Cleaver, J. E., and Boyer, H. W. (1972), *Biochim. Biophys. Acta 262,* 116.
Dean, W. W., Dancis, B. M., and Thomas, C. A., Jr. (1973), *Anal. Biochem. 56,* 417.
Echalier, G., and Ohanessian, A. (1970), *In Vitro 6,* 162.
Freifelder, D. (1971), *Methods Enzymol. 21,* 153.
Friedman, E. A., and Smith, H. O. (1972a), *J. Biol. Chem. 247,* 2846.
Friedman, E. A., and Smith, H. O. (1972b), *J. Biol. Chem. 247,* 2854.
Friedman, E. A., and Smith, H. O. (1972c), *J. Biol. Chem. 247,* 2859.
Gelb, L. D., Kohne, D. E., and Martin, M. A. (1971), *J. Mol. Biol. 57,* 129.
Lee, C. S., Davis, R. W., and Davidson, N. (1970), *J. Mol. Biol. 48,* 1.
Malamy, M. H., Fiandt, M., and Szybalski, W. (1972), *Mol. Gen. Genet. 119,* 207.
Mukai, T., Matsubara, K., and Takagi, Y. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2884.
Richardson, C. C., Lehman, I. R., and Kornberg, A. (1964), *J. Biol. Chem. 239,* 251.
Ritchie, D. A., Thomas, C. A., Jr., MacHattie, L. A., and Wensink, P. C. (1967), *J. Mol. Biol. 23,* 365.
Thomas, C. A., Jr., and Abelson, J. A. (1966), *Proced. Nucleic Acid Res. 1,* 553.
Thomas, C. A., Jr., and Dancis, B. M. (1973), *J. Mol. Biol. 77,* 25.

Europium as a Fluorescent Probe of Transfer RNA Structure†

J. M. Wolfson‡ and D. R. Kearns*

ABSTRACT: The binding of europium(III) to *Escherichia coli* $tRNA^{fMet,Glu}$ and to unfractionated *E. coli* tRNA has been investigated by using the 4-thiouridine sensitization of europium $^5D_0 \rightarrow {}^7F_1$ emission and changes in the lifetime of the $^5D_0$ state of europium reported earlier (J. M. Wolfson and D. R. Kearns (1974), *J. Am. Chem. Soc. 96,* 3653). Binding of the first 3–4 europium ions is independent and sequential, approximately 600 times stronger than the magnesium binding, and the binding sites are located near the 4-thiouridine residue found at position 8 in a number of *E. coli* tRNA. Competition experiments suggest the strong binding sites are the same for magnesium and europium. The europium binding properties of both unfractionated *E. coli* tRNA and purified $tRNA^{fMet}$ are quite similar, indicating that the location of the strong binding sites and their binding constants are nearly the same for a large group of tRNA. The europium binding properties of native and denatured tRNA are quite different, however.

There are a number of experiments which demonstrate that divalent metal ions stabilize tRNA in biologically active conformations (Fresco et al., 1966).

Magnesium fulfills the divalent cation requirement, but other divalent ions (Mn^{2+}, Zn^{2+}) and even trivalent rare earth ions can substitute for Mg^{2+} in the amino acylation of tRNA molecules (Igarashi et al., 1971; Kayne and Cohn, 1972). The rare earth ions are especially well suited for use in studying the metal binding properties of polynucleotides in solution since their optical properties (relative intensity of bands in the absorption and emission spectra, band positions, polarization, lifetime, radiative and nonradiative quantum yields) are sensitive to the nature of attached ligand groups (Gallagher, 1964, 1965). There is additional interest in the rare earth ions since they are currently being used in X-ray diffraction studies of tRNA crystals (Kim et al., 1974; Suddath et al., 1974; Ladner et al., 1972; Pasek et al., 1973; Robertus et al., 1974).

We have already published a preliminary account of optical and nuclear magnetic resonance (NMR) studies of the binding of Eu^{3+} to tRNA molecules in solution (Wolfson and Kearns, 1974; Jones and Kearns, 1974), and Formoso (1973) has reported preliminary studies of the binding of Tb^{3+} to RNA. We now present the results of a comprehensive study of the binding of Eu^{3+} to a number of different tRNA. As we shall show, the binding of Eu^{3+} occurs independently and sequentially under our conditions (0.1 *M* NaCl, pH 7, no Mg^{2+}) rather than cooperatively, as has been reported for Mn^{2+} ions under different experimental conditions (Danchin, 1972; Danchin and Gueron, 1970), and that most *E. coli* tRNA exhibit a similar pattern of metal binding.

The general picture which emerges from these studies is that most tRNA exhibit nearly identical metal binding properties insofar as the strong metal binding sites are concerned, and this result supports the notion that the majority of the tRNA have similar conformations.

Materials and Methods

Biochemicals. Three samples of yeast $tRNA^{Phe}$ (purified by Robert Wang, K. Lim Wong, Donald Lightfoot, and Simon Chang) were used, all having 95% amino acid accep-

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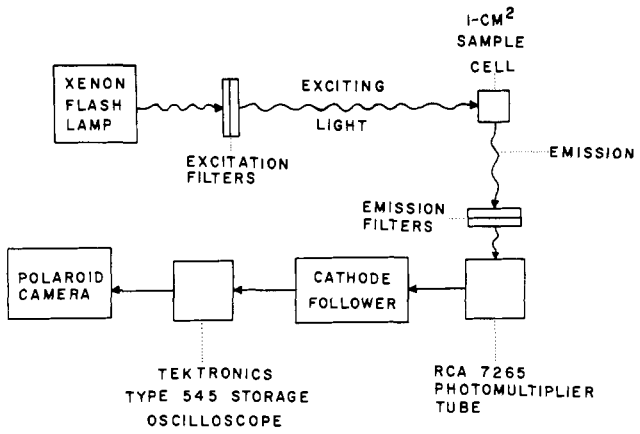

FIGURE 1: Schematic diagram of the apparatus used to measure the europium decay.

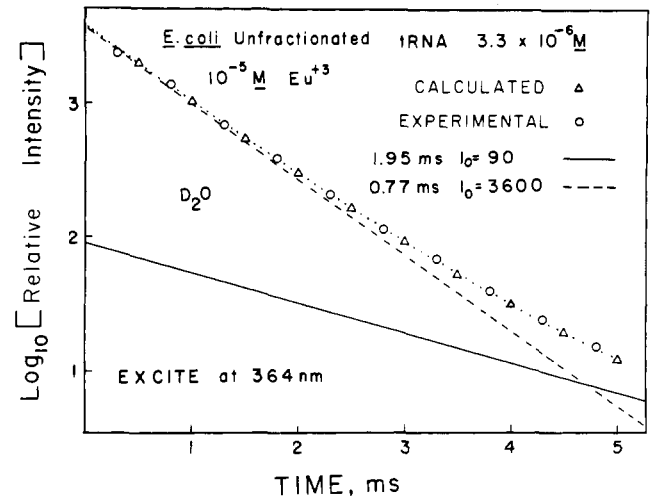

FIGURE 2: An example of nonexponential decay of the europium emission illustrating the way in which it can be resolved into a sum of two exponential decays.

tor activity. Mixed yeast tRNA which had fractions containing $tRNA^{Phe,Lys,Cys}$ removed was a gift from Brian Reid. Unfractionated *E. coli* tRNA was purchased from Plenum (lot 73042). Pure species of *E. coli* $tRNA^{fMet}$ (lot 15290) and for $tRNA_{II}^{Glu}$ (lot 15291) with 95 and 80% amino acid acceptance activities, respectively, were a gift of Oak Ridge National Laboratories. Poly(uridylic acid) (potassium salt, lot VH 617) was purchased from Mann Research Labs.

Chemicals and Solutions. These were purchased from the following sources: europium chloride hexahydrate, Alfa Inorganics; sodium cacodylate, Na_4EDTA, and ethidium bromide, Calbiochem; deuterium oxide, 99%, Aldrich Diaprep. The commonly used buffer (N-buffer) contains 0.1 *M* NaCl–0.05 *M* (sodium) cacodylate (pH 7.0).

tRNA Preparation. Excess salt was removed from the tRNA by dialysis, Sephadex G-25 chromatography, or ethanol precipitation. The tRNA then was put in 0.05 *M* EDTA, 0.1 *M* NaCl, and 0.01 *M* cacodylate (pH 7.0), and the miture was chilled in ice–water, dipped into a 70–75° water bath for 20 sec, and quickly returned to the ice–water (standard heat treatment). EDTA, salt, and buffer were removed completely by repeated Sephadex G-25 chromatography or by dialysis, first against salt and buffer, to remove the EDTA–metal complex, and then against deionized distilled water. tRNA prepared by this method is considered to be "denatured." To "renature" the denatured tRNA an aqueous solution was dialyzed vs. N-buffer at 5° for at least 6 hr. An attempt to produce renatured *E. coli* $tRNA^{Glu}$ by putting denatured material directly into N-buffer followed by heating at 40° for 35 min yielded tRNA which produced the same results as denatured material. All tRNA concentrations were determined from the absorbance of 260 nm in N-buffer assuming that a concentration of 2.00 μM tRNA has an absorbance at 260 nm of 1.00 (Litt, 1969).

Lifetime Measurements. Fluorescence decay was measured using the apparatus shown schematically in Figure 1. Either a Westinghouse FT 230 lamp, with a half-life of about 20 μsec, or a Chadwick Helmuth Model 135 stroboscopic lamp (half-life of ~20 or ~50 μsec) was used. The spectral bandpass of the (nominal) 395-nm excitation filter (two Corning 5-60 glass filters) and the 620-nm emission filter combination (combination of a 650-nm interference filter, Optics Technology; 2-61, 2-62, and 2-73 Corning glass filters) were measured using a Cary Model 14 recording spectrophotometer. For later discussion it is important to note that this "nominal" 395-nm excitation band transmits a considerable amount of light at shorter wavelengths which allows excitation of thiouridine residues in tRNA and thus subsequent energy transfer to Eu^{3+}. The emission filter combination allowed the 618-nm emission peak of europium to pass while effectively screening the 595-nm peak. An alternate excitation filter used was a (Jena Glaswerk) 364-nm monopass filter with a 12-nm half-width and 33% transmittance at the maximum. The signals from several flashes were averaged by superimposition on the oscilloscope storage screen and photographed, and these data were converted into a plot of log (relative fluorescence intensity) vs. time. When this plot was linear it was possible to calculate a decay time from the slope and an initial fluorescence intensity from the zero-time intercept based on the equation of first-order exponential decay:

$$I = I^0 \exp(-t/\tau) \quad (1)$$

where I is the intensity at time t, I^0 is the intensity at $t = 0$, and τ is the decay time. In all cases the lifetime of Eu^{3+} emission was much longer than the decay time of the exciting lamp. Nonexponential decays were fit using an equation for multiple, simultaneous decay:

$$I = \sum_n I_n^0 \exp[-t/\tau_n] \quad (2)$$

where the index n is either 1, 2, or 3 and I_n^0 and τ_n refer to the intensity due to component n, with lifetime τ_n. The method used to fit the curves was primarily trial and error, with educated guesses based on the single component lifetimes found for extreme cases. The quality of the data did not justify the use of more elaborate fitting procedures. The resolution of a typical nonexponential decay is shown in Figure 2. For single component decay (or for a single component in multicomponent decay) the integrated intensity (total intensity) is calculated using

$$I_n = \int_{t=0}^{\infty} I_n^0 \exp[-t/\tau_n]dt = I_n^0\tau_n \quad (3)$$

Steady State Fluorescence Intensity Measurement. All measurements were made using a Turner Spectro 210 spectrofluorimeter unless otherwise noted. To correct for drift in instrument gain standards were used during all titrations. Linear corrections of the intensity for volume changes due to dilution were made in all the reported results.

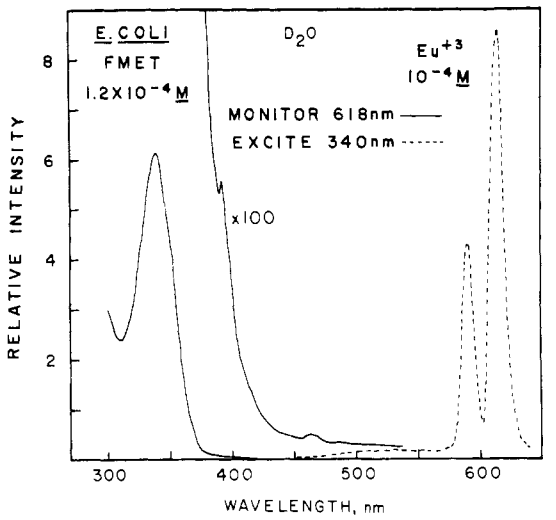

FIGURE 3: Excitation and emission spectra of renatured *E. coli* $tRNA^{fMet}$-europium complex in 25:1 D_2O-H_2O. D_2O was used as the solvent in these experiments to enhance the intensity of the europium emission.

Results and Discussion

Most data were obtained with $tRNA^{fMet}$ so we begin by considering these results first. We then show how these results and those obtained with other tRNAs permit us to draw broader conclusions regarding the metal binding properties of tRNA in general.

(A) E. coli tRNA^{fMet}. (a) EVIDENCE FOR SENSITIZED ENERGY TRANSFER TO EUROPIUM. In initial studies of the effect of complexation with tRNA on the Eu^{3+} emission properties we noticed striking differences between the optical properties of Eu^{3+} bound to *E. coli* tRNA as compared with yeast tRNA (Wolfson and Kearns, 1974). Since many *E. coli* tRNA species (Barrell and Clark, 1974), but no yeast tRNA, contain a 4-thiouridine (S^4U) we suspected that S^4U was responsible for enchancement of the Eu^{3+} emission when bound to *E. coli* tRNA. The excitation spectrum (λ_{max} 340) of the Eu^{3+} emission shown in Figure 3 confirms this. Even with excitation at 395 nm, most of the Eu^{3+} emission is due to S^4U absorption.

(b) STEADY STATE EMISSION AND LIFETIME STUDIES OF EU^{3+} BINDING. When a solution of Eu^{3+} is excited at 395 nm and titrated with increasing amounts of native *E. coli* $tRNA^{fMet}$ the intensity of the Eu^{3+} luminescence increases and passes through a maximum when the metal/tRNA ratio is about 3:1 (Figure 4A). With the addition of more tRNA the intensity diminishes (even though the total amount of light absorbed increases) and eventually plateaus at the value obtained when there is one metal per tRNA.

The complex behavior of the titration curve indicates more than one type of binding site is involved and this is confirmed by measurements of the lifetime of Eu^{3+} emission. In tRNA-free solutions, the decay of the Eu^{3+} emission is exponential (τ = 1.4 msec in D_2O, 0.14 msec in H_2O). The decay in the presence of tRNA was definitely nonexponential, but we found that it could be fit using a single long lifetime of 1.9 msec and a range of shorter lifetimes between 0.8 and 1.3 msec (in D_2O). A plot of the relative contributions of the short and long lived components of the fluorescence decay to the total luminescence intensity is shown in Figure 5A and B. As tRNA is added to the Eu^{3+} solution, there is a sharp increase (factor of 5) in the intensity of the short lived component until the Eu/tRNA ratio is 3:1. Further addition of tRNA causes a decrease in the short lived component, but an increase in the long lived component leveling off at a Eu/tRNA ratio of 1:1.

(c) LOCATION OF Eu^{3+} BINDING SITES. The excitation spectrum shown in Figure 3 clearly demonstrates that energy transfer from the S^4U (λ_{max} 340 nm) at position 8 is responsible for the enhancement of the Eu^{3+} luminescence. The small peak at 395 nm is due to direct excitation of the Eu^{3+}, whereas the much larger peak at 340 nm is due to absorption by S^4U (there is no detectable Eu^{3+} absorption at 340 nm).

Further evidence for energy transfer from S^4U comes from comparison of the titrations of *E. coli* $tRNA^{fMet}$ with Eu^{3+} before and after photolysis at 340 nm (see Figure 6A). This photolysis cross-links the S^4U at position 8 with the cytosine at position 13 (Favre et al., 1971; Leonard et al., 1971; Bergstrom and Leonard, 1972). The reduction in

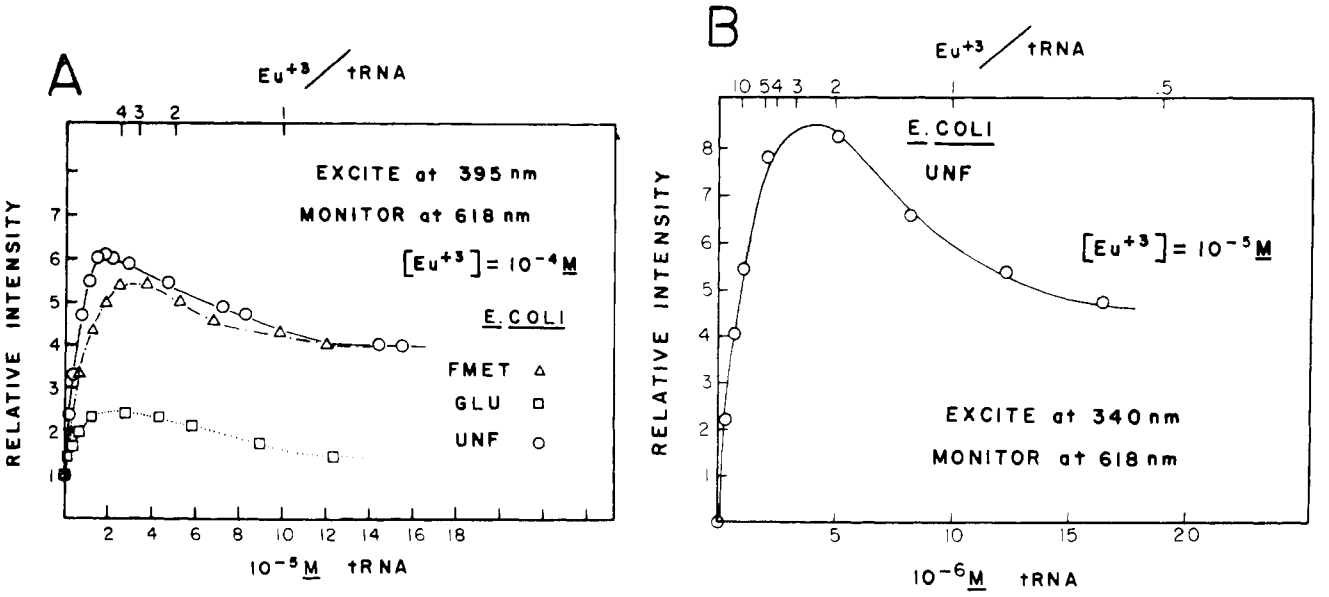

FIGURE 4: Variation of the steady-state europium emission with added tRNA (renatured). (A) Effect of adding *E. coli* $tRNA^{fMet}$, $tRNA^{Glu}$, and unfractionated tRNA, 395-nm excitation. (B) Effect of adding unfractionated *E. coli*, 340-nm excitation.

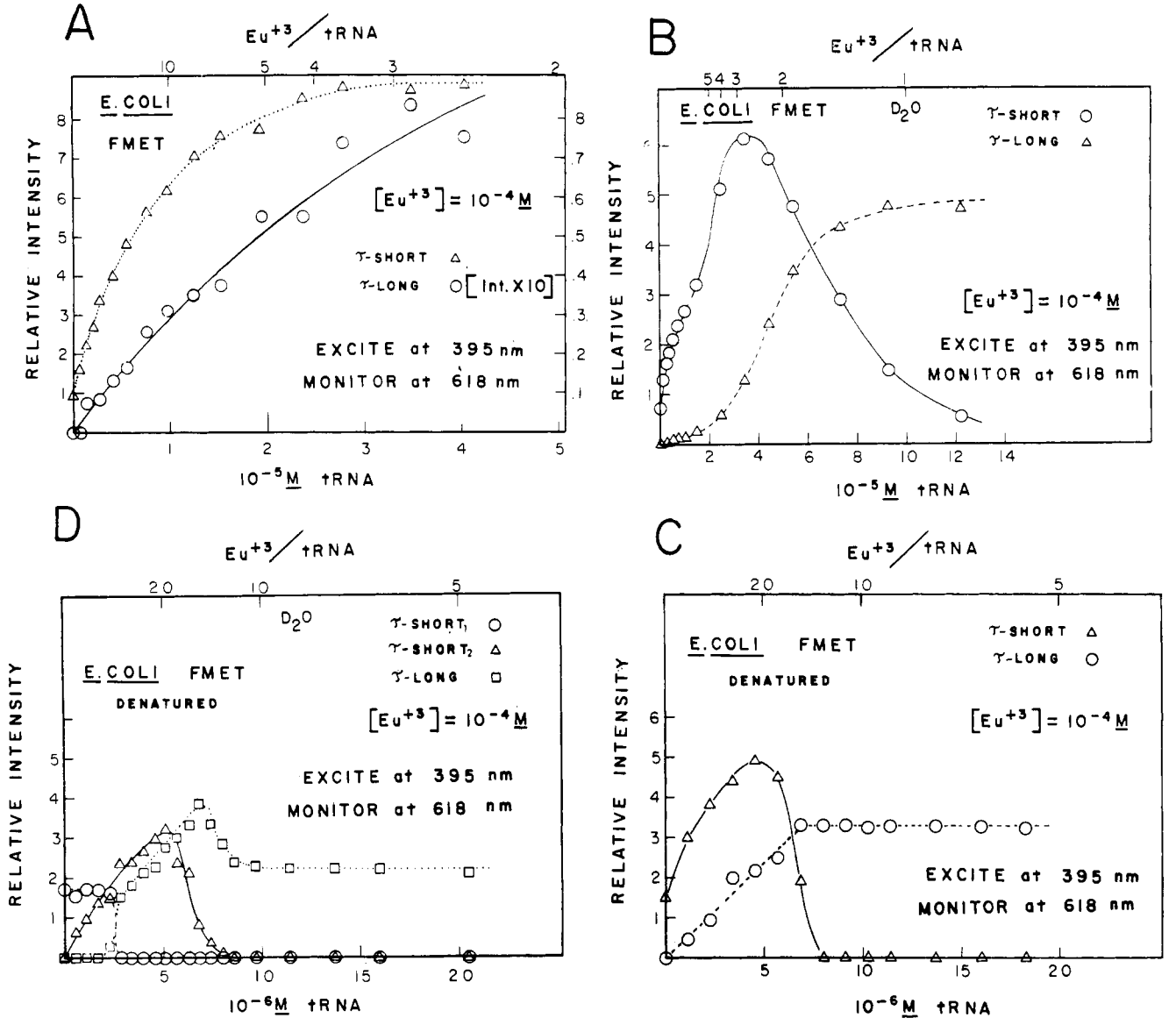

FIGURE 5: Variation in the intensity of the long and short lived components of the europium emission when a fixed amount of europium was titrated with renatured $tRNA^{fMet}$ in (A) H_2O, or (B) D_2O, or with denatured $tRNA^{fMet}$ in (C) H_2O, or (D) D_2O.

intensity of the europium emission in the photolyzed sample shows that S^4U is essential to the sensitization of the Eu^{3+} emission. Other studies of engy transfer from organic molecules to Eu^{3+} permit us to conclude that the emitting ions must be located quite close to the S^4U residue (Heller and Wasserman, 1965; Lamola and Eisinger, 1969).

Although not crucial to our discussion there are several reasons for believing that the sensitization involves energy transfer from the triplet state of the S^4U rather than its lowest single state. First, Lamola and Eisinger (1969) find that quenching of the fluorescence does not lead to a concomitant increase in the Eu^{3+} emission. Second, fluorescence of S^4U is apparently quite weak ($\phi < 10^{-4}$) and this greatly reduces opportunity for energy transfer (Favre, 1974). Third, incorporation of thiocarbonyl groups into a molecule greatly enhances intersystem crossing from the singlet to the triplet state and this too would favor energy transfer from the triplet state (McGlynn et al., 1969).

The lifetime measurements demonstrate that with $tRNA^{fMet}$ in 25:1 D_2O (Figure 5B) there is short lived intensity which peaks at 3:1 Eu^{3+}/tRNA. Since most of the emission intensity comes from energy transfer both at 3:1 and 1:1 europium per tRNA at least two of the three strong binding sites must be near S^4U. The weaker of these two sites has a shorter lifetime and higher overall quantum yield than the other site which is stronger and has a longer lifetime for Eu^{3+}.

(d) RATE OF EXCHANGE OF Eu^{3+} AMONG THE TIGHT BINDING SITES (0-3 Eu^{3+}/tRNA). NMR studies of the binding of Eu^{3+} to tRNA indicate that in the absence of Mg^{2+} the exchange of Eu^{3+} among the tight binding sites (or perhaps between tRNA molecules) is rapid compared to 1-10 msec (Wolfson and Kearns, 1974; Jones and Kearns, 1974). While part of the rapid exchange could be intermolecular these results suggest it is possible for Eu^{3+} ions to occasionally move around the molecule from site to site. Therefore, a Eu^{3+} nominally bound at some site distant to the S^4U could migrate to and pick up energy from the excited state S^4U residue. While this mechanism may contribute to the S^4U sensitization of the Eu^{3+} emission, the fact that the lifetime data show decidedly nonexponential behavior for the strong sites indicates the metals are more or less bound to specific sites for times approaching 1 msec. Migration of the Eu^{3+} ions among the weaker sites (say for

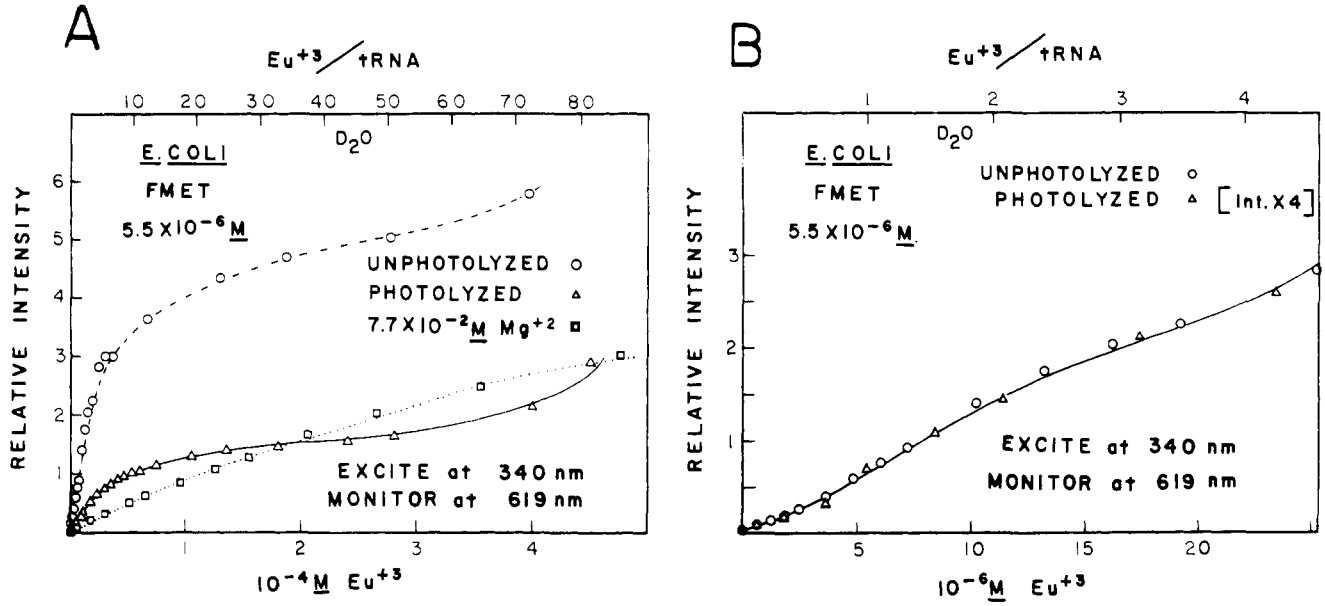

FIGURE 6: Variation in the steady-state europium intensity when renatured *E. coli* $tRNA^{fMet}$ is titrated with europium. (A) Comparison of an unphotolyzed sample with a photolyzed sample, and a sample containing magnesium. (B) Comparison of the unphotolyzed and photolyzed sample in the lower concentration region. The curve for the photolyzed sample has been multiplied by a factor of 4.

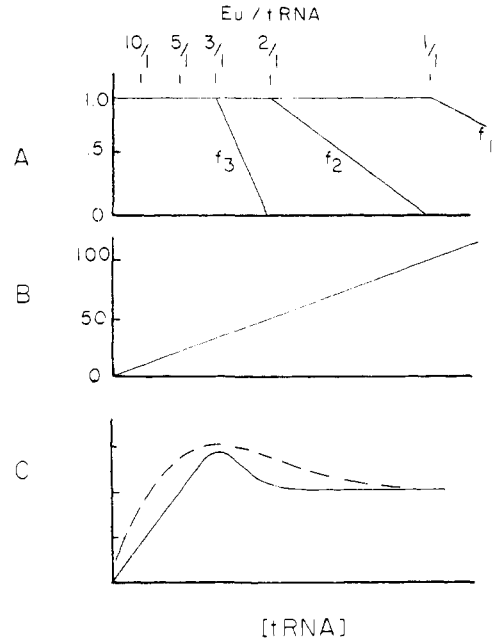

FIGURE 7: A theoretical curve showing the expected variation in the intensity of europium luminescence when a fixed amount of europium is titrated with various amounts of tRNA. In the particular model chosen, all emission is assumed to arise from the first and third most tightly bound europium (see text). Calculated titration curve for a model with three strong binding sites with $K_1 > K_2 > K_3$ and $\phi_3 > \phi_1 \gg \phi_2$. (A) Variation in the fractional occupancy (f_n) of sites 1, 2, and 3 when a fixed amount of europium is titrated with tRNA. (B) Variation in the total light absorbed by the tRNA. (C) Predicted variation in the sensitized Eu^{3+} luminescence (—) compared with the observed variation in the Eu^{3+} luminescence (- - -). The discrepancies are due in part to the fact that more weakly bound metals still contribute to the total luminescence (see Figure 6) and there is undoubtedly some contribution from the metal bound at the second site.

Eu^{3+} to tRNA ratios above 5:1) is probably responsible for the continued increase in the intensity of the Eu^{3+} emission as tRNA is titrated with increasing amounts of Eu^{3+} (see Figure 6A).

(e) INDEPENDENT VS. COOPERATIVE BINDING OF Eu^{3+} TO STRONG SITES. Consider now the titration of Eu^{3+} with $tRNA^{fMet}$ (Figure 4A). The rapid increase in intensity with added tRNA results from more of Eu^{3+} being bound near or having access to S^4U with increasing tRNA concentration. The intensity is maximum for 3 Eu^{3+}/tRNA. These results indicate that the binding is essentially independent since in totally cooperative binding there would be no changes after all the strong sites are filled. This would occur at 3 europiums per tRNA if there are only three strong sites and at an even lower tRNA concentration if there are more than three strong sites. This interpretation is predicated on the assumption that under our experimental conditions all the Eu^{3+} ions in the tRNA containing solutions are bound to the tRNA molecules, and this was demonstrated using unfractionated tRNA (see below).

(f) POSSIBLE MODEL TO ACCOUNT FOR THE TITRATION BEHAVIOR. In view of the fact that the binding does not appear to be cooperative under our experimental conditions (moderately high salt, pH 7) we can assign binding constants K_n to the various Eu^{3+} binding sites, and corresponding efficiency factors, ϕ_n, for the S^4U sensitization of the Eu^{3+} emission. In experiments where a fixed amount of Eu^{3+} is titrated with tRNA, the amount of light absorbed by the S^4U residue is proportional to the tRNA concentration [tRNA], and f_n, the fractional occupancy of site n, will depend upon the Eu/tRNA ratio and the relative values of the K_n. The variation of the S^4U sensitized Eu^{3+} emission with tRNA concentration is then simply given by the expression

$$I = \alpha[\text{tRNA}] \sum_n \varphi_n f_n$$

One possible model consistent with the experimental data indicating three strong binding sites located close to S^4U is: $K_1 > K_2 > K_3$ and $\phi_3 > \phi_1 > \phi_2$. Energy transfer to Eu^{3+} ions bound at other weaker binding sites is assumed to be much less efficient (i.e., $\phi_3 > \phi_n$). This model, which leads to the theoretical curve shown in Figure 7C, qualitatively accounts for the titration curve shown in Figure 4A and B.

(g) RELATION BETWEEN Mg^{2+} AND Eu^{3+} BINDING

SITES. While the biochemical studies indicate that tRNA molecules are functional in the presence of either Mg^{2+} or Eu^{3+} (Kayne and Cohn, 1972), this does not prove that the binding sites for these two metal ions are the same. Our studies indicate the strong Eu^{3+} binding constants are on the order of $\sim 10^8\ M^{-1}$, whereas Lynch and Schimmel (1974) and others find that the strong Mg^{2+} binding constants are on the order of $2 \times 10^5\ M^{-1}$ (Sander and Ts'o, 1971; Danchin and Gueron, 1970). Consequently, if Mg^{2+} and Eu^{3+} compete for the *same* binding sites, a ~400-fold excess of Mg^{2+} should be required to displace 1/2 of the Eu^{3+}. In accord with the above expectations, we find that addition of 600-fold excess of Mg^{2+} reduces the Eu^{3+} luminescence by a factor of ~2, as the results shown in Figure 8A demonstrate. While this result does not prove, it certainly supports the notion that Eu^{3+} and Mg^{2+} compete for the same strong binding sites.

(h) BINDING PROPERTIES OF PHOTOCROSS-LINKED $tRNA^{fMet}$. When the S^4U_8 and ^{13}C residues in $tRNA^{fMet}$ are photocross-linked, there was a substantial reduction in the S^4U sensitized luminescence (Figure 6A), indicating that approximately 80% of the molecules were affected. The fact, however, that this mixture of photolyzed and unphotolyzed molecules still exhibits a titration curve *identical* in shape with the original material (see Figure 6B) provides strong evidence that the binding constant for europium at site(s) near S^4U and elsewhere are not affected by photocross-linking. Specifically, if binding to site(s) near position 8 was significantly weaker after photolysis the experimental intensity curve would have risen much more rapidly for the partly photolyzed mixture, since relatively more europium would be bound near S^4U for the remaining unphotolyzed molecules. On the other hand, if the binding to the site(s) near position 8 was (were) much stronger on the photolyzed than on the unphotolyzed molecules, there would have been a distinct lag in the initial portion of the intensity curve. These observations are also consistent with biochemical studies which show that the degree of aminoacylation of $tRNA^{fMet}$ is unaffected by photocross-linking (Chaffin et al., 1971).

(i) COMPETITION WITH EDTA FOR Eu^{3+} BINDING. The results of the titration of the mixture with 3 Eu^{3+}/tRNA by EDTA (Figure 8C) are consistent with the known strong binding constant of about $10^{17}\ M^{-1}$ for the Eu^{3+}–EDTA complex. The results show that at pH 7 (in N-buffer) two EDTA molecules per Eu^{3+} are required to remove Eu^{3+} from binding sites near S^4U. This fact and the slight S-character of the titration curve indicates that chelation of Eu^{3+} with one EDTA is not adequate to remove it completely from the tRNA.

(j) METAL BINDING PROPERTIES OF DENATURED *E. coli* $tRNA^{fMet}$. The results obtained with *denatured* $tRNA^{fMet}$ were quite different from those obtained with native material (compare Figure 5A,B and 5C,D). The peak intensity in the titration curves of denaturated $tRNA^{fMet}$ occurs when there are about 20 Eu^{3+} per tRNA in contrast to the native material which peaked with about 3 Eu^{3+} per tRNA. These results are especially important in that they illustrate the sensitivity of metal binding properties to the tRNA conformation.

(k) *Comparative Binding Strength of E. coli* $tRNA^{fMet}$ AND YEAST $tRNA^{Phe}$. Because yeast tRNA lack the S^4U residue the enhancement observed with the *E. coli* species is absent when Eu^{3+} binds to yeast tRNA. This difference was used to study the relative binding strengths of *E. coli*

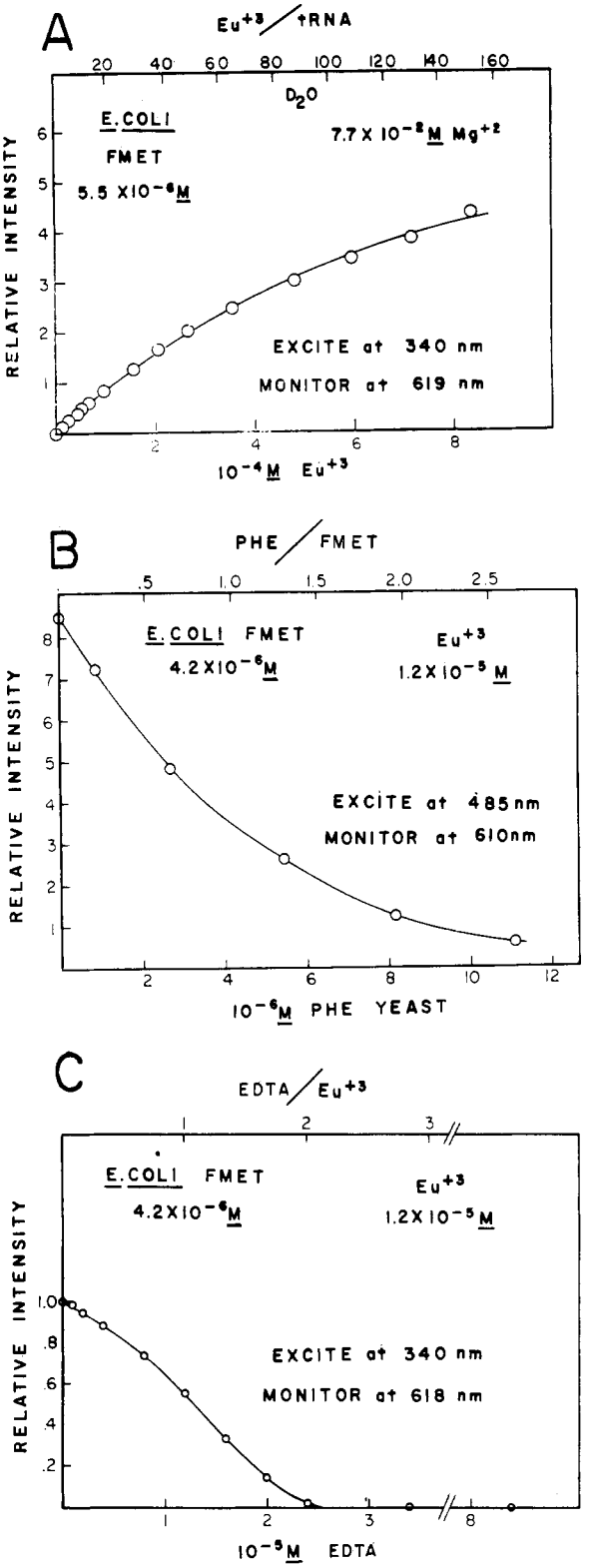

FIGURE 8: (A) Variation in the steady-state europium luminescence when a sample of *E. coli* $tRNA^{fMet}$ containing 77 m*M* Mg^{2+} is titrated with europium. (B) Competition experiment in which $tRNA^{fMet}$ is titrated with yeast $tRNA^{Phe}$ and the intensity of the europium emission monitored. (C) Competition experiment in which $tRNA^{fMet}$ is titrated with EDTA and the intensity of the europium emission monitored.

tRNA and yeast $tRNA^{Phe}$. In these experiments a sample of $tRNA^{fMet}$ containing 3 Eu/tRNA was titrated with aliquots of yeast $tRNA^{Phe}$ with the results shown in Figure 8B. The addition of an approximately equal amount of

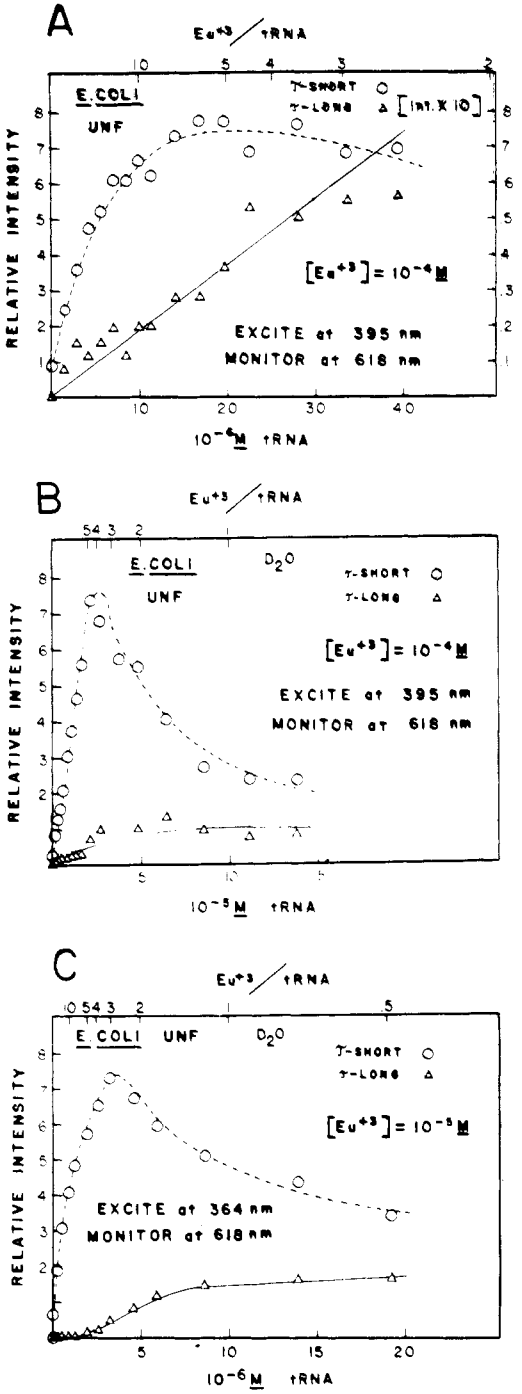

FIGURE 9: Variation in the intensity of the europium emission resulting from addition of unfractionated *E. coli* tRNA to a fixed amount of europium: (A) in H_2O, 395-nm excitation; (B) in D_2O, 395-nm excitation; (C) in D_2O, 364-nm excitation. In these figures, the contributions from the long and short lived decay components to the total intensity are plotted as a function of the tRNA concentration.

$tRNA^{Phe}$ caused a 50% reduction in the Eu^{3+} luminescence, indicating that these two different tRNA species have nearly identical Eu^{3+} binding strengths.

$tRNA^{fMet}$ is unique in that it is the initiator tRNA so it was possible that it might exhibit rather special binding properties. We therefore examined the metal binding properties of other tRNA.

(*B*) *Unfractionated E. coli tRNA.* To the extent that all tRNA have the same general conformation as *E. coli* $tRNA^{fMet}$, they should exhibit analogous metal binding properties. Since most *E. coli* tRNA species that have been sequenced have a S^4U residue at position 8 (Barrell and Clark, 1974), this possibility was examined using the same techniques as in the study of $tRNA^{fMet}$. Both the lifetime measurements (Figure 9A–C) and the titration of the steady-state luminescence (Figure 4B) reveal that the metal binding properties of the unfractionated tRNA are virtually the same as for $tRNA^{fMet}$. As with $tRNA^{fMet}$ the peak in the titration curve (340- or 365-nm excitation) occurs when there are about 3 Eu^{3+}/tRNA. If a significant percentage of the tRNA exhibited very different binding patterns (i.e., bound the Eu^{3+} much more strongly or weakly), the peak in the titration curve would have been shifted from that observed with $tRNA^{fMet}$. The similarities extend to other aspects of the metal binding.

(a) BINDING CONSTANT (STRONG). The intensity of Eu^{3+} emission from a mixture of 10 μM Eu^{3+} with 3.4 μM renatured unfractionated *E. coli* tRNA (in H_2O N-buffer) was decreased by 50% by the addition of about 6 mM $MgCl_2$. Assuming that this represents 50% displacement of Eu^{3+} from tRNA the ratio of binding constants for Eu^{3+} to Mg^{2+} is 600:1. This is in excellent agreement with the value of ~600:1 found with the steady-state titration experiment of $tRNA^{fMet}$ with Eu^{3+}. A dilution of the above mixture to 1:10 with N-buffer showed a tenfold decrease in intensity, indicating very little dissociation. Dilution to 1:100, however, caused more than a 100-fold decrease in intensity, indicating that at 0.1 μM Eu^{3+} and 0.034 μM tRNA there is some dissociation of Eu^{3+}. If the strong binding constant for Mg^{2+} is 2×10^5 M^{-1} (Danchin, 1972; Lynch and Schimmel, 1974), then, based on the competition experiments, it would be about 10^8 M^{-1} for Eu^{3+}. Thus, we expect to see the partial dissociation of Eu^{3+} which is observed at 0.034 μM tRNA. This is good evidence that Eu^{3+} binds to the same sites as Mg^{2+}.

(b) INDEPENDENT VS. COOPERATIVE BINDING OF Eu^{3+} TO STRONG SITES. The steady-state titrations of Eu^{3+} with unfractionated *E. coli* tRNA in H_2O, exciting at both 340 nm (Figure 4B) and 395 nm (Figure 4A), are very similar to the titration with $tRNA^{fMet}$. Consequently, the same independent binding must occur with most *E. coli* tRNA species, and the location of the sites with respect to S^4U is similar to those in $tRNA^{fMet}$.

(*C*) *Eu^{3+} Binding to E. coli $tRNA^{Glu}$.* *E. coli* $tRNA^{Glu}$ has no S^4U residue, but it has a modified 2-thiouridine (S^2U) residue in the anticodon loop (Barrell and Clark, 1974) which is capable of sensitizing Eu^{3+} emission. The long wavelength tail of the absorption drops off more rapidly than does the S^4U peak, so that when $tRNA^{Glu}$ is excited at 395 nm a greater percentage of the Eu^{3+} emission (about 50%) is due to direct excitation of the Eu^{3+} rather than sensitized via S^2U.

The titration of the steady-state Eu^{3+} emission with $tRNA^{Glu}$ (broad maximum around 4 Eu^{3+}/tRNA (Figure 4A)) and the lifetime data (Figure 10A) indicate that the maximum is located close to 4–5 Eu^{3+}/tRNA. This is to be contrasted with $tRNA^{fMet}$ which showed a peak at about 2–3 Eu^{3+}/tRNA. The fact that, relatively, there is such a large europium emission in $tRNA^{Glu}$ with one Eu^{3+}/tRNA is due in part to the large contribution from direct excitation of the europium.

Independent of the exact interpretation of the $tRNA^{Glu}$ binding data, it is evident that the metal binding properties of the denatured $tRNA^{Glu}$ are quite different from the native $tRNA^{Glu}$ as comparison of data shown in Figure 10A

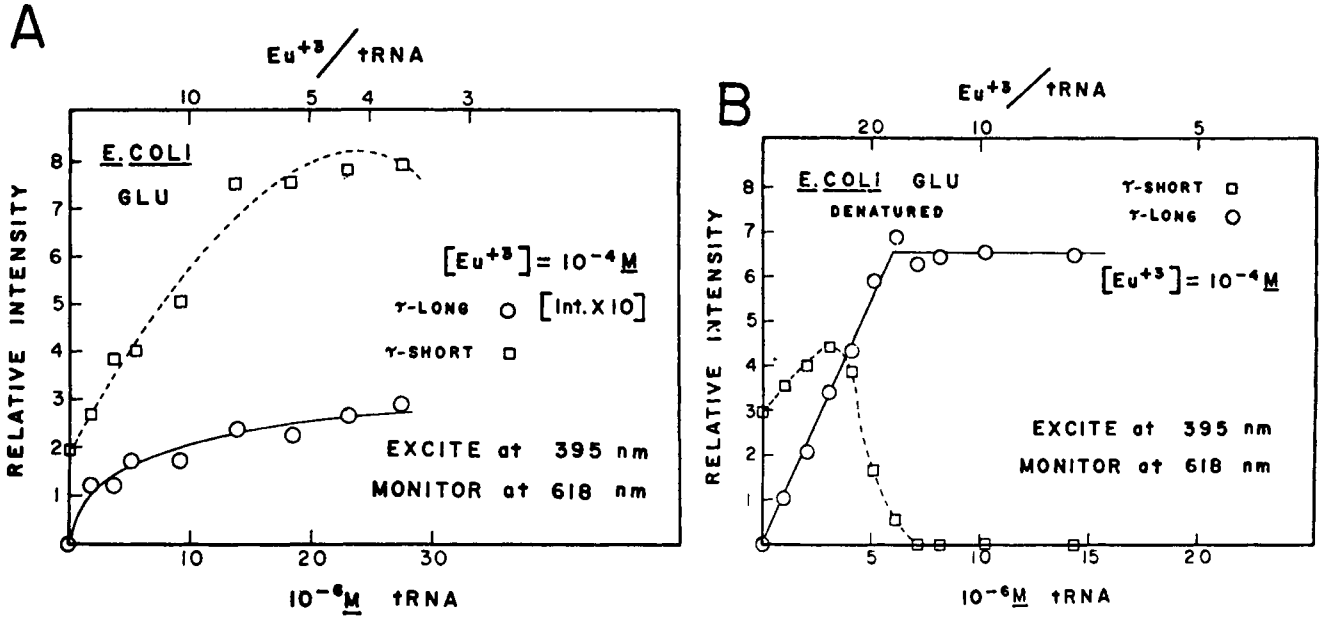

FIGURE 10: Variation in the intensity of the europium emission resulting from the addition of *E. coli* $tRNA^{Glu}$ to a fixed amount of europium. (A) Titration with renatured $tRNA^{Glu}$; (B) titration with denatured $tRNA^{Glu}$. The contributions from the long and short lived decay components to the total luminescence have been plotted as a function of the tRNA concentration.

and B indicates. Whereas the peak in the titration curves with native tRNA is located around 4–5 Eu^{3+}/tRNA, it is displaced to around *20* Eu^{3+}/tRNA with the denatured material. Again, we find the metal binding properties of native and denatured materials are different, and this strengthens our notion that the binding behavior exhibited by the native material requires a rather special conformation for the molecule.

Summary

The above studies demonstrate that there are at least three strong Eu^{3+} binding sites per *E. coli* $tRNA^{fMet}$ with the third strongest and the strongest sites located near S^4U (position 8). The Eu^{3+} bound to the site which has best access to S^4U has a different lifetime and emission intensity than Eu^{3+} bound elsewhere. The three strong sites have a more independent than cooperative binding character and the average strong binding constant is about 10^8 M^{-1}, or ~600 times stronger than competitively binding Mg^{2+}. Photochemical cross-linking of S^4U with cytosine (position 13) does not alter the overall binding of Eu^{3+}. There are significant differences between the binding properties of native and denatured $tRNA^{fMet}$, indicating that the titration curves obtained with native $tRNA^{fMet}$ are characteristic of a special tertiary structure which is different from that obtained using denatured $tRNA^{fMet}$.

Unfractionated *E. coli* tRNA exhibited a binding pattern which was entirely similar to $tRNA^{fMet}$, and competition experiments with $tRNA^{fMet}$ and yeast $tRNA^{Phe}$ show that these two different species have binding constants for the strong sites which are nearly the same. In view of the interpretation given to the $tRNA^{fMet}$ data, we conclude that the majority of the *E. coli* tRNA also have Eu^{3+} binding properties which are very nearly identical with those exhibited by $tRNA^{fMet}$. Since the majority of *E. coli* tRNA species which have been sequenced so far, including $tRNA^{fMet}$, fall into class I (Levitt, 1969) it will be interesting to see whether some of the other tRNA which have large minor stems exhibit different Eu^{3+} binding properties.

The europium binding constant measurement and magnesium competition experiment support the notion that Eu^{3+} and Mg^{2+} are binding at the same sites on the tRNA molecule. However, independent of this point these optical studies very nicely complement X-ray diffraction (Kim et al., 1974; Ladner et al., 1972) and NMR studies (Jones and Kearns, 1974) currently in progress, and provide convenient structural probes of molecules that are biochemically functional. Although there are certain similarities there are important differences between our results obtained with Eu^{3+} and previous Mg^{2+} binding studies. Specifically, the binding of Mg^{2+} is found to be cooperative (Danchin, 1972; Lynch and Schimmel, 1974) whereas our data indicate that the binding of Eu^{3+} is noncooperative (Wolfson and Kearns, 1974; Jones and Kearns, 1974). Part of this difference may be due to differences in the experimental conditions. In experiments where Mg^{2+} binding is cooperative, the concentration of NaCl was low (usually less than 10 m*M*) (Danchin, 1972; Lynch and Schimmel, 1974) whereas in our experiments the NaCl concentration was relatively high (0.1 *M*). It is known that high concentrations of NaCl can induce the same tertiary structure that is stabilized by much lower concentrations of Mg^{2+} (Cole et al., 1972; Wong et al., 1975) and furthermore, Lynch and Schimmel (1974) have shown that increasing the NaCl concentration decreases the degree of cooperativity in the Mg^{2+} binding experiments. Thus, if the proper tertiary structure were preformed by the presence of the high Na^+, the Eu^{3+} binding would not be expected to be cooperative.

After this manuscript was submitted, a paper by Kayne and Cohn (1974) on the binding of terbium and europium to tRNA appeared. They too studied the S^4U sensitization of rare earth luminescence (Wolfson and Kearns, 1974) but arrive at somewhat different conclusions than we do. They interpreted their binding data in terms of a model with four independent strong binding sites for europium, all with approximately equal binding constants of 1.5×10^5 M^{-1}. Since the binding constants for magnesium are 2×10^5 M^{-1} (Danchin, 1972; Lynch and Schimmel, 1974) their data indicate that the binding of europium is no stronger than the binding of magnesium. Although we agree in the

approximate number of strong binding sites (3–4) we find the europium binding constants are considerably larger (10^8 M^{-1}) and that the addition of the first three europium ions appears to be sequential (Jones and Kearns, 1974). The sequential character of the europium addition is most clearly brought out in experiments where a fixed amount of europium is titrated with tRNA (for example, see Figures 4, 6, and 9). When the titration is carried out in the reverse manner (fixed amount of tRNA titrated with europium) differences in the quantum yield associated with the different binding sites are not so readily apparent. Interestingly enough, Kayne and Cohn comment on the possibility that their titration curves may be sigmoidal, but felt their data were not adequate to warrant further analysis.

Kayne and Cohn have presented one model to interpret their binding data (particularly Figure 4, Kayne and Cohn, 1974) but we believe their results can equally well be interpreted in terms of the model we developed above on the basis of more extensive experimental results (several different types of titrations, lifetime measurements, dilution and competition experiments, and NMR). All that would be required is that the addition of more than three europiums per tRNA leads to some further increase in the intensity of the europium emission, and the results shown in Figure 6 confirm that this is true.

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References

Barrell, B. G., and Clark, B. F. C. (1974), "Handbook of Nucleic Acid Sequences", Oxford, England, Johnson-Bruvvers, Ltd.

Bergstrom, D. E., and Leonard, N. J. (1972), *J. Am. Chem. Soc. 94,* 6178.

Chaffin, L., Omilianowski, D. R., and Bock, R. M. (1971), *Science 172,* 854.

Cole, P. E., Yang, S. K., and Crothers, D. M. (1972), *Biochemistry 11,* 4358.

Danchin, A. (1972), *Biopolymers 11,* 1317.

Danchin, A., and Gueron, M. (1970), *Eur. J. Biochem. 16,* 532.

Favre, A. (1974), *Photochem. Photobiol. 19,* 15.

Favre, A., Michelson, A. M., and Yaniv, M. (1971), *J. Mol. Biol. 58,* 381.

Fresco, J. R., Adams, A., Ascione, R., Henley, D., and Lindahl, T. (1966), *Cold Spring Harbor Symp. 31,* 527.

Formoso, C. (1973), *Biochem. Biophys. Res. Commun. 53,* 1084.

Gallagher, P. K. (1964), *J. Chem. Phys. 41,* 3061.

Gallagher, P. K. (1965), *J. Chem. Phys. 43,* 1742.

Heller, A., and Wasserman, E. (1965), *J. Chem. Phys. 42,* 949.

Igarashi, K., Yoh, M., and Takeda, Y. (1971), *Biochim. Biophys. Acta 238,* 314.

Jones, C. R., and Kearns, D. R. (1974), *J. Am. Chem. Soc. 96,* 3651.

Jones, C. R., and Kearns, D. R. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4237.

Kayne, M. S., and Cohn, M. (1972), *Biochem. Biophys. Res. Commun. 46,* 1285.

Kayne, M. S., and Cohn, M. (1974), *Biochemistry 13,* 4159.

Kim, S. H., Suddath, F. L., Quigley, G. J., McPherson, A., Sussman, J. L., Wang, A. H. J., Seeman, N. C., and Rich, A. (1974), *Science 185,* 435.

Ladner, J. E., Finch, J. T., Klug, A., and Clark, B. F. C. (1972), *J. Mol. Biol. 72,* 99.

Lamola, A., and Eisinger, J. (1969), Molecular Luminescence, Lim, E., Ed., New York, N.Y., W. A. Benjamin, p 801.

Leonard, N. J., Bergstrom, D. E., and Tolman, G. L. (1971), *Biochem. Biophys. Res. Commun. 44,* 1524.

Levitt, M. (1969), *Nature (London) 224,* 759.

Litt, M. (1969), *Biochem. Biophys. Res. Commun. 32,* 507.

Lynch, D. C., and Schimmel, P. R. (1974), *Biochemistry 13,* 1841.

McGlynn, S. P., Azumi, T., and Kinoshita, M. (1969), The Triplet State, Englewood Cliffs, N.J., Prentice-Hall.

Pasek, M., Venkatappa, M. P., and Sigler, P. B. (1973), *Biochemistry 12,* 4834.

Robertus, J. D., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. F. C., and Klug, A. (1974), *Nature (London) 250,* 546.

Sander, C., and Ts'o, P. O. P. (1971), *J. Mol. Biol. 55,* 1.

Suddath, F. L., Quigley, G. J., McPherson, A., Sneden, D., Kim, J. J., Kim, S. H., and Rich, A. (1974), *Nature (London) 248,* 20.

Sueoka, N., Sueoka, T., and Gartland, W. J. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31,* 571.

Wolfson, J. M., and Kearns, D. R. (1974), *J. Am. Chem. Soc. 96,* 3653.

Wong, K. L., Wong, Y. P., and Kearns, D. R. (1975), *Biopolymers,* (in press).