Biochemical Pharmacology, Vol. 37, No. 18, pp. 3401–3406, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

STUDIES OF THE ROLE OF DNA FRAGMENTATION IN SELENIUM TOXICITY

PER GARBERG, ANNIKA STÅHL, MARGARETA WARHOLM and JOHAN HÖGBERG
Department of Toxicology, National Institute of Occupational Health, S-171 84 Solna, Sweden

(*Received* 4 *September* 1987; *accepted* 11 *March* 1988)

Abstract—The role of DNA damage in selenite cytotoxicity was studied in isolated hepatocyte model systems. An initial series of experiments, with hepatocytes in suspension, indicated that selenite-induced DNA fragmentation was oxygen dependent and could be inhibited by cyanide, $HgCl_2$ and CuDIPS. These findings were interpreted to imply that selenite-induced redox cycles were involved in this effect. In a second series of experiments, the effect of inhibitors of poly(ADP-ribose)polymerase (3-aminobenzamide and theophylline) and DNA alkylating agents on selenite-induced cellular lysis was studied. These experiments were performed with hepatocytes in primary culture and 20–30 μM selenite lysed the cultured cells after about 20 hr exposure. It was found that alkylators added 20 hr before selenite acted synergistically with selenite, and that inhibitors of poly(ADP-ribose)polymerase antagonized lysis. Further studies also indicated NAD degradation before lysis. These data indicate a modulating role for DNA damage in selenite cytotoxicity mediated by poly(ADP-ribose)polymerase. Taken together with previously published data on, for example, potentially lethal oxidation of NADPH (Anundi *et al.*, *Chem. Biol. Interact.* **50**, 277, 1984) they also suggest that cell death resulted from interactions between several events that may deplete energy supplies. The results are compatible with a selective killing of DNA-damaged hepatocytes by low doses of selenite.

The glutathione-dependent reductive biotransformation of selenite to dimethylselenide may involve redox cycles. In selenite-exposed isolated hepatocytes this was indicated by a transient increase in oxygen consumption [1], induction of hypoxia [2] and a coordinated starting of dimethylselenide formation after an initial lag phase [2]. A previous study of selenite toxicity had shown that rat hepatocytes in suspension were lysed within 2–3 hr by 50 μM selenite [3]. It was concluded that this type of toxicity was related to energy depletion induced by glutathione reductase-dependent redox cycles consuming NADPH [3].

Selenite is an anticarcinogen but also a DNA-damaging compound. Ultimate DNA-reactive species have not been identified [4], but some data suggest that selenite has to undergo biotransformation in order to become active [5, 6]. The involvement of redox cycles can thus be anticipated. The possibility that the DNA damage may modulate selenite toxicity is also of interest. Recent studies have indicated a relationship between DNA damage, caused by, for example, carcinogens [7] or activated oxygen [8], and cytotoxicity which may have the effect of preventing cancer development. Cells suffering excessive DNA damage may be lysed in a suicidal type of response, mediated by the nuclear enzyme poly(ADP-ribose)polymerase. This enzyme is activated by DNA strand breaks and may kill cells via NAD degradation and impaired energy production [9, 10]. It thus seems possible that selenite-induced redox cycles may deplete energy supplies not only via glutathione reductase but also via DNA damage and a subsequent poly(ADP-ribose)polymerase activation.

This study was undertaken in order to determine whether the same mechanisms as those previously implicated in toxic energy depletion could also induce DNA fragmentation, and whether damage to DNA could modulate selenite toxicity. As in previous studies isolated rat hepatocytes in suspension were used. In addition hepatocytes in primary culture have been used in order to carry out longer term toxicity studies. Effects on DNA were measured as single-strand breaks. This alteration is known to arise during DNA repair induced by different types of toxic events and has been implicated in the activation of poly(ADP-ribose)polymerase [10].

MATERIALS AND METHODS

Chemicals. Superoxide dismutase (from bovine erythrocytes), cytochrome *c* (horse heart type III) diethylnitrosamine (DEN) and 3-aminobenzamide (3-ABA) were obtained from Sigma, collagenase (from *Clostridium histolyticum*) from Boehringer Mannheim, ^{75}Se-labelled sodium selenite (2–20 mCi/mg Se) from Amersham, sodium selenite from Merck, methylmethane sulfonate (MMS) and sodium selenide from ICN, K & K Laboratories Inc., New York, hydroxylapatite from Bio-Rad Laboratories and 4′,6-diamidino-2-phenylindole·2HCl (DAPI) From Serva-Feinbiochemica (Heidelberg, F.R.G.). Copper(diisopropylsalicylate)$_2$ (CuDIPS) was synthesized according to Ref 11.

Hepatocyte isolation and incubation. Male Sprague–Dawley rats (about 200 g) were used as donor animals. Hepatocytes were isolated by collagenase perfusion [12]. Cells were prepared in a laminar flow hood with sterilized equipment and

solutions, and were either used as cultured cells or as cells in suspension. Cells to be cultured were seeded on collagen-coated dishes [13] and incubated at 37°, first for 4 hr in RPMI 1640 (GIBCO) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% calf serum. The medium was then changed to RPMI 1640 supplemented with linoleic acid (5 μg/ml), $CuSO_4$ (100 nM), selenite (3 nM), $ZnSO_4$ (50 pM), penicillin (100 units/ml) and streptomycin (100 μg/ml) (cf. Ref. 14).

The number of cells seeded was 1×10^6 per 60 mm plate. Within one day the dishes were covered by cells with hepatocyte morphology. Experiments were usually begun the day after isolation of hepatocytes, and were performed in fresh serum-free medium as described above or in Krebs–Henseleit buffer. Test substances were dissolved in the culture medium.

Hepatocytes in suspension (2×10^6 cells/ml: in 20 ml) were incubated in rotating round-bottom flasks at 37°. The medium used was Krebs–Henseleit buffer pH 7.4 supplemented with methionine (0.5 mM). Carbogen gas (93.5% O_2:6.5% CO_2) was supplied to the surface unless otherwise indicated [12].

Cell viability in cultures was assessed by phase contrast microscopy and by a lactate dehydrogenase (LDH) assay. LDH activity was measured in aliquots from the medium and increased activity was taken as a sign of increased leakage from cells. Viability of hepatocytes in suspension was determined either by Trypan blue exclusion or by NADH penetration assay [12].

Selenium metabolism and nicotinamide excretion. Excretion of the selenium-metabolite dimethyl selenide was measured as the volatilization of ^{75}Se-labelled selenite from suspensions of hepatocytes [1]. Nicotinamide concentration in the medium was determined by HPLC-analysis [15]. In these experiments, cells were incubated in Krebs–Henseleit buffer.

Superoxide analysis. The formation of superoxide was determined as SOD-inhibitable reduction of ferricytochrome *c*, monitored at 550 nm [16] at 30° using a split beam spectrophotometer (Aminco DW-2).

Determination of single-strand breaks. Measurements of single-strand breaks were made according to the DNA unwinding technique described by Ahnström and Erixon [17]. In this assay, the proportion of single-stranded DNA reflects the number of strand breaks and sites susceptible to alkaline hydrolysis present in the DNA.

The amount of DNA was determined by a fluorometric method using DAPI [18]. Fluorescence was monitored on a Perkin Elmer LS-2 Filter Fluorimeter (excitation 375 nm and emission 454 nm).

All experiments were repeated at least three times with different cell batches. Data shown are from typical experiments or the mean of several experiments where indicated.

RESULTS

The induction of single-strand breaks (SSB) by selenite in suspended hepatocytes was dose depen-

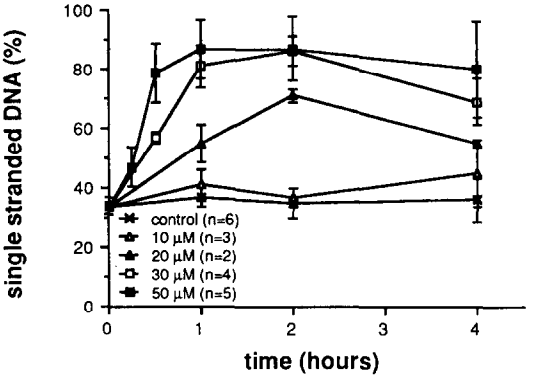

Fig. 1. SSB levels in suspended hepatocytes exposed to selenite. Selenite (0–50 μM) was added at zero time and SSB levels measured in aliquots withdrawn at times indicated. Each point represents the mean ± SD of two to six experiments as indicated.

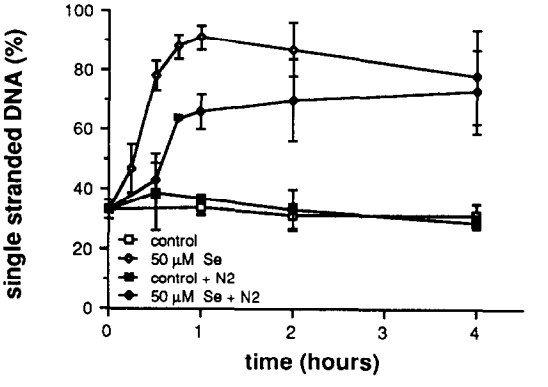

Fig. 2. Effect of oxygen on selenite induced SSB formation. Suspensions of hepatocytes exposed to 50 μM selenite and non-exposed controls were supplied with either nitrogen or carbogen during the first half hour of incubation. All incubates were then supplied with carbogen during the following incubation. Each point represents the mean ± SD from three experiments.

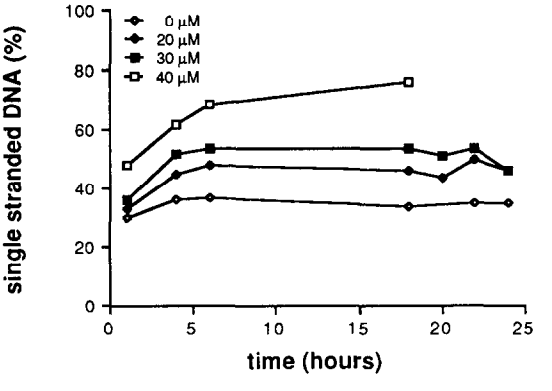

Fig. 3. Effect of selenite on SSB levels in hepatocytes in primary culture. Hepatocytes were exposed to selenite 24 hr after seeding, and SSB levels were measured at times indicated (one dish for each time point).

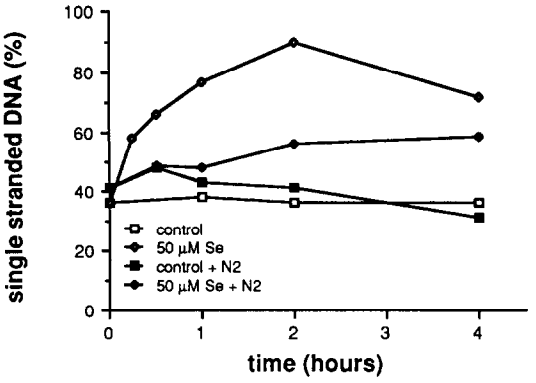

Fig. 4. Selenide (Na_2Se) induced SSB formation in suspended hepatocytes and the effect of oxygen. Selenide exposed hepatocytes (50 μM) and non-exposed controls were supplied with either nitrogen or carbogen during the first half hour of incubation. During the following incubation, all cells were supplied with carbogen.

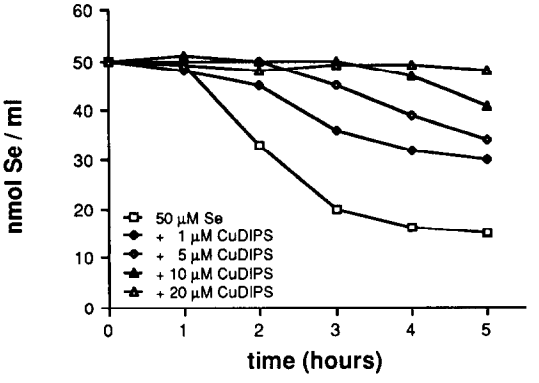

Fig. 6. Effect of CuDIPS on dimethylselenide excretion in suspended hepatocytes. ^{75}Se-selenite (50 μM) and CuDIPS (0–20 μM) were added at zero time. Volatilization of Se was measured as the disappearance of ^{75}Se from the incubates. Aliquots were withdrawn at times indicated.

dent (Fig. 1). Usually, 10 μM had little or no effect, while 20 μM and higher doses always induced a significant response. The increase in SSB was transient and lasted for 1–2 hr, depending on the concentration. It occurred only during the metabolic lag phase, which lasted 1 hr when 50 μM selenite was used (Fig. 6), and 2–3 hr for lower concentrations (cf. Ref. 1). Following this increase, the level of SSB declined but never reached control levels during incubation for 4 hr. Cytotoxic effects were occasionally observed after 3 or 4 hr when 50 μM selenite was used, but not at lower concentrations.

Previous studies have shown that during the lag phase, there is a temporary increase in oxygen uptake in selenite exposed hepatocytes [1, 3]. This may lead to hypoxia, which seems to be a prerequisite for dimethyl selenide production [2]. Inhibitors of dimethyl selenide formation and oxygen uptake, such as cyanide and $HgCl_2$ which selectively trap metabolites of selenite [3], were now tested for their ability to inhibit SSB formation. It was found that both cyanide (25 μM) and $HgCl_2$ (10 μM) decreased the type of response shown in Fig. 1 (data not shown). These data were compatible with an involvement of redox cycles in the SSB response, so experiments with nitrogen gas instead of carbogen were also performed. Figure 2 shows that the effect of selenite on SSB levels was delayed and reduced by supplying nitrogen instead of carbogen for the first half hour of incubation. In similar experiments using methylmethane sulfonate instead of selenite there was no effect of nitrogen on SSB levels.

The effect of selenite on SSB levels in cultured hepatocytes was also measured (Fig. 3). The effects were similar to those recorded in suspended cells. However, the clear decrease seen in suspended cells was not seen in cultured cells. In experiments where the cells were exposed to 20 or 30 μM selenite for 5 hr only, a decrease in SSB levels was seen after the change of medium.

Hydrogen selenite is a reactive intermediate in the selenite biotransformation pathway, and has been suggested as a possible DNA damaging species [5]. However, experiments with nitrogen indicated an oxygen dependency for the induction of SSB by selenide (Fig. 4).

Superoxide (O_2^-) may be generated in auto-oxidation and redox cycles [19]. Its formation during selenite metabolism was tested for in a non-cellular system. It was found (Fig. 5) that superoxide was

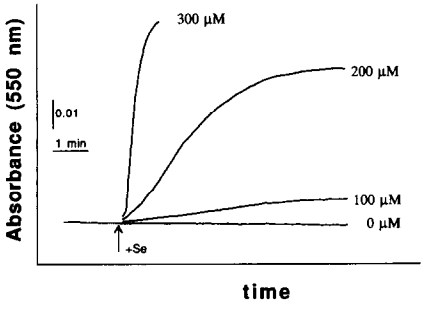

Fig. 5. Selenite dependent formation of superoxide. The difference in absorbance between the two cuvettes was monitored at 550 nm. Both cuvettes contained potassium phosphate buffer (50 mM) pH 7.5, cytochrome *c* (0.2 mg/ml) and reduced glutathione (5 mM). The reference cuvette also contained superoxide dismutase (25 μg/ml). Recordings of the difference in absorbance were started and selenite was added to both cuvettes where indicated.

Table 1. Effect of selenite on hepatocyte leakage

Selenite conc. (μM)	LDH activity (IU/ml)				
	1 hr	2 hr	3 hr	4 hr	24 hr
0	0.016	—	—	0.016	0.097
10	—	—	—	0.016	0.113
20	—	—	—	0.032	0.177
30	—	—	—	0.048	0.289
40	—	—	—	0.032	0.289
50	—	—	0.032	0.048	0.306
75	—	—	0.048	0.129	0.273
100	0.016	0.032	0.064	0.209	0.273

Hepatocytes were exposed to selenite 24 hr after isolation. LDH activity was measured in samples (0.1 ml) taken from medium at times (after selenite addition) indicated. Data taken from a typical experiment.

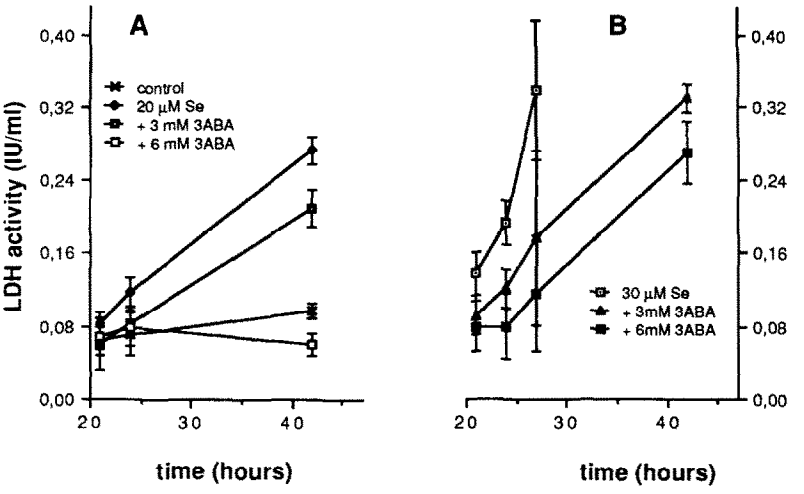

Fig. 7. Effect of 3-ABA on selenite toxicity. Cultured hepatocytes were exposed to selenite and 3-ABA 24 hr after seeding. In panel A all dishes except control dishes were exposed to 20 μM selenite. In panel B all dishes were exposed to 30 μM selenite. LDH activity was measured in aliquots of the medium at time points (after selenite and 3-ABA addition) indicated. Each point represents the mean of three dishes ± SD.

formed by combining selenite with glutathione, a combination which also is known to be genotoxic [5]. In the absence of glutathione selenite (500 μM) did not induce a change in absorbance. To further implicate O_2^- in the genotoxicity of selenite studies with superoxide dismutase and CuDIPS were performed. It was found that extracellular SOD had no effect on SSB levels induced by 50 μM selenite, whereas CuDIPS did. However, CuDIPS also inhibited dimethylselenide formation (Fig. 6) in the same way as cyanide or $HgCl_2$ [3]. In fact, it was found to be a very efficient inhibitor of selenite metabolism, and 1 μM CuDIPS had marked effects.

Known scavengers of the hydroxyl radical, dimethyl sulfoxide (300 mM) and mannitol (10 mM), had no effect on the SSB level induced by 30 μM selenite. Similar results were obtained with mannitol in a recent study on selenite induced DNA alterations in fibroblasts [20].

The possible role of DNA alterations in selenite toxicity was investigated by the use of inhibitors of poly(ADP-ribose)polymerase [8] in toxicity studies on suspended hepatocytes. Selenite concentrations (75–100 μM) which lysed the cells within 2–3 hr [3] were used. However, the inhibitors did not prevent the lysis (data not shown). Lower concentrations of selenite were not tested in this model as it did not permit incubation periods longer than 5–7 hr. Studies were also performed using hepatocytes in primary culture. With this model it was found that lower concentrations of selenite (20–40 μM) induced a more delayed type of toxicity (Table 1). It was also found that inhibitors of poly(ADP-ribose)polymerase could further delay or prevent this toxicity. Figure 7 shows that 3 mM 3-ABA delayed selenite toxicity for about 5 hr, and that 6 mM delayed it for about 10 hr. In other experiments it was found that 0.2 mM delayed the toxicity of selenite for about 30 min. As 0.2 mM inhibits poly(ADP-ribose)polymerase by more than 50% [21] a rapid metabolism of 3-ABA in hepatocytes was also indicated. In all these experiments morphological changes mirrored the changes in leakage, so that a high LDH-activity was obtained in plates where most cells were detached or rounded-up. In plates with unchanged LDH activity the morphology of the cells was the same as in control plates, that is, a hepatocyte morphology was preserved. Theophylline was also used in the toxicity studies and found to have similar effects as 3-ABA.

The involvement of poly(ADP-ribose)polymerase was further indicated by measuring nicotinamide excretion [8]. Table 2 shows that after 6 hr 30 μM selenite increased the concentration of nicotinamide in the medium, indicating activation of poly(ADP-ribose)polymerase and NAD degradation. The mean nicotinamide concentration in 5 selenite exposed plates (30 μM; 6 hr) from three experiments was 1.55 ± 0.36 μM, and in control plates from the same experiments 0.86 ± 0.27 μM. This difference was significant ($P < 0.05$), while the differences between control plates and plates with lower selenite concentrations were not significant.

Table 2. Effect of selenite on nicotinamide release

Selenite conc. (μM)	Nicotinamide conc. (μM)	
	3 hr	6 hr
0	0.58	0.62
10	0.52	0.76
20	0.62	0.77
30	0.55	1.18

Hepatocytes were cultured as described in Materials and Methods for 24 hr. The medium was then changed to a selenite containing Krebs–Henseleit buffer. Samples for HPLC analysis (0.2 ml) were taken after 3 and 6 hr. Data taken from a typical experiment.

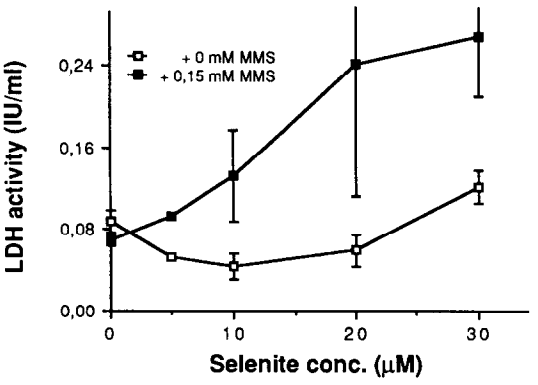

Fig. 8. Effect of MMS pretreatment on selenite toxicity in hepatocytes. Cultured hepatocytes were exposed to MMS (0.15 mM) 4 hr after seeding. Twenty-four hours after seeding the cells were exposed to selenite. LDH activity was measured in aliquots of the medium taken 24 hr after selenite addition. Each point gives the mean ± SD for three dishes.

The effect of DNA alkylating agents on selenite toxicity was also investigated. Cultured cells were exposed to methylmethane sulfonate (MMS) or diethylnitrosamine (DEN) in doses which were not cytotoxic and 20 hr later to selenite. It was found that both MMS (Fig. 8) and DEN (not shown) treatment resulted in an increased sensitivity towards selenite toxicity.

DISCUSSION

This study shows that selenite is a potent inducer of DNA single-strand breaks in oxygenated hepatocytes. Work by others with a similar model shows a comparable potency of for example, menadione [22] and in a fibroblast model selenite has also been shown to induce DNA fragmentation [20]. Other work with selenite have shown that the DNA damaging effect was potentiated by glutathione [5] or erythrocyte lysates [6], suggesting an involvement of metabolic activation. It has also been suggested that intermediates, such as selenopersulfide (GSSeH) and selenide (HSe^-), may react with DNA [5]. Our data confirm an involvement of metabolic activation in the induction of DNA damage by selenite. However, the data also tend to exclude a direct attack of selenide.

The involvement of metabolic activation was indicated by the effects of cyanide, $HgCl_2$, and nitrogen on selenite induced SSB levels. Cyanide was used as a trapper of selenodiglutathione (GSSeSG) and Hg^{2+} as a trapper of Se^{2-} [3]. Nitrogen was used as a hypoxic agent, and may have prevented redox cycling but may also have increased the rate of selenite disappearance. It may thus be argued that the effect of nitrogen was mainly due to an increased rate of metabolism and that selenite was the DNA damaging species. However, previous measurements on selenite disappearance [23] indicate that not more than 20% of the added dose of selenite was lost during the nitrogen exposure, and this limited loss cannot explain the almost complete inhibition by nitrogen. Furthermore, the effect of reoxygenation is not compatible with this hypothesis.

The demonstration of O_2^- production by selenite and glutathione is further evidence for the occurrence of redox cycles during selenite metabolism. Other evidence, such as the temporary increase in oxygen consumption, was presented in the introduction and has been confirmed by others [24]. A relationship between redox cycles and DNA alterations was indicated not only by the effects of nitrogen (Figs 2 and 4), but also by the coordinated rise and fall in SSB levels and oxygen consumption. It was also found that selenite concentrations which increased oxygen consumption [3] affected SSB levels. It may thus be concluded that there is evidence which implicates redox cycles in selenite-induced DNA fragmentation, but that more definite proof is lacking. It is, for example, not possible to identify the type of DNA alterations induced by selenite, mainly due to the lack of effect of SOD, mannitol or dimethylsulfoxide. However, these negative data do not exclude an involvement of redox cycles. The DNA fragmentation can be explained by assuming changes in Ca^{2+} homeostases (cf. Ref. 25) which may activate endonucleases [26], or by assuming that products of redox cycles other than activated oxygen may react with DNA.

Findings of the cytotoxicological studies indicate that DNA alterations, induced either by selenite or by DNA alkylating agents, were involved in the lysis of cultured cells at low concentrations of selenite. In this dose range, inhibitors of poly(ADP-ribose)polymerase prevented the cytotoxic effect whereas alkylators potentiated it. At higher concentrations, the inhibitors did not prevent cytotoxicity, confirming earlier conclusions on a critical role for NADPH oxidation and related events in the cell lysis at a high Se concentration [3, 27]. In hepatocytes in culture, the effect of selenite on DNA was apparently more prolonged. The reason for this is not clear, but may relate to a better access to oxygen and nutrients in cultured cells which could permit redox cycling for longer time periods (cf. Ref. 2). The prolonged effect cannot be explained by a low GSH level in cultured cells, as this was found to be somewhat higher (76 ± 9 nmol/10^6 cells) than the GSH level in suspended cells. A rather late degradation of NAD was also indicated in this model.

Considering the relatively slow repair of DNA damage induced by alkylators in hepatocytes (cf. Refs 28 and 29), the effects of MMS and DEN can best be explained by assuming a sublethal activation of poly(ADP-ribose)polymerase subsequent to DNA alkylation. The lysis induced by selenite alone in the low concentration experiments can either be explained by a lethal activation of poly(ADP-ribose)polymerase or by a sublethal activation in combination with other events. The latter interpretation is favoured by recent data indicating that a poly(ADP-ribose)polymerase activation *per se* was not sufficient to kill hepatocytes [30]. It is also supported by the notion that other potentially lethal effects of the redox cycles, such as NADPH oxidation and induction of hypoxia, are synergistic with the effects mediated by poly(ADP-ribose)polymerase. The activation of this enzyme was thus probably of importance for selenite toxicity, but effects induced in parallel might have been of equal importance

by sensitizing the cells to the killing effect of the poly(ADP-ribose)polymerase.

The type of influence on poly(ADP-ribose)polymerase mediated lysis that can be discerned here may thus be complex and as indicated above, perhaps also specific for selenite. These conditions, as well as the fact that selenite preferentially killed carcinogen-treated cells, raise the question of whether selenite toxicity is of importance in the anticarcinogenic effects of selenite. To our knowledge, this hypothesis has never been tested, but could be of interest, especially when trying to explain the anticarcinogenic effects of a high dietary intake.

REFERENCES

1. A. Ståhl, I. Anundi and J. Högberg, *Biochem. Pharmac.* **33**, 1111 (1984).
2. P. Garberg and J. Högberg, *Biochem. Pharmac.* **36**, 1377 (1987).
3. I. Anundi, A. Ståhl and J. Högberg, *Chem. Biol. Interact.* **50**, 277 (1984).
4. R. J. Shamberger, *Mutat. Res.* **154**, 29 (1985).
5. R. F. Whiting, L. Wei and H. F. Stich, *Mutat. Res.* **78**, 159 (1980).
6. J. H. Ray and L. C. Altenburg, *Mutat. Res.* **54**, 343 (1978).
7. D. A. Carson, S. Seto, D. B. Wasson and C. J. Carrera, *Exp. Cell Res.* **164**, 273 (1986).
8. I. U. Schraufstatter, P. A. Hyslop, D. B. Hinshaw, R. G. Spragg, L. A. Sklar and C. G. Cochrane, *Proc. natn. Acad. Sci. U.S.A.* **83**, 4908 (1986).
9. N. A. Berger, *Radiat. Res.* **101**, 4 (1985).
10. J. C. Gaal, K. R. Smith and C. K. Pearson, *TIBS* **12**, 129 (1987).
11. J. R. J. Sorensen, *J. Med. Chem.* **19**, 135 (1976).
12. J. Högberg and A. Kristofersson, *Eur. J. Biochem.* **74**, 77 (1977).
13. S. C. Strom and G. Michalopoulos, in *Methods in Enzymology*, Vol. 82 (Eds. L. W. Cunningham and D. W. Frederiksen), p. 544. Academic Press, Orlando, FL (1982).
14. R. Enat, D. M. Jefferson, N. Ruiz-Opazo, Z. Gatmaitan, L. A. Leinwand and L. M. Reid, *Proc. natn. Acad. Sci. U.S.A.* **81**, 1411 (1984).
15. S. S. Matsumoto, K. O. Raivio and J. E. Seegmiller, *J. biol. Chem.* **254**, 8956 (1979).
16. M. J. Green and H. A. O. Hill, in *Methods in Enzymology*, Vol. 105 (Ed. L. Packer), p. 3. Academic Press, Orlando, FL (1984).
17. G. Ahnström and K. Erixon, in *DNA Repair* (Eds. E. C. Friedberg and P. C. Hanawalt), p. 403. Marcel Dekker, New York (1981).
18. S. A. S. Walles and K. Erixon, *Carcinogenesis* **5**, 319 (1984).
19. I. Fridovich, in *Ann. Rev. Pharmac. Toxic.*, Vol. 23 (Eds. R. George, R. Okun and A. K. Cho), p. 239. Annual Reviews Inc., Palo Alto, CA (1983).
20. R. D. Snyder, *Cancer Lett.* **34**, 73 (1987).
21. J. E. Cleaver, M. J. Banda, W. Troll and C. Borek, *Carcinogenesis* **7**, 323 (1986).
22. H. Morrison, B. Jernström, M. Nordenskjöld, H. Thor and S. Orrenius, *Biochem. Pharmac.* **33**, 1763 (1984).
23. J. Anundi, J. Högberg and A. Ståhl, *Archs Toxic.* **50**, 113 (1982).
24. G. Batist, A. G. Katki, R. W. Klecker Jr. and C. E. Myers, *Cancer Res.* **46**, 5482 (1986).
25. J. Högberg, A. Ståhl, I. Anundi and A. Götharson, *Nutrition Res. Suppl I*, 674 (1985).
26. H. Nomurra, A. Kitamura, Y. Tanigawa, M. Tsuchiya, M. Heki, O. Sugimoto and M. Shimoyama, *Biochim. biophys. Acta* **781**, 112 (1984).
27. A. A. Vlessis and L. Mela-Riker, *Molec. Pharmac.* **31**, 643 (1987).
28. G. Brambilla, A. Martelli, A. Pino and L. Robbiano, *Cancer Res.* **46**, 3476 (1986).
29. D. L. Stout and F. F. Becker, *Carcinogenesis* **3**, 599 (1982).
30. R. Alvarez-Gonzalez, R. Eichenberger and F. R. Althaus, *Biochem biophys. Res. Commun.* **138**, 1051 (1986).