Effects of Disulfiram, Diethyldithiocarbamate, Bisethylxanthogen, and Benzyl Isothiocyanate on Glutathione Transferase Activities in Mouse Organs

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ABSTRACT

Four sulfur compounds known to inhibit tumorigenic effects of chemical carcinogens were administered to female CD-1 mice at 0.5% of the diet for 14 days, and their effects on cytosolic glutathione transferase (EC 2.5.1.18) specific activities were examined in liver, lung, kidney, urinary bladder, forestomach, proximal small intestine, and colon. Disulfiram, sodium diethylidithiocarbamate, bisethylxanthogen, and benzyl isothiocyanate elevated glutathione transferase specific activities in most of the organs examined. The four sulfur compounds differed in the extents and organ specificities of their effects on these enzyme activities. In the liver, bisethylxanthogen and benzyl isothiocyanate increased glutathione transferase activities to at least 3 times control levels and caused differential increases in the isozyme patterns observed after isoelectric focusing of the cytosols. Bisethylxanthogen also increased immunoreactive glutathione transferase in liver cytosol. Recrystallized disulfiram was less effective in enhancing hepatic glutathione transferase activities than was commercial (97%) disulfiram. Among the six extraha- patic organs examined, the small intestine and the forestomach exhibited the greatest response of glutathione transferase activities to each of the four sulfur compounds. Benzyl isothiocyanate was most effective in these "portal of entry" organs but less effective than bisethylxanthogen in the other extraha- patic organs examined. Bisethylxanthogen elicited significant increases in glu- tathione transferase activities in liver, lung, and small intestine even when administered at 0.01% to 0.05% of the diet, suggesting that this compound may have considerable potential as an inhibitor of carcinogens susceptible to enzymatic conjugation with glutathione.

INTRODUCTION

The glutathione transferases (EC 2.5.1.18) are a family of major detoxification enzymes that conjugate numerous reactive electrophiles with glutathione and may also have a sacrificial function in covalently binding chemically reactive ligands (1–10). These enzymes are inducible by several classes of compounds that protect against carcinogenic and toxic chemicals (11–17). Induction of glutathione transferases by anticarcinogenic compounds is believed to be a part of the mechanism of anticarcinogenesis (11–17). A strong correlation between protection against carcinogenesis and induction of liver and proximal small intestine cytosolic glutathione transferases has led to the sug-

gestion that glutathione transferase induction in these organs may serve as a marker for protection (14, 15).

DSF, sodium DDC, BEX, and BITC have been shown to inhibit tumorigenic and toxic effects of a variety of chemical carcinogens (17–19). Among the rodent tissues in which protection by one or more of these compounds has been demonstrated are the liver, lung, forestomach, small intestine, colon, adrenal, mammary gland, and urinary bladder (17–19). Fiala et al. (20) established that protection by DSF against the colon carcinogen, dimethylhydrazine, was due to inhibition by DSF in vivo of two steps in the oxidative activation of dimethylhydrazine. The mechanisms by which these anticarcinogenic sulfur compounds protect against polycyclic hydrocarbon, nitrosamine, alylamine, and azo dye carcinogens are less completely understood. Inhibition of monooxygenases by thionosulfur compounds and isothiocyanates, which appears to contribute very significantly to protection by these compounds, has been reviewed in detail recently (18). Enhancement of the activities of nonoxidative detoxification en-
zymes, resulting in increased potential for enzymatic inactivation of reactive electrophiles, may also contribute to protection by these sulfur compounds against carcinogenesis and toxicity.

Administration of DSF to mice has been shown to increase glutathione transferase specific activities in the liver and the small intestine and to increase the major hepatic glutathione transferase mRNA (14, 21, 22). Enhancement of glutathione transferase activity by BITC has been observed in mouse forestomach, liver, and small intestine (14, 15, 23). The present report is concerned with the comparative effects of DSF, sodium DDC, BEX, and BITC on glutathione transferases of mouse liver, lung, kidney, urinary bladder, forestomach, proximal small intestine, and colon. The extent to which increased glutathione transferase activity is a common feature of the administration of these four sulfur compounds has been explored, and some of the characteristics of the increases in these enzymes have been examined.

MATERIALS AND METHODS

Sulfur Compounds. BEX was a gift from Fike Chemicals, Nitro, WV. DSF was purchased from Aldrich Chemical Company, Milwaukee, WI. BITC and sodium DDC were from Eastman Kodak Organic Chemicals, Rochester, NY. BEX and DSF were crystallized 5 times from ethanol, and each yielded a single spot upon thin layer chromatography.

Mice and Tissues. Female CD-1 mice, 5 wk old, were purchased from Charles River Breeding Laboratories, Wilmington, MA. The mice were housed in stainless steel wire cages in an environmentally controlled room with a 12-h light-dark cycle. They received ad libitum a basal diet.

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3 The abbreviations used are: DSF, disulfiram (tetraethylthiuram disulfide); sodium DDC, sodium diethylidithiocarbamate; BEX, bisethylxanthogen; BITC, benzyl isothiocyanate; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.
into groups of six and given the experimental diets, containing DSF, sodium DDC, BEX, or BITC at the concentrations specified below. Control groups continued to receive the basal diet. After 14 days on these diets, the mice were killed by cervical dislocation, and organs were excised immediately. Cold 0.15 M KCl in 2 mM EDTA at pH 7.0 was used to perfuse the livers and to rinse the lungs, kidneys, urinary bladders, and forestomachs. The colon and the proximal half of the small intestine were slit lengthwise, food and fecal material were removed, and the mucosa was collected by scraping with a spatula. Each tissue sample was immediately placed in a 12- x 48-mm screw top tube with silicone gasket (Vanguard) and frozen in liquid nitrogen. Tissue samples were stored at −80°C until further processing.

Preparation of Cytosol Fractions. All steps were carried out at 0–4°C. Organs were homogenized individually in a size 22 Dual homogenizer (Kontes) in 0.25 M sucrose (1 ml for urinary bladders, 3 ml per g for other organs). The homogenates were centrifuged at 27,000 x g for 20 min. A 0.1 M solution of CaCl2 in 0.25 M sucrose was added to the supernatants (0.2 ml per ml of supernatant). After 30 min at 0°C, these samples were centrifuged at 27,000 x g for 20 min. Micosomes, aggregated by the CaCl2 treatment, were thus removed, leaving a clear supernatant (cytosol fraction).

Enzyme Assays. Glutathione transferase activities were measured spectrophotometrically at 25°C, with DCNB and DCNB (Eastman) as substrates. The methods of Habig et al. (24) were used, except that the concentration of DCNB in the assay system was 0.5 mw (rather than 1 mw) to facilitate solubility. Androst-5-ene,3,17-dione was prepared by the method of Kawahara (25), and its isomerization was measured as described previously, without addition of exogenous glutathione (11, 26).

Protein concentrations in the cytosol fractions were determined by the procedure of Lowry et al. (27).

Immunoprecipitation of Enzyme Protein. The purification of the major glutathione transferase of mouse liver cytosol, the immunization of rabbits with this antigen, and the preparation of preimmune and immune γ-globulin fractions have been described previously (22). The cytosol preparations evaluated in the present studies were obtained by combining equal portions of liver cytosol from six untreated mice or from six mice that had received a diet containing 0.5% BEX for 14 days. The preparation from BEX-treated mice was diluted with 18.7 mw CaCl2 in 0.25 M sucrose to yield the same CDN conjugating activity per ml as the preparation from untreated mice. Immunoprecipitation was performed as described previously (22).

Examination of Transferase Isozyme Composition. Cytosol fraction derived from 1 g of liver (consisting of equal portions of liver from each of six mice) was applied to a 0.9- x 4-cm column of DEAE-cellulose (Whatman) equilibrated and developed at 4°C with 1 mm dithiothreitol in 15 mm Tris-HCl at pH 6.7. The first 10 ml of effluent were collected, and 9 ml of this solution were subjected to isoelectric focusing (28) for 66 h at 2°C in a 110-mI sucrose gradient containing ampholytes (LKB) of pH 8 to 11. Fractions (1.6 ml) were collected. Measurements of pH were performed immediately at 0°C, and glutathione transferase activities were assayed in the fractions.

RESULTS

Effects of Dietary Additives on Body Weights and Liver Weights. Groups of six mice, with group total initial body weights of 156.5 ± 0.5 (SE) g that received either the control diet or diets containing 0.5% (by weight) DSF, sodium DDC, BEX, or BITC for 14 days, exhibited changes in group body weights of +10% (control diet), 0% (DSF), +4.8% (sodium DDC), +4.4% (BEX), and −7.3% (BITC). The marked weight loss in the group fed the BITC diet appeared to be due to initial aversion to the diet. Liver weights, expressed as percentage of body weight, were 7.35 ± 0.38 (BEX), and 6.26 ± 0.19 (BITC). Only BEX increased the actual mean liver weight, which was 1.93 ± 0.16 g for the BEX group as compared with 1.45 ± 0.08 g for the control group.

Effects of DSF, Sodium DDC, BEX, and BITC on Glutathione Transferase Activities. When administered to mice at 0.5% of the diet for 14 days, each of the four anticarcinogenic sulfur compounds tested caused statistically significant increases in cytosolic glutathione transferase specific activities in most of the organs examined (Table 1). However, the effects of these compounds differed markedly in both magnitude and specificity. Such differences were especially apparent in the liver where BEX and BITC increased glutathione transferase specific activities to at least 3 times control levels, whereas DSF was far less effective, and sodium DDC caused little if any increase. Among the six extrahepatic organs examined, the proximal small intestine and the forestomach exhibited the largest response of glutathione transferase activities to each of the four sulfur compounds. BITC was most effective in these "portal of entry" organs, but less effective than BEX in the other extrahepatic organs. BEX caused the greatest enhancement of glutathione transferase activities in the lung and the kidney and was the only compound to cause a marked increase in these enzyme activities in the colon. In BITC-treated mice, urinary bladder glutathione transferase activities were substantially lower than those in mice fed the control diet. The urinary bladder was the only organ in which a decrease in glutathione transferase activity was observed in response to any of the four sulfur compounds.

Administration of BEX at concentrations in the diet of 0.01% to 0.25% yielded lesser increases in cytosolic glutathione transferase specific activities in liver, lung, and proximal small intestine mucosa (Table 2) than were elicited by 0.5% BEX (Table 1). Significant increases in some of these activities were observed even at 0.01% BEX. All of the activities measured were significantly elevated in response to the diet containing 0.05% BEX.

Immunoprecipitation of Glutathione Transferase. The relative amounts of immunoreactive enzyme protein in liver cytosols from mice fed the control diet or the 0.5% BEX diet were examined by immunoprecipitation with antibody (immune γ-globulin, IgG) prepared from the serum of rabbits immunized against the major mouse hepatic glutathione transferase (22). Calculations were performed as described previously (22). The antibody removed a maximum of 77% of the CDNB conjugating activity of the liver cytosols from each dietary group (Chart 1). More antibody was required to precipitate the glutathione transferase activity from the liver cytosols from BEX-treated mice than from the liver cytosols from mice fed the control diet, indicating that BEX had induced an increase in immunoreactive enzyme protein. The apparent increase in immunoreactive glutathione transferase(s), to 8.3 times control levels (Chart 1), was substantially greater than the BEX-induced increase in glutathione transferase specific activities (Table 1). This suggested that BEX treatment had altered the glutathione transferase isozyme composition of the liver cytosol.

Examination of Hepatic Glutathione Transferase Isozymes by Isoelectric Focusing. Basic protein fractions of liver cytosols from mice fed diets containing 0.5% BEX or 0.5% BITC for 2 wk, or the control diet, were examined by isoelectric focusing (Chart 2). Liver cytosol fractions freshly prepared for this purpose had specific activities toward CDNB of 2.230, 8.311, and 7.182
of IgG from rabbits immunized against the major glutathione transferase of mouse liver. CDNB conjugating activity was measured in the supernatant fractions. By use of a double antibody procedure, glutathione transferase activity was precipitated with varying amounts of protein, respectively, with CDNB as a substrate. Recovery of CDNB conjugating activity in the fractions after isoelectric focusing was 70% for the preparations from mice fed the control diet or the BEX diet and 77% for the preparation from mice fed the BEX diet. The distribution of enzymatic activities in the fractions revealed that BEX and BITC had induced differential increases in some glutathione transferase isozymes. The major CDNB conjugating activity, which focused at pH 8.8, was greatly enhanced by both BEX and BITC, whereas the isozyme(s) focusing at pH 9.2 to 9.4-ene,3,17-dione is catalyzed by some glutathione transferases.

Table 1

<table>
<thead>
<tr>
<th>Organ</th>
<th>Substrate</th>
<th>None (nmol/min/mg)</th>
<th>DSF®</th>
<th>Sodium DDC®</th>
<th>BEX®</th>
<th>BITC®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>CDNB</td>
<td>2535 ± 161c</td>
<td>1.30 ± 0.14c</td>
<td>1.09 ± 0.07d</td>
<td>3.55 ± 0.16c</td>
<td>3.00 ± 0.59f</td>
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<td></td>
<td>DCNB</td>
<td>41.7 ± 3.2</td>
<td>1.58 ± 0.15b</td>
<td>1.23 ± 0.06a</td>
<td>3.09 ± 0.17</td>
<td>3.50 ± 0.31a</td>
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<tr>
<td>Lung</td>
<td>CDNB</td>
<td>423 ± 27</td>
<td>1.40 ± 0.09a</td>
<td>1.29 ± 0.05a</td>
<td>1.86 ± 0.13c</td>
<td>1.83 ± 0.09a</td>
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<tr>
<td></td>
<td>DCNB</td>
<td>4.34 ± 0.39</td>
<td>1.83 ± 0.13c</td>
<td>1.48 ± 0.06bc</td>
<td>2.00 ± 0.06a</td>
<td>1.73 ± 0.04a</td>
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<tr>
<td>kidney</td>
<td>CDNB</td>
<td>603 ± 33</td>
<td>1.25 ± 0.06b</td>
<td>1.18 ± 0.01c</td>
<td>1.70 ± 0.04c</td>
<td>1.37 ± 0.05d</td>
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<td>DCNB</td>
<td>9.18 ± 0.41</td>
<td>1.33 ± 0.05c</td>
<td>1.22 ± 0.06d</td>
<td>1.49 ± 0.06d</td>
<td>1.35 ± 0.05d</td>
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<tr>
<td>Urinary bladder</td>
<td>CDNB</td>
<td>2663 ± 163</td>
<td>1.45 ± 0.12a</td>
<td>1.13 ± 0.07a</td>
<td>1.57 ± 0.07b</td>
<td>0.64 ± 0.04c</td>
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<td></td>
<td>DCNB</td>
<td>85.8 ± 3.2</td>
<td>1.81 ± 0.31a</td>
<td>1.08 ± 0.05a</td>
<td>1.17 ± 0.04fc</td>
<td>0.56 ± 0.04a</td>
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<tr>
<td>Foresomach</td>
<td>CDNB</td>
<td>847 ± 70</td>
<td>1.84 ± 0.12a</td>
<td>1.45 ± 0.06d</td>
<td>2.45 ± 0.14a</td>
<td>3.43 ± 0.17d</td>
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<td></td>
<td>DCNB</td>
<td>9.38 ± 0.92</td>
<td>2.13 ± 0.12b</td>
<td>1.64 ± 0.11b</td>
<td>2.81 ± 0.21b</td>
<td>4.29 ± 0.22b</td>
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<tr>
<td>Small intestine</td>
<td>CDNB</td>
<td>1079 ± 72</td>
<td>2.50 ± 0.26a</td>
<td>3.16 ± 0.19a</td>
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<td>DCNB</td>
<td>8.88 ± 0.73</td>
<td>3.92 ± 0.46c</td>
<td>4.75 ± 0.29c</td>
<td>5.13 ± 0.31a</td>
<td>8.31 ± 0.46a</td>
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<td>Colon</td>
<td>CDNB</td>
<td>562 ± 53</td>
<td>1.40 ± 0.06b</td>
<td>1.20 ± 0.15a</td>
<td>2.17 ± 0.14b</td>
<td>1.25 ± 0.06a</td>
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<td></td>
<td>DCNB</td>
<td>14.3 ± 2.4</td>
<td>1.05 ± 0.18a</td>
<td>1.04 ± 0.14a</td>
<td>2.15 ± 0.25c</td>
<td>0.97 ± 0.14a</td>
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</tbody>
</table>

* The sulfur compounds were fed for 14 days at 0.5% of the diet.
® Specific activity (treated/control ratio).
* Mean ± SE for six mice, except urinary bladders (five mice).
* Mean ± SE for six mice, except urinary bladders (five mice).
* Significantly different from control value (P < 0.01).
* P < 0.01.
* P < 0.02.
* P < 0.05.

Table 2

<table>
<thead>
<tr>
<th>BEX (% of diet)</th>
<th>CDNB (nmol/min/mg)</th>
<th>DCNB (nmol/min/mg)</th>
<th>CDNB (nmol/min/mg)</th>
<th>DCNB (nmol/min/mg)</th>
<th>CDNB (nmol/min/mg)</th>
<th>DCNB (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1857 ± 72c</td>
<td>32.0 ± 1.5</td>
<td>329 ± 15</td>
<td>4.84 ± 0.26</td>
<td>823 ± 27</td>
<td>7.67 ± 0.54</td>
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<tr>
<td>0.05</td>
<td>1979 ± 45c</td>
<td>35.7 ± 0.9</td>
<td>417 ± 17c</td>
<td>6.02 ± 0.16b</td>
<td>947 ± 57</td>
<td>10.7 ± 0.7b</td>
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<tr>
<td>0.05</td>
<td>2423 ± 87c</td>
<td>44.4 ± 0.5d</td>
<td>402 ± 11c</td>
<td>5.89 ± 0.17bc</td>
<td>1155 ± 52d</td>
<td>16.5 ± 1.0d</td>
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<tr>
<td>0.10</td>
<td>3312 ± 69d</td>
<td>53.8 ± 2.2d</td>
<td>530 ± 13c</td>
<td>6.93 ± 0.31c</td>
<td>1945 ± 58d</td>
<td>28.1 ± 2.0d</td>
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<tr>
<td>0.25</td>
<td>3977 ± 105d</td>
<td>69.0 ± 2.2d</td>
<td>525 ± 10d</td>
<td>6.92 ± 0.26d</td>
<td>2266 ± 67d</td>
<td>33.8 ± 2.3d</td>
</tr>
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</table>

* Mean ± SE (n = 6).
® Significant different from control value (P < 0.01).
* P < 0.02.
* P < 0.001.

Chart 1. Immunoprecipitation of glutathione transferase activity from liver cytosol fractions obtained from mice that had received a diet containing 0.5% BEX for 2 wk (©) or a control diet (®). The cytosol preparations were obtained by combining equal portions of liver cytosol from six mice. The preparations from mice fed the control diet or the BEX diet had specific activities of 2.46 or 9.80 μmol/min/mg of protein, respectively, with CDNB as a substrate. By use of a double antibody procedure, glutathione transferase activity was precipitated with varying amounts of IgG from rabbits immunized against the major glutathione transferase of mouse liver. CDNB conjugating activity was measured in the supernatant fractions.
compounds and isothiocyanates inhibit oxidative activation of one transferase activity to a much lesser extent (Table 1) than commercial DSF in response to both concentrations of commercial DSF (Table 3). Differential Effects of Commercial and Recrystallized DSF. Recrystallized DSF at 0.5% of the diet elevated hepatic glutathione transferase activities, whereas recrystallized DSF was more effective at 1.0% of the diet (21, 22). To ascertain whether this was due to the crystallization of the DSF or to the difference in dose, or to a combination of these factors, we examined the effects on hepatic glutathione transferase activities of recrystallized DSF at 1.0% of the diet and of commercial DSF at 0.5% and 1.0% of the diet. Whereas recrystallized DSF was more effective at 1.0% than at 0.5% of the diet in elevating hepatic glutathione transferase activities, greater increases in these activities were observed in response to both concentrations of commercial DSF (Table 3).

**DISCUSSION**

There is much evidence that anticarcinogenic thionosulfur compounds and isothiocyanates inhibit oxidative activation of carcinogens (18). Increased inactivation of reactive metabolites may also contribute to protection against carcinogenesis. We are examining the effects of anticarcinogenic sulfur compounds on the activities of nonoxidative enzymes that inactivate reactive electrophiles. In the present investigation, DSF, sodium DDC, BEX, and BITC were found to have in common the ability to induce substantial increases in glutathione transferase activities, although considerable differences were observed among the four compounds in their organ specificities and in the magnitudes of their effects on glutathione transferases.

The sulfhydryl compound, sodium DDC, and its disulffide analogue, DSF, which are metabolically interchangeable, yielded comparable effects on glutathione transferase activities. Our observation that commercial (97%) DSF was far more effective than recrystallized DSF at elevating hepatic glutathione transferase activities indicates the presence of a potent glutathione transferase inducer as an impurity in commercial DSF. Fiala et al. (29) similarly noted that rat hepatic γ-glutamyltransferase specific activity was markedly increased by diets containing commercial (95%) DSF but not by diets containing recrystallized DSF. It remains unclear whether the same or different impurities in commercial DSF influence hepatic γ-glutamyltransferase and glutathione transferase activities, and whether such impurities may mediate some of the other reported effects of DSF on enzyme activities (cf. Ref. 18). It is clear, however, that recrystallized DSF is an effective inhibitor of carcinogenesis (19, 29).

BEX, which contains an ethoxy moiety in place of the diethylamino group of DSF, may be of particular interest because, unlike DSF and sodium DDC, it cannot yield diethylamine as a metabolite (30). At 0.5% of the diet, BEX was a far more effective inducer of glutathione transferases than were DSF and sodium DDC. Even at concentrations of only 0.01% and 0.05% of the diet, BEX elicited significant increases in glutathione transferase activities in several organs. It would seem that relatively few studies have evaluated the anticarcinogenic potential of BEX (18, 30). Our observations suggest that BEX may have considerably greater potential than DSF or sodium DDC as an inhibitor of carcinogens susceptible to inactivation by glutathione transferases.

**REFERENCES**


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