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An Insect Model for Assessing Arsenic Toxicity: Arsenic Elevated Glutathione Content in the *Musca domestica* and *Trichoplusia ni*

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Throughout history, arsenic has acquired an unparalled reputation as a poison. Arsenic was used as a poison as early as 2000 B.C. The toxicity of arsenic (As) extends to mammals, fish, insects, plants and fungi. Toxic compounds include those which generate arsenous anions, e.g. white arsenic, As_2O_3; sodium arsenites, $NaAsO_2$ and $Na_2As_2O_3$; and Paris green, $(CH_3.COO)_2\ Cu_3.Cu(AsO_2)_2.Ca(OH)_2$. According to epidemiological evidence, inorganic arsenic compounds have been strongly suggested as human carcinogens (Pershagen 1983). Human exposure to arsenic through various means is correlated with an increased incidence of skin, lung, and possibly liver cancers. Inorganic trivalent arsenic is systematically more poisonous than the pentavalent form and it is possible that pentavalent arsenic is reduced to the trivalent form before exerting any toxic effects.

Arsenites and arsenates interconvert in living systems via cytochrome c and cytochrome oxidase (Osborne and Ehrlich 1976). Toxicity is attributed to the trivalent form As^{3+} or arsenous anion, which bind covalently to a protein or enzymes cysteine groups (Squibb and Fowler 1983). This type of affinity for sulfur is even greater for dithiol-containing enzymes. The tripeptide glutathione (GSH) is the major cellular nonprotein thiol reductant which protects cells against free radicals, reactive oxygen species, and other toxicants. We previously reported that arsenic as As^{3+} and As^{5+} altered the activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), its peroxidase (GSTPX) and glutathione reductase (GR), lipid peroxidation and protein oxidation in *M. domestica* and *T. ni* (Zaman *et al.*, 1993a; *Zaman et al., 1995 Arch Insect Biochem Physiol in press*). In the present report, we focus on the potential of utilizing two insect species, the housefly, *Musca domestica* and the cabbage looper moth, *Trichoplusia ni*, as a model for the study of arsenic toxicity. In this study, after 48 hours of exposure to As^{3+} and As^{5+}, a significant induction of GSH level and subsequent decrease in the level of GSSG in both insect species were observed.

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MATERIALS AND METHODS

Housefly pupae of Fales 1958 T-II were obtained from S.C. Johnson and Son (Racine, Wisconsin). Male and female flies were separated within 24 hr of emergence. The males were discarded and the females were maintained *ad libitum* on a dry mixture of sucrose/low-fat powdered milk (1:1, w/w), and in a separate container, drinking water supplied with a paper towel. Flies were held at 24-27^{0}C on a 12:12 hr light/dark photoperiod. Cabbage looper was obtained from Boyce Thompson Institute (Ithaca, NY) and was reared according to the procedure reported earlier (Zaman *et al.*, 1994a; Zaman *et al.*, 1994b; *Zaman et al., 1995 Arch Insect Biochem Physiol in press*; Pritsos *et al.*, 1988; Ahmad and Pardini 1988, 1990). The concentration of As^{3+}, and As^{5+} was 0.005% (w/w) in the diet of *T. ni*, and in the water (w/v) for *M. domestica* respectively which was determined to be the LC_5 (Zaman *et al.*, 1993a; *Zaman et al., 1995 Arch Insect Biochem Physiol in press*). Three concentrations, 0.005, 0.05 and 0.5% for both arsenic compounds (As^{3+}, As^{5+}) and appropriate controls were tested in both adult female *M. domestica* and 4th-instar *T. ni*. Arsenic compounds were admininstered to *M. domestica* on a (w/v) basis in 25 mL drinking water and to *T. ni* on a (w/w) basis in 5 g diet blocks. *M. domestica* was also supplied with 1:1 sugar and powdered milk in a seperate container. Toxicological symptoms were observed and mortalities were recorded at 48 hours for both insects. Mortality data were pooled and the LC_{50}s and LC_5s (lethal to 50% and 5% of population respectively) were calculated using probit analysis (Finney 1964). All glassware was acid leached and rinsed with deionized water to remove contaminating arsenic. All compounds used in these experiments (water, sucrose, powdered milk, diet and paper towels) were free of arsenic contamination. Arsenic stock solutions were prepared with Millipore deionized water. Arsenic in tissue of both insects were detected as the "hydride" using an Atomic Absorption Spectrometer 951. The details of this method have been reported (Zaman *et al.*, 1994a; *Zaman et. al., 1995 Arch Insect Biochem Physiol in press*).

Adult female *M. domestica*, 10 insects/replicate, 3 replicates/ determination, were placed on their respective control (de-ionized drinking water) or 0.005% (w/v) of either As^{3+} or As^{5+} in the drinking water. Fourth-instar *T.ni* larvae, 10 larvae/replicate, 3 replicates/ determination, were placed on their respective control or 0.005% (w/w) As^{3+} or As^{5+}, spiked diets. The treatment level is ca. LC_5, the minimal acute dose, after 48 hr of treatment for both insects. Following approprite exposure times, 25% (w/v) homogenates of surviving insects were prepared with 50 mM potassium phosphate buffer with 1 mM EDTA, pH 7.4, in an ice-chilled glass homogenizer with a motor-driven Teflon pestle for 1 min. The crude homogenates were centrifuged at 1000 g for 15 min. and the sediment consisting of chunks of cuticle, cellular debris, and floating lipids was discarded. The supernatants were mildly sonicated twice for 10s and used directly as the source of glutathione

(GSH/GSSG), and protein assay. GSH and GSSG were measured by DTNB recycling assay according to Anderson (1985). A brief description of the assays is presented. One milliliter of insect supernatant containing 1 mg/mL protein was mixed with 700 µL daily buffer (143 mM sodium phosphate buffer + 6.3 mM EDTA + 0.248 mg/mL NADPH at pH 7.5), 100 µL DTNB solution, and 200 µL double-distilled H_20 and then incubated for 15 min at 30°C. Ten microliters GR was then added to initiate reaction. The absorbance of the each sample was determined at 412 nm. To determine the level of GSSG, S-sulfosalicylic acid was added to the insect protein. Next, 2 µL 2-vinylpyridine was added and mixed well. Six microliters triethanolamine was then added to the side of the tube and mixed. This mixture was kept at room temperature for 60 min. and 10 µL GR was added to initiate reaction. The absorbance of the sample was determined at 412 nm. A standard curve for GSH or GSSG was generated with the assays, and the results are expressed as nanomoles GSH or GSSG per milligram protein. Total protein concentrations of the supernantants were determined with bovine serum albumin as the standard using the BCA protein kit.

Mean ± SEM are derived from a pool of six assays (n=6). The results were analyzed by two-tailed paired t-test to determine the levels of significance. The p values less than 0.05 were taken to indicate significant differences.

RESULTS AND DISCUSSION

M. domestica was highly susceptible to both forms of arsenic following a 48 hr exposure with LC_{50}s of 0.008% and 0.011% (w/v) for As^{3+} and As^{5+}, respectively. *T. ni* larvae were susceptible to As^{3+} with as LC_{50} of 0.032% (w/w) but seem to tolerate As^{5+} well with an LC_{50} of 0.794% (w/w) concentration after 48 hr of exposure (Table 1). The minimally acute LC_5 dose of both As^{3+} and As^{5+} varied considerably but averaged 0.005% for both insects. In *M. domestica* GSH levels significantly induced with As^{3+}, 1.12-fold and insignificantly induced with As^{5+}, 1.06-fold (Fig. 1A). In *T. ni* after 48 hours of exposure with As^{3+} and As^{5+}, the GSH levels significantly induced, 1.46-fold and 1.13-fold respectively (Fig. 1B). The increased GSH levels by As was associated with decreased levels of GSSG. In *M. domestica*, the GSSG levels was decreased with As^{3+} significantly (1.21-fold) and with As^{5+} insignificantly (1.07-fold) respectively (Fig. 2A). In *T. ni*, the GSSG levels was decreased significantly with As^{3+} (1.20-fold) and was not affected by As^{5+} (1.04-fold) (Fig. 2B).

Free radical mediated oxidative stress and associated pathologies may arise from depletion of thiols such as cysteine and glutathione (Squibb and Fowler 1983; Tamki and Frankenberger 1992). Covalent linkage of arsenic with the cysteine thiol groups of enzymes and dithiol co-factor requiring enzymes could inactivate these enzymes causing electron leakage. Such leakage could lead to the formation of superoxide. Once superoxide is

Table 1. Dose-mortality response of *M. domestica* and *T. ni* to arsenic as As^{3+} and As^{5+} at concentration 0.005%, (w/v for *M. domestica* and w/w for *T. ni*, n = 6).

Insect	Arsenic	Regression coefficient	LC_{50}	P
M. domestica	As^{3+}	0.99	.008	<0.001
	As^{5+}	0.90	.011	<0.01
T. ni	As^{3+}	0.99	.032	<0.001
	As^{5+}	0.94	.794	<0.01

generated, its cascade into H_2O_2 and finally the hydroxyl radical would result in serious cellular and nuclear impairments from peroxidizing reactions (Halliwell and Gutteridge 1984). The process of conversion of As^{5+} to As^{3+} involving transfer reactions by mitochondrial cytochromes may also be a site for electron leakage for the generation of oxygen radicals.

Methylation has been demonstrated to be a major pathway for detoxification of inorganic arsenic by mammals; the methylated arsenicals are the major excreted products in urine and in culture medium. Recently, GSH has been shown to be involved in the methylation process of inorganic arsenic (Tamki and Frankenberger 1992). The arsenic/GSH binding would consume the cellular GSH pool and trigger new GSH synthesis, probably by activating γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH biosynthesis. Elevation of GSH and decreased GSSG levels to arsenic exposure may reflect a self-protective mechanism against cellular injury caused by metals.

By binding directly to As, GSH may protect cells by reducing the interaction between arsenic and sulfhydryl groups of essential enzymes and other proteins. It should be noted that arsenic-resistant Chinese hamster ovary cells contain an increased GSH content. GST's have also been proposed to serve as potent binders of a variety of xenobiotics (Lee *et al.*, 1989). Our previous study (Zaman *et al.*, 1993a; *Zaman et al., 1995 Arch Insect Biochem Physiol in press*) showed that GST's activity was significantly induced by As in *M.domestica*. In *M. domestica*, may this enzyme act as a carrier of inorganic arsenic or its methylated products. Our observations strongly suggest that the resistance of arsenic is mediated by the elevation of intracellular GSH and GST levels.

Mitochondria seem to be the most sensitive cellular organelle to arsenic. Numerous *in vitro* studies have demonstrated that addition of arsenic to isolated mitochondria or tissue homogenate or slices, produces an inhibition of cellular respiration, the oxidation of tricarboxylic acid cycle substrates and

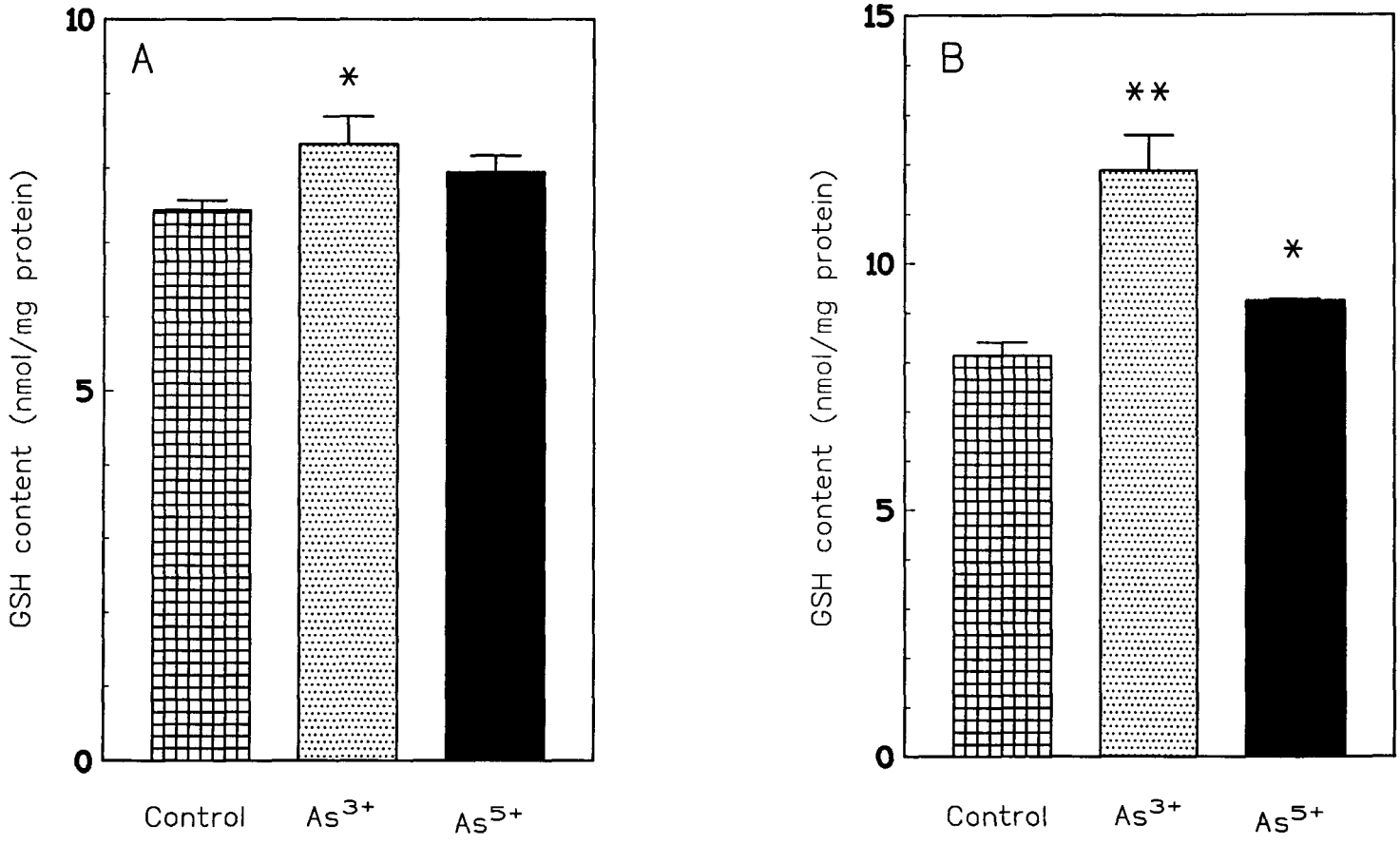

Figure 1 Effect of arsenic as As^{3+} and As^{5+} at concentration 0.005% on glutathione (GSH) content in M. domestica (A) and T. ni (B) Mean ± S.E. n=6; *: $p<0.01$; **: $p<0.001$.

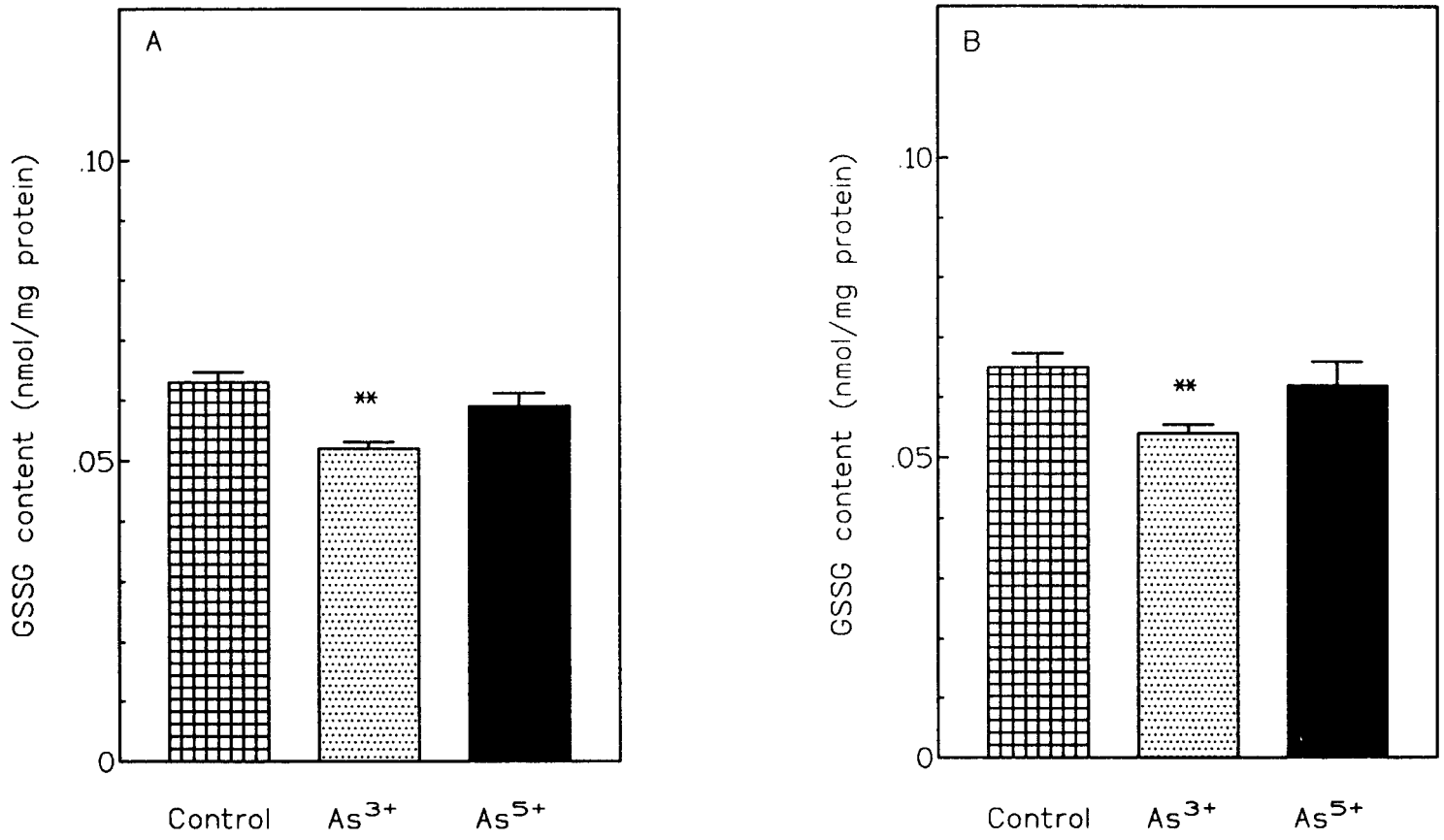

Figure 2 Effect of arsenic as As^{3+} and As^{5+} at concentration 0.005% on glutathione (GSSG) content in M. domestica (A) and T. ni (B) Mean ± S.E. n=6; **: $p<0.001$.

oxidative phosphorylation (Brazy *et al.,* 1980). Yih *et al.,* (1991) showed that treatment with sodium arsenite apparently decreases cellular ATP levels in a dose- and time-dependent manner in HeLa S-3 cells. They suggested that reduction in ATP induced by arsenic was possibly through mitochondrial damage. Deneke (1992) determined that micromolar levels of sodium arsenite increase cystine transport in bovine pulmonary artery endothelial cells, resulting in increases in intracellular glutathione (GSH) levels. Bannai *et al.,* (1991) also reported similar effects of sodium arsenite on cystine transport and intracellular GSH levels in mouse peritoneal macrophages.

Kreppel *et al.,* (1993) demonstrated that arsenic are effective inducers of hepatic metallothionein (MT), which is a sulfhydryl-rich metal-binding protein that provides protection against metal toxicity. The ability of different arsenic compounds to induce MT varied markedly. As^{3+} is a potent MT inducer, approximately 3 times more than As^{5+}. These results indicate the onset of arsenic-induced glutathione content (GSH) in our insect model in a manner analogous to mammalian species. Corbett (1974) had suggested that the toxicity of As^{5+} is due primarily to its conversion to As^{3+}. Our toxicological studies reinforce the statements of Corbett (1974) and Matsumura (1975) that more research is needed to clarify the toxic mode of action of arsenicals in insects and other organisms.

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