Contact Dermatitis 2008: 59: 79–89
Printed in Singapore. All rights reserved

CONTACT DERMATITIS

Zinc diethyldithiocarbamate allergenicity: potential haptenation mechanisms

Itai Chipinda[1], Justin M. Hettick[1], Reuben H. Simoyi[2] and Paul D. Siegel[1]

[1]Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505-2888, USA, and [2]Department of Chemistry, Portland State University, Portland, OR 97201-0751, USA

Background: Zinc diethyldithiocarbamate (ZDEC) and its disulfide, tetraethylthiuram disulfide (TETD), are rubber accelerators and contact allergens that cross-react in some individuals.

Objective: This study explored potential protein haptenation mechanisms of ZDEC and its oxidation products.

Methods: ZDEC oxidation/reduction products and sites of protein binding were assessed using high-performance liquid chromatography and mass spectrometry. The murine local lymph node assay (LLNA) was employed to probe haptenation mechanisms of ZDEC by examining its allergenicity along with its oxidation products and through elimination of oxidation and chelation mechanisms by substituting cobalt for zinc [cobalt (II) dithiocarbamate, CoDEC].

Results: Oxidation of ZDEC by hypochlorous acid (bleach, HOCl), iodine, or hydrogen peroxide resulted in production of TETD, tetraethylthiocarbamoyl disulfide, and tetraethyldicarbamoyl disulfide (TEDCD). Albumin thiols reduced TETD with subsequent mixed disulfide formation/haptenation. ZDEC directly chelated the copper ion on the active site of the superoxide dismutase, whereas CoDEC did not bind to Cu proteins or form mixed disulfides with free thiols. ZDEC, sodium diethyldithiocarbamate, TEDCD, and TETD were all positive in the LLNA except CoDEC, which was non-allergenic.

Conclusion: The thiol is the critical functional group in ZDEC's allergenicity, and haptenation is predominantly through chelation of metalloproteins and formation of mixed disulfides.

Key words: allergenicity; diethyldithiocarbamate; haptenation; local lymph node assay; mechanism; oxidation; thiuram. © Blackwell Munksgaard, 2008.

Accepted for publication 18 March 2008

Dialkyldithiocarbamates and the corresponding disulfides have diverse applications in industry, agriculture, and medicine. The most common industrial use of zinc diethyldithiocarbamate (ZDEC) is to accelerate vulcanization in rubber and latex products manufacturing. Significant amounts of ZDEC and tetraethylthiuram disulfide (TETD) have been found in a number of rubber products ranging from shoes, elastic from underwear, condoms (1), surgical drains (2), and gloves (3, 4). ZDEC and TETD are well documented as contact sensitizers in studies in both animals and humans (5–9). Allergic contact dermatitis (ACD) associated with exposure to ZDEC- and TETD-containing products such as medical examination gloves is widespread (6–8). Cross-reactivity can occur between dithiocarbamates and thiurams, and mixtures of both chemicals can be present in the gloves (10). Oxidation products of these accelerators may also be potential contact sensitizers because it is common in the hospital setting to wear rubber gloves while using strong oxidizing disinfectants, including iodine, hydrogen peroxide, and bleach.

It is commonly accepted that ZDEC, like many low-molecular-weight contact allergens, can only trigger an immune response when bound to a protein in the form of an immunogenic protein–hapten complex (11). Haptenation of epidermally relevant skin proteins by ZDEC has not been

reported despite the numerous studies on the disposition and systemic toxicity of ZDEC, TETD, and their corresponding analogues (12–14). The chelating properties of the dithiocarbamate ligand have been proposed to be responsible for the modification of some metalloproteins and metalloenzymes (15, 16), but these chelation properties have not been investigated with respect to skin sensitization. Dithiocarbamates have also been demonstrated to inhibit cytotoxicity mediated by natural killer cells, and experimental evidence suggests that this is through disulfide bond formation with cellular sulfhydryl components (15).

Despite the reported biological effects and metabolites of dithiocarbamates that are quite varied (12), the mechanism(s) by which ZDEC is able to haptenate proteins resulting in ACD has not been explored. We hypothesized that the thiol carbamate group (**–**C(=S)**–**S**–**) of ZDEC is the critical and reactive functional group responsible for protein haptenation, leading to skin sensitization. A series of experiments were carried out to test the hypothesized pathways outlined in Fig. 1, which were to oxidatively transform ZDEC (pathways I and II) and to study the fate of ZDEC and the oxidation products in the presence of metal ions, metalloproteins (pathways III and IV), and protein thiols (pathway V). ZDEC and its oxidation/transmetallation products were tested in the local lymph node assay (LLNA) to probe the contribution of the chemical pathways in the sensitizing potential of ZDEC.

Materials and Methods

Chemicals

ZDEC, zinc dimethyldithiocarbamate (ZDMC), sodium diethyldithiocarbamate (NaDEC), TETD, bovine erythrocyte copper/zinc superoxide dismutase (SOD), acetone, hypochlorous acid (HClO), iodine, hydrogen peroxide, trichloroacetic acid (TCA), formic acid (FA), phosphate-buffered saline (PBS), acetonitrile (ACN), cobalt (II) chloride, copper (II) chloride, barium (II) chloride, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione, oxidized glutathione, cysteine, cystine, cysteamine, bovine serum albumin (BSA), dithiothreitol (DTT), and hexyl cinnamic aldehyde (HCA) were acquired from Sigma Aldrich (St Louis, MO, USA). Tritiated thymidine (^{3}H-TdR, specific activity 2 Ci/mmol) was from Dupont NEN (Waltham, MA, USA) and scintillation fluid from PerkinElmer (Waltham, MA, USA). Microdialysis tubing (molecular weight cut-off 3.5 kDa) was from Pierce (Rockford, IL, USA). All solutions were prepared using nanopure water (Milli-Q; Millipore, Bedford, MA, USA).

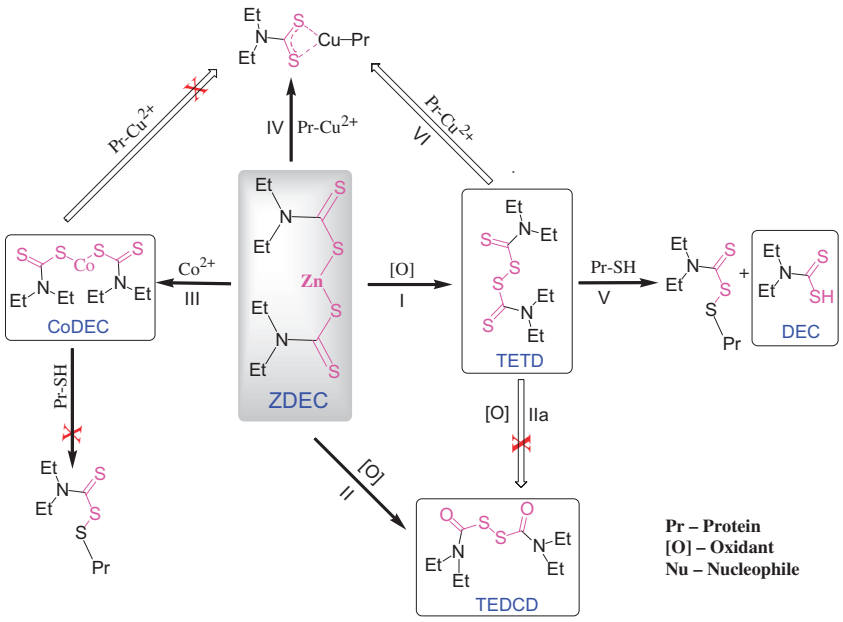


Fig. 1. Summary of the chemical oxidation pathways of ZDEC and possible protein haptenation pathways. Oxidation of ZDEC to TETD (I) with subsequent protein thiol (Pr-SH)-induced reduction of TETD results in protein haptenation via mixed disulfide formation (V). Oxidative desulfuration (II) produces electrophilic carbonyl centres on TEDCD, which are hypothesized to covalently bind to nucleophilic protein residues. ZDEC reacts directly with metalloproteins by chelating the metal ions, resulting in strong co-ordinate bonds. Crossed-out arrows represent non-viable pathways as demonstrated by experimental data. CoDEC, cobalt (II) dithiocarbamate; DEC, diethyldithiocarbamate; TEDCD, tetraethyldicarbamoyl disulfide; TETD, tetraethylthiuram disulfide; ZDEC, zinc diethyldithiocarbamate.

Cobalt (II) dithiocarbamate (CoDEC) was synthesized directly from NaDEC and $CoCl_2$ using a previously reported method (17). Tetraethyldicarbamoyl disulfide (TEDCD) was prepared from 0.1 g/ml NaDEC with 10% (v/v) HClO. The reactants were stirred at 37°C for 15 hr. The resultant precipitate was filtered, washed with ice-cold water, and dried. High-performance liquid chromatography (HPLC) and mass spectrometry (MS) were used to characterize the product before use in the LLNA.

Animals

Female BALB/c mice were purchased from Taconic (Hudson, NY, USA). Animals were 6–8 weeks old upon arrival and allowed to acclimate for a minimum of 10 days. Animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility at National Institute for Occupational Safety and Health (NIOSH), Morgantown, WV, USA. Animals were housed under controlled environmental conditions in High Efficiency Particulate Act (HEPA)-filtered ventilated polycarbonate cages on autoclaved hardwood beta-chip bedding and provided food and autoclaved tap water *ad libitum*. All animal procedures were reviewed and approved by the NIOSH Animal Care and Use Committee.

Experimental Procedures

Instrumentation

Spectrophotometric characterization of ZDEC, TETD, free thiols, oxidation reactions, and protein conjugation was carried out on a Beckman (Fullerton, CA, USA) DU 800 spectrophotometer with a VWR Scientific (Niles, IL, USA) circulating water bath for temperature control.

HPLC analysis was performed on a Shimadzu Prominence system equipped with an SPD-M10A photodiode array detector (PDA) (Shimadzu, Columbia, MD, USA). Separations were performed using a gradient of 5–80% ACN over 20 min at a flow rate of 1 ml/min through a Supelco Discovery (Bellefonte, PA, USA) C18 column (5 μm particle size, 250 mm × 4.6 mm). The column was presaturated with ZDMC for all ZDEC, NaDEC, and TETD analyses as previously described (1). Absorbance at 260 and 420 nm were monitored, and in some cases, the maximum spectral scan mode was employed.

All mass spectra were acquired using a Micromass QTOF-II (Waters Corp., Milford, MA, USA) quadrupole time-of-flight mass spectrometer. Analytes were dissolved in a 50/50 ACN/1% FA mixture and analysed using positive mode electrospray ionization (+ESI). Nitrogen was used as the desolvation gas. Tandem mass spectrometry (MS/MS) data were generated via collision-induced dissociation in argon. The resultant mass spectra for the ZDEC–Cu/Zn–SOD reaction were charge deconvoluted using maximum entropy calculations performed in MASSLYNX (MaxEnt1). The output range was 10–35 kDa at a resolution of 1.0 Da/channel.

Chemistry

Oxidation reactions were carried out by mixing 1 μM ZDEC with a 1- to 10-fold excess of oxidant in 50/50 ACN/H_2O with continuous stirring at 37°C. Oxidants used were ClO^-, H_2O_2, and I_2. Serial spectral scans at 260 nm were performed to observe the loss of ZDEC, and aliquots of the reaction solutions were taken for HPLC-PDA analysis. MS was performed on fractions collected from the HPLC column after diluting each fraction 10^5-fold in 50/50 ACN/0.1% FA.

Formation of mixed disulfides by reduction of TETD was performed by reacting 0.01 mM cysteine or glutathione with 0.01 mM TETD in 50/50 ACN/H_2O with constant stirring for 15 min at 37°C. Fractions from the HPLC column were collected and diluted 10^5-fold in 50/50 ACN/0.1% FA for MS analysis. BSA, which was reduced with DTT according to a previously used method to afford a single reduced thiol on the BSA (BSA–Cys34) (18), was reacted with TETD by incubating 0.8 mg/ml BSA with 0.5 mM TETD in 50 mM ammonium bicarbonate (NH_4HCO_3) buffer (pH 7.4) for 1 hr on a shaker at 37°C. HPLC and MS samples were obtained for the identification and characterization of diethyldithiocarbamate (DEC) and DEC–Cys34–BSA mixed disulfides after dialysis (molecular mass cut-off of 3500 Da) in 50 mM NH_4HCO_3. Loss of the BSA–Cys34–DTNB reactivity (18) was also used to determine loss of thiol reactivity as a result of TETD binding. As controls, BSA thiol concentrations were determined for (i) fresh BSA in buffer that was not incubated or dialysed, (ii) BSA (in the absence of TETD) that had been incubated under the same conditions (as the TETD-treated BSA) and dialysed, and (iii) BSA that had been reduced by DTT and dialysed.

Reaction of ZDEC/TETD with cobalt/copper ions

Transmetallation reactions were performed by reacting 10 μM ZDEC with a onefold to fivefold excess of either $CoCl_2$ or $CuCl_2$ in ACN at room temperature. Chelation of Co(II) and Cu(II) ions with 10 μM TETD was allowed to proceed for

2 days in ACN with HPLC-PDA analysis being performed at intervals. Absorbance measurements were made at 320 nm (CoDEC) and 420 nm (CuDEC). Reactions were performed in sealed test tubes to avoid loss of ACN. MS was used to identify the transmetallation and complexation products.

ZDEC–Cu/Zn–SOD reaction

Haptenation of SOD was performed by incubating 0.16 mg/ml SOD solution with 0.06 mM ZDEC in 50 mM NH_4HCO_3 buffer (pH 7.4, for 1 hr on a shaker at 37°C). A control consisting of SOD in the buffer was also incubated. Dialysis of the reaction solution and control was performed overnight using 3.5 kDa molecular weight cut-off microdialysis tubing against 2 L of 50 mM NH_4HCO_3 with two buffer changes. Absorbance measurements at 420 nm were performed prior to and after dialysis. HPLC and MS samples were obtained after dialysis.

Local lymph node assay

To determine the allergenic potency of test chemicals, the LLNA was performed according to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) standard method (19). After grouping into homogeneous groups ($n = 5$) based on their weight, mice were dosed with 25 μl of a test chemical [ZDEC: 0.5%, 1%, and 5%; NaDEC: 0.5%, 2.5%, and 5%; CoDEC: 0.5%, 2.5%, and 7.5%; TETD: 0.5%, 2.5%, and 7.5%; and TEDCD: 0.5%, 2.5%, and 7.5%; the positive control, 30% HCA or the vehicle (acetone)] applied on the dorsum of both ears. About 0.2 ml of 20 μCi ^{3}H-TdR in 0.01 M PBS was injected into the tail vein on D6, and after 5 hr, the mice were euthanized via CO_2 inhalation. Left and right draining auricular lymph nodes were excised and pooled for each animal. Single cell suspensions were made, and following overnight incubation in 5% TCA, samples were counted using a Packard Tri-Carb 2500TR (Meriden, CT, USA) liquid scintillation analyzer with subtraction of the background. Stimulation indices (SI) were calculated by dividing the mean disintegrations per minute (DPM) per test group by the mean DPM for the vehicle control group. Three concentrations of each test chemical were evaluated by the linear interpolation approach (20) to obtain effective concentration (EC3) inducing a threefold SI over the vehicle control. At 30%, the HCA positive control resulted in an average SI value of 9.47.

Statistical analysis

Evaluation of data for whole animal experiments was conducted with SIGMASTAT 3 from Systat Software, Inc. (San Jose, CA, USA). Data were analysed by one-way ANOVA followed by a *post hoc* Bonferroni test, and data were considered significant at $P < 0.05$.

Results

Oxidation of ZDEC

The direct ZDEC oxidation pathways leading to protein haptenation that were evaluated are pathways I, II, and IIa in Fig. 1. ZDEC was reacted with iodine, hydrogen peroxide, and hypochlorous acid (bleach, HOCl) and found to be oxidized to TETD and two thiocarbamoyl disulfides, tetraethylthiocarbamoyl disulfide (TETCD) and TEDCD. All oxidants reacted rapidly with ZDEC to afford the oxidation products that were common to all oxidants, although the stoichiometry differed. The oxidative transformation of ZDEC depended on the ratio of

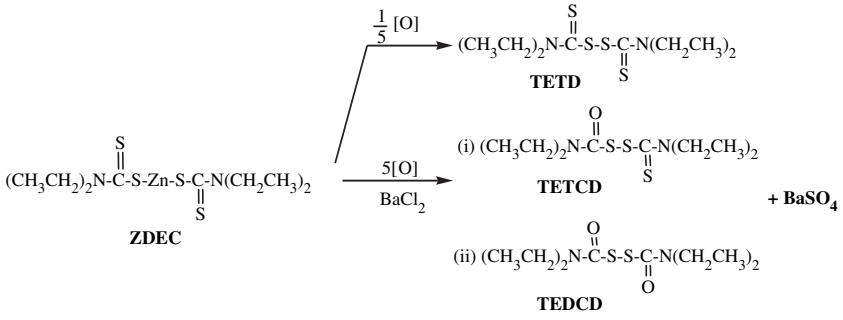


Fig. 2. Hydrogen peroxide, iodine, and bleach were all able to oxidize ZDEC. Depending on the ZDEC-to-oxidant ratio, the products were TETD, TETCD, and TEDCD. $BaCl_2$ was used to confirm the oxidative desulfuration on the thiocarbamates. $BaCl_2$, barium chloride; $BaSO_4$, barium sulfate; TEDCD, tetraethyldicarbamoyl disulfide; TETCD, tetraethylthiocarbamoyl disulfide; TETD, tetraethylthiuram disulfide; ZDEC, zinc diethyldithiocarbamate.

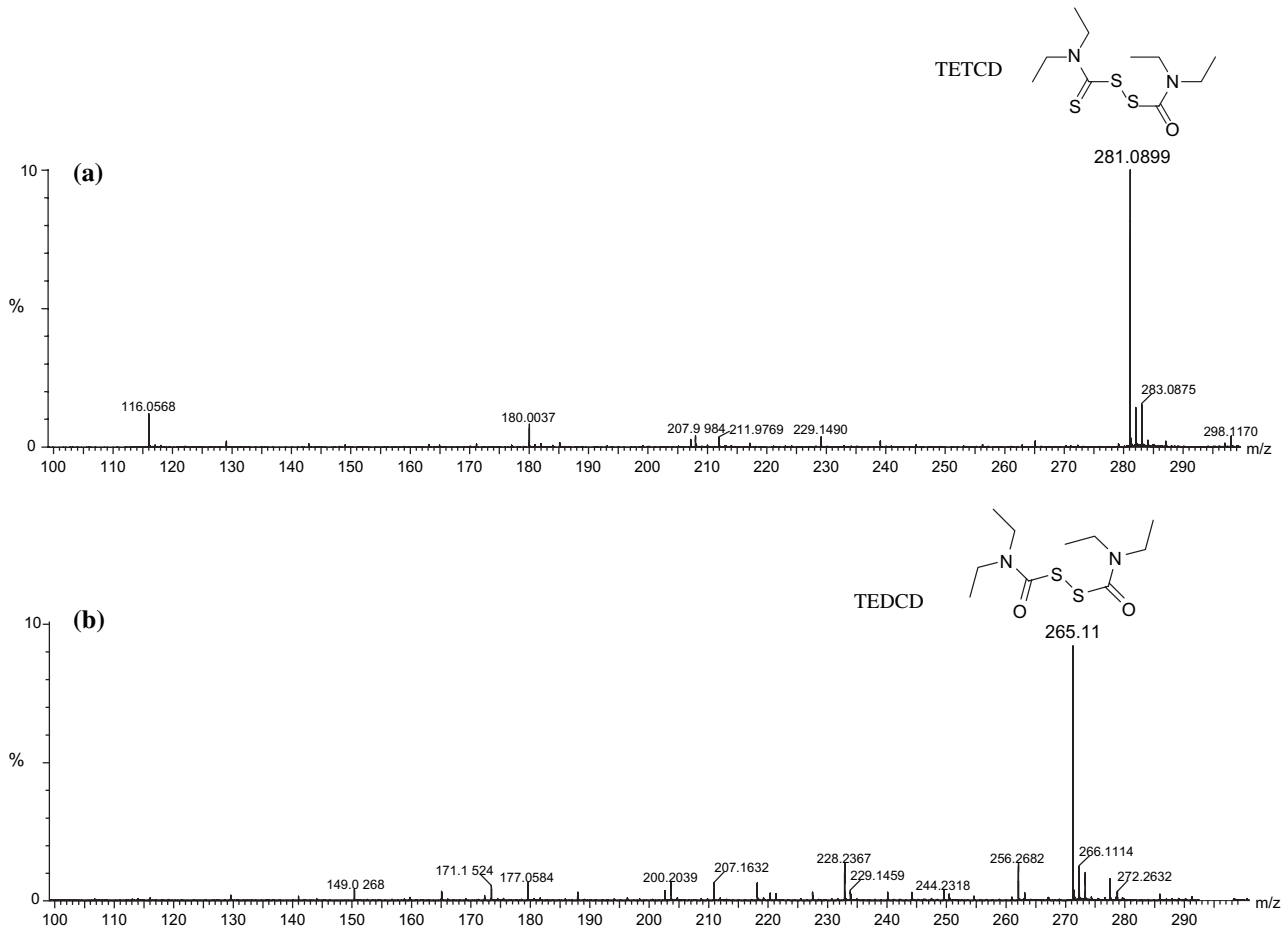


Fig. 3. Mass spectrometry of the high-performance liquid chromatography fractions of 1 μM ZDEC reacted with a fivefold excess of HOCl. ZDEC is oxidized to (a) TETCD (*m*/*z* 281.09) and (b) TEDCD (*m*/*z* 265.11) showing the complete oxidation (on the S atom) and cleavage of the CS bond to yield a CO on one and both sides of the disulfide bond. HOCl, hypochlorous acid; TEDCD, tetraethyldicarbamoyl disulfide; TETCD, tetraethylthiocarbamoyl disulfide; ZDEC, zinc diethyldithiocarbamate.

ZDEC:oxidant as indicated in reaction scheme shown in Fig. 2. Figure 3 shows the MS results for the HPLC fractions of 1 μM ZDEC that had been reacted with a fivefold excess of HOCl. Figure 3a with a *m*/*z* of 281.09 shows the complete oxidation (on the S atom) and cleavage of the C=S bond to yield a C=O on one side of the disulfide bond (=TETCD), and Fig. 3b with *m*/*z* 265.11 shows the same oxidation on both S atoms of the C=S bonds on both sides of the disulfide bridge of TETD (=TEDCD). Thus, excess oxidant resulted in the extrusion of S from the thiocarbamate bond as the sulfate ion. Barium chloride ($BaCl_2$) addition resulted in quantitative formation of the insoluble barium sulfate ($BaSO_4$) from reaction with free sulfate ions, thus confirming the complete oxidation on the S atom and cleavage of the C–S bond. These results demonstrate that the S atoms of C–S moiety are vulnerable to oxidation and subsequent substitution by O atoms (Fig. 1, pathway II). Interestingly, direct oxidation of TETD to TETCD or TEDCD was not observed, suggesting that Zn may be required to catalyse this reaction (Fig. 1, pathway IIa).

Mixed disulfide haptenation through non-enzymatic reduction of TETD

TETD was readily reduced by BSA–Cys34, cysteine, glutathione (GSH), and cysteine ethyl ester (CEE) to afford DEC, a mixed disulfide and the disulfide of the corresponding thiol (Fig. 1, pathway V). When the thiols and their corresponding disulfides were incubated with ZDEC (data not shown) under the same conditions, there was no formation of mixed disulfides, indicating that mixed disulfide hapten formation requires ZDEC to be initially oxidized to TETD. MS results (Fig. 4) demonstrate the formation of DEC–S–S–cysteine with an *m*/*z* of 269.19. Other ions observed in the mass spectrum include DEC (*m*/*z* 148.10), cystine (*m*/*z* 241.16), and unreacted

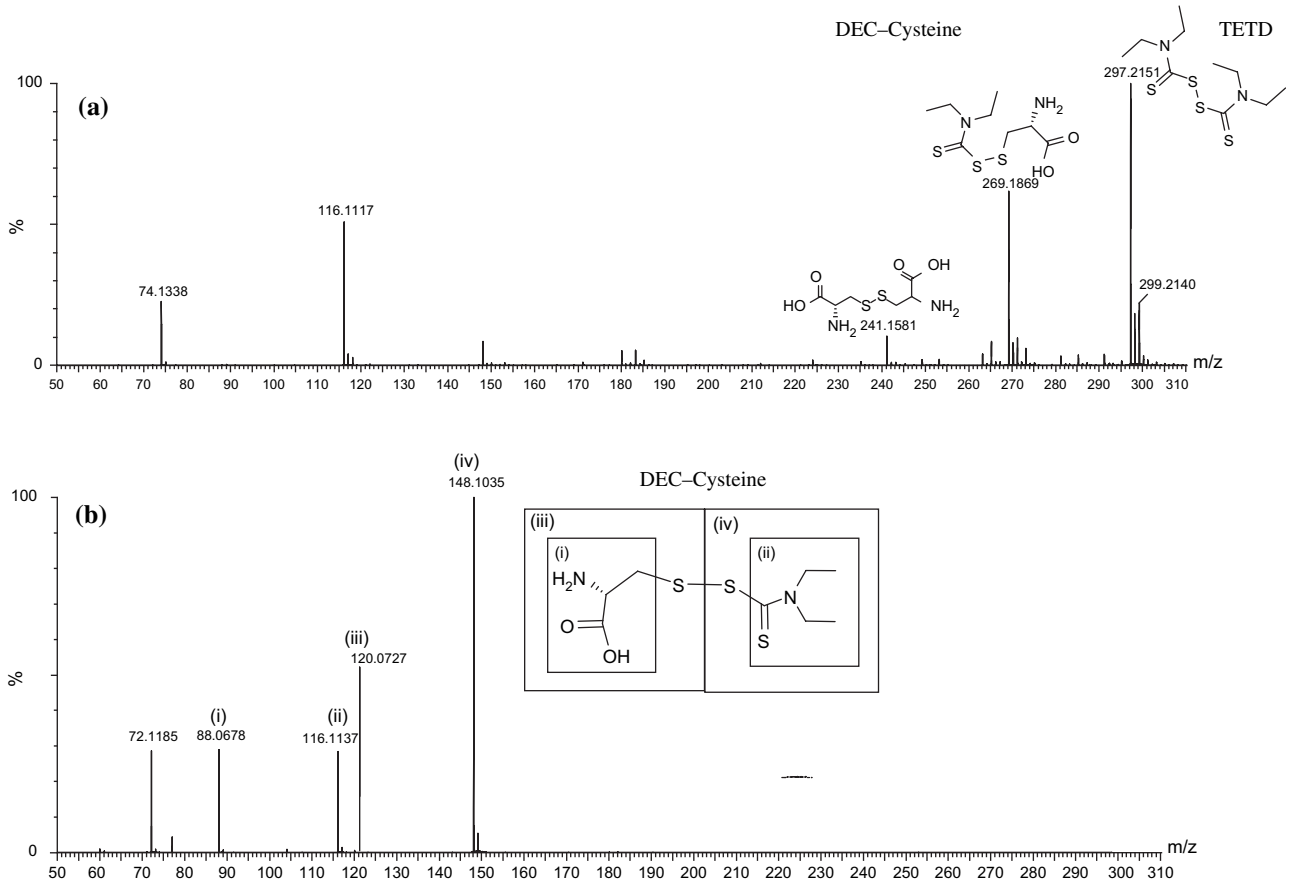


Fig. 4. Quadrupole time-of-flight mass spectrometry of the TETD–cysteine reaction. Mixed disulfide formation results in the DEC–cysteine adduct (a) (*m*/*z* 269.19). Other ions in the reaction include DEC (*m*/*z* 148.10), cystine (*m*/*z* 241.16), and unreacted TETD (*m*/*z* 297.22). Tandem mass spectrometry of the DEC–cysteine adduct ion (*m*/*z* 269.19) as shown in (b) resulted in fragment ions of both DEC (*m*/*z* 148.10) and cysteine (*m*/*z* 120.07), confirming the ion as the mixed disulfide. DEC, diethyldithiocarbamate; TETD, tetraethylthiuram disulfide.

TETD (*m*/*z* 297.22). MS/MS of the ion at *m*/*z* 269.19 [Fig. 4b] resulted in fragment ions because of both DEC (*m*/*z* 148.10) and cysteine (*m*/*z* 120.07), confirming the ion as the mixed disulfide. The DEC–cysteine adduct was thus confirmed as a major product of the thiol-induced reduction of TETD, suggesting the formation of mixed disulfides between the DEC and the thiols. The presence of cystine was a result of the oxidation of cysteine by TETD.

Reaction of TETD with BSA resulted in the blocking of the reduced Cys34 residue on BSA. Figure 5(c) shows the reaction between the reduced BSA–Cys34 with the thiol-specific reagent, DTNB, to produce chromophore, 2-nitro-5-thiobenzoic acid, which absorbs at 412 nm. The Cys34 reactivity to DTNB was lost when BSA was preincubated with TETD at the physiological pH [Fig. 5(b)]. Appropriate controls showed that the blockage of Cys34 was only possible with the disulfide TETD and not DEC. The Cys34–DEC bond was shown to be strong as there was still no reactivity to DTNB after extensive dialysis of the TETD-treated BSA. The BSA–Cys34 was able to reduce TETD in the same way as free cysteine, and covalent modification of the Cys34 by TETD was via mixed disulfide formation.

Reaction of ZDEC/TETD with cobalt/copper ions

To understand the interaction of ZDEC with physiologically relevant metal ions, the kinetics of the complexation reaction between Cu(II)/Co(II) and ZDEC/TETD was studied. The progress of the TETD–Cu(II) and TETD–Co(II) reactions was very slow, taking about 2 days to reach completion at room temperature compared with that of ZDEC with both Cu(II) and Co(II), which was complete in less than 10 seconds. Yellow- and green-coloured complexes were obtained with Cu(II) and Co(II), respectively, when they were incubated with either ZDEC or TETD. The CuDEC had an absorbance peak at 420 nm, whereas the CoDEC complex absorbed at 320 nm. HPLC separation with MS analysis

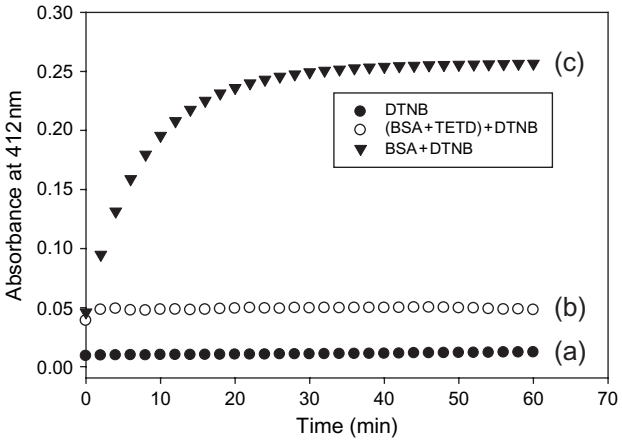


Fig. 5. Reaction of Cys34 on BSA with DTNB: (a) 0.5 mM DTNB, (b) 0.8 mg/ml BSA preincubated with 0.5 mM TETD reacted with 0.5 mM DTNB, (c) 0.8 mg/ml BSA + 0.5 mM DTNB. TETD was able to block the free thiol on Cys34. BSA, bovine serum albumin; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); TETD, tetraethylthiuram disulfide.

confirmed formation of CuDEC and CoDEC, irrespective of whether the metal ion was incubated with ZDEC or TETD. A fast transmetallation reaction where Zn was substituted with either Cu(II) or Co(II) was the predominant reaction for ZDEC. Where TETD was used, instead, it is possible that the disulfide bond on TETD was amenable to reduction with either residual Cu(I) or Co(II), thus availing two DEC ligands that chelated the metal ions.

ZDEC–Cu/Zn–SOD reaction

The addition of 0.06 mM ZDEC to 0.16 mg/ml (≈10.26 μM) SOD resulted in the development of a yellow colour similar in appearance to the complex that was formed between ZDEC and $CuCl_2$ (Fig. 1, pathways IV and VI). Reaction of ZDEC with SOD produced a product with an absorption peak at 420 nm similar to that following Cu^{2+} transmetallation of ZDEC. The ZDEC–SOD reaction product was consistent with the incorporation of DEC by way of a co-ordinate bond formed when a SOD-bound copper ion displaced the zinc on ZDEC. The apoenzyme (metal free) did not react with ZDEC under similar conditions, suggesting that adduct formation between ZDEC and SOD was via the chelation of the SOD copper ion. Modification of the copper-containing active site of the monomeric SOD by ZDEC was confirmed by positive ESI-MS after dialysis against the NH_4HCO_3 buffer (pH 7.4). As shown in Fig. 6 a and b, deconvolution of the multiply charged mass spectra of SOD and ZDEC-treated SOD yields molecular masses of 15 588 and 15 736 Da, respectively. This mass shift of 149 Da, absent in the control SOD (which was dialysed but not treated with ZDEC), corresponds to the addition of one DEC to the SOD. The fact that chelation of the SOD copper by ZDEC resulted in a stable hapten–protein complex was also evident by the yellow colour of the SOD–DEC adduct, which persisted after extensive dialysis. Subsequent absorbance

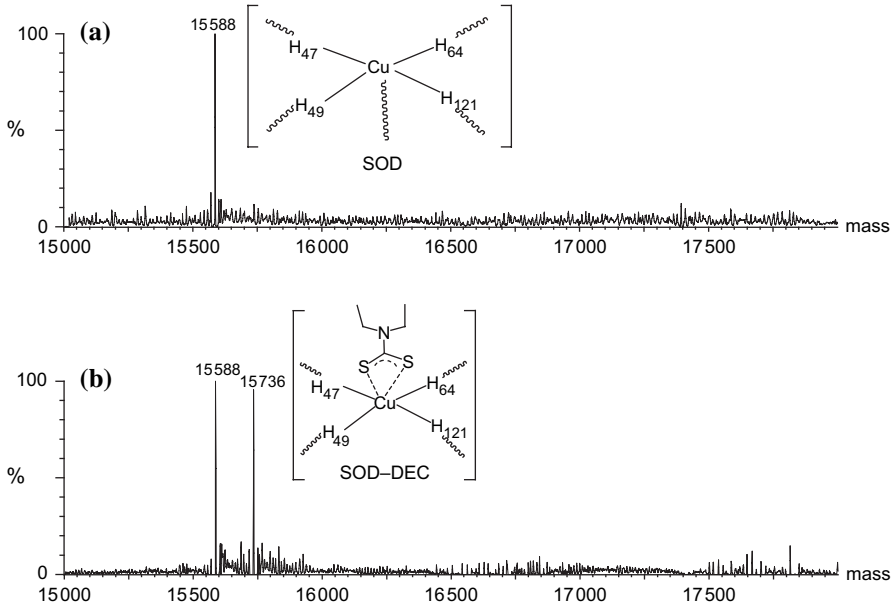


Fig. 6. Deconvolution of the multiply charged mass spectra of SOD and ZDEC-treated SOD. Incubation of (a) 0.16 mg/ml SOD and (b) 0.16 mg/ml SOD + 0.06 mM ZDEC in an ammonium carbonate buffer (pH 7.4) with subsequent dialysis yielded molecular masses of 15 588 and 15 736 Da, respectively. The mass shift of 149 Da, absent in the control SOD, was consistent with the addition of 1 mol of DEC to the monomeric SOD. DEC, diethyldithiocarbamate; SOD, superoxide dismutase; ZDEC, zinc diethyldithiocarbamate.

measurements after dialysis did not show any significant removal of the DEC from the SOD. There was no change in reactivity of the reduced SOD thiols to DTNB when it was used to probe the possibility of a reaction between the ZDEC and the sulfyhydryl groups (Cys6 and Cys111) on SOD. These findings were in agreement with our observation of the lack of reactivity of ZDEC and cysteine, glutathione or BSA–Cys34.

LLNA: allergenicity assessment of ZDEC and its derivatives

Reaction products of ZDEC oxidation and transmetallation were identified and dermal sensitization by these products evaluated using the LLNA. No overt irritation, as indicated by ear swelling and redness, was observed following application of the highest soluble concentrations of the test chemicals (5% for ZDEC and NaDEC and 7.5% for TETD, TEDCD, and CoDEC). Exposed animals showed neither significant changes in body weight and behaviour nor clinical signs, suggesting that exposure did not induce systemic toxicity. The EC3 values were calculated from the SI values (20). ZDEC, TETD, NaDEC, and TEDCD induced dose-dependent lymph node cell proliferation (Fig. 7). CoDEC, which was used to prevent transmetallation of DEC onto metalloproteins or oxidation to form mixed disulfides with proteins (Fig. 1, pathway III), did not produce statistically significant ($P = 0.17$) changes in lymphocyte proliferation at 0.5%, 2.5%, and 7.5% concentrations. Figure 8 summarizes the structural formulae of the test chemicals used for comparison and their activity in the LLNA as judged by ^{3}H-TdR incorporation and the derived EC3 values. Comparison of the sensitizing potential of CoDEC and TEDCD to the known sensitizers ZDEC and TETD (based on EC3 values), respectively, identified CoDEC as a non-sensitizer and TEDCD as a stronger sensitizer than TETD ($P = 0.02$).

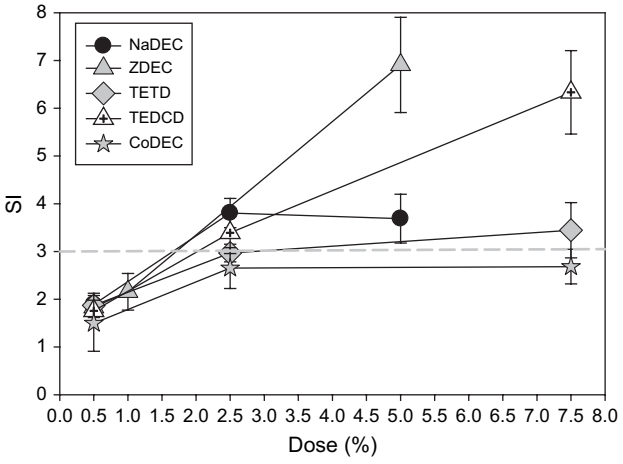


Fig. 7. Lymph node cell proliferation measured as stimulation index (SI). ZDEC, NaDEC, TETD, and TEDCD induced dose-dependent responses, which were statistically significant with P values 0.04, 0.003, 0.035, and 0.0004, respectively. CoDEC, however, did significant stimulate lymph node cell proliferation in 0.5%, 2.5%, and 7.5% dosed mice ($P = 0.17$). CoDEC, cobalt (II) dithiocarbamate; NaDEC, sodium diethyldithiocarbamate; TEDCD, tetraethyldicarbamoyl disulfide; TETD, tetraethylthiuram disulfide; ZDEC, zinc diethyldithiocarbamate.

Discussion

The use of gloves in occupational settings often exposes the vulcanization accelerators within the glove matrix to oxidizing chemicals with potential resultant chemical transformation of the accelerators. Thus, oxidation products of these accelerators need to be considered when examining potential pathways of allergenicity. A recent study (18) in our laboratory demonstrated that mercaptobenzothiazole (MBT, a thiol-based vulcanization accelerator)-containing gloves exposed to bleach result in the conversion of MBT to the disulfide. Both chemistry (protein binding) and animal allergenicity studies point to the disulfide as the active allergenic form of MBT (18, 21). Similar to the potential for oxidation of MBT, the quantitative oxidation of ZDEC (to TETD, TETCD, and TEDCD) using I_2, H_2O_2, and HClO was observed.

The LLNA results, together with the chemical reactivity data, suggest that ZDEC may haptenate proteins either directly or indirectly through NaDEC, TETD, or TEDCD. The observed oxidation of ZDEC to TETD, TETCD, and TEDCD provides indirect pathways through which ZDEC can haptenate proteins. As outlined in Fig. 1, pathway V, TETD can be reduced by both free and protein thiols resulting in mixed disulfide formation and release of a DEC molecule. The reduction of TETD in blood plasma has been previously reported (22). The role of mixed disulfide formation in protein haptenation leading to skin sensitization has only been investigated in a few cases with MBT (18), lipoic acid (23), and diallyl disulfide from garlic (24). BSA, which is 80% homologous to human serum albumin (HSA), was used as an analytical tool to examine protein–TETD disulfide formation. Approximately 40% of extravascular HSA is located in the skin (25), but it is probably not the only dermal protein that can be haptenated through formation of disulfide bonds.

Hepatic metabolism of TETD has been reported in the literature as it is used clinically

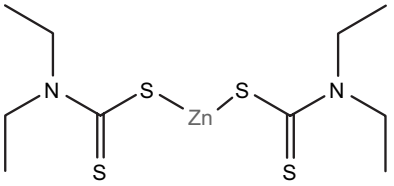
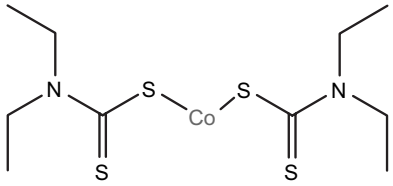
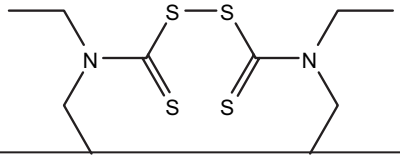
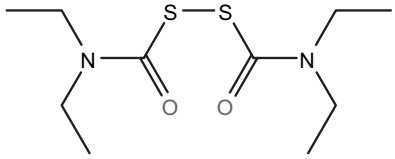
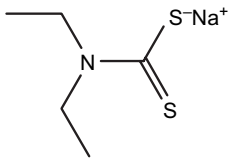
Name	Structure	LLNA EC3 value (%)
ZDEC		1.01
CoDEC		>7.5
TETD		5.42
TEDCD		1.70
NaDEC		1.66

Fig. 8. Structural formulae of the test chemicals used for comparison of their activity in the LLNA as judged by ^{3}H-TdR incorporation and the derived EC3 values. ZDEC, NaDEC, TEDCD, and TETD were all sensitizers. CoDEC did not elicit a significant lymph node cell proliferative response at the concentration tested. CoDEC, cobalt (II) dithiocarbamate; EC, effective concentration; ^{3}H-TdR, tritiated thymidine; LLNA, local lymph node assay; NaDEC, sodium diethyldithiocarbamate; TEDCD, tetraethyldicarbamoyl disulfide; TETD, tetraethylthiuram disulfide; ZDEC, zinc diethyldithiocarbamate.

(as disulfiram) for the treatment of alcoholism (14, 26–31). Oxidative desulfuration, similar to the direct oxidation performed with ZDEC in this study, was observed with rat and human liver microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate (26). The release of DEC from TETD with its subsequent bioactivation through metabolic pathways affords an electrophilic sulfoxide capable of covalently modifying lysine and histidine residues on proteins (27, 28). While hepatic metabolism may not be indicative of the same metabolic processes occurring in the skin, multiple human P-450 enzymes (CYP3A4, CYP1A2, CYP2A6, and CYP2D6) present in both organs (32) are able to metabolize DEC methyl ester, a metabolite of TETD, to S-methyl-*N*,*N*-diethylthiolcarbamate sulfoxide (27). Thus, the potential for ZDEC and TETD bioactivation within the skin cannot be totally excluded. However, the contributory effect of epidermal bioactivation to ZDEC and TETD allergenicity may be minimal given the fact that after reduction of TETD, thiol methyltransferase (TMT)-mediated methylation of one of the thiols on DEC is required prior to sulfoxidation (28). The role of TMT has been outlined in liver (33), but to date, no TMT activity has been reported in the skin (32). The other prevalent metabolic (hepatic) mechanism, which avails an alkyl isothiocyanate capable of acylating nucleophilic protein residues (34), is not possible with ZDEC in both the liver and the skin because of hindrance by the two ethyl groups on the nitrogen atom. Decomposition or metabolism to isothiocynates is only possible when one of the ethyl groups is substituted by a hydrogen atom (35).

The inclusion of CoDEC in the LLNA and reaction of SOD with ZDEC were important in the exploration of transmetallation as a protein haptenation mechanism (Fig. 1, pathway IV). Cobalt, copper, and iron exist in the physiological environment either as free or as bound ions, which can readily transmetallate with zinc on ZDEC. The specificity of the chelation of copper bound on the active site of the monomeric SOD when reacted with ZDEC combined with lack of ZDEC reactivity with reduced Cys6 and Cys111 on SOD supports yet another haptenation mechanism. Dialysis did not remove either the DEC or the Cu(II) ion as a DEC–Cu(II) complex, demonstrating the strength of the DEC–Cu(II) bond as well as the stability of the co-ordination between Cu(II) and the four histidines (His44, His46, His61, and His118) on SOD. Previous SOD inhibition studies have also suggested the stability of the SOD–DEC adduct where the CuDEC could not be extracted from the inactivated SOD with organic solvents (36, 37). In a recent review, co-ordinate bonds have been described to have bond strengths (200–420 kJ/mol) comparable to covalent bonds (38). Chelation of Cu(II) on SOD was not observed when CoDEC was used instead of ZDEC, suggesting that CoDEC is a stable complex with limited reactivity in the presence of competing metal ions. The lack of an observable CoDEC-induced immune proliferative response in the LLNA is in agreement with the chemistry of CoDEC in the presence of SOD and confirms that ZDEC's allergenicity requires haptenation either through metalloprotein chelation or through one of its oxidation products.

It is appealing to simply explain the greater potency of TEDCD (EC3 = 1.70) relative to TETD (EC3 = 5.4) in terms of a more prevalent nucleophile–electrophile interaction. TETD can only modify proteins through mixed disulfide formation with cysteinyl residues, which are less abundant compared with nucleophilic lysines and arginines, or possibly through metabolism to a sulfoxide intermediate. Oxidative desulfuration on the thiocarbamate of TETD seems to impart greater potency relative to TETD, while transmetallation of Zn(II) with Co(II) results in a less reactive CoDEC complex. Paradoxically, TEDCD, while displaying greater potency in the LLNA than TETD, was not subject to haptenation through mixed disulfides or metal chelation as attempts to either reduce the disulfide bond on TEDCD or chelate the Cu(II) and Co(II) ions were not successful. This was in agreement with previous studies (39) where carbamoyl disulfides were shown to be poor ligands relative to thiurams with the central S=S bond in carbamoyl disulfides being much stronger than the corresponding S=S bond in thiuram disulfides. It is worth pointing out that apart from demonstrating the greater potency of TEDCD and enhanced stability of the TEDCD disulfide bond, a direct TEDCD–protein reaction (to albumin) was not observed. Further investigations on CYP-mediated sulfoxidation of TEDCD (27) are warranted and would be insightful in determining whether TEDCD is a direct hapten or a prohapten, requiring metabolic activation to exert its immunogenicity.

In conclusion, formation of mixed disulfides, chelation of metals on metalloproteins, and possibly nucleophilic addition of amino acids onto electrophilic carbamoyl compounds following metabolism are probable protein haptenation mechanisms in ZDEC allergenicity. Given the current data, the relatively greater potency of ZDEC versus TETD in the LLNA may be because of both covalent binding through its oxidative products and direct non-covalent co-ordinate binding.

Acknowledgements

This study was supported by an interagency agreement with the National Institute of Environmental Health Sciences (NIEHS) (Y1-ES0001-06) and received partial funding from NSF grant number CHE 0614924. The findings and conclusion in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

References

1. Depree G J, Bledsoe T A, Siegel P D. Determination of zinc dialkyldithiocarbamates in latex condoms. *J Chromatogr Sci* 2004: 42: 80–84.
2. Nicolaysen P H, Klink K J, Shriver E et al. Local and systemic toxicity in mice following subcutaneous implantation of latex penrose drains. *J Toxicol Cutaneous Ocul Toxicol* 2004: 23: 233–248.
3. Depree G J, Bledsoe T A, Siegel P D. Survey of sulfur-containing rubber accelerator levels in latex and nitrile exam gloves. *Contact Dermatitis* 2005: 53: 107–113.
4. Bergendorff O, Persson C, Hansson C. High-performance liquid chromatography analysis of rubber allergens in protective gloves used in health care. *Contact Dermatitis* 2006: 55: 210–215.
5. Duarte I, Terumi N J, Lazzarini R. Hand eczema: evaluation of 250 patients. *Am J Contact Dermat* 1998: 9: 216–223.
6. Estlander T, Jolanki R, Kanerva L. Dermatitis and urticaria from rubber and plastic gloves. *Contact Dermatitis* 1986: 14: 20–25.
7. Kaniwa M, Isama K, Nakamura A et al. Identification of causative chemicals of allergic contact dermatitis using a combination of patch testing in patients and chemical analysis. Application to cases from rubber gloves. *Contact Dermatitis* 1994: 31: 65–71.
8. Geier J, Lessmann H, Uter W, Schnuch A. Occupational rubber glove allergy: results of the Information Network of

Departments of Dermatology (IVDK), 1995-2001. *Contact Dermatitis* 2003: 48: 39–44.
9. Bergendorff O, Hansson C. Contact dermatitis to a rubber allergen with both dithiocarbamate and benzothiazole structure. *Contact Dermatitis* 2007: 56: 278–280.
10. Knudsen B B, Menne T. Contact allergy and exposure patterns to thiurams and carbamates in consecutive patients. *Contact Dermatitis* 1996: 35: 97–99.
11. Ryan C A, Gerberick G F, Gildea L A et al. Interactions of contact allergens with dendritic cells: opportunities and challenges for the development of novel approaches to hazard assessment. *Toxicol Sci* 2005: 88: 4–11.
12. Johnson D J, Amarnath V, Amarnath K, Valentine H, Valentine W M. Characterizing the influence of structure and route of exposure on the disposition of dithiocarbamates using toluene-3,4-dithiol analysis of blood and urinary carbon disulfide metabolites. *Toxicol Sci* 2003: 76: 65–74.
13. Johnson D J, Graham D G, Amarnath V, Amarnath K, Valentine W M. Release of carbon disulfide is a contributing mechanism in the axonopathy produced by N,N-diethyldithiocarbamate. *Toxicol Appl Pharmacol* 1998: 148: 288–296.
14. Tonkin E G, Valentine H L, Zimmerman L J, Valentine W M. Parenteral N,N-diethyldithiocarbamate produces segmental demyelination in the rat that is not dependent on cysteine carbamylation. *Toxicol Appl Pharmacol* 2003: 189: 139–150.
15. Edwards B S, Merritt J A, Jelen P A, Borden E C. Effects of diethyldithiocarbamate, an inhibitor of interferon antiviral activity, upon human natural killer cells. *J Immunol* 1984: 132: 2868–2875.
16. Morpurgo L, Desideri A, Rigo A, Viglino P, Rotilio G. Reaction of N,N-diethyldithiocarbamate and other bidentate ligands with Zn, Co and Cu bovine carbonic anhydrases. Inhibition of the enzyme activity and evidence for stable ternary enzyme-metal-ligand complexes. *Biochim Biophys Acta* 1983: 746: 168–175.
17. Eagle C T, Farrar D G, Holder G N, Gooden D M, Goodman A B, Wyatt S W. Synthesis and characterization of Tris(diethyldithiocarbamato) cobalt(III) as an undergraduate inorganic laboratory. *Chem Educ* 2000: 5: S1430–S4171 (99)03301-1.
18. Chipinda I, Hettick J M, Simoyi R H, Siegel P D. Oxidation of 2-mercaptobenzothiazole in latex gloves and its possible haptenation pathway. *Chem Res Toxicol* 2007: 20: 1084–1092.
19. Haneke K E, Tice R R, Carson B L, Margolin B H, Stokes W S. ICCVAM evaluation of the murine local lymph node assay. Data analyses completed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods. *Regul Toxicol Pharmacol* 2001: 34: 274–286.
20. Basketter D A, Lea L J, Dickens A et al. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. *J Appl Toxicol* 1999: 19: 261–266.
21. Chipinda I, Siegel P D, Zhang X D, Simoyi R H. Role of the thiol group in MBT's allergenicity. *Cutan Ocul Toxicol* 2008: 27: 103–116.
22. Cobby J, Mayersohn M, Selliah S. The rapid reduction of disulfiram in blood and plasma. *J Pharmacol Exp Ther* 1977: 202: 724–731.
23. Bergqvist-Karlsson A, Thelin I, Bergendorff O. Contact dermatitis to alpha-lipoic acid in an anti-wrinkle cream. *Contact Dermatitis* 2006: 55: 56–57.
24. Jappe U, Bonnekoh B, Hausen B M, Gollnick H. Garlic-related dermatoses: case report and review of the literature. *Am J Contact Dermat* 1999: 10: 37–39.
25. Peters T Jr. *All About Albumin: Biochemistry, Genetics and Medical Applications*, San Diego, Academic Press, 1996.
26. Madan A, Williams T D, Faiman M D. Glutathione- and glutathione-S-transferase-dependent oxidative desulfuration of the thione xenobiotic diethyldithiocarbamate methyl ester. *Mol Pharmacol* 1994: 46: 1217–1225.
27. Madan A, Parkinson A, Faiman M D. Identification of the human P-450 enzymes responsible for the sulfoxidation and thiono-oxidation of diethyldithiocarbamate methyl ester: role of P-450 enzymes in disulfiram bioactivation. *Alcohol Clin Exp Res* 1998: 22: 1212–1219.
28. Madan A, Parkinson A, Faiman M D. Identification of the human and rat P450 enzymes responsible for the sulfoxidation of S-methyl N,N-diethylthiolcarbamate (DETC-ME). The terminal step in the bioactivation of disulfiram. *Drug Metab Dispos* 1995: 23: 1153–1162.
29. Madan A, Parkinson A, Faiman M D. Role of flavin-dependent monooxygenases and cytochrome P450 enzymes in the sulfoxidation of S-methyl N,N-diethylthiolcarbamate. *Biochem Pharmacol* 1993: 46: 2291–2297.
30. Shen M L, Johnson K L, Mays D C, Lipsky J J, Naylor S. Determination of in vivo adducts of disulfiram with mitochondrial aldehyde dehydrogenase. *Biochem Pharmacol* 2001: 61: 537–545.
31. Shen M L, Johnson K L, Mays D C, Lipsky J J, Naylor S. Identification of the protein-drug adduct formed between aldehyde dehydrogenase and S-methyl-N,N-diethylthiocarbamoyl sulfoxide by on-line proteolytic digestion high performance liquid chromatography electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* 2000: 14: 918–923.
32. Smith C K, Hotchkiss S A M. *Allergic Contact Dermatitis; Chemical and Metabolic Mechanisms*, London, Taylor and Francis, 2001.
33. Glauser T A, Nelson A N, Zembower D E, Lipsky J J, Weinshilboum R M. Diethyldithiocarbamate S-methylation: evidence for catalysis by human liver thiol methyltransferase and thiopurine methyltransferase. *J Pharmacol Exp Ther* 1993: 266: 23–32.
34. Thompson R W, Valentine H L, Valentine W M. In vivo and in vitro hepatotoxicity and glutathione interactions of N-methyldithiocarbamate and N,N-dimethyldithiocarbamate in the rat. *Toxicol Sci* 2002: 70: 269–280.
35. Joris S J, Aspila K I, Chakrabarti C L. Decomposition of monoalkyl dithiocarbamates. *Anal Chem* 1970: 42: 647–651.
36. Heikkila R E, Cabbat F S, Cohen G. In vivo inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J Biol Chem* 1976: 251: 2182–2185.
37. Misra H P. Reaction of copper-zinc superoxide dismutase with diethyldithiocarbamate. *J Biol Chem* 1979: 254: 11623–11628.
38. Divkovic M, Pease C K, Gerberick G F, Basketter D A. Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. *Contact Dermatitis* 2005: 53: 189–200.
39. Popielarz R, Clouet G. Synthesis and initiation/transfer properties of carbamoyl disulfides. *Polymer Sci Technol* 1993: 194: 2897–2906.

Address:
Itai Chipinda
Allergy and Clinical Immunology Branch
Health Effects Laboratory Division
National Institute for Occupational Safety and Health
1095 Willowdale Road, Morgantown
WV 26505-2888, USA
Tel: +1-304-285-5721
Fax: +1-304-285-6126
e-mail: ichipinda@cdc.gov