Efflux of Reduced Glutathione after Exposure of Human Lung Epithelial Cells to Crocidolite Asbestos

Scott A. Golladay,* Sun-Hee Park, and Ann E. Aust
Department of Chemistry and Biochemistry, Utah State University, Logan, Utah

This study investigated glutathione (GSH) homeostasis in human lung epithelial cells (A549) exposed to crocidolite. Exposure of A549 cells to 3 μg/cm² crocidolite resulted in a decrease in intracellular reduced glutathione by 36% without a corresponding increase in GSH disulfide. After a 24-hr exposure to crocidolite, 75% of the intracellular GSH lost was recovered in the extracellular medium, of which 50% was in reduced form. Since the half-life of reduced GSH in culture medium was less than 1 hr, this suggests that reduced GSH was released continuously from the cells after treatment. The release of GSH did not appear to result from nonspecific membrane damage, as there was no concomitant release of lactate dehydrogenase or 14C-adenine from loaded cells after crocidolite treatment for 24 hr. Crocidolite exposure resulted in the formation of S-nitrosothiols but no increase in the level of GSH-protein mixed disulfides or GSH conjugates. Exposure of A549 cells to crocidolite for 24 hr decreased gamma glutamylcysteine synthetase (γ-GCS) activity by 47% without changes in the activities of GSH reductase, GSH peroxidase, GSH S-transferase, or glucose-6-phosphate dehydrogenase. Treatment of cells with crocidolite pretreated with the iron chelator desferrioxamine B resulted in the same level of intracellular GSH depletion and efflux and the same decrease in γ-GCS activity as treatment with unmodified crocidolite, which suggests that iron-catalyzed reactions were not responsible for the GSH depletion. — Environ Health Perspect 105(Suppl 5):1273–1277 (1997)

Key words: asbestos, glutathione, glutathione disulfide, reduced glutathione efflux, iron, crocidolite, A549 cells, human lung epithelial cells

Introduction

Exposure to asbestos results in an increased risk of fibrosis, bronchial carcinoma, and mesothelioma of the pleura, pericardium, and peritoneum (1). Although considerable research has been directed toward understanding fiber-related disease, the molecular mechanism by which crocidolite causes disease is still not well understood.

Crocidolite, the most carcinogenic form of asbestos, contains 27% iron by weight (2). There is evidence to suggest that the generation of reactive oxygen species via iron-catalyzed reactions contributes to crocidolite-dependent biological effects (2–4). Iron appears responsible for the biochemical reactivity of crocidolite in vitro, e.g., formation of the highly reactive hydroxyl radical (5,6) and induction of DNA single-strand breaks (7). In cultured cells, iron also appeared involved in 8-hydroxy-2′-deoxyguanosine formation (8,9), synthesis of mRNA for the inducible form of nitric oxide synthase (iNOS) and production of nitrite, a stable oxidation product of nitric oxide (NO) (9).

Redox active iron on the surface or mobilized from phagocytized fibers has the potential to catalyze the formation of reactive oxygen species. One line of defense against these species is glutathione (GSH), the most prevalent thiol-disulfide redox buffer, found in millimolar concentrations in most human cells (10). Decreases in cellular GSH can occur in response to either transition-metal catalyzed formation of hydroperoxides, or to the modulation of key enzymes responsible either for synthesis and maintenance or utilization of GSH.

Several investigators measured intracellular GSH levels in cultured cells exposed to crocidolite (11–13). The experiments reported here measured levels of GSH and several of its alternate forms and examined the activities of enzymes involved in the maintenance and reaction of GSH in human lung epithelial cells (A549) after crocidolite treatment.

Materials and Methods

Asbestos and Reagents

Crocidolite was obtained from R. Griesemer (National Institute of Environmental Health Sciences/National Toxicology Program, Research Triangle Park, NC) and contained 27% iron by weight. Fiber size was characterized; a mean length of 10 μm and mean width of 0.27 μm, determined by scanning electron microscopy, was reported (14). Metaphosphoric acid and 1-heptanesulfonic acid were obtained from Fluka Chemical (Ronkonkoma, NY) and 2-vinylpyridine was obtained from Aldrich Chemical (Milwaukee, WI). 14C-adenine was obtained from NEN Life Science (Boston, MA). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

Cell Culture

Culture Medium and Cells. Ham’s F12 culture medium without FeSO4 (Life Technologies, Grand Island, NY) was used for these experiments. Complete growth medium was composed of F12, 50 μg/ml gentamicin (M.A. Whittaker Byproducts, Walkersville, MD), 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), and 1.18 g NaHCO3/liter medium, to obtain a final pH of 7.4. A549 cell line (ATCC CC18S) was obtained from American Type Culture Collection (Rockville, MD). Cells were cultivated and maintained as previously described (9).

This paper is based on a presentation at The Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles held 15–18 September 1996 in Lake Placid, New York. Manuscript received at EHP 26 March 1997; accepted 10 July 1997.

We are grateful to C.-C. Chao for many helpful comments. This work was supported by a grant from the National Institute of Environmental Health Sciences (ES05814).

Address correspondence to Dr. A.E. Aust, Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300. Telephone: (801) 797-1629. Fax: (801) 797-3590. E-mail: aus@cc.usu.edu

*Present address: Research and Development Division, Anesta Corporation, 4745 Wiley Post Way, Plaza 6, Suite 650, Salt Lake City, UT 84116.

Abbreviations used: A549, human lung epithelial cells; CPM, counts per min; DF, desferrioxamine B; G-6-PDH, glucose-6-phosphate dehydrogenase; γGCS, gamma glutamylcysteine synthetase; GPx, glutathione peroxidase; iNOS, inducible form of nitric oxide synthase; GR, glutathione reductase; GSH, reduced glutathione; GSGG, glutathione disulfide (oxidized glutathione); GST, glutathione-S-transferase; HPLC-EC, high-performance liquid chromatography with dual electrochemical detection; LDH, lactate dehydrogenase; NO, nitric oxide.
Treatment of Cells

Cells were treated with crocidolite or crocidolite from which iron had been partially removed with desferrioxamine B (DF). DF crocidolite was prepared as previously described (9). Crocidolite or DF crocidolite was suspended in sterile 1.18 g/liter NaHCO₃, pH 7.4, immediately before use.

The cells were plated at a culture density of 20,000 cells/cm² and treated after 24 hr with crocidolite or DF crocidolite at the indicated concentrations. The treatment medium was removed at the indicated times and the cells were dislodged and resuspended in the appropriate solution for the analyses described below. Under these treatment conditions, we observed that approximately 60% of the crocidolite fibers was taken up by the epithelial cells (15).

Cytotoxicity Assay

Treated cells were suspended in complete growth medium and the cytotoxicity of crocidolite determined by percent relative cloning ability as previously described by Takeuchi and Morimoto (9). Results are reported as the mean ± SD (n = 3).

Analyses for Intracellular Glutathione Levels

Enzyme Recycling. Treated cells (6.0 × 10⁶) were collected and lysed in 5% metaphosphoric acid for 15 min on ice. The 16,000 × g supernatant of the cell lysate was analyzed for GSH and GSH disulfide (GSSG), using enzymatic recycling, as described by Griffith (16). The results are expressed as nmol GSSG/10⁶ cells or nmol (GSH + GSSG)/10⁶ cells.

High-performance Liquid Chromatography with Dual Electrochemical Detection. Cells were treated and harvested as described for the enzyme recycling assay. GSH and GSSG were separated and quantified simultaneously by high-performance liquid chromatography with dual electrochemical detection (HPLC-EC), as originally described by Richie and Lang (17).

Analysis for Extracellular Glutathione

After cell treatment, the extracellular medium was removed and centrifuged at 250 × g for 10 min. The supernatant was removed and acidified immediately with 5% metaphosphoric acid to determine extracellular GSH and GSSG, as described for the enzyme recycling method.

Determination of Glutathione-Protein Mixed Disulfides and GSH Conjugates

Treated cells (1.2 × 10⁶) were collected and lysed in 500 µl of 1% metaphosphoric acid. Reduction of GSH-protein mixed disulfides or GSH conjugates in the 10,000 × g supernatant was determined using the method of Meredith (18).

Results are expressed as nmol/10⁶ cells.

Determination of S-Nitrosothiols

Treated cells (approximately 1.5 × 10⁶) were collected, resuspended in 200 µl deionized distilled water, and lysed by repeated freezing in liquid nitrogen and thawing. Protein was removed from the 16,000 × g supernatant of the cell lysate by mixing 200 µl of the supernatant with 200 µl 10% ZnSO₄. The content of S-nitrosothiol was determined by the method of Saville (19). All steps were conducted in minimal light to prevent decomposition of S-nitrosothiols (20,21).

Enzyme Assays

Dislodged cells (1.8 × 10⁷) were collected and resuspended in 100 mM phosphate buffer, pH 7.4, containing 1 mM DF. Cell suspensions were lysed by three cycles of freezing and thawing or by the addition of 0.1% Triton X-100. The 105,000 × g supernatants were analyzed immediately or kept frozen at −20°C until analysis. Protein concentrations were assayed using the bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

The activity of gamma glutamylcysteine synthetase (γ-GCS) was determined using the method of Seelig and Meister (22). Enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 nmol nicotinamide adenine dinucleotide (reduced form)/min/mg of protein (U/mg protein). The activity of GSH peroxidase (GPx) was determined by Wendel’s method (23). The activity of GSH reductase (GR) was determined using the method of Carlberg and Mannervik (24). The activity of GSH-S-transferase (GST) was determined using the method of Habig et al. (25). The activity of glucose-6-phosphate dehydrogenase (G6PDH) was determined by Lee’s method (26). The activity of lactate dehydrogenase (LDH) was determined using the method of Welder and Acosta (27).

Membrane Permeability

Twenty-four hours after plating the cells, we treated them with 3 µg/cm² crocidolite for 24 hr and the amount of LDH in the medium or in the cells was determined, as described above. An additional method to determine whether the membrane permeability was altered by crocidolite treatment determined the amount of ¹⁴C-adenine released from the cells after crocidolite treatment. Twenty-four hours after plating the cells, we exposed them to ¹⁴C-adenine (1 µCi) in complete medium for 4 hr. The medium containing ¹⁴C-adenine was removed, cells were washed with phosphate-buffered saline, then treated with 6 µg/cm² crocidolite for 24 hr for comparison with untreated control cells. At the end of the treatment period, counts per minute (cpm) resulting from ¹⁴C in the medium or cells was determined by scintillation counting. Results were compared between treated and untreated samples as cpm in the medium per total cpm (cpm in the medium plus cells).

Results

Effect of Crocidolite Exposure on Intracellular Levels of Glutathione in A549 Cells

A549 cells were used to study levels of GSH and GSSG after exposure to crocidolite because they exhibit characteristics of epithelial type II cells, which are the target cells for asbestos-induced bronchogenic carcinoma. Crocidolite produced a dose-dependent depletion of intracellular GSH without elevation of GSSG (Table 1). There was up to a 59% decrease in intracellular GSH levels in treated cells, and levels remained constant above 6 µg/cm² crocidolite (data not shown). Simultaneous determination of GSH and GSSG levels, using HPLC-EC, confirmed the results observed using the enzyme-recycling method (Table 1). To determine if the GSH decrease resulted from iron-catalyzed oxidative reactions occurring during cell lysis and GSH determination, crocidolite (equivalent to the amount that remained associated with the harvested cells treated with 3 µg/cm² [1.75 µg/cm²]) was added immediately prior to lysis of untreated cells for GSH determination. This did not result in a significant difference in GSH levels (23 ± 2 nmol/10⁶ cells) compared with controls without crocidolite (23 ± 3 nmol/10⁶ cells). These results confirm that the decrease in GSH observed in cells treated for 24 hr was not an artifact of the assay procedure. Treatment of cells with DF crocidolite from which reactive iron had been removed (2,9) resulted in a
Table 1. Effect of crocidolite exposure on intracellular glutathione levels in human lung epithelial cells. *

| Crocidolite, µg/cm² | GSH + GSSG, nmol/10⁶ cells | GSSG, nmol/10⁶ cells | Relative survival, % |
|--------------------|-----------------------------|--------------------|---------------------|
| 0                  | 22 ± 3 (23 ± 3)             | 0.27 ± 0.03 (0.34 ± 0.09) | 100                 |
| 1.5                | 17 ± 2 (17 ± 2)*            | 0.27 ± 0.05 (0.34 ± 0.07) | 63 ± 4              |
| 3.0                | 14 ± 2 (15 ± 2)*            | 0.26 ± 0.03 (0.32 ± 0.08) | 46 ± 5              |
| 6.0                | 9 ± 1 (9 ± 2)*              | 0.32 ± 0.07 (0.30 ± 0.04) | 13 ± 4              |
| DF² 3.0            | 14 ± 1*                     | 0.28 ± 0.03         | 54 ± 4              |
| DF 6.0             | 9 ± 1*                      | 0.30 ± 0.08         | 33 ± 5              |

*As49 cells were treated with crocidolite or DF crocidolite at the indicated concentrations. Total and oxidized glutathione were determined by enzyme recycling or HPLC-EC, as described in "Materials and Methods." Results are expressed as mean ± SD (n=3). *Numbers in parentheses indicate determinations made by HPLC-EC, as described in "Materials and Methods." *Crocidolite from which reactive iron was removed by DF pretreatment. * , significantly different from the untreated control (Student’s t-test, p<0.05).

decrease in intracellular GSH levels comparable to that observed for unmodified crocidolite (Table 1), which suggests that iron was not involved in the decrease of GSH.

Effect of Crocidolite Exposure on Formation of GSH-Protein Mixed Disulfides, GSH Conjugates, and S-Nitrosothiols

Results shown in Table 2 indicate that crocidolite treatment of As49 cells does not lead to elevation of GSH-protein mixed disulfides or GSH conjugates. There was a significant elevation in S-nitrosothiols at the 6 µg/cm² treatment. However, this would represent less than 1% of the GSH depleted from the cells. DF pretreatment of crocidolite fibers prevented the formation of S-nitrosothiols (Table 2), which suggested that iron is involved in S-nitrosothiol formation.

Effect of Time of Crocodolite Exposure on Intracellular and Extracellular GSH Levels

As shown in Figure 1A, incubation of As49 cells with crocidolite (3 µg/cm²) for up to 24 hr resulted in a time-dependent decrease in intracellular total GSH starting 4 hr after treatment. The GSSG levels remained constant during this time period (data not shown).

Exposure of As49 cells to 3 µg/cm² results in a time-dependent increase in extracellular total GSH, as shown in Figure 1B. This is consistent with earlier observations for the depletion of intracellular GSH. Approximately 5 nmol/10⁶ cells of total GSH found in the medium at 12 hr (71% of the total GSH) or 24 hr (60% of the total GSH), respectively, was in the reduced form. To determine the half-life of reduced GSH in this medium at 37°C, 30 nmol reduced GSH, a concentration comparable to that observed after 24-hr treatment with asbestos, was added to the medium. Aliquots were removed and assayed for reduced GSH at designated time intervals up to 24 hr. The half-life of reduced GSH under these conditions was less than 1 hr, which suggests that reduced GSH was released from the cells into the medium continuously during the treatment period.

LDH levels were assessed in samples of extracellular medium to determine whether membrane damage occurred. At 24 hr, medium samples from untreated and 3 µg/cm² crocidolite-treated cells showed no differences in extracellular LDH activity (data not shown). In a separate experiment, cells were loaded with 14C-adenine before 24 hr treatment with 6 µg/cm² crocidolite. There was no increase in 14C found in the medium of the treated compared with control cells. Therefore, no apparent significant cell membrane injury occurred during the 24-hr crocidolite treatment.

Effect of Crocidolite Exposure on GSH-associated Enzymes

Enzymes responsible either for synthesis and maintenance or utilization of GSH were assayed after treatment of cells with 3 µg/cm² for 12 or 24 hr. There were no significant changes in the activity of GR, GPx, GST, or G6PDH as compared with the untreated controls (data not shown). However, there was a significant decrease in the activity of γ-GCS (p<0.01) at the 24 hr time point from 15 ± 1 U/mg protein to 7 ± 1 U/mg protein. When cells were exposed to DF crocidolite, there was an identical reduction in the activity of γ-GCS, which suggested that iron was not responsible for the loss of activity.

Table 2. Effect of crocidolite exposure on the formation of GSH conjugates, GSH-PSG, and S-nitrosothiols in human lung epithelial cells.*

| Crocidolite, µg/cm² | PrSSG, nmol/10⁶ cells | GSH conjugates, nmol/10⁶ cells | S-nitrosothiol, pmol/10⁶ cells |
|--------------------|----------------------|-----------------------------|-----------------------------|
| None               | 1.8 ± 0.3            | 0.3 ± 0.2                   | <10⁶                        |
| 3.0                | 1.6 ± 0.2            | 0.3 ± 0.2                   | 20 ± 10                     |
| 6.0                | 1.6 ± 0.3            | 0.42 ± 0.02                 | 50 ± 20*                    |
| DF² 3.0            | 1.6 ± 0.4            | 0.30 ± 0.1                  | <10                      |
| DF 6.0             | 1.4 ± 0.3            | ND                          | <10                      |

Abbreviations: ND, not determined; PrSSG, protein-mixed disulfides. *As49 cells were exposed to crocidolite or DF crocidolite at the indicated concentrations for 24 hr. PrSSG, GSH conjugates, and low molecular weight S-nitrosothiols were determined, as described in "Materials and Methods." Results are expressed as mean ± SD (n=3). *Below the detection limit of 10 pmol/10⁶ cells. *Crocidolite from which 7% of the iron was removed by DF pretreatment. * , significantly different from the untreated control, assuming an untreated control level of 10±10 (Student's t-test, p<0.01).
Discussion
The data presented here show that exposure of A549 cells to crocidolite resulted in a concentration-dependent decrease in intracellular GSH without a concomitant increase in GSSG. Decreases in cellular GSH have been reported by Janssen et al. (11) in rat pleural mesothelial cells exposed to crocidolite, by Boehme et al. (13) in rat alveolar macrophages exposed to crocidolite, and by Israbian et al. (12) in human pulmonary epithelial-like cells (WI-26) exposed to amosite asbestos. All of these investigators attributed the decrease in GSH to formation of oxygen radicals. However, only Boehme et al. (13) evaluated GSSG concentrations and observed no change in comparison with the untreated controls; our results are consistent with those observations.

Depletion of intracellular GSH did not appear to result from its oxidation and subsequent efflux, as 50% of the intracellular GSH depleted in 24 hr was recovered in the extracellular medium in reduced form. Because the half-life of reduced GSH placed in this medium was less than 1 hr, the presence of this high percentage of reduced GSH in the medium after treatment of the cells with crocidolite suggests that reduced GSH was released from the cells into the medium continuously during the treatment period. Other investigators observed GSH efflux during apoptosis (28). However, GSH release observed after crocidolite treatment did not appear to be the result of non-specific membrane damage or apoptosis because there was no increase in LDH release after crocidolite treatment or in 14C release after crocidolite treatment of 14C- adenine-loaded cells. Boehme et al. (13) reported release of GSH into the extracellular medium after exposure of rat alveolar macrophages to crocidolite. Their results cannot be compared directly with ours due to differences in dose, cell type, and time of exposure. Epithelial type II cells release several compounds to epithelial-lining fluid, which is the first barrier against inhaled oxidants (29,30), and influx of GSH by epithelial cells could be a protective mechanism. In fact, levels of reduced GSH observed in alveolar-lining fluid range from 200 to 800 μM (31). Other investigators observed reduced GSH efflux from hepatocytes exposed to vasopressin (32) or glucagon (33). Investigators subsequently showed that efflux of GSH from hepatocytes was protein mediated (34). The list of tissues identified, which efflux reduced GSH in response to a variety of stimuli, is growing, and speculation on the function for extracellular GSH was recently reviewed by Smith et al. (35).

There was a significant elevation in S-nitrosothiols with 6 μg/cm² treatment. Exposure of A549 cells to crocidolite results in synthesis of mRNA for iNOS and elevation of intracellular nitrite, which is a stable oxidation product of NO (9). Reaction between enzymatically generated NO and thiol-containing proteins or GSH in the presence of a transition metal (36), such as Fe from crocidolite, would produce the S-nitrosothiol detected in this treatment. However, the S-nitrosothiol produced under these conditions would account for less than 1% of the total GSH depleted.

Crocidolite exposure led to a significant decrease in the activity of γ-GCS, the rate-limiting enzyme in GSH synthesis. This would likely lead to decreased synthesis of GSH and may contribute significantly to the dramatic decrease in intracellular GSH observed after crocidolite treatment. The only other GSH-associated enzyme activity reported to change after crocidolite exposure was that of GPx. Janssen et al. (37) observed an increase in GPx from rat lung tissue after rat exposure to crocidolite for 6 days. We did not observe any change in GPx activity or in GR, GST, or G6PDH activity after exposure of A549 cells to crocidolite for 24 hr. GR and G6PDH activity did not change, nor did GSSG increase, which suggest that the GR had sufficient activity and sufficient reducing equivalents (reduced nicotinamide adenine dinucleotide phosphate) to maintain the GSH in the reduced state even though oxygen radicals were likely being generated by Fe from crocidolite.

It did not appear that reactive iron on crocidolite was essential for either intracellular GSH depletion or efflux, or for decrease in the activity of γ-GCS. We showed previously that the removal of iron from crocidolite fibers did not affect the uptake of the fibers into A549 cells (9). This suggests that binding of the fibers to the cell membrane or internalization of the fibers may somehow lead to the efflux of GSH. Experiments are in progress to elucidate the mechanism for reduced GSH efflux stimulated by crocidolite fibers. It is apparent from the studies reported here, however, that iron associated with crocidolite is required for the formation of S-nitrosothiols. Chao et al. (9) showed that iron was required for NO synthesis in A549 cells after crocidolite exposure. The NO produced as a result of this exposure would be required for the formation of S-nitrosothiols. DF crocidolite treatment, resulting in depletion of intracellular GSH levels to 41% of untreated controls (the same level of depletion observed for unmodified crocidolite), did not lead to transcription of mRNA for iNOS (Park and Aust, unpublished observation), synthesis of NO (9), or formation of S-nitrosothiols.

In conclusion, exposure of A549 cells to crocidolite resulted in GSH efflux into the extracellular medium, formation of S-nitrosothiols, and decreased γ-GCS activity, which led to a decrease in intracellular GSH with no detectable oxidation to GSSG. The role that efflux of reduced GSH may play in the damaging effects of crocidolite in the epithelial cells remains unclear.

REFERENCES
1. Selikoff IJ, Lilis R, Nicholson WJ. Asbestos disease in United States shipyards. Ann NY Acad Sci 330:295–311 (1979).
2. Hardy JA, Aust AE. Iron in asbestos chemistry and carcinogenicity. Chem Rev 95:97–118 (1995).
3. Kamp DW, Graceff P, Pryor WA, Weitzman SA. The role of free radicals in asbestos-induced diseases. Free Radic Biol Med 12:293–315 (1992).
4. Shull S, Manohar M, Marsh JP, Jansen YMW, Mossman BT. Role of iron and reactive oxygen species in asbestos-induced lung injury. In: Free Radical Mechanisms of Tissue Injury (Moslen MT, Smith CV, eds). Boca Raton, FLCRC Press, 1992;153–162.
5. Weitzman SA, Graceff P. Asbestos catalyzes hydroxyl and superoxide radical generation from hydrogen peroxide. Arch Biochem Biophys 228:373–376 (1984).
6. Aust AE, Lund LG. Iron mobilization from crocidolite results in enhanced iron-catalyzed oxygen consumption and hydroxyl radical generation in the presence of cysteine. In: Mechanisms in Fibre Carcinogenesis (Brown RC, Hoskins JA, Johnson NF, eds). North Atlantic Treaty Organization Advanced Study Institute Series. New York:Plenum, 1991;397–405.
7. Lund LG, Aust AE. Iron mobilization from crocidolite asbestos greatly enhances crocidolite-dependent formation of DNA single-
GLUTATHIONE EFFLUX FROM CROCIDOLITE-TREATED HUMAN LUNG CELLS

strand breaks in qX174 RFI DNA. Carcinogenesis 13:637–642 (1992).
8. Takeuchi T, Morimoto K. Crocidolite asbestos increased 8-
hydroxydeoxyguanosine levels in cellular DNA of a human
promyelocytic leukemia cell line, HL60. Carcinogenesis
15:635–639 (1994).
9. Chao CC, Park SH, Aust AE. Participation of nitric oxide and
iron in the oxidation of DNA in asbestos-treated human lung
cells. Arch Biochem Biophys 326:152–155 (1996).
10. Meister A, Anderson ME. Glutathione. Annu Rev Biochem
52:711–760 (1983).
11. Janssen YMW, Heintz NH, Mossman BT. Induction of c-fos
and c-jun proto-oncogene expression by asbestos is ameliorated
by N-acetyl-t-cysteine in mesothelial cells. Cancer Res
55:2085–2089 (1993).
12. Isradian VA, Weitzman SA, Kamp DW. Dibutyryl cAMP attenu-
ates asbestos-induced pulmonary epithelial cell cytotoxicity
and decline in ATP levels. Am J Physiol 267:L518–L525 (1994).
13. Boehme DS, Maples KR, Henderson RF. Glutathione release
by pulmonary alveolar macrophages in response to particles in
vitro. Toxicol Lett 60:53–60 (1992).
14. Campbell WJ, Huggins CW, Wylie AG. Chemical and physi-
ological characterization of amosite, chrysotile, crocidolite and non-
fibrous tremolite for oral ingestion studies by the National
Institute of Environmental Health Sciences. Bureau of Mines
Report of Investigations 8452. Washington:United States
Department of the Interior, 1980.
15. Chao C-C, Lund LG, Zinn KR, Aust AE. Iron mobilization
from crocidolite asbestos by human lung carcinoma cells. Arch
Biochem Biophys 314:384–391 (1994).
16. Griffith OW. Determination of glutathione and glutathione
disulfide using glutathione reductase and 2-vinylpyridine. Anal
Biochem 106:207–212 (1980).
17. Richie JP Jr, Lang CA. The determination of glutathione,
cyst(e)ine, and other thiols and disulfides in biological samples
using high-performance liquid chromatography with dual elec-
trochemical detection. Anal Biochem 163:9–15 (1986).
18. Meredith MJ. Analysis of protein-glutathione mixed disulfides
by high performance liquid chromatography. Anal Biochem
131:504–509 (1983).
19. Saville B. A scheme for the colorimetric determination of
microgram amounts of thiols. Analyst 83:670–672 (1958).
20. Sexton DJ, Muruganandam A, McKenney DJ, Murus B.
Visible light photochemical release on nitric oxide from S-
nitrosoglutathione: potential photochemotherapeutic applica-
tions. Photochem Photobiol 59:463–467 (1994).
21. Cappel RE, Gilbert HF. Thiol/disulfide exchange between 3-
hydroxy-3-methylglutaryl-CoA reductase and glutathione. J
Biol Chem 263:12204–12212 (1988).
22. Seelig GF, Meister A. Glutathione biosynthesis: γ-glutamylcys-
teine synthetase from rat kidney. Methods Enzymol
113:379–389 (1985).
23. Wendel A. Glutathione peroxidase. Methods Enzymol 77:324–
332 (1981).
24. Carlborg I, Mannervik B. Glutathione reductase. Methods
Enzymol 113:484–490 (1985).
25. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases.
J Biol Chem 249:7130–7136 (1974).
26. Lee C-Y. Glucose-6-phosphate dehydrogenase from mouse.
Methods Enzymol 89:252–257 (1982).
27. Welder AA, Acosta D. Enzyme leakage as an indicator of cytoto-
icity in cultured cells. In: In Vitro Toxicity Indicators (Tyson CA,
Frazier JM, eds). San Diego:CA:Academic Press, 1994:46–49.
28. van den Dobbelsteen DJ, Nobel CSI, Schlegel J, Cotgreave IA,
Orrenius S, Slater AFG. Rapid and specific efflux of reduced
 glutathione during apoptosis induced by anti-Fas/Apo-1 anti-
body. J Biol Chem 271:15420–15427 (1996).
29. Bridgeham MM, Marsden M, Selby C, Morrison D, MacNee
W. Effect of N-acetyl cysteine on the concentration of thiols in
plasma, bronchoalveolar lavage fluid, and lung tissue. Thorax
49:670–675 (1994).
30. Slade R, Crissman K, Norwood J, Hatch G. Comparison of
antioxidant substances in bronchoalveolar lavage cells and
fluid from humans, guinea pigs, and rats. Exp Lung Res
19:469–484 (1993).
31. Cantin AM, North SL, Hubbard RC, Crystal RG. Normal
alveolar epithelial lining fluid contains high levels of glu-
tathione. J Appl Physiol 63:152–157 (1987).
32. Lu SC, Garcia-Ruiz C, Kuhlenkamp J, Oorkhrens M, Salas-
Prato M, Kaplowitz N. Hormonal regulation of glutathione
efflux. J Biol Chem 265:16088–16095 (1990).
33. Liu J, Sato C, Takano T, Marumo F. Characterization of vaso-
pressor-mediated GSH efflux from HEP G2 cells: significance of
protein kinase C. Life Sci 52:1217–1223 (1993).
34. Garcia-Ruiz C, Fernandez-Checa JC, Kaplowitz N. Bidirec-
tional mechanism of plasma membrane transport of reduced
 glutathione in intact rat hepatocytes and membrane
vesicles. J Biol Chem 267:22256–22264 (1992).
35. Smith CV, Jones DP, Guenthner TM, Lash LH, Lauterburg BH.
Compartmentation of glutathione: implications for the study of
 toxicity and disease. Toxicol Appl Pharmacol 140:1–12 (1996).
36. Wade R, Castro C. Redox reactivity of iron(III) porphyrins and
heme proteins with nitric oxide. Nitrosyl transfer to carbon, oxy-
gen, nitrogen, and sulfur. Chem Res Toxicol 3:289–291 (1990).
37. Janssen YM, Marsh JP, Absher MP, Hemenway D, Vacek PM,
Leslie KO, Borm PJ, Mossman BT. Expression of antioxidant
enzymes in rat lungs after inhalation of asbestos or silica. J Biol
Chem 267:10625–10630 (1992).

Environmental Health Perspectives • Vol 105, Supplement 5 • September 1997