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Binding of Dimethylarsinous Acid to Cys-13α of Rat Hemoglobin Is Responsible for the Retention of Arsenic in Rat Blood

Meiling Lu,[†,‡] Hailin Wang,[‡] Xing-Fang Li,[‡] Lora L. Arnold,[§] Samuel M. Cohen,[§] and X. Chris Le*[,†,‡]

Departments of Chemistry and Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, T6G 2G3 Canada, and Department of Pathology and Microbiology and the Eppley Institute for Cancer Research, University of Nebraska Medical Center, Omaha, Nebraska 68198-3135

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The metabolism, disposition, and carcinogenicity of arsenic differ dramatically between humans and rats. To understand the molecular basis of these differences, we have characterized arsenic species in rats that were treated with inorganic arsenate (iAs^V), monomethylarsonic acid (MMA^V), or dimethylarsinic acid (DMA^V) for up to 15 weeks. Arsenic significantly accumulated in the red blood cells (RBCs) of rats in the form of hemoglobin (Hb) complexed with dimethylarsinous acid (DMA^{III}), regardless of whether the rats were treated with iAs^V, MMA^V, or DMA^V, suggesting rapid methylation of arsenic species followed by strong binding of DMA^{III} to rat Hb. The binding site for DMA^{III} was identified to be cysteine 13 in the α-chain of rat Hb with a stoichiometry of 1:1. Over 99% of the total arsenic (maximum 2.5−3.5 mM) in rat RBCs was bound to Hb for all rats examined ($n = 138$). In contrast, only 40−49% of the total arsenic (maximum ∼10 μM) in rat plasma was bound to proteins. The ratios of the total arsenic in RBCs to that in plasma ranged from 88−423 for rats that were fed iAs^V, 100−680 for rats that were fed MMA^V, and 185−1393 for rats that were fed DMA^V, when samples were obtained over the 15-week exposure duration. Previous studies have shown an increase in urothelial hyperplasia in rats fed DMA^V. This is the first article reporting that treatment with iAs^V in the drinking water also produces urothelial hyperplasia and at an even earlier time point than dietary DMA^V. Dietary MMA^V produced only a slight urothelial response. A correlation between the Hb−DMA^{III} complex and urothelial lesion severity in rats was observed. The lack of cysteine 13α in human Hb may be responsible for the shorter retention of arsenic in human blood. These differences in the disposition of arsenicals may contribute to the observed differences between humans and rats in susceptibility to arsenic carcinogenicity.

Introduction

Arsenic is classified as a human carcinogen by a number of international organizations (*1*, *2*). Human epidemiological studies have demonstrated a consistent association between elevated arsenic exposure and the prevalence of skin, bladder, and lung cancers (*2*−*6*). More than 40 million people around the world may be at risk (*7*, *8*). However, the mechanism or mode of action by which arsenic causes various cancers is very complex and has not been well delineated. Recent studies have investigated multiple plausible mechanisms, including reaction with sulfhydryl groups of critical cellular proteins, generation of oxidative damage by trivalent arsenic metabolites, inhibition of DNA repair, inhibition of several key enzymes, modulation of signal transduction, perturbation of DNA methylation, and alternations in cell-cycle control, differentiation, apoptosis, and gene expression (*2*, *6*, *9*−*15*). Many of these mechanisms are interdependent and may vary with specific arsenic compounds, tumor sites, cell types, and animal species.

The differences in biological response to arsenic exposure among animal models complicate the understanding and evaluation of arsenic carcinogenicity and toxicity. Long-term treatment (e.g., a 2-year bioassay) with high doses of inorganic arsenic or pentavalent arsenic metabolites has not resulted in tumor induction in most experimental animals tested (*2*, *11*) unless there was also co-exposure to other carcinogens (*16*−*19*), the use of more susceptible transgenic mice (*20*, *21*), or an in utero exposure of mice to inorganic arsenic (*18*, *22*, *23*). An intriguing exception is the production of urinary bladder tumors in rats (especially female rats) after long-term treatment with high doses (40−200 μg/g) of dimethylarsinic acid (DMA^{V1}) in the diet (*24*) or drinking water (*25*, *26*). Therefore, there has been much interest in understanding the mode of action for DMA^V-induced bladder tumors in rats (*24*−*29*).

A significant difference in arsenic metabolism and disposition has been demonstrated between rats and most other animal species, including humans (*30*−*35*). In general, inorganic arsenic undergoes biomethylation, which involves the alternate reduction of pentavalent arsenic species to trivalent arsenic species

* To whom correspondence should be addressed. Tel: (780) 492-6416. Fax: (780) 492-7800. E-mail: xc.le@ualberta.ca.

† Department of Chemistry, University of Alberta.

‡ Department of Laboratory Medicine and Pathology, University of Alberta.

§ University of Nebraska Medical Center.

[1] Abbreviations: CID, collision-induced dissociation; Cys, cysteine residue; DMA^{III}, dimethylarsinous acid; DMA^V, dimethylarsinic acid; GFC, gel filtration chromatography; Hb, hemoglobin; HGAFS, hydride generation atomic fluorescence spectrometry; HPLC, high performance liquid chromatography; iAs^V, inorganic arsenate; ICP, inductively coupled plasma; MMA^{III}, monomethylarsonous acid; MMA^V, monomethylarsonic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; nanoESI, nanoelectrospray ionization; PBS, phosphate buffered saline; QTOF/MS, triple quadrupole time-of-flight mass spectrometry; RBCs, red blood cells; SEM, scanning electron microscopy; TMA, trimethylarsine; TMAO, trimethylarsine oxide.

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followed by oxidative methylation of the trivalent arsenic species. In humans, the biomethylation process appears to stop at the dimethyl arsenicals. In rats, however, the biomethylation process proceeds further to the formation of trimethyl arsenicals, mainly trimethylarsine oxide (*24*, *28*−*29*). Furthermore, rats have shown a much longer retention time of arsenic in their blood compared to that in humans (*30*−*34*) primarily because of retention in red blood cells (RBCs). Hemoglobin (Hb), the most abundant protein in RBCs, has been considered a possible target protein (*36*, *37*). Our recent work has suggested that arsenic binding to Hb is a basis for the accumulation of arsenic in rat blood (*38*). However, it is not known what arsenic species binds to the Hb of rats treated with different arsenic species. Here, we show that arsenic can significantly accumulate in rat RBCs because of the binding of the reactive arsenic metabolite, dimethylarsinous acid (DMA^{III}), to a highly reactive cysteine residue in rat Hb (cys-13 in the α chain). We demonstrate that regardless of whether rats are fed arsenic in the form of inorganic arsenate (iAs^{V}), monomethylarsonic acid (MMA^{V}), or dimethylarsinic acid (DMA^{V}), the predominant arsenic species bound to the rat Hb is DMA^{III}, indicating the efficient biomethylation of arsenicals in rats and the high affinity of rat Hb for DMA^{III}.

The discovery of DMA^{III} as the predominant protein-bound arsenic species in rat Hb is fundamentally important because of the cytotoxic and possible indirect genotoxic effects of DMA^{III} (*14*, *39*, *40*). It is generally accepted that the genotoxicity of DMA^{III} does not involve direct interaction with DNA. However, indirect processes involving oxidative damage possibly resulting from reactive oxygen species (*39*−*46*) and inhibition of DNA repair (*15*, *39*, *47*) have been implicated. DMA^{III} is also more cytotoxic (*24*, *48*−*50*) and a more potent enzyme inhibitor (*14*, *51*−*53*) than its pentavalent counterpart and the inorganic arsenic species. Studies of its biochemical interactions *in vivo* will contribute to a further understanding of the mode of action responsible for arsenic toxicity.

Materials and Methods

Caution. *The arsenic species included in this study are toxic and are potential human carcinogens. Caution and care should be exercised when handling these materials.*

Standards and Analytical Reagents. Arsenic standards for speciation analysis and protein binding studies were the same as those described previously (*38*). Methanol, formic acid, ammonium acetate, ammonium hydroxide, and water (all HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Phosphate buffered saline (PBS) was purchased from Sigma (St. Louis, MO). All other reagents used in the experiments were HPLC grade or analytical grade.

Arsenic Compounds Used for Rat Treatment. Sodium arsenate (iAs^{V}) (Sigma, St. Louis, MO) was stored at room temperature without further purification. Dimethylarsinic acid (DMA^{V}) and monomethylarsonic acid (MMA^{V}) (Luxembourg Industries, Ltd., Tel-Aviv, Israel) were stored desiccated at room temperature. DMA^{V} and MMA^{V} of measured concentrations (2−100 μg/g) were mixed into Certified Rodent Diet 5002 (PMI Nutrition International, St. Louis, MO) prior to pelleting by Dyets (Bethlehem, PA).

Rats Treated with iAs^{V}, MMA^{V}, and DMA^{V}. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. One hundred forty female F344 rats, 4 weeks old at the time of arrival, were purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed in the same way as described previously (*24*). They were fed pelleted Certified Rodent Diet 5002. Food and tap water were available *ad libitum* throughout the study. Following quarantine, the rats were randomized into seven groups by a weight stratification method. Group 1 was provided tap water supplemented with 100 μg/g iAs^{V}; all other groups (*2*−*7*) were given untreated tap water. An analysis of the tap water by the Metropolitan Utilities District (Omaha, NE) showed an arsenic level of less than 0.1 μg/L. Group 2 was fed the basal diet supplemented with 100 μg/g MMA^{V}, group 1 and the control group (group 3) were fed the basal diet, and the other four groups (groups 4 to 7) were fed the basal diet supplemented with 2, 10, 40, and 100 μg/g DMA^{V}, respectively. An analysis of the basal diet by Nutrition International showed an arsenic level of less than 0.2 μg/g. The average food and water consumption for each group of rats was recorded. The average achieved dosage of arsenic (mg/kgBW/day) was obtained from the food and water consumption values, the body weight of rats, and the concentration of the arsenic species in water and food. The dosage results are summarized in Table 1.

Table 1. Achieved Dosage (mg/kg BW/day) during Week 2 of Treatment

treatment	arsenic compd (mg/kg)	arsenic (mg/kg)
2 μg/g DMA^{V} in food	0.18	0.10
10 μg/g DMA^{V} in food	0.90	0.49
40 μg/g DMA^{V} in food	3.64	1.98
100 μg/g DMA^{V} in food	9.10	4.94
100 μg/g MMA^{V} in food	9.10	4.88
100 μg/g iAs^{V} in water	10.40	10.40

Bladder Inflation and Blood Collection and Separation. After 1, 2, 6, 8, 10, and 15 weeks of treatment with normal diet or arsenic supplemented diet or drinking water, five rats in groups 1−3, and 7 were sacrificed. The five rats in groups 4−6 were sacrificed after 10 weeks of treatment. All rats were sacrificed between 0900 and 1400 by an overdose of Nembutal (50 mg/kg of body weight, i.p.). At each sacrifice, blood from the abdominal aorta was collected from non-fasted rats into tubes containing lithium heparin and stored on ice until all samples were collected. RBCs and plasma were separated by centrifugation at 3200 rpm at 4 °C for 10 min. The resultant plasma and packed RBCs were immediately frozen in liquid nitrogen, kept on dry ice during shipping, and stored at −80 °C until analysis. At all time points except after 15 weeks of treatment, the urinary bladder was inflated *in situ* with Bouin's fixative, removed, and placed in the same fixative. Half of the bladder from each animal was processed for examination by scanning electron microscopy (SEM) and classified in one of five categories as previously described (*54*). Briefly, class 1 bladders have flat, polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial necrosis; class 3 bladders have numerous small foci of superficial urothelial necrosis; class 4 bladders have extensive superficial urothelial necrosis, especially in the dome of the bladder; and class 5 bladders have necrosis and piling up (hyperplasia) of rounded urothelial cells. Normal rodent urinary bladders are usually class 1 or 2, or occasionally class 3. The other half of the bladder was cut longitudinally into strips and, with a slice of stomach, was embedded in paraffin, stained with hematoxylin and eosin, and histopathologically examined (*54*, *55*).

Red Blood Cell Lysis. The packed RBCs (40 mg, wet weight, about 40 μL) were transferred into a 1.5 mL vial, which contained 200 μL of water and 5 μL of 5% ammonia aqueous solution where they were lysed. Ammonium acetate aqueous solution (100 mM, pH 7.0) and water were added to the vial to construct the cell lysate in 1.0 mL of ammonium acetate solution (20 mM, pH 7.0). The vial was centrifuged at 12 000 rpm at 4 °C for 10 min. The supernatant solution (or cell lysate) was then analyzed as described below.

Gel Filtration Chromatography (GFC) Separation with ICPMS Detection. As previously described (*38*), a Perkin-Elmer 200 series HPLC system (PE Instruments, Shelton, CT) fitted with an autosampler was part of the system used for the quantitative analysis of arsenic species. A Sephadex desalting column (10 mm internal diameter, 26 mm long) with an exclusion limit of 5000 Da (Amersham Biosciences, CA) was used as the gel filtration column to separate the bound protein from the unbound arsenic species.

An ammonium acetate solution (20 mM, pH 7.0) was chosen as the mobile phase, and its flow rate was 1 mL/min. The injection volume of a sample was 30 μL. The effluent from the gel filtration column was introduced directly into the spray chamber of an inductively coupled plasma mass spectrometry (ICPMS) system (Elan 6100 DRC plus, PE Sciex, Concord, ON). The operating parameters of ICPMS were optimized to an RF power of 1150 W, plasma gas flow of 15 L/min, auxiliary gas flow of 1.2 L/min, and nebulizer gas flow of 0.89 L/min.

The rat plasma was diluted 20 times and directly subjected to GFC-ICPMS analysis. The RBC lysate was diluted up to 3000 times and subjected to GFC-ICPMS analysis. The sample from each rat was analyzed twice, and the results were averaged. The results for the five rats from each group were then averaged at each time point.

Mass Spectrometric Analysis of RBC Lysates from Rats Exposed to Arsenic. RBC lysates from all control and treated rats were analyzed by a hybrid triple quadrupole time-of-flight mass spectrometer (QSTAR Pulsar i system, Applied Biosystems/MDS Sciex, Concord, ON) equipped with a nanoelectrospray source (nanoESI-MS) (Protana, Denmark). The RBC lysates were first desalted by a BioSpin-6 column (Bio-Rad Laboratories, CA). Immediately prior to nanoESI-MS analysis, the desalted protein fraction was diluted 10 times with water, methanol, and formic acid to the final methanol concentration of 10% and formic acid concentration of 0.002%. The resultant solution was then loaded onto a nanoelectrospray capillary tip (Protana, Denmark) and subjected to nanoESI-MS analysis. The electrospray voltage was 1000 V. Each spectrum (*m*/*z* 500−2000) was collected for 2 min (120 cycles). The instrument was routinely calibrated by using standard horse skeletal apomyoglobin (Sigma, St. Louis, MO) for protein analysis. The resulting spectra were further deconvoluted using Bioanalyst software (Applied Biosystems/MDS Sciex).

Collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) was used to identify the binding site of DMA^{III} in Hb. The second quadrupole of the hybrid triple quadrupole time-of-flight mass spectrometer was used to select the Hb−DMA^{III} complex as the parent ion, whereas the third quadrupole was used as a CID cell. The fragment ions were detected by the time-of-flight mass spectrometer. Collision energy was 110 eV, and a collision gas flow was set at value 6 (unitless).

Mass Spectrometric Analysis of DMA^{III} Binding to Synthetic Peptides. Three peptides were synthesized as P1, P2, and P3, with their sequences adapted from the local sequences of cys13α, cys104α, and cys111α, respectively. The sequences of P1, P2, and P3 are listed below.

P1: NH_2-**NIKNCWGKI**-COOH (cys13 α)

P2: NH_2-**FLSHCLLVT**-COOH (cys104 α)

P3: NH_2-**VTLACHHPG**-COOH (cys111 α)

The synthesized peptides were incubated with DMA^{III} *in vitro*, and the resultant peptide−DMA^{III} complexes were subjected to MS and MS/MS analysis. The MS/MS spectra of peptide−DMA^{III} complex were compared with those of the peptides themselves. DMA^{III} binding to any of these peptides was shown to generate characteristic dipeptide and tripeptide ions, which were distinctly related to the amino acids neighboring the cysteine residues.

HPLC-HGAFS Analysis of the Arsenic Species in the Protein Complexes. Hydride generation coupled with atomic fluorescence spectrometry (HGAFS) was used for the detection of the arsenic species released from the protein. RBC lysates from control and treated rats were precipitated using acetone, centrifuged, and dissolved in alkaline solutions. Most of the bound arsenic (>90%) was released from the protein, which was examined using GFC-ICPMS as described above. The released arsenic was separated from the protein by ultrafiltration using microcon-3 filters (Millipore, Bedford, MA). The filtrate (with the released arsenic) was then diluted with mobile phase, and 20 μL of resultant solution was injected into the HPLC-HGAFS system that was described previously (*56*, *57*) for arsenic speciation.

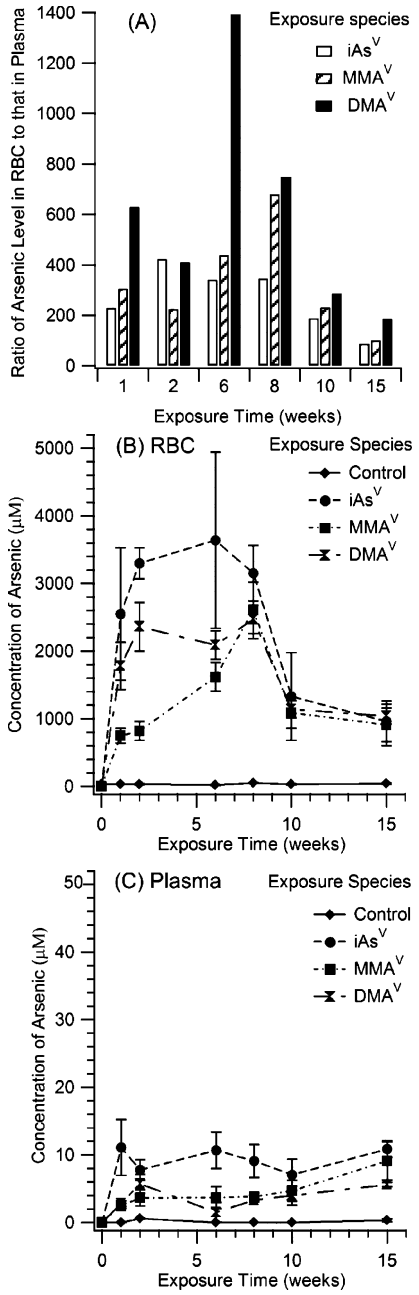

Figure 1. Arsenic concentration in red blood cells (RBCs) and plasma of control rats and rats fed iAs^V, MMA^V, and DMA^V. (A) Ratio of total arsenic in RBCs to that in plasma. (B) Total arsenic concentration in RBCs of rats. (C) Total arsenic concentration in plasma of rats over the 15-week exposure time. The total arsenic concentrations in RBCs and plasma represent the sum of both free and protein-bound arsenic species obtained from the GFC-ICPMS analysis. At each time point, data from five rats in each group were averaged. Each sample from the same rat was analyzed twice, and the data were averaged with a relative standard deviation of <5%. Within each group of five rats, the relative standard deviation from the average value was mostly within 35%.

Results

Distribution and Accumulation of Arsenic in Rat Blood. We first examined the arsenic distribution in blood obtained from rats exposed for up to 15 weeks to three different pentavalent arsenicals (iAs^V, MMA^V, and DMA^V) at a dose of 100 μg/g. We found that arsenic accumulated predominantly in rat RBCs but not in plasma (Figure 1). The ratios of the measured total arsenic in rat RBCs to that in rat plasma were 88−423 for rats fed iAs^V, 100−680 for rats fed MMA^V, and

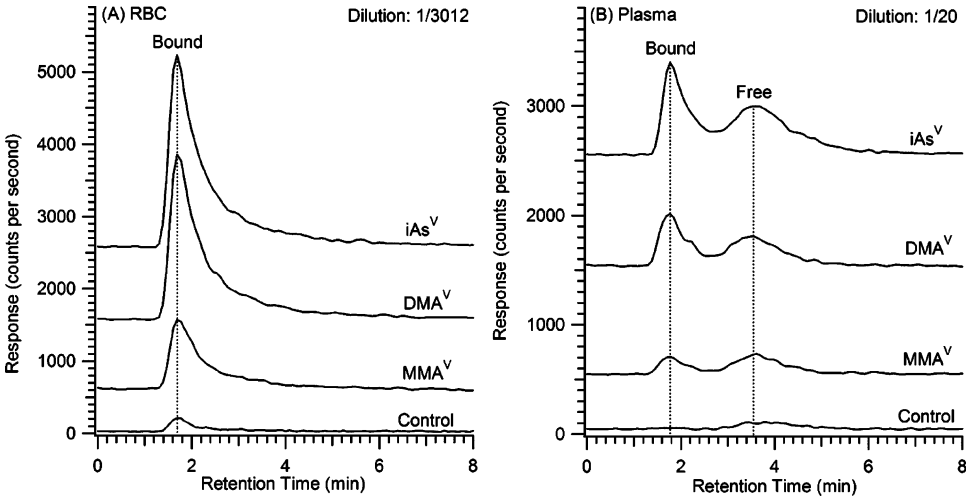

Figure 2. Chromatograms obtained from a GFC-ICPMS analysis of protein-bound and free arsenic in red blood cells (A) and plasma (B) of control rats and rats fed pentavalent arsenic for 2 weeks. After the separation of plasma from red blood cells (RBCs), the plasma was diluted 20-fold and directly subjected to GFC-ICPMS analysis. The RBCs were first lysed and subjected to high-speed centrifugation. The supernatant solution was diluted and then subjected to GFC-ICPMS analysis. The total dilution factor for RBCs was 3012-fold. The detailed experimental conditions are shown in the Materials and Methods section.

185−1393 for rats fed DMA^V, respectively, when sampled over a period of 15 weeks (Figure 1A). The maximum total arsenic in RBCs of rats exposed to 100 μg/g iAs^V in water was about 3.5 (±1.3) mM after 6 weeks of exposure, and 2.6 (±0.4) mM and 2.5 (±0.2) mM in the 100 μg/g MMA^V and DMA^V groups after 8 weeks of exposure, respectively (Figure 1B). In comparison, the arsenic level in the plasma of rats treated with the three pentavalent arsenic compounds at the same dose of 100 μg/g fluctuated around 10 μM during the treatment period of 1 to 15 weeks (Figure 1C). These results indicate that arsenic was effectively arrested in rat RBCs at millimolar concentrations (Figure 1B) but not in plasma (only micromolar concentrations, as shown in Figure 1C) for all three pentavalent arsenic compounds investigated, making the total amount of arsenic found in rat RBCs ~88−1393-fold higher than that found in rat plasma.

To understand the large difference of arsenic concentration in RBCs and plasma, we separately determined the protein-bound and the free arsenic species in RBCs and plasma. This was done using gel filtration chromatography combined with inductively coupled plasma mass spectrometry (GFC-ICPMS) (*38*, *57*, *58*). With this method, both free and protein-bound arsenic in plasma and RBCs can be effectively separated by the fast gel filtration chromatography and sequentially quantified by the selective detection of ICPMS. Figure 2 demonstrates the chromatograms obtained from GFC-ICPMS analysis of arsenic binding and distribution in RBCs and plasma from the rats exposed to arsenic. The protein-bound arsenic migrated out faster (1.8 min) than the free (unbound) arsenic (3.6 min). In rat RBCs, protein-bound arsenic is the predominant fraction for all of the rats exposed to iAs^V, MMA^V, or DMA^V (Figure 2A). Even in control rats fed the basal diet (without supplemental arsenic), a small amount of protein-bound arsenic was detected in RBCs. However, very little free arsenic was observed in control or treated rat RBCs (Figure 2A). In fact, the fraction of arsenic bound to protein in rat RBCs is over 99% for all rats examined (n = 138), regardless of the arsenic species (iAs^V, MMA^V, and DMA^V) administered to the rats, exposure dose (0−100 μg/g), or exposure duration (0−15 week). It is evident that the degree of protein binding of arsenic in RBCs is independent of the speciation of the arsenicals used in the treatment of rats. In contrast, in the plasma of rats exposed to arsenic (100 μg/g iAs^V, MMA^V, or DMA^V, n = 30 for each), the average protein-bound arsenic accounted for 40.1 (±15.6)%, 49.3 (±17.3)%, and 43.5 (±13.7)% of the total arsenic in plasma. These results suggest that the higher degree of protein binding to arsenic in rat RBCs is responsible for the higher concentration of arsenic in rat RBCs.

Effect of Exposure Dose of DMA^V. To investigate the dose−response of arsenic in rat blood, we examined rats that were exposed to DMA^V for 10 weeks with the doses of 0, 2, 10, 40, and 100 μg/g. The results demonstrate that arsenic content both in RBCs and in plasma is dose-dependent. The protein-bound arsenic present in the RBCs of the rats (n = 5) significantly increased from 0.03(±0.01) mM (control level exposure, n = 5) to 1.1 (±0.3) mM (40 μg/g DMA^V exposure, n = 5). There was no further significant increase in the arsenic content of the RBCs of the rats exposed to 100 μg/g DMA^V compared to the level in the 40 μg/g group (Figure 3A). This was probably caused by the decrease in the concentration of protein-bound arsenic in the 100 μg/g DMA^V group, which started after 8 weeks of exposure (Figure 1B). In comparison, the total arsenic content in the plasma increased from undetectable levels to 2.1 (±0.94) μM (n = 5) with increasing exposure doses from 0 to 10 μg/g. It reached over 4.0 (±1.4) μM (n = 5) with increasing exposure doses from 10 to 100 μg/g (Figure 3B). Examination of the separate dose−response curves of the protein-bound arsenic and free arsenic in plasma (Figure 3B) showed that protein-bound arsenic increased with increasing exposure doses only when the doses were under 10 μg/g, and it approached a saturation point when the doses were above 10 μg/g. Unlike protein-bound arsenic, the free arsenic level in the plasma continued to increase with increasing exposure doses. In the entire dose range (0−100 μg/g DMA^V), the concentration of arsenic in the RBCs was much higher (234- to 370-fold) than its concentration in the plasma, consistent with the exposure-time-dependent results shown in Figure 1.

Identification of the Bound Arsenic Species in Rat Hb. To understand the biochemical basis of arsenic arrest and accumulation in rat RBCs, we further identified and characterized the protein-bound arsenic species using two techniques, including nanoelectrospray triple quadrupole time-of-flight mass spectrometry (nanoESI-QTOF/MS) and HPLC coupled with hydride generation atomic fluorescence spectrometry (HPLC-HGAFS). NanoESI-QTOF/MS analysis can provide chemical information on the identities of bound proteins, reactive arsenic

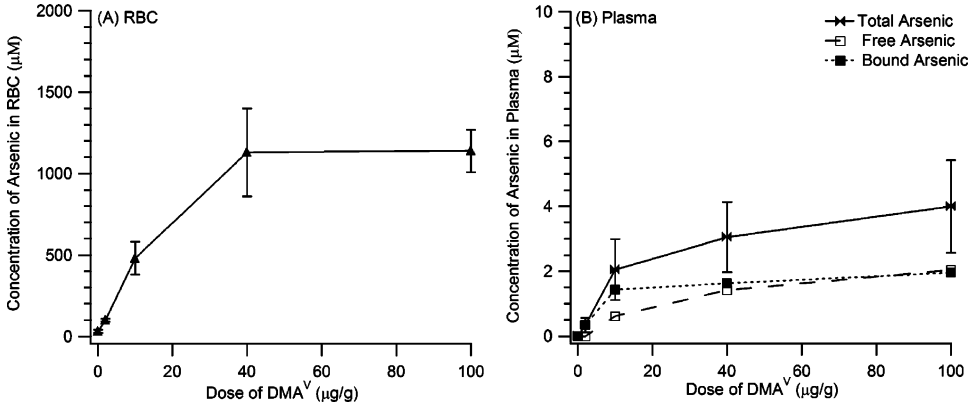

Figure 3. Arsenic concentration in red blood cells (A) and plasma (B) of rats fed either a basal diet or one supplemented with DMA^V. The rats (five in each group) were exposed to control level, 2, 10, 40, or 100 μg/g DMA^V in the diet continuously for 10 weeks and were then sacrificed, and their blood was collected for arsenic analysis by GFC-ICPMS.

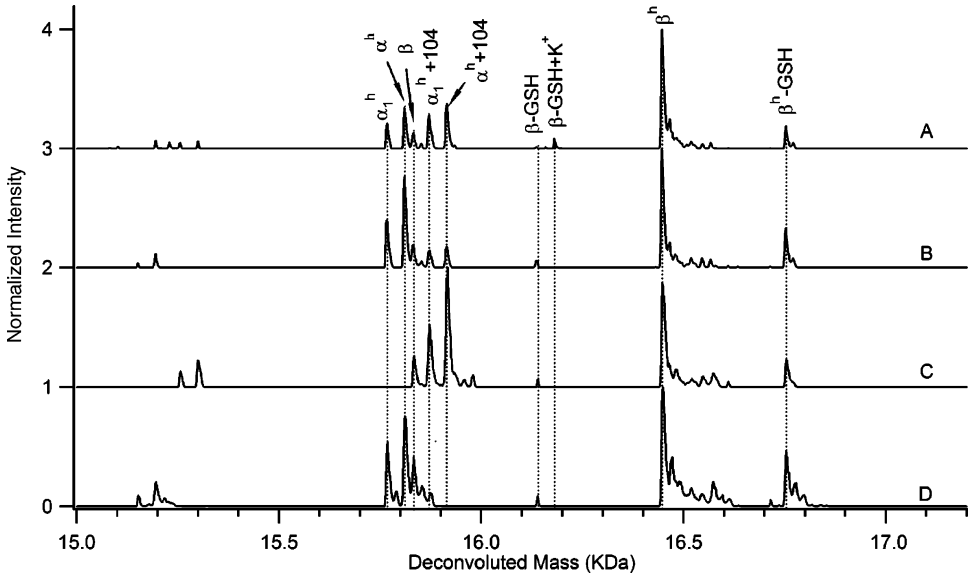

Figure 4. Deconvoluted nanoelectrospray mass spectra from an analysis of lysates of red blood cells from rats exposed to arsenic for 1 week. Rats were fed (A) 100 μg/g DMA^V in the diet; (B) 100 μg/g MMA^V in the diet; (C) 100 μg/g iAs^V in drinking water; and (D) a normal diet as the control. The major peaks related to hemoglobin α and β chains were labeled in the spectra. Peaks of a^h+104 and $a_1{}^h$+104 are the complex of one α unit or α_1 unit (variant of the α unit) bound to one DMA^{III} molecule (a mass increase of 104 Da from the intact heme-α unit) with the heme group attached. As a result of exposure to iAs^V, MMA^V, or DMA^V, a predominant protein complex, DMA^{III}−Hbα with a stoichiometry of 1:1, was observed in rat RBCs. There is no peak with a mass increase of 104 Da from the β chain. However, the glutathione-conjugated β chain (β-GSH and β^h-GSH) was consistently observed in all rats, including the control rats, with a mass increase of 305 Da from the β chain. Note: In trace C showing the rats fed with 100 μg/g iAs^V in water, $\alpha_1{}^h$ and α^h are absent but $\alpha_1{}^h$+104 and α^h+104 are present, indicating that $\alpha_1{}^h$ and α^h are completely bound to DMA^{III}.

Table 2. Identified Species in Figure 4 with Corresponding Molecular Mass

identified species		mass (Da)	present in rats
α^h	α chain with heme (1:1)	15813.4	all rats
β	β chain without heme	15834.3	all rats
β^h	β chain with heme (1:1)	16450.3	all rats
β^h-GSH	glutathione conjugate of β^h (1:1)	16755.4	all rats
$\alpha_1{}^h$	variant of α chain (1:1)	15769.4	all rats
α^h+104	DMA^{III} bound to α^h (1:1)	15917.3	only treated rats
$\alpha_1{}^h$+104	DMA^{III} bound to $\alpha_1{}^h$ (1:1)	15873.3	only treated rats

species, and *in vivo* pertinent binding stoichiometry. Figure 4 demonstrates the deconvoluted mass spectra obtained from an analysis of rat RBC lysates from control rats and rats exposed to 100 μg/g of iAs^V, MMA^V, or DMA^V. The identified mass spectral peaks are summarized in Table 2. In rat RBCs, hemoglobin (Hb) is a major protein component (~95%) and consists of four polypeptide chains (tetramer form, two α and two β) with a complexed heme in each peptide chain. The mass spectrum from a control rat demonstrated two major peaks of rat Hb α and β units with the corresponding molecular masses of 15 813 and 16 450 Da (trace D in Figure 4), resulting from the dissociated Hb tetramer. In the mass spectrum from rats exposed to DMA^V (trace A in Figure 4), a new peak of 15 917 Da was observed, accompanied by a decrease in the peak of the Hb α unit (15 813). This new peak has a mass increase of 104 Da from the intact Hb α unit (15813 Da). The mass increase of 104 Da is characteristic of an addition of one DMA^{III} molecule (121.97 Da) to the protein with a loss of one water molecule (18.01 Da) (*38*). Therefore, the results indicate that the DMA^{III}−Hbα complex with a stoichiometry of 1:1 is the predominant protein-bound arsenic species. Furthermore, no other arsenic−protein complex (different in binding stoichiometry or in bound arsenic species) was observed.

Interestingly, the same protein complex (DMA^{III}−Hbα complexes with a stoichiometry of 1:1) was also observed in rats exposed to MMA^V and iAs^V (traces B and C in Figure 4). There was no detectable complex of MMA^{III} or iAs^{III} with the Hb of RBCs from any of the treated rats. Consecutive monitoring of RBCs from rats exposed to the arsenicals (0−15 weeks)

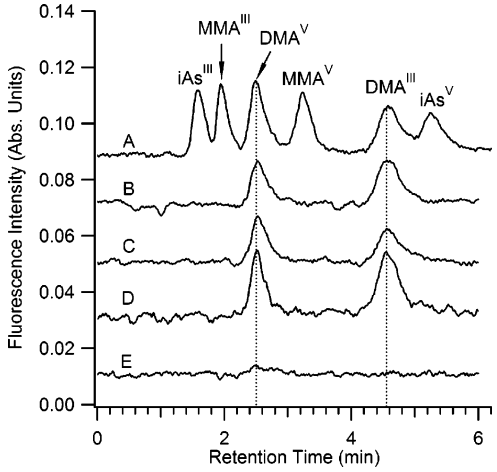

Figure 5. HPLC-HGAFS chromatograms showing the species of arsenic released from hemoglobin in the red blood cell lysate of rats exposed to different arsenic species for 8 weeks. (A) Standard solution containing six arsenic species in deionized water. (B) Rats fed with 100 μg/g iAs^V in their drinking water. (C) Rats fed with 100 μg/g MMA^V in their diet. (D) Rats fed with 100 μg/g DMA^V in their diet. (E) Rats fed with a normal diet as the control. Detailed HPLC separation conditions are described in the Materials and Methods section.

further supported the fact that the DMA^{III}−Hbα complex with the stoichiometry of 1:1 was predominant and persistent.

To confirm that DMA^{III} is the predominant arsenic species bound to the RBCs of rats exposed to three different arsenicals, we further released arsenic from the Hb (release efficiency >90%) of rat RBCs and determined the species using a well-established method: HPLC-HGAFS (*56*, *57*). This method can provide accurate speciation information for a number of arsenicals, including iAs^{III} (arsenite), MMA^{III} (monomethylarsonous acid), DMA^{III}, iAs^V, MMA^V, and DMA^V. As demonstrated in Figure 5, in all of the rats exposed to iAs^V (trace B), MMA^V (trace C), or DMA^V (trace D), only two arsenicals, DMA^V (2.25 min) and DMA^{III} (4.38 min), were observed that had retention times consistent with the corresponding standard arsenicals (trace A). No significant amount of arsenic was detected in the rats fed the basal diet (trace E). Experiments *in vitro* suggest that there is no direct reaction between rat Hb and the pentavalent DMA^V (*38*). The presence of DMA^V in the filtrate after releasing arsenic from the protein may result from the oxidation of unstable DMA^{III} (*59*) during the release and the follow-up separation process. The results further support that DMA^{III} is the predominant arsenic species bound to rat Hb *in vivo* in the rats exposed to the arsenicals.

Identification of the Binding Site of Rat Hb for DMA^{III}. Three cysteine residues (Cys13, Cys104, and Cys111) in the α chain and two cysteine residues (Cys93, and Cys125) in the β chain of rat Hb are the potential binding sites for trivalent arsenicals (*38*). In experiments *in vitro* where excess amounts of DMA^{III} were incubated with rat Hb, DMA^{III} was able to bind to all of the sulfhydryl groups in rat Hb (*38*). However, when rats were dosed with iAs^V, MMA^V, or DMA^V, the reactive metabolite DMA^{III} was found to bind preferentially to the α chain of rat Hb (Figure 4). Because there are three cysteine residues in the α chain of rat Hb, we further examined whether there was a primary binding site for DMA^{III}.

Using electrospray tandem mass spectrometry and a top down proteomic approach (*60*−*62*), we have identified that Cys13 in the α chain of rat Hb is the primary binding site for DMA^{III}. This characterization was achieved by comparing the accurate mass of the molecular and fragment ions from the rat Hb and the Hb−DMA^{III} complex in the blood of control rats and arsenic-treated rats, respectively, as briefly described below. The binding of a DMA^{III} molecule to a cysteine of a protein (with the loss of a water molecule) results in a mass increase of 103.9607 amu. This characteristic can be used to identify fragment ions containing DMA^{III}. Under collision-induced dissociation (CID) conditions, Hb and Hb−DMA^{III} are fragmented to characteristic peptide fragments that are detected by tandem mass spectrometry (MS/MS). Accurate mass measurements of these fragment ions provide information on which amino acids are neighboring the cysteine that binds to DMA^{III}. The sequence of rat Hb indicates that the immediate neighboring amino acids of Cys13 in the α chain are LysAsnCysTrpGlyLys. Table 3 shows examples of the relevant major fragment ions that contain DMA^{III}. They include AsnCys-DMA^{III}, DMA^{III}-CysTrp, DMA^{III}-CysTrpGly, and AsnCys-DMA^{III}Trp. The measured values are in good agreement with the theoretical values for these expected species, with acceptable mass accuracy (<50 ppm). These results suggest that DMA^{III} is bound to Cys13.

Table 3. CID MS/MS Measurements of Fragment Ions Containing DMA^{III}

fragment ions	measured value (amu)	expected value (amu)	mass accuracy (ppm)
AsnCys-DMA^{III}	322.020	322.021	−3
Cys-DMA^{III}Trp	394.057	394.057	0
Cys-DMA^{III}TrpGly	451.090	451.079	24
AsnCys-DMA^{III}Trp	508.085	508.100	−29

The neighboring amino acids of Cys104 are SerHisCysLeuLeu and of Cys111 are LeuAlaCysHisHis. No DMA^{III}-bound species that are specific to these local sequences were detected, suggesting that DMA^{III} did not bind to Cys104 or Cys111.

The identification of Cys13 in the α chain of rat Hb as the DMA^{III} binding site was further confirmed with supporting evidence from experiments with three synthetic peptides (each containing nine amino acids) encompassing each of the cysteine residues in the α chain of rat Hb. Incubation *in vitro* of DMA^{III} with these peptides resulted in the formation of the expected peptide−DMA^{III} complexes, confirming the CID MS/MS methodology for the identification of the Hb binding site for DMA^{III}.

Computer modeling of the rat Hb conformation showed that Cys13α in rat Hb is located in an open hydrophobic pocket (Figure 6). This is particularly suitable for binding with DMA^{III}, which is more hydrophobic than the inorganic As^{III}.

Results of Parameters Measured in-Life in Rats. Treatment with DMA^V and MMA^V had no effect on the weight gain of rats (Table 4). However, weight gain in the group treated with 100 μg/g iAs^V was significantly decreased by the end of week 2 compared to the weight gain in the control group. Although the mean body weight in the iAs^V-treated group remained significantly decreased compared to that of the control group throughout the experiment, the percent weight gain in the iAs^V-treated group was similar to that of the control group at 8 and 13 weeks. Food consumption (g/kg BW/day) was not affected by treatment with any of the three arsenicals (Table 4). Water consumption (g/kg BW/day) was significantly decreased throughout the study in the group treated with iAs^V in water compared to water consumption in the control group (Table 4). The mean achieved dosage for each group during week 2 of treatment is shown in Table 1. Because the rats consumed more water per kg of body weight than the diet, the consumption of arsenic compound (mg) per kg of body weight is higher in the 100 μg/g iAs^V group compared to those of the 100 μg/g MMA^V or

Table 4. Effect of Treatment with iAs^V, MMA^V, or DMA^V on Body Weights, Food Consumption, and Water Consumption[a]

	body weight			food consumption g/kg BW/day			water consumption g/kg BW/day		
treatment	Wk 2	Wk 8	Wk 13	Wk 2	Wk 8	Wk 13	Wk 2[b]	Wk 8	Wk 13
control	128 ± 1	172 ± 2	191 ± 3	92 ± 1	60 ± 1	52[d]		124 ± 3	113[d]
2 μg/g DMA^V in food	129 ± 2	173 ± 3	[e]	90[d]	59[d]	[e]		122[d]	[e]
10 μg/g DMA^V in food	124 ± 2	167 ± 3	[e]	90[d]	63[d]	[e]		133[d]	[e]
40 μg/g DMA^V in food	126 ± 2	171 ± 3	[e]	91[d]	60[d]	[e]		151[c,d]	[e]
100 μg/g DMA^V in food	125 ± 1	170 ± 2	187 ± 2	91 ± 2	63 ± 1	51[b]		145 ± 3[c]	109[d]
100 μg/g MMA^V in food	128 ± 1	171 ± 2	188 ± 4	91 ± 1	60 ± 1	50[d]		124 ± 3	99[c,d]
100 μg/g iAs^V in water	110 ± 1[c]	148 ± 3[c]	160 ± 6[c]	87 ± 1	58 ±2	49[d]	104 ± 4	95 ± 4[c]	66[c,d]

[a] The values are expressed as the mean ± S.E. [b] The Wk 2 water consumption measurements for groups 2−7 were not used because of a procedural error. [c] Statistically significantly different from the control group, $p < 0.05$. [d] Only one cage in the group. [e] All animals in the group were sacrificed after 10 weeks of treatment.

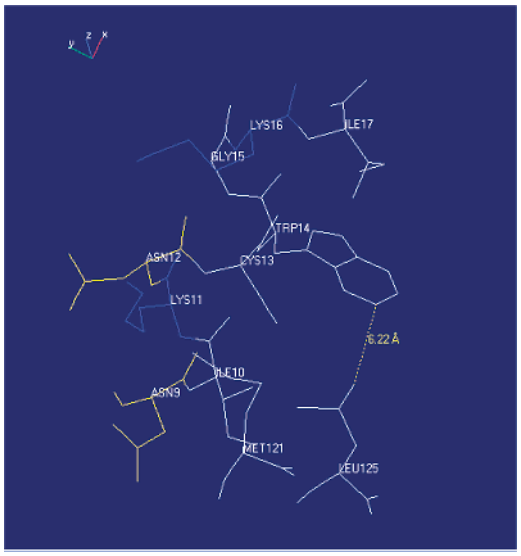

Figure 6. Computer modeling of local environment of Cys 13 (within 4 Å) in the rat hemoglobin α unit (Swiss-Prot code: P01946) using DeepView (Swiss PdbView) from http://swissmodel.expasy.org.

100 μg/g DMA^V groups. When the achieved dosage is calculated for only the arsenic portion of the compounds, the dosage in the iAs^V group, is much higher than that in the MMA^V or DMA^V groups.

Examination by light microscopy showed that treatment with MMA^V and DMA^V did not cause an increase in the incidence of hyperplasia of the bladder epithelium at any time point (Table 5). However, there was an increase in the incidence of hyperplasia, as observed by light microscopy, in the group treated with 100 μg/g iAs^V in drinking water beginning as early as 1 week after the start of treatment and the increase continued at each time point measured through the 10-week treatment period. Treatment with iAs^V has not previously been reported to have an effect on the rat bladder epithelium.

Generally, SEM has been shown to be more sensitive than light microscopy in detecting early proliferative responses in the urothelium. Also, SEM is considerably more sensitive in detecting cytotoxicity of the superficial urothelial cells because (1) the very thin nature of these cells makes them difficult to evaluate by light microscopy, and (2) early cytotoxic changes tend to be in small foci, and SEM permits the evaluation of a much greater portion of the luminal surface than light microscopy. When examined using the more sensitive SEM, the rats treated with iAs^V showed significant changes as did the rats fed 100 μg/g of DMA^V. Only slight changes were detected in the MMA^V-treated group. There was a dose−response for changes detected by SEM in rats fed with various doses of DMA^V with a clear no-effect level at 2 μg/g and only marginal changes at 10 μg/g. The severity of the changes in rats administered DMA^V progressed over time, whereas the changes in rats administered iAs^V did not.

Discussion

We have shown that arsenic significantly accumulated in the RBCs of rats (elevated levels) in the form of hemoglobin (Hb) complexed with DMA^{III}, regardless of whether the rats were treated with iAs^V, MMA^V, or DMA^V. The presence of an Hb−DMA^{III} complex as the predominant arsenic species in rat RBCs is probably because of the combination of the rapid methylation of arsenic species (*31*, *33*) and the strong binding of DMA^{III} to rat hemoglobin (*38*). The iAs^V species undergoes biomethylation, which involves alternate steps of two-electron reduction of pentavalent arsenic to trivalent arsenic followed by oxidative methylation of the trivalent arsenic (*63*). The reactive trivalent arsenic metabolites include inorganic arsenite (iAs^{III}), monomethylarsonous acid (MMA^{III}), and dimethylarsinous acid (DMA^{III}). MMA^V can be metabolized in rats to form MMA^{III}, DMA^V, DMA^{III}, trimethylarsine oxide ($TMAO^V$), and trimethylarsine (TMA^{III}). Although MMA^{III} is an intermediate reactive metabolite that can be formed in rats treated with iAs^V and MMA^V, we did not observe any Hb-bound MMA^{III} in rat blood (Figures 4 and 5). The absence of an Hb−MMA^{III} complex in rats is probably due to the lower affinity of rat Hb for MMA^{III} (5 times lower than that for DMA^{III}) (*38*). An alternative explanation is that MMA^{III} is rapidly metabolized further to DMA^V, DMA^{III}, $TMAO^V$, and TMA^{III} in the liver and kidneys of rats, the major organs for arsenic metabolism, before it is fluxed into the blood stream. Likewise, we did not observe an Hb−iAs^{III} complex in rat blood, although iAs^{III} can be formed in iAs^V-treated rats. The binding affinity of iAs^{III} for rat Hb *in vitro* is 20 and 95 times lower than that for MMA^{III} and DMA^{III}, respectively (*38*). In rats, iAs^{III} undergoes further methylation to MMA^V, MMA^{III}, DMA^V, DMA^{III}, $TMAO^V$, and probably TMA^{III}. Therefore, whether the rats were treated with iAs^V, MMA^V, or DMA^V, the common reactive trivalent arsenic end-metabolite was DMA^{III}, which bound to the Hb of rats.

Much recent research has focused on the carcinogenic effects of DMA^{III} and DMA^V (*24*−*29*, *64*). DMA^{III} has been shown to generate free radicals; such reactive oxygen species may cause oxidative stress *in vivo* (*40*, *41*, *45*, *65*−*67*). DMA^{III} has also

Table 5. Effects of Treatment with iAs^V, MMA^V, or DMA^V on the Bladder Epithelium

	bladder histology		SEM classification[a]				
treatment	normal	hyperplasia	1	2	3	4	5
		after treatment for 1 week					
control	4	1	2	2	1		
100 μg/g DMA^V in food	4	1			3		2
100 μg/g MMA^V in food[b]	5			1			2
100 μg/g iAs^V in water[b]	2	3					3
		after treatment for 2 weeks					
control	4	1		5			
100 μg/g DMA^V in food[c]	4	1					4
100 μg/g MMA^V in food[c]	5			1	3		
100 μg/g iAs^V in water	1	4			1		4
		after treatment for 6 weeks					
control	5		4	1			
100 μg/g DMA^V in food	4	1					5
100 μg /g MMA^V in food	5			2	3		
100 μg/g iAs^V in water		5[d]					5
		after treatment for 8 weeks					
control	5		3	2			
100 μg/g DMA^V in food	5						5
100 μg/g MMA^V in food	5			1	4		
100 μg/g iAs^V in water	2	3					5
		after treatment for 10 weeks					
control	5		3	2			
2 μg/g DMA^V in food	5		1	4			
10 μg/g DMA^V in food	5			2	3[f]		
40 μg/g DMA^V in food	2	3					5
100 μg/g DMA^V in food	4	1					5
100 μg/g MMA^V in food[e]	4	1					2
100 μg/g iAs^V in water		5[d]					5

[a] The use of 1−5 in the SEM classification scheme is arbitrary. Changes in class 5 bladders are not 5 times more severe compared to class 1 bladders. See Materials and Methods for a brief definition of each class. [b] Two bladders were unobservable. [c] One bladder were unobservable. [d] Statistically significantly different from the control group, $p < 0.05$. [e] Three bladders were unobservable by SEM. [f] Moderate cytotoxicity was present.

Table 6. Correlation of the Hb−Arsenic Complex with Urothelial Lesion Severity

treatment	urothelial lesion severity at 10 weeks[a]	Hb−As complexes at 10 weeks (mM)
control	1.4	0.03
2 μg/g DMA^V in food	1.8	0.10
10 μg/g DMA^V in food	2.6	0.48
40 μg/g DMA^V in food	5	1.13
100 μg/g DMA^V in food	5	1.14
100 μg/g MMA^V in food	5	1.09
100 μg/g iAs^V in water	5	1.33

[a] The urothelial lesion severity was calculated as the average SEM value for the rats in each treatment group ($n = 5$ in all groups except the 100 μg/g MMA^V group, where $n = 2$).

been shown to be an indirect genotoxic agent (*68*−*70*). Possible forms of DNA damage caused by DMA^{III} include the formation of single strand DNA breaks, oxidative base damage of DNA, DNA−protein cross-links, and chromosomal aberrations. These indirect genotoxic and oxidative damage effects have generally only occurred at concentrations that produced cytotoxicity. In addition, the binding of reactive trivalent arsenicals to sulfhydryl groups of proteins could significantly alter cellular processes and/or lead to cytotoxicity and cell death (*36*, *37*, *48*−*53*). Therefore, the characterization of DMA^{III} binding to Hb *in vivo* is biochemically and toxicologically significant, although the fate of the Hb−DMA^{III} complex in blood needs further study. Because the binding of DMA^{III} to Hb stabilizes DMA^{III}, the Hb−DMA^{III} complex may serve as a reservoir that gradually releases DMA^{III} when the red blood cells decompose.

We did not directly monitor the reactive oxygen species in RBCs or other tissues. Alternatively, we examined the oxygen binding affinity of Hb from rats treated with and without arsenic compounds. If there is a significant amount of reactive oxygen species present in RBCs, the rat Hb would be a target. The rat Hb would be oxidized to methemoglobin, and the binding affinity of rat Hb would decrease. However, we found that oxygen binding affinity was not significantly changed (Figure 7 in Supporting Information), suggesting that DMA^{III} binding to rat Hb does not affect the oxygen binding domain and the oxidation state of the heme. This result indicates that the reactive oxygen species and free radicals generated in RBCs are limited. Furthermore, because DMA^{III} is predominantly bound to rat hemoglobin, free DMA^{III} is either not available or insufficient for the induction of reactive oxygen species *in vivo*.

Chronic exposure to high levels of arsenic could lead to binding site saturation, such as that observed during the 8−10 weeks of rat treatment, leading to serious health effects. Among the rats treated with three pentavalent arsenic species, the iAs^V-treated group (with the highest arsenic concentration) showed the highest protein-bound arsenic and earliest saturation in RBCs (Figure 1B). The decrease of Hb-bound arsenic after 8−10 weeks is probably related to the lifetime of RBCs, which is about 60 days for rats (*71*). Hyperplasia of the bladder urothelium of iAs^V-treated rats developed as early as one week after the start of exposure as observed by light microscopy (Table 5). This is the first article of rat bladder urothelial proliferation produced by iAs^V administration. DMA^V has previously been demonstrated to produce similar bladder changes in rats at doses of 40 and 100 μg/g in the diet. MMA^V has generally not shown effects on the bladder in such studies (*28*, *29*), although mild hyperplasia has been reported in rats administered 50 and 200 μg/g MMA^V in drinking water in a two-year bioassay (*72*). Additional studies are necessary to confirm these findings. Nonetheless, our results suggest a strong correlation ($R^2 = 0.99$) between Hb-bound arsenic and bladder urothelial lesion severity in rats (Table 6 and Figure 8 in Supporting Information).

The binding of DMA^{III} to Hb may also change arsenic metabolism and the profiles of free arsenic distribution because it changes the biologically available dose and the kinetic properties of the accumulation and excretion of arsenic. It may play an important role in the toxicokinetics and toxicodynamics of arsenic. The significant interception of DMA^{III} by rat Hb may potentially protect the cells from oxidative stress by

reducing the available free DMA^{III} species in the cells. This would be consistent with the fact that rats are much more resistant and tolerant to acute arsenic toxicity than humans.

Previous reports have documented the accumulation of arsenic in rat blood (*31*, *33*, *34*, *36*, *73*−*75*). We demonstrated that the binding of DMA^{III} to rat Hb was responsible for this accumulation. The preservation of the protein-bound arsenic species and the direct mass spectrometric measurement provide clear evidence that the reactive trivalent metabolite DMA^{III} is bound to the α chain of rat Hb. We have further identified that Cys13 in the α chain of rat Hb is the reactive binding site of DMA^{III}. Cys13α is present in rat Hb, but not in human Hb. Although there are nearly 700 human Hb variants, none of these variants contains a Cys13α. This may explain the much weaker binding of human Hb than that of rat Hb to the same trivalent arsenic species (*38*). The capacity for arsenic accumulation in the RBCs of different animal species may be determined by the difference in the affinity of their Hb.

Besides rats, cats can also accumulate arsenic in blood (*74*), and cat hemoglobin also contains cysteine α-13 (swiss-prot ID: P07405). This is quite interesting; however, there has been no thorough toxicity study of arsenic in cats. Mice are widely used in arsenic toxicity studies. Both rats and mice are more tolerant to arsenic toxicity than humans, and both have a higher methylation efficiency than humans (*9*, *31*−*34*). Arsenic in mice has a short retention time (*34*), which is consistent with the fact that mice do not have reactive cysteine 13α in their hemoglobin. Although it is biochemically significant to demonstrate that the reactive cysteine 13α in the hemoglobin is responsible for the binding of DMA^{III} in rat red blood cells, this finding alone cannot explain why mice as well as rats are less sensitive than humans to arsenic-induced carcinogenesis. There are likely other reasons for arsenic-induced carcinogenesis.

In conclusion, results from the GFC-ICPMS analysis of the protein-bound and free arsenicals in RBCs and plasma (Figures 1−3) indicate that arsenic is predominantly accumulated in RBCs. This appears to be due to the higher affinity of rat Hb compared to that of plasma proteins for arsenic species. In rats treated with iAs^{V}, MMA^{V}, and DMA^{V}, the Hb−DMA^{III} complex is the predominant arsenic species in RBCs and is responsible for the accumulation of arsenic in rat blood. This complex species could play an important role in the metabolism of arsenic, the distribution of a biologically effective dose of arsenic, the retention and biochemical interaction of reactive arsenic species, and the overall toxicity of arsenic.

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Supporting Information Available: Results showing the effect of arsenic binding on hemoglobin oxygen binding affinity (Figure 7) and the correlation of Hb-bound arsenic with urothelial lesion severity (Figure 8). This material is available free of charge via the Internet at http://pubs.acs.org.

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