The Ribosomal Proteins of *Escherichia coli*. I. Purification of the 30S Ribosomal Proteins*

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ABSTRACT: The proteins of the 30S ribosomal subunit of *Escherichia coli* were separated from the ribosomal ribonucleic acid by a modification of the standard acetic acid extraction procedure. Chromatography of the proteins on cellulose phosphate columns led to the identification of approximately 20 unique protein fractions. Rechromatography of these proteins either on cellulose phosphate or Sephadex columns yielded 21 proteins that were chromatographically and electrophoretically unique. Control experiments suggest that these proteins are not artifacts produced by disulfide bond aggregation, noncovalent aggregation, enzymatic fragmentation, or supernatant contamination. It is concluded that there are approximately 20 proteins in the 30S subunit of *E. coli*.

The current view that the ribosome has a complicated structure is based principally on the work of Waller who provided evidence suggesting that there are at least 24 different proteins in the ribosomes of *Escherichia coli* (Waller and Harris, 1961; Waller, 1964). Considerable work has reinforced this view (Kaltschmidt *et al.*, 1967; Möller and Castleman, 1967; Möller and Chrambach, 1967; Traub *et al.*, 1967; Traut *et al.*, 1967). The present study was initiated with two distinct objectives: to develop procedures for the preparative scale purification of each of the ribosomal proteins of *E. coli*, and to study the distribution of the proteins among the ribosomal particles in order to decide whether or not the ribosomes of *E. coli* are a homogeneous population of particles. In order to simplify the experiments, we have chosen to first study the 30S ribosomal subunit of the *E. coli* ribosome. A partial enumeration of proteins from the 30S ribosomal subunit has been provided recently by Fogel and Sypherd (1968) and Moore *et al.* (1968) who isolated fourteen different proteins from this subunit. Moore *et al.* (1968) concluded that there is one copy of each of the purified proteins in every 30S particle.

We have purified 21 proteins from 30S ribosomal subunits. The purification procedures as well as experiments directed at controlling the artifacts which complicate this system are described in this paper. The chemical and physical characterization of the purified proteins is presented in the second paper in this series (Craven *et al.*, 1969); these data show that there are at least 19 and at most 22 proteins in 30S ribosomal subunits. The molecular weights of these proteins are such that one copy of each protein cannot be accommodated by a single 30S particle. Preliminary data to be presented in a subsequent paper suggest that the number of copies of the ribosomal proteins in the 30S particles deviates considerably from perfect 1:1 stoichiometry. Our data indicate that the 30S ribosomal subunits of *E. coli* are probably heterogeneous.

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Methods

Ribosomes were prepared from *E. coli* B as described previously (Kurland, 1966) except for a few minor changes. $MgCl_2$ was used throughout instead of magnesium sulfate and the puromycin incubation of the crude extract was omitted. The procedure was scaled up by a factor of 11 and took 1 day longer because the high-speed centrifugations were performed in a Spinco 30 rotor. Overnight dialysis was against 15 l. of TSM (0.01 M Tris–0.003 M succinic acid–0.01 M $MgCl_2$, pH 8.0).

30S Particles. Ribosomes were dialyzed overnight against 0.01 M Tris-HCl, 0.05 M KCl, 0.3 mM $MgCl_2$, and 6 mM mercaptoethanol (pH 7.6) layered on an approximately linear gradient of 5–30% (w/v) sucrose in the same buffer, and centrifuged for 7 hr at 40,000 rpm in a Spinco BIV zonal rotor. Fractions of 50 ml were collected and their A_{260} was measured. Those which contained the 30S particles were dialyzed overnight against TSM and concentrated either by precipitation with $(NH_4)_2SO_4$ (49 g/100 ml of 30S particles) or by high-speed centrifugation. No differences were observed between these two methods of concentration with respect to the chromatographic and electrophoretic patterns obtained with the proteins. The most satisfactory results from the zonal centrifugation are obtained with a sample of approximately 30,000 A_{260} units of ribosomes in 90 ml of buffer applied to the gradient.

Extraction of Proteins. Three different methods of extracting the proteins of the 30S ribosome were used in the course of this work.

SELF-DIGESTION. 30S particles in TSM were dialyzed overnight against the standard urea–phosphate buffer (6 M urea–50 mM NaH_2PO_4–12 mM methylamine, pH 6.5) containing 10 mM mercaptoethanol during which time the RNA was degraded and the low molecular weight products diffused through the membrane, leaving the proteins in the dialysis bag (Spahr, 1962).

LiCl–UREA. 30S particles in TSM were mixed with an equal

volume of 4 M LiCl–8 M urea and allowed to stand at 2–4° overnight (Leboy *et al.*, 1964). After a low-speed centrifugation, the supernatant containing the 30S proteins was removed from the RNA pellet. More than 98% of the protein was recovered when this method was used.

ACETIC ACID. A slight modification of the method described by Waller and Harris (1961) was used. To one volume of vigorously stirred 30S particles in TSM (A_{260} between 200 and 1500) were added one-tenth volume of 1 M $MgCl_2$ and two volumes of glacial acetic acid in rapid succession. The mixture was stirred in an ice bath for 45 min and the RNA precipitate was removed by centrifugation for 10 min at 20,000*g*. The pellet was washed with the same 67% acetic acid mixture and recentrifuged. The combined supernatant contained more than 90% of the ribosomal protein. A high level of magnesium ions was found to increase the yield of protein (95% at 0.1 M, 85% at 0.01 M), while sulfate ions depressed it. Therefore, after ammonium sulfate concentration of 30S particles, the sulfate was removed by very extensive dialysis against TSM before the protein was extracted by this method.

Chromatography. PREPARATION OF BUFFER. Chromatography was carried out in phosphate-buffered 6 M urea, and protein was detected by monitoring the A_{230} of the effluent. Reagent grade 6 M urea (Mallinckrodt or Baker) has a high A_{230} (0.3–3.0) relative to H_2O and this was reduced to a low level (0.05–0.10) by decolorizing the urea with activated charcoal. To a solution of approximately 7.5 M urea was added 50 g/l. of activated charcoal and the mixture was stirred in the cold room for several hours. The charcoal was removed by filtration and washed with distilled water. The decolorized filtrates were pooled and then used to make the standard urea phosphate buffer which was 6 M urea–0.05 M NaH_2PO_4–0.012 M methylamine (pH 6.5). The methylamine was present as a scavenger for cyanate. For most of the work reported here 50 μl of β-mercaptoethanol was added per l. of buffer to prevent disulfide-bond formation in the proteins. If necessary the pH of the buffer was adjusted by the addition of HCl or NaOH.

PREPARATION OF PHOSPHOCELLULOSE. The phosphocellulose used was standard-capacity Mannex P (0.9 mequiv/g, Mann Research Laboratories). High-capacity Mannex P (1.1 mequiv/g) and phosphocellulose obtained from Serva can also be used successfully. Phosphocellulose (50 g) was suspended in 2 l. of distilled water and the fine particles were decanted four or five times until approximately 40% had been discarded. The remainder was suspended for a short time in 0.1 N NaOH, which was removed by washing the phosphocellulose on a filter with four successive 1-l. quantities of water. It was then resuspended in 0.01 N HCl and again washed four times with water. The slight yellow tinge of Mannex P was removed by the basic wash. One of the two Mannex P lots used in this work required 30 min and the other only 15 min in NaOH for complete decolorization. A thorough NaOH wash appears to enhance recovery of small protein samples. After washing, the phosphocellulose was suspended in 1.5 l. of the standard buffer and allowed to equilibrate overnight before the column was poured; 50 g of Mannex P treated in this way was sufficient for a column 90 × 1.8 cm.

PACKING THE COLUMN. Columns were packed under an air pressure of 5 psi at a flow rate approximately ten times that required for chromatography. Flow was controlled from the bottom of the column. When the desired column length was reached a disk of filter paper was placed on the cellulose bed and the column was washed at 4° with the standard buffer until the pH of the effluent was the same as that of the standard buffer.

PREPARING THE SAMPLE. Samples for chromatography were either unfractionated 30S protein or pooled fractions from previous columns. Acetic acid extracts of 30S protein were prepared for chromatography by dialysis against successive 2-l. quantities of standard buffer for a 2- or 3-day period. When the pH of fresh dialysis buffer was unchanged after 12 hr, the protein was considered ready for chromatography. In some cases, the protein was equilibrated with standard buffer by passage through a column of G-25 Sephadex. Samples for rechromatography were dialyzed overnight against standard buffer at the desired pH. In all cases, the dialysis tubing had been heated at 70° for at least 3 days before it was used.

APPLICATION OF THE SAMPLE AND CHROMATOGRAPHY. All operations were carried out at 4°. The sample was allowed to run on to the column followed by (a) one void volume of standard buffer when the sample was whole 30S protein or (b) one third to one-half of the void volume for rechromatography. A linear sodium chloride gradient in the standard buffer was then passed through the column to elute the proteins. The flow rate was kept approximately constant throughout these operations. With unfractionated 30S protein best results were obtained with a column 2.8 × 60 cm, a flow rate of 45 ml/hr, a gradient of 0–0.6 M NaCl in 6 l. of standard buffer (pH 5.8 or 6.5), and about 400 mg of 30S protein. For rechromatography the standard procedure was to load between 4 and 30 mg of partially purified proteins onto a column 60 × 1.6 cm using a flow rate of approximately 15 ml/hr and a 1000-ml linear gradient of 0.2 M NaCl whose starting concentration of NaCl was 0.05 M lower than the concentration at which the sample had been eluted during the initial chromatography. The protein in the eluate from the columns was detected by measuring the A_{230} of each fraction.

RECHROMATOGRAPHY ON SEPHADEX G-100. In some cases the chromatographic fractions obtained from the initial chromatography on phosphocellulose are found to have two or more electrophoretic components that migrate in polyacrylamide gels at very different rates. In such cases it is possible to fractionate those proteins on Sephadex G-100 after concentration of the pooled fractions in a Diaflo Model 50 ultrafiltration cell with a UM-2 membrane (Amicon Corp., Lexington, Mass.). Good results were obtained with a column (85 × 2.8 cm) equilibrated with standard buffer at a flow rate of 15 ml/hr and with a sample volume of 10 ml.

Electrophoresis. Polyacrylamide gel electrophoresis at pH 4.5 was used to analyze both chromatographic fractions and total protein preparations. The electrophoresis was performed by the modification of the techniques of Reisfeld *et al.* (1962) described by Leboy *et al.* (1964). The bottom gel (55 × 6 mm) contained 10% (w/v) acrylamide (electrophoresis grade) and 0.15% (w/v) methylenebisacrylamide. Both these chemicals were obtained from Eastman Organic Chemicals. The upper gel contained 2.5% acrylamide and 0.625% methylenebisacrylamide; 0.1 ml of this gel was used for the spacer and up to 0.2 ml of protein in standard buffer or 65% acetic acid was mixed with 0.1 ml of upper gel for use as the sample. Both the spacer gel and the sample gel mixture were polymerized by exposure to fluorescent light in the presence of riboflavin.

However, good results could be obtained for split gels without polymerizing the sample gel. Electrophoresis was for approximately 3.5 hr with a current of 3 mA/gel and in the absence of tracking dye. At the end of this time the gels were removed from the tubes and stained in 0.25% Buffalo Black (w/v)–7% acetic acid (v/v) for at least 3 hr. Destaining was carried out in 7% acetic acid by electrophoresis with a current of 5 mA/gel. When the split gel technique (Leboy *et al.*, 1964) was used, the septum was a rectangular piece cut from a 22-mm square plastic microcoverslip (Arthur H. Thomas Co.) inserted into an electrophoresis tube and sealed with paraffin. Approximately 5 μg of purified proteins, 25 μg of whole 30S protein, or 75 μg of whole 70S protein was sufficient to give darkly staining bands on single gels. Half these quantities were used for split gels. Gels were scanned directly using a Joyce-Loebl microdensitometer.

Reduction. In some experiments reductions were carried out by bringing the protein solution to pH 8.1 by adding solid Tris or by adding one-quarter the volume of 1 M Tris-HCl. 2-Mercaptoethanol was added to 0.06 M and the solution was incubated for 3 hr at 37°.

Concentration Measurements. Protein concentrations were measured by the method of Lowry *et al.* (1951) using muramidase (Worthington Biochemical Corp.) as a standard. A micro-Kjeldahl (Hiller *et al.*, 1948) nitrogen assay was run on total ribosomal protein. It was found that the Lowry reaction (with muramidase standard) gave 93% of the Kjeldahl value for ribosomal protein concentration. RNA concentrations were measured by the orcinol method (Mejbaum, 1939) using sRNA (General Biochemicals) as a standard. The concentration of the standard was estimated from the absorbance at 260 mμ.

Results

Extraction of Proteins. The LiCl–urea procedure of Leboy *et al.* (1964) is ideal from the point of view of yield because it consistently yields 98% or more of the ribosomal protein but such protein preparations contain 3% or more contamination with RNA. This interferes with the chromatographic fractionation of the proteins. The acetic acid extraction method of Waller and Harris (1961) gives a lower RNA contamination but only 80–85% of the ribosomal protein is recovered with this procedure. After trying a variety of alternative procedures, we found that it is possible to modify the acetic acid extraction by employing high $MgCl_2$ concentrations and thereby obtain 95% or greater recovery of the ribosomal protein with very low RNA contamination (Table I). This was the method routinely used in the present study.

The disc electrophoresis patterns obtained with acetic acid extracted protein, LiCl–urea prepared protein, and the protein obtained from the 30S subunit by enzymatic digestion of the RNA (see Methods) are shown in Figure 1. The patterns are virtually indistinguishable for all three preparations of protein with the exception of the slowest moving component, which is relatively reduced in the acetic acid preparation.

The protein is extensively dialyzed before chromatography but at least 95% of the protein is recovered after dialysis (Table I), and densitometer tracings show that the disc electrophoresis patterns are indistinguishable before and after dialysis.

Chromatography. When the 30S ribosomal proteins are eluted from a phosphocellulose column with a linear salt gradient more than 90% of the protein is recovered in 20 or more chromatographic peaks (Table I, Figure 2). The fractions marked with arrows in Figure 2 were analyzed by disc electrophoresis on split gels which are shown in Figure 3. Clearly proteins 2a, 2, 4a, 5, 8, 9, 12b, 15a, and 16 can be obtained in an electrophoretically homogeneous state after a single passage on phosphoecellulose, if the shoulders of the respective chromatographic peaks are discarded.

The data presented in Figures 2 and 3 suggest that there are approximately 20 different 30S ribosomal proteins. However,

FIGURE 1: Densitometer tracings from single gels of the three different protein preparations described in the text. The very sharp peak on the left of the LiCl–urea tracing is due to a scratch on the gel surface. Electrophoresis is from right to left.

TABLE I: Protein Recoveries and RNA Contamination during Protein Extraction and Fractionation.[a]

Stage	Total Protein (mg)		$\left(\frac{\text{mg of RNA}}{\text{mg of Protein}} \times 100\right)$
30S ribosomes	438		230[b]
Acetic acid extract	426		1.8
Chromatographic starting material	408	2210 A_{230}	<0.5
Chromatographic eluate		2040 A_{230}	

[a] Protein concentrations were measured by the procedure of Lowry *et al.* (1951) using muramidase as a standard, except in the chromatographic eluate where only the absorbance at 230 mμ was measured. This latter method was also used for the chromatographic starting material. RNA concentrations were measured by the orcinol procedure (Mejbaum, 1939) using sRNA as a standard. [b] Estimated from A_{260} of ribosomes.

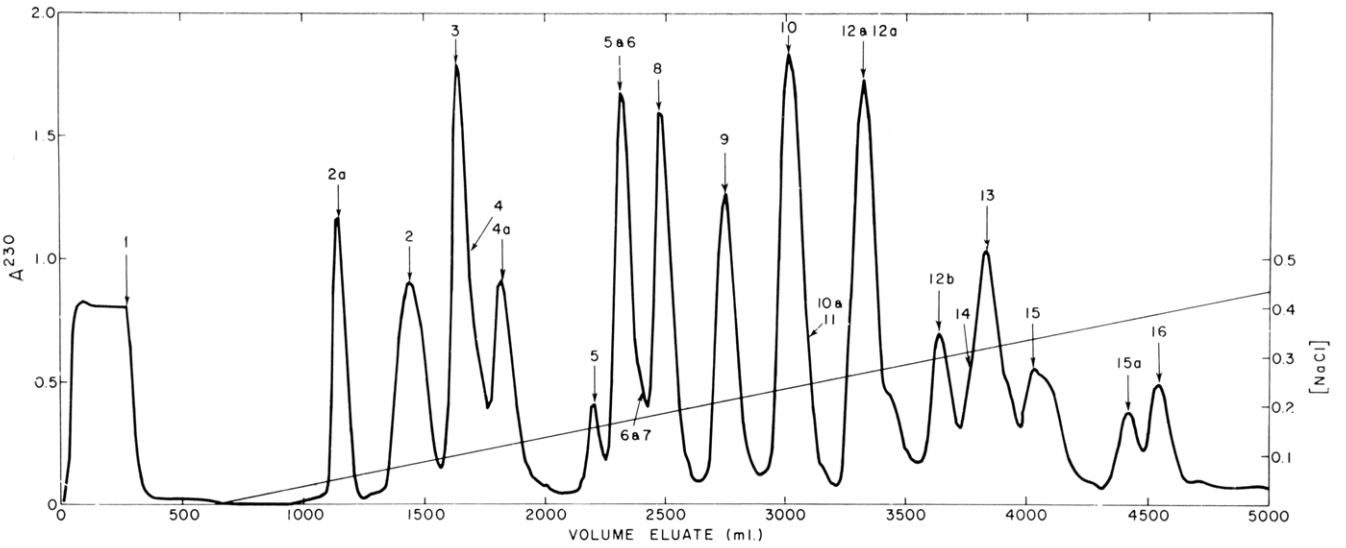

FIGURE 2: Chromatographic profile of unfractionated 30S protein on phosphocellulose at pH 5.8. The column dimensions were 60 × 2.8 cm. The flow rate was 45 ml/hr. The gradient was 0–0.6 M NaCl in 6000 ml. The buffer contained 50 μl of β-mercaptoethanol/l. The arrows point to the fractions analyzed by gel electrophoresis shown in Figure 3. The numbers designate the proteins which are major components of the arrowed fractions.

there are several groups of chromatographically separable components that are indistinguishable by disc electrophoresis (Figure 4). These are: 2a, 3, and 10; 2, 5, and 9; 4 and 8; 6, 11, 14, and 15; and 7 and 12b.

CONTAMINATION with 50S ribosomal proteins does not appear to be a factor which contributes to the apparent heterogeneity of the proteins isolated from the 30S subunit. Schlieren sedimentation analysis of several independent 30S subunit preparations sets an upper limit of 5% contamination with 50S subunits. In addition, we have found that most of the proteins of the 30S and 50S subunits are electrophoretically and chromatographically distinguishable. Finally, we have isolated and partially characterized 16 proteins from the 50S subunit; none of these appears to be related to any of the 30S

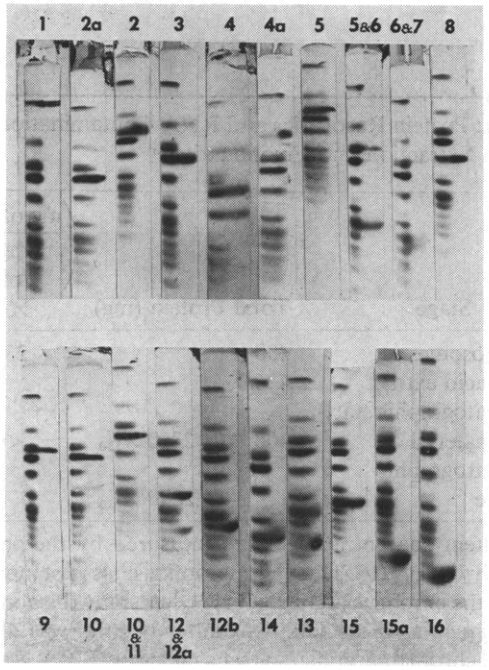

FIGURE 3: Split gel electrophoretic analysis of the arrowed fractions in Figure 2. On the left of each gel is unfractionated 30S protein after dialysis into standard buffer. On the right is an aliquot of the fraction.

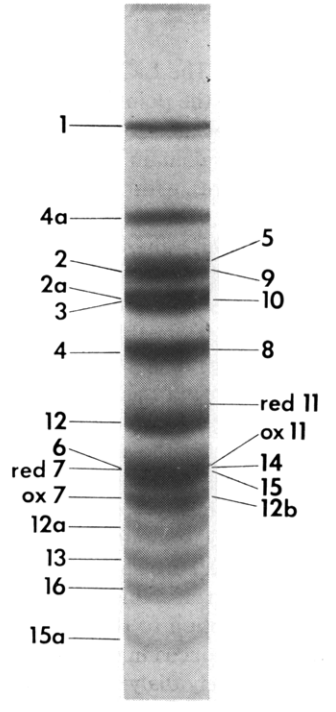

FIGURE 4: Correlation of chromatographic components with electrophoretic bands. The photograph shows the electrophoretic band pattern of unfractionated 30S protein. The numbers on the left and right of the gel indicate which proteins are present in each band. Red and ox are abbreviations for reduced and oxidized, respectively.

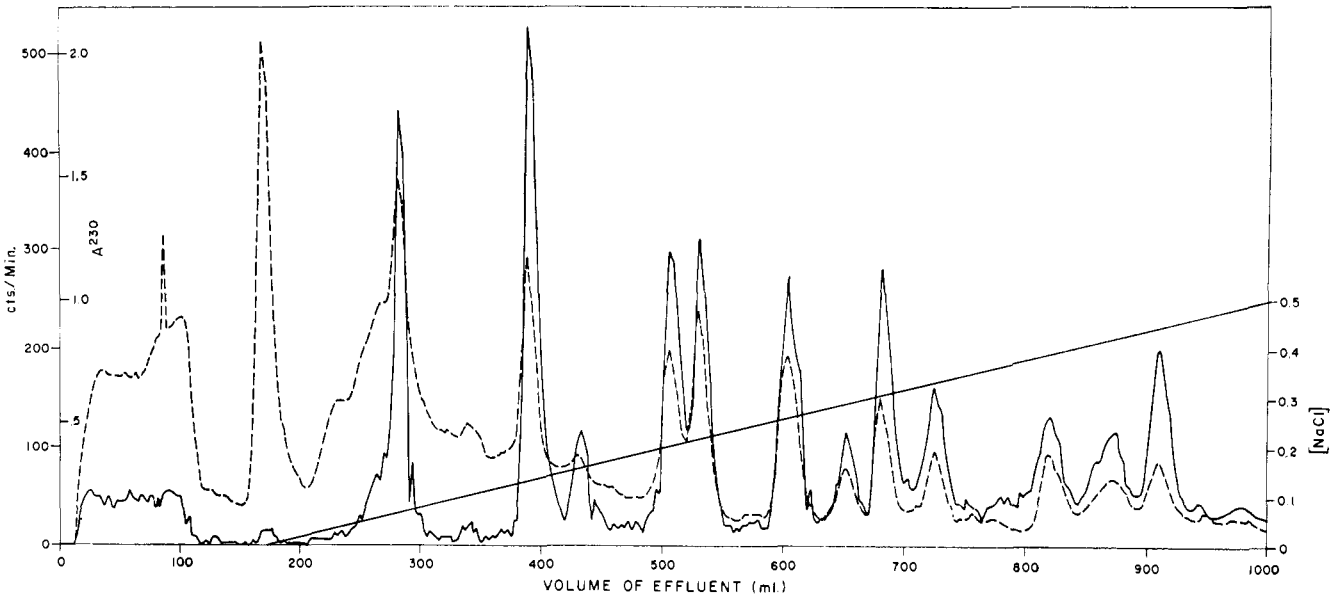

FIGURE 5: Cochromatography of protein from crude 30S ribosomes and [^{14}C]amino acid hydrolysate labeled protein from washed 30S particles. A 0.2-ml aliquot of each sample was counted in a liquid scintillation spectrometer. (----) Ultraviolet absorption and (—) radioactivity. The elution pattern of this column is different from that of Figure 4 because it was developed at pH 6.5 and there was no β-mercaptoethanol in the buffer.

proteins (G. Mora, G. Craven, P. Voynow, S. J. S. Hardy, and C. G. Kurland, unpublished data).

The contribution of supernatant proteins to the apparent heterogeneity of the ribosomal proteins was assessed as follows. An extract (S 30) of *E. coli* was subjected to zone centrifugation in 3×10^{-4} M $MgCl_2$. The fractions containing the 30S ribosomes were pooled (crude ribosomes) and mixed with ^{14}C-labeled 30S subunits that had been prepared by our standard $(NH_4)_2SO_4$ procedure. Then the acetic acid extracted proteins from this mixture were chromatographed on phosphocellulose (Figure 5). The first half of the elution pattern is strikingly different from the latter half, which contains the more basic proteins. The crude 30S particles (ultraviolet absorbance) contain relatively more acidic protein than the $(NH_4)_2SO_4$-prepared ribosomes (radioactivity). As a consequence, the first half of the elution pattern has more peaks from the crude ribosomes than the $(NH_4)_2SO_4$ ribosomes and the base line is much higher in the case of the crude ribosomal protein. Electrophoretic analysis of the unlabeled peaks and shoulders in the first half of the profile showed only faint bands, many of which were not evident in the starting material. This diffuse acidic protein is very likely supernatant contamination (Kurland, 1966; Hardy and Kurland, 1966). In contrast to the first half of the profile, the second half shows a reasonable correlation between the ultraviolet absorbance and the radioactivity. This suggests that the stoichiometry and heterogeneity of the basic proteins are not dramatically altered by employing the $(NH_4)_2SO_4$ procedure.

Degradation of the Proteins. Ribosomes prepared by conventional procedures are known to have proteolytic activity tightly bound to both the 30S and 50S subunits (Matheson and Tsai, 1965; McCorquodale, 1963). Most of this proteolytic activity is removed from the ribosomes if the $(NH_4)_2SO_4$ procedure is employed (A. Matheson, personal communication), but this purification requires at least 1 day. Therefore, it is necessary to determine whether or not some of the electrophoretically identifiable proteins are degradation products resulting from the action of the proteolytic enzymes on the intact ribosomes during purification. This can be determined by taking advantage of the fact that the ribosomal proteins make up the bulk of the basic proteins in *E. coli* (Waller, 1963). One volume of frozen bacteria was thawed in one-tenth volume of 1 M $MgCl_2$ and two volumes of glacial acetic acid. The mixture was stirred for 1 hr in an ice bath, and the precipitate was removed by low-speed centrifugation. The acetic acid soluble protein from the whole bacteria was then compared with the acetic acid extracts of the 70S ribosomes (Figure 6). All of the electrophoretic components seen with the protein from the 70S ribosomes are present in the protein extracted from the whole bacteria. The principal difference between the two samples is the presence of small quantities of diffuse, slowly moving components in the bacterial extract which are not seen in the ribosomal protein. This experiment suggests that the apparent electrophoretic heterogeneity of the ribosomal proteins is not the result of enzymatic degradation of the proteins during the purification of the ribosomes.

Since it takes weeks to purify and analyze the fractions obtained from a single phosphocellulose column eluate of the 30S ribosomal protein, even minute traces of proteolytic activity could cause a variety of artifacts. We have observed that the weight-average molecular weight of the 30S ribosomal protein (measured by the procedure of Yphantis, 1964) drops to one-half its initial value after 3 weeks at room temperature in standard buffer. This degraded protein yields an altered electrophoretic pattern when compared with the starting material on a split gel. The main differences between the two samples are the following: the appearance of new components at the top of the gel, the disappearance of several components at the bottom of the gel, and a fuzziness of the bands in the incubated material. The data indicate the presence of

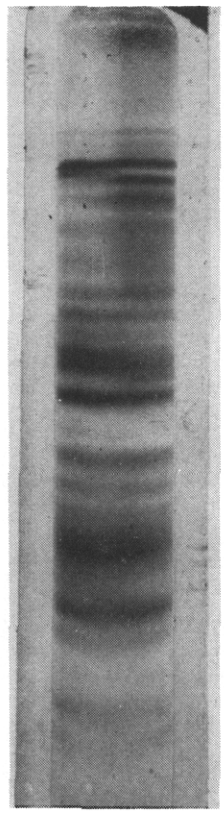

FIGURE 6: Comparison of a split gel of an acetic acid extract of intact cells and an acetic acid extract of 70S ribosomes. On the right side is the acetic acid extract of intact cells prepared as described the text.

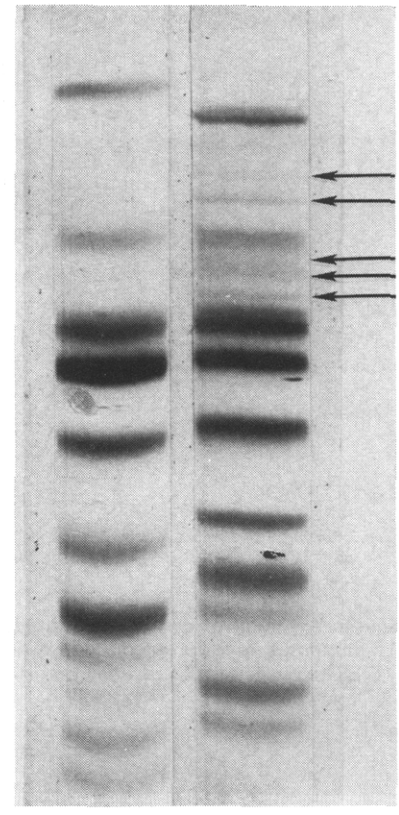

FIGURE 7: Electrophoretic band patterns of reduced and unreduced unfractionated 30S protein. The right-hand gel contains unfractionated protein after several weeks at −20° with repeated freezing and thawing. The left-hand gel contains the same material after reduction with β-mercaptoethanol. Arrows indicate those components which disappear after reduction.

trace amounts of proteolytic enzymes in our protein preparations which could complicate estimates of the heterogeneity of the proteins. Fortunately, we do not usually observe degradation of the purified proteins when they are stored at low temperatures (2–4°) for months at a time.

Aggregation. When chromatographic fractionation of the proteins is carried out with buffers which do not contain a reducing agent, components which are not present in the starting material appear and accumulate with increasing time of storage of the protein. Two of these anomalous chromatographic components are quite reproducible and represent major components. Variable amounts of other minor components also appear in the gels when the protein is stored in the cold or after repeated freezing and thawing of material stored at −20°. The appearance of these anomalous electrophoretic components is accompanied by the disappearance or reduction in intensity of at least one of the original electrophoretic components. Since all of the anomalous components have low electrophoretic mobilities, it is possible that they are aggregates which are retarded in the gels. The addition of mercaptoethanol to unfractionated protein which contained these anomalous components caused them to disappear, and the diminished band to reappear in its original intensity (Figure 7). These results strongly suggest that disulfide-bonded aggregates are formed in the absence of a reducing agent.

Protein 2a is obtained as a single sharp chromatographic peak but in the absence of reducing agents it contains two electrophoretic components, one of which does not correspond to any component seen in the starting material. When protein 2a is rechromatographed at the same pH but with a shallower gradient of salt, it is separated into two components. The first of these contains both electrophoretic bands, while the second contains only the slower migrating component. After reduction with mercaptoethanol, both chromatographic peaks show only the faster moving electrophoretic component. The molecular weight of the first chromatographic peak of protein 2a was measured in the absence of mercaptoethanol. The equilibrium distribution showed molecular weights varying between 20,000 and 27,000 daltons. After reduction with mercaptoethanol, the equilibrium distribution showed a single molecular species of 17,000 daltons. When the protein in the second chromatographic peak of protein 2a was analyzed in the absence of mercaptoethanol, it was found to have a single molecular species of 35,000 daltons. The data suggest that protein 2a can form a stable aggregate, probably a dimer, in the absence of mercaptoethanol; this aggregate is probably disulfide bonded.

The chromatographic and electrophoretic behavior of protein 2a–dimer was very similar to that of one of the anomalous chromatographic components. The amino acid and tryptic peptide analyses of this anomalous component were almost identical with those of 2a. Furthermore, upon reduction with mercaptoethanol, its electrophoretic mobility was the same as that of 2a. It is concluded that this anomalous component is 2a dimer.

TABLE II: Purification Procedures for the Proteins of the 30S Subunit.

Protein	Major Contaminants after Initial Chromatography at pH 5.8	Major Contaminants after Initial Chromatography at pH 6.5	First Rechromatography Conditions	Second Rechromatography Conditions
1	None	None		
2	None	None		
2a	None	None		
3	4a	4a	Sephadex G-100	
4	3 and 4a	3 and 4a	pH 7.5, 0.04–0.24 M NaCl	
4a	4	4	pH 6.5, 0.05–0.25 M NaCl	
5	None or 6 and 7	None or 6 and 7	Sephadex G-100	
6	5 and 7	5 and 7	Sephadex G-100 to remove 5	pH 5.5, 0.1–0.3 M NaCl[a]
7	5 and 6	5 and 6	Sephadex G-100 to remove 5	pH 5.5, 0.1–0.3 M NaCl[a]
8	None	None		
9	None	None		
10	None	None		
11	10 or 12	10	pH 6.5, 0.2–0.4 M NaCl for 12 Sephadex G-100 for 10	
12	12a	None	pH 6.5, 0.2–0.4 M NaCl	
12a	12	None	pH 6.5, 0.2–0.4 M NaCl	
12b	None or 12a	None	pH 6.5, 0.2–0.4 M NaCl	
13	14	14	pH 6.5, 0.26–0.46 M NaCl[a]	
14	13	13	pH 6.5, 0.26–0.46 M NaCl[a]	
15	13	13	pH 6.5, 0.3–0.5 M NaCl[a]	
15a	15	15	Sephadex G-100	
16	None	None		

[a] Reduced with 2-mercaptoethanol before rechromatography.

A parallel relationship exists between the second anomalous chromatographic component and protein 10. The aggregate of protein 10 is eluted from phosphocellulose at a higher salt concentration than the monomer. The molecular weight of the aggregate is approximately twice that of the monomer (46,000 compared with 26,000). After reduction with mercaptoethanol, the aggregate is electrophoretically identical with protein 10. It has been shown that the anomalous component is similar with respect to its amino acid composition and tryptic peptides to protein 10.

If a low level of mercaptoethanol (50 μl/l.) is present in the chromatography buffer, the aggregates of proteins 2a and 10 cannot be detected in the column eluate. Very few components which do not correspond to electrophoretic components of the starting material can be detected in the eluates of phosphocellulose. Those anomalous components which are seen appear as very minor components (less than 1% of total) judged by their intensity of staining on gels and the absorbancy of the column fractions in which they occur. Drastic reduction of each purified 30S protein with 0.06 M mercaptoethanol at pH 8.1 has no effect on its electrophoretic mobility, with the exception of proteins 7 and 11. Both of these proteins electrophorese more slowly after reduction and protein 11 moves to a position on the gels which does not correspond to any component in the unfractionated, unreduced starting material (Figure 4). The cause of these changes in mobility is not known, but the fact that proteins 7 and 11 electrophorese more slowly after reduction is not consistent with the hypothesis that reduction destroys aggregates of proteins 7 and 11. Rather, it is likely that reduction causes a shape change in these proteins. We conclude that none of the purified chromatographic components obtained in the presence of mercaptoethanol is a disulfide-bonded aggregate.

We have observed that the molecular weight distributions of 30S proteins in 6 M urea and in 5 M guanidine hydrochloride are indistinguishable. Since guanidine hydrochloride is a more effective denaturant than urea, this result implies that there is not extensive aggregation of the proteins in 6 M urea; however, it does not eliminate the possibility that a small fraction of the protein is aggregated in the standard buffer.

Purification of the Proteins. Table II summarizes the data from more than 80 chromatographic columns and lists the procedures by which every protein is best obtained in an electrophoretically homogeneous state. Initial chromatography was carried out either at pH 5.8 or 6.5. Chromatographic separations at pH 5.8 are usually a little cleaner than those at pH 6.5 and this pH is preferable for the resolution of the least basic proteins. The advantages of chromatography at pH 6.5 are that it gives a larger number of pure components in one fractionation step and that the more basic proteins are generally better separated. Table II lists the methods by which each protein is separated from its major contaminants. For every protein except 6 and 7 this can be done by carrying out only one fractionation step after initial chromatography.

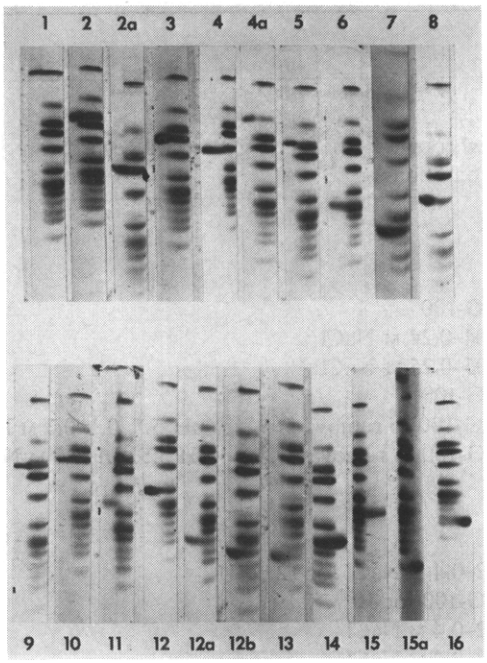

FIGURE 8: Split gels of all the purified proteins. The reference material is unfractionated 30S protein.

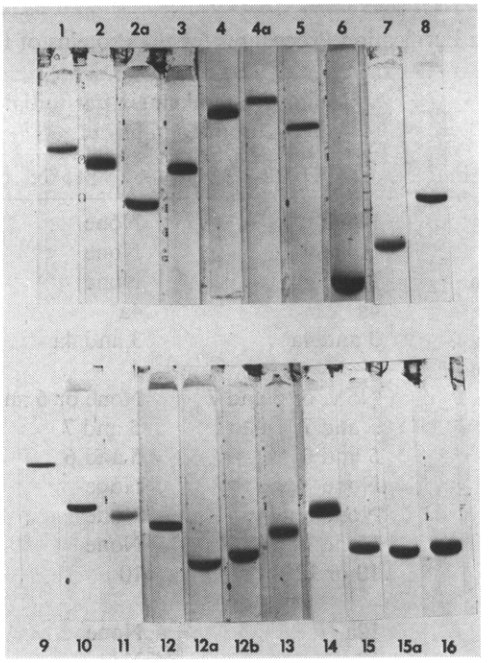

FIGURE 9: Single gels of all the purified proteins.

Protein 5 should be removed from 6 and 7 by rechromatography on Sephadex before the latter two are separated on phosphocellulose.

Figures 8 and 9 show each of 21 purified proteins after electrophoresis on split and single gels. Electrophoretic homogeneity of the purified proteins estimated from microdensitometer tracings of stained single gels is greater than 95%. All these purified proteins except for protein 7 were obtained from the initial chromatography shown in Figure 2. Protein 7 which is a minor component was not obtained because of a low recovery of material from the rechromatography on phosphocellulose in this instance.

Discussion

The chromatographic procedures described in the present paper have enabled us to purify all or almost all of the electrophoretically and chromatographically identifiable proteins in the 30S ribosomal subunit. This has been done under conditions which minimize the contribution of demonstrable artifacts to the apparent heterogeneity of the ribosomal proteins. There is no serious limit to the quantities of protein which can be purified by these procedures except for that imposed by the availability of highly purified 30S subunits. Therefore, one result of the present study is a methodology which should be useful in obtaining purified proteins for detailed analysis of the structure and function of the ribosome.

The objectives of the present study are to enumerate all of the ribosomal proteins and then to determine the number of copies of each protein in the ribosome. The most subtle problem here is to determine the extent to which contaminants are contributing to the apparent heterogeneity of the ribosomal proteins. We have devoted considerable effort to this problem (Hardy and Kurland, 1966; Kurland, 1966; Likover and Kurland, 1967). While our ammonium sulfate procedure yields ribosomes that are as active for *in vitro* polypeptide synthesis as are those prepared by conventional procedures, they contain between 20% and 30% less protein than ribosomes prepared by differential centrifugation. Data presented in this paper show that the proteins which are removed by ammonium sulfate are a heterogeneous population of proteins which are more acidic than most of the remaining ribosomal proteins. There is little if any selective loss of the major basic proteins which remain in the ribosome. In other experiments we have analyzed the proteins which are washed off the crude ribosomes by ammonium sulfate and have found that they are chromatographically and electrophoretically distinguishable from the proteins which remain on the ribosome. Much more work is required to demonstrate that none of the proteins present in the ammonium sulfate washed ribosomes are contaminants, but it seems probable that the ammonium sulfate washed ribosome more nearly approximates an uncontaminated particle than does the ribosome prepared by differential centrifugation.

The artifact which is easily identified and controlled is the aggregation of the ribosomal proteins through disulfide bonds. Although we have demonstrated most clearly the dimerization of homologous proteins through disulfide bonds, the presence of heterologous aggregates is also indicated in some experiments. It is possible to completely eliminate these artifacts by working in the presence of appropriate reducing agents.

We have not been so successful in controlling the effects of proteolytic activity on the ribosomal proteins. The control experiment in which ribosomal proteins were extracted from intact bacteria indicates that the apparent heterogeneity of the ribosomal proteins is not a consequence of enzymatic degradation of a small number of native ribosomal proteins. It is possible to avoid the gross effects of proteolytic activity by using well-washed ribosomes and working as rapidly as possible at low temperatures. Once the proteins are purified

they seem to be quite stable. However, the effects of traces of proteolytic enzymes are still seen on occasion. Thus, we have isolated degraded fragments from at least six of the ribosomal proteins. The fragments have been identified by their tryptic peptides which are related to those of the native proteins and by their reduced molecular weights. Recently, we have employed an esterase inhibitor in the purification of the proteins; there was no reduction in the number of chromatographically separable components.

We have purified 21 proteins which are unique and homogeneous according to electrophoretic and chromatographic criteria. The data in the accompanying paper (Craven *et al.*, 1969) demonstrate that each of these 21 proteins is chemically distinct from every other one. However, there are several ambiguities in the data which force us to conclude that two of these proteins could be enzymatically derived fragments (7 and 9). We have on two occasions detected a contaminant of protein 15 which may be a unique protein that we have not purified. We conclude that there are at least 19 and at most 22 proteins in the 30S subunits purified by our procedures.

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