Mutagenicity of Dimethylated Metabolites of Inorganic Arsenics

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The genotoxic effects of dimethylarsinic acid (DMAA), one of the main metabolites of inorganic arsenics in mammals, and its further metabolites were investigated using *Escherichia coli* B tester strains. When H/r30R (wild-type; Exc$^+$Rec$^+$) and Hs30R (uvrA$^-$; Exc$^-$Rec$^+$) cells were incubated with DMAA for 3 h in liquid NB medium, many more revertants appeared in sealed tubes than in the control, but this was not the case in unsealed tubes, suggesting that volatile metabolites of DMAA caused the mutagenesis. By gas chromatography-mass spectrometry (GC-MS), dimethylarsine and trimethylarsine, known to be volatile metabolites in microorganisms, were detected in the gas phase of DMAA-added tester strain cell suspensions in sealed tubes. Among these arsines, dimethylarsine was mutagenic in WP2 (wild-type; Exc$^+$Rec$^+$) and WP2uvrA (uvrA$^-$; Exc$^-$Rec$^+$), while trimethylarsine was not. The mutagenesis induced by dimethylarsine required oxygen gas in the assay system; the number of revertants markedly increased in an oxygen-replaced system and diminished in a nitrogen-replaced one. These results suggest that the reaction product(s) between dimethylarsine and molecular oxygen is responsible for the mutagenesis. The significance of this mutagenesis in the genetoxic action of inorganic arsenics is discussed.

Keywords dimethylarsinic acid; dimethylarsine; trimethylarsine; inorganic arsenic; inorganic arsenic metabolite; GC-MS; mutagenesis; genotoxicity; *Escherichia coli*

Introduction

Epidemiological studies have indicated that inorganic arsenics are carcinogenic to humans,[1] whereas experimental studies with laboratory animals have not succeeded in giving evidence of carcinogenicity. Studies on the mutagenicity of these compounds in bacterial tester strains have also not always provided positive results. We ourselves have reported that arsenite (AsO_2^-) and arsenate ($HAsO_4^{2-}$) were not mutagenic to *Escherichia coli* B tester strains. Additionally, we found that these arsenics rather enhanced the error-free excision repair of ultraviolt (UV)-damaged deoxyribonucleic acid (DNA) by retarding the DNA replication and thus prolonging the period for excision repair in the tester strains, possibly leading to a reduction in UV-induced mutation.[2]

On the other hand, a number of investigations have indicated that the inorganic arsenics are metabolically reduced and methylated to produce their dimethyl derivatives, such as dimethylarsinic acid (DMAA; $(CH_3)_2$-AsO(OH)) and dimethylarsine ($(CH_3)_2AsH$) in microorganisms and mammals, and trimethyl compounds by further methylation in some aerobic microorganisms (Chart 1).[3-6] It is therefore necessary to investigate the genotoxic effect of methylated metabolites of inorganic arsenics. In the present study, we found that dimethylarsine, a further metabolite of DMAA which itself showed no mutagenicity, has a potent mutagenic activity in *E. coli* tester strains.

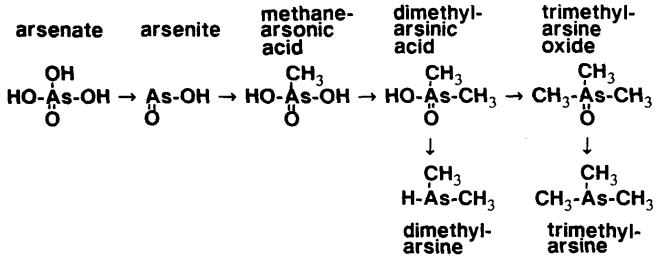

Chart 1. Proposed Metabolic Pathway of Arsenics[3-6]

Materials and Methods

Bacterial Tester Strains and Methylated Arsenics *E. coli* B H/r30R [wild-type (Exc$^+$Rec$^+$)], Hs30R [uvrA$^-$ (Exc$^-$Rec$^+$)], WP2 [wild-type (Exc$^+$Rec$^+$)] and WP2uvrA [uvrA$^-$ (Exc$^-$Rec$^+$)] were donated by Professor S. Kondo of Kinki University. DMAA was obtained from Nakarai Chemical Co., Kyoto. Trimethylarsine was prepared by the reaction of arsenic trichloride with methylmagnesium iodide in diethyl ether,[7] similarly to the method reported for trimethylphosphine.[8] Trimethylarsine oxide was prepared by oxidation of trimethylarsine with aqueous 30% hydrogen peroxide in diethyl ether.[9]

Lethal and Mutagenic Effects of DMAA on H/r30R and Hs30R Cells Examined by a Pre-incubation Method H/r30R and Hs30R cells (*ca.* 10^8 cells/ml) were incubated in 1/15 M phosphate buffer (pH 6.8) or liquid nutrient broth (NB; 0.8% Difco nutrient broth containing 0.4% NaCl, total volume 10 ml) containing 10 mM DMAA in L-type sealed or unsealed tubes at 37 °C. After incubation, aliquots (1.5 ml) of cell culture were transferred to other test tubes and then centrifuged at 3000 rpm for 15 min. The pellets (cells) were washed twice with 1/15 M phosphate buffer (pH 6.8), and resuspended in 1.5 ml of 1/15 M phosphate buffer (pH 6.8). The cell suspension (1 ml) was layered on an SEM (semi-enriched medium; Difco agar (12 g) dissolved in 750 ml of distilled water was added to a mixture of medium E × 50[10] (16 ml), 40% glucose (8 ml) and liquid NB (40 ml)) agar plate. The number of revertant colonies formed after incubation at 37 °C for 2 d was counted. For measurement of viability, 0.1 ml of diluted cell culture (*ca.* 10^4 cells/ml) was layered on an SEM agar plate, and the number of colonies was counted after incubation at 37 °C for 1 d.

Determination of Metabolites of DMAA by Gas Chromatography-Mass Spectrometry (GC-MS) Hs30R cells (*ca.* 10^8 cells/ml) in liquid SEM medium (10 ml) were incubated with 10 mM DMAA at 37 °C in a 40 ml screw-capped vial. After incubation, 1 ml of the head-space gas was withdrawn with a syringe and analyzed by GC-MS (Shimadzu LKB-9000). The conditions for measurement were as follows.

Gas chromatography: column, 5% PEG-20M on Chromosorb 101 (80—100 mesh) in a 2 m × 3 mm glass column; column temperature, 155 °C; injection temperature, 200 °C; carrier gas, He, 25 ml/min. Mass spectrometry: ionizing energy, 20—70 eV; accelerating voltage, 3.5 kV; separator temperature, 290 °C.

Mutagenic Effects of Dimethylarsine and Trimethylarsine on Tester Strains Determined by a Gas Assay Method The tester strains on SEM agar plates were exposed to dimethylarsine or trimethylarsine gas, basically according to the method of de Meester *et al.*,[11] as follows. A solution (1 ml) of 10 mM DMAA or trimethylarsine oxide was added to 10 ml of 6 N HCl, and then 5% $NaBH_4$ (5 ml) was slowly added. The generated gas was introduced into a glass desiccator (*ca.* 4.7 l) through a KOH-pellet trap.

After exposure of the tester strains on the SEM plates to the gas, the plates were cultivated at 37 °C for several days and then the numbers of revertant colonies were counted.

Results

Mutagenic Effect of DMAA on H/r30R and Hs30R Cells

To examine if DMAA is mutagenic for H/r30R and Hs30R cells, the cells were incubated with DMAA in liquid NB medium in L-type tubes. The tubes were tightly sealed to prevent the leakage of volatile arsenic metabolites, *e.g.*, dimethylarsine (bp *ca.* 36 °C) and trimethylarsine (bp *ca.* 70 °C). As shown in Fig. 1, appreciably higher numbers of H/r30R and Hs30R revertants were detected in the presence of 10 mM DMAA than in the control after a 3 h incubation, with no appearent change in the viable cell number of these strains. The induction of mutation by DMAA required a 3 h or longer incubation period. Since DMAA was not mutagenic for both the strains under incubation in 1/15 M phosphate buffer (pH 6.8) for 30 min (Table I), it seemed that the mutagenesis was not caused by DMAA itself but by DMAA derivatives metabolically produced by H/r30R and Hs30R cells. The mutation was not observed when the incubation was carried out in an unsealed tube (Fig. 2). Therefore, volatile metabolites of DMAA might cause the mutation.

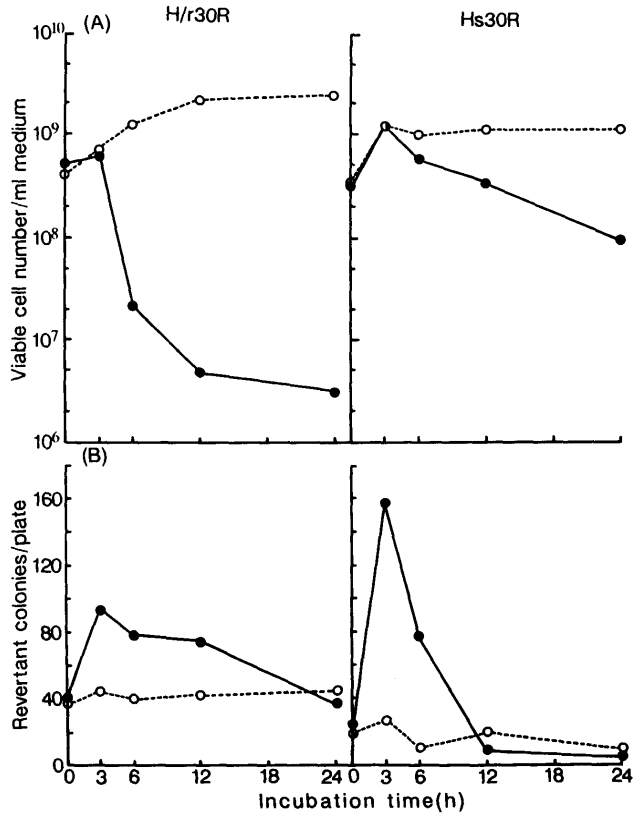

Fig. 1. Lethal and Mutagenic Effects of DMAA on H/r30R and Hs30R Cells in Liquid Medium in a Sealed System

(A) and (B) represent viable cell number and Arg^+ revertant number per ml of medium, respectively, after incubation at 37 °C in NB medium in sealed tubes. ○, control; ●, 10 mM DMAA.

TABLE I. Lethal and Mutagenic Effects of DMAA on H/r30R and Hs30R Cells

	Survival fraction (%)		Revertant colonies/plate	
	H/r30R	Hs30R	H/r30R	Hs30R
Control	100	100	37	26
DMAA	108	100	40	28

The cells were incubated with 10 mM DMAA in 1/15 M phosphate buffer (pH 6.8) for 30 min at 37 °C.

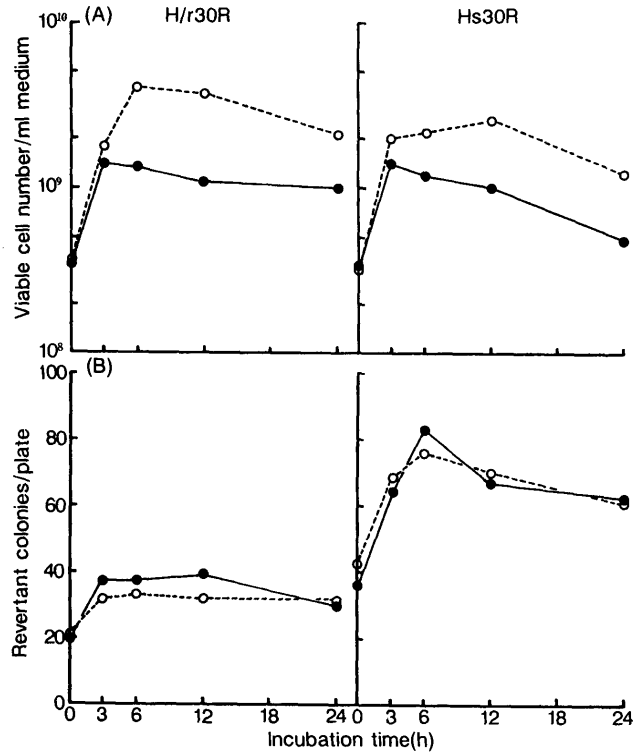

Fig. 2. Lethal and Mutagenic Effects of DMAA on H/r30R and Hs30R Cells in Liquid Medium in an Unsealed System

(A) and (B) represent viable cell number and Arg^+ revertant number per ml of medium, respectively, after incubation at 37 °C in NB medium in unsealed tubes. ○, control; ●, 10 mM DMAA.

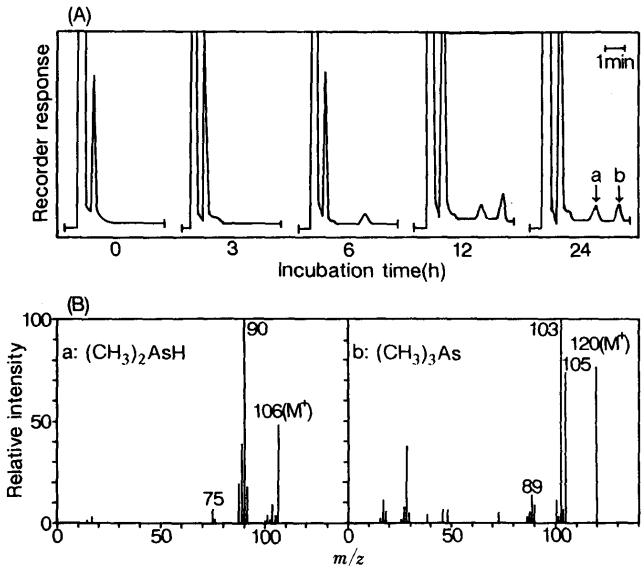

Fig. 3. Gas Chromatogram (A) and Mass Spectrum (B) of Gaseous Arsenics Metabolically Produced from DMAA by Hs30R Cells

(a) and (b) in the mass spectrum correspond to two compounds (a and b) separated by gas chromatography.

DMAA Metabolites Produced in the Cells To identify the mutagenic metabolites of DMAA, we tried to determine the gaseous metabolites by GC-MS analysis. As shown in Fig. 3, two GC peaks (a and b) appeared (Fig. 3A) and were identified as being those of dimethylarsine (a) and trimethylarsine (b), respectively, by MS analysis (Fig. 3B). The longer the incubation time was, the greater the production of these arsines was (Fig. 3A). As these arsines were not produced in a blank experiment containing no cells, they were considered to be cellular metabolites of DMAA.

Mutagenic Activity of Dimethylarsine and Trimethylarsine DMAA is known to be metabolized to dimethylarsine and trimethylarsine in microorganisms (Chart 1).[3,4,6] Because these arsines were detected in Hs30R culture medium containing DMAA, we examined the mutagenic effect of these methylated arsines. Dimethylarsine and trimethylarsine were prepared by reducing DMAA and trimethylarsine oxide, respectively, with $NaBH_4$ in a closed system. As shown in Fig. 4, when dimethylarsine was led into the gas assay apparatus using three tester strains, 3—10 times more revertant colonies appeared than in the control after cultivation for 7—10 d. Although the dose dependency of this effect was not fully determined because of the high killing effect of dimethylarsine and some difficulty with the quantitative gas assay system, the results obtained here strongly suggest that dimethylarsine is mutagenic. On the other hand, trimethylarsine showed no mutagenic effect on WP2 and WP2uvrA cells (Table II).

Effect of Oxygen on the Mutagenic Activity of Dimethylarsine Dimethylarsine readily reacts with atmospheric oxygen and forms a number of oxidative products.[12] We therefore examined the effect of oxygen on the mutagenic activity of dimethylarsine. As shown in Fig. 5, during dimethylarsine exposure of the tester strains on SEM agar plates, replacement of air with oxygen caused a higher mutagenesis, whereas that with nitrogen completely suppressed the mutagenicity. This suggests that oxygen may be

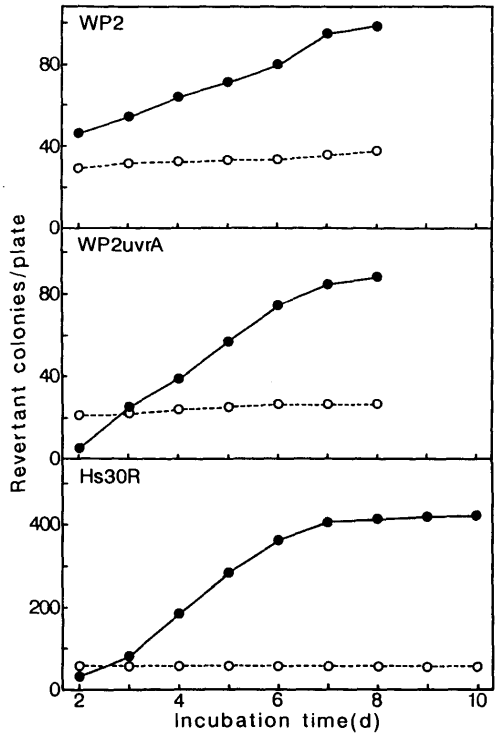

Fig. 4. Mutagenic Activity of Dimethylarsine on *E. coli* B Tester Strains

The tester strains on SEM agar plates were exposed to dimethylarsine in the gas assay apparatus for 15 h at 20 °C, and then incubated at 37 °C. ○, control; ●, dimethylarsine.

TABLE II. Lethal and Mutagenic Effects of Trimethylarsine on WP2 and WP2uvrA Cells

	Survival fraction (%)		Revertant colonies/plate	
	WP2	WP2uvrA	WP2	WP2uvrA
Control	100	100	53	43
Trimethyl-arsine	66	57	41	42

The cells on SEM agar plates were exposed to trimethylarsine for 15 h at 20 °C, and then incubated at 37 °C for 6 d.

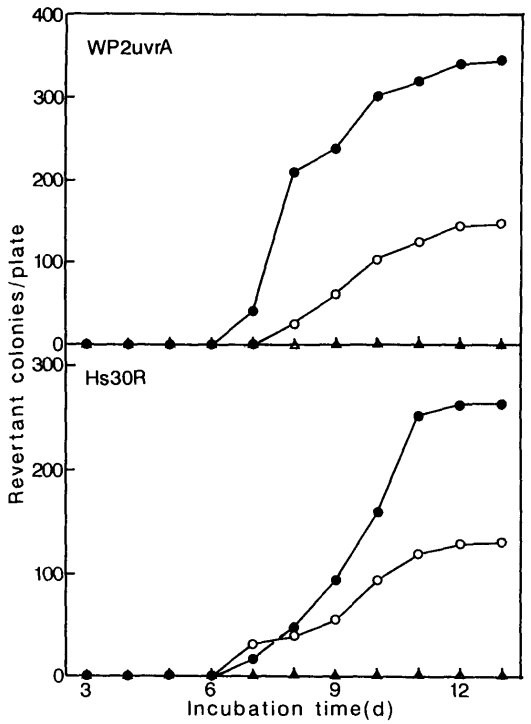

Fig. 5. Effect of Oxygen on Mutagenesis of Dimethylarisine-Exposed WP2uvrA and Hs30R Cells

Oxygen or nitrogen was passed through the apparatus to displace air for 30 min. The cells on SEM agar plates were exposed to dimethylarisine for 3 d at 37 °C, and then incubated at 37 °C. ○, not replaced; ●, replaced with oxygen; △, replaced with nitrogen.

TABLE III. Effect of Exposure Time to Dimethylarsine under Oxygen on Mutagenesis of WP2uvrA

Exposure time to dimethylarsine (h)	Revertant colonies/plate
Control[a]	25
0[b]	46
1	74
3	7667
6	2913
12	1002

Oxygen gas was passed through the apparatus to displace air for 30 min. The cells on SEM agar plates were exposed to dimethylarsine at 20 °C, and then incubated at 37 °C for 2 d. *a*) Control was reduced with 1 ml of distilled water instead of 1 ml of 10 mM DMAA with 5% $NaBH_4$. *b*) Exposure time to dimethylarsine was less than 1 min.

necessary for the mutagenesis of dimethylarsine. We further determined the optimum exposure time, in the oxygen-replaced system, of dimethylarsine for the induction of mutation of WP2uvrA cells on SEM agar plates. As shown in Table III, the highest number of revertant colonies appeared on exposure for 3 h, followed by incubation at 37 °C for 2 d.

Discussion

As one of the experimental approaches to examine inorganic arsenic carcinogenesis, which has been evidenced epidemiologically but not experimentally, bacterial mutation assay is thought to be useful at least for estimation of the initiation step of carcinogenesis. However, data so far obtained on the mutagenicity of inorganic arsenics, such as arsenite and arsenate, are contradictory; some reports indicate that they are mutagenic[13] but others do not.[2,14,15] Thus, our attention was focused on their metabolites. As is well known since McBride and Wolfe[3] proposed a metabolic methylation pathway for arsenics, inorganic arsenics are methylated to DMAA *via* methanearsonic acid and then to dimethylarsine and trimethylarsine in a variety of organisms (Chart 1).[3-6] These methylated arsenics may be worthy of investigation for genotoxic action.

The present study demonstrates that, of these methylated arsenics, dimethylarsine, a volatile metabolite of DMAA, is a potent mutagen in *E. coli* B tester strains, but DMAA and methanearsonic acid are not (data not shown). Dimethylarsine is a volatile (bp 36 °C) compound having a strong odor of garlic. Here, we detected dimethylarsine, together with trimethylarsine, which showed no mutagenicity, in the gas phase of DMAA-added bacterial medium. Solis-Cohen and Githens[16] reported that the respiration of arsenic-intoxicated man had a garlic-like odor, suggesting that arsenics are also metabolized in man to produce dimethylarsine. In fact, we detected dimethylarsine from the respiration of DMAA-administered mice (to be reported elsewhere). Therefore, it is very likely that mammals administered inorganic arsenics produce dimethylarsine, which has mutagenic activity.

The mutagenic activity of dimethylarsine seemed to require oxygen, suggesting that the mutagenesis induced by dimethylarsine was not direct, but was due to a reaction product (s) between dimethylarsine and oxygen. We have obtained some evidence that active oxygen produced by the reaction of dimethylarsine and molecular oxygen causes DNA damage even *in vivo*, as will be reported in the near future.

The methylation pathway of inorganic arsenics has generally been considered to be a metabolic detoxification process. In fact, the acute toxicity of arsenite was much lowered by this pathway. However, the present findings suggest that the genotoxicity may be increased by the methylation pathway.

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References

1) International Agency for Research on Cancer, "IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans," Vol. 23, Lyon, 1980, pp. 39—141.
2) S. Okada, K. Yamanaka, H. Ohba and Y. Kawazoe, *J. Pharmacobio-Dyn.*, **6**, 496 (1983).
3) B. C. McBride and R. S. Wolfe, *Biochemistry*, **10**, 4312 (1971).
4) E. A. Woolson, *Environ. Health Persp.*, **19**, 73 (1977).
5) E. A. Crecelius, *Environ. Health Persp.*, **19**, 147 (1977).
6) W. R. Cullen, C. L. Froese, A. Lui, B. C. McBride, D. J. Patmore and M. Reimer, *J. Organomet. Chem.*, **139**, 61 (1977).
7) R. A. Zingaro and A. Merijanian, *Inorg. Chem.*, **3**, 580 (1964).
8) R. A. Zingaro and R. E. McGlothlin, *J. Chem. Eng. Data*, **8**, 226 (1963).
9) A. Merijanian and R. A. Zingaro, *Inorg. Chem.*, **5**, 187 (1966).
10) H. J. Vogel and D. M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).
11) C. de Meester, M. Duverger-van Bogaert, M. Lambotte-Vandepaer, M. Mercier and F. Poncelet, *Mutat. Res.*, **90**, 443 (1981).
12) W. M. Dehn and B. B. Wilcox, *Am. Chem. J.*, **35**, 1 (1906).
13) H. Nishioka, *Mutat. Res.*, **31**, 185 (1975).
14) G. Lofroth and B. N. Ames, *Mutat. Res.*, **54**, 65 (1978).
15) T. G. Rossman, D. Stone, M. Molina and W. Troll, *Environ. Mutagen.*, **2**, 371 (1980).
16) S. Solis-Cohen and T. S. Githens, "Pharmacotherapeutics, Materia Medica and Drug Action," Appleton and Company, New York, 1928, pp. 594—641.