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Purification of Proteins from the 50S Ribosomal Subunit of *Escherichia coli* by Ion-Exchange Chromatography†

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ABSTRACT: Thirty-three proteins have been isolated from the 50S ribosomal subunit of *Escherichia coli* by a technique based solely upon ion-exchange chromatography. The procedure can be adapted to a wide range of sample sizes, requires no prefractionation of the subunit proteins, and employs readily regenerated chromatographic media. The molecular weights, purity, and immunological properties of the individual proteins have been characterized. More than 20 of the proteins were judged to be at least 95% pure by electrophoretic analysis; the remaining proteins were generally over 90% pure. Methods for the immunological identification of small amounts of ribosomal proteins are described.

From 27 to 34 chemically distinct proteins have been identified in the 50S ribosomal subunit of *Escherichia coli* (Traut et al., 1969; Hindennach et al., 1971; Mora et al., 1971; see review by Wittmann, 1974). Development of techniques for the purification and characterization of these components has contributed significantly to investigations of their role in protein synthesis. A variety of specific 50S proteins have now been implicated in critical subunit functions including tRNA binding, peptidyl transferase and GTPase activities, interaction with elongation and termination factors, and structural integration of the 5S RNA (see reviews by Pongs et al., 1974; Cantor et al., 1974; Stöffler, 1974; Möller, 1974; Monier, 1974). It has also been possible to identify the proteins which lie in or near the binding sites for several antibiotics (Nierhaus and Nierhaus, 1973; Pongs et al., 1973a,b; Sonenberg et al., 1973; Wittmann et al., 1973). Owing to the discovery of conditions for the total reconstitution of the *E. coli* 50S subunit in vitro (Nierhaus and Dohme, 1974), the rapid extension of such studies can be expected.

Since purified 50S subunit proteins are required for many kinds of investigations, efficient means of resolving them are of considerable importance. Several techniques for the isolation of individual 50S subunit proteins have been described, all of which entail prefractionation of the proteins, either by treatment of the subunits with high concentration of LiCl or urea (Traut et al., 1969; Hindennach et al., 1971; Möller et al., 1972; Pearson et al., 1972), or by fractional precipitation of the total 50S protein mixture with ammonium sulfate (Hindennach et al., 1971; Mora et al., 1971) before purification by chromatography or electrophoresis. Prefractionation techniques, however, do not always separate the proteins into mutually exclusive groups and a single protein is frequently found in more than one of the fractions.

We presently describe a method for obtaining more than 30 proteins of the 50S subunit in a state of high purity by ion-exchange chromatography alone. Since no prior fractionation of the proteins is required, losses are reduced to a minimum and any given protein is recovered quantitatively at the appropriate stage of the procedure. The method is applicable to both small and large quantities of material and, because ion-exchange columns are used throughout, sample concentration is not critical. Most of the proteins isolated by this procedure have been correlated with those purified according to Hindennach et al. (1971) by measurements of electrophoretic mobility, molecular weight, and immunological specificity.

Materials and Methods

Bacterium and Culture Cultures. Escherichia coli strain MRE600 (Cammack and Wade, 1965), growing exponentially at 37 °C in 500 ml of medium containing 7 g/l. $NaH_2PO_4 \cdot 2H_2O$, 3 g/l. K_2HPO_4, 0.5 g/l. NaCl, 1.0 g/l. NH_4Cl, 1.32 g/l. $(NH_4)_2SO_4$, 0.25 g/l. $MgSO_4 \cdot 2H_2O$, 0.18 g/l. $CaCl_2 \cdot 4H_2O$, 4 g/l. glucose, and 0.2 g/l. casamino acids (Difco, vitamin-free), was incubated for five generations with 3–6 mCi of [^{3}H]amino acid mixture (New England Nuclear, NET-250). Ribosomal proteins were assumed to be labeled to equal spe-

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cific activity under these conditions. Cells were harvested by centrifugation, washed twice with buffer I, and stored at −70 °C.

Buffers. (i) Extraction and Purification of Ribosomes. The buffers were as follows: buffer I, 0.01 M Tris-acetate (pH 7.8)–0.01 M magnesium acetate–0.06 M ammonium acetate; buffer II, 0.02 M Tris-acetate (pH 7.8)–0.02 M magnesium acetate–0.5 M NH_4Cl; buffer III, 0.01 M Tris-acetate (pH 7.8)–0.0003 M magnesium acetate–0.03 M ammonium acetate. These buffers contained 0.006 M 2-mercaptoethanol as well.

(ii) Chromatography of Ribosomal Proteins. Chromatography buffers were made up in 6 M urea and contained the following components: buffer P, 0.05 M NaH_2PO_4 (titrated to pH 6.5 with methylamine); buffer S, 0.05 M sodium citrate (1 part Na_3 citrate plus 2 parts citric acid: titrated to pH 4.0 with citric acid); buffer D, 0.01 M acetic acid (titrated to pH 5.7 with concentrated NH_4OH); buffer C, 0.05 M sodium acetate (titrated to pH 5.6 with acetic acid). Each of the above buffers received 300 μl of 2-mercaptoethanol per l. to yield a final concentration of about 0.004 M.

Preparation of Ribosomes and Ribosomal Subunits. All manipulations were carried out between 0 and 4 °C. Frozen cells were broken open by grinding with twice their weight of alumina and extracted with 10 volumes of buffer I. Alumina and cell debris were removed from the slurry by centrifugation at 18 000*g* for 10 min, the supernatant was treated with 10 μg/ml DNase (Worthington), and the resulting suspension was recentrifuged at 30 000*g* for 30 min. Ribosomes were sedimented from this extract at 58 000 rev/min for 1 h in an IEC A-321 rotor. The supernatant was discarded and the ribosome pellets were resuspended in buffer II. The suspension was clarified by low-speed centrifugation and the ribosomes were washed by sedimentation through an equal volume of 30% sucrose in buffer II at 58 000 rev/min for 6–8 h in an A-321 rotor. The supernatant was discarded and the pellets were resuspended in buffer III.

For the separation of subunits, the ribosomal suspension was centrifuged through a 5–20% linear sucrose gradient in buffer III at 27 000 rev/min for 9 h in a Spinco SW27 rotor. Sucrose gradient effluents were analyzed for absorbance at 260 nm with the aid of a Gilford Model 2400 recording spectrophotometer equipped with a flow cuvette; fractions containing 50S subunits were pooled according to the optical density profile. Subunits were concentrated by sedimentation at 45 000 rev/min for 16 h in a Spinco Ti60 rotor and resuspended in buffer I. Samples were removed for measurement of optical density and radioactivity; the specific radioactivity of the subunits was calculated using an extinction coefficient of $E_{260\text{-nm}}{}^{1\text{mg/ml}} = 16$.

Preparation of Chromatographic Media. Regular high-capacity phospho(P)-cellulose (Mann) was washed according to Hardy et al. (1969) and equilibrated against buffer P. Sulfopropyl(SP)-Sephadex (Pharmacia C-25) was gently stirred into 10 volumes of buffer S and allowed to settle. The supernatant was then decanted and fresh buffer was added. This procedure was repeated three to four times in the course of 24 h. The ion exchanger was finally collected by filtration and resuspended in buffer S. Diethylaminoethyl(DEAE)-cellulose (Whatman DE52) was precycled by washing with 0.5 N HCl, distilled H_2O, and 0.5 N NaOH. The slurry was collected on a paper filter by aspiration, washed extensively with distilled H_2O, and resuspended in buffer D. Fine particles were decanted and the suspension was degassed under gentle vacuum. Carboxymethyl(CM)-cellulose (Whatman CM52) was precycled in a similar manner, except that the order of acid and base washes was reversed. After thorough rinsing with distilled H_2O, the cellulose was resuspended in buffer C and fine particles were removed.

Column Preparation. All columns were packed in a similar fashion, regardless of the ion exchanger used. A fine-mesh nylon cloth was placed at the bottom of a 3 or 5 mm i.d. × 40 cm glass tube to retain the slurry. Each of the washed, settled ion exchangers was stirred gently in 2–5 volumes of the appropriate buffer, pipetted into the column, and permitted to settle by gravity. When the packed material reached a height of 10–20 cm, the effluent clamp was cracked open to allow a few drops of buffer per minute to flow through the column, but in no case was the rate permitted to exceed that used during column operation. This procedure prevented clogging of the column due to overcompression of the ion exchanger. More of the slurry was pipetted onto the column as needed until the desired height was attained. Buffer was then pumped through the column for at least 24 h. With sufficient care, it is possible to use a single column for 20–30 times without repacking.

Extraction and Chromatography of Ribosomal Proteins. Labeled ribosomal proteins were extracted from 50S subunits with 67% acetic acid and dialyzed to equilibrium against buffer P (Hardy et al., 1969). Initial chromatography was carried out on P-cellulose. From 10 to 15 mg of total 50S proteins (specific activity, 300 000–600 000 cpm per mg) was applied to a 5 mm i.d. × 40 cm column at a flow rate of 4.5 ml/h and washed with 2 volumes of buffer P. The proteins were then eluted with a linear gradient of 0–0.5 M NaCl in 300 ml of the same buffer (gradient, 0.1 M increase per 60 ml). Fractions of 0.9 ml were collected at 12-min intervals and a 25-μl sample was withdrawn from each for radioactive analysis. Proteins from several tubes of each radioactive peak were analyzed by polyacrylamide gel electrophoresis. Components that appeared to be pure by this criterion were pooled; protein mixtures were consolidated and separated by a second cycle of chromatography.

SP-Sephadex. Certain of the protein mixtures that were not resolved on P-cellulose were dialyzed against buffer S and applied to a 3 mm i.d. × 40 cm column of SP-Sephadex at a flow rate of 1.8 ml/h. The column was then washed with buffer S and eluted with a linear NaCl gradient in which the salt concentration increased by 0.1 M per 25 ml of buffer. Fractions of 0.45 ml were collected every 15 min and 25–50 μl from each was counted.

DEAE-Cellulose. The acidic proteins not retained by P-cellulose were dialyzed against buffer D and separated by chromatography on a column of DEAE-cellulose. After washing with buffer D, the column was eluted as described by Möller et al. (1972) with a linear gradient running from 0.01 M ammonium acetate, pH 5.7 (buffer D), to 0.2 M ammonium acetate, pH 5.0 (buffer D made 0.2 M in acetic acid and titrated to pH 5.0 with concentrated NH_4OH). Gradient increment was 0.1 M per 40 ml. Column size, flow rate, sample collection, and radioactive assay were the same as for SP-Sephadex column.

CM-Cellulose. Several protein mixtures from the first cycle of chromatography were equilibrated with buffer C by dialysis and fractionated on a CM-cellulose column (Otaka et al., 1968). Elution was accomplished with linear gradients of sodium acetate, pH 5.6. The salt concentration increased by 0.1 M per 40 ml of buffer. Column size, flow rate, sample collection, and radioactive assay were performed as for the SP-Sephadex column.

Purified proteins were stored at −20 °C in the buffer used to elute them from the columns (6 M urea, 0.02–0.5 M salt,

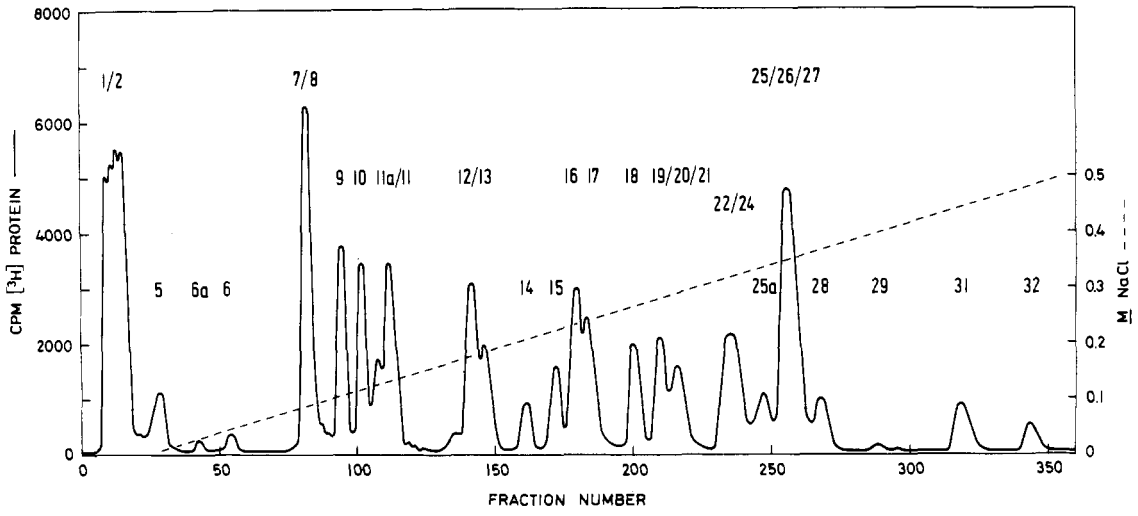

FIGURE 1: Fractionation of total 50S subunit proteins from *E. coli* MRE600 by chromatography on P-cellulose at pH 6.5. The presence of protein in the eluate, indicated by the solid line, was detected by radioactive assay. Salt gradient is denoted by the dashed line. Polyacrylamide gel electrophoresis demonstrated that peaks 1/2, 7/8, 11a/11, 12/13, 16, 19/20/21, 22/24, and 25/26/27 contained more than one protein component.

pH 4-6.5). Protein concentration ranged from 0.02 to 0.2 mg/ml.

Analysis of Radioactivity. Aliquots from column fractions were diluted to 1 ml, 100 μg of bovine serum albumin was added as carrier, and protein was precipitated by the addition of 1 ml of 10% trichloroacetic acid. Precipitates were collected on glass fiber filters (Whatman, GF/C), which were dried, immersed in 5 ml of toluene containing 4 g/l. Omnifluor (New England Nuclear), and counted in a Tri-Carb liquid scintillation spectrometer (Packard).

Concentration Measurements. Protein concentration was estimated by the procedure of Lowry et al. (1951), using egg-white lysozyme and bovine serum albumin as standards.

Gel Electrophoresis. Proteins of the 50S subunit were classified according to their electrophoretic mobilities in polyacrylamide gels containing 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide in 8 M urea, pH 4.5 (Leboy et al., 1964). Protein bands were stained with Coomassie brilliant blue. Gels were scanned in a Gilford Model 2400 spectrophotometer at 560 nm to determine the purity of isolated protein fractions; the relative amounts of major and minor components were determined from the areas under the corresponding peaks. Split gels, in which two samples were electrophoresed in adjacent halves of the same tube, were used to compare purified proteins with each other or with total 50S subunit proteins.

Two-dimensional polyacrylamide gel electrophoresis of isolated ^{3}H-labeled proteins was carried out in the presence of 300 μg of unlabeled 50S proteins as described by Kaltschmidt and Wittmann (1970). Spots containing individual components were excised and oxidized in an automatic sample oxidizer (Packard). After condensation, samples were mixed with Instagel (Packard) and radioactivity was measured by scintillation counting.

Electrophoresis of purified radioactive proteins in cellulose acetate gels was performed by the method of Stöffler (1967). Each sample was mixed with a reference standard consisting of 2-4 μg of the corresponding unlabeled protein prepared according to Hindennach et al. (1971). After the gel was stained and dried, the colored band representing the standard protein was cut out. Both the band and the remaining portions of the gel were counted in Bray's solution (Bray, 1960). The labeled protein was considered to be pure when at least 90% of the radioactivity was detected in the band containing the standard protein.

The molecular weights of individual proteins were determined from their electrophoretic mobilities in polyacrylamide gels containing 15% acrylamide, 0.4% *N,N'*-methylenebisacrylamide, and 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969). The molecular weights of the 50S subunit proteins were estimated from a least-squares plot of log (molecular weight) vs. migration distance for the following standard proteins: leucine aminopeptidase (53 000), ovalbumin (43 000), carboxypeptidase (34 600), carbonic anhydrase (29 000), trypsin (23 300), β-lactoglobulin (18 400), sperm whale myoglobin (17 200), hemoglobin (15 500), egg-white lysozyme (14 300), ribonuclease A (13 700), cytochrome *c* (11 700), ribonuclease T_1 (11 000), and insulin (5700). In addition to the use of external standards, each ribosomal protein was electrophoresed in the presence of several internal standards. Molecular weight values reported in the text represent the average of at least five independent runs.

Immunochemical Procedures. Antisera against individual proteins of the 50S subunit were raised and characterized as previously reported (Stöffler and Wittmann, 1971a,b; Stöffler et al., in preparation). The immunoglobulins of several antisera were concentrated by ammonium sulfate precipitation at 33% of saturation. Double-diffusion tests were carried out according to Ouchterlony (1968) under the conditions described by Stöffler and Wittmann (1971b).

The sensitivity of the immunochemical assay was increased in certain cases by labeling purified proteins with ^{131}I using the lactoperoxidase method of Miller and Sypherd (1973). The iodinated proteins were precipitated in 12.5% trichloroacetic acid and then dissolved in 20 μl of 8 M urea containing a mixture of unfractionated 50S proteins at a concentration of 5 mg/ml. Ouchterlony double-diffusion tests were performed in the usual way. The center well of each plate received a single ^{131}I-labeled component accompanied by 100 μg of unlabeled carrier protein. An antiserum directed against *E. coli* 50S subunits, previously shown to yield a precipitate with 24 of the 34 constituent proteins (Zeichhardt, 1974; Stöffler, 1974), was added to one of the peripheral wells. The other peripheral wells received 100 μl each of two-fold-concentrated antisera specific to various individual 50S proteins. The Ouchterlony plates were developed for 36 h at 4 °C, washed ten times with buff-

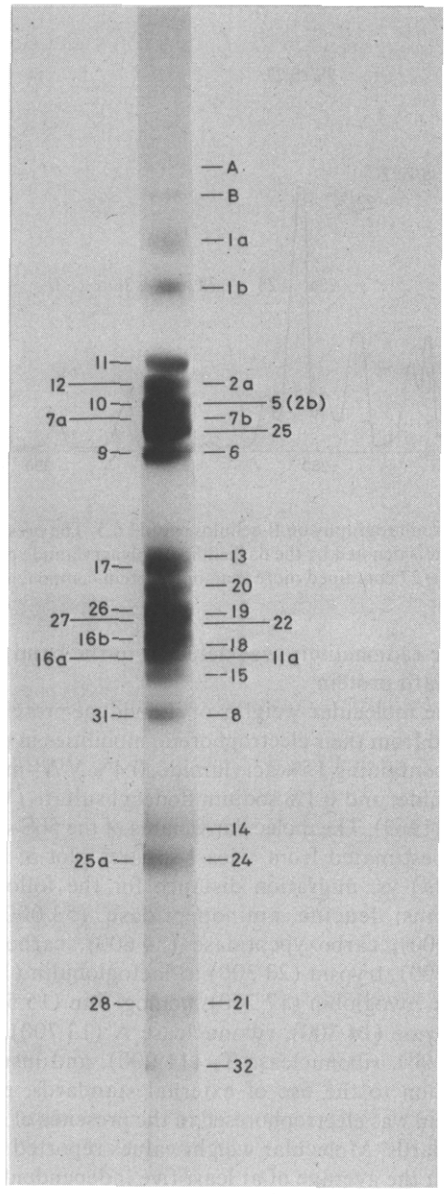

FIGURE 2: Polyacrylamide gel pattern of unfractionated 50S subunit proteins illustrating correspondence between chromatographic peaks and electrophoretic bands. Gels contained 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide in 8 M urea, pH 4.5. Two faint bands at the top of the gel have been designated A and B by analogy with similar components described by Mora et al. (1971).

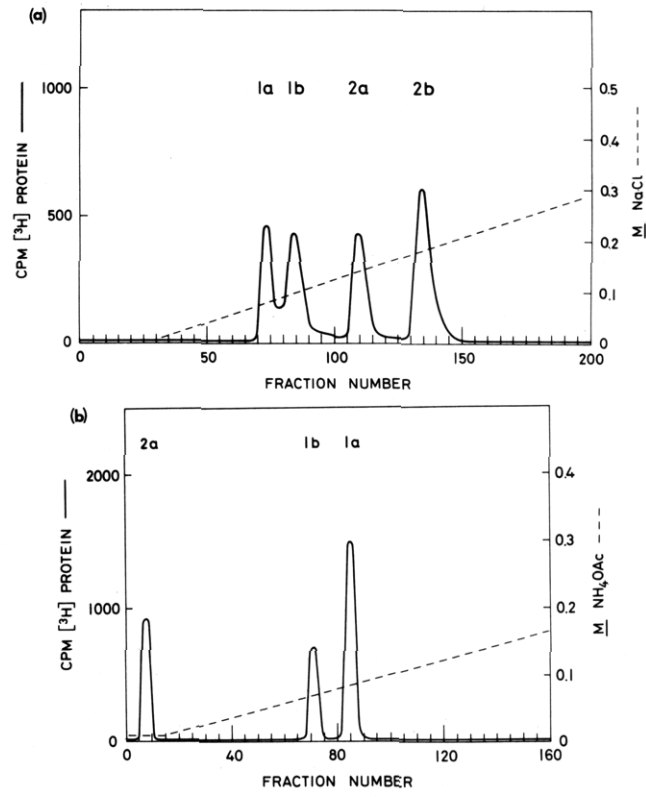

FIGURE 3: Chromatographic resolution of acidic protein fraction 1/2 on (a) SP-Sephadex at pH 4.0 and (b) DEAE-cellulose at pH 5.7 (Möller et al., 1972). Protein radioactivity: solid line. Salt gradient: dashed line.

ered saline, and air-dried. The plates were then subjected to autoradiography in order to identify radioactive precipitin lines.

Results

Chromatography of 50S Subunits Proteins on P-cellulose. The pattern of elution of total 50S proteins from P-cellulose is presented in Figure 1. Aliquots from several fractions of each peak were analyzed by urea–polyacrylamide gel electrophoresis. Those giving a single electrophoretic band were pooled and further tested for homogeneity by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels and, in several instances, by rechromatography at a different pH. Peaks 5, 9, 10, 14, 15, 18, 25a, 28, 31, and 32 each appeared to consist of a unique component in these tests and were therefore judged to be pure after one cycle of chromatography. Immunochemical analysis of these proteins yielded similar estimates of purity, although a low level of contamination was detected in fractions 18 and 25a (see below).

Protein mixtures or incompletely resolved proteins were pooled individually and subjected to a second cycle of chromatography on SP-Sephadex, DEAE-cellulose, or CM-cellulose as described in subsequent sections. The correspondence between chromatographic components and electrophoretic bands is illustrated in Figure 2. The total amounts of protein eluted from each column were estimated by radioactive and colorimetric measurements. Both methods indicated recoveries of 80% or more from P-, DEAE-, or CM-cellulose and about 50% from SP-Sephadex.

The elution profiles obtained from P-cellulose were highly reproducible. No difficulty was encountered in scaling up the preparation as long as sample size, column cross-section, and quantity of elution buffer were increased in proportion. Peaks 6a, 6, and 29 were consistently present, although their low yield suggests that they may correspond to ribosomal protein aggregates or to nonribosomal contaminants. It is also possible, although not likely, that they represent undesignated 50S subunit components. Only in the case of peak 6 was there sufficient material for further characterization.

Purification of Acidic 50S Subunit Proteins. In order to fractionate the acidic proteins of pool 1/2, which were not adsorbed to P-cellulose at pH 6.5, SP-Sephadex and DEAE-cellulose were tested at several pHs. Proteins 1a and 1b, which correspond to L7 and L12, respectively (see Table I), were satisfactorily resolved by chromatography on either SP-Sephadex at pH 4.0 (Figure 3a) or DEAE-cellulose at pH 5.7 (Figure 3b). The latter method, originally described by Möller et al. (1972), was adopted for routine separation of these two proteins since the overall recovery of material was generally higher on DEAE-cellulose than on SP-Sephadex. SP-Sepha-

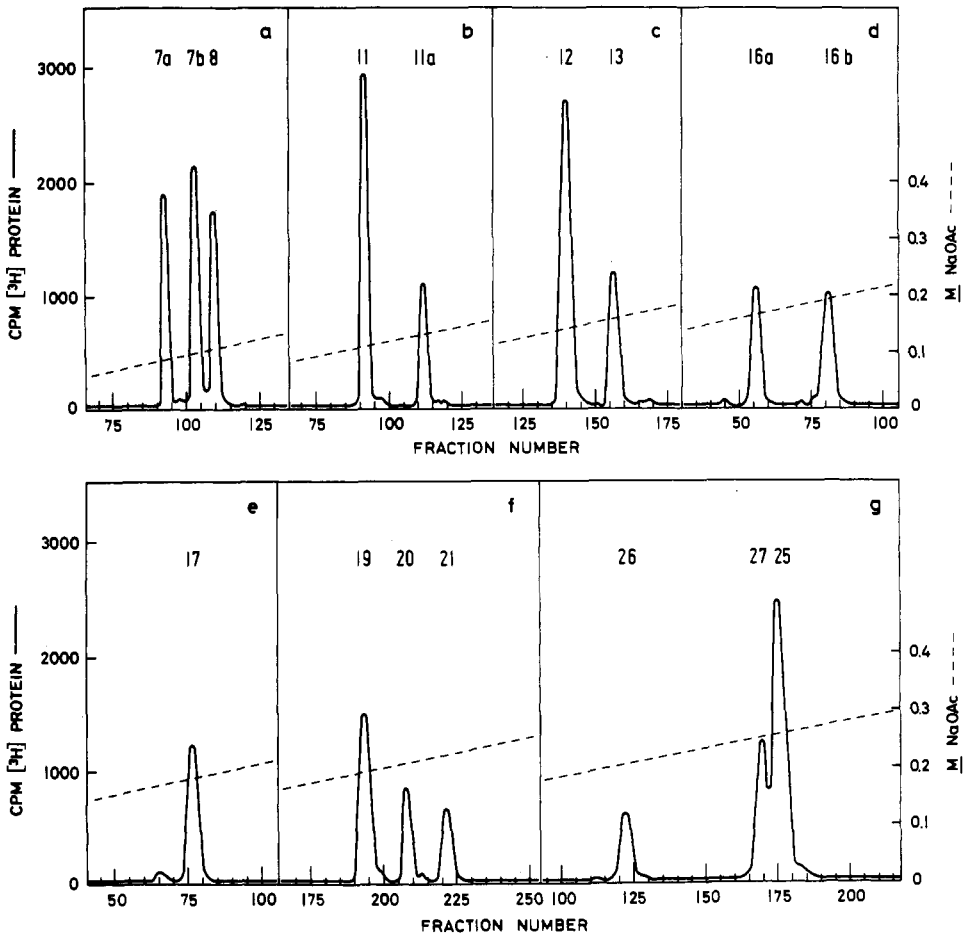

FIGURE 4: Rechromatography of protein mixtures on CM-cellulose at pH 5.6. (a) 7/8, (b) 11a/11, (c) 12/13, (d) 16, (e) 17, (f) 19/20/21, and (g) 25/26/27. Protein radioactivity: solid line. Salt gradient: dashed line.

dex, however, resolved two additional protein fractions, 2a and 2b (Figure 3a). Fraction 2b appeared to contain protein 5, even though the bulk of this component was slightly retarded by P-cellulose (see Figure 1). The presence of protein 5 was later confirmed by immunochemical tests. Unexpectedly, a second protein of nearly identical electrophoretic mobility and molecular weight was found to occur in this fraction as well (see Table I). Chromatography of pool 1/2 on SP-Sephadex at pH 5.6 or on DEAE-cellulose at pH 8.5 (Mora et al., 1971) failed to separate proteins 1a and 1b, although the second procedure yielded a small amount of the high molecular weight components A + B that was apparently free of contamination (Figure 2).

Purification of Basic 50S Subunit Proteins. Most of the protein mixtures unresolved by P-cellulose were separated completely on CM-cellulose at pH 5.6 (Figure 4). Since the constituents of each one generally eluted at different salt concentrations, a considerable saving of time and effort was achieved by chromatographing several of the mixtures together in a single column run. Thus, pools 7/8, 12/13, and 19/21 were combined and fractionated in one step. Similarly, pools 11a/11, 16, and 25/26/27 were all resolved in a single run. Pool 22/24, which could not be separated on CM-cellulose, was readily fractionated on SP-Sephadex at pH 4.0 (Figure 5). Gels of the purified proteins are reproduced in Figure 6.

Alternative strategies were used in the separation of several of the protein mixtures. These results are briefly discussed to illustrate how the techniques described here can be modified to expedite the isolation of any given protein that might be

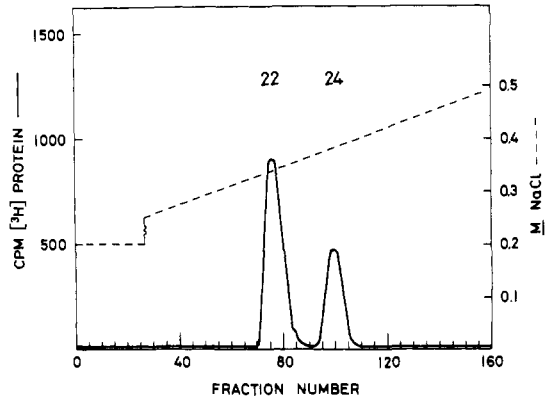

FIGURE 5: Chromatographic separation of fraction 22/24 proteins on SP-Sephadex at pH 4.0. Protein radioactivity: solid line. Salt gradient: dashed line.

required for a specific structural or functional investigation. In addition, they could aid in the design of efficient methods for purifying a mutant protein with altered chromatographic properties.

Peaks 16 and 17. The three proteins contained in fractions 16 and 17 cannot be fractionated in a single step because the elution positions of 16a and 16b coincide on P-cellulose, whereas those of 16b and 17 nearly coincide on CM-cellulose. In the procedure presently employed, each fraction from P-cellulose peaks 16 and 17 was analyzed by polyacrylamide gel electrophoresis. Since protein 17 elutes slightly after proteins

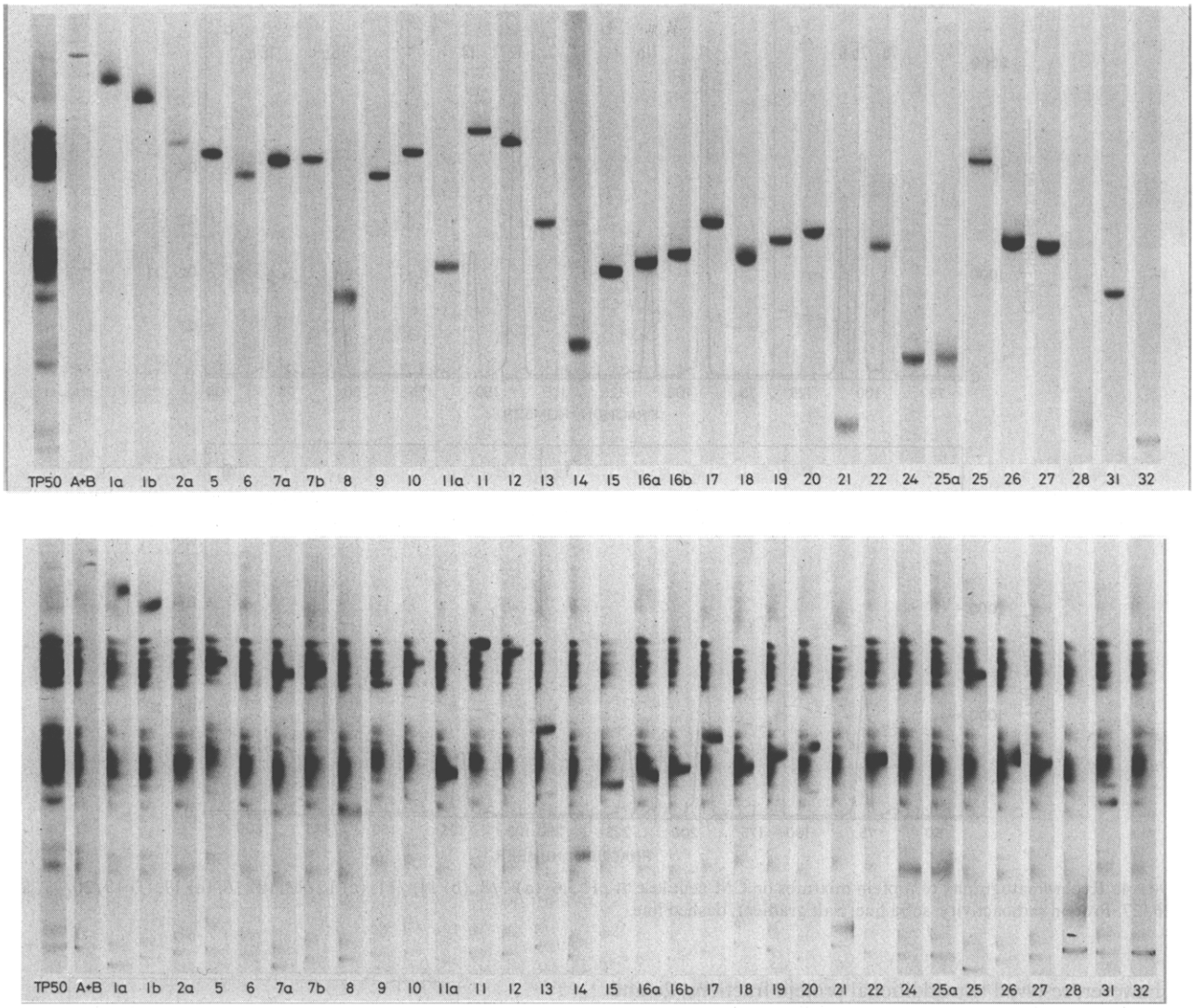

FIGURE 6: Polyacrylamide gel electrophoresis of purified 50S subunit proteins. Upper panel: standard gels of each protein. Lower panel: split gels with total 50S proteins (TP50) at left and individual 50S proteins at right. Gel composition is the same as in Figure 2.

16a and 16b, and since its electrophoretic mobility is distinctive (see Figure 2), fractions containing this protein were selected and pooled separately from those containing 16a and 16b. The latter proteins were then fractionated on CM-cellulose (Figure 4d). Protein 17 generally required chromatography on CM-cellulose as well to remove a small amount of contaminating material (Figure 4e). Alternatively, peaks 16 and 17 were pooled and chromatographed together on CM-cellulose at pH 5.6. This separated protein 16a from proteins 16b and 17; once 16a was removed, the 16b/17 mixture was resolved by another round of chromatography on P-cellulose at pH 6.5 (data not shown).

Peak 25/26/27. Proteins 25, 26, and 27 were satisfactorily fractionated on either CM-cellulose at pH 5.6 (Figure 4g) or SP-Sephadex at pH 4.0 (data not shown). With both methods, the bulk of protein 25 was recovered in pure form. Small quantities of protein 25 frequently contaminated peak 27 but were easily removed by rechromatography of the latter on CM-cellulose to yield pure protein 27. Substantial differences in the molecular weights of proteins 25, 26, and 27 (see Table II) suggested that it would be feasible to resolve them by gel filtration. This expectation was subsequently confirmed and it has been found that good separation of these three proteins can be obtained using Sephadex G-150 equilibrated with buffer P containing 0.5 M NaCl (P. Spierer, personal communication).

Peaks 7/8, 11a/11, and 12/13. The fractionation of pool 11a/11 has also been accomplished by gel filtration and, from the molecular weights summarized in Table II, it is anticipated that pools 7/8 and 12/13 should be separable by this technique as well.

Immunochemical Analysis of the 50S Subunit Proteins. Immunochemical techniques were used to correlate the 50S subunit proteins isolated in the course of this work with those described by Hindennach et al. (1971). The availability of specific noncross-reacting antisera directed against all 34 of the latter proteins (Stöffler and Wittmann, 1971a,b; Stöffler et al., in preparation) permitted efficient and unambiguous identification of most of the proteins prepared by the present method. This approach was particularly advantageous in that the immunological tests generally required no more than 10–20 μg of each purified component. A list of correlations is provided in Table I.

Many of the proteins were tentatively identified by comparing their chromatographic properties, electrophoretic mobilities, and molecular weights with those of 50S subunit proteins characterized by other investigators (Dzionara et al., 1970; Hindennach et al., 1971; Mora et al., 1971; Pearson et

Table I: Isolation, Purity, and Identification of 50S Subunit Proteins.

Protein According to Present System	Rechromatography[a]	Purity		Protein No. According to Ref[d]	Identity and Amount of Contaminating Protein[e]
		Electrophoretic Method[b]	Immunochemical Method[c]		
A + B	DEAE-Cellulose, pH 8.5	90			
1a	SP-Sephadex, pH 4.0 *or* DEAE-cellulose, pH 5.7	>95	>90	L7[h]	
1b	SP-Sephadex, pH 4.0 *or* DEAE-cellulose, pH 5.7	90	>90	L12[h]	
2a	SP-Sephadex, pH 4.0	85	80	L9	L8 (10%); L10 (10%) L10 (40%)
2b	SP-Sephadex, pH 4.0	90	60	L8	
5		>95	>90	L10	
6		90		*i*	
7a	CM-Cellulose, pH 5.6	>95[f]	>90	L11	
7b	CM-Cellulose, pH 5.6	95[g]	>90	L5	
8	CM-Cellulose, pH 5.6	95	>90	L29	
9		90[f]	>90	L6	
10		95	>90	L3	
11a	CM-Cellulose, pH 5.6	95[g]	>90	L25	
11	CM-Cellulose, pH 5.6	>95[g]	>90	L1	
12	CM-Cellulose, pH 5.6	95	>90	L4	
13	CM-Cellulose, pH 5.6	90	>90	L14	
14		95	>90	L30	
15		>95[g]	>90	L24	
16a	CM-Cellulose, pH 5.6	>95	>90	L23	
16b	CM-Cellulose, pH 5.6	>95[f]	>90	L19	
17	CM-Cellulose, pH 5.6	>95[f]	>90	L13	
18		95[g]	>90	L22	L18 (5%)
19	CM-Cellulose, pH 5.6	>95[g]	90	L18	
20	CM-Cellulose, pH 5.6	90	>90	L15	
21	CM-Cellulose, pH 5.6	90	>90	L33	
22	SP-Sephadex, pH 4.0	95	>90	L17	
24	SP-Sephadex, pH 4.0	90	>90	L27	
25a		80[f]	75	L28	L26 (20%)[f]
25	CM-Cellulose, pH 5.6 *or* Sephadex G-150	95[g]	>90	L2	
26	CM-Cellulose, pH 5.6 *or* Sephadex G-150	>95 [g]	>90	L21	
27	CM-Cellulose, pH 5.6 *or* Sephadex G-150	>95	>90	L16	
28		90[f]		L32[j]	
31		90[g]	>90	L20	
32		>95		(L34)[k]	

[a] Procedure required for purification of protein following initial fractionation on P-cellulose at pH 6.5 (see Figures 1 and 3–5). [b] Estimated from scans of one-dimensional polyacrylamide gels. [c] Estimated from quantitative Ouchterlony double-diffusion tests. [d] Nomenclature of Hindennach et al. (1971) based upon electrophoretic mobility of proteins in two-dimensional polyacrylamide gels. [e] Estimated by immunochemical method. [f] Also estimated by analysis of two-dimensional polyacrylamide gels. [g] Also estimated by electrophoresis on cellulose acetate gels. [h] Proteins 1a and 1b both react with anti-L7 and anti-L12 sera; positive correlation established by electrophoretic mobilities. [i] Protein 6 reacted weakly with antiserum specific to 30S subunit protein S5 in some experiments. [j] All preparations of protein 28 reacted with anti-L32 serum, although results were subject to quantitative variation; positive correlation established by analysis of two-dimensional polyacrylamide gels. [k] Tentative correlation of protein 32 with L34 based on electrophoretic mobility; this component did not react with any of the 34 monospecific antisera.

al., 1972). Positive correlations were then made by standard double-diffusion tests in which each protein was developed against a limited number of antisera selected on the basis of the preliminary assignments. In most instances, a given fraction was precipitated by one and only one of the three to six antisera tested (Figure 7). A unique correspondence was thereby established for all single proteins except 1a, 1b, 6, 26, 28, and 32. Fractions 2b and 25a were found to consist of two-component mixtures.

Proteins 1a and 1b both reacted with anti-L7 and anti-L12 sera, but not with any of the other antisera. L7 and L12 were previously shown to be immunologically indistinguishable (Stöffler and Wittmann, 1971a) and it is now known that their amino acid sequences are identical (Terhorst et al., 1973). The N-terminal residue of L7 is acetylated, however, and it can be differentiated from L12 by its slower migration in polyacrylamide gels at acid pH (Möller et al., 1972). From an inspection of Figure 6, it is clear that proteins 1a and 1b correspond to L7 and L12, respectively.

The identification of protein fractions 6, 26, 28, and 32 posed

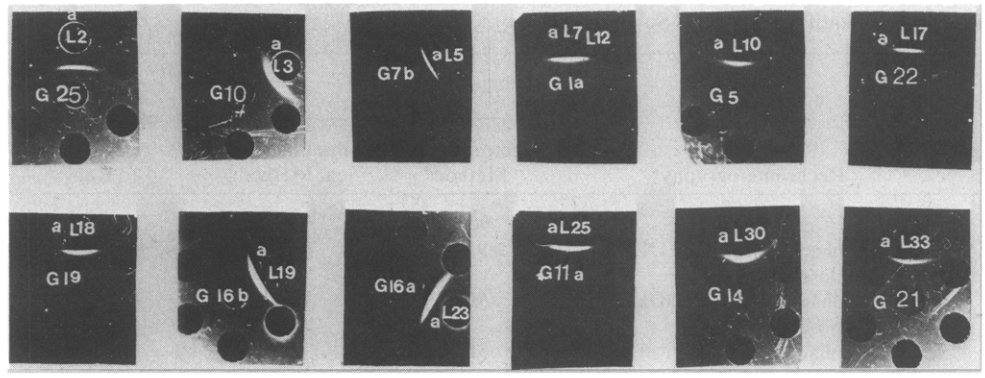

FIGURE 7: Identification of 50S subunit proteins by the Ouchterlony double-diffusion test. Immunodiffusion was performed in gels consisting of 1% agarose in 1 M sodium barbital (pH 8.6)–0.75 M LiCl. The center well of each plate contained 2 μg of a single purified protein (G). Peripheral wells contained 90 μl of undiluted antisera directed against various individual proteins. The antiserum yielding a precipitin line is indicated (a). Plates were photographed without staining after 36 h.

additional problems since tentative assignments could not be made in these cases. Protein 28 was definitively correlated with L32 by a combination of immunochemical and electrophoretic methods. Proteins 6, 26, and 32 were developed against each of the 34 monospecific antisera by the standard technique. This procedure established the correspondence of protein 26 with L21, but neither protein 6 nor protein 32 yielded visible precipitin lines in any of the tests.

In an effort to increase the sensitivity of the immunochemical assay, protein 6 was labeled with ^{131}I at high specific activity. Proteins 15 and 25 were treated in a similar fashion to serve as controls. The iodinated proteins were mixed with unlabeled 50S subunit proteins and reacted individually with the 34 antibody preparations, as well as with an antiserum raised against intact 50S subunits. Since the samples contained a complete set of 50S proteins, a visible line of precipitation was observed with each antiserum. In addition, the precipitin line was expected to be radioactively labeled when the antiserum corresponded to the iodinated test protein. Autoradiography of the plates revealed that proteins 15 and 25 did indeed form radioactive precipitates with antisera directed against L24 and L2, respectively, as anticipated from earlier results (Table I). Both proteins also reacted positively with anti-50S subunit serum. By contrast, ^{131}I-labeled protein 6 did not react with any of the antisera. It is thus unlikely that this component corresponds to any known 50S subunit protein or derivative thereof. Although fraction 6 may be nonribosomal in origin, some preparations responded weakly to an antiserum raised against S5, a protein of the 30S subunit that occasionally partitions with the larger ribosomal subparticle (Tischendorf et al., in preparation). The ^{131}I labeling technique thus served to confirm our earlier conclusions but did not provide any new information on the identity of the proteins discussed here. Nonetheless, this method should prove useful when even smaller amounts of protein are available than in the present case since it is in principle an order of magnitude more sensitive than the standard Ouchterlony double-diffusion test.

Purity of the 50S Subunit Proteins. The homogeneity of each protein fraction was initially assessed from spectrophotometric scans of one-dimensional polyacrylamide gels (Figure 6). The results, presented in Table I, indicate that most of the proteins are at least 90% pure. A number of proteins were also checked for homogeneity by electrophoresis in cellulose acetate or two-dimensional polyacrylamide gels as noted.

Estimates of purity derived from gel scans have proven to be generally consistent with those obtained by other methods. Some care must be exercised in interpreting such data, however, since two proteins that elute from a column at the same salt concentration may also possess identical electrophoretic mobilities. For example, proteins 7a and 7b co-chromatograph on P-cellulose (Figure 1) and co-electrophorese on one-dimensional polyacrylamide gels (Figure 2). They can be distinguished by a variety of other properties, however, such as their chromatographic behavior on CM-cellulose (Figure 4a), their molecular weights (see Table II), their mobilities in bi-dimensional gels (Kaltschmidt and Wittmann, 1970) and their immunological characteristics (Stöffler and Wittmann, 1971a,b). The mixture of proteins 16a and 16b represents a similar case.

Owing to these considerations, several ostensibly pure protein fractions, including 9, 10, and 18, were rechromatographed on CM-cellulose and SP-Sephadex. In each instance, a homogeneous peak was recovered which migrated as a single component upon electrophoresis in two or three different gel systems. Nonetheless, some contamination of protein 18 could still be detected as described below.

Each of the isolated proteins was further tested for purity by immunochemical criteria. Samples of a given protein at three to four different concentrations were added to the center wells of a series of Ouchterlony plates. Each plate was then developed with the homologous antiserum as well as with antisera specific to other proteins that eluted from P-cellulose in the same or in neighboring peaks. Most of the proteins were precipitated exclusively by the homologous antiserum throughout the entire concentration range and were judged to be over 90% pure (Table I). In a few cases, a complete immunochemical assay was performed in which the test protein was challenged with all 34 monospecific antisera. Proteins 17 and 26, for instance, reacted only with anti-L13 and anti-L21 sera, respectively, providing strong evidence of their purity.

In general, contamination of the isolated proteins was held to a minimum by estimating the electrophoretic homogeneity of samples from peak tubes before they were selected for pooling. Despite such precautions, impurities were detected in fractions 2a, 2b, 18, 25a, and 28 even after two cycles of chromatography. Immunological tests showed that fraction 2a consists of 80% L9 and roughly 10% each of two other acidic proteins, L8 and L10. Fraction 2b was found to contain approximately 60% L8 and 40% L10 by the same technique. The presence of L10 in this peak is of some interest since most of the L10 is slightly retarded by P-cellulose and elutes in pure form as peak 5. Fraction 18 elutes from P-cellulose as a single

well-resolved peak and was initially believed to be pure L22. However, immunodiffusion tests revealed that it was consistently contaminated with small amounts of L18. Since the contaminating L18 persists at a level of about 5% following rechromatography of fraction 18, it may differ in structure from the bulk of the L18 which elutes in fraction 19. It is possible that this finding reflects a microheterogeneity in L18, a protein that may occur in two copies per 50S subunit (Weber, 1972). Peak 25a consists mainly of L28 but it was shown to contain about 20% L26 as well by both immunological assay and two-dimensional gel electrophoresis. The immunochemical characterization of fraction 28 was subject to considerable variation and, although L32 was identified as a major constituent, estimates of purity were not conclusive. Electrophoresis on two-dimensional gels confirmed that, in at least one preparation, this fraction contained over 90% L32.

Molecular Weights of the 50S Subunit Proteins. Molecular weights of the purified 50S proteins, determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, are listed in Table II. Components A and B could not be separated from one another and were analyzed as a mixture. The present values are in generally good agreement with those published by Dzionara et al. (1970). Precise molecular weights are now known for L7 (12 260; Terhorst et al., 1973), L12 (12 200; Terhorst et al., 1973), L18 (12 770; Brosius et al., 1975), L25 (10 912; Dovgas et al., 1975; Bitar and Wittmann-Liebold, 1975), L27 (8998; Chen et al., 1975), L29 (7262; Bitar, 1974) L30 (6410; Ritter and Wittmann-Liebold, 1975), and L32 (6316; Wittmann-Liebold et al., 1975) on the basis of amino acid sequence analysis. The values calculated from the sequence data differ by no more than ±15% from the sodium dodecyl sulfate gel values reported here (except for the very small proteins L30 amd L32) and no systematic error in our measurements is apparent.

The total molecular weight of the 50S subunit proteins, exclusive of components A and B, is 475 000 daltons (Table II). Inclusion of A and B brings the total to 580 000 daltons. It is not known whether these high molecular weight components are normal constituents of the 50S particle, but in salt-washed ribosomes they occur in very small amounts and cannot contribute substantially to overall particle mass. Although the protein complement of the 50S subunit has been estimated to lie between 520 000 and 555 000 daltons (Dzionara et al., 1970; Mora et al., 1971), a value of 450 000 daltons is predicted from the difference in molecular weight between salt-washed subunits (Hill et al., 1969) and 23S RNA (Kurland, 1960). The aggregate protein mass of 475 000 thus falls within acceptable limits. This figure assumes one copy of each component per subunit, however. Stoichiometric measurements have revealed that proteins L7 + L12 occur in at least three copies per subunit (Möller et al., 1970; Thammana et al., 1973) and that protein L18 may be present in two copies per subunit [Weber, 1972; see also Figure 4f where protein 19 (L18) can be clearly observed to yield a peak almost twice as large as that of protein 20 (L15) even though its molecular weight is lower]. Correcting the calculation accordingly, the total protein mass of the 50S subunit is estimated to be 495 000 daltons.

Table II: Molecular Weights of 50S Subunit Proteins.[a]

Protein[b]	Mol Wt	Protein[b]	Mol Wt
A + B	54 900 + 48 300	15 (L24)	12 000
1a (L7)	10 200	16a (L23)	11 200
1b (L12)	10 100	16b (L19)	12 800
2a (L9)	16 900	17 (L13)	17 700
2b (L8)	17 700	18 (L22)	12 600
5 (L10)	17 800	19 (L18)	11 300
6	13 000	20 (L15)	16 500
7a (L11)	15 300	21 (L33)	8 100
7b (L5)	21 700	22 (L17)	14 000
8 (L29)	7 100	24 (L27)	10 200
9 (L6)	21 200	25a (L28)	9 500
10 (L3)	25 800	25 (L2)	32 400
11a (L25)	9 800	26 (L21)	11 800
11 (L1)	27 600	27 (L16)	17 400
12 (L4)	26 200	28 (L32)	7 900
13 (L14)	13 800	31 (L20)	16 700
14 (L30)	8 800	32 (L34)	9 000

[a] Determined by sodium dodecyl sulfate gel electrophoresis as described in Materials and Methods. [b] Protein nomenclature according to Hindennach et al. (1971) is given in parentheses.

Discussion

Ion-exchange chromatography has been used to purify 33 proteins from the 50S ribosomal subunit of *E. coli*. The procedure employed is considerably simpler than previous separation schemes (Traut et al., 1969; Hindennach et al., 1971; Mora et al., 1971; Pearson et al., 1972) and offers a number of advantages; it is highly reproducible, equally convenient for small and large quantities of material, and does not require prior separation of the proteins into "split" and "core" fractions by treatment of the subunits with high salt concentrations. These methods should prove particularly useful in the isolation of proteins from eukaryotic ribosomes where prefractionation has not proved practicable (Wool and Stöffler, 1974). The present techniques are also quite efficient since yields are high at each step and no more than two cycles of chromatography are needed to isolate any given protein. Moreover, second-cycle fractionation of several protein mixtures can often be accomplished in a single column run. The purity of a specific component can generally be increased by judiciously selecting column fractions for pooling after first analyzing an aliquot from each one for contaminants by one-dimensional polyacrylamide gel electrophoresis. An additional advantage for small, dilute samples is that they need not be concentrated before rechromatography since all proteins are adsorbed to the same small zone on the ion exchanger. Alternate strategies of purification are proposed in a number of instances to guide in the selection of the most direct means for obtaining a particular protein.

Thirty-one of the proteins described in this report were unambiguously identified with those enumerated by Hindennach et al. (1971) through systematic immunochemical analysis (see Table I). Two other proteins, 6 and 32, did not react with any of the monospecific antisera directed against the 34 individual 50S subunit components. Conversely, anti-L31 and anti-L34 sera failed to precipitate any of the present proteins, and anti-L26 serum precipitated only a minor fraction from peak 25a. L26 is identical with protein S20 of the 30S particle and is believed to partition between the small and large subunits when the 70S ribosome is dissociated (Tischendorf et al., in preparation). The immunodiffusion tests revealed that protein L10 is chromatographically heterogeneous. Whereas most of this protein elutes in peak 5, between 25 and 35% of the total elutes unretarded from P-cellulose in fraction 1/2 and is subsequently recovered in peaks 2a and 2b (see Table I). It is thus possible that L10 is subject to modification or degradation or that it exists in more than one conformation.

Protein 32 has been tentatively correlated with L34 since both are small and both represent the most basic components of their respective sets. Moreover, it did not migrate to the position of L31 in a bidimensional polyacrylamide gel. The failure of this component to react with four different L34-specific antibody preparations may reflect the fact that antisera elicited by small ribosomal proteins are often rather weak and precipitate their antigens only within a narrow range of antigen:antibody ratios. A 50S subunit component of similar chromatographic and electrophoretic properties has been characterized by Traut et al. (1969). We therefore conclude that protein 32 is a genuine constituent of the 50S subunit. Protein 6 may correspond to 30S subunit protein S5, to a previously uncharacterized 50S subunit component, or to a nonribosomal contaminant. No further information on the identity of this protein is presently available. High molecular weight components analogous to A + B were not found by Hindennach et al. (1971), although they have been observed by two other groups (Traut et al., 1969; Mora et al., 1971). Since these proteins occur in very low yield, their status is uncertain and it is possible that they remain adventitiously bound to the ribosome even after washing with 0.5 M salt. L31, which has been reported to occur in well below stoichiometric amounts in isolated 50S subunits (Weber, 1972), was not identified in the protein preparations used for the present studies.

The 50S subunit proteins isolated in a number of laboratories have recently been correlated by an analysis of their immunochemical specificities, amino acid compositions, molecular weights, and electrophoretic mobilities in both one- and two-dimensional polyacrylamide gels (Kurland et al., in preparation). The correspondence among the subunit components is excellent and no strain-specific differences have yet been detected in proteins derived from *E. coli* K12 (Otaka et al., 1968; Hindennach et al., 1971), B (Mora et al., 1971), and MRE600 (Traut et al., 1969; Pearson et al., 1972; present work). Besides the intrinsic interest of this result, correlation of the 50S proteins and adoption of a common nomenclature is expected to facilitate the comparison of experimental results obtained by different investigators. A similar correlation of the 21 components of the *E. coli* 30S subparticle has already proven to be indispensable (Wittmann et al., 1971).

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