CHAPTER - II

MATERIALS AND METHODS

MATERIALS :

I) MAINTENANCE OF THE EXPERIMENTAL ANIMALS :

The albino rats (*R. norvegicus*) were received from Haffkins Institute, Bombay. They were weighing approximately 170 to 190 gms. They were kept in metalic cages, which were made up of galvanized tin sheets and were of size 14"x9"x6". Its cover was made up of iron mesh. Each cage contained one rat. The caging system included the maintenance of rats dry, clean and of relative thermal neutrality. Sufficient space was provided for free movements. Such environment proved to more condusive for the rats to mature and reproduce in a normal way and maintain good health. Their bedding was changed two to three times every week. The animal cage racks and accessory equipments such as feeders and water bottles were washed with soap powder and sanitized to avoid contamination. All cages were arranged in a room where sufficient ventilation and light were available.

The rats were fed daily with Lipton India Ltd. pellets diet according to the laboratory conditions (50gm/rat/day). Sufficient, clean and fresh water was provided.

II) CHEMICALS :

Uranyl nitrate hexahydrate [$UO_2(NO_3)_2.6H_2O$] & Bovine Serum albumin were obtained from BDH, England. Other chemicals used

were obtained from Qualigens, Bombay. The following chemicals were used : Uranyl nitrate hexahydrate - $[UO_2(NO_3)_2.6H_2O]$,

Anaesthetic ether,

Ethylene-diaminetetra acetic acid - (EDTA),

Sodium chloride - (NaCl),

Sodium hydroxide - (NaOH),

Copper Sulfate - $(CuSO_4.5H_2O)$,

Sodium tungestate - (Na_2WO_4),

Sodium tatarate - $([CH(OH), COONa]_2)$,

Sodium Molybdate - $(Na_2\ MoO_4.2H_2O)$,

Hydrochloric acid - (HCl),

Sodium carbonate - (Na_2CO_3),

Phosphoric acid - (H_3PO_4),

Lithium sulfate - $(Li_2SO_4.H_2O)$,

Bromine - (Br),

Tris(hydroxymethyl) - $[NH_2.C(CH_2OH)_3]$

Sodium dodecyl sulfate (SDS)

2-mercaptoethenol - $[CH_2\ 2(SH).CH_2.NH_2.HCl]$

Acrylamide (HI-MEDIA) $(CH_2{:}CH.CO.NH_2)$

Bis-acrylamide (HI-MEDIA),

Glycin $(NH_2.CH_2.COOH)$,

N,N,N',N' tetramethyl ethylene diamine (TEMED),

Ammonium per sulfate - $(NH_4)_2\ S_2O_8$

Bromophenol Blue (Romali-Batch No.110)

Glycerol $(CH_2.COH)$,$CH(OH)$, (H_2OH),

Coomassie Brilliant Blue, and

Acetic acid - (CH_3COOH),

All the chemicals used were of analytical grade.

METHODS :

I) DOSE PREPARATION OF URANYL NITRATE :

A) DOSE CONCENTRATION :

Many investigators have worked on Uranium poisoning by administrating Uranyl nitrate in different dose concentrations. The dose of UN in the present investigation was determined on the LD_{50} value (Venugopal et al., 1978). A sublethal dose of 5 mg/kg body weight of UN was choosen for the experimentation (Gojer and Sawant, 1986). The dose was prepared in 0.9% sodium chloride solution in such a way that each rat received a dose of 5 mg UN/kg body weight. The control rats were given an equivalent dose of saline.

For the administration of UN, separate and sterilized syringe with fine needle(No.26) were used. After the dose each rat was maintained separately.

B) ADMINISTRATION OF UN :

Xenobiotic substance when enters or is introduced into the animal body, usually after absorption from skin, lungs or gastro-intestinal tract they enter the blood stream. For the purpose of studying toxic effects of metals or any other chemical, toxicologists have identified routes for the insult

toxicants into the body. The most common are inhalation, intravenous, intraperitoneal, subcutaneous and intra-muscular routes.

In the inhalation route, the toxic effects were observed in pulmonary structure. The intravenous site of administration introduced the toxic material in the blood stream, thus producing acute toxicity. The intraperitoneal route of administration of toxicant is a very common procedure. It results in a rapid absorption of toxic material due to rich blood supply in the peritoneal region. Through the portal circulation, the toxic material is circulated and brought to kidney for excretion. The toxicants administered intramuscularly and subcutaneously are usually absorbed at slow rate.

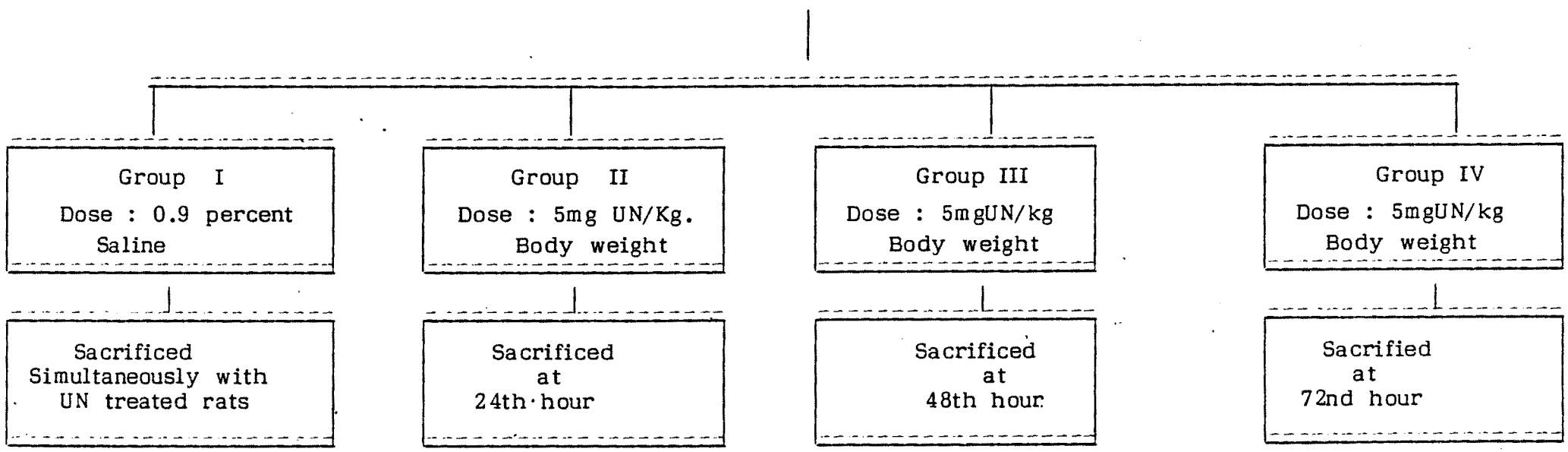
The intraperiotoneal route of administration of uranyl nitrate was employed in the present study in which maximum absorption of injected UN was possible.

C) EXPEIMENTAL PROTOCOL :

To determine the changes in the protein profile after uranyl nitrate intoxication, the duration of the doses were selected as 24th hour, 48th hour and 72nd hour.

EXPERIMENTAL PROTOCOL

Male Albino Rats (Rattus norvegicus)
Body Weight 170 - 190 grams
Pallet Feed, Water ad libitum.



The experiments were carried out in four groups;

GROUP I : Three normal male rats (170-190 g body wt) were maintained as control.

GROUP II : Three healthy male rats were given a dose of UN 5mg/kg body weight and they were scarificed after exactly 24th h.

GROUP III : Three healthy male rats were given a dose of 5mg UN/kg body weight and the rats were sacrificed at 48th hour.

GROUP IV : Three healthy male rats were given a dose of 5 mg UN/kg body weight and were sacrificed at 72nd hour.

EXPERIMENTAL PROTOCOL :

No.of groups	Experimental animal	Treatment of UN	Sacrification hour
I	Control	-	-
II	UN24	5 mg/kg body wt.	24
III	UN48	"	48
IV	UN72	"	72

D) COLLECTION OF BLOOD :

Immediately after the sacrifing animal, the blood was collected directly from ventrical of heart. It is collected

with the help of sterilized syringe, in a centrifuge tube. 0.1 ml EDTA, an anticoagulant was used to prevent coagulation of blood. It was prepared by dissolving 500 mg EDTA in 10 ml distilled water.

E) SEPARATIONOF PLASMA :

The collected blood was centrifuged at 3000 - 5000 g for 10-15 min. using research centrifuge and the plasma was collected by pasture pipettes.

F) PARAMETERS STUDIED :

1) Total proteins from the plasma were estimated by the method of Lowry et al. (1951).

2) Electrophoretic separation of Plasma proteins was done with SDS-Polyacrylamide gel electrophoresis method (Laemmli, 1970).

1) ESTIMATION OF TOTAL PROTEINS FROM PLASMA :

Estimation of total proteins from plasma was carried out as per Lowry et al. (1951) Method.

Out of the collected plasma 0.1 ml was diluted with 9.9 ml of 0.9% Saline and was used for the assay.

a) PREPARATION OF CHEMICALS :

i) Lowry's A : 20% sodium carbonate in 0.1 N NaOH

ii) Lowry's B : 0.5% Copper sulfate in 1% sodium tatarate pH 7.

iii) Lowry's C : 50 ml og Lowry's A + 1 ml of Lowry's B mix at the time of use.

iv) Folin - Ciocalten - Phenol - Reagent :

100 gm sodium tungstate

25 gm sodium molybdate

700 ml - distilled water

100 ml - Concentrated hydrochloric acid - HCl

50 Ml - 85% Phosphoric acid H_3PO_4

The above mixture was refluxed for 10 hours in Soxhlet apparatus.

To the refluxed mixture were added.

150 gm $\underline{\ }$ Lithium Sulfate

50 ml - Double distilled water

5 drops of Bromine water.

To mixture was boiled to remove excess of Bromine.

The boiled mixture was diluted to 1 N acid for use.

b) ASSAY OF TOTAL PROTEINS :

	Sample	Blank
i)	1 ml of Plasma Sample	1 ml of glass distilled water

ii)	5ml of freshly prepared Lowry's C	5 ml of freshly prepared Lowry's C

Mixed well and kept at room temperature for 10 minutes.

iii)	Add 0.5 ml of 1 N Folin-Ciecalten -Phenol-reagent	Add 0.5 ml of 1 N Folin-Ciecalten-Phenol-reagent

Mixed vigorusly and kept at room temperature for 30 minutes

Using blank the optical density of the sample was set 660nm. Using a standard graph of Bovine serum albumin the total amount of Proteins were determined.

III) **ELECTROPHORETIC ANALYSIS OF PLASMA PROTEINS** :

During the studies on molecules of chemical, biochemical or biological interest a separation process is a must, whether the objective is merely to analyse a complex mixture, to describe it's composition, or to separate the constituents for further examination. When the mixture involved is a natural product, such as plant and animal tissues or fluid the separation involves; atleast one of the various forms of electrophoresis. Since last two decades electrophoresis has rapidly evolved from low resolution method of relatively limited application, to a wide variety of analytical and small-scale preparative techniques of unrivalled resolving power and exceptional versality. The tremendous expansion of the method led to improvement and modification, variation, building of new equipment and new areas of exploitation were opened.

The study of biological material of high molecular weights e.g. Proteins, is hampered by difficulties arising at the time of separation and examination of the properties. For the study, it is necessary to cause as little damage as possible to the molecule at the time of separation and isolation. So that their properties are not changed significantly. Thus, current separation methods, e.g. electrophoresis lean heavily on processes which cause minimum disturbance to both physical and chemical properties, and retain the biological activity.

The term electrophoresis describes migration of charged ion under the influence of an electric field. This requires a medium and many factors such as size of particle, viscocity of the medium pH , ionic strength, heat etc. affect the electrophoretic separation pattern, even if the supporting medium such as paper, cellulose acetate, starch gel, polyacrylamide gel etc. are employed. Depending upon the supporting medium used different methods of electrophoresis are as follows -

A) PAPER ELECTROPHORESIS :

Although used in the past for the separation of proteins and other macromolecules, paper is not now-a-days considered suitable for this purpose because it is not very homogenous where pore size is concerned and also because some proteins are absorbed on the paper. The results obtained are broad bands with lack of

zone sharpness. For example, with normal blood serum only five protein bands can be seen distinguished as albumin, α_1-globulin, α_2-globulin, β-globulin and γ - globulin.

Today the use of paper as a medium for electrophoresis is confined to the separation of amino acids, peptides and nucleotides.

B) THIN LAYER ELECTROPHORESIS :

Almost all the analytical separations that can be achieved by electrophoresis on paper can also be done on cellulose or silica gel G. thin-layer plates in shorter time with greater case of handling and the power of resolution is also greater because of the more homogenous nature of thin-layer materials. The electrophoresis is carried out within a short time.

Thin layer electrophoresis (TLE) recently is applied successfully to the quantitation of amino sugars in acid hydrolysates of glycoproteins (Farwell and Dion, 1979).

All the mapping methods in TLE are performed at high and were not suitable for the separation of intact proteins. However this method can be applied as a small scale preparative procedure.

C) CELLULOSE ACETATE ELECTROPHORESIS :

Cellulose acetate is a much homogenous medium for electrophoresis than paper, has a more uniform pore size, and does

not absorb proteins. There is less trailing of protein zones and resolution is better.

A number of advantages are there is cellulose acetate electrophoresis than paper electrophoresis, but polyacrylamide gel is the medium capable of giving the highest degree of resolution particularly by using multiphasic buffer system. Such buffers are not readily utilized with cellulose acetate. Because of the advantages listed above the use of cellulose acetate is very popular in clinical laboratories.

D) STARCH GEL ELECTROPHORESIS

In the native state starch is made up of granules composed of mixture of anylase, which is a polymer of glucose units. When a relatively concentrated suspension of starch granules is heated in water or an aqueous buffer a viscous solution is formed which on cooling sets to a gel. This use of starch was pioneered by Smithies (1955). This method gave a dramatic improvement in resolution compared with earlier methods such as paper and it soon became widely adopted. It's use led to the discovery of genetic variation in blood serum proteins.

Unfortunately being a natural product the product of composition of starch can vary and it may contain differing proportions of amylase and amylopectin which can affect its gelling

ability and resolution. The concentration of starch can be altered somewhat to give gels of various pore size with different degrees of molecular sieving, but it is very difficult to achieve this. A further disadvantage is that quantitative measurement by densitometry is difficult and rather inaccurate.

E) ZONE ELECTROPHORESIS IN FREE SOLUTION : DENSITY GRADIENTS AND SEPHADEX COLUMNS

Electrophoresis in the absence of any inert supporting material is accompanied by considerable diffusion and is now-a-days virually confined to separation of charged particles such as intact cells, colloidal particles etc. This method requires the use of a stationary solvent phase with a density gradient, as well as greatly reducing mixing by convention also results in reduced diffusion owing to the increased viscosity of the medium.

F) TWO DIMENSIONAL MAPPING

TWo-dimensional maps (Finger-prints) were accepted and used for many years in studies involving the structure of proteins or their modifications. The two dimensional maps of intact proteins, nucleic acids, or polysaccharides can be most useful for characterization of tissues, biological fluids, extracts of tissues and organs or any other unknown mixture containing these constituents. They are also potentially useful, for identification

purpose (e.g. taxonomy, forensic work etc.), for studying genetic variations and relationships, for the detection of stages in cellular differentiation and studies of growth cycles, for the examination of pathological stages and the diagnosis of disease and for many other purposes.

Unfortunately histories and other basic proteins are generally not well separated by O'Farrell's method (1975) and use of SDS were usually accompanied by a complete loss of any enzymatic activity, so specific detection methods relaying on such activity are of no use.

G) ISO-ELECTRIC FOCUSING (IEF) AND ISOTACHOPHORESIS (ITP)

These methods are specifically intended for the fractionation of molecular species differing only in net charge. IEF is analytical method of very high resolution. ITP can be used for analytical experiments but resolution is usually less good than IEF or PAGE.

The major disadvantage of the IEF method is lack of solubility of some macromolecules at their isoelctric pH's and loss of enzymatic or biological activity, which may occur owing to low ionic strength conditions. So used preparatively on a relatively small scale.

H) AFFINITY ELECTROPHORESIS :

In this technique sample constitutents, usually proteins are separated not only by their mobility differences under the influences of an applied electric field, but also at the same time by interactions between the sample and specific affinity ligands.

When gelation occur in this process, the ligands formed are physically trapped within the gel. When small ligands are used which would not be trapped in this way, they should either be uncharged so that they do not migrate out of the gel during electrophoresis, or else they should be included in the electrode buffer so that the concentrations within the gels remain at a constant level. This is the only disadvantage.

I) IMMUNO-ELECTROPHORETIC TECHNIQUES

These techniques consist of a combination of an electrophoretic step with the formation of precipitates of antigen-antibody complexes. Single and two dimensional procedures can be used, and with different ways of applying samples. Many of these procedures rely upon the migration of antigens through or into an antibody containing gel. Buffers and pH values are usually chosen so that only the antigens migrate and antibodies either do not move at all or at most migrate very slowly and remain evenly distributed throughout the gel during the whole electrophoresis.

This method is particularly valuable as very small amounts of antigen and antibody are required. Though this method is very attractive for screening work, it's use is confined to a purely qualitative role in the detection of unknown antigens or antibodies. The interpretation of immunodiffusion patterns become rather difficult when complex mixtures are analysed with multi-specific antisera.

J) POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Modified electrophoretic method is polyacrylamide gel electrophoresis which employ a supporting medium. The purpose of a supporting medium is to cut down convection currents and diffusion so that the separated components remain as sharp zones with maximum resolution between them.

1. Pore size effect :

In gel media the passage of any particle is hindered by the structure of the matrix, the extent depending upon the relative sizes of the particle. Pores in the gel network. Therefore both molecular size and charge play a role in the separation process.

2. Chemical Properties of Polyacrylamid gel :

The polymerization of acrylamide monomers can be initiated by natural light so both acrylamide and Bis

should be stored in brown bottles. A low storage temperature (e.g. 4°C) is beneficial. Solid monomer and Ammonium per sulphate are quite stable under these conditions but solutions are less stable and should not be kept for more than 1-2 months. N,N,N',N'-tetramethyl-ethylenediamine (TEMED) is also light sensitive and should be stored in the dark. Both monomers (acrylamide and Bis) are highly toxic and exhibit toxicity via skin absorption or by inhalation. Once polymerization has occurred the resulting gels are relatively non-toxic and can be handled safely.

3) Suitable gel and buffer system :

A mixture of proteins or nucleic acids to be analysed by PAGE almost always consists of molecules differing both in size and in net charge and by these characteristics they can be separated into a number of distinct zones or bands. Many factors such as conditions of pH, concentrations of acrylamide and Bis, ionic strength, potential gradient, running time etc play role in the separation. For the optimum separation number of preliminary runs are carried out. The nature of sample itself usually provides some guide to the conditions which are likely to give good separation. For example, if one is examining mixture of high molecular weight proteins one would choose a large pore gel.

If some components of the mixture are unstable or precipitate at particular pH value, then this should be avoided during separation. It is difficult to determine the precise pH conditions especially when using multiphasic buffers. As a generatization, basic proteins such as histones are best separated at acidic pH values, while the majority of proteins which have isoelectric point within the pH range 4-7.5 and hence are weakly acidic tend to separate best in alkaline gel in the pH range 8-9.

4) Slabs or Cylindrical gels :

With harizontal slabs it is usual for sample to be applied by cutting a slot in the gel into which is inserted a small piece of filter paper having a known valume of sample solution. This has the advantages that the sample can be placed anywhere in the slab and harizontal slabs can be run on simple inexpensive apparatus.

Vertical gels either slabs or rods are easier to use with multiple buffer systems and only components moving towards one electrode are seen. With a gel rod apparatus each sample is run on a separate rod, so for an accurate comparison of different samples conditions must be identical, in all rods throughout the experiment.

5) Gel Formation and Pre-electrophoresis treatment :

The polymarization of monomers requires a free radical mechanism which is initiated by the addition of catalysts. A variety of these have been used, but the most common is ammonium persulphate which produces free - oxygen radicals by base-catalysed mechanis-m. The base most often used to catalyse this reaction is N,N,N',N,-tetramethylethylene-diamine (TEMED).

6) Sample application and the electro-phoresis

It is important to use comparitively small volumes of sample - e.g. 5-25 µl for 5mm diameter gel rod. With most vertical apparatus the sample is applied through buffer in the upper electrode chamber using microsyringe. So sample must be of a higher density than the buffer. This is achieved by adding small amounts of (2-10 per cent) of sucrose to the sample. It is common practice to add a very small amount of tracking dye, usually Bromophenal Blue for alkaline buffer system, to the sample. The purpose of tracking dye is to migrate at a higher rate than any of the macromolecular components in the sample and to indicate when electrophoresis should be terminated. The position of tracking dye is also useful as a reference point and for the purpose of calculating mobilities of components.

7) Gel staining :

Once the tracking dye has reached the desired position the electric power is switched off and the gel is removed from the apparatus and for bathed by suitable stain for localization of separated components.

IV) POLYACRYLAMIDE GEL ELECTROPHORESIS IN THE PRESENCE OF DETERGENT

Many scientists wishing to study the components of tissues, cells or membranes faced the problem of solubilizing the material for further investigations. The method of using detergents such as cationic detergent cetylpyridinium chloride (CPC) and anionic detergent e.g. sodium dodecyl sulphate (SDS) was most suitable. It is beneficial if the solubilized material can then be utilized directly for further procedures.

V) THE USE OF SODIUM DODECYL SULPHATE (SDS) FOR MOLECULAR WEIGHT MEASUREMENT

Methods for measurement of molecular size by gel electrophoresis fall into two main categories, namely those making use of a relationship between mobility and gels of various concentrations and those for which a simple gel concentration is used. The latter is applicable only to a series of molecules with

the same molecular charge density. Thus the use of SDS for molecular weight measurement is an alternative approach to the methods of molecular weight measurement which rely upon a mathematical or physical cancelling of charge effects during electrophoretic separation is to cancel out differences in molecular charge chemically so that all components then migrate solely according to size.

On treatment with an ionic detergent such as SDS surprisingly large amount of components are bound. For proteins the figure of about 1.4g. SDS per gram of protein is often quoted (Pitt-Rivers and Impiombato 1968; Fish, Reynolds, and Tanford 1970). Thus electrophoretic migration is proportional to the effective molecular radius or approximately to the molecular weight of the polypeptide chain. (Shapiro, Vinuela and Naizel 1967). The relationship with some exceptions does hold true for a very large number of proteins (Webber and Osbarn, 1969) and the method has become one of the most widely used for measurement of protein molecular weights.

David M. Neville, Jr.(1971) used discontinuous buffer system to determine the molecular weights of Protein-dodecyl sulfate complexes. This system utilizes a sulfate borate discontinuity which stacks and unstacks protein-SDS complexes over a range of 2,300 to 320,000 daltons providing high resolution fractionation.

A double-lable two dimensional gel electrophoresis procedure specially designed for serum plasma protein analysis (Wheeler, T.T.; Loong, P.C.; Jordan, T.W. and Ford H.C. 1986) is economical because small quantities of inexpensive isotopes are used.

The SDS polyacrylamide gel electrophoresis was followed as this procedure gives good results.

2) **METHOD OF SEPARATIONOF PLASMA PROTEINS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS** :

i) Plasma Sample :

100 µg of proteins, 10% glycerol.

2% sodium dodecyl sulfate,

5% 2-mercapto ethenal,

0.001 % Bromophenal blue,

0.0625 M Tris-HCl buffer of pH 6.8

This mixture was kept at 65°C for 10 min. before loading.

ii) Upper reservoir buffer (Cathod)

0.025 M Tris

0.192 M glycine

0.1% sodium dodecyl sulfate (SDS)

pH 8.6

iii) Stacking gel buffer :

0.125 M Tris adjested to pH 6.8

with HCl

0.1 % SDS

pH 6.8

iv) Running gel buffer :

0.375 M Tris adjested to pH 8.8 with HCl

0.1 % SDS

pH 8.8

v) Lower reservoir buffer (anode) :

0.025d M Tris

0.192 M glycine

0.1% SDS

pH 8.6

vi) Stacking gel (T = 3.08%, C =2.6%)

3 gm Acrylamide

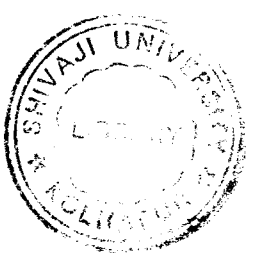
0.080 gm Bis-acrylamide

Buffer to 100 ml

0.025 ml TEMED

0.025 gm Ammonium per-sulfate

pH 6.8



vii) Running gel (T=7.5%, C=3%) :

10 gm Acrylamide

0.267 gm Bis-acrylamide

Buffer to 100 ml

0.025 ml TEMED

0.025 gm Ammonium per sulfate

pH 8.8

viii) Stain :

Coomassie Brilliant Blue in

Methanol, acetic acid, and water

in 5:1:4 v/v ratio.

ix) Acetic acid :

7%

VI) PROCEDURE FOR CARRYING OUT ELECTROPHORESIS

At first the gel tubes of similar length (10 cms) and diameter (4-5 mm) were chosen and fixed vertically in stand. The running gel was poured upto first mark, taking precaution to avoid bubble formation. Few drops of water were added to prevent the miniscus formation and facilitation of the polymerization. The solution was then allowed to polymerise.

After polymerization, water was removed and stacking gel was added to the gel tubes upto the second mark and again water was added and the gel tubes were allowed to stand for polymerization.

After polymerization was complete water was thoroughly removed and the gel tubes were then fixed vertically in the electrophoretic tank. Upper and lower reservoir buffers were added to their respective parts in the electrophoretic tank so that the gel tubes were immersed in the reservoir buffer at both the ends. Each gel tube is then loaded with 2.5 µl sample.

The electrophoresis was run at 3mA per gel tube until the tracking dye reached the bottom of the tube. The gel tubes then were removed from the electrophoretic tank with the help of syringe filled with used reservoir buffer.

The gels were stained with Coomassie Brilliant Blue for 10-12 hours. The destaining was carried out with 7% acetic acid until the protein bands were clearly seen. The gels were then preserved in 7% acetic acid.

The scans were obtained from Shimadzu spectrodensitometer at 595 nm with speed 1:24 specimen drive ratio in 0 to 1 optical density range. The relative mobilities of the separated proteins were calculated (Weber and Osborn, 1969) by formula.

$$\text{Mobility} = \frac{\text{Distance of Protein migration}}{\text{Length of gel after staining}} \times \frac{\text{Length of gel before staining}}{\text{Distance of dye migratio}}$$

From the mobilities of standard proteins, molecular weights were calculated as per Weber and Obsorn (1969) Table-2 and Table-3.

The percent quantity of each protein was estimated as per Webber and Osborn (1969). The alterations in the total plasma proteins content in control and after the uranyl nitrate intoxications during various periods were noted down.