PART - I

Chapter 1

Materials and Methods

SECTION - I

(i) Source of Animals and Maintenance

Medium sized mature specimens of the frog *Rana tigerina* (weighing 40 to 50 gms), originating from wild populations, were collected from village areas of Dabhoi, about 45 kms from Baroda city. Large collections were done only during the post-breeding season (September to January). Small number of specimens were collected in other season also for the experiments on mitotic activity in the bone marrow. The frogs were maintained in an aquaterraria at 20-25 °C. They were fed cockroaches *ad libitum* thrice a week. In all experiments the animals were acclimatized to laboratory conditions at least 7 days before use.

(ii) Experimental Protocol

In each experiment, 5 animals (both sexes) were used as experimental animals. Control animals were kept as a separate group of 15 animals. Only one group of control was kept against a series of experiments.

Bone marrow was selected as the target tissue for the analysis of chromosomal aberration as well as counting the mitotic index. Cells were analyzed after arresting the chromosomes at the metaphase stage by the administration of colchicine 6 hours prior to sacrifice. From each animal 8 slides were prepared and 300 metaphase cells were analyzed for the chromosomal aberration studies.

The chemicals were either injected intraperitoneally or the animals were treated in the chemical media. In all the experiments two sublethal dose/concentrations were used. For the ip administration, three different timings were selected. ie. 6 hrs, 24 hrs and 48 hrs. When the animals were exposed to the treatment media two different treatment periods were selected (7 days and 14 days).

SECTION - II

Treatment agents and dosage/concentrations

Only the sublethal doses/concentrations of the chemicals were used in all the experiments. These dosages were selected on the basis of LD 50/LC 50 of the chemicals as earlier determined on the same or other species of anurans. (Power et al., 1989). Frogs were not used for separate assays to determine the LD 50/LC 50. Chemicals to be administered (ip) were prepared in distilled water and the stock solution was kept in refrigerator. The selected dosages of chemicals for various experiments is as given below.

1. Mercuric chloride. $HgCl_2$
   1.5 mg/kg body weight; 3.0 mg/kg body weight
2. Cadmium chloride. $CdCl_2 . 2H_2O$
   1.5 mg/kg body weight; 3.0 mg/kg body weight
3. Nickel chloride. $NiCl_2$
   1.5 mg/kg body weight; 3.0 mg/kg body weight
4. Lead acetate. $(CH_3 COO)_2 Pb.3H_2O$
   1.5 mg/kg body weight; 3.0 mg/kg body weight

Treatment Media Preparations

Frogs were exposed to the treatment media in three series of experiments with the agents such as Lead acetate, Effluent water and Cobalt sulphate.

Lead acetate: Two different sublethal concentrations (25 ppm and 50 ppm) were used for two different treatment periods (7 days and 14 days).

Effluent water: Effluent water was collected from the Mini River. Analysis of water sample was done by Atomic Absorption Spectrophotometer. The effluent water was diluted with tap water to prepare different concentrations of treatment media. When

frogs were kept in 100% solution, no death were recorded within 20 days. Hence, two different treatment periods, 7 days and 15 days were selected for three different sublethal concentrations 75%, 50% and 25%.

Cobalt sulphate ($CoSo_4.7H_2O$): This experiment was carried out with an aim to know whether the observed clastogenic effect of effluent water was caused by the amount of cobalt present in the water. Hence, the animals were treated in two different Cobalt sulphate solutions which were having the equal amount of cobalt found in the effluent water. A stock solution of cobalt sulphate (1905 ppm; contains the same amount of cobalt as in the effluent water) was made and diluted to 75% and 50% by adding tap water.

Procedure of Chromosome Preparations

Colchicine solutions: Colchicine. (0.02%) aqueous solution was prepared. The solution was injected at a concentration of 0.25 mg/kg body weight 6 hrs prior to sacrifice.
Hypotonic solution: 0.075 molar KCl in distilled water; prepared immediately before use.
Fixative solutions: Carnoy's fixative (methanol: acetic acid, 3:1).
Giemsa stock: 4 gm Giemsa powder(Merck), 250 ml glycerine, 250 ml of absolute methyl alcohol and glass beads mixed for 72 hours on a rotator.
Phosphate buffer: pH 6.2 (18.6 ml of 1/15 *M* KH_2PO_4 and 81.4 ml of 1/15 *M* Na_2HPO_4)
Staining solution: 6 ml filtered stock Giemsa, 6 ml buffer and 138 ml distilled water.

Bone marrow chromosome preparations: Each frog was given an intraperitoneal injection of colchicine solution. Six hours later the frogs were pithed and both the femurs removed. The bone marrow was blown into 5 ml of hypotonic solution and the cells suspended by trituration with a Pasteur pipette. The suspension was allowed to stand for 15-20 min at room temperature after which the cells were collected by centrifugation at 1,000 rpm for seven min. Three and a half ml of the supernatant were removed and the button of cells was resuspended in 4 ml of chilled Carnoy's solution. After 20 min at 4 °C the suspension was recentrifuged and the resultant button was again resuspended in 4 ml of fixative. After an overnight fixation the solution was centrifuged at 1000 rpm for seven minutes. The cell suspension was

suspended in 1 ml of fresh fixative. A drop of the cell suspension was suspended in wet, ice-cold, chemically cleaned slides, at a 45° angle. The suspension was allowed to spread for 10 sec and ignited by passing the slide through a flame. The preparation was kept for one day for drying. Staining was done for 7 minutes in Giemsa solution. Slides were passed through acetone-xylene grades and pure xylene, before mounting in DPX. Photographs were taken on Kodak or Illford (100 ASA/21DIN) film using Zeiss Photomicroscope.

Chromosome preparations from spleen: Pieces of freshly extirpated spleen, each no longer than 0.1 cm^3 were minced as finely as possible with fine scissors in small petridishes. Then 10 ml of hypotonic (0.075M) KCl solution was added and the tissue pieces were vigorously suspended with Pasteur pipettes until the solution was distinctly turbid. Following the hypotonic treatment, the layer of cellular suspension above the deposit layer of tissue particles was carefully removed and centrifuged for 10 min at 1000 rpm. The pellet was fixed with 3-5 ml of glacial acetic acid : methanol (1:3). The fixed material was kept at 4 °C for at least 12 hr, then washed once with fixative. Chromosomes were prepared by flame dry method as described above.

Mitotic Index

Similar method, described above for chromosomes preparations from the bone marrow, was adopted to prepare cell smear preparations of bone marrow for counting the mitotic index. Slides, those prepared from the control animals for the chromosome aberration tests, were also used for counting the mitotic index in those seasons. Mitotic index was calculated as the number of cells in division per thousand cells counted.

Statistical Analysis

Chromosomal aberration tests: Statistical analysis of the data was done by equality proportion test and the value is considered to be significant when $Z \geq 1.96$.

Mitotic index: The significance level between the control and experiments was determined by Student's 't' test. P value of 0.05 or less was accepted as statistically significant.