Biochem, J. (1989) 264, 651–655 (Printed in Great Britain)

Hydrogen peroxide cytotoxicity

Low-temperature enhancement by ascorbate or reduced lipoate

Sonja K. JONAS,* Patrick A. RILEY*‡ and Robin L. WILLSON†

* Department of Chemical Pathology, University College and Middlesex School of Medicine, Cleveland Street, London W1P 6DB, U.K., and † Department of Biochemistry, Brunel University, Uxbridge, Middx. UB8 3PH, U.K.

The principal mechanism of H_2O_2 toxicity is thought to involve the generation of hydroxyl ($HO^{\bullet}$) radicals through its interactions with Fe^{2+} ions by the Fenton reaction. Of particular interest has been the demonstration by Ward, Blakely & Joner [(1985) Radiat. Res. **103**, 383–392] that the cytotoxicity of H_2O_2 is diminished at low temperature. We have now examined this phenomenon further with a mammalian epithelial cell line (CNCMI-221). Resistance of these cells to 100 μM-H_2O_2 added extracellularly exhibits a transition in the temperature range between 27 °C and 22 °C. We have found that the low-temperature resistance to cytotoxic concentrations of H_2O_2 is abolished by preincubation of cells with reductants such as ascorbate or reduced lipoic acid. This implies that the low-temperature resistance to H_2O_2 cytotoxicity may be due to inhibition of cellular reductive processes. The restoration of the cytotoxic action of H_2O_2 at 4 °C by ascorbate is prevented by pre-exposure of cells to desferrioxamine. This is evidence that transition-metal ions (such as iron ions) are involved in the cytotoxicity and is consistent with a mechanism of cell damage that depends on the Fenton reaction and a metal ion in the reduced state. Restoration of H_2O_2 cytotoxicity at low temperature by ascorbate is consistent with the artificial production of an intracellular reducing environment that at normal temperatures is sustained by cellular metabolism.

INTRODUCTION

In recent years there has been a growing interest in H_2O_2 as a source of hydroxyl ($HO^{\bullet}$) radicals in studies of cytotoxic mechanisms. The toxicity of H_2O_2 has been studied in a variety of different cell lines (Ward *et al.*, 1983; Jones & Kennedy, 1983; Hoffmann *et al.*, 1984; Starke & Farber, 1985; Dallergri *et al.*, 1987; Link & Riley, 1988). Several studies have demonstrated that the cytotoxicity is dose-dependent (Hoffmann & Meneghini, 1979; Rubin & Farber, 1984; Spitz *et al.*, 1987).

The principal mechanism of cytotoxicity produced by H_2O_2 is thought to involve $HO^{\bullet}$ radicals generated by the Fenton reaction in close proximity to the DNA strands. Several studies have provided support for this: (i) scavenger studies with chromatin *in vitro* show that the $HO^{\bullet}$ radical is the principal species damaging DNA (Tullis, 1987); (ii) studies with bacterial spores demonstrate that $HO^{\bullet}$ radicals are not damaging when generated extracellularly (Jacobs *et al.*, 1985); (iii) from studies with λ and T bacteriophages it has been proposed that damage to DNA is caused by a site-specific Fenton reaction (Samuni *et al.*, 1983); (iv) in V79 cells (Larramendy *et al.*, 1987) or human fibroblasts (Mello-Filho & Meneghini, 1984) this could be prevented by pretreatment with *o*-1,10-phenanthroline.

Ward *et al.* (1985) have suggested that the cytotoxicity of H_2O_2 is associated with local multiply damaged sites in DNA. This suggestion arose as a result of failure to observe cell killing from singly damaged sites following exposure of Chinese-hamster ovary cells at 0 °C to doses of H_2O_2 that are toxic at 37 °C.

Our findings confirm that at low temperature (4 °C) H_2O_2 is non-toxic in a concentration range shown to produce a dose-dependent diminution of survival at 37 °C. Also, we demonstrate conditions in which H_2O_2 toxicity is restored at low temperature and provide further support for the involvement of reduced transition-metal ions in this effect.

MATERIALS AND METHODS

Materials

H_2O_2 [aq. 30% (w/v) solution, stabilizer-free] was obtained from Aldrich Chemical Co. Sodium dehydroascorbate was obtained from Koch–Light Laboratories. Ascorbic acid was obtained from Sigma Chemical Co. Reduced (sealed ampoules) and oxidized lipoic acid (DL-1,2-dithiolane-3-pentanoic acid) were obtained from Sigma Chemical Co. Desferrioxamine methanesulphonate was obtained from CIBA–GEIGY. [*Me*-^{3}H]-Thymidine (specific radioactivity 5 mCi/nmol) was obtained from Amersham International and diluted in phosphate-buffered saline (see below) to give a stock solution of 20 μCi/ml. Eagle's medium and Earle's salts were obtained from Flow Laboratories.

Sodium dehydroascorbate, ascorbic acid and desferrioxamine methanesulphonate were dissolved in distilled water immediately before each experiment. H_2O_2 was diluted in distilled water immediately before addition to cells. Lipoic acid was dissolved in dilute ethanol. Phosphate-buffered saline was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 0.132 g of $CaCl_2,H_2O$, 0.1 g of $MgCl_2,6H_2O$, 1.5 g of $Na_2PO_4,2H_2O$ and 0.2 g of KH_2PO_4 in 1 litre of distilled water. All reagents were sterilized by filtration through a 0.22 μm-pore-size filter (Millipore).

‡ To whom correspondence should be addressed.

Cell line

An established mammalian epithelial cell line (CNCM I-221) was used for all experiments. To minimize any variation due to repeated subculturing, the experiments were conducted with cells between passage numbers 21 and 30. Cells were grown in multi-well trays, each of diameter 1.5 cm (1.77 cm^2) (Falcon Plastics, Scientific Supplies). The culture medium consisted of Eagle's minimal essential medium with Earle's salts supplemented with 10% (w/v) foetal bovine serum (Imperial Labs), penicillin (10 μg/ml), streptomycin (10 μg/ml) and 20 mM-Hepes.

Delayed-thymidine-incorporation assay for cell survival

The cytotoxic effect of H_2O_2 is not observable directly after treatment and takes several hours to become manifest. Changes in membrane permeability were found to be late in onset and unreliable indicators of the toxic action of H_2O_2. In the present studies we have used the delayed-[^{3}H]thymidine-incorporation assay as an index of toxicity, since this correlates very well with the plating efficiency assay, and is an index of reproductive viability (Jonas *et al.*, 1988). The method for determining cell survival as estimated by the delayed-thymidine-incorporation assay is as follows. After cells had been exposed to the agents to be tested (see Table 1 for protocol), the medium was removed and the cells were washed twice in fresh phosphate-buffered saline and re-incubated in serum-containing medium at 37 °C in an atmosphere of 2% CO_2 in air. After 18–24 h (which permits at least one cell division to occur) the cells were exposed to 1 μCi of [^{3}H]thymidine/ml for 30 min at 37 °C. The cells were then washed in phosphate-buffered saline, fixed with 5% (w/v) trichloroacetic acid and washed twice with phosphate-buffered saline. The multi-wells were then dried and the acid-insoluble material was digested overnight in 250 μl of 1 M-NaOH per well. Samples (100 μl) of the digest were each mixed with 5 ml of scintillation fluid [consisting of 4 g of 2,5-bis-(5-t-butylbenzoxaz-2-oyl)thiophen/l of toluene/napthalene/2-methoxyethanol (55:8:37, by vol.)] and radioactivities were counted in an Intertechnique SL40 counter. The mean radioactivity count from four wells was expressed as a percentage of the radioactivity obtained from control cells that had not been exposed to the agents under test. These data are referred to as the survival index.

Incubation with H_2O_2

The numbers of attached cells at the time of exposure were in the region of 5×10^5 cells/well for different experiments. Cells were incubated for 60 min at 4 °C or 37 °C in phosphate-buffered saline containing H_2O_2. The concentration of H_2O_2 used was between 5 μM and 80 μM, corresponding to a cell concentration of up to 800 fmol/cell.

In a separate experiment cells at a density of 1.5×10^5 cells/ml were incubated in phosphate-buffered saline containing 100 μM-H_2O_2 for 60 min at temperatures ranging from 4 °C to 37 °C.

Preincubation and incubation protocols

A series of experiments were carried out that involved the preincubation of cells with reducing agents at 4 °C or 37 °C in growth medium. This was followed by washing the cells twice with phosphate-buffered saline and replacing the medium by phosphate-buffered saline containing 50 μM-H_2O_2 at either 4 °C or 37 °C.

The sequence of treatment procedures is listed in Table 1. In experiments with reducing agents or their oxidized analogues, the agents were added immediately before preincubation III (stage 5). In experiments with desferrioxamine, the drug was added immediately before preincubation II (stage 3), when the cells had reached a density of approx. 5×10^4 cells/well.

Table 1. Experimental protocol

For full experimental details see the text. Abbreviations: SC MEM, culture medium consisting of Eagle's minimal essential medium with Earle's salts, 10% foetal bovine serum, penicillin (10 μg/ml), streptomycin (10 μg/ml) and 20 mM-Hepes; PBS, phosphate-buffered saline (see the text).

Stage	Procedure	Incubation conditions: Medium	Temperature	Time
1	1 ml of 2×10^4 cells inoculated into multi-wells			
2	Preincubation I	SC MEM	37 °C	24 h
3	Preincubation II (±desferrioxamine)	SC MEM	37 °C	24 h
4	Wash 2× with PBS			
5	Preincubation III (±reductants)	SC MEM	4 °C/37 °C	120 min
6	Wash 2× with PBS			
7	Add 50 μM-H_2O_2			
8	Incubation in H_2O_2	PBS	4 °C/37 °C	60 min
9	Wash 2× with PBS			
10	Post-incubation I	SC MEM	37 °C	24 h
11	Add [^{3}H]thymidine (1 μCi/ml)			
12	Post-incubation II (labelling)	SC MEM	37 °C	30 min
13	Wash 5× with PBS			
14	Add trichloroacetic acid			
15	Wash 2× with PBS			
16	Digest and assay			

Table 2. Survival index of CNCM I-221 cells after exposure to H_2O_2 at different temperatures and cell concentrations

For experimental details see the text. Survival indexes are given as means ± S.D. ($n = 4$).

Temperature (°C)	Concn. of H_2O_2 (μM)	Cell density (cells/ml)	Concn. of H_2O_2 (fmol/cell)	Survival index (%)
37	5	2×10^5	25	88 ± 4
37	10	2×10^5	50	75 ± 7
37	10	1×10^5	100	79 ± 13
37	30	1.5×10^5	200	54 ± 12
37	30	8×10^4	375	22 ± 4
37	40	1.5×10^5	268	28 ± 3
37	50	2×10^5	250	30 ± 13
37	50	1.5×10^5	500	18 ± 7
37	50	1.2×10^5	417	14 ± 3
37	80	1.5×10^5	400	21 ± 4
4	10	4×10^5	25	101 ± 22
4	30	4×10^5	75	94 ± 13
4	50	4×10^5	125	87 ± 13
4	50	1.5×10^5	334	94 ± 11
4	50	6×10^4	834	114 ± 4
4	50	1×10^5	500	104 ± 17
4	50	1×10^5	500	104 ± 16
4	50	6×10^4	834	103 ± 18
4	50	1.5×10^5	334	100 ± 6
4	50	1.5×10^5	334	104 ± 10
4	50	1.6×10^5	313	121 ± 16
4	80	4×10^5	200	88 ± 16

RESULTS

Table 2 gives the data from several experiments that show a dose-dependence of survival at 37 °C but not at 4 °C. The Table also demonstrates the dependency of survival on cell density, which accounts for the large variation in survival index at particular concentrations of H_2O_2. The concentration of H_2O_2 to which the cells were exposed is expressed in terms of fmol/cell to eliminate these effects of cell density, to which the system was found to be very sensitive, as also noted by others (Ziegler-Sylakakis & Andrae, 1987; Spitz *et al.*, 1987). The data show clearly that lowering of the incubation temperature to 4 °C abolishes the cytotoxic effect of H_2O_2 exhibited at 37 °C.

In a further study a range of temperatures between 0 °C and 37 °C was investigated. Cells at a density of 1.5×10^5 cells/ml were exposed to 100 μM-H_2O_2 for 60 min. The data show that there is a slight fall in survival index to 80% as the temperature is raised from 0 °C to 22 °C, as illustrated in Fig. 1. A steep gradient between 22 °C and 27 °C was observed, suggesting a transition temperature at about 25 °C.

Preincubation with ascorbate

Cytotoxicity of H_2O_2 in the concentration range that is effective at 37 °C was observed at 4 °C in cells that had been exposed during preincubation III to 1 mM-ascorbate (Table 3). A 2 h preincubation period was required. Periods of exposure to 1 mM-ascorbate for less than 2 h had little or no effect. Analogous experiments with dehydroascorbate demonstrated that the effect was dependent on the temperature of the preincubation (Table 3). Preincubation with dehydroascorbate at 4 °C exhibited a toxic action independent of H_2O_2, but at 37 °C preincubation with dehydroascorbate had the effect of restoring the toxic action of H_2O_2 at 4 °C in a manner similar to that observed with ascorbate.

Preincubation with lipoic acid

Cells were exposed during preincubation III to 100 μM- or 500 μM-lipoic acid (oxidized or reduced) before treatment with H_2O_2 at 4 °C. Preincubation with

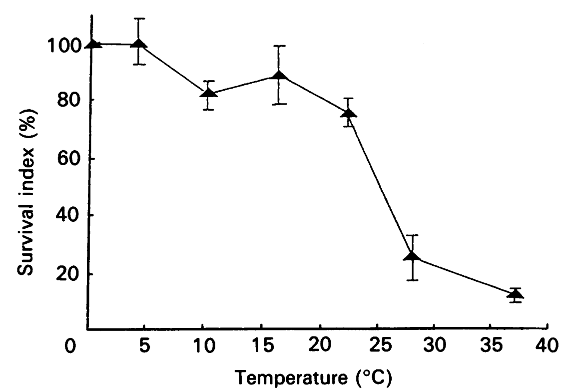

Fig. 1. Survival data for cells exposed to 100 μM-H_2O_2 at temperatures between 0 °C and 37 °C

Cells at a density of 1.5×10^5 cells/ml were exposed for 30 min to 100 μM-H_2O_2 in phosphate-buffered saline at pH 7.4 at the temperatures indicated. The plot shows the variation of survival index with temperature. Means ± S.D. are shown ($n = 4$).

Table 3. Effect of preincubation of cells with reducing agents (120 min) on the cytotoxicity of H_2O_2 (50 μM for 60 min)

Reducing agents were present during preincubation III (stage 5 of the experimental protocol). H_2O_2 incubation was in phosphate-buffered saline in all cases (stage 8 of the experimental protocol). Cell density was 1.5×10^5 cells/ml. Survival indexes are given as means ± S.D. ($n = 4$).

	Incubation temperatures		Preincubation additions...	Survival index (%)				
	Preincubation III	Incubation		Control	Ascorbate (1 mM)	Dehydro-ascorbate (1 mM)	Reduced lipoate (100 μM at 37 °C) (500 μM at 4 °C)	Oxidized lipoate (100 μM at 37 °C) (500 μM at 4 °C)
Control	37 °C	37 °C		100 ± 14	107 ± 20	–	–	–
+ H_2O_2	37 °C	37 °C		46 ± 6	39 ± 8	–	–	–
Control	37 °C	4 °C		100 ± 7	99 ± 14	90 ± 11	113 ± 19	100 ± 10
+ H_2O_2	37 °C	4 °C		97 ± 16	43 ± 4	46 ± 12	71 ± 4	98 ± 12
Control	4 °C	37 °C		100 ± 18	–	–	–	–
+ H_2O_2	4 °C	37 °C		42 ± 16	–	–	–	–
Control	4 °C	4 °C		100 ± 18	108 ± 17	55 ± 13	91 ± 10	83 ± 9
+ H_2O_2	4 °C	4 °C		90 ± 15	89 ± 21	69 ± 20	103 ± 8	76 ± 13

reduced lipoic acid re-established the H_2O_2-induced cytotoxicity at 4 °C (Table 3), but to a lesser extent than ascorbate. The ethanol solvent of lipoic acid limited the maximum concentration that could be used, and 500 μM-lipoic acid applied at 37 °C was found to be toxic by itself (results not shown). Despite the greater lipophilicity of lipoic acid, preincubation at 4 °C with reduced lipoic acid did not restore the cytotoxic action of H_2O_2 even at the higher concentrations used.

Protection by desferrioxamine

Pre-exposure to desferrioxamine for 24 h at 37 °C (preincubation II) before treatment with ascorbate (preincubation III) protected the cells from the toxic effect of H_2O_2 incubation at 4 °C observed with ascorbate preincubation alone (Table 4). This desferrioxamine-induced protection increased in a dose-dependent manner and was complete at a concentration of 200 μM-desferrioxamine. No toxic effect was evident in cells treated with desferrioxamine alone.

Table 4. Effect of desferrioxamine on the restoration of H_2O_2-induced cytotoxicity at 4 °C by ascorbate

Desferrioxamine was present during preincubation II (stage 3 of the experimental protocol), followed by ascorbate during preincubation III (stage 5), followed by incubation with H_2O_2 (stage 7). Survival indexes are given as means ± S.D. ($n = 4$).

Pre-exposure to desferrioxamine (200 μM) for 24 h at 37 °C	Preincubation with ascorbate (1 mM) for 120 min at 37 °C	Exposure to H_2O_2 (50 μM) for 60 min at 4 °C	Survival index (%)
0	0	0	100 ± 11
0	0	+	116 ± 16
0	+	0	109 ± 20 *
0	+	+	46 ± 8 *
+	0	0	100 ± 15
+	0	+	80 ± 14
+	+	0	83 ± 18
+	+	+	112 ± 22

* $P < 0.005$.

DISCUSSION

The addition of ascorbate to cells that were subsequently incubated at 4 °C with H_2O_2 was shown to restore the cytotoxicity to the same degree as was observed with H_2O_2 treatment alone at 37 °C. The requirement for an incubation period of 2 h and the fact that dehydroascorbate at concentrations of 1 mM also restores the cytotoxic effect of H_2O_2 at 4 °C if preincubation takes place at 37 °C suggest that ascorbate autoxidizes extracellularly and is taken up by the cells as dehydroascorbate. The half-life of ascorbate oxidation is less than 2 h (Lewin, 1976). Intracellularly it then becomes metabolically re-reduced to ascorbate. This process has been proposed by Bridges & Hoffmann (1986), who measured the rate of ascorbate uptake by K562 cells. Cells incubated with ^{14}C-labelled ascorbate for 2 h exhibited a linear uptake rate of 0.029 nmol/min per 10^7 cells. Cells were also found to have a high content of ascorbate as measured by h.p.l.c. following exposure to dehydroascorbate. This would explain why dehydroascorbate was equally effective when applied to cells at 37 °C and why it did not restore the cytotoxicity when cells were preincubated with dehydroascorbate at 4 °C.

In the present experiments prolonged preincubation with desferrioxamine abolished the action of ascorbate. The requirement for a 24 h preincubation period suggests a low rate of desferrioxamine uptake, or alternatively a slow release of iron from the cells. Low rates of uptake of desferrioxamine by cells have been reported previously (Halliwell & Gutteridge, 1985). Desferrioxamine specifically binds Fe^{3+} rather than Fe^{2+} and is known to inhibit superoxide-driven reduction of Fe^{3+} (Halliwell & Gutteridge, 1985). It is probable that desferrioxamine also prevents reduction of Fe^{3+} by ascorbate.

Ward *et al.* (1987) have demonstrated that different extents of DNA damage result from exposure to H_2O_2 at 0 °C and 37 °C. Numerous double-strand breaks are observed at 37 °C, whereas only single-strand breaks are found at 0 °C. This could result either from differences in the extent of damage inflicted or differences in the activity of repair processes, or a combination of these factors. Our data on cell survival suggest that less damage is inflicted at low temperature.

The restoration of the full toxic effect of H_2O_2 at 4 °C by reducing agents and its abolition by pretreatment with desferrioxamine are consistent with the proposal that the toxic damage is produced by $HO^{\bullet}$ radicals generated through the agency of the Fenton reaction. The difference in DNA damage produced by H_2O_2 at 37 °C and at 4 °C is a reflection of the amount and degree of reduction of iron ions (or similar transition-metal ions) associated with chromatin. Studies by Imaly *et al.* (1988) indicate that DNA damage in *Escherichia coli* following exposure to low concentrations of H_2O_2 is dependent on cell metabolism and suggest that NADH may be involved.

A similar argument could also be applied to other potential cellular targets in which the density of $HO^{\bullet}$ radicals generated in their vicinity determines the degree and distribution of damage sustained. This may be of relevance in connection with reperfusion injury (Bulkley, 1987; Garlick *et al.*, 1987; Halliwell *et al.*, 1985).

The above interpretation depends on the assumption that intracellular iron tends to be in the oxidized state in cells kept at low temperatures. This might be due to the lack of reducing equivalents normally generated by cellular metabolism. In their absence, the redox potential of the iron–oxygen couple would rapidly convert most of the iron into Fe(III), which is unreactive towards H_2O_2. It cannot be ruled out that superoxide may be a potential reductant that becomes less available at low temperature (Kyle *et al.*, 1988). In the case of damage to the genome some of the most vulnerable sites would be those in the locality of 'zinc finger' domains of DNA-binding proteins (Sunderman & Barber, 1988). Although zinc does not undergo redox reactions, if the zinc were replaced by redox-reactive metal ions then damaging free-radical reactions may result (Willson, 1977, 1988; Williams, 1984). Under normal circumstances the concentration of reduced metal ions in chromatin would be expected to be low. Consequently the production of a high local density of lesions in DNA by $HO^{\bullet}$ radicals derived from the Fenton reaction will depend on a large proportion of the potentially reactive metal ions being in the reduced state or capable of being reduced by an intrinsic mechanism or by reductants introduced from outside the cell.

Although our data on cytotoxicity are consistent with this argument, the mechanism cannot be regarded as simple. The existence of a transition temperature at about 25 °C suggests that membrane-related functions may be involved. Any influence of membrane fluidity on the penetration of H_2O_2 into the cell can be excluded by the re-establishment of H_2O_2 toxicity at low temperature by pre-exposure to the reducing agents. The low-temperature data suggest that any membrane involvement is indirect, since the effect of the lipid-soluble reductant (lipoic acid) was similar to that of ascorbate.

We thank Dr. C. Bell (Brunel University) and Dr. R. B. Wenham (Fisons Pharmaceuticals) for helpful discussion. The financial support of Fisons Pharmaceuticals and the Association for International Cancer Research is gratefully acknowledged.

REFERENCES

Bulkley, G. B. (1987) Br. J. Cancer **55**, Suppl. **8**, 66–73
Bridges, K. R. & Hoffmann, K. E. (1986) J. Biol. Chem. **261**, 14273–14277
Dallergri, F., Ballestrero, A., Frumento, G., Adami, R. & Patrone, F. (1987) J. Clin. Lab. Immonul. **23**, 95–99
Garlick, P. B., Davies, M. J., Hearse D. J. & Slater, T. F. (1987) Circ. Res. **61**, 757–760
Halliwell, B. & Gutteridge, J. M. C. (1985) Free Radicals in Biology and Medicine, pp. 32–33, Oxford University Press, Oxford
Halliwell, B., Gutteridge, J. M. C. & Blake D. (1985) Philos. Trans. R. Soc. London B **311**, 659–671
Hoffmann, M. E. & Meneghini, R. (1979) Photochem. Photobiol. **30**, 151–155
Hoffmann, M. E., Mello-Filho, A. C. & Meneghini, R. (1984) Biochim. Biophys. Acta **781**, 234–238
Imaly, J. A., Chin, S. M. & Linn, S. (1988) Science **240**, 640–641
Jacobs, G. P., Samuni, A. & Czapski, G. (1985) Int. J. Radiat. Biol. **47**, 621–623
Jonas, S. K., Riley, P. A. & Willson, R. L. (1988) in Free Radicals: A Search for New Methodology (Rice-Evans, C. & Halliwell, B., eds.), vol. 4, pp. 495–512, Richelieu Press, London
Jones, P. D. & Kennedy, F. G. (1983) in Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects (Sies, H. & Wendel, A., eds.), pp. 109–116, Raven Press, New York
Kyle, M. E., Nakae, D., Sakaida, I., Miccadei, S. & Farber, J. L. (1988) J. Biol. Chem. **263**, 3784–3789
Larramendy, M., Mello-Filho, A. C., Leme Martins, E. A. & Meneghini, R. (1987) Mutat. Res. **178**, 57–63
Lewin, S. (1976) Vitamin C: Its Molecular Biology and Medical Potential, pp. 75–101, Academic Press, Orlando
Link, E. & Riley, P. A. (1988) Biochem. J. **249**, 391–399
Mello-Filho, A. C. & Meneghini, R. (1984) Biochim. Biophys. Acta **781**, 56–63
Rubin, R. & Farber, J. L. (1984) Arch. Biochem. Biophys. **228**, 450–459
Samuni, A., Aronovitch, J., Godinger, D., Chevion, M. & Czapski, G. (1983) Eur. J. Biochem. **137**, 119–124
Spitz, D. R., Dewey, W. C. & Li, G. C. (1987) J. Cell. Physiol. **131**, 364–373
Starke, P. E. & Farber, J. L. (1985) J. Biol. Chem. **260**, 86–92
Sunderman, F. W. & Barber, A. M. (1988) Ann. Clin. Lab. Sci. **18**, 267–271
Tullis, T. D. (1987) Trends Biochem. Sci. **12**, 297–300
Ward, J. F., Blakely, W. F. & Moberly, J. B. (1983) Radiat. Res. **94**, 629–630
Ward, J. F., Blakely, W. F. & Joner, E. I. (1985) Radiat. Res. **103**, 383–392
Ward, J. F., Evans, J. W., Limoli, C. L. & Calabro-Jones, P. M. (1987) Br. J. Cancer **55**, Suppl. **8**, 105–112
Williams, R. J. P. (1984) Endeavour **8**, 65–70
Willson, R. W. (1977) Ciba Found. Symp. **51**, 333–354
Willson, R. L. (1988) in Zinc in Human Biology (Mills, C. F., ed.), pp. 147–173, Springer-Verlag, Berlin
Ziegler-Sylakakis, K. & Andrae, U. (1987) Mutat. Res. **192**, 65–68

Received 22 February 1989/13 July 1989; accepted 20 July 1989