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Evidence for Photoinduced Cross-Linkage, in Situ, of 30S Ribosomal Proteins to 16S rRNA†

Lester Gorelic

ABSTRACT: The effects of ultraviolet radiation on the 30S ribosomal subunit of *Escherichia coli* were studied. Irradiation in aqueous solution under anaerobic conditions resulted in a dose-dependent decrease in the separability of the rRNA and protein components of the 30S ribosomal subunit in 4 *M* urea–3 *M* LiCl. The results of gel filtration studies of the irradiated ribosomes before and after treatment with pancreatic ribonuclease indicated that the decrease in separability of the ribosome components was a result of the photoinduced formation of covalent RNA–protein cross-links. The number of covalent cross-links was estimated to correspond to less than 3 per 10,000 daltons of ribosomal proteins. One-dimensional gel electrophoresis studies of the course of the photoinduced cross-linkage reaction indicated that cross-linkage of individual 30S ribosomal proteins to the 16S rRNA proceeds in two dose-dependent steps. The first step requires an input of 1×10^{20} quanta of 253.7-nm radiation and results in the cross-linkage of at least five ribosomal proteins to the 16S rRNA. The second step requires a total input of 2×10^{20} quanta of 253.7-nm radiation, and results in the cross-linkage of most of the remaining 30S ribosomal proteins to the 16S rRNA.

Ultraviolet irradiation of stable complexes between proteins and nucleic acids results in the covalent cross-linkage of the proteins to the nucleic acid molecules (Markovitz, 1972; Lin and Riggs, 1974; Schoemaker and Schimmel, 1974; Strniste and Smith, 1974). Since it has been shown that several ribosomal proteins can interact directly with the rRNA components of the ribosomal subunits (Schaup et al., 1970,1971; Gray et al., 1973a,b; Yug and Wittmann, 1973; Garrett et al., 1974; Zimmermann et al., 1974), it is reasonable to expect that ultraviolet (uv) irradiation of the individual ribosomal subunits or of the intact 70S ribosome complex would result in some nucleic acid–protein cross-linkage.

The formation of an RNA–protein cross-link in irradiated ribosomes requires that a ground-state or photoexcited protein molecule be sufficiently close to the heterocyclic rings of a photoexcited or ground-state rRNA base, respectively, to form a stable covalent bond. Consequently, the pattern of photoinduced protein–nucleic acid cross-linkage in the individual ribosomal subunits or in the 70S complex could provide valuable information concerning the relative contribution of direct interactions between the rRNA bases and various ribosomal proteins to ribosome structure. It is also possible that a study of photoinduced protein–nucleic acid cross-linkage in the individual ribosomal subunits may provide an explanation for the known photoinactivation of the functional activity of the ribosomal subunits (Kagawa et al., 1967; Tokimatsu et al., 1968; Yasuda and Fukutome, 1970).

For these reasons, a detailed investigation of the photochemistry of the *Escherichia coli* ribosomal subunits was undertaken. In previous reports (Gorelic, 1974,1975) it was demonstrated that uv irradiation of intact 50S ribosomal subunits resulted in the covalent cross-linkage of specific 50S ribosomal proteins to the rRNA components of the subunit. The data presented in this report indicate that photoinduced covalent cross-linkage of specific ribosomal proteins to rRNA also occurs in irradiated 30S subunits.

Experimental Section

Materials. Pancreatic ribonuclease was obtained from Worthington Biochemicals. Ribonuclease-free deoxyribonuclease I used in the preparation of cell-free extracts of *E. coli* was obtained from Worthington Biochemicals. Ribonuclease-free sucrose was obtained from Schwarz/Mann. Acrylamide and bismethyleneacrylamide were obtained from Eastman Kodak and were recrystallized before use. *N,N,N′,N′*-Tetramethylenediamine was obtained from Eastman Kodak and was distilled over zinc dust in a nitrogen atmosphere and stored over KOH pellets. Sephadex G-100 was obtained from Pharmacia. Bio-Gel A-5.0m was obtained from Bio-Rad Laboratories. Potassium ferrioxalate was prepared by a published procedure (Hatchard and Parker, 1956). Oxygen-free nitrogen was obtained from the Linde Corporation. All other reagents were Analytical Reagent grade.

Ribosomal subunits (30 S and 50 S) were prepared and isolated according to previously published procedures (Tiss-

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received May 15, 1975.* This work was supported by a grant from the Research Corporation, Wayne State University Faculty Research Award, Grant No. CA 18046 from the National Institutes of Health, and U.S. Public Health Service Career Development Award No. CA 70999.

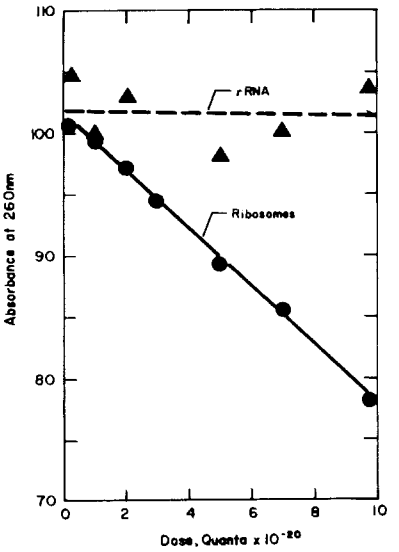

FIGURE 1: Effect of uv radiation on the absorbance of 30S ribosome and 16S rRNA solutions. Solutions of 30S ribosomal subunits or 16S rRNA containing 100 A_{260} units in 100 ml of irradiation buffer were irradiated internally at 253.7 nm. Aliquots were removed at regular intervals and the absorbance at 260 nm recorded. The absorbance readings are expressed as the number of A_{260} units remaining in 100 ml of irradiated solution after a given dose of uv radiation.

ieres et al., 1959; Traub et al., 1971). Cross-contamination of the subunits, as assayed by in vitro polyphenylalanine synthesis (Cannon et al., 1963) and sucrose gradient centrifugation, was less than 5%.

Ribosomal proteins and 16S rRNA were isolated by extraction of 30S ribosomal subunits with 4 *M* urea–3 *M* LiCl according to the procedure of Traub et al. (1971). Gel filtration analysis, under denaturing conditions, of the 16S rRNA prepared in this manner indicated that RNA chain breakage did not occur during isolation.

Irradiation Conditions. Solutions of 30S ribosomal subunits in irradiation buffer [5 m*M* H_3PO_4 (neutralized with KOH to pH 7.4), 10 m*M* $MgCl_2$, and 30 m*M* KCl) were deaerated by purging with oxygen-free nitrogen and placed in a jacketed vessel equipped with a Suprasil dipper well or in a thermostated 1.0-cm path-length Suprasil cuvette. Irradiations were performed at 22.0 ± 0.10°C according to the procedures previously used for the 50S subunits (Gorelic, 1975). Samples were irradiated internally by immersion of the dipper well–lamp assembly into the solution, or externally by positioning the dipper well–lamp assembly a distance of 1.0 cm from a Suprasil cuvette. The output of the lamp at 253.7 nm was determined by ferrioxalate actinometry (Parker, 1953) to be $1.8 \pm 0.04 \times 10^{18}$ quanta sec^{-1}. The intensity of 253.7-nm radiation incident on the face of the cuvette was $4.73 \pm 0.09 \times 10^{16}$ quanta sec^{-1}.

Preparation of Urea–LiCl Supernatant Fractions from Unirradiated and Irradiated 30S Ribosomal Subunits. Ribosomal subunits were either treated with 4 *M* urea–3 *M* LiCl for 48 hr at 4°C according to the method of Traub et al. (1971) or dialyzed against 8 *M* urea–6 *M* LiCl for 48 hr at 4°C, and were separated into soluble and insoluble fractions by low-speed centrifugation. The insoluble fraction was resuspended in Tris buffer (10 m*M* Tris-HCl, pH 7.8) and stored at −70°C. This fraction will be referred to as the urea–LiCl pellet fraction. The soluble fraction was dialyzed exhaustively against Tris–urea buffer (10 m*M* Tris-HCl (pH 7.8), 8 *M* urea, 12 m*M* methylamine-HCl, and 6 m*M* 2-mercaptoethanol) and stored at −70°C. The dialyzed fraction will be referred to as the urea–LiCl supernatant fraction.

Solutions of unirradiated ribosomes were used in the above preparations without a preliminary concentration step. Irradiated ribosomal subunits were concentrated by a combination of ultrafiltration (Amicon No. XM-300 ultrafilter) and ultracentrifugation on a Beckman Model L2-65B ultracentrifuge (Spinco Type 65 rotor; 250,000*g* for 12 hr) prior to preparation of the urea–LiCl supernatant fraction. The methods used to concentrate the ribosome solutions were shown, by the two-dimensional gel technique of Kaltschmidt and Wittmann (1970), not to affect the normal protein composition of the ribosomal subunits.

Gel Filtration Studies. Analysis of samples by gel filtration was performed on 1.3 cm × 37 cm columns of Sephadex G-100 (40–100 μ particle size; mol wt fractionation range of 10^5 to 10^3) or Bio-Gel A-5.0m (200–400 mesh; mol wt fractionation range of 5×10^6 to 10^4). The elution buffer used in these studies was constituted of Tris-HCl (pH 7.4, 10 m*M*), urea (6 *M*), and methylamine-HCl (12 m*M*). The columns were calibrated with Blue Dextran (mol wt 2×10^6) and Bromophenol Blue (mol wt 670). All tested samples were subjected to exhaustive dialysis against the elution buffer prior to application to the gel filtration columns.

Electrophoresis Studies. Samples were electrophoresed at pH 4.5 on 7.5% acrylamide gels according to the method of Traut et al. (1969). The samples were prepared for electrophoresis by dialyzing against the electrophoresis buffer. Methyl Green was added to the dialyzed samples as tracking dye, and the samples were applied to the top of 200 mm × 6 mm gels. The applied samples were then electrophoresed for 16 hr at 4°C at an applied voltage of 6.5 V/cm. Proteins were visualized by staining with Coomassie Brilliant Blue.

RNA and Protein Determinations. RNA was determined by the orcinol method (Mejbaum, 1939) and protein by the Lowry method (Lowry et al., 1951). The resultant data were presented either as weight protein or RNA, or as A_{750} and A_{670} units, respectively. One A_{750} unit is defined as the amount of protein giving an absorbance of 1.0 at 750 nm in the standard Lowry reaction, and one A_{670} unit as the amount of RNA giving an absorbance of 1.0 at 670 nm in the standard orcinol reaction.

Results

Effect of Uv Irradiation on the Uv Absorption Spectrum of Ribosome-Bound 16S rRNA. Irradiation of an aqueous solution of 30S ribosomal subunits at 253.7 nm results in a dose-dependent decrease in the absorbance at 260 nm of the 16S rRNA component. The magnitude of this decrease (2.35×10^{-20} A_{260} units per quanta of 253.7-nm radiation) is comparable to the previously published value for the 50S subunit (Gorelic, 1975). In contrast to ribosome-bound 16S rRNA, the absorbance at 260 nm of solutions of free 16S rRNA is not affected by exposure to doses of from 5×10^{19} to 10^{21} quanta of 253.7-nm radiation (Figure 1).

These results indicate that the effect of uv radiation on ribosome-bound 16S rRNA cannot be simply explained by known photoinduced modifications of the pyrimidine components in free 16S rRNA such as photohydration or photodimer formation (Moore and Thomson, 1957; Wang, 1962; Johns et al., 1965; Wacker et al., 1961). Rather, the data suggest that the 30S ribosomal proteins play an essential

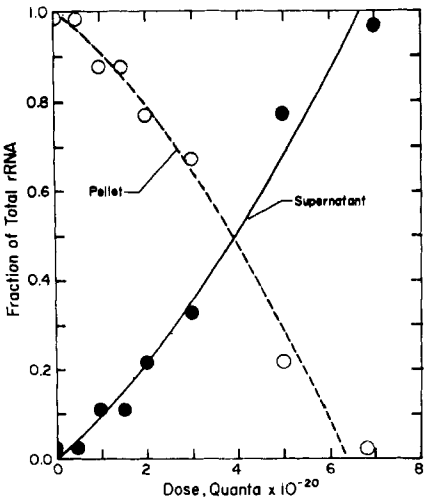

FIGURE 2: Effect of uv radiation on the separability of ribosome components. Six solutions of 30S ribosomal subunits (100 ml each, 1.0 A_{260} unit of 30S ribosomes/ml) were prepared. Each solution was irradiated internally with a different dose of 253.7-nm radiation, and urea-LiCl supernatant and pellet fractions were prepared. The RNA contents of these fractions were determined and expressed as the fraction of the total 16S rRNA. Each data point represents the mean of duplicate determinations run on ribosome samples isolated from two different irradiations. The standard deviation of the data from the mean values was calculated to be ±7%.

role in determining the course of photoinduced changes, probably by forming covalent cross-links to the 16S rRNA in a manner analogous to that found to occur in uv-irradiated 50S ribosomal subunits (Gorelic, 1975).[1]

Separability of Ribosome Components. Treatment of solutions of unirradiated 30S ribosomal subunits with 4 *M* urea-3 *M* LiCl, followed by centrifugation, results in the complete separation of the proteins (supernatant fraction) from the 16S rRNA (pellet fraction) components (Figure 2). When uv-irradiated 30S ribosomal subunits were subjected to the same fractionation procedure, it was found that the amount of RNA in the supernatant fraction increased and the amount in the pellet fraction decreased as the dose of incident radiation was increased. The doses required to "solubilize" 10 and 100% of the 16S rRNA were found to be 1×10^{20} quanta (9.8×10^4 ergs/mm^2) and 7×10^{20} quanta (4.5×10^5 ergs/mm^2), respectively. In contrast to the solubility behavior of ribosome-bound 16S rRNA, when 16S rRNA was first irradiated and mixed with isolated 30S ribosomal proteins and when the resultant mixture was subjected to the same fractionation procedure as the ribosome samples, a dose-dependent loss of rRNA from the pellet fraction was not observed (L. Gorelic, unpublished observation). These results indicate that the photoinduced solubilization of 16S rRNA requires the presence of ribosomal proteins during irradiation, and probably involves covalent cross-linkage of individual ribosomal proteins to the 16S rRNA. The validity of this latter conclusion was tested in the following studies.

[1] One should also consider the possibility that the ribosomal proteins could affect the photochemistry of the 16S rRNA indirectly by forcing the ribosome-bound 16S rRNA into a conformation more reactive toward protein-independent photoreactions than the conformation of free 16S rRNA. However, there is no experimental evidence for such conformational differences, and, in fact, the reported resemblance of the optical rotatory dispersion and circular dichroic spectra of free and ribosome-bound 16S rRNA (Sarker et al., 1967) supports the alternative suggestion that the secondary structures of free and ribosome-bound 16S rRNA are quite similar.

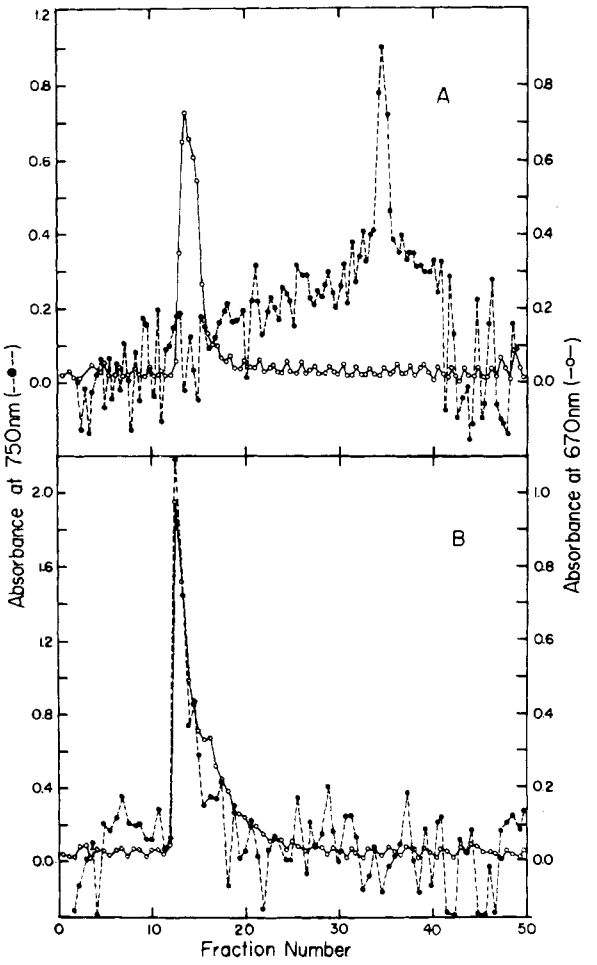

FIGURE 3: Gel filtration properties of the RNA and protein components in urea-LiCl supernatant fractions. Samples (0.5 ml) were applied to a 1.3 × 37 cm column packed with Bio-Gel A-5.0m and were eluted with a Tris-urea buffer (10 m*M* Tris-HCl (pH 7.4), 6 *M* urea, and 12 m*M* methylamine-HCl). Fractions of 0.53 ml were collected every 12 min and were assayed for protein (●) and RNA (○) contents. The resultant data are expressed as A_{750} and A_{670} units per fraction. The void volume of the columns was 11.6 ml; the low molecular weight Bromophenol Blue marker eluted at 42.5 ml. (A) Superposition of elution profiles of 16S rRNA and 30S ribosomal proteins prepared from unirradiated 30S ribosomal subunits. Column inputs were 6.1 A_{670} units (RNA) and 20.1 A_{750} units (protein). (B) Elution profiles of RNA and protein components in a urea-LiCl supernatant fraction prepared from a 30S ribosomal subunit irradiated with 7×10^{20} quanta of 253.7-nm radiation. Column inputs were 6.1 A_{670} units and 20.1 A_{750} units.

Gel Filtration Studies on Bio-Gel Columns. Samples of 16S rRNA and 30S ribosomal proteins were prepared and applied separately to a Bio-Gel A-5.0m column. The 16S rRNA (mol wt 5.5×10^5) was eluted as a single peak near the column void volume of 11.6 ml. The 30S ribosomal proteins, with molecular weights ranging from 6.8×10^4 to 10×10^3, were eluted as multiple overlapping peaks at considerably larger elution columns than the 16S rRNA sample (Figure 3A). In contrast, when a urea-LiCl supernatant fraction prepared from uv-irradiated ribosomes and containing both RNA and protein was applied to such a column, all the proteins co-eluted with the rRNA near the column void volume (Figure 3B). These results indicate that after relatively large doses of uv radiation, all of the 30S ribosomal proteins become stably—or perhaps covalently—bound to the 16S rRNA to form an isolable ribonucleoprotein complex.

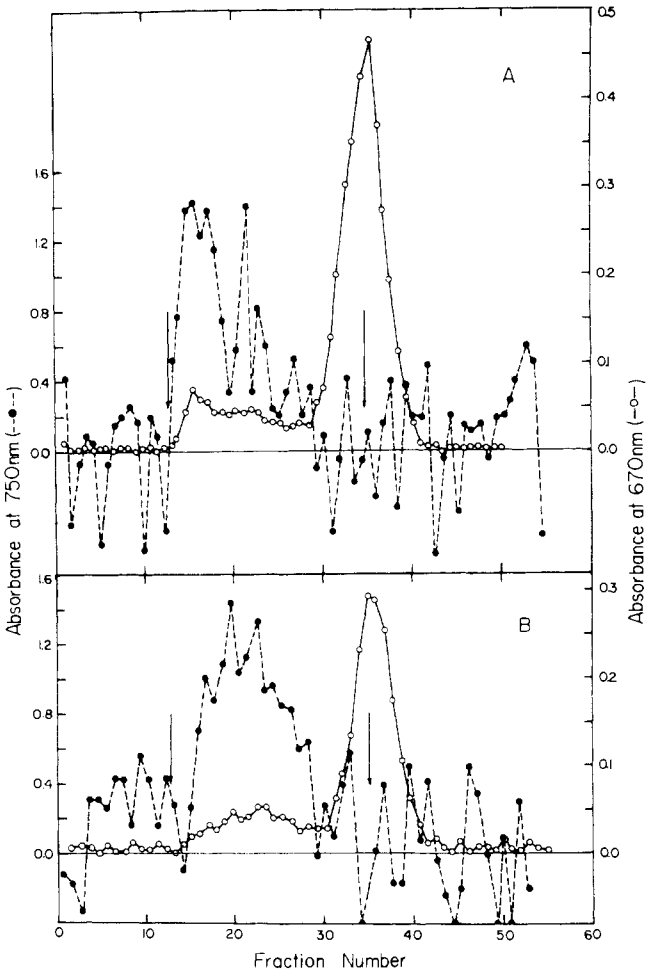

FIGURE 4: Ribonuclease sensitivity of the photochemically cross-linked ribonucleoprotein complex. Samples were applied to 1.3 × 37 cm columns of Sephadex G-100 and were eluted with elution buffer (10 m*M* Tris-HCl (pH 7.4), 6 *M* urea, and 12 m*M* methylamine-HCl). Fractions (0.90 ml) were collected every 7 min and were assayed for protein (●) and RNA (○) contents. The resultant data are expressed as A_{750} and A_{670} units per fraction. The positions of the blue dextran and Bromophenol Blue markers are denoted by the arrows. (A) 30S ribosomal proteins and RNase-digested 16S rRNA. A solution of 16S rRNA (6.1 A_{670} units) in storage buffer (10 m*M* Tris-HCl (pH 7.4), 8 *M* urea, and 12 m*M* methylamine-HCl) was incubated at 30°C for 2 hr with pancreatic ribonuclease (10 μg of ribonuclease per mg of rRNA). The digested sample was then mixed with pure 30S ribosomal proteins (20.1 A_{750} units) and the resultant mixture dialyzed against elution buffer. The dialyzed sample was diluted to 0.5 ml with elution buffer and then subjected to gel filtration. (B) Ribonuclease digest of urea-LiCl supernatant fraction prepared from 30S ribosomal proteins irradiated with 7×10^{20} quanta of 253.7-nm radiation. A sample of the supernatant fraction containing 6.1 A_{670} and 20.1 A_{750} units was incubated in storage buffer as described in the legend to Figure 3A. The digested sample was dialyzed against elution buffer, diluted to 0.5 ml with elution buffer, and then subjected to gel filtration.

Effects of Ribonuclease on the Stable Ribonucleoprotein Complex. A urea-LiCl supernatant fraction containing the stable ribonucleoprotein complex identified in the above studies was incubated with pancreatic ribonuclease, dialyzed to remove small oligonucleotides, and then subjected to gel filtration on Sephadex G-100. A high molecular weight ribonucleoprotein complex eluting at the column void volume was no longer detected (Figure 4B). Instead, multiple overlapping protein peaks were detected at elution volumes less than the column void volume, and an abundance of low molecular weight oligonucleotides were detected near the Bromophenol Blue marker. Some orcinol-reactive material also co-eluted with the proteins.

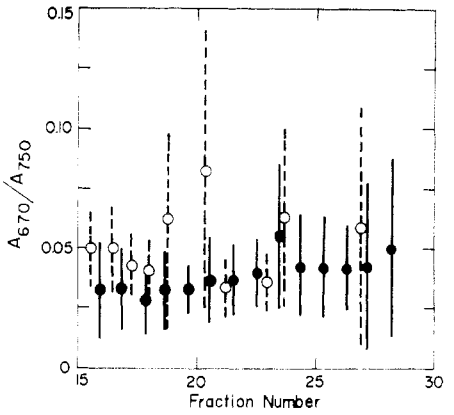

FIGURE 5: Ratio of A_{670}/A_{750} readings in regions of overlap of protein and orcinol-reactive material in gel filtration studies, on Sephadex G-100, of ribonuclease-digested samples of 16S rRNA and the photochemically cross-linked ribonucleoprotein complex. The data points for the ribonuclease digest of the 16S rRNA are represented by the open circles (○), and for the orcinol-reactive material in the ribonuclease digest of the cross-linked ribonucleoprotein complex by closed circles (●), and were taken from the data in Figures 4A and 4B, respectively. The predicted standard deviations of these ratios from mean values are denoted by bars, and were calculated on the basis of the fluctuations of the Lowry and orcinol data around the base lines in Figure 4 at elution volumes where orcinol- and Lowry-reactive material was not present.

As a control to determine whether the orcinol-reactive material co-eluting with the proteins in the above gel filtration studies is due to nucleotides covalently bound to the ribosomal proteins or free oligonucleotides derived from RNase digestion, a mixture of RNase-digested 16S rRNA and 30S ribosomal proteins was prepared, dialyzed, and applied to a Sephadex G-100 column. The resultant elution profiles of the protein and RNA fragments (Figure 4A) are very similar to that observed with the ribonuclease digest of the high molecular weight ribonucleoprotein complex (Figure 4B). The ratios of Lowry-reactive to orcinol-reactive material in each of the collected fractions are presented as A_{670}/A_{750} ratios in Figure 5. As can be seen, the A_{670}/A_{750} ratios in the control and ribonucleoprotein complex samples vary from values of 0.015 to 0.075, around a mean value of 0.04. The relationships between the A_{670} and A_{750} readings and absolute amounts of RNA and protein are 0.004 A_{670} unit/μg of RNA and 0.01 A_{750} unit/μg of protein. From these latter values, it can be readily calculated that an A_{670}/A_{750} ratio of 0.04 corresponds to 750 μg of RNA per 10,000 μg of protein or approximately three nucleotides per 10,000 daltons of protein. Since the A_{670}/A_{750} ratios of the control and ribonucleoprotein complex samples are similar, it may be that there are on the average at most, and probably less than, three nucleotides covalently bound per 10,000 daltons of protein.

One-Dimensional Polyacrylamide Gel Electrophoresis Studies. The 16S rRNA component of the *E. coli* 30S ribosomal subunit is a high molecular weight polyanionic material that would not be expected to exhibit any appreciable electrophoretic mobility toward the cathode in small porosity (7.5% w/v) polyacrylamide gels. Therefore, a radiation-induced RNA-protein complex should not be able to penetrate small porosity gels (7.5% w/v acrylamide) and should not give rise to the usual ribosomal protein bands upon electrophoresis.

Electropherograms on 7.5% polyacrylamide gels of the

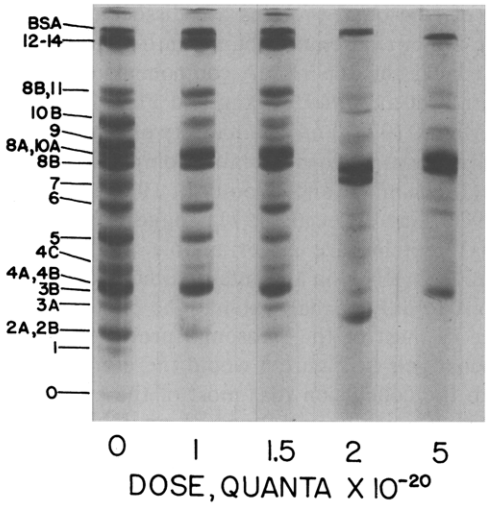

FIGURE 6: Gel electrophoresis studies of the protein components in urea–LiCl supernatant fractions. Urea–LiCl supernatant fractions were prepared from control unirradiated 30S ribosomal subunits, and from 30S ribosomal subunits subjected to different doses of 253.7-nm radiation. Samples of 0.2-ml volume containing 180 μg of protein were then prepared for and subjected to gel electrophoresis according to the procedure described in the Experimental Section. Bovine serum albumin (10 μg per sample) was added to the samples prior to electrophoresis in order to serve as a staining standard. The numbers on the left-hand side of the control gel sample correspond to the band assignments of Traut et al. (1969).

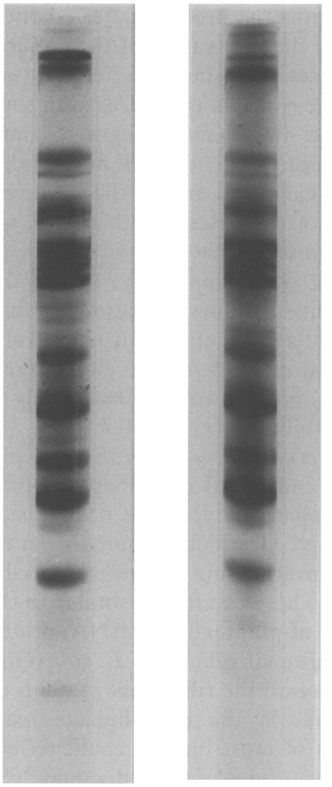

FIGURE 7: Effect of ribonuclease digestion on the electrophoretic patterns of the protein components in urea–LiCl supernatant fractions. Urea–LiCl supernatant fractions were prepared from control unirradiated 30S ribosomal subunits, and from 30S ribosomal subunits irradiated with 5×10^{20} quanta of 253.7-nm radiation. The resultant samples were then digested with pancreatic ribonuclease according to the method described in the legend to Figure 4. The digested samples were dialyzed against electrophoresis buffer as described in the Experimental Section. Incubation of the irradiated sample in the absence of ribonuclease did not affect the electrophoretic pattern of the irradiated sample (data not shown): (A) unirradiated control; (B) irradiated sample.

protein components in urea–LiCl supernatant fractions prepared from ribosomes irradiated with different doses of 253.7-nm radiation are presented in Figure 6. These electropherograms quite clearly indicate that photoinduced disappearance of stainable material from the gels occurs in two distinct, dose-dependent steps. An input of 1×10^{20} quanta (9.8×10^4 ergs/mm^2) of 253.7-nm radiation results in the complete disappearance of 5 bands (no. 0, 1, 3B, 7, and 9), a substantial reduction in the intensity of band no. 4C, and detectable reductions in the intensities of four other bands (2A + 2B, 5, 6, and 10B). A total input of 2×10^{20} quanta (1.96×10^5 ergs/mm^2) results in the complete disappearance of all but three (no. 2A + 2B, 8B, and 8A + 10A) of the remaining bands. On the basis of band assignments made by Traut et al. (1969), these results correspond to the loss of at least five 30S ribosomal proteins in the first step, and a total of 16 proteins after a total input of 2×10^{20} quanta.

As a control for these studies, samples of 30S ribosomal proteins and a urea–LiCl supernatant fraction prepared from heavily irradiated (5×10^{20} quanta) ribosomal subunits were treated with ribonuclease and were then subjected to gel electrophoresis. The resultant electropherograms of the control and irradiated samples were similar (Figure 7), suggesting that the disappearance of bands from the electropherograms of the irradiated samples was probably due to covalent RNA–protein cross-linkage rather than to other photoinduced changes in the primary structure of the ribosomal proteins.

Discussion

The data presented in this report show that exposure of buffered solutions of *E. coli* 30S ribosomal subunits results in the following dose-dependent changes in the properties of the protein and/or rRNA components of the subunit: (1) reduction in the absorbance of the solution at the λ_{max} of the rRNA component of the ribosome that is not seen when solutions of free 16S rRNA are irradiated with the same doses of 253.7-nm radiation; (2) increased solubility of the rRNA component in 4 *M* urea–3 *M* LiCl, a change not seen with free 16S rRNA; (3) coelution, under denaturing conditions (8 *M* urea), of the protein and rRNA components on a Bio-Gel A-5.0m gel filtration column; (4) alterations in the electrophoretic mobilities of several of the ribosomal proteins on one-dimensional polyacrylamide–8 *M* urea gels that are almost completely removed by the action of pancreatic ribonuclease. For the reasons stated in the Results section, these observations are consistent with the conclusion that irradiation of *E. coli* 30S ribosomal subunits with 253.7-nm radiation results in the covalent cross-linkage of the ribosomal proteins to the rRNA.

The observed similarities in the electrophoretic patterns of RNase-treated urea–LiCl supernatant fractions prepared from control and heavily irradiated ribosomes (Figure 7) would seem, prima facie, to be in conflict with the arguments presented above in support of covalent RNA–protein cross-links. One would anticipate that, instead, retention of covalently linked and negatively charged oligonucleotides on the ribosomal proteins after RNase digestion should result in significant changes in the electrophoretic patterns of the proteins "recovered" from the irradiated samples. However, since only a few nucleotides appear to remain cova-

lently attached to the proteins after ribonuclease digestion (Figure 5) and the pK_a values of the amino groups in three of the four bases (cytosine, guanine, and adenine) are approximately the same as the pH used in the electrophoretic studies, it is possible that the negative contributions of the phosphate groups in the attached nucleotides were partially or completely cancelled out by the positive charges on the amino groups of the bases. Furthermore, since the isoelectric points of most of the 30S ribosomal proteins are approximately 12 (Kaltschmidt, 1971), and the number of lysine and arginine residues in these proteins varies from 24 to 40 (Rombauts et al., 1971), it is unlikely that the covalent attachment of a few negatively charged nucleotides to each ribosomal protein would substantially change the electrophoretic mobilities of the proteins.

The data presented also show that the photoinduced disappearance of individual 30S ribosomal proteins from one-dimensional polyacrylamide gels occurs in two dose-dependent steps, each of which presumably involves the cross-linkage of nonoverlapping groups of 30S ribosomal proteins to the rRNA. These results are similar to the previously reported studies of photoinduced RNA–protein cross-linkage in the 50S ribosomal subunit of *E. coli* (Gorelic, 1975) and suggest that not all the ribosomal proteins exhibit the same photoreactivities in the cross-linkage reaction. This observed pattern of cross-linkage could reflect differences in the spatial orientations of the individual 30S ribosomal proteins relative to the 16S rRNA bases or could be a result of differences in the chemical reactivities of the individual 30S ribosomal proteins in the cross-linkage reaction. In the first case, the more reactive group would be presumed to consist of those proteins that are sufficiently close to the photoexcited rRNA based in the native ribosomal subunits to form stable covalent RNA–protein cross-links, whereas the less reactive group would consist of those ribosomal proteins that are inaccessible to the 16S rRNA bases in the native conformational state of the ribosome, but could assume spatial orientations reactive in the cross-linkage after a photoinduced change in ribosome conformation. Alternatively, since a number of pyrimidine photoadducts exhibit finite lifetimes in aqueous media (Johns et al., 1965; Gorelic et al., 1972), it is possible that the less reactive groups of proteins are capable of forming only unstable photoadducts (cross-links) in the native conformational state of the ribosome. In the second case, the more reactive proteins would be anticipated to contain more amino acids reactive in the cross-linkage reaction and/or capable of forming stable RNA–protein cross-links than the less reactive proteins. An unequivocal decision between these alternatives requires a more detailed analysis of the kinetics of photoinduced RNA–protein cross-linkage than presented in this report, and the unequivocal identification of each of the 30S ribosomal proteins participating in the cross-linkage reaction. The results of such studies will be reported shortly (L. Gorelic, manuscript in preparation).

The data presented in this report have been discussed in terms of photoinduced RNA–protein cross-linkage. The photoinduced formation of RNA–RNA cross-links that physically entrap a number of ribosomal proteins in the intact subunit could also give rise to a number of the photoinduced changes in the properties of the ribosomal subunit and its components reported in this study. There are a number of reasons to believe, however, that RNA–RNA cross-linkage may not make a significant contribution to these changes. First of all, the facility with which a substantial number of ribosomal proteins are dissociated by high salt from the intact ribosomal subunits into a split protein fraction that lacks the 16S rRNA components (Nomura et al., 1969; Traut et al., 1969; Chang and Flaks, 1970; Homann and Nierhaus, 1971) and the inability of most of the 30S ribosomal proteins to form isolable complexes with the 16S rRNA (Mizushima and Nomura, 1970; Schaup et al., 1970, 1971; Garrett et al., 1971) suggest that many of the ribosomal proteins are either unable to directly interact with the 16S rRNA in the intact subunit or interact with the rRNA to only a small extent. The photoinduced disappearance of most of the ribosomal proteins from the one-dimensional gels in Figure 6 would therefore not be consistent with the conclusion that most of the affected proteins have been physically entrapped in an RNA–RNA cross-linked complex, since many of these proteins were probably not in extensive physical contact with the 16S rRNA in the unirradiated subunit. Secondly, even if noncovalent entrapment of some proteins did take place in the irradiated native subunit, it seems likely that most (if not all) of the entrapped proteins would be released as a result of the complete denaturation of the secondary structure of the 16S rRNA and the ribosomal proteins by the high concentrations of urea–LiCl used in the preparation of the urea–LiCl supernatant fractions.

In conclusion, the studies presented in this report are consistent with the conclusions that (1) uv irradiation of the 30S ribosomal subunit of *E. coli* results primarily in the covalent cross-linkage of ribosomal proteins to the 16S rRNA, but they do not unequivocally rule out the possibility that a small number of the ribosomal proteins were physically entrapped in the ribosomal subunit as a result of the formation of RNA–RNA cross-links; (2) RNA–protein cross-linkage is specific in the sense that nonoverlapping groups of 30S ribosomal proteins seem to be cross-linked to the 16S rRNA at different doses of uv radiation. Studies are currently in progress to rigorously establish the presence of covalent RNA–protein cross-links in the irradiated 30S ribosomal subunit and to determine the number and locations of such cross-links.

Previous work has led to the general conclusion that the assembly of both *E. coli* ribosomal subunits involves the sequential addition of proteins to the rRNA (Nomura and Traub, 1968; Mizushima and Nomura, 1970; Nashimoto and Nomura, 1970; Nashimoto et al., 1971). One may, therefore, conclude that some ribosomal proteins bind readily to the rRNA, while other ribosomal proteins are assembled into ribosome structure via protein–protein interactions. It is not known, however, whether the first group of ribosomal proteins binds to the rRNA by interaction with the bases and/or the sugar–phosphate backbone. Resolution of the questions unanswered by the results of the present study, and application of the two-dimensional gel technique of Kaltschmidt and Wittmann (1970) to a detailed investigation of the kinetics of photoinduced cross-linkage of individual ribosomal proteins to the rRNA, could complement the earlier work on ribosome assembly and give a fuller understanding of the interactions between rRNA and ribosomal proteins in the intact ribosomal subunits.

Acknowledgment

The author wishes to express his appreciation to Professor T. T. Tchen for his many constructive comments on the contents of the manuscript.

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