

Oxidation of 2-Mercaptobenzothiazole in Latex Gloves and Its Possible Haptenation Pathway

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The rubber accelerator, 2-mercaptobenzothiazole (MBT), has been reported to cause allergic contact dermatitis from gloves and other rubber products, but its chemical fate when exposed to occupational oxidants and the mechanism of its pathogenesis are not known. It was hypothesized that the thiol group is critical to MBT's (its oxidation products or metabolites) covalent binding and/or haptenation to nucleophilic protein residues. Oxidative transformation of MBT to the disulfide 2,2'-dithiobis(benzothiazole) (MBTS) was observed within the glove matrix when hypochlorous acid, iodine, and hydrogen peroxide were used as oxidants. Cysteine reduced MBTS to MBT with subsequent formation of the mixed disulfide 2-amino-3-(benzothiazol-2-yl) disulfanylpropionic acid which was identified and characterized. Spectrophotometry and mass spectrometry experiments demonstrated the simultaneous reduction of MBTS and disulfide formation with Cys34 on bovine serum albumin, suggesting a potential route of protein haptenation through covalent bonding between protein cysteinyl residues and the MBT/MBTS thiol moiety. Metabolism of MBT using isoniazid and dexamethasone-induced rat liver microsomes, to give a protein reactive epoxide intermediate and provide an alternative protein haptenation mechanism, was not observed. The data suggest that the critical functional group on MBT is the thiol, and haptenation is via the formation of mixed disulfides between the thiol group on MBT and a protein sulfhydryl group.

Introduction

Latex and rubber contact allergies, resulting from low-molecular mass chemicals that are used as either accelerants or antioxidants in the latex and rubber manufacturing processes, have been increasing in frequency over the past decade (1–3). This observation may be attributed to the increased use of latex examination gloves and rubber products. One of the latex accelerants known to cause latex allergies culminating in allergic contact dermatitis (ACD) is 2-mercaptobenzothiazole (MBT), an important industrial chemical with an annual production of approximately 40,000 tons in Europe and more than 1 million pounds in the United States. Other industrial applications of MBT include its use as an antioxidant and stabilizer in rubber and plastics manufacturing, a corrosion inhibitor in cutting oils and greases, a floatation agent in metal ore processing, and a fungicide and bactericide (4). The implication of MBT as an etiologic agent of ACD has been demonstrated clinically using patch testing (5). Type IV hypersensitivities, as a result of exposure to MBT, have been reported for subjects who were in contact with latex and rubber products such as tennis shoes (6), elastic underwear, condoms (7, 8), and suction socket prosthesis devices (9). Even though MBT and its oxidative product, MBTS, are known contact allergens, their immunogenic mechanism is still a subject for debate, more so because the ultimate allergenic chemical form of MBT is unknown given the fact that under normal use conditions, in situ glove MBT may come into contact with both industrial (bleach, iodine, peroxide) and biological

oxidants (peroxides). It is thus important to study the chemical fate of MBT within the glove matrix when the glove comes into contact with these occupationally encountered oxidants.

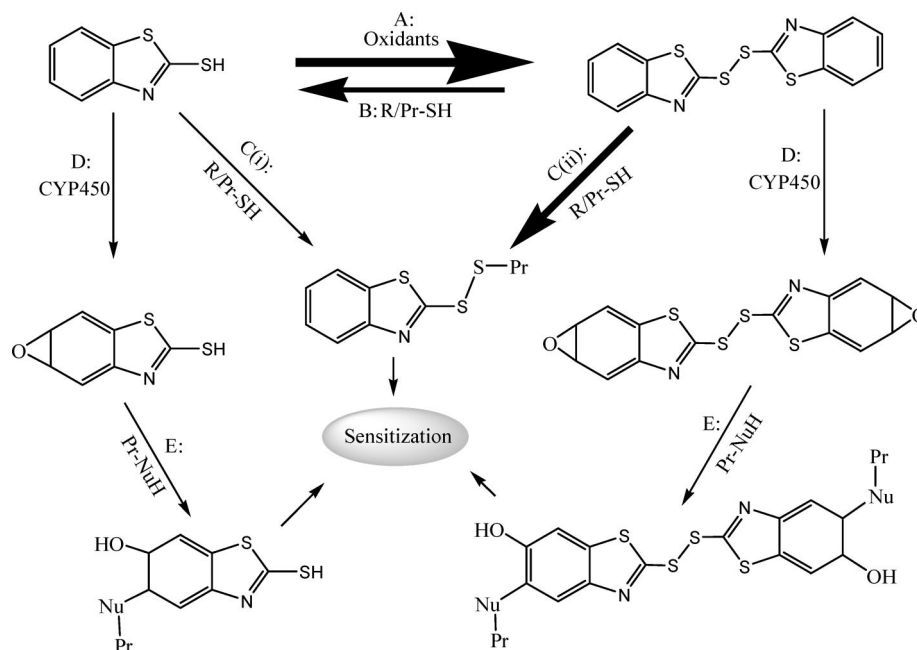
It is generally agreed that low-molecular mass xenobiotics such as MBT (<500 Da) do not have a sufficient molecular volume, once they have penetrated the skin and been absorbed into the epidermis, to stimulate a cellular and/or antibody immune response. MBT, therefore, has to bind to proteins and/or peptides to form macromolecular immunogens in the form of hapten-carrier complexes, a postulation which has come to be commonly termed the "hapten hypothesis" (10, 11). el Dareer reported that MBT and its disulfide, 2-mercaptobenzothiazole disulfide (MBTS), can bind covalently to erythrocyte membranes and soluble proteins, but the nature of the binding was not explored. Further experiments have shown the enzyme-mediated binding of the MBT derivative benzothiazole-2-sulfonamide to glutathione (12, 13). The importance of mixed disulfides as a mechanistic pathway responsible for native protein modification (14) has not received much attention as opposed to the more common nucleophilic attack on electrophilic allergens, examples of which are the 1,4-Michael addition and Schiff base formation mechanisms (15).

Cutaneous xenobiotic metabolism is suspected to be a prevalent mechanism through which chemicals are activated to protein reactive intermediates, as the skin (particularly the epidermis) is now recognized as a metabolically active tissue containing many of the metabolic enzymes that were previously thought to have activity only in the liver (16–18). To this effect, the fate of MBT within the epidermis is unknown. Microsomal hydroxylation of the benzene ring is postulated to occur via reactive epoxide intermediates, and epoxides have been shown to form adducts with nucleophilic residues on proteins (19).

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Scheme 1. Hypothesized Pathways through Which MBT and Its Derivatives Are Able To Elicit T-Cell Proliferation^a

^a Disulfide bridge formation with proteins (Pr-SH) and adduct formation on the ring with proteins (Pr-NuH) via epoxidation are the proposed mechanisms. Experimental data supported pathways A, B, and C(ii). CYP450-mediated metabolism was not observed, ruling out pathways D and E.

Given the lack of exploratory data on the skin sensitization of MBT and its metabolites, a series of experiments were carried out to test the hypothesized pathways outlined in Scheme 1, which were to oxidatively transform MBT within the glove matrix (pathway A); study the fate of MBT and the oxidation products in the presence of endogenous thiols (R-SH) and thiol-containing proteins (Pr-SH) (pathways B and C); and probe the possibility of microsomal (CYP450) phase I metabolism on the aromatic ring with subsequent adduct formation with proteins (pathways D and E).

Experimental Procedures

Materials. 2-Mercaptobenzothiazole, 2,2'-dithiobis(benzothiazole), sodium hypochlorite, soluble starch, sodium thiosulfate, sodium hydroxide, sodium perchlorate, L-cysteine, L-cysteamine, glutathione, oxidized glutathione, phosphate-buffered saline, dithiothreitol (DTT), 5,5-dithiobis(nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM), acetonitrile (ACN), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA, fraction V), formic acid (FA), trifluoroacetic acid (TFA), phosphoric acid, ammonium carbonate, chlorzoxazone (CZ), 5-chloro-2-mercaptobenzothiazole (CMBT), trypsin, chymotrypsin, and dichloromethane were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Latex gloves were purchased from Best Manufacturing Co. (Menlo, GA). Dexamethasone and isoniazid-induced Sprague-Dawley rat liver S9 microsomes were purchased from Invitro Technologies (Baltimore, MD). Dialysis tubing (molecular mass cutoffs of 3.5, 12, and 30 kDa) was purchased from Spectrum Laboratories (Rancho Dominguez, CA). The concentrations of the ClO^- were determined iodometrically and confirmed spectrophotometrically [$\epsilon_{(\text{ClO}^-)292} = 350 \text{ M}^{-1} \text{ cm}^{-1}$]. All solutions were prepared using nanopure water (MilliQ; Millipore, Bedford, MA) from a Millipore system.

Instrumentation. 1. Spectrophotometry. Spectrophotometric characterization of MBT, MBTS, free thiols, and oxidation reactions was carried out on a Beckman (Fullerton, CA) DU 800 spectrophotometer with data acquisition and processing being done using the DU800 spectrometer software. The analytical wavelength range was 200–800 nm. Temperature control was achieved by using a VWR Scientific (Niles, IL) circulating water bath.

2. High-Performance Liquid Chromatography (HPLC).

HPLC analysis was performed on a Shimadzu Prominence system consisting of a LC-20AT solvent delivery module, a DGU-20A5 degasser, a SIL-10AD auto-injector, a CBM-20A interface, and an SPD-M10A photodiode array detector (PDA, Columbia, MD). An injection volume of 10–100 μL was used, and separations were performed on a Supelco (Bellefonte, PA) Discovery C18 column (5 μm particle size, 250 mm $\times$ 4.6 mm) at a flow rate of 1 mL/min. For MBTS/BSA reactions, separations were performed using a Phenomenex (Torrance, CA) Jupiter C18 protein column (5 μm particle size, 250 mm $\times$ 4.6 mm). An isocratic, binary mobile phase consisting of 70% ACN in water was employed. For protein and peptide elution, 0.1% trifluoroacetic acid (TFA) was used instead of water. Absorbance at 324 and 270 nm was routinely monitored, and in some cases, the maximum spectral scan mode was employed.

3. Mass Spectrometry. 1. MBTS–Cysteine Reaction.

All spectra were acquired on a Micromass QTOF-II (Waters Corp., Millford, MA) quadrupole time-of-flight mass spectrometer (qTOF MS). Analytes were dissolved in a 50/50 ACN/1% FA mixture and analyzed using positive-mode electrospray ionization (+ESI) (20, 21) at a capillary voltage of 2.8 kV and a flow rate of 5 $\mu\text{L}/\text{min}$. The source block was maintained at 80 $^\circ\text{C}$, and the nitrogen desolvation gas was maintained at 150 $^\circ\text{C}$ and a flow rate of 400 L/h. Tandem mass spectrometry (MS/MS) data were generated via collision-induced dissociation (CID) in argon at a collision energy of 15 eV. All data were visualized and analyzed using the Micromass MassLynx 4.0 software suite for Windows XP (Waters).

2. BSA–MBTS Reaction. Analytes were diluted 25/1 with the 50/50 ACN/1% FA mixture and analyzed by positive-mode ESI at a capillary voltage of 3.3 kV, a cone voltage of 45 V, and a flow rate of 5 $\mu\text{L}/\text{min}$. The source block was maintained at 80 $^\circ\text{C}$, and the nitrogen desolvation gas was maintained at 150 $^\circ\text{C}$ and a flow rate of 400 L/h. The resultant mass spectra were charge-deconvoluted using maximum entropy calculations performed in MassLynx (MaxEnt1). The output range was 50–80 kDa at a resolution of 1.0 Da/channel.

4. Reaction of MBT with HOCl. Determination of the stoichiometry of the HOCl–MBT reaction was performed in excess HOCl by varying the initial hypochlorous acid concentration. The total remaining oxidizing power of HOCl with Cl_2 was determined by iodimetric titration. Acidified potassium iodide was added, in excess,

to the reaction solutions, and the liberated iodine was titrated with sodium thiosulfate using a starch indicator. The confirmation of the stoichiometry was conducted spectrophotometrically by observing the absorbance of MBT at 324 nm ($\epsilon_{324} = 26,345 \pm 91 \text{ M}^{-1} \text{ cm}^{-1}$).

To oxidize MBT within the glove matrix, each glove was weighed and cut into approximately 1 cm strips. The pieces (1 g) were incubated in 10 mL of 0.01 M ClO^- on a shaker (250 rpm) at room temperature for 1 h, and an aliquot was taken for HPLC analysis. The gloves were subsequently incubated in ACN for 1 h, and a second aliquot was taken for HPLC analysis. Five gloves were used in each set of experiments. As a control, another set of gloves was cut into pieces and incubated with distilled deionized water for 1 h and subsequently extracted with ACN. The control water and ACN samples were analyzed by HPLC-PDA.

5. Nonenzymatic Reduction of MBTS. A stock solution of 5 mM MBTS and fresh solutions of cysteine (5 mM) and DTNB (5 mM) were prepared in a 50/50 ACN/ H_2O mixture. The concentrations of cysteine and DTNB were kept constant, while that of MBTS was varied. Spectral scans were obtained, and absorbances at 412 and 320 nm were monitored to indicate formation of nitrothiobenzene from DTNB and MBT from MBTS, respectively. Preliminary experiments demonstrated that MBT did not react with cysteine or DTNB, and MBTS did not react with DTNB under these conditions, confirming that changes in absorbance were due to the MBTS–cysteine and DTNB–cysteine reactions. MBTS (0.248 mM) was reacted with 0.496 mM cysteine for 1 h at 37 °C. The products of this reaction were monitored by HPLC-PDA using an isocratic 40/60 ACN/ H_2O elution. A white, fluffy precipitate which formed in the reaction solution was recovered by evaporation and dissolved in a 50/50 ACN/0.1% FA mixture for mass spectral analysis.

6. Calibration of Thiol Determination with Cysteine and Quantification of BSA Thiols. The method reported by Schultz (15) was modified. DTNB and cysteine (5 mM) stock solutions were prepared in a 50 mM $(\text{NH}_4)_2\text{CO}_3$ buffer. Cysteine standards from 50 to 250 μM were each reacted with 1 mM DTNB at room temperature in a total volume of 2 mL. Absorbance readings taken at 412 nm ($\epsilon_{412} = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$) after 15 min were plotted against the final cysteine concentration. The calibration curve was used to determine BSA sulphydryl concentration ($[\text{SH}]/[\text{BSA}]$) following reaction of 0.4 mL of 40 mg/mL BSA with 0.2 mL of 5 mM DTNB for 2 h with subsequent absorbance readings at 412 nm. The calibration curve was also used to determine loss of $[\text{SH}]/[\text{BSA}]$ when BSA was incubated with MBTS.

7. Preparation of Reduced BSA. The reconversion of the Cys34 residue on BSA to the free sulphydryl form was performed by incubating equal volumes of 40 mg/mL BSA with 4 mM DTT in $(\text{NH}_4)_2\text{CO}_3$ buffer at room temperature for 3 h, followed by dialysis (molecular mass cutoff of 3500 Da) in 2 L of 50 mM $(\text{NH}_4)_2\text{CO}_3$ for 48 h with three buffer changes during that period to ensure complete removal of DTT. The postdialysis BSA concentration was determined spectrophotometrically ($\epsilon_{278} = 43,000 \text{ M}^{-1} \text{ cm}^{-1}$) (22).

8. Reaction of MBTS with Bovine Serum Albumin (BSA). A stock solution consisting of 40 mg/mL BSA was prepared in 50 mM ammonium carbonate buffer (pH 8.2). Then, 0.04 mL of the stock BSA solution and 1.96 mL of 0.248 mM MBTS were incubated for 1 h on a shaker at 37 °C. A BSA control was also incubated. HPLC and MS samples were obtained after dialysis (molecular mass cutoff of 3500 Da) in 2 L of 50 mM $(\text{NH}_4)_2\text{CO}_3$ for 48 h with three buffer changes. Loss of DTNB reactivity (as described above) was used to determine loss of thiol reactivity as a result of MBT binding. As controls, BSA thiol concentrations were determined for (i) fresh BSA in buffer that was not incubated or dialyzed, (ii) BSA (in the absence of MBTS) that had been incubated under the same conditions (as the MBTS-treated BSA) and dialyzed, and (iii) BSA which had been reduced by dithiothreitol (DTT) and dialyzed. A comparison of the kinetics of the reaction of DTNB with (i) reduced BSA, (ii) reduced and dialyzed BSA, and (iii) reduced BSA incubated with MBTS with subsequent dialysis was performed. The same reactions were performed in a pH 7.4 ammonium carbonate buffer and analyzed by ESI-MS.

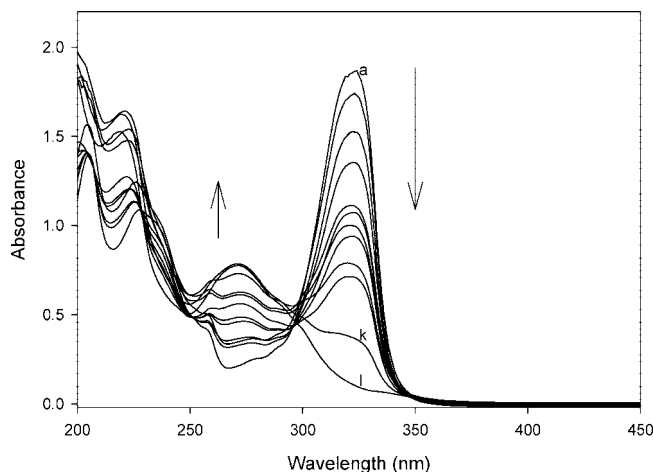


Figure 1. Oxidation of MBT with ClO^- . The reaction solutions were allowed to sit for 15 min before being scanned. MBT is held constant at (a) 0.071 mM with consecutive increments of the oxidant (b–l). When the ratio of MBT to ClO^- was 2/1 (0.071 mM MBT and 0.0355 mM ClO^-) (trace k), almost all the MBT was oxidized, and with a 1/1 ratio, all the MBT is oxidized. Absorbance was monitored at 270 nm.

To ascertain the Cys34 as the MBTS-targeted thiol-containing residue, the thiol specific blocking reagent *N*-ethylmaleimide (NEM) was also reacted with reduced BSA prior to reactions with either DTNB or MBTS. DTT-reduced BSA (0.01 mg/mL, 180 μM) was reacted with 1.8 mM NEM for 30 min in a pH 7.4 $(\text{NH}_4)_2\text{CO}_3$ buffer at room temperature with subsequent dialysis. Aliquots were taken for $[\text{SH}]/[\text{BSA}]$ determination with DTNB and MS analysis. BSA pretreated with NEM and incubated with MBTS was also subjected to MS analysis.

9. Digestion of BSA with Trypsin and Chymotrypsin. Untreated BSA and modified BSA were diluted to 50 μM and thermally denatured at 95 °C for 30 min. Thermal denaturation was quenched by incubating the sample in an ice bath for 30 min. Enzymatic digests were carried out at a protein/enzyme ratio of 40/1 (w/w) at 37 °C overnight in 50 mM ammonium bicarbonate.

10. Accelerant Metabolism Studies. A modified, previously reported method (23) was employed for these studies. A NADPH regenerating system (NRS) was prepared by combining 0.1 g of sodium bicarbonate (NaHCO_3) with 8.5 mg of NADP, 39 mg of glucose 6-phosphate, and 30 units/mL glucose-6-phosphate dehydrogenase in a total volume of 5 mL of deionized water and used immediately after preparation. Three substrates consisting of 0.35 mM stock solutions of MBT, CZ, and CMBT were prepared separately in deionized water. CZ and CMBT were used as positive controls, and the assays were performed in triplicate. One hundred microliters of the 20 mg/mL isoniazid/dexamethasone-induced S9 rat liver microsomes was placed in a test tube in an ice bath followed by 640 μL of 100 mM Tris buffer. Ten microliters of MBT was then added to the test tube. The test tube containing liver microsomes and MBT and a second test tube containing the NRS were placed in a 37 °C water bath with constant shaking for 5 min. Two hundred fifty microliters of the NRS was then added to the test tube containing the 750 μL mixture of MBT and the S9 fraction, and the mixture was incubated for 1 h at 37 °C. The reaction was stopped by addition of 50 μL of 43% H_3PO_4 . Each reaction mixture was then extracted, twice with 1 mL of CH_2Cl_2 . The organic and aqueous layers were separated by centrifugation at 3000g for 10 min. After the combined organic layers were dried under N_2 at ambient temperature, residues were dissolved in 100 μL of a 40/60 ACN/ H_2O mixture by sonication and 20 μL aliquots were injected onto the HPLC column. The control employed heat-inactivated S9 microsomes.

Results

Oxidation of MBT. The stoichiometry of the HOCl –MBT reaction was determined to be consistent with reactions R1 and

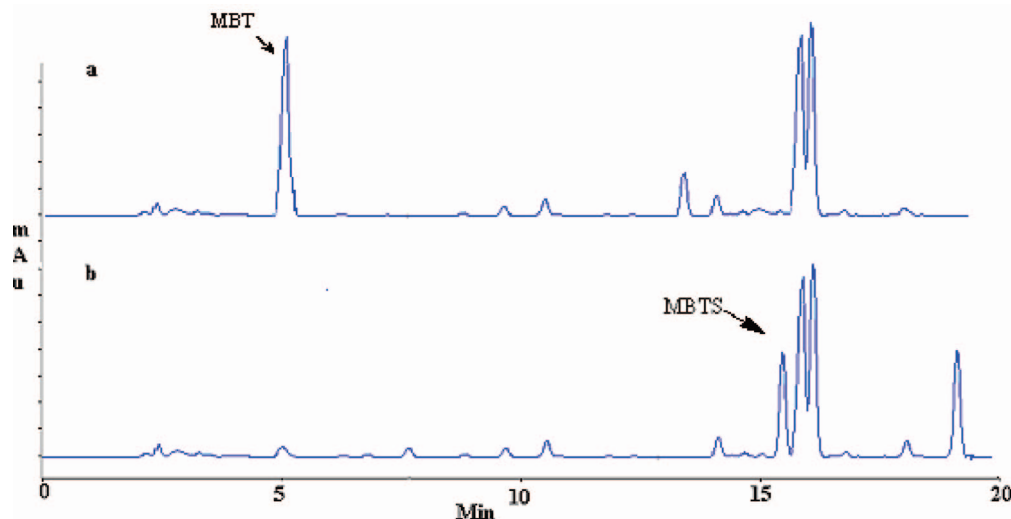


Figure 2. Oxidation of MBT in the glove matrix. (a) HPLC chromatogram of the acetonitrile extract of the unbleached glove showing the presence of MBT. (b) HPLC chromatogram of the acetonitrile extract of the glove after bleach incubation. The MBT is oxidized to the disulfide.

R2. The oxidation of MBT occurs in two stages where Cl_2 is produced in the first stage. The Cl_2 is a reactive intermediate, as it is quickly reduced to the chloride ion, oxidizing more MBT. Hence, the overall oxidation (R3) involves the conversion of 2 mol of MBT to the disulfide, 2,2'-dithiobis(benzothiazole) (MBTS). The stoichiometry was confirmed by a spectrophotometric method (Figure 1) in which a 1/1 ratio of MBT to HOCl (trace I) resulted in complete conversion of MBT to MBTS after 15 min. Under these conditions, the only oxidation product of MBT is the disulfide, and this was confirmed by UV-vis spectrophotometry, HPLC, and mass spectrometry. The formation of the disulfide, which has an absorbance at 270 nm, was a swift reaction that does not appear to involve any acid catalysis.



The commercially available latex glove that was used contained only MBT (and not MBTS), and oxidation of the MBT was readily achieved within the glove matrix. The ACN extract of the control glove contained only MBT, and that of the bleached glove contained mainly MBTS. The HPLC chromatograms in Figure 2 clearly demonstrate that the disulfide is retained within the glove matrix. The disulfide was also detected in the UV-vis spectrum. The HPLC chromatogram (Figure 2b) shows other peaks which could not be identified. Identification of these peaks was beyond the scope and objectives of this study given that the glove matrix may contain multiple oxidizable components.

Reduction of MBTS. There was a marked loss in reactivity between cysteine and DTNB when MBTS was incorporated into the reaction solution. The loss in reactivity between DTNB and cysteine was monitored at 324 and 412 nm, as shown in Figure 3. As the amount of MBTS was increased (traces a–g), there was less cysteine available to react with DTNB and release the chromophore 2-nitro-5-thiobenzoic acid (TNB); hence, a decrease in absorbance at 412 nm was observed. There was no change in the absorbance at 324 and 412 nm under the same conditions (like those for DTNB, cysteine, and MBTS) for the reaction of (i) DTNB with MBTS, (ii) DTNB with MBT, and (iii) DTNB with cysteine. Therefore, the release of the chro-

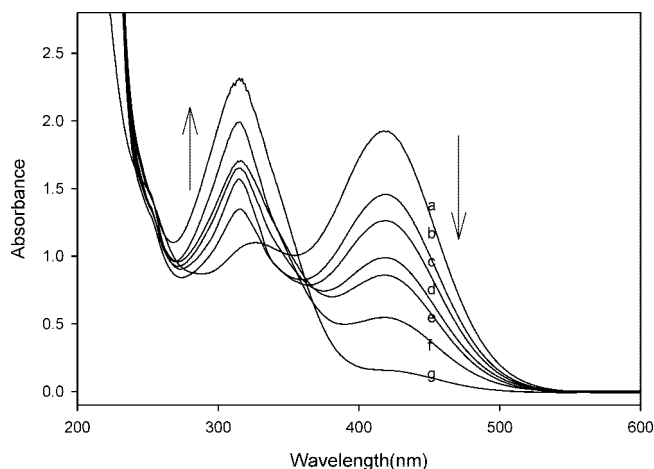


Figure 3. Spectrophotometric use of DTNB to follow the reduction of MBTS with cysteine: 0.15 mM cysteine with 0.15 mM DTNB and (a) 0.025, (b) 0.05, (c) 0.075, (d) 0.1, (e) 0.125, (f) 0.15, or (g) 0.175 mM MBTS. As the MBTS concentration was increased, there was less cysteine available, and consequently, less 2-nitro-5-thiobenzoic acid formed. The amount of free DTNB available also increased.

mophore TNB from DTNB could be attributed only to the reaction of cysteine with DTNB. When the same reaction conditions were employed with other thiols such as cysteamine and glutathione, the same reduction of MBTS was observed.

Chromatographic separation with subsequent mass spectrometry analysis afforded quantitation and characterization of the MBTS reduction products. The formation of the MBT–cysteine adduct through a disulfide bridge was confirmed as the major product of the thiol-induced reduction of MBTS. MS results (Figure 4) demonstrate the formation of 2-amino-3-(benzothiazol-2-yl disulfanyl)propionic acid (MBT–S–S–cysteine) with an m/z value of 286.9969. Other ions observed in the mass spectrum include MBT (m/z 167.9958), cysteine (m/z 241.0325), and unreacted MBTS (m/z 332.9622). The presence of cysteine was a result of the oxidation of cysteine by MBTS (compounds found both in soluble and in precipitant fractions of the reaction mixture). The presence of MBT, MBTS, and cysteine in Figure 4 was due to the fact that no separation and purification steps were performed to isolate unreacted MBTS. The amount of remaining MBTS had to be established to determine the reaction ratios of MBTS to cysteine. There was little evidence of cysteine in the mass spectrum, presumably because MBTS was in excess.

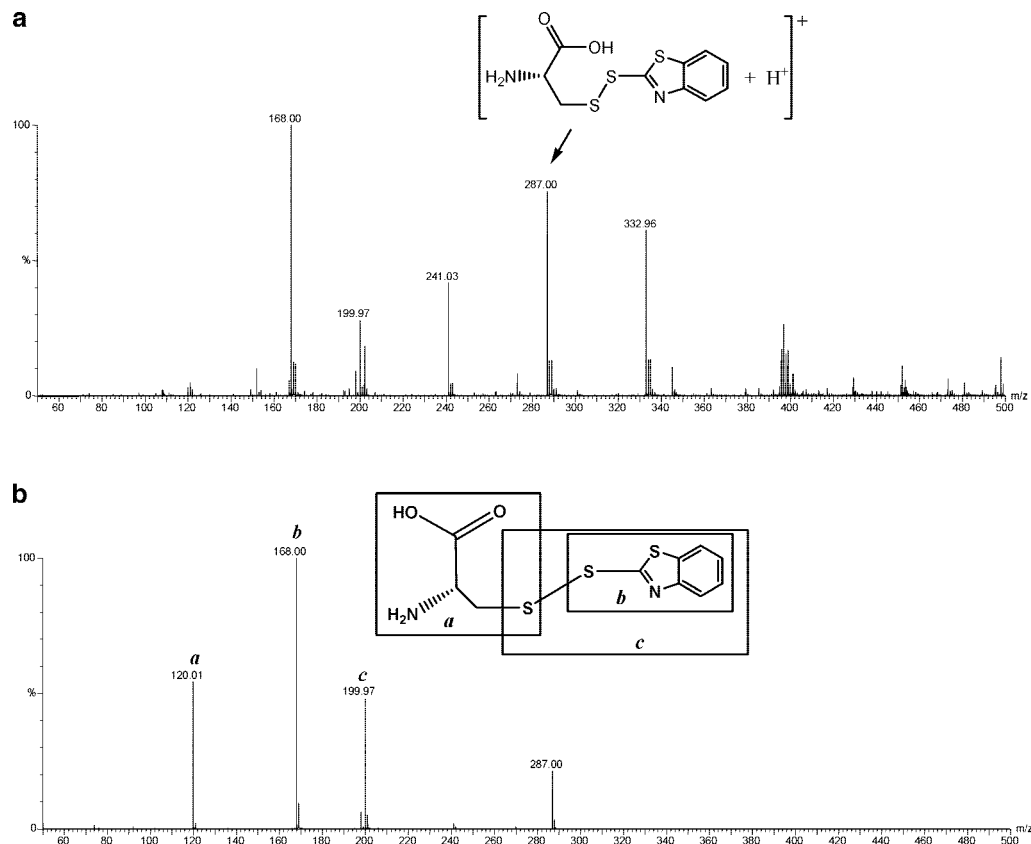
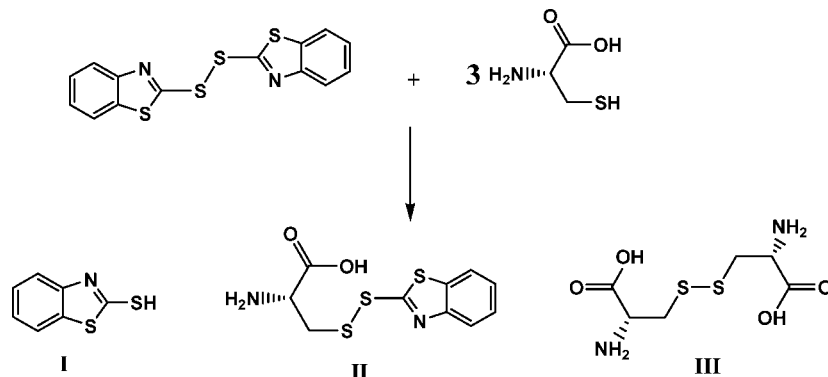


Figure 4. Mass spectrometry of the cysteine–MBTS reaction. Spectrum a confirms the presence of the MBT–cysteine mixed disulfide [2-amino-3-(benzothiazol-2-yl disulfanyl)propionic acid, m/z 286.996], MBT (m/z 167.995), and cystine (m/z 241.032) as the main products in the reduction of MBTS with cysteine. The presence of MBTS (m/z 332.962) and the absence of cysteine are due to the fact that MBTS was in excess. Spectrum b is a collision-induced dissociation (CID) tandem mass spectrum of the MBT–cysteine disulfide. The cleavage of the disulfide bond results in fragment ions indicative of MBT and cysteine.

Scheme 2. Reduction of MBTS with Cysteine To Yield the MBT–S–S–Cysteine Adduct



Tandem mass spectrometry of the ion at m/z 286.9622 (Figure 4b) resulted in fragment ions due to both MBT and cysteine, confirming the ion as the mixed disulfide. The reduction reaction stoichiometry was confirmed by both HPLC and MS to be 1/3 (MBTS/cysteine), and Scheme 2 outlines the reduction and subsequent formation of a mixed disulfide.

BSA–MBTS Reactions. The generation of an easily measurable change in absorbance when the Cys34 residue on BSA reacts with DTNB is well-documented (24, 25). The production of 0.39 equiv of TNB/mol of BSA ($=[\text{SH}]/[\text{BSA}]$) was observed when freshly prepared BSA was reacted with excess DTNB for 2 h. A reductive incubation with DTT prior to reaction with DTNB recovered 83% of the bound thiols, resulting in a $[\text{SH}]/[\text{BSA}]$ ratio of 0.96. MBTS was reduced by BSA, albeit at a slower rate relative to the rate of reduction by free cysteine, under the same reaction conditions that were employed for the

cysteine–MBTS reaction. Both fresh BSA and DTT-treated BSA (with dialysis) showed a complete loss of Cys34 reactivity at both pH 7.4 and 8.2 when they were incubated with MBTS prior to reaction with DTNB. The appearance of an absorption shoulder around 324 nm for the MBTS-treated BSA, even after dialysis, as shown in Figure 5 signified the incorporation of MBT by the protein. Because MBT also absorbs around 278 nm, the MBTS-treated BSA exhibits increased absorbance in that region relative to the control BSA. The loss of the reactivity of the single reduced thiol on BSA coupled with the production of MBT is indicative of a reaction occurring between MBTS and Cys34. To elucidate and confirm if adduct formation with BSA is via the reactive Cys34 residue, dialyzed samples of DTT-reduced BSA incubated with NEM in an $(\text{NH}_4)_2\text{CO}_3$ buffer at pH 7.4 and 8.2 were also reacted with DTNB. Figure 6

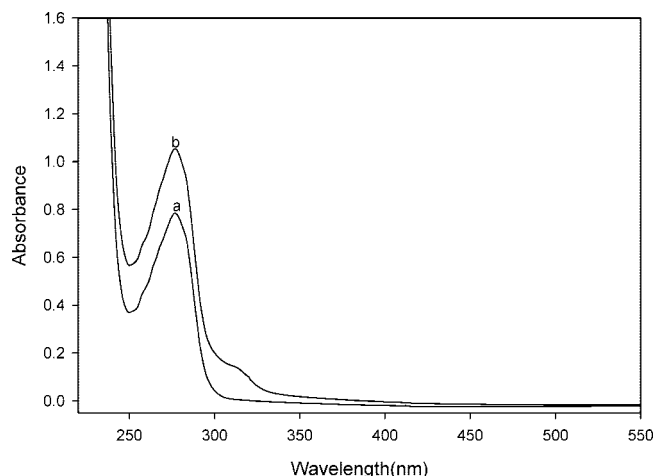


Figure 5. Comparison of the the absorptivities of dialyzed fractions of BSA: (a) 1.21 mg/mL BSA (control) and (b) 1.21 mg/mL BSA with 48.4 mg/mL MBTS incubated for 24 h prior to dialysis against a bicarbonate buffer. The shoulder around 324 nm and the enhanced absorbance around 278 nm for spectrum b are due to the contributing absorbance of the BSA-bound MBT (produced by reduction of MBTS).

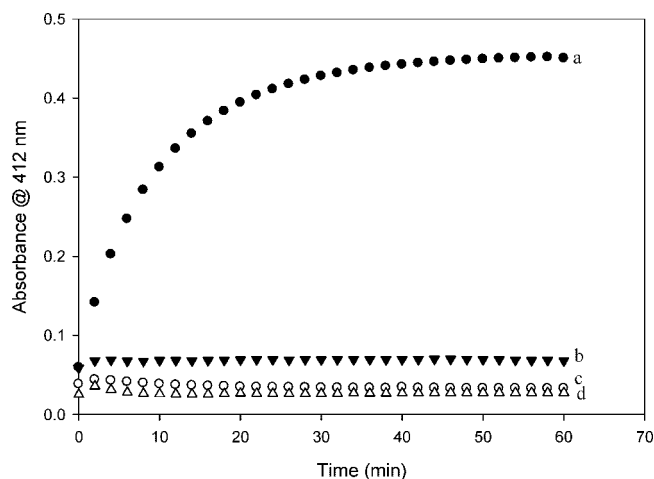


Figure 6. Reaction of Cys34 on BSA with DTNB: (a) 50 μ M BSA, (b) 50 μ M BSA with 0.5 mM MBTS, (c) 50 μ M BSA with 0.5 mM NEM and 227 μ M DTNB, and (d) 50 μ M BSA with 0.5 mM NEM and 227 μ M DTNB. NEM and MBTS are able to block the free thiol on Cys34.

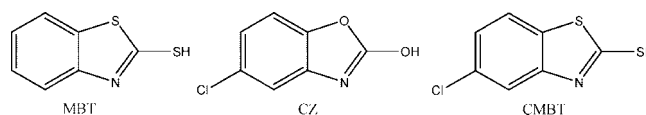
highlights the lack of reaction between the Cys34 residue and DTNB when both NEM and MBTS were used to block the thiol.

The loss of thiol–DTNB reactivity in the presence of MBTS was attributed to the formation of a strong bond between Cys34 and MBT and is supported by the fact that bound MBT cannot be removed by dialysis. In fact, attempts to recover Cys34 reactivity by extensive dialysis were not successful. Even though reactions of the control BSA showed a slight decrease in the amount of total SH content relative to freshly prepared BSA, after dialysis the [SH]/[BSA] ratio did not change, indicating that the dialysis had caused dilution of the whole protein, as expected. Covalent modification of the Cys34 residue of BSA by both NEM and MBT was confirmed by positive ion electrospray mass spectrometry. Deconvolution of the multiply charged mass spectra of BSA and MBTS-treated BSA yields molecular masses of 66,437 and 66,601 Da, respectively, in good agreement with the theoretical average molecular masses of BSA (66,430 Da) and MBT (167 Da). The formation of a mixed disulfide on Cys34 is indicated in Figure 7b in the amino acid sequence RQCPR', a theoretical trypsin fragment where R and

R' are the peptide sequences GLVLIAFSQYLQ and FDEHVK, respectively. Despite the different electronic environment and steric hindrance around Cys34 (compared to the free cysteine), the sulfhydryl group was still accessible to MBTS and reacted in much the same way as the free cysteine. Although the reaction rate was lower relative to that of MBTS–cysteine, both reactions adhered to pseudo-first-order kinetics in excess MBTS (results not shown). The NEM-treated BSA had a mass shift of 125 Da corresponding to the addition of 1 mol of NEM. A control experiment which involved preincubation of reduced BSA with NEM followed by addition of MBTS after extensive dialysis yielded a mass shift similar to that of the NEM-alkylated BSA showing that NEM effectively blocks the MBTS reactive residue on BSA. In a similar manner, preincubation of BSA with MBTS precluded the addition of NEM to BSA, indicating that both NEM and MBTS were targeting the same residue on BSA. It was apparent from the deconvoluted ESI spectra that binding of both the NEM and MBTS to BSA was occurring at pH 7.4 and 8.2 even though the reaction performed at pH 8.2 was more quantitative than that at pH 7.4. MBTS did not react with lysine, arginine, and serine when the free amino acids were incubated with MBTS under the reaction conditions that were employed for the MBTS–cysteine reaction.

Cys34-containing tryptic and chymotryptic peptides G21–K41 (GLVLIAFSQYLQQC PFDEHVK), Q32–F36 (QQCPF), and L31–F36 (LQQCPF) were observed in the peptide mass map of wild-type BSA; however, the MBTS-conjugated peptides were conspicuously absent from the peptide mass maps of derivatized BSA. Comparison of the peptide mass maps of wild-type BSA and conjugated BSA did not show additional peaks which could be attributed to the incorporation of MBT on the protein. Incorporation of MBT in BSA at Cys34 may inhibit digestion of the protein; in fact, this phenomenon is commonly observed in Cys34-modified BSA (26).

Metabolism. Isoniazid and dexamethasone-induced S9 rat liver microsomes were used to assess the potential metabolism of MBT and MBTS by CYP2E1 and CYP3A4, respectively. The chlorine-substituted oxygen analogue of MBT, chlorzoxazone (CZ), was used as a positive control since it has been demonstrated to be a substrate for both CYP2E1 and CYP3A4. 5-Chloro-2-mercaptobenzothiazole (CMBT) was also included as a control due to its structural resemblance to CZ. Thermally inactivated microsomes were employed as a control assay and did not show any activity on the three substrates. Under the experimental conditions that were used, none of the three compounds reacted with NRS, implying that the metabolites were a result of the protein-mediated hydroxylation.



Chromatographic separation with diode array detection of the concentrated organic extracts is presented in Figure 8, demonstrating hydroxylation of both CZ and CMBT. The insertion of the hydroxyl group on the aromatic ring was not observed with MBT, suggesting that (i) MBT might not be a substrate for the CYP2E1 and CYP3A4 isoforms, (ii) if MBT is a substrate then the induction of both CYP2E1 and CYP3A4 might not be sufficient to metabolize MBT to detectable levels, and (iii) the negative inductive effect of the Cl (as in CZ and CMBT) on the aromatic ring renders the compounds susceptible to hydroxylation on the carbon atom adjacent to the Cl.

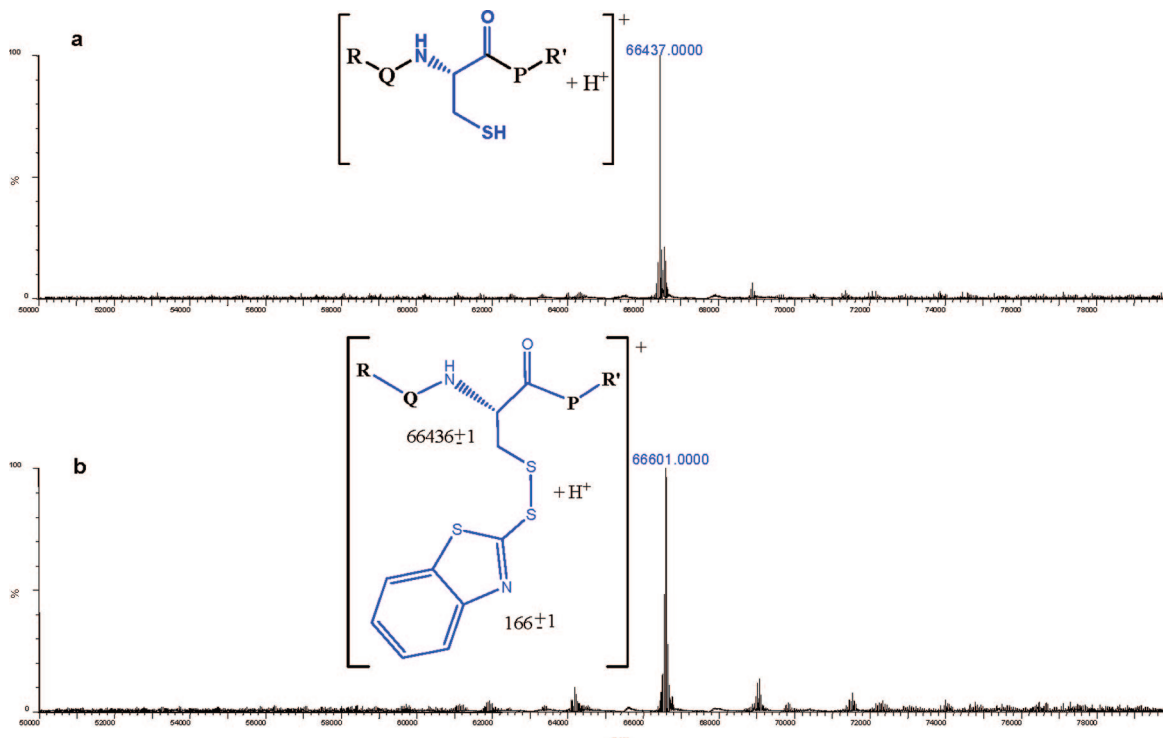


Figure 7. Electrospray mass spectrum of the BSA–MBT adduct. (a) Deconvoluted mass spectra of untreated BSA and (b) the MBTS/BSA incubate. In the experiment, 0.4 mL of 40 mg/mL BSA and 0.2 mL of 0.248 mM MBTS were incubated in an ammonium carbonate buffer (pH 8.2) for 1 h on a shaker at 37 °C with subsequent dialysis. The mass shift between spectra a and b corresponds to the mass of MBT.

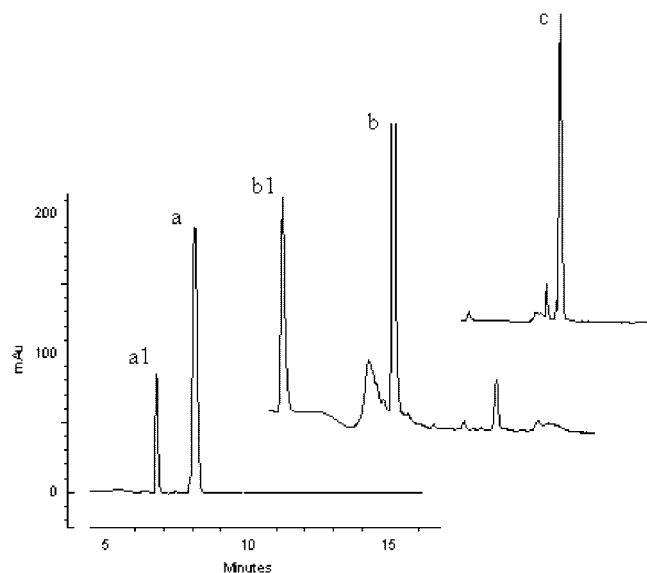


Figure 8. CYP2E1-mediated hydroxylation of (i) chlorzoxazone (a) to 6-hydroxychlorzoxazone (a1) and (ii) 5-chloro-2-mercaptobenzothiazole (b) to 5-chloro-6-hydroxy-2-mercaptobenzothiazole (b1) and (iii) the absence of metabolites with mercaptobenzothiazole (c).

Discussion

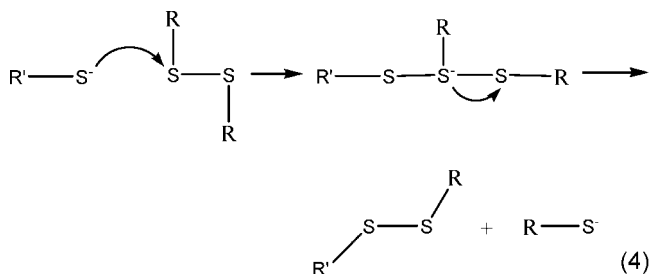
These data support pathway A (Scheme 1) and suggest that exposure of MBT-containing gloves to common occupational/hospital oxidants such as bleach results in quantitative oxidative transformation of MBT to MBTS, which is the sole product. It was also demonstrated that MBTS, once formed, does not leach into alkaline bleach solutions. This suggests a higher chance of the chemical leaching into the skin sebum directly from the glove (vs being lost in the sterilizing solution). The rapid reduction of MBTS with cysteine was shown, and given the abundance of this thiol within the epidermis, the interaction of

MBTS with both protein and nonprotein thiols is probable. Under this scenario, MBT would be allergenic following either exogenous or endogenous oxidation to MBTS, followed by subsequent formation of disulfide linkages with protein thiols.

Disulfide interconversion between MBTS and Cys34 on BSA leading to the incorporation of MBT onto BSA has been demonstrated, herein, by loss of BSA thiol–DTNB/NEM reactivity and mass spectrometry. The modification of BSA and, potentially, other sulfhydryl-containing proteins by thiol-based sensitizers is most likely through disulfide bridge formation and adduct formation on Cys34, which has been shown to exhibit allostericity where distant conformational changes and reactivities were observed on distant amino acid residues (27). Contact allergies to garlic have been shown to be due to diallyl disulfide, a chemical present in garlic, whereby it has been hypothesized that the chemical haptens proteins by forming mixed disulfides (28). The data obtained herein demonstrate the importance of the hapten hypothesis where MBT covalently conjugates to the free cysteine on BSA, and data support the hypothesized pathways B and D in Scheme 1.

Dithiol and disulfide interconversion is a conspicuous property of the S–S bond whose reactivity can be attributed to the presence of lone pairs and low-energy 3d orbitals on S which make the S–S bond polarizable in the presence of nucleophiles. Disulfide formation through an addition–elimination sequence (R4) involving a trigonal bipyramidal, metastable transition state has been proposed, where the incoming thiolate anion places an appreciable charge on all three sulfurs leading to scission of the disulfide bond with formation of a mixed disulfide (29).

Cysteiny groups are only alkylated in the deprotonated thiolate anion state as shown by previous studies where following the alkylation rate as a function of pH has been used to determine the pK value of the reactive cysteine (30, 31). Given an estimated Cys34 pK_a value of ≈7 (32), the use of a carbonate buffer (pH 7.4 and 8.2) was optimal for reduction, as this ensures the deprotonation of Cys34 and promotes the



reaction of the cysteine thiolate with MBTS. It must be pointed out that the three surrounding ionizable groups of Asp38 (carboxylate), His39 (imidazole), and Tyr84 (hydroxyl) tend to make Cys34 inaccessible at acidic pH; however, it has been demonstrated that a pH greater than 7 results in a structural transition from the N (neutral) to B (basic) form of the protein with basic loosening and opening of the shallow crevice enclosing Cys34 (33). The unfolding of BSA and the deprotonation of Cys34 readily occur at these pH values (7.4 and 8.2), and an addition–elimination reaction with MBTS ensues. The inference of the modification at Cys34 was also strengthened by the fact that MBTS did not react with the amino groups of lysine or arginine or with the carboxylic acid terminus of serine under the same conditions as the cysteine–MBTS reaction.

Efforts to understand the links between protein haptenation and the ability of a chemical to cause sensitization have had the limitation of restricting experimental studies to congeneric sets of chemicals, mainly electrophiles, thus overlooking other functional groups that are also protein reactive. A number of approaches to establishing structure–activity relationships (SARs) such as the relative alkylation index (RAI) (mathematical model based on electrophilicity and hydrophobicity parameters) (34) and the deductive estimation of risk from existing knowledge (DEREK) (35) have been built on existing databases on structure–toxicity relationships with a strong emphasis on hapten–protein reaction mechanisms and metabolism. While the electrophile (hapten)–nucleophile (protein) type interactions are common with strong skin sensitizers, other forms of previously ignored protein haptenation mechanisms, such as disulfide formation, may be important in predicting skin sensitization. In a study that was carried out on 355 randomly chosen chemicals that are human sensitizers, Rosenkranz et al. (36) observed that electrophilicity accounted for 30–40% of ACD inducing ability with the remainder being associated with nonelectrophilic structures. Numerous latex accelerants, which are skin sensitizers, have sulfhydryl moieties which react directly with proteins even though they are resistant to enzymatic or nonenzymatic oxidation to amine reactive sulfones or sulfoxides.

The lack of a complete profiling of proteinaceous constituents of the skin limits current xenobiotic–protein haptenation studies for modeling proteins and peptides. Human serum albumin (HSA) and bovine serum albumin (BSA) have been studied as model proteins where chemicals covalently modified Cys34 (37), Lys190 (38), Lys199 (39), His9, His146, and His338 (40). BSA was used as a model protein to evaluate direct haptenation in this study as well as to highlight the importance of disulfide formation as a protein modification pathway. BSA is 80% homologous to HSA, and approximately 40% of extravascular HSA is located in the skin (41). MBT, on the other hand, is a model compound which seems to exert its potency through a mechanism deviating from the 1,4-Michael type addition and Schiff base formation, which are examples of electrophile–nucleophile interactions.

The enzymatic bioactivation of MBT and MBTS to possible reactive epoxide intermediates was not observed following

incubation with CYP2E1- and CYP3A4-induced microsomes. What has been established is the prevalence of phase II hepatic metabolism of MBT, in vivo, resulting in its glucuronidation (12, 42), and MBT has also been cited as an excreted metabolite in the metabolism of 2-(thiocyanomethylthio)benzothiazole (43), implying that it is relatively stable. Hydroxylation of the benzene ring with subsequent formation of the monohydroxyl and dihydroxyl derivatives has only been noted in bacterial biotransformations of MBT (44). Hydroxylation would be indicative of a mechanism which yields a reactive epoxide as an intermediate as previously reported (45). The metabolic activity of CYP2E1 on CZ and CMBT, which are structural analogues of MBT, was observed. This, along with previous studies in which MBT (or MBT glucuronide) was an ultimate metabolite (46, 47), suggests that ring oxidation is unlikely in the absence of electron-withdrawing groups on the aromatic ring. Microsomal isoforms expressed in the epidermis are, therefore, not likely to metabolize MBT. In summary, the probable predominant mechanism of haptenation of proteins by MBT is by first nonenzymatic oxidation to MBTS, followed by disulfide exchange with a protein thiol to form the protein–S–S–MBT adduct. In addition, these results assert the potential importance of disulfide formation as a general haptenation and protein modification mechanism.

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References

- (1) Binkley, H. M., Schroyer, T., and Catalfano, J. (2003) Latex Allergies: A Review of Recognition, Evaluation, Management, Prevention, Education, and Alternative Product Use. *Journal of Athletic Training* 38 (2), 133–140.
- (2) Filon, F. L., and Radman, G. (2006) Latex allergy: A follow up study of 1040 healthcare workers. *Occup. Environ. Med.* 63 (2), 121–125.
- (3) Nardelli, A., Taveirne, M., Drieghe, J., Carbonez, A., Degreef, H., and Goossens, A. (2005) The relation between the localization of foot dermatitis and the causative allergens in shoes: A 13-year retrospective study. *Contact Dermatitis* 53 (4), 201–206.
- (4) Reddy, C. M., and Quinn, J. G. (1997) Environmental chemistry of benzothiazoles derived from rubber. *Environ. Sci. Technol.* 31 (10), 2847–2853.
- (5) Kaniwa, M. A., Isama, K., Nakamura, A., Kantoh, H., Itoh, M., Miyoshi, K., Saito, S., and Shono, M. (1994) Identification of causative chemicals of allergic contact dermatitis using a combination of patch testing in patients and chemical analysis. Application to cases from rubber footwear. *Contact Dermatitis* 30 (1), 26–34.
- (6) Jung, J. H., McLaughlin, J. L., Stannard, J., and Guin, J. D. (1988) Isolation, via activity-directed fractionation, of mercaptobenzothiazole and dibenzothiazyl disulfide as 2 allergens responsible for tennis shoe dermatitis. *Contact Dermatitis* 19 (4), 254–259.
- (7) Harmon, C. B., Connolly, S. M., and Larson, T. R. (1995) Condom-related allergic contact dermatitis. *J. Urol.* 153 (4), 1227–1228.
- (8) Bircher, A. J., Hirsbrunner, P., and Langauer, S. (1993) Allergic contact dermatitis of the genitals from rubber additives in condoms. *Contact Dermatitis* 28 (2), 125–126.
- (9) Conde-Salazar, L., Llinas Volpe, M. G., Guimaraens, D., and Romero, L. (1988) Allergic contact dermatitis from a suction socket prosthesis. *Contact Dermatitis* 19 (4), 305–306.
- (10) Landsteiner, K., and Jacobs, J. (1936) Studies on the sensitization of animals with simple chemical compounds. *J. Exp. Med.* 64, 625–639.
- (11) Dupuis, G., Benezra, C., Schlewer, G., and Stampf, J. L. (1980) Allergic contact dermatitis to α -methylene- γ -butyrolactones. Preparation of alantolactone-protein conjugates and induction of contact sensitivity in the guinea pig by an alantolactone-skin protein conjugate. *Mol. Immunol.* 17 (8), 1045–1051.
- (12) el Dareer, S. M., Kalin, J. R., Tillery, K. F., Hill, D. L., and Barnett, J. W. (1989) Disposition of 2-mercaptobenzothiazole and 2-mercaptobenzothiazole disulfide in rats dosed intravenously, orally, and

- topically and in guinea pigs dosed topically. *J. Toxicol. Environ. Health* 27 (1), 65–84.
- (13) Stijntjes, G. J., te Koppele, J. M., and Vermeulen, N. P. (1992) High-performance liquid chromatography-fluorescence assay of pyruvic acid to determine cysteine conjugate β -lyase activity: Application to S-1,2-dichlorovinyl-L-cysteine and S-2-benzothiazolyl-L-cysteine. *Anal. Biochem.* 206 (2), 334–343.
- (14) Dickerson, J. E., and Lou, M. F. (1993) A new mixed disulfide species in human cataractous and aged lenses. *Biochim. Biophys. Acta* 1157 (2), 141–146.
- (15) Schultz, T. W., Yarbrough, J. W., and Johnson, E. L. (2005) Structure-activity relationships for reactivity of carbonyl-containing compounds with glutathione. *SAR QSAR Environ. Res.* 16 (4), 313–322.
- (16) Pannatier, A., Jenner, P., Testa, B., and Etter, J. C. (1978) The skin as a drug-metabolizing organ. *Drug Metab. Rev.* 8 (2), 319–343.
- (17) Swanson, H. I. (2004) Cytochrome P450 expression in human keratinocytes: An aryl hydrocarbon receptor perspective. *Chem.-Biol. Interact.* 149 (2–3), 69–79.
- (18) Du, L., Hoffman, S. M., and Keeney, D. S. (2004) Epidermal CYP2 family cytochromes P450. *Toxicol. Appl. Pharmacol.* 195 (3), 278–287.
- (19) Bergstrom, M. A., Luthman, K., Nilsson, J. L., and Karlberg, A. T. (2006) Conjugated dienes as prohaptens in contact allergy: In vivo and in vitro studies of structure-activity relationships, sensitizing capacity, and metabolic activation. *Chem. Res. Toxicol.* 19 (6), 760–769.
- (20) Whitehouse, C. M., Dreyer, R. N., Yamashita, M., and Fenn, J. B. (1985) Electrospray interface for liquid chromatographs and mass spectrometers. *Anal. Chem.* 57 (3), 675–679.
- (21) Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246 (4926), 64–71.
- (22) Zhang, Y., and Wilcox, D. E. (2002) Thermodynamic and spectroscopic study of Cu(II) and Ni(II) binding to bovine serum albumin. *J. Biol. Inorg. Chem.* 7 (3), 327–337.
- (23) Peter, R., Bocker, R., Beaune, P. H., Iwasaki, M., Guengerich, F. P., and Yang, C. S. (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem. Res. Toxicol.* 3 (6), 566–573.
- (24) Janatova, J., Fuller, J. K., and Hunter, M. J. (1968) The heterogeneity of bovine albumin with respect to sulfhydryl and dimer content. *J. Biol. Chem.* 243 (13), 3612–3622.
- (25) King, T. P. (1961) On the sulfhydryl group of human plasma albumin. *J. Biol. Chem.* 236, C5.
- (26) Shen, B., and English, A. M. (2005) Mass spectrometric analysis of nitroxyl-mediated protein modification: Comparison of products formed with free and protein-based cysteines. *Biochemistry* 44 (42), 14030–14044.
- (27) Christodoulou, J., Sadler, P. J., and Tucker, A. (1994) A new structural transition of serum albumin dependent on the state of Cys34. Detection by ^1H -NMR spectroscopy. *Eur. J. Biochem.* 225 (1), 363–368.
- (28) Jappe, U., Bonnekoh, B., Hausen, B. M., and Gollnick, H. (1999) Garlic-related dermatoses: Case report and review of the literature. *Am. J. Contact Dermatitis* 10 (1), 37–39.
- (29) Rosenfield, R. E. (1977) Directional Preferences of Nonbonded Atomic Contacts with Divalent Sulfur. 1. Electrophiles and Nucleophiles. *J. Am. Chem. Soc.* 99 (14), 4860–4861.
- (30) Mossner, E., Iwai, H., and Glockshuber, R. (2000) Influence of the pK_a value of the buried, active-site cysteine on the redox properties of thioredoxin-like oxidoreductases. *FEBS Lett.* 477 (1–2), 21–26.
- (31) Kallis, G. B., and Holmgren, A. (1980) Differential reactivity of the functional sulfhydryl groups of cysteine-32 and cysteine-35 present in the reduced form of thioredoxin from *Escherichia coli*. *J. Biol. Chem.* 255 (21), 10261–10265.
- (32) Shaked, Z., Szajewski, R. P., and Whitesides, G. M. (1980) Rates of thiol-disulfide interchange reactions involving proteins and kinetic measurements of thiol pK_a values. *Biochemistry* 19 (18), 4156–4166.
- (33) Bos, O. J., Labro, J. F., Fischer, M. J., Wilting, J., and Janssen, L. H. (1989) The molecular mechanism of the neutral-to-base transition of human serum albumin. Acid/base titration and proton nuclear magnetic resonance studies on a large peptic and a large tryptic fragment of albumin. *J. Biol. Chem.* 264 (2), 953–959.
- (34) Roberts, D. W., Fragnals, R., Lepoittevin, J. P., and Benezra, C. (1991) Refinement of the relative alkylation index (RAI) model for skin sensitization and application to mouse and guinea-pig test data for alkyl alkanesulphonates. *Arch. Dermatol. Res.* 283 (6), 387–394.
- (35) Smith Pease, C. K., Basketter, D. A., and Patlewicz, G. Y. (2003) Contact allergy: The role of skin chemistry and metabolism. *Clin. Exp. Dermatol.* 28 (2), 177–183.
- (36) Rosenkranz, H. S., Klopman, G., Zhang, Y. P., Graham, C., and Karol, M. H. (1999) Relationship between allergic contact dermatitis and electrophilicity. *Environ. Health Perspect.* 107 (2), 129–132.
- (37) Yasuzawa, T., and Tomer, K. B. (1997) Structural determination of the conjugate of human serum albumin with a mitomycin C derivative, KW-2149, by matrix-assisted laser desorption/ionization mass spectrometry. *Bioconjugate Chem.* 8 (3), 391–399.
- (38) Bohnet, J. P., Fonda, M. L., and Feldhoff, R. C. (1992) Identification of Lys190 as the primary binding site for pyridoxal 5'-phosphate in human serum albumin. *FEBS Lett.* 298 (2–3), 266–268.
- (39) Bertucci, C., Ascoli, G., Uccello-Barretta, G., Di Bari, L., and Salvadori, P. (1995) The binding of 5-fluorouracil to native and modified human serum albumin: UV, CD, and ^1H and ^{19}F NMR investigation. *J. Pharm. Biomed. Anal.* 13 (9), 1087–1093.
- (40) Alvarez-Sanchez, R., Divkovic, M., Basketter, D., Pease, C., Panico, M., Dell, A., Morris, H., and Lepoittevin, J. P. (2004) Effect of glutathione on the covalent binding of the ^{13}C -labeled skin sensitizer 5-chloro-2-methylisothiazol-3-one to human serum albumin: Identification of adducts by nuclear magnetic resonance, matrix-assisted laser desorption/ionization mass spectrometry, and nano-electrospray tandem mass spectrometry. *Chem. Res. Toxicol.* 17 (9), 1280–1288.
- (41) Peters, T., Jr. (1996) *All About Albumin: Biochemistry, Genetics and Medical Applications*, Academic Press, San Diego.
- (42) Fukuoka, M., Satoh, M., and Tanaka, A. (1995) Metabolism of 2-thiobenzothiazoles in the rat. Urinary, fecal and biliary metabolites of 2-benzothiazyl sulfenamides. *Arch. Toxicol.* 70 (1), 1–9.
- (43) Manninen, A., Auriola, S., Vartiainen, M., Liesivuori, J., Turunen, T., and Pasanen, M. (1996) Determination of urinary 2-mercaptobenzothiazole (2-MBT), the main metabolite of 2-(thiocyanomethylthio)benzothiazole (TCMTB) in humans and rats. *Arch. Toxicol.* 70 (9), 579–584.
- (44) Haroune, N., Combourieu, B., Besse, P., Sancelme, M., Kloepper, A., Reemtsma, T., De Wever, H., and Delort, A. M. (2004) Metabolism of 2-mercaptobenzothiazole by *Rhodococcus rhodochrous*. *Appl. Environ. Microbiol.* 70 (10), 6315–6319.
- (45) Nilsson, A. M., Bergstrom, M. A., Luthman, K., Nilsson, J. L., and Karlberg, A. T. (2005) A conjugated diene identified as a prohaptens: Contact allergenic activity and chemical reactivity of proposed epoxide metabolites. *Chem. Res. Toxicol.* 18 (2), 308–316.
- (46) Elfarrar, A. A., and Hwang, I. Y. (1990) In vivo metabolites of S-(2-benzothiazolyl)-L-cysteine as markers of in vivo cysteine conjugate β -lyase and thiol glucuronosyl transferase activities. *Drug Metab. Dispos.* 18 (6), 917–922.
- (47) Larsen, G. L., Bakke, J. E., Feil, V. J., and Huwe, J. K. (1988) In vitro metabolism of the methylthio group of 2-methylthiobenzothiazole by rat liver. *Xenobiotica* 18 (3), 313–322.

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