The Partial Extraction of Proteins from Rat Liver Ribosomes, and the Physical Properties of the Residual Nucleoprotein Particles*

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ABSTRACT: Some of the structural proteins were detached from rat liver ribosomes by extraction with LiCl. The residual subribosomal particles were separated from the extracted proteins by sedimentation into 2 M sucrose. Greater amounts of the various proteins were extracted by solvents of higher LiCl concentration or higher pH. The fraction extracted in 0.5 M LiCl–0.2 mM $MgCl_2$ at pH 7.3 contained basic proteins, which migrated rapidly in polyacrylamide gels at pH 4.5, and less basic, slow-moving proteins. Additional proteins of intermediate mobility were extracted in 0.7 or 1.0 M LiCl. Some proteins were completely removed, while others were extracted partially or not at all; a small number of proteins, of intermediate mobilities, remained bound to the residual particles.

The particles remaining after extraction with 0.5 or 0.7 M LiCl were well-defined structures with sedimentation coefficients, densities, and melting-out curves that lay between those of ribosomal subunits and free ribosomal ribonucleic acids. The isolated large subunit was used to study reassociation. When the proteins extracted in 0.5 M LiCl at pH 7.3 were added back particles with a sedimentation coefficient, density, and melting-out curve like those of the isolated large subunit were recovered.

Rat liver ribosomes contain many kinds of proteins, which differ in size as well as in charge (Hamilton and Ruth, 1967). How these proteins are assembled on the RNA framework of each ribosomal subunit is still not understood. One approach to this problem, the stepwise removal of different classes of proteins, has given valuable information about the structure of ribosomes from halophilic bacteria (Bayley, 1966) and *Escherichia coli* (Marcot-Queiroz and Monier, 1966; Nomura and Traub, 1967; Osawa, 1965; Lerman *et al.*, 1966; Staehelin *et al.*, 1967; Itoh *et al.*, 1968). The techniques used on *E. coli* ribosomes are not suitable for studying liver ribosomes, which dissociate completely to RNA and protein in 6 M CsCl or 2 M LiCl. Recently, however, Lerman (1968) has achieved a partial extraction of liver ribosomal proteins in 3 M CsCl containing magnesium ions. Proteins can also be extracted stepwise by passing the ribosomes through Sephadex in lower concentrations of LiCl (Reboud and Petermann, 1967). The present paper describes a centrifugal extraction technique and gives some properties of the extracted proteins and the residual nucleoprotein particles.

Materials and Methods

Ribosomes, Subunits, and RNA. Rat liver ribosomes were isolated as previously described (Petermann and Pavlovec, 1967) with buffer F[1] as the final solvent and stored at −20° in 3% sucrose. Mixed subunits were prepared by passing the ribosomes through G-100 Sephadex in 0.1 M KCl–0.001 M potassium phosphate (pH 7.3) to remove bound magnesium (Petermann and Pavlovec, 1966). Separated subunits were prepared by the method of Hamilton and Ruth (1969). To 100 mg of ribosomes in 8 ml of buffer F were added 1 ml of 0.2 M $KHCO_3$ (pH 8.0) and 1 ml of 0.2 M EDTA (trisodium salt). The large and small subunits were separated by centrifugation in a 5–20% sucrose gradient in 0.001 M potassium phosphate–0.03 M KCl (pH 7.0) in the B-XIV zonal rotor (Anderson, 1966). The fractions containing each subunit were pooled, adjusted to 0.2 mM $MgCl_2$ by adding 0.001 M potassium phosphate–0.8 mM $MgCl_2$, and centrifuged for 15 hr at 150,000*g*. The pellets were suspended in small volumes of buffer F and clarified by low-speed centrifugation.

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[1] Abbreviation: buffer F, 0.001 M potassium phosphate–0.2 mM $MgCl_2$ (pH 7.3).

rRNA was isolated and freed of magnesium as described by Petermann and Pavlovec (1963b) and stored at −20°.

Preparation of the Particles. BUFFERS. The following is a list of buffers: buffer F, 0.001 M potassium phosphate–0.2 mM $MgCl_2$ (pH 7.3); 0.02 M $KHCO_3$ (pH 8.3); 0.5 M LiCl, pH 7.3, containing 0.001 M potassium phosphate and 0.2 mM $MgCl_2$; 0.7 M LiCl, pH 7.3, containing 0.001 M potassium phosphate and 0.2 mM $MgCl_2$; 1.0 M LiCl, pH 7.3, containing 0.001 M potassium phosphate and 0.2 mM $MgCl_2$; and 0.5 M LiCl, pH 9.3, containing 0.0046 M glycine, 0.0023 M KOH, and 2 mM $MgCl_2$.

For each buffer there was a corresponding solution five times as concentrated in LiCl; the pH 9.3 solution also contained 10 mM $MgCl_2$ and five times the concentration of buffer salts. The ribosome solution was adjusted to the salt composition of each dilute buffer by adding one-fourth volume of the corresponding concentrated buffer.

Extraction of Proteins. Samples containing 50 mg of ribosomes or isolated subunits were diluted to 40 ml in one of the solvents described above. Samples (5 ml) were layered over 4 ml of 2 M sucrose (RNase free, from Mann Research Laboratories) containing the corresponding buffer and centrifuged at 150,000*g* for 15 hr (Petermann and Pavlovec, 1963a). The particles, whose density was greater than 1.6 g/ml, sedimented through the 2 M sucrose–LiCl, while the extracted proteins remained above. The supernatants were removed with a syringe and the upper layers, which were characterized by $A_{260}:A_{235}$ of 0.7 and $A_{260}:A_{280}$ of 1.3, were pooled. When whole ribosomes were used, there appeared at the 2 M sucrose boundary a turbid material characterized by $A_{260}:A_{235}$ of 0.90 and $A_{260}:A_{280}$ of 1.55. This fraction was removed separately. The 2 M sucrose was discarded and the pellets were suspended in buffer F or in 0.02 M $KHCO_3$ and dialyzed to remove the sucrose. The preparations from ribosomes will be referred to as 0.5 M LiCl, pH 7 or 9; 0.7 M LiCl, one-step or two-step; or 1.0 M LiCl particles. The subunit preparations will be called 0.5 M LiCl, 0.7 M LiCl (one step), or 0.7 M LiCl (two-step).

Two-Step Experiments. The proteins were removed by centrifugation in two stages at pH 7.3 in 0.2 mM $MgCl_2$. The technique of the first step, extraction with 0.5 M LiCl, has been described above. For the second step, 15 mg of the 0.5 M LiCl pellets from ribosomes or isolated large subunits was suspended in buffer F and dialyzed against the same buffer. The solvent was then adjusted to 0.7 M LiCl and a volume of 10 ml; 5 ml was put into each of two 50-rotor tubes, over 4 ml of the corresponding buffer in 2 M sucrose, and centrifuged as described above. With the large subunit, the proteins extracted at each stage were divided into two parts; one was examined by polyacrylamide gel electrophoresis, and the other was used to study the reversibility of the reaction. The pellets (0.7 M LiCl, two-step particles) were suspended in 0.02 M $KHCO_3$ or in buffer F.

Reassociation of the Large Subunit. To 4 mg of 0.5 M LiCl particles in 1 ml of buffer F was added 1.2 mg of the extracted protein in 6 ml of the same buffer containing 0.5 M LiCl. The material was dialyzed for 1.5 hr against buffer F containing 0.2 M LiCl, then overnight against plain buffer F. The solution was concentrated by burying the dialysis bag in dry G-200 Sephadex and examined in the analytical ultracentrifuge. For isopycnic sedimentation and chemical analysis most of the excess proteins were removed by centrifuging the particles at 150,000*g* for 15 hr, resuspending in buffer F, and clarifying by low-speed contrifugation. For the second reassociation experiment, 3 mg of 0.7 M LiCl, two-step particles, and 0.9 mg of the proteins extracted in the second step were used. The two fractions were mixed in 0.7 M LiCl at pH 7.3, and the mixture was treated as described above.

Protein and RNA Concentrations. Approximate ribosome concentrations were calculated from the absorbance of rat liver ribosomes, with $E_{1\%}^{1cm}$ at 260 mμ of 140 (A. Pavlovec, unpublished data). Whole ribosomes have an $A_{260}:A_{235}$ ratio of about 1.5 (Petermann, 1964); a change in this value served as a rough indication of a change in the protein to RNA ratio of the various fractions obtained. Protein was measured by the method of Lowry *et al.* (1951), bound magnesium by the titan yellow method (Petermann and Pavlovec, 1967), and RNA as described by Hamilton and Ruth (1969).

Electrophoretic Analysis. The total proteins of the ribosomes, subunits, or particles were extracted with 2 M LiCl. The RNA precipitate was removed by centrifugation, and the supernatant was dialyzed against 0.033 M sodium acetate (pH 5.6) containing 6 M urea (Hamilton and Ruth, 1967). The proteins extracted by 0.5–1.0 M LiCl were dialyzed against 2 M LiCl, then against the urea–acetate buffer. The material in the turbid layer was made 2.5 N in HCl, and the precipitate was removed by centrifugation. Both the soluble and insoluble fractions were dialyzed against the urea–acetate buffer. Polyacrylamide gel electrophoresis was carried out by the method of Reisfeld *et al.* (1962) at pH 4.5 in 7.5% gels.

Ultracentrifugal Analysis. Ribosomal subunits, particles, reassociated material, and RNA were dialyzed against 0.02 M $KHCO_3$ or buffer F and examined in the analytical ultracentrifuge in 12- or 30-mm cells at 44,000 rpm at 25°, and occasionally also at 5°, with schlieren optics. The RNA, the turbid fraction, and certain particle preparations were also examined with ultraviolet absorption optics, in 12-mm cells, at 44,000 rpm and 25°.

For isopycnic centrifugation the ribosomal subunits and particles were fixed in 6% formaldehyde for 1 day (Perry and Kelley, 1966) and centrifuged in a CsCl gradient for 46 hr at 36,000 rpm in an SW39 rotor. Samples of 3 drops were collected and diluted, and their absorbance at 260 mμ was measured. The refractive index of every fourth sample was measured, and its density was calculated by the formula of Meselson *et al.* (1964).

Hyperchromicity. Melting-out curves were made on

the ribosomal subunits, RNA, and various particle preparations in 0.02 M $KHCO_3$. The samples were warmed from 10 to 93° at a rate of 1°/min, and the absorbance at 260 mμ was determined as described by Cavalieri *et al.* (1962).

Results

Influence of LiCl Concentration on Protein Extraction. Since 2 M LiCl separates all the proteins from the RNA of rat liver ribosomes (Hamilton and Ruth, 1967), the effects of lower concentrations were examined. The whole ribosomes contained about equal amounts of RNA and protein (Table I). At pH 7.3 extraction with 0.5 M LiCl decreased the protein:RNA ratio to 0.72; if we assume that no RNA was lost, 29% of the protein had been removed. In 0.7 M LiCl additional protein was extracted, and the total amount removed was the same when whole ribosomes were extracted directly (expt 3) as when the particles were extracted first with 0.5 M and then with 0.7 M LiCl (expt 6). Even more protein was removed by 1.0 M LiCl (expt 4). At pH 9.3 (expt 5) 49% of the protein was extracted by 0.5 M LiCl, although the magnesium concentration was unusally high.

The isolated large subunits had a low protein:RNA ratio, 0.81. They lost less protein than the ribosomes in the first extraction (expt 8), but more in the second step (expt 9). The small subunits lost 59% of their protein in 0.5 M LiCl (expt 11); a second extraction was not attempted.

The layer of turbid material that accumulated at the 2 M sucrose boundary was 90% protein and 10% RNA, and represented 3% of the total RNA in the tube. Examination in the analytical ultracentrifuge showed heterogeneous aggregates.

Electrophoretic Analysis of the Extracted and Particle Proteins. The proteins of rat liver ribosomes give very complicated patterns in gel electrophoresis (Hamilton and Ruth, 1967). In the present study both the extracts and the proteins of the residual particles still showed a large number of bands.

Figure 1 illustrates electrophoretic patterns of extracted and particle proteins from whole ribosomes. Qualitative differences are apparent, but the complexity of the patterns makes quantitative accounting difficult. It does appear, however, that the extracted proteins (gels 1 and 3) migrated in all regions of the gel, while the particle proteins (gels 2 and 4) migrated primarily in the central third of the gel. The 0.5 M LiCl selectively removed the most and the least cationic components (gel 1), although traces of the least cationic ones remained in the particles (gel 2). The 0.7 M LiCl extracted additional proteins (gel 3) from the central region, leaving particles with a relatively small number of proteins (gel 4). Treatment with 1 M LiCl gave fractions (not shown) very similar to those obtained in 0.7 M LiCl.

Figure 2 illustrates the two-step extraction of the large subunit. Since the least cationic ribosomal proteins are not found in the large subunit (M. G. Hamilton, unpublished observation), the differences between the protein in the 0.5 M LiCl extract (gel 1) and particles (gel 2) are less marked than they were for the whole ribosomes. Densitometer tracings of the patterns did, however, show selective removal of some bands. The 0.5 M LiCl particles showed 14 major bands. When they were treated with 0.7 M LiCl, the extract showed nine of these bands, while the final particles (gel 4) presented a much simpler pattern with only five major bands. In these crude fractionations, however, most of the proteins were not completely extracted in any one step. Thus with the protein marked A in Figure 2 a large proportion was extracted by 0.5 M LiCl, and the remainder by 0.7 M LiCl. Protein B, on the other hand, was completely resistant to 0.5 M LiCl, but completely extracted by 0.7 M LiCl. Other components, such as C, were partially extracted in each step. Only one protein, D, was completely resistant to 0.7 M LiCl.

TABLE I: The Effect of LiCl Concentration on the Amounts of Protein Removed from Ribosomes and Subunits in 0.2 mM $MgCl_2$ at pH 7.3 except Where Specified.

Expt	Material	LiCl (M)	Protein: RNA Ratio	% Protein Removed
1	Ribosomes		1.01	
2	Ribosomes	0.5	0.72	29
3	Ribosomes	0.7	0.53	48
4	Ribosomes	1.0	0.41	59
5	Ribosomes	0.5[a]	0.51	49
6	Particles from expt 2	0.7	0.53	19[b]
7	Large subunit		0.81	
8	Large subunit	0.5	0.68	15
9	Particles from expt 7	0.7	0.29	49[c]
10	Small subunit		0.98	
11	Small subunit	0.5	0.41	59

[a] pH 9.3, 2 mM $MgCl_2$. [b] Per cent of total ribosomal protein extracted in this step. [c] Per cent of total large-subunit protein extracted in this step.

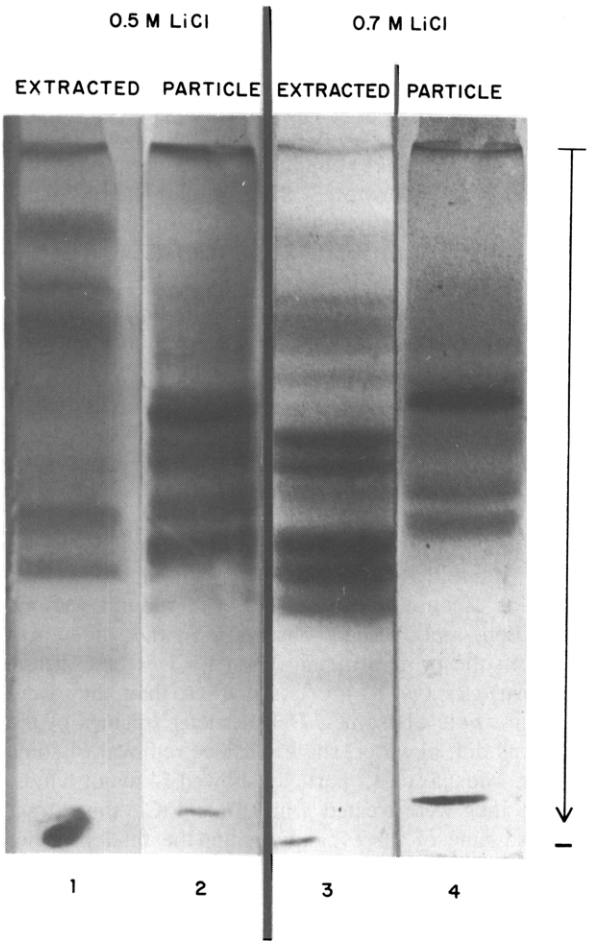

FIGURE 1: Extracted and particle proteins from whole ribosomes, as shown by gel electrophoresis. Migration is from the origin at the top toward the cathode at the bottom. (1) 0.5 M LiCl, pH 7, extracted proteins; (2) 0.5 M LiCl, pH 7, residual particles; (3) 0.7 M LiCl, one step, extracted proteins; and (4) 0.7 M LiCl, one step, residual particles.

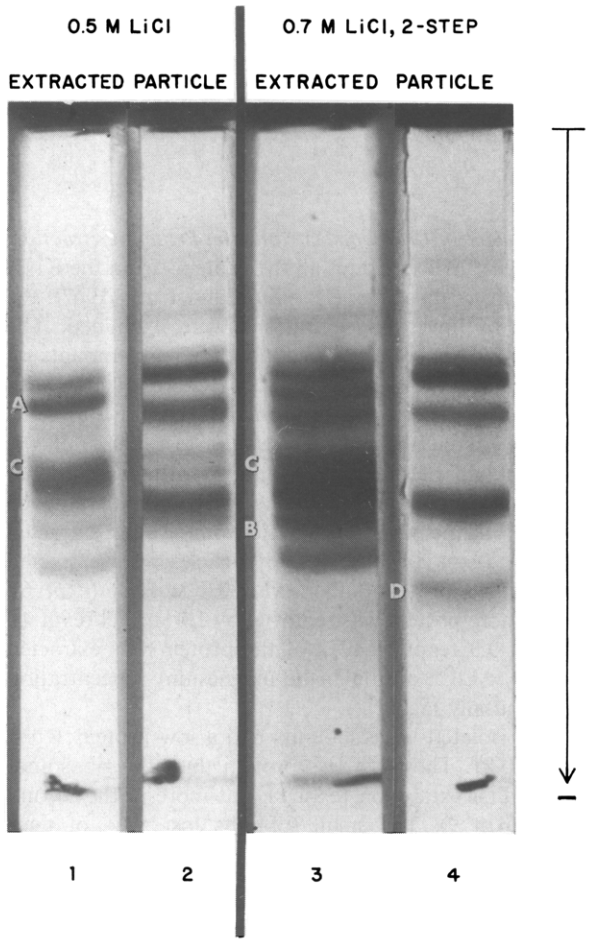

FIGURE 2: Extracted and particle proteins from the large subunit. (1) 0.5 M LiCl, extracted proteins; (2) 0.5 M LiCl, residual particles; (3) 0.7 M LiCl, two step, extracted proteins; (4) 0.7 M LiCl, two step, residual particles; For A–D, see text.

The turbid material obtained by centrifugation of whole ribosomes in 0.5 M LiCl at pH 7.3 (not shown) contained less basic proteins, found in the least cationic third of the gel, that were insoluble in 2.5 N HCl; more basic proteins, found in the central third, that were soluble in HCl; and a small amount of RNA. Before extraction with HCl the more basic proteins did not migrate into the gel, so were probably contained in large complexes of some kind.

Buoyant Density in Cesium Chloride. On isopycnic centrifugation in a cesium chloride gradient the isolated large subunits gave a sharp boundary, of density 1.62 g/ml (Figure 3). When proteins were extracted with 0.5 M LiCl at pH 7, the density of the residual particles increased to 1.66. The reassociated subunits, obtained by adding back the proteins to the particles, had an average density like that of the original subunits, 1.62 g/ml, but gave a wider band. The protein:RNA ratio of this preparation was high, 0.92; since the material had been centrifuged overnight, the pellets probably contained some free protein. The particles obtained by restoring the 0.7 M LiCl, two-step proteins to the corresponding particles had a density like that of the 0.5 M LiCl, pH 7 particles, and gave an equally sharp band.

Melting-Out Curves. The temperature, T_m, at which the increase in hyperchromicity was half its final value was 58° for the ribosomal subunits. It remained at 58° for the particles extracted with 0.5 M LiCl at pH 7.3, but fell to 53° for the 0.7 M LiCl, two-step particles. RNA had a T_m of 51°. For the isolated large subunit T_m was 59°; for the 0.5 M LiCl particles, 55°; and for the 0.7 M LiCl, two-step particles, 50°. Since the particle melting-out curves were reversible, RNase appeared to be absent from these preparations. When the proteins extracted by 0.5 M LiCl at pH 7.3 were added back to the particles, the T_m rose again to 59°.

Ultracentrifugal Behavior. Since the particles tended to aggregate when LiCl or KCl was present, they were studied in solvents of low electrolyte content such as 0.02 M $KHCO_3$ or buffer F. Sedimentation patterns of some of these preparations are shown in Figure 4.

All the sedimentation coefficients were low (see below). The ribosomal subunits showed the usual two boundaries plus some slow material and a small 5S peak, which probably contained both transfer and 5S RNAs. The 0.5 M LiCl, pH 7 particles showed two slightly slower main boundaries plus the 5S peak. The 0.5 M LiCl, pH 9 particles traveled even more slowly, and showed material sedimenting between the two main boundaries, at about 22 S. The particles obtained by extraction with 0.7 M LiCl (two step) showed 8% of heterogeneous aggregates, 31S and 17S boundaries, additional material between the peaks, and the 5S boundary. The pattern of the 0.7 M LiCl particles obtained in one step was similar, but showed less of the slowly sedimenting material. Since this fraction was examined at only one concentration, the sedimentation coefficients could not be extrapolated to infinite dilution. All the particles extracted with 0.7 M LiCl contained the 22S material.

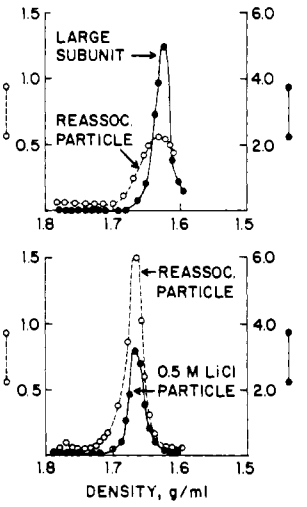

FIGURE 3: Isopycnic sedimentation in cesium chloride gradients of the large subunit and particles derived from it. The upper pattern shows the large subunit (solid line) and the particles formed by reassociation of the proteins extracted by 0.5 M LiCl at pH 7 with the residual particle (dash line). The lower pattern shows the 0.5 M LiCl, pH 7 residual particle (solid line) and the particles formed by reassociation of the proteins extracted by 0.7 M LiCl (two step) with the residual particle (dash line).

The particles extracted with 1.0 M LiCl had a pattern like that of the 0.7 M LiCl, one-step preparation. The RNA showed large 25S and 15S boundaries, a small 22S boundary, and some slowly sedimenting material.

Some of these samples were also centrifuged at 5°. The $s^0_{20,w}$ value of the small ribosomal subunit was unchanged, and that of the large subunit increased only 5%. The 0.5 M LiCl, pH 9 particles, on the other hand, showed definite changes in $s^0_{20,w}$; the value for the small particle increased 11%, while that for the large particle increased 22%.

The particles prepared from isolated large subunits are also shown in Figure 4. The 0.5 M LiCl, pH 7 particles showed 8% of aggregates, a small fast boundary, a large 34S peak, and some 22S material. There was also a trace of 3S to 7S material, although most of the t- and 5S RNAs had already been removed during fractionation in the zonal rotor. The 0.7 M LiCl, two-step particles showed a small fast boundary, a main 29S peak, and an increased amount of the 22S material.

Physical constants calculated for the various types of particles are shown in Table II. The constant, k, is given only for the large subunits and particles,

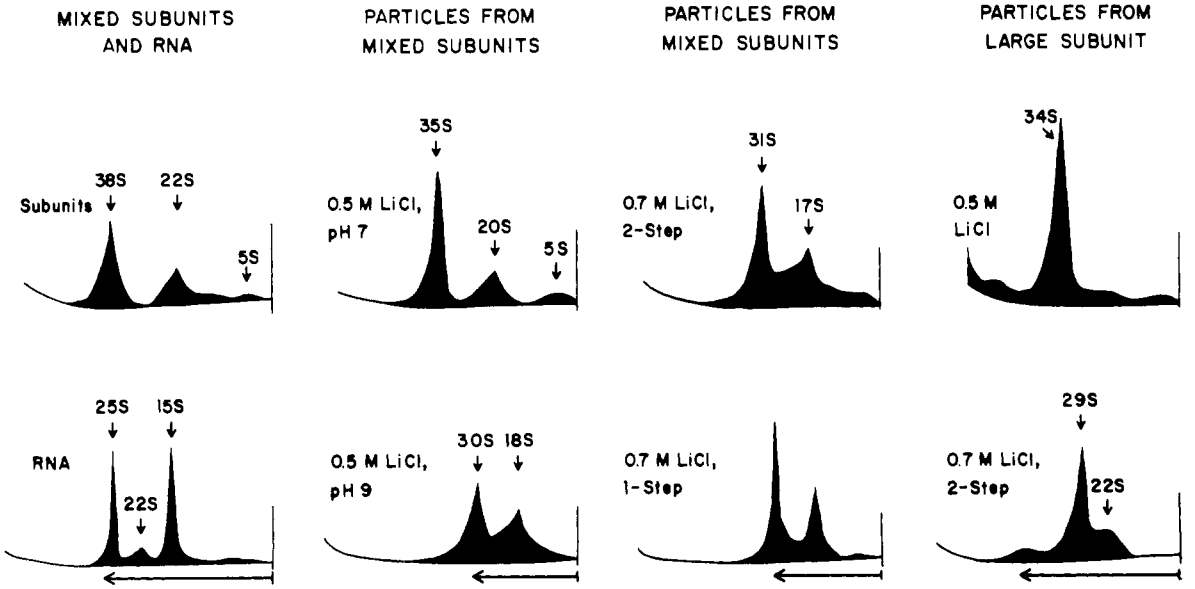

FIGURE 4: Ultracentrifugal patterns of mixed subunits, RNA, and LiCl-extracted particles. All examined in 0.02 M $KHCO_3$ at 25°. The sedimentation coefficients are values at infinite dilution.

TABLE II: Physical Constants of Subunits and Particles.

Preparation	$s^0_{20,w}$[a] (S)	k[a,b] (ml/mg)	$\bar{v}$ (ml/mg)	M × 10^{-6}	f/f_0[a]
Mixed subunits	22.0		0.63[c]	1.18[c]	2.63
	37.5	0.07	0.62[c]	3.12[c]	3.05
0.5 M LiCl, pH 7	19.6		0.62[d]	1.03	2.78
	35.0	0.11		2.92	3.12
0.7 M LiCl, two step	17.0		0.60[d]	0.92	3.17
	31.2	0.09		2.60	3.45
0.5 M LiCl, pH 9	18.0		0.60[d]	0.91	2.97
	30.0			2.57	3.56
Large subunit					
0.5 M LiCl	34.0	0.09	0.61	2.86	3.27
0.7 M LiCl, two step	28.5		0.58	2.19	3.60
Small subunit					
0.5 M LiCl	18.7		0.59	0.85	2.81
RNA	14.7		0.53	0.60[e]	3.37
	24.5	0.28		1.70[e]	4.05

[a] In 0.02 M $KHCO_3$, pH 8.3. [b] k is the constant in the equation $1/s = (1/s^0)(1 + kc)$, where c is concentration in milligram per milliliter. [c] Hamilton and Ruth (1969). [d] Average value calculated for mixture. [e] Petermann and Pavlovec (1963b).

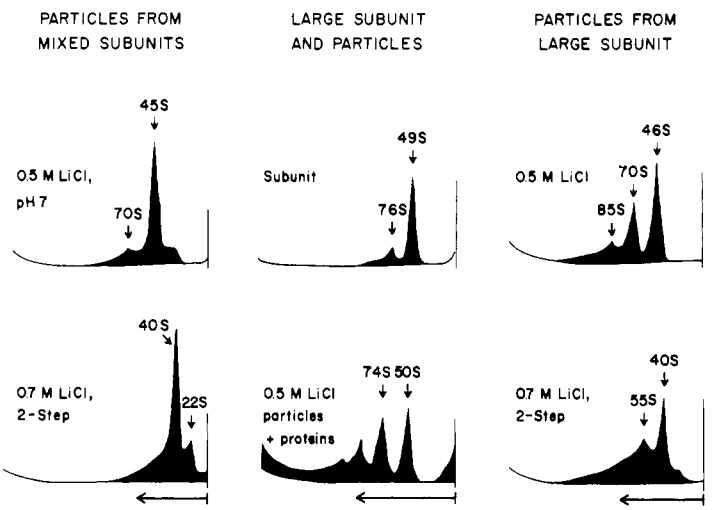

FIGURE 5: Ultracentrifugal patterns of LiCl-extracted particles from mixed subunits; isolated large subunit; LiCl-extracted particles from the large subunit; and 0.5 M LiCl particles plus extracted proteins. All examined in 0.001 M potassium phosphate–0.2 mM $MgCl_2$ (buffer F) at 25°. The sedimentation coefficients are values at infinite dilution.

since the concentrations of the small subunits and particles were always very low. The anhydrous partial specific volumes, $\bar{v}$, of the particles were calculated from their chemical compositions and the values of 0.53 for RNA and 0.74 for protein (Hamilton and Ruth, 1969). The molecular weights were obtained by adding to the molecular weight of the RNA, 0.6×10^6 or 1.7×10^6 Daltons (Petermann and Pavlovec, 1963b), the weight of the proteins calculated from the protein:RNA ratios. The fractional ratios, f/f_0, were calculated from s, $\bar{v}$, and M in the usual way (Edsall, 1953).

The unusually low sedimentation coefficients and high values of k and f/f_0 of the ribosomal subunits can probably be ascribed to the elongation that these structures undergo in an ion-poor solvent in the absence of magnesium (see Discussion). As the protein content of the particles decreased $s^0_{20,w}$ fell even farther; both k and f/f_0 rose, but did not reach the values for free RNA.

In buffer F (Figure 5) most of the 0.5 M LiCl, pH 7 particles from whole ribosomes still sedimented as separate subunits, with 16% of 70S and no 5S peak; the small RNAs were probably bound to the particles. The 0.7 M LiCl, two-step particles showed a larger slow boundary, 22 S, that probably included both the particles derived from small subunits and the 22S component described above; a large 40S boundary; and 25% of heterogeneous aggregates.

The isolated large subunits showed a 49S boundary

plus a small 76S peak that probably represented dimers. The 0.5 M LiCl particles had a lower sedimentation coefficient, 46 S, and showed much more tendency to form dimers and larger aggregates. The 0.7 M LiCl, two-step preparation showed 13% of the 22S fragments described above, plus monomers (40 S), dimers (55 S), and aggregates. The material obtained by reassociation of the 0.5 M LiCl particles and extracted proteins showed monomer and dimer boundaries with sedimentation coefficients close to those of the original subunits, plus trimers, tetramers, and larger aggregates. The slowly sedimenting material was probably excess protein. The 0.5 M LiCl particles derived from the small subunit (not shown) showed a main 19S component plus 20% of aggregated material.

The various particles all had higher sedimentation coefficients in buffer F than in 0.02 M $KHCO_3$. The 0.5 M LiCl, pH 9 particles (not shown) had sedimentation coefficients of about 40 and 22 S. When increasing amounts of KCl were added to the solvent both $s^0_{20,w}$ values rose proportionately.

Discussion

The proteins extracted from the ribosomes by LiCl have been separated from the residual nucleoprotein particles by two methods. The first, gel filtration (Reboud and Petermann, 1967) was less satisfactory because some of the extracted proteins formed large complexes that were excluded from the gel and remained in the particle fraction. The present method, sedimentation into concentrated sucrose, afforded a good separation of these complexes, which accumulated in a turbid layer just above the sucrose boundary. A further advantage of centrifugation was that dilution was greatly reduced.

When the LiCl concentration was raised, increased amounts of protein were extracted from the ribosomes. Other experiments, made by the earlier filtration technique (Reboud and Petermann, 1967), showed that more protein was also extracted at higher pH, and that increasing the magnesium content of the solvent tended to protect the ribosomal structure.

Electrophoretic patterns as complicated as those of ribosomal proteins are difficult to evaluate. Closely spaced major bands seem to coalesce, and trace components may fail to absorb the stain until they are concentrated in a particular fraction. At low pH values carbamylation of amino groups by cyanate in the urea should not occur. Since no reducing agent was used, intermolecular disulfide formation cannot be ruled out, but such effects are probably minor, since carboxymethylation caused little change in the gel pattern of the large subunit (Hamilton and Ruth, 1967). The most easily extractable material, removed by 0.5 M LiCl at pH 7.3, included the least cationic and some of the most cationic proteins. Some of these components may represent extraneous proteins, such as RNase, that were tightly bound to the ribosomes, although these preparations show no dissociable protein on free electrophoresis in 0.1 M salt (Schwartz and Petermann, 1966). Other proteins, like the strongly bound transferase II (Moldave and Skogerson, 1967), may be essential for ribosomal function.

Complexes such as those found in the turbid fraction are frequently noted in extracts of ribosomal proteins. Between pH 5 and 9 the mixed proteins precipitate unless urea is present, and even in 6 M urea moving-boundary electrophoresis shows evidence of complex formation (Hamilton and Ruth, 1966).

In contrast to ribosomal subunits from yeast, which had the same melting-out curve as the isolated ribosomal RNA (Cotter *et al.*, 1967), the liver subunits had a T_m 7° higher than that of the RNA. The ribosomal T_m was unaffected by the removal of 29% of the protein, but when an additional 19% was extracted it fell almost to the RNA value. Although the isolated large subunit had a lower protein:RNA ratio than the ribosomes, it had a slightly higher T_m, which probably reflected the higher guanine–cytosine content of its RNA (Hirsch, 1966). As additional proteins were extracted the T_m of the particles fell progressively.

The 5S boundary seen in the ultracentrifugal patterns of the particles probably represented both tRNA and 5S RNA. Marcot-Queiroz and Monier (1966) found that the 5S RNA was detached from *E. coli* ribosomes by 2 M LiCl, and this RNA is more easily removed from rat liver ribosomes than from those of *E. coli*, being detached merely by removal of magnesium (Petermann and Pavlovec, 1969).

The physical properties of the residual particles were those of uniform structures, not random aggregates of RNA and the remaining proteins. They showed well-defined boundaries in both velocity and isopycnic sedimentation, and the moving-boundary electrophoresis patterns of particles obtained by gel filtration showed neither free RNA, free protein, nor any evidence of dissociable complexes (Reboud and Petermann, 1967). The reassociated particles also appeared to be well-defined structures, although we have no evidence that the extracted proteins returned to their original sites.

Since the ribosomal proteins have low molecular weights (Hamilton and Ruth, 1967), the general conformation of each subunit, compact or unfolded, is determined by that of its RNA. In cation-poor solvents rRNAs show decreased sedimentation coefficients, increased viscosity, and related changes in optical properties. These changes have been ascribed to an unfolding to a less compact state (Cox and Littauer, 1962). Rat liver RNAs show similar sedimentation behavior (Figure 4; Petermann and Pavlovec, 1963b), and the ribosomal subunits and protein-depleted particles behaved in a similar fashion. Subunits of *E. coli* (Spirin *et al.*, 1963) and rat sarcoma (Petermann and Pavlovec, 1966) ribosomes also sediment more slowly in cation-poor solvents.

Liver rRNAs also show increased $s^0_{20,w}$ values on chilling (Petermann and Pavlovec, 1963b), presumably because they become more compact. The ribosomal subunits were not affected by temperature, whereas the particles did show higher $s^0_{20,w}$ values in the cold.

A third property of liver rRNA is the presence of a small amount of a 22S component. This is due to a

break in some of the RNA chains that is masked as long as magnesium is present (Petermann and Pavlovec, 1963b). When the magnesium is removed, and the hydrogen bonds are broken by heat or formamide treatment, the break becomes apparent; about one-third of the RNA chain is detached and sediments with the 16S boundary. The remaining piece sediments as 22S. In the whole large subunit, freed of magnesium, this break was masked by protein. In the particle preparations it remained masked until over 30% of the proteins had been extracted; beyond this point the slower sedimenting fragment appeared.

Since studies on *E. coli* had shown that reconstitution experiments should be carried out on isolated subunits (Nomura and Traub, 1967; Staehelin *et al.*, 1967), the large subunit was used in these experiments. The particles "reconstituted" after step 1 had an ultracentrifugal pattern, an average density, and a melting-out curve like those of the large subunit. Amino acid incorporation was not tested, since the original subunits are inactive. The protein-depleted liver subunits obtained by Lerman (1968) could be reconstituted to active ribosomes.

Ribosomal assembly has been assumed to proceed *in vivo* by the successive addition of specific proteins to the RNA. It now appears, however, that with the small *E. coli* subunit all the proteins can be added at the same time, *in vitro*, and still find their places on the RNA (Traub and Nomura, 1968). Although precursor particles have not yet been found in animal cells (Warner and Soeiro, 1967), animal ribosomes are more complex than bacterial ones, and it is still possible that the proteins of the large subunit are added to the RNA in a definite order, perhaps the reverse of the order in which we have detached them.

Acknowledgment

The authors thank Mrs. Amalia Pavlovec, Miss Mary Ruth, and Miss Isabella Wright for skillful assistance in various phases of this project.

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