Cellular DNA damage by hydrogen peroxide is attenuated by hypotonicity

Elizabeth A. L. MARTINS and Rogerio MENEGHINI*
Department of Biochemistry, Institute of Chemistry, University of São Paulo, C.P. 20780, CEP 01498-470, São Paulo, SP, Brazil

Chinese hamster fibroblasts (line V79) withstand well exposure for 30 min to hypotonic medium, corresponding to 25% physiological phosphate-buffered saline (PBS). Under these conditions, the cells become resistant to two effects of H_2O_2: DNA damage and inhibition of cell clone formation. The normal sensitivity to the DNA-damaging action of H_2O_2 is restored if, after exposure to hypotonic PBS, the cells are incubated in isotonic cell-culture medium. However, restoration of sensitivity is not observed on incubation in isotonic PBS. The normal sensitivity to H_2O_2 is also restored if one of the following reducing agents is added to hypotonic PBS: ascorbate, NADH and NADPH, in this order of decreasing efficiency. The recovery of sensitivity to H_2O_2 by ascorbate is completely inhibited by 1,10-phenanthroline, indicating that ascorbate is mediating the reduction of Fe(III). The decrease in the sensitivity to the DNA-damaging action of H_2O_2 is not a peculiarity of hypotonic PBS, since it appears to be caused by hypo-osmolarity in general: it is also observed in culture medium of 25% the isotonic concentration, and in 0.07 M sucrose. One explanation for this phenomenon is that hypotonic stress leads to a depletion of reducing species, in particular ascorbate. Under these conditions Fe(II) tends to be oxidized to Fe(III) and the Fenton chemistry is mitigated. However, other possibilities are that hypotonicity brings about structural modifications in the chromatin, rendering it less accessible to H_2O_2, or that it attenuates the Ca^{2+}-activation of endonuclease, induced by oxidative stress.

INTRODUCTION

Cellular injury and DNA damage are well-studied effects of a pro-oxidative condition imposed to a cell. Evidence has accumulated that the extremely reactive 'OH radical is generated during a pro-oxidative condition and that it attacks DNA (Meneghini, 1988; Imlay and Linn, 1988; Halliwell and Aruoma, 1991; Dizdaroglu et al., 1991) and other target(s) whose damage causes cell killing (Repine et al., 1981; Mello-Filho et al., 1984; Ward et al., 1985; Starke and Farber, 1985). The most likely mode of 'OH radical production *in vivo* is via Fenton chemistry (Halliwell and Gutteridge, 1992), through reduction of H_2O_2 by ferrous ion: $H_2O_2 + Fe(II) \rightarrow HO^{\cdot} + OH^- + Fe(III)$. Cuprous ion is also an efficient producer of the 'OH radical in the Fenton reaction (Halliwell and Gutteridge, 1992). However, copper is much scarcer than iron in biological systems. In fact, we have obtained evidence that Fe(II) is the relevant metal responsible for 'OH radical *in vivo* production by the Fenton reaction (Mello-Filho and Meneghini, 1991).

Understanding cellular iron metabolism is rather relevant to the comprehension of Fenton chemistry in the cell. Much has been recently learned about the control of cellular iron uptake (Thorstensen and Romslo, 1990; Kuhn, 1991; Klausner et al., 1993) and iron storage (Thorstensen and Romslo, 1990; Kuhn, 1991; Eisenstein et al., 1991; Klausner et al., 1993). It appears that intracellular iron can be separated into ferritin-bound and ferritin-free iron (Rothman et al., 1992). This latter comprises a small (< 2%) and rapidly chased pool of iron, chelatable by desferrioxamine, and is likely to be the iron responsible for cell injury (Rothman et al., 1992). Given the relative abundance of cellular species capable of forming complexes of iron (citrate, ATP, ADP) and given the reductive cellular environment (GSH, NADPH, ascorbate), one would imagine that the prevailing cellular 'free iron' is a low-molecular-mass complex of Fe(II), ready to participate in Fenton chemistry.

We set out to investigate circumstances in which the cell would exhibit a decreased capacity to respond to oxidative stress. We considered that cell manipulation could generate a condition in which cellular participants in the Fenton chemistry would be depleted, leading to an increased resistance to a pro-oxidative status. We found that under hypotonic conditions the cells become more resistant to two effects of H_2O_2, namely inhibition of cell clone formation and DNA-damaging actions. The possibility of restoring the normal cellular response to H_2O_2 by addition of different compounds made it possible to define cellular species that participate in the Fenton reaction.

MATERIALS AND METHODS

Cell culture

V79 Chinese-hamster fibroblasts constitute an established cell line (clone M8) and were routinely grown in Dulbecco's modified Eagle's medium, pH 7.0, supplemented with 10% (v/v) fetal-calf serum, 472 units of penicillin/ml and 94 μg of streptomycin/ml. Cells were incubated in a humidified CO_2/air atmosphere (1:19) at 37 °C as previously detailed (Mello-Filho et al., 1984). Cultures were determined to be mycoplasm-free by staining with Hoechst-33528 (Fox, 1981).

Hypotonic conditions

Hypotonic PBS (25% PBS) is a 1:3 dilution of PBS with double-distilled water. Hypotonic medium (25% medium) is a 1:3 dilution of Dulbecco's modified Eagle's medium with double-distilled water, supplemented with 10% serum. Hypo-osmotic sucrose is 0.07 M sucrose in 8.1 mM Na_2HPO_4/1.47 mM KH_2PO_4 buffer, pH 7.0.

Cell-clone-formation assay

For this, 400 cells were plated on 3 cm-diameter Petri dishes; 8 h later, they were treated with H_2O_2 in isotonic or hypotonic PBS,

Abbreviation used: PBS, phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl, pH 7.0).
* To whom correspondence should be addressed.

at 37 °C for 30 min. Cells were grown in complete medium for 7 days, fixed with 3% formaldehyde and stained with 0.3% Crystal Violet. Cell viability was scored as the ability to form colonies of at least 50 cells.

DNA precipitation assay

The cells were labelled in the presence of 1 μCi/ml [^{3}H]methylthymidine (50 Ci/mmol; Amersham) for 24 h and transferred to multi-wells (Costar; 2 cm^2/16 mm diameter). After 12 h, the cells were washed with PBS and supplied with 1 ml of hypotonic or isotonic PBS with the indicated additions. H_2O_2 concentrations were determined just before the experiment (Cotton and Dunford, 1973). The indicated H_2O_2 concentrations are those at the beginning of the incubation, since cellular enzymes rapidly consume H_2O_2 from the medium (Hoffmann and Meneghini, 1979). Incubation proceeded for 30 min at 37 °C, in the dark, and was interrupted by removal of the medium, and addition of the lysis buffer described below. H_2O_2 and ascorbate were from Merck; 1,10-phenanthroline, NADH and NADPH were from Sigma Chemical Co.

The DNA precipitation assay is a modified version of an assay previously developed (Olive, 1988) and was described in detail elsewhere (Martins et al., 1991). The cells were lysed by adding 300 μl of lysis buffer (10 mM Tris/HCl, 10 mM EDTA, 50 mM NaOH, 2% SDS, pH 12.4) and 300 μl of 120 mM KCl. The lysate was incubated for 10 min at 65 °C, transferred to a 1.5 ml Eppendorf tube and incubated in an ice bath for 10 min. A DNA–protein K–SDS precipitate is formed under these conditions, from which low-molecular-mass single-strand DNA is released. This DNA was recovered in the supernatant from a 10 min centrifugation at 2500 *g* and 10 °C, and was transferred to 1.5 cm × 2.0 cm pieces of thick filter paper. The sediment was dissolved in 100 μl of water at 65 °C and transferred to another piece of filter paper. These were serially washed in 5% trichloroacetic acid, ethanol (twice) and acetone, dried, and their radioactivity was determined in a liquid-scintillation counter. The percentage of solubilized DNA was calculated for each sample and subtracted from the solubilized DNA value in the untreated controls, which averaged 15% in several experiments. The correlation between the amount of precipitated DNA and the frequency of DNA single-strand breaks was previously established (Martins et al., 1991). In all plots, the values represent the mean of three independent determinations with the respective S.D.

RESULTS

The Chinese hamster cell clone (M8) used in the experiments is very resistant to hypotonicity. The maintenance of these cells in the presence of 20% PBS for 30 min at 37 °C did not decrease their ability to form clones (results not shown). During this time the cells swelled and rounded up; however, after re-establishing normal isotonicity, the cells re-acquired their original morphology. Further decreasing tonicity brought about an abrupt decrease in the ability to form clones, from 100% at 20% PBS to 0.5% at 10% PBS (results not shown). The experiment of Figure 1 shows how exposure to H_2O_2 in isotonic PBS and hypotonic PBS affects the ability of the cells to form colonies. In the 20–50 μM H_2O_2 concentration range and under isotonicity, an exponential effect of inhibition of the ability to form clones is observed. Under hypotonic conditions the ability to form clones is sustained at 100%. An increased resistance to H_2O_2 in hypotonic PBS is also observed at the level of DNA strand breaks (Figure 2). The production of strand breaks declines by two-thirds under hypotonic conditions. Apparently, some component of the 'OH radical generation system is being depleted during hypotonicity. We asked whether, after the hypotonic treatment, replacing the cells in normal medium would re-establish the normal sensitivity to H_2O_2. The experiment of Figure 3 shows that the normal level of H_2O_2-induced strand break is attained after 30 min of incubation in normal medium. It is noteworthy that restoring isotonicity with normal PBS did not bring about recovery in the H_2O_2 sensitivity. Therefore, components of the medium are required for the recovery.

We next attempted to add physiological reducing agents to hypotonic PBS, with the expectation that some of these agents might have been depleted by the hypotonic treatment. The more stable form of Fe in an oxygenated milieu is Fe(III). However, Fe(II) is more soluble at physiological pH and is thought to be the form of 'free iron' in the cell, requiring for this a reducing environment. The experiment of Table 1 shows that addition to cells of 1 mM reducing compounds (NADH, NADPH, GSH or ascorbate) in isotonic PBS did not significantly change the level of H_2O_2-induced strand breaks. The only exception was ascorbate, which increased the level of strand breaks by 40%. This may be explained by the low capacity that these compounds have

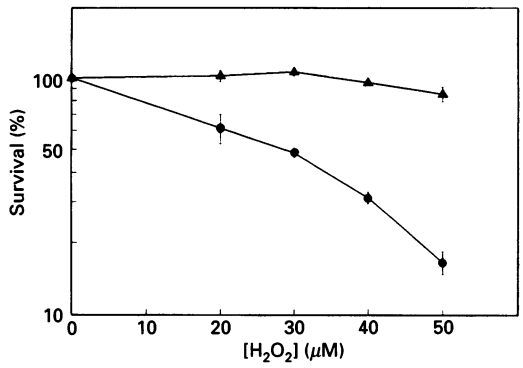

Figure 1 Effect of H_2O_2 on the ability of the cells to form clones in isotonic (●) and hypotonic (▲) conditions

Bars indicate S.D. of the mean of three independent determinations.

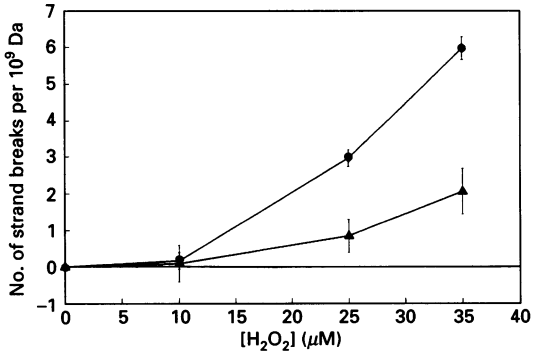

Figure 2 DNA strand breaks by H_2O_2 in isotonic (●) and hypotonic (▲) PBS

Exposure was for 30 min to the indicated concentrations of H_2O_2. Bars indicate S.D. of the mean of three independent determinations.

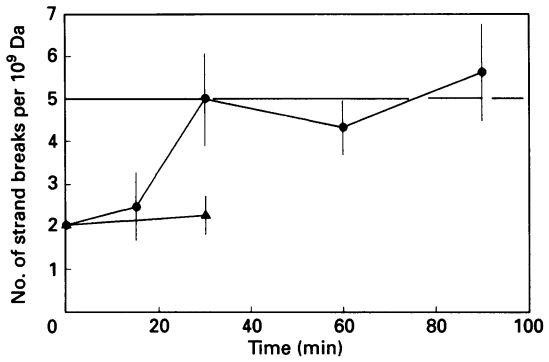

Figure 3 Recovery of sensitivity to H_2O_2 in medium

The cells were exposed to hypotonic PBS for 30 min and then isotonic cell-culture medium (●) or to isotonic PBS (▲) for the indicated times. After that, the cells were exposed to 50 μM H_2O_2 for 30 min in isotonic PBS and processed for determination of strand breaks. The horizontal line indicates the level of DNA strand breaks in cells that were not exposed to hypotonic PBS. Bars indicate S.D. of the mean of three independent determinations.

Table 1 Effect of reducing agents on DNA strand breaks

Treatment was in isotonic or hypotonic PBS for 30 min, at 40 μM H_2O_2, in the presence of 1 mM of the indicated reducing agents. Results represent means ± S.D. of three independent determinations.

Additions to isotonic PBS	Additions to hypotonic PBS	No. of strand breaks per 10^9 Da
H_2O_2	–	6.80 ± 0.77
H_2O_2 + NADH	–	7.92 ± 0.46
H_2O_2 + NADPH	–	7.57 ± 0.49
H_2O_2 + GSH	–	8.08 ± 1.15
H_2O_2 + ascorbate	–	9.69 ± 1.08
–	H_2O_2	1.85 ± 1.00
–	H_2O_2 + NADH	7.38 ± 2.77
–	H_2O_2 + NADPH	4.15 ± 1.85
–	H_2O_2 + GSH	1.62 ± 0.31
–	H_2O_2 + ascorbate	9.88 ± 1.26

to enter cells. GSH, for instance, is known hardly to penetrate the cell membrane (Martensson et al., 1989). However, the same additions to cells in hypotonic PBS modified the pattern of H_2O_2-induced strand breaks. GSH was ineffective, but NADPH, NADH and ascorbate were, in this order, increasingly capable of restoring the normal H_2O_2-sensitivity. Clearly, structural changes on the membrane due to cell swelling caused by hypotonic conditions enabled these compounds to enter cells. The difference between these compounds does not necessarily reflect their capability to reduce Fe(III) to Fe(II), but may be due to differential permeability of the modified plasma membrane.

The most likely interpretation for these results is that these reducing.agents are providing intracellular reducing equivalents for maintaining Fe in the ferrous form, necessary for the Fenton reaction to proceed. The experiment of Table 2 shows that this interpretation is correct in the case of ascorbate. The resumption of the normal response to H_2O_2 in hypotonic conditions caused by ascorbate is completely abrogated by 1,10-phenanthroline, a

Table 2 Inhibition by a Fe(II) chelator of the strand-break production induced by ascorbate

Treatment was in hypotonic PBS for 30 min, at 40 μM H_2O_2, in the presence of the indicated additions; 1,10-phenanthroline was 100 μM and ascorbate was 1 mM. Results represent means ± S.D. of three independent determinations.

Additions	No. of strand breaks per 10^9 Da
H_2O_2	2.90 ± 1.28
Ascorbate	1.96 ± 0.22
H_2O_2 + ascorbate	9.93 ± 3.14
H_2O_2 + phenanthroline	1.71 ± 0.42
H_2O_2 + ascorbate + phenanthroline	1.30 ± 0.28

Table 3 Effect of different hypotonic conditions on the production of DNA strand breaks by H_2O_2

The cells were exposed for 30 min to one of the three different hypotonic or isotonic conditions (defined in the Materials and methods section) and then for 30 min to 40 μM H_2O_2 in isotonic PBS. Results represent means ± S.D. of three independent determinations.

Medium	No. of strand breaks per 10^9 Da
Iso-medium	4.39 ± 0.99
Hypo-medium	2.06 ± 0.87
Iso-PBS	3.29 ± 1.51
Hypo-PBS	1.08 ± 0.72
Iso-sucrose	3.82 ± 0.44
Hypo-sucrose	1.09 ± 0.77

chelator that binds Fe(II) and prevents its oxidation by H_2O_2 (Mello-Filho and Meneghini, 1984, 1985).

In the experiment of Figure 3, we showed that the resumption of sensitivity to H_2O_2 was attained by incubation in isotonic complete medium, but not in isotonic PBS. We then asked whether the resistance to H_2O_2, observed upon incubation in hypotonic PBS, could be also observed under other hypotonic conditions. The experiment of Table 3 shows that H_2O_2-induced DNA strand breaks decrease in three distinct media, all equally hypo-osmotic: 25% PBS (Hypo-PBS), 25% medium (Hypo-medium) and 0.07 M sucrose (Hypo-sucrose). Therefore, the increase in resistance to the DNA-damaging action of H_2O_2 seems to be brought about by the hypo-osmotic condition, irrespective of the nature of the physiological medium used to attain it.

DISCUSSION

To maintain a favourable condition for the Fenton chemistry in the cell, Fe(II) must be present. There is evidence for the presence of cytosolic chelatable iron, which is free of ferritin and transferrin (Richardson and Baker, 1992; Rothman et al., 1992). However, neither the valence nor the ligand(s) of chelatable iron have been established. Recent results indicated that, in order to enter the cell, iron must be first reduced to Fe(II) (Watkins et al., 1992; Dancis et al., 1992; Eide et al., 1992). Once inside the cell, Fe(II) can be oxidized by dioxygen to produce $O_2^{\cdot-}$. However, this tendency for oxidation is likely to be counterbalanced by the reductive cytosol environment. In fact, several compounds rela-

tively abundant in the cell are able to reduce Fe(III) to Fe(II), such as ascorbate, GSH, NADH and NADPH (Winterbourn, 1993). These species are considered to be more relevant than O_2^- as reducing agents of Fe(III) (Winterbourn, 1979). It is necessary to assume that Fe(II) enters the nucleus, where it may bind to DNA and catalyse $^{\cdot}OH$ radical production that can attack DNA (Mello-Filho et al., 1984; Dizdaroglu et al., 1991). The reductive capacity in the nuclear environment is unknown, but clearly a small pool of Fe(II) therein is capable of reacting with H_2O_2.

We showed that under hypotonic conditions the cells exhibited a diminished response to H_2O_2 in terms of DNA strand breaks and inhibition of cell clone formation. The normal response was recovered by addition of one of the reducing agents, ascorbate, NADH or NADPH, to the hypotonic medium. Apparently, the reductive cytosolic milieu became depleted of reducing equivalents by the hypotonic condition. This could have occurred by leaking of these species outwards the swollen cells or by metabolic imbalance between their synthesis and consumption, brought about by the hypotonic stress. In any case, if the depletion of reducing equivalents occurred during exposure to hypotonic PBS, it is interesting that it did not produce major cell injury, as judged by cell survival experiments. Therefore, it is possible that a mild oxidative stress, caused by the hypotonic condition, was protective against a subsequent stronger oxidative stress by H_2O_2, because the depletion of antioxidants, such as ascorbate, NADH and GSH, favoured the decrease in Fe(II) concentration.

These results are reminiscent of those obtained by incubation of cells at 4 °C (Jonas et al., 1989). Under this condition the cells were rendered more resistant to the killing action of H_2O_2, and their sensitivity was restored by preincubation with ascorbate. The ascorbate depletion that occurred at 4 °C was considered to be caused by the diminished metabolic oxidation of dehydroascorbate (Jonas et al., 1989). In our case, ascorbate was the more effective reducing agent, among the compounds tested, to restore sensitivity to H_2O_2. The importance of this compound as a pro-oxidative agent for DNA damage and cell killing was also demonstrated in the case of chromium toxicity (Sugiyama et al., 1991). Cr(VI) was shown to cross the cell membrane, but only became toxic upon reduction to Cr(III) by intracellular ascorbate.

It is noteworthy that the DNA-damaging action of menadione was not affected under hypotonic condition (results not shown). A Fenton reaction seems to be the main mechanism responsible for menadione-induced DNA strand breaks (Cantoni et al., 1991; Calderaro et al., 1993). It is likely that the difference between menadione and H_2O_2 lies in the fact that the former generates the semiquinone radical by metabolic reduction (Nohl et al., 1986); the semiquinone reacts with dioxygen to produce the superoxide radical. Both these radicals can reduce Fe(III), and thus the depletion of ascorbate during hypotonic conditions would not alter the Fe(II) pool.

Other mechanisms to explain the decreased sensitivity to H_2O_2 cannot be ruled out without further investigation. For instance, it is known that difference in cellular water content imposed by anisotonic conditions causes significant alterations in chromatin structure (Raaphorst et al., 1978). It is possible that these alterations render the DNA less accessible to H_2O_2 (Ward et al., 1987). It is also possible that hypotonicity blocks rises in Ca^{2+} and Ca^{2+}-dependent endonuclease activation. This activation occurs under oxidative stress and is another component in the production of DNA damage (Cantoni et al., 1989).

This work was supported in part by grants from FAPESP (Brazil) and from The Tobacco Research Council (U.S.A.). E.A.L.M. holds a post-doctoral fellowship from FAPESP.

REFERENCES

Calderaro, M., Martins, E. A. L. and Meneghini, R. (1993) Mol. Cell. Biochem. **126**, 17–23
Cantoni, O., Sestili, P., Cattabeni, F., Bellomo, G., Pou, S., Cohen, M. and Cerutti, P. (1989) Eur. J. Biochem. **182**, 209–212
Cantoni, O., Fiorani, M., Cattabeni, F. and Bellomo, G. (1991) Biochem. Pharmacol. **42**, S220–S222
Cotton, M. L. and Dunford, H. B. (1973) Can. J. Chem. **51**, 582–587
Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G. and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3869–3873
Dizdaroglu, M., Nackerdien, Z., Chao, B.-C., Gajewski, E. and Rao, G. (1991) Arch. Biochem. Biophys. **285**, 388–390
Eide, D., Davis-Kaplan, S., Jordan, I., Sipe, D. and Kaplan, J. (1992) J. Biol. Chem. **267**, 20774–20781
Eisenstein, R. S., Garcia-Mayol, D., Pettingell, W. and Munro, H. N. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 688–692
Fox, M. (1981) in DNA Repair (Friedberg, E. C. and Hanawalt, P. C., eds.), pp. 523–543, Marcel Dekker, New York
Halliwell, B. and Aruoma, O. I. (1991) FEBS Lett. **281**, 9–19
Halliwell, B. and Gutteridge, J. M. C. (1992) FEBS Lett. **307**, 108–112
Hoffmann, M. E. and Meneghini, R. (1979) Photochem. Photobiol. **30**, 151–155
Imlay, J. A. and Linn, S. (1988) Science **240**, 1302–1309
Jonas, S. K., Riley, P. A. and Willson, R. L. (1989) Biochem. J. **264**, 651–655
Klausner, R. D., Rouault, T. A. and Harford, J. B. (1993) Cell **72**, 19–28
Kuhn, L. C. (1991) Br. J. Haematol. **79**, 1–5
Martensson, G., Stheinherz, R., Jain, A. and Meister, A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 8727–8731
Martins, E. A. L., Chubatsu, L. S. and Meneghini, R. (1991) Mutat. Res. **250**, 95–101
Mello-Filho, A. C. and Meneghini, R. (1984) Biochim. Biophys. Acta **781**, 56–63
Mello-Filho, A. C. and Meneghini, R. (1985) Biochim. Biophys. Acta **847**, 82–89
Mello-Filho, A. C. and Meneghini, R. (1991) Mutat. Res. **251**, 109–113
Mello-Filho, A. C., Hoffmann, M. E. and Meneghini, R. (1984) Biochem. J. **218**, 273–275
Meneghini, R. (1988) Mutat. Res. **195**, 215–230
Nohl, H., Jordan, W. and Youngman, R. J. (1986) Adv. Free Radical Biol. Med. **2**, 211–279
Olive, P. L. (1988) Environ. Mol. Mutagen. **11**, 487–495
Raaphorst, G. P., Law, P. and Kruuv, J. (1978) Physiol. Chem. Phys. **10**, 177–185
Repine, J. E., Fox, R. B. and Berger, E. M. (1981) J. Biol. Chem. **256**, 7094–7096
Richardson, D. R. and Baker, E. (1992) J. Biol. Chem. **267**, 21384–21389
Rothman, R. J., Serroni, A. and Farber, J. L. (1992) Mol. Pharmacol. **42**, 703–710
Starke, P. E. and Farber, J. L. (1985) J. Biol. Chem. **26**, 10099–10104
Sugiyama, M., Tsuzuki, K. and Ogura, R. (1991) J. Biol. Chem. **266**, 3383–3386
Thorstensen, K. and Romslo, I. (1990) Biochem. J. **271**, 1–10
Ward, J. F., Blakely, W. F. and Joner, E. I. (1985) Radiat. Res. **103**, 383–392
Ward, J. F. Evans, J. W., Limoli, C. L. and Calabro-Jones, P. M. (1987) Br. J. Cancer **55**, 105–112
Watkins, J. A., Altazan, J. D., Elder, P., Li, C.-Y., Nunez, M.-T., Cui, X.-X. and Glass, J. (1992) Biochemistry **31**, 5820–5830
Winterbourn, C. C. (1979) Biochem. J. **182**, 625–628
Winterbourn, C. C. (1993) Free Radical Biol. Med. **14**, 85–90

Received 24 May 1993/24 August 1993; accepted 15 September 1993