0026-895X/91/030281-04$03.00/0

MOLECULAR PHARMACOLOGY, 39:281-284



*ACCELERATED COMMUNICATION*

# Inhibition of Cell Colony Formation by Selenite: Involvement of Glutathione

PAULA BARONE CAFFREY and GERALD D. FRENKEL

*Department of Biological Sciences, Rutgers University, Newark, New Jersey 07102*

Received September 25, 1990; Accepted December 21, 1990

**SUMMARY**

Selenium is an essential trace element that has been shown to have anticarcinogenic activity. One mechanism that has been proposed for this activity is a cytotoxic effect of selenium on tumor cells. As a means of assessing its cytotoxicity, we have examined the effect of selenite on tumor cell viability, using as an assay the ability of the cells to form colonies. We have found that brief exposure of HeLa cells to micromolar concentrations of selenite resulted in significant inhibition of colony formation, indicating that this is an assay for selenite cytotoxicity that is more sensitive than those that have been employed previously. In order to investigate the involvement of cellular glutathione in selenite cytotoxicity, we treated cells with buthionine sulfoximine (BSO) before selenite exposure. This treatment, which resulted in a 7-fold reduction in the level of intracellular glutathione, also caused a significant decrease in the inhibitory effect of selenite on colony formation. However, when cells were exposed to selenite that had previously been reacted with glutathione, the BSO-induced decrease in cytotoxicity was eliminated. In contrast, reaction of selenite with other sulfhydryl compounds, such as cysteine and mercaptoethylamine, did not restore its potency in BSO-treated cells. The simplest explanation for these results is that, for selenite to exert its inhibitory effect, it must react with intracellular glutathione to form the selenodiglutathione derivative.

Several hypotheses have been proposed to explain the observed anticarcinogenic activity of selenium compounds (1). One proposed mechanism that is currently being investigated is that selenium compounds exert a cytotoxic effect on tumor cells, thereby inhibiting their proliferation. Selenium compounds do in fact inhibit cell proliferation (2–5), although the precise mechanism of this inhibition has not been elucidated. Selenite has been found to be one of the most potent selenium compounds in this regard, but it is metabolized by cells to a variety of selenium-containing species. The question has, thus, arisen as to whether this metabolism is necessary for the cytotoxic effects of selenite or whether selenite itself can exert these effects (1, 6).

The first step in the metabolism of selenite is believed to be its chemical reaction with glutathione (the predominant cellular SH compound) to form the selenotrisulfide (7):

$$4\ GSH + H_2SeO_3 \rightarrow GSSeSG + GSSG + 3\ H_2O$$

Several studies have suggested the involvement of SH compounds in the cytotoxic effects of selenite (8–15). For example, SH compounds enhance the inhibitory effect of selenite on amino acid transport and protein synthesis (9, 12, 16, 17). Selenotrisulfides have been shown to inhibit both the growth of ascites tumors *in vivo* and their cell viability (10, 13). They have also been shown to inhibit DNA, RNA, and protein synthesis *in vitro* (17, 18). Direct evidence for the involvement of SH compounds in the cytotoxic effects of selenite was provided by studies that demonstrated that depletion of SH compounds from cells resulted in a significant decrease in the inhibitory effect of selenite on cellular DNA and RNA synthesis (19, 20).

These findings have led us to investigate whether reaction with glutathione is required for selenite to have an inhibitory effect on cell viability, a measure of cytotoxicity with obvious relevence to tumor proliferation. As an assay of viability, we have utilized the ability of the cell to form a colony. We have found that selenite is a very potent inhibitor of cell colony formation and that depletion of glutathione from cells results in a significant decrease in the inhibitory effect of selenite.

## Experimental Procedures

**Materials.** Sodium selenite, BSO, glutathione, cysteine, and mercaptoethylamine were obtained from Sigma. Selenotrisulfides were formed by the reaction of selenite (0.25–3.0 $\mu$M) with the appropriate SH compound, at a 1:4 molar ratio (7), in culture medium (without

This work was supported by Grant ES-04087 from the National Institutes of Health. This is Publication 91 from the Department of Biological Sciences, Rutgers-Newark.

**ABBREVIATIONS:** SH, sulfhydryl; BSO, buthionine sulfoximine.

serum) for 30 min at room temperature. The formation of selenotrisulfide was confirmed by UV spectrometry (7, 18). [$^{75}$Se]selenite was purchased from Amersham.

**Cells.** HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). They were grown in Dulbecco's modified Eagle's medium, with 10% fetal calf serum (GIBCO), at 37° in a 5% $CO_2$ atmosphere.

**SH determination.** The level of intracellular SH compounds was determined by the Ellman reaction, as described previously (19).

**Colony assay.** Approximately $2 \times 10^5$ cells were seeded in 100-mm dishes. After incubation for 2 days, the cells were exposed to the selenium compound, as indicated for the individual experiments. Duplicate cultures were exposed to each dose of selenium compound. Where indicated, cells were exposed to BSO (final concentration, 1 mM) for 24 hr; the BSO was then removed and the appropriate concentration of selenium compound was added. After incubation for 1 hr, the cells were trypsinized and counted, and approximately 200 cells were seeded in 60-mm dishes for determination of colony formation. At least four replicate colony determinations were carried out for each culture. After incubation for 10 days, the cells were rinsed with phosphate-buffered saline, fixed with methanol, and stained with Giemsa, and the number of colonies/dish was determined. For each treatment, the mean number of colonies and the standard deviation were calculated.

**Determination of cell-associated selenium.** Cells were exposed to [$^{75}$Se]selenite or [$^{75}$Se]selenodiglutathione (0.9 mCi/μmol) in medium without serum. After 1 hr, the medium was removed, the cells were washed with phosphate-buffered saline, removed from the dish with a rubber policeman, centrifuged, and resuspended in 0.1% sodium dodecyl sulfate containing 10 mM EDTA, and the radioactivity was determined in a Beckman γ-counter.

## Results

The effect of selenite on cell viability was measured using, as an assay, the ability of the cells to form colonies. As shown in Fig. 1, exposure of HeLa cells to selenite for 1 hr resulted in a dose-dependent decrease in the number of cells that form colonies; under these conditions, 50% inhibition occurred with approximately 1.5 μM selenite. This result indicates that the colony assay is one of the most sensitive indicators of selenite-induced cytotoxicity (see Discussion).

In order to examine the role of glutathione in this inhibitory effect of selenite, cells were treated with BSO before exposure to selenite. This compound inhibits an enzyme in the biosynthetic pathway of glutathione, thus causing a specific decrease in its intracellular level. In our experiments, BSO treatment caused a 7-fold decrease in the level of intracellular SH. After selenite exposure, this level increased in cells that had not been treated with BSO, as has been reported for other cell types (21). However, after exposure to selenite there was an even greater (9-fold) difference in SH levels between control and BSO-treated cells. As shown in Fig. 1, exposure to 5 μM selenite had virtually no effect on colony formation by BSO-treated cells, although this concentration of selenite inhibited colony formation by control cells by more than 90% (Fig. 1). Approximately 8 μM selenite was required to achieve 50% inhibition of colony formation by BSO-treated cells.

These results indicate that the level of intracellular glutathione can influence the effect of selenite on cell viability. If this is due to a requirement for the reaction of selenite with glutathione to form selenodiglutathione (7) (see Introduction), then, in contrast to selenite, the potency of selenodiglutathione should not be decreased by pretreatment of cells with BSO. To examine this, selenite was reacted with glutathione (see Experimental Procedures) and cells were exposed to the products of the reaction. The results (Fig. 2) show that BSO treatment did not cause a decrease in the inhibition of colony formation by selenodiglutathione; on the contrary, it appears to have caused an increase in its effect. Thus, the decrease in the potency of selenite that results from depletion of intracellular glutathione is overcome when selenite is reacted with exogenous glutathione to form selenodiglutathione. This effect cannot be due either to unreacted glutathione or to the glutathione disulfide product of the reaction, because these had no significant effect on colony formation (data not shown).

We have examined whether the higher potency of selenodigluthione, compared with selenite, in BSO-treated cells could

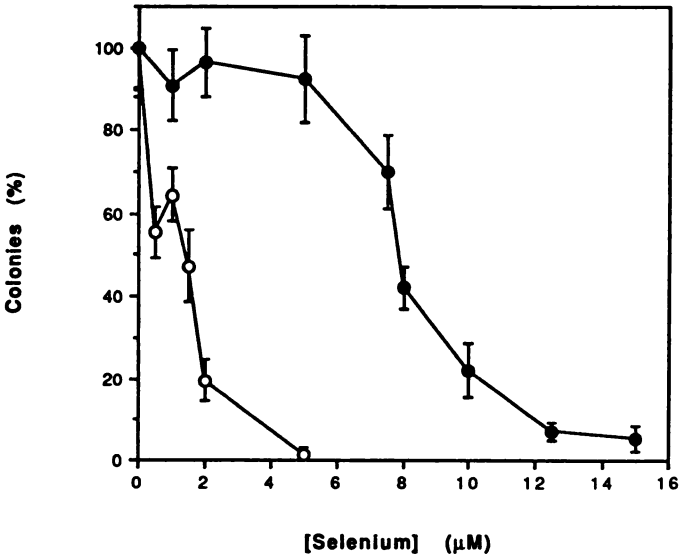


**Fig. 1.** Effect of selenite on colony formation. Cells were exposed to the indicated concentrations of selenite for 1 hr, after which the number of cells that formed colonies was determined as described in Experimental Procedures. The results are presented as a percentage of colonies formed in the absence of selenite. ○, Control cells; ●, BSO-treated cells.

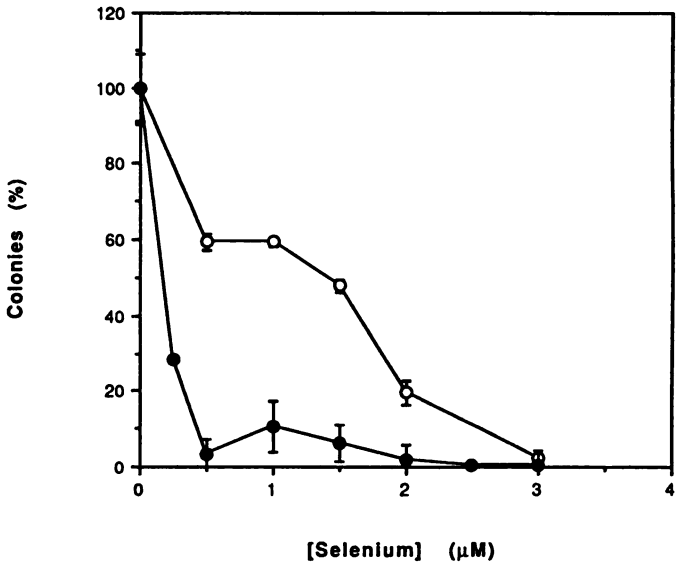


**Fig. 2.** Effect of selenodiglutathione on colony formation. The experiment was carried out as in Fig. 1, except that, before its addition to the cell cultures, selenite was reacted with glutathione, as described in Experimental Procedures. The symbols are the same as in Fig. 1.

be the result of higher levels of cell-associated selenium. As shown in Table 1, after exposure of BSO-treated cells to 5 μM selenite (which had no inhibitory effect on colony formation) there were 3.36 nmol of Se/$10^6$ cells. In contrast, after exposure to only 0.5 μM selenodiglutathione (which did significantly inhibit colony formation) there were only 0.13 nmol of Se/$10^6$ cells. These results clearly demonstrate that in BSO-treated cells the increased potency of selenite after reaction with glutathione is most likely due to the presence of a different form of selenium, rather than a higher level.

Although glutathione is the predominant intracellular low molecular weight SH compound, other intracellular SH compounds, such as cysteine and mercaptoethylamine (22), can react with selenite to form corresponding selenotrisulfides (7). To examine the potential role of these compounds in the inhibitory effect of selenite, cells were exposed to the products of the reaction of selenite with cysteine or mercaptoethylamine. Although colony formation was inhibited by selenodicysteine (Fig. 3) and by selenodimercaptoethylamine (Fig. 4), prior treatment of cells with BSO did not significantly alter their sensitivity to either compound.

A comparison of the potency of the various selenium compounds is presented in Table 2. In cells with normal levels of glutathione, selenodiglutathione had the same potency as selenite. However, in glutathione-depleted cells, selenodiglutathione was considerably more potent than selenite. In contrast, selenodicysteine and selenodimercaptoethanol were less potent than selenite in control cells and were no more potent than selenite

TABLE 1

**Cell-associated selenium in cells exposed to selenite and selenodiglutathione**

| Cells | Medium addition | Cell-associated selenium |
|---|---|---|
| | | *nmol/$10^6$ cells* |
| Control | Selenite (1.5 μM) | 0.38 |
| Control | Selenodiglutathione (1.5 μM) | 0.32 |
| BSO-treated | Selenite (5.0 μM) | 3.36 |
| BSO-treated | Selenodiglutathione (0.5 μM) | 0.13 |

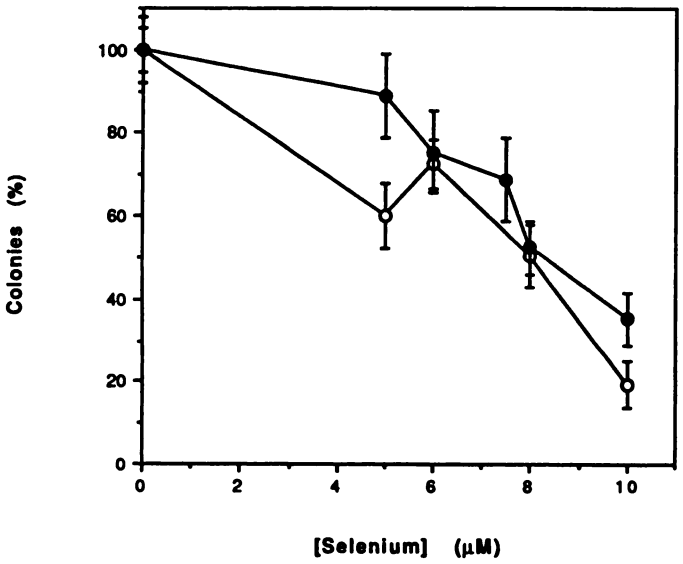


**Fig. 3.** Effect of selenodicysteine on colony formation. The experiment was carried out as in Fig. 1, except that, before its addition to the cell cultures, selenite was reacted with cysteine, as described in Experimental Procedures. The symbols are the same as in Fig. 1.

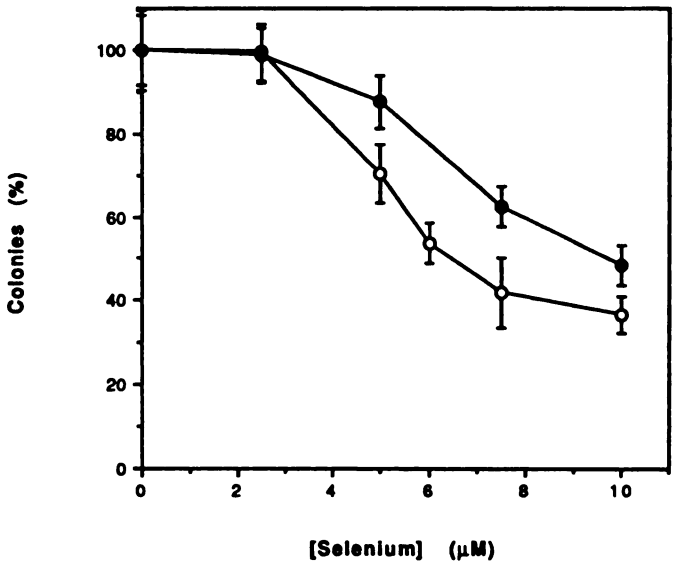


**Fig. 4.** Effect of selenodimercaptoethylamine on colony formation. The experiment was carried out as in Fig. 1, except that, before its addition to the cell cultures, selenite was reacted with mercaptoethylamine, as described in Experimental Procedures. The symbols are the same as in Fig. 1.

TABLE 2

**Inhibition of colony formation by selenium compounds**

$LC_{50}$ is the approximate concentration that produced 50% inhibition of colony formation. The data are taken from Figs. 1–4.

| Compound | $LC_{50}$ | |
|---|---|---|
| | Control cells | BSO-treated cells |
| | *μM Se* | |
| Selenite | 1.5 | 7.5 |
| Selenodiglutathione | 1.5 | <0.25 |
| Selenodicysteine | 8.0 | 8.5 |
| Selenodimercaptoethylamine | 7.0 | 10 |

in glutathione-depleted cells. Thus, neither cysteine nor mercaptoethylamine was able to substitute for glutathione in overcoming the decreased potency of selenite in BSO-treated cells.

## Discussion

Our results have shown that selenite inhibits the ability of cells to form colonies. With this assay, 50% inhibition was obtained with 1.5 μM selenite after an exposure of only 1 hr (Fig. 1). This should be compared with other assays that have been employed to assess selenite cytotoxicity. With cell proliferation as an endpoint, 50% inhibition required exposure to 5 μM selenite for several days; no significant inhibition was detectable with 2 μM selenite (23). With dye exclusion (as a measure of cell viability) as an endpoint, exposure of the cells to 10 μM selenite for 6 hr was required to produce a 50% decrease in viability (24). Thus, the inhibition of colony formation provides a sensitive assay for measuring the cytotoxicity of selenium compounds.

Our results have shown that depletion of glutathione from cells results in a decrease in the potency of selenite in inhibiting cell colony formation but that prior reaction of the selenite with glutathione overcomes this decreased potency; the resulting selenodiglutathione is, in fact, more potent in glutathione-

depleted cells than selenite is in control cells (Table 2). This demonstrates that the decreased potency of selenite in BSO-treated cells is the result of glutathione deficiency and provides direct evidence that glutathione is required for this cytotoxic effect of exogenous selenite. The most likely explanation for this requirement is that exogenous selenite must react with intracellular glutathione to form selenodiglutathione. This reaction is the first step in the metabolic conversion of selenite to several other selenium-containing species (25). Our results do not address the question of whether selenodiglutathione itself exerts a cytotoxic effect directly or whether further metabolism to other selenium compounds and/or incorporation into selenoproteins is required. This question is currently under investigation.

Depletion of glutathione from cells by treatment with BSO reduced the potency of selenite approximately 5-fold (Table 2). Previous studies in this laboratory showed that BSO treatment did not affect the inhibition of cellular DNA or RNA synthesis by selenite, although nonspecific depletion of SH compounds from cells with diethylmaleate did significantly decrease the potency of selenite (19, 20). Recent results have demonstrated that, when selenite is added to BSO-treated cells, the selenotrisulfides of cysteine and mercaptoethylamine are formed instead of the selenodiglutathione that is formed in control cells (26). This suggests that the lack of effect of BSO in this system is most likely due to the inhibition of DNA and RNA synthesis by these alternative selenotrisulfides (or their metabolites). The fact that BSO treatment does decrease the potency of selenite in the inhibition of colony formation suggests that these selenotrisulfides do not exert this effect on cells. This idea is supported by the failure of prior reaction of selenite with cysteine or mercaptoethylamine to overcome the decreased potency of selenite in BSO-treated cells (Figs. 3 and 4).

In cells with a normal level of glutathione, selenodiglutathione had about the same potency as selenite (see Table 2). In contrast, the selenotrisulfides of cysteine and mercaptoethylamine were considerably less effective than selenite in inhibiting colony formation. This difference could be due to the lower permeability of the cells to these selenotrisulfides or to a lower intrinsic activity inside the cell. Consistent with the latter explanation is our recent finding that these selenotrisulfides are formed intracellularly when selenite is added to BSO-treated cells (26). The fact that in BSO-treated cells selenite has the same low potency as these selenotrisulfides suggests that they are relatively inactive intracellularly in the inhibition of colony formation. They do, however, appear to be active in other cytotoxicity assays (12, 18–20).

It is clear from these results that cells are less sensitive to the effect of selenite after they have been depleted of glutathione. There have been reports of variations in the susceptibility of normal and tumor cells to glutathione depletion (27). This suggests that manipulation of intracellular glutathione levels might represent a strategy for achieving selective inhibition by selenite of the growth of tumor cells versus normal cells. This could have important implications for the future development of selenium compounds as chemopreventive agents.

**Acknowledgment**

We thank Christina MacVicar for excellent technical assistance.

**References**

1. Medina, D., and D. Morrison. Current ideas on selenium as a chemopreventative agent. *Pathol. Immunopathol. Res.* **7**:187–199 (1988).
2. LeBoeuf, R., B. Laishes, and W. Hoekstra. Effects of selenium on cell proliferation in rat liver and mammalian cells as indicated by cytokinetic and biochemical analysis. *Cancer Res.* **45**:5496–5504 (1985).
3. Milner, J., and C. Hsu. Inhibitory effects of selenium on the growth of L1210 leukemic cells. *Cancer Res.* **41**:1652–1656 (1981).
4. Petrie, H., L. Klassen, M. Tempero, and H. Kay. *In vitro* regulation of human lymphocyte proliferation by selenium. *Biol. Trace Elements Res.* **11**:129–146 (1986).
5. Medina, D., and C. Oborn. Differential effects of selenium on the growth of mouse mammary cells *in vitro*. *Cancer Lett.* **13**:333–344 (1981).
6. Frenkel, G. Effects of sodium selenite and selenate on DNA and RNA synthesis *in vitro*. *Toxicol. Lett.* **25**:219–223 (1985).
7. Ganther, H. Selenotrisulfides: formation by the reaction of thiols with selenious acid. *Biochemistry* **7**:2898–2905 (1968).
8. Batist, G., A. Katki, G. Klecker, and C. Myers. Selenium-induced cytotoxicity of human leukemia cells: interaction with reduced glutathione. *Cancer Res.* **46**: 5482–5485 (1986).
9. Hogberg, J., and A. Kristoferson. Inhibition of amino acid uptake in isolated hepatocytes by selenite. *FEBS Lett.* **107**:77–80 (1979).
10. Poirier, K., and J. Milner. Factors influencing the antitumorigenic properties of selenium in mice. *J. Nutr.* **113**:2147–2154 (1983).
11. Snyder, R. Effects of sodium selenite on DNA and carcinogen-induced DNA repair in human diploid fibroblasts. *Cancer Lett.* **34**:73–81 (1987).
12. Vernie, L., M. DeVries, L. Karreman, R. Topp, and W. Bont. Inhibition of amino acid incorporation in a cell-free system and inhibition of protein synthesis in cultured cells by reaction products of selenite and thiols. *Biochim. Biophys. Acta.* **739**:1–7 (1983).
13. Vernie, L., C. Homburg, and W. Bont. Inhibition of the growth of malignant mouse lymphoid cells by selenodiglutathione and selenodicysteine. *Cancer Lett.* **14**:303–308 (1981).
14. Whiting, R., L. Wei, and H. Stich. Unscheduled DNA synthesis and chromosome aberrations induced by organic selenium compounds in the presence of glutathione. *Mutat. Res.* **78**:159–169 (1980).
15. Young, J., C. Crowley, and E. Tucker. Haemolysis of normal and glutathione-deficient sheep erythrocytes by selenite and tellurite. *Biochem. Pharmocol.* **30**:2527–2530 (1981).
16. Vernie, L., W. Bont, and P. Emmelot. Inhibition of *in vitro* amino acid incorporation by sodium selenite. *Biochemistry.* **13**:337–341 (1974).
17. Vernie, L., J. Collard, A. Eker, A. DeWildt, and I. Wilders. Studies on the inhibition of protein synthesis by selenodiglutathione. *Biochem. J.* **180**:213–218 (1979).
18. Frenkel, G., A. Walcott, and C. Middleton. Inhibition of RNA and DNA polymerases by the product of the reaction of selenite with sulfhydryl compounds. *Mol. Pharmacol.* **31**:112–116 (1987).
19. Frenkel, G., and D. Falvey. Evidence for the involvement of cellular sulfhydryl compounds in the inhibition of DNA synthesis by selenite. *Mol. Pharmacol.* **34**:573–577 (1988).
20. Frenkel, G., and D. Falvey. Involvement of sulfhydryl compounds in the inhibition of cellular RNA synthesis by selenite. *Biochem. Pharmacol.* **38**:2849–2852 (1989).
21. Kuchan, M., and J. Milner. Intracellular glutathione and selenite toxicity in canine mammary cells in culture. *FASEB J.* **3**:A471 (1989).
22. Jocelyn, P. *Biochemistry of the SH Group.* Academic Press, London (1972).
23. Golczewski, J., and G. Frenkel. Cellular selenoproteins and the effects of selenite on cell proliferation. *Biol. Trace Element Res.* **20**:115–126 (1989).
24. Fico, M., K. Poirier, A. Watrach, M. Watrach, and J. Milner. Differential effects of selenium on normal and neoplastic canine mammary cells. *Cancer Res.* **46**:3384–3388 (1986).
25. Ip, C., and H. Ganther. Activity of methylated forms of selenium in cancer prevention. *Cancer Res.* **50**:1206–1211 (1990).
26. Frenkel, G., D. Falvey, and C. MacVicar. Products of the reaction of selenite with intracellular sulfhydryl compounds. *Biol. Trace Element Res.*, in press.
27. Russo, A., W. DeGraff, N. Friedman, and J. Mitchell. Selective modulation of glutathione levels in human normal *versus* tumor cells and subsequent differential response to chemotherapy drugs. *Cancer Res.* **46**:2845–2848 (1986).

**Send reprint requests to:** Dr. Gerald Frenkel, Department of Biological Sciences, Rutgers University, 101 Warren Street, Newark, NJ 07102.