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Thermodynamics of the As(III)–Thiol Interaction: Arsenite and Monomethylarsenite Complexes with Glutathione, Dihydrolipoic Acid, and Other Thiol Ligands

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Colorimetric (near-UV absorption spectroscopy) and calorimetric (isothermal titration calorimetry) methods have been used to quantify the equilibrium and thermodynamics of arsenite and monomethylarsenite (MMA) coordinating to glutathione (GSH) and the dithiols dimercaptosuccinic acid (DMSA), dihydrolipoic acid (DHLA), and dithiothreitol (DTT). We found that both arsenite and MMA form moderately stable complexes ($\beta = 10^6$–10^7) with GSH; that arsenite forms a particularly stable 2:3 complex ($\beta \approx 10^{18}$) with the biological cofactor DHLA; that MMA has a somewhat higher affinity than arsenite for thiol ligands; and that entropic factors modulate the overall stability of As(III) complexes with thiols, which are favored by the exothermic formation of As(III)–thiolate bonds. The implications of these results for arsenic toxicity are discussed.

Introduction

Human exposure to arsenic in drinking water is a major public health problem in parts of Asia, Africa, and South America, as well as certain regions of the U.S. with arsenic rich aquifers. Populations in these locations, as well as those exposed to anthropogenic sources of arsenic pollution, have a higher risk for skin lesions; peripheral neuropathy; altered heme metabolism; diabetes; and cancer of the skin, liver, lung, kidney, and bladder.[1] This has led the U.S. Agency for Toxic Substances and Disease Registry to rank arsenic, which is found at 1014 of the 1598 U.S. EPA national priority sites, first on a list of 50 chemicals of most concern for human health.[2] However, the biochemical basis for the correlation between chronic consumption of low to moderate levels (1−100 ppb) of arsenic and human disease is not well understood. Thus, there is a need for further investigation of the toxicity of arsenic and its fundamental biological chemistry.

The two environmentally and biologically relevant forms of arsenic in neutral aqueous solution are arsenate [As(V)], $HAsO_4^{-2}$, and arsenite [As(III)], $As(OH)_3$.[3] Arsenate predominates under aerobic conditions, is imported into cells by the anion (phosphate, sulfate) transport pathway, and disrupts cellular processes as a phosphate mimic, but is reduced to arsenite within cells. Arsenite, which typically forms three-coordinate trigonal-pyramidal complexes or, less commonly, tetrahedral complexes, is more toxic than arsenate, and is brought into cells by aqua-glyceroporins that transport water, glycerol, urea, and other small neutral molecules.[4] Arsenite has a high affinity for sulfur-containing ligands and has been shown to disrupt the activity of certain enzymes such as pyruvate dehydrogenase,[5,6] glutathione reductase,[7] and thioredoxin reductase.[8]

Arsenic is metabolized to mono- and dimethylated species, which are detected in the urine of individuals exposed to elevated levels of arsenic.[9] These species are formed through an oxidative addition mechanism involving rate-limiting arsonate/methylarsonate reductases and methyl transfer from S-adenosylmethionine or methylcobalamin, catalyzed by arsenic methyltransferases.[10] This was initially thought to

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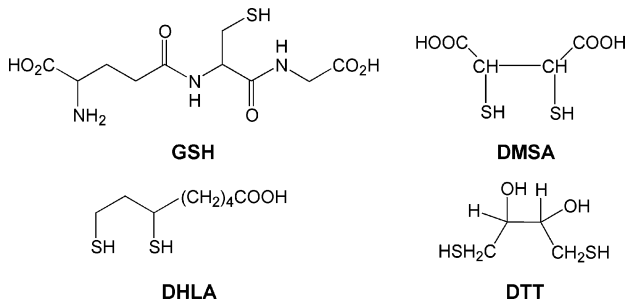
be a detoxification pathway, but recent cell-culture[11−13] and animal[14] toxicity studies suggest that these methylated species, particularly monomethylarsenite (MMA), $(H_3C)As(OH)_2$, are more toxic than arsenite itself.

Two different mechanisms have been found for arsenic detoxification.[15] Certain bacteria and yeast have an *ars* operon, which encodes for proteins that reduce arsenate and export arsenite. Arsenic resistance in eukaryotes is conferred by MRP (multidrug resistance protein) members of the ABC superfamily of transport ATPases,[16] which export arsenite as its glutathione (GSH) complex from cells,[17] including human liver cells that excrete arsenic into the bile.[18]

The toxicity of arsenite can be broadly associated with its affinity for soft ligands, such as Cys residues in proteins, but few studies have quantified As(III) solution chemistry with thiols. Arsenite forms a well-characterized 1:3 complex with the Cys-containing tripeptide GSH, which provides tris-thiolate coordination for the As(III),[19−21] and a recent study reported an unusually high stability constant for this complex.[22] Earlier, the stabilities of arsenite complexes with the common reductant dithiothreitol (DTT) and other dithiols were determined,[23] and it has been shown that the dithiol-chelating agent dimercaptosuccinic acid (DMSA) will displace GSH from its 1:3 complex with As(III).[20]

In this study, we have used colorimetric (near-UV absorption spectroscopy) and calorimetric (isothermal titration calorimetry) methods to quantify the stability and thermodynamics of formation of arsenite and MMA complexes with GSH and the dithiol ligands DMSA, DTT, and dihydrolipoic acid (DHLA), which is an essential biological cofactor (Chart 1). We find that neither arsenite nor MMA forms unusually stable complexes with GSH; that arsenite and DHLA do form a particularly stable 2:3 complex; that MMA forms complexes with thiols that are somewhat more stable than those of arsenite; and that entropic factors modify the overall stability of As(III) complexes with thiols, which are favored by the exothermic formation of As(III)−thiolate bonds.

Chart 1



Experimental Section

Reagents. All reagents were ≥99% pure and used as received. Reduced L-glutathione (GSH, γ-GluCysGly), sodium meta-arsenite $(NaAsO_2)_n$, D,L-dithiothreitol (DTT), meso-2,3-dimercaptosuccinic acid (DMSA), and D,L-6,8-thioctic acid (lipoic acid, LA) were purchased from Sigma (St Louis, MO). Disodium methylarsonate, $Na_2[(H_3C)AsO_3]$, was purchased from ChemService (West Chester, PA).

Hazard: All arsenic compounds are toxic and should be handled with caution; in particular, methyldiiodoarsonous acid has an appreciable volatility under laboratory conditions, and all manipulations should be done with adequate ventilation. Methyldiiodoarsonous acid, $(H_3C)AsI_2$, which forms monomethyl arsonous acid [also known as monomethylarsenite (MMA)] $(H_3C)As(OH)_2$, upon dissolution in aqueous solution, was synthesized according to a modification of the procedure of Chouchane and Snow.[24] $Na_2[(H_3C)AsO_3]$ (13 g) was dissolved in 15.5 mL of warm water, and ca. 5 mL of concentrated HCl was added to lower the pH to ∼3. After 12 h, 7.7 g of NaI that had been dissolved in 7.3 mL of water was added over a period of 1.5 h, as SO_2 was bubbled through the solution. Subsequently, the solution was alternately cooled and warmed until there was complete formation of a yellow-orange oil, which was transferred to a small beaker and from which a light yellow solid precipitated upon washing with ice-cold water three times to remove excess reagents. The somewhat volatile solid was dried over desiccant in a sealed desiccator, and the final yield was 4 g (∼30%). ICP-MS analysis of a ∼30 ppm solution of the product contained 77 ± 1 μM As and 163 ± 9 μM I, which is consistent with the formula $(H_3C)AsI_2{\cdot}1.4H_2O$ (expected values are 76 and 163 μM, respectively). A 300-MHz ^{1}H NMR spectrum of the product dissolved in 50 mM deuterated phosphate buffer (pD 7.1) had a single resonance at 1.24 ppm, which is the literature value for MMA, and no signal for $(H_3C)AsO_3H^{-1}$, the aqueous solution form of the starting As(V) species, at 1.39 ppm.[24]

Dihydrolipoic acid (DHLA) was prepared from oxidized lipoic acid (LA) by a slight modification of the procedure of Walton et al.[25] LA (0.5 g) was dissolved in 10 mL of a 50% aqueous ethanol solution, and the mixture was then deoxygenated with an Ar purge and cooled to 0 °C. $NaBH_4$ (1.0 g) was added in several aliquots, and the solution was then stirred for 4 h. Subsequently, 25 mL of deoxygenated water was added, and the solution was acidified with concentrated HCl until the evolution of gas subsided. The product was extracted with deoxygenated dichloromethane and was then washed three times with deoxygenated water. Solvent was removed under reduced pressure, and the product was stored under an Ar atmosphere. Quantitation of the signature LA absorption at 333 nm (ϵ = 150 $M^{-1}cm^{-1}$) indicated >99% DHLA.

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Methods. Near-UV absorption measurements of titrations of arsenite and MMA with thiols were made in the 190−380 nm range on a Perkin-Elmer λ-9 or Hitachi U3000 spectrophotometer. All titrations were performed under an anaerobic atmosphere in 20 mM HEPES (pH 7.4) buffer solutions with 0.10 M NaCl at 25 °C for DTT, DMSA, and DHLA, but at 37 °C for GSH. Titration spectra are presented as solid and dashed lines for increasing and decreasing intensity, respectively, in the 270−290 nm region. Although the energy of the broad As(III)−thiolate absorption varies somewhat among the complexes, inset plots of intensity versus stoichiometry are all given for the representative wavelength of 270 nm. Titration data were analyzed using BioLogics Specfit software Version 3.0.35, and the reported binding constants are the average of at least two best fits of titrations in the forward (As → thiol) and reverse (thiol → As) directions, in most cases.

Isothermal titration calorimetry (ITC) measurements were carried out at 25 or 37 (±2) °C, as described previously,[26] with either a MicroCal MCS or VP MicroCal titration microcalorimeter, each of which is enclosed in its own custom plexiglass glovebox, which allowed all measurements to be made under an inert atmosphere. Sample and titrant solutions were prepared from the same 20 mM HEPES pH 7.4 buffer solution with 0.10 M NaCl, which had been thoroughly deoxygenated with Ar and stored under an inert atmosphere. The concentration of thiol used in ITC measurements was determined by a free-thiol assay[27] using 2,2′-dithiodipyridine and measuring the absorption intensity of the resulting 2-thiopyridine at 343 nm, using a molar extinction coefficient (ϵ = 8338 M^{-1} cm^{-1}) determined under the same experimental conditions (20 mM HEPES buffer, pH 7.4, 0.10 M NaCl). Titrations involving DHLA required 4% ethanol in both the cell and the syringe to maintain solubility. ITC results are presented by showing the baseline-adjusted experimental titration data (heat flow versus time) on the top and the peak-integrated, background- (dilution-) subtracted, concentration-normalized molar heat flow per aliquot versus the titrant-to-sample molar ratio on the bottom. ITC data were analyzed with either a one-site or a sequential-binding model using the OriginLab's Origin software package Version 7.0. Best-fit values (K; ΔH; and, where appropriate, N) are reported as average values for a statistically significant number of individual titrations or values that give an internally consistent fit to both forward and reverse titrations under optimized experimental conditions.

Results and Analysis

Colorimetric Data. Thiol coordination to As(III) results in new charge-transfer (CT) electronic transitions in the near-UV range (250−320 nm) that can be monitored to determine the stability of As(III)−thiolate complexes. Titrations of arsenite or monomethylarsenite (MMA) into buffered solutions of thiols and reverse titrations of thiols into buffered solutions of arsenite or MMA were performed under anaerobic conditions. Near-UV absorption spectra of the titration solutions were then fit with Specfit multicomponent spectral analysis software to determine stability constants for the species that are formed.

(i) Arsenite. Figure 1 shows the spectral data for a titration of arsenite into a buffered pH 7.4 solution of reduced glutathione (GSH) (arsenite → GSH titration) and a plot of the absorbance at 270 nm versus the As/thiol ratio (inset).

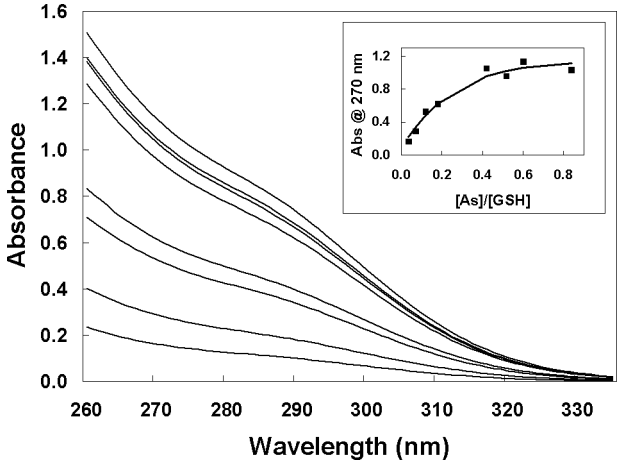

Figure 1. Near-UV absorption spectra of 5.0 mM GSH solutions with increasing concentrations of arsenite after incubation for 3 h at 37 °C. The inset shows a plot of A^{270} versus the As/GSH ratio for these samples and the A^{270} value from the best fit of this spectral data (line) using a sequential-binding model (see text) and values in Table 1.

Because the ligand substitution reaction is slow, solutions were incubated at 37 °C for 3 h after addition of each aliquot to ensure that equilibrium had been achieved. These spectral data can be fit to a simple model that includes only formation of the expected 1:3 complex and gives a stability constant of 1×10^8. However, although the 1:3 complex should be the predominant As(III) species early in the titration when there is an excess of GHS, a 1:1 complex is expected to become the major As(III) species as the titration continues toward an excess of arsenite. These, and related ITC data (vide infra), suggest an alternate sequential-binding model that assumes an initial 1:3 complex, followed by a 1:2 complex, and finally a 1:1 complex. Using near-UV absorption spectra for the 1:1 and 1:3 complexes (obtained under conditions of excess arsenite and excess GSH, respectively) as fixed input spectra and stability constants from the best fit of arsenite + GSH ITC data (vide infra) as initial parameters, the best-fit stability constants for these three species were determined (Table 1).[28]

The broad CT absorption from the interaction of arsenite with the dithiol dimercaptosuccinic acid (DMSA) was used to quantify the stability of As−DMSA species. Figure 2 shows representative near-UV absorption spectra for arsenite → DMSA (forward) and DMSA → arsenite (reverse) titrations, as well as the dependence of A^{270} on the As/DMSA and DMSA/As ratios, respectively (insets). The former titration has maximum absorption at As/DMSA = 0.5, and the latter titration has maximum absorption at DMSA/As = 2, both of which indicate the formation of a 1:2 complex. Both titrations also show evidence (insets in Figure 2) for a 1:1 complex, which would have bis-thiolate As(III) coordination and a lower molar absorption. The large drop in A^{270} at higher As/DMSA ratios in the arsenite → DMSA titration cannot be explained by dilution, and the sigmoidal behavior of A^{270} early in the DMSA → arsenite titration suggests a

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(28) Similar parameters give a good fit to near-UV absorption spectra of GSH → arsenite titrations (Supporting Information), but the fit is poor at larger excess of GSH, which might be due to spectral contributions from an increasing amount of the GSH thiolate anion.

Table 1. Stability Constants Obtained from Best Fits of Near-UV Spectral Titrations[a]

	$As(OH)_3$	MMA
GSH[b]	1:1 → 1:2 → 1:3 $K_1 = 20$ $K_2 = 430$ $K_3 = 1.2 \times 10^3$ $\beta_3 = 1.0\ (\pm 0.2) \times 10^7$	1:1 → 1:2 $K_1 = 1.6 \times 10^4$ $K_2 = 1.9 \times 10^3$ $\beta_2 = 2.3\ (\pm 0.5) \times 10^7$
DMSA[c]	1:1 → 1:2 $K_1 = 3.0 \times 10^4$ $K_2 = 1.6 \times 10^5$ $\beta_2 = 5\ (\pm 2) \times 10^9$	1:1 $K = 2.7\ (\pm 0.2) \times 10^5$
DHLA[c]	2:3 $\beta_{2:3} = 4\ (\pm 3) \times 10^{18}$	1:1 $K = 3.2\ (\pm 0.3) \times 10^6$
DTT[c]	1:1 $K = 1.1\ (\pm 0.5) \times 10^6$	1:1 $K = 2.0\ (\pm 0.8) \times 10^6$

[a] K_i represents the individual stepwise stability constants for coordination of the ith thiol to the As(III) species; β_i represents the overall stability constants for the As(III) complex with i thiols. [b] 37 °C. [c] 25 °C.

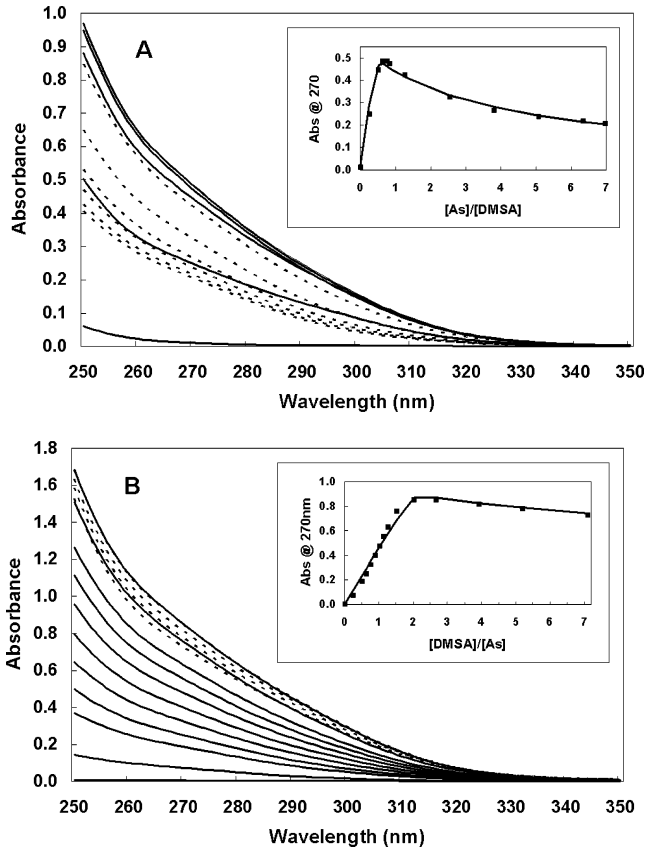

Figure 2. Near-UV absorption spectra of representative (A) arsenite → DMSA (0.40 mM) and (B) DMSA → arsenite (0.40 mM) titrations, with solid and dashed lines for spectra where intensity is increasing and decreasing, respectively, upon addition of titrant. Insets show plots and fits as described for Figure 1.

shift from a lower-absorbing 1:1 complex at low DMSA/As ratios to a higher-absorbing 1:2 complex as the ratio increases. Both titrations can be fit to a sequential-binding model (initially 1:2 followed by 1:1 for arsenite → DMSA and initially 1:1 followed by 1:2 for DMSA → arsenite). However, stability constants for the 1:1 and 1:2 species in Table 1 are reported only from fits of arsenite → DMSA titrations because of the inability of Specfit analysis to capture the early sigmoidal behavior in the reverse titration.

Arsenite titrations with the dithiol dihydrolipoic acid (DHLA) provide near-UV spectral evidence for the formation of only a single species. Analysis of both arsenite → DHLA and DHLA → arsenite titrations (Supporting Information), as well as ITC data (vide infra), indicates that this complex has a stoichiometry of 2:3, which achieves the expected tris-thiolate As(III) coordination, and a stability constant of $(4 \pm 3) \times 10^{18}$.[29]

Both forward and reverse titrations of arsenite with the dithiol dithiothreitol (DTT) provide near-UV spectral data (Supporting Information) for the formation of only a 1:1 species, which would have bis-thiolate As(III) coordination. Analysis of these titrations provides a stability constant of $(1.1 \pm 0.5) \times 10^6$ for this species.

(ii) MMA. Titrations of the As(III) species monomethylarsenite (MMA) with GSH in both the forward and reverse directions provide near-UV spectral data (Supporting Information) that are qualitatively similar, particularly with regard to stoichiometry, to those observed for arsenite titrations with DMSA (Figure 2). These titration data were fit to give stability constants for the 1:1 and 1:2 complexes of MMA with GSH (Table 1).

Titrations of MMA with the dithiols DMSA, DHLA, and DTT in both the forward and reverse directions provide near-UV spectral data (Supporting Information) for the formation of 1:1 complexes with the expected bis-thiolate coordination. Analysis of these data gives best-fit values of $(2.7 \pm 0.2) \times 10^5$, $(3.2 \pm 0.3) \times 10^6$, and $(2.0 \pm 0.8) \times 10^6$ for stability constants of the MMA complex with DMSA, DHLA, and DTT, respectively.

Calorimetric Data. Isothermal titration calorimetry (ITC) was used to measure the heat associated with anaerobic titrations of arsenite or MMA with GSH, DMSA, DHLA, and DTT in buffered pH 7.4 solution. The titration data were then fit to one-site, independent two-site, or sequential-binding models with Origin software to determine best-fit values for the stability constants, K_{ITC}, and the reaction enthalpies, ΔH_{ITC}, which were then used in a thermodynamic analysis of the formation of As(III)−thiolate complexes.

(i) Arsenite. Figure 3 shows 37 °C ITC data for forward and reverse titrations of arsenite and GSH. Because these reactions are slow,[30] the heats of dilution are large, and the stability constants are small, relatively high concentrations were required to obtain data that could be fit accurately. As with the corresponding optical titration (Figure 1), ITC data for the arsenite → GSH titration (Figure 3A) could be interpreted as formation of a single low-stability species. However, fitting these data to a one-site model yielded an unreasonably low value (~0.1) for the stoichiometry of the complex. Further, the GSH → arsenite ITC data (Figure 3B) indicated an initial event with low heat early in the titration when the concentration of GSH is less than that of As(III),

(29) This $\beta_{2:3}$ value corresponds to a clear minimum in the sum of squares residuals in the Specfit analysis of this titration data.

(30) Rapid continuous stirring of the ITC cell led to equilibration of these samples between injections, as indicated by the return to baseline and the absence of baseline drift over the course of As(III) → GSH titrations.

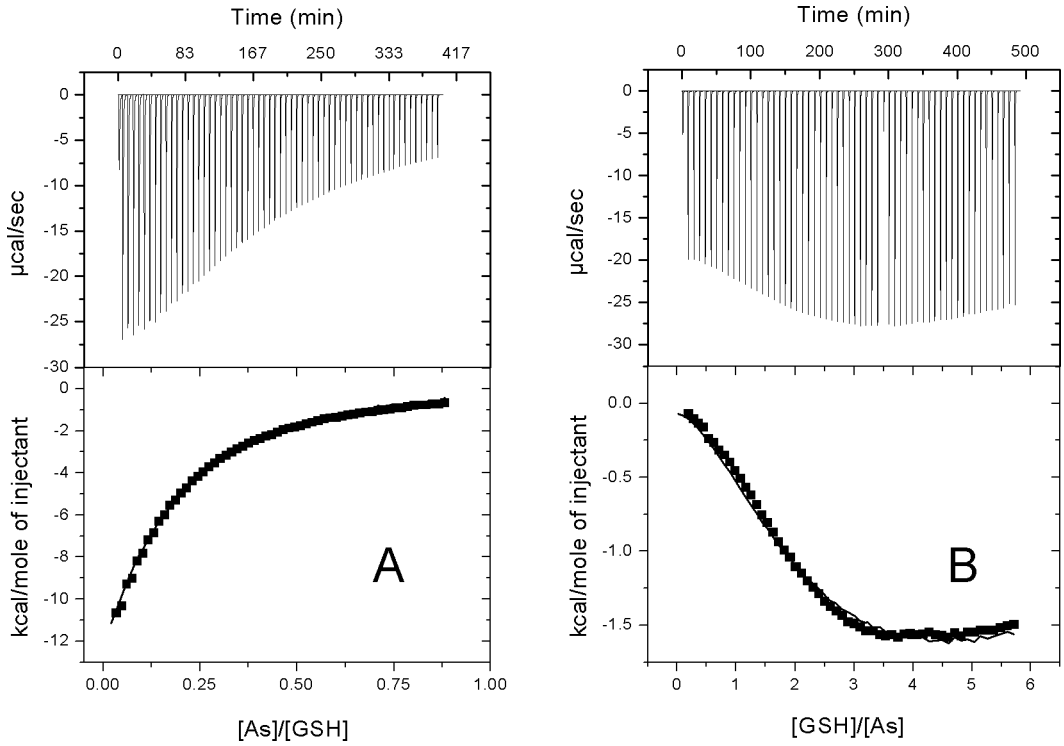

Figure 3. ITC data of (A) arsenite → GSH (7 mM) and (B) GSH → arsenite (1.5 mM) titrations, with best fits to a sequential-binding model using parameters in Table 2.

followed by a second event with greater heat when the concentration of GSH is greater than that of As(III).[31] This suggests the sequential formation of a 1:1 complex, presumably a 1:2 complex, and finally a 1:3 complex in the GSH → arsenite titration; conversely, sequential formation of 1:3, 1:2, and 1:1 complexes is expected for the arsenite → GSH titration. Although independent determination of the stability constants and heats of formation for these three species was not possible, injection of an aliquot of arsenite into a large excess of GSH at 37 °C reproducibly gives $\Delta H_{ITC} = -38.6$ kcal/mol for the overall heat of formation of the 1:3 complex. Constraining the sum of the ΔH parameters for the sequential formation of the three species to this value, iterative fitting with a sequential-binding model converged to a unique set of values that give excellent fits to both the forward and reverse ITC titrations (Table 2).[32]

In parallel with the Figure 2 optical titrations, Figure 4 shows representative ITC data for forward and reverse titrations of arsenite and DMSA. Whereas the arsenite → DMSA titration has only a single inflection at As/DMSA = 0.5, indicating formation of a 1:2 complex, the DMSA → arsenite titration has an initial exothermic event followed by an even more exothermic event with an inflection at DMSA/As = 2. This latter titration indicates initial formation of a 1:1 complex, followed by formation of a 1:2 complex, which is consistent with the optical titration data. As with the arsenite + GSH ITC data, iterative fitting with a sequential-binding model converged to a unique set of parameters that gives excellent fits to both the forward and reverse ITC titrations (Table 2).

Table 2. Best-Fit Values from ITC Measurements at 37 °C[a]

	$As(OH)_3$	MMA
GSH	$K_1 = 20$ $\Delta H_1 = -2.5$ kcal/mol $K_2 = 430$ $\Delta H_2 = -3.1$ kcal/mol $K_3 = 200$ $\Delta H_3 = -33.1$ kcal/mol	$K_1 = 3.1 \times 10^3$ $\Delta H_1 = -5.2$ kcal/mol $K_2 = 4.3 \times 10^3$ $\Delta H_2 = -12.6$ kcal/mol
DMSA	$K_1 = 5 \times 10^4$ $\Delta H_1 = -9.8$ kcal/mol $K_2 = 1.7 \times 10^4$ $\Delta H_2 = -17.5$ kcal/mol	$N = 1.01 \pm 0.01$ $K = 1.0 (\pm 0.3) \times 10^7$ $\Delta H = -13.2 \pm 0.2$ kcal/mol
DHLA	$N = 1.59 \pm 0.02$ $K = 2.1 (\pm 0.2) \times 10^5$ $\Delta H = -14.2 \pm 0.2$ kcal/mol	$N = 1.01 \pm 0.01$ $K = 1.1 (\pm 0.3) \times 10^7$ $\Delta H = -17.0 \pm 0.2$ kcal/mol
DTT	$N = 1.18$ $K = 9.5 \times 10^5$ $\Delta H = -13.7$ kcal/mol	$N = 1.06$ $K = 8.2 \times 10^5$ $\Delta H = -15.9$ kcal/mol

[a] K_i and ΔH_i are the individual stepwise stability constants and enthalpies, respectively, for the *i*th thiol coordinating to the As(III) species; N is the thiol/As(III) stoichiometry ratio.

ITC data for the interaction of arsenite with the dithiols DHLA and DTT in both the forward and reverse directions (Supporting Information) revealed single coordination events with a 2:3 stoichiometry for DHLA and a 1:1 stoichiometry for DTT, as found with the optical titrations. Best fits of these ITC data to a one-site model provide stability constants and enthalpies of formation for these species (Table 2).

(ii) MMA. Both forward and reverse ITC data for the interaction of MMA and GSH (Supporting Information) are qualitatively similar to those for arsenite and DMSA (Figure 4). A sequential-binding model involving initial formation of a 1:2 complex followed by a 1:1 complex was used in

(31) The GSH → arsenite ITC data eventually return to a smaller heat of dilution at higher molar ratios, but there is some discrepancy with the fit, presumably because of the large deviation from ideal solution behavior at high titrant (solute) concentrations.

(32) When K is small, there is a larger uncertainty in the corresponding value of ΔH, which is particularly true for the intermediate 1:2 species.

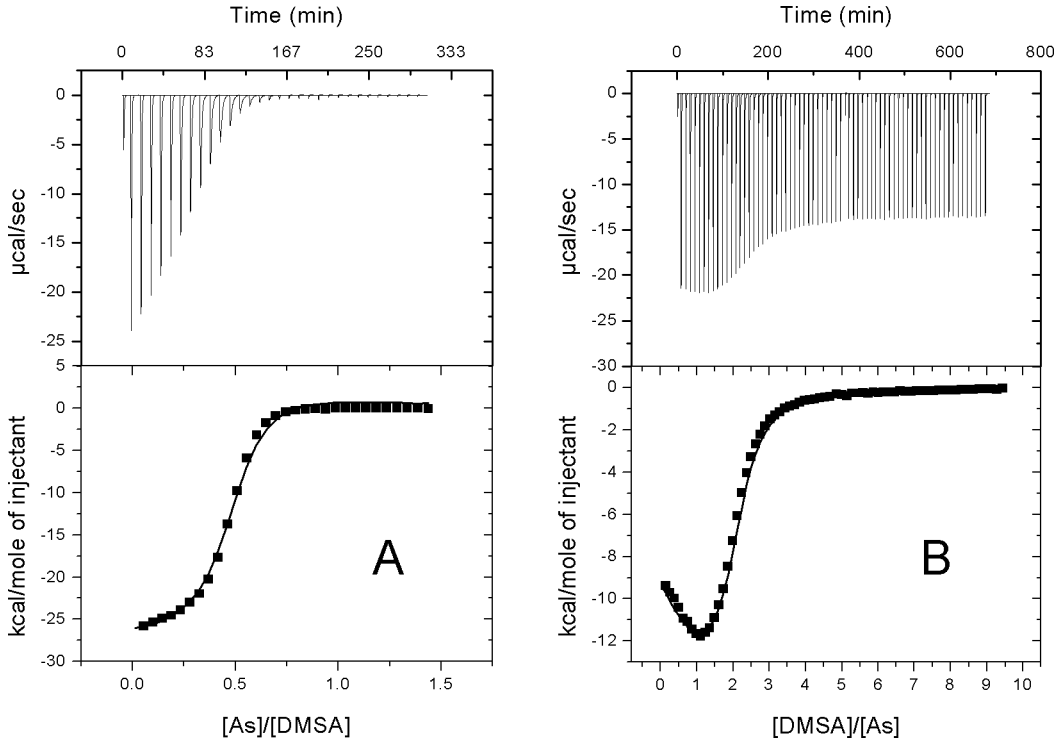

Figure 4. ITC data of (A) arsenite → DMSA (0.8 mM) and (B) DMSA → arsenite (0.4 mM) titrations, with best fits to a sequential-binding model using parameters in Table 2.

fitting the MMA → GSH data to obtain best-fit parameters (Table 2), which also give a good fit to the GSH → MMA data.

ITC data for the interaction of MMA with the dithiols DMSA, DHLA, and DTT in both the forward and reverse directions (Supporting Information) all indicated a single coordination event at a stoichiometry of 1:1. These data were all fit to a one-site model for the formation of MMA−dithiolate complexes, with the best-fit parameters reported in Table 2.

Thermodynamics. Under ITC experimental conditions (20 mM HEPES, pH 7.4, 0.10 M NaCl, 37 °C), arsenite exists as $As(OH)_3$, MMA exists as $(H_3C)As(OH)_2$, the thiols are all protonated, and there is no direct interaction of these species with the buffer. Thiol coordination leads to net formation of an As(III)−thiolate bond and release of water, from deprotonation of the thiol and displacement of a hydroxide ligand (eq 1).

$$As^{III}-OH + RSH \rightleftarrows As^{III}-SR + H_2O \qquad (1)$$

Thus, additional proton equilibria are not expected, and this was confirmed by the identical ΔH values for arsenite + DTT at pH 7.4 in 20 mM HEPES and in 20 mM phosphate, two buffers with significantly different heats of protonation. Therefore, the best-fit K values (Table 2) and their corresponding values of ΔG are directly comparable to the best-fit ΔH values (Table 2), allowing direct calculation of ΔS for these reactions.

Table 3 reports the overall stability constants determined by ITC and the thermodynamic parameters for the formation of $As(GSH)_3^{-3}$, $As(DMSA)_2^{-4}$, $As_2(DHLA)_3^{-3}$ and As(DTT) from arsenite and the thiol and for the formation of $(H_3C)As(GSH)_2^{-2}$, $(H_3C)As(DMSA)^{-2}$, $(H_3C)As(DHLA)^{-1}$, and $(H_3C)As(DTT)$ from MMA and the thiol.

Table 3. Thermodynamic Properties for the Formation of As(III)−Thiolate Complexes

	$As(OH)_3$	MMA
GSH	1:3 $\beta_3 = 1.8 \times 10^6$ $\Delta G = -8.8$ kcal/mol $\Delta H = -38.7$ kcal/mol $\Delta S = -96$ cal/(mol·K)	1:2 $\beta_2 = 1.3 \times 10^7$ $\Delta G = -10.1$ kcal/mol $\Delta H = -17.8$ kcal/mol $\Delta S = -25$ cal/(mol·K)
DMSA (5)[a]	1:2 $\beta_2 = 8.3 \times 10^8$ $\Delta G = -12.7$ kcal/mol $\Delta H = -27.3$ kcal/mol $\Delta S = -47$ cal/(mol·K)	1:1 $K = 1.0 \times 10^7$ $\Delta G = -9.9$ kcal/mol $\Delta H = -13.2$ kcal/mol $\Delta S = -9$ cal/(mol·K)
DHLA (6)[a]	2:3 $\beta_{2:3} = 4 \times 10^{18}$ [b] $\Delta G = -25$ kcal/mol $\Delta H = -43$ kcal/mol[c] $\Delta S = -59$ cal/(mol·K)	1:1 $K = 1.1 \times 10^7$ $\Delta G = -10.0$ kcal/mol $\Delta H = -17.0$ kcal/mol $\Delta S = -20$ cal/(mol·K)
DTT (7)[a]	1:1 $K = 9.5 \times 10^5$ $\Delta G = -8.5$ kcal/mol $\Delta H = -13.7$ kcal/mol $\Delta S = -17$ cal/(mol·K)	1:1 $K = 8.2 \times 10^5$ $\Delta G = -8.4$ kcal/mol $\Delta H = -15.9$ kcal/mol $\Delta S = -24$ cal/(mol·K)

[a] Size of ring formed upon bidentate bis-thiolate coordination to As(III). [b] Value from Specfit analysis of near-UV absorption data. [c] See text.

Discussion

The toxicity of arsenic at low chronic exposure is a public health problem in many countries and in certain regions of the U.S. However, the biochemistry that underlies increased incidence of diseases, such as diabetes and cancer, in these populations is still poorly understood. Arsenite [As(III)],

which is found in certain aquifers used for drinking water and is more toxic than arsenate [As(V)], has a high affinity for sulfur ligands. Although arsenite binding to sulfur-containing sites on biological molecules might be the chemical basis for its toxicity, there is little quantitative evidence to support this hypothesis. Therefore, we have quantified the interaction of arsenite and its in vivo As(III) metabolite monomethylarsenite (MMA) with thiol ligands, including two that are biologically important − glutathione (GSH) and dihydrolipoic acid (DHLA).

(i) This Study. Two very different physical methods were used to quantify thiol binding to As(III): near-UV absorption spectroscopy and isothermal titration calorimetry (ITC). The former measures an electronic property associated with the As(III)−thiolate bond (charge-transfer transitions), and absorption intensity is directly proportional to the amount of complex that is formed. Although As(III)−thiolate CT transitions are broad and generally not useful for distinguishing different species during a titration, analysis of the spectra with Specfit and different stoichiometric models allows stability constants to be determined for these complexes. ITC, on the other hand, measures a thermodynamic property (heat flow, which equals ΔH under isobaric conditions) that is also directly proportional to complex formation. Fitting calorimetric titrations to an appropriate model (one-site, sequential, competition) with Origin also provides stability constants, as well as reaction stoichiometry and enthalpy of complex formation for thermodynamic analysis.

Because the near-UV absorption spectra and ITC data were obtained with identical solutions (20 mM HEPES, pH 7.4, 0.10 M NaCl), the stability constants in Tables 1 and 2 should be directly comparable for arsenite and GSH, where all measurements were made at 37 °C, and similar for other complexes, where there was a small temperature difference (25 versus 37 °C). For most cases, this is true, but the exceptions require further consideration. Greater uncertainty is unavoidable, particularly with near-UV absorption data and Specfit analysis, for situations in which a sequential formation of As(III)−thiolate complexes occurs (arsenite + GSH, arsenite + DMSA, MMA + GSH). A larger error is also associated with optical titrations involving addition of MMA because of the intrinsic near-UV absorption of this As(III) compound and its increasing contribution to the titration spectra. This was particularly true with DMSA, leading to higher confidence in the ITC value (Table 2) for the MMA−DMSA stability constant.

The most glaring discrepancy is the large difference between the two stability constants for the arsenite−DHLA complex, 4×10^{18} from optical data and 2×10^{5} from ITC data. Both methods indicate a 2:3 stoichiometry, and neither gives any evidence for intermediate species in the titrations. Specfit analysis provides the overall stability of this 2:3 species from the near-UV titration data, based on a model that allows any metal-to-ligand stoichiometry. Origin has a limited number of currently available models, and analysis of the ITC data was based on a one-site binding model that does not accurately account for the 2:3 stoichiometry. Thus, only Specfit analysis of the optical titration data employs an appropriate model and accurately determines the stability and free energy of formation of this 2:3 species. The ITC data, though, accurately measure the change in enthalpy per DHLA [DHLA → As(III) titrations] and per As(III) [As(III) → DHLA titrations]. The enthalpy of formation of the 2:3 complex (Table 3) is then found as 3 times the former value (−43 kcal/mol) or 2 times the latter (approximately −44 kcal/mol).

(ii) Comparison to Other Results. Few values for the stability constants of As(III) complexes with thiols have been reported. A recent study used potentiometry, NMR spectroscopy, and near-UV absorption to investigate cysteine and GSH complexes of arsenite, and it contains a near-UV titration similar to that in Figure 1.[22] However, the model used to analyze these data includes only formation of the 1:3 arsenite complex with GSH, and values of 2×10^{33} (near-UV absorption) and 1×10^{32} (potentiometry) are reported for its stability constant. In contrast, our ITC data clearly indicate multiple titration species that must be included in a sequential-binding model to achieve an internally consistent set of parameters that fit both forward and reverse ITC and optical titrations. Our data are qualitatively and quantitatively inconsistent with the large stability constants reported by Rey et al.,[22] and we believe that our ITC value of $\beta_3 = 2 \times 10^6$ is an accurate stability constant for $As(GSH)_3^{-3}$.

Zahler and Cleland quantified the interaction of arsenite with several dithiols, using a procedure involving a free-thiol chemical assay.[23] Considering the very different method and conditions that they used, their value of 3×10^6 for the stability constant of the arsenite−DTT complex is remarkably close to our value of 1×10^6, obtained using two other methods.

Delnomdedieu et al. used NMR spectroscopy to investigate the competition between GSH and dithiols for arsenite.[20,21] The transfer of arsenite from a 1:3 complex with GSH to a complex with DMSA, reported as $As_2(DMSA)_3$, was observed by NMR spectroscopy. Although we find evidence for a 1:2, and not a 2:3, arsenite complex with DMSA, our stability constants for $As(GSH)_3^{-3}$ and $As(DMSA)_2^{-4}$ are consistent with the observation that DMSA displaces GSH from its complex with arsenite. This conclusion is consistent with the effective use of DMSA in chelation therapy for arsenic poisioning.[33]

(iii) Structure and Bonding. Most of the complexes whose formation was quantified by optical and ITC titrations achieve the expected three As(III)−thiolate bonds for arsenite[34] and two As(III)−thiolate bonds for MMA. Titration stoichiometry indicates formation of a 1:2 arsenite complex with DMSA, which would have tris-thiolate coordination with one bidentate DMSA and one monodentate DMSA. Although a tetrahedral tetra-thiolate As(III) complex with two bidentate DMSA ligands cannot be ruled out by these data, EXAFS results are consistent with tris-thiolate As(III) coordination in its complex with DMSA.[35] The 1:1 arsenite

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complex with DTT has only bis-thiolate coordination, but the X-ray structure of this complex[36] reveals that one of the alcohol groups also coordinates to the As(III), making this a tridentate ligand.

The dithiols used in this study form a series with a stepwise change in the separation between the two thiols (bidentate bite angle), which has structural consequences for their interaction with arsenite. Bis-thiolate coordination by DTT forms a seven-membered ring, but this has enough conformational flexibility that one of the alcohols completes the As(III) coordination in the 1:1 complex. DHLA forms a six-membered ring upon bis-thiolate coordination, but the most stable species is a 2:3 complex, presumably with a bridging DHLA that completes tris-thiolate coordination for both As(III)'s. DMSA forms the smallest (five-membered) ring upon bis-thiolate coordination, but a 1:2 complex with a second DMSA and tris-thiolate coordination is more stable than the 2:3 complex found with DHLA. This might be a consequence of DMSA's smaller bite angle and more limited conformational flexibility, or the unfavorable 6− overall charge on a 2:3 complex of arsenite and DMSA.

The enthalpies of formation of these complexes provide new insight about the effect of methylation on As(III) chemistry. Specifically, arsenite coordination to the first GSH, the first DMSA, and DTT can be directly compared to MMA coordination to the analogous thiol. In these three cases, ΔH is more favorable (exothermic) for MMA by 2.6 ± 0.4 kcal/mol. This provides a quantitative measure of the relative effect of hydroxyl and methyl groups on thiol bonding in As(III) complexes and likely reflects the unfavorable π-donor interaction of arsenite's hydroxyl with π-donor thiolate ligands, as MMA's methyl has only a σ-donor bonding interaction.

Comparison of the thermodynamics of As(III) interaction with the three dithiols (Table 3) is best made with MMA because its dithiol complexes all have 1:1 stoichiometry. Increasingly unfavorable entropy of formation (ΔS) correlates with increasing size of the bidentate ring and corresponds to increasing loss of conformational degrees of freedom upon bis-thiolate coordination. The enthalpies of formation (ΔH) are similar for DHLA and DTT, but the value is significantly less for DMSA, which could be due to electronic or electrostatic effects of the two carboxylates that are vicinal to the two DMSA thiols.[37] The sum of enthalpic and entropic contributions leads to a somewhat more favorable free energy of formation for MMA complexes with DMSA and DHLA than with DTT.

Thermodynamic comparison of monothiol and dithiol coordination to As(III) is also best made with MMA because of the similar 1:1 dithiol complexes. Enthalpies of formation for the four MMA complexes give an average value of −8 kcal/mol for each thiol that binds (eq 1). Because the thiol (Cys) deprotonation enthalpy is 8.5 kcal/mol[38] and the enthalpy of formation of water from proton and hydroxide is −13.7 kcal/mol, the enthalpy for thiolate displacement of hydroxide from As(III) (eq 2)

$$As^{III}-OH + RS^- \rightleftarrows As^{III}-SR + OH^- \qquad (2)$$

can be determined from a thermodynamic cycle to be −2.8 kcal/mol. This quantifies the enthalpic contribution from As(III)−thiolate bonding to the stability of these MMA complexes and likely originates from better overlap and energy match of As and S valence orbitals than As and O valence orbitals. Considering entropy of complex formation, the dithiol value becomes increasingly unfavorable with increasing ring size until ΔS for DTT (−24 cal/mol·K), which forms a seven-membered ring with As(III), is similar to ΔS for two GSH monothiols (−25 cal/mol·K). This suggests that the entropy of formation for the DTT complex might represent the unfavorable entropic contribution to As(III) coordination to conformationally unconstrained dithiols.

(iv) Biological Implications. These results for arsenite and MMA binding to GSH and three dithiols have significant biological implications. First, they establish the stability of the important intracellular species $As(GSH)_3^{-3}$ and $(H_3C)As(GSH)_2^{-2}$ under physiologically relevant conditions (pH 7.4, 0.10 M NaCl, 37 °C).[19] Potential cellular As(III) binding sites must have an affinity that is large enough to displace GSH from these complexes. In this regard, the higher stability of the 1:2 complex of arsenite with DMSA reflects its effectiveness in chelation therapy for arsenic poisoning.[33]

The more favorable enthalpy for the formation of MMA−thiolate complexes, relative to arsenite−thiolate complexes, found here supports a generally higher MMA affinity for thiol ligands in biological binding sites. This suggests a fundamental chemical explanation for biological evidence that MMA is more toxic than arsenite.[11−14]

Stabilities of the MMA−dithiolate complexes are consistent with recent results of Cline et al., who measured MMA binding to pairs of Cys residues in helical peptides and found MMA−peptide stabilities ranging from 5.1×10^6 to 3.6×10^7.[39] These values for Cys pairs that are conformationally constrained on a peptide helix are closer to those for DMSA and DHLA, which have a smaller disfavorable entropy term and less conformational freedom than does DTT.

High-affinity protein binding sites for arsenite have been associated with so-called vicinal thiols.[40] The thermodynamics of arsenite and MMA binding to dithiols, however, indicates that entropic factors are an important contribution to the overall stability (affinity). Thus, vicinal Cys residues that are conformationally constrained and favorably positioned by a protein structure will have a higher affinity for

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As(III). On the other hand, conformationally unconstrained-Cys residues, such as those found in zinc fingers, where metal binding is required to stabilize a functional protein structure, will have an entropic (conformational) penalty and, therefore, a lower affinity for As(III). This has direct relevance to arsenite effects on biochemical pathways controlled by the glucocorticoid receptor (GR),[41] which has several conserved Cys residues and two tetra-thiolate Zn^{2+} binding sites in its DNA-binding domain. Ongoing studies with GR peptides and domains are testing this hypothesis.

Finally, both arsenite and MMA have a high affinity for DHLA, which might be the mechanism by which they inhibit DHLA-dependent dehydrogenases.[5,6,14] Arsenite, in particular, forms a stable 2:3 complex with DHLA, although its biological importance is unclear because arsenite is not as potent an inhibitor of pyruvate dehydrogenase as is MMA.[14] Future studies will correlate these thermodynamic results with As(III) binding and inhibition of DHLA-dependent enzymes.

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Supporting Information Available: Representative near-UV absorption spectra and ITC data for forward and reverse titrations of arsenite and MMA with GSH, DMSA, DHLA, and DTT. This material is available free of charge via the Internet at http://pubs.acs.org.

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