CHAPTER III

MATERIALS AND METHODS

3.1 Animals, their feeding and management

Male Wistar rats weighing 120 to 200 gms were used throughout this experimental work. The animals were given stock diet (Hindustan Lever Ltd.) ad libitum.

3.2 Studies on the in vitro interactions of highly polymerized DNA with carcinogens/non carcinogens.

Highly polymerized calf thymus DNA (HP DNA) from Sigma was dissolved in buffered saline (0.15M sodium chloride, 0.015M sodium citrate, pH-7.0) at a concentration of 20 ug/ml for all the experiments. The inorganic and organic compounds used were dissolved in water and DMSO respectively.

3.2.1 Inorganic Compounds

The metal compounds (in water) were added to DNA solution so as to have a final concentration of 10 mM. The samples were then heated to 100°C for 15 min in a boiling water bath. The absorbance at 260 nm was measured immediately after heating. The samples were then allowed to cool slowly and the absorbance was measured at different temperatures while cooling. When the temperature reached 25°C, the samples were transferred to ice bath and were allowed to remain there for 1 hour and the absorbance was again measured at 260 nm.

3.2.2 Organic Compounds
The compounds (in DMSO) were incubated with microsomal suspension at 20°C for 2 hours in the presence of NADPH (1 mM) at a final concentration of 100 µg/ml. The samples were then centrifuged at 10,000 g for 20 min. The pellets were discarded and the supernatant containing the activated compound (ultimate carcinogen) was then added to HP-DNA solution. The samples were heated at different temperatures from 40°C onwards and the absorbance was measured at 260 nm while heating. The Tm was calculated from the graphs plotted as shown in the figures.

Similarly the HP-DNA solutions containing different activated compounds were separately incubated at 25°C and 37°C respectively for different time intervals and their absorbance was measured at 260 nm.

### 3.3 Dosing patterns of Sudan I and Sudan III for studies on drug metabolising enzymes.

27 rats were divided into 6 groups of 3 animals each. Intraperitoneal injections of dyes, Sudan-I and Sudan-III, and promoter phenobarbital were given for three days in the following combinations.

- **Group I** 0.5 ml Normal saline.
- **Group II** 0.5 ml Corn oil.
- **Group III** 80 mg Phenobarbital per k.b.w. per day in PSS.
- **Group IV** 20 mg Sudan I per k.b.w. per day in corn oil.
- **Group V** 20 mg Sudan I per k.b.w. per day + 80 mg phenobarbital per k.b.w. per day in PSS.
- **Group VI** 20 mg Sudan III per k.b.w. per day in corn
oil.

Group VII 20 mg Sudan III per k.b.w. per day in corn oil + 80 mg phenobarbital per k.b.w. per day in PSS.

Group VIII 20 mg Sudan I per k.b.w. per day + 20 mg Sudan III per k.b.w. per day in corn oil.

Group IX 20 mg Sudan I + 20 mg Sudan III per k.b.w. per day in corn oil + 80 mg phenobarbital per day in PSS.

The animals were killed by cervical dislocation 24 hrs after the last dose.

3.4 Dosing patterns of rats with 2-acetylaminofluorene (2-AAF) as carcinogen and calcium and bleomycin as anticarcinogens

60 male rats were divided into five groups of 12 animals each. 2-acetylaminofluorene (2-AAF), calcium chloride and bleomycin were given intraperitoneally (i.p.) two times a week for 16 weeks. Dose patterns were as follows

- Group I 0.5 ml normal saline (0.9%)
- Group II 0.5 ml DMSO
- Group III 50 mg 2-AAF per k.b.w. per week in DMSO
- Group IV 50 mg 2-AAF per k.b.w. per week in DMSO + 100 mg calcium chloride per k.b.w. per week in saline
- Group V 50 mg 2-AAF per k.b.w. per week in DMSO + 5 mg bleomycin per k.b.w. per week in saline.
Three rats from each group were killed every month and the last group was killed after four months by cervical dislocation for the preparation of microsomes.

3.5 Preparation of microsomes (Gupta and Dani, 1979)

After the cervical dislocation of rats, their livers were excised as such or after perfusion with normal saline in situ, when required. The livers were then weighed and homogenized in 2.5 volumes of 0.225M ST (0.225M sucrose + 50 mM Tris) buffer (pH 7.4) in a Potter Elvehjem apparatus at 0-4°C using a motor driven Teflon pestle rotated at 3000 rpm in a glass vessel. The homogenate was centrifuged for 10 min at 9,000 g in a fixed angle Janetzki 6x26 ml rotor at 4°C. The supernatant was removed and was again centrifuged at 10,000 g for 10 min at 4°C. The post mitochondrial supernatant (PMS) was diluted with 2.5 volumes of 0.225 M cold ST buffer. To this calcium chloride was added to a final concentration of 8 mM. The suspension was allowed to stand for 20 min in ice. and then centrifuged at 10,000 g for 20 min at 4°C. The pellets were washed and resuspended in ST buffer to a final protein concentration ranging from 10-20 mg per ml. The microsomal suspension and post-microsomal supernatants were used for degranulation and enzymatic studies.

3.6 Microsomal degranulation (Gupta and Dani, 1979)

Microsomal suspension (5-9 mg/ml protein), NADPH (1 mM) and the compound under test (40 μg/ml final concentration) in a final volume of 1 ml made either with water or DMSO, depending-
upon the solubility of the compound, were incubated at 20°C for 2 hrs, and then chilled in ice for 15 min. The degranulated microsomes or intact vesicles were obtained by centrifuging at 10,000 g for 20 min at 4°C. The supernatant was discarded and pellets resuspended in ST buffer were used for RNA, protein and phospholipid estimations.

For dose response studies different concentrations of 15,30,45 and 60 μg of carcinogen/non-carcinocen per ml of the final volume were used for different compounds for incubating with microsomes as detailed above. The degranulated or intact microsomes were then analysed for RNA, protein and phospholipids.

3.7 Estimation of Proteins

Protein contents of the microsomes and post microsomal supernatants were estimated by the method of Lowry et al. (1951).

Reagents

(1) 1 per cent copper sulphate solution
(2) 2 per cent sodium potassium tartarate solution
(3) 2 per cent sodium carbonate in 0.1 N sodium hydroxide
(4) 1N Folin - Coicalteu reagent (diluted 1:1 with distilled water before use).
(5) Standard bovine serum albumin (1 mg/ml)

Lowry's reagent was prepared by mixing solutions (1),(2) and (3) in the ratio of 1:1:98. The reagent was prepared fresh.

Procedure 10 μl of the sample was added to 1 ml of double
distilled water. This was followed by 3 ml of Lowry's reagent and the mixture was mixed thoroughly. Then it was allowed to stand for 15 min at room temperature. To this 0.3 ml of the Polin's reagent was added and the contents were shaken on vortex mixer and allowed to stand at 37°C for 30 min. Absorbance was measured at 670 nm. Protein concentrations were calculated from the standard curve of BSA.

3.8 Estimation of RNA

The RNA contents were measured according to the method of Munro and Fleck (1966).

Reagents

(1) 0.22 N perchloric acid
(2) 2.2 N perchloric acid
(3) 0.3 N potassium hydroxide

Procedure

To 100 μl of microsomal sample, 2.5 ml of cold 0.22 N perchloric acid was added. The tubes were kept in ice for 15 min. The supernatant was discarded and the tubes were kept in inverted position for the complete removal of perchloric acid. To this 3.2 ml of 0.3 N KOH was added and then the tubes were incubated at 37°C for 1 hr. The contents were shaken on vortex mixer and than 0.8 ml of 2.2 N perchloric acid was added. The tubes were kept in ice for 10-15 min and then centrifuged. The supernatant was diluted with an equal volume of distilled water. Finally the absorbance was read at 260 nm using Spectronic 2000 spectrophotometer.
3.9 Analysis of lipids

3.9.1 Extraction of lipids

The lipids were extracted according to the method of Folch et al (1957).

Reagents

(1) Chloroform + methanol (2:1, v/v)

(2) Upper phase solvent - methanol : water : chloroform (43 : 47 : 3) containing 0.29 per cent sodium chloride.

(3) 0.9 per cent sodium chloride.

Procedure

A known amount of microsomal sample was taken and to it twenty times mixture of chloroform:methanol was added. It was shaken vigorously and kept overnight. The mixture was filtered through G-3 sintered glass funnel and the filtrate was washed twice with 0.2 volumes of normal saline and finally with upper phase solvent. The chloroform layer containing the lipids was withdrawn and made to a desired volume.

3.9.2 Estimation of Phospholipids

Ashing of phosphate and estimation of inorganic phosphorus

Reagents

1. 10 percent magnesium nitrate in 95% ethanol
2. 0.5 N hydrochloric acid
3. 10 per cent ascorbic acid
4. 0.42 per cent ammonium molybdate in 1 N H SO
5. Phosphorus standard (34 mg KH$\_2$PO$_4$/l containing 7.75
μg phosphorus /ml.

Coloring reagent was prepared by mixing reagents 3 and 4 in the ratio of 1:6 respectively.

**Procedure**

To a requisite amount of lipid extract 0.15 ml of magnesium nitrate solution was added. The mixture was heated to dryness till all the brown fumes ceased to appear, giving a white residue at the base. The tubes were cooled and then 0.3 ml of 0.5 N HCl was added to each tube. The contents of the tubes were hydrolysed by keeping them in a boiling water bath for 15 min.

The inorganic phosphorus released was estimated according to the method of Ames (1960). To each tube containing phospholipid hydrolysate, double distilled water was added to make the final volume to 1.5 ml. To this 3.5 ml of coloring reagent was added and the incubation was done at 37°C for 1 hr. Absorbance was measured at 820 nm. Inorganic phosphorus released was calculated from the standard curve. Phospholipids were calculated by multiplying phosphorus content with a factor of 25.

3.10 Enzyme assays

3.10.1 Cytochrome b

Cytochrome b₅ was assayed by the method of Omura and Sato (1964). Microsomal preparation containing 2 mg protein/ml of 0.1M potassium phosphate buffer (pH 7.0) was placed in the reference and sample cuvettes. A few crystals of sodium dithionite were added to the sample cuvette. Scanning of the spectral change from 450 to 400 nm was recorded using spectronic 2000
spectrophotometer. The change in absorbance at 424 and 409 nm was recorded and cytochrome b\textsubscript{5} content was calculated using an extinction coefficient of 185 cm\textsuperscript{-1} mM\textsuperscript{-1} as follows:

\[
\text{n mol of cyt b}_5/ \text{mg microsomal protein} = \frac{\text{Absorbance at 424 - 409 nm}}{185} \times 10 \times \frac{1}{\text{mg protein per ml mixture}}
\]

3.10.2 Cytochrome P-450

The method of Omura and Sato (1964) was used to assay cytochrome P-450 content. Microsomal preparation containing 2 mg protein/ml of 0.1 M potassium phosphate buffer (pH 7;0) was taken in a test tube and a few crystals of sodium dithionite were added to it, so as to reduce cyt P-450. Carbon monoxide gas was bubbled through the sample cuvette and scanning of the spectral change from 500 to 400 nm was immediately recorded on spectronic 2000 spectrophotometer. The absorbance maxima and minima were recorded and difference of absorbance at 450 and 490 nm were determined. Cytochrome P-450 content was calculated using an extinction coefficient of 91 cm\textsuperscript{-1} mM\textsuperscript{-1} as follows:

\[
\text{n mol of cyt P-450/mg microsomal protein} = \frac{\text{absorbance at 450-490 nm}}{91} \times 10 \times \frac{1}{\text{mg protein per ml of mixture}}
\]
3.10.3 NADPH Cytochrome c reductase

The enzyme was assayed according to the method of Omura and Takesue (1970) with slight modifications. The assay mixture in 0.1 M potassium phosphate buffer (pH 7.4) contained 0.2 mM cytochrome c, 0.1 mM KCN and microsomal protein at a final concentration of 0.2 mg/ml. Reaction was started by adding NADPH to a final concentration of 0.1 mM. Change in absorbance was measured at 550 nm after every 15 sec. for 1 min at 25°C. Controls did not contain NADPH.

Enzyme activity was calculated using an extinction coefficient of 18.5 cm⁻¹ mM⁻¹.

\[
\mu \text{ mole NADPH oxidised/min/mg protein} = \frac{\text{absorbance at 550 nm}}{18.5} \times \frac{1}{\text{mg of protein}} \times \frac{1}{\text{Time of reaction in sec.}}
\]

3.10.4 Aniline Hydroxylase

Aniline hydroxylase was assayed by measuring the amount of p-aminophenol released, by the method of Chhabra et al (1972). The reaction mixture contained microsomes (1 mg protein/ml mixture), NADPH regenerating system (25 \( \mu \) moles glucose-6-phosphate, 25 \( \mu \) moles magnesium sulphate, 100 \( \mu \) moles nicotinamide and 0.6 \( \mu \) moles of NADP), post-microsomal supernatant (3 mg protein/ml) and 40 \( \mu \) moles...
aniline in a total volume of 5 ml of 0.1M potassium phosphate buffer (pH 7.4). Control tubes did not contain aniline. The mixture was incubated at 37°C for 20 min with shaking in an atmosphere of oxygen. p-Amino-phenol released was extracted after the addition of 5 g sodium chloride to the mixture containing 5 ml of ether. Complete extraction was achieved by shaking the contents on vortex mixer (Kato and Gillette, 1965). Then the centrifugation was done at 1,000 g for 10 min and 4 ml of the ether phase was mixed with 2.5 ml of the coloring reagent (0.095 g trisodium phosphate and 0.08 g phenol). The mixture was again shaken at room temperature for 15 min in a Dubnoff - metabolic shaker. It was then centrifuged at 1,000 g for 10 min. The ether layer was discarded and the absorbance of the aqueous phase was measured at 620 nm. Different concentrations of p-aminophenol were used for obtaining the standard curve.

Enzyme activity was expressed as n moles p-aminophenol released/min/mg/protein.

3.10.5 Aminopyrine demethylase

The enzyme was assayed according to the method of Gram et al (1968), and the amount of formaldehyde liberated due to aminopyrine -N- demethylation was estimated by the method of Nash (1953). The assay mixture in 0.1 M potassium phosphate buffer (pH 7.4) contained NADPH regenerating system (25 μ moles glucose -6-phosphate, 25 μ moles magnesium sulphate, 100 μ moles nicotinamide and 0.6 μ moles NADP), post microsomal supernatant (3 mg protein/ml), 50 μ moles semicarbazide, 0.5 mM aminopyrine and 1.0 mg/ml microsomal protein. The mixture was preincubated in
a Dubnoff metabolic shaker at 37 C for 30 min in an atmosphere of oxygen.

Formaldehyde formed from aminopyrine was trapped by semicarbazide as semicarbazone and then determined by the addition of coloring reagent in the final supernatant. Each ml of the incubated mixture was mixed with 2 ml of 0.6 N perchloric acid, centrifuged and 1 ml of the protein free supernatant was added to 2 ml of double strength Nash reagent (150 g ammonium acetate and 2 ml of acetyl acetone in 500 ml of double distilled water). The mixture was then heated at 60 °C for 30 min and cooled in ice bath. Change in absorbance was measured at 415 nm. Standards (0.4 ml to 2 ml of 10,000 times diluted formaldehyde solution) were also assayed as above.

Enzyme activity was expressed as n moles formaldehyde liberated per min per mg protein.

3.11 Gel filtration (Renuka et al., 1982)

Sepharose 2 B was diluted with 50 mm Tris-HCl (pH 7.5) containing 0.25 M sucrose, 5 mM MgCl₂ and 25 mM KCl and equilibrated with the same buffer at room temperature with several changes of the buffer. Columns of 1 cm diameter were filled up to a height of 40 cm and were equilibrated at 4 °C and the whole experiment was performed at the same temperature.

Preparation of sample.

Post mitochondrial supernatant (PMS) was prepared from rat liver by the method of Gupta and Dani (1979). This PMS was treated with metal salts at 40μg/ml concentration in the presence
of 1 mM NADPH. The mixture was incubated for 2 hrs at 25°C.

One of these samples was treated with ribonuclease and then cooled in ice for 3 min. The sample was diluted with STKM buffer so as to have a 20 µg/ml final concentration of RNAase. Samples were vortexed and kept in ice for 10 min.

The samples were then applied on different sepharose 2 B columns. 30 Fractions of 2 ml each were collected and assayed individually for RNA by the method of Munro and Fleck (1966) and for proteins by the method of Lowry et al. (1951).

3.12. Interactions of metals with some enzymes.

3.12.1 Trypsin inhibition

The method of Kunitz (1955) was used for the study of trypsin inhibition. The assay mixture contained 1% casein in 0.1 M phosphate buffer (pH 7.6), 500 µg enzyme and different concentrations of metals like 15, 30, 45, 60, 75 µg in a total volume of 1 ml. The mixture was incubated for 20 min. The reaction was stopped by adding 3 ml of 5% TCA. The mixture was then allowed to stand for 1 hr. The O.D. of the supernatant was read at 280 nm against the blank. The standard was also run along with the test sample.

Activity was expressed as increase in absorbance over control at 280 nm per mg protein per hr.

13.12.2 Chymotrypsin inhibition

Chymotrypsin was also assayed by the method of Kunitz (1955). The assay mixture contained 1% casein in 0.1 M borate buffer (pH 8.0) and 50 µg of the enzyme. CaCl₂ was also...
adde\(d\) so as to have a final concentration of 0.005 M in the enzyme substrate mixture. This enzyme substrate mixture was incubated, in the presence of different concentrations (15, 30, 45, 60, 75 \(\mu g\)) of various metal salts, for 20 min. The reaction was stopped by the addition of 5% TCA. The mixture was allowed to stand for 1 hr. The O.D. of the supernatant was read at 280 nm against the blank. Specific activity is expressed in terms of increase or decrease in absorbance of the reaction mixture over control at 280 nm per mg of protein per hour.

3.12.3 RNase inhibition

RNase inhibition was studied by the method of Gray (1974). The enzyme activity was assayed by measuring the absorbance at 260 nm of the acid soluble degradation products of yeast RNA. The incubation mixture contained 2 mg yeast RNA, 0.1 ml Tris HCl (pH 7.5), 0.1 ml of 0.1 mM EDTA, 75 \(\mu g\) enzyme and different concentrations (15, 30, 45, 60 and 75 \(\mu g\)) of metals in a final volume of 1.5 ml. After the incubation at 37 °C for 30 min, the reaction was stopped by the addition of 0.25 ml of 25% (v/v) aqueous perchloric acid at 0 °C. The precipitated protein and nucleic acids were removed by centrifugation and the absorbance at 260 nm of the supernatant was measured after suitable dilution with double distilled water. Controls were treated in the same manner but did not contain the enzyme. Specific activity was expressed in terms of increase or decrease in absorbance of reaction mixture over the control per mg of protein per hr.
3.13 Dose pattern of different metal compounds for their effects on nuclear proteins

10 male rats were divided into 5 groups of 2 rats each. Intraperitoneal injections of different metal compounds were given for seven days once daily. The dose pattern is as given below:

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.5 ml of double distilled water.</td>
</tr>
<tr>
<td>II</td>
<td>1 mg lead mitrate per k.b.w. per day.</td>
</tr>
<tr>
<td>III</td>
<td>500 µg beryllium sulfate per k.b.w. per day.</td>
</tr>
<tr>
<td>IV</td>
<td>100 µg sodium meta arsenite per k.b.w. per day.</td>
</tr>
<tr>
<td>V</td>
<td>1.5 mg Chromium trioxide per k.b.w. per day.</td>
</tr>
</tbody>
</table>

The animals were sacrificed by cervical dislocation 24 hrs after the last dose. The nuclei were prepared as follows:

3.13.1 Isolation of nuclei and histone extraction

Nuclei were isolated by the method of Mandal (1969). Livers were taken after sacrificing the rats and were weighed. The homogenate was prepared with five volumes of 0.25 M sucrose-0.003 M calcium chloride. The homogenate was filtered through flannelette and centrifuged at 800 g for 10 minutes. The crude nuclear pellet was washed twice with the sucrose- CaCl solution followed by centrifugation.

The nuclear histones were prepared by homogenizing the nuclear pellet in four volumes of buffered saline (0.14 M Sodium chloride- 0.01 M Sodium citrate) and then centrifuging at 2000 g for 10 min. The sediment was washed three to four times until the washing was only slightly opalescent.
The histone fractions were extracted from isolated nuclei according to the method of Monty and Dounce (1959). The first fraction of non-histone proteins was extracted by adding five volumes of 0.14 M NaCl and keeping it for 30 minutes.

Total histones were extracted by adding 5 ml of 0.2 HCl to the residue left above and fractionated into precipitable histone with 0.1 volume of concentrated NH₄OH and soluble histones were then precipitated with 3 volumes of 95% ethanol from supernatant. Each nuclear protein fraction was then estimated separately by the method of Lowry, et al. (1951).