Photoinduced Cross-Linkage, in Situ, of *Escherichia coli* 30S Ribosomal Proteins to 16S rRNA: Identification of Cross-linked Proteins and Relationships between Reactivity and Ribosome Structure†

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ABSTRACT: The kinetics of photoinduced cross-linkage of *Escherichia coli* 30S ribosomal proteins to the 16S rRNA molecule in the intact *Escherichia coli* 30S ribosomal subunit was studied in this report. All of the 30S ribosomal proteins become cross-linked to the 16S rRNA before changes in the sedimentation characteristics of the 30S ribosomal subunit can be detected. The proteins exhibit different reactivities in the cross-linkage reaction. One group of proteins—S3, S7–S9, S11, S12, and S15–S19—is cross-linked to the 16S rRNA by single-hit kinetics, or by photoprocesses of nonunity but low multiplicities. A second group of proteins—S1, S2, S4–S6, S10, S13, S14, and S21—is cross-linked to the 16S rRNA by photoprocesses of a complex nature. A comparison of these data with other properties of the individual 30S ribosomal proteins related to ribosome structure indicated that most of the 30S ribosomal proteins cross-linked to the 16S rRNA by photoprocesses of low multiplicities had been classified rRNA-binding proteins by nonphotochemical methods, and most of the proteins cross-linked to the 16S rRNA by photoprocesses of large multiplicities had been classified as nonbinding proteins. There were certain exceptions to these correlations. Proteins S4 and S20, both RNA-binding proteins, become cross-linked to the 16S rRNA by photoprocesses of large multiplicities, and proteins S3, S11, S12, and S18, none of which have been classified RNA-binding proteins, exhibited low multiplicities in the cross-linkage reaction. All of these exceptions could be explained in terms of limitations inherent in the photochemical methods used in this study and in other types of methods that have been used to study RNA–protein interactions in the 30S ribosomal subunit. The data presented here also suggest that labile RNA–protein cross-links are present in the uv-irradiated 30S ribosomal subunits, and that neither peptide-bond cleavage nor photoinduced modification of the charged side-chain groups in the ribosomal proteins accompanied the cross-linkage reaction. However, some photoinduced RNA-chain breakage might have occurred.

The 30S ribosomal subunit of *E. coli* is a ribonucleoprotein complex constituted of a 16S rRNA molecule and 21 proteins. The identities of the individual ribosomal proteins interacting directly with the 16S rRNA molecule in the intact 30S ribosomal subunit have been the subject of numerous investigations. The basic approaches used in these investigations have included identification of (1) the 30S ribosomal proteins able to form specific and isolable 16S rRNA–protein complexes (Mizushima and Nomura, 1970; Schaup et al., 1970), (2) the 30S ribosomal proteins not dissociated from the intact ribosomal subunit in the presence of high concentrations of LiCl or CsCl (Nomura et al., 1969; Homann and Nierhaus, 1971), (3) the 30S ribosomal proteins associated with specific rRNA fragments prepared by ribonuclease treatment of free 16S rRNA (Zimmerman et al., 1974), and (4) identification of the 30S ribosomal proteins required for ribosome assembly (Mizushima and Nomura, 1970; Nashimoto et al., 1971).

There are situations in which the RNA–protein interactions deduced from the approaches described above may not result in identification of all of the possible RNA–protein interactions in the intact 30S ribosomal subunit. For example, a ribosomal protein might be able to interact directly with the 16S rRNA in the intact ribosomal subunit but be characterized as a "nonbinding" protein if the RNA-binding constant of the protein is too small to prevent its dissociation from the ribosome in the presence of LiCl or CsCl and/or too small to permit the formation of a stable 16S rRNA–protein complex. Alternatively, a ribosomal protein might bind directly to the 16S rRNA molecule in the intact ribosomal subunit, and yet be classified as a nonbinding protein if it were found not to be essential for in vitro and/or in vivo ribosome assembly. These shortcomings could be obviated by the development and application of a method for covalently cross-linking in situ the protein components of the 30S ribosomal subunit to the 16S rRNA. In a series of recent reports (Gorelic, 1975a–c), data have been presented consistent with the conclusion that the protein components of the 30S ribosomal subunit can be covalently cross-linked to the 16S rRNA component by exposure of the intact subunit to 253.7-nm radiation. Since the formation of a cross-link between the rRNA component of the 30S subunit and a protein requires the formation of a covalent bond only between a single rRNA base and amino acid, it was felt that the application of such a photochemical reaction to studies of RNA–protein interactions would be useful in the detection of ribosomal proteins able to interact to only a limited extent with the 16S rRNA molecule in the intact 30S ribosomal subunit. Furthermore, since the reactive groups in the individual proteins must be sufficiently close to the photoexcited rRNA bases to form a RNA–protein cross-link, it was also felt that the reactivities of the individual proteins in photoinduced cross-linkage in situ to the 16S rRNA might reflect their accessibilities to the 16S rRNA in the intact ribosomal subunit.

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The photoinduced cross-linkage in situ of the 30S ribosomal proteins to the 16S rRNA in the intact *E. coli* 30S ribosomal subunit has been studied in more detail in this report than in earlier studies. The results of the studies presented here indicate that the photoinduced cross-linkage of individual ribosomal proteins to the 16S rRNA molecule in the intact 30S ribosomal subunit of *E. coli* occurs in a sequential manner, and that there is good correlation between the reactivities of the various proteins in cross-link formation and their abilities to associate with the 16S rRNA in a number of RNA-containing subribosomal particles.

Experimental Section

Materials

Pancreatic ribonuclease was obtained from Worthington Biochemicals. Acrylamide and methylenebisacrylamide were obtained from Eastman Kodak, and were recrystallized prior to use. *N,N,N′,N′*-Tetramethylenediamine was obtained from Eastman Kodak, and was purified as described previously (Gorelic, 1975a). Sodium lauryl sulfate was obtained from Schwarz-Mann, and was recrystallized from ethanol prior to use. Labeled [5,6-^{3}H]uracil, specific activity 40 Ci/mmol, was obtained from Schwarz-Mann. Potassium ferrioxalate was prepared according to the method of Hatchard and Parker (1956). All other chemicals were analytical reagent grade.

Unlabeled ribosomal subunits were prepared from *E. coli* D10 cells grown in complete media and harvested in midlog phase, according to previously described methods (Gorelic, 1975a). Ribosomal subunits labeled in their rRNA components with [5,6-^{3}H]uracil were prepared from *E. coli* D10 cells grown in complete media containing 2μCi/ml of labeled uracil and harvested in midlog phase.

Methods

Irradiation Conditions. Solutions of *E. coli* 30S ribosomal subunits (100 A_{260} units) in 100 ml of irradiation buffer (5 mM H_3PO_4, neutralized to pH 7.4 with KOH; 10 mM $MgCl_2$; 30 mM KCl) were irradiated at 253.7 nm according to methods previously described (Gorelic, 1975a). The temperature of the ribosome solution was maintained at 22.0 ± 0.1 °C during the course of the irradiations by circulation of thermostated water through the dipper well. The output of the light source at 253.7 nm was determined by ferrioxalate actinometry (Parker, 1953) to be $1.53 \pm 0.04 \times 10^{18}$ quanta s^{-1}.

Electrophoretic Conditions. One-dimensional gel electrophoresis was carried out according to the procedure of Traut et al. (1969). Protein samples were dialyzed against the electrophoresis buffer, aliquots (0.2-ml volume, containing 150 μg of protein) were removed, and bovine serum albumin and methyl green were added as staining standards and tracking dye, respectively. The resultant mixtures were then applied to the tops of 6 × 200 mm acrylamide gels, the compositions of which were 7.5% (w/v) acrylamide, 0.8% (w/v) methylenebisacrylamide, and 8 M in urea. Electrophoresis was carried out from cathode to anode at 4 °C and at pH 4.5 for 16 h at an applied voltage of 6.5 V/cm. The protein bands on the gels were visualized by staining with Coomassie brilliant blue. The resultant electrophoretic patterns were then quantitated in two ways. First, the intensities of the stained bands of the gels were determined by scanning the gels on a Gilford Model 2400S spectrophotometer equipped with a linear gel transport system, and then integrating the areas under the resultant peaks using a K&E Model 162002 planimeter. Second, the bands were cut from the gels, eluted with 25% (w/v) pyridine:water according to the method of Traut et al. (1975), and the amount of stain in each of the resultant eluates was determined spectrophotometrically. With the exception of three gel bands (8A, B, and 10A), there was excellent agreement between the two methods. Since the gel-scanning method gave a profile that exhibited extensive overlap in the region of these three bands (see Figure 1), it was assumed that the slice method gave more accurate data for these particular proteins.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using a modification of the procedure of Weber et al. (1972). The gels used in these studies were polymerized at pH 7.2 and contained, at final concentrations, acrylamide (15%, w/v), methylenebisacrylamide (0.2%, w/v), sodium dodecyl sulfate (0.1%, w/v), and urea (6M). The phosphate-containing electrode buffer (pH 7.2) contained sodium dodecyl sulfate (0.1%, w/v) and was modified by the inclusion of urea (6 M final concentration). Protein samples (0.3-ml volume, containing 150 μg of protein and added bovine serum albumin as a high-molecular-weight marker) were reduced by dialysis against the electrode buffer containing 2-mercaptoethanol (1%, w/v) for 12 h at 20 °C. Brom phenol blue was added to the reduced samples as a tracking dye, and the resultant mixtures were applied to the tops of 6 × 120 mm sodium dodecyl sulfate–polyacrylamide gels. Electrophoresis was carried out at 20 °C for 18 h at an applied potential of 3 V/cm. The proteins were visualized by staining the gels with Coomassie brilliant blue.

Electrophoretic analysis of proteins by two-dimensional gel electrophoresis was carried out by a combination of methods. Electrophoresis in the first dimension was performed according to the published procedure of Howard and Traut (1973), using disc gels of 5 × 80 mm dimensions and containing 4% (w/v) acrylamide, 0.13% (w/v) methylenebisacrylamide, and 6 M urea. The protein samples to be analyzed were dialyzed against the electrophoresis buffer used in the first dimension. Aliquots containing 150 μg of protein were then removed, were diluted to 0.2-ml volume with electrophoresis buffer, and the resultant solutions were applied to the origins of each of two one-dimensional disc gels. Electrophoresis was carried out at pH 8.7. One of the samples was electrophoresed for 15 h from cathode to anode at an applied potential of 10 V/cm. The other sample was electrophoresed for 15 h from anode to cathode at an applied potential of 21 V/cm. The gels were then removed from the tubes and prepared for the second dimension by dialysis against the starting buffer of Kaltschmidt and Wittmann (1970). The gels were cut so that the final dimensions were 4.5 cm for the gel run from cathode to anode, and 7.5 cm for the gel run from anode to cathode. The cut gels were then placed into a vertical gel-slab apparatus (E. C. Apparatus, St. Petersburg, Florida), and the gel in the second dimension was prepared according to the method of Kaltschmidt and Wittmann (1970). The final dimensions of the slab were: thickness × top × side = 30 × 120 × 200 mm. Electrophoresis in the second dimension was carried out at acidic pH (4.6) at an applied voltage of 7.5 V/cm for 22 h and at 4 °C. The slab was then removed from the apparatus, and the proteins visualized by staining with Coomassie brilliant blue.

Preparation of Urea–LiCl Supernatant Fractions. Solutions of 30S ribosomal subunits were irradiated individually with different doses of 253.7-nm radiation. The irradiated solutions, as well as a solution of unirradiated ribosomes, were concentrated by ultrafiltration (Amicon XM-300 ultrafilter), and the ribosomal subunits pelleted by ultracentrifugation on a Beckmann Model L2-65B ultracentrifuge (65-type angle rotor; 250 000*g* for 12 h). The ribosome pellets were resuspended in

a small volume of irradiation buffer and the ribosomes separated into a soluble and an insoluble fraction by treatment with 4 M urea–3 M LiCl (Traub et al., 1971). The insoluble material was removed by low-speed centrifugation. The soluble fraction was dialyzed exhaustively against a storage buffer (10 mM Tris-HCl, pH 7.4, 8 M urea, 6 mM 2-mercaptoethanol, 12 mM methylamine-HCl) and stored at −70 °C. The dialyzed, soluble fraction is designated as the urea–LiCl supernatant fraction.

Results

Electrophoresis Studies. The kinetics of photoinduced cross-linkage of the 30S ribosomal proteins to the 16S rRNA was followed in this study by monitoring on one- and two-dimensional polyacrylamide gels the disappearance of proteins from urea–LiCl supernatant fractions prepared from uv[1]-irradiated ribosomes. The basis of this procedure, as has been discussed elsewhere (Gorelic, 1975a,b), is that proteins covalently cross-linked to a nucleic acid molecule will exhibit electrophoretic properties markedly different from the free protein molecules. The electrophoretic patterns, on one-dimensional polyacrylamide gels, of the protein components in urea–LiCl supernatant fractions prepared from irradiated and unirradiated ribosomal subunits are presented in Figure 1. As can readily be seen, exposure of aqueous solutions of the subunits to a dose of 5×10^{19} quanta results in almost complete disappearance of band 7 from the gels, and reductions in intensities of bands 3A, $8B_{ox}$, 9, and 12–14 (Figure 1A). Exposure of solutions of the subunits to a somewhat higher dose of uv radiation ($1–1.5 \times 10^{20}$ quanta) results in the loss of additional bands from the electropherograms (bands 0, 1, and 9) and reductions in the stained intensities of bands 2A + 2B, 4A + 4B + 3B, 4C, 5, and 10B (Figure 1B). Finally, only three bands (4A + 4B + 3B, 8B, 8A + 10A) can still be detected on electropherograms of samples prepared from ribosomes irradiated with a dose of 2×10^{20} quanta (Figure 1C).

The dose dependence of the reduction in initial stained intensities of the bands on the gels in Figure 1 was determined, and the resultant data were plotted in a series of survival-type curves in Figure 2. As can be seen from the resultant plots, the reduction in intensities of the various bands occurs in a sequential manner. Doses of less than 0.5×10^{20} quanta were sufficient to result in statistically significant decreases in the initial intensities of bands 1, 3A, 4B + 4A + 3B, 5, 6, 7, and 8B (corresponding to proteins S20, S17, S15 and S16, S9, S10, S7, and S8, respectively), whereas doses greater than 0.5×10^{20} quanta were required for significant reductions in the intensities of bands 0, 2A + 2B, 4C, 9, and 10B (proteins S21, S18 + S19, S11, S4, and S3, respectively), and greater than 1×10^{20} quanta for significant reductions in the intensities of bands 11 and 12 (proteins S1 and S2, respectively). Finally, the largest doses used in these studies (5×10^{20} quanta) were not sufficient to significantly reduce the initial intensities of band 8A + 10A (proteins S5 and S6, respectively). The data presented in Figure 2 also suggest that there is considerable variation in the complexity of the photoreactions ultimately responsible for the reductions in intensities of the various bands on the one-dimensional gels. The disappearance from the one-dimensional gels of bands corresponding to S3, S7-S9, S11 and S15–S19 quite clearly occurs by low multiplicity processes (extrapolation numbers of 1–3), whereas the disappearance of the remaining bands on the gels requires photoreactions of considerably higher multiplicities. Finally, although the kinetics of disappearance of most of the ribosomal proteins from the one-dimensional gels exhibit a maximum of one discontinuity, the kinetics of disappearance of three of the ribosomal proteins (one-dimensional bands corresponding to proteins S8, S15, and S16) exhibit two discontinuities and these bands are still detected at the highest doses of uv radiation used in these studies.

A number of 30S ribosomal proteins cannot be resolved into distinct bands on the Traut one-dimensional gels, and band assignments have not been made for other proteins (Traut et al. 1969). In addition, the results of model studies of the photoaddition to pyrimidines of compounds containing the same functional groups as in the naturally occurring α-amino acids (Sinsheimer and Hastings, 1949; Wang et al., 1956; Gorelic et al., 1972) suggest that some of the 30S ribosomal proteins might cross-link to the 16S rRNA exclusively by covalent bonds labile under the acidic conditions used in the one-dimensional gel studies. In contrast to the Traut one-dimensional gels, the two-dimensional gel system developed by Kaltschmidt and Wittmann (1970) and subsequently modified by Howard and Traut (1973) permits the resolution of all 21–30S ribosomal proteins into distinct spots. In addition, since electrophoresis in the first dimension of the two-dimensional gels is carried out at a basic pH, the two-dimensional gel system could provide a means for identification of ribosomal proteins cross-linked to the 16S rRNA by covalent bonds labile at acidic pH values (but stable at a basic pH). Consequently, the samples used in the electrophoretic studies represented by the data in Figure 1 were reexamined using the two-dimensional gel system of Kaltschmidt and Wittmann (1970) as modified by Howard and Traut (1973), and the results of the two-dimensional gel studies compared with the results obtained by one-dimensional gel electrophoresis. The results of the two-dimensional gel studies are presented in Figure 3. Gels of duplicate samples have been presented in Figure 3A, A′ to indicate the limits of reproducibility of the electrophoretic data. The electropherogram in Figure 3B indicates that irradiation of aqueous solutions of the 30S ribosomal subunits with a dose of 5×10^{19} quanta results in a marked reduction, on a two-dimensional electropherogram of the derived urea–LiCl supernatant fraction, in the initial intensities of spots corresponding to proteins S7 and S11 + S12, as well as the appearance of three new spots (a–c) that were not detected in an electropherogram of a supernatant fraction prepared from unirradiated ribosomal subunits. The electropherogram in Figure 3C indicates that doses of $1–4 \times 10^{20}$ quanta are sufficient to result in the disappearance of most of the spots from the gels; the intensities of the spots that do remain are either similar to the intensities in the control sample (spots S2, S5, S6, S8, and S10) or are somewhat reduced relative to their initial values (spots S1, S15, and S16). It should be noted that concomitant with the disappearance of a number of spots from the two-dimensional gels of the latter sample is the appearance of a substantial number of new spots exhibiting zero electrophoretic mobilities in the first dimension, but electrophoretic mobilities in the second dimension comparable to those of proteins S2–S4, S9, S11 + S12 and possibly also S17–S21. In addition to the formation of these new spots, streaks emanating horizontally from these spots towards the anode of the first dimension and slanting upwards towards the anode of the second dimension are also observed. Finally, exposure of the ribosomal subunits to a dose of 7×10^{20} quanta of 253.7-nm radiation is seen to result in the almost complete disappearance of spots S15 and S16 from the gels, and a reduction in the in-

[1] Abbreviations used are: uv, ultraviolet; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

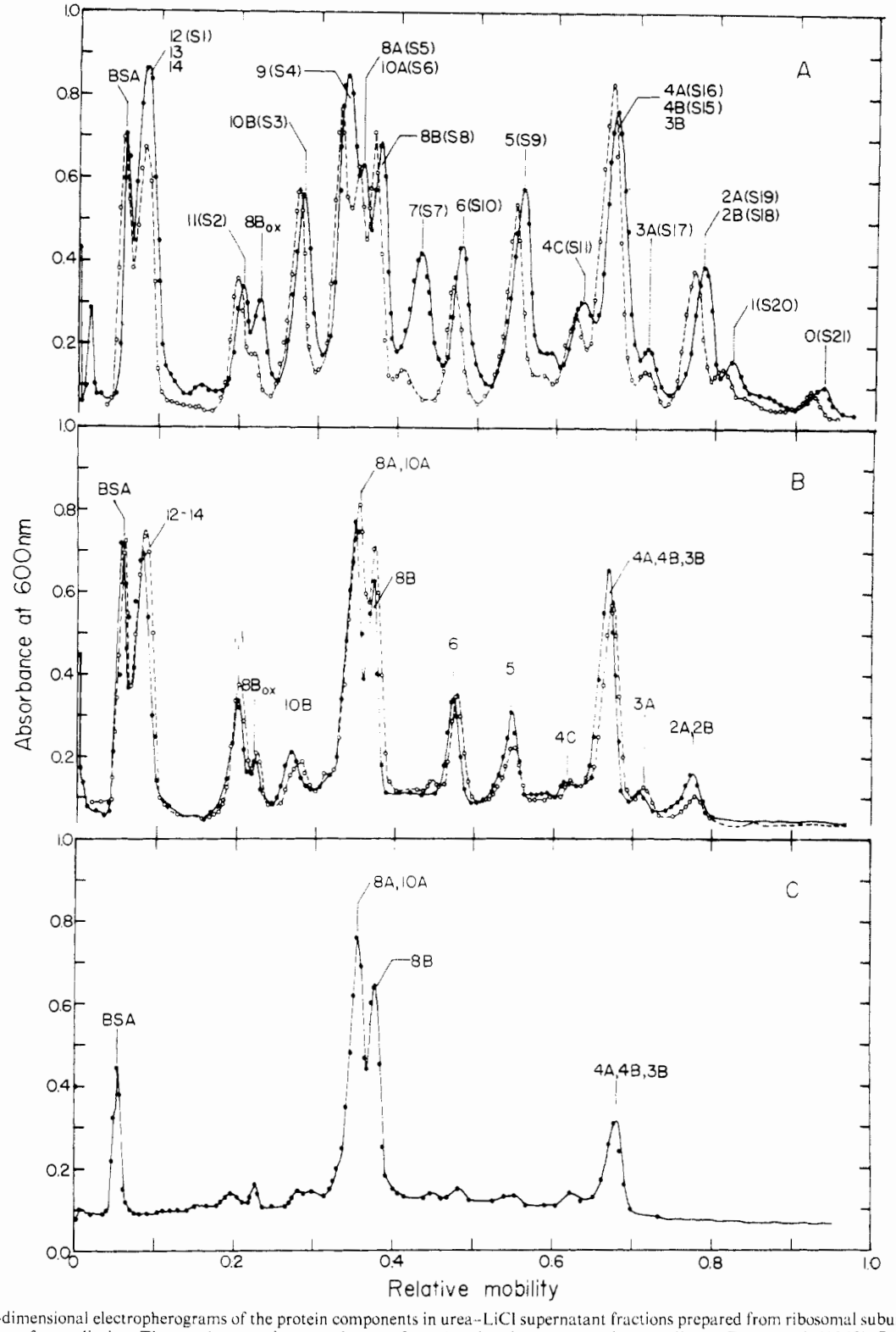

FIGURE 1: One-dimensional electropherograms of the protein components in urea–LiCl supernatant fractions prepared from ribosomal subunits irradiated with various doses of uv radiation. The numbers not in parentheses refer to band assignments made according to Traut et al. (1969). The numbers in parentheses correspond to specific protein assignments made by Kaltschmidt and Wittmann (1970). The doses of 253.7-nm radiation used were: 0 (●) and 5×10^{19} quanta (○), A; 10^{20} (●) and 1.5×10^{20} quanta (○), B; 2×10^{20} quanta (●), C.

tensities of spots S1, S5, S6, and S8 (Figure 3D); this particular dose of uv radiation did not seem to affect the initial stained intensities of spots S2 to S10.

The data on the kinetics of photoinduced cross-linkage of the various 30S ribosomal proteins to the 16S rRNA obtained from the studies on the two-dimensional gels are not strictly comparable to the results obtained on the one-dimensional gels. Bands corresponding to proteins S3, S9, S17, and S18 are detected at somewhat reduced stained intensities relative to their initial values on one-dimensional gels of samples prepared from 30S ribosomal subunits irradiated with doses of $1-1.5 \times 10^{20}$ quanta; but the spots corresponding to these proteins are not detected at their normal positions on the two-dimensional gels (Figures 1B and 3C, respectively). These characteristics

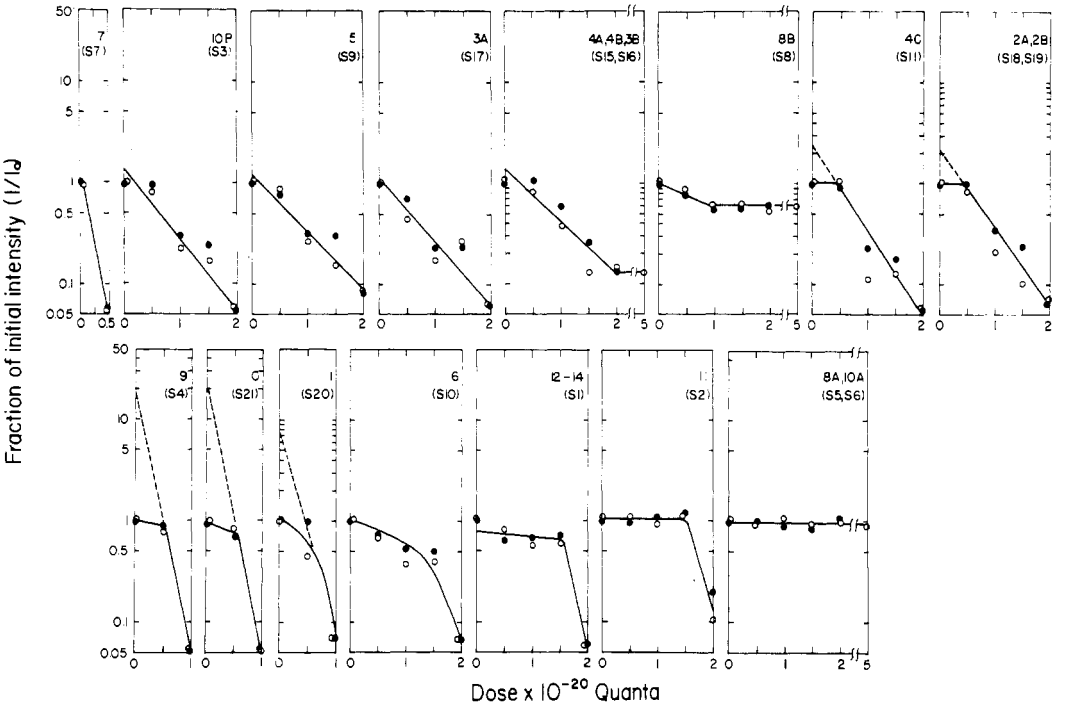

FIGURE 2: Dose dependence of disappearance of proteins from one-dimensional polyacrylamide gels. The intensities of the stained materials in the one-dimensional gels in Figure 1 were determined by integration (●) or by elution of gel slices (○) according to the procedure described under Methods. The resultant data are plotted as the fraction of initial stained intensities remaining at various doses of uv radiation. Each data point represents the average value obtained from two separate irradiation experiments. The deviation of the data between the two experiments was ±10%. The numbers not in parentheses and in parentheses correspond to the band and protein assignments, respectively, made in Figure 1. The dashed lines represent the back-extrapolates of the exponential portions of the fraction-survival curves.

are consistent with the initial cross-linkage of these 30S ribosomal proteins to the 16S rRNA exclusively by covalent bonds labile under the acidic conditions used in the Traut one-dimensional gels, but stable under the basic conditions used in the first dimension of the two-dimensional gels. It is also noted that some of the spots corresponding to proteins S1, S2, S6, and S10 are detected at almost their initial stained intensities on two-dimensional gels of a urea–LiCl supernatant fraction prepared from ribosomes irradiated with a dose of 2×10^{20} quanta but bands corresponding to these proteins were not detected on a one-dimensional electropherogram of the same sample. These latter characteristics are consistent with this particular group of *E. coli* 30S ribosomal proteins having been initially cross-linked to the 16S rRNA by covalent bonds stable under the acidic conditions used in the one-dimensional gels but labile under the basic conditions used in the first dimension of the two-dimensional gels. There is ample precedent for this latter proposal (Sinsheimer and Hastings, 1949; Wang et al., 1956; Gorelic et al., 1972).

Based on arguments developed in earlier reports (Gorelic, 1975a,b), the observed dose-dependent changes in the electrophoretic patterns of the protein components in urea–LiCl supernatant fractions prepared from irradiated ribosomal subunits could be a result of the formation of covalent cross-links between the protein and rRNA components of the subunit. Alternatively, the observed changes in the electrophoretic patterns could have been the result of photoinduced peptide bond cleavage, or photoinduced modifications of the charged groups in the side chains of the amino acid components of the ribosomal proteins. In order to determine whether peptide bond cleavage had occurred, the ribosomal proteins in urea–LiCl supernatant fractions prepared from heavily irradiated ribosomal subunits were "released" from the 16S rRNA by the action of pancreatic ribonuclease, and the resultant digest analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The resultant data are presented in Figure 4. As can readily be seen, the molecular-weight distributions of the irradiated and control samples are virtually identical, indicating that little, if any, peptide bond cleavage had occurred during the course of the irradiation.

The alternative explanation offered above for the dose-dependent changes in the electrophoretic patterns—i.e., side-chain modification—had been tested in a previous study (Gorelic, 1975a) by comparing the electrophoretic mobilities and stained intensities on Traut one-dimensional gels of control ribosomal proteins and proteins released from irradiated ribosomal subunits and the 16S rRNA by a combination of urea–LiCl and RNase treatments. Since the resultant electrophoretic mobilities and stained intensities of the proteins in both samples were found to be quite similar, it was concluded that the charged side chains of the ribosomal proteins had not undergone substantial photoinduced modifications. This question has been reexamined in the report by two-dimensional gel electrophoresis, and the results are presented in Figure 5. As can be seen, most of the spots that are detected on a two-dimensional electropherogram of a control sample (Figure 5A) but absent in an electropherogram of a sample prepared from ribosomal subunits irradiated with a dose of 4×10^{20} quanta (Figure 5B) are detected at their initial intensities in the electropherogram of the RNase-treated sample (Figure 5C). Although spots corresponding to proteins S13 and S18 do not appear to have been fully restored to the intensities observed in Figure 5A, it is likely that the reduced intensities of these spots are a result of inherent deviations in the staining of the proteins corresponding to these spots (see Figure 3A, A′ for an estimate of the variability in duplicate samples of the stained intensities of these spots). In agreement with the earlier one-dimensional studies, ribonuclease treatment of the urea–LiCl

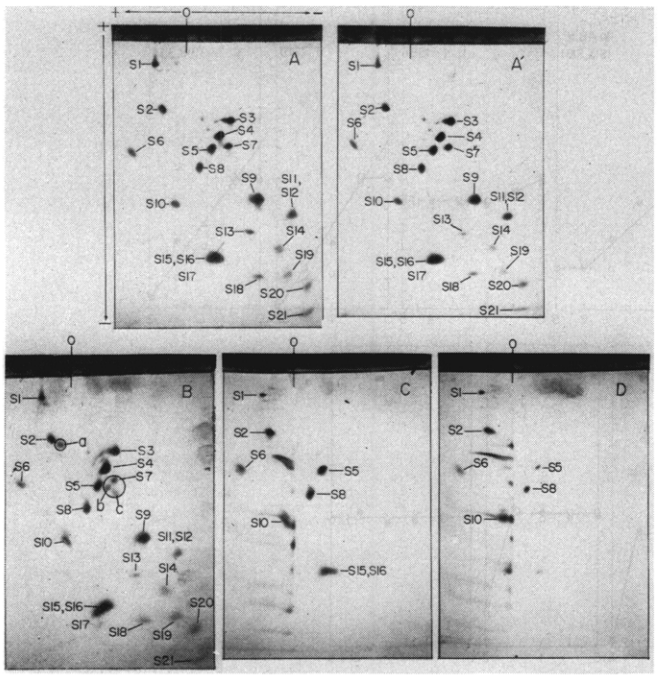

FIGURE 3: Two-dimensional electropherograms of urea–LiCl supernatant fractions prepared from ribosomes irradiated with different doses of uv radiation. Urea–LiCl supernatant fractions were electrophoresed according to the procedures described under Methods, and the proteins visualized by staining the gels with Coomassie brilliant blue. The samples used were obtained from ribosomes irradiated with doses of: 0 quanta (A,A'), 5×10^{19} quanta (B), $1\text{–}4 \times 10^{20}$ quanta (C), and 7×10^{20} quanta (D). Assignments of the spots on the resultant electropherograms were made according to Kaltschmidt and Wittmann (1970).

fractions prepared from irradiated ribosomal subunits did not restore spot S7 to the two-dimensional electropherograms.

Comparison of Electrophoretic Data and Data Obtained from Other Studies of Related Aspects of Ribosome Topography. The electrophoretic data presented in Figures 2 and 3 have been used to estimate the extrapolation numbers (multiplicities) of the photoprocesses required for the cross-linkage of specific ribosomal proteins to the 16S rRNA. This was achieved by determining the back-extrapolates, to zero dose, of the slopes of the exponential portions of the survival curves. The resultant data are presented in Table I, together with data of a nonphotochemical nature presumably related to the ability of the 30S ribosomal proteins to interact directly with the 16S rRNA molecule in the intact *E. coli* 30S ribosomal subunits. Examination of the data in this table suggests that there is a good correlation between the extrapolation numbers for the photoinduced cross-linkage to the 16S rRNA of the individual ribosomal proteins and their presumed abilities to interact directly with the 16S rRNA under a variety of conditions. Proteins S7–S9, S15–S17, and S19, all of which exhibit low to intermediate multiplicities in the cross-linkage reaction, are proteins fractionated by LiCl treatment of the intact 30S ribosomal subunit into an RNA-containing LiCl-core particle, and are able to form specific and isolable complexes with free 16S rRNA. Furthermore, some of these same proteins (S7, S8, S9, S19, and S20) are components of a 21S ribosome precursor accumulated in a ribosome assembly defective *sad* mutant, and, in addition, proteins S7, S8, S9, S16, S17, and possibly S11 and S19, are required for in vitro ribosome assembly. In a related vein, proteins S1, S2, S5, S6, S10, S13, S14, and S21, all of which are dissociated into a split-protein fraction by LiCl treatment of the intact 30S ribosomal subunit and are not able to form stable and isolable complexes with free 16S rRNA, exhibit multiplicities of 10 or greater.

There are a number of exceptions to the correlations mentioned above. Proteins S4 and S20, which appear to become cross-linked to the 16S rRNA by photoprocesses of considerable complexity (extrapolation number of 7) are components of the LiCl-core particle and are able to form specific and isolable complexes with free 16S rRNA. Proteins S3, S11, S12, and S18, which are fractionated into the LiCl-split protein fraction and are not able to form specific complexes with free 16S rRNA, are photochemically cross-linked to the 16S rRNA by photoprocesses of low multiplicities. In addition, although most of the 30S ribosomal proteins required for in vitro and in vivo ribosome assembly become cross-linked to the 16S rRNA by photoprocesses of low multiplicities, many of the ribosomal proteins not required for in vitro or in vivo ribosome assembly also become cross-linked to the 16S rRNA by photoprocesses of low multiplicities.

Sedimentation Characteristics of Irradiated and Unirradiated Ribosomal Subunits. The possible effects of 253.7-nm radiation on the native conformation of the 30S ribosomal subunit were investigated by studying the sedimentation characteristics of the ribosomal subunits as a function of dose of 253.7-nm radiation. The results of these studies are presented in Figure 6, and quite clearly indicate that the highest doses of uv radiation used in the present study are not sufficient to result in substantial changes in the normal sedimentation characteristics of the ribosomal subunit.

Discussion

The kinetics of photoinduced cross-linkage of individual 30S ribosomal proteins to the 16S rRNA in intact 30S ribosomal subunits have been studied in this report by monitoring, on one-dimensional and two-dimensional polyacrylamide gels, dose dependent changes in the electrophoretic properties of individual ribosomal proteins. The basis for this method is that the covalent cross-linkage of the high-molecular-weight and negatively charged 16S rRNA (or oligonucleotide fragments thereof) to a ribosomal protein should markedly alter the physical properties of the protein related to its electrical charge and molecular weight.

Inherent in the above approach is the assumption that any observed changes in electrophoretic mobilities reflect only the formation of covalent bonds between the protein and the rRNA molecule, and that other photoinduced modifications in the primary structures of the ribosomal proteins—viz., peptide bond cleavage, loss of charged groups in the side chains of the amino acid components of the ribosomal proteins, protein–protein cross-linkage—cannot account for these changes. The validity of this assumption is supported by a number of findings. The almost identical molecular weight distributions, on sodium dodecyl sulfate–polyacrylamide gels, of the 30S ribosomal proteins released from unirradiated and irradiated ribosomal subunits and freed from residual rRNA by ribonuclease treatment (Figure 4) suggest that neither extensive photoinduced peptide-bond cleavage nor protein–protein cross-linkage occurs. This conclusion is supported by data in an earlier study indicating that the retention times, on gel filtration media, of ribosomal proteins released from irradiated ribosomal subunits in the manner described above were not substantially shifted towards lower or higher values relative to the retention times of samples prepared from unirradiated ribosomal subunits (Gorelic, 1975b), and from data in this study indicating that a dose-dependent reduction in the stained intensities of the ribosomal proteins on the one-dimensional

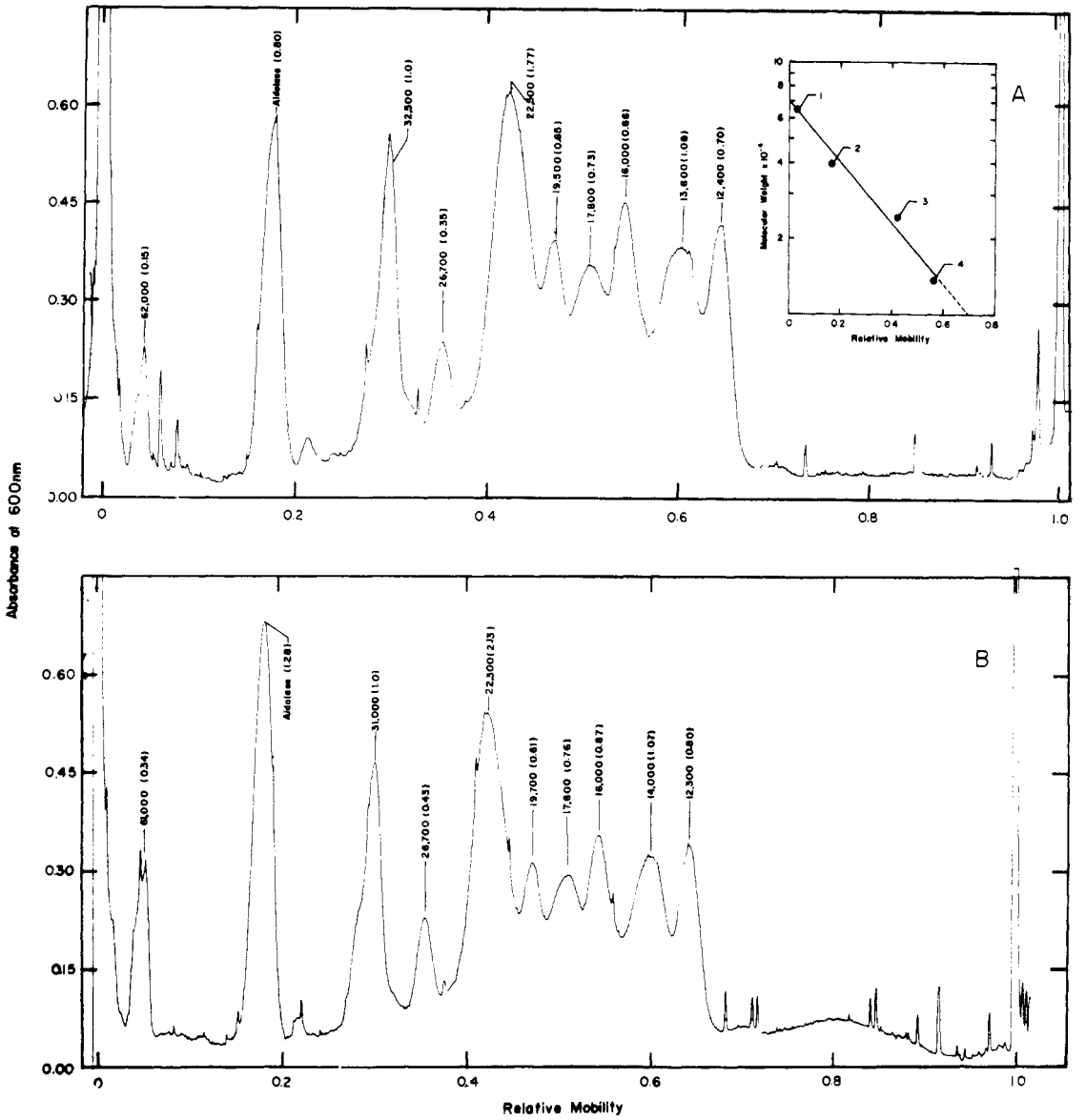

FIGURE 4: Molecular-weight distributions of ribosomal proteins released by ribonuclease digestion of a urea–LiCl supernatant fraction prepared from irradiated ribosomal subunits. A urea–LiCl supernatant fraction was prepared from 30S ribosomal subunits irradiated with a dose of 7×10^{20} quanta, and was incubated for 90 min at 37 °C in the presence of pancreatic ribonuclease (10 μg/mg of rRNA). The resultant digest was prepared for electrophoresis by reductive dialysis according to the procedure under Methods, and was then electrophoresed on sodium dodecyl sulfate–urea–polyacrylamide gels. The bands on the resultant gels were visualized by staining, the gels scanned, and the areas under the peaks in the scans determined by integration. The molecular weights are indicated in the figures by the numbers immediately above each of the peaks in the gel scans. The areas relative to the area of the peak with molecular weight of 31 000–32 500 are given in parentheses above each of the peaks. (A) Control sample, prepared from unirradiated ribosomal subunits; (B) irradiated sample. Insert: calibration curve for the sodium dodecyl sulfate gels. The standards used were pancreatic ribonuclease (4), chymotrypsin (3), aldolase (2), and bovine serum albumin (1) in order of increasing molecular weights.

and two-dimensional gels is largely not accompanied by the appearance of new spots. The only possible exceptions to this conclusion—proteins S7, S11, S12, and spots a-c—will be discussed later. Finally, the finding in this report and in an earlier study (Gorelic, 1975b) that ribonuclease-treatment of a LiCl-supernatant fraction prepared from heavily irradiated 30S ribosomal subunits almost completely reversed the effects of uv radiation on the initial electrophoretic mobilities and stained intensities of the ribosomal proteins suggests that little or no photoinduced modifications of the charged side chains in the individual ribosomal proteins had taken place. Based on these conclusions, the electrophoretic data presented in this report indicate that most of the 30S ribosomal proteins are cross-linked in situ to the 16S rRNA at sufficiently large doses of 253.7-nm radiation. Since the sedimentation studies in this report show that uv irradiation of the intact *E. coli* 30S ribosomal subunit does not result in detectable changes in the native conformation of the ribosome, these results indicate that most of the 30S ribosomal proteins are accessible to the rRNA bases in the native conformational state of the ribosome. This conclusion is in agreement with recent neutron scattering studies, the results of which indicate that there is complete overlap between the scattering centers of the proteins and the 16S rRNA component in the 30S subunit (Moore et al., 1974).

In contrast to the apparent absence of secondary photoreaction involving the protein components of the 30S ribosomal subunit, there are indications in this report that the 16S rRNA component undergoes at least one type of secondary photoreaction: RNA-chain breakage. In addition, it seems as if chain

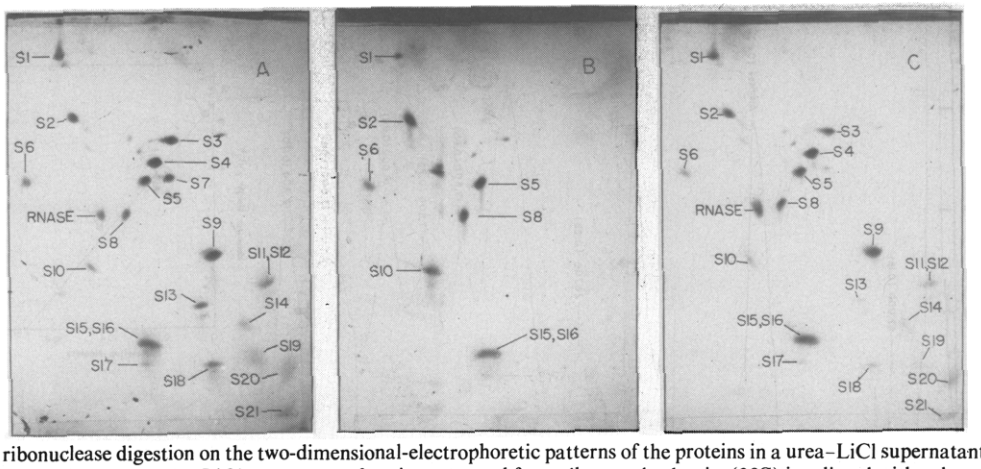

FIGURE 5: Effect of ribonuclease digestion on the two-dimensional-electrophoretic patterns of the proteins in a urea–LiCl supernatant fraction prepared from irradiated ribosomal subunits. A urea–LiCl supernatant fraction prepared from ribosomal subunits (30S) irradiated with a dose of 4×10^{20} quanta of 253.7-nm radiation was incubated with pancreatic ribonuclease (~10 μg/mg of rRNA) for 90 min at 37 °C. The resultant digest was prepared for electrophoresis by dialysis against the electrophoresis buffer and was then subjected to two-dimensional gel electrophoresis. (A) Control sample prepared from unirradiated ribosomal subunits; (B) untreated sample prepared from irradiated ribosomal subunits; (C) ribonuclease-digested sample prepared from irradiated subunits.

TABLE I: Summary of Kinetic Data and Related Properties of 30S Ribosomal Proteins.[a]

Protein		Extrapolation Numbers	LiCl		RNA Binding[d,g]	Present in "sad" 21S Pre-Precursor[e,g]	Required for ribosome assembly[f,g]
1-D[a]	2-D[b]		Split Proteins[c,g]	Core Proteins[c,g]			
7	S7	1		+	+	+	+
8B	S8	1		+	+	+	+
5	S9	1		+	+	+	+
4B	S15	1[h]		+	+	−	−
4A	S16	1[h]		+	+	−	+
3A	S17	1[h]		+	+	−	+
10B	S3	1	+		−	−	−
4C	S11	2	+		−	−	(±)
2B	S18	2	+		−	−	−
2A	S19	2		+	+	+	(±)
–	S12	3[b]	+		−	−	−
1	S20	7		+	+	+	−
12	S1	>10	+		−	−	−
11	S2	>10	+		−	−	−
9	S4	>10		+	+	+	+
8A	S5	>10	+		−	−	−
10A	S6	>10	+		−	−	−
6	S10	>10	+		−	−	−
–	S13	>10[i]	+		−	−	−
–	S14	>10[i]	+		−	−	(±)
0	S21	>10	+		−	−	−

[a] Traut et al., 1969. [b] Kaltschmidt and Wittmann, 1970. [c] Homan and Nierhaus, 1971. [d] Mizushima and Nomura, 1970; Schaup et al., 1971. [e] Nashimoto et al., 1971. [f] Mizushima and Nomura, 1970; Nashimoto et al., 1970. [g] (+), present in or reactive; (−), absent from or unreactive; (±), data is inconclusive. [h] The extrapolation numbers were obtained from the I/I_0 intercepts of the back-extrapolates in Figure 2. [i] Data estimated from changes observed in the two-dimensional gels.

breakage occurs in such a way that proteins initially cross-linked to the intact 16S rRNA molecule are released under the denaturing conditions used in the preparation of the urea–LiCl supernatant fractions, but remain covalently attached to a sufficiently large number of ribonucleotides so that their initial electrophoretic properties are altered. The observed marked reduction in the initial stained intensities on two-dimensional gels of essentially only three proteins—i.e., S7, S11, and S12—at a dose of 5×10^{19} quanta, accompanied by the appearance of three new spots (a–c) of obviously more acidic character than the initial spots (see Figure 3B) would be consistent with such a proposal. The streaks of obviously acidic character that are observed on two-dimensional gels of more heavily irradiated samples (Figures 3C, D) would also be in support of the above proposal if it were assumed that the protein components of these streaks had initially been cross-linked to the 16S rRNA in regions heterogeneous with respect to the numbers and locations of the chain breaks. The explanations that have been offered here for some of the results of the electrophoretic studies of the cross-linkage reaction are conjectural in nature, and consequently do not constitute an unambiguous demonstration of the presence of chain breaks in

the 16S rRNA component of uv-irradiated 30S ribosomes. On the other hand, more recent studies of the structural integrity of the 16S rRNA component in uv-irradiated 30S ribosomes do confirm the existence of chain breaks, and have indicated that chain breakage seems to occur in such a manner that the native tertiary structure of the 16S rRNA seems to be preserved at doses up to 5×10^{20} quanta (Gorelic, manuscript in preparation). It is therefore reasonable to conclude that this secondary photoreaction of the 16S rRNA will not distort conclusions regarding RNA-protein interactions in the native topographical state of the ribosome that are inferred from an analysis of the kinetics of RNA-protein cross-linkage at doses less than 5×10^{20} quanta.

The data presented in this report also indicate that there is considerable variation in the complexities of the photoprocesses required for the cross-linkage of the individual 30S ribosomal proteins to the 16S rRNA. The cross-linkage of 11 of the 30S ribosomal proteins to the 16S rRNA occurs by photoprocesses of low multiplicities, whereas the remaining proteins become cross-linked to the 16S rRNA by photoprocesses of a more complex nature. There are several factors that could account for this variation. The variation could, for example, be due to differences in the accessibilities of the individual 30S ribosomal proteins to the 16S rRNA molecule in the native topographical state of the ribosome. Some of the proteins might normally be able to interact directly with the rRNA bases, whereas other proteins might normally be inaccessible to the rRNA bases for cross-link formation. In the former case, photoexcitation of the bases at the RNA-binding sites would suffice to cross-link the proteins to the 16S rRNA molecule, and, therefore, in this particular case, cross-linkage of the bound proteins to the 16S rRNA should occur by photoprocesses of low multiplicities. On the other hand, prior photoinduced changes in ribosome topography would be required for the cross-linkage of inaccessible proteins to the 16S rRNA molecule, and, therefore, cross-linkage of these proteins to the 16S rRNA should occur by photoprocesses of a more complex nature. Since it was shown (Figure 6) that even the highest doses of uv radiation used in the present study do not result in detectable changes in the sedimentation patterns of the native 30S subunit, the changes in ribosome topography proposed in the above discussion would have to be relatively subtle in nature and could not disrupt the native tertiary structure of the ribosome. One possible candidate meeting these criteria would be localized denaturation of the extensive secondary structure of the 16S rRNA molecule (Thomas, 1969; Thomas and Spencer, 1969; Cox, 1970). Since it has been shown that pyrimidines in helical polynucleotides are inherently less reactive in photoaddition reactions with small molecules than pyrimidines in single-stranded polynucleotides (Grossman, 1968), denaturation of the native secondary structure of the 16S rRNA molecule would be expected to increase the number of base-protein interactions. Denaturation could occur as a result of the cross-linkage to the 16S rRNA of the more accessible proteins, a suggestion that is supported by the finding in this report that most of the more reactive 30S ribosomal proteins are almost completely cross-linked to the 16S rRNA before cross-linkage of the remaining proteins can be detected (Figure 2). Alternatively, denaturation could result from the formation of photoproducts in the helical regions of the 16S rRNA molecule that base pair differently than the parent bases—viz., the photohydrate of uracil, which has been shown to base pair as cytosine (Ottensmeyer and Whitmore, 1968).

There are additional factors that could contribute to the observed difference in reactivities of the individual 30S ribosomal proteins in the cross-linkage reaction. Some of these factors—viz., extent of interactions of different proteins with the rRNA molecule and heterogeneity of the ribosomes with respect to protein composition—represent alternative ways in which the effects of accessibility on reactivity can be expressed. Other factors are independent of accessibility and include the inherent reactivities of the amino acid base pairs formed at the sites of interaction of individual 30S ribosomal proteins with the 16S rRNA and the chemical stabilities of the formed RNA-protein cross-links under the irradiation conditions and/or under the experimental conditions used to determine the kinetics of the cross-linkage reaction. Although the data presented in this report do not permit a quantitative assessment to be made of the relative contributions of each of the factors cited in the above sections to the observed reactivities of the 30S ribosomal proteins in the cross-linkage reaction, some qualitative but definitive statements can be made:

(1) The data presented in this report are supportive of the conclusion that a major factor in determining the reactivity of a ribosomal protein in in situ photoinduced cross-linkage to the 16S rRNA molecule is the accessibilities of the two macromolecules relative to each other in the native conformational state of the ribosome. The basis for this conclusion is that there is a good correlation between the complexities of the photoprocesses required for the cross-linkage of the various 30S ribosomal proteins to the 16S rRNA molecule and the nonphotochemical characteristics of the 30S ribosomal proteins presumed to be indicative of the accessibilities of the ribosomal proteins to the 16S rRNA in the intact 30S ribosomal subunit. Specifically, most of the ribosomal proteins cross-linked to the 16S rRNA by low-multiplicity photoprocesses are able to form specific and stable 16S rRNA-protein complexes, are fractionated into RNA-containing core particles by treatment of the intact 30S ribosomal subunit with high concentrations of LiCl, are essential for the early stages in in vitro ribosome as-

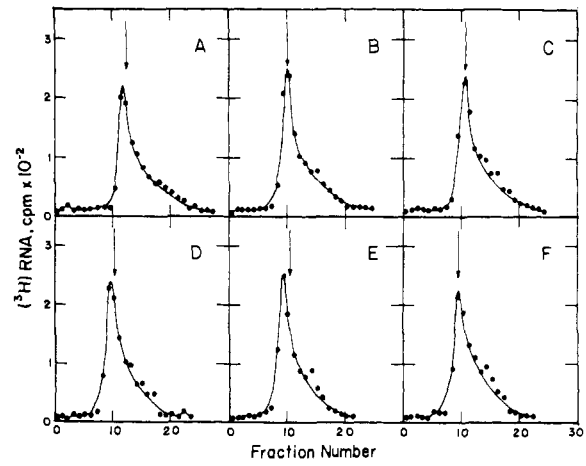

FIGURE 6: Sedimentation properties of irradiated ribosomal subunits. A solution of ^{3}H-labeled ribosomal subunits (30S, 100 A_{260} units) was irradiated at 253.7 nm. Aliquots were removed at regular intervals, unlabeled 30S ribosomes (~2 A_{260} units) added as markers, and the resultant mixtures layered on top of 12-ml 5–20% sucrose gradients. The buffer used in the preparation of these gradients contained Tris (10 mM, pH 7.4), $MgCl_2$ (10 mM), NH_4Cl (30 mM), and 2-mercaptoethanol (~6 mM). The sucrose gradients containing the ribosome samples were then centrifuged in a Beckman SW.41 rotor at 40 500 rpm for 3 h. After centrifugation was complete, fractions were collected dropwise from the individual gradients and assayed for A_{260} and radioactivity. The positions of the marker ribosomes are designated in the figures by the arrows. The doses used were: 0 quanta (A), 5×10^{19} quanta (B), 10^{20} quanta (C), 2×10^{20} quanta (D), 3×10^{20} quanta (E), and 7×10^{20} quanta (F).

sembly, and are components of an in vivo 21S precursor to the the mature 30S ribosomal subunit; and those ribosomal proteins not exhibiting most of the cited nonphotochemical characteristics are cross-linked to the 16S rRNA by photoprocesses of a more complex nature.

(2) Factors in addition to or besides accessibility do contribute to the apparent reactivities of a limited number of 30S ribosomal proteins in photo-induced cross-linkage to the 16S rRNA molecule. The basis for this conclusion is that there are some exceptions to the correlations cited above. Specifically, four of the 30S ribosomal proteins (S3, S11, S12, and S18) not exhibiting the nonphotochemical characteristics indicative of RNA-binding are cross-linked to the 16S rRNA by photoprocesses of low multiplicities, two of the 30S ribosomal proteins exhibiting all of the nonphotochemical characteristics indicative of RNA-binding are cross-linked to the 16S rRNA by photoprocesses of a complex nature, and a number of proteins cross-linked to the 16S rRNA by photoprocesses of low multiplicities are not essential for in vitro ribosome assembly and are not structural components of an in vivo precursor to the mature 30S ribosomal subunit.

(3) The above correlations, and the associated exceptions, are, in fact, consistent with a number of predictions that can be made by a consideration of the types of RNA–protein interactions in the intact 30S ribosomal subunits and the effects of these interactions on the classification of some of the individual ribosomal proteins by nonphotochemical criteria as RNA-binding and on the reactivities of the proteins in the cross-linkage reaction. The basis for such a statement is as follows. It is reasonable to assume that the individual 30S ribosomal proteins might adopt any of four possible orientations relative to the 16S rRNA in the intact 30S ribosomal subunit. First, some proteins might be physically separated from the 16S rRNA molecule. These proteins would not be required for the initial stages of ribosome assembly, would not be components of LiCl-core particles, and would not be able to form specific and isolable 16S rRNA–protein complexes. In addition, since prior photoinduced changes in ribosome topography would be required for cross-link formation, it would be likely that the photoinduced cross-linkage of these proteins to the 16S rRNA would require photoprocesses of a complex nature. Two ribosomal proteins exhibiting all of these characteristics are proteins S5 and S6. Second, some 30S ribosomal proteins might bind to the 16S rRNA only through interactions with the phosphodiester backbone. If these interactions are limited in extent, then the proteins would not meet any of the nonphotochemical criteria for RNA binding. In addition, the cross-linkage of these proteins to the 16S rRNA would be expected to require photoprocesses of a complex nature. However, the complexities of the photoprocesses required for cross-link formation would probably be less than in the situation where the ribosomal proteins were physically separated from the 16S rRNA molecule. The 30S ribosomal proteins exhibiting these characteristics are S1, S2, S5, S6, S10, S13, S14, and S21. If, on the other hand, the interactions with the phosphodiester backbone were quite extensive in nature, then the ribosomal proteins might be required in the initial stages of ribosome assembly, and could be components of the LiCl-core particles. These proteins might also form specific and isolable 16S rRNA–proteins complexes if the basis for site-specific binding to the 16S rRNA molecule resided in the recognition by the bound proteins of unique structural features in the tertiary structure of the 16S rRNA molecule. The photoprocesses required for the formation of RNA–protein cross-links might be quite complex in nature if the basis for inaccessibility to the rRNA bases resulted from spatial constraints placed on the bound proteins by the overall ribosome structure. Alternatively, if the basis for inaccessibility resided in the binding of these proteins to extensively helical regions of the 16S rRNA molecule, then photoprocesses of a less complex nature might be required to cross-link the proteins to the 16S rRNA. The two ribosomal proteins exhibiting the latter photochemical and nonphotochemical characteristics cited here are S4 and S20: both proteins occupy central positions in ribosome assembly (Mizushima and Nomura, 1970; Nashimoto et al., 1971), bind to the 16S rRNA in regions of complex secondary structure (Zimmermann, et al., 1974), and the photoprocesses required for the cross-linkage of these proteins to the 16S rRNA are complex in nature. Third, some of the 30S ribosomal proteins might bind to the 16S rRNA through interactions with the rRNA bases. If the resultant interactions are of limited extent, these proteins might not be required for the initial stages of ribosome assembly, would not be components of LiCl-core particles, and would not be able to form specific and isolable 16S rRNA–protein complexes. The photoprocesses required for the cross-linkage of these proteins to the 16S rRNA would not be of a complex nature if all of the amino acid base pairs at the RNA-binding sites of these proteins were of types reactive in cross-link formation. On the other hand, the required photoprocesses would be complex if most of the "pairs" were of types unreactive in the cross-linkage reaction, or were unable to form stable cross-links. The ribosomal proteins exhibiting the nonphotochemical characteristics cited above and cross-linked to the 16S rRNA by photoprocesses of low multiplicities are S3, S11, S12, and S18, and cross-linked to the 16S rRNA by photoprocesses of a more complex nature are proteins S1, S2, S5, S6, S10, S13, S14, and S21. On the other hand, if there is quite extensive interaction of the bound ribosomal proteins with the 16S rRNA bases, these proteins might be required for the initial stages of ribosome assembly, would be components of the LiCl-core particles, and probably would be able to form specific and isolable 16S rRNA–protein complexes. The basis for site-specific binding to the 16S rRNA in this situation would probably reside in the recognition of specific base sequences in the 16S rRNA molecule by hydrogen-bonding interactions with specific groups in the side chains of the amino acid components of the ribosomal proteins—viz., the hydroxyl groups of serine, threonine, tyrosine, the amino groups of asparagine and glutamine, and the hydrogen on the indole nitrogen of tryptophan. The multiplicities of the photoprocesses required for the cross-linkage of these types of ribosomal proteins to the 16S rRNA would be low (and very possibly unity) if all of the amino acid base pairs at their RNA-binding sites were reactive in cross-link formation and capable of forming stable cross-links, but would be large in the opposite situations. The 30S ribosomal proteins exhibiting the nonphotochemical properties described above and cross-linked to the 16S rRNA by approximately single-hit kinetics are S7–S9, S15–S17, and S19, and cross-linked to the 16S rRNA by photoprocesses of considerable complexity are proteins S4 and S20. Finally, some of the ribosomal proteins might interact with a large number of rRNA bases and be capable of participating in the formation of stable RNA–protein cross-links, but might not be tightly bound to the rRNA bases so that the lifetimes of amino acid and water base pairs were comparable. In such a situation, the bound proteins would be expected to initially cross-link to the 16S rRNA by photoprocesses of low multiplicities, but consumption of the bases at these sites by photoreactions other than cross-linkage—viz., photohydration of the pyrimidine

residues—would result in a situation in which not all of the "bound" protein could cross-link to the 16S rRNA molecule. The observed patterns of cross-linkage of proteins S8 and S15 + S16 would be consistent with a such a situation. It should be noted that an alternative situation might also prevail in which these particular proteins normally assume two different orientations relative to the 16S rRNA bases in the total ribosome population, accessible and inaccessible. Based on the data presented in Figure 2, the ratio accessible to inaccessible would be 2:3 for protein S8 and 85:15 for proteins S15 and S16. However, since two of these proteins (S8 and S16) occupy central positions in ribosome assembly, it would seem unlikely that they would assume such widely different orientations relative to the 16S rRNA molecule in the intact 30S ribosomal subunit. Fourth and finally, portions of certain proteins might not interact with the 16S rRNA, other portions might interact with the rRNA backbone, and the remaining portions of the proteins might interact directly with the 16S rRNA bases. Since this situation is, in reality, a composite of all the situations that have already been discussed, the relationship of this particular situation to the photochemical and nonphotochemical factors discussed above will not be described here.

(4) The patterns of cross-linkage of most of the *E. coli* 30S ribosomal proteins to the 16S rRNA appear to be independent of the electrophoresis conditions used to monitor the cross-linkage reaction. However, the patterns of cross-linkage of seven of the 21-30S ribosomal proteins are dependent upon the electrophoresis condition, but it is unlikely that this dependence affects the assigned relationships between the magnitudes of the extrapolation numbers obtained from the electrophoretic data and the proximities of the proteins to the 16S rRNA in the intact 30S ribosomal subunit. The basis for this conclusion is that the dependence on electrophoresis condition of the patterns of cross-linkage of three of the seven proteins alluded to above—i.e., S1, S2, and S10—is exhibited only at doses larger than those required to make meaningful estimates of the magnitudes of the extrapolation numbers. In addition, although the remaining four of the seven proteins—i.e., S3, S9, S17, and S18—appear to exhibit different rates of cross-linkage on the two gel systems used in the present study, the electrophoresis condition giving the slowest apparent rate of cross-linkage indicates that cross-linkage is occurring by the simplest possible type of kinetics—i.e., single-hit kinetics.

The final comment to be made on the data presented in this report concerns protein S7. This protein is the first to disappear from the two-dimensional gels; it is also the only 30S ribosomal protein that cannot be restored to the two-dimensional gels by ribonuclease treatment, under denaturing conditions, of uv-irradiated 30S ribosomal subunits. Since this particular protein meets all of the nonphotochemical criteria for RNA binding and is cross-linked to the 16S rRNA by what appears to be single-hit kinetics, it would seem reasonable to conclude that protein S7 binds to a substantial number of 16S rRNA bases. The insensitivity to ribonuclease treatment mentioned above suggests that this protein might interact so extensively with the 16S rRNA molecule that the number of nucleotides remaining covalently attached to the protein after ribonuclease treatment is too large to permit detection of the protein on the two-dimensional gels.

In conclusion, the data presented in this report indicate that photochemical methods can be applied to studies of RNA-protein interactions in the intact *E. coli* 30S ribosomal subunit, and that the results of such studies can, in certain cases, complement the results of the application of nonphotochemical methods to studies of a related nature. The basis for these conclusions are: (1) there is a good correlation between conclusions regarding the relative spatial distributions of the rRNA and protein components in the intact 30S ribosomal subunits obtained in this report and by neutron-scattering studies in another laboratory; (2) there is a good correlation between the predicted and observed complexities of the photoprocesses required for the cross-linkage of individual 30S ribosomal proteins to the 16S rRNA and the abilities of the proteins to interact in a specific manner with the 16S rRNA molecule in ribonucleoprotein and subribosomal complexes; (3) ribosomal proteins presumably binding too weakly to the 16S rRNA to meet the nonphotochemical criteria for RNA binding were classified as RNA binding on the basis of the results of the photochemical studies.

The data that have been presented in this report also indicate that there are certain technical limitations in the application of photochemical methods to studies of nucleic acid-protein interactions in the ribosome, and possibly in other types of nucleoprotein complexes. Specifically, although it seems possible to utilize photochemical reactivity alone to assign in a relatively unambiguous manner spatial orientations relative to the nucleic acid component of those proteins exhibiting high reactivity in the cross-linkage reaction, the real and postulated lability of certain types of nucleic acid-protein cross-links prevents unambiguous assignments of the relative spatial orientations of those proteins exhibiting lower reactivities in the cross-linkage reaction. These latter limitations can, however, be at least partially overcome by the availability of other types of data concerning nucleic acid-protein interactions in the nucleoprotein complex in question.

In view of the above conclusions, it would seem reasonable to propose that the *E. coli* ribosomal subunits might be useful as model systems for establishing protocols to be used in the application of photochemical methods to studies of nucleic acid-protein interactions in other large nucleoprotein complexes—viz., deoxyribonucleohistones and chromatin—and for establishing a rationale for the interpretation of the data resulting from such studies. There are a number of findings in this report that are quite clearly useful for such purposes. First, the data presented in this report indicate that too large of a dose of 253.7-nm radiation can result in the cross-linkage to the nucleic acid component of all the protein components in the complex, regardless of their accessibilities to the nucleic acid molecule in the native state of the complex. Consequently, the optimal uv-radiation doses required to obtain data useful in structure elucidation must be determined. Second, the data presented in this report indicate that the experimental conditions used to detect the formation of covalent nucleic acid-protein cross-links must be carefully selected in order to minimize the problems associated with the formation of RNA-protein cross-links labile under the experimental conditions used in the characterization of the irradiated nucleoprotein complex. Third, the results of the photochemical studies must be supplemented with other data pertaining to nucleic acid-protein interactions in the nucleoprotein complex in order to take into account ambiguities arising from unique structural features of the complex and the inability of certain proteins to participate in the formation of nucleic acid-protein cross-links that are stable under the irradiation conditions.

Note Added in Proof

It has been recently shown that three of the 30S ribosomal proteins (S11, S12, S18) not exhibiting the nonphotochemical properties characteristic for RNA-binding proteins but that

became photochemically cross-linked to the 16S rRNA by photoprocesses of low multiplicities do, in fact, form stable complexes with free 16S rRNA (Hochkeppel et al., 1976).

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