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ROLE OF THIOLS IN THE *IN-VITRO* METHYLATION OF INORGANIC ARSENIC BY RAT LIVER CYTOSOL

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Abstract—Rat liver cytosol inactivates inorganic arsenic (Asi) through methylation; *S*-adenosylmethionine is the methyl group donor and reduced glutathione (GSH) is required for full activity. The study of the combined effects of Asi, GSH and other thiols *in vitro* and the results of our previous *in-vivo* studies in humans and rats are consistent with a pathway involving the formation of a monomethylated metabolite which is either rapidly further methylated into a dimethylated derivative or is spontaneously oxidized into monomethylarsonic acid (MMA). The dimethylated metabolite gives rise to dimethylarsinic acid. The first methylation reaction is rate limiting, can be stimulated by GSH and is catalyzed by an enzyme different from that which transfers the second methyl group. The latter is sensitive to inhibition by inorganic arsenic. The stimulation of the first methylation reaction by GSH can only be evidenced at high Asi concentration because under these conditions, the second methylating enzyme can be sufficiently inhibited by Asi to allow some accumulation of MMA. The latter may also slow down the first methylation reaction. A large excess of thiol groups may prevent the methylation reactions probably by decreasing the amount of free trivalent arsenic.

The presence of a methylating activity toward inorganic trivalent arsenic has been previously demonstrated in the cytosol of rat liver. This biotransformation leads to the synthesis of a monomethylated and a dimethylated arsenic derivative which can be determined by flameless atomic absorption as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), respectively; *S*-adenosylmethionine is the methyl group donor and reduced glutathione (GSH) addition is required for full activity. The existence of two distinct enzymatic activities involved in the methylation process has been inferred from the observation that an excess of substrate and the addition of mercuric ions prevent the formation of the dimethylated arsenic derivative without affecting that of the monomethylated compound [1].

In-vivo experiments have confirmed the important role of liver GSH in the metabolism of inorganic arsenic [2]. A depletion of liver glutathione exceeding 90% of the control value leads to a decreased urinary excretion of MMA and DMA and an increased urinary excretion of inorganic arsenic (Asi) following a challenge dose of $NaAsO_2$. When GSH depletion is less severe, the total amount of arsenic excreted in urine after a challenge dose of $NaAsO_2$ is not significantly different from that found in unpretreated animals but the proportion of the three metabolic forms is different: MMA is reduced whereas Asi and DMA tend to increase. These changes resemble those found in patients with liver insufficiency [3].

To gain more insight into the mechanism of inorganic arsenic methylation by rat liver cytosolic enzymes, we have investigated the combined effects of substrate, GSH and other thiol concentrations on the *in-vitro* production of the methylated arsenic metabolites.

MATERIALS AND METHODS

Reagents. *S*-adenosyl-L-methionine (SAMe) in the form of sulfuric and *p*-toluene sulfonic acid salt was kindly offered by Dr. G. Tallone (Bioresearch Co., Liscate, Italy). Vitamin B_{12}, GSH and L-buthionine(*S*,*R*)-sulfoximine (BSO) were purchased from Sigma Chemie GmbH (Deisenhofen, F.R.G.) and phorone (2,6-dimethyl-2,5-heptadien-4-one) from Janssen Chimica (Beerse, Belgium). Other analytical grade chemicals were from Merck (Darmstadt, F.R.G.).

Animals and liver cytosol preparations. Three to four months old Sprague–Dawley rats were used. They had free access to water and a standard diet (AO_3 pellets UAR, Epinay-sur-Orge, France). To decrease the GSH content of the liver before cytosol preparation some animals were fasted overnight and injected i.p. 2 hr before death with phorone [4] dissolved in olive oil at a dose of 250 mg/kg body weight or with BSO [5] in distilled water at a dose of 625 mg/kg. The animals were anesthetized with pentobarbital and after sectioning the inferior vena cava, they were perfused, through a needle introduced in the left ventricle, with 100 ml ice cold 0.9% NaCl containing EDTA 1 mM. The liver was removed, rinsed in the same medium, blotted on filter paper, weighed, transferred in a Potter glass tube kept at 0° and homogenized (30 g/100 ml) in sucrose 0.25 M buffered with Tris–HCl 0.01 M, pH 7.6. The cytosolic fraction was obtained by centrifugation of the whole homogenate at 100,000 *g* for 60 min and diluted twofold with buffered sucrose just before

incubation; the protein content of this preparation was determined by the method of Lowry *et al.* [6] and amounted to about 8 mg/ml. The concentration of reduced glutathione in the cytosol preparation was determined with the Ellman [7] reagent (5,5'-dithiobis(2-nitrobenzoic acid)) after protein precipitation with trichloroacetic acid.

In-vitro *incubation system.* Two milliliters of the cytosolic preparation were mixed with 2 ml of the buffered cofactor solution to have the following final concentrations during incubation: Tris–HCl, 0.05 M, pH 7.6, SAMe 1 mM, vitamin B_{12} 100 μg/ml, Mg^{2+} 1.2 mM and a thiol from 0.1 to 50 mM. The incubation was carried out in stoppered glass tubes with a side arm containing 0.2 ml $NaAsO_2$ solution to obtain a final concentration ranging from 2 to 40 μM. Before the incubation, the tubes were kept in melting ice and the air phase was replaced by nitrogen. The tubes were then transferred to a water bath at 37° and the incubation was initiated by tipping the substrate (As^{3+}) from the side arm. At the end of the incubation period the reaction was stopped by addition of 2 ml 15% trichloroacetic acid. The tubes were centrifuged at 1200 *g* for 10 min. The supernatant was poured in another tube whereas the precipitate was dissolved in 3 ml NaOH 1%. The amounts of MMA or DMA measured in both supernatant and precipitate were summed up. Each incubation was performed in triplicate.

Determination of arsenic derivatives. Inorganic arsenic and its methylated forms MMA and DMA were transformed into their corresponding arsines through reduction with $NaBH_4$ at pH between 0 and 1. The arsines were condensed in a trap cooled in liquid nitrogen, eluted according to their boiling point through warming up of the trap and detected by atomic absorption spectrophotometry using an electrically heated quartz tube. A detailed description of the technique and reproducibility data have been published [8].

Statistical analysis. The results were analyzed by the Student *t*-test or by one way analysis of variance and comparisons between the control mean and the means of the different treatment groups were performed with the Dunnett's test [9]. In case of heterogeneous variance, the *F* statistic was calculated as a pair of weighted mean squares according to Snedecor [10]. The combined effect of substrate and GSH concentration was assessed by a 2-way analysis of variance according to Barlow [11].

RESULTS

Increasing the amount of Asi in the incubation system has an opposite effect on the amount of MMA and DMA which accumulates in the absence as well as in the presence of 1 mM GSH (Fig. 1). The amount of MMA which accumulates in the incubation system increases with substrate concentration until the latter reaches a saturating concentration of about 20 μM; the addition of GSH 1 mM hardly modifies the MMA yield. The amount of DMA produced is optimum for an Asi concentration of 5 μM and the addition of GSH (1 mM) to the incubation medium enhances the amount of the dimethylated arsenical produced. Higher substrate concentrations significantly reduce

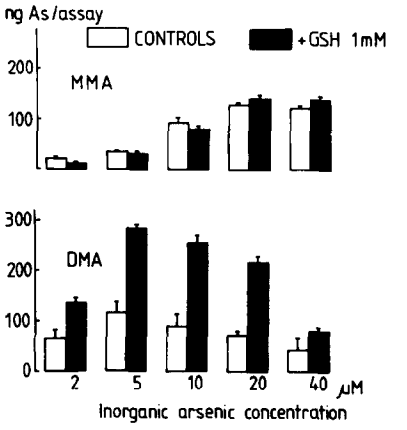

Fig. 1. Influence of substrate concentration on the synthesis of MMA and DMA in the absence and in the presence of GSH 1 mM.

DMA production. A two way variance analysis confirmed the presence of a significant ($P < 0.001$) effect of substrate (Asi) concentration on both MMA and DMA production while GSH addition (1 mM) had a significant effect only on DMA production. Moreover in the latter case a clearcut interaction between GSH and Asi ($P < 0.001$) was also evidenced.

We have compared the time course of MMA and DMA accumulation in the absence and in the presence of GSH 1 and 3 mM (Fig. 2). The latency period for the DMA production is not as marked as found previously [1] probably because in the present work not only the soluble DMA but also the fraction which precipitates with proteins was measured.

Another experiment designed to study further the influence of GSH shows that the amount of MMA which accumulates after 90 min incubation of rat liver cytosol with 10 μM Asi falls progressively when the GSH concentration exceeds 1 mM whereas that of DMA increases progressively to reach a maximum at a GSH concentration of about 3 mM and falls at higher GSH concentration (Table 1).

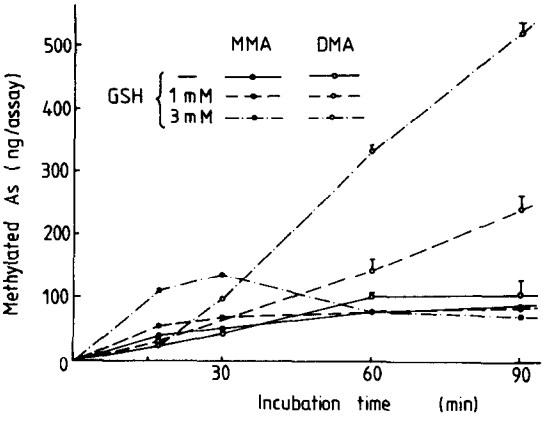

Fig. 2. Influence of GSH and incubation time on the synthesis of MMA and DMA (substrate concentration, 10 μM Asi).

Table 1. Effect of GSH on the synthesis of monomethylarsonic (MMA) and dimethylarsinic (DMA) acid by rat liver cytosol enzymes (substrate concentration, 10 μM Asi)

GSH concentration (mM)	Arsenic (ng/assay·90 min)	
	MMA	DMA
0.65*	93.4 ± 10.6†	91.4 ± 23.5
0.70	95.6 ± 2.2	132.6 ± 20.2‡
1.15	80.4 ± 3.7‡	269.1 ± 12.2‡
3.15	47.7 ± 2.9‡	459.4 ± 46.2‡
5.65	41.6 ± 1.0‡	102.5 ± 6.4‡
10.65	20.5 ± 0.1‡	56.9 ± 2.5‡
25.65	17.2 ± 0.3‡	11.6 ± 1.1‡

* GSH concentration in the incubation system.
† Mean ± SD (N = 3).
‡ Statistically different from control results (i.e. no GSH addition) (Dunnett's test).

The GSH concentration of rat liver cytosol preparations was lowered either by dialysis against the homogenization medium during 4 hr at 4° or by pretreatment of animals with phorone or BSO (see Materials and Methods). The residual GSH level amounted to 42, 20 and 3% of control value in the dialyzed cytosol and that prepared from animals pretreated with BSO and phorone, respectively. Results presented in Table 2 show that GSH depletion decreases the in-vitro production of both arsenic metabolites. The pretreatment with phorone which produces the most important GSH depletion by comparison with that obtained after dialysis or BSO pretreatment leads to a complete inactivation of the arsenic methylating system. The addition of GSH (5 mM) has no effect on the arsenic methylating capacity of the cytosol from phorone pretreated animals and partially reverses that of dialyzed cytosol. After BSO pretreatment, GSH addition to the cytosol has no effect on DMA production and partially reverses the inhibition of MMA accumulation.

Table 3. Effect of thiols on the MMA and DMA production by rat liver cytosolic enzymes (substrate concentration, 10 μM Asi)

Concentration of added thiol (mM)		As in ng/assay·90 min		
		MMA	DMA	Total
GSH	1	90 ± 8†	106 ± 16	196 ± 18
		85 ± 3	242 ± 30‡	327 ± 30‡
DTT	0.005	110 ± 4‡	156 ± 37	266 ± 37‡
	0.05	155 ± 1‡	250 ± 12‡	405 ± 12‡
	0.5	64 ± 8‡	663 ± 77‡	727 ± 77‡
	5	31 ± 2‡	301 ± 9‡	332 ± 9‡
	10	30 ± 1‡	139 ± 16	169 ± 16
ME	0.005	95 ± 4	160 ± 25	255 ± 25‡
	0.05	90 ± 2	159 ± 10	249 ± 10
	0.5	121 ± 1‡	339 ± 8‡	460 ± 8‡
	5	192 ± 7‡	260 ± 52‡	452 ± 52‡
	10	202 ± 1‡	69 ± 10	271 ± 10‡
DMSA	0.05	98 ± 9	14 ± 2*	112 ± 9‡
	0.5	3 ± 1‡	—	3 ± 1‡
DMPS	0.05	10 ± 1‡	—	10 ± 1‡
	0.5	—	—	—

* Not detected.
† Mean ± SD (triplicate measurements).
‡ Statistically different from control values (no thiol added).

The effects of thiols other than glutathione on the production of MMA and DMA by rat liver cytosol was compared (Table 3). At some concentrations, neutral thiols (dithiothreitol and 2-mercaptoethanol) can stimulate MMA and DMA production whereas thiols bearing an acidic group (dimercaptosuccinic and dimercaptopropanesulfonic acids) almost completely abolish the methylation reaction at a concentration of 0.5 or 0.05 mM.

Table 2. Arsenic methylation by enzymes from rat liver cytosol depleted in GSH through dialysis or animal pretreatment with buthionine sulfoximine (BSO) or phorone

Cytosol	Substrate concentration 5 μM			20 μM		
	As in ng/assay·90 min					
	MMA	DMA	Total	MMA	DMA	Total
Control	62 ± 4†	146 ± 20	208 ± 21	141 ± 7	72 ± 12	213 ± 14
id + GSH 5 mM	39 ± 3‡	489 ± 40‡	528 ± 40‡	213 ± 12‡	368 ± 18‡	581 ± 22‡
Dialyzed*	50 ± 1	55 ± 6	105 ± 6	102 ± 9	44 ± 9	146 ± 13
id + GSH 5 mM	58 ± 1‡	169 ± 25‡	227 ± 25‡	146 ± 26‡	61 ± 23	207 ± 35‡
Control	56 ± 1	155 ± 19	211 ± 19	113 ± 6	79 ± 8	192 ± 10
id + GSH 5 mM	31 ± 6‡	572 ± 4‡	603 ± 7‡	183 ± 1‡	357 ± 23‡	540 ± 23‡
BSO*	17 ± 9	20 ± 12	37 ± 13	7 ± 3	14 ± 3	21 ± 4
id + GSH 5 mM	32 ± 8	12 ± 2	44 ± 8	54 ± 3‡	13 ± 1	67 ± 3‡
Phorone*	<1	<1	<1	4 ± 1	22 ± 1	26 ± 1
id + GSH 5 mM	6 ± 1	6 ± 1	12 ± 1	4 ± 1	2 ± 1‡	6 ± 1‡

* Residual GSH concentrations: 42, 20, 3% of control values (0.6 mM) for dialyzed cytosol and those from animals pretreated with BSO and phorone, respectively.
† Mean ± SD (triplicate measurements).
‡ Statistically different from the result obtained in the absence of GSH (Student *t*-test).

Table 4. MMA methylation and competition experiment

Incubation conditions (μM)		Methylated arsenic compound measured after 90 min incubation (μM)	
Asi	MMA	MMA	DMA
0	0.085	0.079 ± 0.002	—
0	0.170	0.162 ± 0.006	—
0	0.255	0.229 ± 0.011	—
10	0	0.069 ± 0.003	5.456 ± 0.370
10	0.085	0.121 ± 0.004	4.167 ± 0.109
10	0.170	0.184 ± 0.003	3.688 ± 0.217

Experiments were also performed in which MMA and DMA were used as substrates in place of Asi. No methylation of MMA into DMA (Table 4) and DMA into a trimethylated derivative (results not shown) was found in the presence of GSH 5 mM. A competition experiment, however, shows that the methylation of inorganic arsenic is slightly inhibited by the prior enrichment of the incubation medium with MMA. If the amount of methylated derivative found when only MMA is added to the incubation medium (0.079 or 0.162 μM MMA and 0 μm DMA) is substracted from that found when both Asi and MMA have been added (0.121 or 0.184 μM MMA and 4.167 or 3.688 μM DMA) and the results are expressed as a percentage of the amounts of methylated derivatives measured in the presence of Asi only (0.069 μM MMA and 5.456 μM DMA), it appears that the production of the monomethylated metabolite is reduced by 39–67% and that of the dimethylated compound by 24 and 32.5% in the presence of 0.085 and 0.170 μM MMA, respectively (Table 4).

DISCUSSION AND CONCLUSIONS

The present investigation underlines the important role of GSH in the methylation of inorganic arsenic into MMA and DMA by rat liver cytosol. GSH may act through different mechanisms: protection of labile thiol groups, activation of methylating enzymes, regulation of free trivalent arsenic concentration. The complete abolition of the methylating activity in the liver cytosol of rats depleted in glutathione by phorone (GSH consumption) or BSO (inhibition of γ-glutamylcysteine synthetase) pretreatment, despite *in-vitro* supplementation with GSH suggests the existence of labile thiol groups which are irreversibly inactivated in an unsatisfactorily reducing environment. This is also consistent with our finding of a complete abolition of the methylating activity in rat liver stored at 4° for 24 hr before cytosol preparation and our failure to detect any methylation of arsenic in human liver collected 24 hr after death. The partial loss of methylating activity when the incubation is performed in open tube and the complete disappearance of the activity when the incubation medium is saturated with oxygen tend to confirm the importance of the integrity of labile sulfhydryl group(s) for the enzymatic process responsible for the methylation of arsenic (results not shown). A similar observation has been reported for the methylation of selenium. In the latter case, however, GSH is necessary for a non-enzymic synthesis of a selenotrisulfide derivative which is enzymatically reduced by NADPH to hydrogen selenide, the latter being the substrate for the methyltransferases. This dithiaselane formation is responsible for a characteristic log-phase in the synthesis of dimethylselenide [12]. With arsenic neither the requirement of a NADPH generation system nor a log-phase is observed for the monomethylated derivative production at least when the incubation is carried out in the absence of oxygen. The reducing character of GSH could also be important for maintaining arsenic in the trivalent state because it seems now well established that arsenic must be reduced to be methylated [13–16].

The results of the present *in-vitro* study on inorganic arsenic biotransformation and those of our previous *in-vivo* studies in humans [3] and in rats [2] are consistent with a pathway involving two consecutive methylation steps (Fig. 3). A monomethylated metabolite is first formed which is either rapidly further methylated into a dimethylated derivative or is spontaneously oxidized into MMA. The dimethylated metabolite gives rise to DMA. We were never able to detect the presence of a trimethylated derivative *in vitro* as well as in urine of human or rats treated with inorganic arsenic.

The latency period for the appearance of DMA following the start of the incubation of rat liver cytosol with inorganic arsenic and the progressive

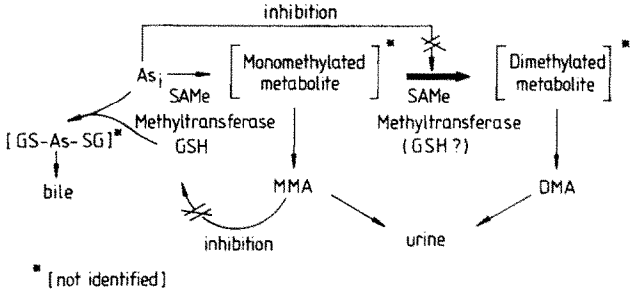

Fig. 3. Pathway of inorganic arsenic metabolism in mammalian liver.

levelling of or even the decrease of MMA accumulation with time (Fig. 2) are in agreement with the scheme depicted in Fig. 3. The first methylation reaction is rate limiting, can be stimulated by GSH (Fig. 2 and Table 2) and is catalyzed by an enzyme different from that which transfers the second methyl group. The latter is sensitive to inhibition by inorganic arsenic (Fig. 1) and also by mercuric ions [1].

The stimulation of the first methylation reaction by GSH can only be evidenced during the first 30 min of incubation (Fig. 2) or at high substrate (Asi) concentrations (Table 2) because under these conditions, the second methylating enzyme can be sufficiently inhibited by Asi to allow some accumulation of MMA. Whether the second methylation reaction can also be stimulated by GSH remains an open question. However, there is no need to assume such an effect of GSH to explain our results. Since the second methylating enzyme is in excess by comparison with the system catalyzing the production of MMA, an increased production of DMA at certain GSH concentrations can simply result from a stimulation of the first methylation step, and furthermore, a decrease of free Asi concentration in the incubation medium through binding by GSH may also facilitate the second methylation reaction. When the latter is inhibited (e.g. by an excess of Asi), the accumulation of MMA which results may reduce the rate of the first methylation reaction. A large excess of thiol groups may also block that reaction probably by decreasing the amount of free trivalent arsenic. It is possible, however, that the inhibition of the methylation reaction by thiol leads to an increased biliary excretion of Asi since some relationship has been shown between the biliary excretion of arsenic and glutathione [17].

It is interesting to mention that Maiorino and Aposhian [18] have observed an influence of dimercaptan metal-binding agents on the biotransformation of arsenite in rabbits largely because following treatment with these chelators, there is less free inorganic arsenic available in tissues for methylation.

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