Extraction and Isolation of Individual Ribosomal Proteins from *Escherichia coli*

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We have described a new method for the quantitative separation of ribosomal proteins and ribosomal ribonucleic acid. A procedure for the preparation of individual ribosomal proteins by polyacrylamide gel electrophoresis is also described. By the use of gels with smaller pores, at least four of the electrophoretic components from the 30S ribosome can be split into additional protein fractions. By the methods described here, it is possible to isolate in high purity at least 15 different proteins from the 30S ribosome of *Escherichia coli*.

The experiments of Waller and Harris (17) and Waller (16) demonstrated the heterogeneity of ribosomal proteins from *Escherichia coli*. Several studies have established the difficulty with which the 20 to 30 proteins are resolved into individual, purified homogeneous fractions (9, 10, 18). Ribosomal proteins are not only quite similar in chemical properties, but are also insoluble in aqueous buffers. This latter property necessitates the use of urea or similar agents to dissolve the proteins. In spite of these difficulties, Traut et al. (15) and Kaltschmidt et al. (4) have reported the preparation of homogeneous fractions of ribosomal proteins.

Our efforts to resolve the proteins of *E. coli* ribosomes were prompted by the need to establish the absolute number of such proteins as a basis for determining the steps in ribosome biosynthesis. Knowledge of the number and properties of these proteins is also essential for an understanding of the specificity of interaction between ribonucleic acid (RNA) and protein in the ribosome, and of the total function performed by the ribosome in protein biosynthesis. In this report, we describe a new method for the extraction of ribosomal proteins and a procedure for the isolation of all of the basic electrophoretic components of the 30S ribosome. We have, in a separate report (2), given amino acid compositions and evidence for the uniqueness of each of these proteins. After the completion of this work, Moore et al. (8) described an ion-exchange method for isolating proteins of the 30S ribosome.

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**Materials and Methods**

Isolation of ribosomes. Ribosomes were isolated from a ribonucleaseless strain of *E. coli* K-12 (3). The bacteria were grown in mineral salts medium (13) supplemented with 0.5\% glucose and 50 \(\mu\)g of l-methionine per ml. The routine isolation of ribosomes was performed on 40 to 50 g (packed wet weight) of cells suspended in 200 ml of tris(hydroxymethyl)-aminoethane (Tris) buffer, 0.01 M, pH 7.4, containing 0.01 M magnesium acetate and 0.5\% Brj 58. Deoxyribonuclease was added at a level of 2 \(\mu\)g/ml.

The suspension was passed through a French pressure cell at 8,000 psi (or a total force of 6,250 lb on the hydraulic press). The effluent from the pressure cell was centrifuged at 10,000 \(\times\) g for 15 min to remove debris and unbroken cells. Ribosomes were recovered from the supernatant liquid by a modification of the ammonium sulfate precipitation method described by Kurland (5). A 42-g amount of solid (NH\(_4\))\(_2\)SO\(_4\) was added to the crude extract, and the mixture was stirred for 3 min at 0 C. The precipitate was removed by centrifugation at 25,000 \(\times\) g for 10 min. This pellet was discarded. An additional 42 g of (NH\(_4\))\(_2\)SO\(_4\) was added to the supernatant liquid, and, after stirring for 3 min at 0 C, the suspension was centrifuged for 10 min at 25,000 \(\times\) g. The pellet from this precipitation contains the ribosomes, and was suspended in 200 ml of Tris buffer, 0.01 M, pH 7.4, containing 0.01 M magnesium acetate (TM/2 buffer). The ammonium sulfate fractionation was repeated on the resuspended ribosomes by treatment with 42 g of (NH\(_4\))\(_2\)SO\(_4\) followed by treatment with 77 g of (NH\(_4\))\(_2\)SO\(_4\). The precipitate obtained after treatment with the smaller amount was discarded.

After the second cycle of precipitation, the ribosomes were suspended in 100 ml of TM/2 buffer containing 0.6 M (NH\(_4\))\(_2\)SO\(_4\). This suspension was centrifuged for 3 hr at 165,000 \(\times\) g in a Spinco type 50 titanium rotor. The ribosome pellets were resuspended in 100 ml of TM/2 and 0.6 M (NH\(_4\))\(_2\)SO\(_4\), and the
centrifugation was repeated. The resulting ribosome pellet is clear and colorless. The pellet was then sus-
pended in TM/2 buffer and dialyzed for at least 12 hr against 100 volumes of the same buffer. The ribo-
somes were then dissociated into 30S and 50S particles by dialyzing for at least 24 hr against Tris buffer containing 10⁻⁴ M magnesium acetate (TM/4). Ribosome concentrations were calculated from 

\[ C_{\text{rib}} = \frac{\text{A}_{260}}{157} \times \text{A}_{260} \]  

(14).

The 30S and 50S subunits were separated by two methods of sucrose gradient centrifugation. In some cases, the SpincO SW 25.2 rotor was used with a 20 to 5% sucrose gradient in TM/4 buffer. The particles were centrifuged for 9 hr at 25,000 rev/min. In later experiments, a SpincO zonal rotor was employed, with a rotor capacity of 1.6 liters of a 20 to 5% gradient. From 0.5 to 1.0 g (dry weight) of ribosomes was ap-
pplied to the gradient, and the particles were centri-
fuged at 34,000 rev/min for 7.5 hr. The fractions corresponding to the 30S subunit were pooled, and a sample was examined by sucrose gradient centrifuga-
tion for contaminating 50S particles. In no case could 50S particles be detected at the 1% level or greater.

**Extraction of ribosomal proteins.** We have developed a new method for the extraction of ribosomal pro-
teins which is described here in detail. Five volumes of cold 2-chloroethanol (Eastman Organic Chemicals, Rochester, N.Y.) were added to 1 volume of ribosomes in TM/4 buffer. HCl was added to a final concentra-
tion of 0.06 N. The mixture was placed at 0°C for at least 2 hr and was homogenized periodically with a hand homogenizer. The precipitated RNA was re-
moved by centrifugation at 5,000 X g for 10 min. The supernatant fluid, containing the protein, was dialedyzed against water to remove the 2-chloroethanol. The dialedyzed solution was then lyophilized to recover the protein. As shown in Results, the protein was re-
covered with an efficiency of greater than 90% and was free from detectable contaminating RNA.

**Analytical acrylamide gel electrophoresis.** Routine analytical disc electrophoresis was performed in gels of 7.5% acrylamide-0.2% bis-acrylamide by a modi-
fication of the procedure described by Reisfeld et al. (11). The formulation of each solution was as follows. Upper buffer contained 3.12 g of 6-aminohexane, 0.8 ml of glacial acetic acid, and water to make 1,000 ml. Lower buffer contained 120 ml of 1 N KOH, 43 ml of glacial acetic acid, and water to make 1,000 ml. Gel stock solutions (for 7.5% gel) had the following compositions: (A) 1 N KOH, 24 ml; glacial acetic, 8.6 ml; N,N',N',N'-tetramethylethylenediamine (TEMED; Eastman Organic Chemicals), 1.0 ml; and 8 M urea to make 100 ml; (B) acrylamide, 30.0 g; bis-acrylamide (N,N'-methylenebisacrylamide; Eastman Organic Chemicals), 0.8 g; and 8 M urea to make 100 ml; (C) ammonium persulfate, 0.28 g and 8 M urea to make 100 ml.

Gels were formed from one part A, one part B, and two parts C. Two other gels were used for ex-
amining the isolated proteins for heterogeneity. These were the high bis (HB) gels, composed of 7.5% acrylamide-0.6% bis-acrylamide, and the high acryl-
amide (HA) gels, consisting of 12% acrylamide-0.3% bis-acrylamide. In these cases, solution B was made so that a fourfold dilution would yield the desired concentration of each monomer. Sample and spacer gels were not used. Gels were 6 mm X 70 mm with a volume of 2.0 ml. Electrophoresis was carried out at 2.5 to 3.2 ma per tube for 3 to 4 hr. The gels were stained in 7% glacial acetic acid containing 0.25% amido black. Destaining was done by electrophoresis in 7% acetic acid.

**Preparative acrylamide gel electrophoresis.** Prepara-
tive electrophoresis of ribosomal proteins was per-
formed by a procedure developed in this laboratory. A gel column 4.4 cm in diameter and 12 cm high was formed in the apparatus shown in Fig. 1. The constitu-
tion of the gel and of the upper and lower buffers was the same as was used for the analytical procedure. Between 20 and 50 mg of ribosomal protein were dissolved in 8 ml urea (no electrolyte) and layered beneath the upper buffer onto the gel column. Elec-
rophoresis was carried out at 25 ma (constant cur-
rrent) for 35 to 40 hr at 8 C. The cooling chamber exten-
sion (Fig. 1) was necessary to dissipate heat gen-
erated during the electrophoresis. Without such heat dissipation, the migrating bands acquire a chevron shape owing to more rapid mobility in the heated interior of the gel.

The protein bands were located by slicing the gel longitudinally and cutting out a 3- to 4-mm strip. The gel strip was stained in the same way that analytical gels were and was destained by electrophoresis in 7% acetic acid. The stained strip swells considerably dur-
ing the staining and destaining procedures and must be shrunk to its original size. This was done by im-
mersing the gel in 70% ethyl alcohol for a few minutes. The shrunken strip was then placed back into the gel column to be used as a guide for cutting out each electrophoretic band. Figure 2 shows a comparison of the separation of 30S ribosomal proteins obtained in the preparative gel (Fig. 2b) and the analytical gel (Fig. 2a).

Protein was recovered from the sliced gels in the apparatus shown in Fig. 3. The gel slices were placed in the mesh basket and then into the apparatus. Acetic acid (0.7%) was used as the electrolyte. A potential of 100 v was applied to the system for 1 hr. The liquid containing the protein was removed and replaced by fresh 0.7% acetic acid. A second electrophoresis was in most instances all that was required for maximal recovery. After electrophoresis, the lucite chamber divider (D) was inserted, and only the liquid on the cathode side was removed. The liquids were pooled, and the entire volume was reduced by flash evapora-
tion.

The protein solutions recovered as outlined above contained variable quantities of nonpolymerized acrylamide. Since the acrylamide seriously affects amino acid determinations, it was necessary to remove it. This was accomplished by gel filtration on Sepha-
dex G-50 in 1 M propionic acid. In some cases, acryl-
amide was removed by adsorbing the protein onto carboxymethylcellulose in 0.05 m sodium acetate buffer, pH 5.6, in 8 ml urea. The acrylamide comes
FIG. 1. Apparatus for preparative polyacrylamide gel electrophoresis. All pieces except the lower chamber (F) are made from glass. Components are: A, cooling chamber reservoir, 200 ml; B, upper electrode; C, upper buffer chamber, 1,000 ml; D, lower electrode; E, cooling chamber extension (outer diameter, 1.35 cm); F, lower buffer chamber (27 cm high; inner diameter, 10.8 cm); G, gel column, 12 cm × 4.5 cm diameter.

2,5-Diphenyloxazole (4 g) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (50 mg) were dissolved in 1 liter of reagent-grade toluene. To this was added 500 ml of absolute ethyl alcohol. Up to 0.4 ml of an aqueous sample was miscible in 20 ml of this solution.

RESULTS AND DISCUSSION

Comparison of extraction methods. Two general methods have been employed for the isolation of ribosomal proteins. One of these, extraction with 66% glacial acetic acid, was first used for ribosomes by Waller and Harris (17). The second widely used procedure is the degradation of ribosomes in a solution of 3 M LiCl-4 M urea (12). Both of these procedures presented difficulties in terms of quantitative recovery and the amount of RNA contamination in the protein preparations. In view of the problems, we developed a new procedure for extracting ribosomal proteins. This method, given in detail in Materials and Methods, is the extraction of ribosomes through in the wash fraction, and the protein can be desorbed with 0.5 M sodium acetate in the same buffer.

Biochemical methods. Protein and RNA were determined by the methods of Lowry et al. (6) and Dische (1), respectively. Bovine serum albumin and yeast RNA were used as standards. Radioactivity was determined on 0.1-ml aqueous samples by scintillation spectroscopy. The samples were counted in a toluene-ethyl alcohol fluid made up as follows.

FIG. 2. Comparison of the distribution of 30S ribosomal proteins in analytical (A) and preparative (B) acrylamide gels. The gel shown in B is a stained strip cut from the preparative gel column. Numbers designate each electrophoretic component. Cathode is at the bottom.
with 80% acidified 2-chloroethanol. Ribosomal proteins are soluble in the solvent, and ribosomal RNA is precipitated. A comparison of the three methods (i.e., acetic acid, LiCl-urea, and 2-chloroethanol) is given in Table 1. For these experiments E. coli cells were grown in minimal medium containing 3H-uracil and 14C-arginine to label the RNA and protein moieties of ribosomes. The ribosomes, purified by (NH4)2SO4 precipitations, were used at 25 mg/ml in each extraction solvent. As the data in Table 1 show, protein was recovered at greater than 90% yield and was free from detectable RNA in the 2-chloroethanol method. Thus, this method gives higher yields of protein than the acetic acid procedure, and it gives a lower degree of RNA contamination than the LiCl-urea method. Moreover, macromolecular RNA can be recovered from the 2-chloroethanol precipitate, whereas it is degraded by LiCl-urea. When lower concentrations of ribosomes were used in any of the three methods, the amount of RNA in the protein fraction increased.

Isolation of individual electrophoretic components by preparative electrophoresis. By use of the 2-chloroethanol extraction procedure, proteins were recovered from 30S ribosomes and separated by preparative disc electrophoresis. The efficiency with which proteins could be recovered from the gel slices was determined by subjecting a sample of radioactive ribosomal protein to the procedure. Table 2 shows the quantitation of each step after electrophoresis. The Sephadex step is responsible for the greatest loss of material, but is necessary to remove nonpolymerized acrylamide.

### Table 1. Comparison of methods for the extraction of ribosomal protein

<table>
<thead>
<tr>
<th>Fraction separated</th>
<th>Counts/min recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloro-ethanol</td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td>0</td>
</tr>
<tr>
<td>RNA-3H</td>
<td>13,000</td>
</tr>
<tr>
<td>Protein-14C</td>
<td>9,060</td>
</tr>
<tr>
<td>Precipitate</td>
<td>1,280</td>
</tr>
<tr>
<td>RNA in supernatant</td>
<td>91.4</td>
</tr>
<tr>
<td>fluidb</td>
<td>0</td>
</tr>
</tbody>
</table>

a Ribosomes were labeled with uracil-3H and arginine-14C. These were diluted with nonradioactive ribosomes to the desired activity. A 25-mg amount of ribosomes was subjected to each extraction procedure.

b Percentages of protein and RNA are expressed relative to the total quantity present in the starting ribosome suspension.

c From the amount which could be detected, this represents less than 0.2% of the total RNA.

### Table 2. Quantitation of recovery and purification steps

<table>
<thead>
<tr>
<th>Step</th>
<th>Counts/min recovered</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery from acrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein in gel</td>
<td>12,428</td>
<td>96</td>
</tr>
<tr>
<td>Total recovered from gel</td>
<td>12,028</td>
<td></td>
</tr>
<tr>
<td>G-50 Sephadex purification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein on column</td>
<td>12,028</td>
<td>96</td>
</tr>
<tr>
<td>Total recovered from column</td>
<td>9,153</td>
<td>76</td>
</tr>
<tr>
<td>Overall recovery</td>
<td></td>
<td>75</td>
</tr>
</tbody>
</table>

a Combined fluids from two cycles of electrophoretic recovery of the protein from gel slices.
urea, and buffer components. When these substances are removed, accurate amino acid analyses can be performed on proteins recovered from the gels. This was demonstrated by subjecting crystalline horse heart cytochrome c to the preparative procedure. Amino acid analyses were performed on samples before and after the entire procedure. Table 3 shows that the analysis for the preparation that was recovered from the gels is in good agreement with literature values. Without the Sephadex step, values for glycine and serine were high and there was excessive destruction of tyrosine.

The procedure just described—preparative disc gel electrophoresis, electrophoretic recovery of protein from the gel, and Sephadex G-50 chromatography—was applied to the separation of proteins from the 30S ribosome. Purified electrophoretic components were examined for purity (Fig. 4) by re-electrophoresis on the standard 7.5% acrylamide-0.2% bis-acrylamide gels (labeled “S”). In addition, the homogeneity of each component was evaluated by electrophoresis on gels of smaller pore size. These gels were made by increasing the acrylamide (i.e., 12% acrylamide-0.3% bis-acrylamide, labeled HA) or the cross-linking component of the standard gel (7.5% acrylamide-0.6% bis-acrylamide, labeled HB). Figure 4 shows that several of the purified bands were resolved into two proteins in either or both of the small-pore gels. Consistent with our previous findings (2), bands 3, 4, and 8 were composed of two different proteins, as shown by the separations on both the high acrylamide (HA) and high bis (HB) gels. It is not easily seen in Fig. 4 that band 9 was also heterogeneous. However, a densitometric trace of the high bis gel in Fig. 4 is shown in Fig. 5, where it can be seen that band 9 is resolved into two components. The heterogeneity

Fig. 4. Analysis of proteins recovered from the preparative gels. Components were examined for heterogeneity by electrophoresis in the standard 7.5% acrylamide-0.2% bis-acrylamide gels (S), 12% acrylamide-0.3% bis-acrylamide (HA), and 7.5% acrylamide-0.6% bis-acrylamide (HB). The pattern at lower right is a standard gel of total 30S protein. Electrophoresis time varied from 2 to 4 hr. Cathode is at the bottom.
TABLE 3. Amino acid composition of cytochrome c after preparative gel electrophoresis

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole per cent amino acid&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Literature&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lysine</td>
<td>18.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>9.8</td>
</tr>
<tr>
<td>Serine</td>
<td>0.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.8</td>
</tr>
<tr>
<td>Proline</td>
<td>3.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>11.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.9</td>
</tr>
<tr>
<td>Valine</td>
<td>2.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Crystalline horse-heart cytochrome c (Mann Research Laboratories, New York, N. Y.).

<sup>b</sup> Results are based on 22-hr hydrolyses, and cysteine was not determined. For purposes of comparison of mole percentages the 2 residues of cysteine were subtracted from the reported 104 residues.

<sup>c</sup> Values reported by Margoliash (7).

<sup>d</sup> This value was estimated from methionine sulfoxide.

The other components that exhibit apparent heterogeneity are band 6 and band 10. In these bands, it can be seen (Fig. 4) that new components are demonstrable in both of the small-pore gels. In this case, however, we believe that bands 6 and 10 are not composed of more than one protein, because band 6 readily develops a second slow-moving component in the standard gel when the purified material is stored in 1 M propionic acid or 8 M urea. Furthermore, there is as yet no evidence for a second component of band 6 isolated from E. coli B. Thus, the slower component may be a modified form of the primary band (e.g., a deamidated species). The apparent heterogeneity of band 10 may also be an artifact, since bands 10 and 11 are present in equal quantities in the mixture of proteins, and both have similar molecular weights (Sypherd, Fogel, and Strnad, unpublished data). This makes it unlikely that 10 is composed of two different proteins. Experiments are now in progress to determine whether bands 6 and 10 are heterogeneous or if the patterns shown in the small-pore gels in Fig. 4 are due to the separation of modified proteins of a single molecular species.

The procedures outlined here were developed for the separation of ribosomal proteins of E. coli. The extraction and separation has also been applied to the ribosomal proteins of other bacteria with complete success. We have shown in a separate report (2) that each protein isolated by these procedures is unique, and we conclude that the protein moieties of the 30S ribosome is composed of no fewer than 15 or 16 different proteins. Our previous data also show that the apparently multiple components of bands 3, 4, and 8 are indeed different proteins. Thus, the single components of these bands recovered from the standard gel can be separated into A and B proteins either by gel filtration (2) or by smaller-pore acrylamide gels.

Finally, it should be pointed out that isolation of 15 to 16 different 30S ribosomal proteins does not indicate the number of active proteins which participate in ribosome structure and function. The use of urea to disperse the proteins results in the isolation of what could be the polypeptide subunits of active proteins. These possibilities can only be assessed by a study of the quarternary structure of these polypeptides.
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LITERATURE CITED


