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Thiodiglycol, the hydrolysis product of sulfur mustard: Analysis of in vitro biotransformation by mammalian alcohol dehydrogenases using nuclear magnetic resonance

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Abstract

Thiodiglycol (2,2'-bis-hydroxyethylsulfide, TDG), the hydrolysis product of the chemical warfare agent sulfur mustard, has been implicated in the toxicity of sulfur mustard through the inhibition of protein phosphatases in mouse liver cytosol. The absence of any inhibitory activity when TDG was present in assays of pure enzymes, however, led us to investigate the possibility for metabolic activation of TDG to inhibitory compound(s) by cytosolic enzymes. We have successfully shown that mammalian alcohol dehydrogenases (ADH) rapidly oxidize TDG in vitro, but the classic spectrophotometric techniques for following this reaction provided no information on the identity of TDG intermediates and products. The use of proton NMR to monitor the oxidative reaction with structural confirmation by independent synthesis allowed us to establish the ultimate product, 2-hydroxyethylthioacetic acid, and to identify an intermediate equilibrium mixture consisting of 2-hydroxyethylthioacetaldehyde, 2-hydroxyethylthioacetalddehyde hydrate and the cyclic 1,4-oxathian-2-ol. The intermediate nature of this mixture was determined spectrophotometrically when it was shown to drive the production of NADH when added to ADH and NAD.

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Keywords: Sulfur mustard; Thiodiglycol; In vitro metabolism; Alcohol dehydrogenase; NMR

Introduction

Sulfur mustard (2,2'-bis-chloroethylsulfide, Fig. 1A) is a vesicant causing delayed injury to human skin that varies in severity from erythema to massive blistering and necrosis in a dose-dependent manner. The actual mechanism by which this occurs remains unexplained although inflammation certainly plays a role. In aqueous medium, pure sulfur mustard undergoes rearrangement by an $\text{S}_{\text{N}}\text{I}$ mechanism to a cyclic sulfonium ion that quickly hydrolyzes (Fig. 2). This process is repeated, due to the bifunctional nature of the molecule, leading to the final hydrolysis product thiodiglycol (2,2'-bis-hydroxyethylsulfide, TDG, Fig. 1B) a symmetric, water-soluble, liquid primary diol with low vapor pressure (Bartlett and Swain, 1949; Yang et al., 1988). TDG itself is relatively non-toxic (Reddy et al., in press) so its formation has been considered a sulfur mustard detoxification step.

In earlier work (Brimfield, 1995), we showed that TDG inhibited non-alkaline phosphatase-related $p$-nitrophenylphosphate phosphatase ($p$-NPP) activity in mouse liver cytosolic preparations. There was strong circumstantial evidence to indicate a mechanistic relationship between $p$-NPP inhibition by TDG in cytosol and the inhibition of one or more protein (serine/threonine) phosphatases by the natural vesicant cantharidin (Li and Casida, 1992). However, cantharidin also inhibited preparations of pure protein phosphatases 1, 2a and 2b while...
Reagents and supplies. Somerville, NJ. Other chemicals and solvents were reagent grade from standard suppliers. A dehydrogenase/pyruvate-based NAD regenerating system in vivo via two cycles of sulfonium ion formation employed catalytic quantities of ADH and NAD and a lactate bifunctional nature of the mustard leads to the formation of a symmetrical diol.

et al., 1993, and Henehan, 1995; Henehan and Oppenheimer, 1993; Henehan et al., 1995). We needed a system by which we could establish metabolite appearance and disappearance. However, monitoring ADH activity by following the absorbance from NADH production provided no information about the structure of enzymatic intermediates and reaction products. The route of the reaction is transparent to spectrophotometric monitoring (Henehan and Oppenheimer, 1993; Abeles and Lee, 1960). The alternative approach using batchwise incubations, isolating the products from the reaction mixture and characterizing them by physicochemical means, permits product identification but raises the risk of failure to identify transient low concentration intermediates and yields little insight into the timing of their comings and goings. We needed a system by which we could establish metabolite identity and dynamically monitor product and intermediate appearance and disappearance.

Oppenheimer's work offered such a system (Oppenheimer and Henehan, 1995; Henehan and Oppenheimer, 1993; Henehan et al., 1993, 1995). He developed an $^1$H NMR procedure that employed catalytic quantities of ADH and NAD and a lactate dehydrogenase/pyruvate-based NAD regenerating system in aqueous buffer. The process is made possible by the application of water suppression using the presaturation technique which reduces the large signal from the protons on water in aqueous media and allows the visualization of the comparatively weak signals from compounds of interest (Hore, 1989).

This report presents the results from a study of the enzyme-mediated transformation of TDG by horse liver ADH and several human isoforms in vitro. The use of $^1$H NMR, with structural confirmation by synthesis, allowed us to identify intermediates and products produced from TDG as the result of ADH oxidation. In the future, we will test these newly identified metabolites for their ability to inhibit pure protein (serine/threonine) phosphatases and determine whether or not TDG is a factor in the mechanism of sulfur mustard-induced vesication.

Materials and methods

Reagents and supplies. Equine liver alcohol dehydrogenase, lactate dehydrogenase (LDH) from rabbit muscle, sodium pyruvate and ß-nicotinamide adenine dinucleotide were purchased from Sigma Chemical Co., St. Louis, MO. Thiodiglycol and thiodiglycolic acid were from Aldrich Chemical Co., Milwaukee, WI, and were used without additional purification. Deuterium oxide (D$_2$O) was supplied by the ACROS Division of Fisher Scientific, Somerville, NJ. Other chemicals and solvents were reagent grade from standard suppliers.

Human $\alpha$ and $\beta_1$ ADH isoforms were the kind gift of Dr. Thomas Hurley, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine. The isoforms were expressed and purified from Escherichia coli (JM105 strain) as described in Choi et al. (2002). Glycerol, added to the purified enzyme preparations before freezing, interfered with the collection of NMR data. It was removed and the buffer corrected by passage through a small Sephadex G25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column followed by repeated concentration and resuspension in fresh buffer at 4 °C using Amicon Microcon centrifugal filtration devices (Millipore Corp., Billerica, MA) with a 10,000 molecular weight cut off.

Enzymology. For NMR, each sample consisted of 10 mM TDG, 2.0 mM NAD, 0.15 U of horse liver ADH and an NAD regenerating system consisting of 250 U of lactate dehydrogenase and 100 mM sodium pyruvate dissolved in 0.1 M sodium phosphate buffer, pH 7.5, in a total volume of 500 µl. Deionized water used to prepare the buffer was made 10% by volume with respect to D$_2$O. All components except ADH were prepared as a mixture and kept at 0 °C. A 480 µl aliquot of the mixture was transferred to a standard 5-mm NMR tube and

Fig. 1. The structures under discussion: (A) sulfur mustard, 2,2'-bis-chloroethyl sulfide; (B) thiodiglycol, TDG, 2,2'-thiodiethanol; (C) 2-hydroxyethylthioacetaldehyde; (D) 2-hydroxyethylthioacetaldehyde hydrate; (E) 1,4-oxathian-2-ol; (F) 2-hydroxyethylthioacetic acid; (G) thiodiglycolic acid, 2,2'-thiobis-acetic acid.

Fig. 2. The rapid S$_2$O$_3$ mediated formation of TDG from sulfur mustard in aqueous medium, via a cyclic sulfonium ion, followed by hydrolysis. The bifunctional nature of the mustard leads to the formation of a symmetrical diol via two cycles of sulfonium ion formation.
brought to 37 °C. The reaction was begun with the addition of 20 μl of ADH (0.15 U) in buffer at 37 °C. Proton NMR monitoring of the enzymatic reaction was done on a Varian Unity Inova 600 MHz instrument equipped with Varian data analysis software version 6.1C (Varian Instruments, Palo Alto, CA). The probe temperature was maintained at 37 °C. Each time point consisted of 64 scans acquired with a spectral width of 10,000 Hz using 32K data points. A 3-s post-acquisition delay was used to allow for full relaxation of the resonances. During monitoring of the enzymatic activity, spectra were collected every 5 to 8 min for up to 16 h.

When recombinant human isoforms were evaluated, activity was normalized to that of the equine enzyme spectrophotometrically by following the increase in optical density at 340 nm using ethanol as a substrate, as described in Dudley et al. (2000), immediately before starting the analysis. The concentration of human ADH used in the NMR evaluation of that enzyme. This allowed us to base our comparisons on equine enzyme systems.

For spectrophotometric evaluation of ADH activity toward the oxathianol (Fig. 1E), we used a mixture in 0.01 M sodium phosphate buffer, pH 7.5 containing 1.0 mg/ml enzyme, 5.0 mM NAD and 10 mM oxathianol. The reaction was run at 30 °C, and the optical density at 340 nm was measured. NADH produced was determined using an extinction coefficient of 6317 mol⁻¹ cm⁻¹ (McComb et al., 1976).

Organic synthesis. The aldehyde (Fig. 1C) was synthesized in its lactol form (Fig. 1E) in a single step from TDG using chromium VI-pyridine reagent according to the method of Corey and Schmidt (1979). To 245 mg of Cr,O₃ stirred in 10 ml of dry CH₂Cl₂ at 0 °C was added 200 μl of anhydrous pyridine drop wise via syringe. The resulting orange/yellow heterogeneous reaction was allowed to stir at 0 °C for 30 min, whereupon 100 mg of thiodiglycol dissolved in 1.5 ml of dry CH₂Cl₂ was added. After stirring for 1.5 h at 0 °C, the brown heterogeneous reaction mixture was warmed to room temperature, diluted with 30 ml of diethyl ether and filtered through a plug of silica gel with 50 ml of diethyl ether being used to complete the transfer. Removal of the solvent in vacuo yielded the crude product mixture as a colorless oil which solidified upon storage at -4 °C. ¹H NMR, Bruker AMX-360 (Buena Vista, NJ) (CDCl₃, ppm) 5.04 (1 H, t, J = 2.8 Hz), 4.28 (1 H, m), 3.87 (1 H, m), 3.59 (1 H, d, J = 7.6 Hz), 2.86 (1 H, dd, J = 13.2 Hz, J = 0.8 Hz), 2.56 (3 H, m); mass spectrum Hewlett-Packard G1800A (Agilent Technologies, Palo Alto, CA) (EI, 70 eV) m/e (relative intensity) C₆H₄O₂S MW = 120.15, observed, 120.1 (M⁺, 35), 102 (8), 91 (21), 74 (51), 61 (73), 46 (100).

Hydroxyethylthioacetic acid (Fig. 1F), used to confirm the structure of the final metabolic product, was synthesized according to the procedures published by Black et al. (1993) and was isolated as the sodium salt. Spectral characterization gave results consistent with the published values.

Results

NMR using ADH from equine liver

The NMR system performed well as a means for evaluating the disappearance of reactants and the appearance of products with the horse liver enzyme. Fig. 3 clearly shows the peaks of significance for evaluating the dynamics of the reaction. The signals associated with the NAD regenerating system were prominent. The methyl protons contributed by the keto form of pyruvate appear as a singlet at 2.36 ppm and for the hydrated form as a singlet at 1.47 ppm. The methyl protons of lactate appear as a doublet at 1.32 ppm. The quartet at 4.12 ppm represented the single proton on the α carbon of lactate. The complex set of signals from protons on the pyridine nucleotide appears above 6.0 ppm. Their intensity was relatively low consistent with the catalytic concentration (2.0 mM) of NAD.

Although the pyruvate peak appeared apparently unchanged during the course of the incubation because of the high starting concentration (100 mM), side by side comparison of consecutive spectra (Fig. 4) and graphing the integrated data (Fig. 5) showed a reduction in pyruvate concentration consistent with the increase in the concentration of the methyl group of lactate. The stoichiometry of lactate production and pyruvate consumption (20 mM) and for TDG disappearance (10 mM) indicated the reduction of 2 mol of NAD per mole of TDG oxidized (Fig. 5) as one would predict for the twofold oxidation of TDG to TDGA (Fig. 1F).

The signals originating with TDG were also prominent. The signal for the methylene protons on the carbon adjacent to sulfur
in TDG (triplet, 2.78 ppm, Fig. 3) and the methylene protons on the hydroxyl-containing carbon in TDG (triplet, 3.78 ppm, Fig. 3) were consistent with a standard spectrum of TDG run under identical conditions of temperature, pH and solvent composition (Fig. 6A). The appearance of a new signal at 3.23 ppm (singlet) representing two equivalent methylene protons on a carbon adjacent to a carboxylic acid group indicated the generation of 2-hydroxyethylthioacetic acid (TDGA, Fig. 1F) as a product. The structure was confirmed by comparison with the NMR spectra of TDG (Fig. 6A), thiodiglycolic acid (Fig. 6B) and 2-hydroxyethylthioacetic acid (Fig. 6C) under the conditions employed in the enzyme work. The terminal nature of the acid in the metabolic pathway was determined by substituting it for TDG in the otherwise complete in vitro horse liver NMR system and finding no evidence of enzymatic turnover (data not shown).

Our initial assumption was that the signal from a free aldehyde would appear in the 9.0–9.5 ppm range where the signal for an aldehydic proton usually appears. This was not the case in the spectra from our experimental metabolic system. However, the appearance of minor transient signals between 2.64 and 2.75 ppm and 3.9 to 4.0 ppm (Fig. 7) caused us to reexamine that expectation. These minor signals seemed to correspond to a single compound based on peak integrals and 2-dimensional correlation spectroscopy (COSY) which allows one to isolate and identify spin systems associated with each compound in a mixture (data not shown). The chemical shifts and splitting patterns suggested a cyclic compound with a hemiacetaldic proton. A chromium (VI)-mediated oxidation (Corey and Schmidt, 1979) of TDG yielded 1,4-oxathian-2-ol (Fig. 1E) a cyclic hemiacetal. NMR analysis of this synthetic product under conditions of temperature, pH and solvent composition identical to those used to investigate the enzymatic activity (Fig. 8) enabled us to identify a doublet of doublets at 2.66 ppm and a multiplet at 2.72 ppm (Fig. 9A). These corresponded to signals found in the spectra made using the horse liver ADH (Fig. 9B). Further analysis showed that these signals arose from the methylene protons on the carbons adjacent to the sulfur in the oxathianol. The complex splitting pattern in the multiplet arose from the diastereotopic nature of the protons imposed by the rigid structure of the oxathianol ring. Each proton is in a unique electronic environment giving each a different chemical shift. The spectra obtained from the pure compound in aqueous buffer and from the experimental enzymatic mixture after 8 min of incubation are compared in Figs. 9A and B. Comparison clearly indicates the presence of the oxathianol in the experimental sample (Fig. 9B). A doublet of doublets between 4.96 and 5.00 ppm, which showed up in the aqueous spectrum of the synthetic product and also in the experimental spectra (Figs. 10A and B), is the signal produced by the proton on the hydroxylated carbon of the oxathianol split by the protons on the methylene group adjacent to the sulfur. The presence of this signal in spectra arising both from the metabolic system and from the synthetic product in our aqueous buffer provided additional confirmation for oxathianol as an intermediate in the metabolism of

Fig. 4. Sequential spectra of TDG acted upon by equine ADH from 2 to 602 min showing the evolution of lactate via the resonance from the protons of its methyl group (1.44 ppm) and the accumulation of the methylene peak from hydroxyethylthioacetic acid (3.23 ppm) over time. There is 50 min separating each spectrum.
The presence of the hydrated aldehyde was consistent with the results of Henehan et al. (1993), who identified acetaldehyde hydrate as the actual intermediate in the metabolism of ethanol to acetic acid. That led us to test the oxathiane-2-ol spectrophotometrically to establish whether it acted as a substrate for the ADH by measuring NADH production using the optical density at 340 nm. The equine enzyme oxidized the oxathiol-related mixture, presumably via the hydrated aldehyde component, at 6 nmol NADH/min/μ.

**NMR using human isoforms**

Additionally, we tested the recombinant human α and β1 isoforms of ADH using the NMR system to test their mechanistic similarity to the horse liver enzyme. TDG seemed to be a poor substrate for human β1/β1. There was little evidence of peaks attributable to either the aldehyde or the acid even after more than 6 h of incubation. This was consistent with results from our spectrophotometric kinetic studies where the β1/β1 ADH had the lowest activity toward TDG among the human class I isoforms capable of using TDG as a substrate (Dudley et al., 2000).

When human-α ADH was used in place of the equine enzyme, the singlet at approximately 3.2 ppm, produced by the protons on the methylene group between the sulfur and the carboxyl group of 2-hydroxyethylthioacetic acid, was observed to form over time (Fig. 11). However, the minor peaks observed with the equine ADH (Fig. 7) were not observed at any time with the human α. The only product detected was the 2-hydroxyethylthioacetic acid, suggesting that the human

TDG by ADH. A set of doublets centered around 2.83 ppm in the spectrum of the synthetic oxathialol in buffer (Fig. 8D) arises from the methylene protons on the carbon adjacent to the ring oxygen. In the experimental enzymatic spectra, these signals are obscured by a large peak related to TDG.

NMR analysis of the synthetic product in deuterchloroform done in conjunction with synthesis (see Materials and methods) indicated only the presence of the oxathialol. In the aqueous medium used for the metabolic spectra, however, the synthetic oxathialol (Fig. 8) exhibited signals not found in the deuterchloroform spectrum. A minor signal at 9.47 ppm (Fig. 8A), observed after expanding the vertical scale, provided evidence of the free acyclic aldehyde. Triplets at 5.15 ppm (Fig. 8B), 3.73 ppm (Fig. 8C) and 2.87 ppm (Fig. 8D) and a doublet centered around 2.81 ppm (Fig. 8D) provided chemical shifts and splitting patterns consistent with the presence of an acyclic aldehyde hydrate.

This conclusion was supported by COSY analysis (data not shown). It appeared that the oxathialol formed an equilibrium mixture with the aldehyde and its hydrate in aqueous medium at pH 7.5.

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Fig. 5. A kinetic plot generated from sequential spectra based on peak integration illustrates the stoichiometric relationship between the pyruvate to lactate and TDG to TDGA conversions. The actual starting concentration of pyruvate + its hydrate was 100 mM. Approximately 9% of the pyruvate was hydrated so the starting concentration of the keto form was 91 mM. The actual starting concentration of TDG was 10 mM. However, since it is a symmetrical molecule the molarity of each methylene group was actually 20 mM. Since only one hydroxyl group is oxidized per molecule, the molarity of the methylene groups drops from 20 mM to 10 mM.

Fig. 6. The spectra of the pure compounds used to illustrate the basis for concluding the peak at 3.27 ppm represented the methylene protons on the carbon adjacent to sulfur in hydroxyethylthioacetic acid. (A) TDG (Fig. 1B); (B) thiodiglycolic acid (Fig. 1G); (C) 2-hydroxyethylthioacetic acid (Fig. 1F). The conditions of solvent and pH used were as outlined in Materials and methods under Enzymology.
enzyme, while processing TDG via a pathway similar to that of the horse liver enzyme, may handle the oxathialon with greater facility. When the activities of the equine enzyme and human-α ADH were compared spectrophotometrically under identical conditions (Materials and methods), the human-α ADH metabolized the equilibrium mixture at the rate of 716 nmol NADH/min/U of enzyme. This can be compared with the much lower rate using horse liver enzyme (above), 6 nmol NADH/min/U of enzyme. As with the equine ADH, the use of 2-hydroxyethylthioacetic acid as a potential substrate in the assays using the human α isoform indicated no activity, suggesting that it is the terminal metabolite with the human α enzyme as well.

**Discussion**

ADH is so inextricably linked with human dietary ethanol that the concept of its action as part of a xenobiotic metabolizing system does not immediately leap to mind. Mammalian ADHs exhibit broad substrate specificity oxidizing most primary and secondary aliphatic and aromatic alcohols. They function as detoxifiers with the digitalis glycosides (Frey and Vallee, 1979) and permethrin (Choi et al., 2002), for example. They can also act as metabolic activators as in the case of 1,4-dihydoxybutane (Besseman and McCabe, 1972), allyl alcohol (Serafini-Cessi, 1972) and 1,3-difluoro-2-propanol, the rodenticide Gliflor (Menon et al., 2001). It was the metabolic activator function that was of interest to us vis-à-vis TDG, protein phosphatases and their participation in the toxicity sulfur mustard.

The facility with which ADH oxidized TDG (Brimfield et al., 1998), the older literature on ethanol metabolism involving aldehyde dehydrogenase (Dalziel and Dickerson, 1965) and speculation from the literature on in vivo TDG metabolism (Black et al., 1993), raised our expectation for the production of a reactive aldehyde intermediate. However, ADH not only oxidizes alcohols to aldehydes but is also capable of oxidizing aldehydes to the corresponding carboxylic acids with comparable efficiency absent any involvement from other enzymes such as aldehyde dehydrogenase (Oppenheimer and Henehan, 1995; Velonia and Smonou, 2000). This is made possible because in aqueous medium most aliphatic aldehydes exist in equilibrium with their hydrates, structural analogs of secondary alcohols, which are also substrates for ADH (Bell and Evans, 1966).

The process can take two pathways depending on the relative affinities of the aldehyde and NADH for the enzyme active site. With longer chain alcohols, such as octanol, the affinity of the active site for the aldehyde product is increased (Oppenheimer and Henehan, 1995; Hinson and Neal, 1975). In this sequence, the aldehyde remains in the active site and is hydrated in situ, setting the stage for oxidation of the hydrated aldehyde to the carboxylic acid. Very little of the aldehyde exists free in solution under these circumstances. Oppenheimer and Henehan (1995) referred to this process as didehydrogenation. Sequential oxidation and didehydrogenation probably represent the extremes in a continuum based on substrate structure.

Identification of the oxathialon in equilibrium with the free aldehyde of TDG and its hydrate at very low levels in our NMR reaction mixture plus the spectrophotometric demonstration that the NADH is formed in the presence of ADH, NAD and the equilibrium mixture provides evidence that this mixture is further oxidized by ADH. The demonstration that 2-hydroxyethylthioacetic acid is not further metabolized allows us to propose the metabolic scheme shown in Fig. 12. Mechanistically TDG falls toward the didehydrogenation end of Oppenheimer and Henehan’s (1995) sequential oxidation–didehydrogenation continuum with both equine liver ADH and the human α enzyme since the free aldehyde and its equilibrium partners are either absent (human α) or present at very low levels (horse liver ADH). The initial step in which TDG (Fig. 12A) is oxidized to its aldehyde (Fig. 12B) is reversible. However, with the NAD being
Fig. 8. The NMR spectrum produced by the synthetic oxathianol under the conditions of solvent and pH used to generate the spectra with the horse liver enzyme (see Enzymology in Materials and methods for details). When the vertical axis was expanded, the minor signal at 9.47 ppm (inset A) produced by the aldehydic proton of the free aldehyde became evident. Additional minor signals at 5.15 ppm (inset B) and 3.73 ppm (inset C), a triplet at 2.87 ppm and a doublet of doublets centered around 2.81 ppm (inset D) were consistent with the presence of the acyclic aldehyde hydrate. This indicated that the oxathianol developed into a mixture containing the cyclic compound, the free aldehyde and the hydrated aldehyde when dissolved in buffer.

Fig. 9. A comparison of the experimental spectrum with the spectrum generated with the synthetic oxathianol in our experimental buffer provided strong evidence for the oxathianol as a metabolic intermediate. Signals between 2.63 and 2.76 ppm produced by methylene protons on the carbons adjacent to the sulfur of the synthetic oxathianol dissolved in our experimental buffer (A) compared with the actual experimental result produced during the metabolism of TDG by the horse liver enzyme (B). It is evident that these two spectra are identical with the exception of the satellite peaks (arrows) in the experimental spectrum (B) arising from TDG due to coupling with protons on naturally occurring $^{13}$C species.

Fig. 10. The signal at 4.98 ppm produced by the proton on the anomeric (hydroxylated) carbon of the synthetic oxathianol interacting with the adjacent methylene protons frozen in axial and equitorial positions by the rigid structure of the ring (A) compared to the actual experimental result produced during the metabolism of TDG by the equine ADH (B). Again, the similarity provides strong evidence for the oxathianol as an intermediate during the action of equine liver ADH on TDG.

The aldehyde exists in equilibrium with its hydrate (Fig. 12C) and the oxathianol (Fig. 12D). The dominant species in the mixture depends on the medium. NMR on the oxathianol dissolved in chloroform during the monitoring of synthesis showed only signals for the cyclic compound. When the solvent was our potassium phosphate buffer, pH 7.5, however, signals rapidly regenerated by lactate dehydrogenase, there is insufficient build-up in NADH concentration to drive the reverse reaction in our system. Oppenheimer and Henchman (1995) showed this by following the NAD recycling on a UV spectrophotometer in parallel with the NMR and observing no changes in the $A_{340}$ during the course of the reaction.
The oxathianol, presumably after ring opening and hydration (Oppenheimer and Henehan, 1995), was a substrate for both the horse liver ADH and the human α ADH identifying it as the precursor for hydroxyethylthioacetic acid. However, the oxathianol-2-ol also has the characteristics of a secondary alcohol and could be the intermediate giving rise to the acid.

The fact that only one end of the TDG is oxidized is evident from the stoichiometry of the pyruvate to lactate conversion by the NADH recycling system. Two moles of pyruvate were converted to lactate for every mole of TDG oxidized to the acid (Fig. 5). This ratio conforms to what has been seen with other symmetric diols such as the conversion of 1,4-butanediol by ADH to 4-hydroxybutyrate (γ-hydroxybutyrate, GHB) the date rape drug (Besseman and McCabe, 1972). This consistent asymmetric attack by ADH brings into question the origin of the reported metabolite of sulfur mustard, thiodiglycolic acid (Fig. 1G), in which both ends of the molecule have been oxidized (Davison et al., 1961).

Also remarkable are the comparative rates of conversion of the oxathianol equilibrium mixture to the acid by the horse liver enzyme versus the class I human α isoform. The two enzymes, with their activity equalized toward ethanol, showed a very different reactivity toward the synthetic oxathianol mixture when the reaction was followed by monitoring NADH generation. The rate of NADH production by the human enzyme was over a hundred times that found with the horse liver ADH. This no doubt explains the lack of signals from any of the aldehyde-related intermediates in the NMR spectra made using the human α isoform. Such a large difference between isoforms and among species in the rate of oxidation for this or other aldehyde equilibrium mixtures may also explain species differences in the toxicity of alcohols.

The ultimate metabolite, TDGA (Fig. 1F), is not inhibitory to pure protein (serine/threonine) phosphatases (Brimfield, unpublished results). Testing the activity of the intermediate equilibrium mixture for its ability to inhibit pure protein (serine/threonine) phosphatases awaits the synthesis of more 1,4-oxathiane-2-ol. In the broader sense, the work has implications for the hydrate and the free aldehyde appeared (Fig. 8). This spectrum did not change over the course of 21 h indicating rapid development of an equilibrium. There was evidence of the oxathianol in the experimental spectra (Figs. 9 and 10). The NMR signals indicating the presence of the hydrated aldehyde in the spectra from the original enzyme mixture are obscured by larger unrelated signals with the exception of a very small peak at 4.37 ppm (Fig. 4). We never observed a signal in any of the experimental spectra for the free aldehyde proton that appeared at 9.47 ppm (Fig. 8) in the spectrum of the synthetic product in buffer. This provided evidence of the low level at which the free aldehyde was present and reinforced the didehydration mechanism.

To test the theory that hydroxyethylthioacetic acid (Fig. 12E) evolved from the hydrated aldehyde (Henehan and Oppenheimer, 1993), we tested the oxathianol-2-ol equilibrium mixture spectrophotometrically for its ability to act as a substrate for ADH. The oxathianol, presumably after ring opening and hydration (Oppenheimer and Henehan, 1995), was a substrate for both the horse liver ADH and the human α ADH identifying it as the precursor for hydroxyethylthioacetic acid. However, the oxathianol-2-ol also has the characteristics of a secondary alcohol and could be the intermediate giving rise to the acid.

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The ultimate metabolite, TDGA (Fig. 1F), is not inhibitory to pure protein (serine/threonine) phosphatases (Brimfield, unpublished results). Testing the activity of the intermediate equilibrium mixture for its ability to inhibit pure protein (serine/threonine) phosphatases awaits the synthesis of more 1,4-oxathiane-2-ol. In the broader sense, the work has implications

![Fig. 12. The proposed generalized metabolic pathway for the conversion of TDG to TDGA by alcohol dehydrogenase](image-url)
for sulfur mustard pharmacokinetics because it identifies previously unknown metabolites and describes how they arise. It sheds light on the mechanism of alcohol dehydrogenase when it is confronted with symmetrical diols and contributes to our knowledge about alcohol dehydrogenases as xenobiotic metabolizing enzymes. Additionally, we introduce a novel and very useful way to study toxicant metabolism in vitro.

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