Ethylenethiourea S-Oxidation Products: Preparation, Degradation, and Reaction with Proteins

Joyce P. James, Gary B. Quistad, and John E. Casida*

Environmental Chemistry and Toxicology Laboratory, Department of Environmental Science, Policy, and Management, University of California, Berkeley, California 94720-3112

Ethylenethiourea (ETU) is a carcinogenic degradation product of major ethylene bis(dithiocarbamate) fungicides with biological activity attributed to poorly characterized oxidation products. The reaction of ETU with H_2O_2 was examined in aqueous medium at pH 5, 7, and 9 by ^1H NMR spectroscopy giving five principal products: sequential formation of sulfenic, sulfinic, and sulfonic acids as well as imidazoline and ethyleneurea. Maximum yields with 2 equiv of H_2O_2 at optimal pH were 10, 71, 5, 53, and 100%, respectively. Oxidation proceeds mainly through the sulfinic acid to imidazoline in acidic medium and the S-oxide to ethyleneurea in basic medium. ^1H NMR of urine from mice treated with ETU revealed ETU and imidazoline (no ethyleneurea or S-oxidation products). Albumin was radiolabeled in ~17% yield by a 10-fold excess of [^{14}C]ETU/H_2O_2 oxidation products (primarily the sulfenic and sulfinic acids), and such protein modification could be prevented by glutathione.

Keywords: *Ethylenethiourea; ethylenethiourea S-oxidation products; hydrogen peroxide; imidazoline; protein adducts; sulfenic acid*

INTRODUCTION

Ethylenethiourea (2-imidazolidinethione or its tautomer 2-imidazolin-2-yl thiol) (ETU or Im-SH) is a degradation product and metabolite of major ethylene bis(dithiocarbamate) fungicides (mancozeb, maneb, metiram, nabam, and zineb) (about 8–12 million pounds used per year) (U.S. Environmental Protection Agency, 1992; Dearfield, 1994). It is also an accelerator in synthetic rubber production. ETU is of special interest and concern because of potential human exposure and adverse health effects. It is an inhibitor of cytochrome P_{450} oxidases (Decker and Doerge, 1991), a genotoxic agent (Dearfield, 1994), a teratogen in rats (Iverson *et al.*, 1980), and a liver and thyroid carcinogen in mice and/or rats (Innes *et al.*, 1969; Ulland *et al.*, 1972; Chhabra *et al.*, 1992). The cancer risk of ETU for man led to a proposal to ban many of the uses of ethylene bis(dithiocarbamate) fungicides (U.S. Environmental Protection Agency, 1987, 1992).

Chemical oxidation of ETU has been studied in potential biomimetic systems with emphasis on hydrogen peroxide (H_2O_2) and hypochlorite (NaOCl); the balance of products is dependent on both the oxidant and the pH (Figure 1). Some of the diverse biological activities of ETU are attributed to biooxidation by cytochrome P_{450} and flavin-containing monooxygenase-linked microsomal oxidases with 2-imidazolin-2-ylsulfenic and -sulfinic acids (Im-SOH and Im-SO_2H, respectively) as the proposed initial reactive intermediates (Poulsen *et al.*, 1979; Decker and Doerge, 1991) (Figure 2). Im-SOH and Im-SO_2H have not been isolated pure, and there is confusion on their NMR assignments and characterization (Marshall and Singh, 1977; Marshall, 1979; Poulsen *et al.*, 1979; Savolainen and Pyysalo, 1979) and the sequence by which they are converted to ethyleneurea (2-imidazolidinone) (EU), 2-imidazoline (Im); and other products (Figure 1). This might be resolved by nuclear magnetic resonance (NMR) spectroscopy studies following the course of the oxidation reaction of ETU with H_2O_2 in aqueous medium. These biomimetic oxidations are considered here over a pH range of 5–9 along with the ability of the oxidized intermediates to covalently derivatize proteins.

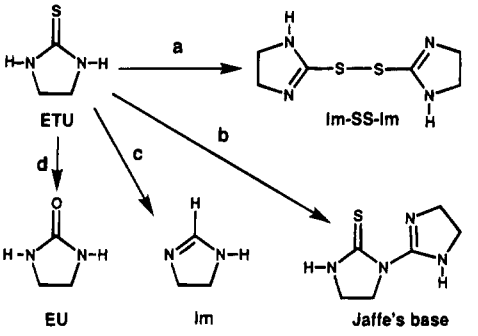

Figure 1. Oxidation of ETU: (*a*) H_2O_2/methanol/HCl (Freedman and Corwin, 1949); (*b*) *N*-chlorosuccinimide/ethanol (Marshall, 1979), NaOCl (Marshall and Singh, 1977) or alcoholic hydrobromous or hydrochlorous acid (Johnson and Edens, 1941); (*c*) H_2O_2 (Marshall, 1979); (*d*) H_2O_2/OH^- or $NaOCl/OH^-$ (Marshall and Singh, 1977; Marshall, 1979).

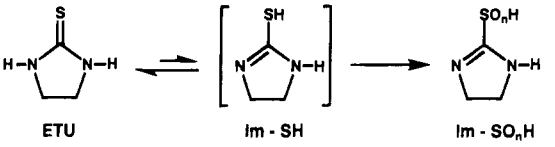

Figure 2. Oxidation of ETU to the corresponding sulfenic (n = 1), sulfinic (n = 2), and sulfonic (n = 3) acids. The sulfenic and sulfinic acids are transitory, and the sulfonic acid is a terminal product at pH 5 or lower.

MATERIALS AND METHODS

Spectroscopy and Chromatography. ^1H and ^{13}C NMR spectra obtained at 300 and 75 MHz, respectively, on a Bruker AM 300 MHz spectrometer at room temperature were referenced to 3-(trimethylsilyl)propanesulfonic acid sodium salt (TSPA) (δ = 0 ppm) in D_2O/H_2O or trace levels of CHD_2OD in CD_3OD (δ = 3.30 ppm). All chemical shifts are given in parts per million (ppm). Mass spectral data were collected on one

* Author to whom correspondence should be addressed [telephone (510) 642-5424; fax (510) 642-6497].

0021-8561/95/1443-2530$09.00/0

or more of four instruments: gas chromatography–mass spectrometry (GC–MS) [chemical ionization with CH_4, Hewlett-Packard 5890 series II GC coupled to a 5971A MS instrument with a DB-5 fused-silica column (30 m × 0.25 mm i.d.)]; direct inlet MS (electron impact, Hewlett-Packard 5985 instrument); liquid chromatography (LC)–MS [University of California at San Francisco, Department of Pharmaceutical Chemistry, VG Platform instrument using electrospray ionization with an Applied Biosystems C_{18} Aquapore column eluted with a 1:1 mixture of 0.1% formic acid and 0.05% formic acid in methanol/propanol (5:2)]; and fast atom bombardment (FAB)-MS (both low and high resolution) (University of California at Berkeley, Department of Chemistry, Fisons 70-SE and Fisons ZAB2-EQ spectrometers).

Ion exchange chromatography involved both cation and anion columns. A strongly acidic cation exchange column (2 cm × 12 cm, Dowex 50 × 8–100 resin) was prepared by washing the resin with 20 mL of 1 M NaOH, followed by 20 mL of 5 M NaCl, and finally 20 mL of 1 M HCl (Newsome and Panopio, 1978). The column was then rinsed with H_2O until the eluent was neutral. A strongly basic anion exchange column (2.5 cm × 15 cm, Dowex 1 X8–100 resin) was prepared by following the above washing procedure in reverse, finishing with the H_2O rinse. High-performance liquid chromatography (HPLC) utilized a Waters 600E system controller coupled to a Waters 994 programmable photodiode array detector monitoring at 220 nm. The Vydac C_4 protein column (0.46 cm × 5 cm, 5 μm) was eluted at 1.5 mL/min with a linear gradient of 0–40% acetonitrile over 5 min followed by 40–60% acetonitrile over 7 min in constant 0.1% trifluoroacetic acid in water.

Thin-layer chromatography (TLC) on silica gel F_{254} involved methanol/glacial acetic acid (9:1) and detection with UV and cinnamaldehyde (Marshall and Singh, 1977) or radioautography (R_f values: ETU, 0.75; EU, 0.65; Im, 0.33; and Im-SO_3H, 0.68).

Sources or Syntheses of Standards. ETU and EU from Aldrich Chemical Co. (Milwaukee, WI) were pure on the basis of ^{1}H NMR and TLC analyses as above. [4,5-^{14}C]ETU (4.9 mCi/mmol) (>99% radiochemical purity by TLC radioautography) was provided by P. C. Kearney, U.S. Department of Agriculture (Beltsville, MD).

Im was synthesized in 80–90% yield according to two methods. In the first, modified from that of Newsome and Panopio (1978), a solution of ETU (10 g) in H_2O (200 mL) was stirred on ice while excess H_2O_2 (30%, 32 g) was added dropwise. The temperature was kept below 30 °C during addition followed by 5 °C for 2 h. The second procedure was based on that of Marshall (1979), modified by using 0.25% trifluoroacetic acid as the solvent, since our findings showed the best yields were obtained under acidic conditions. ETU (40 mM) in 0.25% trifluoroacetic acid in H_2O (500 μL) was treated with H_2O_2 (2 molar equiv) at 25 °C overnight. With both methods, 2-imidazolin-2-ylsulfonic acid (Im-SO_3H) and EU were minor byproducts. For purification, the reaction mixture was subjected to cation exchange chromatography with H_2O (150 mL) and 4 M HCl (100 mL). The HCl fraction was evaporated under vacuum (Speed Vac, Savant Instruments, Inc., Farmingdale, NY) to give Im, identified by direct inlet MS and LC–MS ([MH$^+$] m/z 71), high-resolution FAB-MS (71.0607) (calculated for $C_3H_7N_2$ = 71.0609), ^{1}H NMR [δ 3.94 (4H) and 8.13 (1H)], and ^{13}C NMR (δ 46.57 and 159.82).

Im-SO_3H was the major component in the void volume and H_2O rinse fractions on cation exchange chromatography of the reaction mixture to prepare Im as above. Im-SO_3H was further purified by anion exchange chromatography, eluting with H_2O. The eluate was evaporated under vacuum and the product examined by FAB-MS ([MH$^+$] m/z 151), high-resolution FAB-MS (151.0175) (calculated for $C_3H_7N_2O_3S$ = 151.0177), ^{1}H NMR (δ 4.08), and ^{13}C NMR (δ 48.03). Oxidation of ETU with H_2O_2 in carbon tetrachloride at low temperature [method of Marshall and Singh (1977)] gave quantitatively an Im and Im-SO_3H mixture (10:1).

The disulfide of Im-SH (Im-SS-Im) [prepared according to the method of Freedman and Corwin (1949)] gave a ^{1}H NMR chemical shift in D_2O of 4.05 (referenced to EU at δ 3.52). Addition of a solution of iodine in potassium iodide to an aqueous solution of Im-SS-Im, followed by filtering and drying, gave the periodide first noted by Johnson and Edens (1942) (δ 3.75 in CD_3OD: mp 117–120 °C without purification; literature value 119 °C).

Oxidation Profiles, Rates, and Product Yields Observed by ^{1}H NMR. A solution of ETU (40 mM) was prepared in sodium phosphate buffer (90 mM) at pH 5, 7, or 9 in 90% H_2O/10% D_2O. A 500-μL sample was subjected to NMR analysis, with presaturation of the H_2O peak, and then treated with 4 μL of 30% H_2O_2 (2 molar equiv) with thorough mixing (taken as t = 0). Spectra were collected at least twice up to 5 min and then at 24-min intervals for 4 h followed by normalization and plotting of the relative peak integrations. ETU and EU were referenced to TSPA (δ 0) and subsequent spectra to ETU and EU (δ 3.71 and 3.52, respectively).

Decomposition of Oxidation Products. The stabilities of pure EU, Im, and Im-SO_3H were determined under acidic and basic conditions and in the presence of excess H_2O_2. After an initial ^{1}H NMR spectrum was taken, the pH was adjusted to ~1 with HCl or to ~14 with NaOH, alone or with addition of H_2O_2, and subsequent spectra were collected.

Oxidation of ETU with H_2O_2 in $H_2{}^{18}O$. A solution of ETU (1 mg) in $H_2{}^{18}O$ (70+% enrichment, 178 μL) buffered with sodium phosphate (1 M, 20 μL, pH 9) was treated with 30% H_2O_2 (2 μL) for 17 h. A 0.5-μL aliquot was subjected to GC–MS with monitoring of the base peak [MH$^+$] at m/z 87 and 89 for [^{16}O]EU and [^{18}O]EU, respectively, to determine the origin of the oxygen atom on EU, i.e., whether it comes from oxidant only or from solvent.

Labeling of Proteins by [^{14}C]ETU Oxidation Products. Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) (50 μg) in 100 mM sodium phosphate (pH 7.4) buffer (55 μL final volume) was treated with various molar equivalents of [^{14}C]ETU, H_2O_2, and/or reduced glutathione (GSH). The initial concentration of [^{14}C]ETU was determined by liquid scintillation counting; 30% H_2O_2 was diluted in H_2O as appropriate just before use, and all reagents were kept on ice until combined for incubation. After 30 min at 25 °C, the samples were quickly frozen in an ethanol/dry ice bath and stored at −4 °C until analyzed by HPLC. The BSA peak eluted in ~1.9 mL total volume at ~53% acetonitrile and was quantified either as the whole peak or as 1-min fractions by liquid scintillation counting. The derivatized BSA was considered to be that portion of the ^{14}C coeluting exactly with the protein peak. Background for samples without protein was subtracted from all determinations, and the results were expressed on a percentage basis for molar equiv of ETU bound relative to BSA.

Other proteins examined were dog and human hemoglobin, human GSH S-transferase, and chymotrypsin from Sigma and cytosol from rat and mouse liver (without altering the endogenous level of GSH). Each protein (50 μg) was treated with [^{14}C]ETU and H_2O_2 in the same amounts as in the BSA experiments in which 10 molar equiv of H_2O_2 and ETU were used. The incubations and analyses were as with BSA, with 1-min fractions collected, except the blank samples of [^{14}C]-ETU without H_2O_2 were subtracted.

Metabolites Observed by ^{1}H NMR of Urine from Mice. Male albino mice (~20 g each) were treated by the intraperitoneal or oral route with ETU (100 mg/kg) as an aqueous solution. The urine collected during the period of 0–24 h posttreatment was directly examined (without concentration) by ^{1}H NMR with TSPA as the reference and spectral acquisition times of 5–30 min at room temperature. The urine from ETU-treated mice was compared to that from control mice (administered water only), and the metabolites were identified by chemical shift and (in the case of ETU and EU) by spiking with standard compounds.

RESULTS

Identification of ETU Oxidation Products. Several products from the standard oxidation reactions at pH 5, 7, or 9 were identified by comparison with authentic standards. EU was obtained from ETU and

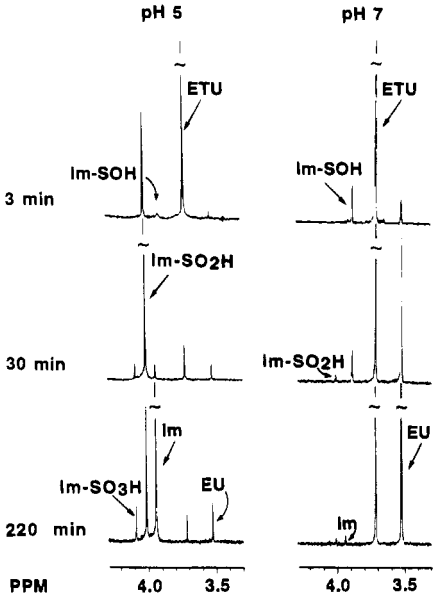

Figure 3. Oxidation profile of ETU with 2 molar equiv of H_2O_2 observed by ^1H NMR. Conditions: 40 mM ETU at pH 5, 7, or 9 in 90 mM phosphate (in 90% H_2O/10% D_2O) incubated for 3, 30, or 220 min at 25 °C. Chemical shifts, for methylene protons only, are as follows: ETU, 3.71 ppm; Im-SOH, 3.89 ppm; Im-SO_2, 4.01 ppm; Im-SO_3H, 4.08 ppm; Im, 3.94 ppm; and EU, 3.52 ppm.

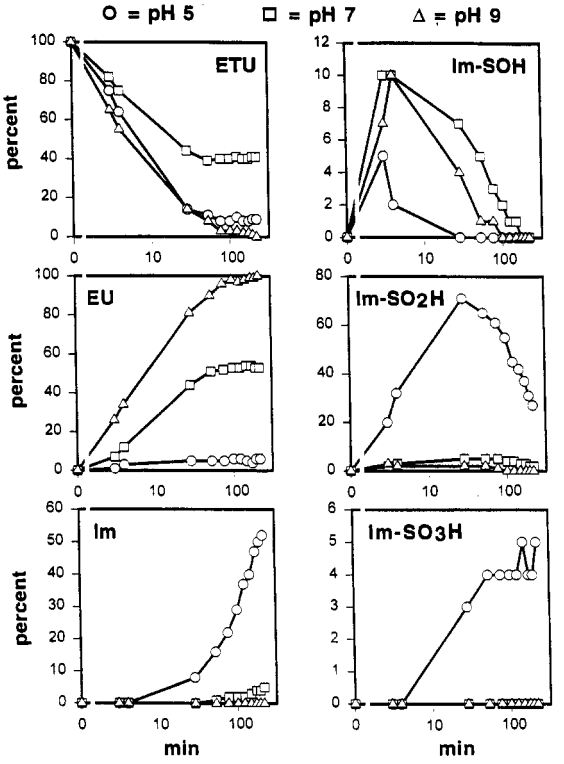

Figure 4. Oxidation rates and product yields for reaction of ETU with 2 molar equiv of H_2O_2. Quantitation is based on integration of methylene signals from the ^1H NMR spectra and is the average of two experiments. The maximum yields in each experiment in sequence were as follows: Im-SOH, 12, 9%; Im-SO_2H, 67, 75%; Im-SO_3H, 4, 7%; Im, 53, 52%; EU, 99, 100%.

H_2O_2 in 53 and 100% yields at pH 7 and 9, respectively. It was identical to an authentic standard by both ^1H NMR (spiked sample) and GC–MS of the pH 7 preparation ([MH^+] *m/z* 87). Im obtained with H_2O_2 in the pH 5 solution was identical in ^1H NMR (spiked sample) to Im purified and dried from the H_2O_2/trifluoroacetic acid reaction [^1H NMR δ 8.13 (s) and 3.94 (s) in a 1:4 ratio (by integration) in 90% H_2O/10% D_2O]. The olefinic proton of Im exchanges with deuterium, as noted by ^1H NMR in D_2O for both the authentic standard and for Im formed from ETU and H_2O_2 at pH 5. In addition, the olefinic proton (standard compound) is canceled in the attached-proton-test ^{13}C spectrum (δ 159.82). Identification of Im was further verified by LC–MS, direct inlet MS, and FAB-MS, each of which gave [MH^+] *m/z* 71. Im-SO_3H in the reaction mixture was identical to the isolated synthetic standard in ^1H NMR (spiked sample) (δ 4.08).

The compounds designated Im-SOH and Im-SO_2H could be optimized for yield (see below) but not stabilized for isolation. Although Im-SOH was most stable at neutral pH, it disproportionates or continues to react with H_2O_2 (even in limiting amount) to form Im-SO_2H. Attempts to separate and identify Im-SOH and Im-SO_2H by GC–MS were not successful when applied both directly to reaction mixtures and following candidate silylation and methylation procedures. The assignments of Im-SOH and Im-SO_2H are therefore based on the sequence of product formation and loss from NMR analysis and on the terminal products they generate as described below.

Im-SS-Im (available as a standard) and Jaffe's base [^1H NMR δ 3.35 (t, 2H), 3.62 (t, 2H), 3.8 (s, 4H); Marshall, 1979] (Figure 1) were not observed by ^1H NMR in the present study.

Oxidation Profiles, Rates, and Product Yields for Reaction of ETU with 2 Molar Equiv of H_2O_2 Observed by ^1H NMR (Figures 3 and 4). The reaction of ETU with 2 molar equiv of H_2O_2 yields five principal products in a ratio dependent on the pH. Although not illustrated, the rate of reaction increases with the oxidant ratio in the range of 0.5–4 molar equiv, but even with 0.5 molar equiv of H_2O_2 the full complement of pH-dependent products is observed with partial consumption of ETU.

The initial rate of reaction of ETU (normalized for that portion that reacts) with 2 molar equiv of H_2O_2 is affected little if any by the pH in the range of 5–9. However, the product ratio is greatly dependent on the pH. ETU reacts completely in about 100 min at pH 5 or 9, whereas about 40% remains unreacted at pH 7. The first product is Im-SOH, reaching a peak of 9–12% at pH 7 and 9 and 5% at pH 5 in 2–5 min. Continuing reaction yields Im-SO_2H, reaching 67–75% yield at pH 5 in 30 min but without buildup at pH 7 or 9. Im-SO_3H does not exceed 4–7% yield after 60 min at pH 5, and it is not observed at pH 7 or 9. EU is the first terminal product observed and accounts for essentially all of the reacting ETU at pH 7 and 9. In contrast, at pH 5 there is major conversion to Im between 30 and 240 min.

Decomposition of Oxidation Products. EU, Im, and Im-SO_3H are all unaffected by a large excess of H_2O_2, and these compounds are stable in strong acid (pH ~1).

Im-SO_3H serves as an intermediate for EU and the compound assigned as *N*-formylethylenediamine (Marshall, 1979). Essentially all of the Im-SO_3H (in a mixture with Im and EU but not H_2O_2) decomposes in D_2O (no buffer) at 25 °C for 8 days or on concentration under vacuum at 40 °C to near dryness. Of the Im-SO_3H in the original reaction mixture (80%), 63% is converted to *N*-formylethylenediamine (δ 3.63 and 3.24 in D_2O) and 36% to EU after 8 days.

In strong base (pH ~14), ETU and EU are stable, Im-SO_3H is converted to EU, and Im is completely con-

Table 1. Labeling of Bovine Serum Albumin by [^{14}C]ETU Oxidation Products

molar equiv			covalent labeling[a]	
ETU	H_2O_2	GSH	n	% ± SE[b]
1	1	0	3	0
1	3	0	3	0
1	10	0	3	0
10	10	0	5	17 ± 3
10	30	0	6	24 ± 4
10	100	0	6	10 ± 2
10	10	3	6	17 ± 2
10	10	10	6	14 ± 4
10	10	30	6	0.5 ± 0.2

[a] Molar equivalent labeling for 50 μg of BSA expressed on a percentage basis. [b] SE, standard error.

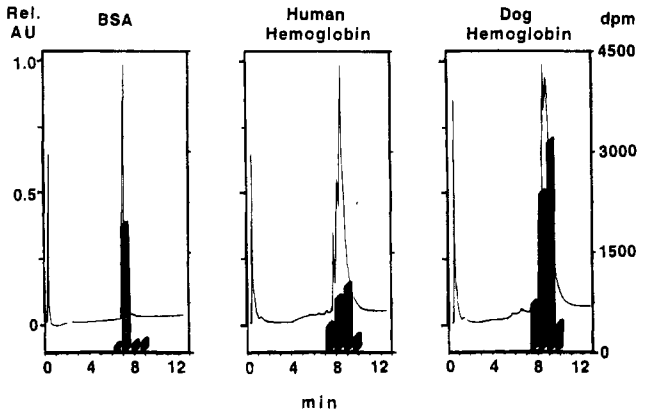

Figure 5. Labeling of proteins by [^{14}C]ETU oxidation products. Conditions: BSA (50 μg) with 10 molar equiv each of [^{14}C]-ETU and H_2O_2 in pH 7.4 100 mM phosphate incubated for 30 min at 25 °C. Human and dog hemoglobin (50 μg) were used in place of BSA without changing the [^{14}C]ETU and H_2O_2 amounts. The number 1.0 on the relative absorbance (Rel. AU) scale is 1.4 for BSA, 0.36 for human hemoglobin, and 0.26 for dog hemoglobin.

verted to *N*-formylethylenediamine (methylenes δ 3.32 and 2.75, formamide δ 8.1, high pH) (based on NMR) and finally to ethylenediamine (methylenes δ 2.65) (spiked sample) and formate (δ 8.45) (based on chemical shift).

Oxidation of ETU with H_2O_2 in $H_2{}^{18}O$. The reaction of ETU with 2 molar equiv of H_2O_2 at pH 9 and in 66+% $H_2{}^{18}O$ gave [^{16}O]EU and [^{18}O]EU in a 97:3 ratio. Hence, the oxygen in EU arises predominantly from H_2O_2 rather than solvent.

Labeling of Proteins by [^{14}C]ETU Oxidation Products (Table 1 and Figure 5). Addition to equimolar BSA and [^{14}C]ETU of 1−10 molar equiv of H_2O_2 does not lead to labeling of BSA. With 10 molar equiv of [^{14}C]-ETU and 10−100 molar equiv of H_2O_2 there is 10−24% labeling of BSA without a major difference with the amount of H_2O_2. Addition of GSH does not affect the labeling of BSA at 10 molar equiv each of [^{14}C]ETU and H_2O_2 until the GSH level exceeds that of the other reactants, *i.e.* 30 molar equiv, at which point the extent of labeling is insignificant.

The labeling of BSA with 10 molar equiv of each of [^{14}C]ETU and H_2O_2 was compared with that of the same mass of other proteins under the same conditions. The extent of labeling on a molar equivalent basis was greater for dog hemoglobin (44%) than for BSA (17%) or human hemoglobin (12%). No significant labeling (<2 times background) was detected for human GSH *S*-transferase, chymotrypsin, and rat or mouse liver cytosolic proteins (although no attempt was made to remove GSH from the cytosol in these experiments).

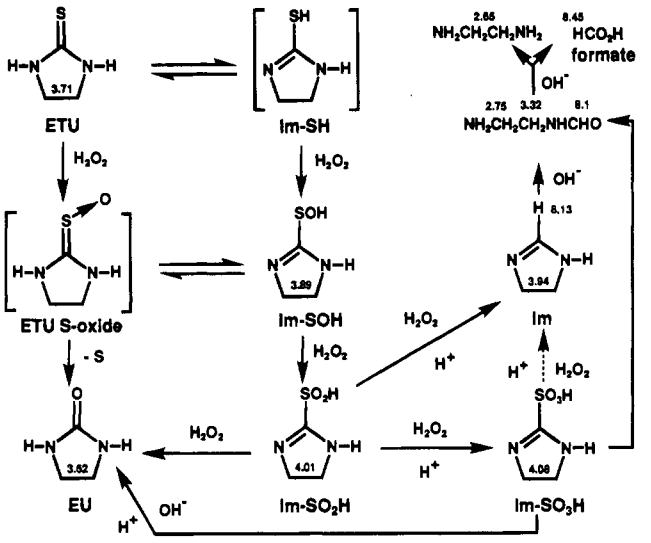

Figure 6. Pathways for oxidation of ETU by H_2O_2 and for decomposition of the oxidation products in acid or base. Chemical shifts are given in H_2O in all cases except for *N*-formylethylenediamine, ethylenediamine, and formate in basic solution. The proton assignments are shown on the Im-SOH and Im-SO_2H structures even though these compounds may exist as the *S*-oxide and *S*,*S*-dioxide tautomers, respectively. The pathway via ETU *S*-oxide to EU predominates at pH 9 and that via Im-SO_2H to Im at pH 5.

Metabolites Observed by ^{1}H NMR of Urine from Mice. ETU and Im were readily detected in the 0−24-h urine in a ratio of 1.0 to 0.3−0.6, respectively, based on ^{1}H NMR integration of the methylene signals. The findings were the same with both intraperitoneal and oral treatments. Products not detected (0.00 to <0.05 relative to ETU as 1.0) were EU, Im-SOH, and Im-SO_2H. The possible presence of Im-SO_3H could not be determined because of an interfering urinary component evident from the control sample.

DISCUSSION

ETU is easily oxidized by H_2O_2 at physiological pH and temperature to give a series of intermediates and terminal products (Figure 6) which might contribute to its biological activity. The sequence of appearance and disappearance of ^{1}H NMR signals in its reaction with H_2O_2 clearly establishes an oxidation sequence of ETU → Im-SOH → Im-SO_2H → Im-SO_3H and clarifies the relationships of other products formed when the oxidation is carried out under acidic and basic conditions.

The reactions of greatest interest are sequential oxidation of ETU to Im-SOH, Im-SO_2H, and Im-SO_3H. It is notable that Im-SOH is directly observed by NMR and appears to have a half-life at pH 7 and 25 °C of ~60 min on the basis of the fact that its rate of loss after formation from ETU is essentially complete. Im-SOH is less persistent at pH 5 or 9, as expected for a sulfenic acid species (Poulsen *et al.*, 1979; Skellern, 1989), due to either decomposition or further oxidation. However, Savolainen and Pyysalo (1979) report that Im-SOH formed by γ irradiation of an ETU solution is sufficiently stable for isolation and spectral characterization. Im-SOH, Im-SO_2H, and Im-SO_3H are previously described as oxidation products of ETU by Poulsen *et al.* (1979) on the basis of HPLC. Although Marshall and Singh (1977) and Marshall (1979) provide ^{1}H NMR evidence for Im-SO_2H and Im-SO_3H, their studies do not state the NMR chemical shift reference and their assignments for Im-SO_2H (and Im as well) are ~0.07

ppm downfield of the current assignments (referenced to TSPA). Im-SO_2H and Im-SO_3H are stabilized at low pH, reaching significant concentrations. On addition of excess H_2O_2 at low pH Im-SO_2H is oxidized to Im and Im-SO_3H, further supporting its assignment as the sulfinic acid.

The terminal products of ETU oxidation by H_2O_2 are dependent on the pH, which determines the amounts, stabilities, and decomposition pathways for the intermediates Im-SOH and Im-SO_2H (Figure 6). Im-SO_3H is not a major product and is only significant at pH 5, at which it has moderate stability. The principal terminal product is Im at low pH and EU at high pH. Under optimal conditions for formation of EU from ETU (pH 9) the reaction with H_2O_2 probably proceeds through the intermediacy of ETU *S*-oxide with loss of sulfur on rearrangement rather than via solvolysis. The low yield of [^{18}O]EU (*i.e.* ~5%) for the reaction in $H_2{}^{18}O$ may involve alternate pathways such as hydrolysis of Im-SO_2H or Im-SO_3H. Two compounds previously reported as products from ETU oxidation with H_2O_2 (Marshall and Singh, 1977), but not observed here, are Im-SS-Im and Jaffe's base; in the earlier study they may have been artifacts formed during isolation for analysis, which was not the case in the present investigation as it utilized direct NMR monitoring. The observed conversion of Im in base to *N*-formylethylenediamine, ethylenediamine, and formate confirms the report of Marshall (1979).

ETU in mammals is partially metabolized to EU and Im (Jordan and Neal, 1979; Iverson *et al.*, 1980) probably by oxidative pathways consistent with those found in the H_2O_2 biomimetic system (Figure 6). Im-SOH is proposed as a major urinary metabolite of ETU in mice (Savolainen and Pyysalo, 1979), which is surprising in light of its apparent instability. The present study confirms Im as a urinary metabolite of ETU in mice but does not reveal any EU, Im-SOH, or Im-SO_2H based on direct ^{1}H NMR examination of the urine.

Oxidation of [^{14}C]ETU by cytochrome P_{450}s and flavin-containing monooxygenases leads to labeling of microsomal protein(s) possibly via Im-SOH (Decker and Doerge, 1991). Comparably, Im-SOH and/or its further oxidation products react with BSA and hemoglobins to form covalent derivatives (probably Im-SS-protein). This process is defused by GSH presumably by reacting directly with Im-SOH to give first Im-SS-G and then ETU plus oxidized GSH (Poulsen *et al.*, 1979; Decker and Doerge, 1991). The reactive intermediate(s) leading to protein derivatization is (are) most likely to be Im-SOH (or Im-SO_2H). ETU is oxidized by thyroid peroxidase/H_2O_2 in the presence of iodide ion to Im and trace EU probably via Im-SO_2H (Doerge and Takazawa, 1990). ETU undergoes facile oxidation at physiological pH, and the initial oxidation products are in themselves reactive intermediates consistent with the diverse types of biological activity of this fungicide metabolite and degradation product.

ABBREVIATIONS USED

BSA, bovine serum albumin; ETU, ethylenethiourea or 2-imidazolidinethione; EU, ethyleneurea or 2-imidazolidinone; FAB, fast atom bombardment; GC, gas chromatography; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; Im, 2-imidazoline; Im-SH, 2-imidazolin-2-yl thiol; Im-SOH, 2-imidazolin-2-ylsulfenic acid; Im-SO_2H, 2-imidazolin-2-ylsulfinic acid; Im-SO_3H, 2-imidazolin-2-ylsulfonic acid; Im-SS-Im, disulfide of Im-SH; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; TLC, thin-layer chromatography; TSPA, 3-(trimethylsilyl)-propanesulfonic acid sodium salt.

ACKNOWLEDGMENT

We thank our laboratory colleagues Susan Sparks for assistance with the biological studies and Qing Li for helpful discussions and advice. LC/MS data were kindly provided by David Maltby of the University of California San Francisco Mass Spectrometry Facility (A. L. Burlingame, Director) supported by the Biomedical Research Technology Program of the Center for Research Resources, NIH, NCRR BRTP RR 01614. FAB-MS spectra were run by Zhongrui Zhou in the MS facility in the Department of Chemistry at the University of California at Berkeley.

LITERATURE CITED

Chhabra, R. S.; Eustis, S.; Haseman, J. K.; Kurtz, P. J.; Carlton, B. D. Comparative carcinogenicity of ethylene thiourea with or without perinatal exposure in rats and mice. *Fundam. Appl. Toxicol.* **1992**, *18*, 405–417.

Dearfield, K. L. Ethylene thiourea (ETU). A review of the genetic toxicity studies. *Mutat. Res.* **1994**, *317*, 111–132.

Decker, C. J.; Doerge, D. R. Rat hepatic microsomal metabolism of ethylenethiourea. Contributions of the flavin-containing monooxygenase and cytochrome P-450 isozymes. *Chem. Res. Toxicol.* **1991**, *4*, 482–489.

Doerge, D. R.; Takazawa, R. S. Mechanism of thyroid peroxidase inhibition by ethylenethiourea. *Chem. Res. Toxicol.* **1990**, *3*, 98–101.

Freedman, L. D.; Corwin, A. H. Oxidation-reduction potentials of thiol-disulfide systems. *J. Biol. Chem.* **1949**, *181*, 601–621.

Innes, J. R. M.; Ulland, B. M.; Valerio, M. G.; Petrucelli, L.; Fishbein, L.; Hart, E. R.; Pallotta, A. J.; Bates, R. R.; Falk, H. L.; Gart, J. J.; Klein, M.; Mitchell, I.; Peters, J. Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: a preliminary note. *J. Natl. Cancer Inst.* **1969**, *42*, 1101–1114.

Iverson, F.; Khera, K. S.; Hierlihy, S. L. *In vivo* and *in vitro* metabolism of ethylenethiourea in the rat and the cat. *Toxicol. Appl. Pharmacol.* **1980**, *52*, 16–21.

Johnson, T. B.; Edens, C. O. The action of 5,5-dibromoxyhydrouracil on ethylene-thiourea. *J. Am. Chem. Soc.* **1941**, *63*, 1058–1060.

Johnson, T. B.; Edens, C. O. Complex formations between iodine and μ-mercapto-dihydroglyoxalines. *J. Am. Chem. Soc.* **1942**, *64*, 2706–2708.

Jordan, L. W.; Neal, R. A. Examination of the *in vivo* metabolism of maneb and zineb to ethylenethiourea (ETU) in mice. *Bull. Environ. Contam. Toxicol.* **1979**, *22*, 271–277.

Marshall, W. D. Oxidative Degradation of ethylenethiourea (ETU) and ETU progenitors by hydrogen peroxide and hypochlorite. *J. Agric. Food Chem.* **1979**, *27*, 295–299.

Marshall, W. D.; Singh, J. Oxidative inactivation of ethylenethiourea by hypochlorite in alkaline medium. *J. Agric. Food Chem.* **1977**, *25*, 1316–1320.

Newsome, W. H.; Panopio, L. G. A method for the determination of 2-imidazoline residues in food crops. *J. Agric. Food Chem.* **1978**, *26*, 638–640.

Poulsen, L. L.; Hyslop, R. M.; Ziegler, D. M. S-Oxygenation of N-substituted thioureas catalyzed by the pig liver microsomal FAD-containing monooxygenase. *Arch. Biochem. Biophys.* **1979**, *198*, 78–88.

Savolainen, K.; Pyysalo, H. Identification of the main metabolite of ethylenethiourea in mice. *J. Agric. Food Chem.* **1979**, *27*, 1177–1181.

Skellern, G. G. Thiocarbamides. In *Sulphur Containing Drugs and Related Organic Compounds: Chemistry, Biochemistry*

and Toxicology; Damani, L. A., Ed.; E. Horwood: Chichester, U.K., 1989; pp 49–89.

Ulland, B. M.; Weisberger, J. H.; Weisberger, E. K.; Rice, J. M.; Cypher, R. Thyroid cancer in rats from ethylene thiourea intake. *J. Natl. Cancer Inst.* **1972**, *49*, 583–584.

U.S. Environmental Protection Agency. EBDC fungicides; initiation of special review. *Fed. Regist.* **1987**, *52*, 27172–27177.

U.S. Environmental Protection Agency. Ethylenebisdithiocarbamates (EBDCs); notice of intent to cancel and conclusion of special review. *Fed. Regist.* **1992**, *57*, 7484–7530.

Received for review March 30, 1995. Accepted July 7, 1995.[⊗] The project described was supported by Grants PO1 ES 00049 and RO1 ES 04863 from the National Institute of Environmental Health Sciences, NIH.

JF950187R

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1995.