J. Phys. Chem. A **2002,** *106,* 8213−8222

Medium Effects on the Stability of Terbium(III) Complexes with Pyridine-2,6-dicarboxylate

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Received: May 23, 2002; In Final Form: June 27, 2002

Systematic investigation of the stability of mono-, bis-, and tris-terbium(III) complexes with dipicolinic acid is reported. The formation constants were extracted from emission titration data. For this purpose, a mathematical formalism for two complexation models was developed. Comparative studies were conducted by variation of the acidity of the medium (from $H_0 = -2$ to pH = 7), the buffer composition (i.e., containing seven different anions), and the salt concentration (from 0.04 to 1 M). It became evident that elevating the pH and lowering the salt concentration were important, not only for increasing the values of the formation constants, but also for forming polynuclear Tb−DPA complexes. Inhibition of the latter phenomenon was observed when acetate or sulfate was present. The main purpose of the present investigation was to establish optimum conditions for conducting terbium(III)-mediated emission assays for bacterial endospore detection.

Introduction

In recent years the need for sensitive methods for quantitative determination of pyridine-2,6-dicarboxylic acid, also known as dipicolinic acid (DPA), has been driven by the demand for detection of bacterial endospores, since calcium dipicolinate contributes substantially to spore coat structure.[1] A highly sensitive approach for spore detection can be realized by measuring the enhancement of the emission of some lanthanide ions upon ligation with DPA.[2−5] The original idea for using Eu^{3+} and Tb^{3+} for such emission assays has been patented;[6] the proposed method has been further developed.[7−9] Despite the demonstrated high sensitivity, speed, and simplicity of the emission assay, there are still demands for optimizing the conditions for conducting such measurements in order (1) to suppress the distorting influence of some impurities and to achieve high reproducibility with samples of various origin, (2) to attain a wide linearity range between measured emission enhancement and DPA concentration, and (3) to increase further the sensitivity without compromising the specificity for DPA detection.

The principal methodology for these assays has been derived from preceding work that employs emission enhancement for quantitative analysis of traces of lanthanide metal ions.[3,10−12] Apparently, for the latter type of analysis, experiments are conducted using excess concentrations of a ligating agent (usually, DPA); therefore, in the case of terbium(III) and DPA, the principal species under observation would be the complex having the largest possible number of DPA ligands (i.e., the tris complex). Furthermore, when DPA is in excess, the measurements are most likely to be performed at neutral pH because DPA then exists in a form of a dianion that ostensibly has the highest ligation propensity. Also, the complexation with DPA would tend to suppress the formation of precipitate that is expected at elevated pHs.[13] The assays for spore detection, however, have to be conducted in the presence of excessive concentrations of terbium(III). Hence, the monoligated Tb−DPA complex, which has been studied less than its tris analogue, would be the primary moiety under observation. Also, at pHs close to neutral (which have been of choice for the recently developed techniques[6,7,9]), the propensity of Tb^{3+} for formation of precipitate[14] and polynuclear complexes[15] can significantly compromise the reliability and reproducibility of the assay experiments.

To attain high sensitivity for DPA detection by means of measurement of terbium(III) emission enhancement, it is essential to achieve conditions where the monoligated complex (1) has a large formation constant, (2) exhibits high emission quantum yield, and (3) demonstrates substantial emission enhancement in comparison to terbium(III) that is not ligated with DPA. Alterations of the conditions of the media can result in about 2-fold increase in the emission quantum yields of the various Tb−DPA complexes.[16] Therefore, an approach to improvement of the sensitivity of a spore detection assay by orders of magnitude involves finding conditions under which the formation of the monoligated Tb−DPA complex is most readily attained.

The photophysical properties of the terbium−DPA system have been extensively studied in the past.[4,5,13,17,18] Also, the formation constants of DPA-ligated lanthanide complexes have been measured, using potentiometric[19] and luminescence[20] techniques. However, most of the investigations failed to address issues about the influence of the media. A spectroscopic investigation of terbium(III) in the presence of DPA with variation of the pH of the environment has been reported.[21] However, it was not evident from their study whether the emission intensities during the titration were the result of alterations in emission quantum yield or due to fluctuations in the state of ligation because of variations in formation constants. Hindle and Hall reported recently an examination of the influence of various media (e.g., pH 5.6 acetate buffer on terbium−DPA complexation with a low level of ligation).[8]

The present article reports for the first time the formation constants for mono, bis, and tris Tb−DPA complexes, obtained from emission titration measurements conducted over a wide acidity range (i.e., from $H_0 = -2$ to pH = 7.0), thus allowing for examination of the four different states of protonation of DPA.[22] Since terbium(III) has high affinity for oxygen-containing ligands,[4,17,23] it is very likely that fluctuation in the formation

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10.1021/jp026168j CCC: $22.00

constants could be observed in the presence of certain anions that might compete for ligation sites. To examine this hypothesis, parallel studies were conducted in buffers that contain seven different anions. In addition, some measurements were conducted with various concentrations of buffer or salt for the purpose of discerning the influence of the ionic strength on the complexation equilibria from the contributions of some specific interactions between terbium(III) and the anions in the solution. As a result of this survey, conditions were identified that are most appropriate for detection of subnanomolar concentrations of DPA in near-neutral water using the Tb emission enhancement method.

Experimental Section

Materials. Pyridine-2,6-dicarboxylic acid (DPA) was purchased from Aldrich and recrystallized from 1,2-dimethoxyethane/water (4/1 v/v) solution. Terbium trichloride hexahydrate (99.9%), as well as the following sodium salts—acetate (99.99%), bromide (99.99+%), chloride (99.999%), perchlorate (99%), nitrate (99.995%), hydrogen phosphate heptahydrate (99.99+%), and sulfate (99.99%)—were also purchased from Aldrich and used as received. The acetic and mineral acids (spectroscopic and/or HPLC grade) were obtained from VWR or Aldrich. Sodium hydroxide and tris(hydroxymethyl)aminomethane (Tris; OmniPur) were from EM Science. All solutions were prepared with MilliQ water (~18 MΩ cm). To prevent precipitation, the pH of the stock solutions of DPA (~100 mM) was adjusted to ~7 with a solution of NaOH; $TbCl_3$ stock solutions (~0.1−1 M) were adjusted to ~4 with HCl.

The neutral-pH buffers were prepared by addition of the corresponding acid to an aqueous solution of Tris base until pH 7 was attained, followed by dilution with water to the designated volume. The acidic buffers (pH 0−4) were prepared by addition of the acids to aqueous solutions of the corresponding sodium salts until the desired pH was reached, and then the buffers were diluted with water to the assigned volumes. The molarities of the acidic buffers designate the total concentration of sodium cations, while the molarities of the neutral buffers correspond to the total Tris concentration (i.e., $[\mathrm{Tris}] + [\mathrm{TrisH}^+]$). The solutions with high acidity, $H_0 = -2$, were prepared by dilution of the corresponding strong acids with water.[24]

Solutions of pH ~2 and above were monitored with a Orion 420A meter equipped with an Orion Comb. pH electrode, while lower pH values were determined via the changes in the UV/visible absorption spectra of strong-acid indicators, e.g., 3,4,5-trimethoxybenzamide ($\mathrm{p}K_a = -1.9$),[25] purchased from Lancaster Synthesis, Inc., and *N*,*N*-diethyl-4-nitroaniline ($\mathrm{p}K_a \sim 0.2-0.3$),[25] obtained from Frinton Laboratories, Vineland, NJ.

Methods. The UV/visible absorption measurements were conducted on a Beckman DU 640B spectrophotometer. The emission spectra were recorded using a PTI Fluorescence System with FeliX software. The samples were excited at either 278 or 266 nm. The former provides maximum emission enhancement and was used for titrations of nanomolar concentrations of DPA. The latter was employed for experiments where DPA concentrations were in the micromolar range. A glass filter, $\lambda_{\mathrm{cutoff}} = 385$ nm, was placed in front of the emission monochromator for all luminescence measurements.

Except for $H_0 = -2$, all titrations presented in this article depict *integrated emission intensity*, *S*, as a function of concentration. The values of *S* for each titration point were obtained via numerical integration of the corresponding emission spectra plotted against frequency (rather than wavelength).[26] A common standard, a solution of rhodamine 6G hexafluorophosphate (R6G) in ethanol, was used in all experiments; i.e., the measured intensities were divided by a correction factor cf, where $\mathrm{cf} = S_{\mathrm{R6G}}/(1 - 10^{-A_{\mathrm{R6G}}(\lambda_{\mathrm{ex}})})$. For all data manipulation and analysis, IgorPro software (version 3.14) from WaveMetrics, Inc., was employed.

Data Manipulation

Emission Enhancement and Titration Curves. Quantitatively, the emission enhancement can be expressed from the ratio between the emission quantum yields of the ligated and nonligated metal ions, $\Phi_{\mathrm{Tb}(\Lambda)_n}$ and Φ_{Tb}, respectively:[27]

$$\text{emission enhancement} = \frac{S_{\mathrm{Tb}+\Lambda}}{S_{\mathrm{Tb}}} = \frac{\Phi_{\mathrm{Tb}(\Lambda)_n}(1 - 10^{-\epsilon_{\mathrm{Tb(L)n}} l [\mathrm{Tb}(\Lambda)_n]}) + \Phi_{\mathrm{Tb}}(1 - 10^{-\epsilon_{\mathrm{Tb}} l [\mathrm{Tb}]})}{\Phi_{\mathrm{Tb}}(1 - 10^{-\epsilon_{\mathrm{Tb}} l C_{\mathrm{Tb}}})} \quad (1)$$

where $S_{\mathrm{Tb}+\Lambda}$ and S_{Tb} are the integrated emission intensities of terbium(III) in the presence and absence of the ligand Λ, respectively;[26] *l* is the optical excitation path length; C_{Tb} is the total concentration of terbium ions; $[\mathrm{Tb}(\Lambda)_n]$ and [Tb] are the equilibrium concentrations of ligated and nonligated terbium, respectively, and $\epsilon_{\mathrm{Tb}(\Lambda)_n}$ and ϵ_{Tb} are the corresponding absorption extinction coefficients at the excitation wavelength.

Analyses were performed on titration data that capture the alteration in the integrated emission intensity, *S*, when Tb^{3+} is added to samples with fixed concentrations of DPA. Correction for the emission of free terbium(III) was applied to the titration data of nanomolar concentrations of DPA where the second term of the numerator of eq 1 could not be neglected (see Supporting Information for details).

Terbium−DPA Complexation Equilibria. For treatment of titration data where the total concentration of DPA was kept below 25 nM, a *one-step equilibrium model* was applied, i.e., a model that represents an equilibrium leading to formation of a polynuclear complex containing *m* terbium ions and *n* DPA ligands, $\mathrm{Tb}_m(\mathrm{DPA})_n$, with the overall formation constant $\beta_{m,n}$:

$$m\mathrm{Tb} + n\mathrm{DPA} \rightleftarrows \mathrm{Tb}_m(\mathrm{DPA})_n$$

$$\beta_{m,n} = \frac{[\mathrm{Tb}_m(\mathrm{DPA})_n]}{[\mathrm{Tb}]^m[\mathrm{DPA}]^n} \quad (2)$$

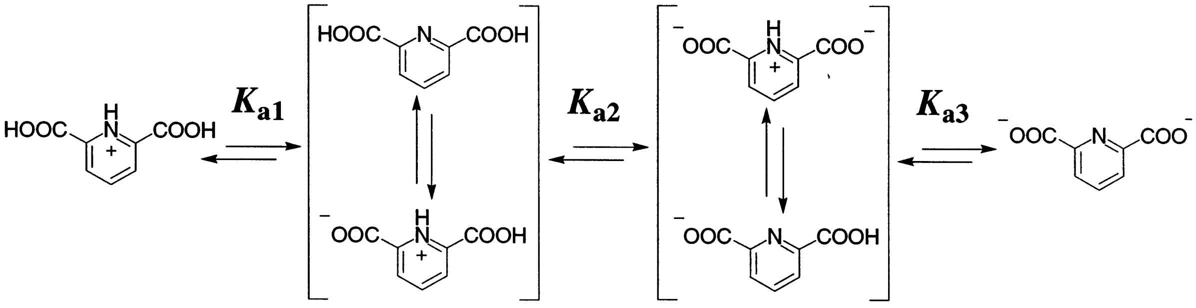
For the titration range where the total terbium concentration, C_{Tb}, exceeds the total DPA concentration, C_{DPA}, i.e., $C_{\mathrm{Tb}} \gg C_{\mathrm{DPA}}$, the one-step equilibrium can be represented with the following linear relationship (see Supporting Information for derivation):

$$\log\left(\frac{R}{{C_{\mathrm{Tb}}}^m}\right) = n \log(1 - R) + \log(\beta_{m,n}) + (n - 1)\log(C_{\mathrm{DPA}}) + \log(n) \quad (3)$$

where *R* is the normalized integrated emission intensity.

For the cases of titration of micromolar concentrations of DPA, however, the one-step equilibrium model was not applicable. Since terbium(III) can ligate up to three moieties of DPA if solely the formation of mononuclear complexes is considered,[17] for treatment of data where the DPA concentration

SCHEME 1: Acid−Base Equilibria for DPA



$$\mathbf{p}K_{\mathbf{a1}} = -1.05$$
$$\mathbf{p}K_{\mathbf{a2}} = 2.22$$
$$\mathbf{p}K_{\mathbf{a3}} = 5.22$$

was relatively high a *stepwise equilibrium model* was used:[28]

$$\mathrm{Tb} + \mathrm{DPA} \rightleftarrows \mathrm{Tb(DPA)} \qquad K_1 = \frac{[\mathrm{Tb(DPA)}]}{[\mathrm{Tb}][\mathrm{DPA}]} \tag{4}$$

$$\mathrm{Tb(DPA)} + \mathrm{DPA} \rightleftarrows \mathrm{Tb(DPA)_2} \quad K_2 = \frac{[\mathrm{Tb(DPA)_2}]}{[\mathrm{Tb(DPA)}][\mathrm{DPA}]} \tag{5}$$

$$\mathrm{Tb(DPA)_2} + \mathrm{DPA} \rightleftarrows \mathrm{Tb(DPA)_3} \quad K_3 = \frac{[\mathrm{Tb(DPA)_3}]}{[\mathrm{Tb(DPA)_2}][\mathrm{DPA}]} \tag{6}$$

The concentrations of the luminophores, i.e., terbium-containing species, can be related to the observed emission intensity via an expression for their quantum yields (similar to eq 1):[27]

$$S = \sum_{i=0}^{n} S_{\mathrm{Tb(DPA)_i}} = lB\frac{1 - 10^{-\sum_j A_j}}{\sum_j A_j}\left(\sum_{i=1}^{n} i\Phi_{\mathrm{Tb(DPA)_i}}\epsilon_{\mathrm{b}} \times [\mathrm{Tb(DPA)}_i] + \Phi_{\mathrm{Tb}}\epsilon_{\mathrm{Tb}}C_{\mathrm{Tb}}\right) \tag{7}$$

where $\sum_j A_j$ represents the total absorption of the sample at the excitation wavelength, B is a proportionality constant that depends on instrument parameters, n is the maximum state of ligation achieved, l is the optical excitation path length, and ϵ_b is the absorption extinction coefficient of DPA moieties that are bound to terbium ions. The equilibrium concentrations of the complexes, $[\mathrm{Tb(DPA)}_i]$, can be readily obtained from numerical solution of a system of polynomial equations derived from eqs 4−6 (see Supporting Information for details).

Results

Due to splitting between the energy levels of its partially occupied 4f orbitals, terbium(III) manifests spectral transitions ($^7F_J \leftrightarrow {}^5D_4$) positioned in the visible region.[4] These transitions, however, are forbidden and, hence, very weakly observable via direct excitation; e.g., $^7F_6 \leftrightarrow {}^5D_4$ transition ($\lambda_{max} = 487$ nm) has an absorption extinction coefficient of ∼0.03 M^{-1} cm^{-1}.[16] Although Tb^{3+} can be excited most efficiently in the far-UV region, ∼220 nm, its excitation can be more readily achieved via energy transfer from ligands whose lowest excited states are with high enough energy.[17] Therefore, the luminescence intensity from terbium(III) solutions can increase several orders of magnitude in the presence of such a ligand, Λ.

By taking under consideration the photophysical properties of terbium−DPA complexes,[16] from eq 1 it is trivial to evaluate that when 10 μM DPA is added to a 100 μM solution of Tb^{3+}, ∼5000-fold emission enhancement is expected ($\lambda_{ex} = 278$ nm). Similarly, when 10 μM DPA is added to 100 nM solution of Tb^{3+}, the expected emission enhancement would amount to about 5 000 000-fold! This phenomenon has been the provenance for the development of numerous analytical techniques for quantitative measurements of lanthanide ions[3,10−12] or for trace detection of DPA.[6−9]

To distinguish the ligation propensity of each of the sites of protonation of DPA[22] (Scheme 1), titration studies at four selected acidities were conducted: $H_0 = -2$ for DPA^+, pH = 0.5 for $DPA^{\pm}$, pH = 3.7 for DPA^-, and pH = 7.0 for DPA^{2-}. Furthermore, it was of significant interest to examine if the presence of various types of negatively charged ions would be of importance to the equilibrium properties of the systems under investigation. Therefore, parallel studies were conducted using three types of aqueous pH buffers, whose negative ions were (1) halides, i.e., not containing oxygen (Br^- and Cl^-), (2) tetrahedral oxygen containing ions ($H_nPO_4^{(3-n)-}$, $H_nSO_4^{(2-n)-}$, and ClO_4^-), and (3) trigonal planar oxygen containing ions (CX_3COO^- and NO_3^-). To better discern the role of these ions, buffers with relatively high concentrations (i.e., 200 mM) were employed. In addition, the influence of the salt/buffer concentration was examined with a buffer with high capacity (e.g., pH 1.9 sulfate buffer; 0.04−1 M).

Complexation Equilibria at Acidity −2. Titrations of 40 μM DPA with terbium(III) in aqueous solutions of sulfuric, hydrochloric, and hydrobromic acids, $H_0 = -2$, were conducted.[24] Emission enhancement was not detected in the presence of these acids. Furthermore, from the shapes of the emission bands at 584 nm ($^7F_5 \leftrightarrow {}^5D_4$ transition),[16] it was determined that in all three cases the observed emission at exceedingly high terbium concentrations was solely from nonligated metal ions. Such observations imply that (1) the cationic DPA species do not ligate terbium(III), or (2) at such a high acidity the Tb−DPA complexes have extremely low emission quantum yields, or, most probably, (3) in the whole titration range the relative amount of ligated terbium ions is negligibly small; hence, their emission cannot be detected against the background of the luminescence from the nonligated Tb^{3+}.

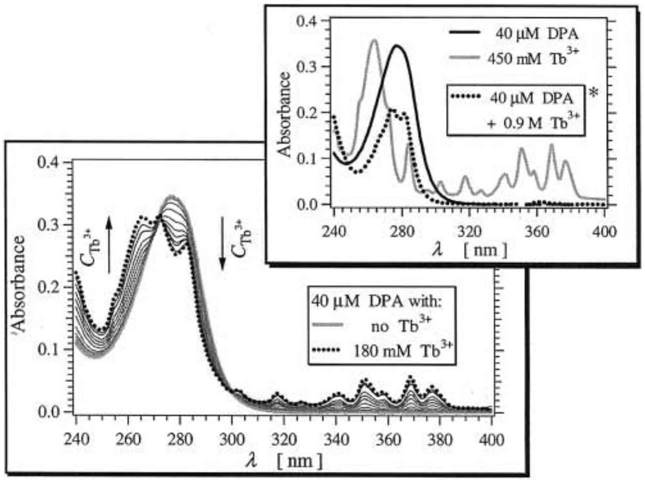


Figure 1. Alteration of the UV absorption spectrum of 40 μM DPA upon addition of $TbCl_3$ in aqueous HCl with $H_0 = -2$. Inset: Spectra of the three species absorbing in this wavelength region. (*0.9 M Tb^{3+} solution was used as a blank for the recording of the spectrum of 40 μM DPA conjugated to terbium.)

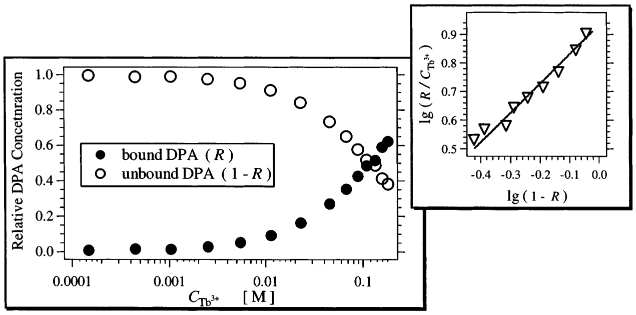


Figure 2. Alteration (upon addition of $TbCl_3$) of the portions of free DPA and DPA conjugated to terbium, obtained from deconvolution analysis of the absorption spectra presented in Figure 3. Inset: The same data converted to the logarithmic function describing the one-step complexation model (eq 3, $m = 1$).

Despite the lack of emission enhancement, further analysis of the titration data for HCl solutions (where considerably higher terbium(III) concentrations can be attained) revealed conspicuous perturbation in the DPA absorption spectra upon addition of Tb^{3+} (Figure 1). The spectra were deconvoluted by fitting to a sum of the spectra of terbium(III), free DPA, and DPA ligated to Tb^{3+} (Figure 1, inset). The results were converted to the corresponding DPA concentrations and normalized, yielding the concentration R values introduced in eq 5 (Figure 2). Consequent conversion and a linear fit, applying eq 3, resulted in $n = 1.0 \pm 0.1$ and $\log(\beta_{1,1}) = 1.0 \pm 0.3$ (Figure 2, inset).

Complexation Equilibria at pH 0.5. Titrations of 23 nM DPA with terbium(III) resulted in significant emission enhancement at pH 0.5 (Figure 3). Since the emission increase occurred at higher C_{Tb} for the sulfate and phosphate buffers, it can be deduced that the Tb−DPA interaction is weaker in the presence of these types of anions. For the titration in the presence of chloride ion, the difference in the steepness of the emission increase with C_{Tb} suggests a type of ligation different from that associated with solutions with other added salts.[29] Converting the data into logarithmic form (eq 3), where m was set to unity for all cases, yielded linear fits with slopes close to unity for all but the chloride titration (Figure 4).[30] The value of n obtained in the presence of chloride ion (Table 1), suggests that, under such conditions, the complexation leads directly to the diligated $Tb(DPA)_2$ complex.

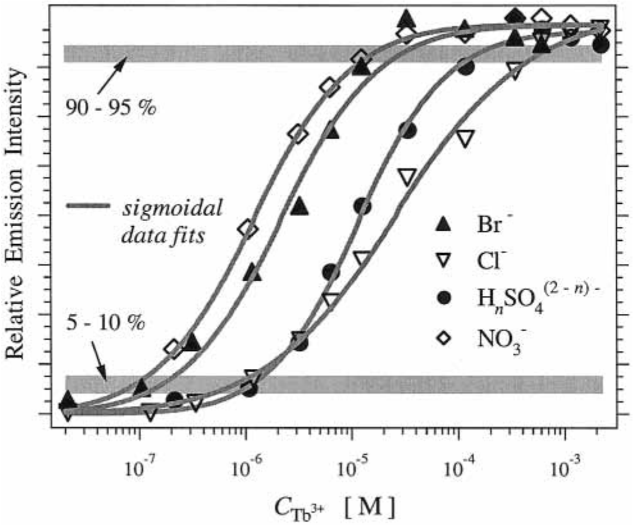


Figure 3. Emission data and corresponding sigmoidal fits for 23 nM DPA titrated with terbium(III) in various 0.2 M buffers, pH = 0.5 (λ_{ex} = 278 nm).

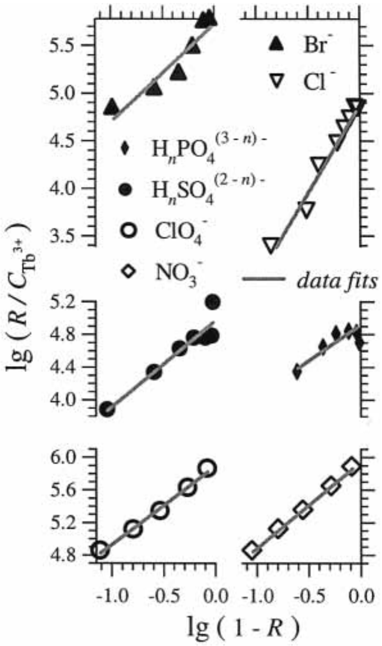


Figure 4. Emission data, converted to the logarithmic function describing the one-step complexation model (eq 3, $m = 1$), and corresponding linear fits for 23 nM DPA titrated with terbium(III) in various 0.2 M buffers, pH = 0.5 (λ_{ex} = 278 nm).

The values for the formation constants, β_n, extracted from the intercepts of the linear fits, shown in Figure 4, not only confirmed the trend that the Tb−DPA interaction tends to be weaker in the presence of tetrahedral oxygen containing anions, but also indicated that the interaction becomes weaker with an increase of the number of protonation sites for these ions (see Table 1, phosphate, sulfate, and perchlorate).

The titration curves for solutions of 20 μM DPA with terbium in the presence of various anions manifested very slight differences that can be discerned only by a fitting of data to a two-step complexation model, eq 7 (Figure 5). The values for the formation constants are shown in Table 1 (the two rightmost columns). Peculiarly enough, in the case of chloride, the first-step formation constant, K_1, is about an order of magnitude smaller than the second-step constant, K_2: note that $\log(K_2) = \log(\beta_2) - \log(K_1)$. Obviously, the considerably higher propensity

TABLE 1: Formation Constants and Complexation Coefficients, *n*, Obtained from Emission Data for DPA Titrated with Terbium(III) in Various Buffers[a]

	results from fitting with			
	one-step model (eq 3) ($C_{DPA} = 23$ nM, $m = 1$)		two-step model (eq 7) ($C_{DPA} = 20\ \mu$M)	
	n	$\log(\beta_n)$	$\log(K_1)$	$\log(\beta_2)$
chloride	1.9 ± 0.1	12 ± 1	5.47 ± 0.002	12.0 ± 0.004
bromide	1.0 ± 0.1	5.9 ± 1.3	5.95 ± 0.001	11.4 ± 0.003
phosphate	0.88 ± 0.16	4.1 ± 1.2	4.29 ± 0.65	10.2 ± 0.002
sulfate	1.1 ± 0.2	5.3 ± 1.2	5.40 ± 0.0003	10.1 ± 0.0001
perchlorate	0.96 ± 0.05	5.6 ± 0.4	5.71 ± 0.00	10.8 ± 0.00
trichloroacetate			5.97 ± 0.003	11.1 ± 0.1
nitrate	1.1 ± 0.02	6.4 ± 0.2	5.98 ± 0.002	11.0 ± 0.08

[a] pH 0.5, 200 mM buffer.

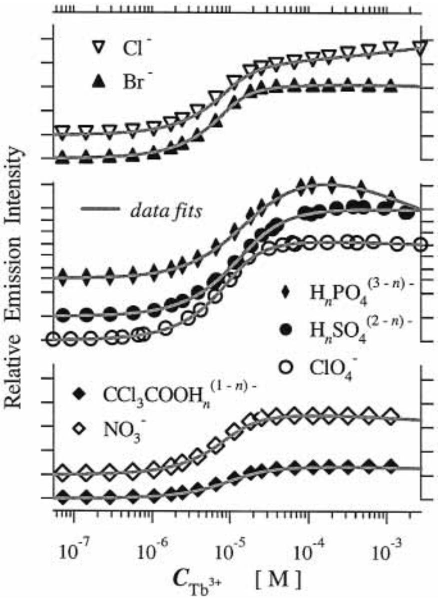


Figure 5. Emission data for 20 μM DPA titrated with terbium(III) in various 0.2 M buffers, pH = 0.5 ($\lambda_{ex} = 266$ nm). The data were fit to a two-step complexation model (eq 7, $n = 2$).

for the second ligation step (eqs 4 and 5) is the principal reason for the preference for direct formation of bis complexes in the presence of Cl$^-$. In the case of phosphate, however, the incipient precipitate formation resulted in larger uncertainty in the determination of $\log(K_1)$. Therefore, within experimental error it can be concluded that $K_1 \approx K_2$, explaining why the titration of diluted DPA in phosphate buffer led directly to monoligated complex (Figure 4).

Excluding the "*abnormality*" in the case of chloride, the titration for other halides and the trigonal oxygen containing ions yielded similar formation constants due to negligible interactions between these anions and terbium(III) under the employed conditions. Overall, the observed complexation trends suggest also that tetrahedral oxygen containing anions compete with DPA for ligation to terbium cations, resulting in a decrease in the formation constants of Tb−DPA complexes. This effect is most conspicuously revealed for solutions containing phosphate ions, where the formation of precipitate is observed at higher terbium concentrations.[31]

At pH 0.5 formation of the tris complex, Tb(DPA)$_3$, was observed when 7 mM terbium(III) was titrated with DPA. The high optical densities at the excitation wavelength attained in the course of such titrations and the propensity of DPA for self-aggregation in aqueous media[32] impeded exact quantitative analysis of the data. However, it was estimated that the value of K_3 (eq 6) at pH 5 is about 10^3 M^{-1} (see Supporting Information).

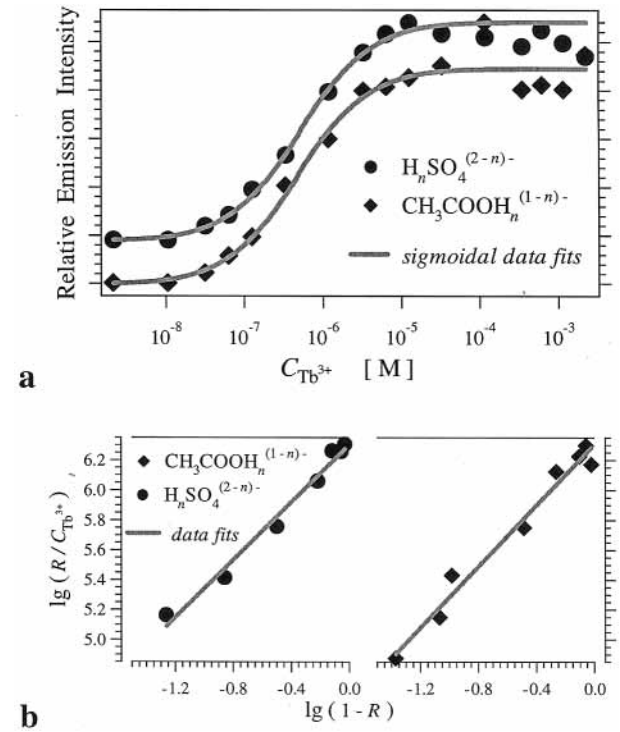


Figure 6. Titration of 2.3 nM DPA with terbium(III) in 0.2 M acetate and sulfate buffers, pH = 3.7 ($\lambda_{ex} = 278$ nm): (a) emission data and corresponding sigmoidal fits; (b) the same data, converted to the logarithmic function describing the one-step complexation model (eq 3, $m = 1$), and the corresponding linear fits.

Complexation Equilibria at pH 3.7. Titration of 2.3 nM DPA with terbium at pH 3.7 also resulted in emission enhancement (Figure 6a). Conversion of the data to the corresponding logarithmic form ($m = 1$, eq 3) revealed distinct linearity (Figure 6b). The unity values of the slopes of the linear fits (Table 2) indicate that only the monoligated Tb−DPA complex is formed under these conditions. Furthermore, the values obtained for the first formation constants, β_1 or K_1, were 3−5-fold larger than the corresponding formation constants measured at pH 0.5.

Fitting data from titration of 20 μM DPA with Tb^{3+} to a three-step complexation model (eq 7) revealed other interesting trends (Figure 7). The first formation constant (eq 4) for solutions with added acetate was slightly larger than the same constant in the presence of sulfate, while both of them were larger than K_1 observed for samples containing phosphate (Table 2). The values obtained for the second-step formation constant (eq 5) revealed a reversed trend; i.e., K_2 was largest for solutions in the presence of phosphate and smallest in the presence of acetate. Furthermore, in the transition from pH 0.5 to pH 3.7, there was about a 2 order-of-magnitude increase in the values for K_2 for added phosphate or sulfate, while in the presence of acetate the same increase was only about 10 times (compare Tables 1 and 2), probably because, at pH 3.7, the solution is buffered with acetate, while at pH 0.5 trichloroacetate is used.

Complexation Equilibria at pH 7.0. At neutral pH, the titration of nanomolar concentrations of DPA with terbium(III) exhibited a pattern that suggests the occurrence of at least two discrete processes (Figure 8). The simplest possible explanation is that the first increase in the observed emission intensity, occurring at $C_{Tb} \sim 10^{-8}-10^{-7}$ M^{-1}, corresponds to the formation of the bis Tb−DPA complex, while the consequent further increase of the measured luminescence intensity, at C_{Tb}

TABLE 2: Formation Constants and Complexation Coefficients, *n*, Obtained from Emission Data for DPA Titrated with Terbium(III) in Sulfate, Acetate, and Phosphate Buffers[a]

	results from fitting with				
	one-step model (eq 3) ($C_{\mathrm{DPA}} = 2.3$ nM, $m = 1$)		three-step model (eq 7) ($C_{\mathrm{DPA}} = 20$ μM)		
	n	$\log(\beta_n)$	$\log(K_1)$	$\log(\beta_2)$	$\log(\beta_3)$
sulfate	0.96 ± 0.06	6.0 ± 0.5	6.20 ± 0.13	13.0 ± 0.28	20.0 ± 0.03
phosphate			3.59 ± 1.33	11.5 ± 0.83	17.5 ± 0.01
acetate	1.0 ± 0.1	6.5 ± 0.6	6.49 ± 0.03	12.6 ± 0.06	18.5 ± 0.12

[a] pH 3.7, 200 mM buffer.

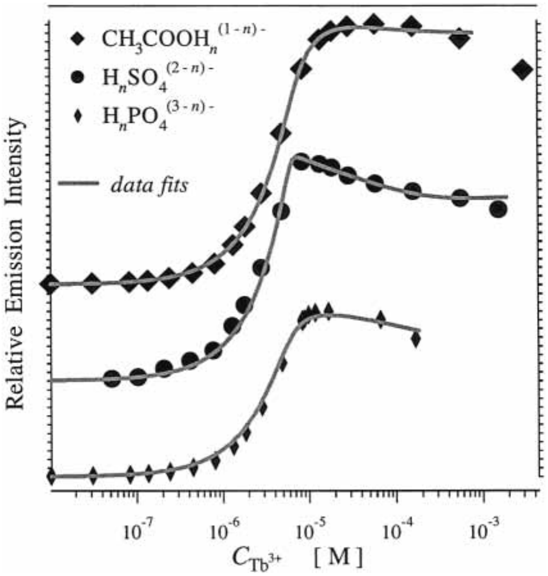


Figure 7. Emission data for 20 μM DPA titrated with terbium(III) in 0.2 M sulfate, phosphate and acetate buffers, pH = 3.7 ($\lambda_{\mathrm{ex}} = 266$ nm). The data were fit to a three-step complexation model (eq 7, $n = 3$).

$\sim 10^{-5}-10^{-3}$ M^{-1}, results from transition from di- to monoligated complex, where the latter exhibits a larger emission quantum yield. Since a fit of the data to a two-step complexation model (eq 7) failed due to the steepness of the measured titration patterns,[29] the titration curves were deconvoluted into two distinct sigmoidal patterns (Figure 8).[33]

Using the one-step equilibrium model,[30] the data collected for solution with acetate unambiguously yielded a line slope that corresponds to formation of monoligated terbium(III), i.e., Tb(DPA). While the data for sulfate-containing solutions suggested formation of $Tb(DPA)_2$, in the presence of halides, nitrate, or perchlorate, the only plausible results were obtained when $m > 1$ (Table 3), which is an indication for formation of polynuclear complexes (see Supporting Information for details). Such a finding is in accord with previous reports of the tendency of terbium(III) to form polynuclear complexes at this particular pH range.[15,34]

The second transition, depicted by the increase of the measured emission at C_{Tb} of $\sim 10^{-5}-10^{-3}$ M^{-1} (Figure 8), has not been quantitatively analyzed (see Supporting Information). The observed emission enhancement is a result of the formation of either fine Tb-containing colloidal particles with DPA bound to them or polynuclear complexes with larger values of m. This finding is in agreement with previously reported increase in emission intensities for DPA-containing complexes upon addition of another rare-earth (or yttrium) ion.[3]

The titration data for 20 μM DPA at neutral pH (Figure 9) closely resembled the data obtained at pH 3.7. Fitting the pH 7 curves to a three-step complexation model (eq 7) resulted in larger formation constants (Table 3); overall, the values for K_1 were about 3−6-fold larger than the values obtained for pH 3.7. For data collected in phosphate, however, the best fits were produced to a model that ignores the first equilibrium step (eq 4); i.e., the value of K_1 was held at zero, indicating that the formation of the monoligated complex is suppressed under such

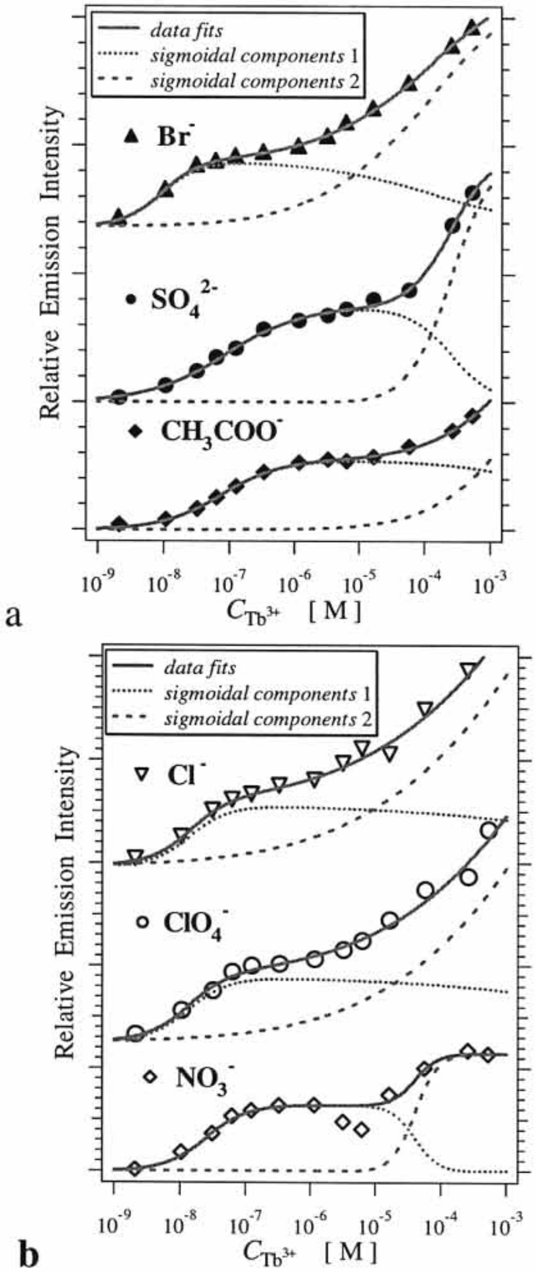


Figure 8. Emission data and the corresponding bisigmoidal fits for 2.3 nM DPA titrated with terbium(III) in various 0.2 M buffers, pH = 7.0 ($\lambda_{\mathrm{ex}} = 278$ nm).

TABLE 3: Formation Constants and Complexation Coefficients, *m* and *n*, Obtained from Emission Data for DPA Titrated with Terbium(III) in Various Buffers[a]

	results from fitting with					
	one-step model (eq 3) (C_{DPA} = 2.3 nM)			three-step model (eq 7) (C_{DPA} = 20 μM)		
	m	n	$\log(\beta_{m,n})$	$\log(K_1)$	$\log(\beta_2)$	$\log(\beta_3)$
chloride	1	0.48 ± 0.05		7.01 ± 0.29	15.0 ± 0.08	23.0 ± 0.04
	2	1.7 ± 0.2[b]	24 ± 1			
	3	2.8 ± 0.3	40 ± 3			
	4	3.5 ± 1.0	54 ± 9			
bromide	1	0.35 ± 0.05		6.99 ± 0.42	14.9 ± 0.6	22.2 ± 1.1
	2	1.0 ± 0.2[b]	16 ± 2			
	3	1.6 ± 0.4	29 ± 4			
	4	2.2 ± 0.6	43 ± 5			
phosphate					15.0 ± 0.03	22.3 ± 0.07
sulfate	1	1.8 ± 0.1[b]	16 ± 0.6	6.99 ± 0.33	15.1 ± 0.02	21.7 ± 0.2
	2	4.5 ± 0.3	45 ± 3			
	3	7.3 ± 0.6	77 ± 5			
	4	10 ± 1	110 ± 8			
perchlorate	1	0.30 ± 0.10		7.26 ± 0.53	15.2 ± 0.1	22.9 ± 0.1
	2	1.1 ± 0.4[b]	16 ± 4			
	3	2.0 ± 0.8	32 ± 7			
	4	2.8 ± 1.1	48 ± 10			
acetate	1	0.92 ± 0.04[b]	7.2 ± 0.4	6.98 ± 0.01	15.0 ± 0.2	21.0 ± 0.9
nitrate	1	0.56 ± 0.03		7.66 ± 0.33	15.4 ± 0.01	22.7 ± 0.2
	2	1.6 ± 0.1				
	3	2.3 ± 0.3[b]	31 ± 2			
	4	3.1 ± 0.4	39 ± 4			

[a] pH 7.0, 200 mM buffer. [b] The italic entries represent the smallest values of *n* that approximate an integer.

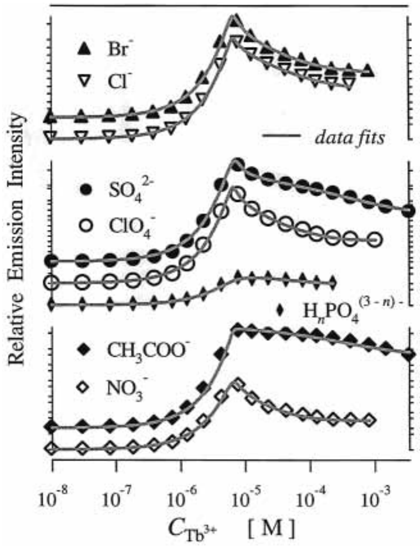


Figure 9. Emission data for 20 μM DPA titrated with terbium(III) in various 0.2 M buffers, pH = 7.0 (λ_{ex} = 266 nm). The data were fit to a three-step complexation model (eq 7).

conditions, most probably due to the incipient formation of the insoluble terbium phosphate.[31,35]

Influence of the Salt Concentration at pH 1.9. The influence of salt concentration on the complexation equilibria between terbium(III) and DPA was examined via measurements using buffer having high capacity (i.e., pH 1.9 sulfate buffer, pK_a(HSO_4^-) = 1.92), and varying the buffer concentration between 40 mM and 1 M. Titrations of nanomolar concentrations of DPA with terbium(III) in 0.2 and 1 M buffer exhibited patterns of formation of monoligated mononuclear Tb−DPA complexes (Figure 10a). Furthermore, the 5-fold increase in salt concentration to 1 M resulted in about a 2 order-of-magnitude drop in the value of the corresponding formation constant, $\beta_{1,1}$ (Table 4). When the buffer concentration was decreased to 40 mM, however, an indication for formation of polynuclear

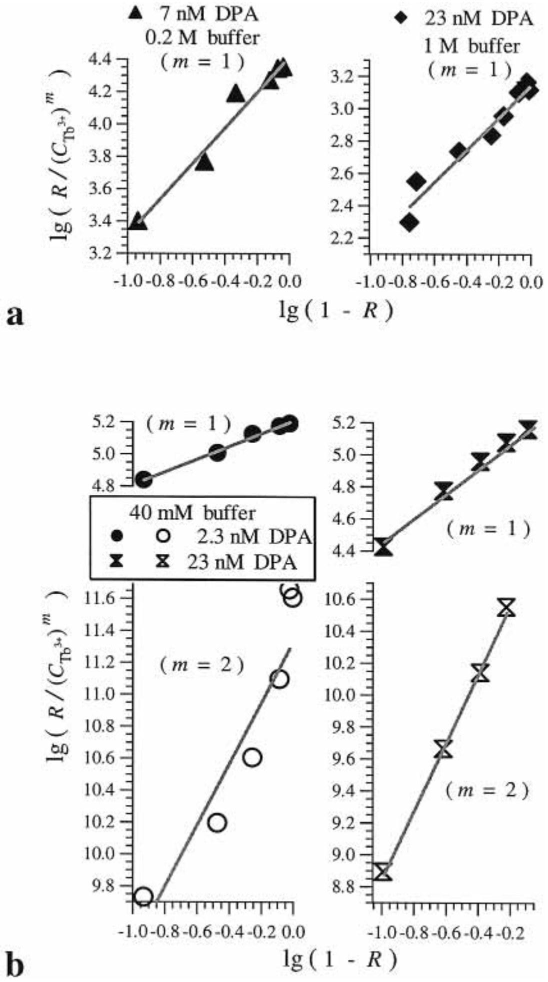
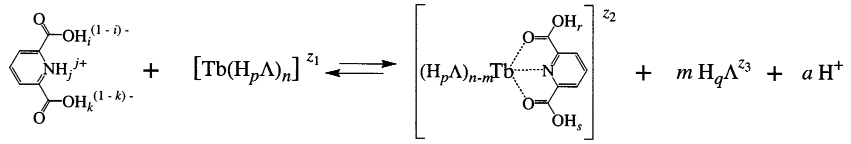


Figure 10. Emission data (λ_{ex} = 278 nm), converted to the logarithmic function describing the one-step complexation model (eq 3, m = 1 or 2) for DPA titrated with terbium(III) in (a) 0.2 and 1 M, and (b) 40 mM sulfate buffer, pH = 1.9.

SCHEME 2: Generalized Complexation Equilibrium for Terbium(III) and DPA



$$z_1 = 3 + n\,(p - \mathrm{x})$$
$$z_2 = 1 + r + s + (n - m)(p - \mathrm{x})$$
$$z_3 = q - \mathrm{x}$$
$$a = i + j + k - r - s + m\,(p - q)$$

TABLE 4: Formation Constants and Complexation Coefficients, *m* and *n*, Obtained from Emission Data for DPA Titrated with Terbium(III) in Sulfate Buffers[a]

	results from fitting with					
	one-step model (eq 3)				two-step model (eq 7) ($C_{\mathrm{DPA}} = 20\ \mu$M)	
	C_{DPA} [nM]	m	n	$\log(\beta_{m,n})$	$\log(K_1)$	$\log(\beta_2)$
1 M buffer	23	1	0.99 ± 0.10[b]	3.1 ± 0.7	3.30 ± 0.003	6.29 ± 2.17
0.2 M buffer	7	1	1.1 ± 0.1[b]	5.2 ± 0.8	5.56 ± 0.34	12.5 ± 0.01
40 mM buffer	2.3	1	0.40 ± 0.02		6.54 ± 0.04	14.0 ± 0.1
		2	1.9 ± 0.4[b]	19 ± 4		
40 mM buffer	23	1	0.75 ± 0.03			
		2	2.1 ± 0.1[b]	19 ± 0.5		
40 mM buffer + 1 M NaCl[c]					6.00 ± 0.96	11.6 ± 0.2
40 mM buffer + 1 M $NaClO_4$[c]					5.98 ± 0.04	11.6 ± 1.3

[a] pH 1.9, 1 M, 200 mM, and 40 mM buffer. [b] The italic entries represent the smallest values of *n* that approximate an integer. [c] 1 M chloride or perchlorate in 40 mM buffer solutions.

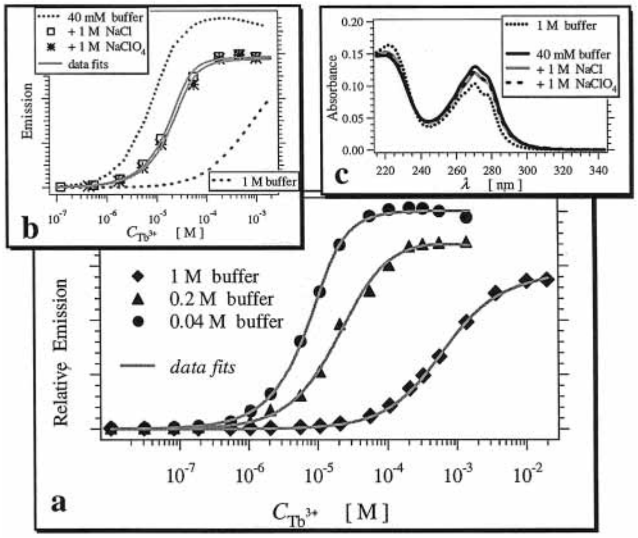


Figure 11. Emission data for 20 μM DPA titrated with terbium(III) in (a) 0.04, 0.2, and 1 M sulfate buffer, pH = 1.9, and (b) 40 mM sulfate buffer, pH 1.9, with 1 M NaCl or $NaClO_4$ ($\lambda_{\mathrm{ex}} = 266$ nm). The data were fit to a two-step complexation model (eq 7). (c) UV absorption spectra of 20 μM DPA in 0.04 and 1 M sulfate buffer, pH 1.9, and in the presence of 1 M chloride and perchlorate.

complexes, $Tb_2(DPA)_2$, was observed (see Figure 10b and Table 4). This result suggests that sulfate ions impede polynucleation. Titration of 20 μM DPA produced patterns that resembled the curves obtained at pH 0.5 (Figure 11a). Furthermore, from the shapes of the observed emission spectra, it was evident that no tris Tb−DPA complexes were formed during the titration courses (see Supporting Information). Fitting the data to a two-step complexation model (eq 7) produced formation constants that exhibit some dramatic trends (Table 4): a 5-fold dilution of the buffer from 1 to 0.2 M forced K_1 to increase 2 orders of magnitude; further 5-fold dilution to 40 mM resulted in another order of magnitude increase in the first-step formation constant. Conspicuously similar trends were exhibited by the second-step formation constant, K_2.

To examine if the alterations in complexation properties resulted indiscriminately from variations in the ionic strength or also from specific interactions with the present sulfate,[36] titrations of 20 μM DPA in 40 mM buffer (sulfate, pH 1.9) in the presence of 1 M chloride or perchlorate were conducted (Figure 11b). The calculated ionic strengths for 1 M sulfate buffer is ~1.33 M, and for 40 mM buffer containing 1 M unibasic salt, it is ~1.05 M.[37] However, the values of the formation constants obtained for the latter cases were significantly closer to the value of the equilibrium constants for 40 mM, rather than for 1 M sulfate buffer with no additional salt (Table 4). These results suggest that complex formation is diminished predominantly by specific interaction with the sulfate species,[36] rather than merely by increase of the ionic strength. Furthermore, it is quite surprising that perchlorate, which contains negatively charged oxygen and is structurally similar to sulfate, exhibits an influence on the complexation equilibria similar to that of chloride (a soft base known to interact weakly with terbium(III)[23]).

Significant perturbations in the absorption spectrum of DPA were observed upon variation of the concentration of the pH 1.9 buffer (Figure 11c), a result that can be ascribed to change of the state of ptotonation of DPA because of the proximity between the pH of the buffers (1.9) and the reported value for pK_{a2} of DPA (Scheme 1).[22] Although the shift of the equilibrium from the anionic to the zwitterionic species will decrease the formation propensity of Tb−DPA complexes, this effect can hardly account for the 3 order-of-magnitude drop in the values of K_1 when the buffer concentration is raised from 40 mM to 1 M (compare values in Tables 1 and 4). Therefore, it can be concluded that interactions of the sulfate ions with terbium(III) are the major impediment for Tb−DPA complexation when sulfate buffer concentration is increased.

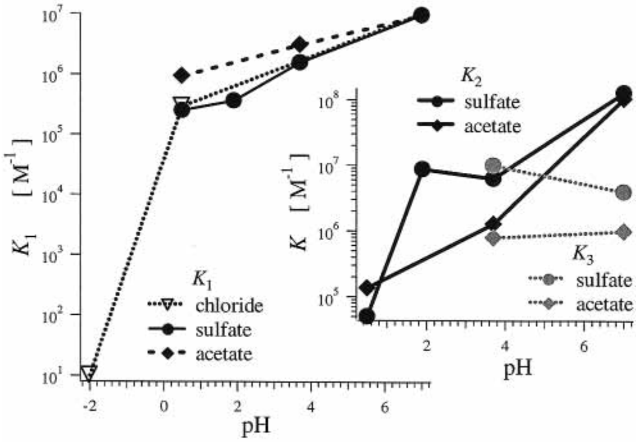


Figure 12. Dependence of the stepwise formation constants, K_1, K_2, and K_3 (see eqs 4−6), on the acidity of the media in the presence of different 200 mM buffers.

Discussion

The goal of this investigation was to find conditions under which terbium(III) exhibits a high propensity for formation of monoligated coordination complexes with dipicolinic acid. Figure 12 shows a summary of the pH dependence for some of the formation constants, K_1. It is expected that an increase of the pH of the media will increase the complexation propensity of terbium(III) with DPA because of the stronger electrostatic attraction exhibited when the ligand is converted to its anionic forms. However, such a simple explanation would fail to account for the dramatic increase of K_1 (more than 4 orders of magnitude) when acidity is changed from −2 to 0.5; in contrast, upon further elevation of pH to 7, the values of K_1 rise only about 2 orders of magnitude (Figure 12).

The dependence of the measured formation constants on the pH of the medium is a reflection of the combined effects of several acid−base and ligation equilibria (Scheme 2). Although electrostatic interactions certainly have a significant effect on the thermodynamics of the complexation processes, to predict the pH dependence of the charge of the participating species, it is essential to have information about the pK_a's not only of the free and ligated DPA, but also of other possible ligands such as water or anionic species present in the solution. For instance, in strongly acidic media (e.g., $H_0 = -2$) the charge of DPA is known to be +1 (Scheme 1);[22] however, the charge of [Tb($H_p\Lambda)_n$] cannot be unambiguously determined without further knowledge about the ligands Λ. However, if it is assumed that most sites are protonated (i.e., $i = j = k = r = s = 1$ and $p \approx q$, Scheme 2), the coefficient in front of H^+, a, will be close to unity. Therefore, the high proton activity (100 M) would favor shifting the equilibrium toward [Tb($H_p\Lambda)_n$] and free DPA species, thus resulting in a relatively small formation constant K_1. Transition from $H_0 = -2$ to pH = 0.5 ($i + j + k = 2$ and $a \leq 0$) causes the equilibrium to shift toward Tb−DPA complexation, a phenomenon reflected by the dramatic increase of the value of K_1 (Figure 12). It is plausible to assume that r or s can be equal to 1 since previously reported crystal structures indicate that the carboxylates of DPA can be protonated when it is coordinated to a metal ion.[38]

At neutral pH, DPA exists as a dianion. However, [Tb($H_p\Lambda)_n$] must be positively charged in order to plausibly argue that the increase of the values of the formation constants when pH is elevated to 7 (Figure 12) is electrostatically driven. On the other hand, at neutral pH, a is expected to be equal to or smaller than 0 (Scheme 2). Hence, an increase in the basicity of the media should favor the dissociation of the complexes. This phenomenon could be the reason for the relatively modest increase in the values of K_1 when the pH is elevated from 0.5 to 7, in comparison with the dramatic increase observed when the acidity is changed from −2 to 0.5 (Figure 12).

Formation of polynuclear Tb−DPA complexes, observed when nanomolar concentrations of DPA are present, can cause complications, not only for development of quantitative assays but also for reproducibility of a low-limit baseline for DPA assay. Therefore, it is essential to establish conditions under which terbium(III) polynucleation is suppressed. The data have provided examples of three species in whose presence polynuclear complex formation is not favored: DPA,[35] acetate, and sulfate. Therefore, it can be concluded that none of these moieties plays the role of a bridging ligand between two terbium ions. The observed increase in the propensity for polynucleation with elevation of pH indicates that hydroxy or oxo ligands are even better bridging agents than water itself, which can play a role at lower pH. Therefore, polynucleation can be suppressed by introducing ligating species that bind to terbium(III) relatively strongly, either displacing water or hydroxyl ligands or (and) introducing steric hindrance to the approach of two terbium ions. Apparently, acetate[39,40] and sulfate[36,40] prove to be good candidates for such polynucleation inhibition.

Phosphate has been of serious concern for terbium(III)-mediated spore detection analyses:[9] about 40−60% of emission quenching of tris Tb−DPA complexes has been observed in the presence of phosphate salt.[12] Also, under conditions where only formation of the monoligated terbium−DPA complex is expected, the emission is completely quenched upon addition of phosphate.[7] The results presented here confirm the high affinity of phosphate for coordination with terbium(III). Precipitate formation was observed at the upper C_{Tb} limit for each of the titrations performed in phosphate buffer, and its formation was considerably more pronounced at higher pH, indicating that PO_4^{3-} ions, rather than HPO_4^{2-} or $H_2PO_4^-$, are the species contributing most to the dissociation of the Tb−DPA complexes. Furthermore, for pH 7 phosphate buffer solutions, even when terbium is in 100-fold excess, the shape of the emission spectrum indicates that predominantly tris Tb−DPA species are present (see Supporting Information for details), and the titration results under the same conditions suggest that no monoligated complex was formed (Table 3). These findings unambiguously imply that the tris Tb−DPA conjugate is more stable in the presence of phosphate than its monoligated analogue, explaining the discrepancy in the extent of emission quenching in the previously reported results.[7,12]

Conclusions

The stability of the monoligated Tb−DPA complex exhibits a nontrivial dependence on the acidity of aqueous media: the expected growth of the values of K_1 in the pH range from 0.5 to 7 is preceded by a dramatic (4 orders of magnitude) increase when acidity is elevated from −2 to 0.5 (Figure 12). The addition of a second DPA ligand is also favored with an increase in pH, while the equilibrium of the third ligation step is not altered when pH is changed from 3.7 to 7.0 (see K_2 and K_3 in Figure 12).

The influence of the various anions on the stability of the Tb−DPA complexes is not easy to discern. However, it was evident that three species can alter the complexation equilibria (most probably via complexing with terbium ions): phosphate,[31] sulfate,[36,40] and acetate.[39,40] The effects of the phosphate were quite conspicuous because of the precipitate that was observed in its presence at high C_{Tb} values. The influence of the addition of excess acetate and sulfate, however, was found to be positive in terms of inhibition of polynucleation and suppression of formation of precipitate of terbium oxide/hydroxide at the upper limit of C_{Tb} at neutral pH. As expected, the presence of high salt concentration compromises the stability of Tb−DPA complexes. This destabilizing effect is hardly a result of high ionic strength alone; rather, it is considerably more pronounced when the excess anion has a strong propensity for interaction with terbium(III).

Consequently, the present results strongly suggest that neutral (or alkaline) pH should be avoided in conducting terbium(III)-mediated emission assays. Although the highest measured values for the formation constants of Tb−DPA complexes at neutral pH present an obvious advantage, the formation of polynuclear Tb complexes under these conditions can compromise any attempt at quantitative analysis. Furthermore, attenuation of high concentrations of Tb^{3+}, C_{Tb}, necessary for achieving lower DPA detection limits will be constrained by the tendency of terbium(III) to form insoluble hydroxide/oxide conjugates at pH >5−6,[14] some of which may even incorporate DPA.[35] Therefore, for spore detection assays, the pH has to be low enough to avoid these interfering effects, without the considerable decrease of the stability of the monoligated Tb−DPA complexes; the presence of acetate and/or sulfate should further improve the conditions for analysis. In addition, sulfate species will eliminate the emission quenching influence of some cations such as Ca^{2+} that are likely to be part of the samples under analysis.[12]

The evidence from the present investigation suggests that optimal conditions for terbium(III)-mediated emission assays (e.g., for bacterial spore detection) can be achieved in the presence of acetate/sulfate buffers (concentration under ∼0.2 M) maintaining the pH of the media in the range of 3−5. Given the irreducible emission intensity associated with uncomplexed terbium(III), a threshold limit for detection of DPA is ca. 500 pM even for conditions that are optimal for equilibrium giving the monoligated Tb(DPA) species.

Acknowledgment. The authors acknowledge with thanks support of this research by the U. S. Army Research Laboratory. Also, we extend our gratitude to Dr. Nicholas Fell for the extensive discussions and productive collaboration.

Supporting Information Available: Derivation of the equilibrium complexation models (eqs 3 and 7) and data manipulation details are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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(28) Since the exact states of protonation of the terbium-containing species are not known, H^+ is not included in eqs 4−6. However, in buffered media the pH is maintained constant; hence, the values of $[H^+]$ modify the pH-dependent stepwise formation constants K_i.

(29) The steepness of the sigmoid patterns of S vs $\log(C_{Tb})$ can be correlated to the m and n coefficients in eq 2: the larger the ratio m/n is, the steeper the titration curve will be.

(30) The linearity of the data was observed when the value for R was larger than 0.05−0.1 and smaller than 0.9−0.95 (see Figure 3). When R approaches zero or unity, even small fluctuations of the experimentally measured values of S will result in logarithms of numbers that are close to or smaller than zero.

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(34) Not taking into account the propensity for formation of polynuclear Tb−DPA complexes could be the reason for higher values of the formation constants for the mono, bis, and tris complexes reported in previous investigations.[19,20]

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