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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF *ESCHERICHIA COLI* RIBOSOMAL PROTEINS

CHARACTERISTICS OF THE SEPARATION OF A COMPLEX PROTEIN MIXTURE

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SUMMARY

We have previously reported the application of reversed-phase high-performance liquid chromatography (RP-HPLC) to the separation of *Escherichia coli* ribosomal proteins (A. R. Kerlavage, L. Kahan and B. S. Cooperman, *Anal. Biochem.*, 123 (1982) 342–348; A. R. Kerlavage, T. Hasan and B. S. Cooperman, *J. Biol. Chem.*, in press). In the present studies RP-HPLC is shown to yield much greater resolution of these proteins than does size-exclusion HPLC. In addition, we report on various aspects of RP-HPLC of ribosomal proteins including column capacity, resolution, reproducibility, recovery, separation of irreversibly denatured protein, and analysis of affinity-labeled ribosomal protein. The capacity of analytical columns was found to range from several micrograms to several milligrams with minimal loss in resolution and highly reproducible retention values. Recovery varied from protein to protein and ranged from 27% to 91%, with an average total protein recovery of 70%. The partitioning of several proteins between two peaks was shown to be due to irreversible denaturation of a small fraction. Finally, the utility of RP-HPLC in the study of the ribosome was demonstrated by analyses of [^{3}H]puromycin-labeled ribosomal proteins, and the demonstration that labeling slightly alters protein elution.

INTRODUCTION

The *E. coli* ribosome is a large (molecular weight, $M_r = 2.3 \cdot 10^6$) ribonucleoprotein complex[1] containing a total of 53 proteins in two dissimilar subunits. The large subunit has a sedimentation coefficient of 50S and contains 33 proteins designated L1-L34 (the species originally designated L8 turned out to be a complex of L10 and L7/L12[2]). The small subunit has a sedimentation coefficient of 30S and contains 21 proteins designated S1-S21. All of the proteins are present in a single copy on the

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ribosome with the exception of the acidic proteins L7 and L12. L7 is the NH_2-terminal acetylated form of L12[3] and a mixture of these proteins (designated L7/L12), consisting of four total copies, is present on the ribosome[2]. Finally, proteins L26 and S20 are identical[4], one copy being partitioned between the 50S and 30S subunits[5]. Resolution of the proteins of the 50S and 30S subunits, designated TP50 and TP30*, respectively, is a challenging problem because of the large number of proteins in each group and because of their general similarities in both molecular weights and isoelectric points (Table I). Elsewhere[7,8] we have shown that reversed-phase high performance liquid chromatography (RP-HPLC) is successful in resolving the 33 proteins of the 50S subunit into 22 peaks and the 21 proteins of the 30S subunit into 17 peaks, and have identified the proteins in these peaks. In the current work we demonstrate the superiority of RP-HPLC to size-exclusion HPLC (HPSEC) and examine in detail several features of RP-HPLC as applied to ribosomal protein separation. These include the effect of sample load on resolution, the extent of protein recovery following HPLC analysis, the resolution of irreversibly denatured from reversibly denatured protein, and the utility of the method in analyzing ribosomal proteins derived from photoaffinity labeled ribosomes.

EXPERIMENTAL

Materials

Sequanal grade trifluoracetic acid and HPLC grade acetonitrile were purchased from Fisher Scientific. Coomassie Brilliant Blue G-250 was purchased from Sigma. [^{3}H]Puromycin (2.7 Ci/mmol) was prepared from [^{3}H]puromycin aminonucleoside (Amsterdam) by a method exactly paralleling that described previously for *p*-azidopuromycin[9]. All other chemicals were reagent grade.

Isolation of ribosomal proteins

70S ribosomes were prepared from *E. coli* Q13 bacteria harvested in mid- or late-log phase using the modification of the Traub *et al.*[10] procedure previously described[11]. Ribosomal subunits were prepared by sucrose-gradient centrifugation as described previously[11], using buffer A [50 m*M* Tris–HCl (pH 7.6 at 4°C); 50 m*M* KCl; 1 m*M* $MgCl_2$; 6 m*M* 2-mercaptoethanol]. Protein was extracted from 50S and 30S particles using the Mg^{2+}/acetic acid procedure of Hardy *et al.*[12] and was precipitated with acetone[13]. Precipitates were dissolved in buffer B [6 *M* urea; 150 m*M* LiCl; 10 m*M* H_3PO_4 (adjusted to pH 8.0 with methylamine); 3 m*M* 2-mercaptoethanol] prior to HPLC injection.

High-performance liquid chromatography

The HPLC system consisted of one 6000 A pump, one M-45 pump, a 660 programmer, and a U6K universal injector, all from Waters Assoc. Column eluate was monitored for UV absorbance using a Waters extended-wavelength module (214 nm) and a Model 440 absorbance detector (280 nm) connected in series. Each was equipped with a 15.5-μl cell with a 1-cm path length.

HPSEC. HPSEC was performed using three different sets of columns: (1) two

* TP50 and TP30 = total protein from 50S and 30S ribosomal subunits, respectively.

Waters I-60 protein analysis columns (each 300 × 7.8 mm I.D.) in series: the eluents were (a) 6 *M* urea, 0.2 *M* formic acid, pH 4.0 or (b) 20% acetic acid, pH 2.0; (2) Waters I-125 and I-60 protein analysis columns (each 300 × 7.8 mm I.D.) in series: the eluent was 10% acetic acid, pH 2.5; (3) a Beckman Spherogel TSK-2000 SW column (600 × 7.5 mm I.D.): the eluents were (a) 6 *M* urea, 0.2 *M* KCl, 6 m*M* 2-mercaptoethanol or (b) 0.02 *M* sodium acetate, 0.1 *M* KCl, pH 4.5, or (c) 0.05 *M* Tris, 0.1 *M* KCl, 1.7 m*M* 2-mercaptoethanol. Chromatography was performed at room temperature. Flow-rates were varied between 0.3 and 1.0 ml/min.

RP-HPLC. RP-HPLC was performed on a SynChropak RP-P C_{18}-silica column (6.5-μm silica, 300 Å pore, 250 × 4.1 mm I.D.; SynChrom). Proteins were eluted at room temperature using gradients as described in the figure legends. Solvents were: (A) 0.1% (w/v) F_3CCOOH in water, pH 2.14; (B) 0.1% (w/v) F_3CCOOH in acetonitrile. The M-45 pump was used to deliver Solvent A and the 6000 A pump was used to deliver Solvent B at a combined constant flow-rate of 0.7 ml/min at column pressure between 500 and 1500 p.s.i. Solvent A was prepared from deionized, reversed-osmosis purified water which was filtered twice under aspirator vacuum through 0.45-μm Metricel filters (Gelman) placed in a 0.5-μm sintered-glass Millipore vacuum filter. F_3CCOOH was added after filtration. Acetonitrile was filtered once through 0.2-μm nylon-66 filters (Rainin Instruments) prior to addition of F_3CCOOH. Solvents were stirred constantly during chromatography and degassed approximately every 24 h by filtration (see above). Columns were returned to initial conditions by a 10-min linear gradient at a flow-rate of 2 ml/min and equilibrated for an additional 10 min at 2 ml/min.

Protein recovery determination

TP50 (2.3 mg) in 40 μl Buffer B was injected onto a SynChropak RP-P column and eluted as described in Fig. 4. Each peak was collected separately into a Polyallomer tube (Thomas Scientific) and lyophilized. The lyophilized protein in each tube was dissolved in 110 μl of 0.1% F_3CCOOH with swirling. An aliquot from each tube (50 μl) was transferred to a second Polyallomer tube and assayed for protein by the Coomassie Brilliant Blue G-250 assay of Bradford[14]. A composite TP50, prepared by combining a second aliquot (50 μl) from each tube (total volume 1.35 ml), was rechromatographed on the same column. Peaks were collected and lyophilized as above. To each tube was added 100 μl of 0.1% F_3CCOOH and protein assays were performed directly in each tube as above. Percent recovery was calculated as the ratio of the amount of protein in a peak after HPLC to that applied to the column. Recoveries of 30S proteins (starting material: 1.0 mg TP30 —see Fig. 5) were determined in the same manner.

Preparation of puromycin-labeled subunits

70S ribosomes (100 A_{260}/ml) were photolyzed for 120 min in the presence of 0.1 m*M* puromycin with 3500-Å lamps as described in Jaynes *et al.*[11]. Subunits were separated on high salt 15–30% sucrose gradients. Proteins were extracted with acetic acid and precipitated with acetone as described by Goldman *et al.*[15]. Care was taken to store material at −80°C when possible, as some loss of ^3H label, especially from S14, occurred on storage of proteins at −20°C. Proteins from 150–250 pmoles of

TABLE I

PHYSICAL PROPERTIES AND ELUTION CHARACTERISTICS OF *E. COLI* RIBOSOMAL PROTEINS

Protein	*Molecular weight**	*Isoelectric point***	$\alpha \pm$ *S.D.****	*Acetonitrile at elution (%)*	*Recovery (%)* [§]
L1	24,599	9.2	1.78 ± 0.02	41.0	63
L2	29,416	>12.0	1.31 ± 0.02	37.0	66
L3	22,258	9.7	1.31 ± 0.02	37.0	66
L4	22,087	7.6	2.60 ± 0.02	48.0	58
L5	20,171	9.4	1.86 ± 0.02	41.5	64
L6	18,832	10.0	1.66 ± 0.02	40.0	66
L7	12,220	4.8	2.79 ± 0.04	57.5	27
L9	15,531	6.4	1.66 ± 0.02	40.0	66
L10	17,737	7.5	2.60 ± 0.02	48.0	58
L11	14,874	9.7	1.72 ± 0.03	40.5	82
L12	12,178	4.9	2.76 ± 0.04	56.0	43
L13	16,019	10.1	1.24 ± 0.02	36.5	84
L14	13,541	12.3	1.17 ± 0.02	36.0	82
L15	14,981	>12.0	1.66 ± 0.02	40.0	66
L16	15,296	>12.0	1.66 ± 0.02	40.0	66
L17	14,364	11.0	1.31 ± 0.02	37.0	66
L18	12,770	12.0	1.35 ± 0.02	37.5	63
L19	13,002	>12.0	1.17 ± 0.02	36.0	82
L20	13,366	>12.0	2.21 ± 0.02	44.0	68
L21	11,565	8.2	1.24 ± 0.02	36.5	84
L22	12,227	11.5	1.43 ± 0.02	38.3	85
L23	11,013	9.6	1.43 ± 0.02	38.3	85
L24	11,185	10.7	0.78 ± 0.01	31.8	90
L25	10,694	9.4	1.03 ± 0.01	34.5	89
L26	9553	n.d. [§§]	1.00	34.3	82
L27	8993	>12.0	0.46 ± 0.01	27.5	81
L28	8875	n.d.	0.84 ± 0.01	32.5	83
L29	7274	10.0	1.48 ± 0.01	38.8	77
L30	6411	>12.0	1.17 ± 0.02	36.0	82
L32	6315	11.3	0.28 ± 0.01	24.0	73
L33	6255	>12.0	0.35 ± 0.01	25.5	83
L34	5381	n.d.	0.23 ± 0.01	23.0	81
S1	61,159	<7.6	2.60 ± n.d.	48.0	53
S2	26,613	6.7	2.11 ± n.d.	43.5	50
S3	25,852	12.0	1.48 ± 0.01	38.8	75
S4	23,137	10.4	1.43 ± 0.01	38.3	71
S5	17,515	9.9	1.69 ± 0.01	40.3	78
S6	15,704	4.9	1.72 ± 0.01	40.5	78
S7	19,732	12.2	1.78 ± 0.02	41.0	71
S8	13,996	9.1	1.43 ± 0.02	38.3	71
S9	14,569	>12.0	1.69 ± 0.02	40.3	78
S10	11,736	7.9	1.38 ± 0.02	37.8	71
S11	13,728	>12.0	1.03 ± 0.00	34.5	78
S12	13,606	>12.0	0.55 ± 0.01	28.8	84
S13	12,968	>12.0	1.48 ± 0.01	38.8	75
S14	11,063	>11.0	0.82 ± 0.01	32.0	82
S15	10,001	>12.0	1.05 ± 0.00	34.8	69
S16	9191	11.6	1.38 ± 0.01	37.8	71

TABLE I *(continued)*

Protein	*Molecular weight**	*Isoelectric point***	$\alpha \pm S.D.$***	*Acetonitrile at elution (%)*	*Recovery (%)* §
S17	9573	9.7	1.17 ± 0.01	36.0	86
S18	8896	>12.0	1.07 ± 0.00	35.0	64
S19	10,299	>12.0	0.94 ± 0.01	33.8	84
S20	9553	>12.0	1.00	34.3	75
S21	8369	>12.0	0.69 ± 0.01	30.8	91

* From ref. 1.

** From ref. 6.

*** Values are relative to S20 (= L26). Standard deviations were calculated from: L proteins, four analyses over a period of several weeks; S proteins, six consecutive analyses.

§ Average reproducibility was ±6%.

§§ n.d. = Not determined.

subunits were taken up in 0.1% F_3CCOOH just prior to chromatography. Eluted fractions were collected in 7-ml polypropylene vials (Wheaton) and adjusted to constant acetonitrile concentration (final volume 0.91 ml)*. Following addition of 5.5 ml of the 25% Triton/toluene cocktail, described by Jaynes *et al.*[11], samples were counted in an Intertechnique SL30 liquid scintillation counter.

RESULTS AND DISCUSSION

Choice of HPLC system

The most commonly used HPLC systems for protein separations are ion exchange (HPIEC), size exclusion (HPSEC), and reversed-phase (RP-HPLC) (as reviewed in refs. 16 and 17). Due to the highly basic nature of ribosomal proteins (Table I), we decided not to attempt HPIEC to resolve ribosomal proteins. Several conditions were explored for resolving proteins from the 30S subunit by HPSEC. An example of the best resolution obtained is shown in Fig. 1, in which seven peaks can be discerned. Because 20 of the 21 30S proteins have molecular weights between 8000 and 27,000 daltons (Table I) our judgement is that resolution by HPSEC superior to that seen in Fig. 1 may prove difficult to achieve.

On the other hand, ribosomal proteins are well resolved by RP-HPLC, in which separation is based on protein hydrophobicity. Chromatograms for TP50 and TP30 are shown in Fig. 2. The 33 50S proteins are resolved into 22 peaks and the 21 30S proteins are resolved into 17 peaks. The identities of the proteins within these peaks have been published elsewhere[7,8]. All of the known ribosomal proteins are identified in these chromatograms, with the exception of L31, a protein which has previously been isolated in only small amounts and is difficult to identify by polyacrylamide gel electrophoresis (PAGE)[5]. Conversely, three peaks, eluted at 24 min, 36 min, and 40 min in the TP50 chromatogram, do not contain known ribosomal

* There is a weak dependency of ^3H-counting efficiency on acetonitrile concentration. Under our conditions, the following dependence was observed (%CH_3CN in samples, ^3H-counting efficiency relative to no CH_3CN (1.00)): 15%, 0.96; 25%, 1.04; 35%, 1.05; 45%, 1.03; 55%, 1.04; 65%, 0.91.

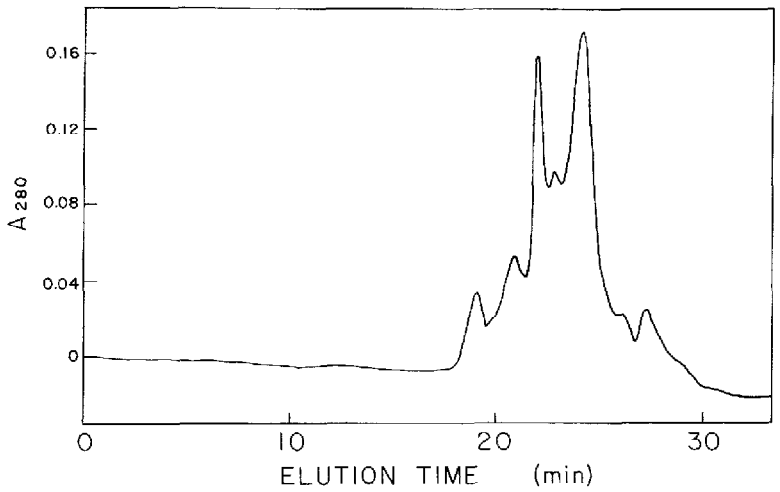

Fig. 1. HPSEC of TP30. A solution of 620 μg of TP30 in 200 μl of 10% acetic acid was chromatographed on Waters protein analysis columns (I-125 followed in tandem by I-60). Proteins were eluted with 10% acetic acid (pH 2.5) at a flow-rate of 1 ml/min.

proteins. The minor, unlabeled peaks seen in the TP30 chromatogram arise from slight contamination with TP50.

Relative retentions (α) and the values for the percent acetonitrile at elution are also listed in Table I. Values of α were generally quite reproducible, having an average standard deviation of ± 0.02. Greater deviations, which were observed occasionally, were always traceable to either variation in solvent composition, in pump performance (*i.e.*, low flow-rate due to an air bubble) or in sample load (see below).

As seen in Fig. 3 (lower panel), there is a clear correlation of molecular weight with the percent acetonitrile at elution, with some notable exceptions. The correlation appears strongest for protein of $M_r < 15{,}000$. Given the similarity of these proteins with respect to their high isoelectric points, their resistance to denaturation, and their abilities to bind other proteins and/or RNA within the ribosome, it is possible that this observed correlation reflects an underlying correlation of M_r with exposed hydrophobic surface. Here it should be noted that the elution of polypeptides containing less than 20 residues is well correlated with hydrophobicity[18,19].

The elution of the high-M_r proteins is not so well correlated with the percent acetonitrile. In particular, the proteins S2, S3, S4, L1, L2 and L3 are eluted earlier than predicted. The elution of the acidic proteins L7 and L12 also is not correlated with M_r, since these proteins appear much later than expected. Other reports have suggested correlations of elution with M_r[20], or with hydrophobicity[21], although numerous exceptions were noted. Our elution results show essentially no correlation with mole% hydrophobic residues (data not shown), leading us to believe that neither M_r nor hydrophobicity is sufficient to predict elution behavior over a wide range of protein structure[22].

Column capacity and resolution

Sample loads ranged from 50 to 200 μg of protein for typical analytical chromatograms on RP-HPLC such as those shown in Fig. 2. Increasing the protein load

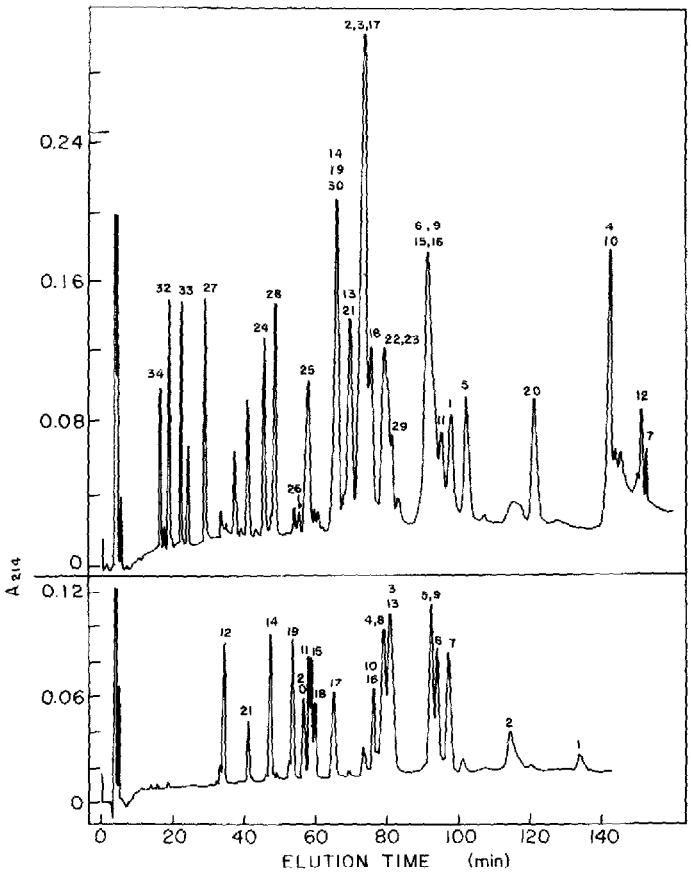

Fig. 2. RP-HPLC of TP50 and TP30. Upper panel: L proteins. A solution of TP50 (217 μg) in 10 μl of Buffer B was applied to a SynChropak RP-P column and eluted with a convex gradient (curve 5, Waters 660 programmer) of 15% to 45% Solvent B in 120 min followed by a 10-min isocratic elution at 45% B and a 30-min linear gradient from 45% B to 72.5% B. No peaks were eluted after 150 min. Lower panel: S proteins. A solution of TP30 (84 μg) in 15 μl of Buffer B was applied to a SynChropak RP-P column and eluted with a convex gradient (as above) of 15% B to 45% B in 120 min followed by an additional 20-min isocratic elution at 45% B. No additional peaks were eluted after 140 min or with an additional gradient of 45% B to 72.5% B in 30 min. Protein identifications are according to Kerlavage and co-workers[7,8].

to 1 mg or more leads to only a minor loss of resolution. A chromatogram of 2.3 mg of TP50 is shown in Fig. 4 (upper tracing). Although the peaks are generally broader when compared with those seen in Fig. 2 (upper panel), the only significant loss of resolution involves the peaks eluted between 65 and 75 min (proteins L13, L21; L2, L3, L17; and L18). Similarly, elution of 1.0 mg of TP30 (Fig. 5, upper tracing) leads to significant loss of resolution only between the peaks containing S10 and S16 and S4 and S8 and between the peaks containing S6 and S7.

Resolution similar to that obtained on analytical columns was obtained when TP50 (2.5 mg) was chromatographed on a preparative C_{18} μBondapak column (300 × 7.8 mm I.D., Waters Assoc.). Further studies are underway to determine maximum capacity and recovery using this column.

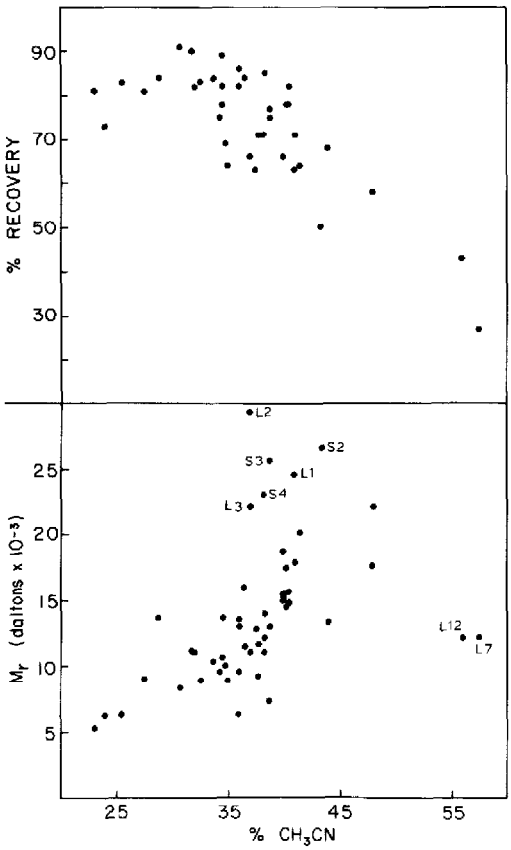

Fig. 3. Upper panel. Correlation of percent recovery with percent acetonitrile at elution. The percent acetonitrile at elution was determined by extrapolation from the output tracing of the gradient programmer and was corrected for the dead volume between the solvent mixing chamber and the column. Lower panel: Correlation of M_r with percent acetonitrile at elution. The least squares linear regression fit for all points has an r of 0.445. When the labeled points are omitted from the fit, $r = 0.754$.

Protein recovery

When elution of milligram or more amounts of either TP50 or TP30 was followed by elution without further injection of protein, an elution profile such as that shown in Fig. 4, lower tracing, or Fig. 5, lower tracing, was obtained. When this was followed by a second elution without further injection of protein, only traces of peaks were observed. Similar "ghost" peaks on blank elutions have been observed previously[21]. These results make clear the importance of column washing during the course of RP-HPLC analysis to avoid contamination of subsequent chromatograms. This was easily accomplished by a 20-min gradient from 15% Solvent B to 65% Solvent B, which was found to be more efficient than washing the column with dimethyl sulfoxide.

The appearance of "ghost" peaks demonstrates that some proteins are retained by the RP-HPLC column to a much greater extent than others, although they may be removed from the column by repeated elution. In order to obtain a more quantitative measure of protein recovery from the column, known amounts of each peak fraction

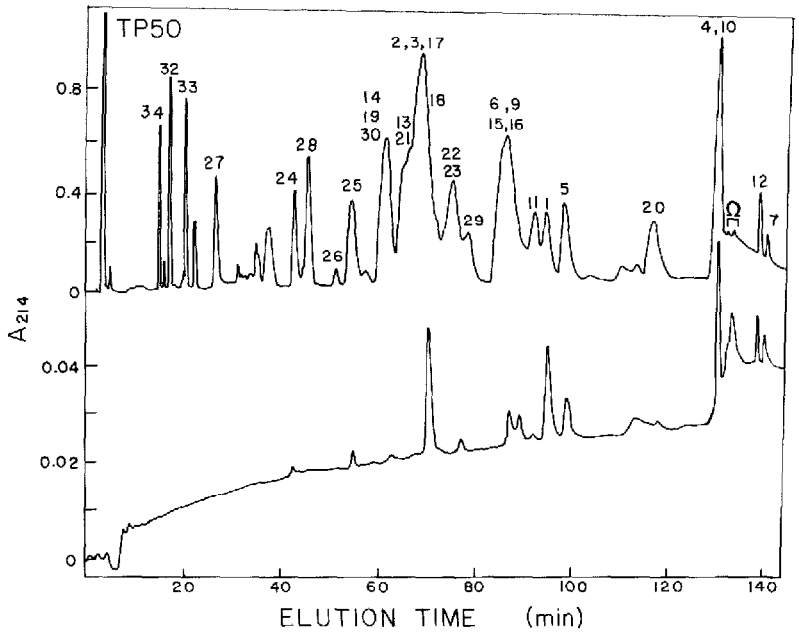

Fig. 4. Large-scale separation of TP50 and 50S proteins retained by C_{18}-silica. Upper tracing: TP50 (2.3 mg) was applied to a SynChropak RP-P column and eluted as described in the legend to Fig. 2, upper panel, except that the 10-min isocratic step at 45% B was omitted. After 150 min the column was returned to 15% B with a 10-min linear gradient at 2 ml/min and the column was equilibrated at 15% B for an additional 10 min at 2 ml/min. Lower tracing: An elution gradient identical to that described above was used without an injection of additional protein. Note change in absorbance scale between upper and lower tracings.

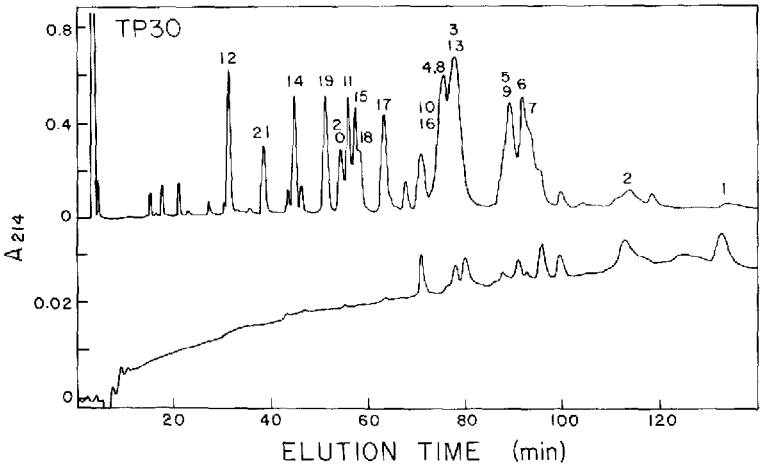

Fig. 5. Large-scale separation of TP-30 and 30S proteins retained by C_{18}-silica. Upper tracing: TP30 (1.0 mg) was applied to a SynChropak RP-P column and eluted as described in the legend to Fig. 2, lower panel. After 140 min the column was re-equilibrated at 15% B as described in the legend to Fig. 2. Lower tracing: An elution gradient identical to that described above was used without injection of additional protein. Note change in absorbance scale between upper and lower tracings.

were pooled and chromatographed, and the resulting peaks were analyzed for protein content as described in Experimental. As may be seen in Table I, the observed recoveries ranged from 27 to 91%. Not unexpectedly, those proteins retained most strongly by the column (L18, L6, L9, L15, L16, L1, L5, L20, L4, L10, L12, L7 —Fig. 4, lower tracing; S2 and S1— Fig. 5, lowering tracing) are also those having some of the lowest percentage of recovery, averaging 58 ± 11% as compared to the overall average recovery of 74 ± 13%*. As may be seen in Fig. 3, upper panel, the proteins eluted at higher concentrations of acetonitrile are more likely to be recovered in lower yield.

It should be noted that for peaks containing more than one protein, the observed recovery was averaged. Thus, for example, proteins L6, L9, L15, and L16 were assigned the same recovery. Similarly, when two peaks were incompletely resolved, an average recovery was assigned to the proteins in both peaks, as for instance to S10, S16, S4, and S8.

The total protein recovery was 71 ± 1% for 50S proteins and was 70 ± 2% for 30S proteins**. We have previously reported a value of 85% for overall 30S protein recovery[7]. That value was calculated by comparing the sum of the protein recovered in each peak with the total amount of TP30 applied to the column. In the current experiments, total protein recovery was calculated by comparing the sum of the protein recovered in each peak to the sum of the protein present in each peak prior to pooling and application to the column. That the current values are somewhat lower than our previously reported value probably reflects the additional manipulations involved in such a peak-by-peak determination of yield, and almost certainly overestimate the losses due to the chromatography itself. Nevertheless, our recoveries compare favorably with other reported RP-HPLC recoveries, that range from 8 to 100%, with most falling between 30 and 80%[18,21,23].

Reversible vs. irreversible denaturation

We presume that ribosomal proteins are denatured under the acidic and mixed solvent conditions of our chromatography, but our ability to reconstitute proteins isolated by HPLC into intact and functional ribosomal particles shows that such denaturation is reversible[8]. On the other hand, we have obtained evidence in the present study for the presence of small amounts of irreversibly denatured ribosomal protein which can be resolved by RP-HPLC from reversibly denatured ribosomal protein.

Elsewhere we have shown[8] that the doublet peak in Fig. 2 labeled Ω and eluted between 130 and 135 min contains a mixture of 50S proteins in the relative proportions L1, L9 > L4 > L3, L5, L10, L15. The relative A_{214} of this peak was quite variable from one experiment to the other, although it was always small, none of the L proteins in peak Ω exceeding 10% compared to the amount of that protein eluted at its standard α value as defined in Table I.

The elution of each of several ribosomal proteins at two different α values is due to the presence of small amounts of irreversibly (or poorly reversibly) denatured protein in a TP50 preparation. Thus, when rechromatographed, virtually all (more

* These values are averages of the recoveries listed in Table I.
** Each value is an average of two experiments.

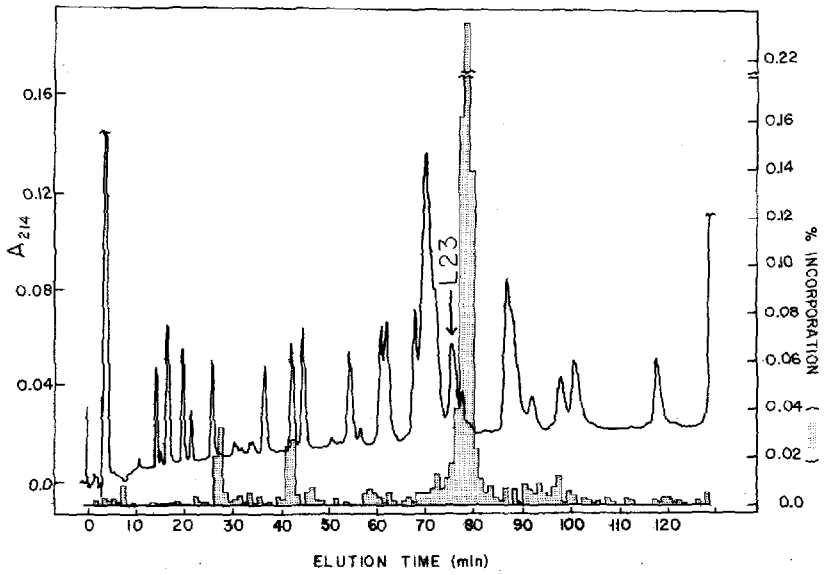

Fig. 6. RP-HPLC analysis of [^{3}H]puromycin-labeled 50S proteins. TP50 (179 pmol) from 70S ribosomes photolyzed in the presence of [^{3}H]puromycin was dissolved in 80 μl of 0.1 % F_3CCOOH and applied to a SynChropak RP-P column. The column was eluted as described in the legend to Fig. 2, lower panel. Fractions (1-min) were collected, adjusted to a constant acetonitrile concentration (0.91 ml final volume), and counted for ^{3}H (see Experimental). The position of unlabeled Protein L23 is indicated by the arrow. Percent incorporation was calculated as mol [^{3}H]puromycin per mol subunit.

than 80 % as judged by A_{214}) of the protein in peak Ω was again eluted in the original position of the Ω peak*, whereas each of the peaks containing separately proteins L1, L9, L4, L3, L5, L10, or L15 was again eluted at its standard α value (Table I), and gave rise to no protein corresponding to peak Ω. Similar results with other proteins have been obtained recently by Cohen *et al.*[24].

Analysis of proteins derived from photoaffinity-labeled ribosomes

Our laboratory has been heavily involved in photoaffinity labeling studies with *E. coli* ribosomes[25]. In our previous work we relied principally on one- and two-dimensional PAGE analysis for the identification of labeled proteins. Since RP-HPLC is clearly superior to PAGE with respect to rapidity and protein recovery, while offering resolution almost as good as two-dimensional PAGE, we decided to explore its use in analyzing proteins from 50S and 30S subunits labeled with [^{3}H]puromycin.

We previously had shown that on puromycin photoincorporation into ribosomes, protein L23 is labeled to by far the highest extent[11]. On RP-HPLC analysis of [^{3}H]puromycin-labeled TP50, the major peak of radioactivity is eluted slightly later than L23, actually together with L29 (Fig. 6). This result demonstrates both the feasibility of using RP-HPLC to analyze labeled proteins as well as the uncertainty in identifications of labeled proteins based solely on α value because of the slight change

* The chromatogram was not qualitatively altered by prior incubation of peak Ω for 3 h at 37°C in Buffer B containing 100 m*M* 2-mercaptoethanol.

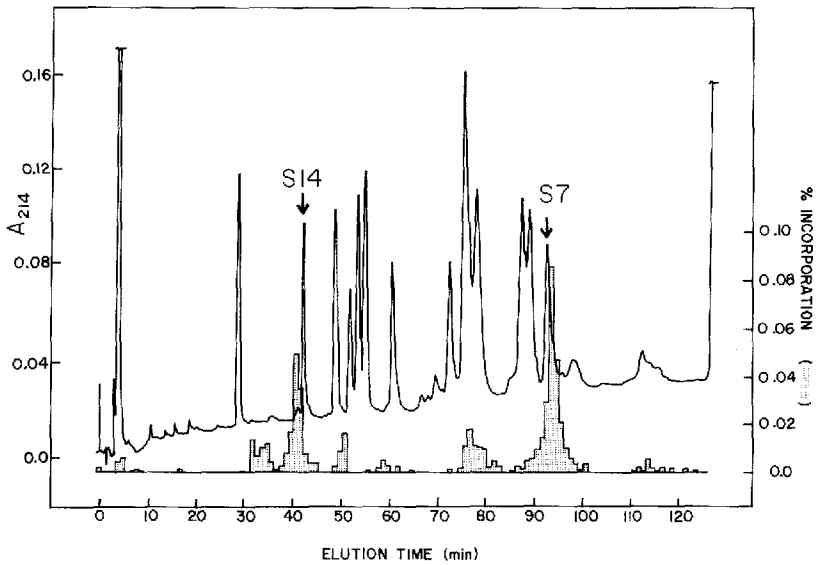

Fig. 7. RP-HPLC analysis of [^{3}H]puromycin-labeled 30S proteins. TP30 (280 pmol) from [^{3}H]puromycin-labeled 70S ribosomes was dissolved in 80 μl of 0.1% F_3CCOOH, chromatographed and fractions were counted as described above. The positions of unlabeled proteins S14 and S7 are indicated by the arrows.

in the normal α value which may accompany protein modification (a similar problem is found on PAGE analysis because of changes in electrophoretic mobility on modification[11]). A parallel RP-HPLC analysis of [^{3}H]puromycin-labeled TP30 (Fig. 7) shows two significant, although much smaller, peaks of radioactivity. Since these two peaks nearly coincide with two well-resolved proteins, identification of the labeled proteins as S14 and S7, a result in full accord with results of PAGE analysis[11,26], is more certain.

It is important to point out that the slight differences in α values which we have noted between puromycin-labeled and unlabeled proteins, while a disadvantage with respect to labeled-protein identification, actually represent an important advantage for another aspect of our work. Elsewhere we have shown that peaks containing multiple components can be resolved into their components by re-elution using a shallow gradient[8]. It should therefore be straightforward to exploit changes in α values to fully resolve puromycin-labeled from unlabeled proteins. Utilization of the purified, stoichiometrically-labeled proteins in reconstitution studies, and subsequent testing of the activity of the reconstituted ribosome, should permit clear answers to the questions of the functional significance of puromycin photoincorporation.

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NOTE ADDED IN PROOF

An alternative HPLC separation of ribosomal proteins using an ion-exchange column and affording lower resolution has recently been reported[27].

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