

Inhibition of human squalene monooxygenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls

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Abstract Squalene monooxygenase is a flavin adenine dinucleotide-containing, microsomal enzyme that catalyzes the second step in the committed pathway for cholesterol biosynthesis. Feeding weanling rats a diet containing 1% elemental tellurium causes a transient, peripheral demyelination due to the disruption of cholesterol synthesis in Schwann cells secondary to inhibition of squalene monooxygenase. The tellurium species responsible for the inhibition is unknown, as is the mechanism of inhibition. To study the potential mechanisms of tellurium toxicity in humans, three likely in vivo metabolites of tellurium (tellurite, dimethyltellurium dichloride, and dimethyltelluride) were tested as inhibitors of purified human squalene monooxygenase. All three inhibitors reacted with the enzyme slowly and the resulting interaction was not freely reversible. The 50% inhibitory concentration for the methyltellurium compounds (~100 nM) after a 30-min preincubation was 100-fold lower than that of tellurite, indicating a role for hydrophobicity in the enzyme-inhibitor interaction. The ability of glutathione and 2,3-dimercaptopropanol to prevent and reverse the inhibition indicated that the tellurium compounds were reacting with sulfhydryls on squalene monooxygenase, and the ability of phenylarsine oxide, which reacts specifically with vicinal sulfhydryls, to inhibit the enzyme indicated that these sulfhydryls are located proximal to one another on the enzyme. These results suggest that the unusual sensitivity of squalene monooxygenase to tellurium compounds is due to the binding of these compounds to vicinal cysteines, and that methylation of tellurium in vivo may enhance the toxicity of tellurium for this enzyme.—Laden, B. P., and T. D. Porter. Inhibition of human squalene monooxygenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls. *J. Lipid Res.* 2001. 42: 235–240.

Supplementary key words cholesterol biosynthesis • demyelination • 2,3-dimercaptopropanol • glutathione • arsenic • selenium • phenylarsine oxide

Squalene monooxygenase catalyzes the second and potentially rate-limiting step in the committed pathway for cholesterol biosynthesis, yet little is known about the basic structure and biochemistry of the enzyme (1). This microsomal, flavin adenine dinucleotide (FAD)-containing monooxygenase catalyzes the epoxidation of squalene across a double bond to form 2,3-oxidosqualene in a reac-

tion more typical of the cytochromes P450. Electrons are passed from NADPH, via cytochrome P450 reductase, to the FAD of squalene monooxygenase, where they are used to reduce one atom of molecular oxygen (O₂) to water, while the other oxygen atom is inserted into the substrate, squalene (2). Oxygen activation has been suggested to proceed via formation of a flavin C(4a)-hydroperoxide (3). Studies of squalene monooxygenase have been somewhat limited because of the difficulties associated with enzyme purification. However, a truncated rat squalene monooxygenase, with an N-terminal deletion removing the putative membrane-binding domain, was cloned and expressed in bacteria (4, 5). On the basis of these studies, our laboratory cloned, expressed, and characterized a truncated human squalene monooxygenase (6) that is used in the present work.

Feeding weanling rats a diet containing 1% elemental tellurium causes a transient demyelination of peripheral nerves due to the inhibition of squalene monooxygenase in Schwann cells (7). This inhibition blocks cholesterol biosynthesis and leads to degradation of the myelin sheath (8). The form of tellurium responsible for inhibition of squalene monooxygenase remains unknown. Tellurite (TeO₃²⁻) inhibits squalene monooxygenase activity in microsomal preparations from rat liver and sciatic nerve, suggesting that it may be the neurotoxic metabolite (7). However, tellurium is methylated in the liver (9), and two methyltellurium compounds, dimethyltellurium dichloride [Te(CH₃)₂Cl₂; DMTDC] and dimethyltelluride [Te(CH₃)₂; DMT], inhibit squalene monooxygenase activity both in cultured Schwann cells and in weanling rats, whereas tellurite does not (10). To determine which tellurium compounds also inhibit the human enzyme we tested the ability of tellurite, DMT, and DMTDC to inhibit the human enzyme in

Abbreviations: DMP, 2,3-dimercaptopropanol; DMT, dimethyltelluride; DMTDC, dimethyltellurium dichloride; FAD, flavin adenine dinucleotide; PAO, phenylarsine oxide; TMT, trimethyltellurium chloride.

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a purified system and investigated the mechanism of this inhibition. The present study provides evidence that the tellurium compounds react with vicinal cysteine sulfhydryl groups on squalene monooxygenase, and that methylation of this element, normally considered a detoxication reaction, may actually yield a more toxic metabolite for this enzyme. Moreover, selenium and arsenic share many similar chemical properties with tellurium and can produce a demyelination similar to that caused by tellurium; the molecular targets responsible for the arsenic and selenium-induced neuropathy are unknown, but the ability of phenylarsine oxide to inhibit squalene monooxygenase suggests that this enzyme may be one such target.

EXPERIMENTAL PROCEDURES

Materials

Sodium tellurite and phenylarsine oxide (PAO) were purchased from Aldrich (Milwaukee, WI); DMT, DMTDC, and trimethyltellurium chloride (TMT) were purchased from Organometallics (East Hampstead, NH). NADPH, FAD, 2,3-dimercaptopropanol, and cytochrome *c* were purchased from Sigma (St. Louis, MO). Radiolabeled squalene was synthesized by the Chemical Synthesis Facility (Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT) at 7 mCi/mmol. Thin-layer chromatography plates were manufactured by Whatman (Clifton, NJ).

Purification of squalene monooxygenase

Squalene monooxygenase expressed from the pTYB4 vector was purified according to the protocol provided by New England BioLabs (Beverly, MA) for expression of proteins with the IMPACT T7 system (intein-chitin binding domain fusion proteins). This purification is described in greater detail by Laden, Tang, and Porter (6). Briefly, bacterial cells were lysed by French press in buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid and 0.1% Triton X-100. The lysate was then centrifuged at 12,000 *g*. After the supernatant fraction was loaded onto a chitin affinity column, the column was washed with 20 column-volumes of the above-described buffer. The column was then incubated overnight at 4°C in cleavage buffer consisting of the French press buffer with 30 mM 2-mercaptoethanol to promote cleavage. The released squalene monooxygenase protein was eluted and the buffer was changed, by centrifugal dialysis, to a storage buffer containing 20 mM Tris-HCl (pH 7.4) with 0.1% Triton X-100 and stored at -80°C until use.

Squalene monooxygenase activity assays

Activity assays were carried out as previously described (6). Standard incubations (200 μ l) contained 20 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 30 μ M FAD, 40 μ M [14 C]squalene, 28 pmol of cytochrome P450 reductase, and 3 μ g (57 pmol) of squalene monooxygenase. Reactions were preincubated for 30 min at 37°C and then started by the addition of NADPH (final concentration, 1 mM) and incubated for an additional 30 min at 37°C. Reactions were stopped by extraction into methylene chloride and fractionated on silica thin-layer plates with 5% ethyl acetate in hexane. The plates were visualized and quantified by electronic autoradiography (InstantImager; Packard, Meriden, CT). Reactions were linear for 60 min. All graphs and kinetic constants were generated with Prism 3.0 (GraphPad Software, San Diego, CA) from measurements made in duplicate or better, with standard errors of the means indicated by error bars where appropriate.

Cytochrome P450 reductase assays

Cytochrome P450 reductase was purified by affinity chromatography from *Escherichia coli* cells expressing the cloned rat cDNA (11) and quantified spectrally, using an extinction coefficient of 21.4/mM at 456 nm. Cytochrome *c* reduction was measured in buffer containing 0.1 mM potassium phosphate (pH 7.7), 50 μ M cytochrome *c*, and 5 pmol of cytochrome P450 reductase after a 30-min preincubation at 37°C with the tellurium or arsenical compound. Reactions were then started by addition of NADPH (50 μ M final concentration) and the reduction of cytochrome *c* was followed at 550 nm for 1 min at room temperature.

RESULTS

Inhibition of squalene monooxygenase by selected tellurium compounds

Figure 1 depicts the inhibition of squalene monooxygenase by tellurite, DMTDC, and DMT. TMT was also tested, but was not inhibitory (data not shown). There are some notable differences between the inhibition profiles of these compounds. First, as indicated by the 50% inhibitory concentration (IC_{50}) values after a 30-min preincubation, the methylated tellurium compounds ($IC_{50} \approx 100$ nM) were approximately 100-fold more inhibitory than tellurite ($IC_{50} \approx 10$ μ M). Second, at a concentration of 100 μ M the methylated tellurium compounds were completely inhibitory, whereas tellurite was only partially inhibitory: at tellurite concentrations as high as 10 mM the enzyme maintains approximately 5% of maximal activity (data not shown). This phenomenon is further detailed in Fig. 2. A graph of inverse velocity versus total inhibitor concentration yielded a hyperbolic curve with a horizontal asymptote, indicative of partial (hyperbolic) inhibition, and consistent with an enzyme-inhibitor complex that is still able to catalyze product formation, but less efficiently (12).

To verify that the tellurium compounds were binding specifically to squalene monooxygenase and not to cytochrome P450 reductase, the ability of P450 reductase to reduce cytochrome *c* in the presence of each tellurium

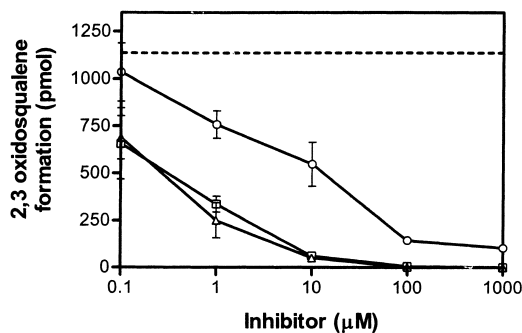


Fig. 1. Inhibition of human squalene monooxygenase by tellurium compounds. Standard assays were preincubated for 30 min at 37°C with increasing concentrations of tellurite (open circles), DMT (open squares), or DMTDC (open triangles). Reactions were then started by addition of NADPH (1 mM final concentration) and incubated for an additional 30 min. The dashed line indicates product formation in the absence of inhibitor. Values represent the average of triplicate determinations \pm standard error.

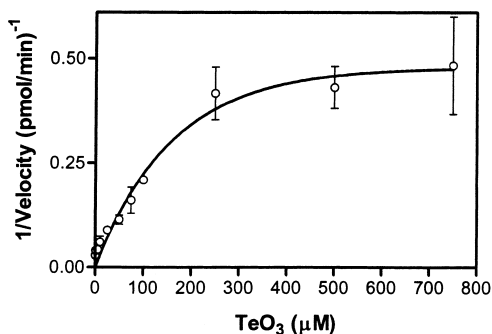


Fig. 2. Hyperbolic (partial) inhibition of human squalene monooxygenase by tellurite. Standard assays were preincubated for 30 min at 37°C with increasing concentrations of tellurite. Reactions were then started by addition of NADPH (1 mM final concentration) and incubated for an additional 30 min. Values represent the average of triplicate determinations, $r^2 = 0.85$.

compound was determined. As previously reported, tellurite had no effect on cytochrome *c* reduction at concentrations up to 1 mM (6). The dimethylated compounds produced 5–10% inhibition at 100 μ M (data not shown), a concentration at which the monooxygenase reaction was completely inhibited. As the reductase concentration exceeds saturation by 20% in the monooxygenase reactions and thus is not rate limiting, it is unlikely that the slight inhibition of reductase by 100 μ M dimethyltellurium contributes to the complete inhibition of the monooxygenase reaction at this concentration.

Several approaches were used to determine whether the tellurium compounds bind squalene monooxygenase in a classic, freely reversible manner. Although active enzyme could not be recovered after either gel-filtration chromatography or centrifugal dialysis, even in the absence of inhibitor, active enzyme could be maintained in dilution assays. Enzyme was preincubated with each tellurium compound for 30 min and then diluted 8-fold and the reaction initiated with NADPH. Activity was not increased after dilution of the inhibitors, indicating that these compounds were not released from the enzyme on dilution and thus bind squalene monooxygenase irreversibly (data not shown).

Progression curves for squalene monooxygenase reactions in the presence and absence of the tellurium compounds are shown in **Fig. 3A**. A replot of these data are presented in **Fig. 3B**, where the extent of inhibition is plotted against time. Inhibition by tellurite was not evident until 20 min, whereas up to 50% inhibition was evident for DMTDC and DMT as early as 10 min. These data demonstrate slow, time-dependent binding of the tellurium compounds to squalene monooxygenase and reveal that the greater potency of the methylated compounds relative to tellurite is due to their higher affinity for the enzyme, rather than to the fact that tellurite is a partial inhibitor.

Effect of sulfhydryls on inhibition of squalene monooxygenase by tellurium compounds

Various sulfhydryl compounds were tested for their ability either to protect against or to reverse inhibition by tel-

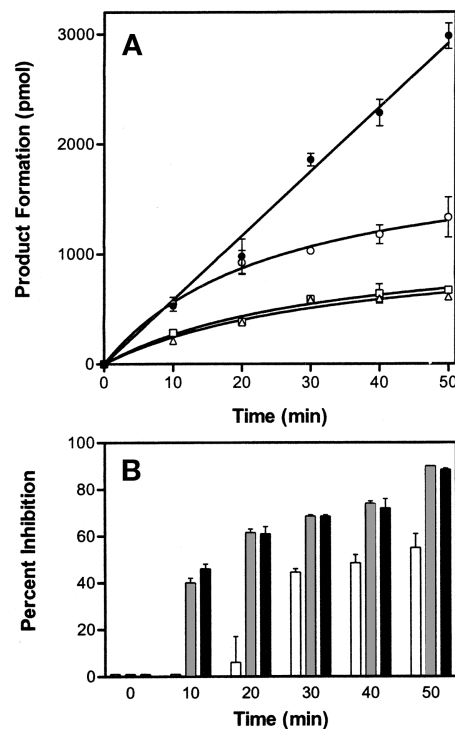


Fig. 3. Slow (time-dependent) binding of tellurium compounds. Standard assays were preincubated for 10 min at 37°C in the presence of 10 μ M tellurite (open circles, open columns), 10 μ M DMTDC (open triangles, shaded columns), or 10 μ M DMT (open squares, solid columns) or no inhibitor (closed circles). The reactions were started by the addition of squalene monooxygenase (57 pmol) and incubated for the indicated times at 37°C. A: Total product formation. B: Inhibition as a percentage of total activity. Values represent the average of duplicate determinations, with error bars indicating the range.

lurium. Both glutathione and 2,3-dimercaptopropanol (DMP), added prior to the 30-min preincubation, afforded nearly complete protection from tellurite, maintaining approximately 90% of the initial activity (**Fig. 4A**). Glutathione was somewhat more effective than DMP in preventing inhibition by the methylated compounds: the addition of 1 mM glutathione to the preincubations maintained 50% of the initial activity, while the presence of 1 mM DMP maintained 20 to 25%. When added after the preincubation, DMP was the better reversal agent for tellurite inhibition (**Fig. 4B**), restoring 85% of initial activity, whereas little reversal was seen with glutathione. In contrast to the protection experiments, neither sulfhydryl agent could reverse the inhibition by the methyltellurium compounds.

To evaluate the effect of the sulfhydryl compounds per se on squalene monooxygenase activity, reactions were carried out in the presence of various concentrations of either glutathione or DMP (data not shown). Both glutathione and DMP activate the enzyme between 10 μ M and 1 mM, with maximum activation at 500 μ M glutathione (140%) and 50 μ M DMP (165%). In addition, cytochrome *c* reduction by P450 reductase was monitored in the presence of increasing concentrations of glutathione

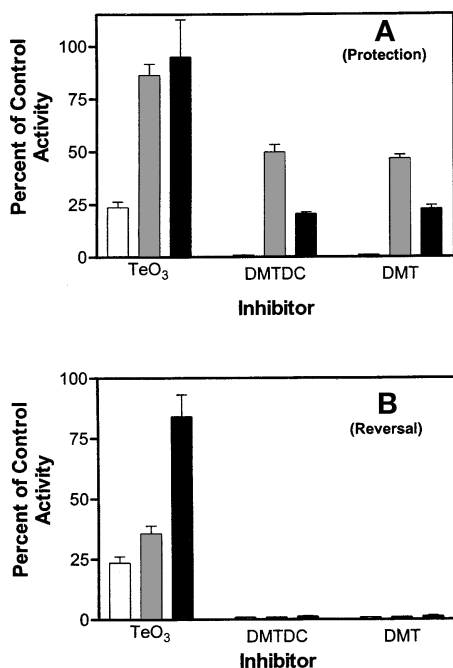


Fig. 4. Effect of sulfhydryls on inhibition of squalene monooxygenase by tellurium compounds. A: Protection experiments. Standard assays were preincubated for 30 min at 37°C with 50 μ M tellurite, 50 μ M DMTDC, or 50 μ M DMT and either no sulfhydryls (open columns), 1 mM glutathione (shaded columns), or 1 mM DMP (solid columns). Reactions were then started by addition of NADPH (1 mM final concentration) and incubated for an additional 30 min. B: Reversal experiments. Neither glutathione nor DMP were included in the preincubation, but were instead added simultaneously with NADPH (1 mM final concentration) to start the reaction. No sulfhydryls (open columns); 1 mM glutathione (shaded columns); 1 mM DMP (solid columns). Values represent the average of triplicate determinations \pm SE.

(data not shown). There was no activation or inhibition of cytochrome *c* reduction at concentrations up to 1 mM glutathione, indicating that the effects of glutathione are on squalene monooxygenase and not on the reductase. It was not possible to measure cytochrome *c* reduction in the presence of DMP, as DMP itself reduced cytochrome *c*.

PAO inhibition of squalene monooxygenase

The arsenical compound PAO reacts with vicinal thiols in proteins (13, 14). **Figure 5** shows that squalene monooxygenase is potently inhibited by PAO, thereby indicating the presence of vicinal sulfhydryls in this enzyme. PAO did not affect cytochrome *c* reduction by P450 reductase, indicating that it specifically affects squalene monooxygenase (data not shown). This inhibition can be prevented and reversed by sulfhydryl reagents (**Fig. 6**). Preincubation with either glutathione or DMP in the presence of PAO resulted in nearly complete protection from inhibition. However, DMP was significantly more effective than glutathione in reversing the inhibition by PAO. PAO reversal experiments containing DMP showed greater than 50% recovery of initial activity, while experiments with glutathione showed essentially no recovery.

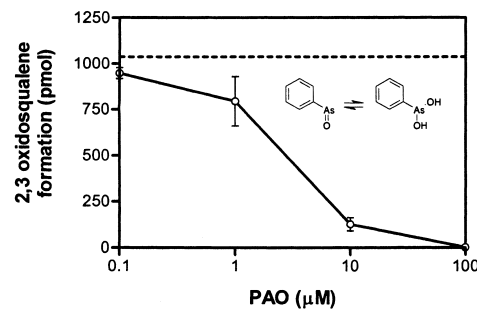


Fig. 5. Effect of PAO on squalene monooxygenase activity. Standard assays were preincubated for 30 min at 37°C with increasing concentrations of PAO. Reactions were then started by addition of NADPH (1 mM final concentration) and incubated for an additional 30 min. The dashed line indicates untreated activity. Values are the average of triplicate determinations \pm SE. The structure of PAO in its native and hydrated state is shown.

DISCUSSION

Feeding weanling rats a diet containing 1% elemental tellurium causes a transient, peripheral demyelination due to the disruption of cholesterol synthesis in Schwann cells secondary to inhibition of squalene monooxygenase (7, 8). However, the tellurium species responsible for the inhibition is unknown. We have previously reported that tellurite inhibits purified human squalene monooxygenase (6); herein we report that two methyltellurium compounds also directly inhibit the purified human enzyme, indicating that any of the three compounds may be the inhibitory tellurium species in vivo. As tellurium is methylated in the liver (9), the dimethyl and, secondarily, the trimethyl species are likely to be the forms of tellurium found in the circulation. Goodrum (10) showed that dimethyl- and trimethyltellurium inhibit squalene monooxygenase in Schwann cells in culture, but that tellurite, either when added to cell cultures or when administered intraperitoneally to rats, was not inhibitory. Thus, it is

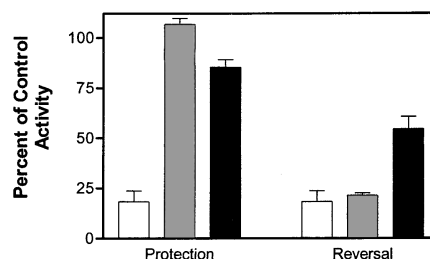


Fig. 6. Protection and reversal of PAO inhibition of squalene monooxygenase by sulfhydryls. Standard assays containing 10 μ M PAO were preincubated for 30 min at 37°C (open columns). Protection assays, containing either 1 mM glutathione (shaded column) or 1 mM DMP (solid column) in the preincubation, were started by the addition of NADPH (1 mM final concentration), while reversal assays were started by the addition of NADPH (1 mM final concentration) and either glutathione (1 mM, shaded column) or DMP (1 mM, solid column). All reactions were carried out at 37°C for an additional 30 min. Values are the average of triplicate observations \pm SE.

likely that a methyltellurium compound is the inhibitory species of tellurium found in vivo. As trimethyltelluronium was essentially inactive with the purified human enzyme in our studies, it further is likely that dimethyltellurium is the toxic species in vivo. This is consistent with the results of Goodrum (10), in which TMT was approximately 100-fold less potent than DMTDC with cultured cells in vivo. The two dimethyltellurium species, DMTDC and DMT, were indistinguishable in our studies, suggesting that they are chemically equivalent in solution; it is likely that both compounds form hydrated, $\text{Te}^{(\text{IV})}$ oxidation species in solution (15).

The present work implicates the involvement of cysteine sulfhydryls in the inhibition of squalene monooxygenase by tellurium compounds. Sulfhydryls have been implicated as targets for tellurite binding in other enzymes (16, 17), and the organotellurium compound AS101 was shown to selectively inhibit cysteine proteases by reacting with active site thiols (18). Indeed, in the present studies, inhibition by both tellurite and the dimethyltellurium compounds could be prevented by preincubation with either glutathione (a monothiol) or DMP (a dithiol). Although the protective effect of thiols was not as great for the methylated compounds as it was for tellurite, it does indicate that these compounds react with thiols. The ability of the specific sulfhydryl reagent *N*-ethylmaleimide to inhibit squalene monooxygenase in rat liver microsomes is also consistent with the presence of essential sulfhydryls on squalene monooxygenase (7).

The fact that the inhibition by tellurite can also be reversed by the addition of thiols further supports the suggestion that enzyme sulfhydryls are the target of tellurite binding in squalene monooxygenase. The thiol compound likely substitutes for the cysteine sulfhydryl, releasing a tellurite-thiol adduct from the enzyme. However, neither glutathione nor DMP reversed the inhibition by the methylated compounds. This may be due to a tighter interaction between the methyltellurium compounds and the monooxygenase relative to tellurite, possibly because the reactive cysteine(s) reside in a hydrophobic pocket. Alternatively, the bulkier methyl groups may impede access of the thiols to the tellurium-cysteine bond, preventing exchange. The existence of a hydrophobic pocket harboring the reactive cysteine(s) would also explain why the methyltellurium compounds react with the enzyme more quickly than tellurite (Fig. 3).

Some notable differences exist between the ability of glutathione and the ability of DMP to protect against and reverse the inhibition by tellurite. Both glutathione and DMP completely prevented inhibition by tellurite, indicating that both a monothiol and dithiol can interact with tellurite to a similar extent when they are free in solution. However, DMP was much more effective than glutathione in reversing the inhibition by tellurite. The fact that a dithiol reversed the inhibition, but a monothiol did not, suggests that tellurite may be reacting with two cysteines on the enzyme (vicinal sulfhydryls). The remarkable sensitivity of squalene monooxygenase to tellurium inhibition in vivo, and the lack of other sulfhydryl-related toxicities

after the intraperitoneal administration of DMTDC (10), argues that the interaction of tellurium with squalene monooxygenase is not characteristic of a typical sulfhydryl reagent. These results raise the possibility that tellurium readily reacts with dithiols to yield an S-Te-S adduct (Cys-Te-Cys), and that squalene monooxygenase contains two appropriately located cysteine sulfhydryls. The weak inhibition by TMT, which has only a single free ligand for exchange and forms a stable trimethyl cation, is consistent with this hypothesis (15).

Trivalent arsenical compounds, such as PAO, react with vicinal sulfhydryl groups on proteins to form thioarsine rings (13, 14). Although these compounds can also react with single sulfhydryls, the product is not stable (19). The interaction of PAO with vicinal sulfhydryls can be efficiently reversed by the addition of the dithiol DMP, but not by monothiols, such as 2-mercaptoethanol or glutathione (13, 14). The inhibition of squalene monooxygenase by PAO (Fig. 5) provides support for the presence of vicinal thiols on this enzyme. As expected, glutathione and DMP prevented the inhibition by PAO, but only the dithiol DMP was capable of reversing the inhibition. This pattern of protection and reversal closely resembles that seen for tellurite, supporting a role for vicinal sulfhydryls in tellurite binding. The facile reversal of PAO inhibition, but not dimethyltellurium inhibition, by DMP may reflect the different valence structures of arsenic and tellurium (PAO is trivalent, whereas dimethyltellurium is tetravalent) and the ability of DMP to approach the cysteine adducts of these compounds.

While the cysteines that react with the tellurium compounds (and PAO) have yet to be identified, they are clearly essential for catalysis. Lee et al. (20) have shown that mutagenesis of either of two cysteines in rat squalene monooxygenase results in complete loss of enzyme activity, and have proposed that these two cysteines form a disulfide bond that is necessary for enzyme activity; mutation of either of two additional cysteines resulted in a 50% loss of activity. We find that high concentrations of squalene partially prevent inhibition by DMT, but not by tellurite (data not shown). In contrast, FAD concentrations have no effect on inhibition by these agents. These results suggest that the reactive cysteines are in or near the squalene binding site, and are consistent with the observation that the more hydrophobic tellurium compounds bind more rapidly to the enzyme (Fig. 3). This may also explain why arsenite was earlier found not to be inhibitory (6), whereas the more hydrophobic PAO was found here to be an effective inhibitor of the enzyme.

In summary, the present studies reveal that methyltellurium compounds directly inhibit squalene monooxygenase, and demonstrate that the inhibition is a result of binding to critical sulfhydryl groups. The inhibition of squalene monooxygenase by PAO argues for the presence of vicinal sulfhydryl groups that are required for catalysis; based on the pattern of protection and reversal by thiols, tellurite likely reacts with the same vicinal sulfhydryls. As inhibition by the methylated compounds is not reversed by dithiols, the involvement of vicinal sulfhydryls is less obvious, al-

though steric constraints may prevent access of dithiols to the dimethyltellurium adducts. Nonetheless, the simplest scenario is that PAO, tellurite, and the dimethyltellurium compounds all bind to the same site, and that the methyltellurium compounds have much greater affinity for the enzyme than tellurite, probably because the reactive cysteines lie in a hydrophobic pocket. It should be noted that gel electrophoretic studies demonstrate that the tellurium compounds are not forming cross-links between molecules (data not shown). The role that these vicinal sulfhydryls play in catalysis is unknown and is the subject of current investigations.

Last, it is noteworthy that the data here can be proposed to explain some of the neurotoxic effects of arsenic and selenium. One of the toxic effects of arsenic is a peripheral neuropathy characterized by a demyelination of long axon nerve fibers (21), similar to the tellurium-induced demyelination in weanling rats (7). The molecular target responsible for initiating the arsenic-induced demyelination is not known. However, as the trivalent arsenical compound PAO has been shown here to inhibit squalene monooxygenase, it is possible that inhibition of squalene monooxygenase contributes wholly or partially to the demyelination observed with chronic, severe arsenic exposure. Selenium is positioned directly above tellurium on the periodic table and has also been shown to produce a demyelinating neuropathy: demyelination occurs in the lumbar ventral horn of the spinal cord in pigs overexposed to selenium (22). We have previously shown that selenite and selenium dioxide are capable of inhibiting the purified human enzyme (6), and it is therefore plausible that this enzyme may be the target bound by selenium to initiate the demyelination in pigs. ■

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