Topography of Ribosomal Proteins of the *Escherichia coli* 30S Subunit as Studied with the Reversible Cross-Linking Reagent Methyl 4-Mercaptobutyrimidate†

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ABSTRACT: Radioactive 30S ribosomal subunits from *Escherichia coli* were allowed to react with methyl 4-mercaptobutyrimidate hydrochloride, a new protein cross-linking reagent cleavable by mild reduction (Traut *et al.* (1973), *Biochemistry 12*, 3266). Following the cross-linking reaction which consists of the formation of disulfide linkages between neighboring proteins, the ribosomal proteins were analyzed by electrophoresis in polyacrylamide gels containing dodecyl sulfate. Specific bands of molecular weight higher than unmodified ribosomal proteins were apparent. These products, the molecular weights of which were estimated from their electrophoretic mobility, were isolated, reduced, and reanalyzed by electrophoresis on gels containing dodecyl sulfate in order to establish the molecular weights of the reductively cleaved protomeric constituents of the putative dimers or higher cross-linked oligomers. In one case, gel analysis alone unambiguously showed the existence of a dimer containing proteins S2 and S3. In other cases, dodecyl sulfate gel molecular weight data were insufficient to provide unambiguous identification of the components of cross-linked products. In these cases, the cross-linked products were analyzed with monospecific antisera to 30S ribosomal proteins. The analysis showed the existence of mixed dimers between proteins S5 and S8, and proteins S13 and S19. These results suggest that S2 and S3, S5 and S8, and S13 and S19 occupy proximal positions in *E. coli* 30S ribosome.

Knowledge of the spatial arrangement of ribosomal proteins in the native structure would contribute to the detailed understanding of ribosomal function in protein synthesis. The study of the topography of ribosomal proteins in the 30S subunit of *Escherichia coli* has received considerable attention during recent years (for review, see Wittmann and Stöffler, 1972, and Kurland, 1972). Several different experimental approaches have provided information on the topography of ribosomal proteins. These include studies on the protein composition of nucleoprotein particles produced by mild nuclease digestion (Schendel *et al.*, 1972; Morgan and Brimacombe, 1973; Roth and Nierhaus, 1973), on ribosome assembly (Mizushima and Nomura, 1970), on the susceptibility of ribosomal proteins and specific ribosomal functions to enzymatic or chemical modifications (Chang and Flaks, 1970; Craven and Gupta, 1970; Crichton and Wittmann, 1971; Kahan and Kaltschmidt, 1972; Huang and Cantor, 1972; Rummel and Noller, 1973), on the functional properties of ribosomes with genetically altered proteins (Birge and Kurland, 1970; Zimmermann *et al.*, 1971), on the inhibitory effect of specific Fab fragments on different ribosomal functions (Lelong *et al.*, 1974), and on the effect of adding proteins back to deficient particles on the restoration of specific ribosomal functions (Randall-Hazelbauer and Kurland, 1972; Marsh and Parmeggiani, 1973).

The information provided by these approaches concerns primarily the gross distribution of the ribosomal proteins; *i.e.*, whether a protein is relatively accessible or inaccessible to specific reagents or whether several proteins may exist in a relatively imprecisely defined neighborhood. By contrast to the approaches cited above, cross-linking of neighboring proteins with bifunctional reagents can give more detailed and chemically precise information on proximity relationships between pairs or groups of proteins.

Early experiments in this and Slobin's laboratory employed bissuberimidate[1] as a cross-linking reagent to study the topography of *E. coli* 30S ribosomal proteins (Bickle *et al.*, 1972; Slobin, 1972). Other laboratories have used bismaleimides (Chang and Flaks, 1972; Lutter *et al.*, 1972), other bisimidoesters (Lutter *et al.*, 1972), and tetranitromethane (Shih and Craven, 1973) as cross-linking reagents to study the same problem. More recently a new reagent, mercaptobutyrimidate was synthesized in this laboratory (Traut *et al.*, 1973). Like bissuberimidate the new reagent produced a number of cross-linked products when allowed to react with the 30S particle, but not with free total 30S proteins. However, unlike bissuberimidate, the protomeric components cross-linked with the new reagent can be regenerated in 100% yield. This is due to the fact that the linkage between proteins formed

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; mercaptobutyrimidate, 4-methyl mercaptobutyrimidate hydrochloride; bissuberimidate, bis(methyl)suberimidate dihydrochloride.

with the new reagent, produced by the formation of disulfide bands, is susceptible to cleavage by mild reduction (see Figure 1). The work reported here describes our initial application of this reagent to the study of the topography of proteins of the *E. coli* 30S ribosome. Both molecular weight data of cross-linked products and their reduced components, and immunochemical analysis of two of the cross-linked products have been employed to show the proximity of the following pairs of proteins: S5 and S8, S2 and S3, and S13 and S19.

Materials and Methods

Preparation of Radioactive Ribosomes. E. coli strain MRE-600 was grown at 37° with shaking in a minimal medium which contained the following components per liter: 2 g of glucose, 13.2 g of $Na_2HPO4 \cdot 7H_2O$, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 30 mg of $MgCl_2 \cdot 6H_2O$, 1.42 mg of Na_2SO_4, 0.8 mg of $FeCl_3$, and 0.11 mg of $CaCl_2$. The initial medium also contained 12.5 mCi of ^{35}S (as carrier free H_2SO_4) per l. After cell growth ceased when sulfate became limiting, cold sodium sulfate was added to make the final concentration 0.1 mM. After one generation of growth with non-radioactive sulfate in excess, the flask was slowly cooled by immersing it in an ice bath for 10 min during which time the temperature fell to 15°. The temperature was held at 15° for a further 10 min and then lowered to 0° by adding crushed ice. A slow cooling process of this kind has been reported to produce runoff ribosomes or 70S particles free of m- and tRNA (Chliamovitch and Anderson, 1972). Ribosomes were extracted by grinding with alumina in buffer containing 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4), and 0.007 M β-mercaptoethanol (Tissieres *et al.*, 1959), and centrifuged by standard procedures. The crude ribosome pellet was resuspended and centrifuged at 50,000 rpm for 12 hr in a buffer containing a high-salt concentration (0.01 M $MgCl_2$, 1 M NH_4Cl, 0.01 M Tris-HCl (pH 7.4), and 0.014 M β-mercaptoethanol). In order to eliminate "native" subunits (defined as those which do not associate to form 70S couples at 10 mM Mg^{2+}), the pellet containing the washed ribosomes was resuspended in a buffer containing a high Mg^{2+} concentration and lowered salt concentration (0.01 M $MgCl_2$, 0.1 M NH_4Cl, 0.01 M Tris-HCl (pH 7.4), and 0.007 M β-mercaptoethanol). The particles were centrifuged in a SW27 rotor through a 7–25% sucrose density gradient of the same composition. Previous work in this laboratory has shown that native 30S subunits sediment slightly slower than normal (derived) 30S ribosomes (T. T. Sun, unpublished results). In addition, they contain what appears to be extra non-ribosomal protein species of high molecular weight, and they contain reduced amounts of certain other ribosomal proteins (Bickle *et al.*, 1973). The 70S ribosomes isolated from the gradient were centrifuged and the pellet was resuspended in a buffer containing a lowered magnesium concentration (0.001 M $MgCl_2$, 0.1 M NH_4Cl, 0.01 M Tris-HCl (pH 7.4), and 0.014 M β-mercaptoethanol). A second centrifugation through a 7–25% sucrose density gradient in the low Mg^{2+} buffer dissociated the 70S ribosomes into "derived" 30S and 50S subunits. The 30S subunits thus prepared were used for cross-linking studies. The specific activity of freshly prepared ribosomal protein varied between 120,000 and 180,000 cpm of $^{35}S/\mu g$ of protein.

Cross-Linking. Radioactive 30S ribosomes at a concentration of 3 mg/ml were incubated with 10 mM mercaptobutyrimidate in 0.001 M $MgCl_2$, 0.05 M KCl, 0.05 M triethanolamine-HCl (pH 8.0 at 25°), and 3% β-mercaptoethanol for 20 min at 0°. The "SH-charged" ribosomes thus formed as a result of reaction of the imidate function of the reagent with lysine amino groups were then dialyzed against the same buffer but without β-mercaptoethanol. Following dialysis, the ribosomes were incubated for 30 min at room temperature in

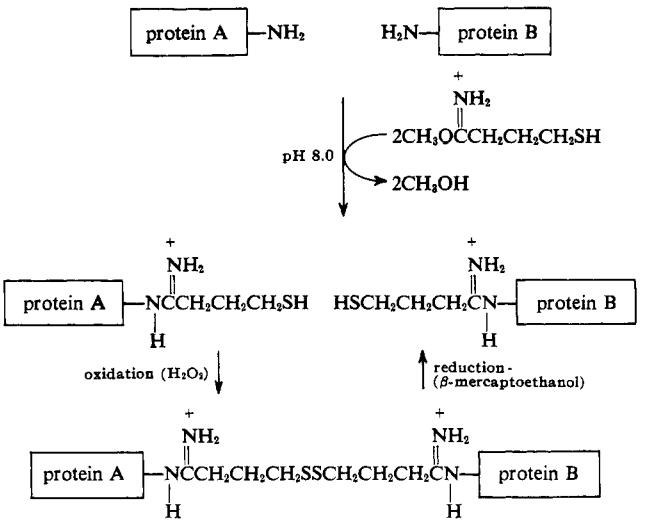

FIGURE 1: The mechanism of reversible cross-linking of neighboring proteins by 4-methyl mercaptobutyrimidate.

the same buffer with the addition of H_2O_2 to a final concentration of 40 mM (Traut *et al.*, 1973). The ribosome structure was destroyed by addition of one-fifth volume of SDS gel sample buffer (0.029 M $NaH_2PO_4 \cdot H_2O$–0.072 M $Na_2HPO_4 \cdot 7H_2O$–5% SDS–40% glycerol). It has been shown previously that the formation of disulfide bonds by this procedure depends completely on the utilization of intact ribosomal subunits, as opposed to dissociated ribosomal proteins, as substrates for the modification and oxidation reactions (Traut *et al.*, 1973). The protein mixture was analyzed by SDS polyacrylamide gel electrophoresis.

The cross-linking of 30S ribosomes by bissuberimidate was performed as described previously (Bickle *et al.*, 1972) with the exception that 1% β-mercaptoethanol was included in the cross-linking buffer (0.001 M $MgCl_2$, 0.05 M KCl, and 0.05 M triethanolamine-HCl, pH 8.0). This was found advantageous in preventing 30S dimer formation which occurs occasionally at pH 8.0 and higher.

Gel Electrophoresis. A discontinuous SDS gel system (Laemmli, 1970) containing 15% acrylamide, 0.087% bisacrylamide, 0.1% SDS, and 0.37 M Tris-HCl (pH 8.7) was used (Traut *et al.*, 1973). Gels were either prepared in a glass tube (gel size 0.6 × 11.5 cm) or as a slab (Studier, 1973).

Tubular gels were used as a preparative technique to fractionate the mixture of cross-linked (radioactive) ribosomal protein. Immediately after electrophoresis, the unstained gel was cut transversely into 1-mm slices. Each slice was ground with a glass rod in a small disposable plastic tube and extracted at 37° for 5–10 hr three times with 200 μl of 1% SDS. The three extracts were combined and an aliquot was counted in a Beckman LS200 liquid scintillation counter.

Slab gels were used for analyzing the individual or pooled fractions obtained from the preparative disk gel. The patterns of total 30S ribosomal proteins obtained by either the slab or the disk gel technique were identical.

The position of each 30S ribosomal protein in the gel system was established using as standards purified single pro-

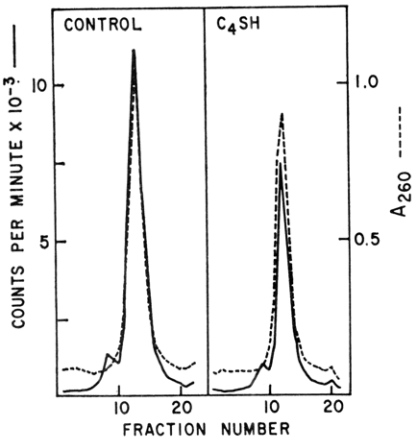

FIGURE 2: Sedimentation analysis of ^{35}S-labeled 30S ribosomes before (left) and after (right) cross-linking with 10 mM mercaptobutyrimidate. Samples were centrifuged in 4.5-ml 7–25% linear sucrose density gradients containing the buffer employed for the reaction with mercaptobutyrimidate (0.05 M KCl–0.05 M triethanolamine-HCl (pH 8.0)–0.001 M $MgCl_2$). Radioactive ribosomes (1 μg) containing approximately 50,000 cpm (^{35}S) were mixed with 100 μg of cold 30S ribosomes which served as absorbancy marker, applied to the gradient and centrifuged (55,000 rpm for 110 min at 4° in a SW56 rotor). After centrifugation, the gradients were collected in 0.2-ml fractions. OD_{260} was measured in a Gilford 2400 spectrophotometer after the sample was diluted to 0.5 ml with H_2O. Radioactivity was measured with a Beckman CS200B liquid scintillation counter using Triton–toluene (1:2) scintillation fluid. Fraction 1 is the bottom of the gradient.

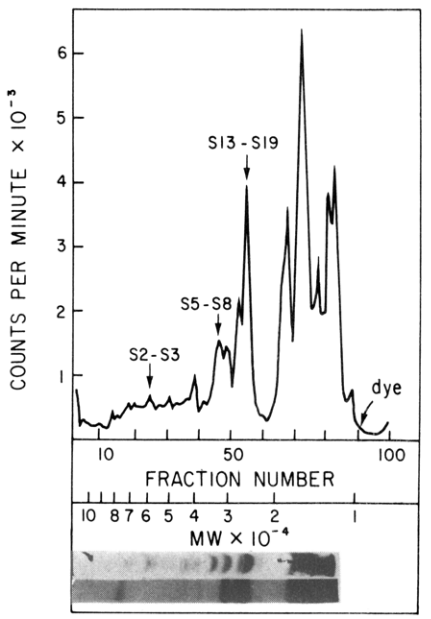

FIGURE 3: Fractionation of 30S ribosomal proteins cross-linked with mercaptobutyrimidate by preparative SDS gel electrophoresis. ^{35}S-labeled 30S ribosomes (1500 μg; 60 × 10^6 cpm) were cross-linked with 10 mM mercaptobutyrimidate as described in Methods. The ribosomes were heated in SDS gel sample buffer, applied to a 0.5 × 11.5 cm tubular SDS gel, and electrophoresed (Traut *et al.*, 1973). The profile of radioactivity was obtained by slicing the gel, extracting, and counting an aliquot of each fraction (see Methods). The lower panel shows the autoradiograms of similar SDS gels of the ribosomal protein from the control and mercaptobutyrimidate-cross-linked ribosomes.

teins (Figure 4). The mobility of free proteins treated with mercaptobutyrimidate is unchanged during analysis by electrophoresis in the SDS gel system employed. A calibration curve of molecular weight *vs.* migration distance was obtained utilizing the established molecular weights of certain well-resolved ribosomal proteins.

Preparation of Antisera against E. coli 30S Ribosomal Proteins. Rabbit antisera against S1, S4, S5, S7, S8, S9, S10, S11, S13, S14, S16, S18, S19, S20, and S21 were prepared by immunization with purified 30S ribosomal proteins as described by Higo *et al.* (1973). Antisera were tested by immunodiffusion against all pure 30S proteins and found to react only with the protein used for immunization, with the exception of anti-S1 which also reacted very weakly with S6 in addition to S1. In the latter case the S1 band was clearly distinguishable in Ouchterlony patterns both by position and by intensity when total proteins from the 30S ribosome were used as the antigen. In some cases antisera were concentrated 5-fold (Higo *et al.*, 1973) before use.

Radioimmunodiffusion. Cross-linked fractions eluted as described above from a preparative SDS disk gel with 1% SDS were first dialyzed against several changes of 6 M urea–0.03 M methylamine acetate (pH 5.6 at 25°) at 4° for 3 days to remove free SDS and then dialyzed into buffer suitable for immunodiffusion analysis (0.02 M $MgCl_2$, 1 M KCl, and 0.03 M Tris-HCl, pH 7.4; Kahan *et al.*, 1973). The possible presence of residual SDS on the proteins thus prepared did not interfere with the reacton between the proteins and antisera (see Results).

The double-diffusion analysis in 1% agar, 0.02 M $MgCl_2$, 1 M KCl, and 0.03 M Tris-HCl (pH 7.4) was performed as described by Kahan *et al.* (1973). Diffusion was carried out for 2–3 days at room temperature. The gels were washed in a large excess of gel buffer (three changes, 24 hr/wash), then washed in distilled water, photographed, covered with a wet sheet of filter paper, and air-dried. Autoradiograms of the dried gels were obtained with Kodak Royal Blue X-Ray film (1–4-days exposure).

Reagents. Mercaptobutyrimidate was prepared as previously described (Traut *et al.*, 1973; now available commercially from Polysciences, Inc., Warrington, Pa.) and stored under vacuum at 4° over P_2O_5. Bissuberimidate was synthesized as described according to McElvain and Schroeder (1949) and was kindly provided by Dr. W. Benisek.

Results

Sedimentation Behavior of the 30S Ribosomes Modified with Mercaptobutyrimidate. When the radioactive 30S ribosomes cross-linked by mercaptobutyrimidate under the conditions described above were analyzed by sucrose density gradient centrifugation (Figure 2), it was found that they cosedimented with untreated nonradioactive 30S ribosomes added as marker. No dimers of the 30S subunits were formed as a result of the cross-linking procedure. Previous experiments had shown that no cross-linking occurs when total 30S protein extracted from the ribosome was treated by the procedures employed (Traut *et al.*, 1973). It was concluded therefore that no major conformational change resulted from the cross-linking reaction, and furthermore that the protein:protein cross-linking which was detected with a result of the proximity of the proteins within individual 30S ribosomal particles, and not due to inter-ribosomal interaction, nor to disulfide-bond formation between free proteins in solution.

Fractionation of the Mercaptobutyrimidate-Cross-Linked 30S Ribosomal Proteins by SDS Gel Electrophoresis. When the total protein mixture from radioactive 30S ribosomes cross-linked with mercaptobutyrimidate was analyzed by SDS polyacrylamide gel electrophoresis, new bands were observed

which migrated more slowly than the protomeric proteins characteristic of the control pattern (Traut *et al.*, 1973). In order to isolate these new bands, 1500 μg of 30S ribosomal subunits labeled with ^{35}S (60×10^6 cpm) were cross-linked with mercaptobutyrimidate and applied to a preparative (tubular) SDS gel. After electrophoresis the gel was cut into 1-mm slices and each slice was extracted and counted as described in Methods. The total recovery of radioactivity from the gel was between 70 and 80%. The radioactivity profile obtained by slicing a gel was similar to the pattern obtained by autoradiography of a dried gel (Figure 3). Partially purified cross-linked species were obtained for further analysis by selecting specific slices or combining adjacent slices.

SDS Gel Analysis of the Isolated High Molecular Weight Fractions Resulting from Cross-Linking. Examination of the fractions isolated from preparative SDS gels by electrophoresis on a second SDS slab gel before and after reduction provided the following information: (1) the apparent molecular weight of the particular cross-linked fraction; (2) the molecular weights and the number of components contained in the cross-linked product after reductive cleavage; (3) unambiguous identification of certain proteins contained in cross-linked products for those proteins which appear as bands known to consist of only one protein species on the SDS gel system employed; *i.e.*, proteins S1, S2, S3, and S4 which can be identified unambiguously from their mobility during SDS gel electrophoresis. For most of the ribosomal proteins with a molecular weight of less than 20,000, SDS gel electrophoresis does not provide sufficient resolution for unambiguous identification of individual proteins. However, since the position of all 30S ribosomal proteins in the gel system was established (Figure 4d), the molecular weights of the reduced protomeric components of all dimers could be determined. In this way the possible candidates for participation in any given cross-linked species could be distinctly limited to a relatively small subset of proteins.

Analysis of the cross-linked material eluted from gel slices was carried out before and after reduction as described in Methods. Nonradioactive 30S total protein (20 μg) was added to the reduced radioactive sample in order that the gel could be stained by Coomassie Blue after electrophoresis. Comparison of the autoradiogram with the stained pattern thus provided both the molecular weight of the radioactive components and their position relative to the established protein pattern (Figure 4d). Figure 4a–c shows the results of this analysis for three different cross-linked fractions.

Molecular Weight 58,000 Fraction. The fraction (Figure 3, fraction 24) had an apparent molecular weight of 58,000 and gave rise after reduction to two major protein components with molecular weights of 32,000 and 29,000 (see Table I and Figure 4c). In addition it contained a number of minor products with molecular weights less than 20,000. The molecular weight data is consistent with dimer formation between the two major monomeric proteins identified after reduction. The two major protein constituents were identified unambiguously by gel electrophoresis in SDS as S2 and S3.

If a dimer consisting of S2 and S3 constitutes the primary constituent of this fraction, then the two components should be present in equimolar amounts. Since the molecular weights of these two proteins are similar (32,000 *vs.* 29,000), the proteins S2 and S3 should also be present in approximately equal mass fractions. Figure 5 shows a scan of the autoradiogram of this fraction after reduction. The areas of the peaks corresponding to proteins S2 and S3 are approximately equal. Control experiments showed the linearity of film image density to the amount of radioactivity present. Assuming that the sulfur content of the two proteins is similar it can be concluded that proteins S2 and S3 are present in fraction 24 in a 1:1 molar ratio. This assumption is justified by the early experiment of Traut (1966) in which a close correspondence of autoradiograms of gels of 30S protein labeled both with ^{35}S and a mixture of [^{14}C]amino acids was demonstrated.

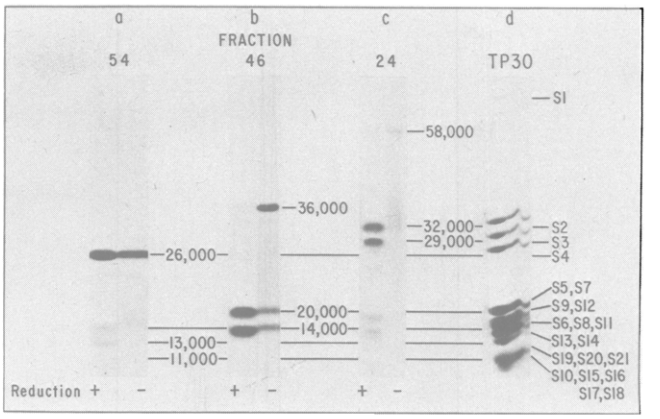

FIGURE 4: SDS gel analysis of the selected cross-linked fractions after reductive cleavage. 50 μl of the fractions extracted from a preparative SDS gel (in Figure 3) containing 5000–12,000 cpm was mixed with 10 μl of SDS gel sample buffer (0.029 M $NaH_2PO_4 \cdot H_2O$–0.072 M $Na_2HPO_4 \cdot 7H_2O$–5% SDS–40% glycerol). Half was applied to the analytical gel without reduction while the other half was mixed with 2 μl of β-mercaptoethanol, 2 μl of ribosomal solution containing about 20 μg of total 30S ribosomal proteins to serve as staining marker, and then applied to the gel. All samples were heated at 65° for 15 min before electrophoresis. After electrophoresis the gel was stained with Coomassie Blue, dried under vacuum, and exposed to X-ray film for autoradiography. (a–c) Autoradiogram of fractions 54, 46, and 24 after and before reductive cleavage. (d) Pattern of 30S total protein stained with Coomassie Brilliant Blue. The position of each 30S ribosomal protein in this gel system was established using purified individual proteins as standards.

Although the molecular weight and stoichiometry data support the hypothesis that this fraction contains an S2–S3 dimer, the possibility exists that S2 and S3 are cross-linked to other ribosomal proteins which are also present in this fraction in minor amounts (Figure 5), instead of having been cross-linked to each other. This possibility was ruled out because (1) the difference between the molecular weights of the cross-linked fraction (58,000) and that of S2 or S3 is approximately 28,000; this difference is too large to be accounted for by any single minor component, all of which have molecular weights of less than 20,000. Therefore, the cross-linked species could not consist of a mixture of dimers, such as [S2–protein A] or S3–protein B]; (2) if cross-linked trimers such as [S2–protein A–protein B] and [S3–protein C–protein D] were the major components present in the fraction, it would be a highly unlikely coincidence that both S2 and S3 would be present in high yield and in stoichiometric amounts. Therefore, it was concluded that fraction 24, although contaminated with higher oligomers of other proteins, contained as its principal component a dimer of S2 and S3.

Molecular Weight 36,000 Fraction. The fraction (Figure 3, fraction 46) had an apparent molecular weight of 36,000 and gave rise after reduction to two major components with lower molecular weights, 20,000 and 14,000 (see Figure 4). The data are consistent with dimer formation between two proteins with the molecular weights observed after reduction of the fraction. From the calibration of the SDS gel system with pure proteins (see Figure 4d), it was established that the band

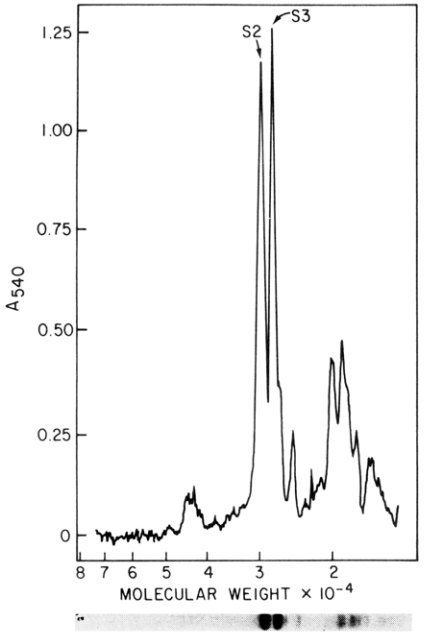

FIGURE 5: Scan of autoradiogram of reduction products formed from fraction 24. The autoradiogram was scanned in a Gilford 2400S spectrophotometer at 540 mμ (Traugh and Traut, 1972).

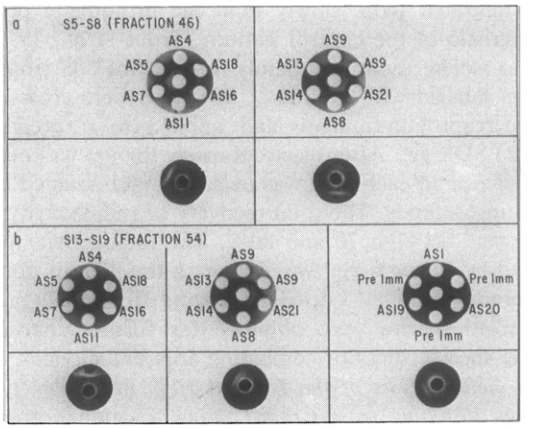

FIGURE 6: Immunochemical identification of the proteins in fractions 46 and 54. The dialyzed sample (20 μl) containing approximately 0.1 μg of cross-linked proteins of each fraction (about 15,000 cpm of ^{25}S) were mixed with 10 μg of cold 30S total protein and applied to the central well. Appropriate amounts of antisera against individual 30S proteins were put in the side wells. Immunodiffusion patterns and autoradiograms of the dried gels were obtained as described in Methods: (a) fraction 46; (b) fraction 54.

corresponding to a molecular weight of 20,000 might contain S5 or S7, and that corresponding to a molecular weight of 14,000 might contain S6, S8, or S11. However, since the SDS gel does not provide resolution adequate to differentiate between these two sets of proteins, additional analysis of the fraction was necessary to identify unambiguously the proteins found by reduction of the dimer. Immunochemical methods, in which the cross-linked products were analyzed with antibodies to single pure 30S proteins, were employed. From this analysis, it was found that fraction 46 (Figure 3) contained S5 and S8, with only negligible amounts of S7 or S11 (see Figure 6a).

To test further whether S5 and S8 were covalently cross-linked together, an intragel cross-absorption experiment (Van Regenmortel, 1967) utilizing antibodies was performed as follows. Antiserum to either S5 or S8 was added to the center well of the Ouchterlony plate and allowed to diffuse into the gel. Then the cross-linked fraction was added to the same center well. Antibodies against S8 and S5, respectively, were added to the outer well and the immunodiffusion reaction carried out. If all the S5 and S8 in this fraction were cross-linked to each other, then antibodies against either of these two proteins added to the center well should absorb both S5 and S8 and prevent their further reaction with either anti-S5 or anti-S8 in the side wells. It was found that a cross-absorption with anti-S5 (Figure 7b) completely eliminated reactivity with anti-S8, and cross-absorption with anti-S8 eliminated 80–90% of the reactivity with anti-S5. It was therefore concluded that most of the S5 in this fraction was in S5-S8 complex, while a small portion of it (less than 20%) was probably crosslinked to some protein other than S8. The yield of cross-linking between this pair of proteins was found to be the highest of those analyzed in the present studies.

Molecular Weight 26,000 Fraction. The fraction (Figure 3, fraction 54) had an apparent molecular weight of 26,000 and coelectrophoresed with purified S4. Following reduction it gave rise to three major bands of lower molecular weights (14,000, 13,000, and 11,000) and in addition to a major component with the original molecular weight unchanged. The latter was interpreted to be S4, uncross-linked, and present in the isolated fraction. The cross-linked material present in the fraction is interpreted to consist of a mixture of two or more dimers since the sum of the molecular weights of the three reduction products substantially exceeds that of the original cross-linked material. When tested with antibodies against pure 30S proteins, the fraction showed strong reactivity toward antibodies against S4, as expected, but also S13 and S19 (Figure 6b). These results alone are consistent with, but do not unambiguously prove, the hypothesis that S13 and S19 were cross-linked, since it is possible that this fraction contains two dimers; *e.g.*, [S13–protein A] and [S19–protein B]. Confirmation of the result, however, was obtained using an independent method (L. Kahan and W. Held, manuscript in preparation). Ribosomes were reconstituted with [^{125}I]S19, cross-linked with glutaraldehyde, and the radioactivity present in the putative S13–[^{125}I]S19 dimer was found to coprecipitate with an S13–anti-S13 complex.

Cross-Linking of 30S Ribosome by Bissuberimidate. When 30S subunits labeled with ^{35}S were cross-linked with bissuberimidate instead of mercaptobutyrimidate, the pattern of high molecular products identified by SDS gel electrophoresis was similar although not identical (Figure 8). In particular, a new band of mol wt 36,000 was formed in high yield just as with mercaptobutyrimidate. Since proteins cross-linked by bissuberimidate cannot be readily cleaved (Bickle *et al.*, 1972), no information was obtained concerning the molecular weights of its component proteins. However, immunochemical analysis (data not shown) of this fraction revealed the presence of both S5 and S8. The existence of this dimer found also with the mercaptobutyrimidate reagent was thus further confirmed.

Discussion

The purpose of the present work was to obtain information about the topographical relationship of the ribosomal proteins of the *E. coli* 30S subunit by using bifunctional reagents to covalently link neighboring proteins. Information thus obtained is complementary to that derived by other more indirect approaches, *e.g.*, genetic studies, studies on ribosome assembly, studies of the protein compositions of the ribosomal subparticles produced by mild nuclease digestion, etc., and

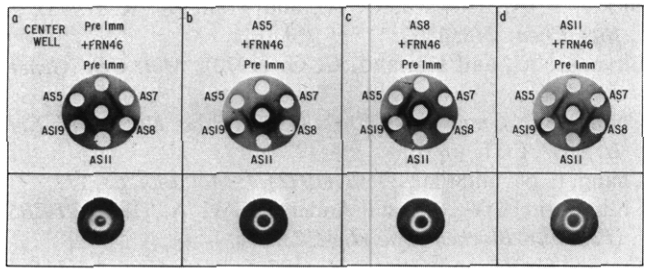

FIGURE 7: Intra-gel cross-absorption experiment of fraction 46 proving the cross-linking between proteins S5 and S8. Appropriate antisera (20 μl) used for cross-absorption were allowed to diffuse from the central well first, then 20 μl of the putative S5–S8 fraction plus 10 μg of cold 30S total proteins were applied to the same well. Antisera were added to the outside wells and radioimmunodiffusion was carried out as described in Methods: (a) preimmune serum, as a control; (b) anti-S5; (c) anti-S8; (d) anti-S11; which served as another control since only negligible amounts of S11 were found present in fraction 46 (see Figure 6a).

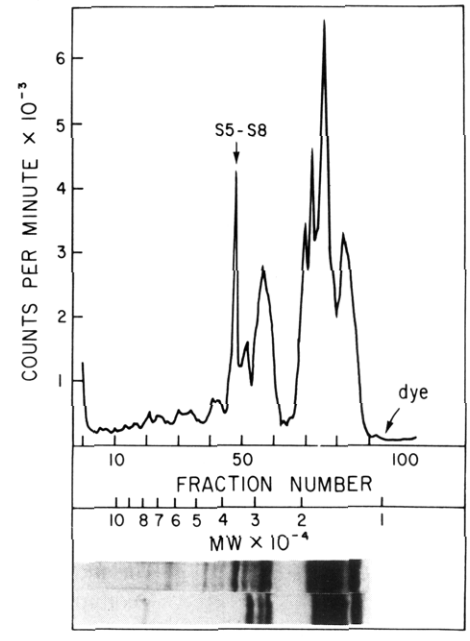

FIGURE 8: Fractionation of 30S ribosomal protein cross-linked by bissuberimidate by preparative SDS gel electrophoresis. Conditions were identical with those described in the legend of Figure 3 except 30S ribosomes were cross-linked with 25 mM bissuberimidate instead of 10 mM mercaptobutyrimidate. Lower panel shows the autoradiogram of similar SDS gels of the ribosomal proteins from the control and bissuberimidate-cross-linked ribosomes.

will contribute directly to a complete understanding of the three-dimensional arrangement of the *E. coli* 30S ribosomal proteins.

Several factors might influence the efficiency of cross-linking between ribosomal proteins: (1) the distance between the closest reactive groups in the proteins, and the length of the reagent used; (2) the presence or absence of rRNA which might sterically or chemically hinder the cross-linking reaction between two neighboring proteins; (3) the chemical nature of the reagent and of the reactive amino acid side chains exposed at points of protein–protein contact; (4) the shape as well as the relative orientation of proximal proteins, *e.g.*, two rod-shaped proteins oriented side-by-side might be cross-linked with higher efficiency than if their orientation were end to end. Detailed information on the shape or axial ratio of ribosomal proteins is not available at this time; (5) the spatial orientation of the potentially reactive amino acid side chains on two proximal proteins; (6) possible ribosome heterogeneity (Bickle *et al.*, 1973; Voynow and Kurland, 1971). The formation of certain dimers may be precluded by the fact that certain pairs of proteins may never simultaneously be present on the same ribosome. Any one or a combination of several of these factors may contribute to the fact that some pairs of 30S ribosomal proteins are cross-linked in higher yields than the other. At present, there is insufficient information to distinguish between these various possibilities. However, recent results in this laboratory (A. Sommer) indicate that most if not all 30S ribosomal proteins can be isolated in dimers or trimers, although the relative yields are variable.

As pointed out by Lutter *et al.* (1972), the fact that two ribosomal proteins become cross-linked does not necessarily reflect the proximity of the proteins in active ribosomal particles. However, the assumption throughout the studies reported here is that since no major conformational change occurred in the cross-linked particles, the relationships which we have found do indeed reflect those present in native 30S particles. Our confirmation of the existence of a S5–S8 dimer, which Lutter and Kurland (1973) have demonstrated can be incorporated as a dimer to an active reconstituted 30S particle lends validity to this assumption.

Comparisons of the results on ribosomal protein neighborhoods obtained by the present cross-linking approach and the results from various experimental approaches in other laboratories are summarized as follows. (1) Comparison with the protein composition of subparticles produced by digestion of the ribosome with ribonuclease. (a) Schendel *et al.* (1972) found that S2, S3, S5, S8, S13, and S19 (all the proteins which we found in different cross-linked pairs) were uniquely found in one of the subparticles formed by treating the 30S ribosome with ribonuclease. Assuming that during the treatment with ribonuclease there was no major rearrangement or redistribution of the ribosomal proteins, this result indicates that all of these components constitute a group of proximal ribosomal proteins. The present finding of dimer formation between S2 and S3, S5 and S8, and S13 and S19 is consistent with this result. (b) Roth and Nierhaus (1973) found S5 and S8 in one of their subparticles which contained S4, S5, S8, S15, S16, and S20; and both S13 and S19 in another subparticle which contained S5, S9, S10, S13, and S19. (c) Morgan and Brimacombe (1973) found the occurrence of S5 and S8, and S13 and S19 in several of their fragments. Again, these results are consistent with the finding reported here of dimers between S5 and S8, and S13 and S19. (2) Comparison with the order of addition of proteins in the assembly map: in the construction of the assembly map Mizushima and Nomura (1970; also M. Nomura, personal communication) obtained results which suggest that the optimum binding of S2 depends on the presence of S3 during ribosome assembly *in vitro* (*cf.* Table III in that reference). Similarly, Mizushima and Nomura (1970) found that S5 binds to 16S RNA only if S8 is bound first, suggesting that these two proteins might interact directly or indirectly with each other during assembly. (3) Comparison with functional studies: Randall-Hazelbauer and Kurland (1972) have reported that S2, S3, and S14 contribute to the tRNA binding site of the 30S ribosome. It is therefore possible that these three proteins are in the same region on 30S ribosomes and the present finding of cross-link formation between S2 and S3 is consistent with these results. (4) Comparison with other cross-linking studies: pairs or groups of ribosomal proteins which have been found to become cross-linked by different bifunctional reagents are summarized in Table I. It is apparent from this comparison that some pairs or groups or proteins,

TABLE I

Dimer	Cross-Linking Reagent	Reference
S5–S8	Mercaptobutyrimidate	Present report
	Bissuberimidate	Present report and Kurland (personal communication)
	Bisadipimidate	Lutter *et al.* (1972)
S5–S9	Bissuberimidate	Bickle *et al.* (1972); Sommer and Traut (unpublished results)
S18–S21	*N,N'-p*-Phenylenebismaleimide	Chang and Flaks (1972)
	N,N'-p-Phenylenebismaleimide	Lutter *et al.* (1972)
S11–S18–S21	Tetranitromethane	Shih and Craven (1973)
S7–S9	Bissuberimidate	Kurland (personal communication)
S13–S19	Mercaptobutyrimidate	Present report
	Bissuberimidate	Kurland (personal communication)
S2–S3	Mercaptobutyrimidate	Present report

despite the differences in reaction conditions and cross-linking reagents, have been found to be crosslinked by several laboratories independently.

Information obtained from the present and other topographical studies have made it possible to construct a preliminary three dimensional model of the *E. coli* 30S ribosome indicating the spatial arrangement of the ribosomal proteins (T. T. Sun, R. L. Heimark, and R. R. Traut, in preparation). The model makes predictions of new neighborhood relationships between other 30S ribosomal proteins which can be tested experimentally.

The results reported here confirm the applicability of the new cleavable cross-linking reagent mercaptobutyrimidate (Traut *et al.*, 1973) to studies on the topography of ribosomal proteins. Experiments are in progress to explore conditions under which the cleaved products of cross-linked materials may be identified by simple gel techniques alone without recourse to immunochemical methods. Obviously such techniques are required if the reagent is to be used in the study of more complex systems in which antibodies to individual constituents may not be available.

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