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Conversion of arsenite and arsenate to methylarsenic and dimethylarsenic compounds by homogenates prepared from livers and kidneys of rats and mice

Miyuki Hirata,* Takami Mohri, Akira Hisanaga and Noburu Ishinishi
Department of Hygiene, Faculty of Medicine, Kyushu University, 1-1, 3 Chome, Maidashi, Higashiku Fukuoka 812, Japan

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Pooled livers and pooled kidneys from rats or mice were homogenized and spiked with arsenite or arsenate in the concentration range 1.3–20 μmol dm^{-3}. Methylarsenic and dimethylarsenic compounds were determined by the hydride generation technique in the homogenates after a 90 min incubation at 37°C. The rat homogenates methylated arsenite and arsenate more efficiently than the mouse homogenates. Monomethylated arsenic was present in larger amounts than dimethylated arsenic in the rat homogenates. In the absence of reduced glutathione (GSH), no methylation occurred. Addition of GSH promoted monomethylation and dimethylation, whereas dithiothreitol and mercaptoethanol (10 mmol dm^{-3}) fostered only monomethylation. The amounts of monomethylated arsenic in the rat liver homogenates increased with increasing arsenite concentration (1.3–20 μmol dm^{-3}) however, the percentage of arsenic that had been methylated decreased. A similar trend, but with much less monomethylarsenic formed, was observed for arsenate-spiked homogenates. Rat kidney homogenates methylated arsenite and arsenate to a much smaller extent than rat liver homogenates. The K_m values for the monomethylation in rat liver homogenates were found to be 5.3 μmol dm^{-3} for arsenite and 59 μmol dm^{-3} for arsenate.

Keywords: Arsenite, arsenate, methylation, liver homogenates, kidney homogenates, rats, mice, thiols

INTRODUCTION

Man and mammals metabolize trivalent and pentavalent inorganic arsenic to the less toxic methylated compounds, methylarsonic acid (MAA) and dimethylarsinic acid (DMAA).[1–15] Whereas DMAA is the main metabolite found in the tissues and the urine of most mammalian species, MAA and DMAA are formed by man in comparable amounts from inorganic arsenic.

Micro-organisms convert arsenate, a pentavalent inorganic arsenic species, to dimethylarsine and trimethylarsine. Arsenite, MAA and DMAA are intermediates in this transformation.[16] A similar sequential methylation was suggested to take place in mammals with DMAA as the highest methylated metabolite. However, recent studies[17,18] indicated that methylation of arsenic may proceed in mammals to trimethylated arsenic compounds. Methylation of arsenic seems to take place mainly in the liver. The enzymatically mediated reactions use *S*-adenosylmethionine as the methyl donor. The methyl groups are added to arsenic species containing trivalent arsenic in an oxidative addition reaction.[14,15,19,20]

An *in vitro* study with tissue homogenates from rats demonstrated that the liver cytosol is the site of methylation of arsenite. The methylation requires the presence of *S*-adenosylmethionine, magnesium (Mg^{2+}), and reduced glutathione (GSH) as cofactors.[19] Mercury(II) chloride ($HgCl_2$) and excess of arsenite inhibited the conversion of arsenite to DMAA. The methylation of arsenate was not investigated.[19] Only one report describes the quantitative differences between the *in vitro* methylation of arsenite and arsenate in rat liver/kidney slices and hepatocytes.[13] Much of the DMAA was found in the medium of the

*Author to whom all correspondence should be addressed. Telephone (092) 641-1151.

liver/kidney slices and hepatocytes exposed to arsenite and in the medium of the kidney slices exposed to arsenate. No attention was given to MAA because MAA was a minor metabolite in the low arsenic exposure animal.[13]

Marked differences between species in the binding of arsenic to red blood cells, and in the absorption and excretion of arsenic, were observed between the rat and several other mammals.[6,21–23]. However, the capacity for *in vivo* methylation of inorganic arsenic was confirmed to be similar in rats and other mammals.[5,12,13]

In vivo studies with rats[24] and hamsters[25] demonstrated that the tissue GSH level is the regulating factor in the methylation of arsenic. Based on observations by Cullen *et al.*,[20] thiols may also have important physiological roles in the methylation process.

This paper describes results of experiments carried out with homogenates (prepared from livers and kidneys of rats and mice) that were spiked with arsenite or arsenate. The influence of thiols and the effect of the arsenic concentration on the production of methylarsenic and dimethylarsenic species were explored.

MATERIALS AND METHODS

Chemicals

Sodium arsenite ($NaAsO_2$), reduced glutathione, and methylcobalamine (CH_3CoB_{12}) were purchased from Merck AG, FRG; sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$), 2-mercaptoethanol and dithiothreitol from Wako Pure Chemical Co., Japan; and NADPH and glutathione reductase from Oriental Yeast Co., Japan. *S*-Adenosyl-L-methionine sulfate *p*-toluenesulfonate was donated by BioResearch Co., Italy. Solutions of these compounds were prepared by dissolving appropriate amounts in distilled water immediately before use. All other chemicals were of the highest purity available.

Preparation of homogenates

Six-week-old male Wister rats and ddY strain mice were maintained on a commercial diet (Oriental NMF, Oriental Co., Japan) with water *ad libitum*. Animals were sacrificed by decapitation. The livers and kidneys were perfused with ice-cold aqueous 0.25 mol dm^{-3} sucrose solution containing 0.05 mol dm^{-3} Hepes buffer solution of pH 7.4 (unless otherwise specified). The livers and kidneys were then removed. Liver or kidney homogenates (15 %, w/v) in the perfusion medium kept at 0–4°C were prepared from organs pooled from several animals in a Potter–Elvehjem homogenizer.

Incubation procedures

Incubations were carried out at 37°C in 10 cm^3 capped polymethylpentene test-tubes. The incubation medium (2.2 cm^3), similar to the one used by Buchet and Lauwery[19] with a slight modification, contained: 0.25 mol dm^{-3} sucrose solution containing 0.05 mol dm^{-3} Hepes buffer of pH 7.4 (unless otherwise specified), 1 mmol dm^{-3} *S*-adenosylmethionine, 10 mmol dm^{-3} GSH, 1.2 mmol dm^{-3} magnesium chloride, 10 μmol dm^{-3} of arsenite or arsenate and 420 μg methylcobalamine. The arsenic-free medium was added to 2 cm^3 freshly prepared homogenate. This mixture was kept at 37°C for 5 min. Arsenite or arsenate solution was then added to a final volume of 4.2 cm^3. The test-tubes were kept sealed with caps during incubation. Incubations were also carried out under anaerobic conditions. The reaction mixture before the addition of arsenic was preincubated in a stream of flowing nitrogen. The mixture in the presence of arsenic was incubated with or without flowing nitrogen. After 90 min, 1.8 cm^3 of 10 mol dm^{-3} sodium hydroxide (NaOH) was added to stop the reaction. The basic mixture was heated at 85°C for 3 h.

Determination of arsenic metabolites

Inorganic arsenic, methyl- (MA), dimethyl- (DMA), and trimethyl-arsenic (TMA) compounds in the alkaline-digested samples were determined by a modification of the hydride generation method described by Braman *et al.*[26] A Nippon Jarrel Ash AA-8200 atomic absorption spectrometer equipped with a Nippon Jarrel Ash ASD-100 control unit was used. Aliquots (0.1–6 cm^3) of the samples were placed in the hydride generator and diluted with distilled water to 10 cm^3. Then 20 cm^3 oxalic acid solution (10 %, w/v) was added. The system was purged with helium for 5 min to remove oxygen. Then 4 cm^3 sodium borohydride solution (10 % in 0.05 mol dm^{-3} sodium hydroxide) was added to the reaction chamber over a period of

1 min. The arsines generated were carried by a helium stream to a U-trap (half-packed with Sigma glass beads, 250–300 μm) immersed in liquid nitrogen. After all the sodium borohydride had been added, the stream of helium was maintained for 5 min. Upon removal of the liquid nitrogen, the trap was heated. The arsines were sequentially volatilized and flushed into a heated quartz cell mounted in an atomic absorption spectrophotometer. A hydrogen–argon mixture flowed with the carrier gas stream of helium through the heated quartz cell for the best sensitivity.[27] Each sample was analyzed three times. The absolute detection limit was near 1 ng arsenic for inorganic arsenic compounds and slightly above 1 ng arsenic for methylarsines. The blanks (amount of each arsenic compound in 2 cm^3 of 15 % homogenate) were subtracted from the results (reported as ng As/4.2 cm^3) obtained with the incubated mixtures.

Data presentation

With the exception of Table 2, the results are means of duplicates which consisted of homogeneous preparations from several animals.

RESULTS AND DISCUSSION

Methylation of arsenite and arsenate

Homogenates prepared from livers or kidneys of rats and mice were spiked with arsenite or arsenate to reach an arsenic concentration of 4.2 μg arsenic per 4.2 cm^3 of the reaction mixture. After incubation for 90 min at 37°C, the arsenic compounds in the reaction mixture were determined by the hydride generation technique. With the exception of the kidney homogenates from mice, all homogenates contained inorganic arsenic, methylarsenic compounds, and dimethylarsenic compounds (Table 1). The values in Table 1 are corrected for background with respect to each arsenic derivative. These homogenates probably do not produce trimethylated arsenic, because all concentrations of trimethylated arsenic were not significantly different from the background values. More arsenite than arsenate was methylated by each homogenate. Rat liver had the highest methylating activity, converting 1.8 % of the arsenite and 1.2 % of the arsenate in the homogenates to methylarsenic derivatives during incubation for 90 min. Liver homogenates were more active than kidney homogenates. The homogenates from mouse

Table 1 Amounts of inorganic arsenic, methylarsenic (MA) and dimethylarsenic (DMA) compounds (expressed as arsenic) in 4.2 cm^3 of incubated mixture[a]

		Arsenic in 4.2 cm^3 incubated mixture (ng)			
Homogenate	Arsenic compound added	Inorg. As[b]	MA[b]	DMA[b]	MA[b] + DMA[b]
Liver (rat)	As(III)	3889	32	42	74
	As(V)	3906	19	30	49
Kidney (rat)	As(III)	3889	20	34	54
	As(V)	4130	8	3	11
Liver (mouse)	As(III)	3839	16	14	30
	As(V)	4129	8	17	25
Kidney (mouse)	As(III)	3881	12	0	12
	As(V)	4133	0	0	0

[a]The reaction mixtures, each containing 2 cm^3 of liver or kidney homogenates from rats and mice, were incubated for 90 min at 37°C in the presence of arsenite or arsenate (13.3 μmol dm^{-3}, 4.2 μg arsenic/4.2 cm^3).
[b]Corrected for background (2 cm^3 of 15 % homogenates). Background values are as follows:

Liver (rat)	Inorganic As 0.4 ng, MA 0 ng, DMA 2.8 ng, TMA 3.7 ng.
Kidney (rat)	Inorganic As 1.6 ng, MA 2.5 ng, DMA 4.0 ng, TMA 0 ng.
Liver (mouse)	Inorganic As 0.9 ng, MA 0 ng, DMA 0 ng, TMA 0 ng.
Kidney (mouse)	Inorganic As 1.0 ng, MA 0 ng, DMA 0 ng, TMA 0 ng.

Table 2 Amount of inorganic arsenic, methylarsenic (MA) and dimethylarsenic (DMA) compounds (expressed as arsenic) in 4.2 cm^3 of incubated mixture[a]

Homogenate	Arsenic compound added[b]	Arsenic in 4.2 cm^3 incubated mixture (ng)				Percentage methylated
		Inorg. As	MA	DMA	MA + DMA	
Liver	As(III) (n = 9)	2961 ± 200	59 ± 15	7 ± 9	66 ± 21	2.1
	As(V) (n = 5)	3021 ± 243	21 ± 5****	9 ± 8	30 ± 9***	1.0
Kidney	As(III) (n = 4)	3040 ± 197	33 ± 18	12 ± 10	45 ± 11	1.4
	As(V) (n = 4)	3011 ± 195	11 ± 3*	15 ± 5	26 ± 8**	0.8

[a]The reaction mixture containing 2 cm^3 of liver and kidney homogenates from rats was incubated for 90 min at 37°C in the presence of arsenite or arsenate (10 μmol dm^{-3}, 3.15 μg arsenic/4.2 cm^3). Results are given as mean ± SD.
[b]Numbers of samples analyzed, each from different preparations of homogenates, are given in parentheses.
*, **, ***, ****Significantly different from corresponding As(III) value of same organ at $P < 0.01$, $P < 0.05$, $P < 0.005$, $P < 0.001$ by t-test.

kidneys converted arsenite only to a monomethylated arsenic compound and did not methylate arsenate at all. Because the homogenates prepared from rat livers and rat kidneys methylated arsenite more efficiently than the corresponding homogenates from mice, all subsequent experiments were carried out with rat homogenates.

The incubation experiments were repeated with liver and kidney homogenates pooled from at least two and at most four rats under an atmosphere of air or nitrogen. The initial arsenic concentrations in these experiments were 10 μmol dm^{-3} (3.15 μg arsenic/4.2 cm^3). Incubation under an atmosphere of nitrogen failed to promote methylation. The averaged results (Table 2) show that arsenite and arsenate were converted to mono- and di-methylated arsenic compounds. The amount of methylated arsenic in the arsenite-spiked homogenates was approximately twice the amount in the arsenate-spiked homogenates. The conversion of inorganic to methylated arsenic compounds did not exceed 2.1 % during the 90 min. The amount of dimethylated arsenic is less than the amount of monomethylated arsenic (with the exception of the arsenate-spiked kidney homogenates) and is not significantly different among the four experimental groups (Table 2).

The amount of monomethylated arsenic (59 ng) produced by arsenite-spiked (10 μmol dm^{-3}) liver homogenates (Table 2) was the same as the amount (58 ng) reported by Buchet and Lauwerys[19] under similar conditions. The amount of dimethylated arsenic (7.0 ng), however, was much less than observed in Buchet's system (230 ng).

The reasons for the differences in dimethylarsenic production are at present obscure. Although excess arsenite and mercury(II) ions are known to inhibit the conversion of monomethyl to dimethylarsenic compounds,[6,10,13,19,28,29] an inhibition by arsenite at 10 μmol dm^{-3} concentration is unlikely, because Buchet and Lauwerys,[19] also using a 10 μmol dm^{-3} solution, observed more dimethyl- than monomethylarsenic species, as we also did in our preliminary experiments (Table 1). Administration of arsenic trioxide to hamsters (4.5 mg arsenic/kg body weight) led to the formation of inorganic arsenic, methylarsenic and dimethylarsenic compounds in the liver and the kidneys, in which total arsenic concentrations of 0.2−1.8 μg arsenic/g tissue were found.[3] Dimethylated arsenic was the major metabolite in the urine of hamsters. However, in the liver and the kidneys the concentration of monomethylated arsenic was higher than that of dimethylated arsenic. The *in vivo* conversion of inorganic arsenic into a dimethylated form seems to be facilitated by the rapid transport of dimethylated arsenic into the urine. In *in vitro* experiments with homogenates, therefore, dimethylation of inorganic arsenic might be depressed by the absence of a transport system.

Influence of thiols on the methylation of arsenite

The *in vitro* methylation of arsenic is promoted by GSH.[19] Reduced glutathione catalyzes the reduction of arsenate to arsenite and may keep thiol groups of enzymes in the reduced form. These findings prompted us to explore the effects of other thiol agents on the methylation of arsenite. Rat liver homogenates in sucrose solution containing 0.01 mol dm^{-3} Tris

Table 3 Amount of methylarsenic (MA) and dimethylarsenic (DMA) compounds (expressed as arsenic) in 4.2 cm^3 of incubated mixture[a]

	Arsenic in 4.2 cm^3 incubated mixture (ng)		
Thiol	MA	DMA	MA + DMA
None	0	0	0
GSH	7	22	29
Dithiothreitol	92	0	92
Mercaptoethanol	45	0	45
GSH + GSH-generating system[b]	35	32	67

[a]The reaction mixture, containing 2 cm^3 of rat liver homogenate in a sucrose solution buffered with 0.01 mol dm^{-3} Tris at pH 7.4, was incubated for 90 min at 37°C in the presence of arsenite (10 μmol dm^{-3}, 3.15 μg arsenic/4.2 cm^3) and thiol reagents (10 mmol dm^{-3}). [b]GSH-generating system consists of 5 mmol dm^{-3} NADPH and 5 mmol dm^{-3} glutathione reductase.

buffer at pH 7.4 were incubated in the presence of arsenite (10 μmol dm^{-3}) and GSH, dithiothreitol, or mercaptoethanol (10 mmol dm^{-3}). Tris buffer (0.01 mol dm^{-3}) decreased the amount of methylated compounds as compared with a Hepes buffer (0.05 mol dm^{-3}) of the same pH 7.4 (Tables 2, 3). In a thiol-free medium no methylation occurred (Table 3). In the presence of dithiothreitol or mercaptoethanol at 10 mmol dm^{-3}, arsenite was methylated but only to the methylarsenic stage. GSH at 10 mmol dm^{-3} caused formation of methylarsenic and dimethylarsenic species in small amounts (29 ng). In the presence of a GSH-generating system that reduced GSSG with NADPH to GSH, the amount of methylated arsenic species increased to 67 ng.

Influence of arsenite or arsenate concentration on arsenic methylation

Homogenates from rat livers or rat kidneys were incubated in a Hepes buffer medium in the presence of arsenite or arsenate in the concentration range 1.3–20 μmol arsenic dm^{-3} (Fig. 1). The amount of monomethylarsenic from arsenite increased from 29 ng at 1.3 μmol dm^{-3} to 135 ng at 20 μmol dm^{-3} in 4.2 cm^3 of a sample containing liver homogenate. The percentage of the arsenic methylated, however, decreased from 6.9 % to 2.1 % in the same sequence (Fig. 1). Dimethylarsenic did not change much, remaining in the range 0–9 ng. The amount of methylarsenic from arsenate increased also with increasing arsenate concentration but was always considerably smaller than the amount generated from arsenite at the same concentration (Fig. 1).

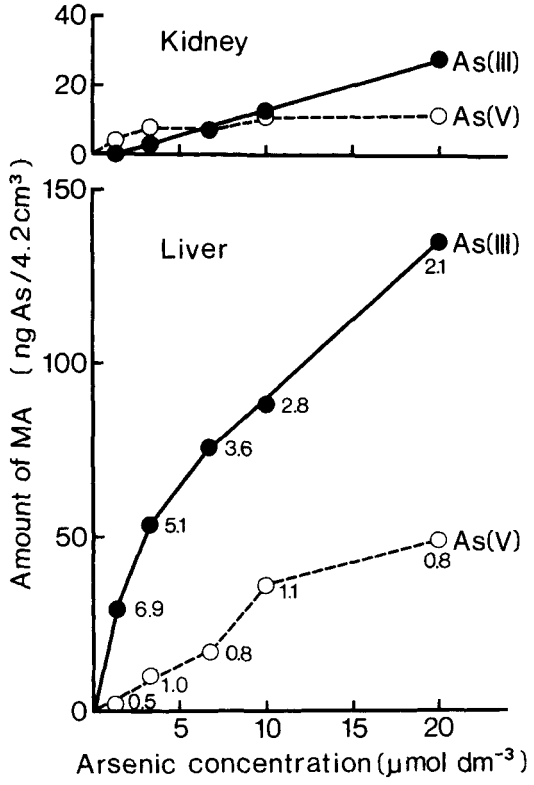


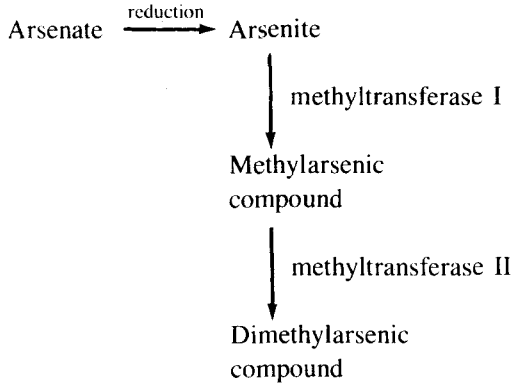
Figure 1 Amounts of methylarsenic compound (MA) in 4.2 cm^3 of incubated mixture (expressed as arsenic). The reaction mixture, containing 2 cm^3 of liver and kidney homogenates from rats, was incubated for 90 min at 37°C in the presence of arsenite and arsenate in the range 1.3–20 μmol dm^{-3}. Numbers inserted by the curves are percentages of MA produced.

Kidney homogenates had a much lower methylating activity than liver homogenates (Fig. 1). This difference is especially pronounced at higher arsenic concentrations. Arsenite and arsenate appear to be methylated to the same extent by kidney homogenates up to arsenic concentrations of 10 μmol dm^{-3}.

To determine the importance of non-enzymic methylation in these reactions, rat homogenates were boiled for 5 min to destroy enzyme activity. The boiled homogenates and the medium without 2 cm^3 of homogenates, but with 2 cm^3 of 0.25 mol dm^{-3} sucrose-containing 0.05 mol dm^{-3} Hepes buffer of pH 7.4, were incubated with arsenite or arsenate (10 μmol dm^{-3}). The following results were obtained:

Boiled homogenates from rats/arsenite	15 ng MA, 6 ng DMA
Boiled homogenates from rats/arsenate	7 ng MA, 2 ng DMA
Medium only/arsenite	15 ng MA, 6 ng DMA
Medium only/arsenate	0 ng MA, 0 ng DMA

These amounts of monomethylarsenic compound are much smaller than the amounts observed from the biologically active homogenates. Therefore, considerable fractions of monomethylarsenic compounds are produced by enzymic reactions, whereas amounts of dimethylarsenic compound appear as an enzymic fraction and a non-enzymic fraction. The conversion of inorganic arsenic to dimethylated arsenic therefore appears to be a two-step reaction catalyzed by two different methyltransferases (Scheme 1). Methyltransferase II was found to be a labile enzyme in *in vitro* experiments and susceptible to inhibition.[19]



Scheme 1

Double-reciprocal plots of the initial rate of the conversion of arsenite and arsenate to a monomethylarsenic species in the liver homogenates (Fig. 2) indicate that arsenite with a K_m value of 5.3 μmol dm^{-3} has an 11-fold higher affinity for the monomethyltransferase (methyltransferase I) than arsenate (K_m = 58.9 μmol dm^{-3}). Arsenite seems to be the arsenic compound that is methylated.[7,20] For arsenate to be methylated, it must first be reduced to arsenite. This reduction reaction remains to be studied by following the change in the concentrations of arsenite and arsenate during the incubation.

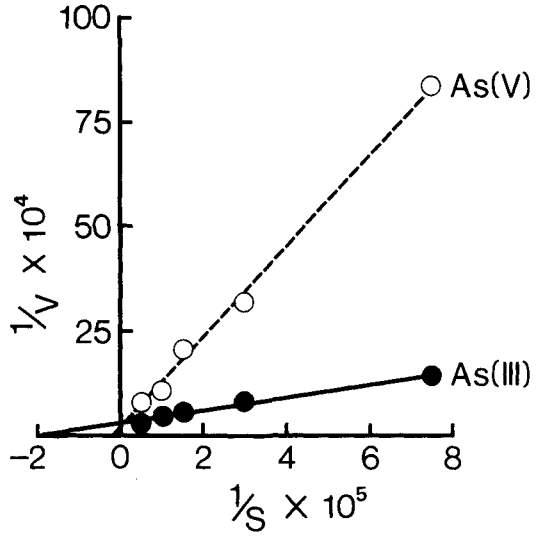


Figure 2 Kinetics of the monomethyltransferase activity of rat liver. V = rate (mol dm^{-3}/90 min); S = inorganic arsenic concentration (mol dm^{-3}).

CONCLUSION

Two inorganic arsenic species, arsenite and arsenate, were partly converted to methylarsenic (MA) and dimethylarsenic (DMA) compounds by rat/mouse homogenates after incubation in the presence of *S*-adenosylmethionine, reduced glutathione (GSH), magnesium (Mg^{2+}) and methylcobalamine. The rat homogenates methylated inorganic arsenic more efficiently than the mouse homogenates. The amount of MA in the rat homogenates was larger than that of DMA. This result is inconsistent with a previous report using rat liver cytosol, *in vivo* studies showing that less MA and more DMA were excreted in the urine of humans and animals. Incubation under nitrogen did not increase the yield of MA and DMA compared with incubation in air. However, the addition of GSH and other thiols activated the methylation ability of the rat homogenates. The amount of MA in rat liver homogenates increased with increasing arsenite or arsenate concentration (1.3–20 μmol dm^{-3}), whereas the amount of DMA was nearly constant in the dose range. Rat kidney homogenates methylated inorganic arsenic to a much smaller extent than rat liver homogenates. The K_m of arsenite (5.3 μmol dm^{-3}) for the monomethyltransferase in rate liver homogenates was 11-fold higher than the corresponding K_m of arsenate (58.9 μmol dm^{-3}). Evidence that arsenate is reduced to arsenite during incubation was not demonstrated but

the difference in the above affinity may have been dependent on the concentration of arsenite present in the incubation medium. This finding contributes to the explanation that arsenite is responsible for sequential methylation.

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