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Interpretation of Inorganic Arsenic Metabolism in Humans in the Light of Observations made *in Vitro* and *in Vivo* in the Rat

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The toxicity of inorganic trivalent arsenic for living organisms is reduced by *in vivo* methylation of the element. In man, this biotransformation leads to the synthesis of monomethylarsonic (MMA) and dimethylarsinic (DMA) acids, which are efficiently eliminated in urine along with the unchanged form (As_i). In order to document the methylation process in humans, the kinetics of As_i, MMA and DMA elimination were studied in volunteers given a single dose of one of these three arsenicals or repeated doses of As_i. The arsenic methylation efficiency was also assessed in subjects acutely intoxicated with arsenic trioxide (As_2O_3) and in patients with liver diseases. Several observations in humans can be explained by the properties of the enzymic systems involved in the methylation process which we have characterized *in vitro* and *in vivo* in rats as follows: (1) production of As_i metabolites is catalyzed by an enzymic system whose activity is highest in liver cytosol; (2) different enzymic activities, using the same methyl group donor (*S*-adenosylmethionine), lead to the production of mono- and di-methylated derivatives which are excreted in urine as MMA and DMA; (3) dimethylating activity is highly sensitive to inhibition by excess of inorganic arsenic; (4) reduced glutathione concentration in liver moderates the arsenic methylation process through several mechanisms, e.g. stimulation of the first methylation reaction leading to MMA, facilitation of As_i uptake by hepatocytes, stimulation of the biliary excretion of the element, reduction of pentavalent forms before methylation, and protection of a reducing environment in the cells necessary to maintain the activity of the enzymic systems.

Keywords: Inorganic arsenic, monomethylarsonic acid, dimethylarsinic acid, human metabolism

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01INTRODUCTION

The acute toxic properties of inorganic arsenic have been known for a long time but its carcinogenic potential has been discovered more recently. Several epidemiological studies have demonstrated that the incidence of skin and lung cancers is significantly increased in subjects who have been chronically exposed to inorganic arsenic compounds by oral or respiratory routes.[1,2] Arsenic in inhaled air or drinking water is present mainly as tri- or penta-valent inorganic species which are much more toxic than the trimethylated arsenicals sometimes found in high amounts in food from marine origin. As the methylation of inorganic arsenic (As_i) in mammals reduces its acute toxicity,[3] partly by reducing its affinity for thiol groups and accelerating its excretion in urine, biotransformations catalyzed by methylating enzymes are also expected to moderate the carcinogenic activity of the element.

Inorganic arsenic toxicokinetics are well documented in animals but fewer studies have been carried out in man.[4–9]

In the present paper we summarize the results of our studies on some aspects of arsenic toxicokinetics in healthy volunteers, in subjects acutely intoxicated by As_i and in patients with liver diseases; finally we attempt to interpret our observations in the light of data collected *in vitro* and *in vivo* in rats.

MATERIALS AND METHODS

Reagents

Common laboratory chemicals were analytical grade reagents purchased from Merck (Darmstadt, Germany), except for sodium methylarso-

nate (99.4%), which was obtained from Carlo Erba (Milan, Italy) and vitamin B_{12} and reduced glutathione, which were purchased from Sigma Chemie GmbH München (Taufkirchen, Germany). *S*-Adenosyl-L-methionine (SAMe) was generously supplied in the stable form of disulfate–di-*p*-toluenesulfonate by BioResearch Co. (Liscate, Milan, Italy).

Arsenic analysis

Sodium arsenite (As_i) and its methylated forms MMA and DMA were determined in urine samples by atomic absorption spectrometry of the corresponding arsines[10] generated by reduction with $NaBH_4$ at $pH < 1$; when needed, samples were ashed with $Mg(NO_3)_2$ in an MgO matrix at 600 °C.[11] The same methodology was applied to incubation media involving rat liver cells or cytosol as a source of the arsenic methylating system.[12,13]

Subjects

The control group included subjects from the laboratory staff: six women (mean age $\pm SD = 41.3 \pm 6.7$ years) and seven men (29.7 ± 12.5 years) without any previous history of hepatic or renal dysfunction. Their serum levels of aspartate aminotransferase (GOT), alanine aminotransferase (GPT) and γ-glutamyltranspeptidase (GGT) were normal and those of HBs antigen and HBc antibodies undetected.

Another control group included 13 women and five men (50.5 ± 16.6 years) without any clinical or biochemical evidence of liver or renal dysfunction but with various non-hepatic diseases, pancreatic cancer or common bile duct stones or stricture. The group of patients comprised 38 subjects, among whom were 20 males, with mean age $\pm SD = 52 \pm 12$ years. On the basis of clinical, sero-biochemical and histological work-up, they were classified into alcoholic cirrhosis ($n = 18$), post-necrotic cirrhosis ($n = 14$) and primary biliary cirrhosis ($n = 6$).

All patients and controls gave informed consent to the study, the design of which was approved by the ethical committee of the Catholic University of Louvain. Details on acutely intoxicated subjects can be found elsewhere.[14]

Administration of arsenicals

For the comparative studies on the fate of As_i, MMA and DMA in the human body, arsenicals were ingested after dissolution in water. For the studies among patients with liver diseases, the intravenous route of administration was used as described before.[15,16] A sample of urine was obtained just before arsenic administration to determine the baseline level of arsenic metabolites. Urine was collected for one or several 24 h periods after treatment and the metabolites were measured as previously described.[10] Care was taken to prevent any additional exposure to As_i, MMA or DMA during the test, e.g. through consumption of wine, mineral water containing arsenic, or food from marine origin.

In vitro methylation system

The cytosolic fraction of rat liver was obtained by direct centrifugation (Beckman Model L3-50 ultracentrifuge) of a whole homogenate (10–30% in 0.25 M sucrose) at 145000 *g* for 30 min in a 50Ti rotor. The incubation system[13] contained (in a final volume of 4.2 cm^3) 2 cm^3 cytosolic preparation, 0.05 M Tris–HCl buffer (pH 7.6), 1 mM SAMe, 10 mM GSH, 100 $\mu g\ cm^{-3}$ vitamin B_{12}, 1.2 mM Mg^{2+} and 0.01 mM $NaAsO_2$. After incubation at 37 °C for 90 min under nitrogen, proteins were precipitated with trichloroacetic acid and arsenic species determined in the supernatant. Similar incubation conditions were used for *in vitro* experiments involving rat tissue slices.[12]

RESULTS

Arsenic species found in urine

In a preliminary study, we confirmed that, in man, exposure to inorganic arsenic leads to the urinary excretion of MMA, DMA and unchanged inorganic arsenic. We have determined the chemical forms of arsenic present in the urine of control subjects and workers occupationally exposed to arsenic trioxide; both groups of subjects had refrained from eating seafood for several days before urine sampling. The total arsenic concentration in urine, determined after a dry-ashing step with an $Mg(NO_3)_2/MgO$ mixture, was not significantly different from the sum of As_i, MMA and DMA measured by the arsine generation technique applied to original samples. An excellent correlation was found between both sets of results ($r = 0.99$; $n = 34$) for values ranging from 2 to 1600 $\mu g\ dm^{-3}$.[11]

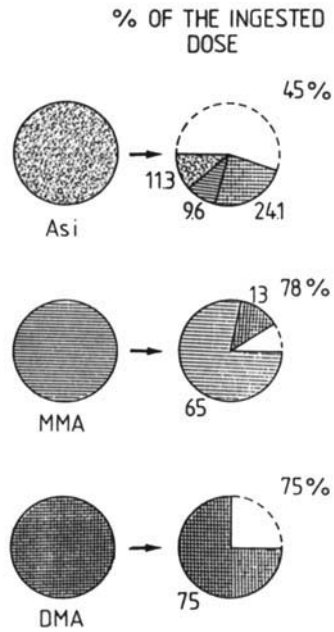

Figure 1 Urinary excretion of As_i and its metabolites within four days after oral administration of 500 μg arsenic as $NaAsO_2$, MMA or DMA.

Arsenic metabolites in urine after ingestion of 500 μg arsenic as sodium arsenite, monomethylarsonate or dimethylarsinate[17]

Four control subjects from the laboratory staff were each given a glass of water containing 500 μg arsenic as either $NaAsO_2$ or MMA or DMA. At least three months elapsed between each test for subjects participating in more than one test. The baseline excretion of As_i, MMA and DMA was determined the day before each experiment and the results were taken into account for the balance calculations. After four days, the mean urinary excretion expressed as the percentage of the dose ingested amounted to 45, 78 and 75% in the case of $NaAsO_2$, MMA and DMA respectively (Fig. 1). The time required for the excretion of half the total amount excreted in four days was 28, 4 and 11 h for As_i, MMA and DMA respectively.[17]

The speciation of arsenic in the urine samples leads to the following conclusions (Fig. 1):

(1) DMA is excreted without any transformation: there is no indication of either the formation of a trimethylated derivative or the demethylation of DMA into MMA or As_i;
(2) MAA also is not demethylated; but a small percentage (13%) can be transformed into DMA;
(3) in the case of As_i ingestion, the fraction of the dose which is excreted in four days amounts to only 45% but involves an important proportion (75%) of methylated metabolites; with time a rapid decrease of the inorganic fraction is observed which parallels the increase of the excretion of the methylated forms of arsenic; MMA increases transiently while the increase in DMA excretion lasts longer.

Arsenic metabolites in urine after repeated ingestion of 500 μg of arsenic as sodium arsenite[18]

When increasing oral doses of sodium arsenite (As_i) (up to 1 mg) are given daily for five days to volunteers, the amount of arsenic excreted in urine reaches a plateau around the fifth day. At that time the total arsenic excreted in urine per 24 h amounts to 60% of the ingested dose. However arsenic speciation in the urine samples collected during the day following the last arsenic administration shows that the amounts of arsenic excreted in the inorganic form are more or less linearly related to the arsenic dose administered; the same observation is made in the case of MMA but the excretion of the dimethylated form tends to level off at high inorganic arsenic dosages, indicating a possible saturation of the DMA production (Fig. 2).

Arsenic metabolites in urine after acute intoxication[14]

The urinary excretion of As_i and of its methylated metabolites has been followed up in five cases of deliberate ingestion of As_2O_3. Depending upon the severity of the intoxication, the major form excreted in urine during the first few days is the unmetabolized inorganic species; thereafter a progressive increase of the proportion excreted as MMA and DMA is observed. By comparison with the excretion pattern observed after ingestion of a small As_i dose, the pattern displayed in acute intoxication cases suggests that the methylation process is delayed until the As_i concentration in the organism has been sufficiently reduced.

Assessment of the liver involvement in As_i methylation

Four volunteers were given the same dose of As_i (500 μg arsenic as $NaAsO_2$) at three-month intervals, the first time by mouth and the second time

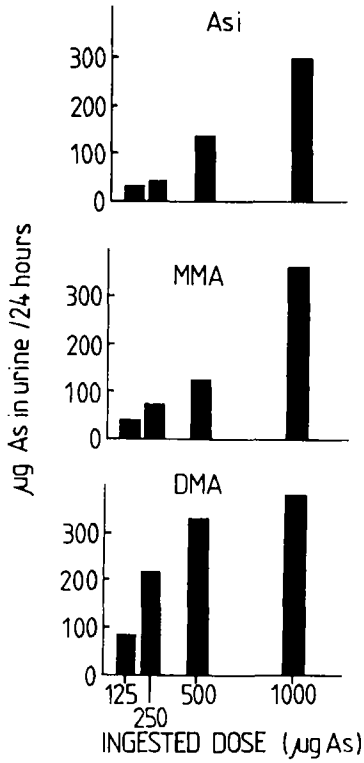

Figure 2 Relationship between the urinary excretion of As_i, MMA and DMA during the last 24 h period following the daily ingestion of 125, 250, 500 or 1000 µg arsenic as $NaAsO_2$ for five days.

intravenously. Urine was collected during the day before and during four days following the administration. Results are given in Table 2. They clearly demonstrate the involvement of the liver in the methylation of inorganic arsenic in man.

Some factors which might influence the arsenic methylation process in man were also investigated: mainly, the effect of a pretreatment with lipotropic agents, and the effect of liver diseases. For the first series of observations, five subjects received during three days before and four days after the arsenic ingestion (500 µg arsenic as $NaAsO_2$), 250 mg methionine, 250 mg choline, 250 mg inositol and 50 µg vitamin B_{12}, twice a day. This trial did not show that it was possible to increase the arsenic methylation capacity by supplementation of precursors of methyl donors.[17] No enhanced methylation capacity could be observed in subjects treated with well-known microsomal enzyme inducers, phenobarbital and diphenylhydantoin.[15]

The effect of liver diseases was assessed by comparing the methylation capacity of patients

Table 1 Urinary excretion of arsenic metabolites in cases of acute intoxication by As_2O_3

Subject	Day	Total As excretion[a] (µg)	As species excreted (% of total)		
			As_i	MMA	DMA
1	1	1900	64	17	19
	3	17600	83	6	11
	9	7400	3	2	95
2	1	16700	76	11	13
	3	10700	62	18	20
	8	4600	2	13	87
3	1	9660	6	11	13
	2	7200	56	22	22
	6	860	10	15	75
4	1	9660	76	14	1
	3	7200	56	2	36
	6	860	10	20	74
5	1	6200	100	0	0
	3	1870	62	7	21
	6	800	25	28	47

[a] µg//24 h for subjects 1 and 2; µg/g creatinine for the others.

Table 2 Arsenic excreted in urine within four days after intravenous or oral administration of 500 µg As_i to four male volunteers

	Arsenic concn in urine (%)[a]	
	Intravenous administration	Oral administration
Total	68.7 ± 4.5	60.9 ± 2.7
As metabolites		
As_i	52.1 ± 4.6	26.8 ± 2.7
MMA	12.0 ± 2.0	17.4 ± 1.9
DMA	35.9 ± 6.3	55.8 ± 3.8

[a] Total as % administered dose; metabolites as % of excreted amount.

with that of healthy controls or of patients with non-hepatic diseases.[16] The intravenous route of administration was used for these studies to avoid misinterpretations possibly due to variable rates of gastrointestinal absorption of inorganic arsenic in controls and in patients. Figure 3 presents the results related to the measurements of As_i and its metabolites in urine collected during the 24 h period following the intravenous administration of arsenic. The percentages of the dose administered which was excreted within 24 h by healthy controls, patients without hepatic diseases and

liver patients, are not statistically different and amount to 30.6±6.0, 29.8±9.1 and 28.7± 13.0%, respectively. Liver diseases have a striking but opposite effect on the excretion of the two methylated arsenic metabolites by comparison with the controls, viz. MMA excretion decreases while that of DMA increases.

Characteristics of the *in vitro* methylating system in the rat[13]

By examining the arsenic-methylating capacity of total homogenates of several organs in the rat, we have previously shown that the liver had by far the highest activity, which was localized in the cytosol.[13] *S*-Adenosylmethionine was a necessary cofactor, while the addition of reduced glutathione (GSH) stimulated the arsenic methylation. The action of mercuric ions on the arsenic methylating system led to the conclusion that two different enzymic activities are responsible for the production of the mono- and the di-methylated arsenic derivatives: indeed, mercury(II) ions up to a final concentration of 10^{-4} M have no influence on MMA production but on the contrary they inhibit DMA synthesis. The substrate concentration also has an opposite influence on MMA and DMA production. With a cytosol prepared from a 15% liver homogenate, DMA production reached an optimum at 5 μM As_i concentration and then decreased progressively with increasing substrate concentration, whereas MMA production had not yet reached its maximum velocity even at 40 μM As_i concentration.

The role of GSH was assessed by comparing the methylation performed by control rat liver cytosol supplemented with various concentrations of exogeneous thiols and by rat liver cytosol from rat treated with GSH-depleting agents such as phorone or buthionine sulfoximine. In the first series of experiments an optimal GSH concentration amounting to 3 mM was shown to be required for full methylating activity while, in the second series of experiments, a decreased or a complete inhibition of arsenic methylation was observed with cytosol from liver depleted in GSH.[19] Likewise, animals pretreated with GSH-depleting agents methylate As_i much less efficiently *in vivo* but the methylation is not improved by a pretreatment of animals with butylated hydroxytoluene, which causes a twofold increase of the liver GSH content.[20]

The study of the arsenic-methylating system in liver slices indicated that arsenic uptake by cells was favorably influenced by GSH, which also enhanced the formation of MMA.[12] Moreover, in this system the *in vitro* methylation of MMA, a process also enhanced by the addition of GSH, could be demonstrated; this was not possible with liver cytosol.

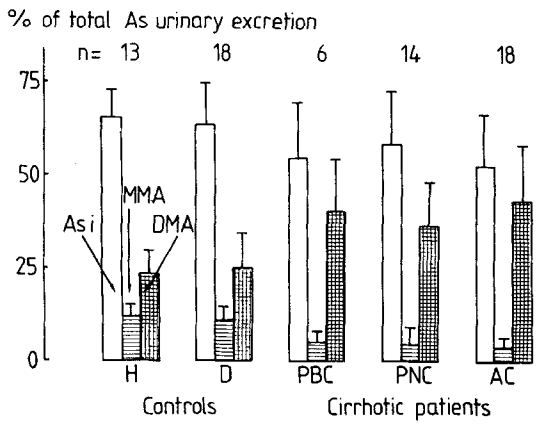

Figure 3 Urinary excretion of As_i, MMA and DMA (% of total amount of arsenic excreted) in controls (H, healthy subjects; D, patients without liver disease) and cirrhotic patients (PBC, primary biliary cirrhosis; PNC, macronodular cirrhosis; AC, alcoholic cirrhosis).

DISCUSSION AND CONCLUSIONS

The results of the *in vitro* study allow us to understand better the observations made on volunteers and patients suffering from liver diseases who were given arsenic(III) and on patients acutely intoxicated by arsenic(III).

First, the *in vitro* studies confirm that in mammals, MMA and DMA (or their mono- or dimethylated precursors) are the only metabolites of inorganic arsenic. No trimethylarsenic derivative was detected; its detection in urine, if any, must be due to interference by arsenic derivatives present in certain seafood.

In the rat, the liver is the main site of arsenic biotransformation. The results of the volunteer studies (influence of liver disease and of the absorption route on the efficiency of arsenic methylation) confirm that in man the liver is also the main site of arsenic biotransformation. The presence of arsenic methylases in the cytosolic fraction of hepatocytes has been demonstrated in

rats, and in man it was shown that microsomal enzyme inducers do not modify the As_i methylation capacity.

The substrate effect on MMA and DMA production explains the fact that in volunteers given increasing amounts of arsenic(III), the urinary excretion of DMA levels off at the highest dose whereas that of MMA increases linearly over the whole dose range. This substrate effect also explains the observation that in subjects acutely intoxicated by arsenic(III), several days may elapse before DMA becomes the preponderant metabolite. A logical hypothesis may also be offered to explain the puzzling observation made in patients with various types of liver diseases in whom the metabolism of arsenic(III) was investigated by measuring the urinary excretion of MMA and DMA following the intravenous administration of a standard dose of As_i. Depending upon the nature of the ailment, a reduction of the monomethylated species production was observed in association with enhanced production of DMA. To explain this we made the hypothesis that in patients with liver diseases, the uptake of As_i by hepatocytes is depressed. Indeed, if this is true the reduction in substrate availability will lead to a reduction of MMA production, but its further methylation into DMA will be stimulated due to the reduction of the inhibitory effect of As_i on the dimethylation reaction.

Results obtained with rat liver slices indicate that liver GSH plays an important role in the metabolism of inorganic arsenic by enhancing the uptake of the trivalent inorganic species by the hepatocytes, and by stimulating the monomethylating activity. It should also be noted that the biliary excretion of inorganic arsenic is regulated by GSH.[21,22] The fact that both the uptake and the excretion of As_i are controlled by GSH concentration makes the regulation of arsenic methylation rather complex; its elucidation will probably be facilitated by the isolation, purification and characterization of the arsenic-methylating proteins.

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