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Trivalent Arsenicals Are Bound to Proteins during Reductive Methylation

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Inorganic arsenic is converted to methylated metabolites, and most is excreted in urine as dimethylarsinic acid in humans and animals. The present study was conducted to investigate the metabolism of arsenic and identify hepatic and renal metabolites of arsenic after an intravenous injection of arsenite (0.5 mg As/kg body weight) in rats. Similar levels of arsenic were found in the soluble (SUP) and nonsoluble sediment (SED) fractions of both organs after 1 h. More than 80% of the SUP arsenic was bound to high molecular weight (HMW) proteins in both organs. Arsenic bound to the HMW and SED proteins were oxidized with H_2O_2 and released in the pentavalent forms (arsenate, monomethylarsonic, and dimethylarsinic acids). The relative ratios of the three arsenicals changed depending on organ, fraction (HMW and SED), and time. Since the arsenic metabolites/intermediates were liberated from proteins by oxidation with H_2O_2 and recovered in the pentavalent forms, and only tri- but not pentavalent arsenicals were bound to proteins in vitro, it was deduced that arsenic metabolites bound to proteins during the successive methylation pathway are in the trivalent forms; that is, successive methylation reaction takes place with simultaneous reductive rather than stepwise oxidative methylation. Thus, on the basis of the present observations, it was proposed that inorganic arsenic was successively methylated reductively in the presence of glutathione, rather than a stepwise oxidative methylation, and pentavalent arsenicals (MMA^V and DMA^V) were present as end products of metabolism, rather than intermediates. We also discussed the in vitro formation of dimethylthioarsenicals after incubating dimethylarsinous acid with liver homogenate.

Introduction

Inorganic arsenic (iAs), which includes arsenate (iAs^V)[1] and arsenite (iAs^{III}), can produce both acute and chronic toxicity (*1*−*7*). Toxicity is associated with the chemicals (inorganic arsenicals) exposed to the body and their metabolites leading to dimethylarsinic acid (DMA^V), the major urinary metabolite, and/or the related reactions (*7*−*11*). Inorganic arsenic is transformed to DMA^V through consecutive reduction and methylation reactions (*12*, *13*). The trivalent forms, rather than the pentavalent forms, are associated with toxicity (*14*−*17*); however, the precise metabolic pathway and mechanisms for formation of specific toxic metabolites are poorly understood.

Glutathione (GSH)-conjugated forms of trivalent arsenic, iAs^{III} and monomethylarsonous acid (MMA^{III}), are excreted in bile; however, biliary excretion occurs primarily at high doses. The major route of metabolism is conversion to dimethylarsinous acid (DMA^{III}), which is released into the bloodstream. The GSH-conjugated forms, arsenotriglutathione [$iAs^{III}(GS)_3$] and monomethylarsenodiglutathione [$MMA^{III}(GS)_2$], are excreted into the bile through the multidrug resistance protein 2/canalicular multispecific organic anion transporter (MRP2/*c*MOAT). This process results in enterohepatic circulation, which occurs during the metabolism of inorganic arsenicals (*18*−*20*). Although $iAs^{III}(GS)_3$ and $MMA^{III}(GS)_2$ were detected in the bile, dimethylarsenoglutathione [$DMA^{III}(GS)$] has not been detected in the bile (*21*, *22*). In addition, none of the GSH-conjugated forms have been detected in the liver (*18*−*23*). Nevertheless, the major portion of ingested arsenic is released into the bloodstream, mostly in the form of dimethylated arsenicals, which are taken up by red blood cells (RBCs), and then finally excreted in urine in the case of rats. Preferential accumulation of DMA^{III} in RBCs was attributed to hemoglobin binding (*24*, *25*). However, the precise mechanism leading to the formation of the dimethylated arsenical (DMA) and the transporter for excretion into the bloodstream have not been identified.

We proposed a procedure for analyzing arsenic metabolites by fractionating organs into soluble (SUP) and nonsoluble sediment (SED) fractions, as shown in Figure 1 (*8*). The SUP fraction (fraction A in Figure 1) is analyzed by HPLC−inductively coupled argon plasma mass spectrometry (ICP MS). Unfortunately, arsenic in the SED fraction cannot be analyzed by HPLC−ICP MS analysis, and arsenic species, such as methylated forms and their electronic charges, cannot be characterized when bound to proteins. Therefore, arsenic in the SED and soluble high molecular weight (HMW) proteins were oxidized to the corresponding pentavalent forms, which were liberated from proteins because pentavalent forms are not bound to proteins through thiol groups.

The present study was conducted to identify the arsenic metabolic pathway by speciating time-dependent changes in arsenic metabolites recovered in the liver and kidneys by focusing on arsenic metabolites bound to HMW and SED proteins (Figure 1). We examined the interaction of arsenicals with soluble and nonsoluble proteins in rat liver homogenate

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[1] Abbreviations: AsB, arsenobetaine; iAs^V, arsenate; iAs^{III}, arsenite; DMA, dimethylated arsenical; DMA^{III}, dimethylarsinous acid; DMA^V, dimethylarsinic acid; MMA, monomethylated arsenical; MMA^{III}, monomethylarsonous acid; MMA^V, monomethylarsonic acid; GSH, glutathione; $iAs^{III}(GS)_3$, arsenotriglutathione; $MMA^{III}(GS)_2$, monomethylarsenodiglutathione; $DMA^{III}(GS)$, dimethylarsenoglutathione; $DMMTA^V$, dimethylmonothioarsinic acid; $DMDTA^V$, dimethyldithioarsinic acid; DMTA, dimethylthioarsenicals; RBCs, red blood cells; HPLC, high-performance liquid chromatography; ICP MS, inductively coupled argon plasma mass spectrometry.

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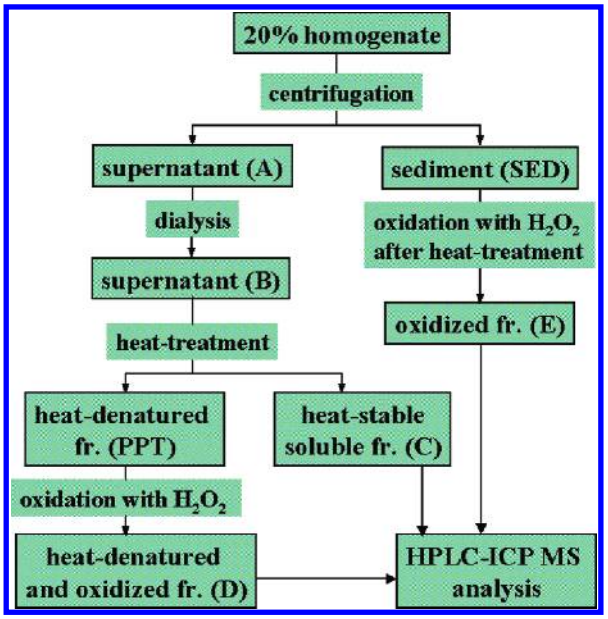

Figure 1. Procedures for the preparation of organ samples and speciation of arsenic. Arsenite was injected intravenously into rats at a dose of 0.5 mg As/kg body weight. Livers and kidneys were removed 5, 10, 30, and 60 min later and homogenized in 4 vol of the extraction buffer to obtain the supernatant (SUP) (A) and sediment (SED) fractions. The supernatant (A) was dialyzed to remove low molecular weight (LMW) constituents (MW < 10 000). The dialyzed supernatant (arsenic bound to high molecular weight (HMW) proteins) (B) was heat-treated at 72 °C for 30 min to denature proteins and to deactivate catalase, chilled in an ice−water bath, and then centrifuged to obtain the heat-stable soluble (C) and heat-denatured fractions (precipitate, PPT). The heat-denatured fraction (PPT) was oxidized with H_2O_2 to give the heat-denatured/precipitated/oxidized fraction (D). The sediment (SED) fraction of the homogenate was heat-treated to deactivate catalase and then oxidized with H_2O_2 to give the sedimented/heated/oxidized (E) fraction. The five fractions were subjected to speciation analysis by HPLC−ICP MS on a gel filtration GS 220 column (A and B fractions) and on an anion exchange ES 502N 7C column (C−E fractions). The distribution profiles of arsenic were depicted for the liver (Figure 3) and kidney fractions (Figure 4).

by incubating various arsenic species (pentavalent iAs^V; MMA^V and DMA^V; and trivalent iAs^{III}, MMA^{III}, and DMA^{III}).

Materials and Methods

Reagents. All reagents were of analytical grade. Milli-Q water (Millipore) was used throughout. Trizma HCl and Trizma Base were purchased from Sigma (St. Louis, MO). Hydrogen peroxide (30% v/v), sodium sulfide (Na_2S), concentrated sulfuric acid, nitric acid, hydrogen chloride, ammonium acetate, acetic acid, 28% ammonia solution, sodium arsenate, sodium arsenite, l-cysteine (Cys), and dimethylarsinic acid [$(CH_3)_2AsO(OH)$] (DMA^V) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Monomethylarsonic acid [$CH_3AsO(OH)_2$] (MMA^V) and arsenobetaine (AsB) were purchased from Tri Chemical Laboratories, Inc. (Yamanashi, Japan). The arsenic standard solution (1000 μg/mL) for ICP MS was purchased from SPEX CentiPrep (Metuchen, NJ). Stock solutions of all arsenic compounds (10 mM) were prepared in purified water, stored in the dark at 4 °C, and diluted daily prior to use.

Preparation of DMA^{III} and MMA^{III} from DMA^V and MMA^V, Respectively. DMA^{III} and MMA^{III} were prepared by reducing DMA^V and MMA^V, respectively, with 5 molar equivalents of l-cysteine in distilled water at 70 °C for 1.5 h or 90 °C for 1 h. The trivalent forms were confirmed by comparison of the respective retention times on a GS 220 gel filtration column by HPLC−ICP MS with those prepared from their iodide forms (*26*) in distilled water under nitrogen atmosphere.

Preparation of Dimethylthioarsenicals from DMA^V. Dimethylmonothioarsinic acid ($DMMTA^V$) was assigned by mistake as dimethylthioarsonous acid ($DMTA^{III}$) (*27*), and then revised based on X-ray analysis data (unpublished observations). The corrected structure was identical to published structures (*28*, *29*). $DMMTA^V$ was prepared by stepwise addition of concentrated H_2SO_4 to an aqueous solution of 38 mM DMA^V and 60 mM Na_2S at the final molar ratio of $DMA^V/Na_2S/H_2SO_4$ = 1:1.6:1.6, and the reaction solution was allowed to stand for 1 h. $DMMTA^V$ was extracted with ethyl ether, and then recrystallized from ethyl ether/methanol in an atmosphere of nitrogen.

Dimethyldithioarsinic acid ($DMDTA^V$) was prepared by stepwise addition of concentrated H_2SO_4 to an aqueous solution of 10 mM DMA^V and 75 mM Na_2S at the final molar ratio of $DMA^V/Na_2S/H_2SO_4$ = 1:7.5:7.5, and the reaction solution was allowed to stand for 1 day (*27*).

HPLC−ICP MS Analysis. The HPLC system consisted of a PU-610 liquid chromatograph solvent delivery pump and a DG 660B-2 degasser (GL Sciences Co., Tokyo, Japan). A polymer-based gel filtration column (Shodex Asahipak GS-220 HQ, 300 mm × 7.6 mm i.d., Showa Denko, Tokyo) with an exclusion limit of 3 kDa, and a silica-based gel filtration column (Shodex Asahipak Protein KW-803, 300 mm × 8.0 mm i.d., Showa Denko) with an exclusion limit of 170 kDa were used to separate low and high molecular weight constituents, respectively. A Shodex Asahipak ES-502N 7C anion exchange column (100 mm × 7.6 mm i.d., Showa Denko) was used to separate arsenic species in protein-free solutions. A 20 μL aliquot of a sample solution was applied to a column, and the column was eluted with 50 mM ammonium acetate buffer (pH 6.5 at 25 °C) or 15 mM citric acid buffer (pH 2.0 at 20 °C) for a gel filtration GS-220 HQ column or an anion exchange column, at the flow rate of 0.6 or 1.0 mL/min. A 200 μL aliquot of a sample solution was applied to a Protein KW-803 column, and the column was eluted with 50 mM Tris-HNO_3 (pH 7.4 at 25 °C). Arsenic in the eluate was monitored with an HP 4500 ICP MS (Yokogawa Analytical Systems, Hachiouji, Japan) at *m*/*z* 75. The signal at *m*/*z* 77 was also monitored to compensate the molecular interference by $ArCl^+$. On-line ICP MS data were processed with software developed in house.

Animal Experiments. Male Wistar rats (Clea Japan Co., Tokyo) were purchased at 5 weeks of age and housed in a humidity-controlled room maintained at 22−25 °C with a 12 h light−dark cycle. Rats were given free access to a commercial diet (CE-2; Clea Japan Co.) and tap water. After a 1-week acclimation period, rats weighed 160−180 g and were assigned to experimental groups. Three rats per group were injected intravenously into a tail vein with arsenite at a single dose of 0.5 mg As/kg body weight. Controls were injected with an equivalent volume of saline vehicle.

Whole Body Perfusion. Since arsenic accumulates preferentially in RBCs of rats in the form of DMA^{III}, blood was removed by whole body perfusion with phosphate-buffered saline (PBS, pH 7.4 at 25 °C) for 10 min at room temperature (*23*). Afterward, the livers and kidneys were excised and stored at −80 °C until use.

Preparation of Liver and Kidney Supernatants for HPLC−ICP MS Analysis. Fresh homogenates (20%, w/v) of livers and kidneys were prepared in 100 mM ammonium acetate solution (pH 6.5 at 25 °C, dissolved oxygen was purged by bubbling with 99.999% nitrogen gas) with a glass-Teflon homogenizer in an atmosphere of nitrogen, and then centrifuged at 105000*g* for 1 h at 4 °C to obtain the supernatant fraction (Figure 1, fraction A) and nonsoluble sediment fraction (Figure 1, SED). A 1.0 mL portion of each supernatant was dialyzed with a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) (cutoff molecular weight, MW < 10 kDa) in 500 mL of 100 mM ammonium acetate (pH 7.4) at 4 °C for 12 h to give a dialyzed fraction (Figure 1, fraction B). The dialysis buffer was changed every 6 h. The dialyzed supernatants (Figure 1, fraction B) were heat-treated at 72 °C for 30 min, chilled in an ice-bath, and centrifuged at 3000*g* for 10 min to obtain the heat-stable soluble (Figure 1, fraction C) and heat-denatured protein (precipitate) (Figure 1, PPT) fractions. After washing with PBS three times, the heat-denatured proteins (precipitate) were oxidized with 0.5 mL of 30% H_2O_2 at room temperature for 3 h to give an oxidized fraction (Figure 1, fraction D). The nonsoluble sediment

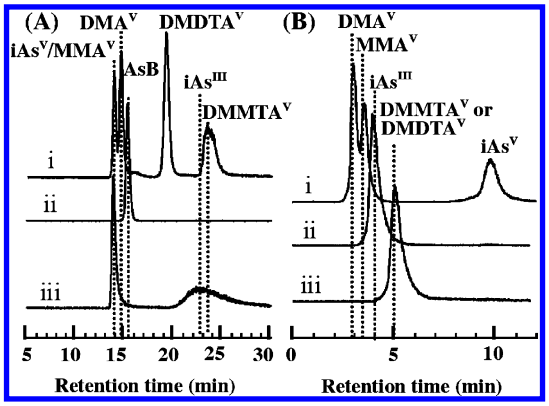

Figure 2. HPLC−ICP MS profiles of authentic arsenicals on gel filtration GS-220 HQ (A) and anion exchange ES-502 7N (B) columns. Solutions (20 μL) of authentic arsenite (iAs^{III}), arsenate (iAs^V), monomethylarsonic acid (MMA^V), dimethylarsinic acid (DMA^V), arsenobetaine (AsB), dimethylmonothioarsinic acid (DMMTA^V), and dimethyldithioarsinic acid (DMDTA^V) (0.1 μg/mL each) were subjected to HPLC−ICP MS analysis on gel filtration GS 220 (A) and anion exchange ES-502 columns (B) by elution with 50 mM ammonium acetate buffer (pH 6.5 at 25 °C) and 15 mM citric acid buffer (pH 2.0 at 20 °C) at the flow rate of 0.6 and 1.0 mL/min, respectively. Mixtures of the following authentic samples were eluted for each chromatogram: (A-i) MMA^V + DMA^V + DMDTA^V + DMMTA^V; (A-ii) AsB; (A-iii) iAs^V + iAs^{III}; (B−i) DMA^V + MMA^V + iAs^V; (B-ii) iAs^{III}; (B-iii) DMDTA^V + DMMTA^V.

fractions of rat liver and kidney homogenates (Figure 1, SED) were washed with PBS three times, heat-treated to deactivate catalase activity, and then oxidized with 0.5 mL of 30% H_2O_2 at room temperature for 3 h to give an oxidized fraction (Figure 1, fraction E). Arsenic metabolites in the nonsoluble (Figure 1, SED) and soluble HMW proteins (Figure 1, B) were determined as the corresponding oxidized arsenicals (iAs^V, MMA^V, and DMA^V) on an anion exchange column.

In Vitro Incubation System. To assay the affinity and reaction of arsenic compounds with liver constituents and to compare those with the arsenic distributions detected in vivo, pentavalent (iAs^V, MMA^V, and DMA^V) and trivalent (iAs^{III}, MMA^{III}, and DMA^{III}) arsenicals were incubated in a 20% (w/v) fresh rat liver homogenate (in 100 mM ammonium acetate solution, pH 7.0, at 25 °C) at the final concentrations of 0.1, 0.5, and 1.0 μg/mL at 37 °C for 30 min. The incubation mixture was centrifuged at 105000*g* at 4 °C for 1 h to give the supernatant (SUP) and sediment (SED) fractions. Arsenic in the SUP fraction was subjected to HPLC−ICP MS analysis on two gel filtration columns (polymer-based GS-220 HQ and silica-based gel Protein KW-803 gel filtration columns for arsenicals of LMW constituents and those bound to HMW proteins, respectively).

Determination of Arsenic Concentrations with ICP MS. Concentrations of arsenic in liver and kidney homogenates and in SUP and HMW proteins fractions were determined with an ICP MS (HP 4500) after wet-ashing with a mixed acid of concentrated nitric acid (HNO_3) and 30% H_2O_2 (v/v = 1/1).

Results

Trivalent arsenicals are readily oxidized and recovered quantitatively as pentavalent arsenicals, such as iAs^V, MMA^V, and DMA^V. Likewise, arsenic bound to proteins can be readily liberated by oxidation with H_2O_2 and recovered quantitatively as free pentavalent arsenicals. Therefore, arsenic in nonsoluble and soluble HMW proteins was oxidized with H_2O_2 to liberate them as soluble pentavalent forms before HPLC−ICP MS analysis (Figure 1). Since H_2O_2 is decomposed by catalase, catalase was deactivated by heat treatment.

We selected a gel filtration GS 220 HQ column for LMW arsenicals because most arsenicals were separated on this column, as demonstrated in Figure 2A). Because arsenate (iAs^V) and MMA^V eluted with identical retention times, they were separated on an anion exchange column (Figure 2B).

Hepatic and renal arsenic metabolites were separated into five fractions (A−E) as outlined in Figure 1, and arsenic species were determined using gel filtration (panels A and B in Figures 3 and 4) and/or anion exchange (panels C−E in Figures 3 and 4) columns. Figures 3A and 4A show that arsenic metabolites in the cytosolic fraction (fraction A in Figure 1) were mostly bound to HMW proteins (void volume) in both livers and kidneys, and minor LMW metabolites were assigned as iAs^V/

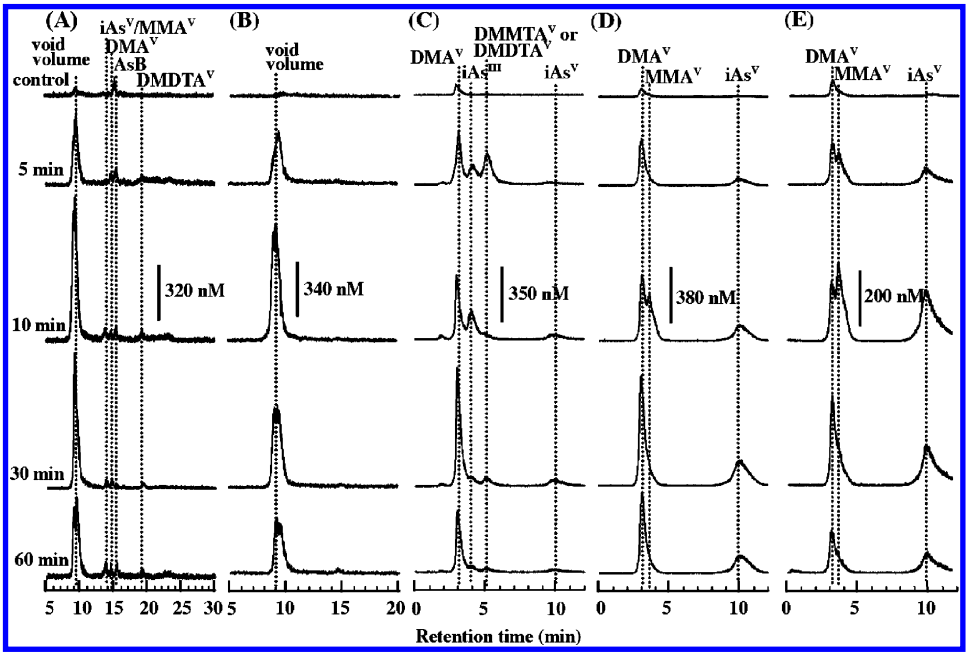

Figure 3. Changes in the distributions of arsenic on gel filtration and anion exchange columns by HPLC−ICP MS in various liver fractions with time after an intravenous injection of arsenite. The livers were excised and processed according to the procedure described in the legend to Figure 1, and subjected to HPLC−ICP MS analysis; fractions A and B, and C−E in Figure 1 on a gel filtration GS 220 column to separate arsenic metabolites into high (HMW) and low molecular weight (LMW) arsenic, and on an anion exchange column to further separate LMW arsenic, respectively. Panels A−E correspond to the fractions A−E in Figure 1, respectively. The vertical bar shows the detection level for arsenic.

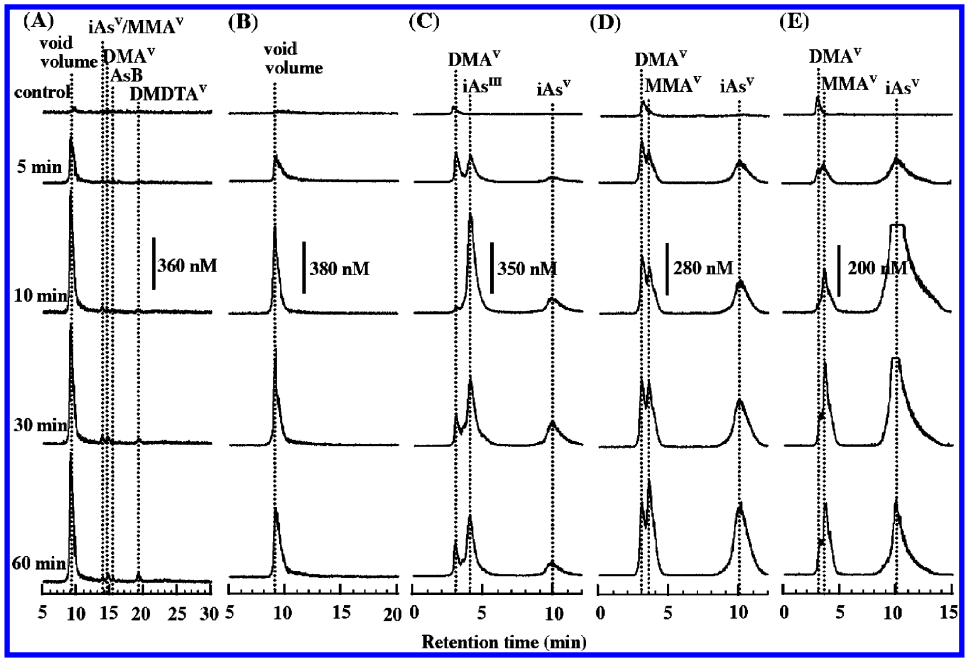

Figure 4. Changes in the distributions of arsenic on gel filtration and anion exchange columns by HPLC−ICP MS in various kidney fractions with time after an intravenous injection of arsenite. The kidneys excised and processed according to the procedure described in the legend to Figure 1 were subjected to HPLC−ICP MS analysis in the same manner as the livers in Figure 3.

MMA^V, DMA^V, and AsB. An arsenic peak corresponding to dimethylthioarsenicals (DMTA) was barely detected.

The minor metabolites in the soluble fraction in Figures 3A and 4A were removed by dialysis (Figures 3B and 4B). The arsenic species bound to HMW proteins were heat-treated to give heat-stable soluble (fraction C in Figure 1) and heat-denatured precipitate fractions (PPT); the latter fraction was oxidized to give fraction D in Figure 1 and recovered as the corresponding pentavalent arsenicals. Although the heat-treatment process was intended only to deactivate catalase, arsenic metabolites in fraction B (panels B in Figures 3 and 4) were separated into tightly and loosely bound arsenic metabolites by this heat treatment (fractions C and D in Figure 1, respectively).

Arsenic in fraction C (Figures 3C and 4C) gave different and curious distributions in the liver and kidneys; DMA^V was the major arsenic in the liver, and iAs^{III} and iAs^V were major arsenic in the kidneys. This curious observation has to be explained; that is, why tri- and pentavalent arsenicals were recovered in Fraction C. Since DMA^V and iAs^V were not present in fraction B (Figures 3B and 4B), we assumed that arsenicals were oxidized in parts during the dialysis and heat-treatment process. Namely, HMW proteins were oxidized during the dialysis process in the absence of GSH without oxidizing arsenicals, and then DMA^{III} and iAs^{III} were liberated from the oxidized proteins, and these free trivalent arsenicals were oxidized in parts during the heat-treatment process. Thus, the curious recovery of tri- and pentavalent arsenicals in fraction C may be explained. However, this assumption requires supporting data.

Arsenic in fraction D (Figures 3D and 4D) also gave different arsenic distributions between the two organs; levels of iAs^V and MMA^V were greater in the kidneys. These observations suggested that arsenic metabolites in the soluble fraction of liver and kidneys were present in the trivalent forms bound to HMW proteins, and these species changed with time depending on organs. It also indicated that methylation of arsenic took place more efficiently in the liver than in the kidneys, and that MMA^{III} was bound to proteins with greater affinity and tenacity than iAs^{III} and DMA^{III}.

The sediment (SED) fraction was first heated to deactivate catalase, and then oxidized with H_2O_2 to give fraction E (Figure 1). Arsenic species in fraction E (Figures 3E and 4E) indicated that arsenic in the nonsoluble sediment (SED) fraction was present as the three trivalent arsenicals (iAs^{III}, MMA^{III}, and DMA^{III}), and their relative ratios and concentrations changed over time, depending on the organ. Namely, arsenic in the SED fraction was present more in the iAs^{III} and MMA^{III} forms in the kidneys than in the livers (Figures 4E and 3E, respectively). These observations suggested that arsenite was bound to nonsoluble SED proteins and monomethylated (first methylation) in the SED fraction and then further methylated (second methylation) in the soluble HMW fraction or DMA^{III} was more readily bound to soluble proteins.

Concentrations of arsenic in homogenates, supernatant (fraction A), and HMW protein fraction (fraction B) were determined to assess the relative distributions of arsenic in the liver and kidneys, as shown in Figure 5. Arsenic was present in forms bound to nonsoluble SED proteins (50%) and soluble HMW proteins (40%). Arsenic recovered as soluble LMW arsenicals, such as arsenate, DMA^V, and $DMDTA^V$, was less than 15%.

The difference in the distribution of arsenic between tri- and pentavalent arsenicals was investigated in vitro by incubating the tri- and pentavalent arsenicals in a 20% liver homogenate (Figures 6 and 7). Although the minor arsenic peak at the void volume was detected at all doses of the three arsenicals, the three pentavalent arsenicals were recovered quantitatively in their original forms (Figure 6). A small but exceptional peak was detected at the retention time corresponding to $DMDTA^V$ after incubation with DMA^V (Figure 6C). This is probably because DMA^V was reduced to DMA^{III}, and then reacted with sulfane sulfur to produce $DMDTA^V$ (sulfuration), as observed by incubation with DMA^{III} in Figure 7.

In contrast to the pentavalent arsenicals, trivalent arsenicals bound to various proteins (Figure 7). Distributions of arsenic in the soluble fraction were determined on two gel filtration columns by HPLC−ICP MS after incubating trivalent arsenicals in a 20% homogenate. One column was a GS-220 column for

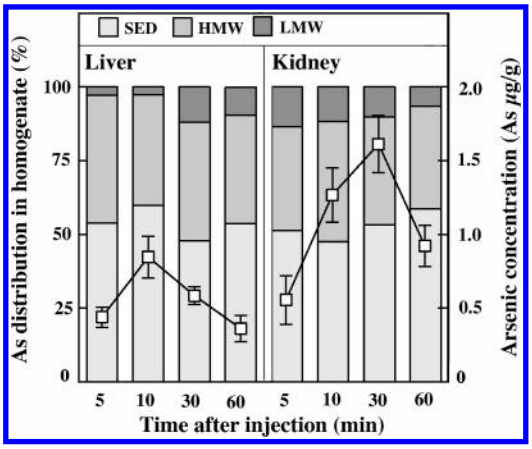

Figure 5. Time-related changes in the distribution ratios of arsenic in the liver and kidneys after an intravenous injection of arsenite. The liver and kidney homogenates (Hom) prepared from the rats ($n = 3$) injected with arsenite were fractionated into the supernatant (SUP) and sediment (SED) fractions as described in the legend to Figure 1. Arsenic in the supernatant (SUP) was processed further to give the high molecular weight (HMW) fraction by dialysis with a dialysis cassette in 50 mM ammonium acid buffer, pH 7.4 (500 mL), at 4 °C for 12 h, and the dialysis buffer was changed every 6 h. Arsenic concentrations in the Hom, SUP, and HMW were determined after acid digestion with HNO_3/H_2O_2, while those in the SED and LMW were calculated (Hom − SUP and SUP − HMW, respectively). Thus, arsenic in the Hom was fractionated into the supernatant (Sup − HMW (medium gray square) and Sup − LMW (dark gray square)) and SED (light gray square) fractions, and expressed as percentile distributions. The arsenic concentration in the Hom (open square symbol) was expressed as mean ± SD ($n = 3$).

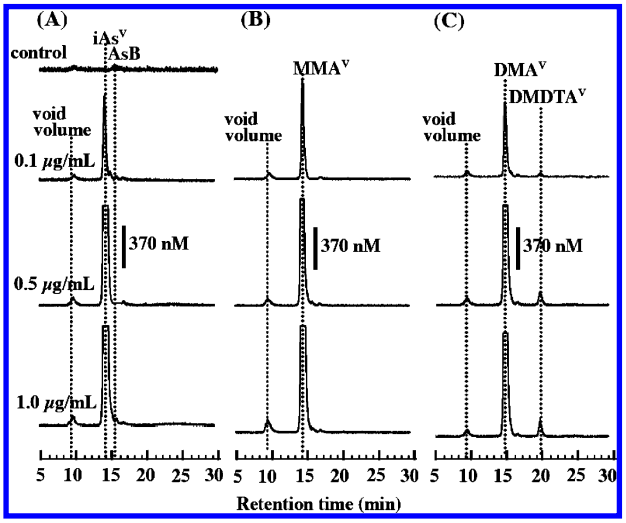

Figure 6. Arsenic distributions in the liver supernatant after incubation in vitro of pentavalent arsenicals in a liver homogenate on a gel filtration column by HPLC−ICP MS. The pentavalent arsenicals, iAs^V (A), MMA^V (B), and DMA^V (C) were incubated in vitro in a 20% liver homogenate at a final concentration of 0.1, 0.5, or 1.0 μg As/mL at 37 °C for 30 min, and then the homogenate was centrifuged at 105 000*g* for 1 h to give the supernatant. A 20 μL portion of the liver supernatant was subjected to HPLC−ICP MS analysis on a gel filtration GS 220 column. The vertical bar shows the detection level of arsenic.

separating LMW arsenicals (Figure 7A−C), and the other was a Protein KW-803 column for separating HMW proteins (Figure 7D,E). Arsenic derived from iAs^{III} was recovered mostly with HMW proteins (void volume) at the lowest dose, and the relative distribution to iAs^{III} (intact form) increased with dose (Figure 7A). After incubation with MMA^{III}, arsenic was also recovered mostly in the void volume (Figure 7B). However, compared with iAs^{III}, the original arsenical MMA^{III} (intact form) was not detected and the arsenic was bound to HMW proteins together

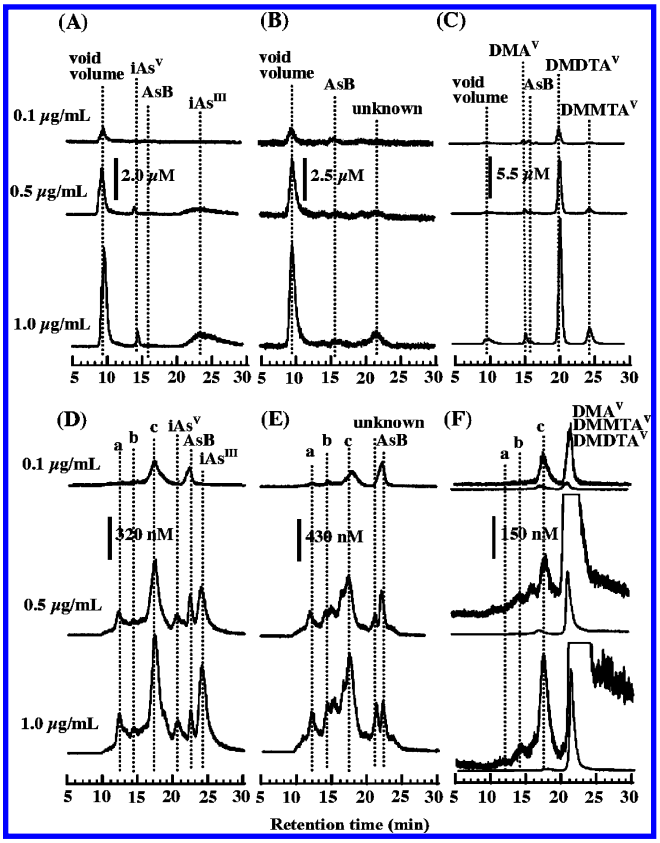

Figure 7. Arsenic distributions in the liver supernatant after incubation in vitro of trivalent arsenicals in a liver homogenate on two gel filtration columns by HPLC−ICP MS. The trivalent arsenicals, iAs^{III} (A and D), MMA^{III} (B and E), and DMA^{III} (C and F) were incubated in vitro in a 20% liver homogenate at a final concentration of 0.1, 0.5, or 1.0 μg As/mL at 37 °C for 30 min, and then the homogenate was centrifuged at 105 000*g* for 1 h to obtain the supernatant. A 20 (A−C) or 200 μL (D−F) portion of the supernatant was subjected to HPLC−ICP MS analysis on gel filtration GS 220 (A−C) and Protein KW-803 (D−F) columns to separate LMW arsenicals (A−C) and arsenicals bound to HMW proteins (D−F), respectively. Panels A, B, C, and F were drawn with two detection levels. The vertical bar shows the detection level of arsenic.

with several unidentified small peaks (Figure 7B), which suggested that MMA^{III} has a higher affinity for HMW proteins than iAs^{III}. In contrast to arsenic derived from iAs^{III} and MMA^{III}, arsenic derived from DMA^{III} produced distinct peaks that corresponded to the two dimethylthioarsenicals ($DMMTA^V$ and $DMDTA^V$) in Figure 7C. Thus, arsenic of DMA^{III} origin was recovered mostly in the soluble fraction, as shown in Figure 8, and only a small percentage was present in the form bound to proteins (Figure 7C).

The trivalent arsenicals bound to HMW proteins were separated on a Protein KW 803 column (Figure 7D−F). Each of the three trivalent arsenicals bound to HMW proteins was separated into three peaks (a−c), and the main peak c was the smallest in molecular size. These distributions were similar to those obtained from in vivo experiments (data not shown). However, we did not attempt to identify these proteins in the present study.

Figure 8 shows the quantitative distributions of arsenic in the liver homogenate after incubation with penta- and trivalent arsenicals. Although the three pentavalent arsenicals were recovered as their original arsenicals in the soluble LMW fraction, the trivalent arsenicals, iAs^{III} and MMA^{III}, were recovered mostly in the nonsoluble sediment (SED) fraction with a similar distribution to that of in vivo observation after injection

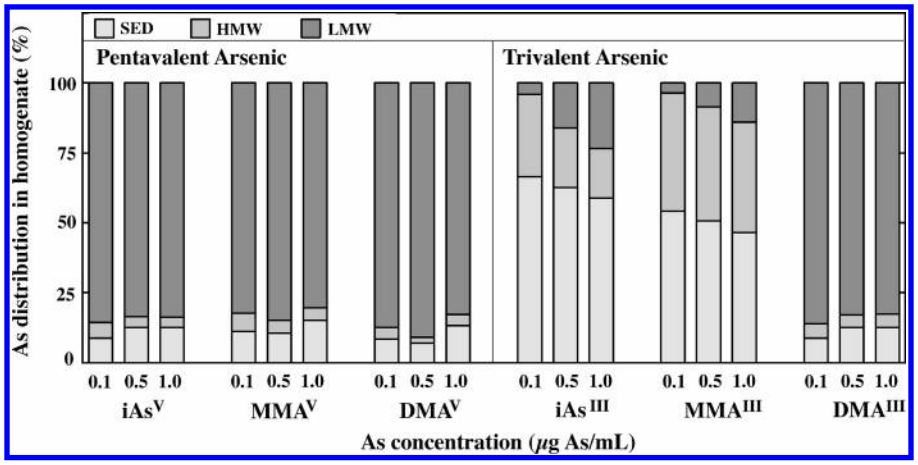

Figure 8. Distribution ratios of arsenic in the liver homogenate after incubation in vitro with penta- and trivalent arsenicals. Penta- and trivalent arsenicals (iAs, MMA, and DMA) were incubated in a 20% liver homogenate at a final concentrations of 0.1, 0.5, or 1.0 μg As/mL, and then the arsenic was fractionated to the supernatant (SUP) and sediment (SED (light gray square)) fractions. Arsenic in the SUP was further fractionated to Sup − HMW (medium gray square) and Sup − LMW (dark gray square) fractions. Arsenic in the three fractions was expressed as mean percentile distributions.

of iAs^{III}. In other words, approximately 90% of the dose was recovered in the nonsoluble SED fraction and soluble HMW proteins. A different distribution was observed after incubating DMA^{III} in a liver homogenate. This was similar to the distribution profiles on the two gel filtration columns in Figure 7C,D since arsenic was recovered in the soluble fraction in the form of two thioarsenicals, DMMTA^{V} and DMDTA^{V}, after incubation of DMA^{III} in a liver homogenate.

Discussion

Metabolites of inorganic arsenicals are either trivalent or pentavalent. Trivalent arsenicals are more reactive and readily bind to thiol groups found on proteins and GSH (*8*, *23*, *30*−*32*). As a result, they inhibit enzymes, such as glutathione reductase, glutathione peroxidases, thioredoxin peroxidase, and thioredoxin reductase (*33*−*37*). In the present study, arsenic metabolites were fractionated (Figure 1) by focusing on metabolites bound to soluble HMW and nonsoluble sediment (SED) proteins. The authentic standards required for our analysis were separated on two columns, and most arsenic peaks were identified. Although arsenic species bound to soluble HMW and nonsoluble SED proteins were identified in their oxidized forms (iAs^{V}, MMA^{V}, and/or DMA^{V}), their proteins were characterized only on a gel filtration column, and no further analysis was conducted.

The present study showed that arsenic found in the liver and kidneys was detected mostly in the forms bound to soluble and nonsoluble proteins. Free pentavalent arsenicals were present only as minor metabolites in both organs. Trivalent but not pentavalent arsenicals were bound to proteins (Figures 6 and 7), and protein-bound arsenicals were recovered quantitatively as pentavalent arsenicals by oxidation with hydrogen peroxide (Figures 3 and 4) as in the case of oxidation of trivalent arsenicals. Thus, protein-bound arsenicals in the soluble and nonsoluble proteins observed in the present study were deduced to be in the trivalent forms. Although this assumption can be supported by the observation that trivalent arsenicals bound to hemoglobin were trivalent forms by mass spectral data (*25*), definitive evidence may be required for arsenicals bound to general proteins.

The relative ratios among the three arsenic metabolites (iAs, MMA, and DMA) were variable depending on organs, soluble/nonsoluble proteins, and time after the administration. Although

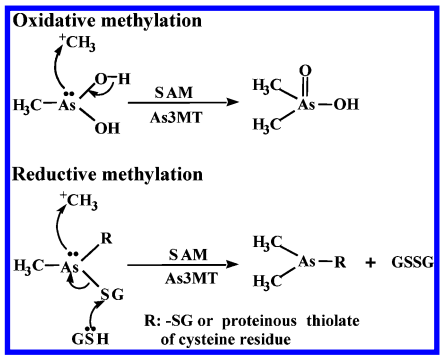

Figure 9. Reaction mechanisms for oxidative and reductive methylation.

some tendencies were observed such as a tendency to decrease with time after distribution in both organs for iAs in the sediment fraction (Figures 3E and 4E), an overall tendency to demonstrate the metabolism from iAs to MMA^{III} and then to DMA^{III} with time was not obvious in the present chromatograms in Figures 3 and 4. This is probably because of the presence of complex arsenic metabolites in each fraction reflecting the capacity of transformation reactions such as the first and second methylation reactions in different organs and subcellular fractions. However, several observations suggested that the metabolic transformation of arsenic takes place more efficiently in the liver than in the kidneys. Namely, although MMA was detected only at an early time point in the liver, it was detected at all time points, increasing over time in the kidneys.

Methylation reaction of arsenicals requires a methyl donor (SAM) and a reductant (GSH), and methylation occurs either oxidatively (oxidative methylation followed by reduction) or reductively (simultaneous methylation and reduction, i.e., reductive methylation). The reaction mechanisms for the two methylation reactions can be schematically presented, as shown in Figure 9. The oxidative methylation is initiated by the nucleophilic attack of an unshared electron pair of arsenic atom to methyl cation followed by the oxidation of arsenic atom. Thus, oxidative methylation occurs on a free trivalent arsenical ($As^{III}(OH)_3$ or $CH_3As^{III}(OH)_2$). The following reduction of the methylated arsenical takes place independently from the oxidative methylation. On the other hand, the reductive methylation is initiated by the transfer of an unshared electron pair of arsenic atom to methyl cation with simultaneous reduction of the arsenic atom by conjugated GSH. $As(R)_n(GS)_m$ conjugates cannot form

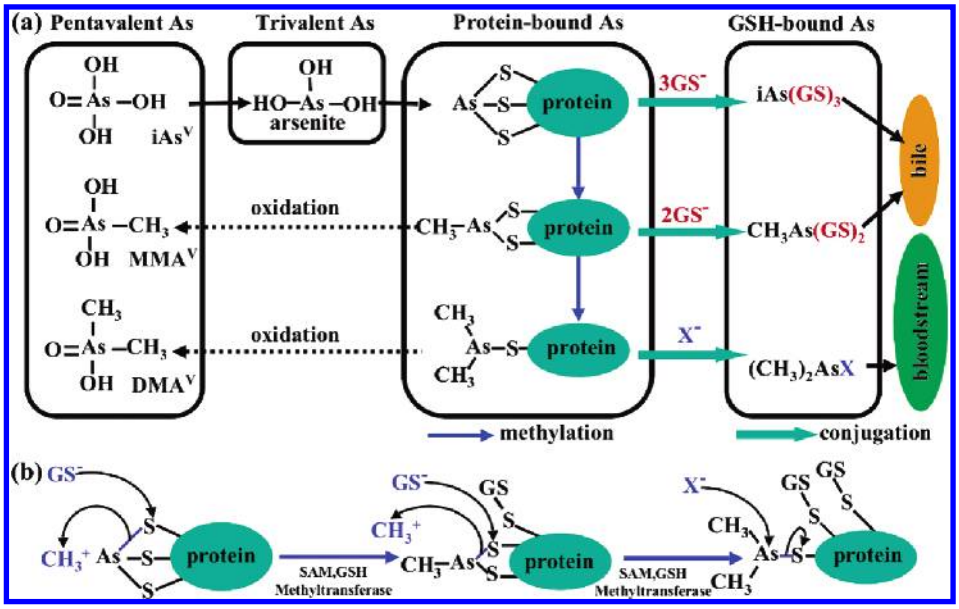

Figure 10. Proposed metabolic pathway for arsenic. Arsenite taken up by the liver is bound to nonsoluble and soluble proteins through thiol groups on a protein or GSH. Methylation takes place on arsenic bound to proteins in the presence of *S*-adenosylmethionine (SAM) and glutathione (GSH). In other words, methylation and reduction take place simultaneously in the presence of SAM and GSH, not separately with oxidative methylation followed by reduction. Arsenic bound to proteins can be liberated from proteins by conjugation with GSH to form $iAs(GS)_3$ and $CH_3As(GS)_2$. However, the major route for arsenic metabolism takes place consecutively by reductive methylation to produce $(CH_3)_2As$-protein, and dimethylated arsenic is liberated by the attack of a nucleophile (X^-) to produce $(CH_3)_2AsX$. One of $(CH_3)_2AsX$ may be the form for excretion into bloodstream. On the other hand, sulfide (sulfane sulfur) can be a nucleophile to produce trivalent dimethylthioarsenical (DMTA) [$(CH_3)_2$-AsSH], and then it is transformed to pentavalent dimethylthioarsenicals ($DMMTA^V$ and $DMDTA^V$) [$(CH_3)_2As(S)OH$ and $(CH_3)_2As(S)SH$, respectively].

pentavalent species as intermediates during methylation reactions because of reductive ability of GSH, but do form methylated trivalent species directly. Another ligand (shown in the scheme as -R) could be -SG or proteinous thiolate of cysteinyl residue in proteins, but proteinous cysteine is more preferable because of its affinity to trivalent arsenic species. Although the enzymes for each reduction and methylation reaction were identified by Aposhian's group (*12*, *38*−*40*), and MMA^V was incorporated and transformed to DMA in rats (*23*), Hayakawa et al. (*41*) demonstrated that $MMA^{III}(GS)_2$ was methylated, but free MMA^{III} was not methylated by Cyt 19 (AS3MT). As trivalent arsenicals were not methylated in the absence of a reductant (*41*) and arsenic metabolites were bound to proteins in the trivalent forms, the oxidative methylation does not occur in the methylation mechanism in Figure 9. Instead, from these two observations, it can be deduced more reasonably that arsenic is reductively methylated consecutively in the forms bound to proteins.

Dimethylthioarsenicals (DMTA) can be detected in vivo in the liver of rats given DMA^V and MMA^V (*23*). DMTA can be prepared chemically by reacting DMA^V with hydrogen sulfide or by incubating it in a reaction medium that generates hydrogen sulfide, such an acidified sodium sulfide solution (*27*). From the chemical and biological properties, we identified two DMTA metabolites as dimethylthioarsonous acid ($DMTA^{III}$) and dimethyldithioarsinic acid ($DMDTA^V$) (*27*). However, after precise re-examinations of the two DMTA, including X-ray crystallographic determination, we agreed with published structures (*28*, *29*). Since DMTA can be prepared only with trivalent DMA (DMA^{III} or a DMA^{III}-generating system) and a sulfide source, $DMTA^{III}$ was assumed to be the direct reaction product of DMA^{III} and sulfide. However, $DMTA^{III}$ was never detected, and the reaction products were pentavalent monothiolated ($DMMTA^V$) and dithiolated ($DMDTA^V$) arsenicals. Their relative ratio changed depending on the reaction conditions, and $DMMTA^V$ was transformed to $DMDTA^V$ in the presence of sufficient sulfide.

The most significant difference between in vivo and in vitro experiments was the subcellular distribution of dimethylated arsenicals. Dimethylated arsenicals of arsenite origin (in vivo) were bound mostly to the nonsoluble SED and soluble HMW proteins (Figures 3 and 4). In contrast, DMA^{III} incubated in the liver homogenate (in vitro) was recovered mostly from the soluble fraction in the form of thioarsenicals ($DMMTA^V$ and $DMDTA^V$) (Figure 7). This difference may be explained as follows: DMTA can be produced in the presence of trivalent (but not pentavalent) dimethylarsenical (DMA^{III}) and sulfane sulfur. DMA^{III} can be produced metabolically after an injection of iAs^{III}, and can be present as free DMA^{III}, GSH-conjugated DMA^{III} ($DMA^{III}(GS)$), and/or protein-bound DMA^{III} (protein-S-DMA^{III}) (in the order of decreasing reactivity). On the other hand, sulfane sulfur may be present in the form of free HS^-, proteinous R−S−SH, and/or trisulfide R−S−S−S−R (in the order of decreasing reactivity), the three forms being convertible depending on the availability of GSH. As observed in the present experiments (Figures 3 and 4), DMA^{III} of iAs^{III} origin was present mostly in the protein-bound forms and reacted poorly with any forms of sulfane sulfur. However, when DMA^{III} was supplied in the form of reactive free DMA^{III}, as in the case of the present in vitro experiment (Figure 7), it seemed to react with all forms of sulfane sulfur to produce DMTA. Thus, as free DMA^{III} was incubated in a liver homogenate in vitro, it reacted readily with sulfane sulfur, resulting in the production of DMTA ($DMMTA^V$ and $DMDTA^V$) (Figure 7). On the other hand, in an in vivo experiment, protein-S-$As(CH_3)_2$ was produced in situ from iAs^{III} (protein-S-iAs^{III}). Since protein-S-$As(CH_3)_2$ conjugate is less reactive than the free or GSH-conjugated form, it remains in the form bound to soluble and nonsoluble proteins without reacting with sulfane sulfur.

Although we did not study further in the present experiments, the three arsenic-binding proteins (a−c in Figure 7D−F) seem to be similar to or identical with those reported by several groups; Vahter and Marafante (*30*) reported three arsenic-binding proteins in the mice and rabbit liver and kidney cytosols

in vivo and in vitro on a Sephacryl S-200 column. Aposhian's group (*31*) also detected three arsenic-binding proteins of >2000, 450, and 100 KDa on a Sepharose 4B-CL column from rabbit liver cytosols. Styblo and Thomas (*32*) detected three arsenic-binding proteins of >1000, 135, and 38 KDa on a Sephacryl S-300 column from rat liver cytosols. Although gel filtration columns used in the three research groups were different from each other and also from the present column, these three arsenic-binding proteins seem to be similar or identical.

On the basis of the present observations and proposed schemes, arsenic administered in the form of arsenite was explained to be metabolized as schematically drawn in Figure 10. Namely, arsenite taken up by organs (liver) is conjugated with soluble and nonsoluble proteins, and then methylated reductively in the presence of SAM by the conjugation of −SH groups (GSH) and/or −SH groups of cysteinyl residues on proteins (reductive methylation) to produce mono- (first methylation) and dimethylated arsenicals (second methylation) consecutively. This reductive methylation mechanism seems to explain the mechanism proposed by Hayakawa et al. (*41*) and Aposhian and Aposhian (*42*). The second methylation reaction seems to be rate-limiting, and excessive doses may produce $iAs^{III}(GS)_3$ and $MMA^{III}(GS)_2$ and induce their excretion into bile (*9*, *18*−*20*). iAs^{V} and MMA^{V} may not be present as intermediates but as end products of metabolism. When DMA^{III} is liberated from proteins in the forms of free DMA^{III} and/or $DMA^{III}(GS)$, they seem to be reacted with sulfane sulfur producing dimethylthioarsenicals ($DMMTA^{V}$ and $DMDTA^{V}$). DMA^{III} bound to proteins is assumed to be excreted into the bloodstream as the major route for the excretion of arsenic from the liver by the participation of an unknown nucleophile (X^{-}). However, the precise identity of X in this excretion form ($((CH_3)_2AsX$) remains to be clarified. Nevertheless, before being excreted from the liver or other organs, the present study showed that arsenic is bound to proteins in the form of DMA^{III} as the stable and less reactive form.

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