35° and sedimented in 10 min at 30,000*g*. They were processed further as described above. Subunits had equal activity when prepared by either method.

Polyuridylic Acid Directed Polyphenylalanine Synthesis. All 40S and 60S peak fractions from each corresponding sedimentation run were assayed separately, and in combination with each other, in a system capable of supporting polyphenylalanine synthesis. A 100-μl incubation mixture contained the following components: 0.2 μmole of GTP, 40 μg of unfractionated *Escherichia coli* tRNA (Schwarz BioResearch), 2.2% charged with [^{3}H]phenylalanine (1000 Ci/mole, Amersham Radiochemical Centre), as described (Matthaei *et al.*, 1966), ribosomes or ribosomal subunits in A_{260} units: 40 S = 0.15, 60 S = 0.35, 40 S + 60 S = 0.15 + 0.35, 80 S = 0.5. Furthermore, it contained 240 μg of protein from fraction III, a 33–70% saturated $(NH_4)_2SO_4$ cut from a S-100 of murine myeloma tissue, containing transferases I and II, basically according to a previous method (Bermek and Matthaei, 1970b), except that fraction III was stored in medium c containing 10% (v/v) double-distilled glycerol (Merck). The final ionic assay conditions were 50 mM Tris-HCl (pH 7.5), 80 mM KCl, 12 mM $MgCl_2$, and 21 mM β-mercaptoethanol. Incubations were done at 37° for 30 min. Aliquots of 75 μl were plated onto Whatman GF/A filters, precipitated for 10 min in ice-cold 10% trichloroacetic acid, and heated for 20 min at 90°. Filters were washed at room temperature twice for 3 min in 5% trichloroacetic acid, twice for 1 min in ethanol–ether (1:1, v/v), and once for 1 min in ether. After drying under an infrared lamp, the filters were counted in 2 ml of scintillation fluid, 4.0 g of 2,5-diphenyloxazole (Packard) per l. of toluene, with a Packard TriCarb liquid scintillation spectrometer having a counting efficiency of about 24%.

RNA Analyses. The subunit peak fractions were examined for homogeneity as follows. The rRNA was extracted three times with cold, water-saturated phenol containing 1% (w/v) sodium dodecyl sulfate. It was precipitated with two volumes of ethanol (−35°) and allowed to stand at −35° overnight. The precipitate was collected by a 10-min centrifugation at 10,000*g*; the ethanol was removed by decantation, and the pellets were three-times extracted with ether and dried. The dry rRNA was subsequently dissolved in 100 μl of borate–Tris buffer (Peacock and Dingman, 1967) and analyzed using a modified agarose–acrylamide gel electrophoresis procedure (Peacock and Dingman, 1967, 1968). Up to 50 μl of an RNA sample prepared as described above and containing 10–100 μg of rRNA was applied to the gel column in 20% (w/v) sucrose. The gels (100 × 5.4 mm) consisted of 0.5% agarose and 2.0% acrylamide. They were electrophoresed at 0–4° and 4 mA/tube for 90 min and stained and destained as described (Peacock and Dingman, 1967). The bands were compared with standards of *E. coli* tRNA and myeloma rRNA previously identified by sucrose gradient centrifugation. The various rRNA species were recorded using a densitometer (Photovolt Corp.).

Results and Discussion

Effect of Preliminary 0.5 M KCl Washing. The first thing observed as a result of the preliminary 0.5 M KCl washing is a shift of the A_{260}:A_{280} ratio from about 1.75 before washing to about 1.85 afterward. This indicates a significant loss of protein from the ribosomes (normally about 8 mg of protein/1000 A_{260} units of crude ribosomes). This high-salt protein wash fraction from the ribosomes has been used to study translational, initiation phenomena (Pritchard *et al.*, 1970; Heywood, 1969; for unpublished results, see C. H. Faust and H. Matthaei). However, the result of this washing is much more significant for the kinds of products one finds upon sucrose gradient analysis (see Figure 1d). It is quite clear that the large diffuse peak of smaller molecules sedimenting after the 40S subunit peak from the crude, unwashed ribosomal preparation is essentially absent from the profile of the washed ribosomes (Figure 1d). The peak probably represents a combination of proteins, tRNA, and mRNA

[2] Calculation based on the approximation of 12.5 A_{260} units/mg of ribosomes.

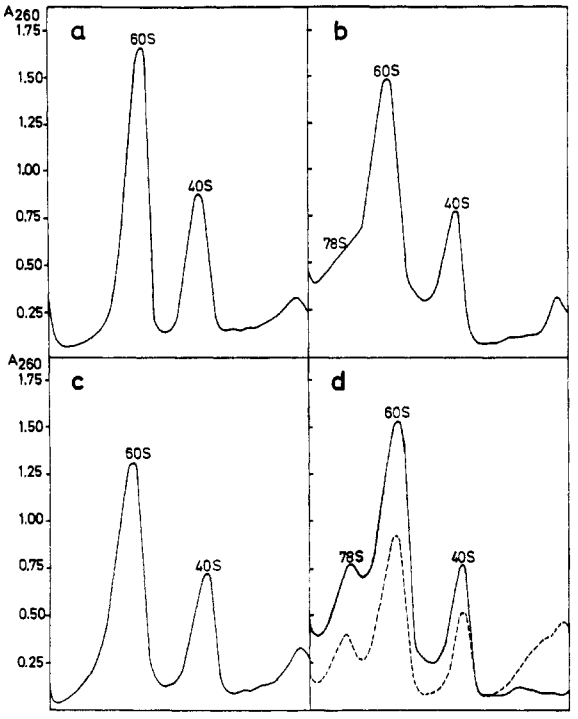


FIGURE 1: The preparation of the gradients is described in the Experimental Section. They contain 525 mM K^+ and 5 mM Mg^{2+}, as do the 300-μl sample overlays. (a) Centrifugation at 20° for 5.5 hr and 27,000 rpm with an SW 27 rotor. Before the preincubation for 15 min at 37° with 0.1 mM puromycin, the ribosomes in the sample overlay were washed with 0.5 M KCl; (b, c, d) centrifugation at 5° for 11.0 hr and 25,000 rpm with an SW 25.3, rotor; (b) sample preparation identical with part a, except the preincubation was at 0° for 15 min; (c) sample preparation identical with part a; (d) the ribosomes were preincubated for 30 min at 37° without puromycin and received a preliminary 0.5 M KCl washing (—) or did not receive one (···). The gradient with the ribosomes receiving the KCl washing has 50% more ribosomes in the 300-μl sample overlay, *i.e.*, about 30 A_{260} units. Fractions were collected and analyzed as described in the Experimental Section.

fragments loosely associated with the ribosomes and normally removed with the preliminary high-salt washing.

Without this preliminary high-salt washing significant A_{260} peaks were observed in the 18S–23S region of the high K^+ and low Mg^{2+} gradients, *e.g.*, 525–1025 mM and 5–10 mM, respectively (figures not shown). This suggested to us a partial degradation of rRNA by RNase loosely associated with the unwashed ribosomes, since no significant amount of this material was observed with the washed ribosomes. Furthermore, such a partial degradation of rRNA may have then facilitated an easier disruption of the subunits into their component rRNA and proteins in the presence of high K^+ and low Mg^{2+} concentrations. This RNase problem has been noted with rat liver ribosomes (Petermann and Pavlovec, 1967).

Effect of Preincubation and Puromycin. With or without preincubation, unwashed or washed ribosomes showed no appreciable dissociation (less than 5%) into subunits when the gradient contained both 25 mM K^+ and 15 mM Mg^{2+} (figures not shown). This was true even if the sample overlay contained $K^+ \leqslant 525$ mM and $Mg^{2+} \geqslant 5$ mM, regardless of any puromycin treatment.[3] However, 100% dissociation into subunits could be easily achieved using 0.1 mM puromycin and 15-min preincubation at 37° (Figure 1a,c). From preliminary experiments, the minimum incubation time necessary for both washed and unwashed ribosomes seemed to be about 10 min, after which neither polysomes nor monosomes could be detected. Later on, it was found that the incubation time at 37° could be reduced to about 5 min with 0.5 mM puromycin. However, when preincubating for as long as 30 min without puromycin at 37°, only about 70% of the ribosomes dissociated into subunits with 525 mM KCl and 5 mM $MgCl_2$ in both the sample overlay and the gradient. The 60S and 40S moieties so released under these conditions gave peak areas in a ratio of about 2.5 to 1.0 in A_{260} tracings (Figure 1d).

This indicated that puromycin transfer was complete and augmented ribosomal dissociation into subunits in the presence of high KCl concentrations. The 80S monomers appeared to be the largest species present after preincubation without puromycin, since no polysomes could be detected presumably due to traces of RNase still present in the sample and not removed after the 0.5 M KCl washing.

In a recent paper (Blobel and Sabatini, 1971) on the dissociation of polysomes aided by puromycin, the nascent polypeptides are shown to be released from the ribosomes by puromycin. This release is enhanced with increasing KCl concentrations. The effect of the K^+:Mg^{2+} ratio on the puromycin-induced dissociation into subunits is also emphasized. At 500 mM K^+, complete dissociation is obtained at 2 mM Mg^{2+}, while it is only partial at 5 mM Mg^{2+} and above. From our work, a similar effect is rather obvious when inspecting Figures 2–7 except that complete dissociation can apparently be achieved at an even higher Mg^{2+} concentration, *i.e.*, 5 mM at 525 mM K^+ (Figure 5c). The profile obtained with incubation at 0° for 15 min and 0.1 mM puromycin is shown in Figure 1b. Here the ribosomes dissociate into 40S and 60S subunits, with the leading edge of the 60S peak skewed into a shoulder at 80 S. This heavy fraction is estimated to be less than 15% of the total A_{260} units. The shoulder completely disappeared if the ribosomes were preincubated at 37° for 15 min (Figure 1a,c). This is in agreement with earlier work (Blobel and Sabatini, 1971). After preincubation at 0° (Figure 1b) the separation between the 40S and 60S peak appeared to be not as good as after preincubation at 37° (Figure 1a,c). The reason for this is not understood.

The data presented here would indicate that the 80 S is stabilized (Figure 1d), probably by peptidyl-tRNA as found by other workers (Blobel and Sabatini, 1970). Even when incubated in the presence of puromycin at 0° to remove this nascent polypeptide chain, the 80S monomer is still stabilized (Figure 1b), although to a lesser degree. This could be due to the bound, deacylated peptidyl-tRNA, which would be removed by a 37° preincubation, as shown earlier (Blobel and Sabatini, 1971) and supported here by Figure 1a,c. Similarly, other workers (Martin *et al.*, 1969) also found that puromycin was absolutely required for complete dissociation of 80S ribosomes. GTP was also tested here, but was found to be without effect in augmenting ribosomal dissociation when using either the crude or washed ribosomes.

Effect of Low and High Centrifugation Temperatures. All sedimentation profiles presented in Figures 2–7 were obtained at 5°. Identical centrifugation experiments were also done at 20°, but no differences in sedimentation profiles were observed at either temperature for a given set of K^+ and Mg^{2+} conditions. The ratios of the areas under the 60S and 40S

[3] When considering the results presented below, this indicated that high K^+ and low Mg^{2+} concentrations were necessary in the gradient to prevent spontaneous subunit reassociation.

260-mμ absorbancy peaks were also the same (Figure 1a,c). This is in contrast to results reported for rat liver (Petermann and Pavlovec, 1967). In this case, ribosomes dissociated to a greater degree at 37° than at 5°, even though the amount of Mg^{2+} bound per mole of phosphate was the same at both temperatures. Part of this dissociation was attributed to RNase action, however. Low-temperature-induced subunit aggregation artifacts were noted also in these rat liver studies and in rat muscle studies (Martin *et al.*, 1969), but not with mouse liver (Falvey and Staehelin, 1970) nor here with myeloma tumor.

However, significant differences were observed when comparing the polyphenylalanine-synthesizing ability of the subunits prepared at the two different centrifugation temperatures (Table I). Those subunits prepared from the 5° run were about 2.5 times more active than the ones prepared from the 20° run. This is probably to be expected, since the disruptive forces of high levels of K^+ would be greater at increased temperatures, and presumably would lead to a marked decrease in biological activity. Nevertheless, this is in direct contrast to the results of others (Bouanou *et al.*, 1968; Bouanou and Arnstein, 1969). They found good polyphenylalanine-synthesizing activity with polysome-derived reticulocyte subunits prepared at high temperature (20°), and no activity when prepared by low-temperature centrifugation (0–5°). However, since significantly more activity can be obtained with the low-temperature preparation, it would appear to be the method of choice for myeloma tumor ribosomes. Even so, the activities reported here are considerably lower than those reported by others (Martin *et al.*, 1969; Falvey and Staehelin, 1970). The reason for this may be any one, or a combination of the following: (1) precharged *E. coli* tRNA was used instead of the homologous tRNA; (2) total 80S ribosomes were used for the subunit preparation and not polysome-derived run-off subunits; (3) a transferase fraction from the S-100 was used which may not have been as active as the S-100 fraction; and (4) possible inactivating effects on the subunits from the long incubation (15 min at 37°) may have resulted at high salt with puromycin. The 80S control in Table I shows data comparable to that obtained in an almost identical test system, using human lymphatic tissue (Bermek *et al.*, 1970a).

Effect of K^+:Mg^{2+} Molar Ratios and Absolute K^+ and Mg^{2+} Concentrations. It was hoped that by investigating a wide range of both K^+ and Mg^{2+} concentrations used in the sample and gradient, a better insight into their antagonistic and/or complementary influence in promoting ribosomal dissociation would be obtained. The influence of Tris-HCl in this regard was not investigated, but has been briefly looked at earlier (Petermann and Pavlovec, 1967) as was the influence of pH (Nolan and Arnstein, 1969). Based on a previous report (Martin *et al.*, 1969), high levels of β-mercaptoethanol were used throughout these studies. The importance of sulfhydryl reagents in augmenting ribosomal dissociation has also been demonstrated (Petermann *et al.*, 1969). The influence of ribosome concentration dependence was not studied, but has been looked at by others (Nolan and Arnstein, 1969). They demonstrated an inverse relationship between the amount of ribosomes dissociated and their concentration for a given K^+:Mg^{2+} ratio. The ribosome concentration used in our studies was within the range investigated by these authors (Nolan and Arnstein, 1969). The effects of various K^+ and Mg^{2+} levels and the influence of their molar ratios (referred to by K^+:Mg^{2+}) upon ribosome dissociation are illustrated in Figures 2–7.

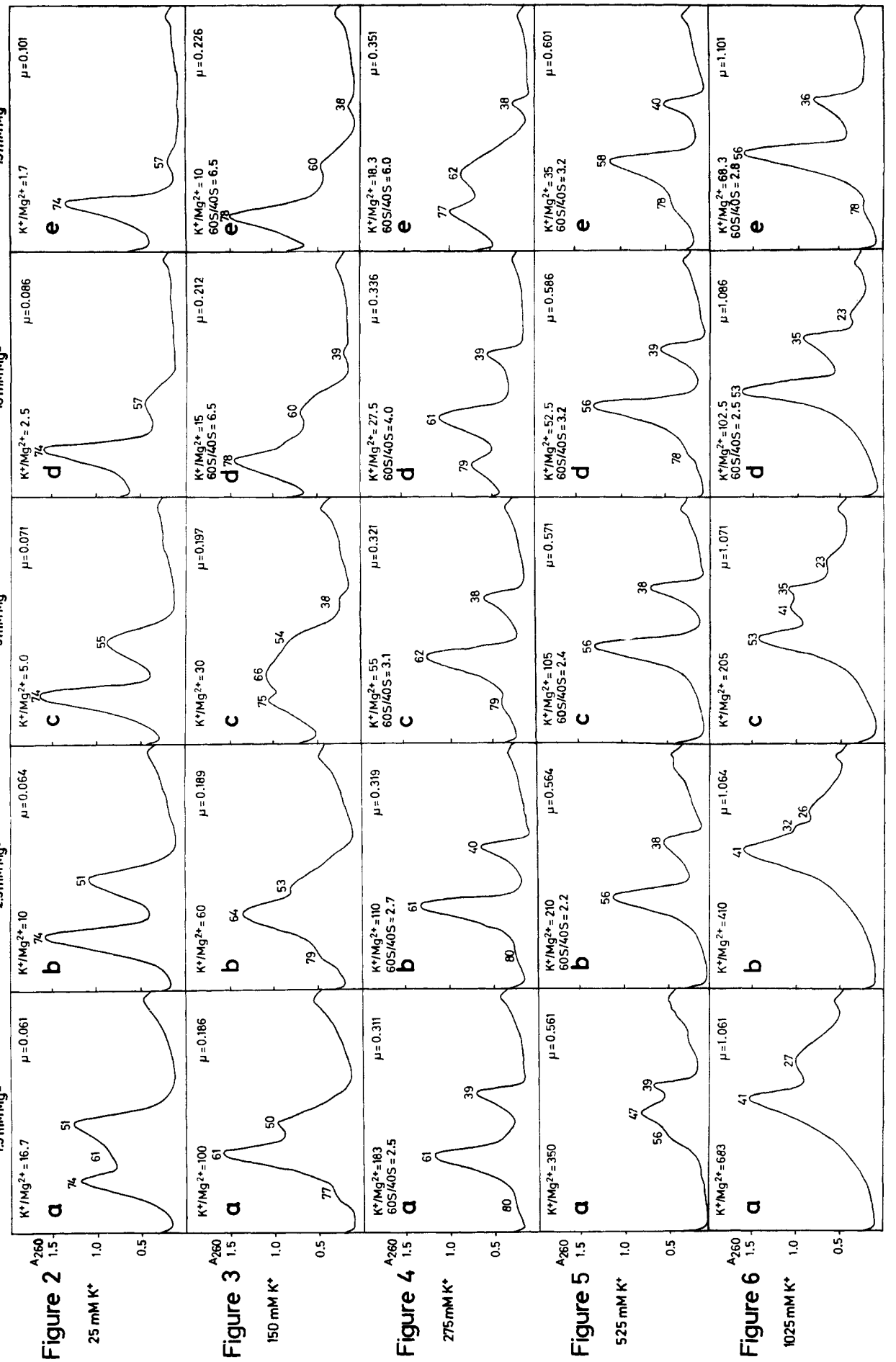
TABLE I: Comparison of the Effect of Centrifugation Temperature on the Polyphenylalanine-Synthesizing Ability of Ribosomal Subunits.[a]

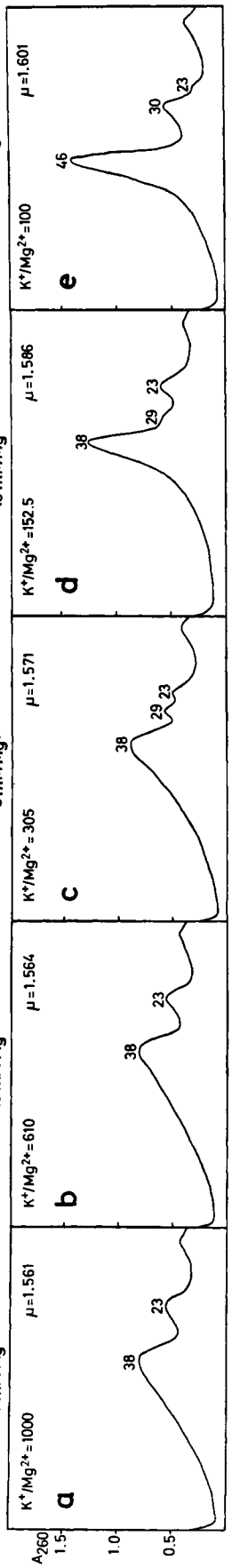
	pmoles of [^{3}H]Phe Incorpd/nmole of Ribosomes or Subunits[b]
Subunits Separated by Centrifugation at 20°[c]	
Complete (40 S + 60 S)	102.0
−40 S	35.4
−60 S	3.4
−GTP	9.7
−Poly(U)	0.0
−Fraction III proteins	0.0
Subunits Prepared by Centrifugation at 5°[d]	
Complete (40 S + 60 S)	258.5
−40 S	27.2
−60 S	3.4
−GTP	4.1
−Poly(U)	0.0
−Fraction III proteins	0.0
0.5 M KCl-Washed 80 S Ribosomes[e]	
Complete	405.0
−80 S	0.0
−GTP	55.8
−Poly(U)	61.2
−Fraction III proteins	0.0

[a] The subunits were prepared and analyzed as described in the Experimental Section. The gradient and sample overlay contained 525 mM K^+ and 5 mM Mg^{2+}. The sample overlay was preincubated at 37° for 15 min in the presence of 0.1 mM puromycin. The preparation of fraction III protein is also described in the text. [b] Where both subunits are present, incorporation is calculated per 40 S moiety. [c] This subunit preparation was centrifuged at 20° in an SW 27 rotor for 5.5 hr at 27,000 rpm with brake. [d] This subunit preparation was centrifuged at 5° in an SW 25.3 rotor for 11 hr at 25,000 rpm with brake. [e] The 80 S ribosomes received the 0.5 M KCl preliminary wash only, with no preincubation, no puromycin, and no sucrose gradient centrifugation.

The *s* coefficients were estimated according to a modified method (Martin and Ames, 1961; Tengerdy and Faust, 1971), using MRE 600 *E. coli* 70S ribosomes and their 50S and 30S subunits as standards.[4] The results obtained from three in-

[4] The standards run at concentrations comparable to those of the myeloma samples were fixed with 4% formaldehyde (Ovchinnikov *et al.*, 1969), or were run at the corresponding K^+ concentrations with $K^+/Mg^{2+} < 100$. From control runs with such *E. coli* ribosomes, the error due to the viscosity and density differences in the range of Mg^{2+} concentrations used with a given K^+ concentration was negligible, *i.e.*, between 1.5 and 15 mM Mg^{2+}. Therefore, we used only one standard containing 70S, 50S, and 30S ribosomes from *E. coli* per one analysis of myeloma ribosomes at one K^+ concentration, *i.e.*, per five different Mg^{2+} concentrations. The following equation was used to estimate $s_{20,w}$: $s_{20,w}^{unknown} = s_{20,w}^{standard}[(\log r_u - \log r_m)/(\log r_s - \log r_m)]$, where r_m = radial distance to the meniscus, r_s = radial distance to the standard, and r_u = radial distance to the unknown. The partial specific volumes of the *E. coli* standards are assumed to be reasonably close to the corresponding myeloma particles and so these differences were neglected, as well as possible hydration differences.


1.5 mM Mg2+
2.5 mM Mg2+
5 mM Mg2+
10 mM Mg2+
15 mM Mg2+
Figure 2
25 mM K+
Figure 3
150 mM K+
Figure 4
275 mM K+
Figure 5
525 mM K+
Figure 6
1025 mM K+
A260
K+/Mg2+ = 16.7
μ = 0.061
K+/Mg2+ = 10
μ = 0.064
K+/Mg2+ = 5.0
μ = 0.071
K+/Mg2+ = 2.5
μ = 0.086
K+/Mg2+ = 1.7
μ = 0.101
K+/Mg2+ = 100
μ = 0.186
K+/Mg2+ = 60
μ = 0.189
K+/Mg2+ = 30
μ = 0.197
K+/Mg2+ = 15
60S/40S = 6.5
μ = 0.212
K+/Mg2+ = 10
60S/40S = 6.5
μ = 0.226
K+/Mg2+ = 183
60S/40S = 2.5
μ = 0.311
K+/Mg2+ = 110
60S/40S = 2.7
μ = 0.319
K+/Mg2+ = 55
60S/40S = 3.1
μ = 0.321
K+/Mg2+ = 27.5
60S/40S = 4.0
μ = 0.336
K+/Mg2+ = 18.3
60S/40S = 6.0
μ = 0.351
K+/Mg2+ = 350
μ = 0.561
K+/Mg2+ = 210
60S/40S = 2.2
μ = 0.564
K+/Mg2+ = 105
60S/40S = 2.4
μ = 0.571
K+/Mg2+ = 52.5
60S/40S = 3.2
μ = 0.586
K+/Mg2+ = 35
60S/40S = 3.2
μ = 0.601
K+/Mg2+ = 683
μ = 1.061
K+/Mg2+ = 410
μ = 1.064
K+/Mg2+ = 205
μ = 1.071
K+/Mg2+ = 102.5
60S/40S = 2.5
μ = 1.086
K+/Mg2+ = 68.3
60S/40S = 2.8
μ = 1.101



FIGURES 2–7: The preparation of the ribosomes is described in the Experimental Section. All runs illustrated here contained the K^+ and Mg^{2+} levels listed in both the gradient and sample overlay. The sample first received the 0.5 M KCl preliminary washing and was preincubated for 15 min at 37° in the presence of 0.1 mM puromycin. The 17-ml linear sucrose gradients of 5 to 25% (w/v) contained 50 mM Tris-HCl (pH 7.5) and 21 mM β-mercaptoethanol, as did the sample overlay. The 320-μl reaction mixture contained 20 A_{260} units of ribosomes and no sucrose, and 300 μl of this was layered on the gradient. The centrifugation was done at 5° for 11.0 hr at 25,000 rpm in an SW 25.3 rotor. Fractions were collected and analyzed as described in the Experimental Section. The ionic strength, μ, was calculated as follows: $\mu = 0.5\Sigma C_i Z_i^2$, where C_i is the concentration of the ionic species, *i*, and Z_i is the charge of ionic species, *i*, using the approximation of about 2.5% to obtain the ionic contribution of Tris-HCl (pH 7.5) (Nolan and Arnstein, 1969).

dependent runs at both 5 and 20° were all within less than ±3% of the mean for a given set of K^+ and Mg^{2+} conditions, attesting to the reliability of the method. Therefore, we believe these experiments accurately reflect in a relative sense, although perhaps not precisely in an absolute one, the events observed. The relative trends seem obvious. The *s* coefficients of both the small and large subunits change nearly 75%, while the *s* value of the 80S monosome changes only about 10%. The $A_{260}:A_{280}$ ratio, which is an approximate measure of the RNA:protein ratio, is dramatically influenced for the small and large subunit-derived particles, increasing nearly 14%, while this ratio for the 80S ribosome is only marginally affected, increasing less than 3%. The general trends of the $A_{260}:A_{280}$ are to decrease with increasing Mg^{2+}, indicating a relative increase in protein to RNA, and to increase with increasing K^+, indicating a relative decrease in protein to RNA. The increase of the $A_{260}:A_{280}$ may also reflect in part a change in the rRNA secondary structure. Similarly, where the large subunit to small subunit ratio (referred to here as 60 S:40 S) could accurately be measured (≤10% error), significant differences were observed. As the $K^+:Mg^{2+}$ increases, the 60 S:40 S tends to decrease, *e.g.*, at $K^+:Mg^{2+}$ = 10, 60 S:40 S ~ 6.5 (Figure 3e) and at $K^+:Mg^{2+}$ = 210, 60 S:40 S ~ 2.2 (Figure 5b), nearly a threefold difference. The ionic strength *per se* seems to have little effect on the yield of subunits.

Monosome. The *s* coefficient of the monosome is only slightly affected through the ranges of K^+ and Mg^{2+} concentrations employed here, *i.e.*, between about 74 and 80 S. In fact, the monosome appears to be marginally affected, if at all, by varying Mg^{2+} levels for a given K^+ level (Table II). An interesting point is that at 25 mM K^+, the $s_{20,w}$ of 74 indicates a somewhat unfolded conformation, regardless of the Mg^{2+} level. The possible significance of this observation is unknown; perhaps this affects the hydration envelope of the ribosome. With increasing K^+ levels for a given Mg^{2+} level, the *s* coefficient of the monosome in all cases increases, suggesting the ribosome assumes a more compact shape (Table II). However, it will also be noted that with increasing K^+ levels, the relative amount of monosomes also decreases. That is, it goes from a maximum of greater than 95% (Figure 2e) to a minimum of about 5% (Figure 5d), where it can be detected. Here the K^+ increases from 25 mM (Figure 2e) to 525 mM (Figure 5d), while $K^+:Mg^{2+}$ goes from 1.7 to 52.5, respectively. There is no detectable amount of monosomes present at 525 mM K^+ and $K^+:Mg^{2+}$ = 105, or any other $K^+ \geq$ 525 mM and $K^+:Mg^{2+} \geq$ 100. At $K^+ \geq$ 525 mM and $K^+:Mg^{2+} <$ 100, detectable amounts of monosomes begin to appear in a manner inversely related to $K^+:Mg^{2+}$. Therefore, in order to achieve 100% dissociation of ribosomes into subunits, not only a $K^+:Mg^{2+}$ molar ratio of approximately 100 is necessary, but also an absolute level of about 500 mM K^+. There appears to be no simple relationship between the relative and/or absolute amounts of K^+ and Mg^{2+} concentrations necessary for maximum ribosome dissociation.

Small Ribosomal Subunit. The *s* coefficient of the smaller subunit decreases from 40 to 23 S (Table II). Similarly, the $A_{260}:A_{280}$ increases from about 1.85 to nearly 2.10 (Table III). These data taken together suggest both a conformational unfolding of the particle and a concomitant loss of protein with both increasing $K^+:Mg^{2+}$ ratios and absolute amounts of K^+. Again, however, no simple relationship seems to be obvious. *s* coefficient data suggest the conformational unfolding is maximal up to 525 mM K^+ before any significant loss of protein is observed, based on the $A_{260}:A_{280}$ (Tables II

TABLE II: *s*-Coefficient Summary.[a]

	A. Monosome (80S Species)					B. Large Subunit Species (60 S)					C. Small Subunit Species (40 S)				
						mM $MgCl_2$									
mM KCl	1.5	2.5	5.0	10	15	1.5	2.5	5.0	10	15	1.5	2.5	5.0	10	15
25	74	74	74	74	74	51 (61)	51	55	57	57					
150	77	79	75	78	78	61 (50)	64 (53)	66 (54)	60	60			38	39	38
275	80	80	79	79	77	61	61	62	61	62	39	40	38	39	38
525				78	78	47 (56)	56	56	56	58	39	38	38	39	40
1025					78	41	41	53 (41)	53	56	27	26 (32)	35 (23)	35 (23)	36
1525						38	38	38	38	46	23	23	23 (29)	23 (29)	30 (23)

[a] Values are tabulated from Figures 2 to 7 in the text. *s* coefficients for forms of a subunit occurring in minor quantity are given in parentheses.

TABLE III: Summary of $A_{260}:A_{280}$ Ratios.[a]

	Small Subunit Derived Particles					Large Subunit Derived Particles				
	mM $MgCl_2$									
mM KCl	1.5	2.5	5.0	10	15	1.5	2.5	5.0	10	15
25	1.88[b]	1.82[b]	1.84[b]	1.86[b]	1.86[b]					
150	1.90[b]	1.90[b]	1.86[b]	1.86	1.85			1.86	1.87	1.87
275	1.85	1.89	1.86	1.84	1.85	1.91	1.88	1.88	1.89	1.84
525	1.86	1.86	1.85	1.83	1.85	1.95	1.89	1.86	1.88	1.89
1025	1.95	1.98	1.91	1.87	1.87	2.03	2.00	1.90	1.96	1.93
1525	2.02	2.10	1.92	1.94	1.91	2.08	2.05	1.98	2.02	2.00

[a] All measurements were done with a Zeiss PMQ II spectrophotometer at a concentration of approximately 0.2–0.4 A_{260} units/ml. [b] These peaks represent a composite of 60 and 40 S (dimers) whose exact composition is unknown.

and III). The possible exceptions are those illustrated in Figures 6d,e and 7e ($K^+:Mg^{2+} \leqslant 100$). These indicate that the increased amount of Mg^{2+} present has an antagonistic stabilizing influence in holding the component parts of the subunit together against the very high K^+ concentrations.

One particularly interesting case is that illustrated in Figure 6c. It appears at this point, $K^+:Mg^{2+}$ approximately 200, that both critical relative amounts of K^+ and Mg^{2+} and a critical absolute amount of K^+ have been reached for ribosomal dissociation and disruption. This is believed to be the case because of the simultaneous existence of four distinct species of particles—23, 35, 41, and 53 S. The 23S particle is a breakdown product of the small subunit with significant loss of protein, while the 35S particle is conformationally more compact than the 23S version of the small subunit with relatively little loss of protein. This becomes clearer when inspecting the other profiles which contain only 23S or 35S particles.

Agarose–acrylamide gel analyses of all fractions from particles listed as small subunit derived (Table II) only indicated the presence of 18S rRNA. This includes these 23S and 35S particles. The minor peaks found consistently in all samples of RNA analyzed were normally present to about the same extent (see Figure 8). Their amounts were slightly variable, but they always appeared in about the same place, *i.e.*, 1.0–1.1 $\times 10^6$ (22–24 S), 3.0–3.1 $\times 10^5$ (11–12 S), and 1.0–1.25 $\times 10^5$ (6.5–8 S). Molecular weights were estimated (Bishop *et al.*, 1967), using myeloma 28S, 18S, 5S rRNA, and *E. coli* 4S tRNA as standards. Such species of rRNA have been reported before for Jensen sarcoma ribosomes (Petermann and Pavlovec, 1966). These most likely represent breakdown products of the 28S and 18S rRNA, perhaps due to trace amounts of RNase, but always well defined.

The polyuridylic acid directed polyphenylalanine synthesis was negative for all particles indicated as derived from small subunits when tested in the absence of large subunit-derived fractions from the same gradients, *i.e.*, less than 0.5% of the control using the complete system with the 80S monosome. Based on the data presented here, we suggest that the small subunit may break down *via* the following steps: 40 S → 35 S → 27 S → 23 S. Similar results have been published on *E. coli* ribosomes as demonstrating a stepwise disruption of the small and large subunits (Weller *et al.*, 1968; Eilam and Elson, 1971). Moreover, the work on Jensen sarcoma ribosomes indicates the same might occur with mammalian ribosomes, since the small subunit was reported to be 33 S when Mg^{2+} was present, and 29 S in its absence (Petermann and Pavlovec, 1966).

Large Ribosomal Subunit. The *s* coefficient of the larger subunit decreases from 66 to 38 S, although this former situation is unclear because of the complicated sedimentation profile (Figure 3c). $A_{260}:A_{280}$ increases from about 1.85 to

TABLE IV: s Values Found in rRNA Analyses of Large Subunit Derived Particles from Peak Fractions, Performed as Described in the Experimental Section.

mM KCl	mM $MgCl_2$ 1.5	2.5	5	10	15
25	5, 18, 28	5, 18, 28	5, 18, 28	5, 18, 28	5, 18, 28
150	5, 18, 28	5, 18, 28	5, 18, 28	5, 18, 28	5, 18, 28
275	5, 28	5, 18, 28	5, 18, 28	5, 18, 28	5, 18, 28
525	5, 28	5, 28	5, 28	5, 28	5, 28
1025	28	28	5, 28	5, 28	5, 18, 28
1525	28	28	28	28	(5?), 28

about 2.10, as with the small subunit (Table III). Again, the conformational change appears to be maximal up to 525 mM K^+ before significant loss of protein from the subunit is observed (Tables II and III). The exceptions again seem to be Figures 6d,e and 7e, where the increased Mg^{2+} evidently plays its antagonistic stabilizing role against the disruptive influence of high K^+ levels (K^+:Mg^{2+} $\leqslant$ 100).

Another notable effect that seems to occur at very high K^+ and low Mg^{2+} levels (Figures 6a,b and 7a–d) is the absence of the 5S rRNA (Table IV). This is based on agarose–acrylamide gel electrophoreses (Figure 8). The apparently missing 5S rRNA of the large subunit may contribute to the conformational unfolding and to a consequently lowered s coefficient. The fate of the 5S rRNA is unknown, since the upper part of the gradients was not examined. It may come off as free rRNA, or as a ribonucleoprotein complex as suggested by others (Petermann *et al.*, 1971), or it may be degraded to oligonucleotide fragments by nascent RNase, while either free, or partially exposed but still bound to the conformationally unfolded large subunit.

These particular ribosomal subunit preparations are inactive in polyuridylic acid directed polyphenylalanine synthesis (Table VI). Possible notable exceptions at these high K^+ levels are illustrated again in Figures 6d,e and 7e (K^+:Mg^{2+} $\leqslant$ 100). The 5S rRNA appears to be present (Table IV) and these preparations do seem to be biologically active (Table VI), except for that described in Figure 7e. The full complement of 5S rRNA may not be present in this case, since only about 50% or less was found here as compared to that normally found, but no quantitation was attempted. However, these preparations do not appear to be as active as those prepared at lower K^+ levels.

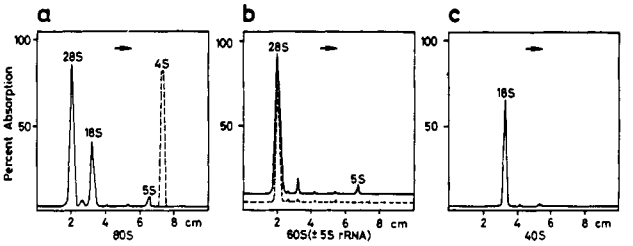


FIGURE 8: The RNA samples were prepared as described in the Experimental Section. The ordinate is an arbitrary scale of per cent absorption, *i.e.*, 100% − % transmission. (a) RNA from 0.5 M KCl washed 80S ribosome. A 4S *E. coli* tRNA marker is also indicated but was run separately. The small peak between the 28S and 18S peaks is 22–24 S, while the two small peaks between the 18S and 5S peaks are about 11–12 S and 8 S (see text); these probably represent RNase degradation products; (b) RNA from the large subunit preparation—525 mM K^+ and 5 mM Mg^{2+} as in Figure 5c, and 1525 mM K^+ and 1.5 mM Mg^{2+} as in Figure 7a. In part b the curve for the rRNA preparation derived from the large subunits prepared at 525 mM K^+ and 5 mM Mg^{2+} was displaced 5 absorption units above the other in order to make both curves visible. (c) RNA from the small subunit preparation—525 mM K^+ and 5 mM Mg^{2+} as in Figure 5c. Relative absorption is not directly comparable among parts a–c.

The peculiar case of Figure 6c is notable again for the particles derived from the large sunuit. Two of the four distinct particles which appear can be directly traced to the large subunit, the 41S and 53S particles. The 41S particle is the breakdown moiety of the large subunit exhibiting significant loss of protein and missing 5S rRNA, while the 53S species is a more compact subparticle still containing the 5S rRNA with not as great a loss of protein. This species present in other preparations presented here contains both the 28S and 5S rRNA. For the large subunit, we also suggest a stepwise disruption approximated as follows: 60 S → 56 S → 46 S → 38 S, with loss of 5S rRNA occurring after the 56S stage. The 46S moiety may be a transition state in which the 5S rRNA is lost from the large subunit. But this is not clear

TABLE V: Polyphenylalanine-Synthesizing Ability of the Large Subunit Derived Particles[a] Shown in Figures 2–7.

mM KCl	mM $MgCl_2$ 1.5	2.5	5.0	10	15
25	37.8	34.5	31.6	31.9	30.7
150	28.3	32.1	27.3	28.7	27.7
275	7.97	14.0	16.2	19.7	24.4
525	0.61	3.62	4.98	15.5	17.4
1025	0.0	0.0	1.48	5.42	7.93
1525	0.0	0.0	0.0	0.0	0.31

[a] Subunits were prepared as described in the Experimental Section, incubating at 37° for 15 min in the presence of 0.1 mM puromycin. The assay is described in the text; values expressed in pmoles of Phe incorporated per 7.0 A_{260} units of ribosomes or subunits, 2 ml, 30 min, 37°.

TABLE VI: Polyphenylalanine-Synthesizing Ability of the Combination of the Small Subunit Derived Particles and Large Subunit Derived Particles[a] Shown in Figures 2–7.

mM KCl	mM $MgCl_2$ 1.5	2.5	5	10	15
25					
150			29.7	32.3	30.3
275	46.5	42.3	38.8	35.2	36.6
525	29.1	39.2	43.7	41.9	33.8
1025	0.0	0.0	12.5	19.6	22.6
1525	0.0	0.0	0.0	0.0	1.05

[a] Subunits combined for the assay as described in the text; values expressed in pmoles of Phe incorporated per 10.0 A_{260} units of ribosomes or subunits, 2 ml, 30 min, 37°.

as the 5S rRNA would appear to be present in the 47S species at 525 mM K^+ and 1.5 mM Mg^{2+} (Figure 5a), and is partially absent from the 46S moiety in Figure 6c. It is not known how similar these 46S and 47S particles are to each other, and this awaits further clarification. The stepwise disruption seen here does not appear to be as clearly defined as that observed with the small subunit. Nevertheless, the literature supports the idea of discrete large subunit-derived subparticles existing under various salt conditions, *e.g.*, 57 S through as low as 30 S in the case of Jensen sarcoma ribosomes (Petermann and Pavlovec, 1966). Similar results have been reported for the large subunit in the case of rat hepatoma (Kuff and Zeigel, 1960), normal rat liver (Petermann, 1964), and normal guinea pig liver (Tashiro and Siekevitz, 1965). It is doubtful that these reduced *s* coefficients for both the large and small subunits could be due to anything other than frictional drag from molecular assymmetry, since at these ionic strengths electrostatic retardation would be small. This indicates a radical change in the frictional ratio ($f:f_0$). Indeed, this was documented for rat liver ribosomes treated with high concentrations of LiCl (0.5–1.0 M) and with varying pH, about 7 to 9 (Rebound *et al.*, 1969).

60S:40S Ratio. The influence of K^+ and Mg^{2+} on the separation of 60S and 40S subunits and the 60 S:40 S is evident from Table IV, and is illustrated in Figures 2–7. 60S:40S ranges from 6.5 to 2.2, so that many of the 60S peaks are contaminated with 40 S (presumably dimers). Functional assays also verified this contamination of 60S peaks with active 40S subunits (Table V). However, the data are not always unequivocal because of the difficulty and error sometimes involved in choosing peak fractions for both the rRNA analyses and polyphenylalanine analyses. Nevertheless, it appears that high K^+ and low Mg^{2+} give the best results for most activity and maximal subunit recovery, with minimal cross-contamination, *e.g.*, 525 mM K^+ with 2.5–5.0 mM Mg^{2+}. The lower levels of 275 mM K^+ with 1.5–2.5 mM Mg^{2+} also look good, but contain measureable amounts of 80S monosomes. Table VI also shows that most of the subunits formed in these above-mentioned K^+ and Mg^{2+} ranges have comparable biological activity.

In contrast, the lower values obtained at 150 mM K^+, or higher K^+ with higher Mg^{2+}, *i.e.*, 275 mM K^+ and 5–15 mM Mg^{2+}, or 525 mM K^+ and 15 mM Mg^{2+}, probably reflect the imbalance of 60 S:40 S in the assay. That is, 40S particles are present in oversaturating amounts, so fewer 80S monosomes would be formed per A_{260} unit. The lower values obtained at 525 mM K^+ and 1.5 mM Mg^{2+} or $K^+ \geqslant 1025$ mM suggest irreversible conformational damage done to the isolated large subunits, *i.e.*, a critical loss of certain necessary protein components as suggested from $A_{260}:A_{280}$ ratios (Table III) and/or loss of 5S rRNA (Table IV). The 50S peak in the cases of 25 mM K^+ (Figure 2a–e) and 150 mM K^+ (Figure 3a–c) was a mixture of large and small ribosomal subunits, as indicated by rRNA analyses (figures not shown) and functional assays in polyphenylalanine synthesis (Table V). The exact nature of this 60S material is unknown, but may be a mixture of small subunits as dimers, possibly unfolded a bit, plus somewhat unfolded large subunits. A similar mixture may be present in the 60S peak at 150 mM K^+ (Figure 3a–e) except that both mixed particles would be a bit more compact, so that both the 40S dimer and the large subunit would presumably exhibit the hydrodynamic properties more commonly reported in the literature. It will be noted that after Figure 3c, 150 mM K^+ and 5 mM Mg^{2+}, no more 50S species is present and it is also at this point that monomers of the small subunit begin to appear (Table II), but this 60S fraction is still contaminated with 40S dimers (Table V).

These K^+ and Mg^{2+} antagonistic influences and their absolute requirements are clearly illustrated with the amounts of ribosomes dissociating into subunits, their *s* coefficients, their $A_{260}:A_{280}$ ratios, possible 5S RNA loss and the 60S:40S ratio. Furthermore, these results suggest a wide variation in the chemical and perhaps biological properties of mammalian ribosomes and their subunits, indicating the need for each investigator to thoroughly look at this aspect of his own system. The subparticles produced in the stepwise subunit disruption may prove to be similar to some *in vivo* occurring ribosomal precursors. The data presented here would also be extremely valuable for experiments in mammalian subunit reconstitution, which thus far has never been done. The many questions these studies have only partially answered, or raised, can only be clarified further by very careful, detailed physicochemical investigations. Such work is presently under way in this laboratory with two-dimensional electrophoresis of ribosomal subunit proteins.

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Glutaraldehyde Reactivity of the Proteins of *Escherichia coli* Ribosomes†

Lawrence Kahan* and Eberhard Kaltschmidt

ABSTRACT: The topography of the small and large subunits of *Escherichia coli* ribosomes has been investigated by use of glutaraldehyde as a chemical probe. All of the ribosomal proteins react with glutaraldehyde when the proteins are present as a concentrated mixture of free proteins to give products which do not give spots in a defined two-dimensional electrophoresis pattern. The proteins of the intact 30S subunit are reactive in the order S1, S10 > S2, S3, S6, S13, S18, S19, S21 > S5, S7, S8, S9–S11 (part), S14 > S4, S9–S11 (part), S12, S15, S16, S17, S20. The proteins of the 50S subunit are reactive in the order L19, L27, L33 > L7, L12, L25, L26, L30, L31 > L4, L5, L6, L8, L9, L10, L11, L14, L18, L22, L23, L24, L29, L32 > L1, L2, L3, L13, L15, L16, L17, L20, L21, L28. Since ribosomes treated with low concentrations of glutaraldehyde retain substantial functional activity and normal sedimentation properties, it is concluded that the most reactive proteins in the intact subunits are at least partly external.

The small and large subunits of *Escherichia coli* ribosomes are complex structures containing about 21 and 33 distinct proteins and 1 and 2 molecules of RNA, respectively. Knowledge of the three-dimensional organization of these structures should be helpful in elucidation of the mechanism of protein synthesis and the control of translation. Some information about the topography of the 30S and 50S subunits has been obtained by modification of the external proteins of the intact ribosome (Craven and Gupta, 1970; Chang and Flaks, 1970, 1971; Moore, 1971; Noller *et al.*, 1971; Spitnik-Elson and Breiman, 1971; Crichton and Wittmann, 1971); however the results of these studies are sometimes ambiguous due to the difficulty of identification and quantitation of native and modified proteins in the complex mixture of proteins obtained after modification and due to the uncertain stability of the ribosome structure during tryptic digestion.

We have developed a simple, generally applicable technique for the modification of some external proteins and the subsequent identification of the unmodified proteins by two-dimensional electrophoresis (Kaltschmidt and Wittmann, 1970a), and have applied this technique to the examination of the topography of the 30S and 50S subunits of *E. coli* ribosomes. Our method consists of derivatization of the external polypeptides with glutaraldehyde, extraction of the protein moiety of the ribosomes into 67% acetic acid, and identification of unmodified proteins by two-dimensional acrylamide gel electrophoresis. Glutaraldehyde reacts almost exclusively with free amino groups and can react with proteins and organelles without causing substantial modification of funtional activity or structure (Avrameas and Ternynck, 1969; Avrameas, 1969; Sabatini *et al.*, 1963). Since modified proteins apparently do not give spots in the gel pattern, they do not interfere with identification of the unmodified proteins (see below).

Materials and Methods

Preparation of Ribosomes. *E. coli* Q13 were broken by alumina grinding, and 70S ribosomes were prepared and dissociated into 30S and 50S subunits as described by Traub *et al.* (1971). 30S subunits were purified by differential centrifugation and density gradient centrifugation (Traub *et al.*, 1971). In some cases 30S ribosomes were further purified by a second density gradient centrifugation step. 50S ribosomes were purified from the material remaining after purification of 30S ribosomes by two cycles of density gradient centrifugation (Traub *et al.*, 1971). Total 70S proteins used as reference proteins were prepared as described by Hardy *et al.* (1969).

Reaction of Ribosomes with Glutaraldehyde (Large Volume Method). Ribosomes were dialyzed against 0.32 M KCl, 0.01 M $MgCl_2$, and 0.01 M sodium cacodylate buffer (pH 7.0) for 18 hr at 4°. The ribosome concentration was adjusted to 0.5

† From the Institute for Enzyme Research, and Departments of Biochemistry and Genetics of the University of Wisconsin, Madison, Wisconsin 53706. The paper is No. 1521 of the Laboratory of Genetics. *Received December 9, 1971.* This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the National Institute of General Medical Sciences (GM-15422), National Science Foundation (GB-31086X), and Petroleum Research Fund.

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