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Thermodynamic Binding Constants for Gallium Transferrin†

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ABSTRACT: Gallium-67 is widely used as an imaging agent for tumors and inflammatory abscesses. It is well established that Ga^{3+} travels through the circulatory system bound to the serum iron transport protein transferrin and that this protein binding is an essential step in tumor localization. However, there have been conflicting reports on the magnitude of the gallium–transferrin binding constants. Therefore, thermodynamic binding constants for gallium complexation at the two specific metal binding sites of human serum transferrin at pH 7.4 and 5 mM $NaHCO_3$ have been determined by UV difference spectroscopy. The conditional constants calculated for 27 mM $NaHCO_3$ are log K_1^* = 20.3 and log K_2^* = 19.3. These results are discussed in relation to the thermodynamics of transferrin binding of Fe^{3+} and to previous reports on gallium binding. The strength of transferrin complexation is also compared to that of a series of low molecular weight ligands by using calculated pM values (pM = −log [Ga($H_2O)_6$]) to express the effective binding strength at pH 7.4.

Gallium-67 is widely used as an imaging agent for a variety of soft tissue tumors and inflammatory abscesses (Hayes, 1978; Welch & Moerlein, 1980), although the mechanism of tumor localization has not been firmly established (Larson, 1978). Even though this radioisotope is commonly administered as a citrate complex, once in the blood the gallium rapidly binds to the iron transport protein transferrin (Clausen et al., 1974; Gunasekera et al., 1972; Vallabhajosula et al., 1980). Since serum transferrin is normally only about 30% saturated with iron (Larson et al., 1979a), it retains a relatively high capacity for binding other metal ions. In addition to this role in serum transport of gallium, it appears that transferrin facilitates gallium movement across tumor cell membranes (Larson et al., 1979a,b; Harris & Sephton, 1977). In some cell culture

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systems, the addition of transferrin is required for appreciable uptake of gallium (Larson et al., 1979a; Harris & Sephton, 1977), and optimization of the transferrin concentration leads to as much as an 8–10-fold cell to medium concentration ratio (Larson et al., 1979a). This uptake appears to be mediated by specific cellular receptors for transferrin (Hayes, 1978; Larson et al., 1979a), possibly followed by endocytosis of the intact gallium–transferrin complex. However, once inside the cell, the gallium binds to other cytoplasmic proteins (Hayes & Carlton, 1973; Lawless et al., 1978) and the lysosomal subcellular fraction (Swartzendruber et al., 1971; Takeda et al., 1977). Other data indicate that lactoferrin, a second member of the transferrin group of proteins, is involved in the binding of gallium in some types of tumors (Hoffer et al., 1977, 1979).

Because of the obvious importance of gallium–transferrin interactions in the tumor localization process, there have been previous attempts to determine gallium binding constants. Clausen et al. (1974) reported that transferrin bound 14 gallium ions with an average binding constant of only 1.77. Since it is well established that transferrin has only two binding sites, these workers appear to be measuring nonspecific metal binding. In addition, an equilibrium constant of 1.77 is inconsistent with the qualitative observations on the strength of gallium binding (Gunasekera et al., 1972; Vallabhajosula et al., 1980; Raiszadeh et al., 1981). Later Larson et al. (1978) studied the binding of trace amounts of ^{67}Ga and reported a log K value of 5.4. However, even this value would seem too low, considering that the iron binding constants are $\sim 10^{20}$ (Aisen et al., 1978) and that gallium is also trivalent and has an ionic radius very similar to that of Fe^{3+}. At the other extreme, Kulprathipanja et al. (1979) reported an apparent binding constant of $\sim 10^{24}$, which is several orders of magnitude *greater* than the iron binding constant determined by Aisen et al. (1978).

Because of the apparent confusion in this important area, we have conducted our own investigation of gallium binding by transferrin, and we have determined the macroscopic gallium binding constants for the two specific metal binding sites of human serum transferrin. A unique feature of the transferrins is that metal binding requires the concomitant binding of a synergistic anion (Bates & Schlabach, 1975), which in vivo is bicarbonate. Thus, all measurements were made with 5 mM freshly added $NaHCO_3$. Constants were determined by UV difference spectroscopy, using the 242-nm band as an indicator of metal binding. We now report that transferrin binds gallium more weakly than ferric ion by a factor of about 300 and that the ratio of the constants for the first and second gallium ion is about 9, which is similar to the value of 20 reported for the iron binding constants (Aisen et al., 1978). These results are compared to previous reports, and possible reasons for the variations in observed gallium log K values are discussed.

Experimental Procedures

Materials. Purified human serum transferrin was purchased from Sigma and further purified by chromatogaphy on a 30 × 2 cm Sephadex G-15 (Pharmacia) column with 0.1 M $NaClO_4$–0.05 M tris(hydroxymethyl)aminomethane (Tris); pH 7.4, as an eluent. Fractions with an absorbance >2 at 278 nm were combined and rechromatographed on the same column by using 0.10 M Tris buffer (pH 7.4) as the eluent. Fractions with an absorbance >1.5 at 278 nm were combined and stored at 4 °C.

The concentration of transferrin in each new stock solution was determined by titration with standard solutions of ferric nitrilotriacetic acid, assuming that two ferric ions bind per transferrin with a molar absorptivity of 2500 M^{-1} cm^{-1} per iron at 470 nm (Chasteen, 1977). On the basis of this titration, the molar absorptivity of the apotransferrin at 278 nm was calculated, and this UV peak was then used on a daily basis to measure the transferrin concentration of each sample. The calculated values were routinely within 10% of the literature value of 93 000 M^{-1} cm^{-1} for the molar absorptivity of apotransferrin (Chasteen, 1977).

Gallium chloride stock solutions were prepared by carefully weighing pieces of pure gallium metal into a 30-mL beaker, adding 10–15 mL of concentrated HCl, covering, and gently heating until all the metal dissolved. More HCl was added periodically as needed to maintain vigorous hydrogen evolution at the gallium surface. This solution was then diluted to 100 mL with a final pH of 0.9. The gallium concentration calculated from the initial weight of the metal was confirmed by atomic absorption spectroscopy.

Solutions of $\sim 5 \times 10^{-4}$ M gallium nitrilotriacetic acid and gallium ethylenediaminediacetic acid were prepared by dissolving the appropriate amount of ligand in ~90 mL of distilled water, adding a measured volume of the gallium stock solution, and diluting to 100 mL with distilled water. For solutions where the ratio of ligand to gallium was less than one, 3 mL of 0.1 M HCl was included prior to the addition of the gallium to prevent hydrolysis.

Apparatus. UV–vis spectra were recorded on a Cary 219 spectrophotometer equipped with a jacketed sample compartment which was kept at 25 °C by an external circulating water bath. All pH measurements were made with a Lazar pH meter (±0.01 pH unit) equipped with a Corning "tris" combination microelectrode.

Procedures. Samples were prepared by diluting aliquots of the stock transferrin solution to $\sim 1.2 \times 10^{-5}$ M with 0.1 M pH 7.4 Tris buffer. Immediately before the titration, sufficient $NaHCO_3$ was added to produce a 5 mM HCO_3^- concentration. Using published constants (Smith & Martell, 1976), we calculated that the $[HCO_3^-]$ in equilibrium with air is 0.14 mM at 25 °C. Thus, the initial bicarbonate concentration varied from 5.0 to 5.14 mM, depending on the degree of air saturation of the various buffer solutions. Although there was undoubtedly some loss of CO_2 during the course of the experiment, the rather small (<0.05) changes in pH indicate that this loss is probably not a limiting source of error in the calculations of the metal binding constants.

Three milliliters of an apotransferrin solution was added to both the sample and reference cuvettes, and a base line was recorded. Gallium was then added in 10–30-μL aliquots to the sample cuvette, while equal volumes of distilled water were added to the reference cuvette. From 10 to 40 min was required to reach equilibrium after each addition. The spectrum from 320 to 235 nm was recorded after each addition, and the absorbance at 242 nm was divided by the total transferrin concentration to give a value of $\Delta\epsilon$. Titration curves were prepared by plotting $\Delta\epsilon$ vs. r, where r is the ratio of the total gallium concentration to the total transferrin concentration.

Such titrations were performed by using $GaCl_3$, Ga(NTA), and Ga(EDDA) as the titrant (NTA = nitrilotriacetic acid; EDDA = *N,N'*-ethylenediaminediacetic acid). In addition, back-reactions were run by adding either NTA or EDDA to the preformed gallium–transferrin complex, which was prepared in the cuvette by adding 2 equiv of $GaCl_3$ to a transferrin–$NaHCO_3$ solution. Although complex formation appeared to be very rapid, 2–3 h was allowed for complete equilibration of the gallium–transferrin complex. Then 10–

30-μL aliquots of NTA or EDDA in pH 7.4 Tris buffer were added, and the decrease in the absorbance at 242 nm was measured.

Calculations. Standard nonlinear least-squares calculations were performed on a Data General Nova 840 minicomputer. The gallium–transferrin–ligand equilibrium system is described by the set of equations

$$T_{Ga} = \alpha_{Ga}[Ga] + \alpha_{GaL}[GaL] + [GaTr] + 2[Ga_2Tr] \quad (1)$$

$$T_{Tr} = [Tr] + [GaTr] + [Ga_2Tr] \quad (2)$$

$$T_L = \alpha_{GaL}[GaL] + \alpha_L[L] \quad (3)$$

where T_x represents the total analytical concentrations of gallium (Ga), transferrin (Tr), and the competing ligand (L) and the α_x terms are pH-dependent functions which account for the various protonated and hydrolyzed forms of the species within brackets. Of particular importance is the gallium ion hydrolysis series

$$\beta_n^{OH} = \frac{[Ga(OH)_n][H]^n}{[Ga]} \quad (4)$$

$$\alpha_{Ga} = 1 + \frac{\beta_1^{OH}}{[H]} + \frac{\beta_2^{OH}}{[H]^2} + \frac{\beta_3^{OH}}{[H]^3} + \frac{\beta_4^{OH}}{[H]^4} \quad (5)$$

Successive log β_n^{OH} values, taken from Baes & Mesmer (1976), were −2.9, −6.6, −11.0, and −16.60 for n = 1–4. This gives an α_{Ga} value of 1.02×10^{13} at pH 7.4. At this pH, gallium is presnt in solution as 98.4% $Ga(OH)_4^-$, 1.6% $Ga(OH)_3$, and only traces of the remaining species. The α_L term accounts for protonated forms of the ligands and has the general formula for H_nL of $\alpha_L = 1 \times \beta_1^H[H] + \beta_2^H[H]^2 + ... + \beta_n^H[H]^n$, where the β's are ligand protonation constants taken from Martell & Smith (1974).

At any given pH, all the α_x terms are fixed, so that we can define new variables and constants as

$$[Ga]' = \alpha_{Ga}[Ga] \quad (6)$$

$$[GaL]' = \alpha_{GaL}[GaL] \quad (7)$$

$$[L]' = \alpha_L[L] \quad (8)$$

$$\log K_{ML}' = \frac{[GaL]'}{[Ga]'[L]'} \quad (9)$$

$$\log K_1' = \frac{[GaTr]}{[Ga]'[Tr]'} \quad (10)$$

$$\log K_2' = \frac{[Ga_2Tr]}{[Ga]'[GaTr]'} \quad (11)$$

Values for the necessary constants for ligand protonation and gallium complexation were taken from the literature (Smith & Martell, 1976). It is now possible to rewrite eq 1–3 in terms of three components, [Ga]′, [L]′, and [Tr], and the appropriate equilibrium constants

$$T_{Ga} = [Ga]' + K_{ML}'[Ga]'[L]' + K_1'[Ga]'[Tr] + 2K_1'K_2'([Ga]')^2[Tr] \quad (12)$$

$$T_{Tr} = [Tr] + K_1'[Ga]'[Tr] + K_1'K_2'([Ga]')^2[Tr] \quad (13)$$

$$T_L = [L]' + K_{ML}'[Ga]'[L]' \quad (14)$$

For a given set of K_{ML}', K_1', and K_2', the concentrations of the three components were calculated by an iterative procedure which varies [Ga]′, [L]′, and [Tr] for an individual titration point to minimize the differences between calculated and analytical values of T_{Ga}, T_{Tr}, and T_L. A value of $\Delta\epsilon_{calcd}$ for the point was then calculated as

$$\Delta\epsilon_{calcd} = \Delta\epsilon_M K_1'[Ga]'[Tr] + 2\Delta\epsilon_M K_1'K_2'([Ga]')^2[Tr] \quad (15)$$

where $\Delta\epsilon_M$ is the molar absorptivity per bound gallium ion for the gallium transferrin difference spectrum at 242 nm. Such values of $\Delta\epsilon_{calcd}$ were obtained for each point in the titration curve. Values of log K_1', log K_2', and $\Delta\epsilon_M$ were then varied to minimize the sum of the squares of the residuals between the observed and calculated $\Delta\epsilon$ values.

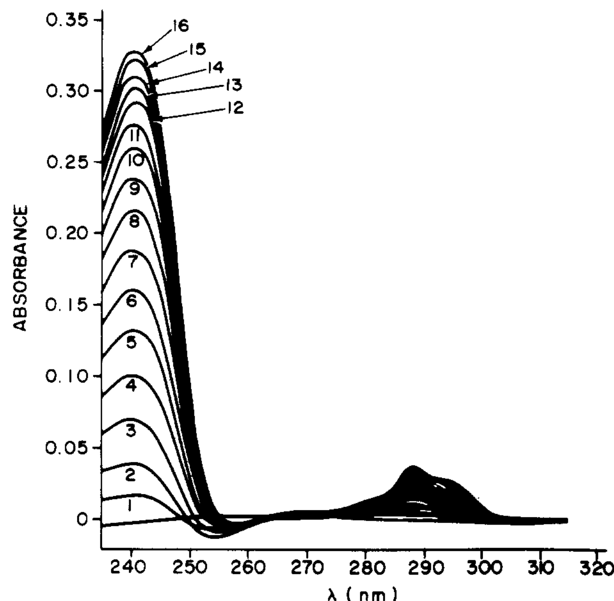

FIGURE 1: UV difference spectra resulting from additions of Ga(NTA) to apotransferrin at pH 7.4. [Tr] = 1.323×10^{-5} M; [Ga(NTA)] = 4.86×10^{-4} M; V = 3.0 mL; curve 1, 10 μL of Ga(NTA); 2, 20 μL; 3, 30 μL; 4, 40 μL; 5, 50 μL; 6, 60 μL; 7, 70 μL; 8, 80 μL; 9, 90 μL; 10, 100 μL; 11, 110 μL; 12, 120 μL; 13, 130 μL; 14, 140 μL; 15, 160 μL; 16, 180 μL.

Results

Gallium solutions containing various ratios of NTA:Ga were used to titrate apotransferrin. A typical series of UV spectra is shown in Figure 1. Values of $\Delta\epsilon$ were calculated from the absorbance maximum at 242 nm and plotted as a function of r, the ratio of total gallium concentration to total transferrin concentration, as shown in Figure 2. The continued increase in $\Delta\epsilon$ for points beyond r = 1 is a strong indication that gallium occupies both of the specific metal binding sites of transferrin. Even when a gallium chloride solution containing no NTA was used as the titrant, the plot of $\Delta\epsilon$ vs. r still begins to curve downward around r = 1.5. Such curvature implies that binding of the second gallium ion to transferrin is rather weak, so that excess gallium is needed to push the binding toward saturation of both binding sites of transferrin. Such an interpretation is supported further by the observation that a stoichiometric amount of NTA significantly reduces gallium binding, especially binding of the second gallium ion. Such results differ from previous data on other highly charged cations. A 10-fold excess of NTA does not reduce Th^{4+} binding to transferrin (Harris et al., 1981), and a large excess of ethylenediaminetetraacetic acid (EDTA) is required to remove Fe^{3+} from transferrin (Bates et al., 1976).

Such apparently weak binding in the absence of any competing ligand is almost certainly related to the hydrolytic tendencies of the Ga^{3+} ion. At pH 7.4, unchelated gallium exists in aqueous solution almost entirely as the relatively

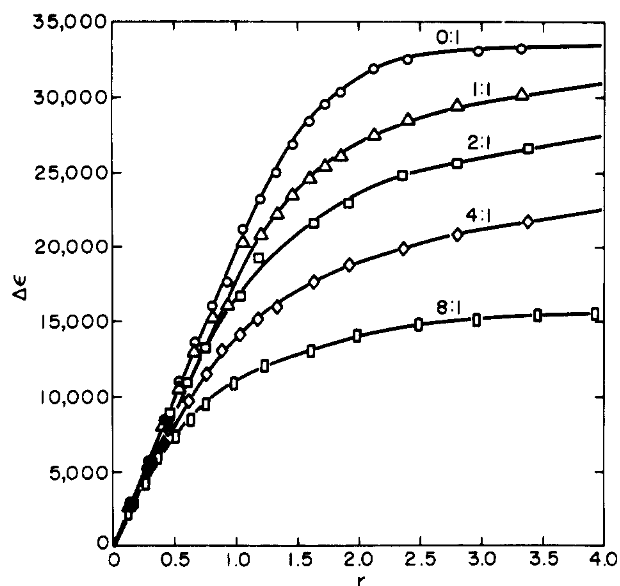

FIGURE 2: Titration curves for additions of $Ga(NTA)_x$ to apotransferrin for $x = 0, 1, 2, 4$, and 8; $\Delta\epsilon$ equals the absorbance at 242 nm divided by the transferrin concentration, and r is the ratio of [Ga] to [Tr].

soluble gallate ion, $Ga(OH)_4^-$, so that binding to transferrin involves the displacement of most, if not all, of these coordinated OH^- ions. Thus, the curvature in the $GaCl_3$ titration curve is actually due to a competition between transferrin and OH^-.

The initial portions of most of the Ga:NTA titration curves are linear, indicating that essentially all of the added gallium binds to transferrin at low values of r. As the titrations proceed, both the accumulation of free NTA in the cuvette and the partial saturation of the transferrin gradually shift the equilibrium so that smaller fractions of each additional dose of gallium bind to the transferrin.

The initial linear portion of these curves should have a slope close to $\Delta\epsilon_M$, the molar absorptivity of the gallium–transferrin complex. The initial slopes for the 0:1 and 1:1 curves are about 20 000 M^{-1} cm^{-1}. Thus, complete saturation of the two transferrin binding sites would produce an observed $\Delta\epsilon$ of about 40 000 M^{-1} cm^{-1}. It is very clear from the curves in Figure 2 that no reasonable amount of gallium, with or without NTA, will produce a $\Delta\epsilon$ of 40 000 M^{-1} cm^{-1}. In systems with NTA present, the accumulation of NTA is the limiting factor. In systems with no NTA, the solubility of unchelated gallium appears to be the limiting factor. At pH >7.4, the free gallium is split roughly 49:1 between the soluble $Ga(OH)_4^-$ and the relatively insoluble $Ga(OH)_3$ [solubility = 4×10^{-8} M (Smith & Martell, 1976)]. Thus, the maximum concentration of $Ga(OH)_4^-$ one can obtain without exceeding the solubility of $Ga(OH)_3$ is 2.5×10^{-6} M. The calculations of gallium binding constants indicated that this level is in fact exceeded in the 0:1 system. The data refinement is satisfactory up to [Ga] $\sim 6 \times 10^{-6}$ M. Inclusion of data points with larger [Ga] values invariably produces $\Delta\epsilon_{calcd}$ values which lie well above the observed $\Delta\epsilon$ values. This is the expected trend, since our calculation assumes that all gallium is soluble and binds to transferrin as dictated by K_2' rather than forming insoluble hydroxo species.

The Ga:NTA titration data were used to calculate values of log K_1*, log K_2*, and $\Delta\epsilon_M$, where K_n* is defined as

$$K_n^* = \frac{[Ga_nTr]}{[Ga][Ga_{n-1}Tr]} \quad (16)$$

Table I: Average Values of K_1* and K_2* for Gallium Transferrin[a]

method	n_1[b]	log K_1* ± 2 SEM[c]	n_2[b]	log K_2* ± 2 SEM[c]
Ga(NTA) + apoTr	11	19.61 ± 0.16	16	18.20 ± 0.10
NTA + Ga_2Tr	5	18.82 ± 0.10	5	18.53 ± 0.14
Ga(EDDA) + apoTr	8	19.69 ± 0.12	8	18.74 ± 0.12
EDDA + Ga_2Tr	5	19.27 ± 0.08	4	18.87 ± 0.33
$Ga(OH)_4$ + apoTr			5	18.70 ± 0.21
grand means		19.53 ± 0.25		18.58 ± 0.25

[a] For definitions of K_1* and K_2*, see equation 16. [b] Number of replicate titrations. [c] Standard error of the mean; $\sigma/n^{1/2}$.

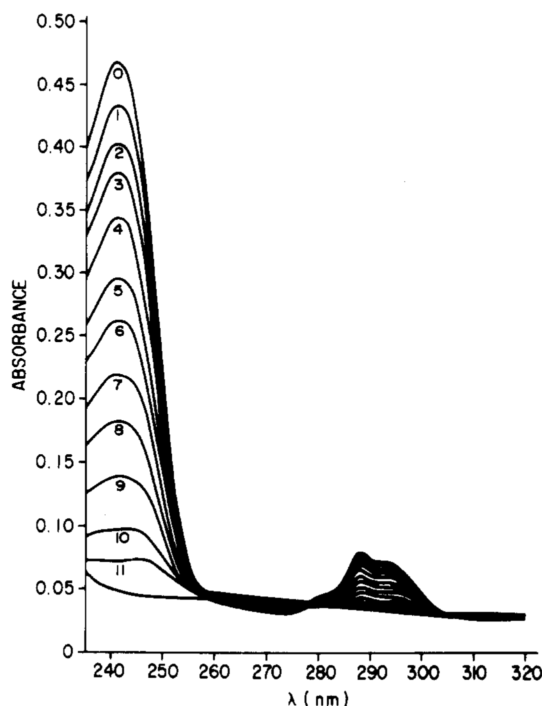

FIGURE 3: UV difference spectra resulting from the addition of free NTA to a solution of gallium transferrin. [Tr] = 1.324×10^{-5} M; [Ga] = 2.647×10^{-5} M; [NTA] = 7.99×10^{-3} M; V = 3.0 mL; curve 0, 0 μL of NTA; 1, 5 μL; 2, 10 μL; 3, 15 μL; 4, 25 μL; 5, 45 μL; 6, 65 μL; 7, 100 μL; 8, 150 μL; 9, 240 μL; 10, 420 μL; 11, 620 μL.

These constants are related to the K_n' values defined by eq 10 and 11, but the [Ga] term in eq 16 represents free hexaaquogallium(III), whereas the [Ga]′ term in eq 10 and 11 represents the sum of all free aquo- and hydroxogallium species. Thus, the two are related by α_{Ga} (eq 5), where $K_n^* = \alpha_{Ga}K_n'$. Gallium binding involves the release of protons and the concomitant binding of bicarbonate, but these species are not included in eq 16. Thus, K_n* is a conditional constant, valid only for pH 7.4 and 5 mM bicarbonate.

Values of K_n* are listed in Table I. The K_1* value is based on titrations where the ratio of NTA:Ga was ≥2. Lower concentrations of NTA appear to have a minimal impact on transferrin binding of the first gallium ion, so these data cannot be used to calculate K_1*. Values of K_2* were calculated in two ways. For eleven solutions with NTA:Ga ≥2, both K_1* and K_2* were varied simultaneously. The average value of log K_1* was then calculated. For an additional five data sets where NTA:Ga <2, the value of K_1* was not varied but was fixed at the average value of the first eleven data sets. The data were fit by varying only K_2* and $\Delta\epsilon_M$. The overall average of log K_2* (18.20 ± 0.10) is not significantly different from the individual means (18.18 and 18.24).

In a separate series of experiments, free NTA was added to solutions of preformed gallium transferrin. The removal

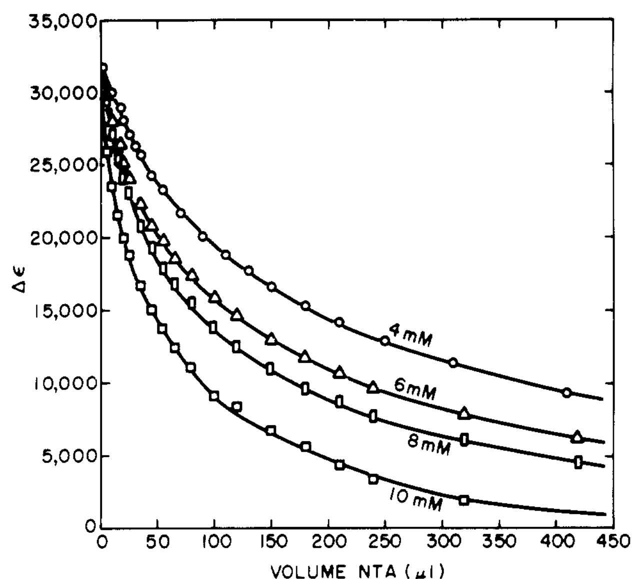

FIGURE 4: Titration curves for the addition of free NTA to gallium transferrin. [Ga] = 2.65×10^{-5} M; [Tr] = 1.32×10^{-5} M; $V = 3.0$ mL; curve 1, [NTA] = 4.03×10^{-3} M; 2, 6.01×10^{-3} M; 3, 7.99×10^{-3} M; 4, 9.97×10^{-3} M.

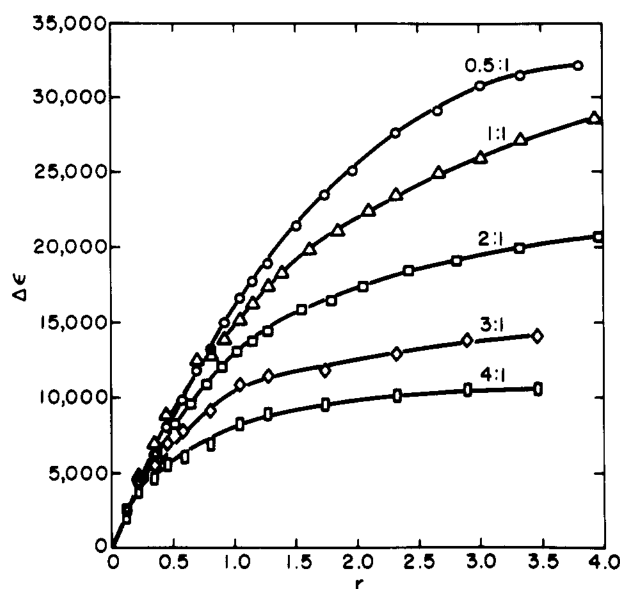

FIGURE 5: Titration curves for the addition of $Ga(EDDA)_x$ to apotransferrin at pH 7.4, where x = 0.5, 1, 2, 3, and 4. $\Delta\epsilon$ is equal to the absorbance at 242 nm divided by the [Tr], and r is equal to the ratio of [Ga] to [Tr].

of gallium from the transferrin complex was followed spectrophotometrically, as shown in Figure 3. Titration curves of $\Delta\epsilon$ vs. the volume of added ligand for various concentrations of NTA are shown in Figure 4. The drop in $\Delta\epsilon$ following the addition of small amounts of NTA provides further evidence for the rather weak binding of the second gallium ion to transferrin. The binding of the first gallium ion to transferrin appears to be much stronger. It takes only about 20 μL of 10 mM NTA to bring $\Delta\epsilon$ down to 20 000 M^{-1} cm^{-1}, which would correspond to an average of one gallium per transferrin. however, it takes over 300 μL to strip the remaining gallium from the protein. Since all concentrations of NTA were able to remove gallium from both metal binding sites, values for K_1* and K_2* could be calculated from all five data sets. Average values of K_1* and K_2* calculated from these data are listed in Table I.

These same types of forward and reverse titrations were repeated by using *N,N'*-ethylenediaminediacetic acid (EDDA) as the competing ligand. The difference spectra are essentially identical with those observed in the NTA experiments, and the resulting titration curves for Ga:EDDA + apotransferrin are shown in Figure 5. The EDDA data are similar to the NTA plots, but they are more curved; that is, there is a larger drop in $\Delta\epsilon$ at any given ratio of ligand to gallium. This is consistent with the larger formation constant for Ga(EDDA) (K_{ML} = 18.15) compared to Ga(NTA) (log K_{ML} = 16.20). In fact, ratios of EDDA:Ga >4 completely block the binding of the second gallium ion to transferrin. Average values of K_1* and K_2* for both forward and reverse titrations with EDDA are listed in Table I.

As discussed above, the titration curve for the addition of free gallium chloride to apotransferrin is linear out to an r value of about 1.5. This indicates that the reaction forming monogallium transferrin is going essentially to completion. However, the curvature at higher r values indicates a measurable distribution of species in the second complexation equilibrium between $Ga(OH)_4^-$ and monogallium transferrin. By treating OH^- as the competing ligand, it was possible to calculate a value of K_2* based on the literature value of β_4OH = $10^{-16.6}$ (Baes & Mesmer, 1976). For these calculations, K_1* was fixed at the grand mean value from the NTA and EDDA titrations. The average value of K_2* obtained by this method is listed in Table I.

Table II: Average Values of $\Delta\epsilon_M$ for the Gallium-Transferrin Complex

method	n	$\Delta\epsilon_M \pm 2$ SEM[a] (M^{-1} cm^{-1})
Ga(NTA) + apoTr	16	20 900 ± 350
NTA + Ga_2Tr	5	19 800 ± 650
Ga(EDDA) + Tr	8	20 700 ± 640
EDDA + GaTr	5	19 800 ± 800
$Ga(OH)_4$ + apoTr	5	20 500 ± 850
grand mean		20 300 ± 600

[a] Standard error of the mean; $\sigma/n^{1/2}$.

From the initial linear portions of the titration curves for GaL + apotransferrin, especially where NTA:Ga <2, one can calculate a slope of $\Delta\epsilon_M$ ~20 000 M^{-1} cm^{-1}. Rather than fix $\Delta\epsilon_M$, at this value during calculations of K_1' and K_2', $\Delta\epsilon_M$ was treated as a third adjustable parameter and was varied along with K_1' and K_2' to minimize the sum of the squares of the residuals of $\Delta\epsilon$. The average values of $\Delta\epsilon_M$ obtained with the various titration methods are listed in Table II. The $\Delta\epsilon_M$ values are more consistent than the K_1* and K_2* values, and an analysis of variance showed that there is no significant difference among the set of average $\Delta\epsilon_M$ values. An overall average $\Delta\epsilon_M$ (±2 SEM) was calculated to be 20 300 ± 600 M^{-1} cm^{-1}, in excellent agreement with our preliminary estimate.

Discussion

Statistical Analysis. Because metal-exchange reactions involving transferrin can be quite slow (Carrano & Raymond, 1979; Pollack et al., 1977; Baldwin, 1980), special care must be taken in binding studies such as this one to ensure that the results reflect thermodynamic equilibrium, rather than kinetic factors. This is the reason for making duplicate measurements of K_1* and K_2* by using both forward and reverse reactions and two different competing ligands. The precision of the overall average binding constant from the various methods is an indicator of whether or not equilibrium has been attained.

Table III: Conditional Iron- and Gallium-Transferrin Binding Constants Calculated for Air-Saturated and Serum Bicarbonate Concentrations

	$[HCO_3^-]$ (mM)	Ga^{3+}	Fe^{3+} [a]	Δ
log K_1*	0.14	18.1	20.7	2.6
log K_2*	0.14	17.1	19.4	2.3
log K_1*	27.0	20.3	22.8	
log K_2*	27.0	19.3	21.5	

[a] Iron values are taken from Aisen et al. (1978), converted to $[HCO_3^-]$ rather than pCO_2.

An analysis of variance (ANOVA) (Johnson, 1973) was run on the sets of average values of both K_1* and K_2* from the various experimental methods. Simple examination of the set of four K_1* values shows that the average obtained by reaction of free NTA with Ga_2Tr varies from other three values. The ANOVA confirms that there is a significant difference among the four means at a 98% confidence level. Further analysis showed that this mean, 18.82, differed from each of the other values but that the three remaining values of K_1* did not differ from each other.

A similar result was obtained with the K_2* values, where a lower number is obtained from the reaction of Ga(NTA) with apotransferrin. An analysis of variance confirms that there is a significant difference among the means at >99% confidence level due to the low value for Ga(NTA) + apotransferrin. There were no significant differences among the remaining values of K_2*. We feel that such agreement in three of four and four of five measurements of K_1* and K_2*, respectively, is a strong indication that our results do indeed reflect thermodynamic equilibrium.

Although the differences between these two low values and the remaining averages are statistically significant, we have serious doubts as to the chemical significance of these variations and are thus reluctant to discard these data. Therefore, we have calculated grand mean values of both K_1* and K_2*, based on all the data, of log K_1* = 19.53 ± 0.25 and log K_2* = 18.58 ± 0.25. If one chose to discard the two data sets based on the ANOVA, these average values would become log K_1* = 19.65 and log K_2* = 18.74. This is a relatively minor shift in log K* values, and it changes the difference between the successive constants by only 0.04, from 0.95 to 0.91.

Thermodynamic Constants. The constants K_1* and K_2* as defined by eq 16 are conditional constants, and the values discussed above apply only at pH 7.4 and a 5 mM bicarbonate concentration. Previous studies have shown that three protons are released for every gallium ion that binds to transferrin (Gelb & Harris, 1980) and that bicarbonate is required for metal binding (Raiszadeh et al., 1981). Therefore, we can define thermodynamic stability constants in terms of the complete set of reactants and products as

$$K_1 = \frac{[\text{Ga-Tr-HCO}_3][\text{H}]^3}{[\text{Ga}][\text{Tr}][\text{HCO}_3]} \quad (17)$$

$$K_2 = \frac{[\text{Ga}_2\text{-Tr-(HCO}_3)_2][\text{H}]^3}{[\text{Ga-Tr-HCO}_3][\text{HCO}_3][\text{Ga}]} \quad (18)$$

and values of K_1 and K_2 can be calculated as $K_n = K_n^*([\text{H}]^3/[\text{HCO}_3])$. Because of the third-order term in hydrogen ion concentration, the numerical values of K_1 and K_2 are quite small, with log K_1 = −0.37 and log K_2 = −1.32.

Given these values of K_1 and K_2, eq 17 and 18 can be used to calculate effective binding constants for bicarbonate concentrations other than the 5 mM level at which these measurements were actually made. The two levels of greatest interest are 0.14 mM (air saturation), since many experiments are run under this condition, and the serum bicarbonate level of 27 mM (Lewis, 1970). Values of such derived constants are listed in Table III, along with published iron binding constants (Aisen et al., 1978). We would note that eq 17 and 18 should not be used to calculate conditional binding constants for other pHs, since Aisen's data on iron binding indicate that K_1 and K_2 are themselves pH dependent (Aisen et al., 1978), presumably due to conformation changes in the protein or protonation of noncoordinated groups near the metal binding sites.

As one expects, transferrin binds iron much more strongly than it binds gallium, by a factor of 400 for K_1* and a factor of 200 for K_2*. However, the ratio of K_1*:K_2* is rather similar for both iron (K_1*:K_2* = 20) and gallium (K_1*:K_2* = 9). On the basis of statistical factors alone, the ratio of successive binding constants for two equivalent binding sites is only 4. Thus, some additional factor is influencing the relative binding affinities.

Aisen et al. (1978) have determined site-specific microconstants for ferric transferrin. When 1 equiv of iron binds to transferrin, about 85% of this iron occupies the a site (C terminal) and only 15% goes to the b site (N terminal). Thus, the spread in successive binding constants beyond purely statistical factors for ferric ion is due primarily to a difference in the intrinsic binding affinities of the two sites. This is consistent with several other studies which indicate nonequivalent binding sites, involving such factors as site differences in the electron paramagnetic resonance (EPR) spectra of the vanadyl (Chasteen et al., 1977) and chromic (Harris, 1977) complexes, the iron binding kinetic accessibility (Aisen et al., 1978), size restrictions on metal binding (Harris et al., 1981; Luk, 1971; Chasteen & Williams, 1981), and the pH dependence of metal binding (Aisen et al., 1978; Harris et al., 1981; Harris, 1977; Chasteen & Williams, 1981).

The successive gallium binding constants differ by a factor of 9. We feel that this probably reflects the same intrinsic differences in the metal binding affinities of the two sites that affect iron binding. If the site preference for gallium is the same as that for iron (Aisen et al., 1978), then K_1 would reflect binding primarily at the a site and K_2 would represent binding primarily at the b site. However, the data reported here do not prove such an explanation. Our data could just as easily be explained by an allosteric mechanism, where binding of the first gallium at either the a or the b site caused a decrease in the binding affinity of the remaining vacant site. Because of the rather large separation between the two sites (Meares & Ledbetter, 1977), it seems very unlikely that binding constants would be strongly affected by direct site–site interaction.

No matter what the basis for the decrease in successive gallium binding constants, a factor of 9 is too small for the macroscopic constant K_1 to represent gallium binding exclusively to a single site. Thus, the addition of 1 equiv of gallium to transferrin would result in some gallium binding at each of the two specific metal binding sites. Additional experiments which would separate the two monogallium transferrins are necessary to establish the ratio of a to b site population.

Other Gallium Studies. Larson and co-workers previously attempted to determine a gallium–transferrin binding constant by equilibrium dialysis using ^{67}Ga in the presence of a large excess of transferrin in Waymouth's tissue culture medium (Larson et al., 1978). They reported a value of log K = 5.40, which is quite different from our log K_1* of 19.5. However, these workers failed to consider the effects of gallium hydrolysis, so that their constant corresponds to our K_1' (eq 10).

If their value of log K is converted to our K^* formalism, by multiplying by the metal hydrolysis term α_{Ga} (eq 5), then the constant becomes log K = 18.4, which is in excellent agreement with our value of 18.1 calculated for solutions air saturated with CO_2 (since Larson's paper did not report a bicarbonate concentration, we assume their solutions were air saturated with CO_2). Since Larson et al. worked only with a large excess of transferrin, they reported only one binding constant which would correspond to K_1.

Larson et al. (1978) also constructed a linear free-energy plot of log K_{Ga} vs. log K_{Fe} for a series of seven low molecular weight ligands and on the basis of an early reported iron–transferrin binding constant of $10^{30.5}$ (Davis et al., 1962) predicted a transferrin log K_{Ga} value of 27.1, which is much higher than the measured log K values. However, Aisen et al. (1978) have recently reported more reliable iron binding data which indicate an iron log K_1^* of only 20.7. Using this lower value of the iron binding constant, one predicts a gallium binding constant of log K_{Ga} = 18.5. Thus, there is excellent consistency between our results, the data of Larson et al. (1978) on gallium, and the data of Aisen et al. (1978) on iron binding. However, we must point out that the uncertainties in the predicted log K values can be rather large.

Kulprathipanja and co-workers (Kulprathipanja et al., 1979) also used equilibrium dialysis against EDTA to measure gallium binding constants and reported values of log K_1^* = 23.7 and log K_2^* = 22.3 in 0.04 M $NaHCO_3$ at pH 7.3. Such values are almost certainly too high, since even Fe^{3+} does not have such large binding constants. However, from our examination of the experimental data reported by Kulprathipanja et al. (1979), it appears that these workers neglected to consider the protonation of EDTA in their calculation. Since the gallium–EDTA stability constant is expressed in terms of $EDTA^{4-}$, the fully deprotonated form of EDTA, one must consider in any type of ligand competition experiment that at physiological pH, only a tiny fraction of the EDTA exists in this form. At pH 7.3, the [$EDTA^{4-}$] is only 0.27% of the analytical concentration of EDTA. The effect of this modification is to subtract 2.9 from the reported log K values of Kulprathipanja et al., so that now log K_1^* = 20.8 and log K_2^* = 19.4. Using our values of K_1 and K_2, we can calculate values of log K_n^* of 20.1 and 19.2 for n = 1 and 2, respectively, at pH 7.3 and 0.04 M $NaHCO_3$. This is in reasonable agreement, considering the uncertainties in the log K values for Ga–EDTA and the differences in experimental conditions. Since it is known that the kinetics for metal ion removal from transferrin by EDTA are rather slow (Pollack et al., 1977; Baldwin, 1980), and since these workers approached equilibrium only from one direction, starting with the Ga–transferrin complex, one might expect their constants to be somewhat larger than those reported here.

It is interesting to compare the gallium binding affinity of transferrin with that of low molecular weight synthetic ligands. The stability constants per se are often not the most useful criterion for comparing the sequestering abilities of a series of ligands at physiological pH. Therefore, we have adopted the practice of using stability constants to calculate pM values (pM = −log [M]), where [M] is the concentration of unchelated, unhydrolyzed metal ion which would be present at equilibrium in a pH 7.4 solution of 1 μM gallium and 10 μM ligand. The advantage of this approach is that pM values reflect the influence of factors such as ligand basicity, chelate protonation and hydrolysis, differences in metal–ligand stoichiometrics, and dilution effects. Values of both K_{ML} and pM are listed in Table IV. Comparisons of various ligands are straightforward; the larger the pM value, the more effective the ligand under the specified conditions. It must be remembered that the relative order of stabilities may shift if a different set of conditions (i.e., concentration, pH, [CO_3^{2-}]) are used for the pM calculations.

Table IV: pM and log K_{ML} Values for Gallium Chelating Agents[a]

ligand[b]	pM	log K_{ML}	ref
HBED	30.9	39.57	c
MECAMS	26.3	38.0	d
3,4-LICAMS	26.0	38.5	d
EHPG	23.5	31.61	c
DTPA	22.8	23.32	c
EDTA	21.7	21.7	c
transferrin (K_1^*)	21.3	20.3	g
transferrin (K_2^*)	20.3	19.3	g
EDDA	20.3	19.3	c
Tiron	19.4		e
NTA	19.04	16.2	c
OH^-	19.01	39.4	f

[a] Calculated for 1 μM Ga, 10 μM ligand, and 27 mM carbonate, pH 7.4. [b] Abbreviations: HBED, *N,N'*-bis(hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid; MECAMS, *N,N',N''*-tris(2,3-dihydroxy-5-sulfobenzoyl)-1,3,5-benzenetrimethanamine; 3,4-LICAMS, *N,N',N''*-tris(2,3-dihydroxy-5-sulfobenzoyl)-1,5,10-triazadecane; EHPG, ethylenebis[(hydroxyphenyl)glycine]; DTPA, diethylenetriaminepentaacetic acid; Tiron, 3,5-disulfo-1,2-dihydroxybenzene. [c] Harris & Martell (1976). [d] Pecoraro et al. (1982). [e] Letkeman et al. (1980). [f] Baes & Mesmer (1976). [g] This work.

Transferrin pM values were calculated for both K_1^* and K_2^* by assuming a 27 mM bicarbonate concentration. The transferrin pM values fall within the range of the common aminocarboxylic acids. On the basis of K_1^*, transferrin is more effective than NTA or EDDA but is less effective than EDTA or diethylenetriaminepentaacetic acid (DTPA). Table IV also includes pM values for catecholates (MECAMS, 3,4-LICAMS, Tiron; see definitions in Table IV) which are known to form very stable gallium complexes, and phenolates (HBED and EHPG; see definitions in Table IV). The phenolates and multidentate catecholates are clearly superior to transferrin as gallium binding agents. The phenolic ligand HBED is the most effective chelator, with a pM value that is over nine log units greater than the transferrin pM. The catecholate ligands MECAMS and 3,4-LICAMS are also very effective, with pM values five log units greater than that of transferrin.

Such high pM values indicate that phenolate and catecholate ligands may compete in vivo with transferrin for gallium. However, pM values do not depend on kinetic factors and cannot be used to predict the rate at which gallium will exchange between transferrin and the synthetic ligands. Thus, reactions which are highly favored thermodynamically may proceed at rates which are too slow to be physiologically important. However, it has been shown that the catecholates can indeed remove gallium from transferrin in vivo and facilitate its excretion via the kidneys (Moerlein et al., 1981). Comparable studies on phenolate ligands have not been carried out.

In the absence of any chelating agent, gallium forms a stable gallate ion, $Ga(OH)_4^-$, at pH 7.4. The gallate species sets a minimum pM value of 19.01. At higher bicarbonate concentrations, transferrin can compete reasonably well with OH^-. However, at air-saturated bicarbonate levels, the pM values drop to 19.3 and 19.05 for K_1^* and K_2^*, respectively. Thus, under dilute conditions with low bicarbonate levels, the dissociation of about 75% of the gallium from digallium transferrin to form $Ga(OH)_4^-$ is thermodynamically favored. This effect must be considered in preparations of gallium transferrin solutions for subsequent experiments. Separation procedures

such as dialysis and chromatography, which are commonly used to separate excess metal ions from their protein complexes, are likely to cause partial dissociation of the transferrin complex. Thus, it is likely to be rather difficult to isolate pure digallium transferrin, particularly in the presence of lower bicarbonate concentrations.

The ligands Tiron and NTA both have pM values very near 19.01, and thus in these systems, almost all of the gallium exists as $Ga(OH)_4^-$ rather than GaL. The calculation that NTA cannot compete with OH^- for gallium may seem inconsistent with our use of this compound as a competing ligand in this study. However, this is a simple dilution effect. At 1 μM gallium and 10 μM ligand, gallium–NTA will dissociate to produce $Ga(OH)_4^-$. At 100 μM Ga and 1 mM ligand, the equilibrium shifts such that 99.2% of the gallium is bound to NTA. Thus, NTA is a reasonably effective ligand at the concentrations used in the specroscopy studies.

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