CHAPTER II

MATERIALS AND METHODS

MATERIAL AND METHODS :

A) CHEMICALS :

Leishman's stain and uranyl nitrate [$UO_2(NO_3)_3:6H_2O$] were purchased from BDH, Poole, England. Other chemicals used during hematological studies were reagent grade and obtained from BDH.

Dithiozone and Diphenyl carbazid were obtained from E. Merk Dermsteadt, Germany. Tris (hydroxymethyl) amino methane was from BDH, Olive oil was from P.Sasso figli oneglia; Italy.

The solvents chloroform, methanol, Diethyl ether were of analytical grade and obtained from E. Merck and Co., BDH. Solvent were redistilled under undhydrous condition before use.

Various chemicals used for detection of Blood urea nitrogen were also reagent grade and obtained from BDH.

B) EXPERIMENTAL ANIMALS :

Male albino rats (Rattus Norvogicus) weighing 200-225 gms were obtained from 'Haffkine Bio-pharmaceutical Corporation Ltd., Bombay. They were kept in separate metabolic cages, one rat in single cage. The cageing system included the keeping of animals dry, clean; maintaining the animal in the state of relative thermal neutrality; provided sufficient space to assume freedom of movement, such type of comfortable housing provide the animal to maintain good health. The cages were made up of galvanized tin with weld-wash top having size of 14" X 9" X 6". All cages were arranged in a room where sufficient ventilation and light was present.

Rats were fed daily with 'Hind Liver' pelleted diet according to their requirements. The food was clean, free of contaminants; palatable and nutritionally adequate. They were also provided with sufficient, clean water through the watering devices. Sanity was maintained through out the experimentation.

C) SELECTION OF DOSES AND ROUTE OF ADMINISTRATION :

A sublethal dose of 5 mg/kg body weight uranyl nitrate and a lethal dose of 10 mg/kg body weight of uranyl nitrate were chosen for the experimentation. The above doses were selected with the help of experimental data on mortality and LD_{50} values.

From the various routes of administration like subcutaneous, intramuscular, intraperitoneal, intravenous etc., intraperitoneal route was found suitable for out study. The intraperitoneal route of administration of toxicant is most prevalently used; it results in rapid absorption of toxic material due to rich blood supply in peritoneal region. While in other routes of toxicants administration; it absorbed comparatively at slower rate. The intravenous route of administration is also advisible in such studies; which introduces the toxic material directly in blood but this technique needs lot of practice, to perform this in small animal like rat.

D) SELECTION OF DOSE INTERVAL :

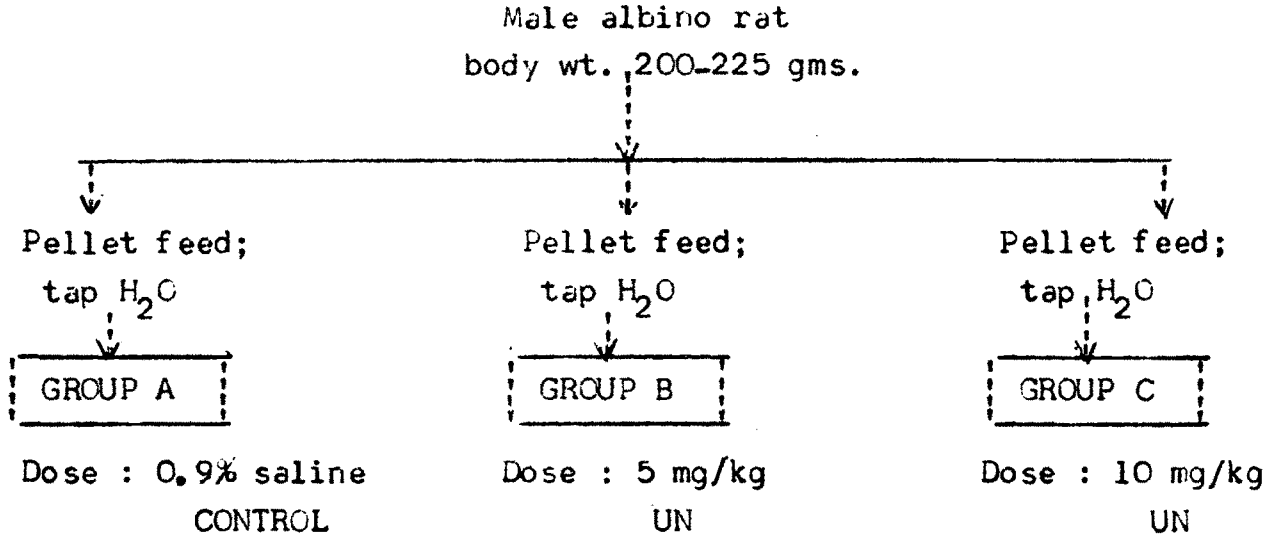
For the hematological study in rat; under the influence of uranyl nitrate, induced acute renal failure, dose intervals were chosen which are in general practice in such studies. The uranyl nitrate induced acute renal failure study is generally divided

under 3 different phases i) Early initiation phase (2,4 hrs), ii) Late initiation phase (8,12 hrs), iii) Maintenance phase (48,72 hrs). This type of phase programme study is followed by many investigators.

Hence, for our experimental studies, dose intervals from 2, 4, 8, 12, 48, 72 were selected and expressed as early initiation phase (2,4 hrs), late initiation phase (8,12 hrs) and maintenance phase (48,72 hrs).

E) ACUTE TOXICITY STUDIES :

EXPERIMENTAL PROTOCOL

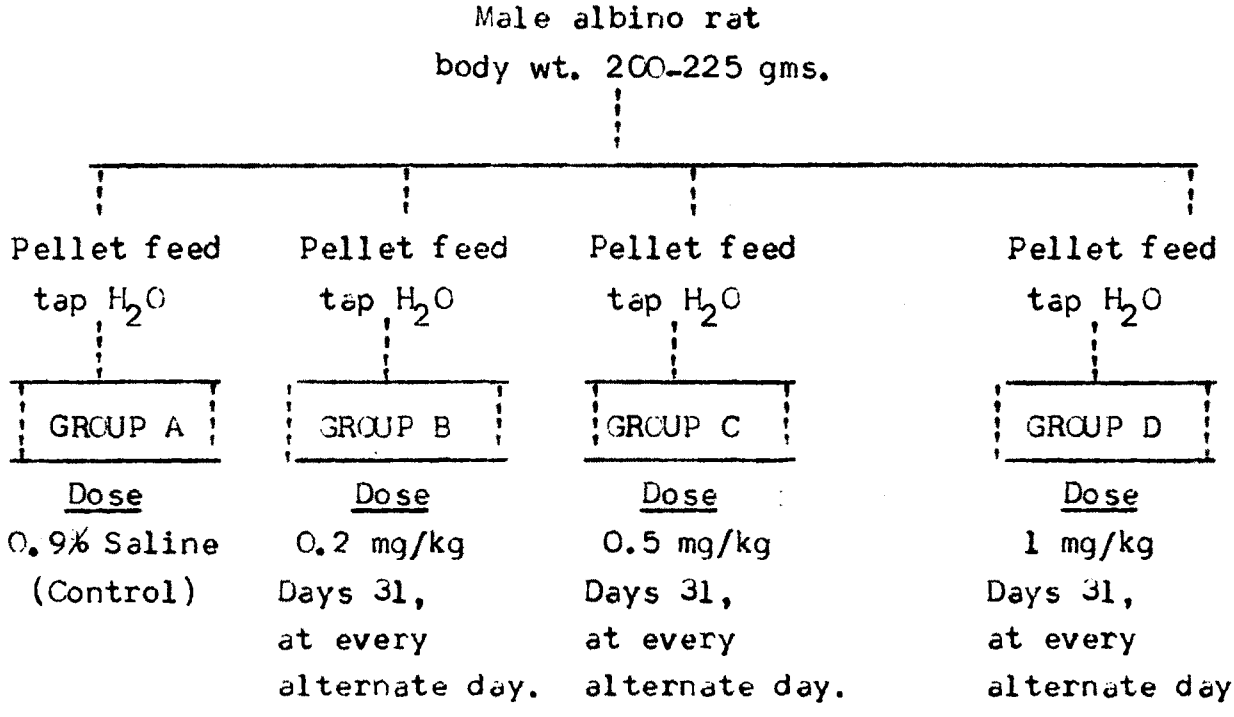


Animals were divided into 3 groups as above; Group B receiving 5 mg/kg dose of uranyl nitrate; Group C receiving 10 mg/kg dose of uranyl nitrate and third Group A of control animals received a equivalent volume of isotonic saline intraperitoneally. Uranyl nitrate was dissolved in a concentration such that each animal received 1 ml. of intraperitonial

solution per kg body wt. After the injection, animals were returned to the cages and sacrificed after specified time interval.

F) PROLONGED TOXICITY STUDIES :

EXPERIMENTAL PROTOCOL



Animals were divided in 4 groups, each group containing 3 animals. One group received a small dose of 0.2 mg/kg uranyl nitrate intraperitoneally every alternate day for 31 days. Second group received a dose 0.5 mg/kg uranyl nitrate through the same route for the same duration. Third group received a dose of 1 mg/kg uranyl nitrate intraperitoneally every alternate day for the same duration. With every experimental group one group of control was maintained throughout the chronic prolonged studies; the control group received a equivalent dose of 0.9 % saline for

placebo effect. The uranyl nitrate treated animal received a 1 ml. Solution of uranyl nitrate every alternate day. After the long term intoxication of uranyl nitrate for 31 days animals were utilized for routine hematological studies.

HEMATOLOGY :

1) DETERMINATION OF CLOTTING TIME :

Measurement of clotting time was done by Wright's capillary tube method (1966). The capillarly tube was dipped in the drop of blood oozing from the tail cut. The blood was drawn up in the tube, in the form of small column of blood due to capillary action. Every one minute interval the capillary tube was broken off, and the broken ends of the tube are separated carefully. The thread like structure; fibrin, appeared between the gap of the broken ends. Simultaneously time was recorded on the stopwatch and taken in triplicate.

2) ERYTHROCYTE SEDIMENTATION RATE AND HEMATOCRIT VALUES :

The blood was diluted; 4 part to 1 part of 3 % sodium citrate solution. The blood was drawn into sedimentation tube and fixed in the vertical position. A special rack was made for this purpose, but the tube was pressed into a paraffin block and held vertically. The distance of the cell level was measured in mm. after every hours.

After recording the sedimentation rate, the tube was centrifuged at 3000 x g for at least 15 minutes. The cells were completely packed. The packed cell volume was measured on graduated tube and recorded as hematocrit value.

3) HEMOGLOBIN CONCENTRATION :

The percentage of hemoglobin was determined using a Sahli's haemometer as described by Wright (1966). The graduated diluting tube was filled upto 2 gm. mark by decinormal solution of hydrochloric acid. The micropipette was filled with blood upto 20 cmm. The tip of the micropipette was cleaned carefully and introduced in to the graduated tube. The micropipette was passed right upto the bottom into the decinormal solution of hydrochloric acid. The blood slowly expelled into the acidic solution. The micropipette was rinsed with decinormal hydrochloric acid. The tube was allowed to stand for 20 to 30 minutes. The mixture was then diluted with distilled water, by adding drop by drop, at every time stirred by flat end of the glass rod, till the colour of mixture matches with standard colour. Reading was recorded in gms/100 ml on verticle index. Results were taken in triplicate for accuracy.

4) RED BLOOD CORPUSCLES COUNT :

Enumeration of red blood corpuscles was done by Neubauer's counting chamber. The R.B.C. pipette was filled upto 0.5 mark with blood. The tip of the pipette was wiped carefully. The tip of the stem was then immediately placed into the dilution fluid. The fluid was sucked upto mark 101. Then the mixing of the dilution fluid and blood was done by shaking the pipette well. First few drops of diluted fluid was discarded before filling the Chamber. A drop of diluted blood was allowed to flow through the narrow margin between the counting chamber and coverslip.

The haemocytometer was placed under microscope. First it was observed under low power then to high power. The cells were counted in 100 squares in triplicate.

5) WHITE BLOOD CORPUSCLES COUNT :

Total white blood cells count was taken by Neubauer's counting chamber. Blood was sucked up by the W.B.C. pipette upto 0.5 mark and diluting fluid upto 11 mark. Mixing of blood and dilution fluid was done by shaking the pipette well. Then the counting chamber was filled by diluted blood and cells were counted under the microscope.

6) DIFFERENTIAL COUNT :

Differential count was carried out using Leishman's stain. The drop of blood was placed about $\frac{1}{4}$ inch from the one end of cleaned slide. Another cleaned slide was held on the first slide at 30 to 45 degree angle. The second slide was withdrawn towards the drop of blood till the touched end of slide just touches the drop and it spread along slide. The second slide then pushed smoothly toward end of previous slide. The smear was then allowed to dry. It was fixed in 70 % alcohol, and allowed to dry again. The smear was covered by Leishman's stain for one minute. An equal volume of distilled water was added. The stain was mixed with water by gentle shaking. The diluted stain was poured off and slide was held under running tap water and dried with filter paper. The smear was examined under low power and then high power of microscope for identification of cells.

COLLECTION OF BLOOD SAMPLE :

The rat was slightly anaesthetized with ether. The tip of the tail was cleaned by alcohol. The tail was cut by sterilized scalpel. Initial few drops of blood were discarded. The blood was taken to total RBC count; total WBC count; determination of hemoglobin; differential count and for clotting time counting.

The animal was then sacrificed and blood was taken through cardiac puncture for fragility test, hematocrit, erythrocyte sedimentation rate; lipolytic activity; and blood-urea nitrogen determination.

SEPARATION OF SERUM :

Two ml blood was taken in a centrifuge tube and centrifuged at 5000 x g for 15 minute. The upper layer of serum was taken out with the help of dropper in another test-tube and stored, at room temperature. Serum was suitably diluted with the addition of 0.9 % saline.

LIPOLYTIC ACTIVITY :

The lipase (Tri-acyl-glycerol hydrolase, TAGH) was assayed by the method described by Hayashi and Tappel (1970) except for free fatty acids. The assay system contained 0.25 ml substrate, 1 ml. of 0.1 M Tris-HCl buffer (pH 7.2) and 0.25 ml serum. The incubation was carried out in a metabolic shaker with constant shaking of the rate of 160 stroke per minute with 4 cm amplitude for 10 minute at 37°C. The enzyme reaction was terminated at the end of incubation by adding 2 ml copper TEA [1 N. acetic acid,

1 M 2', 2" - trinitrollo ethanol; 6.45 % $Cu(NO_3)_2$; 1:9:10 v/v/v] reagent. 10 ml of chloroform was added to above content shaked vigrously and allowed to settle down for $1\frac{1}{2}$ hr. After $1\frac{1}{2}$ hr., 5 ml sample was taken out from each flask and centrifuged for 5 minutes. Two ml of centrifuged sample was taken in stoppered test-tube. The colour was developed by the addition of 1 ml of 2.5 % solution of a mixture of diphenyl carbazid and diphenyl carbazone (5:95 w/w) in methanol. The liberated fatty acids were measured according to Itaya, K. (1977) at 550 nm with Speckol, Carl-Zeiss, Jena Dermsteadt DDR.

Tri-acyl-glycerol hydrolase (EC 3:1:1:3) (IUPAC-IUB commission, 1979) is an enzyme which hydrolyzes esters of long chain aliphatic acids from glycerol at oil/water interfaces. Fat bodies forming emulsion globule with water usually provide interface and these have been termed as supersubstrates (Brockerhoff and Jensen 1974). The more polar products such as monoglycerides, diglycerides, free fatty acids and glycerol produced as a result of lipolytic activity are absorbed as mixed micelles into the target cell, except the glycerol the free fatty acids are transported to blood forming a complex with the blood proteins. The oxidation of Co-A esters is activated by the free fatty acids, or else they are converted into other physiolcgically active compounds or otherwise metabolized. These are some of the important roles ascribed to the lipolytic enzymes.

Apart from their biological significance these enzymes are especially important in the field of nutrition; food technology;

clinical medicine, analytical and preparative lipid chemistry and biochemistry; in research on disturbances of lipid metabolism and the disease of circulatory and nervous systems, or in research field like disturbances in lipid metabolism under the toxic stress.

PURIFICATION OF CLIVE CIL :

Triglycerols from olive oil were purified on a column of neutral alumina gel (Jensen et al., 1966,b). The gel was activated at 260°C for 12 hrs. The activated gel was washed with the mixture of petroleum ether and diethyl ether (9:1 v/v). The olive oil was dissolved into the minimum quantity of solvent mixture and loaded on a column. Triglycerols were eluted with petroleum ether-diethyl ether (9:1 v/v). One gram of alumina per two grams of olive oil to be separated was used for the column.

PREPARATION OF OLIVE OIL EMULSION :

The emulsion of olive oil was prepared according to Fraser and Nicol (1966). 5 grams of triacylglycerols and 5 ml of ringer solution were used.

ENZYME UNIT :

One unit of lipase was defined as the amount of enzyme which produced one mole of free fatty acids per minute under the appropriate assay conditions as described by Matsmura et al.(1976).

METHOD EMPLOYED FOR DETECTION OF BUN :

The blood-urea-nitrogen level was detected by the method described by Levine et al. (1961). One ml of blood was taken in

test-tube and to it four ml 10 % TCA was added. After five minutes it was filtered with 0.5g charcol through filter paper, 2.5 ml of filtrate was taken and 0.5 ml Ehrlich's reagent was added to it. And colour thus produced was measured at 425 nm with speckol.

For the control, 4 ml 10 % TCA was taken in test-tube and 1 ml distilled water was added to it. Then 0.5 ml Ehrlich's reagent was added and read at 425 nm; on speckol.