Enzymatic Reduction of Arsenic Compounds in Mammalian Systems: The Rate-Limiting Enzyme of Rabbit Liver Arsenic Biotransformation Is MMA^V Reductase

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A unique enzyme, MMA^V reductase, has been partially purified from rabbit liver by using DEAE-cellulose, carboxymethylcellulose, and red dye ligand chromatography. The enzyme is unique since it is the rate-limiting enzyme in the biotransformation of inorganic arsenite in rabbit liver. The K_m and V_{max} values were 2.16×10^{-3} M and 10.3 μmol h^{-1} (mg of protein)$^{-1}$. When DMA^V or arsenate was tested as a substrate, the K_m was 20.9×10^{-3} or 109×10^{-3} M, respectively. The enzyme has an absolute requirement for GSH. Other thiols such as DTT or L-cysteine were inactive alone. At a pH below the physiological pH, GSH carried out this reduction, but this GSH reduction in the absence of the enzyme had little if any value at pH 7.4. When the K_m values of rabbit liver arsenite methyltransferase (5.5×10^{-6} M) and MMA^{III} methyltransferase (9.2×10^{-6}) were compared to that of MMA^V reductase (2.16×10^{-3} M), it can be concluded that MMA^V reductase was the rate-limiting enzyme of inorganic arsenite biotransformation. MMA^V reductase was also present in surgically removed human liver.

Introduction

Inorganic arsenic in the drinking water of millions of people has become a major public health problem of global proportions (*1*). This chronic exposure to inorganic arsenic can cause cancer of the skin or some internal organs as well as cardiovascular disorders. The problem can be expected to increase since with limited water supplies, deeper wells need to be dug. Such wells, for example, artisian wells, often have a high arsenic content. Yet an understanding of the basic mechanisms responsible for arsenic carcinogenesis and even the biotransformation of inorganic arsenic are not understood at the molecular level.

On the basis of the chemistry of the reactions, the biotransformation of inorganic arsenate and arsenite in mammals theoretically involves four steps. A two-electron reduction of arsenate to arsenite is followed by methylation of arsenite to form MMA^V.[1,2] Another two-electron reduction of MMA^V to form MMA^{III} occurs, which is methylated to form DMA^V. Only recently, however, have some of the enzymes involved in these mammalian reactions been purified to the extent where interfering inhibitory or stimulatory factors have been removed (*2*−*8*). For example, a human liver arsenate reductase (Scheme 1) which catalyzed the first reaction in Scheme 1 has been partially purified (*2*), as have the arsenite methyltransferases from rabbit liver (*5*), hamster liver (*6*), rhesus monkey liver (*7*), and Chang human hepatocytes (*8*). The MMA^{III} methyltransferase from the rabbit liver and human hepatocytes have also been partially purified (*8*). Whether an enzyme is needed for the reduction of MMA^V, however, has received little, if any, attention in the scientific literature. It has been suggested that glutathione (GSH) plays an important role in the reductive mechanisms of arsenic biotransformation (*9*).

In the study presented here, a MMA^V reductase from the livers of rabbits has been isolated and characterized and also has been identified in human liver. At physiological pH, the enzyme and GSH were required for this reduction. The purpose of this preliminary communication is to alert others working in the arsenic area to the importance of this enzyme in how the rabbit, which has been considered an appropriate model for humans (*10*, *11*), processes inorganic arsenic. In the paper presented here, evidence is presented for the enzymatic reduction of MMA^V to form MMA^{III}, a reaction necessary to produce the substrate for the methyltransferase reaction that produces DMA^V. This reaction appeared to be the rate-limiting reaction in the biotransformation of inorganic arsenic in the rabbit and perhaps other mammals.

Experimental Procedures

Caution: *Inorganic arsenic is classified as a group 1 carcinogen for humans (12). Exposure to inorganic arsenic can lead to development of skin and other cancers.*

Reagents. [^{14}C]Methylarsonic acid, disodium salt (MMA^V) (specific activity of 4.5 mCi/mmol), was purchased from ARC-

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[1] Abbreviations: MMA, generic term including MMA^{III} and MMA^V; MMA^{III}, monomethylarsonous acid; MMA^V, monomethylarsonic acid; DMA, generic term including DMA^{III} and DMA^V; DMA^V, dimethylarsinic acid; DTT, dithiothreitol; SAM, *S*-adenosylmethionine; GSH, glutathione; SAHC, *S*-adenosylhomocysteine.

[2] Radabaugh, T. R., and Aposhian, H. V. (1999) Arsenate reductase of human liver. *Toxicol. Appl. Pharmacol.* (submitted for publication).

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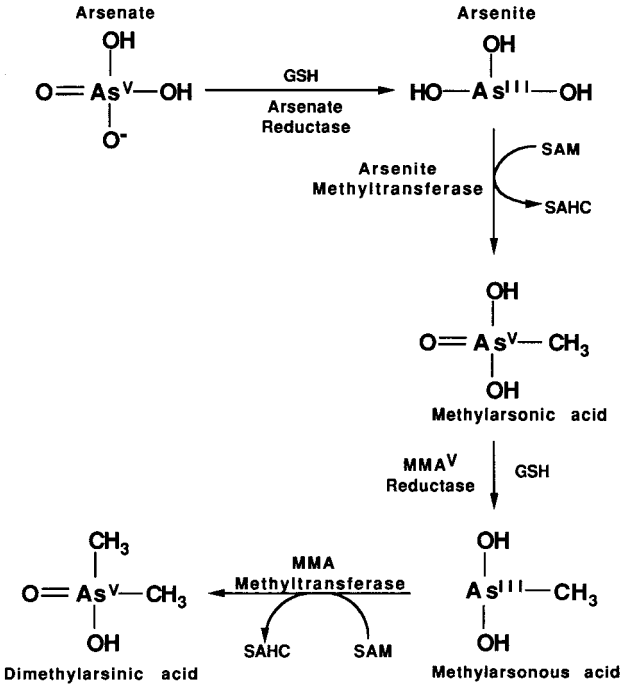
Scheme 1. One of the Pathways for the Biotransformation of Inorganic Arsenic in Mammalian Systems



Inc. (St. Louis, MO). Carrier-free [^{73}As]arsenate (1.3 μCi/μL) was purchased from Los Alamos National Laboratory (Los Alamos, NM). [^{14}C]Dimethylarsinic acid (specific activity of 11.2 mCi/mmol) was a generous gift from Management Technology (Research Triangle Park, NC). American Chemical Society reagent grade sodium arsenate was purchased from MCB Reagents (Cincinnati, OH). MMA^V was obtained from Pfaltz and Bauer Inc. Glutathione, DEAE-cellulose, carboxymethylcellulose, and red dye for chromatography were purchased from Sigma Chemical Co. (St. Louis, MO). Monoflow-3 scintillation cocktail was from National Diagnostics (Atlanta, GA). Carbon tetrachloride and diethylammonium diethyldithiocarbamate was from Aldrich (Milwaukee, WI).

All other chemicals were analytical reagent grade or of the highest quality available.

Animals and Tissues. Viral antigen-free New Zealand white male rabbits (2.5 kg body weight) were from Myrtle's Rabbitry, Inc. (Thompson Station, TN), and were acclimatized, for 1−2 weeks prior to use, in an environmentally controlled animal facility with a 12 h dark/light cycle at 22−24 °C. Animals were provided Harlan Teklad 0533 Rabbit Diet 7008 and water ad libitum.

Human liver was obtained from The Association of Human Tissue Users (Tucson, AZ). The liver had been removed from a 52-year-old male who died of an intracranial hemorrhage. The cold ischemia time was 16 h. Cytosol was prepared in the same manner as rabbit liver cytosol.

MMA^V Reductase Assay. The reaction mixture contained 0.10 M Tris-HCl (pH 8.0), 5 mM GSH, 22 nmol and 3.4 × 10^5 cpm of [^{14}C]MMA^V, and the enzyme in a final volume of 250 μL. The reaction mixture was incubated for 60 min at 37 °C.

Samples were then placed on ice; 0.50 mL of carbon tetrachloride containing 10^{-2} M diethylammonium diethyldithiocarbamate (DDDC) was added, and the mixture was shaken vigorously for 15 min at room temperature. After centrifugation at 3000*g*, the organic phase was separated and the MMA^{III} was back-extracted from the organic phase with 0.50 mL of 0.10 M sodium hydroxide. After centrifugation at 3000*g*, the aqueous phase was separated and transferred into scintillation vials containing 120 μL of 0.5 M HCl. National Diagnostics Monoflow-3 scintillation cocktail (5 mL) was added, and the contents of the vials were counted in a Beckman (Fullerton, CA) model LS7800 liquid scintillation counter. Separate experiments have shown that the presence of 5−10 mM glutathione in the reaction mixture did not influence the extraction of formed trivalent arsenic into the organic phase.

Confirmation of the Reaction Product. The extraction method was a modification of that previously described (*13*) which allowed quantitative extraction and separation of all the trivalent species of arsenic (As^{III}, MMA^{III}, and DMA^{III}) from pentavalent species into carbon tetrachloride in the presence of diethylammonium diethyldithiocarbamate over a pH range of 2−10. Additional confirmation that MMA^{III} was formed was obtained by using a PRP-x100 anion-exchange column (Hamilton, Reno, NV) (25 cm × 4.1 mm i.d.). The column consists of spherical 10 μM particles of a styrene−divinylbenzene copolymer with triethylammonium exchange sites for detecting compounds, such as [^{14}C]MMA^{III} and [^{14}C]MMA^V. Detection was achieved with a Beckman 171 radioisotope detector. The column was eluted with 30 mM sodium phosphate (pH 5.0) (*14*).

The [^{14}C]MMA^{III} used as a standard was prepared by reduction of [^{14}C]MMA^V with metabisulfite-thiosulfate reagent using a procedure described by Reay and Asher (*15*).

Purification of MMA^V Reductase. Rabbit liver cytosol was prepared at 4 °C as described by Zakharyan et al. (*5*) and stored at −70 °C until it was used. The MMA^V reductase was purified by DEAE-cellulose, CM-cellulose, and red dye 120 chromatography. Rabbit liver cytosol fraction I (5.9 mL) was loaded onto a DEAE-cellulose column (10.5 cm × 1.5 cm) which had been equilibrated with 10 mM Tris-HCl (pH 7.8 at 4 °C) at a flow rate of 30 mL/h. The column was washed with the same buffer until no additional protein or MMA^V reductase activity was eluted. Elution was then begun with a gradient of 100 mL of 0.01 M Tris-HCl (pH 7.8) to 100 mL of 0.50 M NaCl in 0.01 M Tris-HCl (pH 7.8). The fractions with MMA^V reductase activity were collected, combined, precipitated by the addition of ammonium sulfate (100% saturation), and centrifuged at 15000*g* for 15 min. The precipitate was dissolved in 10 mM sodium phosphate (pH 6.5) and dialyzed for 24 h at 4 °C against two changes of 2 L of the same buffer, containing 1 mM GSH. The dialysis tubing (Spectrum) had a molecular mass cutoff of 15 000 Da.

The dialyzed preparation (9 mL) was applied to a carboxymethyl-cellulose column (13 cm × 1.5 cm) equilibrated with the 10 mM phosphate buffer (pH 6.5), and the column was washed with the equilibration buffer until no protein was detected in the wash. For elution from the CMC column, a linear gradient from 0.01 M sodium phosphate (pH 6.5) and 0.50 M NaCl to 0.01 M sodium phosphate buffer (pH 6.5) was used. Fractions containing MMA^V reductase were collected, combined, and diluted to a final NaCl concentration of 0.06 M and a final phosphate buffer concentration of 10 mM (pH 6.5) and loaded onto a red dye 120 column (1 cm × 2 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.5). The column was washed with the starting buffer until no additional protein was eluted, followed by stepwise elutions with 0.50 M NaCl in 10 mM Tris-HCl (pH 7.5), and then 1 M NaCl and 10 mM Tris-HCl (pH 7.5). MMA^V reductase activity was eluted with 0.50 M NaCl.

Other Methods. Protein concentrations were determined by the method of Bradford (*16*) using bovine serum albumin as the standard. GSH peroxidase was assayed by the method of Wendel (*17*) and glyoxalase by the method of Racker (*18*).

Results

Partial Purification of MMA^V Reductase from Rabbit Liver. MMA^V reductase was partially purified from rabbit liver using DEAE-cellulose, carboxymethylcellulose, and red dye 120 chromatography (Table 1 and Figure 2). On the basis of the specific activity of the enzyme in the liver cytosol, a 118-fold purification with a 15% recovery was obtained. For the assays used in the purification procedure, the MMA^V concentration was 88

Table 1. Partial Purification of MMA^V Reductase from Rabbit Liver Cytosol[a]

fraction	volume (mL)	[protein] (mg/mL)	activity (nmol/mL)	specific activity (nmol/mg)	total activity (nmol)	purification (*x*-fold)
(I) cytosol	5.9	29.7	77.9	2.62	460	–
(II) DEAE-cellulose	42	0.21	6.9	33.0	290	12
(III) ammonium sulfate precipitation	9	0.55	23.4	42.5	211	16
(IV) carboxymethylcellulose	36	0.033	4.1	124	147	47
(V) red dye 120	18	0.012	3.7	308	67	118

[a] This table summarizes the second partial purification of this enzyme. The first purification produced similar results.

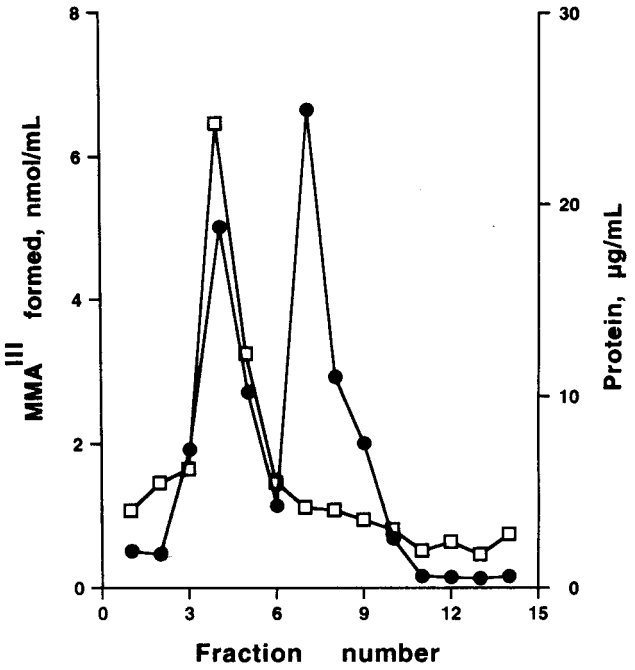


Figure 1. Red dye 120 chromatography of rabbit liver MMA^V reductase. Fraction IV (1.2 mg of protein) was diluted to a final NaCl concentration of 0.06 M and a final sodium phosphate buffer concentration of 10 mM (pH 6.5) and loaded onto a 1 cm × 2 cm column of red dye 120 and the column washed with 6 mL of 10 mM sodium phosphate buffer (pH 6.5). The column was eluted with 24 mL of 0.50 M NaCl/10 mM Tris-HCl (pH 7.5) and then with 40 mL of 1.0 M NaCl/10 mM Tris-HCl (pH 7.5). Fractions had volumes of 6 mL: **(□)** MMA^{III} and **(●)** protein.

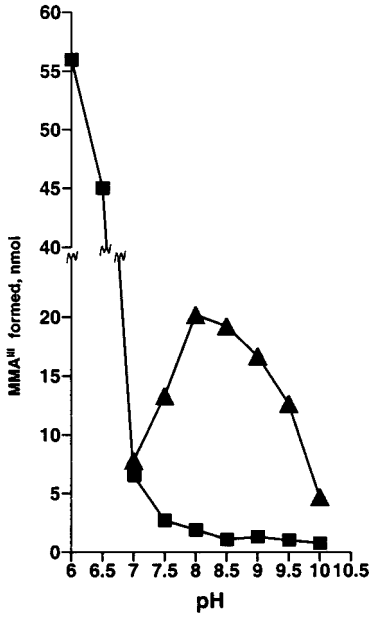


Figure 2. Optimum pH. **(▲)** pH curve for MMA^V reductase of rabbit liver. Assay conditions are as described in Experimental Procedures. Fraction V (1.2 μg of protein) was used per assay. **(■)** pH curve for MMA^V reduction in the absence of enzyme and in the presence of GSH (5 mM). Assay conditions are as described in Experimental Procedures.

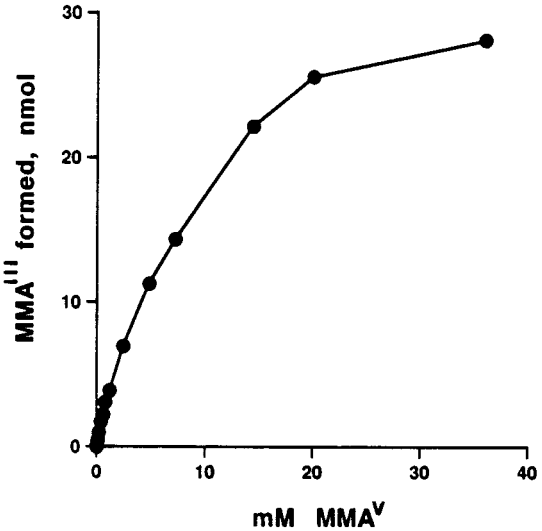


Figure 3. Substrate (MMA^V) saturation for MMA^V reductase. Fraction V (1.2 μg of protein) was used for each assay. The assay procedure is described in Experimental Procedures.

nmol/mL. This concentration, which is not the substrate saturating concentration, was used because of the limited supply of [^{14}C]MMA^V. The cytosol specific activity and fraction V specific activity were 130 and 16800 nmol/mg, respectively, when the MMA^V concentration was saturating at 14.4 mM. This partial purification has allowed a number of important properties of this enzyme to be elucidated without the confounding influence of inhibitory and/or stimulating activities of unknown contaminating factors that very often occur in crude cellular extracts.

Confirmation That MMA^{III} Is the Product of the Reaction. HPLC retention times for standard [^{14}C]-MMA^{III} and [^{14}C]MMA^V were 2.6 ± 0.06 and 3.956 ± 0.02 min, respectively. The ^{14}C-labeled product of the MMA^V reductase reaction had a retention time of 2.56 ± 0.04 min.

Optimum pH and Chemical versus Enzymatic Reduction of MMA^V. The pH optimum of the rabbit liver MMA^V reductase appeared to be 8.0 (Figure 3). At this pH and in the presence of the enzyme, the rate of the reaction (20.2 nmol of MMA^{III} produced/h) was 11 times greater than that in the absence of this enzyme (1.9 nmol of MMA^{III} produced/h). In both cases, 5 mM GSH was present. The pH optimum for the nonenzymatic chemical reduction of MMA^V by GSH was pH ≤6.0. In the absence of the enzyme and above pH 7.0, GSH reduced MMA^V but at a much slower rate. At pH 7.5, only 2.7 nmol of MMA^{III} was produced, whereas 13.32 nmol was produced in the presence of the enzyme at this

Table 2. Thiols and the Reduction of MMA^V by Rabbit Liver MMA^V Reductase[a]

thiol present in the assay	MMA^{III} formed (nmol ± SE)
5 mM GSH	21.2 ± 1.4
1 mM DTT	0.166 ± 0.04
5 mM GSH and 1 mM DTT	40.6 ± 2.6
5 mM CySH	0.47 ± 0.05
5 mM GSH and 5 mM CySH	21.8 ± 0.78
without GSH	<0.01

[a] Fraction V (1.2 μg of protein) was used in each assay. The substrate [^{14}C]MMA^V had a specific activity of 1.58×10^3 cpm/μg and was present at a concentration of 14.4 mM. The experiments were repeated four times.

pH. Above this pH, the rate of reduction in the absence of the enzyme rapidly approached the limit of detection.

GSH Requirement. MMA^V reductase has an absolute requirement for GSH (Table 2). Neither DTT nor L-cysteine alone satisfied this requirement, but in the presence of 5 mM GSH, DTT exhibited stimulatory activity.

Substrate Saturation. The enzyme appeared to be saturated with MMA^V at approximately 15 mM (Figure 4).

Enzyme Kinetics and Specificity. Using the partially purified MMA^V reductase preparation and with MMA^V as the substrate, the K_m was 2.1×10^{-3} M and the V_{max} was 10 μmol mg^{-1} h^{-1}. If DMA^V was tested as the substrate, the K_m was 20.9×10^{-3} M and the V_{max} was 11.9 μmol mg^{-1} h^{-1}. When arsenate was in the reaction in place of MMA^V, the K_m value increased approximately 55-fold to 109×10^{-3} M and the V_{max} was 166 μmol mg^{-1} h^{-1}. Thus, DMA^V and arsenate were reduced by this enzyme (Figure 5). Such a reduction of these latter two arsenicals must not be considered to be due to contaminating enzymes since each of these arsenicals competes with MMA^V as the substrate (Figure 5). In the presence of MMA^V and 80 mM arsenate, the K_m was 14.1×10^{-3} M and the V_{max} was 12 μmol mg^{-1} h^{-1}. When 80 mM DMA^V was present with MMA^V, the K_m was 28×10^{-3} M and the V_{max} was 14 μmol mg^{-1} h^{-1} compared to the values of 3.0×10^{-3} M and 13 μmol mg^{-1} h^{-1} in the presence of only MMA^V.

The MMA^V reductase (fraction V) lacked GSH peroxidase activity (*17*) and was inactive in catalyzing the formation of thiol esters between methylglyoxale and GSH (glyoxalase activity) (*18*).

This enzyme activity was also found in the cytosol prepared in the same manner from surgically removed human liver. The specific activity of such a cytosol was 112 nmol/mg of protein as assayed in the presence of 14.4 mM MMA^V.

Discussion

Arsenite methyltransferase, MMA^V reductase, and MMA^{III} methyltransferase have all been partially purified to the extent that they were free of any known inhibitory and/or stimulatory contaminating activity (*2*, *5*, *8*). The MMA^V reductase has been purified to the extent that only a single band appeared after SDS−PAGE, and the amino acid sequence is now being determined.[3] Thus, it is pertinent to discuss the significance of the K_m values of these enzymes and their relative importance in mammalian arsenite metabolism.

[3] R. A. Zakharyan and H. V. Aposhian, unpublished results.

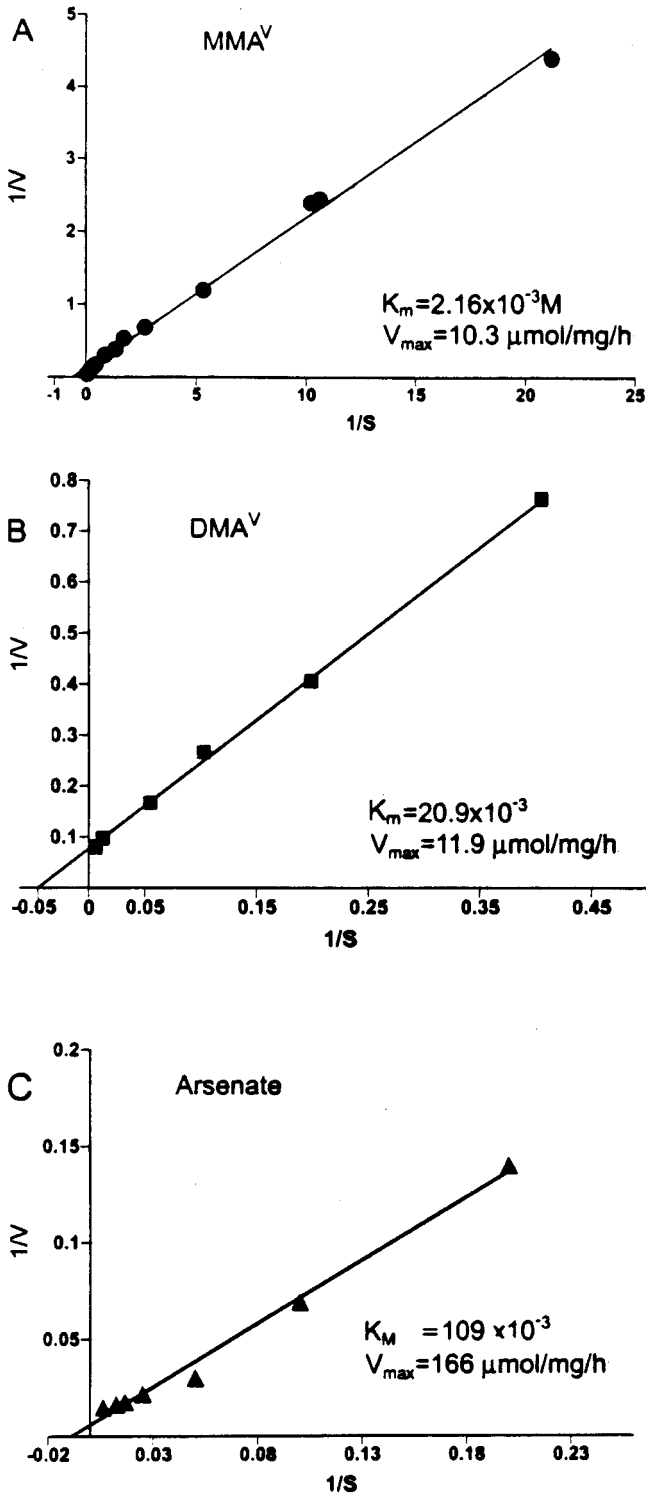


Figure 4. Michaelis−Menten kinetics for rabbit liver MMA^V reductase. (A) MMA^V as the substrate. (B) DMA^V as the substrate. (C) Inorganic arsenate as the substrate. Fraction V (1.2 μg of protein) was used for each assay. The assay procedure is described in Experimental Procedures.

First, MMA^V reductase is the rate-limiting enzyme of arsenic methylation metabolism of rabbit liver. Whereas the K_m of rabbit liver MMA^V reductase was in the millimolar range, the arsenite and MMA^{III} methyltransferase activities (Figure 1) had K_m values in the micromolar range (Table 3). The K_m is an approximation of the affinity of an enzyme for its substrate. The larger the K_m, the weaker the affinity. Thus, it is reasonable to conclude that the rate-limiting reaction in this biotrans-

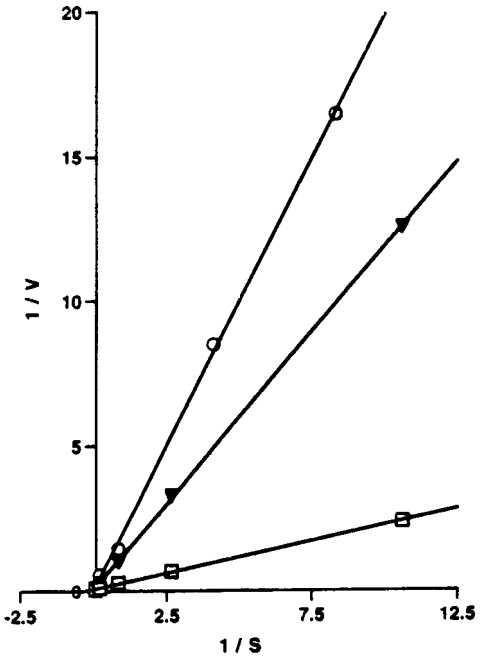


Figure 5. Michaelis−Menten kinetics for rabbit liver MMA^V reductase. **(□)** MMA^V as the substrate. The MMA^V concentration was varied. **(○)** MMA^V in the presence of 80 mM DMA^V. **(▼)** MMA^V in the presence of 80 mM arsenate. Fraction V (1.2 μg of protein) was used for each assay. The assay procedure is described in Experimental Procedures.

Table 3. Comparison of Kinetic Values of Rabbit Liver Enzymes Involved in the Biotransformation of Inorganic Arsenite

enzyme	K_m (M)
arsenite methyltransferase (*5*, *8*)	5.5×10^{-6}
MMA^V reductase (Figure 4)	2.16×10^{-3}
MMA^{III} methyltransferase (*8*)	9.2×10^{-6}

formation pathway for arsenite (Figure 1) was the reduction of MMA^V by this reductase. Since the K_m of MMA^V reductase was in the millimolar range, it would be necessary in vivo that millimolar concentrations of MMA^V be reached before significant amounts of it would be reduced and MMA^{III} would be produced.

Second, it would appear that the concentration of MMA^V needed for reduction to occur was larger than the concentration needed for urinary excretion of it to proceed. This appeared to be why significant amounts of MMA^V were excreted in the urine of some species in addition to the excretion of DMA^V, the end product of inorganic arsenic biotransformation. It explains why MMA^V appeared in the urine of rabbits, hamsters, and humans, even though it was not the end product of inorganic arsenic metabolism. DMA^V production, of course, depends on MMA^{III} availability.

Third, once sufficient levels of MMA^V in the cells were produced by arsenite methyltransferase, the reductase may produce highly toxic MMA^{III},[4] which must be methylated by MMA^{III} methyltransferase to DMA^V to prevent toxic levels of MMA^{III} from accumulating in the cell.

Fourth, it is well-known that there are large interspecies variations in the relative amounts of inorganic arsenic, MMA, and DMA excreted in urine after exposure to inorganic arsenic. These large variations are known to occur among individuals of the same species (*19*). Polymorphism of this rate-limiting MMA^V reductase may be the reason for these variations. The amount of MMA available for urinary excretion by a specific individual or a given species may be the result of or may depend on the K_m of the MMA^V reductase of that individual and that species. The rabbit, hamster, and human excrete in their urine significant amounts of MMA (*19*, *20*), while other species such as the rat and mouse do not, even though they all excrete DMA (*21*, *22*). The National Research Council (NRC) has recommended for many years that the rat not be used to study inorganic arsenic metabolism (*23*) because its red cells bind DMA.

Finally, there is still disagreement as to the mechanisms for the detoxication of inorganic arsenic. Although methylation has been proposed as one mechanism (*19*), certainly it is not so for the marmoset monkey, chimpanzee, guinea pig, and a number of other animals. These three species lack these arsenic methyltransferases (*2*, *7*, *24*, *25*) and do not excrete MMA or DMA in their urine (*24*−*26*). So the question still remains as to how such species detoxify inorganic arsenic. Protein binding of arsenic species must be considered as a possibility (*27*) even though its role in arsenic detoxication is still to be proven unequivocally.

In some bacterial systems, the Ars operon contains an arsenate reductase gene. The protein product of that gene is part of another arsenic detoxication system that decreased arsenic toxicity by reducing arsenate to arsenite, which subsequently was effluxed from the bacterial cell as shown by the experiments of the Rosen group (*28*) and Silver group (*29*). Although an arsenate reductase has been partially purified from surgically removed human liver (*4*), such an arsenite efflux system has not been identified in human systems. Nor have arsenic methyltransferases been found in surgically removed human liver (*4*). In the latter case, it may be due to enzyme instability caused by ischemia. Arsenic methyltransferases have been found in cultured Chang human hepatocytes where the enzymes have been stable to freezing and thawing (*8*).

In summary, on the basis of the results of the experiments described here, MMA^V reductase appears to be the rate-limiting enzyme in the biotransformation of inorganic arsenite in the rabbit and perhaps other species that methylate inorganic arsenite.

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