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Separation of Ribosomal Proteins from *Escherichia coli* and Rabbit Reticulocytes Using Reverse-Phase High-Performance Liquid Chromatography†

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ABSTRACT: Reverse-phase high-performance liquid chromatography has been used to fractionate ribosomal proteins from *Escherichia coli* and rabbit reticulocytes. Different column packing materials and solvent systems were compared for their effectiveness with bacterial proteins. A large-pore (300 Å) short alkyl chain support (Altex RPSC) in conjunction with a triethylamine phosphate (pH 2.2)/acetonitrile solvent system was particularly effective and separated mixtures of total protein from each ribosomal subunit into a number of peaks approaching the actual number of proteins present. For example, with the use of the Altex RPSC column, the 21 proteins of 30S subunits were resolved into 18 distinct peaks, and the 33 proteins of the 50S subunits were resolved into 28 peaks. Overall recovery varied from 75% to 90% in different experiments. The composition of each peak was established by two-dimensional gel electrophoresis. Relatively acidic proteins, for example, S1 and L7/L12 of *Escherichia coli*, were bound more tightly to the column and recovered in lower yields than the other more basic proteins. Proteins that were incompletely resolved in a single step could be obtained in pure form by rechromatography on the same column with an altered gradient or with a different type of reverse-phase packing material. Ribosomal proteins from rabbit reticulocytes were also separated with good resolution and yield by using the RPSC column.

The determination of the protein topography of ribosomes from *Escherichia coli* using cross-linking with the reagent 2-iminothiolane is a major goal in this laboratory. The substantial number of cross-linked protein dimers established for 30S, 50S, and 70S particles (Sommer & Traut, 1976; Lambert & Traut, 1981; Lambert et al., 1983) represents an exhaustive investigation of the cross-links formed with this lysine-specific reagent. Identification of sites of cross-link formation within individual proteins can provide topographic information at higher resolution. This approach entails the purification of individual cross-linked dimers from the complex heterogeneous mixture of chemically modified ribosomal proteins. Such mixtures have proven difficult to resolve by using methods applied in the past for purifying ribosomal proteins such as ion-exchange chromatography. Before investigating the utility of reverse-phase high-performance liquid chromatography (HPLC)[1] for fractionating mixtures of cross-linked proteins, we first conducted an investigation of reverse-phase HPLC as a method for purifying non-cross-linked ribosomal proteins, and these results are presented here. We compared different column supports and solvent systems for the fractionation of ribosomal proteins from *Escherichia coli*. A system consisting of TEAP, pH 2.2, and acetonitrile on large-pore (300 Å) short alkyl chain supports (RPSC) gave the best results for most proteins. While this work was in progress, very similar results were published by Kerlavage et al. (1982, 1983). They employed a solvent system consisting of TFA and acetonitrile. We compared the relative efficacy of TFA and TEAP and found the latter slightly superior in some regards. The present work complements that of the other group, and the two systems represent alternative strategies for the purification of individual proteins. In addition, the applicability of the method for fractionating cross-linked proteins and eukaryotic ribosomal proteins is demonstrated.

Experimental Procedures

Triethylamine and TFA were from Aldrich. Triethylamine was redistilled twice from ninhydrin and stored under nitrogen at −20 °C. Trifluoroacetic acid was refluxed over CrO_3 (Allen, 1981) followed by distillation and storage under nitrogen at −20 °C. HPLC-grade acetonitrile, 1-propanol, and 2-propanol were from Burdick and Jackson. 2-Iminothiolane was from Serva. All other chemicals were reagent grade.

Isolation of Prokaryotic Ribosomal Proteins. Ribosomes were prepared from *Escherichia coli* strain MRE 600 as described by Hershey et al. (1977). Zonal centrifugation, using the method of Eikenberry et al. (1970), was used to prepare

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[1] Abbreviations: HPLC, high-performance liquid chromatography; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Bis-Tris, [bis-(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

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pure 30S and 50S subunits. Purified subunits, dissolved in 10 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 14 mM 2-mercaptoethanol, were extracted with 66% acetic acid according to the method of Hardy et al. (1969), and the supernatant protein was dialyzed exhaustively against 6% acetic acid and lyophilized.

Isolation of Eukaryotic Ribosomal Proteins. Ribosomal subunits were prepared from rabbit reticulocytes as described by Howard et al. (1975). Ribosomal proteins were extracted as described above.

High-Performance Liquid Chromatography. HPLC was performed on a system consisting of two Altex 100A pumps, a 421 controller, an Altex Model 155-00 variable-wavelength spectrophotometer, and a Kipp & Zonen BD 41 strip-chart recorder. The reverse-phase columns employed were the following: (1) Synchropak RP-P (C18) and RP-8 (C8) (0.41 × 25 cm); (2) Altex C18, C8, cyanopropyl, and RPSC (C3) (0.46 × 25 cm); (3) Baker diphenyl (0.46 × 25 cm); (4) Waters C18 (0.39 × 30 cm). Aqueous solvent systems consisted of either 25 mM TEAP, pH 2.2, or 0.1% TFA. Organic solvents used included acetonitrile, 1-propanol, and 2-propanol. In some cases, the organic solvents were made 0.1% in either H_3PO_4 or trifluoroacetic acid. All aqueous solvents were passed through a 1 × 24 cm Lichroprep RP-8 reverse-phase column (Alltech Associates) to remove trace contaminants, followed by filtration through a 0.2-μm Nylon 66 membrane (Alltech Associates) and thoroughly degassed. Additional details on solvent systems, flow rates, and gradient shapes used are given in the figure legends.

Lyophilized ribosomal protein samples were solubilized in 66% acetic acid, 6 M guanidine hydrochloride, or 6 M urea and injected immediately onto reverse-phase columns. The elution profiles and recoveries were identical. However, storage of samples dissolved in urea even for 12 h led to substantial loss of resolution and yield. Carbamylation of samples was excluded as a possible explanation of the effect of urea since treating the sample with 1% sodium dodecyl sulfate (65 °C for 15 min) followed by removal of the sodium dodecyl sulfate with acetone/HCl (Barritault et al., 1976) led to improved resolution. In all subsequent experiments lyophilized ribosomal proteins were resuspended in 66% acetic acid.

Ribosomal proteins collected from reverse-phase separations employing TEAP were desalted by drying the samples in a Speed Vac concentrator (Savant Instruments), resuspending them in 250 μL of water, and injecting them into a short (0.32 × 5 cm) Synchropak RP-8 column. A solvent system consisting of 0.1% TFA and 2-propanol with a gradient from 0% to 100% solvent B in 10 min was employed (Bradley et al., 1982). The salt-free protein eluted as a sharp peak following the TEAP front. Recoveries were quantitative.

Samples for rechromatography were not desalted but injected directly onto the reverse-phase column. Volumes varied from 50 to 500 μL.

The recovery was determined by collecting the total eluate from injected samples of 1 mg. Aliquots were removed and evaporated to dryness in a Speed Vac concentrator, resuspended in 0.8 mL of water, and assayed by using the method of Bradford (1976) with bovine serum albumin as the standard.

Identification of Proteins by Polyacrylamide Gel Electrophoresis. Ribosomal proteins fractionated by HPLC using the TEAP/acetonitrile solvent system were dialyzed overnight against 6% acetic acid by using a 28-chamber microdialysis unit (Bethesda Research Laboratories, Inc.) and lyophilized. When the TFA/acetonitrile solvent system was used, the protein fractions were taken to dryness in the Speed Vac concentrator. The samples were dissolved in 50 μL of freshly prepared 8 M urea, 20 mM Bis-Tris/acetate, pH 3.7, and 1% (v/v) 2-mercaptoethanol containing small amounts of 30S (25 μg), 50S (40 μg), 40S (40 μg), and 60S (80 μg) proteins to serve as markers. The 30S and 50S ribosomal proteins from *Escherichia coli* were identified according to the two-dimensional gel electrophoresis system of Kenny et al. (1979) except that the upper electrode buffer for the first dimension (20 mM Bis-Tris/acetate, pH 3.7) contained 0.02% (w/v) 2-mercaptoethylamine hydrochloride. Rabbit retiulocyte 40S ribosomal proteins were identified by using the two-dimensional gel electrophoresis system described by Tolan & Traut (1981) which employs 4% acrylamide in the first dimension and 15% acrylamide in the second dimension. Rabbit reticulocyte 60S ribosomal proteins were identified with a 4% acrylamide/urea gel in the first dimension and a 17.5% acrylamide/sodium dodecyl sulfate gel in the second dimension.

Cross-linked ribosomal dimers fractionated by HPLC were dialyzed, lyophilized, and then analyzed by two-dimensional diagonal sodium dodecyl sulfate gel electrophoresis (Kenny et al., 1979) with 13.5% (w/v) acrylamide in both the first and second dimensions. The identities of spots beneath the diagonal were deduced from the positions of known cross-links (Lambert et al., 1983).

Cross-Linking 30S Subunits. 30S subunits (3 mg/mL) solubilized in 50 mM triethanolamine hydrochloride, pH 8, 50 mM KCl, 1 mM $MgCl_2$, and 10 mM dithioerythritol were made 12 mM in 2-iminothiolane (from a 0.5 M stock consisting of equimolar 0.5 M triethanolamine and 0.5 M triethanolamine hydrochloride, pH 8). After 2.5 h at 0 °C, the reaction mixture was diluted to 1.5 mg/mL by the addition of a solution of 50 mM triethanolamine hydrochloride, pH 8, 50 mM KCl, 1 mM $MgCl_2$, and 10 mM dithioerythritol. The solution was then oxidized with 40 mM H_2O_2 for 0.5 h. Excess H_2O_2 was removed with catalase (10 μg/μL). After 0.25 h, 1 volume of a solution consisting of 80 mM iodoacetamide, 8 M urea, and 6 M LiCl was added and the mixture allowed to stand for 2 h. Ribosomal proteins were extracted with 66% acetic acid, dialyzed exhaustively against 6% acetic acid, and lyophilized.

Partial Reconstitution of 50S Subunits. Proteins L7/L12 were removed from 50S subunits by the method of Hamel et al. (1972). Approximately 250-μg aliquots of L7/L12 were dialyzed against 6% acetic acid and lyophilized. Aliquots were resuspended for 1 h in reconstitution buffer (10 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 14 mM 2-mercaptoethanol) containing 30% acetonitrile (70% 25 mM TEAP:30% acetonitrile) or 60% acetonitrile (40% 25 mM TEAP:60% acetonitrile). Samples were then dialyzed exhaustively against reconstitution buffer and recombined with aliquots of 50S subunits lacking L7/L12 (50S cores). The molar ratio of L7/L12 to 50S cores was 8, a 2-fold excess of L7/L12 compared to the original 50S particle. After 1 h at 0 °C, the samples were pelleted through a 20% (w/v) sucrose cushion (in reconstitution buffer), resuspended in reconstitution buffer, and extracted with 66% acetic acid. The reconstituted particles were analyzed by two-dimensional gel electrophoresis (Kenny et al., 1979) for the presence of proteins L7/L12.

Results

A number of column packings, including Waters C18 (60–100-Å pore), Baker diphenyl (300-Å pore), Synchropak RP-P and RP-8 (300-Å pore), Altex C18, C8, and cyanopropyl (each 60–100-Å pore), and Altex RPSC (300-Å pore), were tested. Columns with large-pore packings (300 Å) resolved

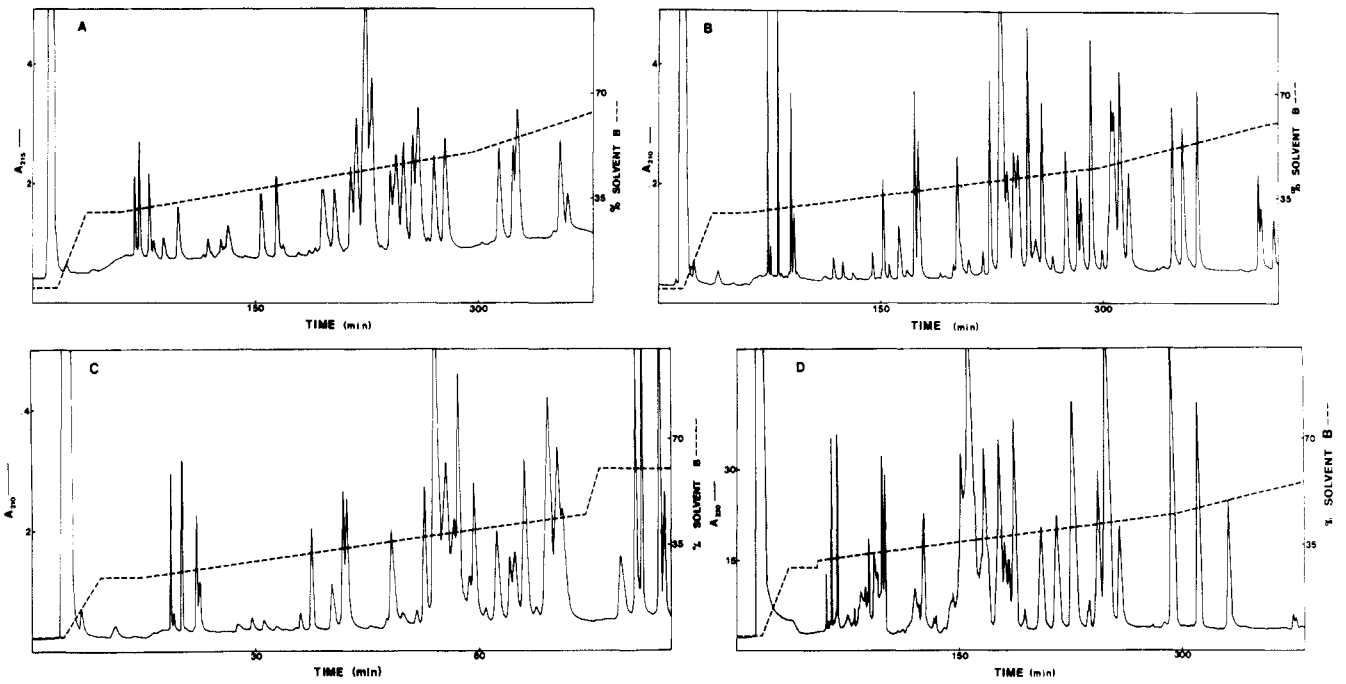


FIGURE 1: Reverse-phase chromatography of total 50S ribosomal proteins on an Altex RPSC column. (A) Solvent system A, 0.1% TFA; solvent system B, acetonitrile/0.1% TFA. Flow rate 0.25 mL/min. Sample load 300 μg. (B) Solvent system A, 25 mM TEAP, pH 2.2; solvent system B, acetonitrile/0.1% H_3PO_4. Flow rate 0.25 mL/min. Sample load 300 μg. (C) Solvent system same as (B). Flow rate 1 mL/min. Sample load 300 μg. (D) Solvent system same as (B). Flow rate 0.25 mL/min. Sample load 3 mg.

mixtures of ribosomal proteins better than small-pore packings (60–100 Å) of the same type and gave higher recoveries. The Altex RPSC column containing a short C3 alkyl chain packing material gave the best overall resolution of ribosomal proteins from the 30S and 50S subunits of *Escherichia coli*. Recoveries of 85% were obtained by using the Altex RPSC column compared to 70% by using the RP-P and RP-8 columns. Useful separations were also obtained by using the RP-P, RP-8, diphenyl, and cyanopropyl columns (see Figure 7 discussed later).

Acetonitrile, 1-propanol, and 2-propanol were tested as organic solvents. Acetonitrile was chosen as the most effective solvent on the basis of superior peak symmetry and resolving capacity (data not shown) and ultraviolet transparency (190-nm cutoff). Attention was focused on aqueous solvents based on phosphoric acid. The addition of ion pairing agents such as dodecylammonium, dimethylethylammonium, and triethylammonium gave improved resolution, and of these, triethylammonium was most effective. The ionic strength of TEAP used was important for obtaining optimum fractionation of ribosomal proteins. The best results were obtained at concentrations between 12.5 and 50 mM. Concentrations above 50 mM gave decreased retention times, lowered recoveries, and, in some cases, loss of the larger less basic ribosomal proteins (e.g., S1, S2, S3, and S4). Concentrations below 12.5 mM gave increased retention times and broadened peaks. Inclusion of 0.1% H_3PO_4 in the acetonitrile improved the resolution, in particular for late-eluting peaks. This standard TEAP/acetonitrile solvent system was used in all subsequent chromatographic runs.

TFA, an aqueous solvent known to be effective in resolving large peptides and proteins (Mahoney & Hermodson, 1980; Hermodson & Mahoney, 1981; Henderson et al., 1981), was also tested. We compared the two solvents under the same conditions (identical sample load, column type, flow rate, organic solvent). The TEAP solvent system was superior with respect to peak symmetry and resolving capacity (Figure 1A,B). A disadvantage of TEAP compared to TFA, its lack of volatility, is obviated by the ease with which protein samples containing TEAP can be rapidly desalted (less than 15 min) on small reverse-phase columns using a TFA/2-propanol solvent system (see Experimental Procedures).

Flow rates were varied between 0.1 and 1 mL/min. A flow rate of 1 mL/min was adequate for the resolution of many ribosomal proteins (Figure 1C), but significant improvement in protein peak separation resulted from a lower flow rate such as 0.25 mL/min (Figure 1B). At this flow rate, one separation took 7 h. Flow rates of less than 0.25 mL/min were judged too time consuming relative to the small increase in resolution obtained. In some instances, a flow rate of 0.1 mL/min resulted in improved resolution in one part of the chromatogram but decreased resolution in another. The lower flow rates were advantageous when large amounts of sample were injected.

The effect of increasing the amount of sample from 0.3 to 3 mg is shown in Figure 1D. There are losses in resolution, but the overall profile and peak separation remain unchanged. Samples of 5 mg of 30S ribosomal proteins were chromatographed without serious deterioration in the resolution of peaks (results not shown).

Chromatography on the Altex RPSC column of the total protein from *Escherichia coli* 30S subunits, which contain 21 different proteins, yielded 18 clearly separated peaks (Figure 2). The composition of each peak was determined by pooling fractions, dialyzing them against 6% acetic acid, and lyophilizing them. Lyophilized samples were mixed with a small amount (ca. 25 μg) of total 30S ribosomal proteins and analyzed by two-dimensional polyacrylamide gel electrophoresis used for the identification of ribosomal proteins. The purified proteins appeared as darkly stained spots relative to a lightly stained pattern of marker proteins. An example showing the identification of protein S2 is given in Figure 3A. All 21 30S ribosomal proteins were eluted from the RPSC column with an overall recovery of 85%. Qualitative analysis of individual peaks by gel electrophoresis indicated low recoveries for proteins S1, S2, S8, and S10. A quantitative analysis of the recovery of S1 was made with the protein radiolabeled with

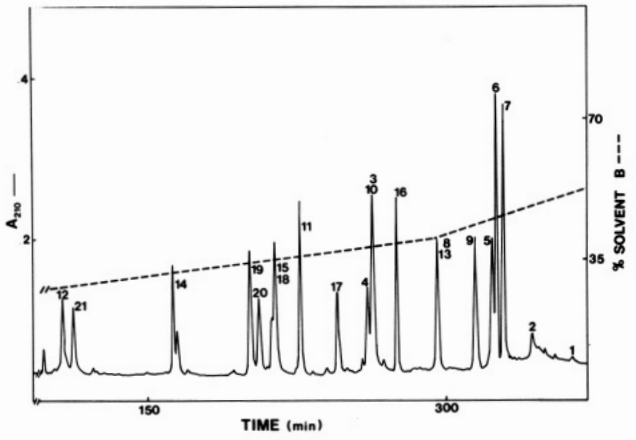


FIGURE 2: Fractionation of total 30S ribosomal proteins using an Altex RPSC column. Solvent system A, 25 mM TEAP, pH 2.2; solvent system B, acetonitrile/0.1% H_3PO_4. Flow rate 0.25 mL/min. Sample load 200 μg. Peaks were identified as described under Experimental Procedures.

iodo[^{14}C]acetamide and showed that the recovery from the RP-8 column was approximately 5%. The recovery of proteins S2, S8, and S10 was much improved by using diphenyl and cyanopropyl columns with TEAP/acetonitrile (data not shown).

Chromatography on the Altex RPSC column of the total protein from *Escherichia coli* 50S subunits, which contain 33 different proteins, yielded 28 clearly resolved peaks (Figure 4). The constituents in each peak were identified as described above. An example showing the identification of L20 is given in Figure 3B. All of the 50S proteins were recovered from the column with an overall recovery of 85%. Gel electrophoresis of individual peaks indicated much lower recoveries for L7/L12, L9, and L27. Substitution of TFA for TEAP improved the recovery of L9 and L27 (data not shown); chromatography on the cyanopaopyl and diphenyl columns with TEAP also resolved them with good yield (data not shown). The recovery of proteins L7/L12 was sensitive to the flow rate and gradient slope; recoveries were higher with steeper gradients (data not shown) and with flow rates of 1 mL/min as compared to 0.25 mL/min (Figure 1B,C).

Not all ribosomal proteins were obtained in pure form in a single chromatographic run. Impure proteins could be further purified by rechromatography on the same column using a more shallow gradient or on a different reverse-phase column. For example, proteins S5 and S6 are so close together in the chromatogram shown in Figure 5A that each is contaminated with the other; Figure 5B shows the complete separation of S5 and S6 obtained by rechromatography with a more shallow gradient. Some protein mixtures could not be resolved by rechromatography on the same column. For example, the peak (Figure 5C) containing ribosomal proteins L2, L13, L14, and L30 was not further resolved; however, chromatography on the diphenyl column resolved L30 and L2 from L13 and L14 (Figure 5D). L13 and L14 were separated by rechromatography on the Altex RPSC using a more shallow gradient (data not shown). Figure 6 illustrates a typical profile obtained with 30S and 50S ribosomal proteins on the Baker diphenyl andd Altex cyanopropyl columns. Strategies to purify mixed peaks can be deduced by comparing these separations with those for the RPSC column. For example, S13 and S8 can be resolved on the Altex cyanopropyl column while proteins S15 and S18, L1 and 5, and L15 and L16 can be resolved on the diphenyl column.

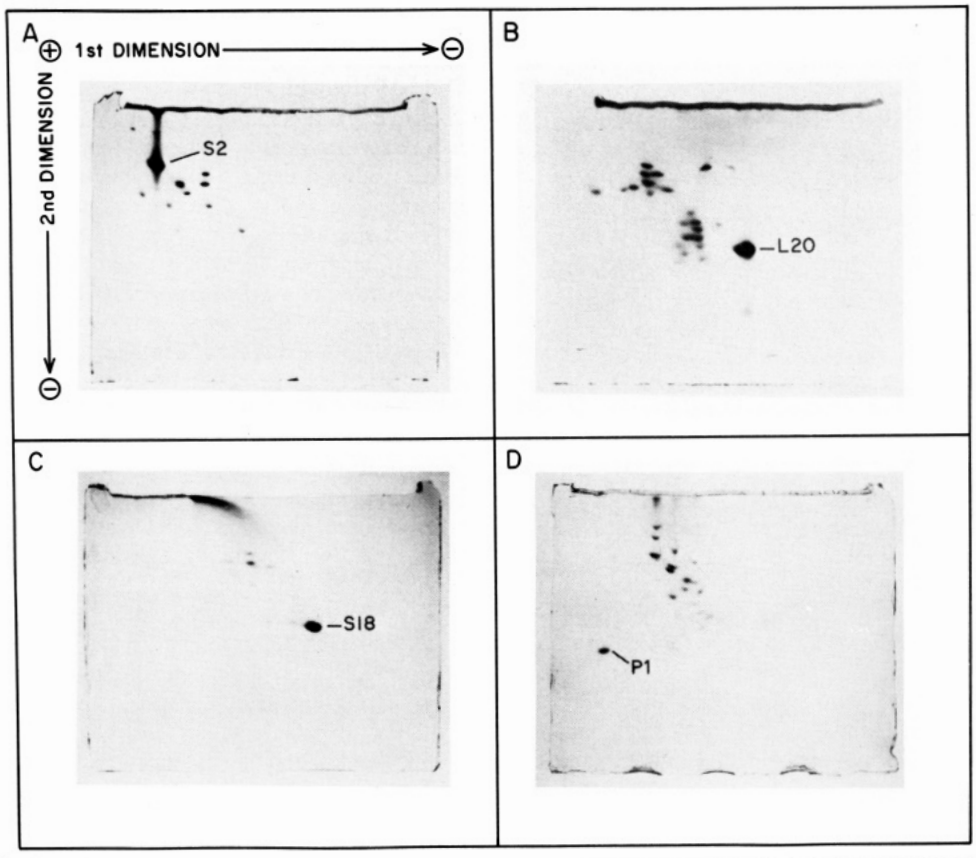


FIGURE 3: Identification of *Escherichia coli* ribosomal proteins S2 (A) and L20 (B) and rabbit reticulocyte ribosomal proteins S18 (C) and P1 (D) by two-dimensional polyacrylamide gel electrophoresis as described under Experimental Procedures. Overloaded spots represent the identified proteins. A light background of marker 30S, 50S, 40S, and 60S ribosomal proteins was added to calibrate the gels. In panel D, although protein P1 appears as dark as the added marker proteins, the identification is unambiguous because P1 is known to bind Coomassie blue very poorly (Madjar & Traut, 1980).

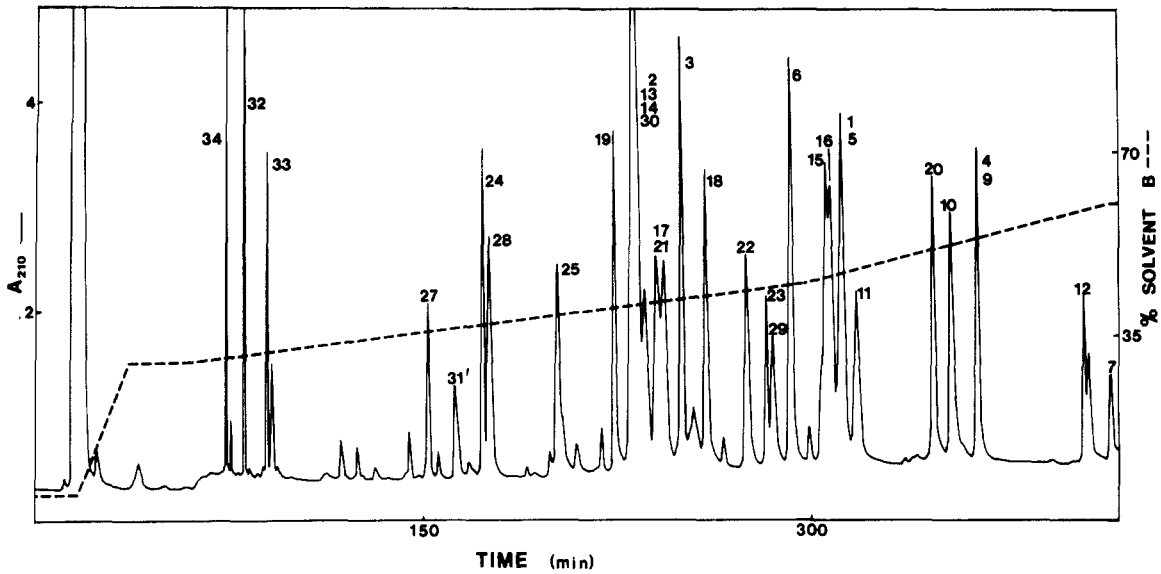


FIGURE 4: Fractionation of total 50S ribosomal proteins on an Altex RPSC column. Solvent system A, 25 mM TEAP, pH 2.2; solvent system B, acetonitrile/0.1% H_3PO_4. Flow rate 0.25 mL/min. Sample load 300 μg. Peaks were identified as described under Experimental Procedures.

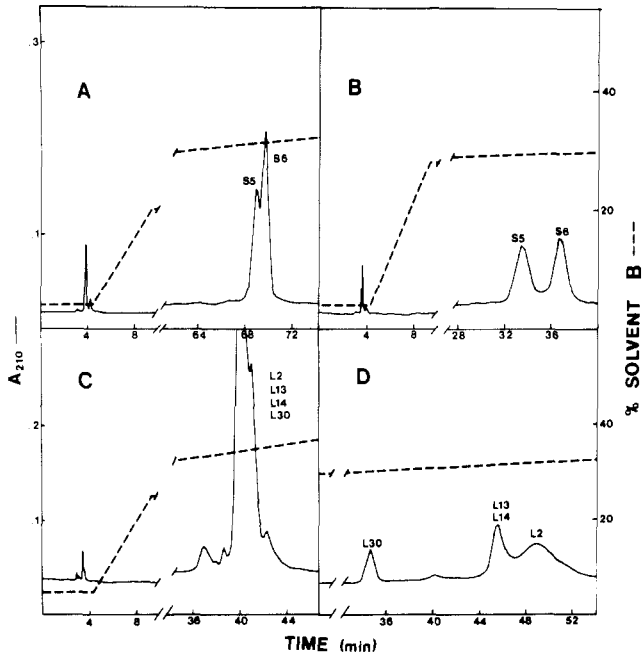


FIGURE 5: Rechromatography of incompletely resolved ribosomal protein peaks. Solvent system A, 25 mM TEAP, pH 2.2; solvent system B, acetonitrile/0.1% H_3PO_4. Flow rate 1 mL/min. (A) Incomplete resolution of 30S ribosomal proteins S5 and S6 on an Altex RPSC column. (B) Complete separation of S5 and S6 by rechromatography on the same column using a modified gradient. (C) Coelution of 50S ribosomal proteins L2, L13, L14, and L30 on an Altex RPSC column. (D) Partial separation of L2, (L13, L14), and L30 on a Baker diphenyl column.

Reverse-phase chromatography employs solvent systems which are potential protein denaturants. We investigated the question of whether native proteins could be recovered following their exposure to high concentrations of acetonitrile by assaying the capacity of proteins L7/L12 thus exposed to bind to 50S cores from which they were absent. Pure L7/L12 was dissolved in solutions of 30% acetonitrile and 60% acetonitrile for 1 h, and then the mixtures were tested for their activity in reassociating with 50S cores as described under Experimental Procedures. The putative reconstituted 50S particles were analyzed by two-dimensional polyacrylamide gel electrophoresis for the presence of L7/L12. Panels A and B of Figure 7 show proteins from L7/L12-deficient cores and from cores reconstituted with untreated L7/L12, respectively. The position of L7/L12 is indicated. Panels C and D show that L7/L12 exposed to 30% and 60% acetonitrile is still able to recombine with 50S cores to approximately the same extent as the untreated protein.

The applicability of reverse-phase HLPC for separating eukaryotic ribosomal proteins was investigated. Total proteins from 40S and 60S subunits from rabbit reticulocytes were separated by using the Altex RPSC column. Both TEAP/acetonitrile and TFA/acetonitrile solvent systems were effective in resolving the eukaryotic proteins. Recoveries of 40S and 60S ribosomal proteins varied between 85% and 95% on the RPSC column.

The separation of rabbit reticulocyte 40S proteins with the RPSC column using a TFA/acetonitrile solvent system is shown in Figure 8A. There are about 22 well-resolved peaks representing the 35 40S proteins. Many of these 22 peaks clearly contain more than one component. The peaks were analyzed in the same manner described for *Escherichia coli* proteins except for changes in the two-dimensional gel system. Figure 3C illustrates the identification of ribosomal protein S18. The elution position of 16 proteins could be assigned unambiguously while 8 others could only be approximately assigned due to coelution or overlap with other proteins on the identification gel. Nine proteins were not found in the eluate presumably because they bind tightly to the column in the manner of *Escherichia coli* S1 and are not eluted by the conditions thus far tested. The locations of the 24 proteins identified are shown in Figure 8A.

The 60S proteins separated by HPLC with the RPSC column using a TFA/acetonitrile solvent systems are shown in Figure 8B. There are approximately 25 well-resolved peaks representing the 45 60S proteins. Many of these 25 peaks clearly contain several components as indicated by poorly resolved peaks and asymmetric shoulders. Again, the peaks separated by valleys near the base line were analyzed by two-dimensional electrophoresis. An example showing the identification of ribosomal protein P1 is given in Figure 3D. The elution positions of some proteins were unambiguously assigned while others could only be approximately placed due to overlap on the identification gel. As with the 40S proteins, eight proteins could not be recovered presumably due to ag-

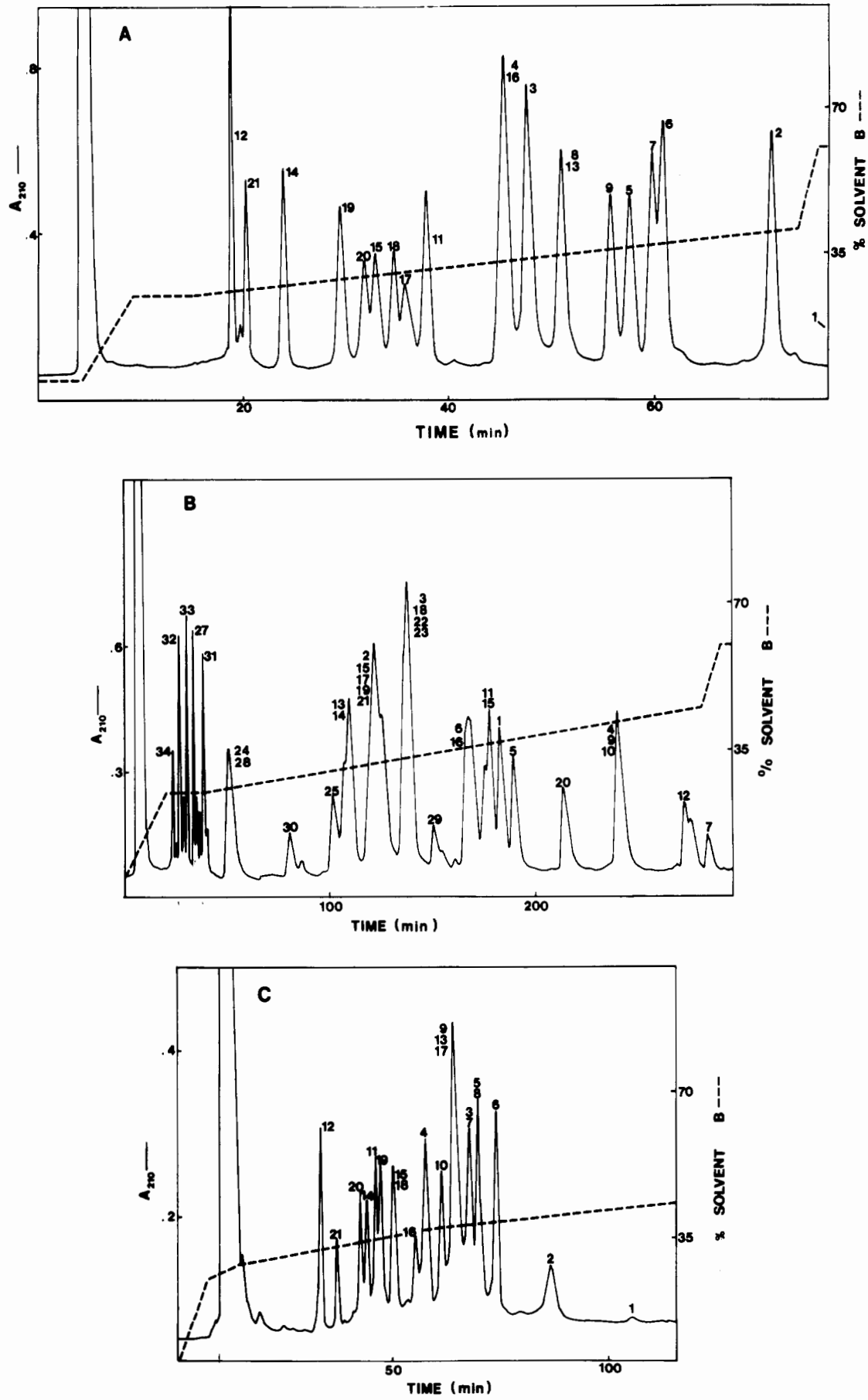


FIGURE 6: Alternative strategies for the fractionation of ribosomal proteins. (A) Total 30S ribosomal proteins (400 μg) chromatographed on a Baker diphenyl column. Solvent system A, 25 mM TEAP, pH 2.2; solvent system B, acetonitrile. Flow rate 1 mL/min. (B) Total 50S ribosomal proteins (1.5 mg) chromatographed on a Baker diphenyl column. Solvent system same as for (A). Flow rate 0.75 mL/min. (C) Total 30S ribosomal proteins (200 μg) chromatographed on an Altex cyanopropyl column. Solvent system A, 50 mM TEAP, pH 2.2; solvent system B, acetonitrile. Flow rate 0.7 mL/min.

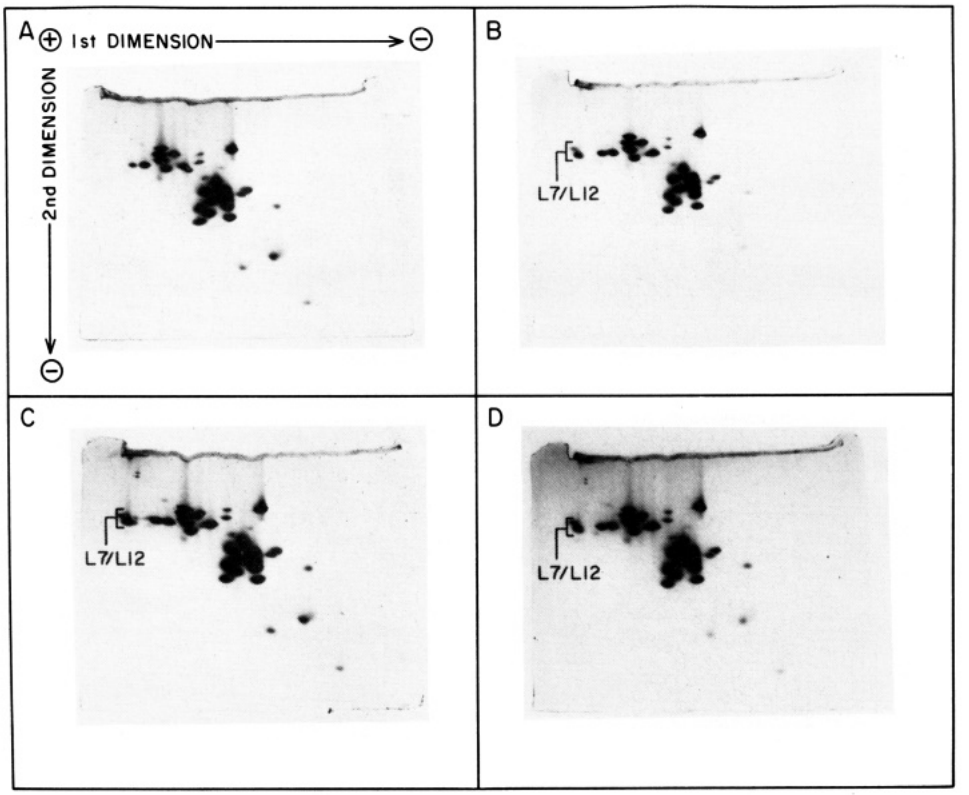


FIGURE 7: Two-dimensional gel electrophoresis of proteins from 50S particles reconstituted from 50S cores and TEAP/acetonitrile-treated L7/L12. (A) Proteins from 50S cores devoid of L7/L12. (B) Proteins from 50S particles reconstituted from 50S cores and untreated L7/L12. (C) Proteins from 50S particles reconstituted from 50S cores and L7/L12 treated with 70% TEAP and 30% acetonitrile. (D) same as (C) except L7/L12 was treated with 40% TEAP and 60% acetonitrile.

gregation or binding so tightly as to reduce recovery below the sensitivity of the electrophoretic analysis. The locations of the 37 proteins identified are shown in Figure 8B.

There were problems encountered with eukaryotic proteins not observed with the *Escherichia coli* proteins. There was evidence of aggregation of proteins, and sample amounts in excess of 1 mg gave increased overall losses. Many more proteins were not recovered from the column. Several proteins (i.e., L27, L27a, L32, and L35) eluted in more than one position, due likely to multiple interactions with other proteins. The brackets indicate proteins which were not separated by the gel system. This behavior has been observed with proteins of both ribosomal subunits not only from rabbit reticulocytes but also from yeast and *Artemia salina*. The phenomenon was sensitive to the starting percent of acetonitrile and decreased notably when the starting concentration was raised from 5% to 15% (results not shown).

Modification of ribosomal proteins with 2-iminothiolane and oxidation to form disulfide cross-links produce a highly complex mixture due to the heterogeneity of modification and cross-link formation. The addition of the relatively hydrophobic modifying groups increases the tendency of the proteins to aggregate and stick tightly to many of the solid supports used in conventional ion-exchange and molecular sieve chromatography. The reverse-phase HPLC systems developed with unmodified ribosomal proteins were tested with modified and cross-linked 30S ribosomal proteins from the *Escherichia coli*. The results are shown in Figure 9. Modification (panel A), which should be compared to Figure 2 run under the same conditions, results in deterioration of peak separation with peak broadening but maintains a pattern which can be related to that of the control. Oxidation (panel B) produces additional change in the profile, further loss of resolution with concomitant broadening, formation of new peaks, and elevation of base-line absorbance. A sample containing 5 mg of cross-linked 30S protein was chromatographed on the Synchropak RP-P column. The eluate was divided into fractions as illustrated in Figure 9. Each fraction was dialyzed overnight against 6% acetic acid and lyophilized. Samples were resuspended in 100 mM Tris-HCl, pH 6.8, and 4% sodium dodecyl sulfate and analyzed by diagonal polyacrylamide gel electrophoresis. Examples of a diagonal gel analyses of fractions F, M, O, and S are shown in Figure 10, panels A, B, C, and D, respectively. The patterns are considerably simplified compared to that of total cross-linked proteins. The identities of certain pairs are clear by comparison to complete diagonal analyses of cross-links published previously (Lambert et al., 1983). The protein pairs S18–S21 (panel A), S13–S19 (panel B), S6–S18 (panel C), and S7–S9 (panel D) are indicated by the arrows.

Discussion

While time and costs precluded a complete systematic testing of all combinations of several parameters, we have shown the general efficacy of reverse-phase HPLC and defined conditions for separating ribosomal proteins from *Escherichia coli* and rabbit reticulocytes.

We compared reverse-phase column packing materials of different types and pore sizes for their effectiveness in separating mixtures of ribosomal proteins. Short (C8 and C3) alkyl chain columns of large pore size gave increased resolving capacity, due to greater effective mass transfer, as well as higher loading capacities when compared to long-chain (C18), small pore size (60–100 Å), and alternative packing materials (cyanopropyl, diphenyl). The usefulness of these columns in fractionating proteins and peptides has been demonstrated (Pearson et al., 1981; Nice et al., 1981; Wilson et al., 1982; O'Hare et al., 1982; Cooke et al., 1983). We compared two

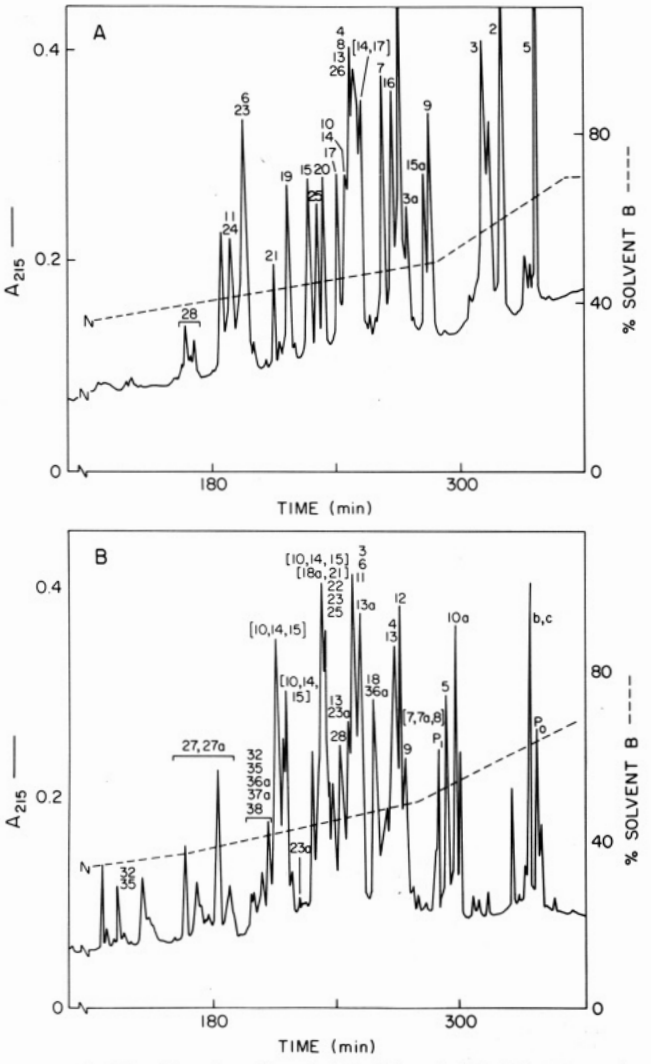


FIGURE 8: Fractionation of total 40S (A) and 60S (B) eukaryotic ribosomal proteins on an Altex RPSC column. Solvent system A, 0.1% TFA; solvent system B, acetonitrile/0.1% TFA. Flow rate 0.25 mL/min. Sample load 400 (A) and 500 μg (B). Peaks were identified as described under Experimental Procedures. Peaks bounded by horizontal brackets contain proteins which elute in several peaks, possibly due to aggregation. Protein identifications grouped within square brackets, e.g., [10,14,15], indicate ambiguity in the identification of a particular peak due to the inability of the two-dimensional gel system to adequately resolve these proteins. Peaks labeled b and c are proteins found in our preparation of rabbit reticulocytes and have not been observed elsewhere in the literature.

aqueous solvents, TFA and TEAP, with acetonitrile as the organic solvent. Both systems were effective; the TEAP system gave slightly superior overall separations of 30S and 50S total protein when tested with the RPSC (C3, 300 Å) column as judged by the number and sharpness of individual peaks. The two methods present useful alternatives for separating specific proteins, since the composition of peaks that contain multiple components is often different. Useful separations at high flow rates (1 mL/min) were obtained; however, slower flow rates (0.25 mL/min) gave substantially sharper peaks, consistent with the results of Lewis et al. (1980) and Cooke et al. (1983). Up to 5 mg of total 30S or 50S protein has been separated with resolution essentially the same as that shown in Figure 1D with 3 mg of protein.

Chromatography of the 21 proteins of total 30S protein with the TEAP/acetonitrile solvent and the RPSC column gave 18 clear peaks, from which 11 proteins could be obtained in pure form. Chromatography of the 33 proteins of total 50S protein gave 28 clear peaks, from which 21 proteins could be recovered

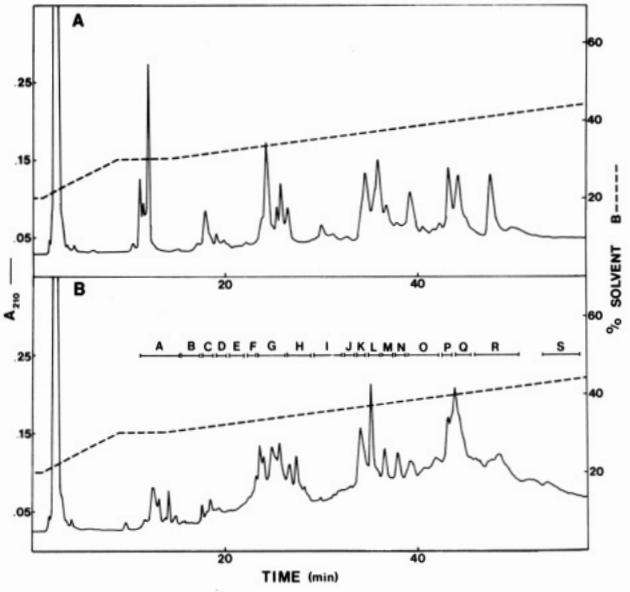


FIGURE 9: Fractionation of 200 μg of 30S ribosomal proteins modified with 2-iminothiolane (A) and 30S ribosomal proteins modified with 2-iminothiolane and oxidized (B) on a Sychropak RP-P column. Solvent system A, 25 mM TEAP, pH 2.2; solvent system B, acetonitrile. Flow rate 0.25 mL/min. (B) Fractions were collected, pooled, and analyzed by diagonal gel electrophoresis as described under Experimental Procedures.

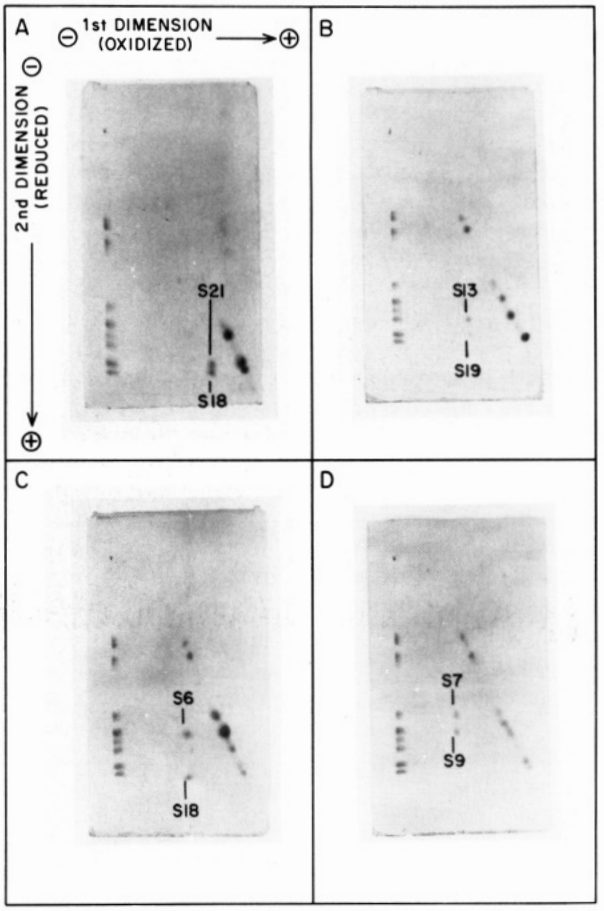
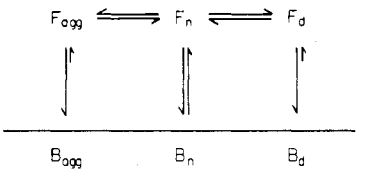


FIGURE 10: Diagonal gel electrophoresis of aliquots F (A), M (B), O (C), and S (D) from Figure 9 illustrating the presence of cross-links S18–S21 (A), S13–S19 (B), S6–S18 (C), and S7–S9 (D).

in pure form. Rechromatography on the same or a different column afforded purification of unresolved peaks.

Protein recovery is influenced by both flow rate and gradient slope (O'Hare et al., 1982). L7/L12 are examples of ribo-

somal proteins markedly affected by these two parameters. The following scheme is an attempt to rationalize the effects of gradient slope and flow rate on recovery:



Native protein initially bound to the column support (B_n) is released as free native protein (F_n) by organic solvent. The native form can be converted to a denatured form (F_d) or to an aggregated form (F_{agg}) which binds more tightly (B_d and F_{agg}), if not irreversibly, to the stationary phase. If the gradient slope is too shallow or the flow rate is slow relative to the rate at which conversion takes place, then recoveries of protein will be low. With steeper gradient slopes or higher flow rates, F_n may effectively be removed from the stationary phase before appreciable conversion to high-affinity conformers occurs.[2]

Reverse-phase HPLC of ribosomal protein presents diverse applications which include the analysis and identification of radiolabeled and affinity-labeled proteins (Kerlavage et al., 1982, 1983), the rapid purification of individual proteins for chemical analysis or reconstitution, the fractionation of cross-linked protein dimers within the mixture of cross-linked and monomeric proteins extracted from cross-linked ribosomes, and the comparison with respect to retention times of protein from different eukaryotic sources.

Kerlavage et al. (1983) have shown that total 30S protein eluted with TFA/acetonitrile was active in the reconstitution of 30S ribosomal subunits. We have shown here that protein L7/L12 retain the capacity to reconstitute protein-deficient cores following prolonged exposure to 60% acetonitrile. The total reconstitution of 50S particles has not yet been attempted. The loss or recovery in low yield of certain proteins limits the success of such total reconstitution experiments.

Preliminary experiments presented here have shown the applicability of HPLC for the fractionation of ribosomal proteins from rabbit reticulocytes. We have also obtained good separations of ribosomal proteins from *Artemia salina* and yeast. Presently, the purification of eukaryotic ribosomal proteins entails ion-exchange chromatography in the presence of urea (Tsuragi et al., 1976, 1977), a very time-consuming procedure. Reverse-phase HPLC represents a rapid and simple method for analytical investigations as well as semipreparative purification of eukaryotic ribosomal proteins of interest.

Preliminary results show the feasibility of using HPLC for fractionating mixtures of cross-linked proteins from both *Escherichia coli* and rabbit reticulocyte ribosomes. We have not found conditions for obtaining individual dimers separated from monomeric proteins by reverse-phase HPLC alone, but significant purification has been achieved. It seems likely that other procedures such as high-performance gel permeation chromatography prior to reverse-phase HPLC will be useful in obtaining pure cross-links.

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[2] Private communication from Dr. Ben Archer.

Registry No. Altex RPSC, 90669-96-8.

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