Journal of Biochemistry and Molecular Biology, Vol. 39, No. 4, July 2006, pp. 452-456

JOURNAL OF Biochemistry and Molecular Biology

Oxidative Damage of DNA Induced by the Cytochrome *c* and Hydrogen Peroxide System

Nam Hoon Kim and Jung Hoon Kang*
Department of Genetic Engineering, Cheongju University, Cheongju 360-764, Korea

Received 18 April 2006, Accepted 19 May 2006

To elaborate the peroxidase activity of cytochrome *c* in the generation of free radicals from H_2O_2, the mechanism of DNA cleavage mediated by the cytochrome *c*/H_2O_2 system was investigated. When plasmid DNA was incubated with cytochrome *c* and H_2O_2, the cleavage of DNA was proportional to the cytochrome *c* and H_2O_2 concentrations. Radical scavengers, such as azide, mannitol, and ethanol, significantly inhibited the cytochrome *c*/H_2O_2 system-mediated DNA cleavage. These results indicated that free radicals might participate in the DNA cleavage by the cytochrome *c* and H_2O_2 system. Incubation of cytochrome *c* with H_2O_2 resulted in a time-dependent release of iron ions from the cytochrome *c* molecule. During the incubation of deoxyribose with cytochrome *c* and H_2O_2, the damage to deoxyribose increased in a time-dependent manner, suggesting that the released iron ions may participate in a Fenton-like reaction to produce ·OH radicals that may cause the DNA cleavage. Evidence that the iron-specific chelator, desferoxamine (DFX), prevented the DNA cleavage induced by the cytochrome *c*/H_2O_2 system supports this mechanism. Thus we suggest that DNA cleavage is mediated via the generation of ·OH by a combination of the peroxidase reaction of cytochrome *c* and the Fenton-like reaction of free iron ions released from oxidatively damaged cytochrome *c* in the cytochrome *c*/H_2O_2 system.

Keywords: Cytochrome *c*, DNA, Fenton Reaction, Peroxidase

Introduction

Cytochrome *c* has been studied extensively because of its central role in electron transfer in living organisms. The protein is localized on the intermembrane-space side of the inner mitochondrial membrane and participates in mitochondrial electron transport (Wilkstrom and Saraste, 1984). Recent reports implicate cytochrome *c* in oxidative stress, which results from the run-away production of reactive oxygen species. In addition, cytochrome *c* acts as a mediator of apoptotic cell death signals (Cai *et al*., 1998). The oxidative stress-mediated cellular damage and apoptotic cell death have been associated with neurodegenerative disorders, Parkinson's disease (PD) (Hashimoto *et al*., 1999), and cancer (Park and Kim, 2005).

During exposure to hydrogen peroxide, many proteins with metal binding sites are susceptible to oxidative damage, and free metal ions could be released (Kang and Kim, 1997). It has been reported that the reaction of hydrogen peroxide with heme proteins, such as cytochrome *c*, produces highly reactive ferryl-heme species that are capable of oxidizing biomolecules and initiating lipid peroxidation (Radi *et al*., 1991a; 1993b). Recently, it was reported that hydrogen peroxide oxidized cytochrome *c* to a peroxidase compound I-type intermediate, in which one oxidizing equivalent is present as an oxoferryl heme species and the other is present as the protein tyrosyl radical (Lawrence *et al*., 2003). The reaction of cytochrome *c* with H_2O_2 may lead to release of iron ions from cytochrome *c*. Therefore, the transition metal, iron, may react with H_2O_2 to produce ·OH through a Fenton-like reaction. ·OH is the most powerful oxidizing species among several reactive oxygen radicals, and is able to oxidize most macromolecules including DNA, protein, lipid, and carbohydrate (Frank *et al*., 1989; Breen and Murphy, 1995; Dean *et al*., 1997). Oxidative DNA damage from reactive oxygen species (ROS) has been hypothesized to play a critical role in several diverse biological processes including mutagenesis, aging, carcinogenesis, radiation effects, and cancer chemotherapy (Ames, 1983; Cerruti, 1984; von Sonntage, 1987).

In this study, we examined the DNA cleavage caused by cytochrome *c* and H_2O_2. Our results suggest that the DNA cleavage induced by cytochrome *c* and H_2O_2 is due to the oxidative damage resulting from ·OH generated by a

*To whom correspondence should be addressed.
Tel: 82-43-229-8562; Fax: 82-43-229-8432
E-mail: jhkang@cju.ac.kr

combination of the peroxidase activity of cytochrome *c* and the Fenton-like reaction of free iron ions released from oxidatively damaged cytochrome *c*.

Materials and Methods

Materials. pUC19 plasmid DNA was prepared and purified from *E. coli* cultures using QIAGEN plasmid kits (Santa Clarita). Bovine cytochrome *c*, catalase, 2-deoxy-D-ribose, thiobarbituric acid, bathophenanthroline sulfonate and deferoxamine (DFX) were purchased from Sigma. Chelex 100 resin (sodium form) was obtained from Bio-Rad. All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

Analysis of DNA cleavage. Supercoiled plasmid pUC19 DNA (1 μg) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 2 h at 37°C with different cytochrome *c* and H_2O_2 concentrations in a total volume of 20 μl. The reactions were stopped by freezing at −80°C. 5 μl of loading buffer (0.25% bromophenolblue, 40% sucrose) was added and the samples were analyzed by electrophoresis in 0.8% agarose in TBE buffer (2 mM EDTA, 89 mM boric acid and 89 mM Tris at pH 8.3). The resulting gel was stained with ethidium bromide. Bands of DNA were detected and photographed under UV light in a dark room.

Measurement of damage to deoxyribose. Detection of damage to deoxyribose was determined by measuring thiobarbituric acid-reactive 2-deoxy-D-ribose oxidation products (Kang, 2004). The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4), 10 mM 2-deoxy-D-ribose, 1 mM H_2O_2 and 100 μM protein in a total volume of 100 μl. The reaction was initiated by addition of H_2O_2 and incubated for 1 h at 37°C. The reaction was terminated by addition of 2.8% trichloroacetic acid (200 μl), PBS (200 μl), and 1% thiobarbituric acid (200 μl), then boiled at 100°C for 15 min, after which the samples were cooled and centrifuged at 15,000 rpm for 10 min. Results were read by uv/vis spectrophotometer (Shimadzu, UV-1601) at 532 nm.

Determination of free iron ion concentration. The concentration of iron ions released from oxidatively damaged cytochrome *c* was measured using bathophenanthroline sulfonate by the method described previously (Pieroni *et al*., 1994). The reaction mixture contained 100 μM cytochrome *c*, 1 mM H_2O_2 and 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 ml. The reaction was initiated by addition of H_2O_2 and incubated for 1 h at 37°C. After the incubation, the mixture was then placed into Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h. The colorimetic reagent was added for analysis by a uv/vis spectrophotometer at 535 nm. The final concentrations in the color reagent were 1% ascorbate, 0.02% bathophenanthroline sulfonate and 1% acetic acid-acetate buffer (pH 4.5).

Results and Discussion

Untreated DNA showed a major band corresponding to the supercoiled form (form I) and a minor band corresponding to nicked circular form (form II) (Fig. 1, lane 1). Plasmid DNA remained intact after incubation with 50 μM cytochrome *c* (Fig. 1, lane 2) or 0.3 mM H_2O_2 (Fig. 1, lane 3) alone. However, when DNA was incubated in a mixture of cytochrome *c* and H_2O_2, the DNA damage occurred (Fig. 1, lane 4). This indicates that both cytochrome *c* and H_2O_2 were required to cause strand breaks in the DNA. The effect of the concentrations of cytochrome *c* and H_2O_2 on the production of DNA strand breaks was then studied. The DNA cleavage increased dose-dependently with doses of up to 100 μM cytochrome *c* (Fig. 2A) and 1 mM H_2O_2 (Fig. 2B), respectively. Previous studies have shown that the reaction of cytochrome *c* with H_2O_2 generates a free radical that oxidizes amino acid residues at or near the cation-binding site, which then introduces carbonyl groups. Such an oxidative modification is an indicator of oxidative stress and may be significant in several physiological and pathological processes (Davies, 1986; Oliver, *et al*., 1987).

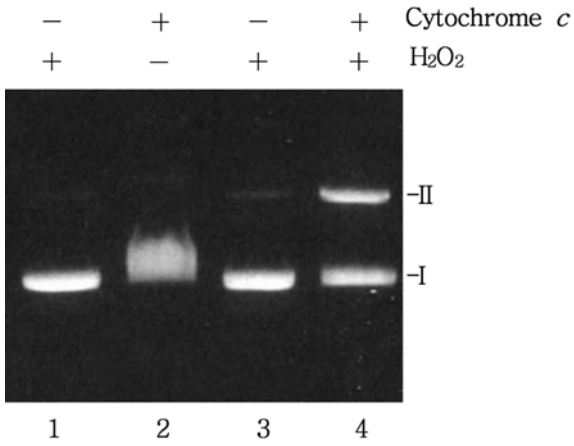

Fig. 1. DNA strand breakage after incubation with cytochrome *c* and H_2O_2. pUC 19 DNA (1 μg) was incubated at 37°C for 2 h: Lane 1, pUC 19 DNA control; lane 2, DNA + 50 μM cytochrome *c*; lane 3, DNA + 0.3 mM H_2O_2; lane 4, DNA + 50 μM cytochrome *c* + 0.3 mM H_2O_2. The reactions were stopped by freezing at −80°C. Loading buffer was added and the samples were analyzed by electrophoresis on 0.8% agarose gel. I and II indicate the positions of the supercoiled and nicked circular DNA plasmid forms, respectively.

The participation of free radicals in the DNA damage by the cytochrome *c*/H_2O_2 system was studied by examining the protective effect of radical scavengers. When plasmid DNA was incubated with cytochrome *c* and H_2O_2 in the presence of azide, mannitol, or ethanol at 37°C for 2 h, all scavengers significantly prevented the DNA cleavage (Fig. 3). The ability of radical scavengers to protect DNA from damage indicates that free radicals may participate in the mechanism of strand breaks produced by the cytochrome *c* and H_2O_2 system.

Cellular metabolism has been shown to generate the reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and superoxide radical (Cerruti, 1984). Trace metals

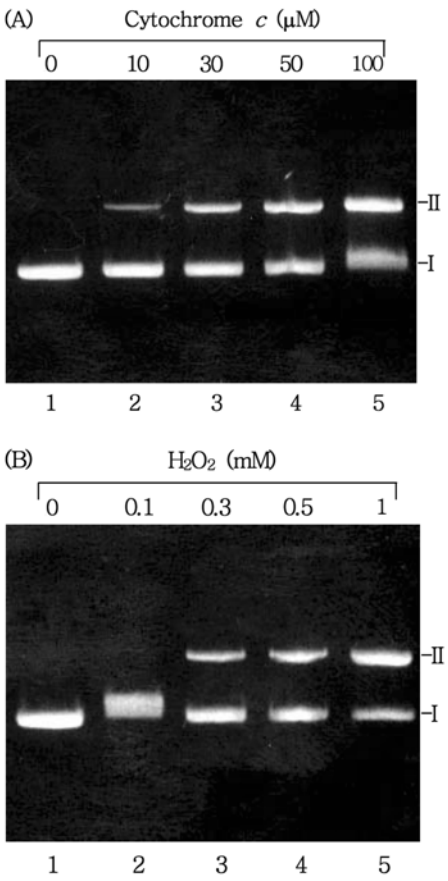

Fig. 2. Effects of cytochrome *c* and H_2O_2 concentrations on DNA strand breakage. (A) pUC 19 DNA (1 μg) was incubated with increasing doses (0-100 μM) of cytochrome *c* and 0.3 mM H_2O_2 at 37°C for 2 h. (B) pUC 19 DNA (1 μg) was incubated with 50 μM cytochrome *c* and increasing doses (0-1 mM) of H_2O_2 at 37°C for 2 h.

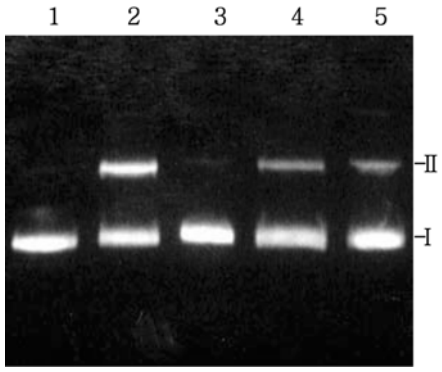

Fig. 3. Effect of radical scavengers on DNA strand breakage induced by cytochrome *c* and H_2O_2. pUC 19 DNA was incubated with 50 μM cytochrome *c* + 0.3 mM H_2O_2 in the presence of a radical scavenger. Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 200 mM azide; lane 4, 200 mM mannitol; lane 5, 500 mM ethanol.

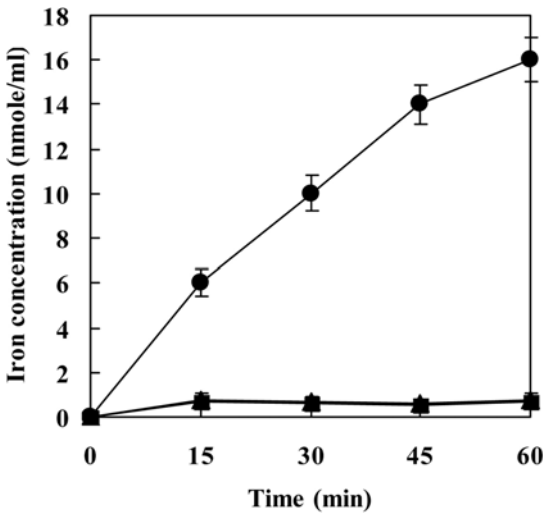

Fig. 4. Iron release in the cytochrome *c* and H_2O_2 system. The samples were incubated in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for various incubation periods with the following: 100 μM cytochrome *c* alone (■); 1 mM H_2O_2 alone (▲); 100 μM cytochrome *c* plus 1 mM H_2O_2 (●). Free iron ion concentrations were determined by a colorimetic reagent utilizing bathophenanthroline sulfonate.

such as copper and iron that are present in biological systems may interact with active oxygen species, ionizing radiation, or microwaves to damage macromolecules (Prutz, 1984; Samuni *et al.*, 1984; Goldstein and Czapski, 1986; Sagripanti *et al.*, 1987; von Sonntage, 1987; Imlay *et al.*, 1988). The cleavage of metalloproteins by oxidative damage may lead to increases in the levels of metal ions in biological cells. We investigated the release of iron in the reaction of cytochrome *c* with H_2O_2. During incubation of 100 μM cytochrome *c* with 1 mM H_2O_2, free iron ions gradually increased as a function of time (Fig. 4). It has been reported that iron ions could stimulate the Fenton-like reaction to produce ·OH, which mediates DNA strand breakage (Tachon, 1989). Attack of ·OH on the sugar, 2-deoxyribose, produces a huge variety of products, some of which are mutagenic in bacterial systems. Some of the fragmentation products can be detected by adding thiobarbituric acid (TBA) to the reaction mixture, resulting in formation of a pink $(TBA)_2$-MDA chromogen (Halliwell and Gutteridge, 1981). This can be used to detect ·OH production, although it is unclear whether or not some other ROS can also degrade deoxyribose. Our results showed that damage to deoxyribose was induced by the cytochrome *c* and H_2O_2 system (Fig. 5). The result suggests that ·OH may participate in the cytochrome *c*/ H_2O_2-mediated DNA cleavage. It has been reported that berberine inhibited the cytochrome *c*/H_2O_2-mediated DNA strand breakage through the scavenging of superoxide anion (Choi *et al.*, 2001). In this study, however, superoxide dismutase could not inhibit the cytochrome *c*/ H_2O_2-mediated DNA strand breakage (data not shown). Therefore, we assumed that the cytochrome *c*/H_2O_2-mediated DNA strand breakage might occur through a Fenton-like

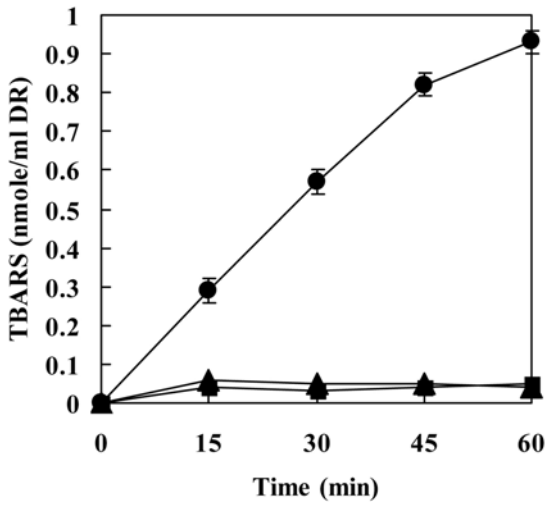

Fig. 5. 2-Deoxy-D-ribose degradation in the cytochrome *c* and H_2O_2 system. 10 mM 2-deoxy-D-ribose was incubated in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for various incubation periods with the following: 100 μM cytochrome *c* alone (■); 1 mM H_2O_2 alone (▲); 100 μM cytochrome *c* plus 1 mM H_2O_2 (●).

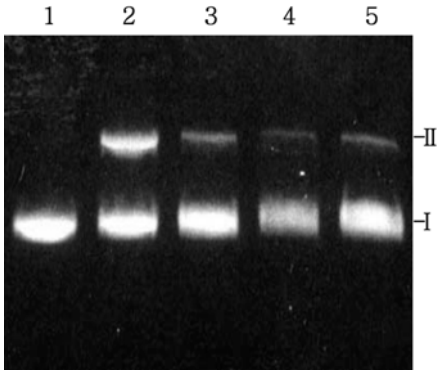

Fig. 6. Effect of DFX on DNA strand breakage induced by the cytochrome *c* and O_2 system. pUC 19 DNA was incubated with 50 μM cytochrome *c* and 0.3 mM H_2O_2 in the presence of various concentrations of DFX. Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 1 mM DFX; lane 4, 5 mM DFX; lane 5, 10 mM DFX.

reaction. The participation of iron ions in the production of DNA strand breaks was studied by examining the protective effect of the iron chelator, DFX. The DNA cleavage was effectively inhibited by 1 mM DFX (Fig. 6). The results indicate that free iron ions are involved in DNA cleavage by the cytochrome c/H_2O_2 system.

In conclusion, the present results indicated that DNA cleavage was induced by the reaction of cytochrome *c* with H_2O_2, involving ·OH generation from H_2O_2. The ·OH radicals were generated through a combination of the peroxidase reaction of cytochrome *c* and the Fenton-like reaction of free iron ions released from oxidatively damaged cytochrome *c*. DNA damage caused by the cytochrome c/H_2O_2 system could be relevant in diseases where mitochondrial dysfunction is elevated, such as Parkinson's disease (Schapira, 1994). It has been reported that at least 0.1 mM/min H_2O_2 will be produced continuously under physiological conditions; this rate increases in adverse conditions such as hyperoxia or ischemia and reperfusion (Boveries *et al*., 1972; Britton *et al*., 1987). Hence, in these abnormal conditions, the modification of human cytochrome *c* using H_2O_2 as a substrate could be potentiated, resulting in DNA damage.

References

Ames, B. N. (1983) Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science* **221**, 1256-1264.

Boveries, A., Oshino, N. and Chance, B. (1972) The cellular production of hydrogen peroxide. *Biochem. J.* **128**, 617-630.

Breen, A. P. and Murphy, J. A. (1995) Reactions of oxyl radicals with DNA *Free Radic. Biol. Med.* **18**, 1033-1077.

Britton, R. S., Bacon, B. R. and Recknagel, R. O. (1987) Lipid peroxidation and associated hepatic organelle dysfunction in iron overload. *Chem. Phys. Lipids* **45**, 207-239.

Cai, J., Yang, J. and Jones, D. P. (1998) Mitochondrial control of apoptosis: the role of cytochrome *c*. *Biochim. Biophys. Acta* **1366**, 139-149.

Cerruti, P. A. (1994) Oxy-radicals and cancer. *Lancet* **344**, 862-863.

Choi, D.-S., Kim, S.-J. and Jung, M. Y. (2001) Inhibitory activity of berberine on DNA strand cleavage induced by hydrogen peroxide and cytochrome *c*. *Biosci. Biotechnol. Biochem.* **65**, 452-455.

Davies, K. J. (1986) Intracellular proteolytic systems may function as secondary antioxidant defenses: a hypothesis. *J. Free Radic. Biol. Med.* **2**, 155-173.

Dean, R. T., Fu, S., Stocker, R. and Davies, M. J. (1997) Biochemistry and pathology of radical-mediated protein oxidation. *Biochem. J.* **324**, 1-18.

Frank, H., Thiel, D. and MacLoad, J. (1989) Mass spectrometric detection of cross-linked fatty acids formed during radical-induced lesion of lipid membranes. *Biochem. J.* **260**, 873-878.

Goldstein, S. and Czapski, G. (1986) The role and mechanism of metal ions and their complexes in enhancing damage in biological systems or in protecting these system. *J. Free Radic. Biol. Med.* **2**, 3-11.

Halliwell, B. and Gutteridge, J. M. (1981) Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. *FEBS Lett.* **128**, 347-352.

Hashimoto, M., Takeda, A., Hsu, L. J., Takenouchi, T. and Masliah, E. (1999) Role of cytochrome *c* as a stimulator of alpha-synuclein aggregation in Lewy body disease. *J. Biol. Chem.* **274**, 28849-28852.

Imlay, J. A., Chin, S. M. and Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* **240**, 640-642.

Kang, J. H. (2004) Modification of Cu,Zn-superoxide dismutase by oxidized catecholamines. *J. Biochem. Mol. Biol.* **37**, 325-329.

Kang, J. H. and Kim, S. M. (1997) Fragmentation of human Cu,Zn-superoxide dismutase by peroxidative reaction. *Mol. Cells* **7**, 553-558.

Lawrence, A., Jones, C. M., Wardman, P. and Burkitt, M. J. (2003) Evidence for the role of a peroxidase compound I-type intermediate in the oxidation of glutathione, NADH, ascorbate, and dichlorofluorescin by cytochrome *c*/H_2O_2. Implications for oxidative stress during apoptosis. *J. Biol. Chem.* **278**, 29410-29419.

Oliver, C. N., Levine, R. L. and Stadtman, E. R. (1987) A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging. *J. Am. Geriatr. Soc.* **35**, 947-956.

Park, J.-H. and Kim, T,-H. (2005) Release of cytochrome *c* from isolated mitochondria by etoposide. *J. Biochem. Mol. Biol.* **38**, 619-623.

Pieroni, L., Khalil, L., Charlotte, F., Poynard, T., Piton, A., Hainque, B. and Imbert-Bismut, F. (2001) Comparison of bathophenanthroline sulfonate and ferene as chromogens in colorimetric measurement of low hepatic iron concentration. *Clin. Chem.* **47**, 2059-2061.

Prutz, W. A. (1984) Inhibition of DNA-ethidium bromide intercalation due to free radical attack upon DNA. II. Copper(II)-catalysed DNA damage by $O_2^{-\cdot}$. *Radiat. Environ. Biophys.* **23**, 7-18.

Radi, R, Thomson, L., Rubbo, H. and Prodanov, E. (1991a) Cytochrome *c*-catalyzed oxidation of organic molecules by hydrogen peroxide. *Arch. Biochem. Biophys.* **288**, 112-117.

Radi, R., Sims, S., Cassina, A. and Turrens, J. F. (1993b) Roles of catalase and cytochrome *c* in hydroperoxide-dependent lipid peroxidation and chemiluminescence in rat heart and kidney mitochondria. *Free Radic. Biol. Med.* **15**, 653-659.

Sagripanti, J. L., Swicord, M. L. and Davis, C. C. (1987) Microwave effects on plasmid DNA. *Radiat. Res.* **110**, 219-231.

Samuni, A., Chevion, M. and Czapski, G. (1984) Roles of copper and O_2 in the radiation-induced inactivation of T7 bacteriophage. *Radiat. Res.* **99**, 562-572.

Schapira, A. H. (1994) Evidence for mitochondrial dysfunction in Parkinson's disease - a critical appraisal. *Mov. Disord.* **9**, 125-138.

Tachon, P. (1989) Ferric and cupric ions requirement for DNA single-strand breakage by H_2O_2. *Free Radic. Res. Commun.* **7**, 1-10.

von Sonntag, C. (1987) The Chemical Basis of Radiation Biology, Taylor & Francis, New York, USA.

Wikstrom, M. and Saraste, M. (1984) Cytochrome oxidase; in *Bioenegetics*, Ernster, L., (ed.) pp. 49-94, Elsevier Science Publishers B. V., Amsterdam, Netherlands.