Comparative Inhibition of Yeast Glutathione Reductase by Arsenicals and Arsenothiols

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Tri(γ-glutamylcysteinylglycinyl)trithioarsenite ($As^{III}(GS)_3$) is formed in cells and is a more potent mixed-type inhibitor of the reduction of glutathione disulfide (GSSG) by yeast glutathione (GSH) reductase than either arsenite (As^{III}) or GSH. The present work examines the effects of valence and complexation of arsenicals with GSH or L-cysteine (Cys) upon potency as competitive inhibitors of the reduction of GSH disulfide (GSSG) by yeast GSH reductase. Trivalent arsenicals were more potent inhibitors than their pentavalent analogs, and methylated trivalent arsenicals were more potent inhibitors than was inorganic trivalent As. Complexation of either inorganic trivalent As or methylarsonous diiodide ($CH_3As^{III}I_2$) with Cys or GSH produced inhibitors of GSH reductase that were severalfold more potent than the parent arsenicals. In contrast, dimethylarsinous iodide ($(CH_3)_2As^{III}I$) was a more potent inhibitor than its complexes with either GSH or Cys. Complexes of CH_3As^{III} with GSH ($CH_3As^{III}(GS)_2$) or with Cys ($CH_3As^{III}(Cys)_2$) were the most potent inhibitors, with K_i's of 0.009 and 0.018 mM, respectively. Inhibition of GSH reductase by arsenicals or arsenothiols was prevented by addition of *meso*-2,3-dimercaptosuccinic acid (DMSA) to a mixture of enzyme, GSSG, and inhibitor before addition of NADPH. DMSA added to the reaction mixture after NADPH reversed inhibition by $(CH_3)_2As^{III}I$ but had little effect on inhibition by $CH_3As^{III}I_2$, $CH_3As^{III}(GS)_2$, $CH_3As^{III}(Cys)_2$, or $As^{III}(GS)_3$. Partial redox inactivation of the enzyme with NADPH increased the inhibitory potency of $CH_3As^{III}I_2$ and $(CH_3)_2As^{III}I$ and changed the mode of inhibition for $CH_3As^{III}I_2$ from competitive to noncompetitive. The greater potency of methylated trivalent arsenicals and arsenothiols than of inorganic trivalent As suggests that biomethylation of As could yield species that inhibit reduction of GSSG and alter the redox status of cells.

Introduction

The tripeptide GSH (γ-glutamylcysteinylglycine) plays critical roles in the reduction of pentavalent arsenicals to trivalency and in the complexation of arsenicals to form arsenothiols. The conversion of pentavalent inorganic As (As^V) to trivalency by the oxidation of GSH and the complexation of trivalent inorganic As (As^{III}) by GSH is described by the reaction scheme,

$$As^V + 5\ GSH \rightarrow As^{III}(GS)_3 + GSSG \qquad (1)$$

This reaction occurs in aqueous solution and in intact rabbit erythrocytes (*1*−*4*). GSH reduces As^V in CH_3As[1] and $(CH_3)_2As$ from pentavalency to trivalency and forms stable complexes with $(CH_3)_2As^{III}$ (*1*, *5*) and phenyldichlorarsine ($C_6H_5As^{III}Cl_2$) (*6*, *7*). A complex of $C_6H_5As^{III}$ and GSH is the predominant arsenical present in human erythrocytes following *in vitro* exposure to $C_6H_5As^{III}Cl_2$ (*8*). Given the high intracellular concentration of GSH and the high affinity of As^{III} for thiols, it is likely that arsenothiols are commonly present in cells. Because trivalent arsenicals are more acutely toxic than pentavalent arsenicals (*9*), GSH-dependent reduction to trivalency may increase the potential toxicity of ingested As. Several fates have been identified for arsenothiol complexes formed by the GSH-dependent reduction of arsenicals. For example, $As^{III}(GS)_3$ donates As^{III} to dithiol-containing molecules (*10*) and is a substrate for the enzyme from rat liver cytosol that catalyzes the production of CH_3As (*11*).

The redox cycling of GSH is central to the cellular response to oxidative stress. In the reaction scheme,

$$2GSH + H_2O_2 \rightarrow 2H_2O + GSSG \qquad (2)$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \qquad (3)$$

the cycling between GSH and GSSG is linked to the scavenging of hydroperoxides generated by the disproportionation of $O_2^{\bullet}$ to O_2 and H_2O_2 by superoxide dismutase (*12*). Reaction 2 is catalyzed by GSH peroxidase (EC

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[1] Abbreviations: tri(γ-glutamylcysteinylglycinyl)trithioarsenite, $As^{III}(GS)_3$; methylarsonous diiodide, $CH_3As^{III}I_2$; dimethylarsinous iodide, $(CH_3)_2As^{III}I$; di(γ-glutamylcysteinylglycinyl)methyldithioarsonite, $CH_3As^{III}(GS)_2$; dicysteinylmethyldithioarsenite, $CH_3As^{III}(Cys)_2$; *meso*-2,3-dimercaptosuccinic acid, DMSA; phenyldichlorarsine, $C_6H_5As^{III}Cl_2$; arsenic acid disodium salt, $Na_2HAs^VO_4$; sodium *m*-arsenite, $NaAs^{III}O_2$; methylarsonic acid, disodium salt, $CH_3As^VO(ONa)_2$; dimethylarsinic acid, $(CH_3)_2As^VO(OH)$; tricysteinyltrithioarsenite, $As^{III}(Cys)_3$; cysteinyldimethylthioarsinite, $(CH_3)_2As^{III}(Cys)$; (γ-glutamylcysteinylglycinyl)dimethylthioarsinite, $(CH_3)_2As^{III}(GS)$.

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1.11.1.9), and reaction 3 is catalyzed by GSH reductase (EC 1.6.4.2). The balance between the oxidation of GSH to GSSG and the rapid reduction of GSSG by GSH reductase contributes to the maintenance of a cellular GSH:GSSG ratio of about 300:1 (*13*, *14*). Increasing the intracellular concentration of GSSG by oxidative stress is positively correlated with an increase in protein−GSH mixed disulfides (*15*). Formation of mixed disulfides of protein thiols and GSH may affect protein structure and serve a regulatory function (*16*, *17*). For example, S-glutathiolation of Cys residues by this process has been shown to modulate the phosphatase activity of carbonic anhydrase III (*18*). Previous work has shown that $As^{III}(GS)_3$ is a mixed-type inhibitor of the reduction of GSSG by GSH reductase (*19*). Using purified yeast GSH reductase, the work reported here examines the dependence of potency of organoarsenicals on valence and the effect of complexes of organoarsenicals with GSH or Cys upon NADPH-dependent reduction of GSSG. These studies demonstrate that trivalent organoarsenicals are more potent inhibitors than their pentavalent analogs and that arsenothiols are a novel class of potent inhibitors of GSH reductase. Either trivalent organoarsenicals generated during biomethylation of As or arsenothiols formed in the cell by reaction with GSH or Cys could alter the cellular GSH:GSSG ratio by the inhibition of GSH reductase.

Experimental Procedures

Caution: *Inorganic arsenic is classified as a human carcinogen (20), and appropriate precautions should be taken in its handling.*

Chemicals. GSH reductase (type IV prepared from baker's yeast), arsenic acid disodium salt ($Na_2HAs^VO_4$), sodium *m*-arsenite ($NaAs^{III}O_2$), Cys, GSH, GSSG, NADPH, and DMSA were obtained from Sigma (St Louis, MO). Methylarsonic acid, disodium salt ($CH_3As^VO(ONa)_2$), was obtained from Chem Service (West Chester, PA) and dimethylarsinic acid ($(CH_3)_2As^VO(OH)$) from Strem (Newburyport, MA).

Chemical Synthesis. Synthesis of Methylarsonous Diiodide ($CH_3As^{III}I_2$). This compound was prepared by the method of Goddard (*21*). To a solution of $CH_3AsO(OH)_2$ (4.4 g, 24 mmol) in 17 mL of water, KI (8.76 g, 26 mmol) and then 3 mL of concentrated HCl were added. SO_2 was bubbled through the mixture for 4 h. The yellow solid that appeared in the mixture was collected by filtration, washed with cold water, and dried *in vacuo* for 1 h. Fractional distillation of the solid under reduced pressure gave $CH_3As^{III}I_2$ (4.2 g, 56% yield) as a yellow crystalline solid (bp 128 °C/16 mmHg). Identity was confirmed by ^{1}H-NMR ($CDCl_3$): δ 3.1 (s). To prevent oxidation, the compound was stored in sealed ampules.

Synthesis of Dimethylarsinous Iodide ($(CH_3)_2As^{III}I$). This compound was prepared by the method of Goddard (*21*). To a solution of $(CH_3)_2AsO(OH)$ (4.14 g, 30 mmol) in 30 mL of water, KI (5.48 g, 33 mmol) and then 1.7 mL of concentrated H_2SO_4 were added. SO_2 was bubbled through the mixture for 3 h. During this period, a yellow oil separated. The oil was collected and dried over Na_2SO_4. Fractional distillation of the oil under reduced pressure gave $(CH_3)_2As^{III}I$ (5.3 g, 76% yield) as a yellow-orange oil (bp 155 °C). Identity was confirmed by ^{1}H-NMR ($CDCl_3$): δ 2.01 (s). To prevent oxidation, the compound was stored in sealed ampules.

Synthesis of Tricysteinyltrithioarsenite ($As^{III}(Cys)_3$) and Tri(γ-glutamylcysteinylglycinyl)trithioarsenite ($As^{III}(GS)_3$). $As^{III}(Cys)_3$ was prepared by the procedure of Serves and associates (*22*). Identity and purity were confirmed by mp, ^{1}H-NMR, and TLC. $As^{III}(GS)_3$ was prepared as described by Styblo and Thomas (*19*). The stability of this complex in the standard reaction mixture has been demonstrated (*19*).

Synthesis of Dicysteinylmethyldithioarsenite ($CH_3As^{III}(Cys)_2$), Di(γ-glutamylcysteinylglycinyl)methyldithioarsonite ($CH_3As^{III}(GS)_2$), Cysteinyldimethylthioarsinite ($(CH_3)_2As^{III}(Cys)$), and (γ-Glutamylcysteinylglycinyl)dimethylthioarsinite ($(CH_3)_2As^{III}(GS)$). These compounds were prepared as previously described (*5*). The recrystallization of $(CH_3)_2As^{III}(GS)$ was modified to increase purity and ease of preparation. Briefly, the compound was dissolved in methanol/water (1:1 v/v), and acetone was added until the solution was turbid. The solution was stored at 4 °C overnight, and the white precipitate was filtered and washed twice with methanol/water (1:1 v/v). The washed filtrate was dried *in vacuo* for 1 day. Identity and purity were confirmed by mp, ^{1}H-NMR, and TLC.

Preparation of Stock Solutions. Stock solutions of arsenicals, arsenothiols, GSH, and Cys were prepared in distilled deionized water at 130−520 mM immediately before use. Dropwise addition of concentrated HCl was needed to increase solubility of some compounds. A stock solution of 260 mM $CH_3As^{III}I_2$ was prepared in 70% ethanol, or 260 mM $(CH_3)_2As^{III}I$ was prepared in 50% ethanol. DMSA was dissolved by dropwise addition of 5 N NaOH, and the resulting neutral solution was diluted in distilled deionized water to a final concentration of 260 mM.

GSH Reductase Assay. Standard assay conditions for GSH reductase activity were those described by Styblo and Thomas (*19*). Here, 0.3 μg of yeast GSH reductase was added to 0.15 M phosphate buffer (pH 7) containing 6 mM EDTA and 0.1−1.0 mM GSSG. Inhibitor (arsenical, arsenothiol, Cys, or GSH) and/or DMSA were added as required for the assay. After a 2 min preincubation at 37 °C, the reaction was initiated by addition of NADPH to a final concentration of 0.23 mM. The final volume of the assay mixture was 2.6 mL. The 340 nm absorbance of the reaction mixture was monitored at 20-s intervals for 3 min in a cuvette with a 1 cm light path. To determine the rate of nonenzymatic oxidation of NADPH, a blank reaction containing all components of the reaction mixture except enzyme was monitored at 20-s intervals for 3 min. The net rate of enzymatic oxidation of NADPH (i.e., the difference between the rates of the total and nonenzymatic reactions) was used to calculate enzyme activity (nmol of NADPH consumed/min). Duplicate assays were performed for all experimental conditions, and mean data were used in all calculations.

Results

Characterization of Inhibitors of the Activity of GSH Reductase. The effects of arsenicals, arsenothiols, Cys, and GSH on the activity of yeast GSH reductase were measured for substrate concentrations from 0.1 to 1.0 mM GSSG. All trivalent arsenicals and arsenothiols tested were inhibitors of the enzymatic reduction of GSSG. A double-reciprocal plot of 1/*v* versus 1/[GSSG] for each compound was used to determine the mode of inhibition. A K_i constant for each compound was calculated from a replot of slopes of the reciprocal plots versus the corresponding concentrations of the inhibitor. As an illustration, Figure 1 shows the double-reciprocal plot and the replot of slopes for $CH_3As^{III}(GS)_2$, the most potent inhibitor tested in the present study. The estimated K_i's and the mode of inhibition for each compound are summarized in Table 1.

All trivalent arsenicals and arsenothiols, except $As^{III}(GS)_3$, were competitive inhibitors of GSH reductase with K_i's that ranged from 0.009 to 5.7 mM (Table 1). In contrast, $As^{III}(GS)_3$ was a mixed-type inhibitor ($K_i = 0.325$ mM). $CH_3As^{III}(Cys)_2$ and $CH_3As^{III}(GS)_2$ were the most potent inhibitors, with K_i's of 0.018 and 0.009 mM, respectively. Both $CH_3As^{III}I_2$ and $(CH_3)_2As^{III}I$ were substantially more potent inhibitors than $NaAs^{III}O_2$ and $As^{III}(GS)_3$. Cys was found to be a relatively potent competitive inhibitor ($K_i = 0.165$ mM). In contrast, GSH was a

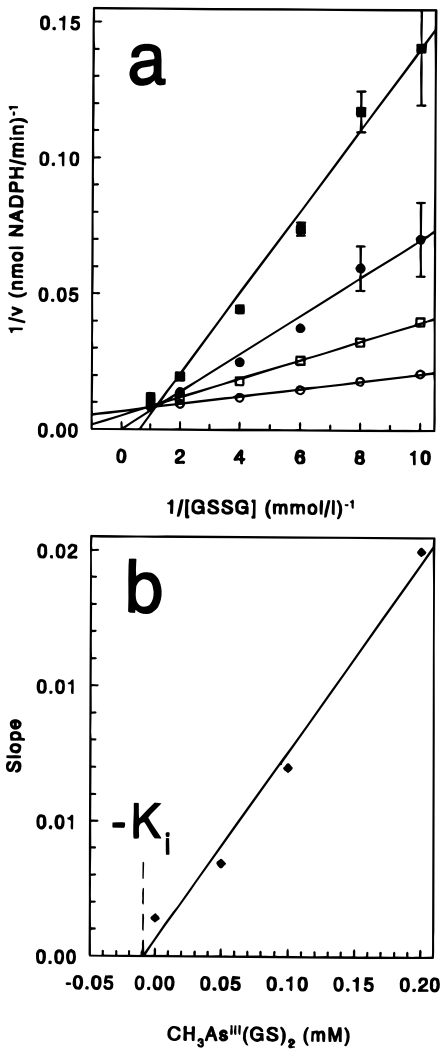

Figure 1. Inhibition of GSH reductase by $CH_3As^{III}(GS)_2$. (a) Double-reciprocal plots of 1/*v* vs 1/[GSSG] for 0 mM (○), 0.05 mM (□), 0.1 mM (●), and 0.2 mM (■) $CH_3As^{III}(GS)_2$. Mean and range of duplicate assays shown. (b) Replot of slopes of the double-reciprocal plots vs corresponding concentrations of $CH_3As^{III}(GS)_2$ for the determination of K_i.

Table 1. Characteristics of the Inhibition of GSH Reductase by Arsenicals and Arsenothiol Complexes

compound	K_i (mM)[a]	mode of inhibition
$NaAs^{III}O_2$	5.71	competitive
$As^{III}(Cys)_3$	0.076	competitive
$As^{III}(GS)_3$	0.325	mixed-type
$CH_3As^{III}I_2$	0.074	competitive
$CH_3As^{III}(Cys)_2$	0.018	competitive
$CH_3As^{III}(GS)_2$	0.009	competitive
$(CH_3)_2As^{III}I$	0.056	competitive
$(CH_3)_2As^{III}(Cys)$	0.834	competitive
$(CH_3)_2As^{III}(GS)$	0.732	competitive
Cys	0.165	competitive
GSH	3.46	uncompetitive

[a] K_i value was determined from the replots of the slope of each reciprocal plot versus the corresponding inhibitor concentration (see Figure 1).

weak uncompetitive inhibitor of GSSG reduction (K_i = 3.456 mM).

Because of its relatively low potency, the IC_{50} value and not the K_i was estimated for the pentavalent arsenical, $Na_2HAs^{V}O_4$. Figure 2 compares the IC_{50} value for $Na_2HAs^{V}O_4$ (~30 mM) with those determined for trivalent arsenicals and arsenothiols in the presence of 0.1 mM GSSG. Addition of 20 mM $CH_3As^{V}O(ONa)_2$ or

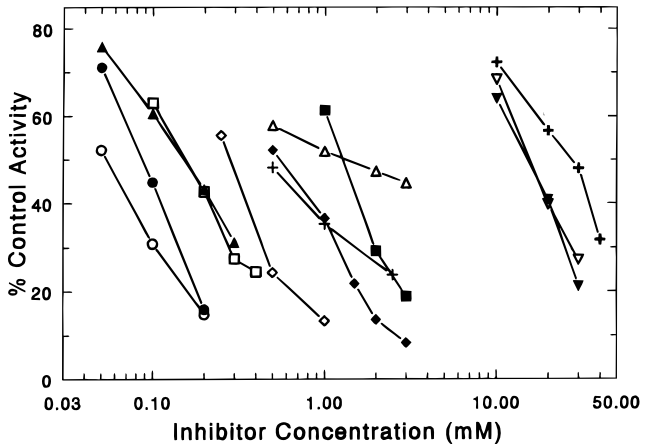

Figure 2. Relative potency for pentavalent and trivalent arsenicals, arsenothiols, and thiols as inhibitors of GSSG reduction by GSH reductase in the presence of 0.1 mM GSSG. ○, $CH_3As^{III}(GS)_2$; ●, $CH_3As^{III}(Cys)_2$; ▲, $CH_3As^{III}I_2$; □, $(CH_3)_2As^{III}I$; ◇, $As^{III}(Cys)_3$; +, $(CH_3)_2As^{III}(Cys)$; ♦, Cys; △, $(CH_3)_2As^{III}GS$; ■, $As^{III}(GS)_3$; ▼, $NaAs^{III}O_2$; ▽, GSH; +, $Na_2HAs^{V}O_4$.

$(CH_3)_2As^{V}O(OH)$ did not inhibit GSH reductase activity (data not shown). To determine whether I^- contributed to the inhibition of GSH reductase by $CH_3As^{III}I_2$ or $(CH_3)_2As^{III}I$, NaI was tested as an inhibitor of enzyme activity. Up to 10 mM NaI did not inhibit the enzymatic reduction of 0.1 mM GSSG (data not shown).

Effect of GSH on the Inhibition of GSH Reductase by $CH_3As^{III}I_2$, $(CH_3)_2As^{III}I$, and $NaAs^{III}O_2$. To examine further the interaction between GSH and organoarsenicals and its effect on the activity of GSH reductase, GSH was added to reaction mixtures (buffer, enzyme, 0.1 mM GSSG, arsenical). To promote the formation of complexes, GSH was added at a molar ratio of 2:1 for $CH_3As^{III}I_2$ and of 1:1 for $(CH_3)_2As^{III}I$. After a 2-min preincubation at 37 °C, the reaction was started by addition of NADPH. The concentration dependency of inhibition by simultaneously-added trivalent organoarsenical and GSH was compared with that for the parent trivalent organoarsenical or authentic organoarsenothiols (Figure 3). The inhibitory effect of $CH_3As^{III}I_2$ was increased by the addition of a 2-fold molar excess of GSH (Figure 3a), yielding a pattern of the inhibition that was identical to that for $CH_3As^{III}(GS)_2$. The comparable potency of authentic complex and the mixture of components suggested that the complex was formed quickly in the reaction mixture. In contrast, addition of GSH to a reaction mixture containing $(CH_3)_2As^{III}I$ resulted in a decreased inhibition as compared with authentic $(CH_3)_2As^{III}GS$ (Figure 3b). The reduced effect of $(CH_3)_2As^{III}I$ in the presence of GSH suggests that this tripeptide can protect the enzyme against the inhibitory effect of this organoarsenical. In the absence of arsenicals, addition of up to 2 mM GSH did not inhibit the reduction of GSSG by GSH reductase (data not shown).

Assay conditions were further modified, and the addition of GSH to the reaction mixture (buffer, enzyme, 0.1 mM GSSG, inhibitor) was delayed until 1 min after the addition of NADPH. Figure 4 shows the time-dependent changes in 340 nm absorbance of the reaction mixtures before and after addition of GSH. Addition of 9 mM GSH to an assay mixture containing 3 mM $NaAs^{III}O_2$ slowed the rate of NADPH oxidation (Figure 4a), suggesting that $As^{III}(GS)_3$, a more potent inhibitor than $NaAs^{III}O_2$, was formed in the reaction mixture. Addition of 0.2 mM GSH to an assay mixture containing

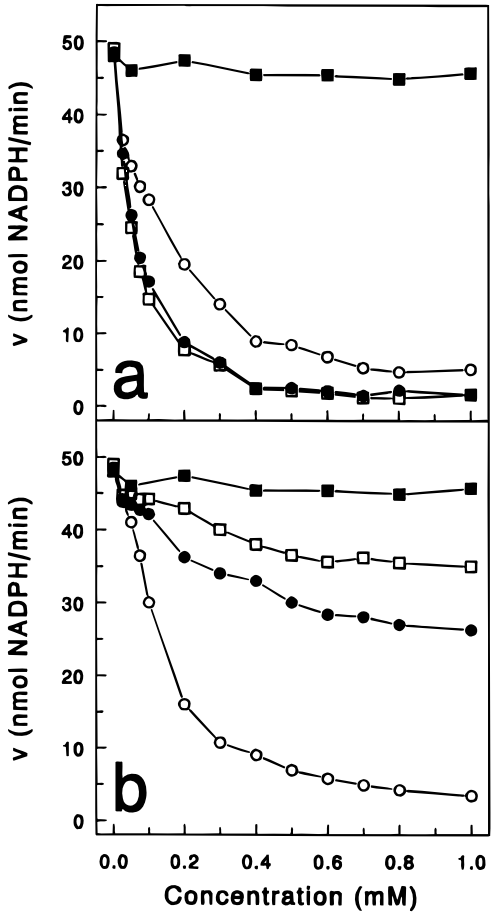

Figure 3. Concentration-dependent inhibition of GSH reductase by concurrently-added trivalent organoarsenicals and GSH compared with the effects of authentic organoarsenothiols, parent organoarsenicals, or GSH. Arsenicals and/or GSH were added to the reaction mixture during the 2-min preincubation period, and reactions were started by addition of 0.23 mM NADPH. Concentration of GSSG in the assay mixtures was 0.1 mM. (a) Effect of concurrent addition of $CH_3As^{III}I_2$ and GSH (1:2 molar ratio) (□); $CH_3As^{III}(GS)_2$ (●); $CH_3As^{III}I_2$ (○); or GSH (■). (b) Effect of concurrent addition of $(CH_3)_2As^{III}I$ and GSH (1:1 molar ratio) (□); $(CH_3)_2As^{III}(GS)$ (●); $(CH_3)_2As^{III}I$ (○); or GSH (■).

0.1 mM $CH_3As^{III}I_2$ did not affect the rate of NADPH oxidation (Figure 4b), suggesting that $CH_3As^{III}(GS)_2$, a potent inhibitor of GSH reductase, was not formed under these conditions. Addition of 0.3 mM GSH to an assay mixture containing 0.3 mM $(CH_3)_2As^{III}I$ increased the rate of NADPH oxidation to a rate comparable to that found in uninhibited reaction mixtures (Figure 4c), suggesting that $(CH_3)_2As^{III}GS$ was formed under these conditions.

Effect of DMSA on the Inhibition of GSH Reductase. The effects of DMSA on the inhibition of GSH reductase by arsenicals and arsenothiols were examined. In the first experiment, DMSA was added to the reaction mixture (buffer, enzyme, 0.1 mM GSSG, arsenical, or arsenothiol) during the preincubation period. Increasing concentrations of DMSA added before NADPH antagonized the inhibitory effects of 0.5 mM $CH_3As^{III}I_2$, 0.5 mM $(CH_3)_2As^{III}I$, 0.2 mM $CH_3As^{III}(Cys)_2$, and 0.1 mM $CH_3As^{III}(GS)_2$ (Figure 5). The percentage of the recovered enzymatic activity was proportional to the DMSA concentration in the assay mixture. The recovery of activity was nearly complete at a molar ratio of DMSA:arsenical of ~0.5 for $(CH_3)_2As^{III}I$ and of ~1 to 1.2 for $CH_3As^{III}I_2$ or $CH_3As^{III}(GS)_2$. The inhibitory effect of 0.2 mM $CH_3As^{III}(Cys)_2$ could not be fully reversed by addition of up to 0.32 mM DMSA (molar ratio = 1.6). Notably, in the absence of arsenicals or arsenothiols, addition of greater than 1 mM DMSA to the reaction mixture was required to inhibit GSH reductase (data not shown).

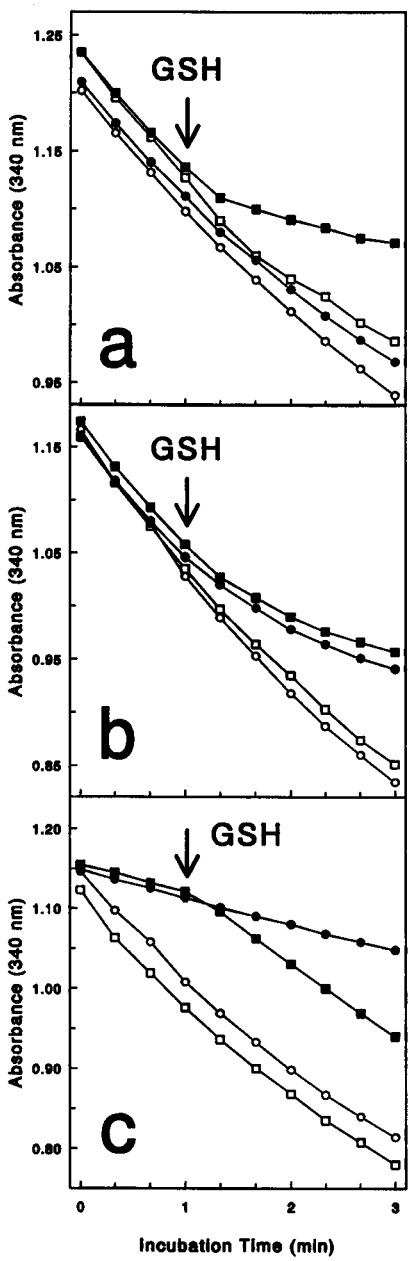

Figure 4. Effect of delayed addition of GSH on the rate of NADPH oxidation in reaction mixtures that contained 3 mM $NaAs^{III}O_2$ (a), 0.1 mM $CH_3As^{III}I_2$ (b), or 0.3 mM $(CH_3)_2As^{III}I$ (c). Following preincubation of enzyme and 0.1 mM GSSG with or without arsenical for 2 min, reactions were started by addition of 0.23 mM NADPH. One minute after addition of NADPH, 9 mM GSH was added to (a), 0.2 mM GSH to (b), and 0.3 mM GSH to (c). Rates of NADPH oxidation (A_{340}) were measured before and after addition of GSH. ○, assay mixture without arsenical or GSH added; □, assay mixture with only GSH added; ●, assay mixture with only arsenical added; ■, assay mixture with arsenical and GSH added.

In other experiments, DMSA was added to the reaction mixture (buffer, enzyme, 0.1 mM GSSG, arsenical, or arsenothiol) 1 min after the addition of NADPH. Figure 6 shows time-dependent changes in A_{340} for reaction mixtures before and after the addition of DMSA. Addition of 0.5 or 1.0 mM DMSA to reaction mixtures that contained 0.5 mM $CH_3As^{III}I_2$ increased the rate of NAD-

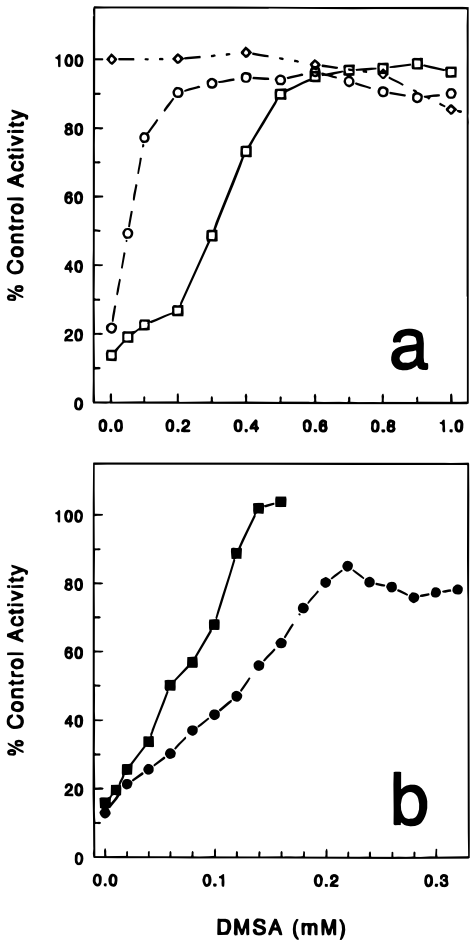

Figure 5. Concentration-dependent effects of DMSA on inhibition of GSH reductase by trivalent organoarsenicals (a) or organoarsenothiols (b) in the presence of 0.1 mM GSSG. Arsenicals and/or DMSA were added to the reaction mixtures during the 2-min preincubation period, and reactions were started by addition of 0.23 mM NADPH. ◊, DMSA alone; ○, 0.5 mM $(CH_3)_2As^{III}I$; □, 0.5 mM $CH_3As^{III}I_2$; ■, 0.1 mM $CH_3As^{III}(GS)_2$; ●, 0.2 mM $CH_3As^{III}(Cys)_2$.

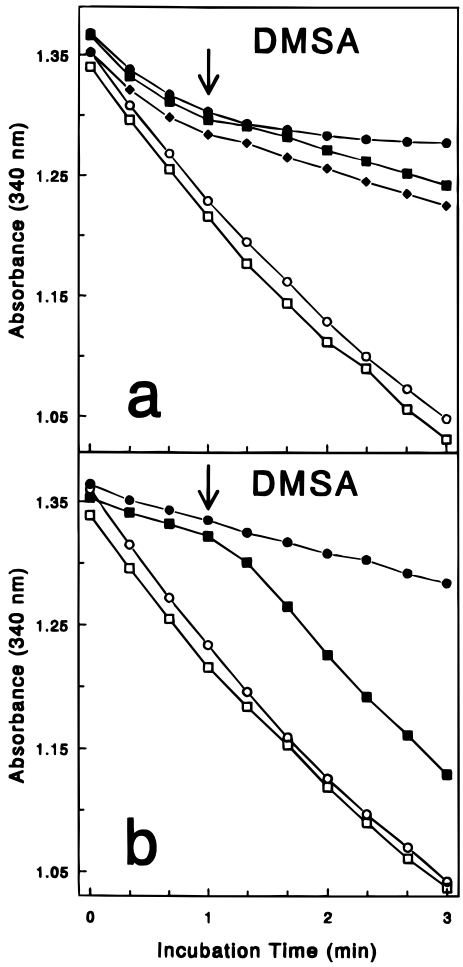

Figure 6. Effect of delayed addition of DMSA on the rate of NADPH oxidation in reaction mixtures that contained 0.5 mM $CH_3As^{III}I_2$ (a) or 0.5 mM $(CH_3)_2As^{III}I$ (b). Following preincubation of enzyme and 0.1 mM GSSG with or without arsenical for 2 min, reactions were started by addition of 0.23 mM NADPH. One minute after addition of NADPH, DMSA was added to the reaction mixture. Rates of NADPH oxidation were determined before and after addition of DMSA. ○, assay mixture without arsenical or DMSA added; □, assay mixture with only DMSA added; ●, assay mixture with only arsenical added; ■, assay mixture with arsenical and 0.5 mM DMSA added; ♦, assay mixture with arsenical and 1.0 mM DMSA added.

PH consumption by about 35% (Figure 6a). Similarly, only partial reversibility of inhibition was observed when DMSA was added at a molar ratio (DMSA:arsenical) of 4:1 to 10:1 to reaction mixtures containing 1 mM $As^{III}(GS)_3$, 0.1 mM $CH_3As^{III}(GS)_2$, or 0.2 mM $CH_3As^{III}(Cys)_2$ (data not shown). In contrast, addition of 0.5 mM DMSA to a reaction mixture that contained 0.5 mM $(CH_3)_2$ $As^{III}I$ completely reversed inhibition (Figure 6b).

Effect of Preincubation with NADPH on Inhibition by GSH Reductase. The preincubation of purified GSH reductase with low concentrations of NADPH produces a time-, temperature-, and concentration-dependent reduction in the rate of GSSG reduction (*23*). The effects of preincubation with NADPH on the inhibition of enzymatic activity by organoarsenicals were examined. Here, the procedure was modified to include 10 μM NADPH in the reaction mixture (buffer, enzyme, 0.1 mM GSSG, and arsenical) during the 2-min preincubation period. Assays were started by the addition of NADPH to a final concentration of 0.23 mM, and time-dependent changes in absorbance at 340 nm were monitored. Over the substrate concentration range of 0.1−1 mM GSSG, pretreatment with NADPH reduced the rate of GSSG reduction to about 50% of the rate found in control assays. Figure 7 shows double-reciprocal plots of 1/v versus 1/[GSSG] for different concentrations of $CH_3As^{III}I_2$ or $(CH_3)_2As^{III}I$ in an assay system preincubated with 10 μM NADPH. Pretreatment with NADPH decreased the K_i's for both organoarsenicals. The estimated K_i's for $CH_3As^{III}I_2$ and $(CH_3)_2As^{III}I$ following preincubation with NADPH were 0.034 mM and 0.014 mM, respectively. For $(CH_3)_2As^{III}I$, the mode of inhibition was competitive for both the native enzyme and the NADPH-pretreated enzyme (Figure 7b). For $CH_3As^{III}I_2$, NADPH pretreatment changed the mode of inhibition to noncompetitive (Figure 7a).

Discussion

The work reported here demonstrates that valence and complexation with thiols affect the potency of arsenicals as inhibitors of the NADPH-dependent reduction of GSSG by GSH reductase. These findings suggest that the biomethylation of As, a process catalyzed by cytosolic AdoMet-requiring enzyme(s) (*11*, *24*), yields organoarsenicals that are more potent inhibitors of GSH reductase than the parent inorganic As. The commonly-held belief that biomethylation is a mechanism for the detoxication

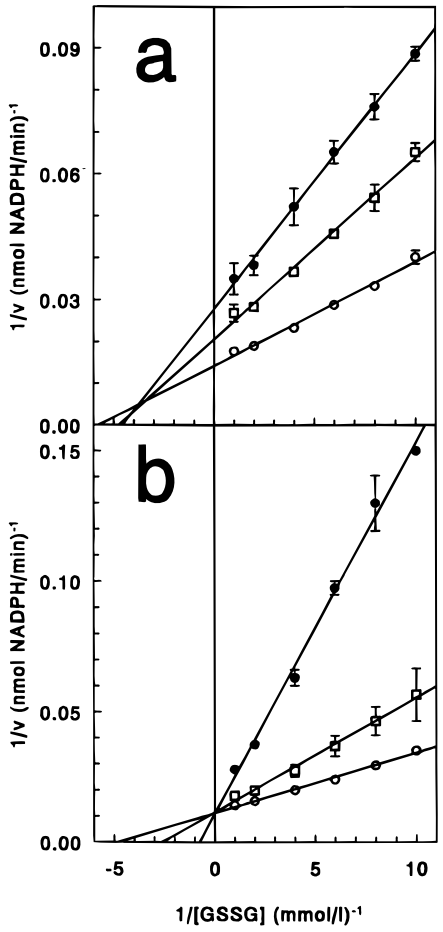

Figure 7. Effect of preincubation with NADPH on inhibition of GSH reductase by organoarsenicals. Double-reciprocal plots of 1/*v* vs 1/[GSSG] for 0 mM (○), 0.025 mM (□), and 0.05 mM (●) $CH_3As^{III}I_2$ (a) and for 0 mM (○), 0.05 mM (□), and 0.1 mM (●) $(CH_3)_2As^{III}I$ (b) in a reaction mixture preincubated with 10 μM NADPH. Following preincubation of enzyme, arsenical, 10 μM NADPH, and 0.1 mM GSSG for 2 min, the reaction was started by addition of 0.23 mM NADPH.

of As (*25*) may be altered by results of the current study that demonstrate the products of the methylation of As to be potent and specific inhibitors of GSH reductase.

As has been reported for GSH reductases from other species (*14*, *26*), GSH was found to be an uncompetitive inhibitor of yeast GSH reductase. The complexation of arsenicals by GSH or Cys had striking effects on their potency as inhibitors of GSH reductase. GSH- or Cys-containing complexes of As^{III} and CH_3As^{III} were more potent inhibitors than the parent arsenicals. In contrast, either thiol-containing complex with $(CH_3)_2As^{III}$ was less potent than $(CH_3)_2As^{III}I$. Addition of GSH to the reaction mixture before addition of NADPH to initiate GSSG reduction resulted in patterns and magnitudes of inhibition consistent with those observed for authentic complexes of GSH with As^{III}, $CH_3As^{III}(GS)_2$, or $(CH_3)_2As^{III}$. This finding suggested that arsenothiol complexes could be formed in the reaction mixture. However, if the addition of GSH was delayed until after preincubation with NADPH, then the pattern and magnitude of inhibition for $CH_3As^{III}I_2$ differ from those observed for authentic $CH_3As^{III}(GS)_2$. This finding suggested that the presence of NADPH prevented the interaction of the arsenothiol complex formed in the reaction mixture with some critical site in the enzyme.

The effects of addition of DMSA on the potency of arsenicals as inhibitors of GSH reductase can be compared to those observed after addition of GSH to the reaction mixture. Previous work has shown that As^{III} is readily donated from $As^{III}(GS)_3$ to DMSA and that DMSA and its analogs are effective antidotes for acute As intoxication (*27*, *28*). Addition of DMSA to the reaction mixture before the addition of NADPH antagonized the inhibitory effect of $CH_3As^{III}I_2$, $CH_3As^{III}(GS)_2$, $CH_3As^{III}(Cys)_2$, or $(CH_3)_2As^{III}I$. Activity was almost fully restored for assays containing $CH_3As^{III}I_2$, $CH_3As^{III}(GS)_2$, or $(CH_3)_2As^{III}I$. Previous work has shown that addition of DMSA to the reaction mixture before addition of NADPH antagonized the inhibitory effect of $As^{III}(GS)_3$ (*19*). The failure of DMSA to restore fully the activity of reaction mixtures that contained $CH_3As^{III}(Cys)_2$ could be due to the liberation of Cys during the decomposition of the complex. Because Cys is a fairly potent competitive inhibitor of GSH reductase, its release could affect the restoration of enzymatic activity. Alternatively, a mixed GSH-DMSA disulfide could be formed and could be an inhibitor of GSH reductase. For reaction mixtures that contained $As(GS)_3$, $CH_3As^{III}I_2$, $CH_3As^{III}(GS)_2$, or $CH_3As^{III}(Cys)_2$, the delayed addition of a molar excess of DMSA after addition of NADPH only partly restored activity. The inhibitory effect of $(CH_3)_2As^{III}I$ was fully reversed by addition of an equimolar amount of DMSA. The failure of DMSA to reverse inhibition of GSSG reduction by some trivalent arsenicals and their thiol complexes suggests that in the presence of NADPH these species are bound to sites in the enzyme that are of higher affinity than those provided by DMSA. These high-affinity binding sites could be provided by the 5 Cys residues in each subunit of dimeric GSH reductase of *Saccharomyces cerevisiae* (*29*).

Finally, the nature of inhibition by arsenicals was examined using GSH reductase that was exposed to a low concentration of NADPH before assay of its capacity to reduce GSSG. GSH reductase is inactivated by NADPH by an intramolecular modification of the dimeric protein (*30*). Inactivation is thought to involve the formation of an "erroneous disulfide" between a critical Cys residue of the redox active site of the enzyme and a neighboring Cys. This effect is reversed by exposure to mono- and dithiols. Coexposure to NADPH and As^{III} results in greater inhibition of GSH reductase than is produced by NADPH alone. The effect of both inhibitors could be reversed with mono- and dithiols (*30*). The experiments reported have examined the influence of NADPH inactivation on the characteristics of inhibition by $CH_3As^{III}I_2$ and $(CH_3)_2As^{III}I$. For both organoarsenicals, the NADPH-inactivated enzyme yielded lower K_i's, and, for $CH_3As^{III}I_2$ but not $(CH_3)_2As^{III}I$, changed the mode of inhibition from competitive to noncompetitive. These findings suggest that NADPH inactivation alters the structure of the enzyme in a manner that affects the nature of its interactions with organoarsenicals. Because NADPH may modify a site adjacent to the redox active center of the enzyme, these findings suggest that the organoarsenicals may be interacting with a site in close proximity to the active center of the enzyme.

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References

(1) Scott, N., Hatlelid, K. M., MacKenzie, N. E., and Carter, D. E. (1993) Reaction of arsenic(III) and arsenic(V) species with glutathione. *Chem. Res. Toxicol.* **6**, 102−106.

(2) Delnomdedieu, M., Basti, M. M., Otvos, J. D., and Thomas, D. J. (1994) Reduction and binding of arsenate and dimethylarsenate by glutathione: A multinuclear magnetic resonance study. *Chem.-Biol. Interact.* **90**, 139−155.

(3) Delnomdedieu, M., Basti, M. M., Styblo, M., Otvos, J. D., and Thomas, D. J. (1994) Complexation of arsenic species in rabbit erythrocytes. *Chem. Res. Toxicol.* **7**, 621−627.

(4) Delnomdedieu, M., Styblo, M., and Thomas, D. J. (1995) Time dependence of accumulation and binding of inorganic and organic arsenic species in rabbit erythrocytes. *Chem-Biol. Interact.* **98**, 69−83.

(5) Cullen, W. R., McBride, B. C., and Reglinski, J. (1984) The reaction of methylarsenicals with thiols: Some biological implications. *J. Inorg. Biochem.* **21**, 179−194.

(6) Dill, K., Adams, E. R., O'Connor, R. J., and McGown, E. L. (1987) One-dimensional and two-dimensional nuclear magnetic resonance studies of the reaction of phenyldichloroarsine with glutathione. *Arch. Biochem. Biophys.* **257**, 293−301.

(7) Dill, K., O'Connor, R. J., and McGown, E. L. (1987) Spin-echo NMR investigation of the interaction of phenyldichloroarsine with glutathione in intact erythrocytes. *Inorg. Chim. Acta* **138**, 95−97.

(8) Chong, S., Dill, K., and McGown, E. (1989) The interaction of phenyldichloroarsine with erythrocytes. *J. Biochem. Toxicol.* **4**, 39−45.

(9) Yamauchi, H., and Fowler, B. A. (1994) Toxicity and metabolism of inorganic and methylated arsenicals. In *Arsenic in the environment. Part II: Human Health and Ecosystem Effects* (Nriagu, J. O., Ed.) pp 35−54, John Wiley and Sons, New York.

(10) Delnomdedieu, M., Basti, M. M., Otvos, J. O., and Thomas, D. J. (1993) Transfer of arsenite from glutathione to dithiol: a model of interaction. *Chem. Res. Toxicol.* **6**, 598−602.

(11) Styblo, M., Delnomdedieu, M., and Thomas, D. J. (1996) Mono- and dimethylation of arsenic in rat liver cytosol in vitro. *Chem.-Biol. Interact.* **99**, 147−161.

(12) Fridovich, I. (1995) Superoxide radical and superoxide dismutase. *Annu. Rev. Biochem.* **64**, 97−112.

(13) Alpert A. J., and Gilbert, H. F. (1985) Detection of oxidized and reduced glutathione with a recycling postcolumn reaction. *Anal. Biochem.* **144**, 553−562.

(14) Chung, P. M., Cappel, R. E., and Gilbert, H. F. (1991) Inhibition of glutathione disulfide reductase by glutathione. *Arch. Biochem. Biophys.* **288**, 48−53.

(15) Brigelius, R., Muckel, C., Akerboom, T. P., and Sies, H. (1983) Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem. Pharmacol.* **32**, 2529−2534.

(16) Gilbert, H. F. (1982) Biological disulfides: The third messenger? Modulation of phosphofructokinase activity by thiol/disulfide exchange. *J. Biol. Chem.* **257**, 12086−12091.

(17) Zeigler, D. M. (1985) Role of reversible oxidation−reduction of enzyme thiols−disulfides in metabolic regulation. *Annu. Rev. Biochem.* **54**, 305−329.

(18) Cabiscol, E., and Levine, R. L. (1996) The phosphatase activity of carbonic anhydrase III in reversibly regulated by glutathiolation. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4170−4174.

(19) Styblo, M., and Thomas, D. J. (1995) *In vitro* inhibition of glutathione reductase by arsenotriglutathione. *Biochem. Pharmacol.* **49**, 971−974.

(20) International Agency for Research on Cancer (1987) Arsenic and arsenic compounds. In *IARC Monograph on the Evaluation of Carcinogenic Risks to Humans−Overall Evaluations of Carcinogenicity: An Update of IARC Monographs 1 to 42*, Suppl. 7, p 100, Lyon.

(21) Goddard, A. D. (1930) in *A Textbook of Inorganic Chemistry, Vol. XI, Organometallic Compounds, Part II, Derivatives of Arsenic* (Friend, J. N., Ed.) pp 21 and 30, Griffin and Co., London.

(22) Serves, S. V., Charalambidis, Y. C., Sotiropoulos, D. N., and Ioannou, P. V. (1995) Reaction of arsenic(III) oxide, arsenous, and arsenic acid with thiols. *Phosphorus, Sulfur Silicon Relat. Elem.* **105**, 109−116.

(23) Pinto, M. C., Mata, A. M., and Lopez-Barea, J. (1984) Reversible inactivation of *Saccharomyces cerevesiae* glutathione reductase under reducing conditions. *Arch. Biochem. Biophys.* **228**, 1−12.

(24) Zakharyan, R., Wu, Y., Bogdan, G. M., and Aposhian, H. V. (1995) Enzymatic methylation of arsenic compounds: assay, partial purification, and properties of arsenite methyltransferase and monomethylarsonic acid methyltransferase from rabbit liver. *Chem. Res. Toxicol.* **8**, 1029−1038.

(25) Styblo, M., Delnomdedieu, M., and Thomas, D. J. (1995) Biological mechanisms and toxicological consequences of the methylation of arsenic. In *Toxicology of Metals−Biochemical Aspects, Handbook of Experimental Pharmacology* (Cherian, M. G., and Goyer, R. A., Eds.) Vol. 115, pp 407−433, Springer Verlag, Berlin.

(26) Sexton, D. J., and Mutus, B. (1992) Glutathione reductases from a variety of sources are inhibited by physiological levels of glutathione. *Comp. Biochem. Physiol.* **103B**, 897−901.

(27) Tadlock, C. H., and Aposhian, H. V. (1980) Protection of mice against the lethal effects of sodium arsenite by 2,3-dimercapto-1-propane sulfonic acid and dimercaptosuccinic acid. *Biochem. Biophys. Res. Commun.* **94**, 501−507.

(28) Kreppel, H., Paepcke, U., Thiermann, H., Szinicz, L., Reichl, F. X., Singh, P. K., and Jones, M. M. (1993) Therapeutic efficacy of new dimercaptosuccinic acid (DMSA) analogues in acute arsenic trioxide intoxication in mice. *Arch. Toxicol.* **67**, 580−585.

(29) Collinson, L. P., and Dawes, I. W. (1995) Isolation, characterization and overexpression of the yeast gene, *GLR1*, encoding glutathione reductase. *Gene* **156**, 123−127.

(30) Pinto, M. C., Mata, A. M., and Lopez-Barea, J. (1985) The redox interconversion mechanism of *Saccharomyces cerevesiae* glutathione reductase. *Eur. J. Biochem.* **151**, 275−281.

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