Materials & Methods
2.1 Animals

Male Swiss albino mice 6-8 weeks old were used. They were random-bred and kept in the air-conditioned (at 25±3°C) Central Animal House of Jawaharlal Nehru University, New Delhi. Food and water were provided *ad libitum*. Food pellets were obtained from Hindustan Lever Ltd., New Delhi. The studies were conducted according to the ethical guidelines of the Indian National Science Academy (INSA) on the use of animals for scientific research.

2.2 Chemicals

Tris-HCl, Reduced Nicotinamide Adenine dinucleotide (NADH), Reduced Nicotinamide Adenine dinucleotide phosphate (NADPH), Dichlorophenolindophenol (DCPIP), Potassium ferricyanide, Triton X-100, Xanthine, Cytochrome-C, Thiobarbituric acid (TBA), Caffeine, Mitomycin-C (obtained from Sigma Chemical Co., USA). Brilliant Blue G-250 (Coomassie Blue) from E.Merck Co., Germany. All other chemicals were of analytical grade and of highest purity from BDH, Mumbai and SRL, Mumbai.
2.3 Irradiation of animals

Animals were irradiated with gamma-rays in air, at room temperature, in the gamma-chamber (TBq $^{60}$Co Model 4000A) obtained from Bhabha Atomic Research Centre, Mumbai. The animals were sacrificed after different time intervals of irradiation. Liver samples collected and the enzyme assays carried out as described.

2.4 Treatment of animals

Mice were injected with caffeine, mitomycin-c and xanthine intraperitoneally 1 hour before irradiation. Caffeine and mitomycin-c were dissolved in water, xanthine was dissolved in 0.01 N NaOH.

2.5 Isolation of enzymes

Mice of 6-8 weeks old were starved for 20 hours before sacrifice. Mice were sacrificed by cervical dislocation. The liver was perfused in situ with ice cold normal saline (0.9%). The liver was dissected out, weighed and homogenized in 0.25M sucrose (10% homogenate was prepared) using Potter Elvehjem homogeniser. The homogenate was centrifuged first at 25000g (10000 rpm) for 10 minutes at 4°C in RC5C Sorvall
centrifuge, to remove the debris. The pellet was discarded and the supernatant was taken and ultracentrifuged at 105,000g (38,700 rpm) for 60 minutes in Beckman’s ultracentrifuge (Beckman-Type 50 Ti rotor), at 4°C.

The cytosol (supernatant) and the microsomes (pellet) were separated. The cytosolic fraction was used for DT-diaphorase and xanthine oxidase enzyme assay. For preparation of sample for xanthine oxidase enzyme DTT (dithiothreitol) was added to the homogenising mixture before homogenisation. The microsomal pellet was rinsed thoroughly with Tris-KCl buffer (pH 7.5) and suspended in Tris-KCl buffer (0.15 M KCl and 10mM Tris HCl buffer, pH 7.5) and used for NADH-cytochrome b5 reductase and NADPH-cytochrome p450 reductase enzyme assays. The microsomal pellet was also used for lipid peroxidation determination.

2.6 Protein Estimation

Protein determination was done by Bradford’s method (1976) using BSA as standard. Coomassie Brilliant Blue G-250 (Dye) binds to the protein causing a shift in the absorption maximum of the dye from 465 to 595 nm. The increase in absorption at 595 nm was monitored.
2.7 Assay for DT-diaphorase

Enzyme assay for DT-Diaphorase was carried out as described by Lind *et al.* (1990). DT-Diaphorase activity was measured with NADH as the electron donor and DCPIP as the electron acceptor.

The standard assay system contains 50 mM Tris-HCl (pH 7.5), 0.08% Triton X-100, 0.5 mM NADH, and 40 μM DCPIP. The reaction was started by the addition of the cytosolic fraction containing the enzyme at room temperature. The reduction of DCPIP was followed at 600 nm spectrophotometrically. The reaction was monitored for 3 minutes in Hitachi UV-2000 spectrophotometer. The specific activity was calculated using the extinction coefficient $\varepsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ which is expressed as units of enzyme activity per mg of protein. One unit of activity is the amount of enzyme that catalyzes the reduction of 1 μmole of DCPIP per minute.

2.8 Assay for xanthine oxidase

The enzyme assay was done as described by Kaminski and Jezewska (1979).

The standard assay mixture contained 50 mM Tris-HCl (pH 8.0),
50 µM Xanthine, in a final volume of 3 ml. Oxygen was passed for 1 minute. The reaction was started at room temperature by the addition of the cell fraction containing the enzyme and followed at 292 nm for 3 minutes spectrophotometrically, in Hitachi UV-2000 spectrophotometer. The extinction coefficient $\varepsilon = 11$ mM$^{-1}$ cm$^{-1}$ was used to calculate the specific activity, expressed in Units/mg protein. One unit of activity is defined as 1 µmole of uric acid formed per minute.

2.9 Assay for NADH-cytochrome b5 reductase

The enzyme assay was carried out as described by Omura and Takesue (1970). NADH is the electron donor and potassium ferricyanide is the electron acceptor in this enzyme assay.

The reaction mixture contains 0.1 M Potassium phosphate, pH 7.5, 0.3 mM NADH, 1 mM Potassium ferricyanide in final volume of 2 ml. The reaction was started by the addition of the microsomal fraction with the enzyme. The reduction of Potassium ferricyanide was followed at 420 nm in Hitachi UV-2000 spectrophotometer for 3 minutes. The specific activity was calculated using extinction coefficient $\varepsilon = 1.02$ mM$^{-1}$ cm$^{-1}$ and expressed in Units/mg protein, where one unit of activity is defined as that causing the reduction of 1 µmol of ferricyanide per minute.
2.10 Assay for NADPH- cytochrome p450 reductase

The enzyme assay was done as described by Gibson and Skett (1994). The p450R activity was measured using NADPH as electron donor and cytochrome-c as the electron acceptor.

The reaction mixture contains 0.1 M Potassium phosphate, 40 μM Cytochrome-c, 2% NADPH. The reaction was started and followed after the addition of the microsomal fraction with the enzyme at 550 nm for 5 minutes spectrophotometrically (Hitachi UV-2000 spectrophotometer. The specific activity is calculated using the extinction coefficient 19.5 mM⁻¹cm⁻¹. One unit of activity is defined as 1 μmol of cytochrome reduced per minute per mg protein.

2.11 Estimation of lipid peroxidation

Lipid peroxidation in the microsomes was estimated by the method as described by Varshney and Kale (1990) spectrophotometrically and expressed in terms of malondialdehyde (MDA) formed per mg of protein.

To determine the concentration of MDA in the suspension, 0.4ml of
microsomes were mixed in a centrifuge tube with 1.6 ml of suspension medium (0.15 m KCl + 10 mM Tris HCl) to which 0.5 ml of 30% TCA was added. To it 0.5 ml of 52 mM TBA was added. The tubes were covered with aluminium foil and placed in a water bath for 30 minutes at 80°C. The tubes were then cooled in ice for 5 minutes and centrifuged at room temperature for 5 minutes at 3,000 rpm in a REMI-T8 table top centrifuge. The absorbance of supernatant was measured at 531.8 nm against reference blank of distilled water in UV-2000 spectrophotometer. The amount of MDA formed in a sample was estimated according to the equation:

\[ \text{nmoles of MDA} = \frac{V \times \text{OD}}{0.152} \]

where, \( V \) = final volume of test solution
\( \text{OD} \) = optical density.

### 2.12 Determination of Dose-rate

The dose rate was determined by using Fricke’s dosimetry as described by Schested (1970). The solution consisted of 0.01M ferrous ammonium sulphate (Fe(NH₄)₂SO₄), 0.8N H₂SO₄ and 0.01 M NaCl and was prepared as follows:

4.4 ml of sulphuric acid was added to 200 ml distilled water first
and allowed to cool at room temperature. 80 mg of ferrous ammonium sulphate and 12 mg of NaCl were then added and the solution was shaken vigorously with a glass rod to dissolve the salts completely and quickly.

5 ml of the dosimeter solution was irradiated for small time periods (1,2,3,4, and 5 minutes). The optical density if the Ferric ions (Fe$^{3+}$) formed, was measured spectrophotometrically at 305nm. At this wavelength the molar extinction coefficient of the ferrous ion (Fe$^{2+}$) is particularly zero and that of the Fe$^{3+}$, 2197 M$^{-1}$ cm$^{-1}$ at 25° C. Another Fe$^{3+}$ peak is located at 244 nm where the molar extinction coefficient is about twice i.e., 4565 M$^{-1}$ cm$^{-1}$. However, at this wavelength the absorption of the ferrous ions cannot be neglected (about 20 M$^{-1}$ cm$^{-1}$). The optical density (OD) of the sample after irradiation is compared with that of the blank (unirradiated solution). A graph of optical density (OD irradiated - OD unirradiated) vs time is plotted and the slope (OD / min) measured.

According to the definition of the G value

$$G = \frac{\text{molecules/ml} \times 100}{D(\text{eV})}$$

where D(eV) is the dose in eV/ml. Therefore,

$$D(\text{eV}) = \frac{\text{molecules/ml} \times 100}{(G)}$$
In case of spectrophotometry, we have

\[
molecules / ml = \frac{\Delta OD \times N \times 10^{-3}}{d \times e \times l}
\]

The conversion of D(eV) to D in rad is

\[
D(eV) = 6.245 \times 10^{13} D \text{ in rad}
\]

and the dose equation can be now written as

\[
D \text{ rad} = \frac{OD \times N \times 10^{-3} \times 100}{G \times d \times e \times l \times 6.245 \times 10^{13}}
\]

where,

\[
\Delta OD = \text{difference in optical density between the irradiated sample and the blank.}
\]

\[
N = \text{Avagadro's number} \ (6.02 \times 10^{23} \text{ molecules / mole})
\]

\[
G = \text{no. of ferrous ions oxidised per 100 ev of absorbed energy.} \ (G \ Fe^{3+} = 15.6)
\]

\[
d = \text{specific density of the dosimeter solution}
\]

\[
(=1.024 \text{ mg/cm}^3 \text{ for } 0.8 \text{N } \text{H}_2\text{SO}_4)
\]

\[
e = \text{molar extinction coefficient} \ (=2197 \text{ M}^{-1} \text{ cm}^{-1} \text{ at } 25^\circ \text{C})
\]

\[
l = \text{optical path length} \ (1\text{cm})
\]

After substituting the values

\[
\text{Dose rate} = 2.75 \times 10^4 \times \Delta OD \text{ in rad}
\]

where,
OD is slope i.e, $OD \text{ / min}$,

Rad being the old unit of absorbed radiation it was converted to gray by the following relation:

$$100 \text{ rad} = 1 \text{ Gy (Gray)}$$