MATERIALS AND METHODS

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3.1 Materials

Animal Materials

Swiss albino mice (5-6 weeks old)	Provided by animal house, Jawaharlal Nehru University New Delhi 110067.
Plasmid	pUC19 construct contains human Gly I cDNA Gifted by Prof. S.K. Sopory International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Road, New Delhi, 110067.

Chemicals

- Acrylamide: Accurate Biochem and Gibco BRL
- Agarose: SeaKem LE (FMC corporation)
- Agar: Bacteriological grade (Qualigens), Phyta-agar (Gibco BRL)
- APS: Sigma
- Amberlite Serva
- Antibiotics: Ampicilin (Sigma), Tetracyclin (Sigma), Chloramphenicol (Sigma), Kanamycin (Sigma)
- Bacterial culture media: Luria Broth (Hi-Media) and Nutrient Broth (Difco)
- Bis-acrylamide: Sigma and Gibco BRL
- Bromophenol blue: Sigma
- BSA: Sigma
- DEPC: USB, Jersey lab and Sigma
- DNase I: RQ1 RNase free DNase I (Promega)
- EDTA: Sigma
- Ethanol: Bengal Chemicals
- Ethidium bromide: Sigma
- Formamide: USB
- Guanidium thiocynide: USB

- IPTG: Biosynth AG
- Lysozyme: Sigma
- β-Mercaptoethanol: Sigma
- MOPS: Gibco BRL
- Nylon and Nitrocellulose membranes: Hybond-N and Hybond-N^+ (Amersham)
- dNTP: Perkin Elme
- PEG: PEG 8000 (Sigma)
- Phenol: Qualigens (Redistilled in the lab)
- PIPES: Sigma
- Proteinase K: Sigma and Gibco BRL
- PVP: Sigma
- Radioactive chemicals: α-^{32}P dATP, α-^{32}P dCTP, γ-32P dATP and ^{35}S-dATP from BARC, India
- Labeling Kit: Promega
- Restriction enzymes and DNA-modifying enzymes: New England Biolabs, Promega, Amersham Life Sciences, Gibco BRL.

- RNaseA: Sigma
- RNasin: Recombinant RNasin (Promega)
- Sarcosyl: Sigma
- SDS: Sigma
- Sephadex G-50: Sigma
- Sephadex G-25: Sigma
- Taq DNA polymerase: Taq polymerase (Perkin Elmer)
- Tris: USB, Gibco BRL
- Urea: Gibco BRL
- X-gal: Biosynth AG
- X-ray films: Amersham (MP)
- Xylene cyanol: Sigma

- Potassium ferricyanide Sigma
- Pyragallol Sigma
- Reduced glutathione(GSH) Sigma
- 1-Chloro-2-4-dinitrobenzene (CDNB) Sigma
- 2-Dichlorophenolindophenol (DCPIP) Sigma
- Xanthine Sigma
- Dithiothreitol Sigma
- Thiobarbaturic acid (TBA) Sigma
- Bovine serum albumin (BSA) Sigma
- Ethylenediaminetetra acetic acid (EDTA)Sigma
- Tris-HCl Sigma

Various other chemicals were procured from indigenous sources like Merck, Qualigens, BDH, CDH, SRL, SD-Fine Chemicals, Spectrochem and HiMedia and were of highest purity available.

3.2 Methods

3.2.1 Preparation of Plasmid DNA

Mini Preparation

The protocol of mini preparation of plasmid DNA (pUC19 construct) was adapted from Sambrook et al. (1989). A single bacterial colony was grown in 2 ml of LB medium containing the appropriate antibiotics in a loosely capped 15 ml tube. The tube was incubated at 37°C with vigorous shaking. One and half ml of culture was transferred in a microfuge tube The cells were pelleted by centrifugation at 10,000 rpm for 30 sec at 4°C. Supernatant was removed and 100 µl of solution I (50 mM glucose, 10 mM EDTA and 25 mM Tris.Cl, pH 8.0) was added. The cells were lysed by the addition of 200 µl of freshly prepared solution II (0.2 N NaOH and 1% SDS). The vial was closed and mixed the content of the tube by inverting tube a few times. This was stored on ice for 5 min. One hundred and fifty µl of solution III (3M potassium acetate, pH 4.8) was added and contents mixed by vortexing. The tube was incubated on ice for 5 min and centrifuged at

13,000 rpm for 2 min at 4°C. The supernatant was transferred to a fresh tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed thoroughly. The phenol and aqueous phases were separated by centrifugation at 13,000 rpm for 2 min at 4°C in a microfuge. Aqueous phase was transferred to a fresh tube and the extraction was repeated once. DNA was precipitated by addition of 2 vol of ice-cold ethanol and kept at -20°C for 30 min. The precipitate was collected by centrifugation at 13,000 rpm for 5 min at 4°C. The supernatant was removed, pellet was washed with 70% ethanol and dried briefly under vacuum. Pellet was dissolved in 50 µl of TE (10 mM Tris.Cl, pH 8.0 and 1 mM EDTA) containing DNase free RNase A. The tube was incubated at 37°C for 1 h and stored the DNA at 4°C. Four µl of this was used for restriction enzyme digestion.

3.2.3 Preparation of *E. coli* Competent Cells and Transformation

The competent cells of DH5α Ecoli Strain was prepared by the $CaCl_2$ treatment according to Sambrook et al. (1989) with minor modifications. Bacterial cells were grown at 37°C, overnight. Flask containing 100 ml of LB medium was inoculated with one ml of overnight culture and grown with vigorous shaking at 37°C till OD_{600} was 0.3-0.4. The culture was chilled on ice for 15-20 min, transferred to a 50 ml Oak-ridge tube and centrifuged at 5000 rpm for 5 min at 4°C. The pellet was gently suspended in half of the original volume of ice-cold 100 mM $CaCl_2$ by gently swirling the tube and was incubated on ice for 30 min. The cells were collected by centrifugation at 5000 rpm for 5 min at 4°C and were resuspended in 1/10 the original volume of ice-cold 100 mM $CaCl_2$ by gently swirling the tube, and stored on ice till use. Competent cells (0.2 ml) were aliquoted into a 1.5 ml Eppendrof tubes for transformation. Appropriate volume of DNA (pUC19 construct) was mixed with the cells and incubated on ice for 10 min. Then, the cells were subjected to heat-shock for 90 sec at 42°C. The tubes were again chilled for 10 min and 0.8 ml of LB medium was added. Tubes were incubated for 45 min at 37°C with gentle shaking in order to express the antibiotic marker. 0.1 ml of cells was plated on LB agar plates containing appropriate antibiotics. After 16 h the colonies appeared on the plates which were analyzed by small-scale DNA preparation.

3.2.4 Purification of DNA from Agarose Gel by Phenol-Freeze-Fracture Method

pUC19 construct plasmidwas digested with EcoRI and DNA fragments of 550bp (human Gly I cDNA) were mainly purified from agarose gel essentially using phenol-freeze-fracture method (Huff, 1991). The ethidium bromide stained agarose gel was visualized under long wavelength UV light on a transilluminator. The desired band was cut and placed into a Eppendorf tube. An equal volume of phenol was added to the gel slice. The tube was vortexed and placed at -80°C until frozen. Further, it was thawed at 37°C and this process was repeated once. It was again frozen and centrifuged at 4°C in the frozen condition for 20min. Supernatant was collected and the aqueous phase was extracted once with chloroform. DNA was precipitated by adding 1/10 vol. of 3 M sodium acetate (pH 5.2) and 2 vol. of ethanol for 30 min at -80°C. The tubes were centrifuged at 13,000 rpm at 4°C for 15 min and the resulting pellet was washed with 70% ethanol and resuspended in 20 µl of TE.

3.2.5 Isolation of RNA

The RNA was isolated from liver of *Swiss albino* mice essentially using acid-phenol guanidium thiocyanide method (Chomczynski and Sacchi, 1987). All types of precautions were taken to avoid RNase contamination. Glassware was baked at 180°C for at least six hours. All solutions were treated with 0.1% (v/v) diethylpyrocarbonate (DEPC) at 37°C overnight and the autoclaved to remove traces of DEPC. Disposable gloves were also worn at all the times during the isolation and analysis of RNA.

One gram of liver tissues frozen in liquid nitrogen was ground to fine powder with mortar and pestle. A mortar and pestle was used to make fine powder of the tissue. It was thawed in 10 ml of solution D (4 M guanidium thiocynate, 25 mM tri-sodium citrate, 0.5% Sarkosyl and 0.1 M β-mercaptoethanol). The homogenate was transferred to an Oak-ridge centrifuge tube. Successively, 1 ml of 2 M sodium acetate, pH 4.0, 10 ml of DEPC-treated water saturated phenol and 2 ml of chloroform:isoamyl alcohol (49:1) were added to it. After each addition, the tubes were shaken for 10 sec and then incubated on ice for 10 min. Following incubation each tube centrifuged at 10,000 rpm for 10 min at 4°C and

the aqueous phase transferred to a fresh tube. The RNA was precipitated by addition of equal volume of isopropanol and stored at -20°C for at least 2 h. Tubes were centrifuged at 10,000 rpm for 20 min at 4°C and the pellet was dissolved in 1 ml of solution D. It was again precipitated by adding 1 ml of isopropanol and stored at -20°C for 1-2 h. The RNA was recovered by centrifugation at 10,000 rpm for 20 min at 4°C. The pellet was suspended in 300 µl of 3 M sodium acetate, pH 5.5 and incubated on ice for 10 min. This salt wash was repeated once more and then pellet was washed with 75% ethanol. Pellet was briefly dried and then suspended in 100 µl of DEPC treated water. Ten µl of 3M sodium acetate pH 5.5 was added to get final concentration of 0.3M and RNA was precipitated with 200 µl of cold ethanol by incubating on ice for 20 min.. RNA was recovered by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was washed with 75% ethanol and dried briefly. Finally, the pellet was dissolved in 50 µl of DEPC-treated water. RNA samples were diluted 200 times in DEPC-treated water and the absorbance at 260 nm, 280 nm and 230 nm were measured. The quality of RNA was determined by OD_{260}/OD_{280} ratio (samples having ratio > 1.7 were taken for experiment). Final quality was determined on formaldehyde agarose gel electrophoresis.

3.2.6 Formaldehyde gel electrophoresis

Fifty ml of 1.5% agarose formaldehyde gel was prepared by adding 0.75 g of agarose in 31.9 ml of DEPC-treated water. This was boiled and cooled to 60°C. Further, 10 ml of 5x MOPS (0.1 M MOPS, pH 7.0, 40 mM sodium acetate, 5 mM EDTA) buffer was added to it, followed by 8.1 ml of 37% formaldehyde solution. The gel was run in DEPC-treated electrophoresis apparatus using 1x MOPS running buffer. RNA samples were prepared by mixing 4.5 µl of RNA (10-20 µg) with 2 µl of 5x MOPS buffer, 3.5 µl of formaldehyde and 10 µl of formamide. The samples were incubated at 65°C for 15 min and then chilled on ice. Further, 2 µl of DEPC-treated formaldehyde gel loading buffer (50% glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol FF) was added. The samples were loaded and electrophoresed at 2-3 V/cm. The gel was stained in DEPC-treated water containing 0.5 µg/ml of ethidium bromide and 0.1 M

ammonium acetate. Subsequently, the gel was destained with several changes of DEPC-treated water and visualized under UV.

3.2.7 Preparation of Radiolabelled DNA Probe

3.2.7.1 Random-Primer Method of Labeling

The random-primer kit (USB, NEB or Promega) of DNA labeling was used according to manufacturer's instructions. Twenty five ng/µl of DNA (purified human Gly I cDNA as mentioned in Methods 3) was denatured by heating in a boiling water bath for 10 min and quickly chilled on ice water. In another microcentrifuge tube 5 µl of 10x labeling mix (containing random primer), 6 µl of dNTP mix (2 µl each of dATP, dTTP, dGTP) and 3 µl (30 µCi) α-^{32}P-dCTP was added. The volume was made up to 47 µl with sterile water. Two µl of denatured DNA and 1 µl of Klenow fragment (5 units) was added and mixed thoroughly. The tube was incubated at 37°C for 1 h and the reaction was terminated by adding 0.2 M EDTA (pH 8.0).

3.2.7.2 Measurement of Specific Activity of the Probe

The incorporation and specific activity was determined by using tri-chloro acetic acid (TCA) precipitation method. One µl of total reaction mixture was diluted to 10 times with sterile water and 1 µl of this diluted sample was mixed with 35 µl of calf thymus DNA (10 µg/ml). This was added to 5 ml of ice cold TCA in a pre-siliconized test-tube. It was incubated on ice for 15 min. After 15 min incubation, the sample was filtered through Whatman GF/C filter. The TCA precipitate was washed with ice-cold ethanol and dried under heating lamp. In another piece of filter paper, 1 µl of diluted sample was straightaway spotted and dried under heating lamp. This represents the total count in 1 µl of the diluted sample while TCA precipitate represents the final incorporated radioactivity. The dried filters were transferred to a scintillation vial containing cocktail 'O' scintillation fluid. The amount of radioactivity was measured in liquid scintillation counter. The specific activity was calculated in the following way:

1. In the case of 100% incorporation of 3 µl of label:

$$\frac{\mu Ci\ dNTP\ added \times 3 \times 330\ ng/nmol}{Sp.\ activity\ of\ dNTP\ (\mu Ci/nmol)} = ng\ theoritical\ yield$$

2. $$\frac{cpm\ in\ washed\ filter}{cpm\ in\ unwashed\ filter} \times 100 = \%\ incorporation$$

3. % incorporation x 0.01 x theoretical yield = ng DNA synthesized

4. $$\frac{total\ cpm\ incorporated\ (cpm\ incorporated \times 10 \times 50)}{(ng\ DNA\ synthesis + ng\ input\ DNA) \times 0.001 \mu g/ng}$$

= Specific activity (cpm/μg)

Here the factor 10 and 50 derived from using 1 μl of 1:10 dilution for TCA precipitation and having to convert this back to a 50 μl total reaction volume, respectively.

3.2.7.3 Purification of the Labelled Probe

The labelled probe was purified by using Sephadex G-50 spun column. Sephadex G-50 was swollen in 50 mM Tris.Cl, pH 8.0, 1 mM EDTA and 0.1% SDS. The swelling was performed by standing this mixture overnight at room temperature. The Sephadex was packed in a 1 ml disposable syringe which has been plugged with siliconizing glass wool. Sephadex was added with the help of a plastic transfer pipette until the bed reaches top of the syringe. This syringe column was hanged in a 15 ml centrifuge tube. The whole assembly was centrifuged in a clinical centrifuge at 3,000 rpm for 3 min. The labeled DNA was loaded on the top and again the centrifuge was run at 3,000 rpm for 3 min in the tube containing 1.5 ml microcentrifuge tube. The labeled DNA was collected in the microcentrifuge tube placed below the syringe, leaving all impurities and unincorporated label in the bed. The purified DNA was directly used for hybridization after denaturation.

3.2.8 Northern Blotting and Hybridization

The RNA isolated from liver of *Swiss albino* mice was electrophoresed on 1.4% agarose formaldehyde gel. It was further treated with DEPC-treated water to remove traces of formaldehyde. The gel was then soaked in 20x SSC solution for 45 min with gentle

agitation. The RNA was transferred to the membrane exactly described by Sambrook et al. A solid support larger than the size of the gel was placed in a glass-baking tray and transfer buffer (20x SSC) was added around it to one half of its height. 3MM paper was folded over the solid support and allowed to hang on either side of the support so that it would form a proper wick allowing efficient movement of transfer buffer. The gel was then placed upside down on it. A piece of nylon filter cut to the size of the gel was floated on a solution of 2x SSC until it becomes completely wet. The wet nylon filter was then placed carefully on the top of the gel, such that one end extended just over the lines of slots at the top of the gel and avoiding trapping of air bubbles between the gel and the filter. All four edges of the nylon filter were covered by strips of Saran wrap in order to prevent any short circuit of the fluid between the gel and stack of filter paper. Three pieces of 3MM-paper cut to the size of the gel were placed over the membrane filterafter wetting them in 2x SSC. A stalk of blotting papers of 1-2 inch height was placed over 3MM papers and on the top a glass plate was kept. A weight of ~500 g was kept on the glass plate. The transfer of the RNA was allowed for about 16 h. At the end of transfer, the paper towels and 3MM paper were removed and the position of gel slots on nylon membrane was marked with a pencil. The membrane filter was peeled off from the gel using a forceps, and transferred to a 3MM paper soaked in 6x SSC. The DNA was crosslinked to the filter paper by UV irradiation in UV Stratalinker^{TM} with 1.2 x 10^5 μJoules of energy. During irradiation, the nucleic acid side was kept up. The filters were dried at the room temperature on a sheet of 3MM paper.The crosslinked filters was floated on 6x SSC till it became wet and then soaked in the same solution for 2 min. It was transferred to a hybridization tube containing the prehybridizing solution [3 ml 1M phosphate buffer (pH 7.2), 7 ml 10% SDS, 0.02 ml EDTA] was used and the prehybridization was carried out at 60°C for 6h. Hybridization was carried out by addition of ^{32}P labeled denatured DNA (human Gly I cDNA) probe (specific activity: 10^8-10^9 cpm/μg of DNA) 16-20 h at 42°C in the hybridization incubator (Robbin Scientific). The filter was washed three times with 2x SSC and 10% SDS at room temperature for 5 min for three times. Further, it was washed with 0.4x SSC and 0.1% SDS at 42°C for 30 min and this process was repeated once. The stringency of washing was increased, if needed. After washing, the filter was wrapped in Saran wrap and

exposed to X-ray film with an intensifying screen for autoradiography and kept at -80°C. Northern blots were analyzed by densitometry. Image Guage V2.54 (Fujifilm) software was used for densitometry analysis.

3.2.9 Staining RNA after transfer to Nitrocellulose filters

After cross-linking of the transferred RNA to the nitrocellulose membrane the, the filter was stained with methylene blue. For this purpose, the filter was soaked in 5% acetic acid for 15 minutes at room temperature. Then the filter was transferred to a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue for 5 minutes at room temperature. The filter was rinsed in water for 5-10 minutes.

3.2.10 Determination of dose rate of γ- rays

Dose rate was determined by using Fricke's chemical dosimetry as described by Schested (1970). To prepare a dosimetry solution (0.01 M Fe[NH4]2 SO4, 0.8 N H2SO4 and 0.01 MnaCl), sulphuric acid (4.4 ml) was added to water (200 ml) first and allowed to cool at room temperature. Ferrous ammonium sulphate (80mg) and sodium chloride (12mg) were then added. The solution was shaken vigorously with a glass rod to dissolve the salts completely as quickly as possible.

The dosimetry solution (5ml) was irradiated for small time period (1, 2, 3, 4, and 5 min.). The optical density of Fe^{3+} formed was measured spectrophotometricallly at 305nm. The molar extinction coefficient of Fe^{2+} formed was measured spectrophotometrically at 305nm. The molar extinction coefficient of Fe^{2+} is 2197 M-1cm-1 at 305nm at 25 C. Another Fe^{2+} is zero and that of Fe^{3+} peak is located at 244nm where the molar coefficient is 4565 M-1cm-1. However, at this wavelenght the absorption of the Fe^{2+} cannot be neglected (about20 M-1 cm-1). The optical density (OD) of the sample after irradiation was compared with that of the blank (unirradiatied solution). The optical density (OD) of the sample after irradiation was compared with that of the blank (unirradiated solution). A graph of ? OD (OD irradiated - OD unirradiated) vs. time was plotted and the slope (? OD/min) measured.

According to the definition of G value

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

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

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

In case of spectrophotometry, we have

$$\text{molecules/ml} = \frac{? \text{ OD} \times N \times 10^{-3}}{d \quad e \quad l}$$

The conversion of D (eV) $= 6.245 \times 10^{13}$ D in rad

and the dose equation can now be written as

$$D \text{ (rad)} = \frac{? \text{ OD} \times N \times 10^{-3} \times 100}{G \; d \; e \; l \times 6.245 \times 10^{13}}$$

where,

? OD = difference in optical density between the irradiated sample and the blank

N = Avagador's number (=6.023×10^{23} molecules/mole)

G = no. of ferrous ions (Fe^{2+}) ions oxidized / 100 eV of absorbed energy (G Fe^{2+} = 15.6)

d = specific density of the dosimetry solution

(= 1.024 mg/cm^3 for 0.8 N H_2SO_4)

e = molar extiction coefficient

(= 2197 $M^{-1}cm^{-1}$ at 25° C)

l = optical path length of the spectrophotometric cell

(= 1.0 cm)

After substituting these values,

Dose rate = 2.75×10^4 x ? OD in rad

where

? OD is a slope i.e. OD/min

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

100 rad = 1 Gy (gray)

3.2.11 Irradiation of Swiss albino mice with γ-rays and Scarifice

Swiss albino male mice (5-6 week old) were used in the present study. Animals were maintained in the air-conditioning animal house of Jawaharlal Nehru University. Standard feed (Hindustan Lever Ltd.) and waters were being provided *ad libitum.* The studies were conducted according to ethical guidelines of the Indian National Academy (INSA) on the use of animals for scientific research.

Whole body irradiation of animals with different doses of gamma- rays were carried out in air at room temperature in a gamma chamber (240TBq ^{60}Co Model 4000 A) obtained from Isotope Division, Bhabha Atomic Centre (BARC), Mumbai, India.

Animals were sacrificed by cervical dislocation. Blood was drawn from heart of mice with the help of a syringe in vials containing heparin as anticoagulants. Liver was perfused *in situ* with 0.9% saline and quickly excised and washed in chilled homogenising solution.

3.2.12 Preparation of tissue homogenates, cytosolic and microsomal fractions

A 10% homogenate (w/v) of liver was prepared using Potter-Elvehjem homogeniser in homogenising solution (250mM sucrose containing 1mM EDTA, pH 7.5). This homogenate was centrifuged at 14000g for 20min using RC5C Sorvall Centrifuge. The pellet was discarded and supernatant was recentrifuged at 105,000g for 60min (Beckmann Type 50 Ti rotor). The resulting supwernatant used for assay of cytosolic enzymes such as Glyoxalase I, superoxide dismutase, glutathiones-S- transferase and catalase. The pellet (microsomal fraction) was washed with 0.15M KCl and 10mM Tris HCl buffer (pH 7.5), and the homogenous suspension of microsomes was prepared in 0.1M potassium phophate buffer (pH 7.5) (Dallner 1963). Microsomes were used to determine the lipid peroxidation.

3.2.13 Determination of specific activity of Glyoxalase I

Glyoxalase I was assayed according to the method of Racker as described by Thornalley (1993).

Principle: Methylglyoxal and glutathione react non enzymatically to form hemithioacetal. Glyoxalase I catalysis the isomerization of the hemithioacetal to S-lactoylglutathione (SLG). The activity is assayed by measuring the initial rate of formation of the SLG.

$$\underset{\text{Methylglyoxal}}{CH_3COCHO} + GSH \longrightarrow \underset{\text{Hemithioacetal}}{CH_3COCH(OH)\text{-}SG}$$

$$\underset{\text{Hemithioacetal}}{CH_3COCH(OH)\text{-}SG} \xrightarrow{GlyI} \underset{\text{S-lactoylglutathione}}{CH_3CH(OH)CO\text{-}SG}$$

Assay: The assay mixture, contained in a volume of 1ml, was 50mM sodium phosphate buffer (pH-7.0), 3.5mM methylgyloxal, 1.7mM reduced glutathione. It was incubated for 10-min. followed by the addition of the supernatant and increase in absorbance was measured for 5 min. at 240nm. The activity is calculated using the extinction coefficient value 2.86 $mM^{-1}cm^{-1}$. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the formation of 1μmol of SLG/min.

3.2.14 Determination of specific activity of Glyoxalase II

Glyoxalalse II was assayed according to the method of McLellan and Thornalley (1992).

Principle: Glyoxalase II catalysis the hydrolysis of SLG to D-lactate and reduced glutathione. The activity is assayed by measuring the decrease in concentration of SLG.

$$CH_3CH(OH)CO\text{-}SG + H_2O \longrightarrow CH3CH(OH)COO^- + GSH + H^+$$

Assay: The assay mixture (1ml) contained Tris-HCl buffer (pH-7.2) and 0.2mM S-lactoylglutathione. Supernatant was added to the above reaction mixture and decrease in absorbance was measured at 240nm. The activity calculated using extinction coefficient 3.1 $mM^{-1}cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme required to catalyse the hydrolysis of 1μmol of SLG/min.

3.2.15 Determination of specific activity of glutathione-S-transferase

Glutathione-S-transferase (GST) was assayed using the method of Habig *et al* (1974).

Principle: The assay is based on the reaction of GSH-CDNB conjugate formation, which is catalyzed by GST

$$\text{CDNB} + \text{GSH} \longrightarrow \text{CDNB-GSH conjugate}$$

Assay: Reaction volume (1ml) contained final concentration of 0.1 M phosphate buffer (pH 6.5), 1mM CDNB in ethanol, 1mM GSH and was incubated at 37°C for 5 min. The reaction was initiated by the addition of enzyme sample and activity was measured for 5 min at 340nm. The specific activity was calculated using extinction coefficient 9.6 $mM^{-1}cm^{-1}$at 340nm and expressed in terms of μmol of CDNB-GSH conjugate formed/mg protein.

3.2.16 Determination of specific activity of superoxide dismutase

Superoxide dismutase was assayed by the method of Marklund and Marklund (1974).

Principle: It catalyses the dismutation of superoxide radical to yield hydrogen peroxide and oxygen. The assay is based on the ability of enzyme to inhibit autooxidation of pyrogallol.

$$\text{Pyrogallol} + O_2 \longrightarrow O_{-2} + \text{Oxidation product}$$

Assay: Supernatant treated with Triton X-100 (1%), on ice for 30 min and was added to assay (1ml) which contained 0.05M sodium phosphate buffer (pH-8.0), 0.1mM EDTA, 0.27mM pyrogallol and absorbance was measured for 5min at 420nm. Stock solution of pyragallol was made in 10ml HCl. One unit of enzyme was defined as amount of SOD required to produce half-maximal inhibition of auto-oxidation.

3.2.17 Determination of specific activity of catalase

Catalase activity was performed by the method of Aebi (1984).

Principle: It catalyses the decomposition of H_2O_2 to give oxygen and water as shown below

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Assay: The supernatant was treated with ethanol (10ml/ml) for 30min on ice. Then Triton X-100 (1%) was added and again kept for 30min on ice. The treated supernatant

was added to the assay mixture which contained 0.05M sodium phosphate buffer (pH 7.0), 10mM H_2O_2 and decrease in absorbance was measured at 240nm. The activity was calculated using extinction coefficient 0.04 $mmole^{-1}cm^{-1}$. One unit of catalase activity was defined as amount of enzyme required to decompose one mole of H_2O_2 per min.

3.2.18 Estimation of lipid peroxidation

Lipid peroxidation in microsomes prepared from liver was estimated spectrophotometrically by thiobarbituric acid - reactive substances (TBARS) method as described by Varshney and Kale (1990) and is expressed in terms of malondialdehyde (MDA) formed per mg protein.

In brief, 0.4 ml of microsomal sample was mixed with 1.6ml Tris KCl (0.15 M KCl + 10mM Tris-HCl, pH 7.4) buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 52mM TBA was added. The tubes were covered with almunium foil and placed in a water bath for 45 min at 80°C, cooled and centrifuged at room temperature for 10 min at 3,000rpm in REMI-T8 table top centrifuge. The absorbance of the clear supernatant was measured against reference blank of distilled water at 531.8 nm in spectrophotometer (Hitachi 2000).

The amount of MDA formed in a sample was estimated according to the equation

$$\text{nmoles of MDA} = V \times OD / 0.152$$

where

V = final volume of test solution

OD = optical density

3.2.19 Protein determination

Protein was determined according to the method of Lowry *et al.* (1951).

Principle: Proteins react with Folin-ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the phosphomolybdate with tyrosine and tryptophan.

Assay: Alkaline solution (50ml of 2% Na_2CO_3 in 0.1 N NaOH + 1ml of 0.5% $CuSO_4$ in 1% Na^+, K^+ tartarate) was prepared freshly when required. This alkaline solution (5ml) was added to the 1.0 ml of test solution, which contains BSA or other protein. It was

mixed thoroughly and allowed to stand at room temperature for 10 min. Subsequently 1N Folin's reagent (0.5ml) was added and allowed to stand for another 30 min. The absorbance was measured at 660nm against the reference blank. The protein content of each sample was evaluated from the standard curve with BSA and was expressed in mg/ml.

3.2.20 Statistical analysis

The statistical difference between the data pairs was evaluated by analysis of variance followed by Student's t-test.