LBL-11878 c.2
Preprint

Lawrence Berkeley Laboratory
UNIVERSITY OF CALIFORNIA

Materials & Molecular
Research Division

Submitted to the Journal of the American Chemical Society

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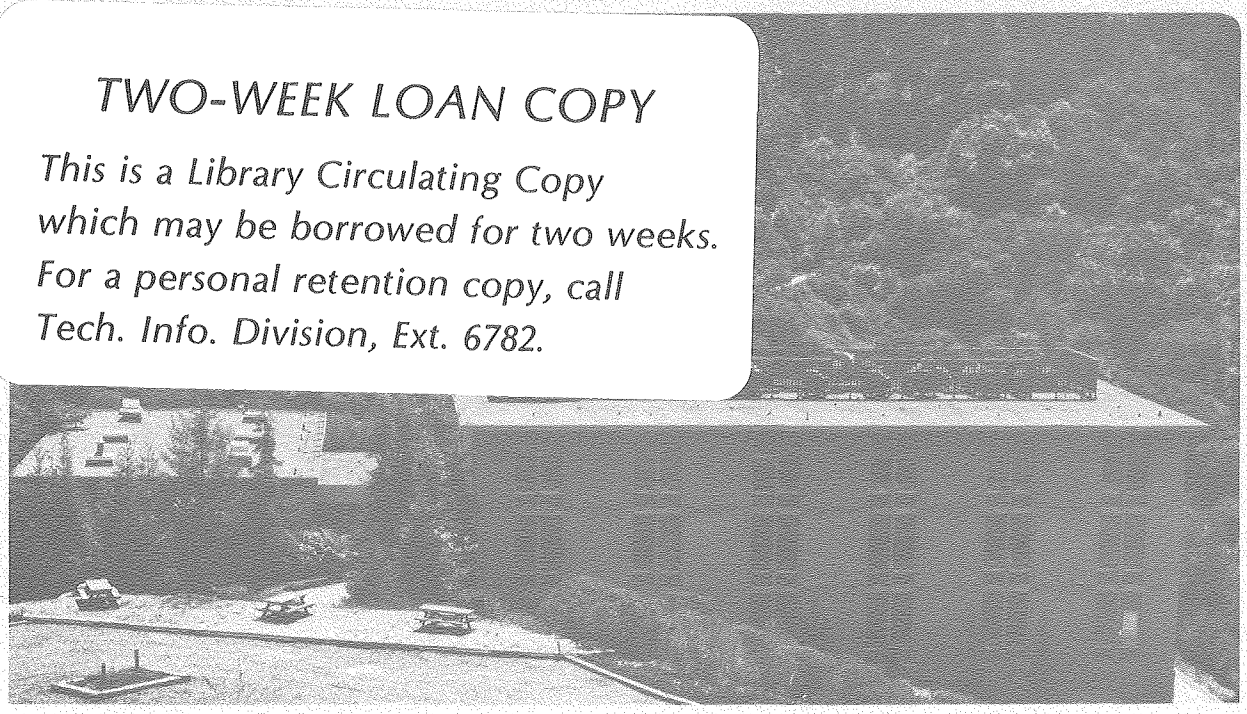
August 1980



Prepared for the U.S. Department of Energy under Contract W-7405-ENG-48

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LBL-11878

Siderophilin Metal Coordination. 1. Complexation of Thorium by Transferrin: Structure-Function Implications

By

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Abstract

As part of a program to develop actinide-specific sequestering agents, the coordination of actinide ions by human transferrin is being investigated. Therapeutically useful synthetic ligands must be able to compete with this iron-transport protein for the bound actinide ion. As in the Fe(III) complex of the native protein, two Th(IV) ions bind at pH 7. This coordination has been monitored at several pH values using difference ultraviolet spectroscopy. The corresponding coordination of a phenolic ligand, ethylene-bis-(o-hydroxyphenylglycine) [EHPG], has been used to determine the $\Delta\varepsilon$ for a tyrosyl group coordinated to Th(IV), in contrast to the common practice of assuming the $\Delta\varepsilon$ for protons and all metal ions are the same. This in turn is used to determine, from the observed $\Delta\varepsilon$ upon protein coordination, the number of transferrin tyrosine residues that coordinate. Maxima in the Th(IV) + EHPG difference UV spectra occur at 292 and 238 nm, with corresponding $\Delta\varepsilon$ values per phenolic group of 2330 and 8680 cm^{-1} M^{-1}, respectively. At pH 7.2, the Th(IV) transferrin spectrum is closely similar to the TH(IV) EHPG spectrum, with maxima at 292 and 240 nm. The $\Delta\varepsilon$ at 240 nm reaches a maximum of 24,700 cm^{-1} M^{-1}, which corresponds to coordination of three tyrosine residues in the dithorium transferrin complex; the stronger binding site ("A" or C-terminal) coordinates via two tyrosines and

the weaker ("B" or N-terminal) via one. There is evidence suggesting that the N-terminal site is slightly smaller than the C-terminal site. While Th(IV) easily fits into the C-terminal site, the large ionic radius of Th(IV) makes this ion of borderline size to fit into the N-terminal site. This may be an important biological difference between Th(IV) and the slightly smaller Pu(IV), which should easily fit into both sites. At pH values below 7, the complexation of Th(IV) by transferrin decreases rapidly. At pH 6 and a Th(IV)/transferrin ratio of 2, only $\sim$ 0.3 Th(IV) are bound per protein ($[Tr] = 10^{-5}$ M). The N-terminal site is more rapidly affected by lowering the pH, so that coordination is entirely at the C-terminal site at low pH. Above pH 9, the conformation at the C-terminal site (two tyrosines) changes such that only one tyrosine is bound, the same that pertains at the N-terminal site at neutral pH. In addition to the three protons released by the coordinating tyrosine residues, the complexation of two Th(IV) ions releases two more protons at pH 8.6, which are ascribed to hydrolysis, so that the metal is bound as a monohydroxo species. It is suggested that diferric transferrin undergoes a similar reaction, and the other implications of these results for the structure and function of the native ferric transferrin are discussed.

Introduction

A major concern associated with nuclear power has been the radiation hazards associated with the nuclear fuel cycle. The major long-term hazards are the trans-uranium elements, especially plutonium. Previous biological studies have shown that Pu^{4+} tends to follow the ferric ion metabolic pathways in mammalian systems. When plutonium first enters the blood, it binds to the iron transport protein transferrin.[4-6] It then circulates as the protein complex until its eventual deposition onto bone surfaces or incorporation into the iron storage proteins ferritin and hemosiderin.[7,8]

We are currently conducting a program to design, synthesize, and evaluate actinide-specific sequestering agents for use in the treatment of plutonium poisoning.[9-12] These ligands incorporate dihydroxybenzene (catechol) moieties as the ligating groups, and recent biological studies have demonstrated their effectiveness in removing plutonium from rats.[13] Since these synthetic compounds must compete with biological ligands for plutonium, we are also interested in the coordination chemistry of the plutonium-protein complexes. We therefore have begun a study on the binding of actinides by human serum transferrin (as a class these iron transport proteins are called siderophilins), which begins with this report on the transferrin complex of Th^{4+}, an ion we use as a convenient model of Pu^{4+}.

Transferrin circulates in the blood as the principal iron transport protein in humans.[13,14] It is responsible

both for carrying iron from absorption and storage sites to areas of utilization, and for complexing the iron as it is released from senescent red blood cells. Transferrin binds a wide variety of metal ions, and in every case there is a simultaneous binding of a synergistic anion to form a ternary metal-protein-anion complex.[14] Carbonate (or bicarbonate) forms the most stable complexes and serves as the synergistic anion in vivo. The protein is comprised of a single polypeptide chain and has two metal binding sites which are very similar, but apparently not identical. The two sites are widely separated,[15,16] and there appears to be little if any interaction between them. The donor groups which comprise the metal binding sites have not yet been positively identified. There has been considerable controversy over the years about the differences, if any, between these two sites. The first site, which is called "A", has now been shown to bind Fe(III) somewhat more strongly at physiological pH than does the second "B" site.[17]

With regard to chelation therapy for the decorporation of metal ions, one of the most important features of the transferrin complexes is their relative lability compared to other protein complexes such as ferritin. Thus, it seems likely that most of the readily chelatable plutonium would come from transferrin, particularly if treatment was begun shortly after exposure. It is interesting to note that previous studies on decorporation by DTPA[18] have shown that

delaying therapy reduces the amount of metal which can be removed.[19] Thus, actinide transferrin complexes are of particular clinical interest.

One of the most useful techniques for evaluating metal binding by proteins is difference UV spectroscopy. Although the details of the transferrin binding site are still unsettled, it is clear that two or three tyrosyl residues are directly bound to each metal ion.[15,20-22] The coordination and concomitant deprotonation of the tyrosyl phenolic groups perturbs the π to π^* transition of the aromatic ring. Thus, one can detect metal binding from the difference spectrum of the metallotransferrin complex versus the apoprotein. This technique is particularly well suited for protein work because it effectively blanks out the contribution of nonbonding groups. In contrast, potentiometric titration (the most useful technique for low molecular weight ligands), is almost useless for protein studies because of the large number of ionizable groups which are not involved in metal binding.

In order to quantitate the extent of metal binding or to determine the number of tyrosines coordinated to each metal ion, it is necessary to know the change in extinction coefficient ($\Delta\varepsilon$) for a coordinated phenolic group versus a protonated phenolic group. In the past it has been common practice to estimate this value as the $\Delta\varepsilon$ (10,000 cm^{-1} M^{-1}), between protonated and deprotonated N-acetyl-tyrosine.[15,20,21] However, this practice does not allow for any variation in

$\Delta\varepsilon$ for coordination of different metal ions and has led some to question the usefulness of this technique.[21] This is an important point which we address in some detail in a separate paper; however, for the purposes of this study, we have determined the molar $\Delta\varepsilon$ per phenol bound to Th^{4+} from spectroscopic data on the thorium complex of ethylene-bis-(o-hydroxyphenylglycine) (Figure 1), abbreviated as EHPG.[18] Based on these results, we discuss the degree and manner of binding of Th(IV) by transferrin.

Experimental

Materials. Purified human serum transferrin (M.W. 77,000)[23] was purchased from Sigma and further purified by gel permeation chromatography on Sephadex G-25 (Pharmacia) using 0.1 M $NaClO_4$, 0.05 M tris, pH 7.4 as an eluant. The fractions containing transferrin were combined and rechromatographed using a 0.05 M solution of the appropriate buffer, either MES,[18] tris,[18] or HEPES.[18] Fractions with an absorbance at 280 nm > 1.3 were combined and stored at 4°C. For spectroscopic studies, aliquots of apotransferrin were diluted and quantitated from the absorbance at 280 nm using an ε of 92,300 $cm^{-1}\ M^{-1}$.[23] Monoferric transferrin was prepared as previously described by D. C. Harris,[24] using MES rather than succinate buffer. The EHPG (90%) was purchased from Sigma and purified by dissolving the material in dilute base and precipitating the ligand by acidifying to pH 4 with the addition of HCl.

The molecular weight of the purified ligand was determined by potentiometric titration with standardized KOH.

Thorium stock solutions (0.01 M) were prepared from $ThCl_4$ and were adjusted to a final acidity of 0.02 M HCl by the addition of standardized acid. The actual Th^{4+} concentration was determined by complexometric titration with EDTA[18] using xylenol orange as an indicator.[25] Dilute solutions of $Th(NTA)_2$ were prepared by adding two equivalents of NTA[18] to aliquots of the stock $ThCl_4$ and diluting to volume.

Apparatus. Spectra were recorded on a Cary 118 spectrophotometer at 25°C. Routine pH measurements were made with a Beckman model 104 pH meter equipped with a combination microelectrode which could be inserted directly into the cuvettes. For proton release studies, pH was recorded to the nearest 0.001 pH unit with a Corning model 130 pH meter equipped with separate glass and saturated calomel electrodes. This meter was also used for the potentiometric equilibrium titrations of EHPG and 1:1 EHPG-Th^{4+} solutions. The meter was standardized to read hydrogen ion concentration directly, not activity, as described elsewhere.[26]

Procedures. A buffered transferrin solution was split between the reference and sample cuvettes. The $Th(NTA)_2$ was added to the sample cuvette in 10 - 30 µℓ aliquots using a 2.0 mℓ Gilmont Micrometer buret, and matching volumes of distilled water were added to the reference cuvette. It was

determined that free NTA had no significant absorbance over the wavelength range of interest. The absorbance measured after each addition of thorium was divided by the total ligand concentration to give $\Delta\varepsilon$. Titration curves were then constructed by plotting $\Delta\varepsilon$ versus r, the ratio of total metal to total ligand. The slope of such a plot is equal to the molar $\Delta\varepsilon$ of the thorium complex.

Proton release studies were conducted by adding aliquots of 4.24×10^{-3} M $Th(NTA)_2$ to a pH 8.6 self-buffered 2.12×10^{-5} M transferrin solution, then readjusting the pH back to 8.6 by the addition of 4.16×10^{-3} M KOH. Corrections were made for the free acid (0.29 H^+/Th^{4+}) contained in the $Th(NTA)_2$ solution and for the partial protonation of the tertiary amine of the dissociated NTA (1.86 H^+/Th^{4+}) using the ligand protonation constants of Martell and Smith.[27]

Results

EHPG titrations. Previous studies have described the EHPG complexation and protonation equilibria with the first row transition metals.[28,29] No data have been reported on actinide complexes, although a lanthanide complex has recently been examined.[30] Therefore, potentiometric equilibrium curves were recorded for Th^{4+}:EHPG to determine the pH range over which both phenolic groups of the ligand are coordinated to thorium. Results for the free ligand and for the 1:1 metal-ligand solution are shown in Figure 2. A zwitterionic H_2L ligand, EHPG is protonated at the two amines and the two

phenolic oxygens. The two buffer regions from a = 0 to a = 2, where a is the ratio of total hydrogen to total ligand, represent the deprotonation of the two amino groups, which have pK_a's of 6.32 and 8.64.[29] The reported pK_a's for the two phenols are 10.24 and 11.68,[29] so as expected there are no discernable features in the titration curve beyond a = 2.

The addition of Th^{4+} to an EHPG solution results in the almost complete displacement of the four dissociable ligand protons. Thus, the metal-ligand titration curve from a = 0 to a = 4 is essentially that of a strong acid. A colorless solid, presumably thorium hydroxide, precipitates shortly past the break at a = 4. These results indicate that EHPG is most likely hexadentate, with both phenols coordinated to Th^{4+} over the pH range 2.5 to 7.

EHPG spectra. Difference UV spectra at pH 6 for Th^{4+}: EHPG versus EHPG alone are shown in Figure 3. There are three extrema: two maxima at 292 and 238 nm and a single minimum at 269 nm. A plot of $\Delta\varepsilon$ versus r, the ratio of added thorium to total EHPG, is shown in Figure 4. The plot has a sharp break near r = 1, confirming the presumed 1:1 ligand stoichiometry of the thorium-EHPG complex, with a final $\Delta\varepsilon$ of 17,360 cm^{-1} M^{-1}. Since this total $\Delta\varepsilon$ represents the coordination of two phenols, the molar $\Delta\varepsilon$ per phenolic group is 8680 cm^{-1} M^{-1}.

Transferrin spectra. Difference UV spectra of the thorium-transferrin complex versus apotransferrin are shown in Figure 5. As in the EHPG difference spectra, there are maxima at 292 and 240 nm; but the transferrin spectra do not have the minimum at 260 nm seen in the Th-EHPG system. A plot of $\Delta\varepsilon$ at 240 nm versus r for pH 7.2 is shown in Figure 6. The data are linear up to an r value of $\sim$ 1.3, then curve downward, eventually leveling off at r = 2.0 and a $\Delta\varepsilon$ = 24,700 M^{-1} cm^{-1}. The two linear segments extrapolate to an intersection of r $\sim$ 1.55.

Curvature in a spectrophotometric titration curve such as that seen in Figure 5 is usually indicative of weak complexation. Since the thorium must be added as the bis(NTA) chelate, it is possible that as the titration progresses, the free NTA which accumulates in solution begins to compete effectively with transferrin for the Th(IV). However, if this were the case, one would expect $\Delta\varepsilon$ to continue to increase beyond r = 2, since the two transferrin binding sites should eventually be filled by the addition of excess $Th(NTA)_2$. Instead, $\Delta\varepsilon$ levels off at r = 2, which is consistent with strong complexation of Th^{4+} by transferrin.

To determine if NTA does compete with the apotransferrin for thorium at pH 7.2, a second set of difference spectra were recorded in the presence of a tenfold excess of NTA. At any point in the titration curve, the excess ligand has almost no effect on $\Delta\varepsilon$, proving

that competition from NTA is not a significant factor over most of the titration. There may be some competition at the end of the titration, when the ratio of free NTA to vacant transferrin binding sites rises to 20 - 30.

The effect of pH on thorium binding was also examined. As the pH decreases from 7.2, the maximum value of $\Delta\varepsilon$ decreases sharply. Titration curves from pH 7.2 to 6.0 are shown in Figure 6 and from pH 9.5 to 8 in Figure 7. The curves at lower pH do not level off as sharply; it appears that the NTA is able to compete with transferrin better under these more acidic conditions. This was confirmed by repeating the titration at pH 6.25 with a tenfold excess of NTA, which resulted in a significant decrease in $\Delta\varepsilon$ value at r values greater than 1.0.

Observed $\Delta\varepsilon$ values at r = 2 as a function of pH from pH 6 to 9.5 are shown in Figure 8. As discussed above, the decrease in $\Delta\varepsilon$ below pH 7 is due to a shift in the effective binding constants of NTA and transferrin, such that NTA begins to compete strongly with transferrin for the thorium. The titration curves at high pH indicate that two thorium ions are still tightly bound over the pH range, therefore the decrease in $\Delta\varepsilon$ must reflect a decrease in the number of tyrosyl residues bound per thorium. The $\Delta\varepsilon$ at pH 9.5 is 16,000 M^{-1} cm^{-1}, which would correspond to only one tyrosine per thorium.

Monoferric transferrin spectra. Monoferric transferrin was prepared by the method of D. C. Harris,[24] such that the one equivalent of iron was bound exclusively at the C-terminal binding site.[31] This complex was then titrated with $Th(NTA)_2$ to give the curve shown in Figure 9. As expected, only a single equivalent of thorium is bound. Since we have observed that iron is more tightly bound than thorium, it is not likely that the thorium is displacing the iron from the C-terminal site. Thus, the thorium is presumably occupying the vacant N-terminal site. The initial slope of the monoferric transferrin curve in Figure 9 is $\sim$ 8,000, indicating the coordination of a single tyrosyl residue.

Proton release studies. At low r values, $\sim$ 2.6 protons are displaced per thorium. This ratio gradually drops to a constant value of 2.0 at high r values. NTA will bind 1.86 H^+ ions per addition of thorium(IV), whereas complexation of the metal by transferrin in the second binding site should liberate two protons per thorium. For this reason, a sharp leveling of the titration is not observed. The total number of protons released has been calculated from the total volume of KOH at a $[Th^{4+}]/[Tr]$ ratio of two. A total of 4.5 protons are released by the coordination of two thorium ions to apotransferrin. The number of protons expected to be released is pH dependent, as the number of tyrosines bound to the metal decrease at high pH. A correction based

on this observation is discussed below. Thus, the proton release studies also indicate that the thorium binding sites are inequivalent. Actual data and detailed reduction procedures are given in a supplementary section.

Discussion

Given the value of 8680 M^{-1} cm^{-1} for each coordinated phenol, the $\Delta\varepsilon$ of 25,000 measured for the dithorium transferrin corresponds to three coordinated tyrosyl residues. Thus, the thorium binding at sites A and B[31] must be non-equivalent, with two tyrosines at one site and a single tyrosine at the second site. It is this intrinsic inequivalence in the two binding sites, rather than weak complexation, which is responsible for the curvature seen in the spectrophotometric titration curves. Furthermore, the shape of these curves is affected by the relative binding strengths of the two sites. This is illustrated in Figure 10, which shows calculated titration curves, assuming complete binding of Th^{4+} by transferrin, for three different ratios of metal binding at the two sites, one with $\Delta\varepsilon$ of 17,000 and the other with $\Delta\varepsilon$ of 8,500.

If the relative binding affinities were so different that the addition of an aliquot of $Th(NTA)_2$ resulted in 100% binding at the $\Delta\varepsilon$ = 17,000 site, then curve 1 in Figure 10, results. For this curve there is a linear segment from r = 0 to r = 1 with a slope of 17,000. From r = 1 to r = 2,

only the second ($\Delta\varepsilon$ = 8,500) sites are available to the thorium, so the slope over this range is 8,500.

If the relative binding affinities of the two sites were such that thorium complexation at the sites overlapped, the shape of the titration curve changes. Curve 2 is calculated assuming a 75:25 ratio of thorium binding at the two sites. The initial linear segment has a slope of 15,250 — which is simply the weighted average of $\Delta\varepsilon$ for each site. The linear portion of this curve extends to r = 1.33, since the excess $Th(NTA)_2$ must be added to saturate the first site. The second linear segment in curve 2 again represents binding at the second site, so the slope is still 8,500.

Only if the thorium is evenly distributed between the two sites would there be a single straight line from r = 0 to r = 2, as shown by curve 3. Since the two transferrin binding sites do not have the same number of tyrosines coordinated to Th^{4+}, it is highly unlikely that their binding affinities would be identical. Thus one would expect curvature in $\Delta\varepsilon$ versus r plots for transferrin.

The distribution ratio of thorium between the two binding sites also affects the r value at which extrapolations of the initial linear segment and the flat line past r = 2 intersect. This value varies from r = 1.5 for exclusive binding at the first site to r = 2 for an even distribution between the two sites. The observed intersection in the

transferrin system is ∿ 1.55 indicating an approximate 90:10 ratio between the two sites. This is consistent with the observed slope of the initial segments which are ∿ 15,000 - 16,000. The data are not suitable for a quantitative calculation of the relative binding affinities; however, it is clear that the two-tyrosine site has a significantly greater affinity for Th^{4+} than does the one-tyrosine site.

The NTA competition studies have shown that the decrease in $\Delta\varepsilon$ below pH 7 is due to the decrease in the number of thorium ions bound to transferrin. This probably reflects increased competition from hydrogen ion for the very basic phenolic oxygens. Since the overall basicity of the NTA is much less, this ligand retains its effectiveness at lower pH's. Thus, as the pH decreases, there is an increase in the relative binding strength of NTA versus transferrin. This is not a surprising result, since even iron binding by transferrin is considerably weakened at pH 6.[24]

The data in Figure 8 show that $\Delta\varepsilon$ also decreases at high pH, even though the titration curves indicate that two thorium ions are tightly bound to transferrin. The $\Delta\varepsilon$ at pH 9.5 is only 16,000 M^{-1} cm^{-1}, corresponding to one coordinated tyrosine per thorium. These changes are presumably due to a conformational change in the tertiary structure of transferrin which alters one of the metal binding sites. Chasteen *et al.* have reported that at pH 7.5 there are two sets of epr resonances for divanadyl transferrin, due

to magnetically nonequivalent environments, which they have assigned to "A" and "B" sites (C- and N-terminal, respectively).[37] Above pH 9, where two VO^{2+} ions remain bound, only one set of epr resonances are observed, which correspond to the N-terminal site. It was proposed that this C-to-N terminal conformational change is coupled to the ionization of a non-coordinating protein functional group with a pK_a of $\sim$ 10. It has also been shown that both divanadyl and diferric transferrin readily lose the metal ion from the N-terminal site when the pH is decreased.[37]

We propose that this same conformational change converts the two-tyrosine thorium site to a one-tyrosine site, thus reducing the total number of coordinated tyrosine groups from three to two. This requires that the two-tyrosine site correspond to the C-terminal site. To establish the actual relationship between the different metal ion sites, monoferric transferrin was titrated with $Th(NTA)_2$. Monoferric transferrin prepared at pH 6 contains at least 90% of the iron in the C-terminal binding site,[24] leaving the N-terminal site open for thorium. The thorium titration was then carried out at pH 7.2. The observed $\Delta\varepsilon$ was $\sim$ 8,000, which corresponds to the coordination of a single tyrosyl residue. Thus the N-terminal site does correspond to the one-tyrosine site, while the C-terminal site corresponds to the two-tyrosine site. That is, it appears that the changes in the $\Delta\varepsilon$ of thorium transferrin at higher pH reflect the same C-terminal

to N-terminal conformational change reported earlier by Chasteen for the vanadyl system.[37] In addition, at low pH thorium is lost primarily from the N-terminal site, as is observed for both the vanadyl and iron systems.[37]

Luk[15] has reported $\Delta\varepsilon$ values for a series of transferrin lanthanide complexes which indicate that there is a size restriction for transferrin binding of very large cations. The observed lanthanide $\Delta\varepsilon$ values at r = 2 are plotted as a function of ionic radius in Figure 11, where it can be seen that the thorium results are consistent with the lanthanide data.[38] Similar plots are obtained whether one assumes that the metals are either six- or eight-coordinate. The smaller lanthanides appear to coordinate four tyrosines, presumably two at each site. However, as the ionic radius increases, there is a sharp decrease in $\Delta\varepsilon$. With thorium there are two distinct sites, the one-and-two-tyrosine sites, which give a total $\Delta\varepsilon$ of 25,000. Luk's results indicate that only one Nd^{3+} ion is bound to transferrin, and that the binding involves two tyrosines. An early lanthanide, Nd^{3+} is apparently too large to fit into the N-terminal site. For the still larger Pr^{3+} ion, even the C-terminal site binding is weakened, so that $\Delta\varepsilon$ drops further.

This critical size dependence has important implications for this study, since we have used Th^{4+} as a model for Pu^{4+}. Since the ionic radius of Pu^{4+} is $\sim$ 0.07 Å smaller than Th^{4+},[38]

and because the curve in Figure 11 breaks so sharply, this difference in size could substantially alter the nature of the transferrin binding of Pu^{4+} compared to Th^{4+}. An indication that this may be the case is the observation that plutonium is found in blood as the transferrin complex, whereas thorium is primarily associated with serum albumin.[4] However, we must point out the titration curves reported for the lanthanides by Luk[15] suggest that there may have been unrecognized difficulties with hydrolysis and nonspecific binding to transferrin. We have repeated the Holmium-transferrin titration using $Ho(NTA)_2$ as the titrant rather than $HoCl_3$ and obtained a $\Delta\varepsilon$ significantly less than that reported by Luk. Although the basic conclusions discussed above regarding size discrimination are probably valid, the uncertainty in the lanthanide data is such that it is imprudent to make predictions based on small differences in ionic radii.

The proton release studies also reflect the inequivalence of the two thorium binding sites, with a total of 4.5 protons being released by the coordination of two thorium ions. Since the data were collected at pH 8.6 (well below the typical pK_a of tyrosine), coordination of each tyrosyl group must involve the release of a proton. The observed $\Delta\varepsilon$ of 24,000 for di(thorium) transferrin at pH 8.6 corresponds to only $\sim$ 2.8 coordinated tyrosines. Thus coordination of tyrosine accounts for 2.8 of the 4.5 protons released upon complexation of thorium by transferrin. This leaves roughly two protons

which must be accounted for. It is generally accepted that histidine residues are also present in the metal binding site of transferrin,[39,40] but since the pH was well above the imidazole pK_a of histidine, it is unlikely that these are the source of the additional one proton per thorium. Instead, protons are probably produced by the hydrolysis of coordinated water molecules. This explanation is consistent with the well-known hydrolytic tendencies of Th^{4+},[41] especially considering that it is highly unlikely that transferrin provides sufficient ligating groups to occupy all the available coordination sites of thorium. Given that water molecules are coordinated to Th^{4+}, complete hydrolysis is expected at pH 8.6. Thus with one model, one expects a total of 4.8 protons released from transferrin at pH 8.6. The observation of 4.5 protons released is in good agreement with the predicted value of 4.8.

Bates et al. have demonstrated that the affinity of bicarbonate to act as the synergistic anion in the ferric transferrin complex is much greater than that of NTA.[42] It has been presumed here that bicarbonate is the synergistic anion in the thorium transferrin complex, since this has invariably proven to be the case for all other metal ions unless special precautions are made to exclude HCO_3^-. It is therefore possible that the complex actually binds CO_3^{2-}, and that the two extra protons released upon thorium complexation are due to the deprotonation of HCO_3^-. Based upon the

anion binding studies with other metals, as well as data with oxalate substituted for bicarbonate as the synergistic anion,[21] we do not favor this explanation, but the data reported here would not contradict such a proposal.

The apparent hydrolysis of both metal ions in di(thorium) transferrin is significant in view of the continuing uncertainty about the identities of the ligating groups in diferric transferrin. The release of six protons upon complexation of two ferric ions to transferrin is often attributed to the coordination of three phenolic groups per ferric ion.[21-22,43] However, interpretation of difference UV spectra of diferric transferrin, based on the absolute magnitude of $\Delta\varepsilon$, are more consistent with the coordination of only two phenolic groups per iron(III).[20] Because of the similarity in the hydrolytic tendencies of Fe^{3+} and Th^{4+}, one must strongly consider the possibility that two of the six protons released by complexation of iron(III) are due to hydrolysis of the ferric ion rather than deprotonation of a protein functional group. We propose that "hard" metals such as Fe^{3+}, Ga^{3+} or Al^{3+} hydrolyze, whereas "soft" metals such as Zr^{2+} or Zn^{2+}, due to their softer nature, are not prone to hydrolysis.

One could argue that because of its large size and poor fit into the transferrin binding site, thorium is more exposed to solvent and thus more likely to hydrolyze than iron. However, Koenig and Schillinger[45] have reported ^{1}H nmr data which indicate that at least one water molecule is directly

coordinated to each metal ion in diferric transferrin. Numerous studies on simple complexes clearly show that ferric ion has a very strong tendency to hydrolyze when the ligand is less than six-coordinate and one or more of the regular octahedral sites is occupied by H_2O. Common hydrolysis constants for the reaction $FeX_5H_2O + OH^- \rightarrow FeX_5OH + H_2O$ are on the order 10^{-4} to 10^{-5} in such situations. Ferric EDTA is seven-coordinate, including four carboxylate anions, yet exhibits a hydrolysis constant of $10^{-7.49}$. Thus based on the known solution chemistry of Fe^{3+}, one might expect the iron in diferric transferrin to hydrolyze at neutral pH. The three protons released upon complexation of ferric ion would then correspond to deprotonation of two tyrosines and the hydrolysis of one water molecule.

Summary

At physiological pH transferrin binds two thorium ions at nonequivalent sites. The stronger binding site has two tyrosines coordinated to thorium and corresponds to the C-terminal site in the native ferric protein complex. The weaker thorium-binding site has only one coordinated tyrosine and corresponds to the N-terminal site. The nonequivalence of the two sites results in curved spectrophotometric titration plots.

The smaller number of coordinated tyrosines at the N-terminal site is due to the large ionic radius of Th^{4+}. It appears that the N-terminal site is slightly smaller than the

C-terminal site and cannot readily accommodate cations larger than Eu^{3+}. This size discrimination may be important when comparing Th^{4+} complexation to the binding of the slightly smaller Pu^{4+} ion.

At pH values below 7, the binding affinity of transferrin for Th^{4+} decreases rapidly, such that by pH 6, only about 0.3 thorium ions are bound to transferrin when the metal is added as the bis(NTA) complex. The N-terminal site is more strongly affected by acidic conditions, so that the binding at pH 6 is probably entirely at the C-terminal site. Above pH 9 there is a conformational change in the protein which converts the C-terminal site (two tyrosine) into a second N-terminal site (one tyrosine). The spectrophotometric titration curve for this species is linear, as expected for equivalent sites.

In addition to the three protons displaced from the coordinated tyrosine residues, the binding of two thorium ions releases two additional protons at pH 8.6; these are ascribed to metal ion hydrolysis reactions. Thus thorium is actually bound as a monohydroxo species. It is suggested that diferric transferrin may undergo an analogous reaction, and that the three protons displaced by each ferric ion could represent two tyrosyl protons and one H^+ from hydrolysis of coordinated water, rather than the coordination of three tyrosines.

Acknowledgment

This work is supported by the National Institutes of Health and the Division of Nuclear Sciences, Office of Basic Energy Sciences, U.S. Department of Energy under Contract No. W-7405-Eng-48.

Supplementary Material

Tabulation of proton release data for $Th(NTA)_2$-transferrin titration. Ordering information is given on current masthead page.

References

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18. Abbreviations used: DTPA = diethylenetriaminepentaacetic acid; EHPG = ethylene-bis-(o-hydroxyphenylglycine); MES = [2(N-morpholino)ethane sulfonic acid]; TRIS = [tris-(hydroxymethyl)aminomethane hydrochloride]; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA = ethylenediaminetetraacetic acid; NTA = nitrilo-triacetic acid.

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Figure Captions

Figure 1. Structural formula of ethylene-bis(o-hydroxyphenyl)glycine [EHPG].

Figure 2. Potentiometric equilibrium curves of EHPG alone and a 1:1 solution of EHPG and $Th(NO_3)_4$. Ionic strength - 0.100 (KNO_3), t = 25°C.

Figure 3. Difference UV spectra of Th^{4+} + EHPG versus EHPG alone, where r is the ratio of added thorium to total ligand. [EHPG] = 1.6×10^{-4}; 0.10 M MES; pH = 6.0.

Figure 4. Difference UV titration curve for Th^{4+} + EHPG at pH 6.0. Data reflect the coordination of two phenolic groups per Th^{4+}; at λ = 238, $\Delta\varepsilon$/phenol = 8680 M^{-1} cm^{-1}; at λ = 292, $\Delta\varepsilon$/phenol = 2330 M^{-1} cm^{-1}.

Figure 5. Difference UV spectra of Th^{4+} + apotransferrin versus apotransferrin at pH 7.2, where r is the ratio of $[Th^{4+}]/[Tr]$. [Tr] = 1.0×10^{-5} M, 0.10 M Tris; pH = 7.2. The thorium was added as $Th(NTA)_2$.

Figure 6. Difference UV titration curves of $Th(NTA)_2$ + apotransferrin as a function of pH (6.0 to 7.2).

Figure 7. Difference UV titration curves of $Th(NTA)_2$ + apotransferrin as a function of pH (8.0 to 9.5).

Figure 8. Values of $\Delta\varepsilon$ at r = 2.0 as a function of pH. The right-hand ordinate shows the apparent number of coordinated tyrosyl groups based on $\Delta\varepsilon = 8630\ cm^{-1}\ M^{-1}$ per coordinated tyrosine.

Figure 9. Difference UV titration curve for $Th(NTA)_2$ + monoferric transferrin versus monoferric transferrin. The monoferric transferrin contains the Fe^{3+} in the "A" (C-terminal) binding site. [Tr-Fe] = 1.4×10^{-5} M; 0.1 M Tris; pH 7.4.

Figure 10. Calculated difference UV titration curves assuming the stipulated relative affinities for the "A" and "B" binding sites, as well as individual $\Delta\varepsilon$ values of 17,000 and 8,500 for thorium binding at the "A: (C-terminal) and "B" (N-terminal) sites, respectively.

Figure 11. Plots of the observed $\Delta\varepsilon$ of dimetallo transferrin as a function of the metal ionic radius (assuming coordination numbers of six and eight). The number of tyrosines is calculated based on a value of 8680 cm^{-1} M^{-1} per coordinated phenolic group. The point for Pu(IV) is placed on the curve at the appropriate ionic radius; no experimental $\Delta\varepsilon$ has been measured.

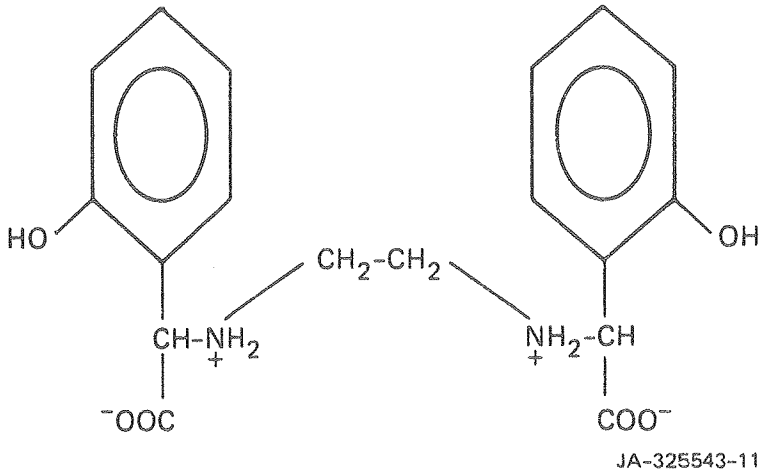

Fig. 1

POTENTIOMETRIC TITRATION CURVE
FOR Th^{4+}:EHPG

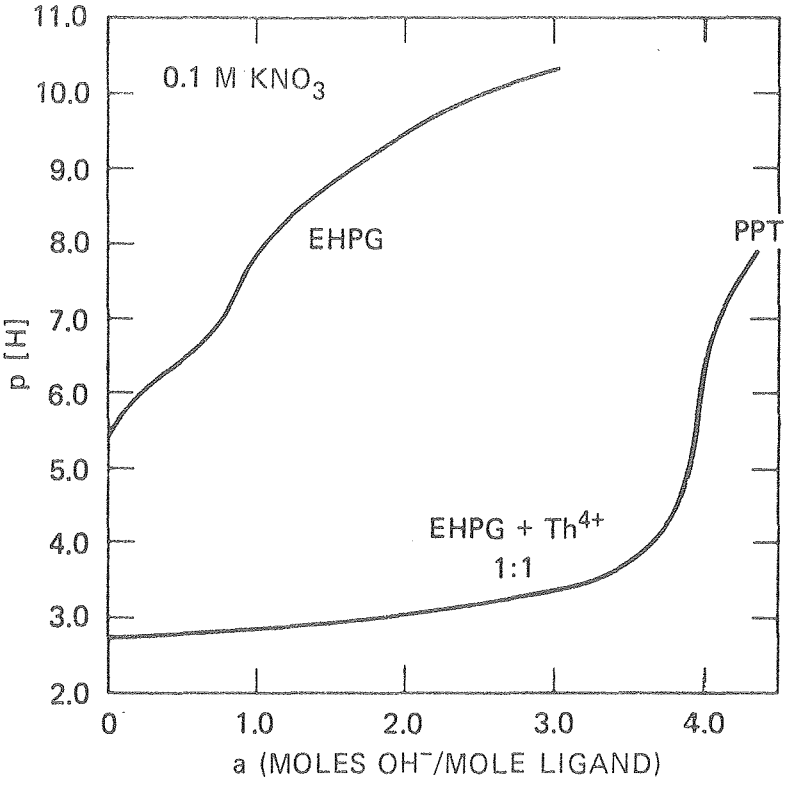

Fig. 2

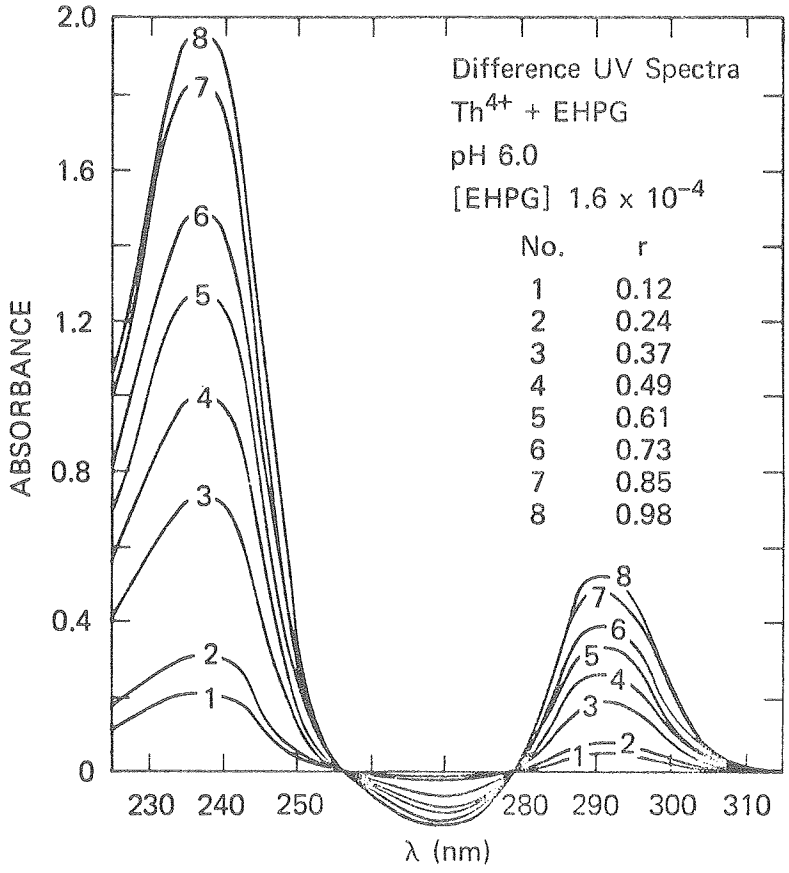
Difference UV Spectra
Th4+ + EHPG
pH 6.0
[EHPG] 1.6 x 10-4
No. r
1 0.12
2 0.24
3 0.37
4 0.49
5 0.61
6 0.73
7 0.85
8 0.98
ABSORBANCE
2.0
1.6
1.2
0.8
0.4
0
230 240 250 280 290 300 310
λ (nm)
JA-325543-7

Fig. 3

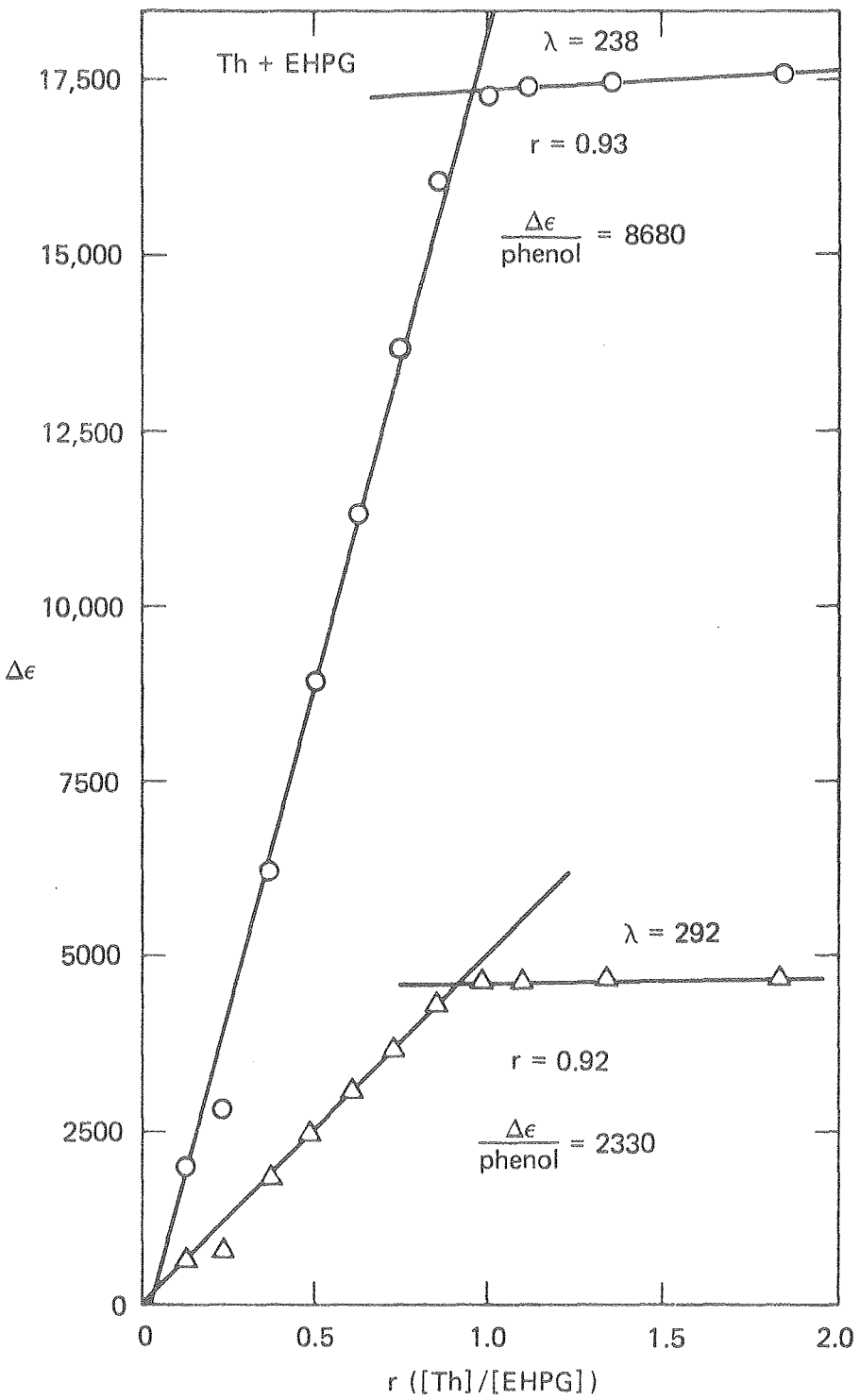
Th + EHPG
λ = 238
r = 0.93
Δε / phenol = 8680
λ = 292
r = 0.92
Δε / phenol = 2330
17,500
15,000
12,500
10,000
7500
5000
2500
0
Δε
0
0.5
1.0
1.5
2.0
r ([Th]/[EHPG])
JA-325543-1

Fig. 4

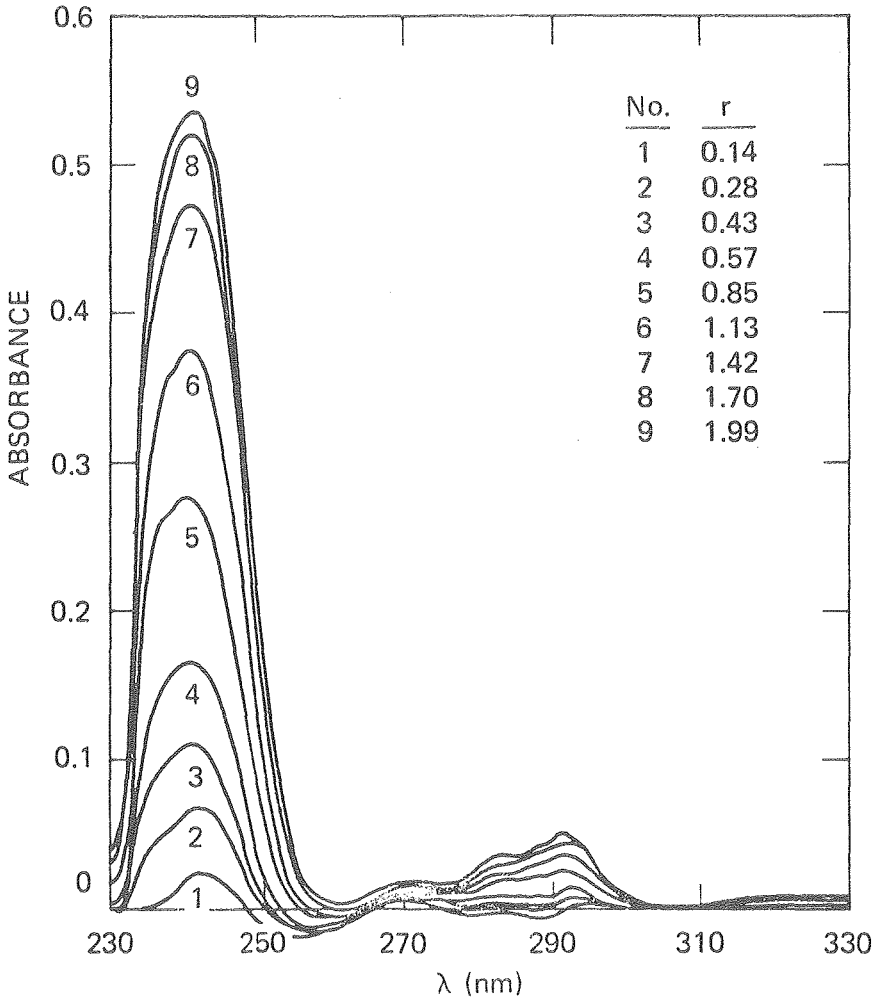

Fig. 5

JA-325583-1

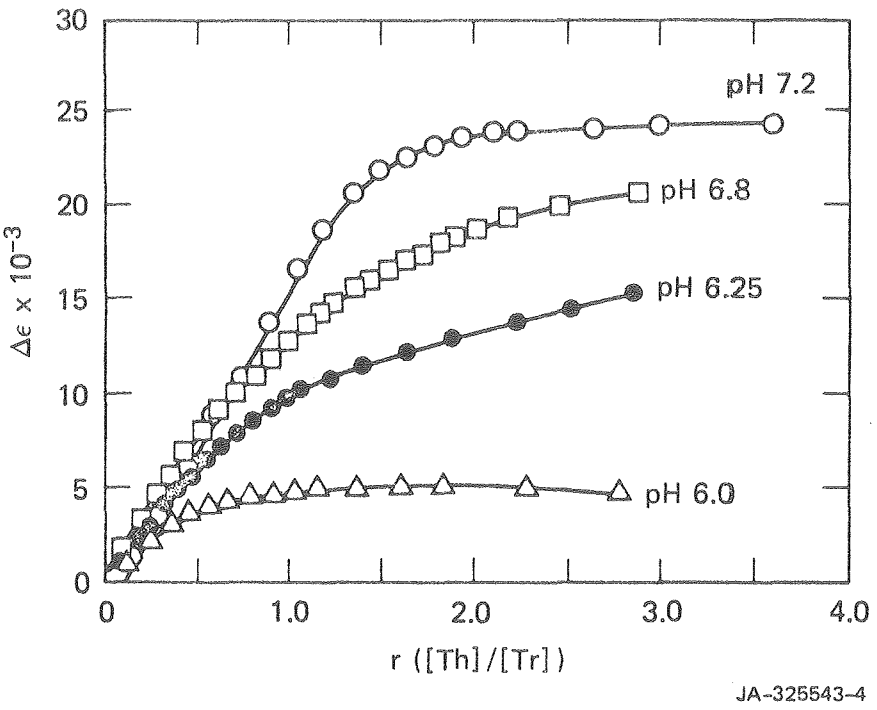
30
25
20
15
10
5
0
$\Delta\epsilon \times 10^{-3}$
0
1.0
2.0
3.0
4.0
r ([Th]/[Tr])
pH 7.2
pH 6.8
pH 6.25
pH 6.0
JA-325543-4

Fig. 6

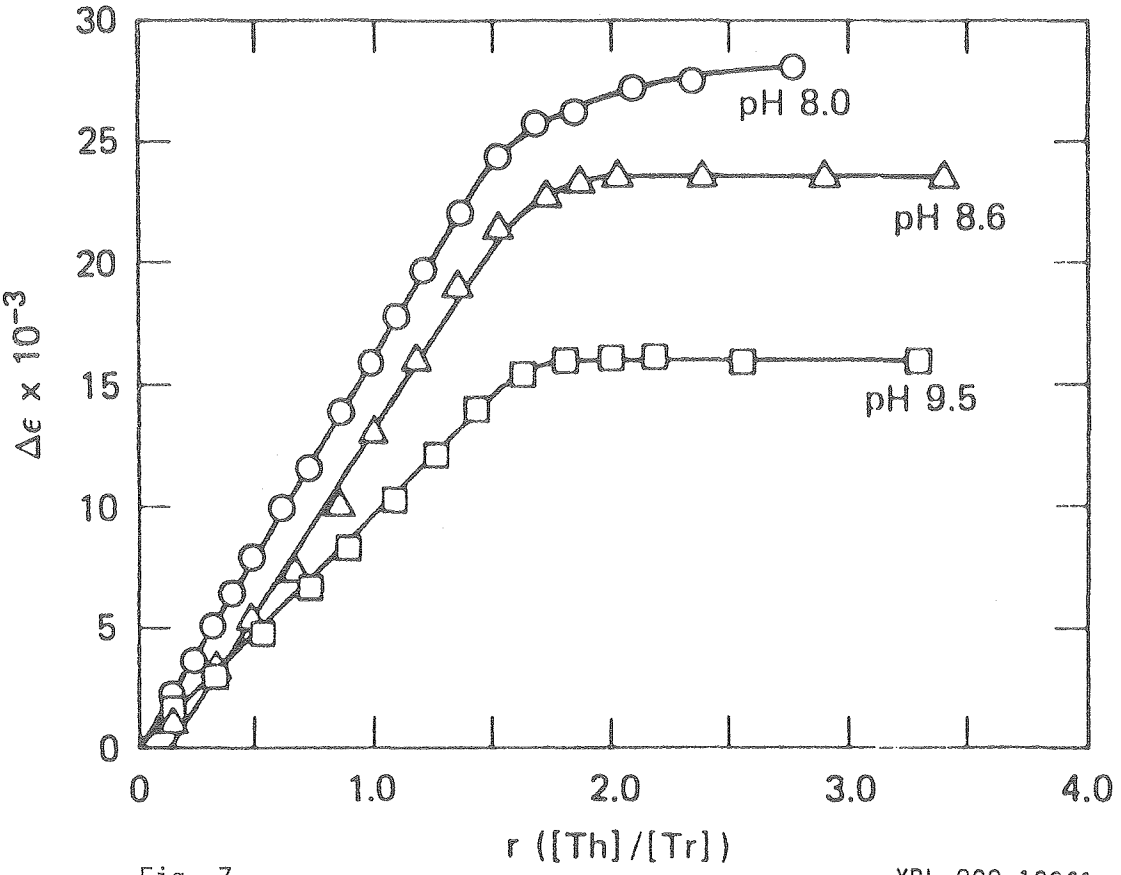

Fig. 7

XBL 808-10961

DIFFERENCE UV TITRATION CURVES FOR THORIUM-TRANSFERRIN FROM pH 8 TO pH 9.5

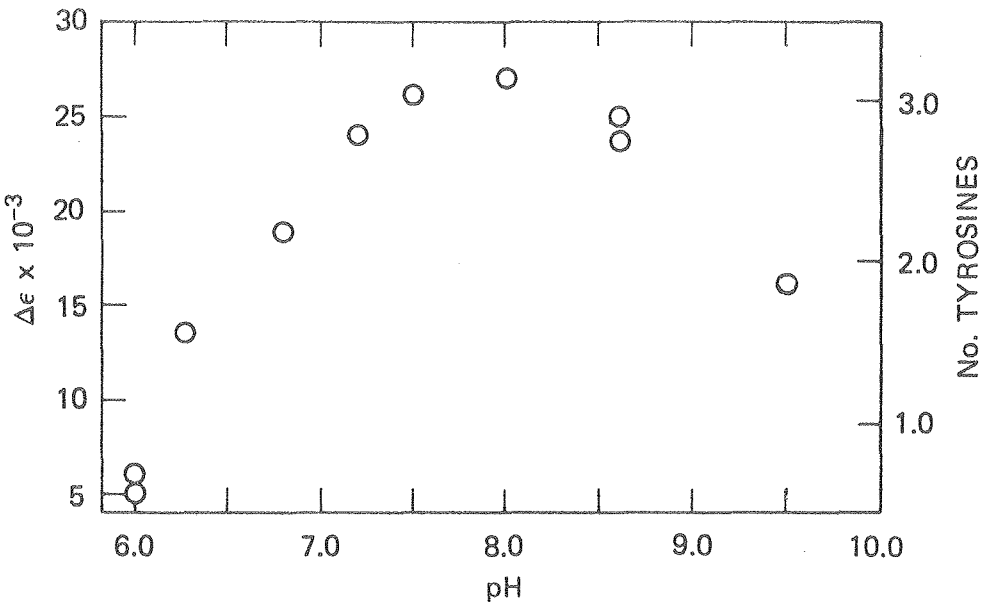
30
25
20
15
10
5
Δε x 10⁻³
3.0
2.0
1.0
No. TYROSINES
6.0
7.0
8.0
9.0
10.0
pH
JA-325543-8

Fig. 8

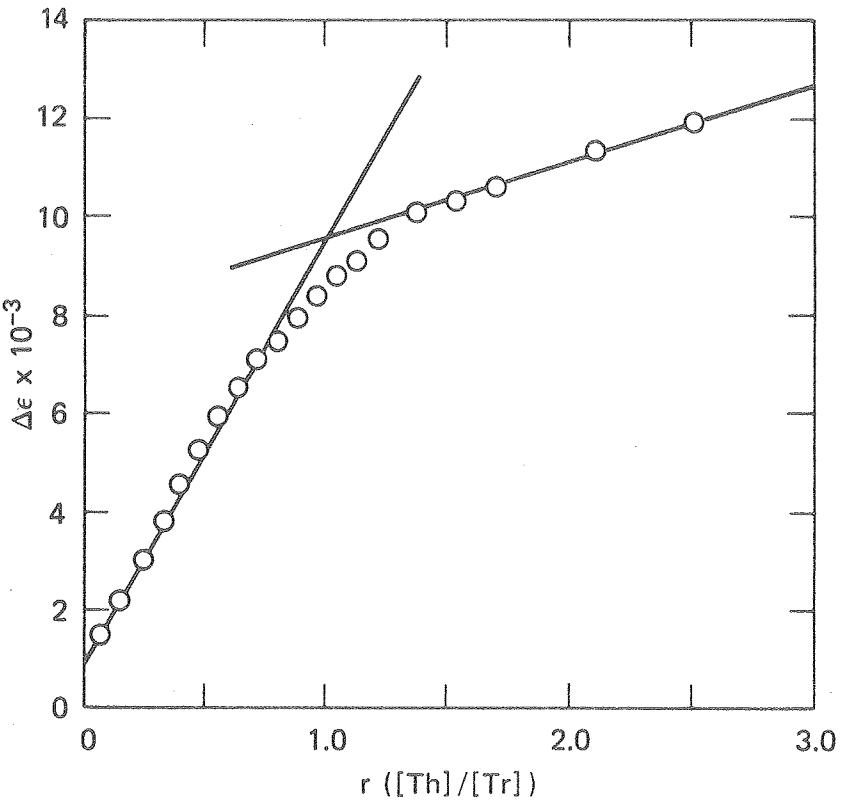

Fig. 9

JA-325543-9

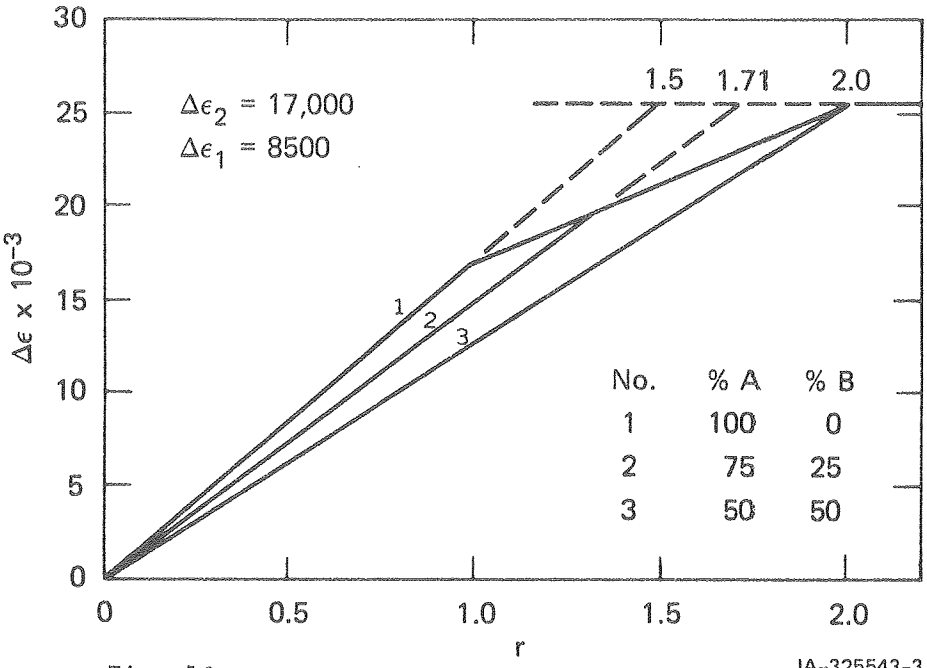
30
25
20
15
10
5
0
0
0.5
1.0
1.5
2.0
r
$\Delta\epsilon \times 10^{-3}$
$\Delta\epsilon_2$ = 17,000
$\Delta\epsilon_1$ = 8500
1.5
1.71
2.0
1
2
3
No. % A % B
1 100 0
2 75 25
3 50 50
JA-325543-3

Fig. 10

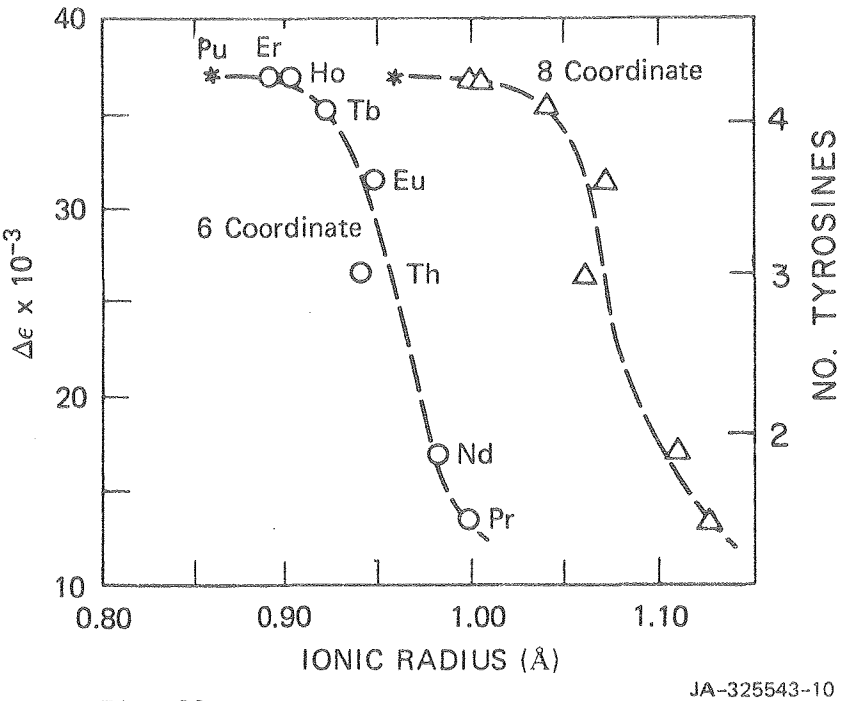
40
30
20
10
$\Delta\epsilon \times 10^{-3}$
Pu
Er
Ho
Tb
Eu
Th
Nd
Pr
6 Coordinate
8 Coordinate
4
3
2
NO. TYROSINES
0.80
0.90
1.00
1.10
IONIC RADIUS (Å)
JA-325543-10

Fig. 11