Protection of superoxide dismutase by caffeine in rat liver mitochondria against γ-irradiation

J. P. Kamat[†], K. K. Boloor[†], T. P. A. Devasagayam[†] and P. C. Kesavan[†,*,§]

[†]Biosciences Group, Bhabha Atomic Research Centre, Trombay Mumbai 400 085, India
*M. S. Swaminathan Research Foundation, 3rd Cross Street, Taramani Institutional Area, Chennai 600 113, India

Radiation is one of the physical agents that induce oxidative stress. Exposure of rat liver mitochondria to high doses of ^{60}Co γ-rays (45–600 Gy) results in the loss of activity of superoxide dismutase (SOD). Presence of caffeine, even in micromolar amounts, during exposure prevents loss of SOD activity. Caffeine, at a concentration of 1 mM also showed protection against radiation-induced inhibition of two other mitochondrial enzymes, namely succinate dehydrogenase and cytochrome c oxidase. The observed radioprotective ability of caffeine may be due to its ability to scavenge the reactive oxygen species generated by radiation and to inhibit radiation-induced membrane damage, as assessed by lipid peroxidation and protein oxidation.

OXIDATIVE stress that results from the increased generation of reactive oxygen species (ROS) has been implicated in major human ailments like cardiovascular disease, cancer and neural disorders and in the process of aging[1,2]. Ionizing radiation is a physical agent that induces oxidative stress by excess generation of ROS. With low Linear Energy Transfer (LET) radiation such as γ-rays, most of the damage induced in biological systems is indirect and is mediated by ROS generated by the radiolytic products of water. These include hydroxyl radical ($^{\bullet}OH$), hydrogen atom ($^{\bullet}H$), hydrated electron ($e^{-\bullet}_{aq}$), superoxide radicals ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) (ref. 3). These reactive species are known to cause degradation of important macromolecules including DNA and membranes[4,5]. Among the sub-cellular organelles, mitochondria form some of the key components of cell killing induced by oxidative stress or radiation[6,7]. Though there are several antioxidant defences in the form of chemicals or enzymes, including those present in the mitochondria they are overwhelmed by high levels of pro-oxidants or radiation.

In the intracellular milieu, oxygen enhances radiation damage. The radiation-induced electrons react with oxygen to form superoxide anions ($O_2^{-\bullet}$) (ref. 8) which have been implicated as important pathologic mediators in various disorders including cancer, inflammation or ischemia[9]. Superoxide dismutase (SOD) catalyses the dismutation of $O_2^{-\bullet}$ to H_2O_2 and O_2, and thus it constitutes an early cellular defence against radiation and other forms of oxidative stress[10,11]. SOD has a high turnover and this provides a basis for maintaining the enzyme in active form during irradiation at moderate doses. However, upon exposure to large radiation doses, the enzyme loses measurable amounts of activity due to reactions with $O_2^{-\bullet}$ (ref. 12). It is therefore of interest to assess whether dietary agents which scavenge electrons in competition with oxygen and/or $O_2^{-\bullet}$ would protect mitochondria against radiation damage.

Caffeine (1,3,7-trimethylxanthine) is a component of coffee, tea and several cola-containing beverages[13]. Though it has been shown to have several pharmacologically useful effects, it is largely regarded as a radiosensitizer by virtue of its inhibition of DNA repair, especially as a post-treatment after UV-irradiation[14,15]. However, several recent studies suggest that if caffeine is present during radiation exposure, it can afford significant protection[16,17]. Our earlier studies have also shown that it protects against radiation-induced oxic pathway leading to seedling injury in barley[18], loss of clonogenic ability in *Bacillus megaterium* spores[19] and micronuclei induction in mice[20]. One recent study also showed that oral feeding of caffeine to mice protected them against radiation-induced lethality[21]. These studies as well as those reported earlier[13,14,22] also indicate that caffeine is effective *in vivo* and that caffeine reaches different tissues including liver after oral feeding. It has also been shown to be metabolized by the hepatic microsomal cytochrome P-450 system[23]. We have shown that radioprotective action of caffeine arises from its effective scavenging of $^{\bullet}OH$, $e^{-\bullet}_{aq}$, $^1O_2$ (refs 16, 17, 19, 24). The present study shows that even with low concentrations, caffeine can protect the antioxidant enzyme SOD against high doses of γ-irradiation as compared to two other mitochondrial enzymes which are not involved in scavenging of radicals generated during radiation such as superoxide.

Mitochondria isolated from livers of male Wistar rats, suspended in 5 mM KPO_4 buffer, pH 7.4, were used for these studies[25]. The above buffer was oxygenated for 30 min at room temperature prior to radiation exposure. In a 5 ml glass culture tube, mitochondria equivalent to 4 mg protein in 2 ml of the above buffer, with or without caffeine, were exposed to γ-rays using Gamma-cell (^{60}Co source, Atomic Energy of Canada Ltd.). In this set-up, the entire tube containing the sample was exposed to radiation at room temperature without stirring. The dose rate used was 15 Gy/min. The exposure times varied with the amount of dose required. After irradia-

[§]For correspondence. (email: dae_hbc@mssrf.org)

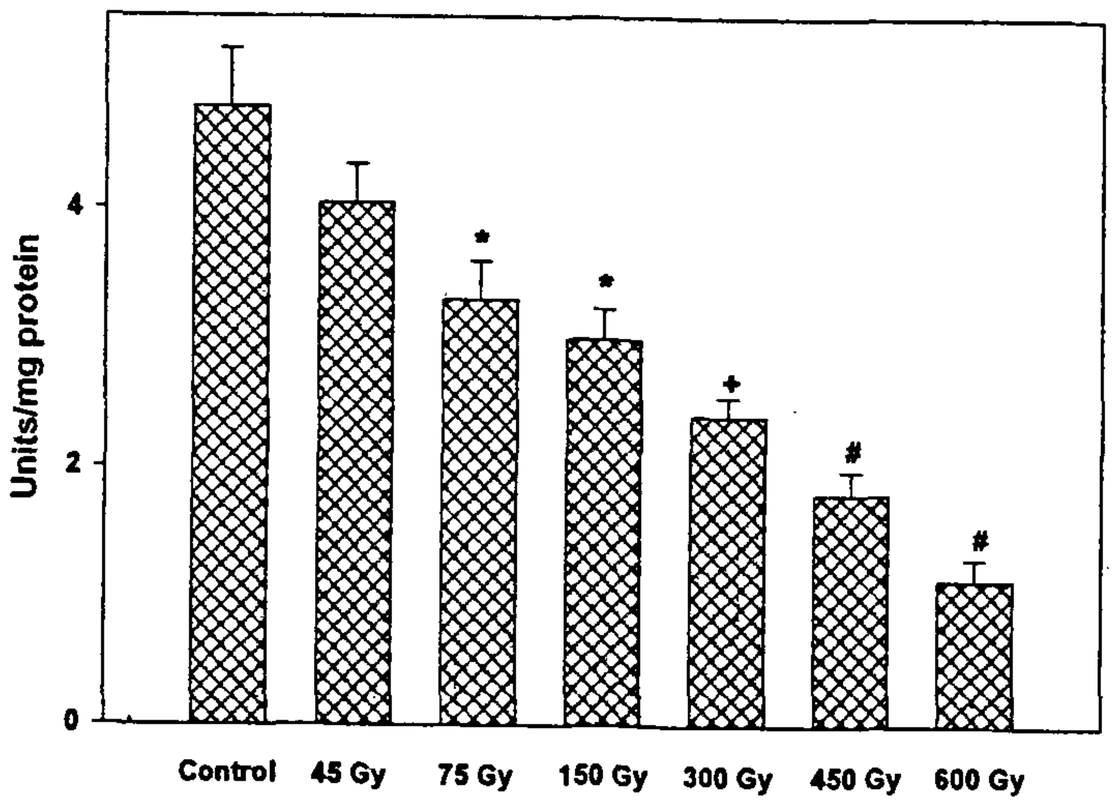

Figure 1. *Inhibition of superoxide dismutase with higher doses of γ-radiation in rat liver mitochondria.* Rat liver mitochondria (2 mg protein/ml) were exposed to γ-radiation (45–600 Gy) as a function of dose and the activity of superoxide dismutase was measured. Values are mean ± SEm from 4 experiments. $^{*}P < 0.05$; $^{+}P < 0.01$; $^{\#}P < 0.001$, as compared to control.

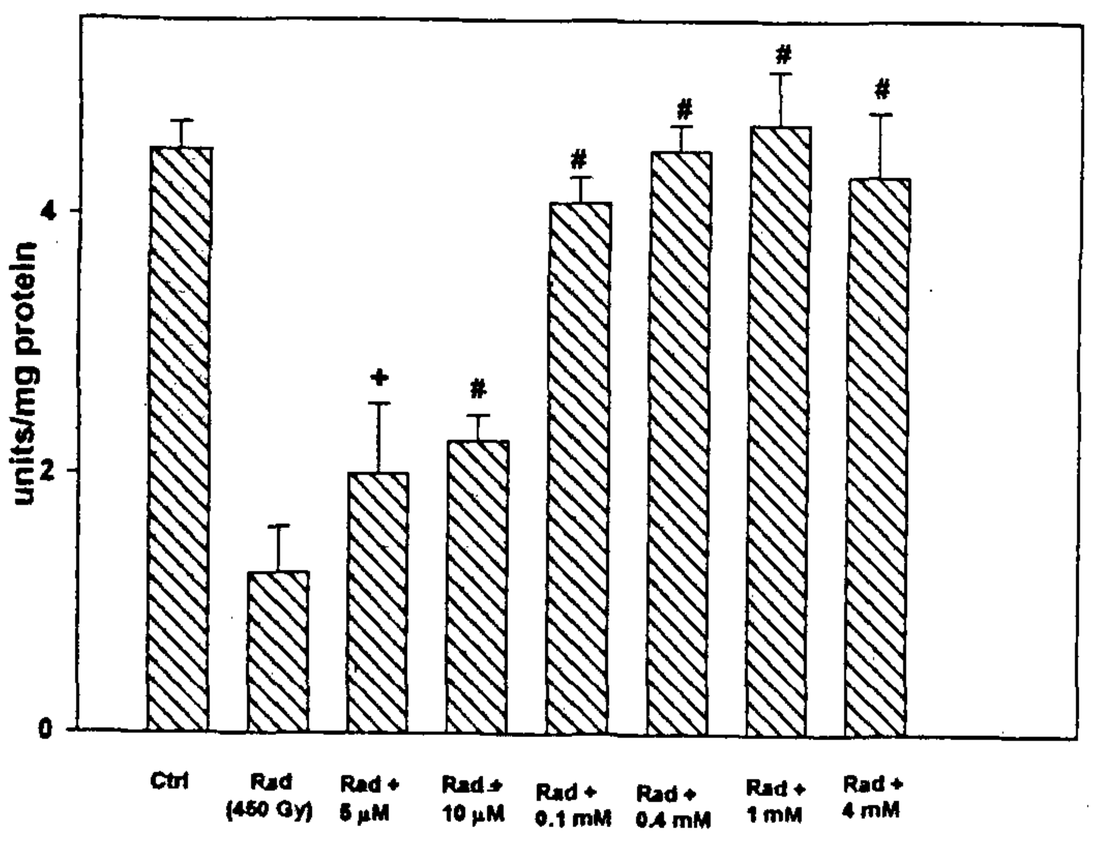

Figure 2. Concentration-dependent effect of caffeine on radiation-induced depletion of superoxide dismutase in rat liver mitochondria. Mitochondria (2 mg protein/ml) were exposed to γ-radiation at 450 Gy with and without caffeine (5 μM–4 mM) and the activity of superoxide dismutase was measured. Values are mean ± SEm from 4 experiments. $^{+}P < 0.01$, $^{\#}P < 0.001$, as compared to radiation treated.

tion the activity of SOD[26], succinate dehydrogenase[25] and cytochrome c oxidase[27] was measured. Lipid peroxidation was assessed by measuring lipid hydroperoxide (LOOH) and thiobarbituric acid reactive substances (TBARS), and protein oxidation was assessed by measuring protein carbonyls[28]. Data were subjected to statistical analysis by Student's *t* test.

Table 1. Possible factors responsible for the radioprotective effect of caffeine

Treatment	Lipid peroxidation		Protein oxidation
	TBARS^	LOOH^	Protein carbonyls^
None (Control)	1.41 ± 0.10	25.78 ± 1.10	1.03 ± 0.10
Radiation (450 Gy)	2.95 ± 0.21^{+}	41.69 ± 3.67^{+}	1.32 ± 0.02
Radiation (450 Gy) + Caffeine (1 mM)	1.58 ± 0.07*	33.06 ± 1.88	0.95 ± 0.03*

Values, expressed as ^nmoles product formed/mg protein are mean ± SEm from 4 experiments. TBARS – thiobarbituric acid reactive substances; LOOH – lipid hydroperoxide. $^{+}P < 0.05$, as compared to control; $^{*}P < 0.01$, as compared to radiation treated.

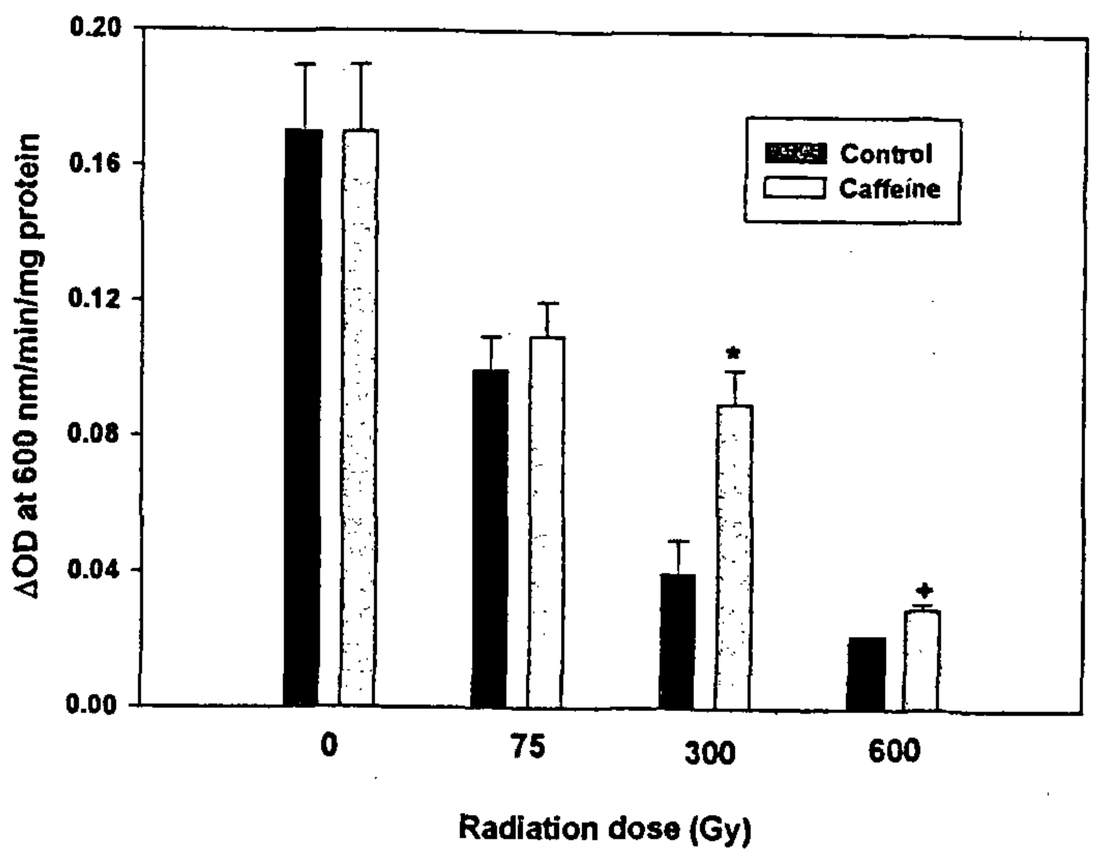

Figure 3. Radiation-induced inhibition of succinate dehydrogenase, and its prevention by caffeine. Mitochondria (2 mg protein/ml) were exposed to γ-radiation at different doses with and without caffeine (1 mM) and the activity of succinate dehydrogenase was measured. Values are mean ± SEm from 4 experiments. $^{*}P < 0.05$, $^{+}P < 0.01$, as compared to radiation treated.

Exposure of mitochondria to radiation, from 45 to 600 Gy, decreased the activity of SOD as a function of dose (Figure 1). A radiation dose of 150 Gy resulted in the loss of enzyme activity by 37.5%, while 450 Gy and 600 Gy caused a loss by 62.5% and 76%, respectively. For examining the possible protective effect of caffeine, mitochondria were exposed to 450 Gy with or without caffeine. Caffeine showed significant protection ($P < 0.001$) even at the low concentration of 10 μM (Figure 2). Caffeine at a concentration of 100 μM showed 87% protection while higher doses did not alter the radiomodulation further. The effect was much less under anaerobic condition (our unpublished data).

To examine whether the protection observed with SOD is likely to be due to protection against generalized oxidation of proteins, we have carried out experiments to examine the possible protective properties against

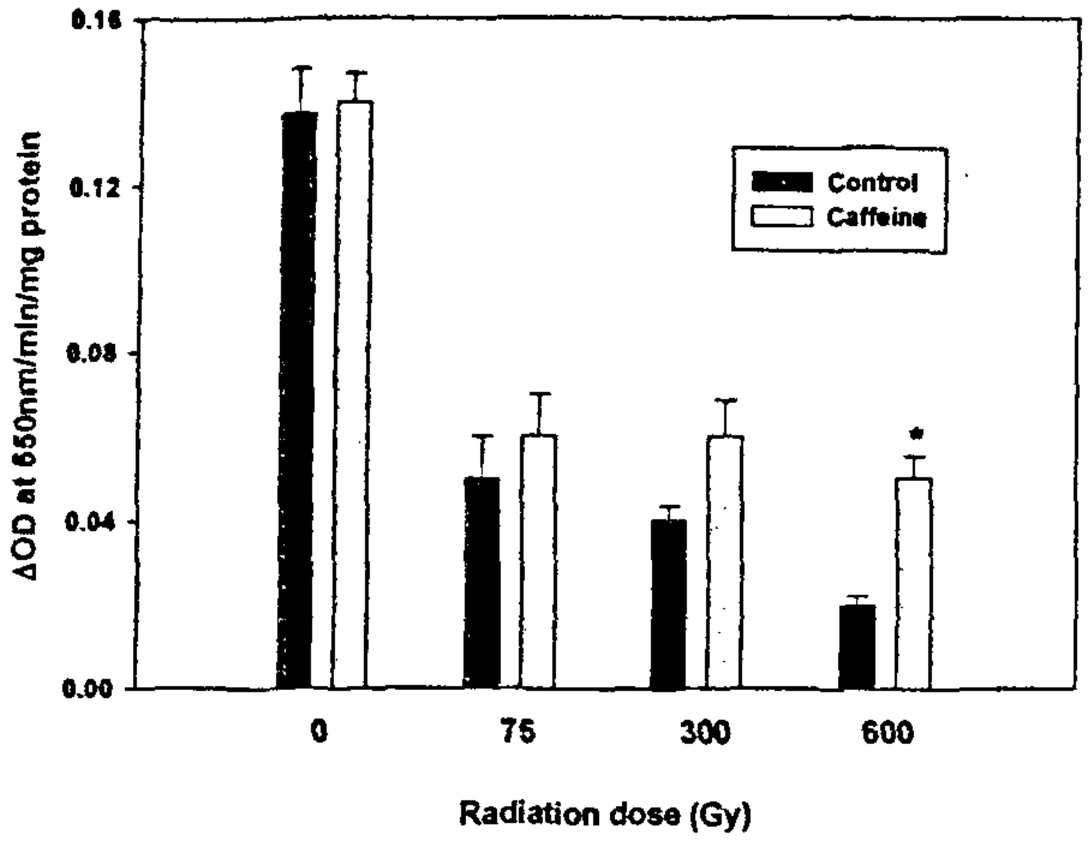

Figure 4. Radiation-induced inhibition of cytochrome c oxidase, and its prevention by caffeine. Mitochondria (2 mg protein/ml) were exposed to γ-radiation at different doses with and without caffeine (1 mM) and the activity of cytochrome c oxidase was measured. Values are mean ± SEm from 4 experiments. $*P < 0.05$ as compared to radiation treated.

two other mitochondrial enzymes, namely succinate dehydrogenase and cytochrome C oxidase. An optimum concentration of 1 mM caffeine was used. Our results show that radiation inhibits succinate dehydrogenase in a dose-dependent manner (Figure 3). Caffeine at 1 mM significantly protects against inhibition at 300 and 600 Gy. Similar results are also observed with another mitochondrial enzyme, cytochrome c oxidase, but only with a radiation dose of 600 Gy (Figure 4). With both the enzymes, however, the extent of protection was much less than that observed with SOD. It is worth noting that these two enzymes are not directly involved in detoxifying superoxide radical. Caffeine as such, has no inhibitory effect on both these enzymes.

To examine possible mechanisms responsible for the observed radioprotection of SOD and other enzymes by caffeine, we have studied the effect of caffeine on radiation-induced membrane damage, as assessed by lipid peroxidation and protein oxidation (Table 1). Exposure of mitochondria to radiation increased the peroxidation of lipids as measured by the TBARS and LOOHs. Protein oxidation also showed a significant increase, as can be seen by the formation of protein carbonyls. Caffeine, at a concentration of 1 mM decreased radiation-induced lipid peroxidation (formation of TBARS by 89% and of LOOH by 54.2%) as well as protein oxidation (almost 100%).

Though there are several antioxidants described in the literature, not all of them give radioprotection under similar conditions. Vitamin C but not E has been shown to be effective *in vivo*, under certain conditions, against γ-ray-induced chromosomal aberrations[29]. Epidemiological studies also did not substantiate the possible radioprotective action of Vitamin E[30]. Other prominent antioxidants credited with radioprotective ability includes β-carotene and glutathione[3,31]. Our earlier studies have shown that caffeine was more effective than both vitamin C and E in protection against radiation-induced micronuclei in mouse bone marrow[20,32]. Caffeine was also more potent in inhibiting the microsomal lipid peroxidation induced by various ROS[24]. The inhibitory effect was much less under anaerobic conditions in mitochondria (our unpublished results).

Earlier, Chelack and Petkau[12] suggested that inactivation of SOD by high doses of irradiation may involve interaction of the enzyme with the radiolytic products. When present during irradiation, caffeine molecules also undergo degradation by both direct and indirect action of radiation[16]. Under *in vitro* conditions and in the presence of O_2, radiation exposure generates various ROS such as $^{\bullet}OH$, $O_2^{-\bullet}$, H_2O_2, singlet oxygen ($^1O_2$) and peroxyl radicals ($ROO^{\bullet}$). It is likely that radioprotection by caffeine observed in the present studies may involve scavenging of these reactive species by caffeine. Our earlier studies[16,24] have shown that caffeine has relatively high rate constants with $^{\bullet}OH$, $e^{-\bullet}_{aq}$ and $^1O_2$. Radiation-induced membrane alterations as well as the level of oxidative stress may have a serious effect on the enzyme activity. Recent studies have shown that the level of SOD may be regulated by the extent of oxidative stress and cytokines[33]. Caffeine can protect against both lipid peroxidation and protein oxidation induced by radiation and this may possibly explain the observed protection of SOD. Both these phenomena are known to seriously affect membrane integrity as well as membrane-related functions[34,35].

In conclusion, the present study provides one more evidence to the suggested radioprotective and antioxidant role of caffeine. Caffeine consumption in the form of tea, coffee or cola-containing beverages is considerable in most human populations. Thus, our present study suggests an additional positive attribute of this compound.

1. Ames, B. N., Shigenaga, M. K. and Hagen, T. M., *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 7915–7922.
2. Sies, H. (ed.), *Antioxidants in Disease, Mechanisms and Therapy*, Academic Press, New York, 1996.
3. Von Sonntag, C., *The Chemical Basis of Radiation Biology*, Taylor and Francis, 1987.
4. Yukawa, O., Miyahara, M., Shiraishi, N. and Nakazawa, T., *Int. J. Radiat. Biol.*, 1985, **48**, 107–115.
5. Green, A., Prager, A., Stoudt, P. M. and Murray, D., *Int. J. Radiat. Biol.*, 1992, **61**, 465–472.
6. Salet, C. and Moreno, G., *J. Photochem. Photobiol. B. Biol.*, 1990, **51**, 133–150.

7. Slyshenkov, V. S., Moiseenok, A. G. and Wojtczak, L., *Free Radical Biol. Med.*, 1996, **20**, 793–800.
8. Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M. and Harman, D., *Ann. Intern. Med.*, 1987, **107**, 526–545.
9. Fridovich, I., *J. Biol. Chem.*, 1989, **264**, 7761–7764.
10. Taniguchi, N., *Adv. Clin. Chem.*, 1992, **29**, 1–59.
11. Przedborski, S. and Schon, E. A., *Nature Genet.*, 1998, **13**, 99–100.
12. Chelack, W. S. and Petkau, A., *Biochim. Biophys. Acta*, 1981, **660**, 83–90.
13. James, J. E., *Caffeine and Health*, Academic Press, London, 1991.
14. Kihlman, B. A. (ed.), *Caffeine and Chromosomes*, Elsevier, New York, 1977.
15. Selby, C. P. and Sancer, A., *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 3522–3525.
16. Kesavan, P. C., *Curr. Sci.*, 1992, **12**, 791–797.
17. Devasagayam, T. P. A. and Kesavan, P. C., *Indian J. Exp. Biol.*, 1996, **34**, 291–297.
18. Kesavan, P. C., Trasi, S. and Ahmad, A., *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, 1973, **24**, 581–587.
19. Kesavan, P. C. and Powers, E. L., *Int. J. Radiat. Biol.*, 1985, **48**, 223–233.
20. Farooqi, Z. and Kesavan, P. C., *Mutat. Res.*, 1992, **269**, 225–230.
21. George, K. C., Hebbar, S. A. and Kesavan, P. C., *J. Radiol. Protection*, 1999, **19**, 171.
22. Abraham, S. K., *Mutat. Res.*, 1991, **262**, 109–114.
23. Sanz, F., Lopez-deBrinas, Rodriguez, J. and Manaut, M., *Quant. Struct-Act. Relat.*, 1994, **13**, 281–284.
24. Devasagayam, T. P. A., Kamat, J. P., Mohan, H. and Kesavan, P. C., *Biochim. Biophys. Acta*, 1996, **1282**, 63–70.
25. Chatterjee, S. R., Srivastava, T. S., Kamat, J. P. and Devasagayam, T. P. A., *Mol. Cell. Biochem.*, 1997, **166**, 25–33.
26. Sun, M. and Zigman, S., *Anal. Biochem.*, 1978, **90**, 81–89.
27. Sajan, M. P., Satav, J. G. and Bhattacharya, R. K., *Toxicol. Lett.*, 1995, **80**, 55–60.
28. Kamat, J. P., Sarma, H. D., Devasagayam, T. P. A., Nesaretnam, K. and Basiron, Y., *Mol. Cell. Biochem.*, 1997, **170**, 131–138.
29. El-Nahas, S. M., Mattar, F. E. and Mohamed, A. A., *Mutat. Res.*, 1993, **301**, 143–147.
30. Neriishi, K., in *Vitamin E – Its Usefulness in Health and in Curing Diseases* (ed. Mino, M.), Japanese Science Society Press, Tokyo, 1993, pp. 187–194.
31. Ben-Amotz, A., Rachmilevich, B., Greenberg, S., Sela, M. and Wesler, Z., *Radiat. Environ. Biophys.*, 1996, **35**, 285–288.
32. Sarma, L. and Kesavan, P. C., *Int. J. Radiat. Biol.*, 1993, **63**, 759–764.
33. Isoherranen, K., Peltola, V., Laurkainen, L., Punnonen, J., Laihia, J., Ahotupa, M. and Punnonen, K., *J. Photochem. Photobiol. B. Biol.*, 1997, **40**, 288–293.
34. Rice-Evans, C. and Burdon, R., *Prog. Lipid Res.*, 1993, **32**, 71–110.
35. Dean, R. T., Fu, S., Stocker, R. and Davies, M. J., *Biochem. J.*, 1997, **324**, 1–18.

Received 26 August 1998; revised accepted 10 May 1999

Cell population growth during the formation of the chick eye lens

Mahendra S. Sonawane[†], A. Therwath* and Sohan P. Modak[†,]**

[†]Molecular Embryology Research Laboratory, Department of Zoology, University of Pune, Pune 411 007, India
*Université Paris 7-DENIS DIDEROT, Laboratoire d'Oncology Moléculaire, 2 Place Jussieu, 75005 Paris, France

The eye lens, a radially symmetrical and autonomously growing organ, contains dividing cells in the epithelium while fibre cells do not divide. Between day 7 and day 15 of chick embryogenesis, the annular pad extending from the peripheral epithelium is formed which acts as a reservoir of proto-differentiated fibres that are then transferred to the fibre area. Around the optical axis, a wave of apoptosis is generated in terminally differentiated fibre cell nuclei. We have determined cell numbers in the epithelium, the annular pad and fibre compartments and the data fit a logistic model the best, indicating a saturating growth. The growth rates for various lens compartments change with time.

THE evaginating optic vesicle comes in contact with the head ectoderm and induces the lens placode[1] that invaginates to form the lens vesicle[2]. The primitive lens contains prospective lens fibres in the inner hemisphere and prospective epithelium in the outer hemisphere[3,4] and becomes an autonomously growing organ. The prospective fibre cells stop dividing[4], enter a G_{01} phase[5] and synthesize lens crystallins[6–8]. The prospective epithelium contains dividing cells[4]. Progressively, the peripheral epithelial cells establish a distinct germinative zone[9,10] beyond which a reservoir, the annular pad, of non-dividing proto-differentiated fibres appears. These cells also contain fibre-specific δ crystallins[8] and are transferred to the fibre area throughout the life span[5,10]. The lens offers the best model for programmed cell death or apoptosis as elongated terminally differentiated primary fibre cells, located along and around the optical axis, exhibit a temporally and spatially specific pattern of nuclear pycnosis and loss[5]. The apoptotic lens cell nuclei lose DNA *in situ*[5], undergo DNA strand scission[11] liberating free 3^1OH ends detected as initiators *in situ* for calf thymus terminal deoxynucleotidyl transferase[12–15]. In both native[16,17] and 2-D (native and denaturing) agarose gels, it was shown[18] that fibre nuclear DNA breaks down and forms a DNA ladder respecting the polynucleosomal structure of chromatin, and loses the histone H-1 (refs 17,19). The fibre cell bodies devoid of nuclei and packed with crystallins, pile around the optical axis. Throughout the life span, the lens cell

**For correspondence.