Terbium(III) and Europium(III) Ions as Luminescent Probes and Stains for Biomolecular Systems

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Contents

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I. Introduction

Luminescence spectroscopy is widely used in the study of biomolecular structure. Applications include the study of (1) discrete equilibrium structures under specific physical and chemical conditions, (2) distributions of structure types in heterogeneous systems, (3) changes in structure and in distributions of structure types as a function of varying conditions, (4) biomolecule–solvent interactions, and (5) the binding of ligands (including metal ions) to biomolecular systems. When the system contains a luminescent *constituent* chromophore (or a set of such chromophores), the *intrinsic* luminescence of the system may be used to elicit structural information. When this condition is not satisfied, a luminescent molecule or ion may be bound to the system, providing an *extrinsic* luminescent probe or stain for examining structure. In some cases, it is advantageous to monitor simultaneously the intrinsic *and* extrinsic luminescence from a biomolecule–ligand (lumiphore) complex. The latter type of study is especially useful for investigating possible structural perturbations caused by the attached (extrinsic) lumiphore and for investigating spectroscopic interactions between the intrinsic and extrinsic luminescent chromophores. An example of a *spectroscopic* interaction would be a coupling betwee the vibronic states of the intrinsic and extrinsic lumiphores leading to nonradiative (electronic) energy transfer between the two types of chromophores. In general, such an interaction is manifested by an observed quenching of the donor species' luminescence and an enhancement of the acceptor species' luminescence.

Site specificity is extremely important in the selection and use of extrinsic lumiphores for investigating biomolecular structure. Different sites probe different aspects of structure and, in many cases, function. Furthermore, the location and chemical nature of the binding sites often have pronounced effects on the luminescence properties of the extrinsic lumiphore. A great deal of progress has been made in the design of organic-type molecules and ions suitable for use as extrinsic lumiphores in studies on a wide variety of biomolecular systems. In some cases these lumiphores are designed to be covalently attached at specific sites on the host system, and in other cases they bind via purely ionic linkages or they associate via weak nonbonding type interactions. In general, these organic lumiphores are *not* used as *substitutional* luminescent probes, although in some instances their structural and binding characteristics are chosen to mimic those of known, biologically important substrates for the host system.

Until relatively recently there has been little use made of metal ion luminescence in the study of biomolecular structure. The reason for this is that among the metal ions known to be essential or important to biological structure and function, *none* are luminescent under biological conditions. Furthermore, few of the metal ions and metal ion complexes used as absorption or magnetic resonance probes for biomolecular systems are luminescent in solution media at temperatures above 77 K. This includes nearly all of the transition-metal ions and their complexes. In fact, in many cases these metal ions act as quenchers of intrinsic biomolecular luminescence when bound close to the intrinsic lumiphores in the host system. The most prominent exceptions to this nonluminescent behavior of metal ions are trivalent europium (Eu^{3+}) and terbium (Tb^{3+}). Both Eu^{3+} and Tb^{3+} are luminescent in aqueous solution, and both generally retain their luminescence when bound to complex ligand systems. Furthermore, both exhibit multiple emissions (due to several electronic transitions) whose relative intensities and line splitting

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patterns (band structuring) are sensitive to the detailed nature of the ligand environment about the metal ion.

The luminescence properties of Eu^{3+} and Tb^{3+} make these ions obvious candidates for use as luminescent probes of metal binding in biomolecular systems. However, to be useful in this role it is also necessary that their coordination chemistry be compatible with the metal ion binding properties of the system to be studied, and it is desirable that their binding properties exhibit some degree of site selectivity. These latter criteria *are* satisfied for Eu^{3+} and Tb^{3+} in a wide variety of biomolecular systems, and this has led in recent years to considerable interest in the use of Eu^{3+} and Tb^{3+} as luminescent probes and stains for investigating biomolecular structure.[1-3]

The principal objective of this review is to give a composite picture and comparative analysis of the spectroscopic properties and techniques most relevant to the use of Eu^{3+} and Tb^{3+} as luminescent probes of biomolecular structure. No overview account of this field has yet appeared in the literature, yet such an account is important to selecting the proper techniques to apply in solving specific structure problems. All of the techniques discussed in this review have been applied, in our laboratory and in others, to a wide variety of systems (in crystals and glasses as well as in solution media). Although emphasis is placed on the general aspects of the field, specific examples of applications are also included. We shall first examine the coordination properties of Eu^{3+} and Tb^{3+} and discuss how these properties may be exploited in the study of various types of biomolecular systems. Second, we shall discuss those aspects of Eu^{3+} and Tb^{3+} luminescence and excitation spectra that are relevant to the use of these ions as luminescent probes and stains. Finally, we shall summarize and compare the measurement techniques most suitable to this purpose and present examples of a variety of specific applications.

II. Coordination Properties of Eu^{3+} and Tb^{3+}

All of the trivalent lanthanide ions (Ln^{3+}) share a number of common coordination properties. Their coordination chemistry exhibits some variability across the lanthanide series, but this variability is much less pronounced than is found among transition-metal ions. All of the Ln^{3+} ions may be classified as type "a" cations in the Ahrland, Chatt, and Davies classification scheme[4] and as "hard" acids in the Pearson classification scheme.[5] Therefore, it is expected (and observed) that among the donor atoms most commonly found in biomolecular systems the binding preference is O > N > S. Furthermore, it is generally agreed that Ln^{3+}–ligand coordination occurs predominantly via ionic bonding interactions, leading to a strong preference for negatively charged donor groups that are also "hard" bases. Water molecules and hydroxide ions (OH^-) are particularly strong Ln^{3+} ligands, so that in aqueous solution only ligands containing donor groups having negatively charged oxygens (as, for example, in carboxylate moieties) can bind strongly. In aqueous solution, donor groups containing neutral oxygen or nitrogen atoms generally bind (or occupy Ln^{3+} coordination sites) *only* when present in multidentate ligands that contain at least one or two other donor groups having negatively charged oxygens. Exceptions to the latter condition occur (e.g., in the binding of carbohydrates and nucleosides to Ln^{3+} ions), but in these cases the binding is invariably observed to be very weak in aqueous solution. Lanthanide complexes prepared under anhydrous conditions are generally found to undergo at least partial hydrolysis in the presence of even small amounts of water.

The predominantly ionic character of Ln^{3+}–ligand interactions and the relatively low charge-to-ionic radius ratios (ionic potentials) of the Ln^{3+} ions can account, in large part, for several additional aspects of lanthanide coordination chemistry. First, there is little or no "directionality" in the Ln^{3+}–ligand interactions so that primary coordination numbers and complex geometries are determined almost entirely by ligand characteristics (conformational properties and the number, sizes, and charged nature of donor groups). The only Ln^{3+} property of importance in this regard is the ionic radius. Lanthanide complexes exhibit coordination numbers ranging from six to twelve (in the solid state and in solution), with eight and nine being the most common.[6,7] In aqueous solution, the aquo ions are believed to have primary hydration numbers ranging from nine (at the beginning of the series) to eight (at the end of the series). The "average" primary hydration numbers for Eu^{3+} and Tb^{3+} in water are estimated to be *between* eight and nine.[8,9] The geometries found for lanthanide complexes are quite varied, being determined principally by ligand conformation, ligand donor group–donor group interactions, competition between ligand donor groups and solvent molecules for available coordination sites, crystal packing forces (in the solid state) and, in some cases, the size of the Ln^{3+} ion.[6,7]

The coordination properties most germane to the study of Ln^{3+}–biomolecular interactions are summarized in Table I. Eu^{3+} and Tb^{3+} exhibit all of the general lanthanide coordination properties listed here. Their respective solution chemistries *do* show some differences,[7] but these differences are small and are not likely to be of any significant consequence in their binding to biomolecular systems. With respect to Eu^{3+} and Tb^{3+} binding to biomolecular systems, the following points are of special importance:

(*1*) *Site Selectivity.* The preferred binding sites will be those with donor groups containing negatively charged oxygen atoms (e.g., carboxylate or phosphate groups). In aqueous solution, only these types of sites can effectively compete with water molecules for Ln^{3+} coordination.

(*2*) *Size and Geometry of Binding Sites.* In complexes of 8-fold or 9-fold coordination, Eu^{3+} and Tb^{3+} are generally found to have *effective* ionic radii of ~1.07–1.12 Å (for Eu^{3+}) and ~1.04–1.10 Å (for Tb^{3+}).[10] These numbers provide estimates of the size requirements for Eu^{3+} and Tb^{3+} binding sites in complex biomolecular systems. No strong preference is expected for site symmetry or geometry except that it be compatible with the donor atom preference of the Ln^{3+} ion.

(*3*) *Binding Strengths.* Binding strengths are expected to be much less than those of transition-metal ions but slightly greater than those of alkali and alkalline earth metal ions.

(*4*) *Site Distortions.* Given the low polarizing ability of Ln^{3+} ions and the ionic nature of their interactions with ligands, it is unlikely that they would induce sig-

TABLE I. Major Coordination Properties of Trivalent Lanthanide Ions

A. strong preference for negatively charged donor groups which are also "hard" bases; with neutral ligands, the order of preference for donor atoms is O > N > S

B. lack of "directionality" in the predominantly ionic Ln^{3+}-ligand interactions, leading to stereochemical preferences within the inner coordination sphere being determined almost entirely by ligand characteristics (conformational properties and the number, sizes, and charged nature of donor groups) and solvation effects

C. variable coordination numbers ranging from six to twelve (in the solid state and in solution), with eight and nine being most common;[a] for complexes of a given structure type, variability of coordination numbers among *different* Ln^{3+} ions is generally ±1, reflecting differences in ionic radii across the lanthanide series

D. water molecules and hydroxide ions (OH^-) are strongly coordinating ligands
   1. in neutral to basic aqueous solution, only ligands containing donor groups having negatively charged oxygens can bind sufficiently strongly to prevent $Ln(OH)_3$ precipitation
   2. in aqueous solution, neutral oxygen or nitrogen ligand donor atoms generally bind (or occupy Ln^{3+} coordination sites) *only* when present in multidentate ligands that contain at least one or two other donor groups having negatively charged oxygens
   3. lanthanide complexes are highly labile in aqueous solution
   4. in the absence of strongly coordinating ligands, the "average" primary hydration numbers for Eu^{3+} and Tb^{3+} in water are estimated to be *between* eight and nine[b]
   5. lanthanide complexes prepared under anhydrous conditions are generally found to undergo at least hydrolysis in the presence of even small amounts of water

[a] See ref 6 and 7. [b] See ref 8 and 9.

nificant changes in the electronic charge distributions at binding sites. However, mechanical type distortions required to accommodate steric interactions in the coordination site are possible.

The points discussed above suggest that Eu^{3+} and Tb^{3+} should bind to a wide variety of systems of biological interest and that this binding should exhibit a high degree of site selectivity. Furthermore, this binding is not expected to result in major perturbations on the inherent structural features of complex host systems. Finally, most of the coordination properties cited above for Ln^{3+} ions are recognized as being very similar to those characteristic of divalent alkaline earth metal ions (especially Ca^{2+}). In fact, most of the recent interest in Ln^{3+} ions as biomolecular structure probes has focused on their use as *substitutional* (or *replacement*) probes for Ca^{2+} and, in a few cases, Mg^{2+}.[1-3,11] Of special interest is the observation that biological activity is often retained, at least partially, by systems in which Ca^{2+} has been replaced by Ln^{3+} ions.[2,3,11] Ca^{2+} and Mg^{2+} are two of the most abundant metal ions found in living systems, yet investigations of their binding and transport properties are inhibited by their lack of spectral characteristics appropriate for structure elucidation. Their isomorphous replacement by Ln^{3+} ions holds out the promise of exploiting the rich and varied spectroscopic properties of the Ln^{3+} ions for obtaining information on the structural aspects of Ca^{2+} and Mg^{3+} biochemistry.

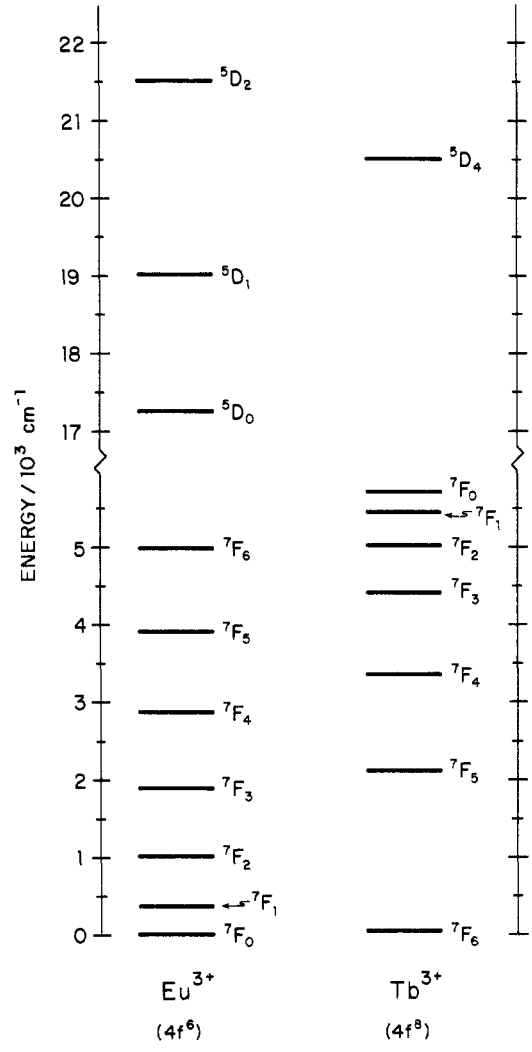


Figure 1. Energy level diagram depicting the approximate locations of baricenters for the lowest-energy multiplet levels of Eu^{3+} ($4f^6$) and Tb^{3+} ($4f^8$). Each level is labeled according to its principal $^{2S+1}L_J$ Russell-Saunders component.

III. Luminescence Properties of Eu^{3+} and Tb^{3+} in Solution Media

The lowest-energy multiplets associated with the $4f^6$ electronic configuration of Eu^{3+} and the $4f^8$ electronic configuration of Tb^{3+} are shown in Figure 1 (each labeled according to its dominant $^{2S+1}L_J$ component). For europium(III) complexes in aqueous solution, essentially all emission emanates from the nondegenerate $^5D_0$ level when excitation is at $\bar{\nu} > 17\,300$ cm^{-1}. The strongest emissions are invariably observed in the $^5D_0 \rightarrow {}^7F_1$ and $^7F_2$ transition regions, and $^5D_0 \rightarrow {}^7F_4$ emission is frequently observed to have a moderately strong intensity. Emission intensities in the remaining $^5D_0 \rightarrow {}^7F_J$ transition regions are generally either very weak or unobservable. The *relative* intensities of the $^5D_0 \rightarrow {}^7F_1$ and $^7F_2$ emissions are very sensitive to the detailed nature of the ligand environment, reflecting the *hypersensitive* character of the $^5D_0 \rightarrow {}^7F_2$ transition. Even in low-symmetry systems the $^5D_0 \rightarrow {}^7F_1$ transition retains its magnetic dipole character, and its radiative transition probability is not much affected by the ligand environment. In contrast, the $^5D_0 \rightarrow {}^7F_2$ and $^7F_4$ transitions are predominantly electric dipole in character, and their radiative transition probabilities are very sensitive to the detailed nature of the ligand environment. The $^5D_0$

TABLE II. Major Characteristics of Tb^{3+} and Eu^{3+} Emission Intensity Spectra for Complexes in Aqueous Solution[a]

transition	spectral region, nm	relative intensities	optical activity[b]	other characteristics
terbium				
$^5D_4 \rightarrow {}^7F_6$	485-500	medium-strong	medium	intensity shows moderate sensitivity to ligand environment
$\rightarrow {}^7F_5$	540-555	strongest	strongest	very strong and exhibits some structuring under high resolution; *best probe transition*
$\rightarrow {}^7F_4$	580-595	medium	medium	intensity shows moderate sensitivity to ligand environment
$\rightarrow {}^7F_3$	615-625	medium-weak	strong	exhibits some structuring under high resolution
$\rightarrow {}^7F_2$	645-655	weak	weak	intensity shows moderate sensitivity to ligand environment
europium				
$^5D_0 \rightarrow {}^7F_0$	578-580	weak	very weak	nondegenerate transition; appears as a single, sharp line *except* when the sample contains a heterogeneous population of Eu^{3+} emitting species
$\rightarrow {}^7F_1$	585-600	strongest	strongest	strong magnetic dipole character; generally sharp and structured under high resolution
$\rightarrow {}^7F_2$	610-630		medium	intensity exhibits *hypersensitivity* to ligand environment
$\rightarrow {}^7F_3$	645-660	weak	weak	always very weak
$\rightarrow {}^7F_4$	680-705	medium	weak	intensity and structuring very sensitive to ligand environment

[a] Low-symmetry or noncentrosymmetric structures are assumed. [b] When bound to chiral ligands.

$\rightarrow {}^7F_0$ transition is formally allowed *only* in low-symmetry systems, and it acquires its intensity via an electric dipole mechanism. Although $^5D_0 \rightarrow {}^7F_0$ emission is generally observed to be relatively weak, its intensity is quite sensitive to the details of the ligand environment.

For europium(III) complexes in *nonaqueous* solutions, it is often possible to observe emissions from the $^5D_1$ and $^5D_2$ levels when excitation is at $\bar{\nu} > 19\,100$ cm^{-1} (for $^5D_1$ emission) or $\bar{\nu} > 21\,500$ cm^{-1} (for $^5D_2$ emission). Although these emissions are generally weak compared to those originating from the $^5D_0$ level, their presence can complicate the interpretation of emission decay results and spectral slitting patterns.

For terbium(III) complexes in aqueous solution, essentially all emission emanates from the $^5D_4$ level when excitation is at $\bar{\nu} > 20\,400$ cm^{-1}. Emission can be seen in each of the $^5D_4 \rightarrow {}^7F_J$ (J = 0, 1, 2, 3, 4, 5, or 6) transition regions, but invariably the most intense emission is observed in the $^5D_4 \rightarrow {}^7F_5$ transition region occurring around 540–555 nm. The $^5D_4 \rightarrow {}^7F_{0,1}$ emissions are always weak, and the relative intensities of the remaining emissions fall in the order $^5D_4 \rightarrow {}^7F_6 > {}^7F_4 > {}^7F_3 > {}^7F_2$. Under high resolution, the room temperature emission spectra show some structuring in each of the transition regions, with the $^5D_4 \rightarrow {}^7F_5$ and $^7F_3$ regions exhibiting the sharpest structure. This structuring within the $^5D_4 \rightarrow {}^7F_J$ emission bands is sensitive to the detailed nature of the ligand environment, but it does not provide the basis for a *reliable* diagnostic probe of complex structure. The *relative* intensities of the $^5D_4 \rightarrow {}^7F_J$ emissions are also sensitive (but not *hypersensitive*) to the detailed nature of the ligand environment, with the $^5D_4 \rightarrow {}^7F_6$, $^7F_4$, and $^7F_2$ emissions showing the greatest sensitivity. The ligand-modulated $^5D_4 \rightarrow {}^7F_J$ emission intensities and band structuring can be attributed to ligand-field-induced changes in the inherent transition probabilities of the respective $^5D_4 \rightarrow {}^7F_J$ transition processes.

When the ligand environment about either Eu^{3+} or Tb^{3+} is chiral or contains chiral centers, the $^5D_J \rightarrow {}^7F_J$ transitions each exhibit some partial circular polarization in their luminescence. That is, the luminescence contains unequal amounts of left- and right-circularly polarized light. This phenomenon is generally referred to as either emission optical activity or circularly polarized luminescence (CPL). CPL is the emission analogue of circular dichroism, and it probes the chirality and natural optical activity of the emitting system.[12] The largest degree of circular polarization is invariably observed in the $^5D_0 \rightarrow {}^7F_1$ transition of Eu^{3+} and the $^5D_4 \rightarrow {}^7F_5$ and $^7F_3$ transition regions of Tb^{3+}, reflecting the relatively strong magnetic dipole nature of these transitions.[13-19] (Recall that in isotropic samples the optical activity or chiroptical strength of an electronic transition is governed by the scalar product of its electric and magnetic dipole transition vectors.[12,16,17]) Europium(III) and terbium(III) CPL spectra exhibit an extraordinary sensitivity to the stereochemical characteristics of the ligand environment,[20-28] although *detailed* spectra–structure relationships remain in a rather primitive state of development.[16-19] Since nearly all systems of biological interest have some degree of chirality, CPL is an especially attractive probe for investigating Eu^{3+}- and Tb^{3+}-biomolecular interactions.[29-32]

In Table II are listed those characteristics of Tb^{3+} and Eu^{3+} emission intensity spectra most relevant to the study of Ln^{3+}–biomolecular systems. Among the Tb^{3+} transitions, $^5D_4 \rightarrow {}^7F_5$ is clearly the best probe transition. It is remarkably intense under a wide variety of solution conditions, and it exhibits an especially strong emission optical activity when Tb^{3+} is bound to chiral ligands.

The most important probe transitions for Eu^{3+} complexes are $^5D_0 \rightarrow {}^7F_1$ and $^7F_2$. In addition to being the most intense, their emission splitting patterns remain relatively simple even in low-symmetry systems. Since $^5D_0$ is nondegenerate, no "hot" crystal field transitions can contribute to the $^5D_0 \rightarrow {}^7F_J$ emission spectra (in contrast to what happens in the $^5D_4 \rightarrow {}^7F_J$ emission spectra of Tb^{3+} complexes). For systems in which there is only one type of Eu^{3+} binding site or only one type of Eu^{3+} complex, the $^5D_0 \rightarrow {}^7F_1$ emission band can split at most into just three components and the $^5D_0 \rightarrow {}^7F_2$ emission band can split at most into just five components (assuming the absence of any resolved vibronic lines).[33,34] The number, energy spacings, and relative intensities of these components can, in principle, provide detailed information about the symmetry and structural nature of the Eu^{3+} coordination site (i.e., the "crystal field" about the Eu^{3+} ion can be character-

TABLE III. Major Characteristics of Tb^{3+} and Eu^{3+} Emission Excitation Spectra for Complexes in Aqueous Solution

excitation mode	major characteristics	
	Tb^{3+} complexes	Eu^{3+} complexes
direct excitation in the near-ultraviolet and visible regions in the *absence* of suitable ligand energy donors	(1) excitation and emission spectra are qualitatively similar to those observed for Tb^{3+}(aquo); quantitative differences may be attributed to crystal field effects (2) excitation spectra mimic (qualitatively) the absorption spectrum of Tb^{3+}(aquo)	(1) excitation in the $^7F_0 \rightarrow {}^5D_{0,1,2}$ absorption regions (visible) yields spectra qualitatively similar to those observed for Eu^{3+}(aquo); the visible excitation spectra mimic the absorption spectra (2) near-ultraviolet excitation spectra may or may not mimic the near-ultraviolet absorption spectra, depending upon where the ligand-to-Eu^{3+} CT states are located and how they relax (3) $^7F_0 \rightarrow {}^5D_0$ excitation line splitting can be used to probe heterogeneity in the Eu^{3+} population of the sample
indirect excitation via ligand-to-Ln^{3+} electronic energy transfer	(1) Tb^{3+} has suitable energy acceptor levels throughout the 26 000-42 000-cm^{-1} region (as well as the $^5D_4$ acceptor level at ~ 20 000-21 000 cm^{-1}); energy transfer to any of these levels is effective in sensitizing $^5D_4 \rightarrow {}^7F_J$ emission (2) sensitization of $^5D_4 \rightarrow {}^7F_J$ emission by energy transfer from strongly absorbing ligands containing near-ultraviolet or blue-visible lumiphores leads to large enhancements of emission intensity (over what is observed for Tb^{3+} aquo ions) (3) excitation spectra mimic the absorption spectra when the absorbing chromophores in the ligand environment are also the energy donor species *or* are strongly coupled to the energy donor species	(1) Eu^{3+} has suitable energy acceptor levels throughout the near-ultraviolet and visible regions (at $\bar{\nu} > 17\,300$ cm^{-1}); energy transfer to the $^5D_2$, $^5D_1$, or $^5D_0$ levels will generally lead to sensitization and enhancement of $^5D_0 \rightarrow {}^7F_J$ emission; however, energy transfer to a CT state generally does not sensitize $^5D_0 \rightarrow {}^7F_J$ emission, being lost instead by thermal relaxation to the ground state (2) when the CT state is the principal acceptor level, the near-ultraviolet absorption and excitation spectra will bear little resemblance to one another

ized).[33,34] The appearance of more than three components in the $^5D_0 \rightarrow {}^7F_1$ emission region or five components in the $^5D_0 \rightarrow {}^7F_2$ emission region is diagnostic of sample heterogeneity with respect to the types of Eu^{3+} complexes or Eu^{3+} binding sites present. When it can be observed with sufficient resolution, the $^5D_0 \rightarrow {}^7F_0$ emission provides even a better diagnostic probe for Eu^{3+} coordination heterogeneity. This emission can exhibit multiple lines *only* when there are at least two different types of Eu^{3+} species present in the sample. Some caution must be exercised in using $^5D_0 \rightarrow {}^7F_{0,1,2}$ line splittings as diagnostic probes. The observation of line splittings *always* carry information; however, the failure to observe such splittings may be due to spectral resolution limitations rather than to inherent structural properties of the system.

The luminescence lifetime of the $^5D_0$ emitting state of europium(III) complexes in aqueous solution, denoted by $\tau_{Eu}(^5D_0)$, generally falls in the 0.1–1.0-ms range. The luminescence lifetime of the $^5D_4$ emitting state of terbium(III) complexes in aqueous solution, denoted by $\tau_{Tb}(^5D_4)$, generally falls in the 0.4–5.0-ms range. These lifetimes are quite sensitive to the detailed nature of the ligand environment, and especially to the number of water molecules occupying inner coordination sites. The modulation of $\tau_{Tb}(^5D_4)$ and $\tau_{Eu}(^5D_0)$ by the ligand environment can be attributed to ligand-dependent effects on both the radiative and nonradiative $^5D_4$ and $^5D_0 \rightarrow {}^7F_J$ transition probabilities. Water (H_2O) molecules (and OH^- ions) are generally much more effective nonradiative *relaxers* of the $^5D_4$ and $^5D_0$ excited states than are other ligands or ligand donor groups.[35-39] On the other hand, D_2O molecules (and OD^- ions) are generally *less* effective nonradiative *relaxers* of these excited states than are most other ligands or ligand donor groups.[35-39] This difference in the effects of H_2O vs. D_2O upon luminescence lifetimes can be exploited in determining the number of water molecules coordinated to Tb^{3+} or Eu^{3+} in the presence of other ligands.[40-43] This is especially important in the study of Ln^{3+}–biomolecular complexes, where knowing the number of bound water molecules is often of significant interest. In general, $\tau_{Tb}(^5D_4)$ and $\tau_{Eu}(^5D_0)$ have very similar probe characteristics for complexes in aqueous solution.

IV. Excitation Spectra and Energy Transfer

The major characteristics of Tb^{3+} and Eu^{3+} emission excitation spectra (for complexes in aqueous solution) are listed in Table III. Excitation spectra involving *direct radiative* excitation of Tb^{3+} ions generally do not provide any structural information not obtainable from absorption measurements. The only advantages offered by excitation measurements (over absorption measurements) in this case are the greater sensitivities and, therefore, lower concentration limits achievable with excitation spectra—especially when laser sources can be used. On the other hand, excitation spectra involving *direct radiative* excitation of Eu^{3+} ions have the potential for providing very useful and sometimes unique structure information. In the near-ultraviolet and blue-visible regions, the excitation and absorption spectra may or may not mimic one another, depending upon the location and relaxation pathways of ligand-to-Eu^{3+} charge-transfer (CT) states. Since the properties of these CT states generally have an intimate dependence on the detailed nature of the ligand envi-

ronment, differences observed between the excitation and absorption spectra of europium(III) complexes have structural implications. However, for probe purposes the most important Eu^{3+} excitation mode involves *direct* excitation of the $^5D_0$ emitting level via the $^7F_0 \rightarrow {}^5D_0$ absorptive transition using a high-resolution (narrow-band) excitation source.[44]

The $^7F_0 \rightarrow {}^5D_0$ transition has the special virtue of being entirely nondegenerate (in crystal fields of *any* symmetry). Therefore, for single complexes containing just one Eu^{3+} ion or for samples containing a homogeneous population of Eu^{3+} ions, excitation spectra obtained within the $^7F_0 \rightarrow {}^5D_0$ transition region can show just one line. The appearance of a split line or of multiple lines within this excitation region can occur *only* if (1) the sample contains a heterogeneous population of Eu^{3+} ions (due to the presence of different complex species or to different binding sites on a single ligand) or (2) two or more Eu^{3+} ions are strongly coupled via an exciton or exchange interaction mechanism. The latter is unlikely to be important for Eu^{3+}–biomolecular complexes in solution media since the resulting interaction energies would lead to band splittings less than homogeneous broadening halfwidths. However, band splittings due to sample heterogeneity (with respect to Eu^{3+} populations) can be expected to be of the order of 15–30 cm^{-1} (or ~5–10 Å), which should be readily observable by use of narrow-line excitation radiation produced by a tunable dye laser. In fact, such line splittings *have* been observed for a number of Eu^{3+}–protein complexes in which the protein molecules are known to bind Ln^{3+} ions at two or more distinguishably different sites.[45-49] The application of this site-selection excitation technique to Eu^{3+}–biomolecular systems has enormous potential for investigating the existence and nature of multiple Ln^{3+} binding sites.[3] Its only drawback is the necessity of using a narrow-line, tunable excitation source capable of delivering high power in the 577–581-nm spectra region. High excitation power is needed due to the very low absorptivity of the $^7F_0 \rightarrow {}^5D_0$ transition, and narrow-line excitation is required to resolve the small line splittings.

When the ligand environment contains chromophores that are highly absorptive *and* luminescent in the near-ultraviolet or blue-visible regions of the spectrum, it is possible to sensitize Eu^{3+} or Tb^{3+} luminescence via ligand-to-Ln^{3+} nonradiative energy transfer processes. These processes are the bases of the *indirect excitation* mode referred to in Table III. Making no assumptions about the *detailed* mechanism of the energy transfer process *except* that it involve a *resonant* coupling between the donor (ligand chromophore) and acceptor (Ln^{3+}) species, two major criteria must be satisfied for this indirect excitation mode to be effective: (1) there must be some overlap between the luminescence spectrum of the donor (ligand) and the absorption spectrum of the acceptor (Ln^{3+}); (2) the acceptor states of the Ln^{3+} ion must be identical with, or strongly coupled to, the emitting states ($^5D_0$ for Eu^{3+} and $^5D_4$ for Tb^{3+}). The first criterion is required to satisfy the resonance condition for energy transfer, while the second criterion ensures that the transferred energy will lead to population of the Ln^{3+} emitting levels.

The luminescent chromophores most commonly found in biomolecular systems (e.g., the nucleic acid bases and the tryptophan, tyrosine, and phenylalanine amino acid residues in proteins) generally exhibit broad-band luminescence spectra falling within the 280–500-nm (35 700–20 000 cm^{-1}) region.[50] Both Eu^{3+} and Tb^{3+} exhibit a multitude of absorption bands in this region,[8] thus indicating the availability of acceptor states that are isoenergetic with the ligand donor states and ensuring large spectral overlaps between the donor luminescence and acceptor absorption spectra. This satisfies the first criterion (cited above) for sensitization of Ln^{3+} luminescence, and it suggests that both Eu^{3+} and Tb^{3+} should be effective quenchers of the ligands' *intrinsic* luminescence. Horrocks and co-workers[45,51] have reported data on a number of Eu^{3+}– and Tb^{3+}–protein complexes that demonstrate that the intrinsic protein luminescence *is* at least partially quenched when the Ln^{3+} is bound close to a protein lumiphore. Moreover, Eu^{3+} is generally found to be a more effective quencher than Tb^{3+}. We have obtained similar results in our laboratory for Eu^{3+} and Tb^{3+} complexes formed with proteins and with smaller ligands containing luminescent chromophoric moieties.

The spectral overlap criterion for energy transfer is satisfied for *both* Eu^{3+} and Tb^{3+} complexes when the ligands luminesce in the near-ultraviolet or blue-visible regions. However, whereas ligand-to-Tb^{3+} energy transfer *nearly always* leads to sensitization and enhancement of $^5D_4 \rightarrow {}^7F_J$ terbium luminescence (i.e., enhancement over that observed for Tb^{3+}(aquo) systems), sensitization of $^5D_0 \rightarrow {}^7F_J$ europium luminescence via ligand-to-Eu^{3+} energy transfer generally occurs *only* when the ligand donor state is located at $\bar{\nu} < 24\,000$ cm^{-1}. This difference between terbium and europium luminescence sensitization can be attributed to differences between the excited states of Tb^{3+} and Eu^{3+} at $\bar{\nu} > 25\,000$ cm^{-1}. Direct or indirect excitation of Tb^{3+} over the 20 500–40 000-cm^{-1} frequency range nearly always leads to the formation of localized 4f-electron excited states that follow a relaxation pathway through the $^5D_4$ emitting level. If the indirect excitation is by ligand-to-Tb^{3+} energy transfer, then we have sensitization of $^5D_4 \rightarrow {}^7F_J$ luminescence. Similarly, direct or indirect excitation of Eu^{3+} over the 17 200–25 000-cm^{-1} frequency range nearly always leads to the formation of localized 4f-electron states that generally (but not always) follow a relaxation pathway through the $^5D_0$ emitting level. (In addition to the multiplet levels shown in Figure 1, Eu^{3+} has a $^5D_3$ level centered at ~24200 cm^{-1}.) However, the excitation of Eu^{3+} $^5D_0 \rightarrow {}^7F_J$ luminescence above ~25 000 cm^{-1} is often complicated by the tendency of Eu^{3+} to undergo $Eu^{3+} \rightarrow Eu^{2+}$ photoreduction via a ligand-to-metal charge-transfer (CT) process.

Most europium complexes comprised of ligands of biological interest exhibit broad, relatively intense ligand-to-metal charge-transfer (CT) absorption bands in the ultraviolet region of the spectrum. Although these CT transitions are generally centered at $\bar{\nu}_{abs} > 35\,000$ cm^{-1}, their absorption bands usually exhibit very broad Franck–Condon profiles with red tails extending out to (or beyond) 25 000 cm^{-1}. Furthermore, the absorptivities of the CT transitions are generally 1 to 4 orders of magnitude greater than those of the 4f → 4f transitions. Consequently, the CT states are expected to be by far the most effective acceptor states in any lig-

TABLE IV. Constituents of Biomolecular Systems Containing Donor Chromophores for Ligand-to-Tb^{3+} Energy Transfer

constituent	λ_E^{max}, nm[a]	λ_F^{max}, nm[a]	λ_P^{max}, nm[a]	comments
tryptophan (Trp)	280[b]	348[b]	435[c]	assuming a singlet (fluorescent) donor state, quantum yields and spectral overlap factors suggest Trp* as the most effective donor and Phe* as the least effective
tyrosine (Tyr)	275[b]	303[b]	395[c]	
phenylalanine (Phe)	258[b]	282[b]	385[c]	
salicylate (Sal)	295[d]	395[d]		a very efficient donor when Tb^{3+} is coordinated to the Sal carboxylate moiety
common mononucleotides (of DNA and RNA)	250-275[e]	319-325[e]	400-430[e]	the photophysics of mononucleotides is significantly altered upon formation of polynucleotides, leading to complications in sorting out the most likely nucleotide-to-Tb^{3+} energy transfer pathways

[a] λ_E^{max} = maximum in fluorescence excitation spectrum; λ_F^{max} = maximum in fluorescence spectrum; λ_P^{max} = maximum in phosphorescence spectrum. [b] In water at pH 7; T = 293 K. [c] In an aqueous 0.5% glucose glass; T = 77 K. [d] Salicylic acid in methanol at T = 296 K. [e] In EGW glass at 80 K.

and-to-Eu^{3+} electronic energy transfer process occurring in the near-ultraviolet frequency range, and their existence accounts for the relatively large quenching efficiencies of Eu^{3+} (toward intrinsic ligand luminescence). However, for most systems studied in aqueous solution, the nonradiative relaxation pathways of these CT states do not pass through the ^5D_J excited-state manifold of Eu^{3+} and, therefore, do not lead to sensitized $^5D_0 \rightarrow {}^7F_J$ emission. Little of the energy deposited in a CT state, either radiatively or nonradiatively by energy transfer, finds its way to the $^5D_0$ emitting level. For this reason, sensitization and enhancement of Eu^{3+} emission via near-ultraviolet ligand excitation are not likely to be a generally useful probe for Ln^{3+}–biomolecular interactions. Exceptions to this general rule are those cases in which the ligands contain a lumiphore with a relatively low energy (<25 000 cm^{-1}) emitting state. In these cases, energy transfer will be to the lower energy ^5D_J levels of Eu^{3+}, leading to sensitization of $^5D_0 \rightarrow {}^7F_J$ emission.

A wide variety of Eu^{3+}–protein, –nucleic acid, and –nucleotide complexes have been studied in our laboratory and by others. In most cases, bound Eu^{3+} has been found to be an effective quencher of intrinsic ligand luminescence. However, sensitization and enhancement of Eu^{3+} emission by ligand-to-Eu^{3+} energy transfer have been observed in just one system, Eu^{3+} bound to *E. coli* tRNA.[52,53] The exceptional behavior of the Eu^{3+}–tRNA (*E. coli*) complexes can be attributed to the presence in tRNA of a special nucleotide residue, 4-thiouridine, which exhibits a relatively low frequency fluorescence. This fluorescence spans the 460–570-nm wavelength region, with a maximum around 510 nm. Spectral overlap between this fluorescence and the higher-energy CT absorption band of Eu^{3+} will be very small, thus eliminating the CT state as an effective energy transfer acceptor level. However, this fluorescence *completely* overlaps the Eu^{3+} $^7F_0 \rightarrow {}^5D_1$ and $^5D_2$ absorptions, allowing for resonant energy transfer from the base donor state directly to the $^5D_2$ and $^5D_1$ excited states of Eu^{3+} with subsequent relaxation to the $^5D_0$ emitting level.

Terbium luminescence can be sensitized by ligand-to-Tb^{3+} energy transfer over the entire near-ultraviolet excitation region, subject only to donor (ligand)–acceptor (Tb^{3+}) distance constraints. Excitation spectra of the sensitized terbium luminescence will, in most cases, mimic the emission excitation spectra of the ligand energy donor moieties. This provides a basis for identifying the ligand groups responsible for the sensitization and, thereby, characterizing certain aspects of the Tb^{3+} binding site.[11,29,54,55] The utility of this information in structure studies, however, depends on how large or how small the effective energy-transfer distances can be. Assuming *either* a Forster-type Coulombic (*nonexchange*) mechanism *or* an overlap (*exchange*) mechanism for the ligand-to-Tb^{3+} energy-transfer processes leads to predictions of relatively short "effective" transfer distances (*critical* transfer distances of $R_0 < 8$ Å in most relevant cases).[56] This is due to the low absorptivities of the Tb^{3+} 4f → 4f transitions (when the Coulombic mechanism is applied) and to the small overlap densities of the Tb^{3+} and ligand valence orbitals (when the exchange mechanism is applied). Furthermore, since the valence-electron charge distributions in both the ground and 4f-electron excited states of Tb^{3+} are expected to be nearly isotropic, both mechanisms lead to predictions that the energy-transfer efficiency will be independent of donor–acceptor orientation.

These predictions of relatively short effective transfer distances suggest that the sensitized luminescence excitation spectra can be used as *selective regional* probes of the Tb^{3+} binding sites. That is, only a select set of ligand groups (the energy donors) located within a relatively small region about the Tb^{3+} binding site are probed. The most common donor chromophores in protein molecules are the indole, phenolic (or phenolate), and phenyl side-chain moieties of the tryptophan, tyrosine, and phenylalanine amino acid residues. Some properties of these donor chromophores are listed in Table IV. Their relative efficiencies as energy donors to Tb^{3+} reflect, to a very large extent, their relative quantum efficiencies as *intrinsic* protein lumiphores. The differences in their fluorescence excitation maxima (denoted by λ_E^{max} in Table IV) provide the basis for identifying which type of chromophore is acting as the energy donor in a Tb^{3+}–protein complex exhibiting ligand-sensitized terbium luminescence. In most cases, the near-ultraviolet excitation spectrum of the complex (monitoring terbium emission intensity) will qualitatively match the fluorescence excitation spectrum of the (free) donor chromophore. This method of comparing the excitation spectra obtained for Tb^{3+}–protein complexes with those characteristic of the constituent lumiphores of proteins has been exploited extensively by Martin and co-workers[11,29–31,54,55] for characterizing Tb^{3+} binding sites in terms of their juxtaposition with specific aromatic amino acid residues in the protein backbone.

The energy donor moieties in nucleic acid systems are the nucleic bases. The excitation, excited-state photophysics, and emission properties of these chromophores are considerably more complex (and variable)

than those of the protein donor chromophores. However, for the *common* nucleotide constituents of DNA and RNA systems, empirical studies have revealed a definite hierarchy with respect to their relative effectiveness as sensitizers and enhancers of terbium emission.[57] This hierarchy is G >> C ~ T >> A. Furthermore, the G-to-Tb^{3+} sensitization and enhancement are observed to be extremely sensitive to a number of structure-related factors, such as (1) chemical modification of the guanine ring, (2) the degree of base pairing and/or base stacking, and (3) single-stranded vs. double-stranded compositions.[57,58] Tb^{3+} provides, then, a guanine-specific luminescent probe for the study of nuclei acid structure. Furthermore, its luminescence sensitivity to single-stranded vs. double-stranded structure is complementary to the luminescence sensitivity exhibited by ethidium bromide to such structure. Terbium luminescence enhancement is observed *only* for single-stranded structures, whereas ethidium bromide fluoresces strongly only when bound to double-stranded structures.[59]

The efficiencies (or quantum yields) of ligand-to-Ln^{3+} (Eu^{3+} or Tb^{3+}) energy-transfer processes can be determined from quenching data (i.e., the quenching of *intrinsic* ligand luminescence by Ln^{3+} acceptors), Ln^{3+} luminescence enhancement data, and the luminescence quantum yields of Ln^{3+} (aquo) and the free (unbound) ligand donor moieties. Horrocks and co-workers[3,45,51] have reported a number of such determinations for several Eu^{3+}- and Tb^{3+}-protein systems and found Trp-to-Eu^{3+} energy-transfer efficiencies to be several orders of magnitude greater than those for Trp-to-Tb^{3+}. However, sensitization and enhancement of Ln^{3+} emission were observed *only* for the Tb^{3+} complexes, indicating that the principal Eu^{3+} acceptor state in the Trp → Eu^{3+} energy-transfer process is charge transfer in character. Assuming a Forster-type dipole–dipole Coulombic mechanism for the Trp-to-Ln^{3+} energy-transfer processes, Horrocks further estimated the *critical transfer distances* (R_0) to be 3–4 Å for Tb^{3+} and ~10 Å for Eu^{3+} (these are the donor–acceptor separation distances required for 50% energy transfer efficiency). For Tb^{3+} coordinated to the ionophore, lasalocid A, in methanol solution, we have determined a ligand-to-Tb^{3+} energy-transfer efficiency of ~0.67.[25] This value is at least 2 orders of magnitude larger than those reported for Trp-to-Tb^{3+} in the protein complexes examined by Horrocks.[3,45,51] This is possibly attributable to the fact that in the Tb^{3+}–lasalocid complex, the Tb^{3+} is bound *directly* to the energy donor chromophore (a salicylate group).

Just as Tb^{3+} may act as an energy acceptor in the processes cited above, it may also function as an electronic energy donor to species having absorption spectra overlapping terbium emission bands. In the present context, the most important such acceptor species for Tb^{3+}-to-acceptor energy-transfer processes are transition-metal ions and other lanthanide ions. Nearly all transition-metal ions are potential acceptors (i.e., they have suitable electronic excited-state properties), and among the Ln^{3+} ions the following can act as acceptors (for Tb^{3+} donor ions): Pr^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, and Tm^{3+}. With the exception of Eu^{3+}, none of these acceptor species exhibits significant luminescence in aqueous solution. For the nonluminescent acceptors, the measurement of Tb^{3+}-to-acceptor energy transfer must be made by monitoring changes in terbium emission lifetimes or the quenching of terbium $^5D_4 \rightarrow {}^7F_J$ emission intensities. In the case of Eu^{3+}, one may also monitor the appearance of Eu^{3+} $^5D_0 \rightarrow {}^7F_J$ emission. As an electronic energy donor to other metal ions, Tb^{3+} may be used to probe the existence of, and interactions between, different metal binding sites in complex systems. Quantitation of the emission lifetime and intensity quenching data obtained in such studies can be used to determine intermetal distances.[47,48,60,61]

As an electronic energy donor to other metal ions, Eu^{3+} can function much like Tb^{3+} (vide supra) except that it is somewhat more restricted due to its emissions lying significantly to the red of the Tb^{3+} emissions. The Eu^{3+} $^5D_0 \rightarrow {}^7F_J$ transitions have fewer resonances with other Ln^{3+} absorptive transitions than do the Tb^{3+} $^5D_4 \rightarrow {}^7F_J$ transitions. Eu^{3+} has been shown to be an effective donor species for Pr^{3+} and Nd^{3+} in several protein systems[47,48,60] and an especially effective acceptor species for Tb^{3+} in a wide variety of smaller model systems.[20,21,26,62]

One final aspect of Tb^{3+} and Eu^{3+} as structure probes that has not yet been mentioned concerns their role as *unbound* energy donors to bound metal ion acceptors. In this role, the Tb^{3+} or Eu^{3+} ions exist in small, electrically neutral chelate structures that are stable in solution. These chelates are designed to be nonbinding with respect to the biomolecular systems under study and to be freely diffusing in solution. Under these conditions, chelate–biomolecule interactions are under diffusion control. When the biomolecule contains an acceptor chromophore for chelate-to-acceptor energy transfer, the intrinsic chelate emission intensity will be quenched and its lifetime altered when chelate–biomolecule collisions occur. The extent of these emission perturbations will depend upon the nature of the acceptor species *and* its location in the biomolecular structure (especially with respect to its accessibility to the chelate in the collision complex). The utility of this technique is enhanced by the use of chelates with especially long emission lifetimes. This can be achieved by using complexes having few water molecules in the inner coordination sphere (recalling that water molecules are very effective nonradiative "relaxers" of the $^5D_4$ and $^5D_0$ emitting states of Tb^{3+} and Eu^{3+}). Also, in this regard, terbium chelates are to be preferred over europium chelates. This technique, often referred to as "diffusion-enhanced energy transfer", has been beautifully exploited by Claude Meares, Lubert Stryer, and their co-workers in ascertaining the locations of chromophores (with respect to their proximity to the surface) in biological macromolecules and membrane systems.[63–66]

V. Summary of Applicable Techniques

A list of the luminescence techniques most applicable to the study of Tb^{3+} and Eu^{3+} complexes with biomolecules is given in Table V, along with comments on their specific applications. The techniques numbered as (1), (2), (3), (6), and (8) are accessible to use with commercially available emission instrumentation (steady state and time resolved) *without* a laser excitation source. The measurement of emission optical

TABLE V. Summary of Luminescence Techniques Applicable to the Study of Tb^{3+} and Eu^{3+} Complexes with Biomolecules

technique	comments
(1) excitation spectra	(a) Tests for sensitization of Ln^{3+} emission via ligand-to-Ln^{3+} energy transfer (b) Identifies ligand donor groups involved in energy transfer and sensitization
(2) luminescence enhancement (under ligand excitation)	(a) can be used to follow binding in emission titration studies (b) can be used to determine ligand donor group-Ln^{3+} distances (donor here refers to an energy donor) (c) most appropriate for terbium complexes
(3) quenching of intrinsic ligand luminescence by Ln^{3+}	Same as for (2) *except* that it is most appropriate for europium complexes
(4) quenching of Tb^{3+} or Eu^{3+} luminescence by other bound metal ions	(a) Probes multiple site binding (b) Can be used to determine intermetal distances
(5) excitation spectra in the $^7F_0 \rightarrow {}^5D_0$ transition region of Eu^{3+}	probes for heterogeneity in Eu^{3+} ion population
(6) measurements of emission lifetimes in H_2O-D_2O solvent mixtures	Can be used to estimate number of water molecules in the inner coordination sphere
(7) emission optical activity (CPL spectra)	(a) most sensitive indicator of binding (b) probe for site symmetry and stereochemistry in chiral systems
(8) relative intensities and structuring of luminescence bands	(a) diagnostic of ligand-field symmetries and ligand conformational properties at Ln^{3+} binding sites (b) most applicable to the Eu^{3+} $^5D_0 \rightarrow {}^7F_0$, $^7F_1$, and $^7F_2$ emission regions

activity requires special-purpose instrumentation capable of detecting and differentiating between the left and right circularly polarized components of the sample luminescence,[12] and $^7F_0 \rightarrow {}^5D_0$ excitation studies require the use of a laser source with tunable output over the 577–580-nm spectral region.

Tb^{3+} is superior to Eu^{3+} as a luminescent stain due to the versatility it offers for either direct or indirect excitation over the near-ultraviolet and blue-visible spectral regions. In addition, the Tb^{3+} $^5D_4 \rightarrow {}^7F_5$ emission is remarkably intense under a wide variety of solution conditions, and it exhibits an especially strong CPL when Tb^{3+} is bound to a chiral ligand. As detailed structure probes, both Tb^{3+} and Eu^{3+} offer special attributes. Near-ultraviolet excitation leading to the sensitization and enhancement of Tb^{3+} emission via ligand-to-Tb^{3+} energy transfer makes Tb^{3+} an excellent probe for determining the types of ligand donor chromophores located close to the Tb^{3+} binding sites. The magnitudes of the observed enhancements may also be used to obtain estimates of Tb^{3+}–donor chromophore distances. The positive attributes of Eu^{3+} as a luminescent structure probe derive mainly from its effectiveness as a quencher ion and the simplicity of the states involved in $^5D_0 \rightarrow {}^7F_{0,1,2}$ emission and in $^7F_0 \rightarrow {}^5D_0$ excitation. The splitting and intensity patterns observed in these emissions (*and* in $^7F_0 \rightarrow {}^5D_0$ excitation) are relatively simple, and offer the promise of being interpretable in terms of site symmetries, coordination geometries, and multiple site distributions.

To be useful as luminescent stains, it is necessary that Tb^{3+} and Eu^{3+} be capable of binding strongly and of retaining their binding properties and luminescence properties under sample assay conditions. As was noted previously, Tb^{3+} and Eu^{3+} exhibit a strong propensity for binding to negatively charged groups that contain oxygen ligating atoms, and such binding generally serves to enhance their luminescence (over that observed for the Ln^{3+}(aquo) ions). Given the ubiquity of such donor groups in biomolecular systems (e.g., carboxylate, phosphate, phenolate, and hydroxylate groups), Tb^{3+} and Eu^{3+} binding to a wide variety of these systems is assured. Binding stoichiometries remain a problem in carrying *quantitative* staining studies, but when the Tb^{3+} or Eu^{3+} luminescence exhibits significant sensitivity to binding, luminescence titration measurements may be used to determine (or estimate) stoichiometries.[2,11,44-48,54,55,57,58,67-69]

VI. Weak Interactions Probed by Emission Optical Activity

In our studies of Eu^{3+} and Tb^{3+} luminescence, emission optical activity (CPL spectra) has proved to be the most sensitive probe of Ln^{3+} binding to weakly coordinating ligands or to ligand donor groups with little coordinating ability in aqueous solution. Examples of weakly coordinating groups (in an aqueous environment) are the neutral carbonyl, hydroxyl, amide, and amino groups—all major constituents of biological macromolecular structures. Although it is unlikely that any of these groups play a *major* role in binding Ln^{3+} ions to proteins and nucleic acids (given the abundance of negatively charged oxygen donor groups in these systems), it *is* likely that they occupy at least some of the inner-sphere coordination sites of the bound Ln^{3+} ions and that they contribute to the coordination process by completing chelate bridges (with the strongly coordinating groups serving as "anchors"). Evidence that these weakly coordinating groups do, in fact, bind Ln^{3+} ions in aqueous solution can be obtained from studies on simple carbohydrates (monosaccharide and disaccharide sugars), nucleosides, and amino acids. In the case of amino acids (and sugar acids), which contain the strongly coordinating carboxylate group, one looks for evidence of bidentate chelation involving the amino (or hydroxyl) groups.

Proton and carbon-13 NMR studies have already provided some evidence for multidentate chelation of sugars, nucleosides, and amino acids to Ln^{3+} ions in aqueous solution,[2] and emission optical activity measurements have been used to follow the multidentate chelation of amino acids to Tb^{3+} and Eu^{3+} in aqueous solution as a function of pH.[20-22] Emission optical activity is an especially suitable technique for investigating these expectedly weak Ln^{3+}–ligand chelations since it combines the stereochemical sensitivity and specificity of natural optical activity and the measure-

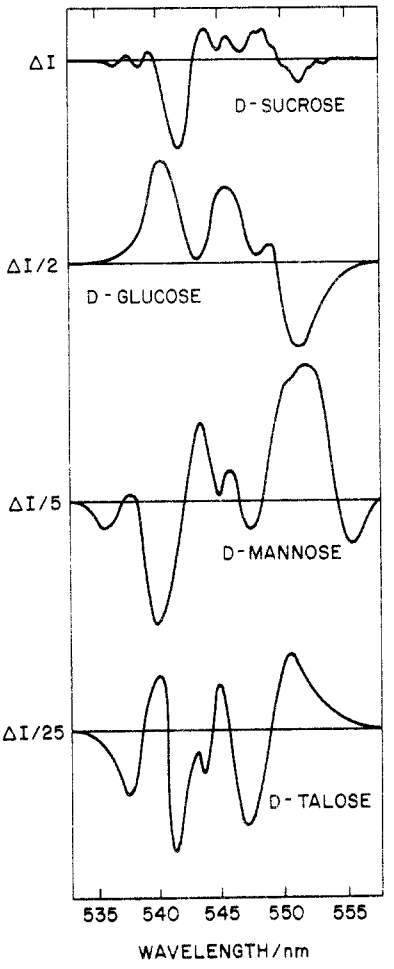


Figure 2. CPL spectra obtained in the $^5D_4 \rightarrow {}^7F_5$ transition region of Tb^{3+}. Spectra were obtained on aqueous solutions at pH ~6.8 with 1:3 [Tb^{3+}]:[sugar] ratios and [Tb^{3+}] = 10 mM.

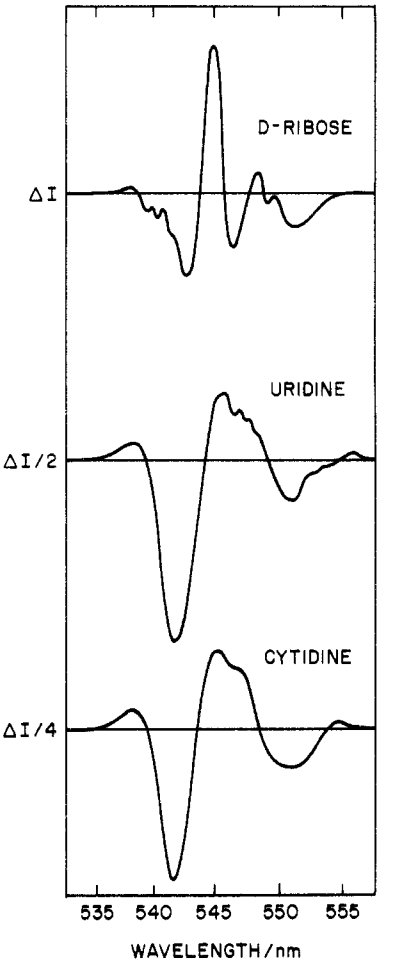


Figure 3. CPL spectra obtained in the $^5D_4 \rightarrow {}^7F_5$ transition region of Tb^{3+}. Spectra were obtained on aqueous solutions at pH ~6.8 with 1:3 [Tb^{3+}]:[ligand] ratios and [Tb^{3+}] = 10 mM.

ment sensitivity inherent to emission techniques. The latter permits studies to be done at low concentrations, and the former ensures that small structural changes in the ligand environment about the Ln^{3+} ions can be monitored.

Examples of emission optical activity induced in the $^5D_4 \rightarrow {}^7F_5$ transition of Tb^{3+} and in the $^5D_0 \rightarrow {}^7F_{0,1,2}$ transitions of Eu^{3+} by sugars and ribonucleosides are shown in Figures 2–4. The CPL spectra shown in these figures were obtained on 1:3 [Ln^{3+}]:[ligand] systems in aqueous solution at pH ~6.8. The *relative* strengths of emission optical activity induced by a series of simple sugars and sugar acids are given in Table VI. The relative emission optical activity strengths exhibited by the neutral sugars correlate closely with the ligands' abilities to coordinate via a terdentate chelation mode involving three hydroxyl groups disposed in an axial–equatorial–axial arrangement on three consecutive carbon atoms of a pyranose ring. This arrangement of three cis hydroxyl groups gives the optimum geometry for Ln^{3+}–ligand terdentate binding. Talose, allose, and ribose each have *three* pyranose anomers possessing this favorable arrangement of OH groups; tagatose, lyxose, and mannose each have *one* such pyranose anomer; and the remaining sugars have no anomeric forms with this arrangement. The strong emission optical activity exhibited by each of the sugar acids reflects the strong coordinating ability of the carboxylate group in these ligands.

All the common ribonucleosides that contain at least one carbonyl group in the base moiety induce at least weak Eu^{3+} and Tb^{3+} emission optical activity in aqueous solution at neutral pH. Inosine, cytidine, and uridine induce the strongest emission optical activity, while adenosine (which contains no carbonyl groups in its base moiety) does not induce an observable emission optical activity. Furthermore, none of the common 2′-deoxyribonucleosides induce an observable emission optical activity. These results imply that the necessary conditions for inducing Ln^{3+} optical activity in Ln^{3+}–nucleoside systems are the availability of (1) at least two *cis*-OH groups on the sugar moiety, and (2) one carbonyl group on the base moiety. Further evidence that the base moiety is involved in Ln^{3+} binding is the observation that Tb^{3+} CPL/emission can be sensitized and enhanced by direct ultraviolet excitation of the base. This sensitization is observed, however, *only* when the base contains a carbonyl group.

The CPL/emission results obtained on sugars and ribonucleosides demonstrate that emission optical activity is a very sensitive probe of the relative binding strengths of these weakly coordinating ligands and of structural differences between the complexes they form with the Ln^{3+} lumiphore. Spectra–structure relationships for this technique are not yet sufficiently refined to yield *quantitative* stereochemical structure information, but even the qualitative aspects of emission optical activity can provide information not readily

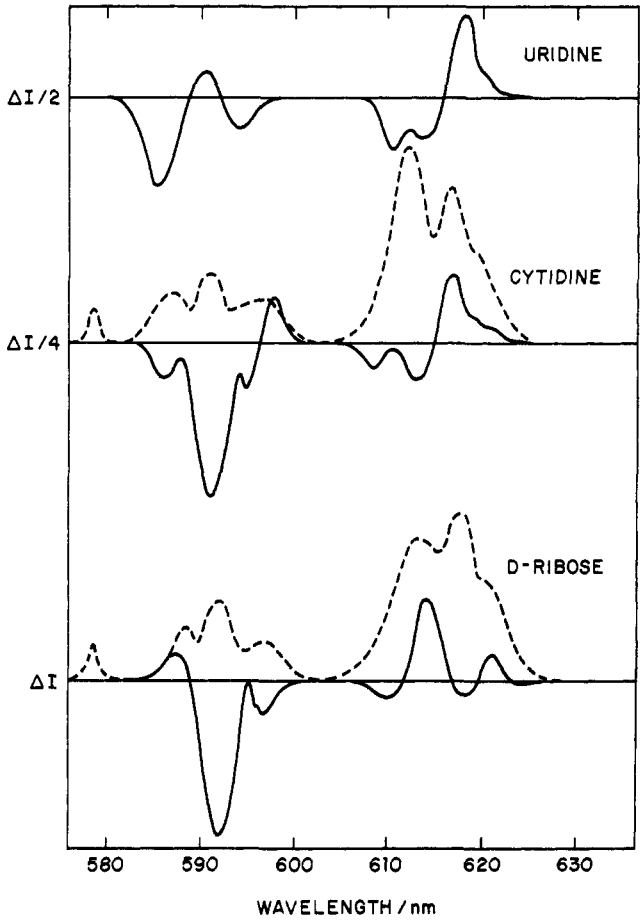
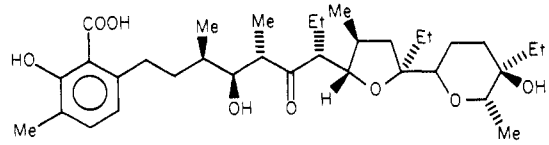


Figure 4. CPL spectra (solid line traces) and unpolarized emission spectra (broken line traces) obtained in the $^5D_0 \rightarrow {}^7F_0$, $^7F_1$, and $^7F_2$ transition regions of Eu^{3+}. Spectra were obtained on aqueous solutions at pH ~6.8 with 1:3 [Eu^{3+}]:[ligand] ratios and [Eu^{3+}] = 10 mM.

obtainable from other kinds of measurements.

A final example of how emission optical activity may be used to prove Ln^{3+} binding to the weakly coordinating groups of a complex ligand is provided by our recent study[25] of the ionophore lasalocid A in methanol



lasalocid A (X537A)

and methanol–water solutions with Tb^{3+} and Eu^{3+}. The strongest ligating group in this molecule is the carboxylate moiety of the salicylic acid heat group. Tb^{3+} binding to this group leads to sensitization and enhancement of terbium emission via salicylate-to-Tb^{3+} energy transfer, but it does *not* produce emission optical activity in the $^5D_4 \rightarrow {}^7F_J$ terbium transitions since the chiral centers of the ligand are too far removed from the Tb^{3+}. However, under solution conditions in which the ligand may wrap around the metal ion and coordinate via the five oxygen atoms contained in the non-salicylate parts of the structure, a very strong terbium emission optical activity is observed. The six-coordinate chelate structure formed under these conditions places the nine chiral centers of the ligand in close (and rigid) proximity to the Tb^{3+} lumiphore.

TABLE VI. Examples of Sugars and Sugar Acids Acting as Chiral Ligands to Eu^{3+} and Tb^{3+} in Aqueous Solution

ligands	emission optical activity[a]	comments
monosaccharides		
D-talose	strongest (among neutral sugars)	exhibit CPL between pH 4 (weak) and pH 9 (strong)
D-allose	strong	
D-ribose	strong	
D-tagatose	medium-strong	
D-lyxose	medium-weak	exhibit CPL between pH 4 (weak) and pH 8 (medium)
D-mannose	medium-weak	
D-arabinose	weak	exhibit CPL *only* between pH 5.5 and pH 7.5
D-galactose	weak	
D-glucose	weak	
D-fructose	weak	
D-xylose	weak	
disaccharides		
lactose	very weak	exhibit CPL *only* between pH 5.5 and pH 7.5
maltose	very weak	
sucrose	weakest	
sugar acids		
sialic acid	strongest (among acids)	exhibit strong CPL over the 2-10 pH range; details of spectra show strong pH dependence
galacturonic acid	very strong	
glucuronic acid	very strong	

[a] Emission optical activity here refers to the *relative* $\Delta I/I$ values observed in CPL/emission spectra obtained for 1:3 [Ln^{3+}]:[ligand] systems in neutral aqueous solution (pH ~6.5-7.0). $\Delta I = (I_L - I_R)$ and $I = (I_L + I_R)/2$, where I_L and I_R denote, respectively, the intensities of the left and right circularly polarized components of the emitted radiation.[12]

In this case, emission optical activity and sensitization of Tb^{3+} luminescence provide very sensitive means for monitoring the formation and conformational properties of Tb^{3+}–lasalocid complexes in solution. The sensitivity of these techniques permits studies to be carried out with [Tb^{3+}] in the range 1–10 μM.

VII. Concluding Remarks

The luminescence properties of Tb^{3+} and Eu^{3+} make these ions quite versatile in their applications to biomolecular structure examination. Their characteristic binding properties coupled with the persistence of detectable emission intensity under variable sample conditions support their use as *stains* or *markers*. Perhaps even more importantly, the sensitivity of their excitation and emission spectra to specific structural features or binding properties of complex ligand systems make them especially useful as *specific structure probes*. In general, Tb^{3+} and Eu^{3+} are complementary in their applications as probes. The techniques appropriate to Eu^{3+} offer the most promise for obtaining detailed structure information, but these techniques are more demanding with regard to measurement procedures and instrumentation requirements. Terbium excitation and emission spectra are relatively easy to measure by use of readily available instrumentation.

The qualitative and semiquantitative aspects of this field are now fairly well delineated and, at this level, most of the techniques listed in Table V can be exploited and applied to a wide variety of systems. Studies at a more quantitative level suffer from lack of detailed understanding regarding ligand photophysics, ligand-to-metal energy transfer mechanisms and path-

ways, and ligand field effects on Tb^{3+} and Eu^{3+} radiative and nonradiative processes. These problem areas are of special interest in our research efforts at the University of Virginia.

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