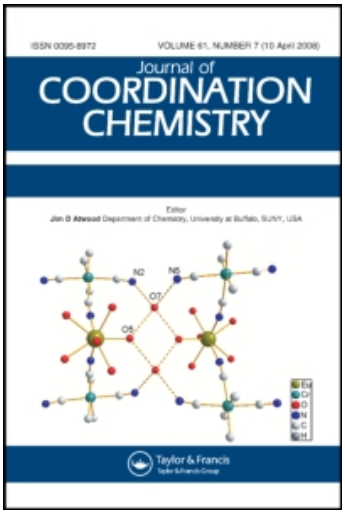

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STABILITY CONSTANTS FOR DIMERCAPTOSUCCINIC ACID WITH BISMUTH(III), ZINC(II), AND LEAD(II)

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Stability constants for the complexation of zinc(II), lead(II), and bismuth(III) by the vicinal dithiolate chelating agent *meso*-dimercaptosuccinic acid (DMSA) have been determined by a combination of potentiometric titration and spectrophotometric competition at 25°C and 0.1 M ionic strength. The spectrophotometric studies use the shifts in the ultraviolet bands of the thiol groups to quantitate metal binding to DMSA in the presence of competitive aminocarboxylic acids. Bismuth(III) forms a bis(DMSA) chelate with an exceptionally high stability constant of $10^{43.87}$. This complex undergoes a series of protonations over the pH range 10 to 2, but there appears to be no measurable dissociation of ligand over this pH range. The zinc–DMSA system is dominated by a $Zn_2(DMSA)_2$ dimer, which has a protonation constant of 10^6 and dissociates completely at lower pH. No more than 20% of total zinc exists as a monomeric complex at any pH. Lead forms a 1:1 complex with a stability constant of $10^{17.4}$. Insoluble protonated lead complexes precipitate at pH < 6.5. Speciation calculations have been used to evaluate the potential competition from serum zinc to the binding of Pb^{2+} and Bi^{3+} by DMSA. The results indicate that DMSA should be relatively effective for the *in vivo* chelation of both Bi^{3+} and Pb^{2+}.

Keywords: *Meso*-dimercaptosuccinic acid, zinc, lead, bismuth, stability constants

INTRODUCTION

Alpha particles are especially toxic to cells because they lose a large amount of energy over a relatively short distance.[1,2] Only 10–20 α particles are required to kill a cell,[3] but the particles travel only 40 to 80 μm through tissues.[1] This is an attractive combination for radiotherapy using internal emitters, since the target cells could be killed with minimal damage to surrounding tissue. However, the toxicity of α-emitters requires a very high degree of target specificity. Recent developments in the area of monoclonal antibodies now offer the possibility of such specificity,[1,2] provided that the radionuclide can be firmly attached to the antibody.

Bismuth-212 is an attractive nuclide for radioimmunotherapy. It can be obtained from a ^{224}Ra generator, has a short physical half-life of 60 min, and decays to stable ^{208}Pb.[1] Initial attempts to link Bi^{3+} to antibodies have involved conjugation of diethylenetriaminepentaacetic acid to the antibody.[1,2] Macrocyclic aminocarboxylates are also under investigation for binding both ^{212}Bi and its parent nuclide ^{212}Pb ($t_{1/2}$ = 10.6 h) to antibodies.[4]

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We have begun an investigation of the complexation of Bi^{3+} by thiol-based chelating agents. To date no stability constants for bismuth thiolate complexes have been reported. However, there are reports that thiolate compounds have a strong influence on the absorption of ingested bismuth,[5] and that bismuth binds to metallothionein-like proteins in kidneys.[6,7] In addition, thiolates are effective antidotes for bismuth toxicity.[8,9]

The initial studies reported here involve the well-studied chelating agent *meso*-dimercaptosuccinate. Dimercaptosuccinate and related dithiolates are already of considerable interest as possible drugs for the treatment of lead toxicity,[10-13] and ^{99m}Tc-DMSA is used as a renal imaging agent.[14] The results of this work demonstrate the exceptional stability of bismuth–thiolate complexes.

EXPERIMENTAL

Reagents

Meso-dimercaptosuccinic acid (DMSA) was purchased from Aldrich and used as received. Solutions of bismuth(III) were prepared by the dissolution of 99.9999% pure bismuth shot in concentrated HNO_3. The bismuth stock solution was standardized by spectrophotometric titration with EDTA using the intense absorbance band of Bi-EDTA at 265 nm to monitor the formation of Bi-EDTA. The concentration of excess acid in the bismuth solution was determined by potentiometric titration of a 1:1 ratio of Bi:EDTA by detecting the position of the inflection at pH 7 and subtracting the effect of the two protons released from the EDTA. Zinc stock solutions were prepared from reagent grade nitrate salts and standardized by complexiometric titration.[15] Lead stock solutions were prepared from DILUT-IT® analytical standards.

Carbonate-free KOH solutions were prepared from DILUT-IT® ampoules using freshly boiled deionized water. The KOH solutions were kept under an ascarite CO_2-scrubber in the reservoir of a Metrohm model 655 autoburette. The absence of carbonate was confirmed for each KOH solution by Gran plots.[16]

Potentiometric Titrations

Potentiometric titrations were performed under an atmosphere of N_2 that was passed through ascarite and 0.1 M KNO_3 scrubbers. The jacketted cell was maintained at 25°C by an external circulating water bath. The ionic strength of all solutions was set at 0.1 M by the addition of 1.0 M KNO_3. The pH was determined using separate glass and calomel electrodes calibrated to read $-\log [H^+]$ directly rather than hydrogen ion activity. Solutions of 5 mM HNO_3 and a pH 7 phosphoric acid buffer were used for a two-point calibration of the electrodes. The $p[H^+]$ of the phosphoric acid buffer was calculated using the phosphate protonation constants from Mesmer and Baes.[17] A dissociation constant for water of $-\log K_w = 13.781 \pm 0.006$ was used in all calculations.[18]

Titrations were conducted using a computer-controlled autotitrator. The system was operated from an IBM XT computer, which used a Techmar Lab-Master interface to control the cell stirring motor, to prompt the Metrohm model 655 autoburette to deliver titrant, and to read pH from a Beckman model 4500 pH meter. The software to control the system was written in Pascal at the University of Idaho.

The titrator adds an aliquot of titrant, stirs the cell for approximately 30 s, and then monitors pH *versus* time. When the pH drift falls below a preset minimum, usually 0.001 pH/min, the final pH is recorded and the burette is prompted for the addition of another aliquot for titrant.

Spectrophotometric Competition

Solutions of metal ion, DMSA, and a competitive ligand were mixed in 0.1 M KCl and allowed to equilibrate at 25°C. The pH of the solutions was selected to obtain a measurable distribution of the metal between the two ligands. Competitions between EDTA and DMSA for bismuth were run at pH 2.3. Competitions between DMSA and HEDTA for complexation of lead were run at pH 7.4. Absorbances were monitored over 24 hrs to ensure that equilibrium had been reached. Electronic spectra were recorded on a Varian 2200 spectrophotometer.

Infrared Spectra

Solid samples of metal DMSA complexes were used to prepare KBr pellets. Infrared spectra were recorded on a Perkin Elmer model 1600 Fourier transform spectrophotometer.

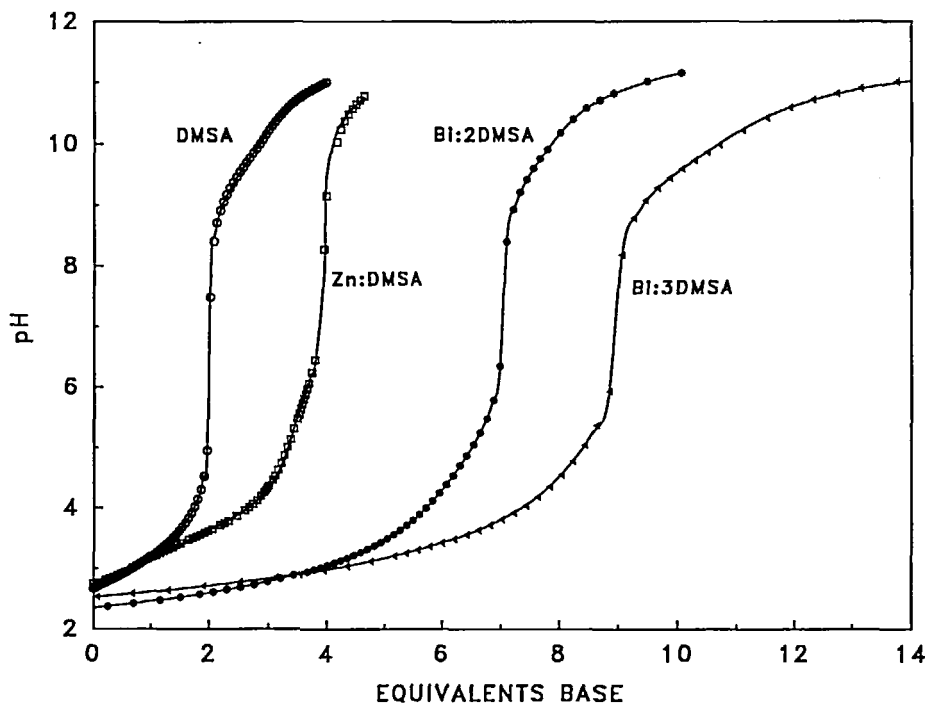

FIGURE 1 Potentiometric equilibrium curves for the titration of free DMSA, a 1:1 mixture of Zn^{2+} and DMSA, a 1:2 mixture of Bi^{3+} and DMSA, and a 1:3 mixture of Bi^{3+} and DMSA. Free ligand and metal ion concentrations are approximately 1–2 mM. Solutions were adjusted to 0.1 M ionic strength with KNO_3 and maintained at 25°C.

RESULTS

Ligand Protonation Constants

The potentiometric equilibrium curve for the protonation of DMSA is shown in Figure 1. The low pH buffer region reflects the protonations of the two carboxylic acid groups, while the upper buffer region reflects the protonations of the two thiolate groups. Protonation constants calculated from four replicate potentiometric titrations are 11.50 ± 0.16, 9.62 ± 0.04, 3.45 ± 0.05, and 2.17 ± 0.20. The largest protonation constant is difficult to determine from potentiometric data. The value of 11.50 was calculated from titration data below pH 11 to avoid potential problems of nonlinearity in the electrode response at very high pH.

Potentiometric Studies of Bi^{3+}

Mixing of equimolar amounts of bismuth and DMSA leads to the immediate precipitation of a yellow solid. Although this solid redissolves after the addition of 4 equiv. of base, no potentiometric data on 1:1 solutions were collected.

When the ratio of DMSA:Bi is increased to 2:1, the resulting bismuth complexes are soluble over the entire pH range from 2 to 11. Potentiometric equilibrium curves for both 2:1 and 3:1 DMSA:Bi solutions are shown in Figure 1. The x-axis represents moles of base per mole of metal ion, so that formation of the fully deprotonated $Bi(DMSA)_2^{5-}$ species should occur at 8 equiv. of base. The equilibrium curves were fitted by an iterative nonlinear least-squares program based on the general least-squares program ORGLES.[19] In this procedure the equilibrium system is described by the following mass balance equations for the three components metal ion (M), ligand (L), and dissociable protons (H).

$$[M]_{tot} = [M] + \sum_i \sum_j \sum_k i[M_iL_jH_k] \tag{1}$$

$$[L]_{tot} = [L] + \sum_i \sum_j \sum_k j[M_iL_jH_k] \tag{2}$$

$$[H]_{tot} = [H] + \sum_i \sum_j \sum_k k[M_iL_jH_k] \tag{3}$$

Equilibrium constants are defined as in (*4*) and (*5*).

$$iM + jL + kH \overset{\beta_{ijk}}{\rightleftharpoons} M_iL_jH_k \tag{4}$$

$$\beta_{ijk} = \frac{[M_iL_jH_k]}{[M]^i[L]^j[H]^k} \tag{5}$$

The chelate concentration terms in equations (*1–3*) are replaced with products of the appropriate equilibrium constants and [M], [L], and [H]. For a trial set of β values, the molarities of these three components are then varied to minimize the residuals between calculated and analytical values for $[M]_{tot}$, $[L]_{tot}$, and $[H]_{tot}$. Numerical derivatives are then used to calculate new values for the β's, so as to minimize the residuals between observed and calculated pH values.

In the 2:1 DMSA:Bi^{3+} system it was not possible to refine values for either β_{110} or β_{120} from the titration data. It appears that the complexation of bismuth is so strong, that the 2:1 complex is completely formed upon mixing. This model is consistent with the lack of precipitation of any of the sparingly soluble 1:1 complex at the acidic extreme of the titration.

The 2:1 curves were fitted by assuming a large, fixed value or β_{120} and refining a series of protonation constants for the 2:1 complex. The chelate protonation constants were defined as in (*6*).

$$K_n^H = \frac{[BiL_2H_n]}{[BiL_2H_{(n-1)}][H]} \tag{6}$$

Protonated species were added to the model one at a time. The fits for successive models were compared using the *R* factor ratio test[20,21] to determine whether the inclusion of a new species resulted in a statistically significant improvement in the fit of the pH data. Models were also characterized by a goodness-of-fit parameter (GOF), defined in (*7*),

$$GOF = \left[\frac{\sum (pH_{obs} - pH_{calc})^2}{(n_{obs} - n_{par})}\right]^{1/2} \tag{7}$$

where n_{obs} is the number of pH observations in a titration and n_{par} the number of independent parameters (β values) allowed to vary during the refinement of the data.

The final model for the 2:1 DMSA:Bi system included a series of five protonation constants for the $Bi(DMSA)_2^{5-}$ species. The inflection in the 3:1 curve is shifted by two equiv., which would correspond to the loss of the two carboxylate groups of the excess ligand. It was possible to fit the 3:1 titration curves with the same species used for the 2:1 curves. Values of the K_n^H values for $Bi(DMSA)_2^{5-}$ are listed in Table I.

TABLE I
Stability and protonation constants for metal complexes of DMSA[a]

Metal Ion	i	j	k	log β_{ijk}[b]	log K_n^H[c]
Bi^{3+}	1	2	0	43.87 ± 0.16	
	1	2	1	53.5 ± 0.3	9.6 ± 0.2
	1	2	2	58.5 ± 0.3	4.96 ± 0.09
	1	2	3	62.0 ± 0.3	3.57 ± 0.07
	1	2	4	64.8 ± 0.3	2.76 ± 0.07
	1	2	5	67.0 ± 0.3	2.20 ± 0.12
Zn^{2+}	1	1	1	20.08 ± 0.15	
	2	2	−1	23.6 ± 0.8	10.3 ± 0.6
	2	2	0	33.6 ± 0.5	
	2	2	1	39.6 ± 0.3	5.94 ± 0.25
Pb^{2+}	1	1	0	17.4 ± 0.2	

[a] All values for 25°C and 0.1 M ionic strength. [b] Constant defined by (*5*). [c] Constant defined by (*6*).

Potentiometric Studies on Zn^{2+}–DMSA

The potentiometric equilibrium curve for a 1:1 ratio of DMSA:Zn is shown in Figure 1. There is a strong inflection at 4 equiv. of base, corresponding to loss of all

four ligand protons. A weak buffer region preceding the major inflection is barely visible in the potentiometric equilibrium curve. The buffer region is more clearly visible as a shoulder at 3.5 equiv. of base in the first derivative plot of the potentiometric data. This non-integral value is strongly indicative of polynuclear metal complexes.

Potentiometric data for both 1:1 and 2:1 DMSA:Zn titrations have been refined by both mononuclear and dinuclear models. The mononuclear model consisted of 110 and 111 complexes. This model produced rather poor GOF values of 0.016 to 0.048 for six independent data sets. Adding additional protonated chelates did not produce any significant improvement in the fit. Several dinuclear models were evaluated. The final model consisted of the 220, 221, 22-1, and 111 species. This model gave GOF values that ranged from 0.002 to 0.008. The dinuclear model improved the fit relative to the mononuclear model for all data sets. The final set of zinc binding constants is listed in Table I. Although titrations were also run with a 2-fold excess of DMSA, it was not necessary to include any 2:1 DMSA:Zn complexes to obtain a satisfactory fit of the data.

Lead DMSA Titrations

Potentiometric equilibrium studies on the Pb–DMSA system were prevented by the precipitation of a yellow solid below pH 6, even in the presence of an excess of DMSA. This solid dissolves after the addition of about 3.5 equiv. of base, and there is an inflection at 4 equiv. of base. The range of solubility was too narrow to allow the calculation of a protonation constant for the Pb–DMSA complex.

Spectrophotometric Competition

Stability constants for both the Pb(DMSA) and the $Bi(DMSA)_2$ complexes were determined by spectrophotometric competition. Spectra for the bismuth system are shown in Figure 2. The competition between EDTA and DMSA was conducted at pH 2.3. At this pH the absorbance of free DMSA is negligible above 280 nm. Coordination of DMSA to bismuth results in an intense absorbance across the uv range, with a shoulder 335 nm. The bismuth–EDTA complex has a very intense absorbance band at 265 nm. However, this band is rather narrow, such that Bi–EDTA has no significant absorbance at 335 nm. Thus for mixtures of Bi, EDTA, and DMSA, it is a simple matter to calculate the concentration of $Bi(DMSA)_2$ from the absorbance at 330 nm.

A typical spectrum for a competition experiment is shown in Figure 2. These spectra are used to calculate effective binding constants of DMSA at pH 2.3 based on the following mass balance equations,

$$[Bi]_{tot} = \alpha_{BiL}[Bi(DMSA)_2] + \alpha_{BiL'}[Bi(EDTA)] \quad (8)$$

$$[DMSA]_{tot} = \alpha_{L}[DMSA] + 2\alpha_{BiL}[Bi(DMSA)_2] \quad (9)$$

$$[EDTA]_{tot} = \alpha_{L'}[EDTA] + \alpha_{BiL'}[Bi(EDTA)] \quad (10)$$

where α values are the usual protonation functions for the free ligands and the bismuth chelates. The presence of excess ligand in these solutions allows one to delete free metal ion as a significant species.

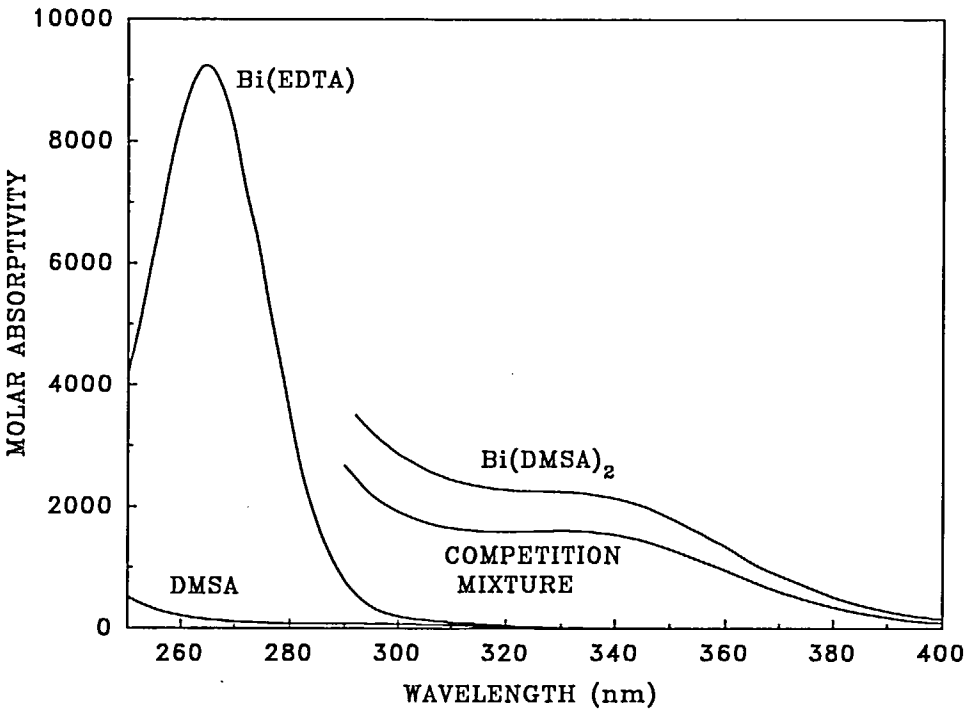

FIGURE 2 Electronic spectra of the components involved in the spectrophotometric competition between DMSA and EDTA in 0.1 M KCl solution at pH 2.3 and 25°C. For the competition experiment, [Bi] = 3×10^{-5} M; [EDTA] = 3×10^{-4} M; [DMSA] = 6×10^{-4} M. The apparent absorptivity is expressed per molarity of Bi.

The apparent extinction coefficient for $Bi(DMSA)_2$ at pH 2.3 reflects the sum of the absorbances of several protonated bismuth chelates and changes slightly as a function of pH. Thus the effective extinction coefficient of this complex was measured at the competition pH of 2.3. Dividing the absorbance of the competition solutions by this effective extinction coefficient gives a value for $\alpha_{BiL}[Bi(DMSA)_2]$. This reduces the problem to three equations and three unknowns, and equations (*8–10*) can be solved algebraically for [DMSA], [Bi(EDTA)], and [EDTA]. One can define an exchange constant K_X as in (*11*),

$$K_X = \frac{[Bi(DMSA)_2][EDTA]}{[DMSA]^2[Bi(EDTA)]} = \frac{\beta_{120}(DMSA)}{\beta_{110}(EDTA)} \qquad (11)$$

and calculate β_{120} for bismuth DMSA from the known value of β_{110} for Bi(EDTA).[22] This procedure gives a log β_{120} value of 43.87 ± 0.16.

Similar competition experiments were run using solutions of Pb^{2+}, DMSA, and *N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid (HEDTA). These competitions were run at pH 7.4, which corresponds to the inflection at 4 equiv. of base in the potentiometric titration. Thus one is assured that there will be no protonated or hydrolyzed forms of the Pb–DMSA chelate present in solution.

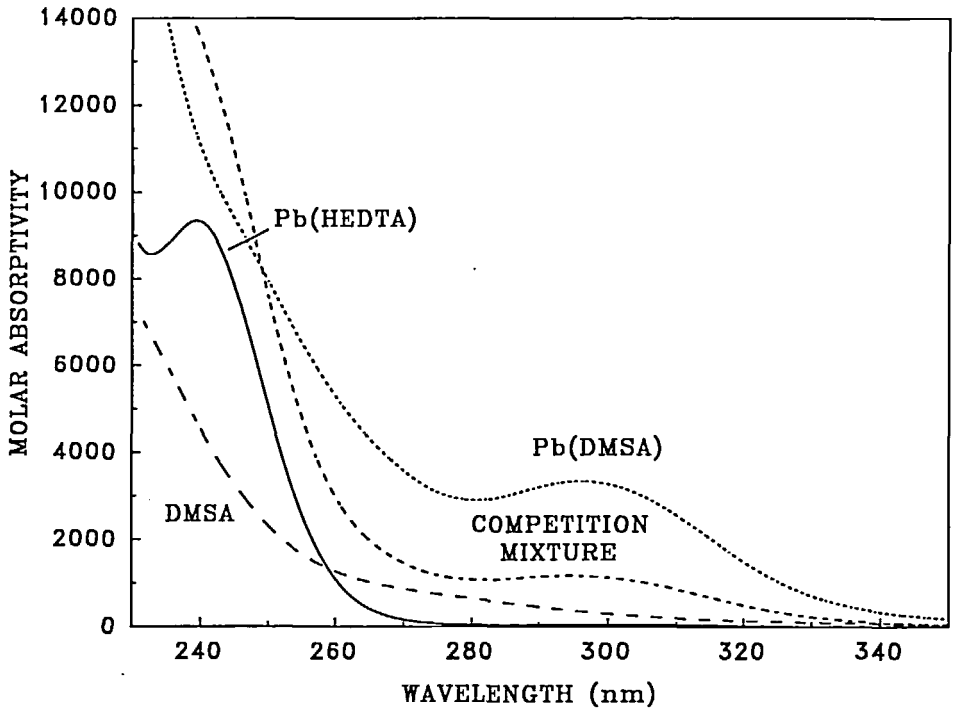

FIGURE 3 Electronic spectra of the components involved in the spectrophotometric competition between DMSA and HEDTA in 0.1 M KCl solution at pH 7.4 and 25°C. For the competition experiment, $[Pb] = 2 \times 10^{-5}$ M; $[HEDTA] = 2 \times 10^{-4}$ M; $[DMSA] = 2 \times 10^{-3}$ M. The apparent absorptivity is expressed per molarity of Pb.

Spectra for the components of the Pb competition experiments are shown in Figure 3. The absorbance of free DMSA is greater at pH 7.4 compared to the pH of 2.3 used in the bismuth studies. Complexation of Pb^{2+} produces a clearly defined peak at 295 nm. The Pb-HEDTA complex has an intense absorbance at 245 nm, but has no significant absorbance at 295 nm. Thus the absorbance at 295 can be described as in (*12*).

$$ABS = \varepsilon_{Pb}[Pb(DMSA)] + \varepsilon_{L}[DMSA] \qquad (12)$$

Equation (*12*) was combined with mass balance equations for Pb, HEDTA, and DMSA to give a system of four equations and four unknowns, which were solved algebraically to determine [Pb(DMSA)], [DMSA], [Pb(HEDTA)], and [HEDTA]. The stability constant of Pb(DMSA) was calculated as described above for bismuth.

There was some concern that 2:1 DMSA:Pb complexes might form in the presence of excess ligand. Therefore, a simple spectrophotometric titration of Pb^{2+} with DMSA at pH 7.4 was conducted. There was a clear end point at one equiv. of DMSA. A small increase in absorbance beyond one equiv. could be accounted for from the absorbance of free DMSA at this pH.

Infrared Spectra of Metal Complexes

DMSA can coordinate *via* carboxylate or thiolate donor groups. Molecular models show that the ligand can coordinate through no more than three of the four possible donor groups. Infrared spectra were obtained on solid samples of the zinc and bismuth complexes to establish the mode of bonding. The IR spectrum of the free ligand is essentially identical to that reported by Rivera *et al.*,[23] with sharp but weak S–H stretching bands at 2552 and 2537 cm^{-1}, and a strong carbonyl stretch at 1700 cm^{-1} for the protonated carboxylic acids.

The addition of bismuth to a solution of DMSA at acidic pH produces an insoluble yellow solid. This material was isolated, dried, and used to prepare a KBr pellet. The infrared spectrum of this material is shown in Figure 4. The carbonyl stretching band for a protonated carboxylic acid is observed at 1705 cm^{-1}. The absence of any S–H bands near 2500 cm^{-1} indicates coordination of both thiolates. The antisymmetric and symmetric stretches of a deprotonated carboxylate group are observed at 1549 and 1367 cm^{-1}.

Solutions containing 2:1 ratios of DMSA:Bi were adjusted to pH 8, which corresponds to the strong inflection in the potentiometric equilibrium curve at 7 equiv. of base. The titration data establish the proton stoichiometry of this species as $Bi(HL)(L)^{4-}$. The addition of Ba^{2+} to this solution leads to the immediate precipitation of a yellow solid. No precipitate forms on addition of Ba^{2+} to free DMSA at this pH. The yellow bismuth compound was used to prepare KBr pellets, and the IR spectrum is shown in Figure 4.

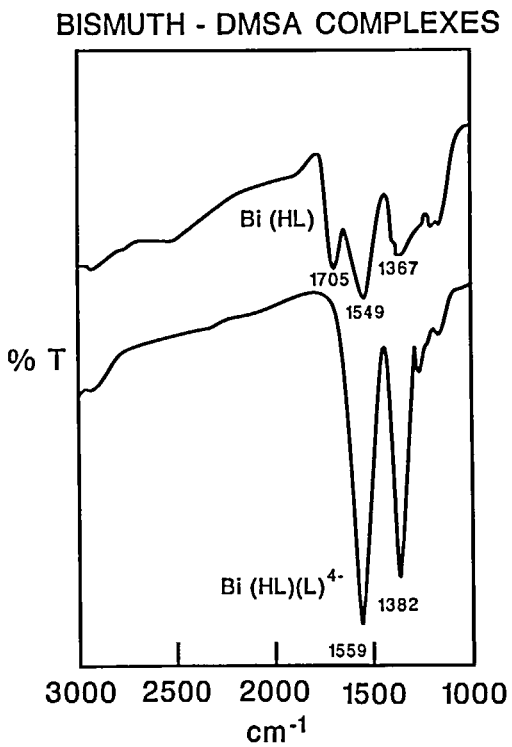

FIGURE 4 Infrared spectra (KBr pellets) of the neutral solid Bi(HDMSA) and the barium salt of $[Bi(DMSA)(HDMSA)]^{4-}$.

One would expect the carboxylates to be deprotonated at this pH regardless of the mode of bonding, and indeed one observes very simple spectra, with antisymmetric and symmetric carboxylate stretches at 1559 and 1382 cm^{-1}. No S–H stretching bands are observed near 2500 cm^{-1}. Even though the titration clearly indicates a monoprotonated species, there is no evidence of protonation of either the carboxylate or the thiolate groups from the IR spectrum.

The 1:1 solution of Zn:DMSA was also adjusted to pH 8, which corresponds to the inflection at 4 equiv. of base, so that the only complex in solution is the $Zn_2(L)_2^{4-}$ species. Addition of Ba^{2+} to this solution produced the immediate precipitation of a white solid having the IR spectrum very similar to that of Bi(HL)$(L)^{4-}$. There were no S–H bands and only two strong bands in the carbonyl region at 1568 and 1384 cm^{-1}.

Speciation Calculations

The distribution of Zn–DMSA complexes as a function of pH for a solution of 2 mM DMSA and 2 mM Zn^{2+} has been calculated using the program SPE.[24] The speciation curves are shown in Figure 5. There is virtually no complexation of zinc at low pH. The 111 complex begins to form at pH 2.5. However, this species accumulates to only about 20%. Between pH 3 and 4, the 221 species begins to accumulate and becomes the dominant species at pH 5. The 221 species then loses a proton to give virtually 100% of the 220 species from pH 7 to 9.

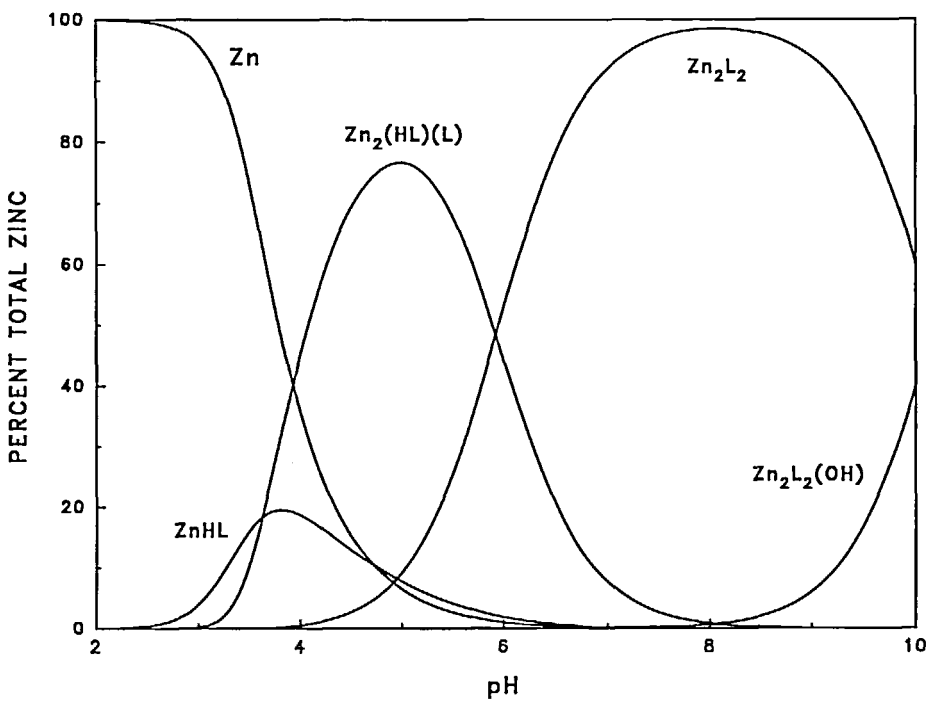

FIGURE 5 Calculated species distribution of the zinc species in a solution of 2 mM Zn^{2+} and 2 mM DMSA as a function of pH.

An initial objective of this work was to determine whether DMSA would be able to retain Bi in competition from hydroxide and from physiological metal ions, especially Zn. Speciation calculations on Bi–Zn–DMSA mixtures at pH 7.4 were performed using the computer program ECCLES.[25] There are not nearly enough bismuth stability constants available to construct a full serum model for Bi^{3+}. Therefore, a simple competition between Zn and 1 nM Bi was evaluated at a series of DMSA concentrations ranging from 2 nM to 1 mM. The concentration of free zinc was set to 2×10^{-9} M, which is the best estimate for the equilibrium level of free zinc in normal serum.[25,26] There is no significant dissociation of the Bi(HL)(L) chelate until the DMSA concentration drops below 4 nM. The primary driving force for dissociation appears to be hydrolysis of bismuth.

A second speciation calculation was made to assess the competition between zinc and lead for complexation by DMSA at physiologically relevant concentrations. The concentration of free zinc was again set to 2 nM,[25,26] while the concentrations of total DMSA and total lead were set to 10^{-6} and 10^{-7} M, respectively. The pH was set to 7.4.

These calculations showed that virtually 100% of the lead would be bound by the DMSA. The avidity of lead complexation is best measured by the pM value, where pM is the negative logarithm of the concentration of free, uncomplexed Pb^{2+}. The pM value corresponding to 10^{-7} M total lead and 10^{-6} M DMSA is 12.05. The concentration of Zn bound to DMSA is approximately 10^{-8} M, distributed between the 220 dimer and the 111 monomer. Almost 90% of the DMSA remains as free, diprotonated DMSA.

The speciation calculations were repeated using 10^{-6} M EDTA in place of DMSA and including both Zn and Ca as competitive metal ions. In contrast to DMSA, the EDTA exists entirely as a mixture of the Zn and Ca complexes. Competition from these two metal ion lowers the pM value for lead to 10.75. Thus we estimate that DMSA should be about 20-times more effective than EDTA for binding lead *in vivo*.

DISCUSSION

Protonation constants of DMSA have been measured several times.[27–30] Although there is general agreement between the reported values, a detailed comparison is difficult because of variations in temperatures and ionic strengths. The first protonation constant is almost too high to measure using a glass electrode. Indeed, Jones *et al.*[30] simply estimate a value of 10^{12} for this constant. The value of this constant will affect the absolute magnitude of the metal–ligand binding constants. However, as pointed out by Jones *et al.*,[30] the choice of this constant has no effect on calculations of species distributions and competitive interactions among ligands and metal ions at physiological pH.

This is the first report of stability constants for a Bi^{3+}-thiolate complex. The overall stability constant of $10^{43.87}$ is exceptionally high and clearly indicates an enormous affinity of thiolate groups for the Bi^{3+} ion. Indeed, the accumulation of a net -5 charge on the complex due to the deprotonated carboxylates would have been expected to diminish the stability of the 2:1 complex. The unfavourable accumulation of an even higher negative charge may account for the absence of any 3:1 complexes.

This accumulation of charge may also be responsible for the unusual protonation behaviour of the $Bi(DMSA)_2^{5-}$ complex. The first protonation constant of this

species is $10^{9.6}$. One would expect that this protonation would have to involve the more basic thiolate groups, since it is well above the protonation constant of a carboxylate. However, the infrared spectra do not show any S–H stretching bands for the monoprotonated Bi(HL) $(L)^{4-}$ species or for the Bi(HL) species.

In principle the IR spectra can be used to differentiate between protonated, coordinated, and deprotonated-noncoordinating carboxylate groups. It has been proposed that the most sensitive parameter for this type of differentiation is the separation ($\Delta\nu$) between the symmetric and antisymmetric carboxylate stretches.[31] One expects $\Delta\nu$ to increase for the coordinated carboxylate relative to a free carboxylate. Previous studies on zinc cysteine complexes showed a substantial shift from $\Delta\nu = 184\,cm^{-1}$ for the N,S-bonded complex having an uncoordinated carboxylate to $\Delta\nu = 260\,cm^{-1}$ for S,O-bonded zinc cysteine complex.[32] However, the IR spectra of the monothiosuccinate complexes of both Pb^{2+} and Zn^{2+}, in which the metal is presumably coordinated to one carboxylate group, show $\Delta\nu$ values of only 135 and 148 cm^{-1}, respectively.[33] In contrast, the spectrum of Pb(DMSA) has a $\Delta\nu$ of 174 cm^{-1}, which was assigned as a coordinated carboxylate.[23] Studies on the IR spectra of amino acids such as EDTA have shown that the differentiation between coordinated and deprotonated, free carboxylate groups is possible for very strongly bound metal ions such as cobalt(III), but such differentiation is more difficult for more weakly bound metal ions such as Zn(II).[34]

The $\Delta\nu$ reported here for the DMSA complexes stays essentially constant at $\sim 180\,cm^{-1}$ for all the metal complexes examined. This relatively small value indicates that there is no strong interaction between the carboxylates and the tervalent Bi^{3+} ion. This is consistent with CPK molecular models, which indicate that it is difficult to achieve simultaneous coordination of both thiol and carboxylate groups in mononuclear DMSA complexes.

The DMSA carboxylates could be coordinated in the 220 zinc dimer. However, the IR data are inconclusive. The range of stretching frequencies for coordinated and deprotonated carboxylates overlap for divalent metal ions such as Zn.[34] Detailed comparisons between free DMSA and the zinc complex are difficult because the carboxylate and thiolate frequencies of the disodium salt of DMSA appear to be perturbed by hydrogen bonding between these two groups (W.R. Harris, unpublished results).

Jones *et al.*[30] measured Zn–DMSA binding constants and also report a series of dimeric species. Similarly, dimers have recently been proposed as the dominant Zn complexes with thiomalic acid.[35] Such species were not reported in earlier studies on DMSA[27–29] and thiomalic acid,[28] but this can be attributed to the simpler computational methods available prior to the widespread access to relatively powerful computers.

Jones *et al.*[30] based their calculations of zinc DMSA binding constants on an assumed value of 12.0 for log β_{011}. The measured value in this study is log β_{011} = 11.50. If this difference in the first ligand protonation constant is taken into account, the values for β_{220} and β_{221} reported by Jones *et al.* are very similar to those reported here. The only significant variation between the two studies is the inclusion of a 222 species by Jones *et al.* compared to a 111 species in this study. The titration data from this study have been evaluated using the Jones model of 222, 221, and 222 species. The fits are very similar to those obtained using the 222, 221, and 111 set of species. However, there is a slightly better fit using the 111 species in place of 222. Putting both species in the model does not lead to any further improvement in the fit.

It was not possible to measure the deprotonation constant for the 111 species at

the mM concentrations of reactants needed for the potentiometric titrations because the complex dimerizes prior to deprotonation. If one assumes that the 110 species is unlikely to account for more than 10% of the zinc without being detectable in the refinement of the potentiometric data, this places a lower limit of $10^{5.5}$ on the protonation constant of the 110 species and an upper limit $10^{14.6}$ on β_{110}. At lower concentrations, where the dimer is destabilized by the squared dependence on both metal and ligand concentrations, one might expect the accumulation of the 110 species at higher pH. Speciation calculations similar to those shown in Figure 5 were repeated including a 110 species with $\log \beta_{110} = 14.6$. At the 2 mM concentrations of zinc and DMSA representative of the titration solutions, the 110 species accounts for less than 10% of total zinc. However, when the concentrations of Zn and DMSA are both set to 2 μM, the 110 species accounts for 90% of the zinc at pH 7–8.

Although this hypothetical 110 species may be a primary zinc species under dilute conditions, speciation calculations indicate that a zinc 110 complex with a $\log \beta_{110}$ of 14.6 would have relatively little effect on the competition between zinc and lead under physiological conditions. When the 110 species is inserted in the speciation calculations, the marginal increase in zinc-binding affinity lowers the lead pM value only from 12.05 to 11.88. Thus the uncertainties surrounding the 110 species do not weaken the conclusion that DMSA is selective for lead at physiological pH.

We had not expected zinc to be a major competitor to Bi^{3+} for binding to DMSA. However, Bi^{3+} also has very large hydrolysis constants, and it was possible that competition from Zn would be sufficient to promote the formation of $Bi(OH)_3$. However, the speciation calculations, although still at a very crude level for bismuth solutions, indicate that DMSA is likely to retain nM concentrations of Bi at physiological pH.

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