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Use of Photochemically Induced Cross-Linking as a Conformational Probe of the Tertiary Structure of Certain Regions in Transfer Ribonucleic Acid†

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ABSTRACT: Photochemically induced cross-linking between $^4Srd_8$ and Cyd_{13} in unfractionated tRNA, $tRNA^{fMet}$, and $tRNA^{Val}$ of *Escherichia coli* was studied in terms of its use as a probe of the tertiary structure of tRNA. The rate of cross-linking, a measure of the proximity of the ^{4}Srd and Cyd residues to each other in a tRNA, was influenced by the nature of the tRNA and required Mg^{2+} for optimal cross-linking. The $T_{1/2}$ for cross-linking of $tRNA^{fMet}$ was 1.31 min, and for $tRNA^{Val}$ 2.35 min. Cross-link formation was measured by reduction of the binucleotide product with $NaBH_4$ to the dihydro derivative, Pdo(4–5)hCyd, which has an ultraviolet absorbance band at 377 to 385 nm, and is strongly fluorescent. Both spectral parameters were influenced by tRNA conformation. Fluorescence was markedly dependent on the surrounding tRNA structure. Removal of Mg^{2+} had a quenching effect, nuclease digestion to various limit products quenched the fluorescence to a degree defined by the nuclease–tRNA combination studied, and a transient increase in fluorescence was observed upon hydrolysis of $tRNA^{fMet}$ with pancreatic RNase. The absorption spectra, on the other hand, showed only small changes after complete hydrolysis with pancreatic, T_1, or T_2 RNase and Mg^{2+} depletion had almost no effect. To bypass the influence of structure on the measurement of cross-link formation, ultraviolet absorbance after complete nuclease digestion was adopted as the method of choice. Under appropriate conditions, ultraviolet absorbance without prior hydrolysis, and fluorescence measurements were also shown to be valid measures of cross-link formation.

Irradiation of *Escherichia coli* tRNA at 335 nm induces a specific and quantitative photochemical reaction which results in the covalent cross-linking of two arms of the tRNA cloverleaf *via* $^4Srd_8$ and Cyd_{13} (Favre *et al.*, 1969; Yaniv *et al.*, 1969) due to the formation of the binucleotide I (Figure 1) which can subsequently be converted into the strongly fluorescent compound II (Figure 1) by reduction with $NaBH_4$ under mild conditions while still part of the tRNA (Favre and Yaniv, 1971; Krauskopf *et al.*, 1972). These two nonadjacent positions are within bonding distance of each other in the X-ray structure for yeast $tRNA^{Phe}$ recently proposed by Kim *et al.* (1973).

Formation of the cross-link has little functional effect. The affinity for Val-, Arg-, and Phe-tRNA synthetases is not greatly reduced (Yaniv *et al.*, 1971; Chaffin *et al.*, 1971) although heterologous aminoacylation of cross-linked Val-tRNA by yeast Phe-tRNA synthetase is blocked (Kumar *et al.*, in preparation), ternary complex formation with EFTu-GTP is not detectably altered (Krauskopf *et al.*, 1972), and overall protein synthesis is not markedly inhibited (Yaniv *et al.*, 1971; Chaffin *et al.*, 1971). Because of these facts and additional evidence that a denatured tRNA structure cannot be cross-linked (Favre *et al.*, 1971) it is believed that cross-linking requires the existence of a structure similar to or identical with the biologically active one, at least in the ^{4}Srd-containing region of the molecule.

The usefulness of this reaction for probing the conformation of tRNA lies in the fact that (a) the ability to form the cross-link is dependent on the conformational state of the tRNA being irradiated due to the requirement for proper juxtaposition of the two residues in space before reaction can take place (Favre *et al.*, 1971; Krauskopf and Ofengand, 1971; Siddiqui and Ofengand, 1971; Bergstrom and Leonard, 1972a) and (b) the intensity of fluorescence of the cross-linked and reduced tRNA is highly dependent on its environment (Favre and Yaniv, 1971).

An additional feature of the reduction product II that permits direct measurement of the amount of reduced binucleotide in tRNA independent of its fluorescence properties is its ultraviolet absorption maximum at 380–385 nm (Favre and Yaniv, 1971; Krauskopf *et al.*, 1972) well removed from the main 260-nm absorption band of tRNA.

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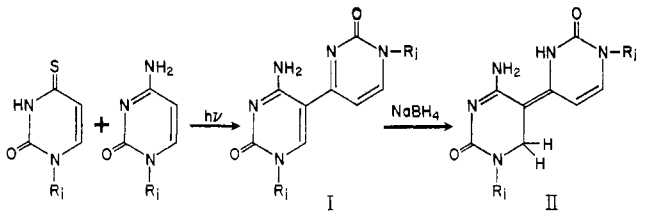

FIGURE 1: Reaction sequence for cross-linking and reduction. The scheme shows the structures of the ^{4}S-C dimer (Leonard *et al.*, 1971; Bergstrom and Leonard, 1972a; Favre *et al.*, 1972) and of its reduction product (Bergstrom and Leonard, 1972b; Favre *et al.*, 1972).

In this communication we present a detailed study of the parameters involved in cross-linking tRNA, in reducing I to II in tRNA, and in measuring the amount of cross-link by ultraviolet (uv) absorption or fluorescence. The influence of secondary structure on the quantitation of this reaction has been bypassed by nuclease digestion prior to analysis, and the methodology has been tested for generality by a comparative study of *E. coli* unfractionated tRNA, $tRNA^{fMet}$, and $tRNA^{Val}$.

Experimental Section

Irradiation of tRNA solutions was carried out by a modification of previously described procedures (Krauskopf and Ofengand, 1971) at an A_{260} up to 26.6 in 20 mM Bicine buffer (pH 7.5), 10 mM $Mg(OAc)_2$ at 1–4°. The Rayonet RPR-100 Photochemical Reactor was equipped with 350-nm lamps which emit less than 0.1% of the maximum light intensity below 305 nm. The solutions were stirred magnetically or by bubbling argon gas through the mixture to remove H_2S generated during the reaction (Bergstrom and Leonard, 1972a). Both procedures gave equivalent results. The cross-linked product was stable for at least several months at −30° and neutral pH.

Reduction of the cross-linked tRNA to a highly fluorescent derivative was carried out by a modification of a previously reported method (Krauskopf *et al.*, 1972). The reaction mixture consisted of 300 μl of sample, 100 μl of 0.5 M Na_3BO_3, 0.5 M KCl (pH 9.5 at 0.1 M), 50 μl of 0.18 M NaEDTA (pH 9.9 at 0.18 M), and 50 μl of $NaBH_4$ (20 mg/ml freshly dissolved in pH 11 ice water). After incubation in the dark for 60 min at room temperature, the excess $NaBH_4$ was destroyed by addition of 80 μl of 3.5 M HOAc and incubation for 10 min at room temperature. The pH was adjusted to neutrality by addition of 400 μl of Bicine–NaOH buffer and then 20 μl of either 2 M $Mg(OAc)_2$ or of H_2O was added. The final volume was 1.0 ml. Cross-linked and reduced tRNAs were completely stable in this solution for at least several weeks when kept frozen. Bicine–NaOH buffer was made from 145 ml of 0.5 M Bicine (pH 7.5) and 55 ml of 2.0 M standard NaOH. In the case of $tRNA^{fMet}$ (see Figure 4), stronger reducing conditions were needed. For this tRNA, 100 μl of $NaBH_4$ was used, and the sample size was proportionately reduced to 250 μl. For the unirradiated-unreduced tRNA controls, the procedure was modified as follows. The proper proportions of $NaBH_4$ and HOAc were mixed separately to decompose the $NaBH_4$. After a 5–10-min incubation, a suitable aliquot was added to the rest of the standard reducing mixture containing the tRNA.

Fluorescence measurements were made on dilutions of the above mixture to 1.5-ml final volume. Standard diluting buffer (FDB) was 0.15 M Bicine (pH 7.5)–0.04 M $Mg(OAc)_2$. The measured fluorescent intensity was directly proportional to the amount of cross-linked and reduced tRNA solution added up to 50% of the final volume and a final A_{260} of 2.2. Above these limits, there was a slight departure from linearity due either to the inner filter effect of added tRNA or to salt quenching. Fluorescent intensity and fluorescence spectra were measured at room temperature in an Aminco-Bowman spectrofluorometer equipped with ratio photometer and operated in the ratio mode. Excitation was at 395 nm and emission at 440 nm. Day-to-day variation in instrument readings were monitored with a solution of quinine sulfate in 0.1 N H_2SO_4.

Ultraviolet absorption spectra were measured at room temperature in a Cary Model 15 spectrophotometer using the 0–0.1 slide-wire and 0.1- to 1.0-ml microcells. The buffer blank did not change in the presence or absence of RNase, Mg^{2+}, irradiation, or real *vs.* sham reduction in the region 340–380 nm. All A_{260} measurements on tRNA were made at pH 7 in 0.01 M Mg^{2+}.

tRNA from *E. coli* was an unfractionated mixture obtained from Schwarz BioResearch. It was desalted by gel filtration on Sephadex G-25 in 20 mM EDTA (pH 8) followed by a second gel filtration over G-25 in water. $tRNA^{fMet}$ (1135 pmol/A_{260}) and $tRNA^{Val1}$ (1160 pmol/A_{260}) were purified from *E. coli* as previously described (Siddiqui and Ofengand, 1971; Krauskopf *et al.*, 1972). Pancreatic RNase was obtained from Worthington, and RNase T1 and T2 were purchased from Sankyo.

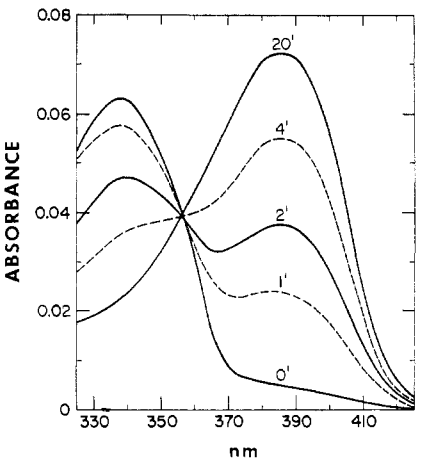

FIGURE 2: Rate of cross-linking as measured by the ultraviolet absorption spectrum of reduced tRNA. $tRNA^{mixed}$ was irradiated under standard conditions at an A_{260} of 26.6 for the times indicated on the figure. The samples were then reduced by the standard procedure and the uv spectra were recorded after addition of 40 mM Mg^{2+}. Final A_{260} was 7.3.

Results

Spectral Characteristics of Cross-Linked and Reduced tRNA. When ^{4}Srd containing tRNA was irradiated and then reduced with $NaBH_4$, the uv absorption spectrum in the ^{4}Srd region changed drastically (Figure 2). The peak at 337 nm corresponding to ^{4}Srd which was present at zero time disappeared with increasing time of irradiation and was replaced by a new band with an absorption maximum at 385 nm. This band was not found if the $NaBH_4$ reduction was omitted (Favre and Yaniv, 1971; Krauskopf *et al.*, 1972). Similar spectra were reported for $tRNA^{Val}$ by Favre and Yaniv (1971) who used

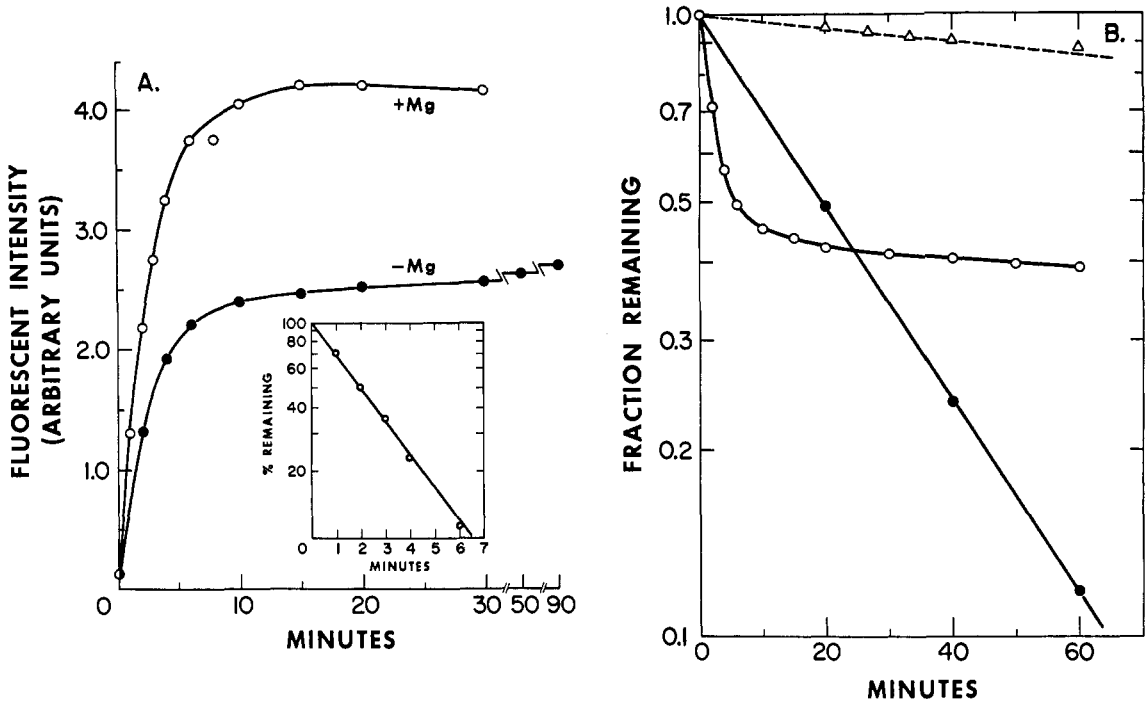

FIGURE 3: Effect of Mg^{2+} on the rate of cross-linking of tRNA. *E. coli* mixed tRNA desalted as described in the Experimental Section was irradiated at an A_{260} of 14.8 containing 20 mM Bicine (pH 7.5) in the presence and absence of 10 mM $Mg(OAc)_2$. The uv lights were turned on for the appropriate times, and 150-μl samples were taken and processed by the standard procedure. (A) Rate of cross-linking as a function of time of irradiation in the presence (○) and absence (●) of 10 mM Mg^{2+}. The inset shows the rate of loss of uncross-linked tRNA in the presence of Mg^{2+}. The fraction remaining was calculated as $F_\infty - F_t/F_\infty - F_0$. The $T_{1/2}$ was 2.0 min. (B) (○) Semi-log plot of the minus Mg^{2+} rate data from part A. Resolution of the curve into a fast and slow component was accomplished by standard methods. (●) Fast part of the curve. Time axis is 0.1 of that shown; $T_{1/2}$ is 2.0 min; (△) slow part of the resolved curve (43% of the total by extrapolation to zero time). Time axis is 1.5 × that shown; $T_{1/2}$ is 417 min.

these spectral changes to follow the kinetics of reduction of I to II. They can also be used to monitor the rate of cross-linking as this figure shows.

The other characteristic of cross-linked and reduced tRNA is its fluorescence. The excitation and emission spectra for this fluorescence were previously reported by Favre and Yaniv (1971) for $tRNA^{Val}$ and are the same for *E. coli* mixed tRNA, for $tRNA^{fMet}$, and for samples whose fluorescence had decreased because of limiting [Mg^{2+}] concentration (data not shown). There was only a slight shift in λ_{max} consistent with the absorption λ_{max} shift after treatment with nuclease such that the fluorescent intensity had decreased to 5%.

Rate of the Cross-Linking Reaction. Previous work (Siddiqui and Ofengand, 1971; Krauskopf *et al.*, 1972) had shown that under our conditions, cross-linking of $tRNA^{Val}$ and $tRNA^{fMet}$ proceeded very rapidly with a $T_{1/2}$ of approximately 2 min. These kinetics were verified in the present experiments using the modified conditions described in this paper. Figure 3 shows, using the fluorescence of the cross-linked and reduced tRNA as an assay, that the rate of reaction was indeed rapid. When Mg^{2+} was present (Figure 3A and inset), a first-order reaction was observed with a $T_{1/2}$ of 2.0 min which was very similar to the previously reported value for $tRNA^{Val}$ of 2.2 min.

Since it had been reported by Favre *et al.* (1971) that the concentration of mono- and divalent cations affected the degree of cross-linking, the rate of cross-linking was also examined in the absence of added Mg^{2+}. Assuming that the absence of Mg^{2+} decreased the probability of proper orientation in space of the ^{4}Srd and Cyd residues, the *rate* of cross-linking should have been decreased but the eventual *yield* of cross-linked product should not have been affected. Surprisingly this was not the result obtained. Figure 3 shows that there was an initial rapid increase in cross-linked product even when Mg^{2+} was omitted, but that the extent never approached that achieved in the presence of Mg^{2+}. Resolution of the minus Mg^{2+} rate curve into its two apparent components (Figure 3B) showed that the fast component reacted as rapidly as the Mg^{2+}-containing solution ($T_{1/2}$ of 2.0 min) but that the slow component (43% of the total) was less than 0.5% as reactive. In the event that the slow component represented a "denatured fraction" trapped in the "denatured" state because of the low temperature used for irradiation, the tRNA sample was heated to 85° for 3 min in 20 mM Bicine (pH 7.5) at an A_{260} of 14.8, quickly chilled in ice, and then irradiated. The same curve was obtained with a fast $T_{1/2}$ of 2.1 min, and almost no slow reaction with an increase in the inactive fraction from 43 to 55%. While the explanation for this effect is not clear, the results do show that Mg^{2+} or other stabilizing cations (Favre *et al.*, 1971) are important in the irradiation step.

The rate of cross-linking in the presence of excess Mg^{2+} for the three tRNAs studied in this work is shown in Table I. In each case, the amount of cross-linked product reached a stable plateau value with increase in time and greater than 90% reacted as a single kinetic species. $tRNA^{fMet}$ reacted 1.8-fold more rapidly than $tRNA^{Val}$, apparently due to a more favorable spatial orientation of the $^4Srd_8$ and Cyd_{13} residues in this tRNA. The value for $tRNA^{Val}$ was very similar to that previously determined for this tRNA (Krauskopf *et al.*, 1972) and is slightly slower than the rate for mixed tRNA. Mixed tRNA behaved as a single kinetic species down

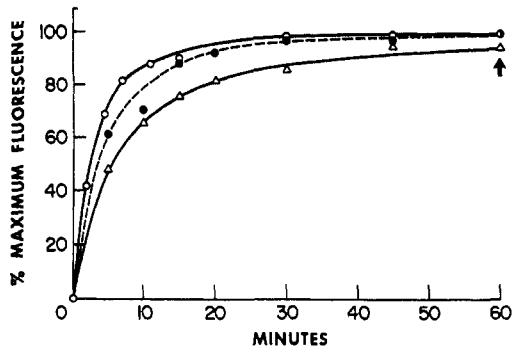

FIGURE 4: Time course of reduction of cross-linked tRNA by $NaBH_4$. tRNA samples irradiated for 20 min were incubated in the standard reduction assay for the times indicated instead of for 60 min, and the amount of reduction was measured by fluorescence. The arrow shows the standard time of reduction. (○) Mixed tRNA (8.85 A_{260} unit/ml of reduction solution). 100% fluorescence was 3.25 arbitrary units; (△) $tRNA^{fMet}$ (6.53 A_{260} unit/ml of reduction solution). 100% fluorescence was 1.88 units. (●) $tRNA^{fMet}$ as above but with twice the concentration of $NaBH_4$, as described in the Experimental Section.

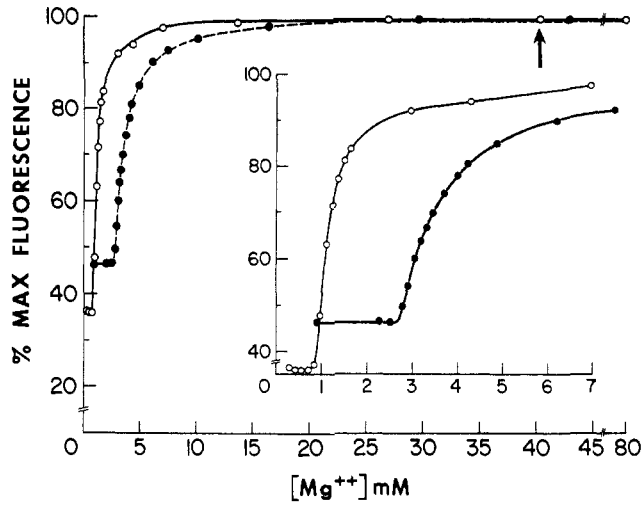

FIGURE 5: Effect of Mg^{2+} concentration on fluorescence of cross-linked and reduced tRNA. *E. coli* mixed tRNA was fully cross-linked by irradiation for 20 min and then completely reduced by the standard procedure described in the Experimental Section. The final solution was diluted with 0.15 M Bicine (pH 7.5) to be either 0.3 mM in Mg^{2+}, 0.9 mM in EDTA, A_{260} of 0.44 (○) or 0.9 mM in Mg^{2+}, 2.7 mM in EDTA, A_{260} of 1.33 (●). Small aliquots of Mg^{2+} were then added to the solution and fluorescence recorded at each $[Mg^{2+}]$ concentration. Corrections for dilution (maximally 5%) were applied to the fluorescence readings and the final concentration of *total* Mg^{2+} is shown on the abscissa. Note that 0.9 mM (○) and 2.7 mM (●) EDTA were also present. The arrow shows the final concentration of $[Mg^{2+}]$ employed in the standard assay, and the inset is an expansion of the early part of the curve.

to the 3% level even though it was a mixture of all *E. coli* tRNAs including $tRNA^{Val}$ and $tRNA^{fMet}$. Apparently, the small (3–5) per cent of these individual tRNAs did not noticeably affect the overall rate.

Rate of Reduction. The time course of reduction of $tRNA^{mixed}$ and $tRNA^{fMet}$ with $NaBH_4$ is shown in Figure 4. Reduction was carried out in the presence of EDTA since preliminary experiments showed that the rate of reduction was decreased when Mg^{2+} was present. Assays were carried out by the fluorescence method. Complete reduction of $tRNA^{fMet}$ in 60 min required a higher concentration of $NaBH_4$. The plateau did not represent incomplete reduction due to decomposition of $NaBH_4$ since when 50% more $NaBH_4$ was added at 15, 30, and 45 min to each of the reduction mixtures, no increase in fluorescence was obtained.

Effect of Mg^{2+} and pH on the Fluorescence and Absorption Properties of Cross-Linked and Reduced tRNA. Because of a statement that divalent cations influenced the fluorescence of II when in tRNA (Favre and Yaniv, 1971) a detailed investigation of the need for Mg^{2+} was made (Figure 5). In 0.3 mM Mg^{2+}–0.9 mM EDTA, only about 37% of the maximum fluorescence was obtained. When Mg^{2+} was added, the fluorescence rapidly increased as soon as the EDTA present was titrated and reached its maximum value well before the concentration of Mg^{2+} used in the standard assay was reached (see arrow). A threefold higher concentration of reduced

TABLE I: Rate of Cross-Linking Different tRNAs.[a]

tRNA	Concn A_{260}	$T_{1/2}$ (min)
$tRNA^{mixed}$	14.8	1.85
$tRNA^{Val\ 1}$	3.7	2.35
$tRNA^{fMet}$	6.8	1.31

[a] The indicated tRNAs were irradiated by the general method described in the Experimental Section at the concentrations indicated in the table. All three samples were irradiated simultaneously and aliquots were taken at 0, 1, 2, 3, 4, 6, 10, 15, 20, 30, and 40 min and processed for fluorescence measurements by the standard procedure described in Experimental. The first-order decay curve was calculated as described in the legend to Figure 3A and the $T_{1/2}$ values were obtained from the slope of these lines by standard methods.

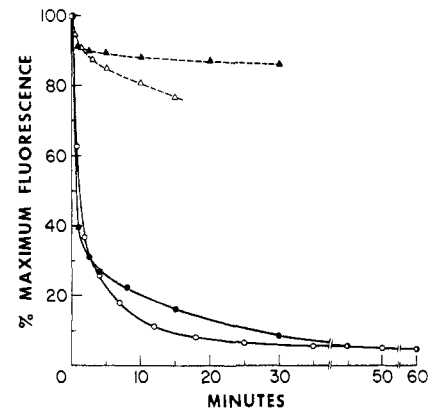

FIGURE 6: Effect of pancreatic RNase on the fluorescence of cross-linked and reduced $tRNA^{mixed}$ and $tRNA^{Val}$. tRNA samples irradiated for 20 min were completely reduced by the standard procedure except that the final addition after the Bicine–NaOH was water instead of $Mg(OAc)_2$. (A) Solid curves: $tRNA^{mixed}$ (○) at an A_{260} of 4.46 was incubated with 27 μg/ml of pancreatic RNase at 37°, and $tRNA^{Val}$ (●) at an A_{260} of 2.52 was incubated with 16 μg/ml of RNase. Both solutions were 9 mM in EDTA and 3 ($tRNA^{mixed}$) or 2 ($tRNA^{Val}$) mM in Mg^{2+}. At the indicated times, aliquots were removed, diluted to 1.5 ml with FDB and the fluorescence was measured immediately. (B) Dashed curves: $tRNA^{mixed}$ (△) at an A_{260} of 4.53, 5 mM EDTA, and 1.7 mM Mg^{2+} was incubated with 0.026 μg/ml of pancreatic RNase at 37°; $tRNA^{Val}$ (▲) at an A_{260} of 0.30, 9 mM EDTA, and 3 mM Mg^{2+} was incubated with 0.017 μg/ml of RNase. At the times indicated aliquots were diluted to 1.5 ml with FDB and the fluorescence was measured immediately.

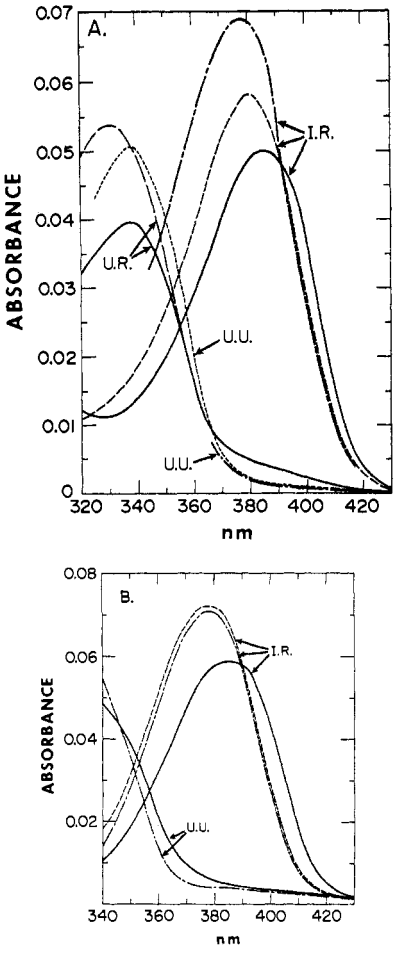

FIGURE 7: Effect of RNase digestion on the ultraviolet absorption spectra of $tRNA^{mixed}$ and $tRNA^{Val}$. Ultraviolet absorption spectra were recorded as described in the Experimental Section. (—) Before RNase; (- - - -) after pancreatic RNase digestion; (— — — —) after pancreatic plus T1 plus T2 RNase digestion; IR, irradiated and reduced tRNA; UR, unirradiated but reduced tRNA; UU, unirradiated and unreduced tRNA. (A) $tRNA^{mixed}$: samples were prepared and digested with nucleases as described in Table II. Before spectral measurements, the $[Mg^{2+}]$ concentration was adjusted to 40 mM. A_{260} was 4.52; (- - - -) UU without RNase treatment; (— — — —) UU after combined RNase treatment. (B) $tRNA^{Val}$; samples were prepared and digested with nucleases as described in Table II. Before spectral measurements, the $[Mg^{2+}]$ concentration was adjusted to 40 mM. A_{260} was 2.19.

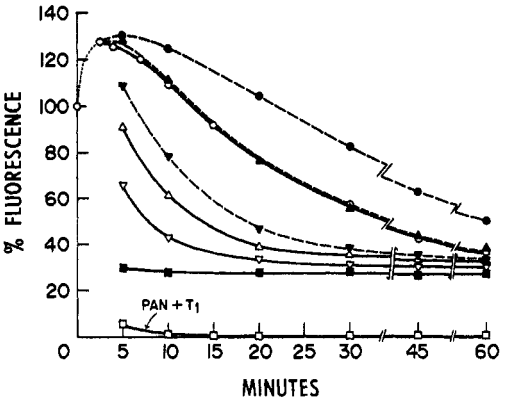

FIGURE 8: Effect of pancreatic and T1 RNase on the fluorescence of cross-linked and reduced $tRNA^{fMet}$. $tRNA^{fMet}$ was irradiated for 20 min and then completely reduced by the standard procedure for $tRNA^{fMet}$ as described in the Experimental Section except that the final addition was H_2O instead of $Mg(OAc)_2$. Digestions were performed at a tRNA concentration of 2.7 A_{260} units/ml at the temperature and nuclease concentration indicated below. The mixtures also contained 8 mM EDTA and 2 mM Mg^{2+}, except ●, ▲, ▼ (dashed curves) which were 40 mM in Mg^{2+}. At the indicated times, 50-μl aliquots were removed, diluted with 1.5 ml of FDB, and the fluorescence was measured immediately. (●) 19 μg/ml of pancreatic RNase at 37° plus 40 mM Mg^{2+}; (▲) 38 μg/ml of pancreatic RNase at 37° plus 40 mM Mg^{2+}; (▼) 95 μg/ml of pancreatic RNase at 37° plus 40 mM Mg^{2+}; (○) 17 μg/ml of pancreatic RNase at 37°; (△) 61 μg/ml of pancreatic RNase at 37°; (▽) 182 μg/ml of pancreatic RNase at 37°; (■) 182 μg/ml of pancreatic RNase at 45°; (□) 62 μg/ml of pancreatic RNase plus 460 units/ml of T1 RNase at 37°.

sample only shifted the curve to correspond to the additional amount of EDTA being titrated.

Similar changes in Mg^{2+} did *not* appreciably affect the intensity of the 385-nm absorption band of cross-linked and reduced tRNA. When Mg^{2+} was added in small aliquots to a solution of cross-linked and reduced mixed tRNA at 3 mM Mg^{2+}–9 mM EDTA in a spectrophotometer cell, the absorption at 385 nm did not change up to 10 mM $[Mg^{2+}]$, decreased by 3% at 14 mM $[Mg^{2+}]$, and remained constant to 74 mM $[Mg^{2}]$. In other experiments, comparing 3 to 43 mM $[Mg^{2+}]$, the decrease in absorption was not more than 5%.

The effect of pH on the absorption spectrum of cross-linked and reduced tRNA was also investigated. Over the range of pH 5–9 no change occurred in either the λ_{max} or intensity of the 385 nm band. Changes in the absorption spectrum of synthetic II have been reported at the more extreme pH values of 2 and 12 (Favre *et al.*, 1972).

Effect of Nucleases on the Fluorescence and Absorption Spectra. The effect of nucleolytic digestion on the ultraviolet absorption as well as on the fluorescence properties of irradiated-reduced tRNA was investigated because of an earlier report (Favre and Yaniv, 1971) which stated that T1 or pancreatic RNase digestion in the presence of Mg^{2+} subsequent to irradiation and reduction reduced the quantum yield of fluorescence of $tRNA^{Val}$ by a factor of 3 and 15, respectively, while isolated compound II had only 0.25% of its fluorescence when in $tRNA^{Val}$. Cross-linked and reduced tRNA was prepared in the absence of Mg^{2+} and then digested because preliminary experiments (see, for example, Figure 8) showed that the rate of digestion was markedly affected by the presence of this cation. Mg^{2+} was added at the end of the digestion because of its requirement for maximal fluorescence. Figure 6 shows the effect of pancreatic RNase on the fluorescence of both $tRNA^{mixed}$ and $tRNA^{Val}$. In both cases a stable plateau value of fluorescence was reached at about 5% of the initial value. A relatively impure preparation of $tRNA^{Val}$ was used in this particular experiment which may account for the higher plateau value of fluorescence observed here compared to that in Table II.

The corresponding absorption spectra are illustrated in Figure 7 which also shows the effect of pancreatic plus T1 plus T2 RNase. There was only a small increase in the 385-nm absorption band after digestion compared to the large decrease in fluorescence, and the extent of digestion varied depending on the nuclease used and the tRNA species involved. Some cross-linked product was evident even in unirradiated samples (compare UU to UR) which probably arose during isolation of the tRNA, and the λ_{max} shift from 385 to 377 nm is in keeping with the absorption maximum for pure compound II (Leonard *et al.*, 1971; Favre *et al.*, 1972).

A summary of the effects of digestion with various nucleases on the fluorescence and absorption characteristics of $tRNA^{mixed}$ and $tRNA^{Val}$ is given in Table II, which includes

TABLE II: Effect of Nuclease Treatment on the Fluorescence and Ultraviolet Absorption of Cross-Linked and Reduced $tRNA^{mixed}$ and $tRNA^{Val}$.

Treatment[a]	$tRNA^{mixed}$		$tRNA^{Val}$	
	Rel Fluorescence (%)	Rel Absorption	Rel Fluorescence (%)	Rel Absorbance
(1) No nuclease	100	1.00 (385)[b]	100	1.00 (385)
(2) Pancreatic RNase (90 μg/ml)	5.1	1.22 (380)	0.6	1.22 (377)
(3) T1 RNase (435 units/ml)	9.4	1.04 (383)	20	0.94 (383)
(4) T2 RNase (87 units/ml)	0.9	1.29 (380)	<0.5	1.15 (379)
(5) Steps 2 + 3 + 4	0.2	1.39 (377)	<0.5	1.23 (377)

[a] tRNA samples were irradiated and reduced as described in the legend to Figure 6, and then digested at an A_{260} of 6.9 ($tRNA^{mixed}$) or 2.2 ($tRNA^{Val}$) in 7.8 mM EDTA, 2.6 mM Mg^{2+} for 60 min at 37° with the amount of enzyme specified. At the end of the reaction, the [Mg^{2+}] concentration was adjusted to 40 mM, and uv spectra were recorded. The absorbance values at the peak were corrected for the absorbance of an unirradiated-unreduced (UU) tRNA control prepared as described in the Experimental Section, and normalized to treatment 1 as 1.00. After the absorbance spectra were taken, 0.2-ml aliquots were diluted to 1.5 ml with FDB and the fluorescence was measured. [b] Wavelength at which the peak absorption occurred.

data not shown in Figure 7. It is clear from inspection of the table that the decrease in fluorescence is not correlated with the increase in absorption although both changes no doubt arise from changes in the molecular environment around the reduced ^{4}Srd-Cyd binucleotide.

Similar results were obtained with $tRNA^{fMet}$ (Figure 8). The presence of Mg^{2+} slowed the rate of loss of fluorescence although the same limit value was reached. A more complete decrease in fluorescence was achieved by the combined action of T1 and pancreatic RNase. The transient increase in fluorescence up to 130% of the original value upon enzymatic digestion which then disappeared as the polynucleotide was further hydrolyzed probably represents a reorientation of the structure following the first few nucleolytic cleavages so as to increase the hydrophobic nature of the environment. The hydrophobicity of the environment has been proposed as the feature determining the intensity of the fluorescence of compound II (Favre and Yaniv, 1971; Bergstrom and Leonard, 1972b). This transient increase was not observed in either $tRNA^{Val}$ or $tRNA^{mixed}$ although it would have readily been detected at the very slow rates of hydrolysis used in the experiment (Figure 6, dashed curves). Possibly this effect is related to the unique structure of the ^{4}Srd region in $tRNA^{fMet}$ previously noted by other workers (Scott and Schofield, 1969; Seno *et al.*, 1969).

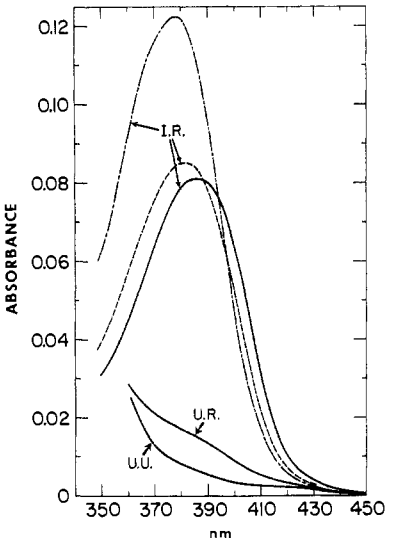

FIGURE 9: Effect of pancreatic and T1 RNase on the ultraviolet absorption spectrum of $tRNA^{fMet}$. Ultraviolet absorption spectra were recorded on 100-μl samples as described in the Experimental Section. The tRNA samples were irradiated, reduced, and digested as described in the legend to Figure 8 for 60 min. Before spectral measurements, the [Mg^{2+}] concentration was adjusted to 40 mM. (——) Undigested tRNA; (- - - -) pancreatic RNase (182 μg/ml) at 45°; (— — —) pancreatic RNase (62 μg/ml) plus T1 RNase (460 units/ml) at 37°. IR, irradiated and reduced tRNA; UR, unirradiated but reduced tRNA; UU, unirradiated and unreduced tRNA. Neither the UR nor the UU spectra were altered over the range of interest after any of the nuclease treatments. All curves were normalized to an A_{260} of 2.66.

The effect of these nucleases on the absorption spectrum of $tRNA^{fMet}$ is illustrated in Figure 9. The same points regarding Figure 7 can be made about this figure. In particular, the hyperchromicity of the 385-nm band after combined enzyme digestion is considerably greater than in the other tRNAs examined. A summary of the effect of nucleases on the fluorescence and absorption properties of $tRNA^{fMet}$ is given in Table III. In general, the effects are similar to those observed for $tRNA^{mixed}$ and $tRNA^{Val}$ in Table II. Each nuclease digest resulted in a final digestion product which possessed a characteristic degree of fluorescence and hyperchromicity but the decrease in fluorescence was not correlated with the increase in absorption.

The effects observed here and in Table II can be broadly understood in terms of the digestion products expected from the sequences of $tRNA^{Val}$ and $tRNA^{fMet}$. Thus, the pancreatic RNase limit product of $tRNA^{fMet}$ should be

$$GGGG^4Sp\quad GGAGCp$$

(26% fluorescence), the T1 RNase limit product should be

$$^4SGp\quad CAGp$$

(16% fluorescence) and after both enzymes only

TABLE III: Effect of Nuclease Treatment on the Fluorescence and Ultraviolet Absorbance of Cross-Linked and Reduced $tRNA^{fMet}$.

Treatment[a]	Rel Fluorescence (%)	Rel Absorbance
(1) No nuclease	100	1.00 (385)
(2) Pancreatic RNase (130 μg/ml)	25.6	1.05 (382)
(3) T1 RNase (267–1330 units/ml)	16.2[b]	
(4) Steps 2 + 3	0.2[c]	1.46 (377)

[a] $tRNA^{fMet}$ was irradiated and reduced as described in the legend to Figure 8, and then digested at an A_{260} of 2.7, 8 mM EDTA–2 mM Mg^{2+}, for 60 min at 37° with the amount of enzyme specified. At the end of the reaction, aliquots were appropriately diluted in FDB and the fluorescent intensity was measured immediately. [b] The actual values were for 267, 667, and 1330 units per ml; 15.1, 17.3, and 16.5%, respectively. [c] The actual values were for 267, 667, and 1330 units of T1 RNase per ml; 0.2, 0.1, and 0.2%, respectively.

$$^4\overbrace{Sp\quad Cp}$$

(0.2% fluorescence) should be obtained. For $tRNA^{Val}$, the T1 RNase limit product should be

$$AU^4\overbrace{SAGp\quad CUCAGp}$$

(Yaniv *et al.*, 1969) (20% fluorescence) while pancreatic or T2 RNase should alone be able to digest all the way to the binucleotide

$$^4\overbrace{Sp\quad Cp}$$

($\leqslant$ 0.5% fluorescence with either enzyme).

Suitability as an Analytical Tool. As shown above, fluorescence can only be used as a quantitative measure of the formation of I when structural alterations of the tRNA are absent. In order to verify that measurement of the uv absorption band at 377 or 385 nm is a valid alternative, we examined tRNA preparations with different degrees of cross-linking induced by irradiation for different lengths of time. The fluorescence, the uv absorption at 385 nm before nuclease digestion, and the uv absorption after nuclease digestion were measured, and the results plotted in Figure 10. All three methods gave the same rate of cross-linking.

A summary of the spectral parameters for the tRNAs used in this study is given in Table IV. The values for $tRNA^{mixed}$ are expected to be lower since all tRNAs do not have ^{4}Srd (the A_{337}:A_{260} ratio, a measure of ^{4}Srd in tRNA, was about half that of $tRNA^{Val}$ or $tRNA^{fMet}$). The A_{377}:A_{260} and A_{385}:A_{260} ratios were expected to be the same for $tRNA^{Val}$ and $tRNA^{fMet}$ since the A_{337}:A_{260} ratios were about equal, but the $tRNA^{Val}$ values were 20 and 10% lower, respectively. The reason for this is not understood. However, the ratio of fluorescence to absorption at 385 or 377 nm was relatively constant for all three tRNA preparations. It appears that under our standard conditions for measuring fluorescence, the maximum intensity

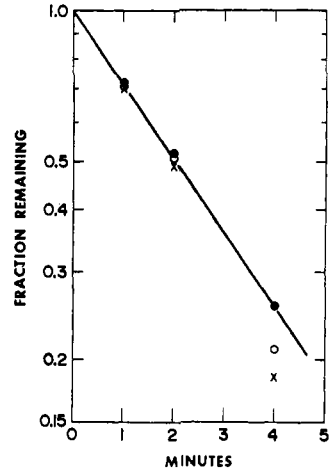

FIGURE 10: Comparison of fluorescence, uv absorption, and uv absorption after RNase as assays for cross-linking in tRNA. The tRNA samples were those of Figure 2. Ultraviolet absorption before RNase was taken from the spectra of Figure 2 at the peak wavelength of 385 nm. Ultraviolet absorption after pancreatic RNase digestion was taken from analogous spectra (data not shown) at the peak wavelength of 380 nm. Aliquots (A_{260} of 7.3) were digested with 150 μg/ml of pancreatic RNase in a solution 9 mM in EDTA and 2 mM in Mg^{2+}. After 60 min at 37° when the fluorescence had decreased to 5% of the original value, the solution was made 40 mM in Mg^{2+}, and the spectra were recorded. Fluorescence was measured on separate aliquots of the same samples before RNase treatment. 100-μl aliquots were diluted to 1.5 ml with FDB and the fluorescence measured in the standard manner. Fraction remaining was in all cases calculated as the absorption or fluorescence at 20 min minus that at time t divided by the quantity at 20 min minus that at 0 time. (●) Uv absorption at 385 nm before RNase; (○) uv absorption at 380 nm after RNase; (×) fluorescence.

is produced even in widely different tRNAs. However, this ratio can be readily changed, for example, by nucleolytic cleavage or by Mg^{2+} removal, or presumably by other denaturing conditions as well.

Discussion

Photochemically induced cross-linking can be used as a monitor of conformational changes in the ^{4}Srd region of the tRNA molecule in two ways. In the first place, since the rate of cross-linking is a measure of the probability that the two residues in question will be within covalent bonding distance

TABLE IV: Summary of Spectral Parameters of Different Cross-Linked tRNAs.[a]

	$tRNA^{mixed}$	$tRNA^{Val}$	$tRNA^{fMet}$
Fluorescence:A_{260}	6.55	14.5	18.2
A_{385}:A_{260} × 10^2	1.07	2.59	2.86
A_{377}:A_{260} × 10^2	1.50	3.33	4.17
Fluorescence:A_{385}	612	560	636
Fluorescence:A_{377}	436	435	436

[a] Data are taken from the previous experiments. The A_{377} values were the absorbance peak values after combined nuclease digestion as described in Table II for $tRNA^{mixed}$ and $tRNA^{Val}$ and in Table III for $tRNA^{fMet}$.

of each other, this rate becomes a means of probing which tRNA configurations cause these two nucleotides to move relative to each other. The effect is illustrated in Table I which shows the rate of cross-linking in $tRNA^{fMet}$ to be about two times that of $tRNA^{Val}$. A more extreme example of this effect was our previous demonstration that 5′–1/4 and 5′–1/2 molecules of tRNA which contain both reactive residues had a zero rate of cross-linking unless supplied with their complementary partners under conditions which allowed reannealing of the fragments to take place (Siddiqui and Ofengand, 1971; Krauskopf and Ofengand, 1971). Moreover, the effect can be used to determine how much of the complementary 3′-part molecule is actually needed for induction of the proper orientation of this part of a tRNA (Siddiqui and Ofengand, 1972). The technique has also been used to test for any changes in structure of $tRNA^{Val}$ upon aminoacylation (Krauskopf *et al.*, 1972) and of $tRNA^{fMet}$ upon acylation with formylmethionine (Berthelot *et al.*, 1972).

In a number of experimental situations, it is necessary to know not only the rate of cross-linking but also the amount of cross-link which was formed. This situation may arise when there is more than one conformational state as, for example, when modification reactions are incomplete. It may not be possible to assess the degree of cross-linking if the chemically modified tRNA shows a decreased fluorescence yield due to a conformational alteration induced by the chemical modification itself. In the examples described in the body of this paper the Mg^{2+} concentration, the extent of primary structure around the fluorescent binucleotide, and the type of tertiary structure (typified by the transient increase in fluorescence upon digestion of $tRNA^{fMet}$ as illustrated in Figure 8) all strongly affected the fluorescence but had little or no effect on the uv absorbance. For this reason, the uv absorbance method described in this paper was developed in order to supplement fluorescence measurements by a technique which is relatively insensitive to conformational changes.

As an illustration of its utility, we have confirmed our previous report (Siddiqui and Ofengand, 1971) that the 5′–1/4 molecule of $tRNA^{fMet}$ is unable to cross-link unless supplemented with its complementary fragment. The earlier measurements were made by the fluorescence method, but did not take into account the possibility that phosphodiester-bond cleavage in the dihydrouridine loop might have had a severe quenching effect on the fluorescence of II. By using the uv absorbance method, we were able to confirm our earlier conclusions. The method is also being used as a means of probing the conformation in the region of the cross-link of variously modified tRNAs. Since these studies involve conformationally altered tRNAs, failure to detect II by fluorescence must be verified by the uv absorbance method.

Changes in fluorescence of a preformed cross-link due to changes in the conformation of tRNA molecules can also be used as a probe. This approach is crudely illustrated by the action of nucleases as described in this paper. Another approach would be to ask whether known conformational changes such as are involved in generating the stable denatured form of some tRNAs affect the fluorescence of a preformed cross-link. Such studies, which would give information on the conformation of the tRNA in the ^{4}Srd region in these denatured forms, are now in progress. Quenching of this fluorescence could in fact be used to compare changes in this region for a number of tRNAs under a variety of denaturing conditions as well as to study complex formation between cross-linked tRNA and specific proteins such as AA-tRNA synthetases, EFTu factors, ribosomes, and the like. These latter studies are also in progess.

Since the degree of fluorescence of the binucleotide II depends upon the primary structure of the nucleotides surrounding it (see Tables II and III), a study of this effect should be rewarding from the standpoint of understanding the molecular nature of the fluorescence enhancement. The fluorescence changes resulting from various treatments of intact tRNAs would then become more explicable in terms of specific conformational changes.

The structure-dependent uv absorption procedure for measuring cross-linking that is described in this paper does not require large amounts of tRNA. A fluorescence measurement >20 times the blank uncertainty requires 0.02 A_{260} unit of tRNA in 1.5 ml while an A_{277} of 0.02 requires 0.05 A_{260} unit in 100 μl. The 3-fold greater amount of tRNA required by the uv absorption procedure is compensated for by the elimination of secondary conformational effects.

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