Keister, D. L., & Yike, N. J. (1966) *Biochem. Biophys. Res. Commun. 24*, 519–525.
Keister, D. L., & Yike, N. J. (1967) *Biochemistry 6*, 3847–3857.
Koenings, A. W. T., & Guillory, R. J. (1973) *J. Biol. Chem. 248*, 1045–1050.
McFadden, B. J., & Fisher, R. R. (1978) *Arch. Biochem. Biophys. 190*, 820–828.
Mitchell, P. (1972) *J. Bioenerg. 3*, 5–24.
Moyle, J., & Mitchell, P. (1973) *Biochem. J. 132*, 571–585.
O'Neal, S. G., & Fisher, R. R. (1977) *J. Biol. Chem. 252*, 4552–4556.
Ormerod, J. G., Ormerod, K. G., & Gest, H. (1961) *Arch. Biochem. Biophys. 94*, 449–463.
Rydström, J. (1977) *Biochim. Biophys. Acta 463*, 155–184.
Sedmak, J. J., & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.
Skulachev, V. P. (1971) *Curr. Top. Bioenerg. 4*, 127–189.
Skulachev, V. P. (1972) *J. Bioenerg. 3*, 25–38.
Skulachev, V. P., Donostov, A. E., Grinius, L. L., Jasaitis, A. A., & Severina, J. J. (1972) *J. Bioenerg. 3*, 277–303.
Stein, A. M., Kaplan, N. O., & Ciotti, H. M. (1959) *J. Biol. Chem. 234*, 979–986.
Suhadolnik, R. J., Lennon, M. G., Uematsu, T., Monahan, J. E., & Baur, R. (1977) *J. Biol. Chem. 252*, 4125–4133.
You, K., Arnold, L. J., Allison, W. S., & Kaplan, N. O. (1978) *Trends Biochem. Sci. 3*, 265–268.

# Covalent Cross-Linking of Transfer Ribonucleic Acid to the Ribosomal P Site. Mechanism and Site of Reaction in Transfer Ribonucleic Acid†

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ABSTRACT: The covalent cross-linking of unmodified *Escherichia coli N*-acetylvalyl-tRNA to the 16S RNA of *Escherichia coli* ribosomes upon near-UV irradiation previously reported by us [Schwartz, I., & Ofengand, J. (1978) *Biochemistry 17*, 2524–2530] has been studied further. Up to 70% of the unmodified tRNA, nonenzymatically bound to tight-couple ribosomes at 7 mM $Mg^{2+}$, could be cross-linked by 310–335-nm light. Covalent attachment was solely to the 16S RNA. It was dependent upon both irradiation and the presence of mRNA but was unaffected by the presence or absence of 4-thiouridine in the tRNA. The kinetics of cross-linking showed single-hit behavior. Twofold more cross-linking was obtained with tight-couple ribosomes than with salt-washed particles. Puromycin treatment after irradiation released the bound *N*-acetyl[$^3$H]valine, demonstrating that the tRNA was covalently bound at the P site and that irradiation and covalent linking did not affect the peptidyl transferase reaction. Cross-linking was unaffected by the presence of $O_2$, argon, ascorbate (1 mM), or mercaptoethanol (10 mM). Prephotolysis of a mixture of tRNA and ribosomes in the absence of poly($U_2$,G) did not block subsequent cross-linking in its presence nor did it generate any long-lived chemically reactive species. There was a strong tRNA specificity. *E. coli* $tRNA_1^{Val}$ and $tRNA_1^{Ser}$ and *Bacillus subtilis* $tRNA^{Val}$ and $tRNA^{Thr}$ could be cross-linked, but *E. coli* $tRNA_2^{Val}$, 5-fluorouracil-substituted $tRNA_1^{Val}$, $tRNA^{Phe}$, or $tRNA_f^{Met}$ could not. By sequence comparison of the reactive and nonreactive tRNAs, the site of attachment in the tRNA was deduced to be the 5′-anticodon base, $cmo^5U$, or $mo^5U$ in all of the reactive tRNAs. The attachment site in 16S RNA is described in the accompanying paper [Zimmerman, R. A., Gates, S. M., Schwartz, I., & Ofengand, J. (1979) *Biochemistry* (following paper in this issue)]. The link between tRNA and 16S RNA is either direct or involves mRNA bases at most two nucleotides apart since use of the trinucleotide GpUpU in place of poly($U_2$,G) to direct the binding and cross-linking of *N*-acetylvalyl-tRNA to the P site did not affect either the rate or yield of cross-linking. Both *B. subtilis* $tRNA^{Val}$ ($mo^5U$) and *E. coli* $tRNA_1^{Val}$ ($cmo^5U$) gave the same rate and yield of cross-linking when directed by the trinucleotide GpUpU. Therefore, the presence of the charged carboxyl group in the $cmo^5U$-containing tRNA apparently does not markedly perturb the orientation of this base with respect to its reaction partner in the 16S RNA. The cross-linking of AcVal-tRNA only takes place from the P site. At 75 mM KCl and 75 mM $NH_4Cl$, <0.4% cross-linking was found at the A site, while 55.5% was obtained at the P site. However, when the salt concentration was lowered to 50 mM $NH_4Cl$, 5% cross-linking to the A site was detected, compared to 49% at the P site. Thus, a simple change in the ionic strength of the incubation mixture was able to alter the affinity labeling pattern of the ribosome.

Protein biosynthesis demands the close functional cooperation of a remarkable variety of macromolecules and macromolecular assemblies, including mRNA, tRNA, a number of soluble protein factors, and the 70S ribosome (Weissbach & Pestka, 1977). Physical coordination of this process is accomplished by the ribosomal particle which provides specific sites of association for the nonribosomal components and thereby assures their correct mutual orientation during initiation, elongation, and termination of the polypeptide chain. A clear understanding of ribosome function requires that the position and topography of these sites be delineated as fully as possible. One means of elucidating such features entails the covalent attachment of normal or chemically modified

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substrates to the ribosome after formation of an active complex. In principle, the location of the cross-linked residues can be determined by established procedures, and, in conjunction with other structural information, a detailed description of functional sites on the ribosomal surface can be derived.

In order to probe the molecular basis for the specific recognition of tRNA ribosomal binding sites by aminoacyl- and peptidyl-tRNA, we have over the past several years studied the topography of ribosome–tRNA complexes by the technique of photoaffinity labeling, using tRNAs derivatized with aryl azides at specific bases located in the central part of the tRNA (Schwartz & Ofengand, 1974, 1978; Schwartz et al., 1975; Ofengand et al., 1977).

During the course of these studies, it became clear that certain tRNAs were capable of direct cross-linking at the ribosomal P site when irradiated with near-UV light (310–400 nm) without the intervention of an added chemical probe (Schwartz & Ofengand, 1978). Covalent attachment was P site specific, was wholly to the 16S ribosomal RNA, and did not require the presence of 4-thiouridine in the tRNA despite the fact that this nucleotide is the only one present in the tRNA molecule which has a strong absorption band at the same wavelengths used for irradiation. Moreover, while *Escherichia coli* $tRNA_1^{Val}$ could be cross-linked, $tRNA^{Phe}$ and $tRNA_f^{Met}$, both of which contain 4-thiouridine at the same position, could not. Since direct cross-linking means that tRNA must be within 4 Å (Scheffer & Dzakpasu, 1978) of 16S rRNA when bound at the P site, it was of obvious importance to determine the site of cross-linking in both RNA molecules.

In the present paper, this reaction has been studied further in order to determine the nature of this unusual light-induced reaction as well as the site of cross-linking in the tRNA molecule. In the accompanying paper, the site of attachment to the 16S ribosomal RNA has been examined. A preliminary account of this work has appeared (Ofengand et al., 1978).

## Experimental Section

*Chemicals. E. coli* $tRNA_1^{Val}$, $tRNA_f^{Met}$, and $tRNA^{Phe}$ were obtained as described (Schwartz & Ofengand, 1978). FU[1]-substituted $tRNA_1^{Val}$ was prepared according to Horowitz et al. (1977), and $tRNA_2^{Val}$, a mixture of $tRNA_{2A}^{Val}$ and $tRNA_{2B}^{Val}$, was a gift from Dr. B. Dudock. *Bacillus subtilis* $tRNA^{Val}$ and $tRNA^{Thr}$ were kindly donated by Dr. H. Ishikura. Both the *B. subtilis* $tRNA^{Val}$ and $tRNA^{Thr}$ were >90% pure by structural analysis, and >81% of the molecules contained $mo^5U$ (H. Ishikura, personal communication). *E. coli* $tRNA_1^{Ser}$ was purchased from Plenum Scientific. 4-Thiouridine-free $tRNA_1^{Val}$, made by treatment with $H_2O_2$, was the same preparation previously described (Schwartz & Ofengand, 1978). Spectral analysis after aminoacylation confirmed the absence of 4-thiouridine. Salt-washed ribosomes were prepared according to Chinali et al. (1978) up to but not including the subunit dissociation step. Tight-couple ribosomes were prepared by the procedures of Noll et al. (1973). They contained less than 10% excess 50S subunits by sucrose gradient analysis under dissociating conditions and were 25–50% active in the binding of AcVal-tRNA to the P site. EFTu was prepared as described (Chinali et al., 1978). Poly($U_2$,G), poly(U), and poly(A,U,G) were obtained from Boehringer-Mannheim, and poly(A,C,G) and GpUpU were from Miles Laboratories, Inc. Poly(A,U,C) was obtained from P–L Biochemicals, Inc. Sephacryl S-200 was from Pharmacia, [$^3$H]valine, serine, phenylalanine, and methionine were from Amersham/Searle, New England Nuclear, or Schwartz/Mann, and membrane filters were from Gelman (GN-6), Schleicher & Schuell (BA85), or Millipore (HA).

*Acetylaminoacyl-tRNA.* Aminoacylation of $tRNA^{Val}$, $tRNA^{Phe}$, and $tRNA_f^{Met}$, formylation of Met-$tRNA_f^{Met}$, acetylation, and purification by gel filtration were carried out as described previously (Schwartz & Ofengand, 1978). Aminoacylation of $tRNA_1^{Ser}$ and $tRNA^{Thr}$ was performed in 100 mM Hepes, pH 7.5, 10 mM KCl, 10 mM $Mg(OAc)_2$, 5 mM ATP, 1 mM dithiothreitol, 10–20 μM amino acid, and enzyme at 30 °C for sufficient time to reach saturation. The extent of acetylation was measured by the $Cu^{2+}$–Tris assay of Schofield & Zamecnik (1968) as modified by Schwartz & Ofengand (1978). In this assay, the $t_{1/2}$ values for hydrolysis of Ser-tRNA and AcSer-tRNA were 0.47 and 56 min, respectively. For Thr-tRNA and AcThr-tRNA, the values were 1 and 125 min, respectively. In all cases, complete acetylation was obtained. The following were the acylation levels for the various aminoacyl-tRNAs: $Val_1$-, 1.4; $Val_2$-, 1.0; FU-$Val_1$-, 1.3; Phe-, 1.2; $Met_f$-, 1.2; *B. subtilis* Val-, 0.8; *B. subtilis* Thr-, 1.3; $Ser_1$-tRNA, 1.0 nmol/$A_{260}$ unit, respectively. The acylation levels of both $H_2O_2$-treated $tRNA^{Val}$ and its control were 1.1 nmol/$A_{260}$ unit.

*Irradiation Procedure.* Samples were irradiated at 0 °C in a Rayonet photochemical reactor using either the 300- or 350-nm lamps supplied with the instrument with or without the filters described below. Up to six flint glass sample tubes, 10 × 75 mm, were grouped in the center of a styrofoam support which floated on cooling water contained in a Pyrex chamber and surrounded by a Pyrex cooling water jacket. The top of the chamber was covered with aluminum foil. The contents of each tube were stirred during irradiation by magnetic fleas. The Mylar polyester plastic sheet filter (Ramabhadran & Jagger, 1976), 92-gauge Type S from Du Pont, was formed into a cylinder which completely shielded the reaction vessel from the lamps. When measured vs. air, the wavelengths for 10 and 1% transmission through the Mylar sheet were 312.1 and 309.8 nm, respectively. As the plastic tends to solarize with use, it was replaced after 15 h of exposure. Solution filters were 4% naphthalene (scintillation grade, No. 6340, Mallinckrodt) in hexane (Spectro grade, No. 13049, Eastman) and 1.19 M $Pb(NO_3)_2$ in water. When measured against water, the wavelengths for 10 and 1% transmission for a 1-cm path were 324.2 and 322.6 nm, respectively, for the naphthalene solution and 335.2 and 331.2 nm, respectively, for the $Pb(NO_3)_2$ solution. When these solutions were used, they filled the inner annular space (1 cm thick) of a double-jacketed reaction vessel like that described above. The top and bottom of the vessel were masked off to insure that only filtered light could reach the sample tubes. The increased glass thickness of this vessel compared to the previous one had little effect on the total light transmitted (see Table I) and certainly would not affect the light transmitted through the solution filters since the wavelength cutoff for Pyrex (5% transmission, 278 nm, 1 mm thick; Jagger, 1967) is much lower.

*Actinometry.* The amount of light reaching the tubes under the various conditions used was measured by potassium ferrioxalate actinometry (Jagger, 1967). In order to eliminate stray long-wavelength light which was irrelevant to the reaction

[1] Abbreviations used: FU, fluorouracil; $f^5U$, 5-fluorouridine; $mo^5U$, 5-methoxyuridine; $cmo^5U$, 5-(carboxymethoxy)uridine; $acp^3U$, 3-(3-amino-3-carboxypropyl)uridine; AcVal-, AcPhe-, AcSer-, and fMet-, *N*-acetylvalyl-, *N*-acetylphenylalanyl-, *N*-acetylseryl-, and *N*-formylmethionyl-, respectively; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

studied in this paper, we replaced the cooling water in the central well of the chamber by 0.43 M $CoSO_4$. This reduced the light transmission to 10% at 423 and 429 nm in the single- and double-jacketed chambers, respectively. Because of the geometry of the irradiation apparatus, the amount of light reaching the tubes was proportional to volume as well as to irradiation time over the range of sample volumes used in this work. That is, irradiation of the actinometer solution for a given time generated a constant concentration of ferrous ion over the range of 0.5–1.5 mL.

*Ribosomal P Site Binding. $tRNA^{Val}$.* Reaction mixtures contained 50 mM Hepes buffer, pH 7.5, 50 mM $NH_4Cl$, 7 mM $MgCl_2$ or as specified, 24 μg/mL poly($U_2$,G) (6-times saturation) or as specified, Ac[$^3$H]Val-tRNA, and tight-couple ribosomes as indicated for each experiment. Mixtures were incubated at 37 °C for 5 min (maximal binding which stayed constant for at least 60 min) and then chilled to 0 °C for subsequent treatment.

*$tRNA_1^{Ser}$.* Binding was performed in 50 mM Hepes buffer, pH 7.5, 50 mM $NH_4Cl$, 9 mM $MgCl_2$, 100 μg/mL poly(U,C,A), 6.3 $A_{260}$ units/mL tight-couple ribosomes, and 104 pmol/mL AcSer-$tRNA_1^{Ser}$ for 10 min at 37 °C (maximal binding was reached in <5 min and was constant to 30 min). Poly(U,C,I) and poly(U,C) were equally as effective as poly(U,C,A) while poly(A,U,G) was inactive.

*$tRNA^{Thr}$.* Binding was performed in 50 mM Hepes, pH 7.5, 50 mM $NH_4Cl$, 7 mM $MgCl_2$, 40 μg/mL poly(U,C,A), 6.1 $A_{260}$ units/mL ribosomes, and 62 pmol/mL AcThr-tRNA for 10 min at 37 °C. Poly(A,C,I) and poly($C_2$,A) were also effective as mRNAs. Polynucleotide-dependent binding was 24 times that in its absence. Eighty-one percent of the added tRNA was noncovalently bound, of which 92% was released by puromycin treatment. More than 75% of the covalently bound AcThr-tRNA was also reactive with puromycin.

*Filter Binding Assay.* Noncovalent and covalent binding of acyl[$^3$H]aminoacyl-tRNA to ribosomes was measured by membrane filter adsorption immediately after dilution in 50 mM Tris, pH 7.4, 50 mM KCl, and 20 or 0.1 mM $Mg^{2+}$, respectively, as reported previously (Schwartz & Ofengand, 1974, 1978). The values for irradiated control mixtures lacking ribosomes, filtered at both $Mg^{2+}$ concentrations, did not change as a function of irradiation dose and were subtracted from the sample values. At 0.1 mM $Mg^{2+}$ this blank value was usually the same as that of the sample tube at 0 time of irradiation. Where the 0-time value was higher, it is noted in the legend and has been subtracted.

In all cases, the noncovalent binding value did not change during the course of the irradiation. The percent cross-linking was calculated as the ratio of filter-bound radioactivity at 0.1 mM $Mg^{2+}$ to that bound at 20 mM $Mg^{2+}$, after blank subtraction as described above. When polynucleotide was omitted, the 20 mM $Mg^{2+}$ value used was that of the tube containing polynucleotide.

## Results

*Preliminary Characterization of the Cross-Linking Reaction.* In our previous study of the attachment of tRNA to the ribosomal P site (Schwartz & Ofengand, 1978), we used mainly salt-washed ribosomes irradiated with 350-nm lamps, although we did observe that irradiation with 300-nm lamps gave the same yield of cross-linked products more quickly. As the first step in the present work, we tested tight-couple ribosomes (Noll et al., 1973) for their activity in the cross-linking reaction. We also carried out a simplified version of an action spectrum analysis in order to obtain information about the wavelength dependence for the reaction and about the nature

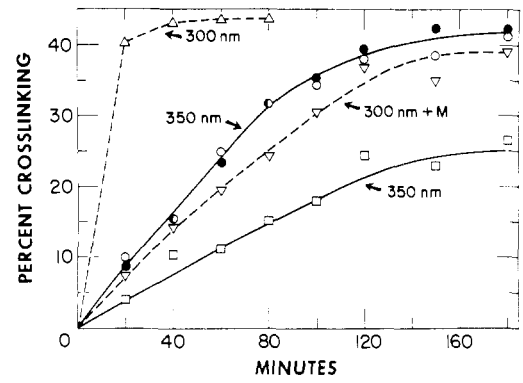


FIGURE 1: Effect of spectral band, absence of 4-thiouridine, and nature of ribosomes on the rate and extent of cross-linking. Incubation, irradiation, and assay for cross-linking as a function of irradiation time were as described under Experimental Section with 6.2 $A_{260}$ units/mL tight couples or 6.9 $A_{260}$ units/mL salt-washed ribosomes and 45 pmol/mL 4-thiouridine-free AcVal-tRNA or 48 pmol/mL control AcVal-tRNA. Either 300- or 350-nm lamps with and without the Mylar filter (M) were used as indicated. One hundred percent cross-linking corresponds to 23.4 or 23.8 pmol/mL 4-thiouridine-free or control AcVal-tRNA noncovalently bound to tight couples and 4.2 pmol/mL for salt-washed ribosomes. (△, ▽, ○) 4-Thiouridine-free AcVal-tRNA and tight couples; (□) 4-thiouridine-free AcVal-tRNA and salt-washed ribosomes; (●) control AcVal-tRNA and tight couples.

of the reactive residues in either the tRNA or the ribosome. As shown in Figure 1, when tight couples were used in place of salt-washed ribosomes at 350 nm, there was, in addition to a sixfold increase in the extent of noncovalent binding, an increase in the fraction of covalent product formed, from 25 to 42%. This effect explains, at least in part, the marked increase in percent cross-linking observed in the current experiments compared to the value of 13% that was found in the earlier work with a different preparation of salt-washed ribosomes. This figure also shows, in verification of our previous observations, that 300-nm irradiation leads to a more rapid rate of cross-linking than does 350-nm irradiation, although the final yield of cross-linked product is the same in both cases. Thus, while the rate of cross-linking was at least four times faster at 300 nm than at 350 nm, the extent of cross-linking was essentially the same, being 44, 39, and 42% at 300 nm, 300 nm plus the Mylar filter, and 350 nm, respectively. This figure also shows that there was no difference in either the rate or extent of cross-linking between control and 4-thiouridine-free AcVal-tRNA when irradiated with 350-nm lamps, just as had been previously found by using 300-nm lamps with the 310-nm cutoff filter (Schwartz & Ofengand, 1978).

The relative rates of reaction at 300 nm with and without the cutoff filter at 310 nm are shown more clearly in Figure 2. The time for 50% reaction without the filter was 9 min, calculated from a semilog plot of the data (Figure 2, inset), which also shows the single-hit character of the linking reaction. With the Mylar filter, single-hit kinetics were again obtained with a half-time of 57 min. Single-hit kinetics were also found at 350 nm with or without the Mylar filter with half-times of 224 and 45 min, respectively. Figure 2 also shows that thiouridine-free AcVal-tRNA was as active as control tRNA when irradiated at 300 nm without a cutoff filter, making it quite clear that 4-thiouridine plays no role in cross-linking from the P site when irradiated with 300-nm lamps plus or minus a cutoff at 310 nm or with the 350-nm lamps.

Figure 2 and Table I show clearly that the shorter wavelengths are more effective in producing tRNA–ribosome cross-links. First, the 310-nm filter depressed the reaction rate sevenfold while only decreasing the incident light intensity by

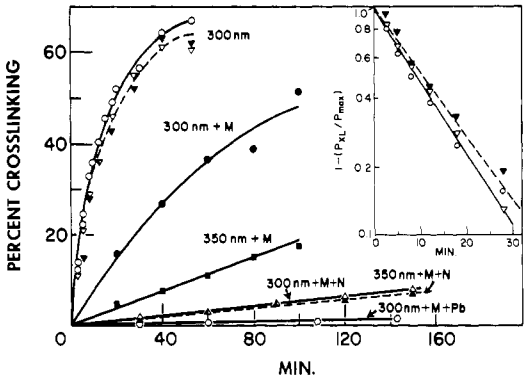


FIGURE 2: Further effects of spectral band and the absence of 4-thiouridine on the rate and extent of cross-linking. Incubation, irradiation, and assay were as described in the legend to Figure 1 except with 68 pmol/mL AcVal-tRNA (preparation 1) (○, ●, ■, △, ▲, ○), AcVal-tRNA (preparation 2) (▼), or 4-thiouridine-free AcVal-tRNA (▼); M, Mylar filter; N, napthalene solution filter; Pb, lead nitrate solution filter. One hundred percent cross-linking corresponds to 44.4, 32.8, or 34.4 pmol/mL AcVal-tRNA (preparation 1), AcVal-tRNA (preparation 2), or 4-thiouridine-free AcVal-tRNA, respectively, which was noncovalently bound to tight couples. The inset is a semilog plot of the 300-nm data where $P_{XL}$ is percent cross-linking and $P_{max}$ is the maximum percent cross-linking.

Table I: Rate of Cross-Linking under Different Irradiation Conditions[d]

| irradiation condition | light intensity[b] [(μEinsteins/min)/mL] | initial rate of cross-linking[a] (% of maximum per h) |
|---|---|---|
| 300 nm | 0.66,[c] 0.59 | 408[c] |
| 300 nm + M | 0.44,[c] 0.43 | 57[c] |
| 300 nm + M + N | 0.39 | 5 |
| 300 nm + M + Pb | 0.27 | 1 |
| 350 nm | 2.8,[c] 3.0 | 58[c] |
| 350 nm + M | 3.0 | 17[c] |
| 350 nm + M + N | 2.1 | 4 |

[a] Initial slope of the curves in Figures 1 and 2, divided by the maximum percent cross-linking obtained, times 100. [b] Measured by ferrioxalate actinometry and corrected for a long-wavelength (>405 nm) output of 0.020 and 0.005 (μEinsteins/min)/mL for the 300- and 350-nm lamp, respectively. This blank value was obtained by using an approximately saturated solution of $NaNO_2$ ($A_{405}^{1cm} = 2.0$) as the solution filter in the inner annular chamber. [c] Irradiated in the single-jacketed apparatus. [d] Samples were irradiated in the double-jacketed chamber, except as indicated, and assayed for cross-link formation. Wavelength of peak output of lamp used, 300 or 350 nm; M, Mylar filter; N, naphthalene; Pb, lead nitrate.

a third. Second, 300-nm + M irradiation was more than 3 times faster than 350-nm + M irradiation, even though there were 6 times more photons supplied by the longer wavelength lamps. Third, when the 323–324-nm filter (N) was used with the 300-nm lamps, the reaction rate decreased 80-fold with only a 40% decrease in incident light intensity. Finally, light above 331 nm (Pb filter) did not induce cross-linking.

Taken together, these data suggest that the optimum wavelengths for cross-linking lie well below 310 nm and that wavelengths above 324 nm are ineffective. These results further support the view that 4-thiouridine is not involved since the $\lambda_{max}$ for this residue in tRNA is at 337 nm. Since reasonable rates of reaction could be obtained at >310 nm, we chose to conduct most of the subsequent experiments under these conditions in order to minimize any potential short-wavelength UV damage to the tRNA or the ribosomes.

Figures 1 and 2 also show the variability in the extent of cross-linking from experiment to experiment. Although, in these experiments, the comparable rates were constant within 10%, there was an almost twofold variation in extent of cross-linking in the two sets of experiments. This effect appears to be due to both an inherent variability among tight-couple preparations as well as to a variable efficiency of ribosome adsorption at low $Mg^{2+}$ concentrations among different lots of the membrane filters used for the assay. The nature of the tight-couple variability is not known, but it must reflect subtle conformational changes since even different aliquots of the same preparation, handled to different extents, have shown significant differences in the extent of cross-linking although the extent of noncovalent binding remained unchanged.

*Site of Cross-Linking in the Ribosome.* When complexes containing Val-$tRNA_1^{Val}$ and salt-washed ribosomes were irradiated with 350-nm lamps, the tRNA became attached exclusively to the 16S ribosomal RNA (Schwartz & Ofengand, 1978). Since the tight-couple ribosomes used in the present experiments gave substantially greater covalent cross-linking and since different irradiation conditions were employed, it was necessary to verify this point. The same methods described previously were used. Covalent complexes of tight-couple ribosomes and AcVal-tRNA formed by 300-nm irradiation with and without the Mylar filter were analyzed on sucrose gradients in 0.3 mM $Mg^{2+}$. All of the ribosome-bound radioactive valine was found in the 30S peak. When this material was recovered from the gradient and analyzed by $NaDodSO_4$–sucrose gradient centrifugation, over 93% of the radioactivity sedimented with the 16S RNA peak (data not shown). These results are exactly analogous to those obtained previously (Schwartz & Ofengand, 1978), ensuring that the basic nature of this reaction, rRNA–tRNA cross-linking, is independent of the exact state of the ribosome as well as of the spectral band used for irradiation.

*Nature of the Light-Induced Reaction.* The most unusual aspect of this cross-linking reaction was the absence of any obvious nucleotide candidates for excitation by the spectral band used. None of the modified bases in $tRNA_1^{Val}$ except 4-thiouridine absorbs appreciably above 310 nm, and there are no known sulfur-containing or hypermodified bases in 16S rRNA (Fellner, 1974). Furthermore, as shown in Table II, agents such as thiols and oxygen which are known to quench free radicals and triplet excited states did not influence the rate of cross-linking either with or without the 310-nm filter. Ascorbic acid, another good free radical quenching agent, had no effect, and purging with argon also did not affect the cross-linking rate compared to the stirred control. We conclude that diffusible free radicals are not likely to take part in this reaction, nor is it likely that radical formation on exposed residues of either RNA molecule is involved, since they should have been readily quenched by the small molecule reagents used.

The mechanism of covalent cross-linking could follow one of several general pathways, following excitation of the reactive base by irradiation. The excited base could react directly with another macromolecule, react with the solvent, decay to the ground state, or decay unimolecularly to an inactive altered molecule. Alternatively, it could be transformed to a short- or long-lived chemically reactive species which could either react with the macromolecule or solvent or else decay to an inactive form. These possibilities were tested by the prephotolysis experiments illustrated in Figures 3 and 4.

Prephotolysis is particularly simple to carry out in this system since noncovalent ribosomal binding, as well as covalent linking, was completely dependent on the presence of poly-($U_2$,G) (Figure 3). Consequently, irradiation of the mixture

Table II: Effect of Oxygen, Mercaptoethanol, or Ascorbic Acid on the Rate of Cross-Linking of AcVal-tRNA to Ribosomes[a]

| | | % covalent linking | | | | |
|---|---|---|---|---|---|---|
| | irradiation time (min) | control | $O_2$ | argon | plus mercaptoethanol | plus ascorbic acid |
| expt 1 (minus filter) | 20 | 17.8 | 10.0 | 11.8 | | |
| | 42 | 19.1 | 17.9 | – | | |
| | 60 | 25.5 | 23.4 | 24.2 | | |
| expt 2 (plus Mylar filter) | 30 | 11.8 | 9.8 | 11.2 | | |
| | 63 | 16.0 | 12.8 | 13.5 | | |
| expt 3 (plus Mylar filter) | 20 | 7.9 | | | 6.9 | |
| | 40 | 13.1 | | | 9.8 | |
| | 60 | 17.1 | | | 14.0 | |
| | 80 | 22.8 | | | 19.4 | |
| | 120 | 29.5 | | | 22.0 | |
| expt 4 (plus Mylar filter) | 30 | 11.5 | | | | 12.9 |
| | 60 | 11.9 | | | | 16.3 |
| | 90 | 18.0 | | | | 20.2 |
| | 120 | 23.5 | | | | 30.1 |
| | 150 | 27.0 | | | | 30.5 |
| | 180 | 29.3 | | | | 33.5 |

[a] Ribosomal binding, irradiation with 300-nm lamps, and assay for cross-linking were done as described under Experimental Section except as noted. Experiment 1 was performed at 15 mM $MgCl_2$, 7.5 $A_{260}$ units/mL tight-couple ribosomes, and 42 pmol/mL AcVal-tRNA of which 31, 31, and 35 pmol/mL were noncovalently bound in the $O_2$, argon, and control samples, respectively. The $O_2$ and argon samples were gassed out for 5 min at 0 °C before irradiation by gentle bubbling from a fine capillary tube. Bubbling was continued during irradiation. The control sample was stirred in a closed tube. Experiment 2 was performed as above but with addition of the Mylar filter, 5.7 $A_{260}$ units/mL of a different tight-couple preparation, and 38 pmol/mL AcVal-tRNA. Reaction mixtures were gassed out by vigorous bubbling for 5 min at 23 °C before addition of ribosomes, incubated 3 min at 37 °C in small stoppered tubes, and then irradiated at 0 °C with gentle bubbling as above. Twenty-two pmol/mL tRNA was noncovalently bound in each sample. Experiment 3 was performed under the standard binding and irradiation conditions given under Experimental Section with 6.0 $A_{260}$ units/mL of a third ribosome preparation and 138 pmol/mL AcVal-tRNA. Where indicated, 10 mM mercaptoethanol was present. Sixty-seven pmol/mL tRNA was noncovalently bound in both the sample and control. Experiment 4 was performed as described in experiment 3 with 74 pmol/mL AcVal-tRNA. Where indicated, 1 mM sodium ascorbate was added after incubation but before irradiation at 0 °C. Thirty-three pmol/mL tRNA was noncovalently bound in both the sample and control.

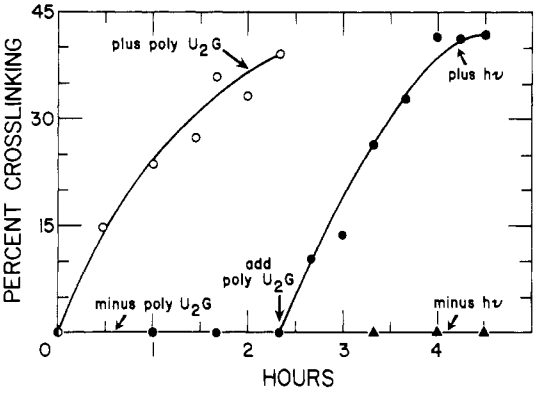


FIGURE 3: Effect of prephotolysis of AcVal-tRNA plus ribosomes in the absence of poly($U_2$,G). The first incubation was as described under Experimental Section with 6.6 $A_{260}$ units/mL tight-couple ribosomes and 80 pmol/mL AcVal-tRNA. (○) Plus poly($U_2$,G); (●) poly($U_2$,G) omitted. One hundred percent cross-linking was equal to 75.8 pmol/mL, and there was <1% noncovalent linking in the absence of poly($U_2$,G). Both tubes were irradiated with 300-nm lamps plus the Mylar filter for the times indicated. At 140 min of irradiation, poly($U_2$,G) was quickly added to the tube lacking poly($U_2$,G) to give the standard concentration, and the tube was incubated 5 min at 37 °C and then chilled to 0 °C. One part was reirradiated (●) while the other part was kept on ice in the dark (▲), and samples were taken at the indicated times. One hundred percent cross-linking for the second incubation corresponded to 62 pmol bound per mL (82% of the first incubation). The total 4.5-h irradiation had no effect (<4%) on the $Cl_3AcOH$ precipitability of the AcVal-tRNA in the mixture.

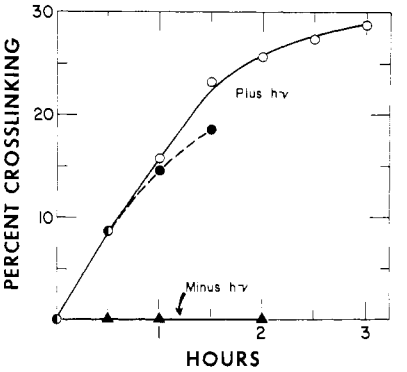


FIGURE 4: Effect of prephotolysis of poly($U_2$,G) plus ribosomes in the absence of AcVal-tRNA. The first incubation was as described under Experimental Section with 6.2 $A_{260}$ units/mL tight-couple ribosomes and 70 pmol/mL (tube A, open circles) or no (tube B) AcVal-tRNA. Both tubes A and B were irradiated with 300-nm lamps plus the Mylar filter. Tube A was sampled at the indicated times. At 2.5 h, 70 pmol of AcVal-tRNA was added to 1 mL of tube B, and tube B was incubated 5 min at 37 °C, chilled to 0 °C, and divided into two parts. One part was reirradiated (solid circles) while the other was kept in the dark at 0 °C (solid triangles). Both parts were then sampled at the indicated times of the second irradiation. One hundred percent cross-linking corresponds to 45 and 39.4 pmol/mL for the first and second incubations, respectively.

of ribosomes and AcVal-tRNA in the absence of poly($U_2$,G) could be performed under exactly the same conditions as for the normal reaction with no corrections necessary for inner filter effects. The contribution of the polynucleotide to the overall absorption of the solution was less than 10%. It is quite clear from Figure 3 that prephotolysis of both the ribosomes and tRNA together but in uncomplexed form had no effect on either the rate or yield of cross-linking when the complexes were subsequently irradiated after the addition of poly($U_2$,G). Prior irradiation of the ribosomes and tRNA separately inhibited noncovalent binding by less than 20%. This result immediately rules out all mechanisms which lead to production of an inactive final product in the absence of a suitable acceptor. Furthermore, the fact that a second irradiation was required in order to obtain cross-linking after complex formation was induced shows that no stable chemical intermediates were produced by the first irradiation which reacted

after subsequent complex formation. Less than 1 min elapsed between the termination of irradiation and addition of poly($U_2$,G), and complex formation is known to be complete in less than 5.0 min under these conditions. Thus, the only general mechanism of reaction not ruled out by this experiment is that in which the excited base, failing to react with another macromolecule, decays back to the original ground state or to some other state still capable of reaction when excited.

Because there is no competing destructive reaction, a 100% yield of cross-linking was expected, with only the rate being affected by steric or other factors. Since this was not the case, we considered slightly more complex possibilities such as one in which the reactive base might only become photoexcitable when placed into a new conformation brought about by tRNA–ribosome complex formation. Thus, cross-linking or photodecomposition could only occur upon irradiation of the complex but not of the separate components. Obviously, the complete system could not be tested, and ribosomes and AcVal-tRNA do not form a functionally significant complex in the absence of mRNA. However, it is known that ribosomes and polynucleotides do form complexes in the absence of tRNA although they are weaker than when tRNA is present (McLaughlin et al., 1966). Consequently, a second prephotolysis experiment was carried out in which the ribosomes and mRNA were present at all times with AcVal-tRNA being added after the first irradiation step (Figure 4).

As before, prephotolysis sufficient to give maximal cross-linking had no effect on subsequent irradiation-induced cross-linking after addition of the AcVal-tRNA, and no stable chemical intermediates could have been produced since light was required for the second reaction. Here also, irradiation for 3 h had a minimal effect on noncovalent binding; only a 12% decrease was found. It seems clear from this experiment that even transient binding of mRNA does not induce photosensitivity in the ribosome. Alternative explanations for the failure to attain 100% cross-linking are deferred to Discussion.

*The cross-linked tRNA is located at the ribosomal P site.* The generally accepted operational definition of P site bound aminoacyl-tRNA is that whose amino acid is reactive with puromycin (Ofengand et al., 1977; Weissbach & Pestka, 1977). We previously showed that the cross-linking of AcVal-tRNA to salt-washed ribosomes could be abolished by prior treatment with puromycin (Schwartz & Ofengand, 1978). This was also the case in the present experiments with tight couples. In an experiment in which 30% cross-linking was observed, treatment with puromycin before irradiation inhibited 86% of the noncovalent binding and 76% of the covalent linking. We could also test whether AcVal-tRNA cross-linked to the P site was still capable of peptide-bond formation with puromycin since in our experiments the tRNA was not attached to the ribosome by its amino acid moiety. Figure 5 shows that this was indeed the case. Puromycin reacted readily with both the covalently and noncovalently bound AcVal-tRNA, showing that at least 80% of the covalently bound AcVal-tRNA was located in the P site. Since approximately the same fraction of AcVal-tRNA was unable to react with puromycin whether noncovalently or covalently bound, the failure to react completely likely reflects some intrinsic loss of activity of the ribosome–tRNA complex rather than covalent linking from a puromycin-unreactive site (a site-bound Val-tRNA does not cross-link to the ribosome; see below).

*Reaction at the Ribosomal A Site.* Our previous studies with either Val-tRNA whose 4-thiouridine was derivatized with the phenacyl group (Schwartz et al., 1975) or unmodified

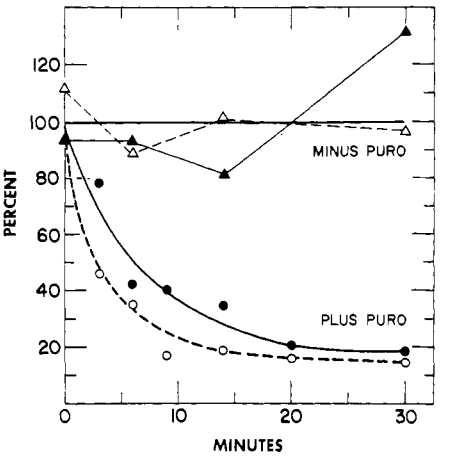


FIGURE 5: Puromycin reactivity of the covalent AcVal-tRNA–ribosomal complex. Ribosomal P site binding was performed as described under Experimental Section except at 12 μg/mL poly($U_2$,G) and 15 mM $Mg^{2+}$ with 5.7 $A_{260}$ units/mL tight-couple ribosomes and 38 pmol/mL AcVal-tRNA. All of the tRNA became ribosome bound. After 90-min irradiation with 300-nm lamps (no filter), 40% (15.1 pmol/mL) became cross-linked. The sample was then divided into two parts; one part was given puromycin (0.6 mM), and both samples were incubated at 37 °C for the times indicated on the figure. At intervals, aliquots were removed and assayed for noncovalent binding (dashed line, open symbols) and for covalent binding (solid line, filled symbols). Percent refers to the fraction of covalently or noncovalently bound AcVal-tRNA remaining. One hundred percent for noncovalent binding was 38 pmol/mL and for covalent binding was 15.1 pmol/mL. (△, ▲) Minus puromycin; (○, ●) plus puromycin.

Val-tRNA (Hsu and Ofengand, unpublished experiments) showed that the type of cross-linking described in this paper does not occur at the A site. Despite this, we considered it necessary to directly compare cross-linking from both A and P sites under equivalent conditions (Figure 6). It is quite clear from Figure 6 that anticodon cross-linking is strictly a P site phenomenon. However, the figure also shows a striking effect of small changes in the concentration of salt in the binding assay mixture. While the change from 50 mM $NH_4Cl$ to 75 mM $NH_4Cl$ and 75 mM KCl had little effect on either the noncovalent binding or covalent linking at the P site, it completely repressed covalent linking at the A site without markedly decreasing the extent of noncovalent binding at this site. In another experiment, there was no effect on noncovalent binding although covalent cross-linking was again completely inhibited. The A site covalent linking at 50 mM $NH_4Cl$ was not due to a small amount of P site binding since (a) all of the A site binding results were first corrected for EFTu·GTP-independent binding and cross-linking and (b) addition of 120 μM EFG and 9 mM puromycin released only 9% of the amino acid covalently bound via tRNA to the A site, while 80% of the amino acid noncovalently bound to the A site was released. EFG was not required for the putative P site bound tRNA, but it was included in both cases since the noncovalent control reaction required translocation of the noncovalently bound Val-tRNA to the P site before reaction with puromycin could take place.

To our knowledge this is the first direct demonstration that small changes in the ionic conditions of in vitro reaction mixtures can markedly affect ribosome affinity labeling results. Such effects have been suggested recently as a partial explanation for contradictory affinity labeling results in the literature (Cooperman, 1978), although differences in the method of preparing and handling the ribosome preparations were not ruled out in these cases.

*tRNA Specificity for Cross-Linking at the P Site.* Although we have shown in the previous sections that unmodified

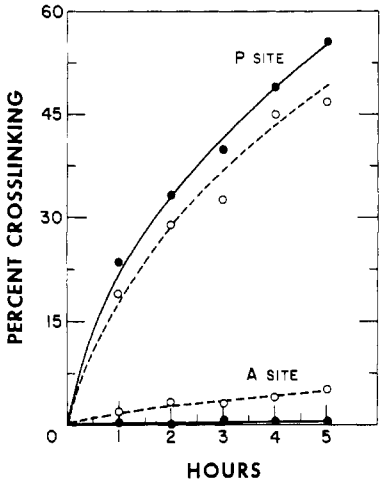


FIGURE 6: Cross-linking activity at the ribosomal A and P sites and the effect of varying salt concentration. Ribosomal P site binding was carried out as described under Experimental Section except at 10 mM $Mg(OAc)_2$ and 40 $\mu$g/mL poly($U_2$,G) with 6.7 $A_{260}$ units/mL tight-couple ribosomes and 36 pmol/mL Ac[$^3$H]Val-tRNA for 15 min at 30 °C. Ribosomal A site binding was carried out in the same mixture except that the Ac[$^3$H]Val-tRNA was replaced by [$^3$H]-Val-tRNA, and 0.1 mM GTP, 1.9 $A_{260}$ units/mL unacylated $tRNA^{Val}$, and 0.35 $\mu$M EFTu were added. In both assays, the ribosomes were added last. (○) With 50 mM $NH_4Cl$ as described above; (●) with 75 mM $NH_4Cl$ and 75 mM KCl in place of the 50 mM $NH_4Cl$. One hundred percent cross-linking of tRNA at the P site corresponded to 26.7 and 25.3 pmol/mL for the open circles and filled circles, respectively. One hundred percent cross-linking at the A site was 30.0 pmol/mL for the open circles and 15.5 pmol/mL for the filled circles. Irradiation and assay were as described under Experimental Section with 300-nm lamps plus the Mylar filter. The unirradiated control value (equal to the irradiated, minus ribosome control) was subtracted in all cases. EFTu-dependent binding and cross-linking was measured by omitting EFTu and GTP from one A site binding mixture and subtracting these values from those of the complete A site mixture. Added EFTu and GTP stimulated noncovalent A site binding 7–14-fold.

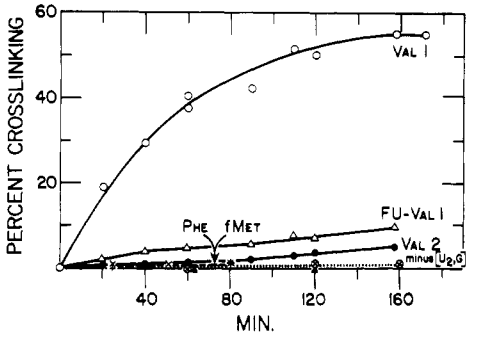


FIGURE 7: tRNA specificity for cross-linking activity. Ribosome incubations were performed as described under Experimental Section at 7 mM $Mg^{2+}$ and 12 $\mu$g/mL poly($U_2$,G) with 5.6 $A_{260}$ units/mL tight-couple ribosomes and 46 pmol/mL AcVal-tRNA. 95, 98, and 100% of the FU-$tRNA_1^{Val}$, $tRNA_2^{Val}$, and $tRNA_1^{Val}$, respectively, were bound. For AcPhe-tRNA (40 pmol/mL) and fMet-tRNA (31 pmol/mL), 20 $\mu$g/mL poly(U) and 20 $\mu$g/mL poly(A,U,G), respectively, were used at 15 mM $Mg^{2+}$. One hundred percent of the AcPhe-tRNA and 23% of the fMet-tRNA were bound noncovalently. Irradiation was with 300-nm lamps plus the Mylar filter. At the indicated times, samples were removed and assayed for the extent of cross-linking by the filter method. The following zero-time values were subtracted: 0.3%, $tRNA_1^{Val}$; 0.2%, $tRNA_2^{Val}$; 0.8%, FU-$tRNA_1^{Val}$; 0.4%, $tRNA^{Phe}$. (○, ⊗) AcVal-$tRNA_1^{Val}$; (●, ⊗) AcVal-$tRNA_2^{Val}$; (△, ▲) AcVal-FU-$tRNA_1^{Val}$; (+) fMet-tRNA; (×) AcPhe-tRNA; (▲, ⊗) minus poly($U_2$,G).

AcVal-$tRNA_1^{Val}$ could readily cross-link to the ribosome, neither AcPhe-tRNA nor fMet-RNA were able to do so (Figure 7). This result was analogous to our previous findings with the *S*-(*p*-azidophenacyl) derivatives of the same tRNAs (Schwartz & Ofengand, 1978). To further explore the specificity of this reaction, we tested two additional valine tRNAs, the minor $tRNA_2^{Val}$ of *E. coli* and $tRNA_1^{Val}$ whose uridine and uridine-derived residues had been substituted by 5-fluorouridine. Neither of these tRNAs could be cross-linked. The slight activity of the FU-$tRNA^{Val}$ is consistent with the residual 10% unsubstituted uridine-derived residues remaining in the preparation (Horowitz et al., 1977). Figure 7 also verifies the strong dependence of covalent linking on the presence of a polynucleotide messenger RNA while Table III shows that any polynucleotide will not suffice for covalent linking, but only one containing the appropriate codons.

Additional tRNA specificity studies are shown in Figure 8. Panel A shows that the cross-linking of $tRNA^{Val}$ from *B. subtilis* was almost as efficient as that of *E. coli* $tRNA_1^{Val}$, and panel B shows that *E. coli* $tRNA_1^{Ser}$ could also be

Table III: Specificity of the Polynucleotide Dependence for Cross-Linking[a]

| polynucleotide added | concn ($\mu$g/mL) | irradiation time (min) | % covalent linking |
|---|---|---|---|
| poly($U_2$,G) | 12 | 60 | 28.8 |
| | | 120 | 36.4 |
| poly(U) | 12 | 60 | 2.2 |
| | | 120 | 4.1 |
| poly(A,C,G) | 13 | 60 | 0.1 |
| | | 120 | 0.1 |

[a] The standard incubation mixture contained 12.3 $A_{260}$ units/mL tight-couple ribosomes, 77 pmol/mL AcVal-tRNA, and polynucleotide as indicated. Irradiation was as described under Experimental Section with 300-nm lamps plus the Mylar filter. Aliquots were removed at the indicated times for assay by the filter method. In this experiment, the blank values for minus ribosomes, 0 time of irradiation, and minus polynucleotide were all the same, equivalent to 0.5 %, and have been subtracted.

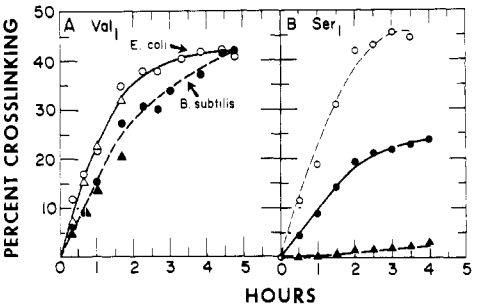


FIGURE 8: Cross-linking activity of *B. subtilis* $tRNA^{Val}$ and *E. coli* $tRNA_1^{Ser}$. (A) Ribosomal binding was carried out as described under Experimental Section with 12 $\mu$g/mL poly($U_2$,G), 6.2 $A_{260}$ units/mL tight-couple ribosomes, and 62 pmol/mL *E. coli* AcVal-$tRNA_1^{Val}$ (○, △) or 64 pmol/mL *B. subtilis* AcVal-tRNA (●, ▲). Ninety-four percent of the *E. coli* and seventy-three percent of the *B. subtilis* tRNA were noncovalently bound. Irradiation with 300-nm lamps plus the Mylar filter was carried out as described above, and samples were taken at intervals for analysis by the filter method. The different symbols are two different experiments. A zero-time value of 0.2% for both tRNAs has been subtracted. (B) Ribosomal binding was carried out as described under Experimental Section. In the presence of poly(U,C,A) (solid circles), 73% of the added AcSer-tRNA was bound noncovalently, while in the absence of added polynucleotide (solid triangles), only 2.2% was bound. Irradiation was as described under Experimental Section with 300-nm lamps plus the Mylar filter for the indicated times. The following zero-time blank values were subtracted: plus poly(U,C,A), 1.1%; minus poly(U,C,A), 0.5%. The dashed line shows a reference incubation with 115 pmol/mL AcVal-tRNA under the same conditions except with 20 $\mu$g/mL poly($U_2$,G) at 7 mM $Mg^{2+}$. Forty-seven percent of the AcVal-tRNA added was bound noncovalently.

Table IV: Comparison of tRNA Sequences Tested for the Ability to Cross-Link to the Ribosomal P Site[a]

| tRNA | cross-linking (%) | residue no. 7 | 29 | 34 | 47 | 59 | 64 | 65 |
|---|---|---|---|---|---|---|---|---|
| $Val_1$ (*E. coli*) | 45 | U | U | $cmo^5U$ | U | U | U | U |
| Val (*B. subtilis*) | 45 | U | U | $mo^5U$ | U | G | U | U |
| $Val_2$ (*E. coli*) | 1.3 | G/A | A | G | $acp^3U$ | C | C/A | G |
| $Val_1^{FU}$ (*E. coli*) | 5.0 | F | F | F | F | F | F | F |
| Phe (*E. coli*) | 0.5 | A | G | G | $acp^3U$ | U | A | C |
| $Met_f$ (*E. coli*) | 0.5 | G | G | C | U | A | G | C |
| $Ser_1$ (*E. coli*) | 24 | G | G | $cmo^5U$ | U | A | U | G |
| Thr (*B. subtilis*) | 17 | G | U | $mo^5U$ | U | G | C | G |

[a] The cross-linking activity was abstracted from Figures 7 and 8 and data not shown (for Thr-tRNA). Residues are listed according to a scheme in which the nucleotide positions common to yeast $tRNA^{Phe}$ are numbered consecutively from the 5′ end (Siddiqui et al., 1979). tRNA sequence data were taken from Siddiqui et al. (1979) except for FU-$tRNA_1^{Val}$ (Horowitz et al., 1974) and *B. subtilis* $tRNA^{Val}$ (H. Ishikura, personal communication).

cross-linked. The mRNA dependence of the $tRNA_1^{Ser}$ cross-linking is also shown, and it is quite clear that Ac-Ser-tRNA also required mRNA to covalently link at the P site.

*Site of Cross-Linking in tRNA.* The marked tRNA specificity shown above has been correlated with the nucleotide sequence for each tRNA (Table IV). This table also includes summary results for Thr-tRNA (the complete set of data is not shown). From this, we have deduced the site of attachment in the tRNA molecule. We assume that there is a single site on the tRNA molecule which can cross-link and that it is located at the same place in all the tRNAs. The evidence for a single cross-link is circumstantial, being based on the single-hit (pseudo-first-order) kinetics of cross-linking which have been found in all of our experiments; for example, see the inset to Figure 2. The evidence that the single cross-link is always in the same place is that all three active tRNAs cross-link exclusively to 16S RNA, to the 8S fragment of 16S RNA, and to the same-sized denatured fragment of the 8S piece (Zimmermann et al., 1979). We also assume that 4-thiouridine is not involved. This has been tested only for *E. coli* $tRNA_1^{Val}$ [Figures 1 and 2; also see Schwartz & Ofengand (1978)], but if the first two assumptions are valid, then the lack of involvement of 4-thiouridine should be true for all tRNAs. It is clearly valid for FU-$tRNA_1^{Val}$, $tRNA^{Val}$ (*B. subtilis*), and $tRNA^{Thr}$ (*B. subtilis*) which do not contain 4-thiouridine (Horowitz et al., 1974, 1977; Hasegawa & Ishikura, 1978; Ishikura, personal communication). We further assume that the observed specificity is due to some common feature of nucleotide sequence and is not a consequence of a unique conformational property in some particular region of the tRNA molecule which is shared by a particular subset of tRNAs.

*Method A.* The failure of FU-$tRNA_1^{Val}$ to react strongly suggested that the reactive residue in the tRNA was uridine-derived, as all uridine and uridine-derived residues are replaced by 5-fluorouridine in this tRNA (Horowitz et al., 1974, 1977), and no gross conformational changes occur upon fluorouracil substitution (Horowitz et al., 1974). When the 4-thiouridine and uridine residues also found in $tRNA_2^{Val}$ were eliminated, the seven positions listed in Table IV remained. Comparison with $tRNA_f^{Met}$ and $tRNA^{Phe}$ eliminated residues 47 and 59. Comparison of $tRNA_1^{Ser}$ with $tRNA^{Phe}$, $tRNA_f^{Met}$, and $tRNA_2^{Val}$ for common residues eliminated all of the original seven positions except for residues 34 and 64. A similar comparison of $tRNA^{Thr}$ vs. the inactive tRNAs eliminated all but residues 29, 34, and 59. The only residue common to all three lists was residue 34.

*Method B.* First, we compared the sequence of *E. coli* $tRNA_1^{Val}$ with $tRNA_2^{Val}$, $tRNA^{Phe}$, or $tRNA_f^{Met}$, selecting only those *positions* whose nucleotide in $tRNA_1^{Val}$ was not found in one of the inactive tRNAs ($tRNA_{2A}^{Val}$, tested subsequently, was also inactive). There were twelve such residues. *B. subtilis* $tRNA^{Val}$ had the same nucleotides at these positions as *E. coli* $tRNA_1^{Val}$. A similar comparison of $tRNA_1^{Ser}$ and *B. subtilis* $tRNA^{Thr}$ with $tRNA_2^{Val}$, $tRNA^{Phe}$, and $tRNA_f^{Met}$ was then made, and the two lists so generated were compared with the list of twelve positions from the $tRNA_1^{Val}$ comparison. Only three positions, residues 6, 32, and 34, were found in both lists. In two of these, residues 6 and 32, the same nucleotide was found in *E. coli* $tRNA_1^{Val}$ (active) as in FU-$tRNA_1^{Val}$ (inactive), leaving only (c)$mo^5U_{34}$ as the candidate for the reactive residue, the same position determined by method A above. Thus, the available evidence, while circumstantial, nevertheless strongly favors the anticodon base as being the site of reaction in tRNA.

*Is the tRNA directly linked to ribosomal RNA*? In any complex system in which cross-linking occurs, it is important to ask whether the two components studied are directly attached to each other or are attached via a third undetected molecule, in our case either the ribosomal protein(s) or the polynucleotide mRNA. The evidence against ribosomal proteins being the spacer link between 16S RNA and tRNA is that protease treatment of the denatured tRNA–8S rRNA fragment derived from 16S rRNA had no effect on the size of the fragment complex (Zimmermann et al., 1979).

If mRNA were the spacer, the polynucleotide could in turn be cross-linked to the 16S RNA at almost any site, and little topographical information about tRNA would be obtainable from this study. We tested this possibility by the use of the trinucleotide codon GpUpU as a substitute for the polynucleotide. In order to obtain a suitable degree of noncovalent binding with GpUpU, it was necessary to increase the $Mg^{2+}$ concentration to 20 mM and the trinucleotide concentration to 5 times that of the polynucleotide. Under these conditions, 73 and 54% of the binding with polynucleotide at 7 and 20 mM $Mg^{2+}$, respectively, were obtained. As shown in Figure 9, irradiation of both types of complexes gave the same rate of cross-linking. This experiment shows, therefore, that, if the codon is part of the covalent complex, the two linkage points can be at most two nucleotides apart.

The same results were obtained when *B. subtilis* AcVal-tRNA with $mo^5U$ at residue 34 was added instead of *E. coli* AcVal-$tRNA_1^{Val}$, which has $cmo^5U$ at this position (data not shown). Furthermore, there was no difference in either the rate or extent of GpUpU-dependent cross-linking when the $mo^5U$- and $cmo^5U$-containing tRNAs were directly compared at 20 mM $Mg^{2+}$ (data not shown). Thus, in a situation in which either $mo^5U$ or the carboxyl group containing $cmo^5U$ was capable of base pairing with U at the wobble position,

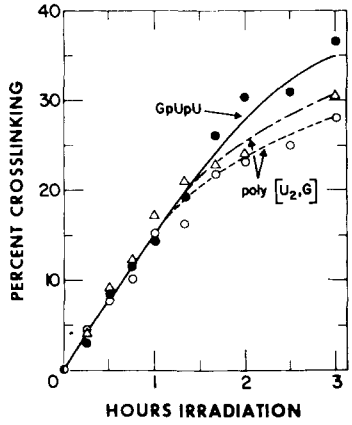


FIGURE 9: Cross-linking activity of complexes with poly($U_2$,G) replaced by GpUpU. Ribosomal P site binding was carried out as described under Experimental Section with 6.7 $A_{260}$ units/mL tight-couple ribosomes and 98 pmol/mL AcVal-tRNA for 10 min at 37 °C. Poly($U_2$,G) was used at 20 μg/mL at 7 (○) or 20 (△) mM $Mg^{2+}$, and GpUpU was used at 89 μg/mL at 20 mM $Mg^{2+}$ (●). One hundred percent cross-linking of tRNA corresponds to 50.4 and 68.8 pmol/mL noncovalently bound for poly($U_2$,G) at 7 or 20 mM $Mg^{2+}$, respectively, and 37 pmol/mL for GpUpU. In the absence of oligo- or polynucleotide, the noncovalent binding was 4.8 pmol/mL at 20 mM $Mg^{2+}$. Irradiation was carried out as described under Experimental Section with 300-nm lamps plus the Mylar filter. Samples were assayed for cross-linking at the indicated times.

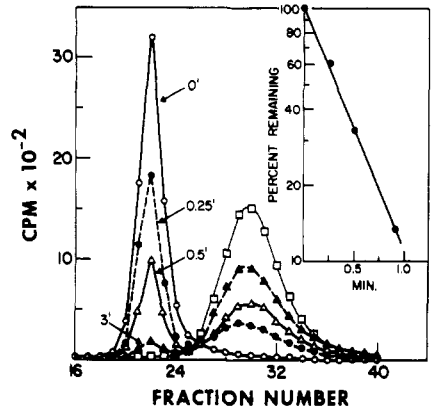


FIGURE 10: Light-induced splitting of the cross-link. 70S ribosome–tRNA cross-linked complexes prepared by irradiation of the standard reaction mixture for 210 min were extracted with an equal volume of redistilled phenol, the phenol layer was washed, and the combined aqueous phases were precipitated with 2.5 volumes of EtOH. The precipitate was dissolved in water and freed of unbound tRNA by gel filtration on a 1.5 × 28 cm column of Sephacryl S-200 equilibrated in 10 mM potassium phosphate and 1 mM EDTA, pH 6.5. The ribosomal RNA peak eluting at fractions 20–23 was pooled. Aliquots (1.1 pmol of complex; $A_{260}$ of 0.16) were irradiated as 600-μL drops (2 mm thick) on a sheet of plastic placed on a water-cooled metal block. The 2537-nm low-pressure argon–mercury lamps were mounted 15.5 cm above the plastic sheet. This configuration produced a dose rate of 1.6 μEinsteins/min as determined by ferrioxalate actinometry (Jagger, 1967). After irradiation the samples were chromatographed on the same Sephacryl S-200 column. The percent of 16S RNA–tRNA remaining was calculated from the fraction of radioactivity in each peak and plotted in the inset. A residual level of cross-linking of 6.2% which remained after 3- or 30-min irradiation has been subtracted from each data point. Ninety-seven percent of the $Cl_3AcOH$-precipitable radioactivity of the original Ac[$^3$H]-Val-tRNA–16S RNA complex remained after 3-min irradiation. (○) 0; (●) 0.25; (△) 0.5; (▲) 3.0 min. The curves for 0.92 and 30 min are not shown. $t_{1/2}$ = 0.31 min. (□) Marker AcVal-tRNA.

there was no shift in spatial orientation of the 5′-anticodon base that was large enough to be detected as a change in the rate of cross-linking.

*Photolysis of the Cross-Link.* Since the ability to cleave the covalent bond at will would greatly facilitate further analysis of this system, we examined the stability of the cross-link to irradiation. We chose 254-nm light for this experiment since the well-known reversible cleavage of thymine dimers in DNA can be effectively carried out at this wavelength (Fisher & Johns, 1976a). For this experiment, we prepared tRNA–16S RNA complexes by irradiation, isolated the RNA free of proteins, and then irradiated the RNA preparation in a denaturing buffer. The reaction mixture was analyzed by gel filtration since cleavage of the Ac[$^3$H]Val-tRNA from the 16S RNA was expected to markedly decrease the molecular weight of the radioactive species. As shown in Figure 10, these expectations were realized. There was a rapid and virtually complete photolysis of the complex, which obeyed single-hit kinetics for at least 85% of the reaction.

Photolysis of tRNA–ribosome covalent complexes at 0.3 mM $Mg^{2+}$ obeyed similar first-order kinetics, and in this case we were able to show that both the tRNA and the ribosome could be recovered unchanged after such photolysis. That is, both the photolyzed ribosomes and the photolyzed tRNA were capable of re-cross-linking when reirradiated with 310–330-nm light (J. Ofengand, unpublished experiments).

## Discussion

This report extends our previous study (Schwartz & Ofengand, 1978) of the light-induced cross-linking of underivatized tRNA to the ribosome. The cross-linking reaction is highly specific for both the tRNA and the ribosomal site and is probably due to the formation of a direct covalent bond between the two RNA species. The pseudo-first-order kinetics of cross-linking (Figure 2, inset) are indicative of a single-hit reaction, as is the detection of a single tRNA–8S RNA fragment derived from the tRNA–16S RNA complex (Zimmermann et al., 1979). No evidence for the intermediary presence of a protein spacer could be obtained (Zimmermann et al., 1979), and, although the presence of an mRNA spacer was not ruled out, use of the trinucleotide GpUpU showed that the topological conclusions would not be affected even if this were the case.

The tRNA specificity observed probably reflects the chemistry of the cross-linking reaction rather than any selectivity of the P site for specific tRNAs since all tRNAs, with essentially similar geometry, must pass through this site. The cross-link may be a cyclobutane dimer between the 5′-anticodon pyrimidine of the tRNA and a uridine or cytidine of 16S RNA since irradiation at 254 nm not only breaks the covalent link (Figure 10) but also regenerates the original reactants (J. Ofengand, unpublished experiments). Photolysis at 254 nm with regeneration of reactants is a characteristic property of pyrimidine–pyrimidine cyclobutane dimers (Fisher & Johns, 1976a). Cyclobutane dimer formation might also explain why $tRNA_f^{Met}$ with cytidine in the 5′-anticodon position was not cross-linked since dimerization of cytidine is known to be much less efficient than that of thymidine (Setlow & Carrier, 1966; Fisher & John, 1976a). The lack of cross-linking when $f^5U$ is at the 5′-anticodon position also can be explained in this way since this substituted pyrimidine does not form a dimer in detectable amounts (Lozeron et al., 1964), probably as a result of its relatively high rate of UV-induced hydration (Fisher & Johns, 1976b).

The failure of $O_2$ to quench cross-link formation (Table II) is not incompatible with cyclobutane dimer formation even though this reaction usually proceeds from the triplet state and is quenched in the presence of $O_2$ (Fisher & Johns, 1976a). When the two bases are sufficiently close, as in a suitably stacked polymer or molecular aggregate, dimerization can

occur in the presence of $O_2$ (Fisher & Johns, 1976a; Leonard et al., 1969; Golankiewicz & Strekowski, 1972). Presumably, the reaction proceeds in this case from the singlet state, although a triplet-state reaction would also be possible if the reaction rate were faster than the diffusion rate of $O_2$.

The failure of prephotolysis in air (Figures 3 and 4) to block cross-linking is likely due to the fact that the only common photochemical reaction of pyrimidines that is not oxygen-quenched is hydrate formation (Fisher & Johns, 1976b). However, in contrast to uridine, thymidine and 5-ethyluridine, and presumably (c)mo$^5$U also, do not form hydrates upon irradiation (<1% of uridine hydrates) (Fisher & Johns, 1976b). The reason for the failure of prephotolysis to hydrate the presumptive uridine or cytidine partner in the 16S RNA is less clear but may be due to its environment, since base pairing (Pearson & Johns, 1966) or base stacking (Leonov & Elad, 1974) strongly reduces the rate of hydration in such instances.

Although we used light of wavelength >310 nm for our studies in order to minimize the potential for nonspecific UV-induced cross-linking, it is clear from Figures 1 and 2 that shorter wavelengths are considerably more effective. This result is in keeping with the proposal that the cross-link is via (c)mo$^5$U since this modification only shifts the uridine absorption band 16–19 nm to the red (Murao et al., 1970, 1976). Indeed, (c)mo$^5$U has very little absorption above 310 nm when in aqueous solution at pH 7. However, a further red shift may be induced by the environment supplied by the ribosome–mRNA complex when (c)mo$^5$U is bound at the P site as part of an anticodon. Thus, the reactivity of (c)mo$^5$U$_{34}$ which we have observed could be due to an intrinsic greater photoreactivity of these nucleotides over f$^5$U$_{34}$ or C$_{34}$, to a fortuitous coincidence of excitation and irradiation wavelengths, or to both effects. This aspect has to be investigated further.

The site of cross-linking in tRNA was deduced indirectly by comparison of the sequences of those tRNAs that could be cross-linked with those that could not. This analysis localized the site to the 5′-anticodon base at the tip of the anticodon, one of the most exposed residues in the entire three-dimensional tRNA structure (Kim, 1976). Nevertheless, as the evidence is circumstantial, efforts are currently underway to confirm the site of cross-linking by direct methods. The corresponding site of linking in the 16S RNA has been localized to the 3′ third of the molecule (Zimmermann et al., 1979).

The marked preference for cross-linking from the P site vs. the A site must be due to some special structural arrangement which exists between the 5′ end of the anticodon and 16S RNA at this site on the 30S subunit. An attractive hypothesis for this interaction which would still allow codon–anticodon recognition to occur is to postulate that the highly exposed 5′-anticodon base of the tRNA (Kim, 1976) stacks on a similarly exposed sequence of 16S RNA. Such a configuration would effectively extend the five-base stack of the tRNA anticodon loop into the 16S RNA and thereby provide added stability to the tRNA–ribosome interaction. Moreover, if base pairing is incomplete (Mitra et al., 1977; Weissenbach et al., 1977) or nonexistent at the P site, this sort of stabilization could be important in maintaining the alignment of peptidyl-tRNA on the ribosome. Assuming that the bases are properly oriented, photoexcitation could then bring about the formation of a pyrimidine–pyrimidine dimer linking the two RNAs (see above discussion). This hypothesis implies further that the cross-linked base in the 16S RNA is strictly defined by its stereochemical relationship to the tRNA and should therefore be the same in all of the tRNA–16S RNA complexes described here. The presence of a tRNA in the A site is not expected to affect the above model. On the one hand, simultaneous codon–anticodon recognition in both P and A sites may not exist. On the other hand, even if it does, it is possible for a base of 16S RNA to stack on the P site 5′-anticodon base even though it is located in the center of the six base-pair codon–anticodon complex by suitable manipulation of the conformation of the anticodon loops and/or the diester link between the mRNA codons.

Grosjean et al. (1978) have recently suggested that interaction of the negatively charged carboxymethoxy group of cmo$^5$U with its own 5′-phosphate can occur when this residue is present in a tRNA anticodon at the 5′ position and have suggested that a conformational change in the anticodon loop backbone occurs to alleviate this situation. Our results (Figure 9) do not support this proposal, assuming that a similar interaction occurs at the P site, since we observed no difference in cross-linking, a sensitive measure of spatial orientation of the uracil ring, whether cmo$^5$U or mo$^5$U (lacking the charged carboxyl group) was available at the 5′-anticodon position for pairing with U in the codon GpUpU.

In the course of comparing the P and A site reactions, we observed a marked effect of ionic strength on EFTu-dependent labeling at the A site. While 50 mM salt allowed some A site reaction to occur, 150 mM salt completely blocked the A site reaction but had virtually no effect on covalent linking at the P site. This is the first example known to us where such salt effects have been unambiguously demonstrated. It supports the proposal of Cooperman (1978), who sought to explain some of the contradictory photoaffinity labeling studies in the literature on the basis of different assay conditions.

The state of the ribosomes also affected our results. Our standard procedure yielded preparations with variable capacity for cross-linking which in no case approached 100% even though no photochemical side reactions could be detected (Figures 3 and 4). One interpretation of these results would be that ribosomes exist in two conformational states, both capable of noncovalent binding, but only one of which has the stereochemical orientation of its 16S RNA allowing covalent cross-link formation. If the ratio of the two sets of conformations varied according to some unknown parameter of the preparation and/or handling, a variable yield could easily be obtained. An alternative explanation for the less than complete reaction may be that, as in the case of thymine dimer formation, a wavelength-dependent equilibrium is reached between formation and splitting which will vary according to the wavelength dependence of the rate constants for the two opposing reactions (Fisher & Johns, 1976a). Since dimer formation also depends on the orientation of the reactants, while the splitting reaction does not, variation in the net equilibrium position could easily occur at a given wavelength since it would depend on the precise orientation of 16S RNA in the decoding region. This model differs from the first one in that existence of simultaneous multiple conformations of the ribosomes need not be postulated. In either case, it seems clear from these results that affinity labeling is in fact a rather subtle tool for exploring ribosome conformation around ligand binding sites.

The irradiation conditions used in these experiments did not inactivate the ribosomes either for P site binding or for peptide-bond formation. As shown in Figures 3 and 4, there was a less than 20% decrease in the level of binding after irradiation with the 300-nm lamps plus the Mylar filter, and in other experiments the same results were obtained in the absence of the filter. The rate of reaction with puromycin,

a measure of the activity of the peptidyl transferase center, was also unchanged after irradiation with 300-nm lamps without the Mylar filter (Figure 5 and unpublished experiments).

In summary, the results reported in this paper indicate that the 5′-anticodon base of tRNA in the ribosomal P site is within at most two nucleotides of a residue in the 3′ third of the 16S ribosomal RNA. It is highly likely that such a close contact is not fortuitous and, therefore, that ribosomal RNA plays an important role in the P site binding of the anticodon region of peptidyl-tRNA.

References


Chinali, G., Horowitz, J., & Ofengand, J. (1978) *Biochemistry 17*, 2755–2760.

Cooperman, B. S. (1978) in *Bioorganic Chemistry* (Van Tamelen, E., Ed.) Vol. 4, pp 81–115, Academic Press, New York.

Fellner, P. (1974) in *Ribosomes* (Nomura, M., Tissières, A., & Lengyel, P., Eds.) pp 169–191, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Fisher, G. J., & Johns, H. E. (1976a) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. I, pp 225–294, Academic Press, New York.

Fisher, G. J., & Johns, H. E. (1976b) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. I, pp 169–224, Academic Press, New York.

Golankiewicz, K., & Strekowski, L. (1972) *Mol. Photochem. 4*, 189–203.

Grosjean, H. J., de Heanu, S., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A 75*, 610–614.

Hasegawa, T., & Ishikura, H. (1978) *Nucleic Acids Res. 5*, 537–548.

Horowitz, J., Ou, C.-N., Ishaq, M., Ofengand, J., & Bierbaum, J. (1974) *J. Mol. Biol. 88*, 301–312.

Horowitz, J., Ofengand, J., Daniel, W. E., Jr., & Cohn, M. (1977) *J. Biol. Chem. 252*, 4418–4420.

Jagger, J. (1967) *Introduction to Research in Ultraviolet Photobiology*, Prentice-Hall, Englewood Cliffs, NJ.

Kim, S. H. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 17*, 181–216.

Leonard, N. J., Golankiewicz, K., McCredie, R. S., Johnson, S. M., & Paul, I. C. (1969) *J. Am. Chem. Soc. 91*, 5855–5862.

Leonov, D., & Elad, D. (1974) *J. Am. Chem. Soc. 96*, 5635–5637.

Lozeron, H. A., Gordon, M. P., Gabriel, T., Tantz, W., & Duschinsky, R. (1964) *Biochemistry 3*, 1844–1850.

McLaughlin, C. S., Dondon, J., Grunberg-Manago, M., Michelson, A. M., & Saunders, G. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 601–610.

Mitra, S. K., Lustig, F., Akesson, B., & Lagerkvist, U. (1977) *J. Biol. Chem. 252*, 471–478.

Murao, K., Saneyoshi, M., Harada, F., & Nishimura, S. (1970) *Biochem. Biophys. Res. Commun. 38*, 657–662.

Murao, K., Hasegawa, T., & Ishikura, H. (1976) *Nucleic Acids Res. 3*, 2851–2860.

Noll, M., Hapke, B., Schreier, M. H., & Noll, H. (1973) *J. Mol. Biol. 75*, 281–294.

Ofengand, J., Schwartz, I., Chinali, G., Hixson, S. S., & Hixson, S. H. (1977) *Methods Enzymol. 46*, 683–702.

Ofengand, J., Schwartz, I., Zimmermann, R. A., Gates, S. M., & Liou, R. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1658.

Pearson, M. L., & Johns, H. E. (1966) *J. Mol. Biol. 20*, 215–229.

Ramabhadran, T. V., & Jagger, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 59–63.

Scheffer, J. R., & Dzakpasu, A. A. (1978) *J. Am. Chem. Soc. 100*, 2163–2173.

Schofield, P., & Zamecnik, P. C. (1968) *Biochim. Biophys. Acta 155*, 410–416.

Schwartz, I., & Ofengand, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3951–3955.

Schwartz, I., & Ofengand, J. (1978) *Biochemistry 17*, 2524–2530.

Schwartz, I., Gordon, E., & Ofengand, J. (1975) *Biochemistry 14*, 2907–2914.

Setlow, R. B., & Carrier, W. L. (1966) *J. Mol. Biol. 17*, 237–254.

Siddiqui, M. A. Q., Ofengand, J., Garel, J.-P., & Drabkin, H. (1979) in *CRC Handbook of Microbiology* (Laskin, A. I., & Lechevalier, H. A., Eds.) (in press) CRC Press, West Palm Beach, FL.

Weissbach, H., & Pestka, S., Eds. (1977) *Molecular Mechanisms of Protein Biosynthesis*, Academic Press, New York.

Weissenbach, J., Dirheimer, G., Falcoff, R., Sanceau, J., & Falcoff, E. (1977) *FEBS Lett. 82*, 71–76.

Zimmermann, R. A., Gates, S. M., Schwartz, I., & Ofengand, J. (1979) *Biochemistry* (following paper in this issue).