A New Method for the Purification of 30S Ribosomal Proteins from *Escherichia coli* Using Nondenaturing Conditions†

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ABSTRACT: A new method for the purification of *Escherichia coli* (A19) 30S ribosomal proteins has been developed that avoids the use of denaturing conditions such as urea, acetic acid, and lyophilization. In this way the majority of the proteins from the small ribosomal subunit can be obtained in 5–100 mg quantities and at ≥90% purity. This has been achieved by the initial "splitting" of the proteins into two main groups with LiCl followed by fractionating on ion-exchange and gel-filtration columns, in the absence of urea and in the presence of salt. The proteins prepared by this nondenaturing procedure were soluble at high ionic strength and less soluble, being aggregated, at low salt concentrations. This behavior was exactly the opposite of that exhibited by proteins prepared with methods using denaturing conditions. These new methods have enabled additional ribosomal RNA-binding proteins to be found and potential protein–protein complexes to be isolated. Preliminary evidence that these proteins may retain a more native structure is presented.

A detailed study of the topography of the *Escherichia coli* ribosome could be greatly facilitated by a knowledge of the structure of the individual components. The small subunit of the *Escherichia coli* ribosome is a complex structure containing ribosomal RNA and 21 different proteins. Study of these ribosomal proteins has been greatly aided by the development of analytical methods such as the two-dimensional gel-electrophoresis system of Kaltschmidt and Wittman (1970) and preparative methods to isolate the numerous proteins as reviewed by Wittman (1974). Proteins prepared by these methods have been used for amino acid sequence studies (Wittmann and Wittmann-Liebold, 1974; Stöffler and Wittmann, 1977), rRNA binding (Zimmermann, 1974; Brimacombe et al., 1976), ribosome reconstitution (Nomura and Held, 1974), and for production of specific antibodies for topological studies (Tischendorf et al., 1975; Lake and Kahan, 1975).

This paper describes methods of protein isolation which seek to maintain the proteins in a more "native" state than previously achieved. The term "native" does not necessarily imply that the proteins are in the same state as that found in the ribosome, since under these conditions their interaction with other proteins and ribosomal RNA is obviously important. Nevertheless, in an effort to study the structure of isolated ribosomal proteins and RNA–protein complexes by physical methods we felt that it was important to use proteins that were in as "native" a state as possible. Previously, the ribosomal proteins have always been isolated under conditions that could lead to denaturation such as the use of urea, acetic acid, temperatures above 4 °C, and lyophilization. We have set out to develop methods of isolation under milder conditions avoiding the use of the above procedures.

By using LiCl to split the 21 30S proteins into two main groups, it has been possible using ion-exchange chromatography followed by Sephadex gel filtration to isolate most of the small ribosomal subunit proteins in quantities sufficient to initiate some physical studies. In addition to the purified proteins, several potential protein–protein complexes of some of the proteins have been isolated. These so-called salt-extracted proteins are more soluble at high ionic strength and less soluble at low salt concentrations. This is the reverse of the solubility exhibited by previously prepared ribosomal proteins.

Proton magnetic resonance studies reported in a separate communication (Morrison et al., 1977) show that proteins isolated by these gentler methods retain more structure than those isolated with urea and acetic acid. We therefore hope that they will be useful for physical studies in an effort to gain some insight into the shape and structure of the individual components of the ribosome.

Materials and Methods

Solutions and Buffers. Stock LiCl and $MgCl_2$ solutions were treated with purified bentonite (Fraenkel-Conrat et al., 1961) and activated charcoal. This was followed by filtration first through standard Whatman filters and then through Millipore HAPW filters (0.45-μm pore size).

The following buffers were used: (A) 0.01 M Tris-HCl[1] (pH 7.5), 0.1 M KCl, 0.02 M $MgCl_2$; (B) 0.01 M potassium phosphate (pH 7.5), 0.001 M $MgCl_2$; (C) 0.01 M Tris-HCl (pH 7.5), 0.07 M KCl, 0.001 M $MgCl_2$; (D) 0.05 M sodium acetate (pH 5.6); (E) 0.05 M sodium acetate (pH 5.6), 0.4 M LiCl. To all buffers, 2-mercaptoethanol, $PhCH_2SO_2F$, and BAM were added, just before use, in final concentrations of 0.006, 5×10^{-5}, and 1×10^{-4} M, respectively.

Bacteria. E. coli A19 cells were grown in rich medium at 37 °C to midlog phase (5 g/L wet weight). They were harvested in a continuous-flow centrifuge and stored at −80 °C.

Ribosomes and Subunits. A 500-g batch of cells was thawed at 4 °C and washed with 500 mL of buffer A. The pellet was

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[1] Abbreviations used are: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; BAM, benzamidine hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; DTE, dithioerythritol; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine; EDTA, (ethylenedinitrilo)tetraacetic acid; CM, carboxymethyl. One A_{260} unit is the amount of substance in 1 mL of a solution giving an absorbance of 1.0 when measured at 260 nm with a path length of 1.0 cm. TP-30 is the total protein extracted from the 30S ribosomal subunit with acetic acid according to Hardy et al. (1969).

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collected by centrifugation at 10 000 rpm for 30 min and resuspended in a further 500 mL of buffer A using a Waring Blendor with the addition of 3 mg of DNase. The cells were broken by passing them twice through a Manton-Gaulin press at 10 000 psi, with cooling in between each cycle. The resultant suspension was centrifuged at 30 000*g* for 10 min to remove unbroken cells, followed by a further centrifugation at 30 000*g* for 30 min to remove cell debris. The supernatant was then centrifuged at 150 000*g* for 2 h in 4 Ti-60 rotors to pellet the ribosomes. After resuspension of this pellet into 200 mL of buffer A, the preparation was clarified and the ribosomes were pelleted from the resultant supernatant as described above. The pellets were then resuspended into 100 mL of buffer A and frozen at −80 °C until needed. Subunits were obtained by dialysis of the unfrozen monosome suspension, diluted to 250 A_{260} units/mL with buffer B, and dialyzed against two changes of this buffer. Samples equivalent to 10 000 A_{260} units were applied to a 15–38% sucrose gradient using a Ti-15 zonal rotor. The resultant subunit peaks were collected and precipitated by a modification of the procedure of Expert-Bezançon et al. (1974). Poly(ethylene glycol) 6000 (Merck) was added to the subunits at a concentration of 11% (w/v) in the presence of 20 mM $MgCl_2$, the mixture was stirred for 30 min, and the subunits were pelleted by centrifugation for 1 h at 15 000*g*. The pellets were redissolved in buffer C and stored at −80 °C.

Extraction of Ribosomal Proteins. The proteins were split into groups using LiCl (Atsmon et al., 1967; Itoh et al., 1968; Homann and Nierhaus, 1971). Frozen subunit preparations were thawed at 4 °C and pelleted by centrifugation at 100 000*g* for 10 h to remove residual poly(ethylene glycol). The pellets were resuspended into buffer C at 100 A_{260} units/mL. To this suspension an equal volume of 2 M LiCl in buffer C was added, and the mixture was made 0.001 M with respect to EDTA. After stirring for 10 h, the core particles were pelleted by centrifugation at 100 000*g* for a further 10 h. The supernatant was diluted with an equal volume of buffer D and dialyzed against two changes of this buffer. After clarification at 15 000*g* for 30 min, the soluble sample was applied to a CM-Sephadex column preequilibrated with buffer D. Usually an amount equivalent to 150 000 A_{260} units of 30S subunits was processed at one time. Hence, due to a limitation of volume during the centrifugation step the procedure was repeated three times using 1 L of extract on each occasion. The resultant core particles were stored at −80 °C until a further extraction was performed. The CM-Sephadex column (3 × 40 cm) was eluted with a linear gradient of LiCl (0.15 → 0.8 M); volume 7 L. Fractions of 15 mL were collected and analyzed for protein quantity by measurement of the A_{235}, and for protein content by cylindrical polyacrylamide gel electrophoresis in 6 M urea at pH 4.5 (Hindennach et al., 1971) or by $NaDodSO_4$-polyacrylamide slab gel electrophoresis as described by Laemmli and Favre (1973).

For the second extraction the frozen core particles were thawed and reextracted in a similar manner but on this occasion were directly resuspended into 2 M LiCl in buffer C at a concentration of 50 A_{260} units/mL with the addition of EDTA, pH 7.0, to a final concentration of 0.01 M. The resulting supernatant was applied to a preequilibrated CM-Sephadex column as described above and eluted with a convex gradient of LiCl (0.15 → 1.0 M); volume 7 L. The resultant core particles were frozen at −80 °C. A final extraction of these cores was performed by resuspending them into 4 M LiCl in buffer C with the addition of EDTA, pH 7.0, to a final concentration of 0.01 M. An extraction with 67% acetic acid as described by Hardy et al. (1969) was performed on the cores remaining after the 4 M LiCl treatment. This served to remove any remaining protein which was present on the RNA core particles.

Proteins were further purified by pooling the appropriate fractions from the ion-exchange columns followed by concentration before they were applied to a Sephadex G-100 column (5 × 100 cm), equilibrated with buffer E.

Concentration Procedures. The proteins were concentrated by the following methods: (1) dialysis against dry Sephadex G-150; (2) dialysis against 15% poly(ethylene glycol) 20 000 (Aquacide III, Calbiochem) in buffer E; (3) pressure filtration using Millipore concentration cells (75- or 3-mL capacity) using Pellicon (PSAC 01310 and PSAC 04710) or Amicon (Diaflo UM-2) membrane filters both with a molecular weight cutoff of 1000; (4) reabsorption onto small CM-Sephadex columns followed by a stepwise elution at high ionic strength. For methods 1 and 2 dialysis tubing (Spectrapor 1, 3, or 6, molecular weight cutoff 4000–6000, 3500 and 2000, respectively) from Spectrum Medical Industries, Los Angeles, was used.

Purified proteins in buffer E were made 1 mM DTE or DTT and stored in small aliquots at −80 °C. Proteins were analyzed for purity and identity by (1) a two-dimensional polyacrylamide gel electrophoresis system as described by Kaltschmidt and Wittmann (1970) using the stain Amido Black; (2) a two-dimensional electrophoresis system using $NaDodSO_4$ in the second dimension (Mets and Bogorad, 1974; Kyriakopoulos and Subramanian, 1977); (3) $NaDodSO_4$ slab gel electrophoresis as described above. Both $NaDodSO_4$ gels were stained with Coomassie brilliant blue stain R250 (Serva). Protein samples too dilute for direct application to the gel were precipitated by the addition of an equal volume of 10% trichloroacetic acid in the presence of 10 μg/mL sodium deoxycholate. After 1 h at 0 °C, the precipitate was collected by centrifugation in a Beckman Microfuge Model B for 1–2 min at 12 000 rpm. The pellet was either washed with diethyl ether to remove residual trichloroacetic acid or dissolved in a sample solution for the electrophoresis system which was at a higher pH to compensate for the acid in the sample pellet. Protein concentrations were determined by two methods. The first method was a modified fluorescamine assay (Böhlen et al., 1973) in which 40 μL of the protein solution was mixed with 260 μL of 0.05 M sodium tetraborate buffer (pH 9.0) followed by the rapid addition of 50 μL of a fluorescamine (Serva) solution in acetone (0.6 mg/mL). The fluorescence was measured using a 250-μL cuvette in an Aminco-Bowman fluorimeter. Lysozyme was used to obtain a calibration curve in the range of 1–100 μg. The second method was a nitrogen assay (Jaenicke, 1974) where ammonium sulfate was used as a standard and the nitrogen content of each protein was calculated from its amino acid composition (Kaltschmidt et al., 1970; Stöffler and Wittmann, 1977). All operations throughout the ribosome and protein extraction steps were carried out at 4 °C or below.

Enzymes and Proteins. Bovine serum albumin was obtained from Calbiochem; glyceraldehyde-3-phosphate dehydrogenase, myoglobin, lysozyme, chymotrypsinogen, carbonic anhydrase, cytochrome *c*, and pyruvate kinase were from Boehringer; aldolase (rabbit) and ovalbumin were from Serva, and DNase I (RNase free) was from Worthington. Haemerythrin extracted from *Golfingia gouldii* (a Sipunculid worm) was a gift from Dr. Subramanian.

Chemicals. BAM, DTE, DTT, Hepes, $PhCH_2SO_2F$, and deoxycholate were purchased from Calbiochem. CM-Sephadex C-25 and Sephadex G-100 and G-150 were obtained from Pharmacia, and sucrose (RNase free) was from Schwarz-Mann. All other chemicals were from E. Merck and were of the highest purity available (zur Analyse).

TABLE I: Distribution of the Ribosomal Proteins from the 30S Subunit over the Three Extracts.[a]

	1 M LiCl, 0.001 M EDTA, buffer C	2 M LiCl, 0.01 M EDTA, buffer C	4 M LiCl, 0.01 M EDTA, buffer C
S1	++	(±)	
S2	++	−	
S3	++	−	
S4	++	(±)	(±)
S5	++	−	
S6	+	−	
S7	(±)	(±)	(±)
S8	(±)	++	
S9	+	−	
S10	+	−	
S13	−	++	
S14	+	−	
S15	(±)	++	
S16	++	−	
S17	(±)	+	
S19	−	++	
S20	+	++	
S21	++	−	

[a] The presence of large amounts of protein is indicated by ++, of smaller amounts by +, and neglegible amounts by (±).

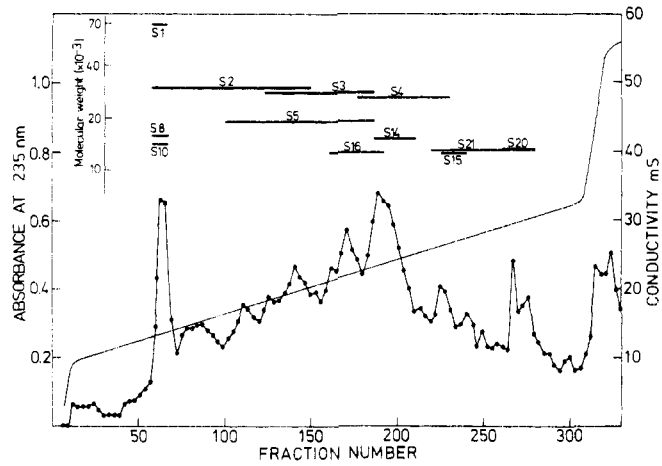

FIGURE 1: Fractionation of proteins obtained from the 1 M LiCl extraction of 30S subunits from *E. coli* A19, by chromatography on CM-Sephadex C-25 in buffer D, pH 5.6. A salt gradient of LiCl 0.15–0.8 M, volume 7 L, was used for elution, followed by a high salt wash of 2 M LiCl. This resulted in a mixture of aggregated protein (less than 10% of the total) being eluted from the column which was representative of all of the proteins in the initial extract. Polyacrylamide gel electrophoresis demonstrates that the elution peaks contain the proteins indicated. The molecular weights used for these proteins are obtained from the $NaDodSO_4$-acrylamide gel system described under Materials and Methods.

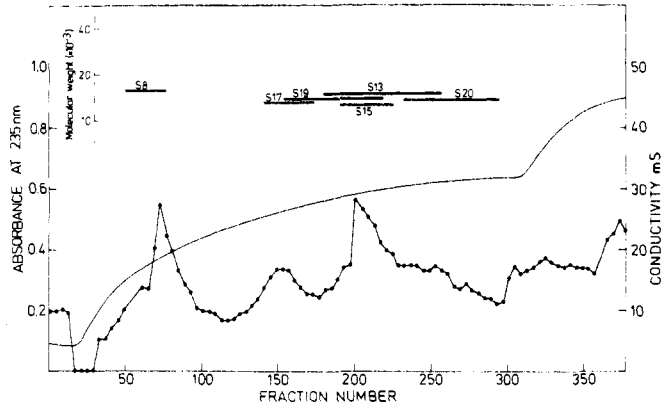

FIGURE 2: Fractionation of proteins obtained from the 2 M LiCl extraction of 30S subunits from *E. coli* A19, by chromatography on CM-Sephadex C-25 in buffer D, pH 5.6. An exponential gradient of LiCl, 0.15–1.0 M, volume 7 L, was used for elution, followed by a further short gradient of LiCl, 1.0–2.0 M. Polyacrylamide gel electrophoresis demonstrates that the elution peaks contain the proteins indicated. The molecular weights used for these proteins were obtained as in Figure 1.

Results

The purification of the 30S ribosomal proteins was greatly facilitated by the initial fractionation of the proteins into two main groups by the use of LiCl. The proteins S1, S2, S3, S4, S5, S14, S15, S16, and S21 were obtained during the first extraction, proteins S9, S10, and S6 forming insoluble material which was removed by centrifugation. The second extraction allowed the purification of proteins S8, S13, S15, S17, S19, and S20 (Table I). This does not agree in detail with the previously published results of Homann and Nierhaus (1971), probably due to the different buffer conditions and ribosome subunit preparation employed. Attempts to increase the number of groups into which the proteins could originally be split proved unsuccessful, since one protein would be present in several groups, hence, making any further purification more tedious. For the same reason, the concentration of ribosomes in the extraction was not increased above 50 A_{260} units/mL, since this resulted in a similar problem of group overlap.

Each extract was dialyzed against buffer D, the starting buffer for the CM-Sephadex column. The extracts were diluted with an equal volume of this buffer before dialysis, but the removal of LiCl still resulted in some precipitation (~10% of the protein) in the first extraction. This was removed by centrifugation and was found to be rich in proteins S9, S10, and S6. Resolubilizing this precipitate with high salt (2 M LiCl) and high concentrations of DTE (up to 0.01 M) was possible to a limited degree. This precipitation problem was hardly present with the second extraction. The elution profiles for the 1 and 2 M LiCl extractions are shown in Figures 1 and 2. Chromatography was carried out at pH 5.6 in acetate buffer as described under Materials and Methods. The convex gradient used in the second extraction increased the separation of proteins in the latter part of the elution. It was found that, with a few exceptions, most fractions from the ion-exchange columns had to be subjected to a second purification step on Sephadex G-100.

The isolated proteins are listed in Table II together with their yield and the chromatographic steps used during their purification. Proteins which were obtained pure after one chromatographic step have increased yields. The reason why some proteins are absent from the lists is attributed to the fact that they have limited solubility under the conditions used for fractionation. Once a protein precipitates from a mixture, other proteins tend to stick to this precipitate and hence their yield is reduced. This is supported by the fact that when the precipitated proteins were not removed from the first extraction before application to the ion-exchange column, with the idea that they might solubilize with the increasing LiCl gradient, a large amount of protein was left on the top of the column which, when redissolved, was representative of the total protein content of the extract and not just of the precipitated proteins. Also, small amounts of proteins S9 and S10 which did redissolve were smeared across the column profile and did not elute as a single protein peak. This effect was also observed with protein S6 even when precipitated material was removed from the extract. Traces of this protein were found associated with proteins S4 and S5. A small amount of protein S7 was often

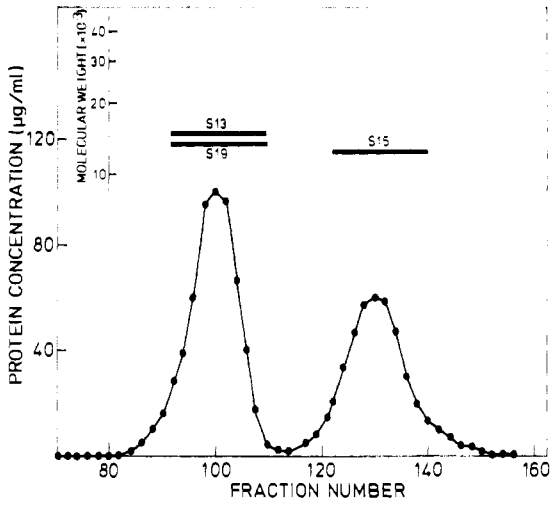

FIGURE 3: Elution profile of proteins S13, S15, and S19 on Sephadex G-100 gel filtration in buffer E at pH 5.6. The molecular weights for the proteins were obtained as in Figure 1.

found associated with proteins S13 and S19 during the second extraction procedure. This S7 could not be separated from S13–S19 by Sephadex G-100 chromatography. After extraction of the RNA core with 67% acetic acid, a little of protein S7 and a trace of S4 were removed.

The yields obtained of most proteins with this new purification method are similar to those obtained by the acetic acid/urea isolation procedure of Hindennach et al. (1971). The yields of proteins S2 and S19 were higher; proteins S6, S7, S9, and S10, which were less soluble, were obtained in lower yields, and proteins S11, S12, and S18 were not found at all.

The fluorescamine assay for protein determination described under Materials and Methods usually gave an overestimation of the protein concentration. Therefore, nitrogen assays were performed on samples where an absolute protein concentration was required. The former method, which was rapid and convenient to perform when many samples had to be analyzed, probably resulted in higher values for the protein concentration due to the relatively high content of lysine found in the ribosomal proteins. The Lowry reaction (Lowry et al., 1951) generally gave lower concentrations than determined by nitrogen assays. Also, the presence of thiol reagents in the protein solutions created errors in the assay even though a modification of the original method (Geiger and Bessman, 1972) was used. Protein concentrations were routinely determined by the fluorescamine assay. By comparison with data obtained from nitrogen assays, conversion factors were established for each protein, which ranged from 1.5 for the larger proteins to 3.5 for the smaller ones.

Several proteins appeared to migrate together after the two chromatographic procedures employed. A list of these is given in Table III, and they are assumed to be potential protein-protein complexes. These proteins migrate together to a varying extent on the CM-Sephadex ion-exchange column and always during gel filtration on Sephadex G-100. Hence, if they are not fractionated on the ion-exchange column, little further separation occurs. Three examples of this effect are (1) approximately 30% of the proteins S3, S4, and S5 comigrate on the ion-exchange column, although this amount varies from one extraction to another. Some S4 separates from the S3–S5 mixture, but no further separation of these three proteins occurs on Sephadex G-100. (2) Most of protein S13 migrates with protein S19 on the ion-exchange column along with protein S15 which is separated from S13–S19 on Sephadex G-100

TABLE II: Purified 30S Ribosomal Proteins.[a]

protein	yield (%)	mg	procedure[b]
S1		26	CM-S, G-100
S2	31	75	CM-S
S3	15	34	CM-S, G-100
S4	26	44	CM-S, G-100
S5	35	47	CM-S, G-100
S8	35	33	CM-S
S13	18	19	CM-S, G-100
S14	9	10	CM-S, G-100
S15	21	16	CM-S, G-100
S16	13	12	CM-S, G-100
S17	10	9	CM-S, G-100
S19	19	19	CM-S, G-100
S20	16	12	CM-S (G-100)
S21	15	9	CM-S, G-100

[a] The yield of each protein calculated for 100 000 A_{260} units of 30S subunits using a protein concentration as determined by the nitrogen assay. The amounts given in milligrams of protein include that contained in the putative protein-protein complexes as shown in Table III. [b] Abbreviation used: CM-S, carboxymethyl-Sephadex.

TABLE III: Potential Protein-Protein Complexes.[a]

potential complex	yield (mg)	procedure[b]
S2-S3	3	CM-S, G-100
S3-S5	37	CM-S, G-100
S3-S4-S5	40	CM-S, G-100
S13-S19	19	CM-S, G-100
S13-S20	12	CM-S, G-100

[a] The yield of each putative complex is calculated for 100 000 A_{260} units of 30S subunits using a protein concentration as determined by the nitrogen assay. [b] Abbreviation used: CM-S, carboxymethyl-Sephadex.

(Figure 3). The S13–S19 elutes as a single peak with an even distribution of the two proteins. (3) Some of S13 also comigrates with S20 on the CM-Sephadex column. When this mixture is put onto Sephadex G-100, some S20 migrates ahead of the S13–S20 mixture, indicating some protein aggregation.

All proteins were identified, and their purity was assessed by the two-dimensional gel-electrophoresis systems described under Materials and Methods. The two-dimensional NaDodSO$_4$ gel system was especially useful for the identification of protein S1 and for distinguishing between proteins S15, S16, and S17. Protein S1 was identical to that described by Subramanian et al. (1976). The purity and extent of any degradation occurring in the proteins were also observed by the use of the discontinuous NaDodSO$_4$-acrylamide slab gel (Figures 4 and 5). This method did not separate all of the 21 ribosomal proteins but was especially useful for the direct identification of several of the higher molecular-weight proteins. The stacking effect of the discontinuous system and the sensitivity of the Coomassie brilliant blue stain revealed components which could not be seen by the other gel methods employed. Most of the individual proteins contained faint traces of other proteins which could be detected on NaDodSO$_4$ slab gels but not on urea two-dimensional gels. The majority of the proteins were considered to be at least 90% pure.

For the most of the ribosomal proteins, an overestimation of the molecular weight is observed from the NaDodSO$_4$ gel as compared with that obtained from the primary sequence.

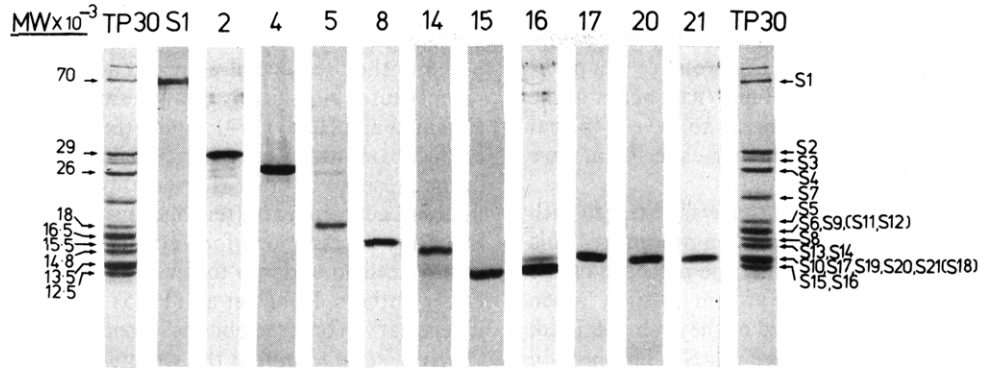

FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of the purified proteins obtained by the nondenaturing isolation procedure. Their positions relative to that of total TP-30 [extracted as described by Hardy et al. (1969)] are shown, together with the molecular weight obtained with reference to the standard proteins: bovine serum albumin (mol wt 68 000), pyruvate kinase (mol wt 57 000), ovalbumin (mol wt 43 500), aldolase (mol wt 40 000), glyceraldehydephosphate dehydrogenase (mol wt 36 000), carbonic anhydrase (mol wt 29 200), chymotrypsinogen (mol wt 25 700), myoglobin (mol wt 16 957), lysozyme (mol wt 14 319), haemerythrin (mol wt 13 600), and cytochrome *c* (mol wt 11 748).

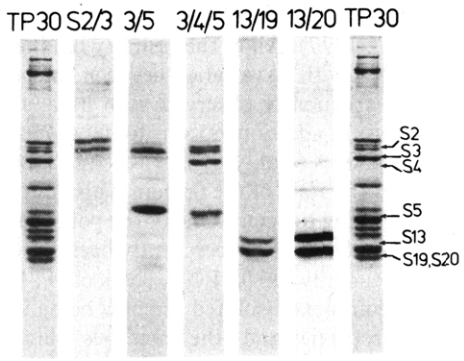

FIGURE 5: NaDodSO$_4$–polyacrylamide gel electrophoresis of potential protein–protein complexes obtained during the isolation procedure. These proteins could only be separated to a limited degree as described in the text. Their positions relative to the TP-30 are shown.

It should be possible, with so many of the amino acid sequences completed for the proteins from the ribosome, to make a calibration based on these absolute molecular weights. This approach also presents some complications, since several of the proteins do not migrate as expected on the basis of their molecular weight. A good example of this point is the protein S21, whose molecular weight based on the sequence is 8359. This protein migrates more slowly on the NaDodSO$_4$ gel than protein S15, which has a molecular weight of 10 000. Proteins S11, S12, and S18, which were not found during this method of isolation, were located on the NaDodSO$_4$ gel with proteins extracted with urea and acetic acid which were kindly provided by Dr. H. G. Wittmann.

A high-molecular-weight component (~90 000 mol wt) seen at the top of the gel containing TP-30 is thought to be a large acidic protein which is present on the 30S subunits. This has also been observed by Dr. Subramanian (personal communication), although its function is not known. Another protein band is observed in the TP-30 mixture which migrates between protein S2 and S3. There is no other known ribosomal protein with this molecular weight and it could be a degradation product of protein S2. The identity of this extra component is under investigation.

The purified proteins and protein mixtures were concentrated by the three methods described. Ultrafiltration methods were used initially but this method had the tendency to cause protein aggregation and, in addition, many of the proteins passed totally or partially through membranes with a reported molecular weight cutoff of 1000. Protein S4 with a molecular weight of 22 550 (Reinbolt and Schlitz, 1973) was the largest protein to pass through the membrane, along with proteins S13, S15, S16, S19, S20, and S21. This effect could be related to the shape of the protein. Protein S4 has been reported to be elongated (Paradies and Franz, 1976; Österberg et al., 1977) and could therefore pass through a membrane more easily than a more globular protein of the same molecular weight. Dialysis of protein solutions against poly(ethylene glycol) was avoided, except in cases where large volumes were involved. This was the most rapid method and the proteins tended to remain more soluble. However, even with the use of poly(ethylene glycol) 20 000 and Spectrapor 3 dialysis tubing, a certain amount of poly(ethylene glycol) passed the dialysis membrane and could not be removed by subsequent dialysis against buffer solutions. Dialysis against dry Sephadex G-150 was found to be very slow and time consuming but was the gentlest method employed. Very little precipitated protein was observed with this last method and hence it was used whenever feasible. Good results were also obtained by adsorbing the proteins at low ionic strength onto an ion-exchange gel and eluting them in a small volume of a high ionic strength buffer. This method was very useful for the concentration of the large volumes of protein solution obtained after the first CM-Sephadex column.

The use of standard Visking dialysis tubing with a molecular weight cutoff of 14 000 resulted in a loss of protein which passed across the membrane. Hence, Spectrapor 3 dialysis tubing with a molecular weight cutoff of 3500 was used for most samples. This greatly increased the yield of small proteins such as S20 and S21. Dialysis of the extracts before application to the ion-exchange columns was performed in 6-cm diameter Visking dialysis tubing from Serva because of the large volume of liquid involved. Spectrapor 3 was not made with such a large diameter. Not so much loss of small proteins through the membrane was observed with the mixture of proteins as was seen with dialysis of the pure protein sample.

Purified proteins were concentrated in buffer E to a protein concentration of 1–5 mg/mL. Proteins were fairly soluble at these concentrations even after freezing and thawing. Protein S2 was usually dialyzed against a buffer at pH 8.5 containing 0.05 M Bicine, 0.4 M LiCl, 0.006 M 2-mercaptoethanol, 2×10^{-5} M $PhCH_2SO_2F$, 4×10^{-5} M BAM before concentration, since the isoelectric point of this protein is at pH 6.7 (Kaltschmidt, 1971). Precipitated protein was seen in S2 samples on standing at 4 °C for 1 week at pH 5.6. Protein S8, at concentrations above 1 mg/mL, was more soluble if the salt concentration in buffer E was raised to 0.6 M LiCl.

Individual proteins were found to be much more soluble in

high than in low ionic strength buffers. A difference in the buffer salt affected the solubility of some of the proteins; for example, several were more soluble in the presence of a phosphate/KCl buffer at pH 7.0 than in a Hepes/KCl buffer at the same pH. The phosphate buffer appeared to have a special solubilizing effect on the protein. Further experiments are being performed to investigate this point.

A problem with proteolytic degradation was observed with several of the proteins during the extraction procedure. This could be overcome in most instances by the presence of the protease inhibitors used throughout the protein isolation. When the level of these inhibitors was reduced or they were omitted, the most susceptible protein to cleavage was S5. This protein was degraded into several discrete bands, as seen on the NaDodSO$_4$ slab gel. Migration on the two-dimensional gel system was affected slightly, since the degraded S5 produced an extended spot which moved slightly faster and further toward the negative side of the slab gel than the intact molecule. The other protein found to give a discrete fragment was S8. This produced a band migrating faster on the NaDodSO$_4$ gel with a molecular weight of 10 000. The problem of proteolysis was not as pronounced with the 30S proteins isolated as described above as was found with a similar isolation procedure for 50S proteins (Dijk, J., and Ackerman, I., manuscript in preparation). This could be attributed to a protease activity associated with the 50S ribosome subunit and not present on the 30S subunit or is more likely due to the difference in pH employed during the protein isolation, pH 5.6 opposed to pH 7.0. When a phosphate buffer system at neutral pH was used for 30S protein fractionation, proteolytic cleavage was substantially increased so that some proteins, for example, S4, were impossible to isolate as an intact molecule (Littlechild, J. A., manuscript in preparation).

Discussion

Using the method described it has been possible to isolate sufficient quantities of many of the 30S ribosomal proteins to initiate several physical studies. In addition, several potential protein–protein complexes were obtained in substantial amounts. A few proteins, however, such as S11, S12, and S18, were not obtained at all. It is not clear where these proteins are lost. All three appear to be very faintly stained on a two-dimensional gel of total protein extracted from our 30S subunits. Therefore, the possibility that they are lost before the protein-extraction step is being investigated.

The most striking difference between these so-called "native" proteins and those isolated with urea and acetic acid is their reversed solubility behavior. Proteins prepared by the former method were more soluble in high ionic strength (0.4–0.6 M LiCl) and less soluble in low ionic strength buffers, whereas proteins prepared by the denaturing procedure were more soluble at low ionic strength. This effect is indicative of a conformational change occurring in the protein molecules upon exposure to urea and acetic acid, possibly resulting in a change in the environment of hydrophobic residues. Removal of the denaturing agents could fail to result in a reversion to the original conformation, with the proteins continuing to have an increased number of exposed hydrophobic groups causing increased aggregation and reduced solubility at high ionic strengths. Proteins prepared by both methods have a tendency to aggregate, although this is more pronounced with the urea-treated proteins. The histones which could have properties similar to ribosomal proteins show similar solubility effects (Roark et al., 1974).

The increased solubility of the salt-extracted ribosomal proteins at high ionic strength enables physical studies to be performed under reconstitution conditions where the proteins are assumed to be in an active state. Ribosomal proteins prepared in the presence of urea and acetic acid can be used to reconstitute biologically active small ribosomal subunits (Nomura and Held, 1974). Under these conditions the proteins are incubated at low concentration and at elevated temperature in the presence of other ribosomal proteins, 16S rRNA, and high salt containing buffer solutions. These conditions would favor any necessary conformational change that the proteins would need to undergo to revert back to their native state. Other authors, Rohde et al. (1975) and Aune (1977), found it necessary in order to obtain ribosomal proteins in a so-called "native" state to unfold the structure with guanidinium hydrochloride before incubation at 37 °C in a reconstitution buffer. This procedure would unfold the polypeptide chain of the protein, which might previously have been trapped in an inactive state, so that the protein could be refolded into its native conformation.

By testing proteins prepared by the methods described in this paper, we have found several new RNA-binding proteins (Littlechild et al., 1977). All of the primary binding proteins bound to 16S RNA with no variation between one preparation and another, as frequently observed with urea/acetic acid extracted proteins. In addition, S5 was found to bind strongly and S2, S13, and S19 weakly to 16S RNA. These findings demonstrate that the state of the protein is also important for specific RNA binding to occur. One of the potential protein–protein complexes, S13–S19, has recently been shown to bind strongly and specifically to 16S RNA (Dijk et al., 1977). The other potential complexes isolated are now being studied for RNA-binding properties and other methods which will give evidence of a "native" protein association as present in the intact ribosome subunit. Recent analytical studies (Rohde et al., 1975; Aune, 1977) show the existence of complex formation between proteins S3–S5, S3–S4, and S4–S5. Much other evidence obtained with chemical cross-linking studies (Lutter et al., 1974; Sommer and Traut, 1976; Expert-Bezançon et al., 1977), fluorescent probes (Huang et al., 1975), and immunological location on the surface of the ribosome (Tischendorf et al., 1975; Lake and Kahan, 1975) of the potential protein–protein complexes is available. The ability to obtain these complexes using this gentle technique of protein isolation avoids, to some degree, the possibility that the structure of isolated proteins will be different from that present in situ on the 30S subunit, regardless of how gentle their extraction. Hence, the use of such protein complexes should aid our understanding of the three-dimensional arrangement of proteins in the ribosome.

Recent studies using protein magnetic resonance and circular dichroism measurements reveal that many of the salt-extracted proteins have a high degree of structure. This can be seen especially in the case of S4, S8, S14, S16, and S17 by ring-current-shifted resonances in the high-field region of the NMR spectrum, indicating the interaction of apolar methyl groups with aromatic residues which are involved in tertiary structure interaction within the protein molecule. These shifts are not observed, or only to a limited degree, with the respective proteins isolated by the conventional acetic acid/urea extraction procedure (Morrison et al., 1977; Morrison, C. A., and Littlechild, J. A., 1978, submitted for publication). We would like to interpret these results as an indication of the more "native" protein structure preserved in these salt-extracted proteins.

Low-angle X-ray diffraction studies, ultracentrifugation, diffusion, viscosity, and density measurements are being employed to investigate the shape of isolated proteins. A study on

protein S4 has been completed which shows this molecule to have a conformation very similar to a flat, elongated ellipsoid (Österberg et al., 1977). Many of the other ribosomal proteins have also been found to be elongated, such as S1 (Laugrea and Moore, 1977; Giri and Subramanian, 1977; Österberg et al., 1978), S15, and S20 (Österberg et al., 1978). Two exceptions to this rule are proteins S8 and S16 which can be classed as globular having axial ratios of less than 4:1 (Giri et al., 1977; Österberg et al., 1978).

This purification procedure for ribosomal proteins involves the handling of large volumes of subunit and protein solutions and is therefore time consuming. Despite these problems, the results obtained to date demonstrate that the proteins so obtained do retain more of their native structure. High salt concentrations especially of LiCl are known to perturb the tertiary structure of proteins (Maruyama et al., 1977). The procedure described employs concentrations of salt below that known to cause protein denaturation at pH values of 5.6–9.0.

Other physical studies and attempts to crystallize these proteins are now underway in the hope that they may aid our understanding of the three-dimensional structure of the small subunit from the *E. coli* ribosome.

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