Introduction
Radiation has accompanied the evolution, if not guided. Mankind has always been exposed to radiation. All of us are exposed to natural as well as man-made radiation. The sun, the outer space, natural radioactive materials present in earth, the buildings we inhabit, the food and water we consume and the air we breathe are all sources of radiation. Man is exposed to substantial doses of radiation from external sources like cosmic-rays (0.4mSv) and terrestrial sources (2mSv) annually. Man gets a significant amount of radiation exposure from his own body. Sub-lethal doses of radiation would induce a variety of changes which might be expressed in different forms of damage including death. On the other hand, low doses of radiation induce cancer. The mutations caused by radiation exposure may be carried from generation to generation. The biological systems consist of about 80% water, thus to a great extent biological effects are mediated through the action of radiation on water. Important products of radiolysis of water are primary free radicals and molecular species like H₂ and H₂O₂ (Arnikar 1983). The overall equation of radiolysis of water is as follows:

\[ \text{H}_2\text{O} \rightarrow e^- \text{aq}, \text{H}, \text{OH}, \text{O}_2^-, \text{HO}_2^-, \text{H}_2, \text{H}_2\text{O}_2 \]

Free radicals are unstable and highly reactive (Hughes 1973). Biological effects resulting from the interaction of free radicals with target molecules is called indirect action of radiation. The damage occurred due to direct interaction or absorption of radiation by target sites is known as the direct effect of radiation. More than 50% of the lethal effects of radiation were found to occur due to indirect action (Roots and Okada 1972; Chapman et al 1973; Banura and Smith 1976; Miller et al 1981; Roots et al 1982; Skov 1984). However, radiation is very useful and also beneficial. The use of radioisotopes in research, medicine, agriculture and industry has not only
unfolded the hidden facts of science, but also significantly contributed to an overall progress of man. To exploit the radiation for benefit of human beings, it is necessary to understand the risk associated with radiation.

Radiation is detrimental to cell. The sensitivity of the cells is directly proportional to their reproductive activity and inversely proportional to their degree of differentiation (Bergonie and Tribondeau 1959). Since the cancerous cells have a great reproductive activity and are less differentiated than the surrounding healthy tissue, they are expected to be vulnerable to the killing action of the ionising radiation. Thus the finding of Bergonie and Tribondeau laid the foundation of radiation therapy of cancer. Radiation therapy is considered to be one of the most important and popular tools to cure cancer.

The extent of radiation damage in a tissue is directly related to the degree of oxygenation of that tissue is well established now. The findings of Thomlinson and Gray (1955), triggered tremendous interest in oxygen as a factor in radiation-therapy. Due to the rapid fall of the concentration of oxygen with the increase in the distance from the blood capillaries with their abundant supply of oxygen, there is a relatively small portion of hypoxic cells in tumours. Hypoxic cells are a common feature of human tumors (Brown and Giaccia 1994).

Since the hypoxic cells are radioresistant, the presence of hypoxic cells in tumour was suggested to limit the success of radiation therapy of cancer. To overcome this difficulty much effort has been expended in the search an agent which will either selectively potentiate the radiation effect, or conversely protect the surrounding normal tissue, and enable the application
of relatively higher dose of radiation so as to favour the killing of cancer cells than normal. The possibility of use of radiomodifiers have opened newer avenues in the radiotherapy of cancer (Workman and Stratford 1993; Workman 1992; Stratford et al. 1994). The use of radioprotective and sensitizing agents, either alone or in combination in case of ionizing radiation, is theoretically advantageous particularly in terms of cost/benefit ratio. Moreover, chemical modifiers would be very useful when cancers are already in advanced stage at the time of diagnosis. Importantly such situations exist in India. One of the methods of overcoming hypoxic cell resistance to radiation would be to wholly eliminate that cell population (Kale 1996)

1.1 Radiomodification

Numerous drugs have been tested for their ability to potentiate or to protect against radiation effect (von Sonntag 1987; Yuhas et al. 1977; Koch 1985; Sugahara 1984). Some of them have shown promising results. Despite showing initial differential modifying effects these chemicals have not been widely accepted for clinical use. A survey of literature suggests that without addressing or understanding the non-acceptability, further work on most of the chemical modifiers was either abandoned or diverted towards the search and testing of new agents. This kind of endeavour helped very little, if at all, in improving the radiation therapy. Therefore, the gap between the present state of knowledge on chemical modifiers and radiation therapy of cancer remains to be bridged. Understanding the mechanistic pathways/biochemical events in radiomodification is essential to minimize this gap.
On administration of radiomodifiers, apart from other events, they are likely to be metabolized by the oxidoreductive enzymes. Therefore, it seems that the oxidoreductive enzymes might also be involved in the determination of radiomodifying ability of the radioprotectors and sensitizers. However, not much is known about this aspect. Moreover very little information is available, if at all, on the radiation effects on the oxidoreductive enzymes themselves. Therefore an attempt has been made in the present work to examine the influence of ionising radiation on the oxidoreductive enzyme system and their modification by caffeine, xanthine and mitomycin-c.

Caffeine has been shown to exert differential modification of oxic and anoxic components of radiation damage using mainly, barley seeds and bacterial spores. Caffeine being a derivative of xanthine, an attempt has also been made to study the modulatory effect of the parent compound. In addition mitomycin-c which is quinone in nature has also been used as a radiomodifier.

1.2 Caffeine

In the early 1820s, caffeine was isolated from green coffee beans and was shown to have basic properties. Caffeine was also found in different parts of the plants like the leaves, fruits, seeds and bark (Kihlman 1977). Chemically it is 1,3,7-trimethylxanthine, and is used as adjuvant analgesic in combination with acetaminophen, aspirin and ibuprofen (Castaneda-hernandez et al 1994). Importantly caffeine is consumed daily by a large population throughout the world. As human beings have been exposed to ionising radiation since long, caffeine might have modified at least partly, its detrimental effects.
It was reported that caffeine potentiated the UV-induced damage in a variety of prokaryotic and eukaryotic cells (Sideropoulois and Shankel 1968; Domon and Rauth 1973). Caffeine was found to increase the UV-induced cell killing in all strains of *Salmonella typhimurium* (Williams and Clarke 1971, 1974) and *Escherichia coli* cells (Witkin, 1959).

In mammalian systems the UV-induced reduction of colony-forming ability of mouse L cells was increased when the cells were irradiated immediately after incubation in growth medium containing caffeine (Rauth 1967). In CHO cells and cell line V79 from lung tissue, caffeine strongly reduced the survival of UV-irradiated cells (Arlett and Harcourt 1972). In keratinocytes, caffeine potentiated the induction of micronuclei by UV-B (Weller et al. 1996). Chromosomal aberrations were produced by caffeine treatments in the root tips of *Tradescantia palutem* (Tanaka and Sinoto 1951), *Allium proliferum* (Kihlman et al. 1971) and *Vicia faba* (Kaul and Zutshi 1973; Kihlman et al. 1974).

Caffeine also potentiated the mutagenic and lethal effects of genotoxic agents. *Micrococcus luteus* UV endonuclease (Kaplan et al. 1971), *Escherichia coli* 3-methyladenine DNA glycosylase I (Bjelland and Seeberg 1987), and human placental AP endonuclease (Kane and Linn 1981) were inhibited by caffeine. Caffeine is suggested to inhibit photoreactivation, excision repair and also the recombinational repair (Selby and Sancer 1990).

Oral administration of caffeine had a substantial inhibitory effect on UV-B induced carcinogenesis in SKH-1 mice. Caffeine when added to the decaffeinated tea restored the inhibitory effect of the decaffeinated tea on
UVB-induced carcinogenesis (Huang et al 1997). It may be remembered that mode and magnitude of absorption of UV is qualitatively different from that of ionising radiations like X-rays and gamma-rays.

Barley seeds have been used extensively as experimental system to study the influence of caffeine on the damage induced by ionising radiation. In germinating seeds and other metabolizing systems caffeine was found to be a potent radiosensitizer (Domon and Rauth 1969; Ahnstrom and Natarajan 1971). Ahnstrom and Natarajan (1971) also found that when barley seeds were exposed to gamma rays and then exposed to caffeine for the first 5 hours of the germination period, the frequency of gamma-ray induced dicentrics and rings doubled. Yamamoto and Yamaguchi (1969) suggested that caffeine increases the frequency of gamma rays-induced fragments by inhibiting the rejoicing of chromatid breaks in root-tip mitosis of germinating barley seeds.

It has been reported that caffeine reduces the level of radiation-induced seedling injury and chromosomal aberration and enhances the magnitude of DNA synthesis under oxygenated condition. The level of seedling injury and chromosomal aberration and the magnitude of DNA synthesis were however, enhanced under O₂-free condition by caffeine (Sah and Kesavan 1986). Caffeine applied during anoxic hydration reduces the seedling injury to a much greater extent than it reduces the frequency of chlorophyll mutations (Kesavan and Ahmad 1974). Several studies suggested that if caffeine is present during the time of occurrence of damage induced by radiation or other agents, it affords significant protection. It has been reported that caffeine protects barley seeds (Kesavan et al 1973; Kesavan and Ahmad 1974; Kesavan et al 1978), Baccillus megaterium
spores (Kesavan and Powers 1985), CHO cells (Kesavan and Natarajan 1985) against oxygen-dependent damage.

Caffeine gives radioprotection against oxic damage by mutual annihilation with the oxidizing peroxy radical intermediates (e.g. superoxide, hydroxyperoxyl radicals) and peroxides which are formed as a result of reaction of oxygen-sensitive sites with oxygen (Kesavan et al. 1973).

80% of the studies involved post-treatment with caffeine. Not much could be answered about the effects of caffeine administered just before irradiation, so that its influence, if any, when present during the exposure of actively metabolizing cells and tissues to irradiation, could have been known. This is important because radiation induced free-radicals in metabolizing systems, are very short-lived with lifetimes of the order of $10^{-9} - 10^{-6}$ sec. So the possible reactions of caffeine with these free radicals and their overall influence on survival, growth rate, chromosomal aberrations and mutations, would be totally missed out in studies with caffeine administered post-irradiation unless the test systems used are dry spores which are metabolically inert.

In animal systems most studies have been performed on cells in culture, but some data from whole-animal experiments are also available. In human TK4 lymphoblastoid cells, caffeine, either before or 3 hr after irradiation almost completely prevented radiation-induced apoptosis. The percentage of cells showing apoptosis decreased when cells were treated with caffeine after irradiation than when exposed to radiation alone (Zhen and Vaughan 1995). Caffeine also prevented apoptosis induced by camptothecin and topotecan or topoisomerase inhibitors by formation of caffeine-drug
complexes (Traganos et al. 1993). However, on the other hand in human leukemic cells caffeine synergistically potentiated cytotoxicity and apoptosis induced by ionising radiation (Efferth et al. 1995).

Treatment of HeLa cells with caffeine has been shown to shorten the G2 delay after irradiation and to decrease their survival. This decrease in the radiation-induced G2 delay mediated by caffeine is accompanied by a shift in the pathway of cell death from mitotic to apoptotic death (Bernhard et al. 1996). Although in irradiated HeLa cells, caffeine greatly reduces the G2-phase delay, it does not alter the progression of cells through the cell cycle in unirradiated cells (Bernhard et al. 1994). Thus, caffeine releases the radiation-induced G2-phase cell cycle block (Cromton et al. 1993).

Effect of caffeine on G2-phase arrest and cyclin B1 expression in human skin fibroblasts (HSF), and transformed HeLa cells following gamma-irradiation, were also examined. Caffeine was effective in attenuating both the radiation-induced increase in cyclin B1 expression and the prolongation of G2 in synchronous and asynchronous HeLa cell populations. The increase in cyclin B1 expression observed in irradiated HSF cells (synchronous and asynchronous) decreased when the cells were treated with caffeine. Treatment of irradiated synchronous and asynchronous HSF cells with caffeine significantly reduced the prolongation of the G2-phase (Narayanan et al. 1997).

G2 arrest induced by radiation was reduced by caffeine also in CHO cells (Masunaga and Keng 1996). Delays in the S- (and G2- phase) induced by X-radiation in HeLa cells were decreased by caffeine (Kiriillova and Spivak
Caffeine reduced the radiation-induced G2/M-phase arrest, but enhanced the degree of DNA fragmentation (Palayoor et al 1995).

In irradiated human lymphocytes the potentiation or protection of chromosomal aberrations caused by caffeine depended on the concentration and temperature (Stoi lov et al 1994). In human squamous cell carcinoma, induction of double strand breaks (dsb) was reduced by caffeine and no effect on dsb repair was observed (Smeets et al 1994). In cultured mammalian AL human-hamster hybrid cells, caffeine increased the induction of S1-mutant by radiation (McGuiness et al 1995). It was reported that acute pre-treatment with caffeine reduced radiation-induced frequency of chromosomal aberrations discernibly, whereas the chronic pre-treatment afforded a much more significant degree of radioprotection in whole-body irradiated mice (Farooqi and Kesavan 1992).

The combined effect of radiation and caffeine has been studied in the mouse embryos. Malformation such as cleft palate and defects of forelegs and hindlegs appeared frequently in the fetuses treated with both radiation and caffeine. The combined action of radiation and caffeine on intrauterine death and malformations was synergistic (Kusama et al 1989).

Studies on the enzyme systems showed that caffeine increased the radiation-induced activity of glyoxalase I, but enhanced the radiation -induced inactivation of glyoxalase II. Since the glyoxalase system play an important role in the regulation of cell division and differentiation, radiation effects on this systems are suggested to have serious biochemical consequences (Sharma and Kale 1993). Not much information is available on the radiomodification of enzyme systems by caffeine.
Caffeine (1,3,7 trimethyl xanthine)

Xanthine (2,6-dioxopurine)

Mitomycin-C
Caffeine has significant abilities to scavenge highly reactive free radicals and excited states of oxygen and to protect crucial biological molecules against these species. Caffeine reacts with both hydrated electrons, and hydroxyl radicals (Kesavan and Powers 1985). The structure of caffeine suggests that it could readily accept electrons. Caffeine in total anoxia or extreme hypoxia acts only as a sensitizer (Kesavan et al 1973; Kesavan and Ahmad 1974; Afzal and Kesavan 1979; Singh and Kesavan 1991). At an oxygen concentration of \(9 \times 10^{-4} \text{ M}\), caffeine is neither a sensitizer, nor a protector. Caffeine becomes an effective radioprotector when the \(O_2^-\) concentration is about \(1.4 \times 10^{-3} \text{ M}\) and above; it is a radiosensitizer at or below an \(O_2^-\)-concentration of \(5.0 \times 10^{-4} \text{ M}\) (Kesavan et al 1991). All aerobic cells contain around \(1.0 \times 10^{-3} \text{ M}\) of oxygen, but the deep seated tumor cells are hypoxic. A combination treatment with caffeine and radiation might help not only in reducing damage to normal cells, but also in preferentially enhancing damage to the hypoxic tumor cells in cancer treatment (Kesavan 1992).

It has been reported recently that caffeine is an effective inhibitor of lipid peroxidation at millimolar concentrations against ‘OH, \(O_2^-\) and \(\text{ROO}^-\), three reactive oxygen species inducing membrane damage in-vivo in biological systems (Devasagayam and Kesavan 1996).

1.3 Mitomycin-C

Mitomycin-C (MMC) is an antibiotic. It has a quinone structure, an aziridine ring and carbamate moieties in its structure. The aziridine ring is an alkylating group, and is similar to those present in antitumor mustards and
the quinone. The carbamate moieties are like those found in some mitotic inhibitors (Schwartz et al 1963). The aziridine ring is primarily responsible for the biological effects of mitomycin-c. MMC requires bioreductive activation to become an alkylating species (Kennedy et al 1980). MMC has preferential toxicity towards hypoxic cells as compared to aerobic cells. The preferential toxicity is due to the enhanced metabolic reduction of the drug to toxic species at low levels of oxygen (Powis 1987).

MMC is activated to an alkylating species by reduction of the quinone moiety and subsequent loss of the methoxy group (Moore 1977; Kennedy et al 1980; Tomasz and Lipman 1981). MMC is a clinically used antitumor antibiotic that binds covalently to deoxyribonucleic acid under reductive or acidic catalysis by linking to the N-2 position of the 2'-deoxyguanosine (Tomasz and Lipman 1986). Spontaneous rearrangement of the reduced molecule leads to the production of a highly reactive quinone intermediate which can alkylate DNA, RNA and protein. MMC also undergoes reductive activation by certain chemicals like Na₂S₂O₄ (sodium dithionite) (Tomasz et al 1987).

Two pathways have been proposed for the cytotoxicity mediated by reductive activation of MMC. The first pathway involves the one electron reduction of MMC to its semiquinone radical MMC', which is a potent DNA-alkylating species. This reduction is catalyzed by enzymes such as NADPH-cytochrome p450 reductase (Bachur et al 1979; Pan et al 1984), NADH xanthine oxidase and NADH dehydrogenase (Doroshow 1983). The second proposed bioactivation pathway involves a two electron reduction of MMC by DT-diaphorase to the corresponding hydroquinone directly without an intermediate semiquinone. The hydroquinone activates the aziridine ring
by elimination of the methoxy group, which in turn increases the susceptibility to nucleophilic attack by DNA (Dorr et al 1985). This pathway, which is a major detoxification route for a variety of quinones in mammalian cells, has been implicated as an important determinant in the MMC induced toxicity (Begleiter et al 1989; Marshall et al 1991). The two electron reduction of MMC by DT-diaphorase is considered to be a determinant of MMC induced genotoxicity and cytotoxicity (Siegel et al 1990).

MMC is being used along with ionizing radiation in the treatment of solid tumors. Both one and two electron reduction products of MMC may be alkylating species (Cinzia et al 1989). MMC was reductively activated by DT-diaphorase in rat liver carcinoma cells which forms an adduct with DNA (Kumar et al 1997). The inhibitor of DT-diaphorase dicoumarol, reduces aerobic MMC toxicity (Keyes et al 1984; Keyes et al 1985; Rockwell et al 1988). This implies that the inhibition of the enzyme decreases the production of toxic metabolites. Paradoxically, in hypoxic cells the MMC induced cytotoxicity is enhanced by dicoumarol (Keyes et al 1985; Rockwell et al 1988). DT-diaphorase deficient cells have been isolated from a cancer prone family, and these cells are resistant to MMC under aerobic conditions and dicumorol does not reduce their aerobic MMC sensitivity (Marshall et al 1989).

Dulhunty and Whitmore reported the isolation of CHO cell lines resistant to mitomycin-C under aerobic conditions of drug exposure. They also reported that these cell lines have the same response to mitomycin-C under hypoxic conditions as do the controls. The cells have a lower level of DT-diaphorase activity than controls, but similar levels of NADPH-cytochrome p450 reductase activity. The deficient cell lines show molecular
defects in the DT-diaphorase gene or gene transcript. DT-diaphorase is involved in MMC activation under aerobic conditions, but this activity is relatively insignificant to MMC activation under hypoxic conditions (Dulhunty and Whitmore 1991). It was reported that MMC was not a substrate for DT-diaphorase and was actually a weak inhibitor by the studies on the metabolism of MMC by purified human kidney DT-diaphorase (Schlager and Powis 1988).

It has been reported that high fat intake increased the tumor growth and also increased the tumor response to MMC treatment as compared to the control low fat diet. At the same time the enzymes which activate MMC, such as NADH-cytochrome b5 reductase and DT-diaphorase, were also induced in the tumor by high dietary fat intake (Shoa et al 1994). MMC treatment of HT 29 cancer cells have demonstrated that it induces DT-diaphorase at the transcriptional level (Yao et al 1997). Dicumarol an inhibitor of DT-diaphorase was shown to protect HT-29 cells from MMC toxicity (Seigel et al 1990). The cell lines with non existent DT-diaphorase activity or marginal activity show resistance to MMC in comparison to ones with DT-diaphorase activity (Mikami et al 1996).

Bioreductive enzymes are known to play an important role in radiomodification by radiomodifiers. Although several compounds are tested, very few are shown to have potential for clinical use. However, MMC and caffeine are considered to be useful in improving the radiation therapy of cancer, but their radiomodifying property is not examined completely and critically. Therefore to exploit MMC and caffeine for the therapy, biochemical events involved in their radiomodification ability need to be understood.
On administration MMC and caffeine are expected to be metabolised which could contribute to the outcome of radiation therapy. Their interactions with bioreductive enzymes e.g DT-diaphorase, NADPH-cytochrome p450 reductase, NADH-cytochrome b5 reductase and xanthine oxidase are not understood in radiolytic systems. Apart from their own metabolism, it might be quite possible that these compounds influence the bioreductive enzymes and alter the metabolising capacity of the cell. Therefore, an attempt has been made to examine the radiomodifying effect of MMC and caffeine on the above mentioned enzymes. In addition, caffeine being a derivative of xanthine, radiomodifying property of xanthine was also examined. Till date, no information is available on this aspect regarding xanthine.

1.4 DT-DIAPHORASE

DT-diaphorase has been extensively studied due to its protective role against the cytotoxicity and mutagenecity of quinone-derived oxygen radicals, and its involvement in the vitamin K-dependent protein carboxylation (Lind et al 1990).

Ernster and Navazio in 1958, reported DT-diaphorase (DTD) in the soluble fraction of the rat liver homogenates which catalyzed the oxidation of NADH and NADPH at equal rates. The enzyme DT-diaphorase is widely distributed in the animal kingdom. Pigeon is the only exception which seems to lack DT-diaphorase. Among animal tissues, liver is one of the richest sources of this enzyme. Relatively high activities are also found in the kidney and brain, whereas heart and skeletal muscle are relatively poor in DT-diaphorase activity (Martius 1963). On fractionation
of the liver homogenates, the bulk (>90%) of DT-diaphorase is recovered in the cytosolic fraction, whereas minor portions of the enzyme are associated with the mitochondria and microsomes (Ernster 1967). Appreciable DT-diaphorase activities are found in different tumors (Beyer et al 1988).

DT-diaphorase is a FAD-containing flavoprotein, which catalyzes the oxidation of NADH and NADPH by various dyes and quinones (Ernster 1987; Buffinton et al 1989; Lind et al 1990). DT-diaphorase [NAD(P)H: quinone oxidoreductase] catalyses the two-electron reduction of quinoid compounds (Q) to hydroquinones (QH):

\[
Q + \text{NAD(P)H} + H^+ \rightarrow \text{QH}_2 + \text{NAD(P)}^+, 
\]

Hydroquinones produced are quite stable and may undergo conjugation reactions (Iyanagi and Yamazaki 1970). This feature of the DT-diaphorase appears to be unique among NAD(P)H oxidizing flavoproteins, which in general are one-electron transfer enzymes and give rise to semiquinones that undergo auto-oxidation to form superoxide radical (O$_{2}^-$) (Lind et al 1982; Iyanagi et al 1984). When both one-electron and two-electron transferring quinone reductases are present, the latter would be expected to limit auto-oxidation and concomitant O$_{2}^-$ formation, by converting the available quinone into the stable hydroquinone. This enzyme plays an important role in the prevention of quinone-mediated toxicity and mutagenesis, since hydroquinones can be conjugated with UDP-glucuronate by means of UDP glucuronic acid (UDPGA)-glucuronosyl or -sulphate transferases that facilitate the excretion of these
compounds from the cell (Cadenas et al 1992). This enzyme also protects the cell from oxidative damage by preventing oxyradical formation from one-electron reduction of quinones and the subsequent formation of superoxide radicals via dismutation of the semiquinone (Lind et al 1982; Thor et al 1982). Certain group of anticancer drugs require bioactivation. The enzyme DT-diaphorase has been reported to play a major role in bioactivation of such drugs. This capacity together with dismutating capacity of quinone radicals has led to its been labeled an "anticancer enzyme" (Beyer et al 1988).

Human blood mononuclear cells exposed to UVB showed an increase in DT-diaphorase activity as compared to the control. It has been suggested that an inducible antioxidant defense mechanism operates on photo-oxidative stress and DT-diaphorase has an antioxidant role (Alvarez and Boveris 1997). DT-diaphorase activity was shown to enhance when human blood mononuclear cells were exposed to visible light (Alvarez and Boveris 1993) and human colon adenocarcinoma cells treated with heat (Yao et al 1994).

As mentioned earlier, DT-diaphorase is an inducible enzyme (Huggins et al, 1964). DT-diphorase could be induced in experimental animals by compounds that are chemoprotective against carcinogenesis (Talalay and Benson 1982). There are several reports which support the antioxidant property of DT-diaphorase. It is induced by antioxidant compounds such as 2(3)-tert-butyl-4-hydroxyanisole (BHA) suggesting the mechanism by which antioxidants such as BHA protect against the mutagenic and carcinogenic effects of a variety of chemical compounds (Benson et al 1980). The treatment of lipid vesicles with DT-diaphorase
show protection against lipid peroxidation. This protective ability was suggested to be linked with the reduced state of CoQ (ubiquinone) (Landi et al 1997).

It has been recently reported that DT-diaphorase maintains membrane bound coenzyme Q (CoQ) in its reduced antioxidant state, thereby providing protection from free radical damage in artificial and natural membrane systems by acting as a two-electron CoQ reductase. It was suggested that DT-diaphorase has been selected during evolution to act as a CoQ reductase to protect cellular membrane components from free radical damage (Beyer et al 1996).

Enhanced activity of DT-diaphorase has been suggested to protect cells against the cellular toxicity and carcinogenicity of quinones (Talalay and Benson 1982). Lung tumors from non-smokers or past smokers showed considerably elevated DT-diaphorase activity compared to the corresponding normal tissue, but tumors from smokers showed no increase in tumor DT-diaphorase (Schlager and Powis 1990). DT-diaphorase did not show any significant age-dependent changes (Gus et al 1993).

Human tumour cell lines with low levels of DT-diaphorase activity showed an increased sensitivity to indoloquinone E09 under hypoxic conditions. The sensitivity decreased with increasing DT-diaphorase activity demonstrating an inverse correlation between the cellular activity of DT-diaphorase and hypoxic sensitivity to E09. The results suggested that DT-diaphorase protected cells from hypoxic cytotoxicity (Plumb and Workman 1994). It has been reported that during ischemia in skin DT-

17
diaphorase activity increases after exposure to oxidants. These reports provide evidence that DT-diaphorase enzyme activity is inducible after oxidant stress. It was also suggested that the elevated level of DT-diaphorase activity during ischemia may be a potential source of antioxidant activity in ischemic skin (Rees et al 1994).

DT-diaphorase reductively activates Mitomycin-C, an anticancer agent, which forms an adduct with DNA. Cells lines with non existent DT-diaphorase activity or marginal activity show resistance to MMC in comparison to ones with DT-diaphorase activity (Mikami et al 1996). Apoptosis induced by quinone drugs are also found to be mediated mainly through DT-diaphorase in some cancer cell lines (Sun and Ross 1996). There are reports that the genotoxicity and cytotoxicity of streptonigrin is high in cell lines having rich DT-diaphorase than DT-diaphorase deficent cells. Streptonigrin was proposed to produce higher incidences of DNA breaks mediated probably through ·OH radicals (Beall et al 1996).

1.5 XANTHINE OXIDASE

Xanthine Oxidase (XO) is widely distributed among species (from bacteria to man) and within various tissues of mammals. In all mammals the liver and intestine have highest xanthine oxidase activity. The mammalian enzyme exists mainly as a dehydrogenase which utilizes NAD* as the electron acceptor, but can be converted into an oxidase form both in-vivo and in-vitro, which utilizes molecular O₂ as the electron acceptor and releases substantial amounts of reactive oxygen metabolites
under various pathophysiological conditions and during tissue reoxygenation after hypoxia.

This enzyme participates in the oxidation of a wide variety of endogenous (e.g. purines) and exogenous (e.g. ethanol) substrates. Xanthine oxidase is mostly recognised for its role as the rate limiting enzyme in the nucleic acid degradation through which all purines are channeled for terminal oxidation. Evidence from various experiments show that it may serve as a source of oxidising species such as \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) radical. There is also growing evidence that \( \text{O}_2^- \) radicals generated by xanthine oxidase are primarily responsible for cellular damage associated with reoxygenation of hypoxic tissues.

Xanthine oxidase exists in-vivo predominantly as a NAD\(^+\) dependent dehydrogenase (XDH) (D type) which can be converted to an O2- dependent oxidase (XO) (O type) by a variety of conditions like (1) proteolysis (2) heating at 37°C (3) storage at -20°C (4) anaerobiasis (5) sulfydryl reagents (Stripe and Dela corte 1969) (6) incubation with subcellular fractions (7) and certain organic solvents.

The physiological importance of xanthine oxidase is exemplified by the disorders of gout and xanthinuria. Gout is characterized by high activity of xanthine oxidase leading to excess urate accumulation. Xanthinuria, a hereditary disorder in man is found to be due to the absence of xanthine oxidase and the rise in extracellular xanthine concentration. Xanthine oxidase also plays a role in dietary iron absorption in intestine and release of iron from liver cells (Topham et al 1982).
Xanthine oxidase is capable of oxidizing a variety of substrates like pyridines, other heterocyclic bases. It catalyses the oxidation of acetaldehyde to acetate. It also participates in oxidation of sulfhydryl groups as in glutathione, fatty acids and phospholipids, alcohols, tryptophan and epinephrine. It also catalyses the iodination of proteins by inorganic iodides (Fried et al 1973). It was also proposed to be a source of oxygen radicals in polymorphonuclear leucocytes (Parks and Granger 1986).

Xanthine oxidase has been implicated in cell damage and injury. It has been reported to play a role in cardiac injury (Downey 1990), intestinal mucosa injury (Granger et al 1986), cerebral injury (Kontos 1989; Lindsey et al 1991) and catalyses hemolysis in RBC'S (Tamai et al 1986). Xanthine oxidase activity increases with age in rats (Janssen et al 1993). Zimmerman and Cerutti (1984) reported that xanthine oxidase activity promotes transformation in mouse fibroblast cells. It has been reported to be responsible for initial events in diabetes development (Sakurai et al 1988; Burkart et al 1992).

Indoloquinone E09 undergoes activation by xanthine oxidase and causes DNA interstrand cross-linking. It was suggested that the anti-tumour activity of E09 may be mediated by interstrand DNA cross-link formation and reducing enzyme xanthine oxidase may be important for activation of E09 in vitro and in vivo (Maliepaard et al 1995).

The role played by xanthine oxidase in pathogenesis of ischemia repurfusion (IR)-injury was first proposed for the intestine (Granger et al
1981) which was later extended to include oxygen free radical (OFR) mediated IR-injury in other organs like the brain, kidney, liver, skeletal muscle, skin and stomach (Korthuis et al 1986). The metabolism during this period include the catabolism of ATP stepwisely to hypoxanthine which gets accumulated. Simultaneously there is conversion of D type to O type during prolonged period of ischemia. On reperfusion sudden burst of O$_2^-$ leads to higher activity of the O - form resulting in the conversion of hypoxanthine to xanthine and finally to uric acid. The byproduct of this reaction gives O$_2^-$ radical and H$_2$O$_2$ which ultimately leads to formation of OH. The importance of xanthine oxidase as a major source of oxyradicals in ischemia repurfusion injury in myocardium is most likely to species specific (Dorion et al 1993).

1.6 Cytochrome p450 system

Most tissues of mammals contain an electron transport complex, generally associated with the endoplasmic reticulum, which functions in the oxidative transformation of a wide variety of both endogenous and exogenous complex organic compounds. Concomitant with the oxidation of reduced pyridine nucleotide (Scheme 1) (Estabrook et al 1977), the molecular oxygen is activated for insertion into the substrate rendering it polar, i.e. more water soluble, permitting its subsequent conjugation for transport and excretion.

The types of substrates metabolized in this manner include a wide variety of organic compounds. In case of many compounds, such as large number of drugs, their enzymatic conversion is considered a detoxification reaction (Scheme 2) (Estabrook et al 1977). In contrast, with
\[ 2e^- + \text{Substrate} + O_2 \xrightarrow{\text{oxygenase}} \text{Product} + H_2O \]

(hydrophobic) (hydroxylated)

Conjugation

Excretion

Scheme 1: General representation of microsomal-catalyzed oxygenase or mixed-function oxidation reactions.

\[
\begin{align*}
\text{Active drug} & \xrightarrow{O_2, e^-} \text{Inactive Drug} \\
\text{Precarcinogen} & \xrightarrow{O_2, e^-} \text{Carcinogen}
\end{align*}
\]

Scheme 2: The duality of cytochrome-p450 catalyzed reactions.
many compounds, the product formed is found to be of significantly higher toxicity than the original substrate. Thus, the dualism of the enzyme system may be looked upon as both beneficial or detrimental to an organism.

The cyclic pattern (Figure 1) (Estabrook et al. 1977) of reduction and oxygenation of cytochrome P-450, as it interacts with substrate molecules, electron donors and oxygen can be described as:
1) The ferric hemoprotein ($\text{Fe}^{3+}$) interacts with a molecule of substrate ($R$) resulting in a complex ($\text{Fe}^{3+}.R$), substrate complex.
2) The substrate complex of ferric-cytochrome P-450 ($\text{Fe}^{3+}.R$) undergoes a one electron reduction to form a ferrous hemoprotein-substrate complex ($\text{Fe}^{2+}.R$) by electrons originating from NADPH and transferred by the flavoprotein NADPH-cytochrome P-450 reductase.
3) This reduced cytochrome P-450 ($\text{Fe}^{2+}.R$) reacts with oxygen to form a complex, termed as oxycytochrome P-450 ($\text{Fe}^{2+}.\text{O}_2.R$), a ternary complex of substrate, oxygen and ferrous cytochrome P-450.
4) The complex of ternary complex undergoes a second one electron reduction via cytochrome b5 forming a complex containing substrate, a peroxide anion with the ferric heme or a perferryl form of cytochrome P-450 with reduced oxygen (peroxide anion complex). Alternatively the oxychrome P-450 ($\text{Fe}^{2+}.\text{O}_2.R$) can dissociate to give a superoxide anion ($\text{O}_2.^-$), concomitant with the regeneration of the ferric hemoprotein. The $\text{O}_2.^-$ then results in hydrogen peroxide.
5) The oxygen is activated and the water is removed from the peroxide anion complex. This complex undergoes protonation and dissociates as hydrogen peroxide or it may rearrange to form an oxene derivative.
Figure 1: Schematic of the proposed cyclic function of cytochrome p-450. The substrate molecule is designated R. The valence state of heme iron of cytochrome p-450 is indicated.
and undergoes electron rearrangement resulting in the introduction of the oxene into the substrate molecule.

6) The resulting oxidized substrate (product) then dissociates from the ferric hemoprotein regenerating the ferric cytochrome P-450.

In such kind of biotransformations, which take place through mixed function oxidation reactions, a class of hemoproteins termed cytochrome P-450 play a very important role. In mammalian tissues, cytochrome P-450 is a membrane bound pigment functioning as the terminal electron transport oxidase. The microsomal enzymes involved in the metabolism of exogenous compounds in the cytochrome P-450 cycle are NADPH-cytochrome P-450 reductase and NADH-cytochrome b5 reductase.

**NADH-Cytochrome b5 Reductase:** NADH-cytochrome b5 reductase (EC 1.6.2.2) is a flavin containing dehydrogenase. NADH-cytochrome b5 reductase catalyzes the reduction of cytochrome b5, using FAD as a prosthetic group. Two forms of this enzyme are known, one is a membrane-bound form in the somatic cells and the other is the soluble form, which is localized in a soluble fraction of erythrocytes.

NADH-cytochrome b5 reductase is present in many tissues such as erythrocytes, brain, (Tamura *et al* 1987); as an integral membrane component, on endoplasmic reticulum and outer mitochondrial membranes of liver (Borgese and Pietrini 1986). Studies show an uneven distribution of the enzyme among different cellular membranes.

The components of the NADH-dependent system and those of the NADPH-dependent system are known to be closely associated (Cohen
It has been proposed that one of the electrons required by cytochrome P-450 for hydroxylation or oxidative N-demethylation is supplied by NADH via NADH-cytochrome b5 reductase and cytochrome b5 (Strittmatter 1965; Cohen and Estabrook 1971; Hildebrandt and Estabrook 1971).

The possible interactions are:

NADPH → NADPH-cytochrome P-450 reductase → cytochrome P-450 → O₂

NADH → NADH-cytochrome b5 reductase → cytochrome b5 → CN-sensitive factor → O₂

NADPH-CYTOCHROME p450 reductase: NADPH-cytochrome p450 reductase occurs in almost all eukaryotic cells, is a flavoprotein component of the endoplasmic reticulum of the liver and other organs (Masters et al 1967). It is localized in human liver, lung, kidney, adrenals, gastrointestinal tract, pancreas and gall bladder (Hall et al 1989). It catalyzes the transfer of electrons from NADPH to cytochrome p450. Cytochrome p450 is the terminal oxidase of the drug metabolism system which hydroxylates a variety of compounds, such as alkanes, fatty acids, drugs, and steroids.

The enzyme is specific for NADPH, but is relatively non-specific for electron acceptor. The enzyme transfers electrons to its native
acceptor, cytochrome p450, as well as a number of artificial electron acceptors such as cytochrome-c, dichlorophenolindophenol (DCPIP), ferricyanide etc (Strobel and Dignam 1977).

NADPH-cytochrome p450 reductase catalyzes the single-electron reduction of several quinone antibiotics to a semiquinone (Iyanagi and Yamazaki 1969; Bachur et al 1979). Quinone antibiotics of the benzanthraquinone, N-heterocyclic quinone and napthoquinone families also function as single-electron receptors for NADPH-cytochrome p450 reductase electron transfer to yield free radical intermediates. The free radical forms of the antibiotics transfer their single electrons to molecular oxygen (Bachur et al 1979), to generate \( \text{O}_2^- \) and in turn free radicals. These free radicals interact with various components of the cell and bring about change in their structure and function.

There is not much work reported on NADPH-cytochrome p450 reductase in relation to radiation effect. However, recently it was reported that when pregnant rats when irradiated (whole-body) with 2.6 Gy gamma-rays, the NADPH-cytochrome p450 reductase activity reduces to 36% of the control value in the four month old pups (Inano et al 1990).

It is quite clear that all these bioreductive enzymes play a critical role to determine the biological effectiveness of various chemical compounds. The same may be applicable to the radiomodifiers. However, these enzymes were not used as biochemical indicators in radiomodification studies or their involvement in radiomodification. Surprisingly, very little, if at all, has been carried out on the effect of ionising radiation on the bioreductive enzymes. Therefore, we have also
examined the radiation induced modulation of specific activities of the enzymes involved in bioreduction of compounds.