Enhancement of Tb(III) and Eu(III) Fluorescence in Complexes with *Escherichia coli* tRNA†

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ABSTRACT: Addition of the fluorescent lanthanide ions, Tb(III) and Eu(III), to *Escherichia coli* tRNA results in an alteration in their respective excitation spectra with no change in their emission spectra. The excitation spectra of both ions change from several sharp distinct bands in the unbound form to one major band at 345 nm and minor bands in the 295–305- and 410-nm regions in the bound form. Tb(III) and Eu(III) fluorescence is enhanced several hundred fold upon binding to tRNA. The 345-nm excitation band is identical with that of the uncommon fluorescent base, 4-thiouridine, which is present in 50% of all *E. coli* tRNA species. The Tb(III) fluorescence enhancement phenomenon (λ_{ex} 345 nm) is observed with *E. coli* $tRNA^{Phe}$, $tRNA^{Glu}$, and $tRNA^{fMet}$, each of which contains one thiouridine residue located at the number 8 position in the polynucleotide chain. Yeast $tRNA^{Phe}$, which contains no 4-thiouridine, does not show this phenomenon. Addition of Tb(III) and Eu(III) cations to *E. coli* tRNA results in quenching of 4-thiouridine emission at 460 and 510 nm. Destruction of the 4-thiouridine residue by ultraviolet (uv) irradiation results in quenching of the enhanced fluorescence of bound Tb(III) ions. The studies indicate that the fluorescence phenomenon observed results from energy transfer from the 4-thiouridine residue to bound lanthanide ions. Titration of partially purified $tRNA^{Met}$ with Eu(III) indicates that Eu(III) fluorescence intensity increases until approximately 4 mol of Eu(III) have been bound per mol of tRNA with an apparent dissociation constant of 6×10^{-6} M for each site. Upon addition of Tb(III) to $tRNA^{fMet}$, Tb(III) fluorescence increases continuously until Tb(III) reaches its solubility limit at approximately 10 mol of Tb(III) added per mol of tRNA at pH 7.5. Supporting evidence for the tight binding of the lanthanide ions in the tRNA complex comes from the observation that Tb(III)- and Eu(III)-tRNA, after a 16-hr dialysis against low ionic strength buffer, still exhibit fluorescence which is characteristic of the complex. Enhancement and quenching of *E. coli* tRNA emission at 460 and 510 nm by various metal ions have also been determined. The nonfluorescent lanthanide ions, Yb(III), Sm(III), and Gd(III), behave like Mg(II) in enhancing 4-thiouridine fluorescence while bound Mn(II) ions quench the fluorescence.

Previous studies in our laboratory have shown that trivalent lanthanide ions will replace the divalent metal ion requirement for the aminoacylation of $tRNA^{Ile}$ by *Escherichia coli* isoleucyl-tRNA synthetase, under certain experimental conditions (Kayne and Cohn, 1972). One of the substrates of this reaction, tRNA, is known to bind metal ions, reportedly required for conformational stability (Sueoka *et al.*, 1966; Fresco *et al.*, 1966). Tb(III) and Eu(III), two members of the lanthanide series of cations, exhibit fluorescence at room temperature in aqueous solution (Sinha, 1966). The fluorescent properties of Tb(III) and Eu(III) offer a unique opportunity to probe the environment of cation binding sites on macromolecules. For example, lanthanide ions have recently been used as fluorescent probes in studying the metal-ion binding sites in transferrin (Luk, 1971). The present paper reports on the interaction of lanthanide ions with the common and uncommon bases in *E. coli* tRNA as reflected in fluorescence and phosphorescence phenomena.

Materials and Methods

Stripped, unfractionated *E. coli* B tRNA was purchased from General Biochemicals, Plenum, and Schwarz-Mann. *E. coli* (MRE600) $tRNA^{Phe}$ (lot 7332102) and $tRNA^{fMet}$ (lot 7103303) and brewers yeast $tRNA^{Phe}$ were purchased from Boehringer Mannheim GmbH. *E. coli* (K-12 MO) $tRNA^{Glu}$ was obtained from Oak Ridge National Laboratory. tRNA(Plenum) was enriched in methionine acceptance activity ($tRNA^{Met}$) (60 pmol/A_{260} unit compared with 10 pmol/A_{260} unit for unfractionated tRNA) by BD-cellulose[1] salt gradient chromatography (Roy *et al.*, 1971). Purified *E. coli* IRS was prepared at the New England Enzyme Center under the supervision of Dr. P. Schimmel and Dr. E. Eldred of the Massachusetts Institute of Technology (Eldred and Schimmel, 1972). A partially purified mixture of aminoacyl-tRNA synthetases (designated synthetase I) was prepared according to the method of Pillinger *et al.* (1969).

[^{3}H]Phenylalanine and [^{3}H]isoleucine were purchased from New England Nuclear and [^{3}H]methionine and [^{14}C]valine from Schwarz-Mann. Lanthanide ions obtained from Ventron as the chloride or oxide were added to solutions in the form of chloride salts. $NaBH_4$ and Tris were purchased from Sigma, Chelex-100 from Bio-Rad, and BD-cellulose from Schwarz-Mann. Tris was treated with Chelex to remove contaminating metal ions.

tRNA concentrations were determined spectrophotometrically at 260 nm in 10 mM NaOH, using an absorbancy

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[1] Abbreviations used are: BD-cellulose, benzoyl diethylaminoethylcellulose; s^4U, 4-thiouridine: IRS, *Escherichia coli* isoleucyl-tRNA synthetase; A_{260} unit, the quantity of tRNA contained in 1 ml of solution which has an absorbance of 1 at 260 nm when measured in a 1-cm pathlength cell; τ, the mean decay time for the luminescent emission process.

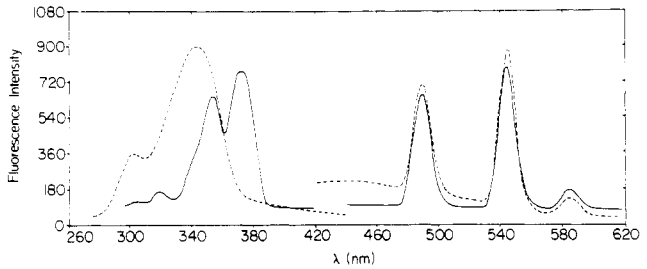

FIGURE 1: Tb(III) fluorescence. Excitation and emission spectra of 46 mM $TbCl_3$ (pH 3.0) (λ_{ex} 374 nm, λ_{em} 545 nm) (—) and 80 μM $TbCl_3$–20 μM tRNA(II)–buffer I (- - -) (λ_{ex} 345 nm, λ_{em} 545 nm).

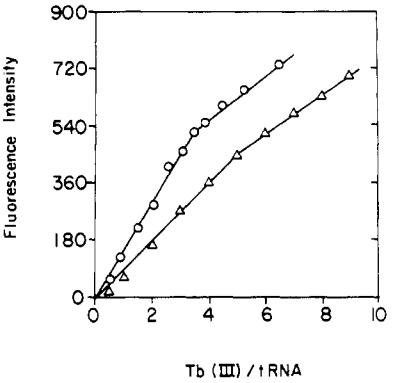

FIGURE 2: Tb(III) fluorescence enhancement upon addition to unfractionated tRNA(II) and $tRNA^{fMet}$(III). Tb(III) fluorescence intensity at 545 nm (λ_{ex} 345 nm) is plotted as a function of [Tb]/[tRNA]. The solutions contained 15 μM *E. coli* $tRNA^{fMet}$ in 10 mM Tris-HCl (pH 7.0), 23° (○), and 20 μM tRNA(II) (unfractionated) in buffer I, 23° (△).

value of 8.3 for a solution 1 mM in nucleotide units (Cohn *et al.*, 1969) and assuming a value of 80 nucleotides per tRNA and a molecular weight of 27,000 daltons. Since polynucleotide chains are cleaved in the presence of lanthanide ions (Bamann *et al.*, 1954; Eichhorn and Butzow, 1965), high temperatures and prolonged incubation of the lanthanide–tRNA complex at room temperature were avoided. In order to avoid the loss of tRNA during dialysis, Visking dialysis tubing was boiled for 3 min in 1% Na_2CO_3 and then dried in an oven at 95° and washed with water. Divalent cation concentrations were measured with a Varian Techtron Model AA-5 atomic absorption spectrophotometer. All vessels containing *E. coli* tRNA were shielded from light to avoid photodimerization of s^4U.

Removal of Tightly Bound Divalent Cations from tRNA. Commercially available, unfractionated tRNA (designated tRNA I) contains approximately 2 mol of Mg(II)/mol of tRNA as determined by atomic absorption. Mg(II) was removed from tRNA I (2 mg/ml) by dialysis for 24 hr at 4° against a solution containing 30 mM Tris-HCl (pH 7.0) and 0.5 M NaCl with suspended Chelex, followed by a dialysis for 24 hr against a solution of 30 mM Tris-HCl (pH 7.0) with suspended Chelex to reduce the NaCl concentration. The amount of Mg(II) remaining bound to tRNA was 0.05 mol/mol of tRNA (designated tRNA II). Since extensive dialysis of tRNA I results in a 10–20% loss of material, a different procedure was used to remove metal ions from fractionated tRNA preparations. In the alternate procedure, divalent cations were removed by heating tRNA (0.5 mg/ml) in 0.2 M NaCl, 30 mM Tris-HCl (pH 7.0), and Chelex for 5 min at 60°. After quick cooling in a water–ice bath, the Chelex was removed by repeated centrifugation, and the tRNA (designated tRNA III) recovered by ethanol precipitation. The procedure allowed complete recovery of the tRNA (as measured by A_{260} absorbance) and the Mg(II) content was 0.3 mol/mol of tRNA.

Irradiation of E. coli tRNA. tRNA(II) or -(III) in the presence of $TbCl_3$ or $EuCl_3$ was irradiated at 340 nm at 23° for 2–4 hr with a Perkin-Elmer MPF-2A fluorimeter using a 40-nm excitation slit setting. Formation of photodimer was monitored spectrophotometrically at 335 nm. The photodimer thus produced was reduced with $NaBH_4$ according to the method of Favre *et al.* (1971).

Purification of $tRNA^{Ile}$. Partially purified $tRNA^{Ile}$ was prepared by BD-cellulose chromatography of *E. coli* B tRNA (Plenum) as described by Roy *et al.* (1971). Fractions enriched in $tRNA^{Ile}$ were pooled and the [^{3}H]Ile-$tRNA^{Ile}$ and its *N*-phenoxyacetyl derivative were prepared according to Gillam *et al.* (1968) using the synthetase I preparation. The derivatized $tRNA^{Ile}$ was then isolated by chromatography on BD-cellulose (Schreier, 1973) and deacylated by incubation in 2 M Tris-HCl (pH 8.8) for 2 hr at 30°. The purified $tRNA^{Ile}$ has an amino acid acceptor activity of 1235 pmol of Ile/A_{260} unit which compares favorably with the value of 1050 pmol of Ile/A_{260} unit determined for other preparations (Schreier and Schimmel, 1972). [^{3}H]Ile-$tRNA^{Ile}$ was purified by BD-cellulose chromatography (Friedman, 1973).

Fluorescence Measurements. A Perkin-Elmer MPF-2A fluorimeter equipped with a Hitachi QPD 33 recorder and thermostated cuvet holder was used for fluorescence measurements. tRNA fluorescence was measured in 30 mM Tris-HCl (pH 7.0) (designated buffer I) in 3-ml quartz cuvets at 23° unless otherwise stated. Since s^4U is destroyed upon irradiation at 334 nm (Favre *et al.*, 1971), prolonged exposure of the tRNA samples at this wavelength was avoided. Fluorescence and phosphorescence measurements were made 5–10 min following addition of di- or trivalent ions to tRNA. Fluorescent lifetimes of short duration (2 nsec–40 μsec) were measured with an Ortec photon counting, fluorescence lifetime instrument. For lifetimes of the order of 40 msec or longer, a Xenon flash lamp was used in conjunction with the Ortec instrument. Excitation and emission spectra have not been corrected for monochromator transmission, and lamp or photomultiplier responses.

Phosphorescence Measurements. Phosphorescence measurements were made with a Perkin-Elmer fluorimeter equipped with a phosphorescence accessory. tRNA phosphorescence was measured in 30 mM Tris-HCl (pH 7.0) and 50% glycerol (designated buffer II) at 77°K in 2 mm (i.d.) quartz tubes. The phosphorescence decay curves for s^4U were accumulated with a Varian C-1024 computer of average transients.

Results

Fluorescence of Lanthanide Ion–tRNA Complexes

Tb(III)–E. coli tRNA Complexes. The trivalent lanthanide ion, Tb(III), exhibits fluorescence in aqueous solution (Sinha, 1966) having major excitation and emission bands at 374 and 545 nm, respectively (Figure 1). When $TbCl_3$ is added to unfractionated *E. coli* tRNA (I,II) in 30 mM ammonium acetate (pH 5.0) or 30 mM Tris-HCl (pH 7.0), a marked change in the Tb(III) excitation spectrum occurs which is accompanied by a several hundred fold in-

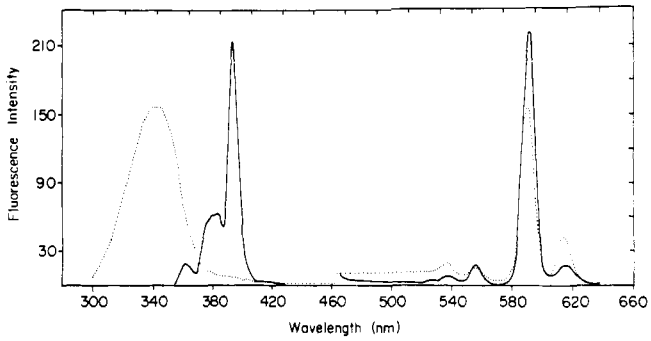

FIGURE 3: Eu(III) fluorescence. Excitation and emission spectra of 44 mM $EuCl_3$ (pH 1.5) (λ_{ex} 394 nm, λ_{em} 585 nm) (—) and of 0.15 mM $EuCl_3$, 0.035 mM tRNA(II), and 30 mM Tris-HCl (pH 7.0) (- - -) (λ_{ex} 345 nm, λ_{em} 585 nm).

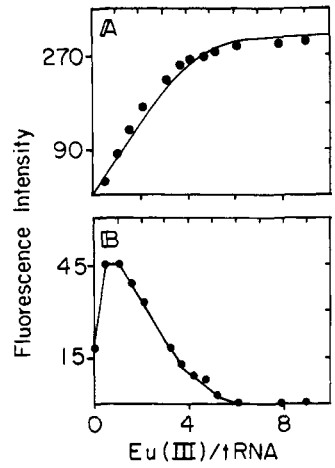

FIGURE 4: Enhancement of Eu(III) and quenching of s^4U fluorescence. The enhancement of Eu(III) fluorescence (λ_{ex} 345 nm, λ_{em} 590 nm) (A) and the quenching of s^4U fluorescence (λ_{ex} 345 nm, λ_{em} 510 nm) (B) is plotted as a function of Eu(III)/tRNA. The solid line in A is a theoretical curve based on four equivalent binding sites, a value of 318 for the fluorescence intensity of the complex, and a K_d of 6.5×10^{-6} M. The solution contained in addition to Eu(III), partially purified $tRNA^{Met}$(II)(30 μM), 30 mM Tris-HCl (pH 7.0), at 23°.

crease in Tb(III) fluorescence intensity (Figure 1). The Tb(III) emission spectrum and its fluorescence lifetime of 543 μsec (Dieke and Hall, 1957) remain unaltered. Analogous spectral changes are obtained when $TbCl_3$ is added to *E. coli* $tRNA^{Phe}$, $tRNA^{Glu}$, $tRNA^{Ile}$, or $tRNA^{fMet}$ in buffer I. The Tb(III) excitation spectrum in the Tb(III)-tRNA complex consists of a major band centered at 345 nm; in several tRNA species, an additional one or two minor bands are also present at 295–305 nm and 410 nm (Figure 1, *cf.* Figure 7).

Tb(III) fluorescence intensity at 490, 540, and 585 nm (λ_{ex} 345 nm) increases as $TbCl_3$ is added to unfractionated tRNA(II) or to $tRNA^{fMet}$(III) as shown in Figure 2. The titration curve is limited in concentration range due to the insolubility of Tb(III) ions at a Tb(III)/tRNA ratio of approximately 10 or greater.

There appears to be a break in the titration curve for both unfractionated and fractionated tRNA samples after 4–6 mol of Tb(III) is added per mol of tRNA. This break is more pronounced in the case of $tRNA^{fMet}$ than it is for unfractionated tRNA. When 4 mol of Tb(III) is added per mol of tRNA, there is a 500-fold enhancement of fluorscence (λ_{ex} 345 nm) relative to Tb(III) in aqueous solution (λ_{ex} 374 nm). In view of the limited titration data, neither a binding constant nor the number of Tb(III) binding sites could be evaluated.

Eu(III)-E. coli tRNA Complexes. The fluorescent lanthanide ion Eu(III) has its major excitation and emission bands at 394 and 585 nm, respectively, as shown in Figure 3. Upon addition of $EuCl_3$ to unfractionated *E. coli* tRNA(II) or to partially purified *E. coli* $tRNA^{Met}$(II) in buffer I or 30 mM sodium acetate (pH 5.0), the Eu(III) emission bands remain unaltered while the observed excitation spectra consist of one broad band centered at 345 nm (Figure 3). The fluorescence intensity of the Eu(III)-tRNA complex is 300-fold greater than that observed for Eu(III) ions in aqueous solution (λ_{ex} 394 nm).

When unfractionated tRNA or $tRNA^{Met}$ in buffer I is titrated with $EuCl_3$, a rapid increase in Eu(III) fluorescence occurs (λ_{ex} 345 nm) upon the addition of 4–6 mol of Eu(III) per mol of tRNA (Figure 4A). Intersection of the extrapolated straight-line portions of the titration curve yielded a value of approximately 3.75 Eu(III) ions bound per tRNA (*cf.* Figure 4A). The difference between the actual fluorescence intensity and the extrapolated values was used to calculate a dissociation constant, K_d, for the Eu(III)-tRNA complex (Reilley and Sawyer, 1961). Assuming four independent binding sites, the apparent K_d was determined to be 6.5 ± 2.2 μM. The solid line in Figure 4A is a theoretical curve based on four equivalent binding sites, a value of 318 for the fluorescence intensity of the complex, and a K_d of 6.5 μM.

Eu(III) and Tb(III) ions have long been known to form energy transfer complexes with a variety of excited molecules (Weissman, 1942). The marked changes in Eu(III) and Tb(III) excitation spectra upon binding to tRNA are indicative of energy transfer complexes comprised of bound lanthanide ions and the base residues of the polynucleotide chain. Studies were therefore undertaken to identify the base residues which were responsible for the observed Tb(III) and Eu(III) excitation band at 345 nm.

4-Thiouridine-Lanthanide Ion Energy Transfer

4-Thiouridine is an uncommon fluorescent base present in 50% of all *E. coli* tRNA species (Lipsett, 1965). With the exception of $tRNA^{Tyr}$ (Lipsett and Doctor, 1967), only one s^4U residue is present per tRNA molecule, and it occupies an identical position in all tRNA species *viz.* the number 8 position from the 5′ end of the polynucleotide chain.

Quenching of s^4U Fluorescence and Phosphorescence by Eu(III) and Tb(III). The s^4U residue of tRNA absorbs energy in the 335-nm region, and when excited at 345 nm, exhibits a broad fluorescence emission centered at 510 nm (Figure 5), with a lifetime equal to or less than 2 nsec (our experiments and Pochon *et al.*, 1971). The broad fluorescence excitation band at 345 nm is identical with that exhibited by Tb(III) and Eu(III) ions when bound to tRNA (*cf.* Figures 1 and 3).

When s^4U fluorescence intensity (λ_{em} 510 nm) in $tRNA^{Met}$ is measured as a function of Eu(III)/tRNA (Figure 4B), there is an initial enhancement in s^4U fluorescence upon the addition of 0.5–1.0 mol of Eu(III)/mol of tRNA which is followed by a decrease in s^4U fluorescence in the presence of additional Eu(III) ions. s^4U fluorescence is no longer observed after 6 mol of Eu(III) is present per mol of tRNA. The quenching of s^4U fluorescence, upon addition of Eu(III) ions, approximately parallels increases in Eu(III)

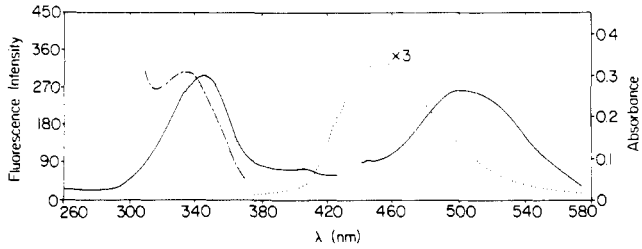

FIGURE 5: Absorption, fluorescence, and phosphorescence spectra of *E. coli* tRNA. Excitation and fluorescence emission spectra of 27 μM tRNA(II), 10 mM Tris-HCl (pH 7.5), and 0.85 mM $MgCl_2$ at 23° (—); phosphorescence spectra (λ_{ex} 345 nm) of 0.18 mM tRNA(I), 30 mM Tris-HCl (pH 7.0), and 50% glycerol at 77°K (- - -); absorption spectra of 46 μM tRNA(I) and 50 mM Tris-HCl (pH 8.0) at 23° (- - -).

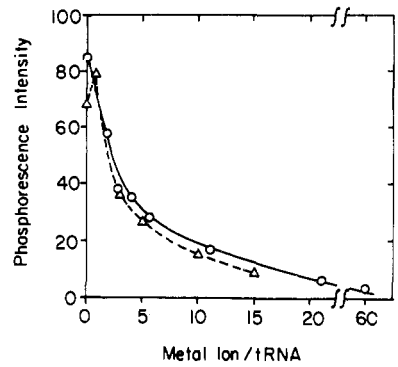

FIGURE 6: Tb(III) and Mn(II) quenching of s^4U phosphorescence. tRNA phosphorescence intensity at 450 nm (λ_{ex} 345 nm) is plotted as a function of Tb(III)/tRNA (Δ- - -Δ) and Mn(II)/tRNA (O—O). The emission slit apertures were 40 and 7.5 nm for Tb(III) and Mn(III), respectively. The solutions contained 0.144 mM tRNA(I) in 10 mM sodium acetate (pH 5.0) and 0.34 mM tRNA(II) in 30 mM Tris-HCl (pH 7.0) for Tb(III) and Mn(II), respectively.

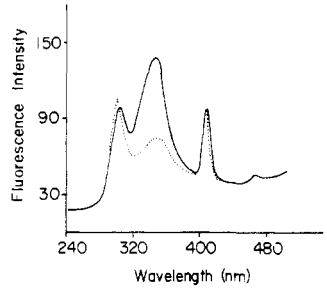

FIGURE 7: Quenching of Tb(III) fluorescence upon uv irradiation. The intensity of the Tb(III) excitation spectra (λ_{em} 545 nm) was measured before (—) and after (- - -) 2-hr irradiation at 340 nm. The solution contained 142 μM $TbCl_3$, 52 μM *E. coli* $tRNA^{Ile}$, and 30 mM Tris-HCl (pH 7.0).

fluorescence as shown in the titration in Figure 4A.

In addition to fluorescence emission at 510 nm, excitation at 345 nm at 77°K in buffer II results in s^4U emission at 460 nm (Figure 5). This emission has been ascribed to s^4U phosphorescence, and is characterized by a relatively short phosphorescent lifetime of 4 msec[2] (our experiments, and Hélène *et al.*, 1968; Pochon *et al.*, 1971).

When s^4U phosphorescence is measured as a function of $TbCl_3$ concentration (Figure 6), it is observed that the addition of $TbCl_3$ to *E. coli* tRNA(I) in 10 mM sodium acetate (pH 5.0) results in an initial enhancement of s^4U phosphorescence emission after 1 mol of Tb(III) is added per mol of tRNA. After addition of approximately 2.5 mol of Tb(III)/mol of tRNA, s^4U emission is quenched to 50% of its original value (Figure 6). The fluorescence of s^4U-$tRNA^{fMet}$ (buffer I) also decreases upon the addition of $TbCl_3$ but quantitation is not easily attainable since s^4U fluorescence is obscured by the spectral overlap of Tb(III) fluorescence.

The striking similarities in the fluorescence excitation spectra of bound Eu(III) and Tb(III) ions and the corresponding decrease in s^4U phosphorescence and fluorescence with increased lanthanide ion fluorescence (λ_{ex} 345 nm) indicate that these ions are binding to tRNA in such a manner as to allow energy transfer to occur from s^4U to the bound ions.

Ultraviolet Irradiated E. coli tRNA. Further evidence for the existence of a Tb(III)-s^4U energy transfer complex in tRNA was obtained by modification of the s^4U residue. Irradiation of *E. coli* tRNA at 334 nm results in formation of an intramolecular dimer of s^4U and a nonadjacent cytosine (Favre *et al.*, 1971). The resulting photodimer does not exhibit an absorption maximum at 335 nm nor fluorescence emission at 510 nm (λ_{ex} 345 nm). Disappearance of the intact s^4U residue can therefore be followed by monitoring 335-nm absorbance and 510-nm fluorescence.

When *E. coli* $tRNA^{fMet}$, $tRNA^{Phe}$, $tRNA^{Glu}$, or $tRNA^{Ile}$ [3] in buffer I is irradiated at 340 nm in the presence of $TbCl_3$, the decrease in 335-nm absorbance of s^4U parallels the decrease in Tb(III) fluorescence due to excitation at 340 nm. Tb(III) fluorescence resulting from excitation at 300 and 410 nm remains unchanged (Figure 7). In the exceptional case of $tRNA^{Phe}$, a twofold increase in Tb(III) fluorescence due to excitation at 305 nm occurs after uv irradiation. $NaBH_4$ reduction of the s^4U photoproduct results in the appearance of a new highly fluorescent species (λ_{ex} 387 nm, λ_{em} 440 nm). However, addition of $TbCl_3$ to reduced $tRNA^{Glu}$ photoproduct does not elicit Tb(III) fluorescence due to excitation at 387 nm.

The decrease in Tb(III) fluorescence (λ_{ex} 345 nm) upon irradiation of s^4U thus further supports the conclusion that energy transfer between s^4U and Tb(III) is responsible for the enhanced fluorescence observed in Tb(III)-tRNA complexes.

Yeast $tRNA^{Phe}$ Complexes. Because of the absence of an s^4U residue in yeast $tRNA^{Phe}$ (Raj Bhandary *et al.*, 1967), this tRNA species could be used to investigate the s^4U-Tb(III) interaction by examining the fluorescent properties of Tb(III) ions when bound to a non-s^4U-containing tRNA. Upon the addition of 142 μM $TbCl_3$ to 11.1 μM $tRNA^{Phe}$ in 10 mM ammonium acetate (pH 5.0), Tb(III) fluorescence exhibits only one excitation band centered at 300 nm. There is a 300-fold increase in Tb(III) fluorescence (λ_{ex} 300 nm) when 10 mol of Tb(III) is added per mol of $tRNA^{Phe}$ relative to Tb(III) in aqueous solution (λ_{ex} 370 nm). No Tb(III) fluorescence is found upon excitation at 345 nm.

Extensive Dialysis of Lanthanide Ion-tRNA Complexes. In order to ascertain whether a diffusion-controlled process (Heller and Wasserman, 1965) dominates the interaction between s^4U and lanthanide ions, the Eu(III)- and Tb(III)-tRNA complexes were dialyzed extensively to remove any weakly bound lanthanide ions. tRNA II or III (1 mg/ml) was incubated for 30 min at room temperature with a ten-

[2] Although all aspects of s^4U luminescence are not fully understood, the 460-nm emission (τ = 4 msec) and 510-nm emission ($\tau \leq 2$ nsec) will be referred to as phosphorescence and fluorescence, respectively.

[3] A minor species of $tRNA^{Ile}$ has recently been isolated which has a s^4U residue located at the number 8 position in the polynucleotide chain (Harada and Nishimura, 1974).

fold molar excess of $TbCl_3$ or $EuCl_3$ in 30 mM Tris-HCl (pH 7.0) or 10 mM ammonium acetate (pH 5.0). Excess lanthanide ions were removed by dialysis for approximately 16 hr at 4° against the same buffer. The intensity of Tb(III) fluorescence (λ_{ex} 345 nm) remaining in the tRNA complex is equivalent to the value measured when 5 mol of $TbCl_3$ is added per mol of tRNA. Tb(III) fluorescence excitation at 300 nm is also retained after dialysis, and in some samples the fluorescence intensity increases relative to that excited at 345 nm.

The fluorescence intensity of Eu(III) ions (λ_{ex} 345 nm) which remain bound to $tRNA^{Met}$ after 16 hr of dialysis corresponds to that observed when 2 mol of Eu(III) has been added per mol of tRNA.

Other Properties of Tb(III)-tRNA Complexes

Interaction with the Common Bases of E. coli tRNA. Previous studies indicate that Tb(III) and Eu(III) ions will form energy transfer complexes with the common nucleotide monophosphates, AMP, CMP, UMP, and GMP (Formoso, 1973; Lamola and Eisinger, 1971), which absorb energy in the 250–300-nm region of the spectrum. The 300-nm Tb(III) excitation peak observed with *E. coli* and yeast tRNA may therefore be due to the interaction of Tb(III) ions with the common base residues in tRNA. The 300-nm excitation band may be a discrete excitation band or may represent only a part of a larger band which is obscured due to the strong absorption properties of tRNA in the 250–280-nm region of the spectrum.

In order to determine if Tb(III) ions are interacting with the common base residues in tRNA, tRNA phosphorescence due to adenine and guanine residues (Steiner *et al.*, 1967) was measured as a function of $TbCl_3$ concentration. Upon the addition of 2.2 mM $TbCl_3$ to 0.18 mM *E. coli* tRNA(I) (buffer II), 89% of purine phosphorescence (λ_{ex} 280 nm, λ_{em} 410 nm) was quenched. Thus, Tb(III) ions affect the luminescent properties of the common purine base residues, in addition to their effect on s^4U.

The 410-nm excitation band observed upon the binding of Tb(III) to $tRNA^{Ile}$ (*cf.* Figure 7) is analogous to that observed for an AMP-Tb(III) complex (Formoso, 1973). Preliminary experiments indicate that the 410-nm excitation band is a characteristic band of the unbound Tb(III) ion and that the resulting Tb(III) emission is enhanced several hundred fold upon the interaction of Tb(III) ions with tRNA.

Interaction of the Y Base of Yeast $tRNA^{Phe}$. Yeast $tRNA^{Phe}$ contains an uncommon fluorescent base, Y, which is located directly adjacent to the anticodon (Raj Bhandary *et al.*, 1967). Its excitation and emission maxima occur at 318 and 445 nm, respectively (Yoshikami *et al.*, 1968). The addition of a tenfold excess of $TbCl_3$ to 11.1 μM $tRNA^{Phe}$ in 10 mM ammonium acetate (pH 5.0) results in a twofold enhancement in Y-base fluorescence. This is comparable to the effect observed upon binding of Mg(II) ions to $tRNA^{Phe}$ (Robinson and Zimmerman, 1971). No energy transfer from Y base to bound Tb(III) was observed.

Addition of an excess of Mg(II) ions (14 mM) to a solution of 0.14 mM $TbCl_3$, 0.011 mM $tRNA^{Phe}$, and 10 mM ammonium acetate (pH 5.0) causes a 50% decrease in Tb(III) fluorescence (λ_{ex} 300 nm) but no significant change in enhanced Y-base fluorescence. Thus, in the case of $tRNA^{Phe}$, Mg(II) ions will displace bound Tb(III) ions, but the overall effect on Y-base fluorescence remains the same.

Effect of Mg(II) Ions on Tb(III)- and Eu(III)-tRNA Complexes. It has been reported that tRNA possesses both weak and tight binding sites for Mn(II) (Danchin and Guéron, 1970) and Mg(II) ions (Schreier and Schimmel, 1974). It was therefore of interest to determine if Mg(II) ions could effectively compete with bound lanthanide ions for the metal ion binding sites on tRNA.

When $MgCl_2$ is added to a solution of extensively dialyzed Tb(III)-tRNA in buffer I, in which the Tb(III) fluorescence corresponds to 5 mol of Tb(III) added per mol of tRNA, a 300-fold excess of Mg(II) ions is required to reduce Tb(III) fluorescence (λ_{ex} 345 nm) by 10%. Likewise, a 40-fold excess of Mg(II) ions is required to decrease Eu(III) fluorescence by 10% when 2 mol of tightly bound Eu(III) is present per mol of tRNA. The results indicate that Mg(II) ions have little or no effect on the s^4U-Tb(III) complex. This may be due to either the inability of Mg(II) to compete effectively with bound lanthanide ions for the same binding site or to the presence of independent Mg(II) and Tb(III) ion binding sites.

Effect of Other Metal Ion-tRNA Complexes on s^4U Luminescence. Since tRNA binds a variety of metal ions, it was of interest to determine the effect of these metal ions on s^4U luminescence and to compare these effects to those observed in the presence of $TbCl_3$ and $EuCl_3$. In agreement with previous reports (Pochon and Cohen, 1972), a threefold enhancement in s^4U fluorescence is observed after 10 mol of Mg(II) is added per mol of *E. coli* tRNA(II) in buffer I. However, no change in s^4U phosphorescence intensity is observed at 77°K in buffer II under similar conditions.

The lanthanide ions, Sm(III), Yb(III), and Gd(III), which do not exhibit luminescent properties in aqueous solution, also enhance s^4U fluorescence. Preliminary results indicate a threefold enhancement of s^4U fluorescence when 0.5 mol of Yb(III) or 0.25 mol of Gd(III) is added per mol of tRNA and approximately a twofold enhancement when 0.25 mol of Sm(III) is present per mol of tRNA.

The effect of the paramagnetic Mn(II) ion on s^4U luminescence is quite similar to that observed upon the addition of Tb(III) and Eu(III) ions to tRNA. Upon addition to tRNA, Mn(II) quenches both s^4U emission at 460 and 510 nm. When the decrease of s^4U phosphorescence is measured as a function of Mn(II)-tRNA, a titration curve is obtained which is quite similar to that observed for Tb(III) ions under similar conditions (Figure 6). Likewise, addition of 60 μM $MnCl_2$ to 28 μM tRNA(II) results in quenching of 50% of s^4U fluorescence, which is comparable to that observed in the presence of Eu(III) ions (*cf.* Figure 4B). Complete quenching of 410-nm emission is achieved at a Mn(II)/tRNA ratio of 6/1.

Discussion

The fluorescent lanthanide ions, Tb(III) and Eu(III), in aqueous solution exhibit sharp excitation bands in the uv region and corresponding emission lines in the visible region of the spectrum. Upon addition of these trivalent ions to unfractionated *E. coli* tRNA, their excitation spectra are completely altered while their emission spectra remain the same. Both ions now have a broad excitation band at 345 nm and no excitation at wavelengths characteristic of Tb(III) and Eu(III) in aqueous solution. Alterations in excitation spectra are accompanied by a 300–500-fold increase in fluorescence intensity. This marked change in the excitation spectrum is indicative of the formation of an energy transfer complex (Weissman, 1942) consisting of lan-

thanide ions and a chromophore which absorbs energy at 345 nm.

A modified purine base, 4-thiouridine, present in 50% of all *E. coli* tRNA species (Lipsett, 1965) exhibits an absorption band at 335 nm which corresponds to the excitation spectra observed for Tb(III) and Eu(III) ions when bound to *E. coli* tRNA. Excitation of s^4U containing tRNA at 340 nm results in s^4U phosphorescence (460 nm) and fluorescence (510 nm)[2] (Hélène *et al.*, 1968; Pochon *et al.*, 1971). With one exception, there is only one s^4U residue per tRNA and it is always located in the same position in the polynucleotide chain.

The observed quenching of s^4U phosphorescence and fluorescence upon the addition of Tb(III) or Eu(III), and the concomitant increase in Tb(III) and Eu(III) fluorescence intensity upon excitation at the absorption maximum for s^4U, support the conclusion that one or more of the lanthanide ions are forming an energy transfer complex with the 4-thiouridine residue of tRNA. Purified $tRNA^{Phe}$, $tRNA^{fMet}$, and $tRNA^{Glu}$ all contain an s^4U base residue, and the Tb(III) and Eu(III) complexes of these tRNAs show the fluorescence energy transfer phenomenon. On the other hand, energy transfer is not observed for the Tb(III) and Eu(III) complexes of purified yeast $tRNA^{Phe}$, which does not contain s^4U. Further evidence of complex formation is adduced from the observation that destruction of s^4U by uv irradiation results in the disappearance of bound Tb(III) fluorescence.

The Tb(III)- and Eu(III)-s^4U energy transfer interaction may arise from ions directly bound to tRNA (Weissman, 1942) or from collisional processes (Heller and Wasserman, 1965). Extensive dialysis of the Tb(III)- and Eu(III)-tRNA solutions against low ionic strength buffer fails to eliminate the fluorescence excitation or emission spectra of the complexes. Therefore, the energy transfer process must involve lanthanide ions which are tightly bound to tRNA.

Difficulties are encountered in attempting to determine the number of lanthanide ions bound in the s^4U energy transfer complex because the insolubility of these ions permits only a limited concentration range to be covered in titrations. For Tb(III), saturation of fluorescence intensity was not reached but in the case of Eu(III) saturation was approached and analysis of the data by the slope-ratio method yielded a value of four for the number of binding sites. On the assumption that there is no interaction between the four binding sites, the apparent dissociation constant for each of the four metal ions bound was calculated to be 6.5 ± 2.2 μM. A comparison of the theoretical titration curve based on this equilibrium of four independent, equivalent sites with the experimental data (Figure 4A) suggests that there may be some cooperativity among the binding sites. However, further analysis is not warranted since the slight amount of curvature observed in the titration of *E. coli* tRNA with Tb(III) (Figure 2) and Eu(III) (Figure 4A) occurs at low cation concentrations where precise measurements of the fluorescence intensity are difficult. Cooperative binding has been observed in the more extensive investigations of Mn(II) and Mg(II) binding to tRNA (Danchin and Guéron, 1970; Lynch and Schimmel, 1974).

It is unlikely that all four lanthanide ions interact equally with s^4U but rather that only one site is the dominant energy acceptor. However, if this binding site has an affinity comparable to that of the three other tight binding sites, 4 mol of Eu(III) is required to achieve complete occupancy of the dominant interacting site (*cf.* Figure 4A).

The initial enhancement of s^4U fluorescence and phosphorescence upon the addition of 0.5–1.0 mol of Eu(III) or Tb(III) per mol of tRNA may be ascribed to the same phenomenon as observed with the nonfluorescent lanthanide ions. Binding of the latter to tRNA results in enhanced s^4U fluorescence. Only after a significant fraction of the binding sites involved in energy transfer is occupied does the quenching effect of Tb(III) or Eu(III) overcome the enhancement effect, thus causing the total overall fluorescence intensity to decrease.

In addition to the energy transfer complexes formed by Tb(III) with s^4U in *E. coli* tRNA, Tb(III) fluorescence is excited at wavelengths (λ_{ex} 300 nm) which are characteristic of complex formation with the common base residues of tRNA, *i.e.* AMP, GMP, UMP, and CMP (Lamola and Eisinger, 1971; Formoso, 1973). The 300-nm excitation band may represent only a small portion of a large excitation band which is obscured by the presence of tRNA which absorbs strongly in the 250–280-nm region of the spectrum. The absence of a Eu(III) excitation band in the region of common base residue absorption (250–300 nm) may also be due to the tRNA absorption. Indeed, previous investigators have studied Eu(III)–nucleotide energy transfer complexes using 265-nm excitation (Lamola and Eisinger, 1971).

A further indication of Tb(III) ion complex formation with the common nucleotide residues in tRNA is the quenching of adenine and guanine phosphorescence in tRNA upon the addition of Tb(III) cations. The phenomenon involves direct binding of Tb(III) ions to tRNA since the quenching persists after extensive dialysis. At a tenfold excess of Tb(III) to yeast $tRNA^{Phe}$ there is a 300-fold enhancement of Tb(III) fluorescence upon excitation at 300 nm. The enhancement is comparable to that observed for the Tb(III)–s^4U complex upon excitation at 340 nm. Since the contribution of the common base type of interaction to overall Tb(III) fluorescence varies with the tRNA species and the experimental conditions (*cf.* Figure 1, Figure 6), Tb(III) may prove a useful general probe of tRNA conformation.

A threefold enhancement of s^4U fluorescence upon addition of 10 mol of Mg(II)/mol of *E. coli* tRNA has been reported by Pochon and Cohen (1972). A similar enhancement is observed with the nonfluorescent lanthanide ions, Yb(III), Sm(III), and Gd(III) but at cation/tRNA ratios of less than 1.

The paramagnetic Mn(II) ion is more effective in quenching fluorescence and phosphorescence than are the paramagnetic lanthanide ions (Rahn *et al.*, 1966). Mn(II) quenches 50% of s^4U fluorescence and phosphorescence at a Mn(II)/tRNA ratio of 2.5. At this Mn(II)/tRNA ratio, very little of the Mn(II) remains free in solution (Danchin and Guéron, 1970). Thus, quenching of s^4U fluorescence and phosphorescence by Mn(II) arises from Mn(II) directly bound to tRNA. All of these experiments support the hypothesis that one or more tightly bound divalent or trivalent cations reside in the vicinity of the s^4U residue of tRNA. That this region of the tRNA molecule is involved in cation binding in tRNAs without s^4U is indicated by results obtained in the X-ray analysis of yeast $tRNA^{Phe}$ (Kim *et al.*, 1972). Thus, in crystals of $tRNA^{Phe}$, a Sm(III) ion is bound in the vicinity of the uridine residue at position 8 of the polynucleotide chain (A. Rich, 1973, personal communication).

Interactions between specific chromophores on the tRNA molecule such as reported here for lanthanides and s^4U should be useful probes of the structure of tRNA in complexes with aminoacylating enzymes and with ribosomes as well. Studies with aminoacylating enzymes are in progress.

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