C H A P T E R - II

MATERIALS AND METHODS

MATERIAL

1 EXPERIMENTAL ANIMAL

The animals used in these experiments were adult male albino rats (Rattus norvegicus) weighing aobut 250 to 300 gm obtained from 'Hindustan antibiotics Ltd., Pune. The rats were kept in metalic cages with enough space for their free movements. The cages were kept in animal house in a seperate room with sufficient ventilation and day light. The temperature of the room was maintained to $25^{o}C \pm 1^{o}C$. The cages were cleaned everyday and paddy bed was replaced after cleaning thrice a week. The animals were fed with standard pellet feed and water was supplied ad libitum. The food was clean dry and nutritionally adequate. The feeders and water bottles were changed every day.

Four groups of rats were established. Each group consisted of five animals the control group also contained 5 animals. The groups of animals undergoing treatment for different periods were labelled as A,B,C and seperately maintained.

2 SELECTION OF DOSES AND ROUTE OF ADMINISTRATION

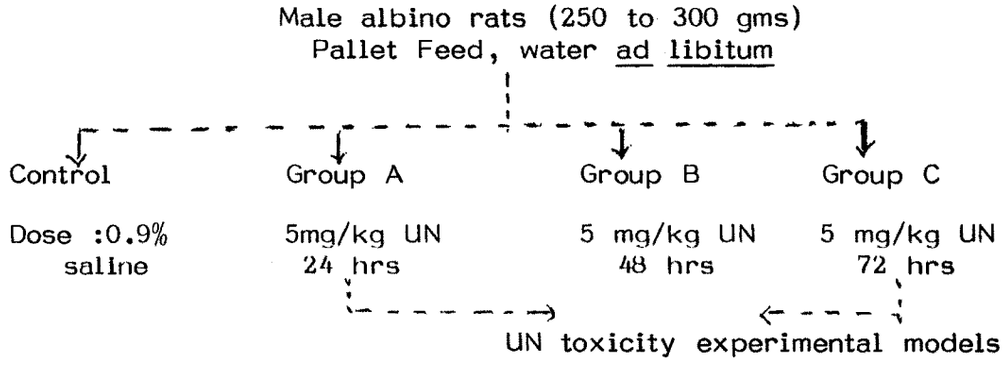
In the recent days UN has been successfully used as tool for inducing experimental ARF and CRF (Flamenbaum et al., 1972 a,b). The doses selected are based on the LD_{50} values. In case of rats the dose of UN used in between 5-10 mg/kg Uranyl nitrate dissolved in physiologic saline. In the present investigation a dose of 5 mg/kg body weight UN has been selected. The selection

of present dose is based on the LD_{50} values obtained in rats and a subacute dose of 5 mg/kg was found to be sufficient enough to bring about adverse changes in renal function. The associated change in RBC structure and function can be detected if at all UN exerts its toxic effects on circulating blood cells. The 5 mg/kg dose of UN was prepared as usual in 0.9% saline.

From the various routes of administration like subcutaneous, intramuscular, intraperitoneal, intravenous etc., intraperitoneal route was found suitable for our study. The most prevalently used route of administration of toxicant is intraperitoneal route it results in rapid absorption of toxic material due to rich blood supply in peritoneal region. 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.

For the penetration uranyl nitrate was dissolved in 0.9% saline so that each animal received 1 ml of respective dose intraperitoneally for different doses separate syringes were used and needles of gauge no.26 were used for injecting UN dose.

EXPERIMENTAL PROTOCOL



Dose : Uranyl nitrate (5mg/kg bw) intraperitoneally for following different time intervals.

Early initiation phase - 24 hrs.

Late initiation phase - 48 hrs

Maintenance phase - 72 hrs.

* Control group received an equivalent volume of 0.9% Physiological saline and sacrificed along with experimental models
* Group A,B,C,D consisted of 5 animals

  In which control group - Group A is control
* The phasewise protocol has been adopted by most of the investigators in order to maintain sequential record of ongoing physiological, biochemical and histological events.

After the desired time interval experimental animals were sacrificed and blood was collected from heart with the help of seringe.

CHEMICALS

All solvents were reagent grade and were obtained from E.Merck, Co., BDH and N.J.USA unless otherwise indicated. They included chloroform methanol, acetic acid, n-Hexane, Diethyl etc. solvents were redistilled in the laboratory under undhydrous condition before use.

DRAWING OF BLOOD SAMPLES

a) Preparation of blood containers

The withdrawal of blood for scientific purposes is an old experimental biology. Early methods were crude and often envolved simply cutting the skin or vein of the experimental subject (Dreyfus 1957); small samples of blood can be drawn directly into evacuated centrifuge tubes. A drop of heparin solution or a few crystals of heparin can be added to the tubes to prevent coagulation samples of less than 20 ml total volume are often drawn directly into syringes (Darmady and Davenport, 1954); to which the anticoagulant is added before the sample is drawn method of obtaining blood bottles or plastic blood bags are preferred for samples in a range of 50 to 500 ml. The plastic blood bags has almost entirly replaced within the last few years by glass blood storage bottle (Strumia, et al., 1955); Methods for obtaining large samples of blood, when a larger animal is to be exanguinated can not be standardized.

Several anticoagulants are available to prevent clotting and the destruction of erythrocytes during blood sampling. Isotonic citrate dextrose solution, a good choice when whole blood must be presurved for metabolic studies as in infusion experiments (Gibson et al., 1965; Wintrobe, 1967). It has disadvantage that it dilutes the blood and significantly increases the volume. The preferred anticoagulants are ethylene-diaminetetra acetic acid

(EDTA) and heparin (Hadley and Weiss, 1955; Schmidt, Hane and Gomez, 1953). EDTA is a reliable anticoagulant, it sequesters the divalent metallic ions that promotes the autoxidation of unsaturated fatty acids in blood lipids (Roy, Davisson and Crespi, 1954). The use of antioxident is also recommended for blood lipid samples during lipid extraction by addition to the extracting solvents.

b) SAMPLING OF ANIMAL BLOOD

The choice of sampling method depends on the size of experimental animal and on the size of the desired sample. Blood is often obtained from small animals by sacrificing and exsanguinating them. Heart puncture recovers much of the total blood from rat or mouse (Stubbs, 1953) small samples can be drawn from rat or mice by slicing off the tip of the tail and collecting the blood (Leahy and Borrow, 1953). Small samples can be drawn from large animals by syringes without anesthesia (Schalm, 1965; Hillyard, Exteman, Peinberg and Chaikoff, 1955).

Samples from several animals are usually pooled for lipid analysis because the amount of lipids present in the circulation is small (O'Amour, F.R.and Blood, 1954). Ten milliliters is frequently the minimum needed for the chromatographic techniques.

c) SEPARATION OF BLOOD FRACTIONS

i) Seperations of plasma

Centrifugation is the standard method for seperating cell from plasma. Samples less than 15 ml can be processed in 15 ml

centrifuge tubes in a small clinical centrifuge; whereas larger volumes like 40 ml are handled in 40 ml plastic centrifuge tube. A refrigerated centrifuge is preferable because the speed and duration of centrifugation otherwise heat the sample to undesirable temperatures. The centrifugal force necessary to sediments, the erythrocytes is relatively small. Adequate seperations can be obtained at less than 1000 g Hanahan et al., (1960). At greater forces such as 1600 g. Cells are well seperated from plasma in within 15 to 20 min. Cleaner seperation is obtained by greater centrifugal force and provides complete removal of platelets from plasma (Nelson, 1967). A disadvantage of high speed centrifugation is that differences in red cell density have more influence at greater centrifugal forces, there is much more pronounced seperation of young and old cells (Westerman, pierce and Jensen, 1963; Van Gastel, et al., 1965). A low-speed centrifugation to remove most of the red blood cell and leukocytes from the sample followed by high speed contrifugation to remove the remaining cells and platelets yields a cleaner plasma sample.

ii) Isolation and purification of red blood cells

Plasma contamination of red blood cells has been a perticularly vexing problem in the analysis of neutral lipids from red cells(Nelson 1967). The cells must be washed at least three times with isotonic buffer before they can be considered to be free of plasma lipoproteins and four washes are preferable

(Nelson, 1967). Several buffer systems are available for washing red cells (Chanutin, Curnish, 1966; Neerhout, 1968). The most common wash is isotonic saline. Another wash medium that is frequently used is isotonic phosphate buffer (Nelson,1967).

Some investigators (Anderson and Turner, 1960; Lovelock, 1955), have claimed that lipids are lost during the washing of intact cells with isotonic solutions, Lipids bound to the other surface of plasma membrane can be removed by extensive washing. Multiple washing is necessary to remove the leukocytes and platelets from red cells. The washing technique is usually adequate if it reduces the leukocytes to less than one per 10^4 red cells a level considered acceptable for red cell lipid analysis or metabolic experiments (Donabedian and Karmen, 1967). The platelet preperation of red cell is difficult to estimate. A good way of removing platelets is to spin the whole blood for 10 min at 400 g and then remove plasma.

5 PREPARATION OF RED CELL GHOST

Red blood cells are made freed of haemoglobin by lysis; the end product of lysing is the red cell 'ghost' which is essentially the intact membrane of red cell. The ghost has most of the properties of intact membrane except that some membrane proteins are lost (Anderson and Turner, 1960). All extraction procedures extract some pigments from the intact red cell.

Extracts prepared from ghosts are free of heme pigments which are undersirable because they promote the autoxication of lipids (Dodge and Phillips, 1966). Many hemolysis procedures have been reported in the literature (Anderson and Turner, 1960; Berstein, Jones, Erickson, Williams, Avrin and Macy, 1938; Danon, Nevo and Marikovsky, 1956). In the simplest method the washed cells are added directly to a large volume of distilled water (Berstein et al., 1938). This fragments the membrane, but does not yield ghost that are completely free of hemoglobin (Hiller and Haffman, 1953). For the production of hemoglobin free ghost, the ionic strength and pH of hypotonic medium are very important, that still retain most, if not all, of the characteristics of the original cell membrane (Dodge, Mitchell and Hanahan, 1963). The forces greater than 1000 g are necessary to sediment and pack the ghosts, firmly enough to allow the removal of the supernatant without disturbing them.

In older methods ghost were prepared by the action of chemical lysins such as digitonin and saponin (Ponder, 1955), or 0.1% sodium chloride saturated with carbondioxide (Ponder, 1948) or successively freezing and thawing as proposed by Jorpes (1932). these techniques are failed to yield haemoglobin free preparations and can also fragment the cell membranes lowering the yield of intact ghosts. The hemolysis of the cells is also carried out by passing them through a series of successively more

dilute solutions of saline, starting with 100 mM, followed by 60 and then 30 mM solutions (Hillior and Hoffman, 1953) but this is time consuming procedure. Wood, Rood and Berg in 1963; found that the addition of 1 mM Na_2 EDTA to the hypotonic solutions enhances the removal of haemoglobin from the cells.

Dodge, Mitchell, and Hanahan (1963), have reported a method for producing ghosts from human red cells with only a single lysing medium. The solution consists of 20 milliosmolar phosphate buffer at pH 7.4 which controls the ionic strength and pH of the solution. According to Dodge and Co-workers three washes of this hypotonic medium are required to yield intact ghosts that are essentially free of hemoglobin, with no loss of membrane lipids.

Hemoglobin-free ghosts are prepared by hypotonic hemolysis at 4 ^{o}C in 40 volumes 10 mM Tris pH 7.4 and isolated by centrifugation at 16,000 g X 15 min (Hanahan and Ekholm, 1974). Dodge, Mitchell and Hanahans 1963). Method for ghost preparation have been modified by Steck and Kant (1974). According to them problem arises for thepersistance of a small volume of some what more dense, red coloured material due to successive washes. The method of Steck and Kant (1974) was applied by Burton, Ingold and Thompson 1981, so as to improve the above situation. In this method red blood cells are hemolyzed by rapid and through mixing with chilled 5 mM phosphate buffer pH 8.0. The ghost

pellet obtained after centirifugation and aspiration of supernatant is washed by resuspending it in 2.5 mM phosphate buffer pH 8.0 and centrifuging as before. The ghosts are washed a second time by an identical procedure using 1.25 mM phosphate buffer, pH 8.0. At this point, the ghosts are free of all visible red material.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is perticularly useful in the seperation of mixtures of lipids and is employed in many forms for the seperation of wide range of compounds open layer of adsorbent for chromatography was first used by Izmailov and Schraiber (1938) who placed thin loose layers of adsorbent on horizontal glass strips Membard and Hall (1949) avoided the problems associated with loose layers of adsorbent by using starch as a binding agent to produce cohesive films adhering to glass Kirchrer et al., (1951) extended the process by coating narrow glass strips with adsorbent bonded with starch or calcium sulfate. These chromatostrips permitted terpenes to seperated by ascending development. The wider plates are coated and used as a chrmatoplates to seperate several samples alongside each other (Reitsema, 1954). The rapid extension of thin layer chromatography must be attributed to the investigations of Stahl (1956, 1958a, 1958 b, 1969), who advised, tested and standardized a system for preparing plates by using special adsorbents. The

methods was applied to the many organic compounds and named the technique "thin layer chromatography". Since TLC has been widely accepted and many books and reviews have been written on the subject.

1 Adsorbents

Silica gel is the most frequently used adsorbent for the thin layer chromatography of lipids. In addition to this aluminium oxide, magnesium silicate, kieselguhr and hydroxyapatite also have been employed. Silica gel is natural or acidic it has a high adsorption capacity. The various adsorbents have been discussed by Mongold (1961). So as to produce thin firm, stable layers adhering to the glass, it is some times necessary to add binders such as starch and calcium sulfate hemihydrate (Plaster of paris) (Meinhard and Hall, 1949; Kirchner etal., 1951). In some cases 10% $MgSiO_2$ was added to the adsorbent and then the sample was mixed in ball mill for 25 min to insure even distribution of the components (Gary J.Nelson, 1967). Most frequently used adsorbent is the calcium sulfate because it is not affected by corrosive reagents such as the strong acids employed for the detection of spots. Special grades of adsorbents are available for preparative thin layer chromatography with properties desined to minimize cracking of thick layers during drying.

2 PREPARATION OF PLATES

The backing plates which are flat, rigid and uniform in thickness are used for spreading the thin layers. Glass does not react with corrosive detection reagents, so that the glass plates are commonly chosen. The dimensions of the glass plates have now become standerdized in the following sizes :5 X 20, 10 X 20, 20 X 20 and 40 X 20 cm. The thickness is often 2-4 mm, variations in this dimension affects the layer thickness, so that with the help of some spreading apparatus only a narrow range of total thickness is permitted.

There are various methods for the application of adsorbent layer, such as by using a water or by smoothening out the dry adsorbent or a solvent slurry which may be sprayed on, coated on by deeping, poured and labelled or spread on most frequently with an aplicator. The accepted std. thickness of the layer for qualitative seperation is 250 μm of wet slurry, which after drying will shrink to less than two third of its thickness layers of 1-5 mm are used for preparative separations of large amounts. According to Bazan and Joel, 1970 in order to take the advantage of both the types of seperation, layers have been spread with a thickness gradient changing from 1000 μm at the origin to 125 μm at the solvent front.

3 PREPARATION OF SLURRY

For the preparation of slurry ratio of adsorbent to water varies for different adsorbents and spreading techniques. The

ratio is usually 2 ml water, to 1 gm adsorbent for silica gel G. Reifer (1962) developed a most suitable system for rapid small scale thin layer chromatography. According to this method the layers are spread on two microscopic slides by depping them back to back in suspension of silica gel G. in chloroform methanol 2:1 . The plates should be allowed to stand until drying which causes the layer become opaque and white. Such plates require small container to seperate mixture and also only few minutes for solvent development. For adsorption chromatography on silica gel, the layers are usually activated by heating to 100 to 110 ^{o}C for one hour. Thin layer plates are commercially available with silica gel or aluminimum oxide layers coated into glass aluminium or plastic backing sheets.

4 SAMPLE APPLICATION

Relatively non polar solvents are utilized for the dissolution of lipids, so as to apply them to thin layers. Polar solvents will cause local deactivation of the adsorbent, allowing the applied spots of lipid to spread widely; this will results in poor resolution. The polar lipids will require chloroform or chloroform-methanol mixtures, where as neutral lipids such as esters and acylglycerols may be dissolved in petroleum ether samples were plotted by using a camag 200 ml (1 mm spot diameter) or by Naromat autospotter but frequently calibrated micropipettes are used. The sample is usually applied as a spot 1.5-2.5 cm from the edge of the layer.Loads of 10 to 500 µg of lipid per spot

are usually applied and should be dissolved in 1-50 µl of solvent. Low loading results in to better seperation of closely running compounds. High loading of the total mixture will be required to enable them to be detected.

5 SOLVENTS AND DEVELOPMENT

The selection of solvent system for thin layer chromatography involves a choice of solvent system in which the solvents are of required polarity and miscibility. Chromatograms may be developed by ascending, descending or radial flow of the solvent. Development can be carried out discontinuously using one pass or multiple passes of the same solvent, in a stepwise fashion with different solvents, with a continuously changing solvent composition gradient or by the so called continuous process, in which the solvent or the compounds are removed when they arrive at the distant end of the thin layer chromatographic plate.

The process may be carried out by two dimensional development of single spot of compound along axis at 90^{o}, employing a different solvent system in the second direction. The development is usually carried out in rectangular glass chamber with lids. They are just large enough to accept the plates, with the developing solvent as a shallow layer at the bottom. The development chamber must be saturated with solvent vapour before development of chromatogram and it is accomplished by lining the walls of the chamber with filter paper. Vapour saturation will

reduce development time by one-third, and will produce straighter solvent fronts and rounder spots.

6 DETECTION AND IDENTIFICATION

There are three common methods utilized for the detection of various lipid components after seperation- (a) Detecting agents may be added to the layer before development of the chromatogram b) The agents may be added after development of the chromatogram c) The radioactivity of isotopically labelled mixtures may be utilized to locate the positions of the zones. The most commonly used technique is the spraying of reagents on the layer after development. The reagents may be destructive or non-distructive may also be specific , reacting with specific lipid classes or nonspecific interacting with most lipids.

The first examination of an unknown mixture by thin layer chromatography should be made by non specific reagents which detect all poossible components on the layer. The approximate amounts of lipids especially miner components of mixtures, may be visually estimated by taking into account the amount of mixture applied and the sensitivity of the various detection reagents (Mangold, 1961; Sims and Larose, 1962; Vroman and Baker, 1965). Extremely high sensitivity of detection of steroids may be obtained through the fluorescence observed after spraying with sulfuric acid (Neher, 1964). In analytical thin layer chromatography the charring agents such as perchloric acid, phosphoric, chromic or sulfuric acids with or without oxidative agents are perticularly used for

detection of lipids.

The specific reagent especially iondine in the form of vapour or as chloroform solution is a good agent for detection of lipids, it is chiefly used in analytical work, but unsaturated fatty chains react with it. It can later be eliminated from the plates simply by subliming in vacuo and then specific sprays can be applied. A number of common phosphotides, neutral lipids and glycolipids have similar mobilities on silica gel G so that it is essential to differentiate at least between these two large families of lipids. There are number of several good ester sensitive reagents for neutral lipids, phosphorus specific reagents for phosphatides and carbohydrate reagents for glycolipids. The individual phosphatides again identified by using specific test reagents for choline, serine; ethanolamine, sphingosine and inositol, individual neutral lipids may also be identified by using specific test reagents for cholesterol free and ester forms glycerides and free fatty acids several authors have discribed critically the diagnostic importance and details of sprays in the thin layer chromatography (Marinetti, 1962; Skipski and Barclay, 1969; Renkonen and Varo, 1967; Krebs et al., 1969; [illegible], 1971).

Specific methods for quantitative analysis of neutral lipids

Neutral lipids like triglycerides, diglycerides and monoglycerides on the elutes from thin layer chromatogram, analysed quantitatively, by means of a hydroxamic acid calorimetric test for ester groups (Vioque and Holman, 1962 and Walsh et al., 1965).

Selected classes of neutral lipids, such as triglycerides, cholesterol esters, cholesterol and free fatty acids, analysed by standard chemical methods on elutes obtained after thin layer chromatographic seperation of tissue lipid extracts. (Angelico et al., 1965; Vacikova et al., 1962; Gloster and Fletcher, 1966; and Badzio and Boczon, 1966). Chloroform methanol (4:1 v/v) elutes all lipids (i.e. triglycerides, diglycerides monoglycerides, free fatty acids, cholesterol, cholesterol ester and hydrocarbons) as well as rhodamine 6G, which may interfere with titration. However diglycerides and triglycerides also fatty acids eluted by diethyl ether, and it gives complete recovery of these compounds. But it does not gives the complete recovery of monoglycerides, cholesterol and cholesterol esters.

When iodine vapours are used for detection of lipid bands, then it affects slightly the recovery of diglycerides and in much greater extent monoglycerides also, but cholesterol and cholesterol esters are fully recovered. The quantitative procedures for analysis of neutral lipids have been applied successfully by several workers.

b) PHOSPHOLIPIDS

There are several steps for quantitative determination of phospholipids by thin layer chromatography.

a) Direct digestion of phospholipids adhering to silica gel or after the elution of phospholipids from the silica gel and so that the phosphorus of phospholipids is converted into the

inorganic phosphate (b) seperation of phospholipids by thin layer chromatography and positive identification of individual lipid spots (c) quantitative determination of phosphorus in the digest (d) silica gel with adhering lipids removed quantitatively from chromatogram and this material is transfered into an appropriate container.

QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS ON THE ELUTES FROM THIN LAYER CHROMATOGRAMS

After development of chromatogram in the choosen solvent, the plate is air dried at room temperature,for about 15 to 20 minutes. The spots on chromatogram are detected with iodine vapours and encircled with a fine needle. Most of the iodine is allowed to evaporate before spot removal a small amount of iodine will not interfere with the phosphorus determination.

The areas of the silica gel above the solvent front and undneath the origin are removed with razor blade. Bigining with the origin of any one running lane, a drop of water is placed on the silica gel area to be removed for analysis and silica gel is transfered at once. To ensure complete transfer of material, the remmaining periphery of the spot is scrapped off and the plate is placed vertically and trapped to allow scrappings to fall into glazed paper. This powder is combined with the previously removed material.

The powder is suspended in tube containing eluting solvent by vigorously shaking the tube and each phospholipid is eluted from silica gel. The first and second elutions are performed with

the solvent mixture choloroform-methanol-acetic acid-water (25:15:4:2,v/v), by using 3 and 2 ml portions of it for each elution. To facilitate the elution process, the silica gel suspension in elution solvent is shaken for 10 minutes. Then, the silica gel is centrifuged and the elute is removed with capillary pipette. The third elution is performed with 2 ml of methanol and fourth with 2 ml of methanol-acetic acid-water (94:1:5, v/v). All the extracts are combined and assayed for phosphorus. Samples with an expected phosphorus content within the standard curve range (0.4-5.0 g) are transfered to the digestion tubes directly, those with greater phosphorus content are transfered to 10 ml Volumetric flask and a sample is taken for analysis. Prior to digestion all sample elutes are evaporated to approximately 2.0 ml or less, convenient volume of digestion.

PHOSPHORUS DETERMINATION

The most generally accepted technique for determination of phosphorus from lipids, on the molybdenum blue reaction has been suggested by Bell and Diosay (1920) and also further developed by Fiske annd Subbrow(1925).Later on there are many variations and modifications of the molybdenum blue method. Though the several methods which are in practice,basically modifications of Fiske and Subbaraw's method (Harri's and Popat, 1954; Bartlett 1954 ; Marinetti 1962;Shin,1962;Rouser 1966;Rosenthal and Chngttsien Han,1969). The perchloric acid digesction method of Bartlett is highly recommended (Skipski etal.,1964),recommended the combination

of the modified procedures of Beveridge and Johnson (1949) for digestion and colour development and Bartlett's suggestion for the measurement of extinction at the wavelength 830 m .

ACTUAL METHODS EMPLOYED IN THE PRESENT INVESTIGATION

After having taken a critical survey of thin layer chromatography blood sampling techniques for study of lipids, the following techniques are employed for the study of lipid alterations in the red cell membrane, of rat after 24, 48 and 72 hrs of UN toxication.

a) SAMPLING OF ANIMAL BLOOD

Blood is obtained by sacrificing the animal by heart puncture which recovers much of the total blood (Stubbs, 1953). Blood is taken in a 15 ml centrifuge tube by using isotonic citrate dextrose solution as an anticoagulant (Gibson and Evans, 1965; Wintrobe, 1967).

b) SEPERATION OF PLASMA

Centrifugation is the standard method for seperating cells from plasma. So that sample is centrifuged in a small clinical centrifuge and red cells were seperated from plasma for complete seperation of red cells centrifugation is carried out again in refrigerater ultracentrifuge at 750 g for 7 min (Nelson and Freeman, 1959; Dise, Goodman and Rasmussen, 1980).

c) ISOLATION AND PURIFICATION OF RED CELLS

Multiple washing is necessary to remove leukocytes and platelets from red cells. The red cells are washed in isotonic

saline solutions and centrifuged two times at 4^{o}C for 15 minutes each and at 2500 rpm. After each centrifugation buffy layer discarded to insure complete removal of leukocytes and platelates .

d) PREPARATION OF RED CELL GHOST

Red cell ghosts are prepared essentially according to method of Dodge et al., which is applied by John D.Turner in 1973 where cold hypotonic phosphate buffer 25 mosm having 7.4 pH is mixed 14:1 (v/v) with cells are centrifuged at 20,000 xg for 30 min, the supernatent decanted care fully process repeated three times and milky white ghosts are used for further processes.

2 BIOCHEMICAL TECHNIQUES

A) EXTRACTION OF LIPIDS

Folch's improved method (Folch et al., 1957) applied for extraction and purification of lipids. To our experience the Folch's method was found to be rapid, convenient and reproducible for extraction of lipids and also resulted in complete extraction of the lipids without degration.

The red cell ghosts are added in 10 volumes of chloroform-methanol (2:1 v/v) at room temperature, and mixture was kept as it is for 2 hrs at 4^{o}C and then filtered through the sintered funnel into a glass stoppered container, the filterate was shaken well with 0.2 volume of glass distilled water. Extracts were allowed to seperate into two distinct phases by centrifugation with the help of small clinical centrifuge. The upper phase which generally contains major part of the nonlipid contaminants was

removed as completely as possible with the helf of fine tipped pipette. The lower phase which mainly contains purified lipid fraction was transfered quantitatively into a glass stoppered container and evaporated in vacuo at 40 °C . The lipid samples thus obtained were weighed and preserved at 20° C until further use.

2 THIN LAYER CHROMATOGRAPHY OF NEUTRAL LIPIDS

A) PREPARATION OF PLATES

About 20 grams of silica gel G (About 200 mesh; containing $CaSO_4$ as a binder, E. Marck, Germany) was slurried with 40 ml of distilled water. The slurry was transferred to the applicator immediately and applied to the plates (20 X 20 cm). The applicater was set at 0.25 mm. The plates were activated by heating in a oven at 110-115° to one hour cooled and pressurved in a desicator for further use.

B) APPLICATION OF SAMPLE

The edges of the plates were trimmed of excess silica gel. To each chromatoplate lipid extracts of known composition dissolved in chloroform were applied with Hamilton Micro Syringe (No.8206-B) 2.5 to 3.0 cm from the bottom edge of the plates.

C) DEVELOPMENT OF CHROMATOGRAMS

The chromatographic chamber (length 25 cm, height 30 cm and width 10 cm) were prepared 20 minutes before the insertion

of the plates. The chambers were lined on three sides with Whatman No.3 filter paper wetted with developing solvent. One step development system was followed. The plates were developed in hexane;diethylether; acetic acid (85:15:2,v/v) as solvent system as recommended by Gloseter and Fletcher(1966).The solvent system was allowed to move approximately 13-15 cm from the bottom of the plate (approximately 40-50 minutes).The plates were dried at room temperature.

D DETECTION AND IDENTIFICATION OF SPOTS

Iodine vapour (Sims and Larose,Jocs,1962) 2'7'-dichlorofluorescein spray (0.2% ethanol) mangold and Malins, 1960; Mangold 1961, 40% H_2SO_4 spsray (Privett and Blank 1962 a; Skipski, et al., 1963),dichromate sulfuric acid spray (Blank et al., 1964) were used for general detection of all lipids.

Spots of extracts on chromatograms were identified according o their positions with respect to reference compoudns.Identification of individual neutral lipid component was carried out by using specific chemical tests directly on the plates. The detection of cholesterol was further confiormed by employing antimany trichlorode spray (Weicker 1959).

1 ELUTION OF CHOLESTEROL

For the elution of cholesterol chloroform-methanol(4:1, v/v) used as an elution mixture,5 ml of elution mixture was added

in the test-tubes containing silica gel with adhered lipid moiety the tube was shaken vigorously for ten minutes. The elutes were removed with pipettes. The elution procedure was repeated twice in the same manner and the three elutes were combined.

2 ANALYSIS OF LIPIDS IN THE ELUTES

Cholesterol was analysed by the method of Abell et al., (1952). The elutes from the cholesterol scrappings were evaporated to dryness.Lipids were redissolved in 10 ml chloroform. 5 ml of water were added and the tubes were shaken vigorously for one minutes. They were then centrifuged at slow speed for 5 minutes, until the emulsion broke and two clear layers were formed. A suitable aliquote of the petroleum ether layer was transfered to a small dry bottle. the petroleum ether was then evaporated by placing the bottles in a water bath at 60 C and blowing a gentle stream of air into them. After cooling to room temperature, the bettles were stoppered with clear dry corks and were taken for colour development with the Liebermann-Burchard reagent. The standard cholesterol solutions were also treated simultaneously identical to that of samples. the bottles containing dry residues from the samples and the standards and a clean empty bottle to receive blank were arranged in a series. 6 ml of the modified Lieberman-Burchard reagent were added first to the empty bottle and then at the regular interals to the other samples care was taken to wash down the entire inner surface of the bottle with the reagent. the bottles were tightly corked, shaken and returned

to the bath. The optical density of each sample was read against the blank in a spectronic 20 at 620 mμ , 30-35 minutes after the reagent was added.

3 THIN LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS

Schlemmer (1961) and Wagner et al., (1961) introduced the use of silica gel G and the solvent system of chloroform: methanol : water for the thin layer chromatography of phospholipids. The prominent phospholipid component phosphotidyl serine, is not properly seperated with the neutral chromatographic plates. For that purposse the use of basic plates has been recommended by Skipski et al., (1962). In present investigation seperation of phospholipids has been carried out on basic plates i.e. by preparing adsorbent slurry in 0.01 M Na_2CO_3 instead of water. Rest of the conditions for the preparation of plates and application of the samples were maintained as described earlier.

A) DEVELOPMENT OF CHROMATOGRAMS

For the seperation of phospholipids from red cell ghosts one dimentional one step thin layer chromatography was followed. The chromatographic chambers (length 28.5 cm, height 34 cm and width 11 cm) were lined on three sides with Whatman No.3 paper wetted with developing solvent.

During present investigation, solvent system recommended by Skipski for the seperation of phospholipids (Skipski et al., 1963) was slightly modified (Sawant et al., 1975) i.e. the

concentration of glacial acetic acid and water was lessened and the volume of methanol was maintained nearly half of the chloroform. So chloroform methanol-glacial acetic acid-water in a proportion 100 : 50 :10 : 4 (v/v) were found to form a suitable solvent system for the ghost. All the phospholipid components from the tissue samples were found to resolve by this solvent system and all the components were found to be quite distinct from each other without tailing.

B) DETECTION AND IDENTIFICATION

Phospholipids are detected on the dried plates by exposing the plates to iodine vapour (sims and Larose, 1962). The phospholipid spots were further identified by employing the molybdenum blue spray (Dittmer and Lester, 1964), Vasakovasky's modified spray (Vasakovasky and Kostesky, 1968). For general phospholipids, ninydrin (0.2% in acetone-lutidine, 9:1 v/v) (Skipski et al., 1962), Dragendroff reagent (Wagner et al., 1961). For choline phospholipids, P-benzoquinone for phosphotidyl ethanolamine, ammonium silver nitrate spray and mercuric oxide barium acetate spray for inositol, colox-benzidine spray for sphingomyelin (Bischel and Austin, 1963; Skipski et al., 1967). The diagnostic importance and details of these sprays in thin layer chromatography are described by Marinetti (1962) Krebs et al., (1969) and Skipski and Barclay (1969).

C) QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS

A) ELUTION OF PHOSPHOLIPIDS

The elution mixture utilized for phospholipids was mixture of chloroform-methanol-acetic acid-water (100:50:10:4,v/v), 2 to 3 ml

of elution mixture is added in a tube containing silica gel powder with phospholipids. Two elutions are performed with this mixture, the silica gel suspension in elution was shaken for 10 minutes to facilitate the elution process. The silica gel was centrifuged and the elute was removed with a pipette. The third elution was performed with 2 ml of methanol and the fourth with 2 ml of methanol-acetic acid-water (94:1:5,v/v). The samples then transfered to digestion tube. All the sample elute prior to digestion were evaporated to approximately 2 ml.

B) PHOSPHORUS DETERMINATIONS

For the determination of lipid phosphorus Bartlett's method (Bartlett, 1959) modified by Mrinetti (1962) was followed. Sample elutes were digested with 0.9 ml of 70% perchloric acid. Digestion was carried out for 15 minutes on a medium gas flame. The tubes were cooled 7.0 ml of distilled water, 1.5 ml of 2.5% ammonium molybdate and 0.2 ml of the amino napthol reagent described by Bartlett (1959) were added. Then the tubes were placed in boiling water bath for exactly seven minutes, removed and allowed to cool for 20 minutes. The optical densities were determined at 830 mμ . Approximate blanks and standards were also run simultaneously.

For the determination of lipid phosphorus from total lipids, Marinetti's semimicrophosphorus method was followed. This method was confirmed more suitable than the technique using amino-napthol-sulfonic acid in the semimicro analysis of the total phosphorus (Varute and Sawant, 1972). Digestion was carried out with 0.5 ml

of 70% perchloric acid and two drops of nitric acid for 15 minutes. After the flasks were cooled, 7.0 ml of distilled water were added. then 1.0 ml of 2.5% ammonium molybdate and 1 ml of elon reagent were added in each flask.The solutions were mixed and left at room temperature for 30 min. The optical density of each solution was determined at 820 mμ by using spectronic 20. The values of phospholipids were calculated in terms of μ mol per mg of proteins.

PROTEIN ASSAY

The membrane protein was determined by the method of Lowry et al., (1951) after solubilization in 1 N NaOH for 45 min.

RED CELL MORPHOLOGY

To study the corpuscular derangement the red cell morphology was observed in control rats as well as nephrotoxic rats by observing the peripherial blood smears in early initiation late initiation and maintenance phase.

HEMATOLOGIC PROFILE

Some of the hematological parameters such as haemoglobin concentration, red cell count, reticulocyte count was observed to find out the intensity of hemolysis under the toxic influence of Uranyl nitrate.