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Metabolism of dimethylarsinic acid in rats: production of unidentified metabolites *in vivo*

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Our previous study revealed that two unidentified metabolites, M-1 and M-2, were excreted in urine after long-term oral administration of dimethylarsinic acid (DMA), the main metabolite of inorganic arsenic. In the present study, we attempted to clarify the mechanism of production of these unknown metabolites. Male F344/DuCrj rats were administered a single dose of DMA (50 mg kg^{-1}) orally or intraperitoneally with or without pretreatment with L-buthionine-SR-sulfoximine (BSO), which inhibits glutathione (GSH) synthesis. Urine was collected by forced urination at various time points after administration of DMA. Arsenic metabolites in urine were analyzed by ion chromatography with inductively coupled plasma mass spectrometry (IC–ICP-MS). The unidentified metabolites M-1 and M-2 were excreted later than elimination of DMA and trimethylarsine oxide (TMAO). GSH depletion decreased in TMAO elimination, suggesting that GSH plays important roles in the methylation of DMA to TMAO in rats. There was no difference in the amount of production of either M-1 or M-2 between BSO-pretreated rats and controls, suggesting that M-1 and M-2 cannot be formed during methylation in the liver. The amounts of elimination of M-1 and M-2 were less after intraperitoneal administration than after oral administration.

Male F344/DuCrj rats were given 100 mg As l^{-1} DMA via drinking water for 20 weeks. Urine and feces were collected forcibly and were analyzed by IC–ICP-MS. A new unidentified metabolite, M-3, was detected only in feces as a metabolite of DMA after 20 weeks exposure to DMA, although M-1 and M-2 were found in both urine and feces. The unidentified metabolites M-1, M-2, and M-3 were excreted mainly as fecal metabolites along with unmetabolized DMA. This finding also suggests that M-1, M-2, and M-3 might be produced in the intestinal tract.

Keywords: dimethylarsinic acid; arsenic; metabolism; urine; feces; rats; microorganism; unidentified metabolite; IC-ICP-MS

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INTRODUCTION

Arsenic is widely distributed in water, air, and soil. There are several different forms of arsenic in the environment. The physical, chemical and toxicological properties of the various arsenic compounds depend on their chemical forms. Several epidemiological studies have indicated that long-term ingestion or inhalation of arsenic can increase the risk of development of skin, lung, bladder, kidney, and liver cancers.[1,2]

Dimethylarsinic acid (DMA) is used as a silvicide, a nonselective herbicide and a cotton defoliator.[3] Humans may be exposed to DMA by ingestion of food that has been contaminated with DMA or by certain types of seaweed that contain DMA naturally.[4] DMA is the major urinary metabolite of inorganic arsenic.[5–8] Most mammals

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methylate inorganic arsenic to methylarsonic acid (MMA) and DMA. *In vitro*[9–12] and *in vivo* experiments[13,14] have shown that methylation of arsenic takes place mainly in the liver, that reduced glutathione (GSH) is required for the reductive mechanism of arsenic methylation, and that *S*-adenosylmethionine is the donor of methylated groups to arsenic in its trivalent oxidation state.

In general, the acute toxicity of organic arsenic compounds is much lower than that of inorganic arsenic.[15,16] Methylation can be considered a mechanism of detoxification of arsenic, since it renders arsenic less reactive to tissue and, therefore, facilitates its elimination from the body.[17] However, whether methylation is a mechanism of detoxification for inorganic arsenic is becoming increasingly controversial, since the evidence for decrease in the toxicity of methylated arsenic compounds compared with inorganic arsenic is limited to findings related to acute toxicity such as the LD50.[18] The carcinogenic potential of DMA in rats was recently demonstrated by our group,[19–22] although little evidence has been obtained of the carcinogenicity of inorganic arsenic in animals. Yamamoto *et al.*[19] reported that 24 weeks exposure to DMA promoted carcinogenesis of urinary bladder, kidney, liver and thyroid gland in F344 rats at concentrations of 50, 100, 200, and 400 ppm in drinking water after initiation with a carcinogen(s). Li *et al.*[20] revealed that DMA, at a level of 100 ppm, has promoting effects on urinary bladder carcinogenesis even in NBR rats. Wanibuchi *et al.*[21] indicated that DMA showed promoting activities in carcinogenesis of the urinary bladder in F344 rats in a dose-dependent manner from a dose of 10 ppm in drinking water. A recent study by Wei *et al.*[22] demonstrated that long-term p.o. administration of DMA at levels of 50 and 200 ppm induced urinary bladder carcinomas in F344 rats. These findings indicated that DMA or its metabolites are a promoter or a carcinogen in rats and may provide clues to the carcinogenic mechanism of arsenic in humans.

The excretion and tissue distribution of arsenic in rats have been reported to differ from that in many other species, including humans. Arsenic was found to accumulate in the red blood cells of rats after exposure to inorganic arsenic[23,24] or DMA.[25,26] However, there are no major differences between rats and other species in the ratio of urinary methylated arsenic metabolites following exposure to inorganic arsenic.[23] Thus, Rowland and Davies[24] suggested that the biotransformation of arsenic in the rat is similar to other animals and may serve as an appropriate model for human metabolic studies.

The proportion of urinary arsenic species is considered a reliable indicator of metabolism in mammals.[27] In addition, the carcinogenic effects on the urinary bladder in rats were reported.[19–22] Thus, for a better understanding of the mechanism for urinary bladder carcinogenecity of DMA, it is important to investigate the urinary elimination in rats chronically exposed to DMA.

The metabolism of arsenic compounds is influenced by duration of administration. Recently, our studies[28] of long-term exposure of rats to arsenite (As(III)), MMA, DMA or trimethylarsine oxide (TMAO) demonstrated that chronically exposed rats had altered patterns of urinary excretion of arsenic species, with long-term exposure decreasing the proportion of TMAO in urine and increasing that of DMA. Two unidentified metabolites were also detected in urine following long-term exposure to arsenic species; the amounts of these metabolites increased in the order DMA > MMA > TMAO, with only small quantities of these detected in the As(III)-treated group. In a previous study, it was reported that DMA was further methylated to TMAO to a small extent in mice and hamsters, with TMAO being the ultimate methylated metabolite.[29,30] However, it has been shown that further methylation of TMAO to tetramethylarsonium ion (TeMA) does occur to a slight extent following long-term exposure to arsenic.[28]

The possibility that some metabolism of arsenicals takes place in the gut of animals, with the involvement of associated microorganisms, cannot be entirely ruled out. Cullen *et al.*[31] showed that homogenates of mouse ceca, sites of high microbiological activity, methylated methylarsine oxide to dimethylarsinate and demethylated it to arsenate. It is possible that the microflora present in the ceca are responsible for methylation or demethylation of arsenic compounds. In previous studies, no evidence was obtained that demethylation of DMA to inorganic arsenic occurs to any significant extent *in vivo*.[25,26,29,30] However, we recently reported that demethylation of DMA or TMAO occurred in rats after long-term exposure.[28]

In efforts to extend the findings of earlier studies on the metabolism of arsenic and to elucidate the biotransformation of arsenic, special attention should be paid to the analytical methods used to characterize methylated arsenic compounds in urine. Ion chromatography with inductively coupled plasma mass spectrometry (IC–ICP-MS) permits examination of the pattern of elimination

and concentrations of several urinary arsenic metabolites simultaneously and at low concentrations.[32,33] The IC–ICP-MS methodology eliminates analytical artifacts and enables measurement of unknown metabolites.

The purpose of the present study was to find the ultimate carcinogenic chemicals after long-term administration of DMA in rats. A further objective of the present study was to clarify the mechanism of production of the unknown metabolites after administration of DMA.

MATERIALS AND METHODS

Reagents

Sodium arsenite, sodium arsenate, MMA, DMA, TMAO, tetramethylarsonium iodide, and arsenobetaine (AsBe), used for analytical standard solutions and with purities of at least 99.99%, were obtained from Tri Chemical Lab. (Yamanashi, Japan). DMA for administration, with a purity of at least 99.9%, was obtained from Wako Pure Chemical Industry (Osaka, Japan). The purity of DMA was confirmed by IC–ICP-MS. DMA for administration contained no other arsenic compounds as impurities. L-Buthionine-SR-sulfoximine (BSO) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Other chemicals (analytical grade) were also from Wako Pure Chemical Industry (Osaka, Japan).

Animals

Five-week-old male F344/DuCrj rats to be administered a single dose of DMA were obtained from Charles River Japan (Hino, Japan) and allowed to acclimate for 1 week. Five-week-old male F344/DuCrj rats to undergo 20 weeks exposure to DMA were obtained from Charles River Japan (Hino, Japan) and allowed to acclimate for 4 weeks. Rats were housed five per box cage with wood-chip bedding and provided with a standard diet (CE2, Clea Japan, Tokyo, Japan) and water *ad libitum*. The room was kept on a 12/12 h light/dark cycle at a temperature of 23 ± 1 °C.

Arsenic metabolites following a single administration of DMA in rats depleted of GSH

Treated rats were injected intraperitoneally 2 h prior to the administration of DMA with BSO, which inhibits GSH synthesis, in distilled water at a dose of 4 mmol kg^{-1} body weight. The treated and untreated rats (seven rats per group) were given DMA orally or intraperitoneally at a dose of 50 mg kg^{-1} body weight. Urine was collected by forced urination at 0, 2, 4, 6, 8, 10, 24 and 48 h after administration. The urine samples were centrifuged to remove particulate materials and stored at −20 °C until analysis.

Arsenic metabolites following 20 weeks oral administration of DMA

Treated rats (ten rats per group) were given 100 mg As l^{-1} DMA in drinking water. For ten control rats, untreated water was given. Urine and feces were collected forcibly in the morning after 20 weeks of treatment. The urine samples were centrifuged to remove particulate materials and stored at −20 °C until analysis. The fecal samples were also stored at −20 °C until analysis.

Preparation for analysis of feces

25 mg of feces were placed in a polypropylene tube and mixed with 0.5 ml of 0.05 M ammonium acetate. The samples were sonicated for 30 min. After centrifugation, the supernatants were ultrafiltered using Ultrafree-MC (Millipore, MA, USA) with a cut-off value of 10 000. The filtrate was stored at −20 °C until analysis.

Instrumentation

A model HP4500 ICP-MS (Hewlett-Packard, DE, USA) was used for arsenic-specific detection. The operating conditions for ICP-MS were established in accordance with those reported by Inoue *et al.*[32] The ion chromatograph was a model IC7000 from Yokogawa Analytical Systems (Tokyo, Japan). For separations of arsenic compounds, two separation modes, cation- and anion-exchange, were used. The cation-mode experiment, using a Showdex NN-614 column (150 mm × 4.6 mm i.d.) packed with cation-exchange resin (Showadenko, Tokyo, Japan), was performed under the following conditions: mobile phase 5 mM HNO_3–6 mM NH_4NO_3, flow rate 0.8 ml min^{-1}, and injection volume 50 µl. The anion-mode experiment, using two Excelpack ICS-A35 columns (150 mm × 4.6 mm i.d.) packed with polymer-based hydrophilic anion-exchange resin (Yokogawa Analytical Systems), was performed under the following conditions: mobile

phase 0.01 M tartaric acid, flow rate 1 ml min^{-1}, and injection volume 50 µl. An outlet from the separation column was connected directly to the nebulizer of the ICP-MS using an ethylenetetrafluoroethylene (ETFE) tube of 0.3 mm i.d.

IC–ICP-MS analysis

Measurements using IC–ICP-MS were performed by the method established by Inoue *et al.*[32] with modifications. Stock standard solutions of sodium arsenite, sodium arsenate, MMA, DMA, TMAO, tetramethylarsonium iodide, and AsBe were prepared by dissolving each compound in pure water at a concentration of 100 mg As l^{-1}. The final diluted aqueous standard mixtures were prepared from each stock standard just before use. The urine samples were thawed and diluted 20–50 times with distilled water just before measurement by IC–ICP-MS. The samples and standards were injected into the ion chromatograph using a 50 µl loop. In order to obtain precise measurements, 1 mg l^{-1} of germanium solution was used as the internal standard for ICP-MS; the internal standard was added to the eluate from IC through a mixing joint prior to introduction to the ICP mass spectrometer.[28] The ICP-MS detection mass was set to *m/z* 75 ($^{75}As^{+}$), *m/z* 72 ($^{72}Ge^{+}$), and *m/z* 77 ($^{40}Ar^{37}Cl$). The ion intensity at *m/z* 77 was of diagnostic value only in the examination for possible occurrence of $^{40}Ar^{35}Cl^{+}$ interference at *m/z* 75. The reproducibility (RSD) for 0.01 mg As l^{-1} standard arsenic compound was about 2%. The amounts of each arsenic peak appearing on the chromatogram were summed as total arsenic. The detection limits for all arsenic species in urine and feces were set at 0.005 mg As l^{-1}.

RESULTS

Urinary excretion after a single administration of DMA in rats depleted of GSH

We tested whether GSH influences the *in vivo* biotransformation of DMA. The animals were pretreated with BSO to decrease the hepatic GSH content before a single p.o. or i.p. administration of DMA. Figures 1 and 2 show the time course of urinary elimination of DMA, TMAO, and two unidentified metabolites, M-1 and M-2, following oral administration of DMA with or without BSO

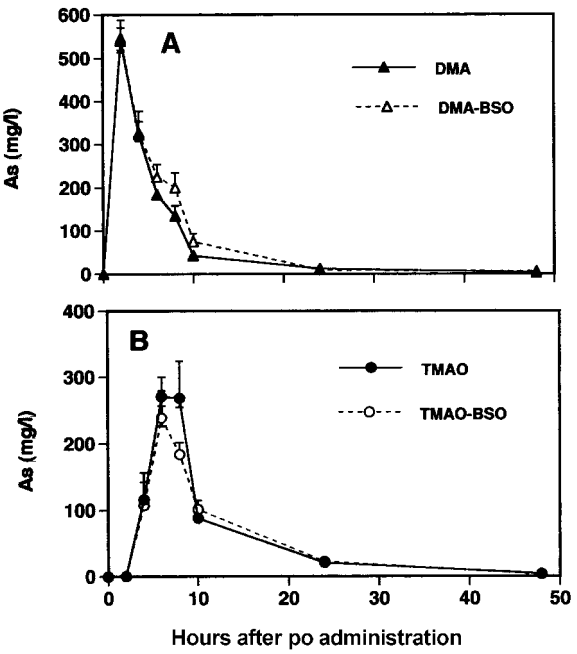

Figure 1 Excretion of (A) DMA and (B) TMAO in urine after a single 50 mg kg^{-1} oral administration of DMA with or without BSO pretreatment.

pretreatment. Most elimination of unchanged DMA occurred within 10 h, with peak elimination between 0 and 2 h in both the BSO-pretreated rats and control rats without pretreatment with BSO. There was no significant difference in DMA elimination between BSO-pretreated rats and controls. Elimination of the methylated metabolite, TMAO, occurred after 2 h, with peak elimination between 6 and 8 h in both groups of animals. The amount of TMAO elimination was lower in BSO-pretreated animals than in controls. A small amount of MMA elimination was detected between 2 and 4 h in both groups (data not shown). Urinary elimination of the unidentified metabolite M-1 occurred after 4 h in controls, with peak elimination at 8 h, but occurred after 2 h with peak excretion at 8 h in the BSO-pretreated rats. Elimination of the unidentified metabolite M-2 appeared after 2 h with peak elimination at 10 h in both groups. The amount of M-2 elimination increased or decreased more slowly compared with the others. The amounts of urinary M-1 and M-2 elimination were slightly higher in the BSO-pretreated group than in controls.

Rats were also given an i.p. dose of DMA with or without pretreatment of BSO. Figures 3 and 4 show

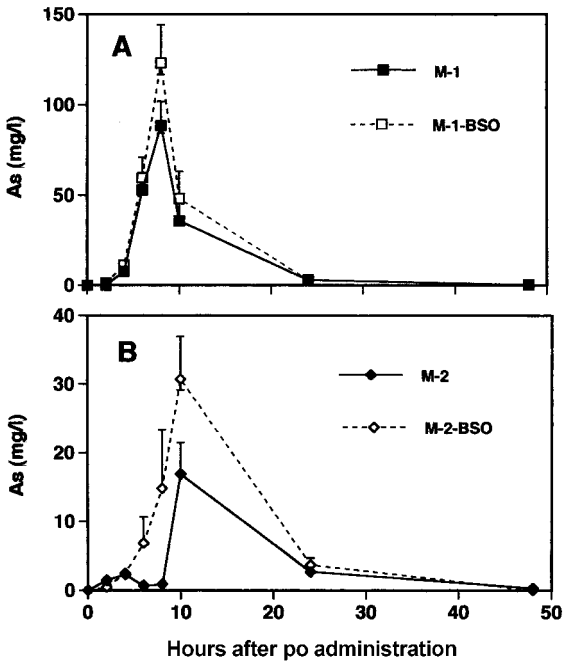

Figure 2 Excretion of unidentified arsenic metabolites, (A) M-1 and (B) M-2, in urine after a single 50 mg kg^{-1} oral administration of DMA with or without BSO pretreatment.

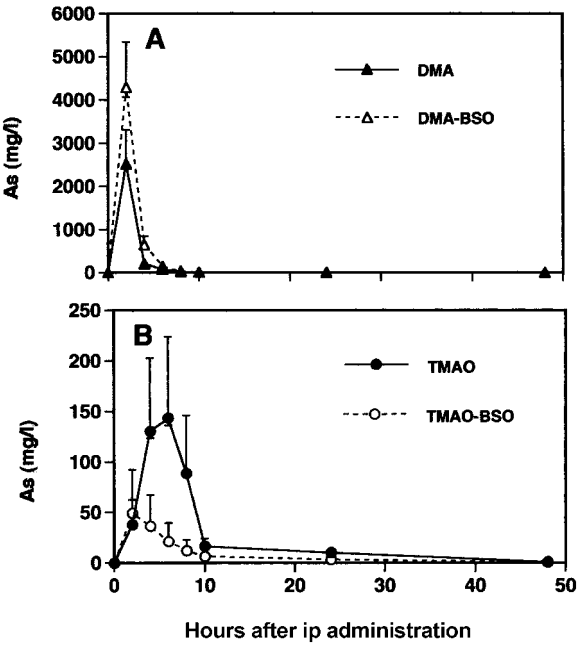

Figure 3 Excretion of (A) DMA and (B) TMAO in urine after a single 50 mg kg^{-1} intraperitoneal administration of DMA with or without BSO pretreatment.

the effect of a single i.p. administration of BSO 2 h before a single i.p. dose of DMA on the urinary excretion of arsenic metabolites. In the BSO-pretreated rats and controls, DMA was excreted mainly as unchanged DMA during the first 4 h and a rapid decrease in the DMA fraction occurred, but this was followed quickly by a progressive increase in TMAO, M-1, and M-2 elimination. Urinary elimination of M-2 was biphasic, with two peaks at 2 h and 10 h. The amount of DMA excreted at 2 h after i.p. administration was about twice as high in BSO-pretreated animals as in controls. In contrast, TMAO excretion was much greater in the controls than in the BSO-pretreated group between 4 and 24 h. There was a wide variety of amounts of M-1 and M-2 elimination, with no apparent difference in excretion of M-1 and M-2 between the two groups.

Urinary and fecal excretion following 20 weeks administration of DMA

It is possible that a portion of metabolism of arsenic compounds takes place in the gut. We therefore compared arsenic metabolites in urine with those in feces after 20 weeks administration of DMA. The

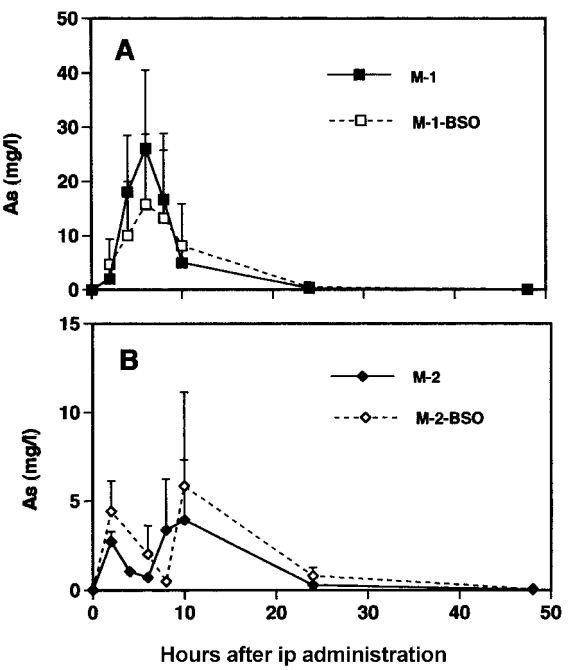

Figure 4 Excretion of unidentified arsenic metabolites, (A) M-1 and (B) M-2, in urine after a single 50 mg kg^{-1} intraperitoneal administration of DMA with or without BSO pretreatment.

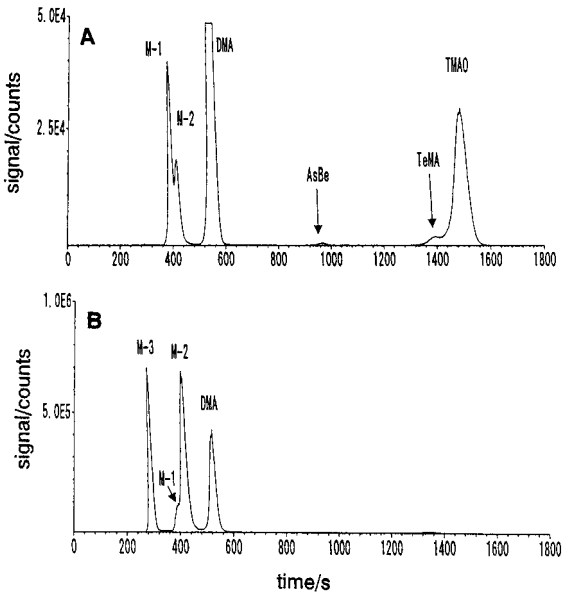

Figure 5 Cation-exchange IC–ICP-MS chromatograms of (A) urine and (B) feces after 20 weeks administration of DMA. Column: NN-614 (150 mm × 4.6 mm i.d.); mobile phase: 5 mM HNO_3–6 mM NH_4NO_3; flow rate: 0.8 ml min^{-1}.

IC–ICP-MS chromatograms of arsenic species in urine and feces after 20 weeks administration of DMA are shown in Fig. 5. As shown in Table 1, DMA, MMA, TMAO, TeMA, AsBe and three unidentified peaks, M-1, M-2, and M-3, were detected in the urine after 20 weeks exposure to DMA. AsBe and M-3 detected in the urine after 20 weeks exposure to DMA may have been due to the presence of arsenic in feed, because their amounts were the same as the basal excretion of arsenic in urine of the nontreated control group (Table 1). Elimination of five arsenic compounds as metabolites was found in feces after chronic oral exposure to DMA, including DMA, TMAO, and the three unidentified arsenic peaks M-1, M-2, and M-3 (Table 2). A trace amount of MMA was also detected in feces after 20 weeks exposure to DMA, but this elimination of MMA in feces may have been due to the presence of this agent in the feed, since the amount of MMA detected was the same as in the controls (Table 2).

Table 3 shows a comparison of metabolites eliminated in urine and feces after 20 weeks administration of DMA. Each mean basal excretion of arsenic species in the nontreated control group was subtracted from the amounts excreted after the 20 week treatments. The mean excretion was expressed as the relative proportion of urinary and fecal metabolites to total arsenic. The proportion of unchanged DMA elimination was twice as high in urine as in feces. The proportion of TMAO elimination was much higher in urine than in feces. On the other hand, the proportions of unidentified M-2 and M-3 elimination were much higher in feces than in urine.

Table 1 Arsenic metabolites in urine excreted after 20 weeks exposure to DMA

	Arsenic in urine (mg As l^{-1})[a]	
Metabolites	Control	20 weeks exposure
As(V)	<0.005	<0.005
As(III)	<0.005	<0.005
MMA	<0.005	0.014 ± 0.004
DMA	0.187 ± 0.011	35.1 ± 3.48
TMAO	0.014 ± 0.001	17.2 ± 1.41
TeMA	0.008 ± 0.001	0.807 ± 0.047
AsBe	0.284 ± 0.026	0.198 ± 0.011
M-1	<0.005	7.40 ± 0.431
M-2	<0.005	2.36 ± 0.400
M-3	0.014 ± 0.001	0.017 ± 0.004
Total As	0.522 ± 0.032	63.2 ± 3.62

[a] Values are means ± standard error for ten rats.

DISCUSSION

The mechanism of methylation of arsenicals is a sequence of reactions in which a trivalent arsenical

Table 2 Arsenic metabolites in feces excreted after 20 weeks exposure to DMA

	Arsenic in feces (mg As kg^{-1})[a]	
Metabolites	Control	20 weeks exposure
As(V)	0.006 ± 0.002	<0.005
As(III)	0.008 ± 0.002	<0.005
MMA	0.010 ± 0.002	0.007 ± 0.003
DMA	0.009 ± 0.003	35.2 ± 1.65
TMAO	<0.005	1.23 ± 0.237
TeMA	<0.005	<0.005
AsBe	<0.005	<0.005
M-1	0.028 ± 0.022	6.15 ± 0.54
M-2	<0.005	58.7 ± 7.34
M-3	<0.005	28.3 ± 7.27
Total As	0.066 ± 0.034	130 ± 11.8

[a] Values are means ± standard error for ten rats.

Table 3 Comparison of arsenic metabolites in urine and feces after 20 weeks exposure to DMA

	Percentage of total As[a]	
Metabolites	Urine	Feces
As(V)	<0.01	<0.01
As(III)	<0.01	<0.01
MMA	0.01 ± 0.00	<0.01
DMA	55.2 ± 2.94	27.4 ± 1.31
TMAO	27.7 ± 2.47	0.98 ± 0.29
TeMA	1.29 ± 0.11	< 0.01
AsBe	<0.01	< 0.01
M-1	11.9 ± 0.68	4.82 ± 0.84
M-2	3.96 ± 0.87	45.3 ± 3.69
M-3	<0.01	21.5 ± 4.09

[a] Each mean basal excretion of arsenic species in the control group was subtracted from the amounts excreted after 20 weeks treatment, and the mean urinary excretion was expressed as the percentage of total arsenic concentration. Values are means ± standard error for ten rats.

is made pentavalent by methyltransferase-catalyzed addition of a methyl group.[34] GSH in the liver plays an important role in the reduction of arsenicals from pentavalency to trivalency. Hirata *et al.*[13] showed that depletion of liver GSH through use of BSO prior to treatment with inorganic arsenic leads to inhibition of methylation in the liver and a significant decrease in the rate of elimination of the methylated arsenic metabolites MMA, DMA, and TMAO. In the present study, urinary metabolites were measured following forced urination at various time points after administration of DMA to BSO-pretreated rats. The results revealed that GSH depletion decreased TMAO elimination (Figs 1B, and 3B), suggesting that GSH plays an important role in the methylation of DMA to TMAO in rats. Our results were consistent with those of Hirata *et al.*[13] They also reported that BSO-pretreated animals exhibited decreased amounts of unchanged metabolite, As(III), and total arsenic elimination.[13] It is well known that elimination of inorganic arsenic from the body depends on its rate of methylation, because methylated metabolites are eliminated more rapidly than inorganic arsenic.[17] Hirata *et al.*[13] concluded that GSH is involved in the detoxification of inorganic arsenic, and that reduction of GSH level is associated with a marked accumulation of inorganic arsenic in the liver. On the other hand, in our study, BSO pretreatment resulted in increasing urinary excretion of DMA following i.p. administration (Fig. 3A). This result suggests that GSH might be associated with an accumulation of DMA, resulting in increased toxicity. This hypothesis is supported by the finding by Ochi *et al.*[35] that GSH markedly enhanced the cytotoxic effects of DMA. It would be of great intrerest to elucidate the toxicity of DMA.

Methylarsenicals, which exist as pentavalent species in neutral solution, are easily reduced to their trivalent derivatives with sulfhydryls such as GSH, cysteine, and lipoic acid.[36–38] Trivalent arsenicals, including dimethylarsinous acid (DMA(III)), are considered as sulfhydryl reagents. Consequently, interaction of DMA(III) with sulfhydryl groups might result in higher tissue accumulation of arsenicals. There have been many reports that trivalent arsenic compounds are more toxic than pentavalent compounds or inorganic arsenics. Dimethylarsine, a volatile trivalent metabolite of DMA, was a potent mutagen in *Escherichia coli* tester strains, although DMA and MMA were not mutagens.[39] Methylated trivalent arsenicals, DMA(III) and methylarsonous acid (MMA(III)) were more potent inhibitors of sulfhydryl enzyme than their pentavalent analogs or inorganic trivalent arsenic.[40] Petrick *et al.*[41] reported that results of cytotoxicity assays revealed the following order of toxicity in Chng human hepatocytes: MMA(III) > As(III) > As(V) > MMA(V) = DMA(V).

In our previous study,[28] two unidentified metabolites, M-1 and M-2, were detected in urine after long-term oral administration of DMA, MMA, or TMAO. In the present study, M-1 and M-2 were also found in urine following a single oral or intraperitoneal administration (Figs 2 and 4) and in urine and feces following 20 weeks administration of DMA (Tables 1 and 2). Earlier studies of the metabolism of DMA in mice or hamsters detected complexes of DMA in urine, feces, liver, and kidneys.[26,30] It has been suggested that such DMA complexes might be intermediates in further methylation to TMAO, since DMA can react with SH-containing compounds, the last being the reducing step which is followed by oxidative methylation.[30] However, it seems unlikely that the unidentified metabolites M-1 and M-2 can be formed during methylation in the liver, since no difference was found in production of either M-1 or M-2 between BSO-pretreated rats and controls (Figs 2 and 4). The delay in M-1 or M-2 elimination compared with that of TMAO supports our hypothesis that M-1 and M-2 are not intermediates in the further methylation to TMAO in liver. The amounts of elimination of M-1 and M-2 after intraperitoneal administration were less than those

after oral administration (Figs 2 and 4). This finding suggests that intestinal bacteria may participate in the production of M-1 and M-2.

A new unidentified metabolite, M-3, was detected only in feces as a metabolite of DMA after 20 weeks exposure to DMA, although M-1 and M-2 were found in both urine and feces (Table 3). Interestingly, the fecal elimination pattern differed significantly from the urinary one. The unidentified metabolites M-1, M-2, and M-3 were excreted mainly as fecal metabolites other than unmetabolized DMA after chronic oral administration of DMA. This finding also suggests that M-1, M-2, and M-3 might be produced in the intestinal tract. Further support for this hypothesis was recently obtained from the findings by Kuroda *et al.*,[42] which showed that DMA was converted to M-2 and M-3 in DMA-containing GAM medium with *E. coli* added under aerobic conditions and that TMAO was converted to M-1.[42] Kuroda *et al.*[42] also reported that the conversion of DMA to M-2 and M-3 by *E. coli* in bouillon medium required cysteine, suggesting that cysteine was involved in the conversion of DMA to M-2 or M-3. Furthermore, it can be hypothesized that DMA or TMAO can be reduced to trivalent derivatives with cysteine in the presence of *E. coli*, resulting in the production of M-1, M-2, and M-3. Yamanaka *et al.*[39] reported that volatile trivalent metabolites, dimethylarsine and trimethylarsine, were detected in the gas phase of DMA-added *E. coli* strain cell suspensions in sealed tubes. These results support our hypothesis. M-1 and M-2 produced by *E. coli* might be absorbed from the intestinal tract and enter entero-hepatic circulation, whereas most of the M-3 might be excreted into the feces without absorption. In the present study, the difference in TMAO elimination between the BSO-pretreated group and controls was less after p.o. administration than after i.p. administration (Figs 1B and 3B). This difference may be due to absorption of M-1 and M-2 from intestine and involvement of M-1 and M-2 in the methylation to TMAO in p.o.-administered rats.

The production of methylated trivalent arsenicals or their complexes is an attractive mechanism for triggering of the carcinogenic effects of DMA in rats. The chemical structures of unidentified metabolites M-1, M-2, and M-3 are now under investigation in our laboratory.

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