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Dimethylarsine and Trimethylarsine Are Potent Genotoxins In Vitro

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The mechanism of arsenic carcinogenesis is unclear. A complicating factor receiving increasing attention is that arsenic is biomethylated to form various metabolites. Eleven different arsenicals were studied for in vitro genotoxicity to supercoiled DNA (pBR 322 and ϕX174). Five arsenicals showed various degrees of positivity—monomethylarsonous acid, dimethylarsinous acid, monomethylarsine, dimethylarsine, and trimethylarsine. Supercoiled DNA, blotted on nitrocellulose filter paper, was exposed to gaseous arsines by suspending the filter paper above aqueous reaction mixtures of sodium borohydride and an appropriate arsenical. All three methylated arsines damaged DNA; inorganic arsine did not. Arsines were generated in situ in reaction mixtures containing DNA by reaction of sodium borohydride with arsenite, monomethylarsonous acid, dimethylarsinous acid, and trimethylarsine oxide, at pH 8.0. Both dimethylarsine and trimethylarsine (generated from 200 μM dimethylarsinous acid and trimethylarsine oxide, respectively) damaged DNA in less than 30 min. Under certain conditions, the two most potent genotoxic arsines, trimethylarsine and dimethylarsine, are about 100 times more potent than dimethylarsinous acid (the most potent genotoxic arsenical previously known). There was no evidence to suggest that anything other than the arsines caused the DNA damage. Possible models for the biological production of arsines were examined. The coenzymes, NADH and NADPH, are biological hydride donors. When NADH or NADPH (5 mM) were incubated with dimethylarsinous acid (0−2 mM) for 2 h, DNA damage was increased by at least 10-fold. A possible explanation for this result is that these compounds react with dimethylarsinous acid to generate dimethylarsine. DNA was incubated with a dithiol compound, dithioerythritol (5 mM), and trimethylarsine oxide (0.5 mM) for 2 h, and the reduction of trimethylarsine oxide to trimethylarsine resulted in DNA damage.

Introduction

Elevated levels of inorganic arsenic in drinking water have been shown to cause an increased risk of cancers of the urinary bladder, lung, and skin in men and women (*1*−*3*). There are extremely large numbers of people exposed to high arsenic concentrations (>50 ppb) in drinking water in West Bengal, India (6 million people) and Bangladesh (25 million people) (*4*).

The genetic toxicology of arsenic was recently reviewed (*1*, *3*, *5*). There is no consensus as to the mechanism of arsenic carcinogenesis. A complicating factor that has only recently come under close scrutiny is that inorganic arsenic is metabolized in humans to produce the major methylated metabolites: monomethylarsenic(V), dimethylarsenic(V), monomethylarsenic(III), and dimethylarsenic(III) species (*6*, *7*). Because the analytical techniques commonly used to examine arsenic metabolites did not target monomethylarsenic(III) and dimethylarsenic(III) species, these were overlooked until recently when they were shown to occur in the urine of arsenic-exposed humans (*8*−*12*) and in hamster liver (*13*). As analytical methodology evolves, other methylated arsenic metabolites might be detected in humans. Hence, all arsenic metabolites that could reasonably be formed in humans should be evaluated for toxicity and genotoxicity regardless of their known occurrence in humans.

The genotoxicities of organoarsenicals have not been examined in as great a detail as inorganic arsenicals, with the possible exception of DMA(V)[1] and its salts, which were once widely used in industry (*14*). Examination of this pentavalent metabolite has led to some important insights into the mechanism of arsenic's carcinogenicity. In a pioneering series of studies, Yamanaka et al. extensively examined the mutagenicity of DMA(V) (*15*−*24*). They found that DMA(V) (1500 mg/kg oral dose) induced DNA damage in rat and mouse lung. They proposed that this was ultimately caused by dimethylarsine (Me_2AsH) generated in vivo. Dimethylarsine was detected in the expired air of both rats and mice after oral administration of DMA(V) (1500 mg/kg) (*15*, *25*, *26*). Dimethylarsine caused DNA damage in an in vitro alkaline elution assay (*26*). Dimethylarsine was also shown to be mutagenic in *Escherichia coli* B tester strains (*16*). In either case, the dose or concentration of dimethylarsine to which the test systems were exposed was not

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[1] Abbreviations: TE, 10 mM Tris-HCl/1 mM EDTA, pH 8.0; MMA(V), monomethylarsonic acid; MMA(III), monomethylarsonous acid; DMA(V), dimethylarsinic acid; DMA(III), dimethylarsinous acid; TMAO, trimethylarsine oxide; DTE, dithioerythritol; ROS, reactive oxygen species; SC, supercoiled DNA; OC, open circular DNA (after a single-strand break); L, linear DNA (after a double-strand break);

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determined. It was proposed that the dimethylarsine causes damage via ROS (*27*). This was supported in part by the fact that addition of superoxide dismutase and catalase significantly inhibited DNA damage. Also, free radical species (presumed to be dimethylarsine peroxy radical) were detected by using electron spin resonance (ESR). Furthermore, a specific biomarker of DNA oxidation (8-oxo-2′-deoxyguanosine) was detected in the lung, liver, and urinary bladder of mice after oral exposure to 720 mg/kg of DMA(V) (*23*).

In this laboratory, it has been demonstrated that the methylated trivalent arsenicals, MMA(III) and DMA(III), cause damage to DNA in nicking assays and in single cell gel (comet) assays (*28*−*32*). In the same assays, arsenite, arsenate, and methylated pentavalent arsenicals did not cause DNA damage even at very high concentrations (300 mM) (*28*). It was also shown that MMA(III) and DMA(III) are potent clastogens, in human lymphocytes and mouse lymphoma cells (*32*). However, MMA(III) and DMA(III) were not point mutagens or potent sister chromatid exchange inducers (*32*). Similar to dimethylarsine, MMA(III) and DMA(III) appear to act via the generation of ROS. ROS inhibitors were found to inhibit the DNA nicking activities of methylated trivalent arsenicals, and the DMPO (a spin trapping reagent) adduct of hydroxyl radical was detected in solutions of DMA(III) by using ESR (*29*). Furthermore, it was recently shown that nanomolar concentrations of arsenite, MMA(III), and DMA(III) and micromolar concentrations of MMA(V) and DMA(V) induced DNA damage in a human cell line (HeLa S3); the action of MMA(V) and DMA(V) was most likely a result of reduction to their trivalent counterparts (*33*). The DNA damage was in the form of formamidopyrimidine−DNA glycosylase sensitive sites, which is indicative of oxidative DNA damage caused by ROS (*33*).

Thus, to date, it appears that the genotoxicity of arsenic might be attributed to MMA(III), DMA(III), dimethylarsine, or a combination of all three compounds. Ultimately, the importance of each of these arsenicals is determined by their tissue concentrations, pharmacokinetics, and intrinsic genotoxicity. We report here our comparison of the genotoxicity of MMA(III), DMA(III), and dimethylarsine. We additionally investigated the genotoxicity of the following arsines: arsine (AsH_3), monomethylarsine ($MeAsH_2$), dimethylarsine (Me_2AsH), and trimethylarsine (Me_3As), in comparison with the other known arsenic metabolites. Finally, a metabolic scheme for the biological production of arsines is proposed.

Materials and Methods

Caution: *Many arsenic compounds are toxic and carcinogenic. Arsines are reactive volatile liquids and gases. These compounds should be handled using appropriate safety measures.*

Materials. Monomethylarsine oxide (*34*), iododimethylarsine (dimethylarsine iodide) (*35*), and TMAO (*36*) were synthesized using previously described methods (all compounds >99% purity). MMA(V) (99.2% purity) was obtained from Chem Service (West Chester, PA), and DMA(V) (99.5% purity) was obtained from Ansul (Weslaco, TX). Concentrated working solutions of the arsenic compounds (100 mM) were prepared by dissolving the appropriate amount of compound in water and adjusting the pH with sodium hydroxide to 8 (as determined by pH indicator strips). In solution at this pH, monomethylarsine oxide and iododimethylarsine yield MMA(III) and DMA(III). These solutions were stored at − 80 °C. Working solutions were thawed and diluted to lower appropriate concentrations on the day an experiment was performed. Sodium borohydride, NADH, NADPH, and DTE solutions were prepared fresh each time an experiment was performed by dissolving the appropriate amount of solid in water. An appropriate amount of NAD was dissolved in water, and the pH was adjusted to 8. SC DNA was purchased from Roche (pBR 322) and New England Biolabs (ϕX174).

Agarose Gel Electrophoresis. The amounts of damaged (nicked) and intact DNA were determined by using agarose gel electrophoresis methods described elsewhere (*29*, *30*). Briefly, DNA (either ϕX174 or PBR 322) was suspended in 45 μL of TE buffer and mixed with 5 μL of gel loading solution (0.05% bromophenol blue, 50% v/v sucrose). The mixtures were loaded in 1% agarose gels containing 0.5 μg/mL ethidium bromide (8−12 lanes per gel). SC fast migrating DNA was separated from L DNA with moderate electrophorectic mobility and OC slow migrating DNA by electrophoresis for 2−3 h at 50 V (constant voltage). Ethidium bromide-stained DNA bands were visualized under UV light. In some experiments, gels were run with 12 lanes, and in these cases, reagent quantities and volumes were adjusted to compensate for the volume of each well being reduced by half.

Nicking of DNA Exposed to Gaseous Arsines. Cellulose nitrate filter paper (Whatman, 0.2 μm pore size) was rinsed with TE buffer and briefly allowed to dry. The filter paper was cut into squares ~5 mm × 5 mm. A pushpin was pushed through the center of an Eppendorf tube (500 μL) cap and through a filter paper square. The filter paper was gently pushed into the roof of the cap. Immediately prior to generating the arsine, the target DNA solution (1 μL of 250 ng/μL) was pipetted onto the filter paper square. An arsenical (50 μL of 100 mM MMA(V), DMA(V), or TMAO) and HCl (10 μL, 1 M) were pipetted into the bottom of the Eppendorf tube. The Eppendorf tube was held almost horizontally so that 10 μL of sodium borohydride (500 mM) could be pipetted onto the Eppendorf side wall without mixing with the other reagents. The Eppendorf was capped with the filter paper containing cap and then held vertically and gently tapped so that the sodium borohydride drop would mix with the other reagents and generate the arsine. After 30 min, the filter paper was removed and placed in another Eppendorf tube to which TE buffer (50 μL) was added. This was then thoroughly agitated for 1 min to rinse the DNA from the filter paper. The amount of nicking of the DNA in the TE solution was then determined by using agarose gel electrophoresis as described above. A control was also run where the arsenical solution was replaced with distilled water.

Another series of experiments were performed where target DNA samples were both suspended above the reaction mixture on filter paper and also DNA was added to the reaction mixture. In this case, 39 μL of a solution containing either arsenite (5 mM), MMA(III) (50 mM), or DMA(III) (5 mM) in TE was mixed with 1 μL of DNA (250 ng/μL) in a 500 μL Eppendorf tube. In the same manner as described above, sodium borohydride (5 μL of 500 mM) was added without mixing with the other reagents. The Eppendorf was capped with a filter paper containing cap and tilted so that the sodium borohydride solution would mix with the other reagents. After 10 min, the filter paper was removed and the DNA was rinsed from the filter paper as described above. The amount of nicking for both the DNA in the solution and the DNA on the filter paper was determined by using agarose gel electrophoresis as described above.

Nicking of DNA Exposed to Arsenicals in Solution. Solutions were prepared containing 250 ng of DNA (either pBR 322 or ϕX174) and assorted arsenical species in TE buffer. The total volume of the reaction mixture was 45 μL. In the experiments where arsines were investigated, these arsines were generated in situ by using excess sodium borohydride (5 mM) and arsenite, MMA(III), DMA(III), and TMAO to generate arsine, monomethylarsine, dimethylarsine, and trimethylarsine, respectively. The precursor arsenical concentrations ranged from

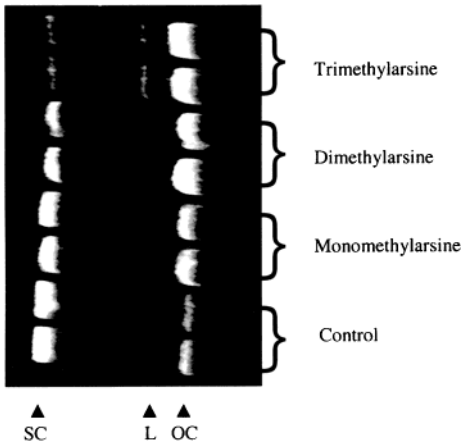


Figure 1. Agarose gel electrophoresis of pBR 322 plasmid DNA that was blotted onto nitrocellulose filter paper and suspended above reaction mixtures containing sodium borohydride (5 μmol) and 5 μmol of MMA(V), DMA(V), and TMAO (reaction mixture pH < 2) that generated monomethylarsine, dimethylarsine, and trimethylarsine, respectively. The vial volume was 500 μL so the theoretical maximum possible arsine concentration was 10 mmol/L.

5 μM to 5 mM. Sodium borohydride was always the last component added to the reaction mixtures. A variety of controls were also run where the arsenical and/or the borohydride was omitted. In other cases, arsenicals were supplemented with various other reducing agents (such as NADH or DTE) and corresponding controls were run. Various incubation times were used. Further details are given in the Results section. DNA nicking was determined by using agarose gel electrophoresis as described above.

Results

The figures and results presented here are representative of a number of experiments that were performed using both ϕX174 and pBR 322 DNA. These experiments were done in duplicate or triplicate. In all cases, the results were reproducible and similar results were obtained for both ϕX174 and pBR 322 DNA. However, the results presented here are for the pBR 322 plasmid only. No evidence was seen in any of the controls to suggest that nuclease contamination was responsible for DNA damage. In this paper, DNA damage was considered to have occurred when the intensity of the OC DNA band in the sample lane was clearly greater than the intensity of the OC DNA band in the control lane as determined by visual examination.

Nicking of DNA Exposed to Gaseous Arsines. DNA was blotted onto filter paper and suspended above acidic solutions (pH < 2) of MMA(V), DMA(V), and TMAO. The addition of sodium borohydride to these solutions generated monomethylarsine, dimethylarsine, and trimethylarsine, respectively, which rapidly diffused from the liquid phase into the headspace of the reaction vial. The DNA was rinsed from the filter paper, and nicking was determined by using agarose gel electrophoresis. DNA damage was observed for all three arsines (Figure 1) in comparison to a control (where DNA was suspended over a mixture of hydrochloric acid and sodium borohydride). It is difficult to estimate the amount of gaseous arsine to which the DNA on the filter paper was exposed. However, if we assume 100% production of the arsine and all of the arsine enters the gas phase and is not lost, the maximum concentration of arsine in the gas phase would have been 10 mmol/L.

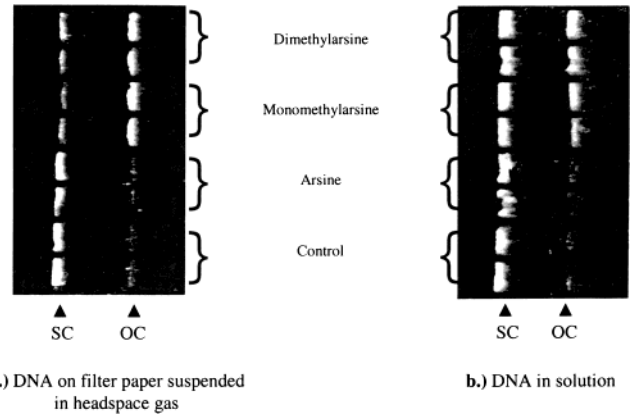


Figure 2. Agarose gel electrophoresis of pBR 322 plasmid DNA that was blotted onto nitrocellulose filter paper and suspended above reaction mixtures containing sodium borohydride (2.5 μmol) and 0.25 μmol of arsenite, 2.5 μmol of MMA(III), and 0.25 μmol of DMA(III) that generated arsine, monomethylarsine, and dimethylarsine (A) and agarose gel electrophoresis of pBR 322 plasmid DNA that was dissolved in those reaction mixtures (reaction mixture pH 8.0) (B). The vial volume was 500 μL, and the liquid volume was 50 μL so the maximum possible concentrations of arsine and dimethylarsine that could have occurred in the gas and liquid phases were 0.5 mmol/L and 5 mM, respectively. The maximum possible concentration of monomethylarsine that could have occurred in the gas and liquid phases was 5 mmol/L and 50 mM, respectively.

When fluorescein was incorporated into reactions of sodium borohydride with arsenicals, fluorescein was not transferred onto the filter paper. Thus, we believe no arsenicals reached the filter paper via aerosolization. Furthermore, when DNA on filter paper was suspended above a solution of DMA(III) (5 mM) without adding any sodium borohydride and incubated for 1 h, no damage occurred to the DNA on the filter paper, although DNA added to the DMA(III) solution was damaged. DMA(III) is relatively involatile (boiling point Me_2AsI, 154 °C), and it would only damage DNA on the filter paper if it reached the filter paper via aerosolization.

In another experiment, DNA was blotted onto filter paper and suspended above solutions of arsenite (5 mM), MMA(III) (50 mM), and DMA(III) (5 mM) at pH 8.0. The addition of sodium borohydride to these solutions generated arsine, monomethylarsine, and dimethylarsine, respectively, and these rapidly diffused into the headspace of the reaction vial. Again, it is difficult to estimate the amount of gaseous arsine to which the DNA on the filter paper was exposed. The DNA was rinsed from the filter paper, and nicking was determined by using agarose gel electrophoresis (Figure 2a). DNA damage occurred for dimethylarsine and monomethylarsine. Thus, dimethylarsine and monomethylarsine generated from two different arsenicals (DMA(V) and MMA(V), Figure 1) and (DMA(III) and MMA(III), Figure 2) caused DNA damage. No damage was observed for arsine. Because the filter paper was only suspended in the gaseous arsines for 10 min, the DNA damage must have occurred in less than 10 min. Because the arsines were generated at pH 8.0, it was possible to also add DNA directly to the reaction mixture (unlike the first experiment where the low pH would have rapidly degraded the DNA). Analysis of DNA from the reaction mixtures, by using agarose gel electrophoresis (Figure 2b), gave similar results to those observed for the DNA exposed to headspace gases (Figure 2a). No damage was observed for arsine, and comparable damage was observed for mono-

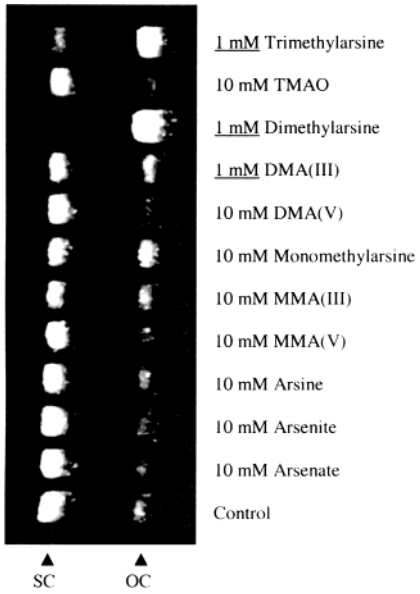


Figure 3. Agarose gel electrophoresis of pBR 322 plasmid DNA after incubation with 11 different arsenicals for 1 h at 37 °C at pH 8.0. The arsines were generated by reaction of arsenite, MMA(III), DMA(III), and TMAO with sodium borohydride (10 mM). The control lane is of DNA that was incubated with 10 mM sodium borohydride.

methylarsine (50 mM) and dimethylarsine (5 mM). Furthermore, the advantage of adding the DNA directly to the reaction mixture is that the maximum possible concentration of the generated arsine to which the DNA in solution is exposed is known (i.e., in this case, 5 mM for arsine and dimethylarsine, 50 mM for monomethylarsine). For this reason, the remainder of the results described are for experiments where DNA was added directly to the reaction mixture.

Nicking of DNA Exposed to Arsenicals in Solution. We examined DNA nicking using the following pure arsenic compounds (obtained from either commercial suppliers or synthesized): arsenite, arsenate, MMA(III), MMA(V), DMA(III), DMA(V), and TMAO by adding solutions of the compounds directly to TE buffer containing DNA. The nicking of DNA by these compounds, except TMAO, has already been investigated by other researchers (*28*−*30*). We obtained similar results, which we present here in order to make a comparison to the arsines.

Arsines are highly reactive and volatile compounds (boiling points: AsH_3, −55 °C; $MeAsH_2$, 2 °C; Me_2AsH, 36 °C; Me_3As, 50 °C), which are difficult, or impossible, to obtain through commercial sources. Thus, it was necessary to generate the four arsines—arsine, monomethylarsine, dimethylarsine, and trimethylarsine—by reacting sodium borohydride with arsenite, MMA(III), DMA(III), and TMAO, respectively. Because these four arsine precursors react with sodium borohydride at pH 8.0, it was possible to mix DNA with the appropriate precursor arsenical and then add sodium borohydride. This in situ generation of arsines proved to be the most effective means of delivering a known maximum dose (i.e., assuming 100% reaction yield) of arsine to the DNA. With the exception of TMAO, it was not possible to use in situ generation of arsines with arsenic(V) compounds at pH 8.0. This is because arsenate, MMA(V), and DMA(V) do not react with sodium borohydride at pH 8.0 (*12*).

To assess the relative genotoxicity of the 11 arsenicals examined in this study, all of the compounds were incubated with pBR 322 plasmid DNA in TE buffer (pH 8.0) for 1 h at 37 °C and DNA nicking was then determined by using agarose gel electrophoresis (Figure 3). Dimethylarsine and trimethylarsine were clearly the most DNA damaging arsenic compounds.

For all 11 arsenicals, DNA damage was determined over a range of concentrations and the minimal concentrations required to cause damage were determined for various incubation times and reducing agents (Table 1).

Table 1. DNA Damaging Action of Arsenical Solutions Either Alone (Third Column) or after Reaction with Sodium Borohydride (Fourth Column) to Generate the Corresponding Arsine Shown[a]

			reductant		
arsenical	incubation time (h)	no supplement	sodium borohydride	NADH or NADPH	DTE
arsenite(III)			arsine		not done
	2	−	−	−	
MMA(III)			monomethylarsine	(monomethylarsine)	no reaction
	2	+	+	+	+
DMA(III)			dimethylarsine	(dimethylarsine)	no reaction
	0	+	+++++	+	+
	2	+++	+++++	+++++	+++
	24	+++++	+++++	note 1	+++++
arsenate(V)	2	−	−	−	not done
MMA(V)	2				MMA(III)
		−	−	−	−
DMA(V)	2				DMA(III)
		−	−	−	+
TMAO			trimethylarsine	no reaction	trimethylarsine
	0	−	+++++	−	−
	2	−	+++++	−	+
	24	−	+++++	−	+++

[a] Also shown are the DNA damaging activities of arsenical solutions when incubated with NADH or NADPH (fifth column); in this case, the presumed reaction products are shown in parentheses. The DNA damaging activities of arsenical solutions when incubated with DTE are shown in the final column along with the reaction product of the DTE and the arsenical. Sodium borohydride alone and DTE alone did not cause DNA damage in any experiment. Both NADH and NADPH alone caused measurable DNA damage but only in the 24 h incubation (note 1). No significant DNA damage was observed in the shorter incubations with NADH or NADPH alone. Key: −, no DNA damage observed at 5 mM or greater; +, DNA damage observed at 5 mM or greater; +++, DNA damage observed at less than 5 mM; +++++, DNA damage observed at less than 0.5 mM. Note 1: When a 24 h incubation was performed using either NADH or NADPH as the reductant, DNA damage occurred in the controls (containing only NADH or NADPH). This high background interfered with the determination of DNA damage in the samples.

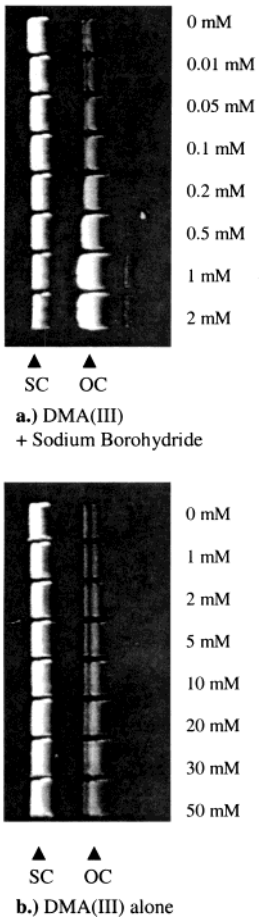


Figure 4. Agarose gel electrophoresis of pBR 322 plasmid DNA after mixing the plasmid DNA with a decreasing concentration of DMA(III) (0.01−2 mM) and sodium borohydride (5 mM) at pH 8.0 and immediately loading the gel and starting the electrophoresis, i.e., no incubation (A), in comparison to electrophoresis of pBR 322 incubated with DMA(III) (1−50 mM) under the same conditions but with no sodium borohydride added (B).

All three inorganic arsenic compounds—arsenate, arsenite, and arsine—caused no damage to DNA at concentrations of 5 mM or greater. This is in agreement with other published studies (*28*).

Monomethylarsine was generated by sodium borohydride reaction with MMA(III). Monomethylarsine caused DNA damage at a concentration of 5 mM or above and was slightly more damaging to DNA than MMA(III). The fact that the potency of monomethylarsine is only slightly higher than MMA(III) (whereas dimethylarsine is 100-fold more potent than DMA(III); see below) might be partially due to the high volatility of monomethylarsine (boiling point 2 °C); that is, most monomethylarsine is lost from the system before it can do any damage.

DNA was mixed with DMA(III) followed by sodium borohydride to generate dimethylarsine, and then, the mixture was immediately added to an agarose gel and the DNA was separated by using electrophoresis (Figure 4a). Thus, the maximum time that the DNA is exposed to the dimethylarsine would be the handling time (5 min or less) and the amount of time it takes the DNA to move from the gel loading well into the gel matrix (at most 25 min). The neutral arsine should remain in the gel loading well. Thus, in this experiment, DNA damage must occur in 30 min or less. The minimum concentration of dimethylarsine required to cause DNA damage was 200 μM (Figure 4a). Increasing the incubation time to 24 h did not result in more DNA damage, presumably because of the high reactivity and volatility (boiling point 36 °C) of dimethylarsine. The reaction of sodium borohydride with DMA(III) is effectively instantaneous. Once dimethylarsine is generated, it will rapidly be lost from the liquid phase by volatilization and oxidation. Oxidation of dimethylarsine will form mostly dimethylarsenic(V) species. The dimethylarsine oxidation products will not regenerate dimethylarsine by further reaction with excess sodium borohydride because they do not react with sodium borohydride at pH 8.0 (*12*).

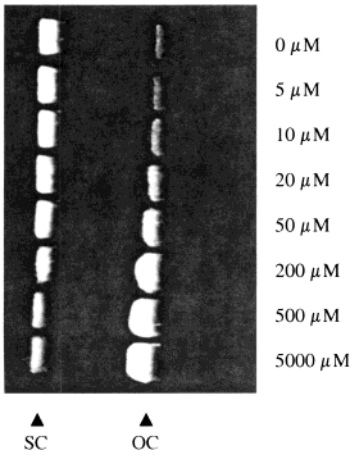


Figure 5. Agarose gel electrophoresis of pBR 322 plasmid DNA immediately after reaction with a decreasing concentration of trimethylarsine, generated by in situ reaction of TMAO (5−5000 μM) and sodium borohydride (5 mM) at pH 8.0.

DMA(III) was considered to be the most potent direct DNA damaging arsenical known (*28*−*30*). However, dimethylarsine is at least 100 times more reactive toward DNA than DMA(III) (comparing Figure 4a,b). This potency comparison is based on the minimum concentration of arsenical required to cause DNA damage relative to the control. In these experiments, DNA was mixed with DMA(III) and then immediately added to an agarose gel and the DNA separated by using electrophoresis, i.e., minimal incubation time (Figure 4b).

When a 2 h incubation period was used, dimethylarsine was 10 times more reactive than DMA(III). When a 24 h incubation was used, dimethylarsine and DMA(III) caused an equal amount of damage to DNA. It is assumed that the dimethylarsine (boiling point 36 °C) caused all of its DNA damage in the first 30 min or less of the incubation period, whereas the DMA(III) (low volatility) damaged DNA at a slower rate over the entire exposure period.

Trimethylarsine was generated from TMAO by using sodium borohydride at pH 8.0. Trimethylarsine caused damage to pBR 322 plasmid DNA at a concentration of 200 μM (Figure 5). As was seen with dimethylarsine, increasing the incubation time did not cause a significant increase in the DNA damage by trimethylarsine. Again, this is most likely due to the high volatility (boiling point 50 °C) and reactivity of trimethylarsine; that is, trimethylarsine is rapidly lost (volatilization and oxidation) from the solution once it is formed.

Nicking of DNA by Arsenicals in the Presence of Biological Reducing Agents. We have demonstrated that arsines cause DNA damage when generated by

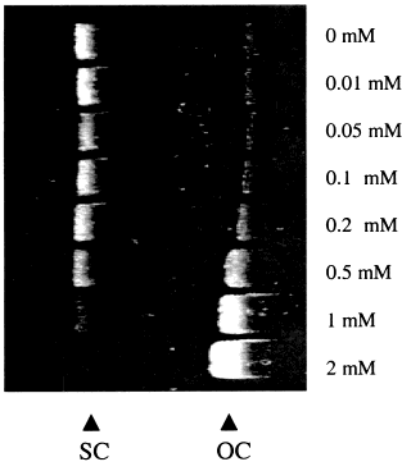


Figure 6. Agarose gel electrophoresis of pBR 322 plasmid DNA after incubation with 5 mM NADH and a decreasing concentration of DMA(III) (0.01−2 mM) for 2 h at 37 °C at pH 8.0 (A) in comparison to electrophoresis of pBR 322 incubated with DMA(III) (1−50 mM) under the same conditions but with no NADH added (B).

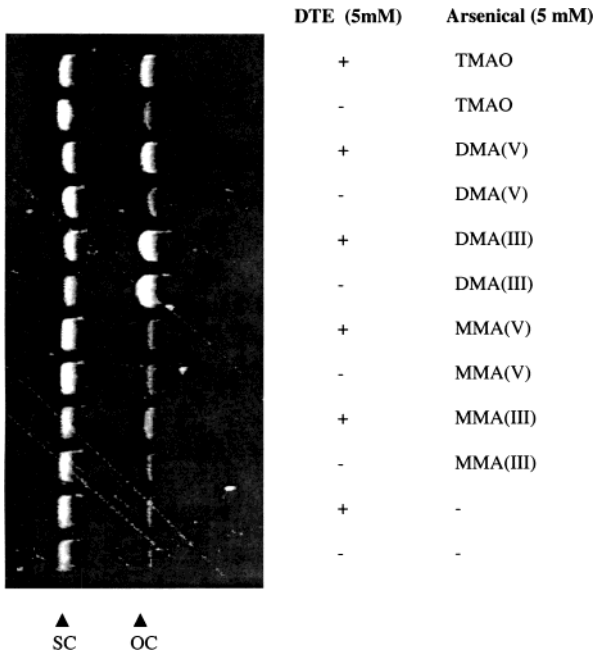


Figure 7. Agarose gel electrophoresis of pBR 322 plasmid DNA after a 2 h incubation (37 °C, pH 8.0) with five different methylated arsenicals (5 mM) with or without DTE (5 mM). Two controls were also run that contained either DTE (5 mM) with DNA or only DNA.

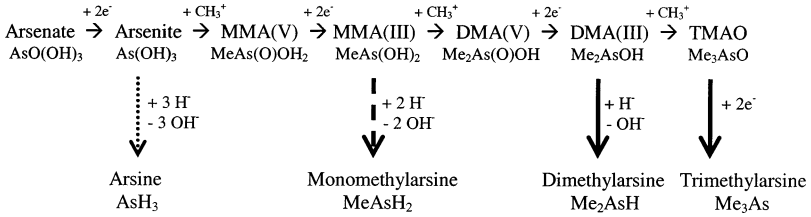
reacting arsenicals with sodium borohydride. Sodium borohydride acts as both a reducing agent and a hydride donor. In this section, we report on experiments where these tasks were accomplished by other more physiologically relevant compounds.

The coenzymes, NADH and NADPH, are biological hydride donors. When NADH (5 mM) was incubated with DMA(III) (0−2 mM) for 2 h, DNA damage was increased by at least 10-fold (Figure 6). A possible explanation for this result is that NADH reacts with DMA(III) to generate dimethylarsine. With DMA(III) (0.2 mM), the lowest concentration of NADH that caused an increase in DNA damage was 50 μM (data not shown). Controls were run either with NAD or with NAD and DMA(III). No DNA damage occurred for NAD alone, and no increase in DNA damage was observed when NAD was incubated with DMA(III) (data not shown). This demonstrates that the action of NADH is due to its role as a hydride source and not due to some other functional group in the molecule. When the incubation time was reduced to a minimum, no increase in DNA damage was observed when NADH was added to DMA(III). These results differ from those observed with sodium borohydride because NADH is much less reactive than sodium borohydride, especially in the absence of an enzyme. The experiments described above were repeated using NADPH, and the same results were obtained.

When NADH was incubated with arsenite for 2 h, no DNA damage was observed (Table 1). When NADH was incubated with MMA(III) for 2 h, there was no significant increase in the amount of DNA damage by MMA(III) (Table 1). NADH and NADPH did not reduce TMAO to trimethylarsine (trimethylarsine has a distinct and intense garlic odor with an odor threshold of 0.01 nM (*37*)). Thus, no DNA damage was observed for mixtures of NAD(P)H and TMAO (Table 1).

It is known that the thiols, GSH and L-Cys, reduce TMAO to trimethylarsine (*38*). When we incubated these reductants with TMAO and DNA, no DNA damage was observed (data not shown) even though the distinct odor of trimethylarsine in the reaction mixtures was detected. The lack of damage in the presence of GSH and L-Cys is possibly due to their ROS scavenging properties, which was demonstrated by GSH inhibiting DNA damage by DMA(III) (data not shown). It also is known that the dithiol compound DTE reduces TMAO to trimethylarsine (*38*). When DNA was incubated with DTE and TMAO for 24 h at 37 °C, significant DNA damage was observed for TMAO concentrations of 500 μM and above (Table 1) and in these mixtures the odor of trimethylarsine was clearly present. DTE (5000 μM) or TMAO (5000 μM) alone did not damage DNA. DTE (5 mM) was incubated with TMAO (5 mM) for 2 h, and DNA damage was observed (Figure 7). Likewise, when DTE was incubated with DMA(V), DNA damage was observed (Figure 7), presumed to be due to the reduction of DMA(V) to DMA(III). DTE did not increase or decrease the amount of DNA damage caused by DMA(III). Thus, DTE does not

Scheme 1. Biological Metabolism and Chemical Reactions of Arsenic Leading to Arsines[a]



[a] All steps shown by a vertical arrow can be achieved in vitro using sodium borohydride at pH 8.0 (as in this study). All steps shown with solid arrows have been demonstrated in mammals and in microorganisms. The transformation of MMA(III) to monomethylarsine (dashed arrow) occurs in microorganisms but has not been demonstrated in mammals. The transformation of arsenite to arsine is readily achieved in vitro using sodium borohydride but is very seldom observed in biology or the environment.

exhibit ROS scavenging properties under these conditions, in contrast to GSH. The amount of DNA damage done by TMAO in the presence of DTE is much less than the amount of DNA damage done by TMAO in the presence of sodium borohydride (i.e., comparing Figures 5 and 7). This is probably because the reduction of TMAO with DTE is not very efficient.

Discussion

The metabolism of arsenic in biological systems has been extensively investigated (*7*, *39*). Underlying this research is the Challenger (*40*) mechanism for arsenic biomethylation (Scheme 1), which is based on the nucleophilic addition of trivalent arsenic species to a carbocation. Such metabolism in humans was once believed to be a detoxification pathway because researchers focused almost exclusively on pentavalent arsenic species, namely MMA(V) and DMA(V), which are readily detected in the urine of arsenic-exposed humans (*1*). In most assays, the methylated pentavalent arsenic compounds were found to be much less toxic (*41*) and genotoxic (*42*) than inorganic arsenic compounds. The recent detection of methylated trivalent arsenicals in human urine prompted investigation of the mammalian toxicity (*43*), cytotoxicity (*44*, *45*), and genotoxicity (*28*−*30*, *32*, *33*) of methylated trivalent arsenicals. The high cytotoxicity and genotoxicity of methylated trivalent arsenicals as compared to other arsenicals suggests that arsenic metabolism may be a toxification pathway (*8*, *20*, *28*, *30*, *32*, *43*−*48*). Therefore, it is clear that the approach of focusing attention only on arsenic metabolites that are easily detected rather than all possible arsenic metabolites has led to significant scientific oversights in the past.

Pursuing this line of inquiry further, we have found monomethylarsine, dimethylarsine, and trimethylarsine to be damaging to DNA. We generated the arsines in situ using sodium borohydride. When filter papers blotted with DNA were exposed to gaseous arsines, DNA damage occurred for monomethylarsine (from MMA(III) or MMA(V)), dimethylarsine (from DMA(III) or DMA(V)), and trimethylarsine (from TMAO). In all of these experiments, the DNA on the filter paper was only exposed to the arsines for 30 min or less. In one experiment with dimethylarsine and monomethylarsine (Figure 2), the exposure time was less than 10 min. Thus, DNA damage occurs extremely rapidly. In the case of target DNA on filter paper exposed only to gaseous arsines, using multiple arsenical sources, it is clear that only the gaseous arsines could be responsible for the damage; however, the concentration of arsine in the vicinity of the DNA on the filter paper was unknown. Further experiments were performed with the DNA placed directly in the aqueous arsine generating reaction mixtures, allowing comparison of arsines with nonvolatile arsenicals.

When the DNA is placed directly in the reaction mixture with the arsenical and sodium borohydride, the possibility exists that the DNA damage is also due to (i) sodium borohydride, (ii) byproducts of the sodium borohydride reaction, or (iii) impurities in the arsenical compounds. The following observations strongly argue against these possibilities.

(i) DNA Damage by Sodium Borohydride. Sodium borohydride alone, even at concentrations an order of magnitude greater (50 mM) than those used in the experiments reported here (5 mM), did not damage DNA.

(ii) DNA Damage Due to Byproducts of the Borohydride Reaction. The major byproduct of the sodium borohydride reaction, borate, does not damage DNA. Indeed, it is a component of the gel electrophoresis buffer (∼90 mM). Any pH change that might occur during the reaction is compensated for by the TE buffer. In some experiments, we checked the pH and saw no change due to the reaction. The reaction of arsenite and MMA(III) with sodium borohydride resulted in no DNA damage and very little DNA damage, respectively. If the DNA damage observed for dimethylarsine and trimethylarsine was merely due to heat generation or reactive intermediate generation by the borohydride reaction, then a similar amount of damage should have been observed for the generation of arsine and monomethylarsine from arsenite and MMA(III). Arsenite and MMA(III) react with borohydride just as efficiently as DMA(III) and TMAO (*12*). When DTE was used instead of sodium borohydride to reduce TMAO to trimethylarsine, DNA damage was still observed.

(iii) DNA Damage Due to Impurities in the Arsenical Compounds. The reaction stoichiometry is consistent with the DNA damaging compound being the in situ generated arsine and not an impurity in the original arsenical solution that became activated by borohydride. That is, the minimum concentration of sodium borohydride required to cause DNA nicking when the arsenical was in excess was equimolar to the minimum concentration of arsenical required to do damage when the borohydride was in excess. If an impurity in the original arsenical solution, activated by borohydride, was responsible for the damage, DNA damage would have occurred at much lower borohydride concentrations. All methylated arsenicals used in this study were greater than 99% pure.

In conclusion, there was no evidence to suggest that DNA damage observed in the presence of sodium borohydride and arsenic compounds was due to anything other than the generated arsines, which were shown to

cause DNA damage as discussed above (nicking of DNA exposed to gaseous arsines).

Dimethylarsine and trimethylarsine were particularly potent at damaging DNA. Dimethylarsine is at least 100 times more DNA damaging than the corresponding DMA(III). In less than 30 min, both dimethylarsine (200 μM) and trimethylarsine (200 μM) caused DNA damage. Although this is a high concentration, it should be realized that it represents an upper bound of the concentration of arsine to which the DNA was exposed assuming that the borohydride reaction gave a 100% yield. Also, the time that the DNA would be exposed to this concentration is extremely short because the arsines rapidly vaporize into the vial headspace. As a result, it is quite likely that we have significantly underestimated the reactivity of the methylated arsines toward DNA. The in vitro arsenic test concentrations used in our study ranged from 5 μM to 50 mM. Arsenic levels found in blood or in organs of arsenic-exposed humans range from nanomolar to low micromolar (*1*). In the most sensitive assay to date for DNA damage by methylated arsenicals described by Schwerdtle et al. (*33*) (alkaline unwinding assay in a human cell line (HeLa S3) after formamidopyrimidine−DNA glycosylase treatment), DNA damage was induced by nanomolar concentrations of DMA(III). If the rank order of DNA damaging potency that we have shown in this work (trimethylarsine and dimethylarsine are about 100 times more potent than DMA(III)) remains true in more sensitive test systems, like that described by Schwerdtle et al., then arsines could be shown to be damaging at physiologically relevant concentrations. Thus, more sensitive physiologically relevant assays need to be performed in the future.

The genotoxicity of MMA(III) and DMA(III) in DNA nicking assays was confirmed by data obtained from cellular assays with arsenical concentrations comparable or less than those used in the DNA nicking assays (*28*, *32*, *33*). No arsines were tested in those studies. It was demonstrated that dimethylarsine increased the number of revertant colonies of *E. coli* B tester strains in a mutagenicity assay, whereas trimethylarsine did not (the doses of arsines were unknown) (*16*). Our results suggest that the in vivo genotoxicity and mutagenicity of arsines should be further evaluated although this will be complicated by the high volatility and reactivity of the four arsines. It may be worth exploring the genotoxicity of TMAO in vivo, because this compound would be easily reduced to trimethylarsine in most systems. In one of the few studies using TMAO, it was as effective as DMA(V) and MMA(V) at promoting formation of GSH S-transferase placental form positive foci in rat liver, initiated by prior diethylnitrosamine administration (*49*). Also, in a two year study with male Fischer 344 rats, TMAO (0−200 ppm administered ad libitum in drinking water) induced hepatocellular adenomas in a dose-dependent manner (*50*). In both studies, evidence was found implicating a higher level of ROS in the TMAO-treated groups as compared to the controls.

In previous research by Yamanaka et al., dimethylarsine was generated externally and passed through a DNA solution (*27*) and DNA damage was analyzed by using alkaline elution. Unfortunately, the dose of dimethylarsine required to cause damage was unknown (as in our gas phase work) and there was no way of comparing the damaging action of the arsine with other nongaseous arsenic compounds. We were able to make comparisons between arsines and nonvolatile arsenicals by generating arsines in situ. Yamanaka et al. obtained evidence that the genotoxicity of dimethylarsine is due to the formation of ROS (*15*, *25*, *27*).

By examining trimethylarsine, we have shown that there is a clear trend in the reactivity of methylated trivalent arsenicals toward DNA in vitro. The DNA reactivity of trivalent arsenicals increases as the number of methyl substituents increase. Trivalent arsenicals with zero and one methyl group are nonreactive or only slightly reactive, respectively, while trivalent arsenicals with two or three methyl groups are considerably more reactive. As the mechanism of the DNA damage induced by these compounds is further examined, this trend might be explained or might serve as a clue in establishing the mechanism of DNA damage. For instance, it is notable that the chemical stability of the arsines is as follows: $AsH_3 > MeAsH_2 > Me_2AsH > Me_3As$ (*51*). This suggests that DNA damaging potency might be directly related to arsine instability. Unstable arsines react rapidly with oxygen to generate ROS, as suggested by Yamanaka (*27*) for dimethylarsine. The role of oxidative stress in arsenic carcinogenesis has recently been reviewed (*52*).

Although the metabolism of arsenic to trimethylarsine in microorganisms is well-known (*37*), it is uncertain if arsenic is metabolized to trimethylarsine in humans or other mammals. Even if trimethylarsine (or other arsines) is produced in humans, the detection of arsines is difficult. The production of arsines in humans certainly has not been ruled out, and dimethylarsine (*15*, *25*, *26*) and trimethylarsine (*41*, *53*) production have been demonstrated in mammals, but there are clear differences in arsenic metabolism between humans and other animals (*1*). There is anecdotal evidence of human victims of arsenic poisoning having a garlic breath odor consistent with the odor of trimethylarsine (*54*).

The production of trimethylarsine is predicted by the Challenger mechanism (Scheme 1) and must occur via the production of trimethylarsenic(V) species (e.g., TMAO). TMAO has been detected in the urine of some arsenic-exposed humans (*55*). Hamsters treated with a single oral dose of arsenic trioxide (4.5 mg/kg) excreted a small amount of TMAO in urine, and trace amounts of TMAO were detected in their livers (*56*). Mice, hamsters, and humans exposed to DMA(V) excrete small quantities (<6%) of urinary arsenic as TMAO (*14*, *55*). In rats dosed with either arsenite or MMA(V), about 1−5% of the total arsenic metabolites was TMAO (Dr. Shoji Fukushima, personal communication). However, after administration of DMA(V) to rats, about 30% of the total arsenic urinary metabolites was TMAO (Dr. Shoji Fukushima, personal communication). In one study, a human volunteer excreted 3.5% of a single oral dose (0.1 mg As/kg) of DMA(V) as TMAO (*55*). TMAO is readily reduced to trimethylarsine. Hamsters given 50 mg/kg arsenic as TMAO orally or intraperitoneally excreted trimethylarsine in expired air approximately 15 min after administration (*53*). Also, mice administered a single lethal oral dose of 14.4 g/kg TMAO excreted trimethylarsine in expired air (*41*) before death. In total, the available evidence suggests that trimethylarsine production in humans is possible although the amounts would be small as compared to monomethylated and dimethylated arsenic species. However, given the high potency of trimethylarsine demonstrated in these studies (at least 100× more potent than

DMA(III)), trimethylarsine production may still play an important role in arsenic genotoxicity, mutagenicity, and carcinogenicity.

The reduction of TMAO in vivo probably involves thiol compounds as reductants (*38*). Whether or not this requires an enzyme is unknown. However, the in vitro reduction of TMAO is readily achieved using a variety of thiol and dithiol compounds (*38*) and the protein metallothionein (*57*). For example, we found that DTE reduced TMAO to trimethylarsine as indicated by the intense garliclike odor of trimethylarsine. When DTE was incubated with both TMAO and DNA, DNA damage occurred. In our system, the amount of DNA damage that occurs is determined by balancing (i) the ability of the thiol compound to reduce TMAO against (ii) the ability of the thiol compound to scavenge ROS or otherwise inhibit DNA damage. In our studies, Cys and GSH did not cause DNA damage when incubated with TMAO even though trimethylarsine was produced (the odor of trimethylarsine was clearly present). In a cell, there are hundreds of thiol compounds (e.g., peptides, proteins including metallothionein (*57*)) that could reduce TMAO to trimethylarsine. Whether this trimethylarsine would then reach some DNA and cause DNA damage is unknown. Trimethylarsine is neutral and lipophilic, and these favorable pharmacokinetic factors might facilitate transport to the interior of the cell and interaction with DNA.

Both rats and mice administered DMA(V) (1500 mg/kg) excreted dimethylarsine in expired air (*15*, *25*, *26*). Both NADH and NADPH increased the DNA damage done by DMA(III) 10-fold. This is possibly because NADH and NADPH react with DMA(III) to form dimethylarsine. Further experiments should be performed to determine if DMA(III) reacts with NADH or NADPH to form dimethylarsine. Because of the high DNA damaging potency of dimethylarsine, its production may be of biological significance, even if only small amounts are produced.

It has been suggested that individuals who are able to methylate arsenic most efficiently are at greater risk of genotoxic damage from ingested arsenic (*28*). The current work further supports that hypothesis with the additional consideration that those individuals that methylate arsenic beyond dimethylarsenic species to trimethylarsenic species and those individuals that produce arsines suffer greater risks of genotoxic damage.

To date, five arsenical compounds (MMA(III), monomethylarsine, DMA(III), dimethylarsine, and trimethylarsine) have been shown to be directly damaging to DNA in vitro. However, given the demonstrated high potency of arsines, one possibility to consider is that the apparent genotoxicity of MMA(III) and DMA(III) is due to arsine formation by chemical disproportionation (e.g., DMA(III) → dimethylarsine + DMA(V)). For instance, because dimethylarsine is at least 100-fold more potent than DMA(III), less than 1% of the DMA(III) would need to undergo disproportionation to the arsine to account for 100% of the DNA damaging activity of DMA(III). This hypothesis would be consistent with both DMA(III) (*29*) and dimethylarsine (*27*) acting via ROS. The relative importance of direct DNA damage by arsenicals (in particular dimethylarsine and trimethylarsine) relative to other proposed mechanisms of arsenic carcinogenesis (i.e., indirect mechanisms (*47*)) is yet to be established.

More study is needed to more fully understand the metabolic activation pathways of arsenicals, the chemical reaction mechanisms underlying arsenic carcinogenesis, and the association of key metabolites or intermediates with adverse health outcomes. Examination of known and proposed reactive arsenicals can play an important role in such analysis by clarifying issues of chemical and biological stability and reactivity that determine biological activity. Our results implicate arsines as highly reactive intermediates in arsenic metabolism that should be the subject of more focused investigation in physiologically relevant assays.

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