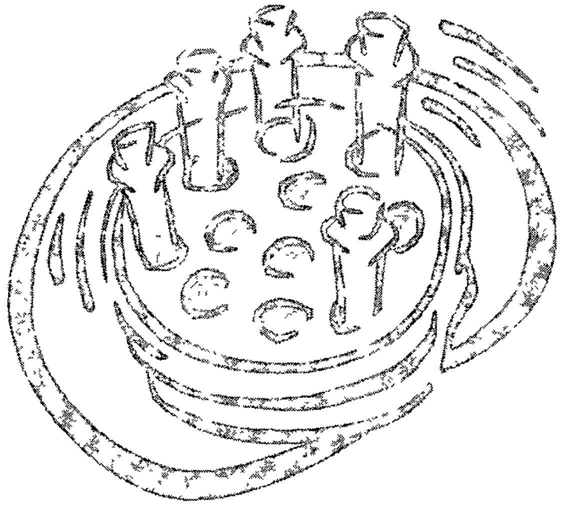
CHAPTER – 3



MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

Adult albino rats (Wistar strain) of either sex weighing 200-250gm were used in the entire study. Each pair of animal was housed in a spacious polypropylene cage containing wood shavings as nesting material, which was maintained at 25 ± 3°C in a well-ventilated animal house under natural photoperiod condition.

3.1.2 Sources of Fine Chemicals

I) 1,1,3,3-tetraethoxy propane, cumene hydroperoxide, octadecandinoic acid conjugated methyl ester, crystalline beef liver catalase, superoxide dismutase, urethane and egg lecithin, were obtained from Sigma Chemicals, St. Louis, M.O., U.S.A.

II) Thiobarbituric acid, tris-buffer, α-tocopherol acetate, cadmium acetate, sucrose, ATP, ferric chloride hexahydrate, triolein, NAD, reduced glutathione, NADP, G-6-PD, digitonin, AMP, 1-amino-2-naphthol-4-sulphonic acid, 5,5'-dithiobis (2-nitro benzoic acid) and bovine serum albumin were obtained from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

III) Lead acetate, cadmium chloride, ascorbic acid, trichloroacetic acid, potassium iodide, acetic acid, cyclohexane, ammonium molybdate, acetylacetone, perchloric acid, alanine, 2-oxoglutarate, 2,4-dinitrophenyl hydrazine, sodium pyruvate, aspartic acid, disodium phenyl phosphate, citric acid monohydrate, sodium nitrate, sulfanilic acid, bilirubin, lithium lactate, hydrogen peroxide, potassium dichromate, triethanolamine, sodium phosphate, copper sulphate, sodium potassium tartarate, sodium metaperiodate and xylene, were obtained from S.D. Fine Chemicals, Mumbai, India.

IV) Sodium hydroxide, sodium carbonate, sodium bicarbonate, magnesium chloride, manganous chloride, sodium chloride, potassium chloride, calcium chloride, potassium hydroxide, sodium dihydrogen orthophosphate, potassium dihydrogen orthophosphate, chloroform, methanol, hydrochloric acid, sulphuric acid, nitric acid, Folin's phenol reagent, phenol, glacial acetic acid and isopropanol were purchased from Qualigens Chemicals Ltd., Mumbai, India.

V) Ethylenediaminetetraacetic acid disodium salt, glycine, epinephrine bitartarate, cholesterol, were obtained from BDH Chemicals, Mumbai, India.

VI) *Spirulina fusiformis* was obtained commercially from Indon Healthcare Ltd., Aslali, Ahmedabad, India.

All the reagents and chemicals used in the entire study were of analytical grade.

3.2 EXPERIMENTAL DESIGN

The study was designed in two sets (lead and cadmium) of experiments. Each set of experiment was divided into biochemical, pharmacological and toxicological studies. After **30 days** of several treatments in rats as mentioned below, various investigations were carried out (page 91-117).

SET – I: LEAD ACETATE

BIOCHEMICAL STUDY:

Group 1 (Control)	Animals received double de-ionized water (DDW) and a normal diet.
Group 2 (10LA)	Animals received 10 ppm of lead acetate dissolved in DDW along with normal diet.
Group 3 (30LA)	Animals received 30 ppm of lead acetate dissolved in DDW along with normal diet.
Group 4 (100LA)	Animals received 100 ppm of lead acetate dissolved in DDW along with normal diet.

PHARMACOLOGICAL STUDY:

Group 1 (Control)	Animals received double de-ionized water (DDW) and a normal diet.
Group 2 (100LA)	Animals received 100 ppm of lead acetate dissolved in DDW along with normal diet.
Group 3 (E)	Animals received vitamin E mixed with food and DDW.
Group 4 (E+100LA)	Animals received vitamin E mixed with food and 100 ppm lead acetate in DDW.
Group 5 (C)	Animals received vitamin C mixed with food and DDW.
Group 6 (C+100LA)	Animals received vitamin C mixed with food and 100 ppm lead acetate in DDW.
Group 7 (SP)	Animals received *spirulina* mixed with food and DDW.
Group 8 (SP+100LA)	Animals received *spirulina* mixed with food and 100 ppm lead acetate in DDW.

TOXICOLOGICAL STUDY:

The deposition of metal in liver, kidney, lung, heart and brain along with histopathological changes in these organs were investigated in animals exposed to lead acetate alone and in combination with vitamin E, vitamin C or *spirulina.*

SET – II: CADMIUM CHLORIDE

BIOCHEMICAL STUDY:

Group 1 (Control)	Animals received double deionized water (DDW) and a normal diet.
Group 2 (10CD)	Animals received 10 ppm of cadmium chloride dissolved in DDW along with normal diet.
Group 3 (30CD)	Animals received 30 ppm of cadmium chloride dissolved in DDW along with normal diet.
Group 4 (100CD)	Animals received 100 ppm of cadmium chloride dissolved in DDW along with normal diet.

PHARMACOLOGICAL STUDY:

Group 1 (Control)	Animals received double deionized water (DDW) and a normal diet.
Group 2 (100CD)	Animals received 100 ppm of cadmium chloride dissolved in DDW along with normal diet.
Group 3 (E)	Animals received vitamin E mixed with food and DDW.
Group 4 (E+100CD)	Animals received vitamin E mixed with food and 100 ppm cadmium chloride in DDW.
Group 5 (C)	Animals received vitamin C mixed with food and DDW.
Group 6 (C+100CD)	Animals received vitamin C mixed with food and 100 ppm cadmium chloride in DDW.
Group 7 (SP)	Animals received *spirulina* mixed with food and DDW.
Group 8 (SP+100CD)	Animals received *spirulina* mixed with food and 100 ppm cadmium chloride in DDW.

TOXICOLOGICAL STUDY:

The deposition of metal in liver, kidney, lung, heart and brain along with histopathological changes in these organs were investigated in animals exposed to cadmium chloride alone and in combination with vitamin E, vitamin C or *spirulina.*

Solutions of 10 ppm, 30 ppm and 100 ppm of lead acetate or cadmium chloride were prepared by dissolving them in double de-ionized water and given as *ad libitum* (Schroeder and Vinton, 1962).

Vitamin E, in the form of α-tocopherol acetate (50 IU/kg/day), Vitamin C, as ascorbic acid (800mg/kg/day) and *spirulina* (1500 mg/kg/day) were given by mixing them with food. The dose of vitamin E, vitamin C and *spirulina* selected in this study for the various treatments is based on the earlier reports by several authors (Sleet and Soares, 1979; Krajcovicova-Kudlackova and Ozdin, 1995; Paritha-Ithayarasi and Shyamala Devi, 1998; Suzuki and Yoshida, 1979 and Murugan, 1995). A standardized food consisting of untreated seed rye flour (60%), powdered skimmed milk (30%), corn oil (9%), mineral premix (0.7%) and added vitamin premix (0.3%) was given throughout the study (Schroeder et al., 1963).

3.2.1 Investigations

The following parameters were investigated after thirty days of treatment with lead or cadmium alone and its combination with vitamin E, vitamin C or *spirulina*.

In Tissues

Name of Investigation	**Liver**	**Kidney**	**Lung**	**Heart**	**Brain**
1. Malondialdehyde formation	✓	✓	✓	✓	✓
2. Hydroperoxide formation	✓	✓	✓	✓	✓
3. Conjugated diene formation	✓	✓	✓	✓	✓
4. Superoxide dismutase	✓	✓	✓	✓	✓
5. Catalase	✓	✓	✓	✓	✓
6. Reduced glutathione	✓	✓	✓	✓	✓
7. Na^+-K^+-ATPase	✓	✓	-	-	-
8. Ca^{++}-ATPase	✓	✓	-	-	-
9. Mg^{++}-ATPase	✓	✓	-	-	-
10. Inorganic phosphorous	✓	✓	✓	✓	-
11. Total proteins	✓	✓	✓	✓	✓
12.Glucose-6-phosphate dehydrogenase	✓	-	-	-	-
13. Cholesterol	✓	✓	✓	✓	-
14. Triglyceride	✓	✓	✓	✓	-
15. Phospholipid	✓	✓	✓	✓	-
16. Metals	✓	✓	✓	✓	✓

In Serum

1. Serum glutamate pyruvate transaminase
2. Serum glutamate oxaloacetate transaminase
3. Alkaline phosphatase
4. Acid phosphatase
5. Lactate dehydrogenase
6. Total bilirubin

7. Total protein
8. Cholesterol
9. Triglyceride
10. Phospholipid

Apart from these, body weight, organ weight, histopathological studies were also carried out.

3.2.2 Removal and Processing of Serum and Tissues for Various Investigations

At the end of 30 days, animals were anaesthetized with urethane (120mg per 100gm, i.p.). Blood was collected directly from the aortic bifurcation without any anticoagulant and allowed to clot for 10 minutes at room temperature. It was then centrifuged at 2500 X g for 20 minutes. The serum obtained was kept at 4°C till further use.

Reagents

1. *Sucrose solution (0.25M)*

   85.58gm of sucrose was dissolved in 200ml of water and diluted to 1000ml with distilled water.

2. *Tris hydrochloride buffer (10mM pH 7.4)*

   1.21gm Tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

Procedure

Liver, lung, heart and kidney were isolated carefully in cooled petridishes. The organs were blotted free of blood and tissue fluids, weighed on a Single Pan Electronic Balance (Precisa 205ASCS) and the weights were recorded. The organs were cross-chopped with surgical scalpel into fine slices, suspended in chilled 0.25M sucrose solution and quickly blotted on a filter paper. The tissues were then minced and homogenized in chilled 10mM Tris-HCl buffer (pH 7.4) to a concentration of 10% w/v with 25 strokes of Teflon pestle in glass homogenizer at a speed of 2500 rpm. Prolonged homogenization under hypotonic condition was designed to disrupt, as far as possible, the structure of the cells so as to release soluble proteins. Suitable aliquots of homogenates were taken out separately for the estimation of metals by atomic absorption spectrophotometry. The remaining homogenates were then centrifuged at 10,000 X g at 4°C for 20 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assay of lipid peroxidation, endogenous antioxidants and Glucose-6-phosphate dehydrogenase. The sediment was re-suspended in ice-cold Tris-HCl buffer (pH 7.4) to get a final concentration of 10% w/v and was used for the estimation of different membrane bound enzymes and proteins.

3.3 TISSUE ESTIMATIONS

3.3.1 Determination of Lipid Peroxidation

3.3.1.1 Lipid peroxidation or malondialdehyde (MDA) formation

It was estimated using the method described by Slater and Sawyer (1971).

Reagents

1. *Thiobarbituric acid (0.67% w/v, in Tris-HCl 1M, pH 7.0)*

   0.67gm of thiobarbituric acid was dissolved in 50ml of Tris-HCl and the final volume was adjusted to 100ml with Tris-HCl buffer pH 7.0.

2. *Trichloroacetic acid (10% w/v)*

   10gm of trichloroacetic acid was dissolved in 60ml of distilled water and the final volume was adjusted to 100ml with distilled water.

3. *Standard Malondialdehyde stock solution (50mM)*

   A standard malondialdehyde solution was prepared by mixing 1.10ml of 1,1,3,3-tetraethoxy propane up to 100ml with tris buffer.

   1.0ml of this stock solution was diluted up to 1000ml to get a working standard solution containing 50nM of malondialdehyde/ml.

Procedure

2.0ml of the tissue homogenate was added to 2.0ml of 10% w/v trichloroacetic acid and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitates were separated by centrifugation and 2.0ml of clear supernatant solution was mixed with 2.0ml of thiobarbituric acid. The resulting solution was heated in a boiling water bath for 10 minutes. It was then cooled in an ice bath for 5 minutes for color development. The absorbance was measured at 532nm against reagent blank.

The values are expressed as nM of MDA formed /mg protein.

3.3.1.2 Hydroperoxide (HYPX)

Tissue hydroperoxide was estimated as described by Buege and Aust (1984). Oxidation of iodide by peroxides is a convenient method for determining the amount of lipid hydroperoxides in a membrane sample. The procedure is based on the ability of iodide to reduce hydroperoxides with the formation of malondialdehyde.

Reagents

1. *Potassium iodide solution (1M)*

   16.6gm of potassium iodide was dissolved in 60ml of distilled water and the volume was adjusted to 100ml with distilled water.
2. *Cadmium acetate solution (0.5% w/v)*

   0.5gm of cadmium acetate was dissolved in 50ml of distilled water and the final volume was made up to 100ml with distilled water.
3. *Chloroform-methanol solvent mixture (2:1)*

   2 parts of chloroform and 1 part of methanol were mixed together.
4. *Acetic acid-chloroform solvent mixture (3:2)*

   3 parts of acetic acid and 2 parts of chloroform were mixed together.
5. *Cumene hydroperoxide solution (1M)*

   1.52ml of cumene hydroperoxide was added to 10ml of chloroform and was solubilized using cyclo mixer.

Procedure

1ml of tissue homogenate was mixed thoroughly with 5ml of chloroform-methanol mixture in screw capped tube and purged with nitrogen followed by centrifugation at 100 X g for 5 minutes to separate the phases. Upper layer was removed and 3ml of the lower chloroform layer was placed in a test-tube and taken to dryness by heating it on a water bath. After drying, 1ml of acetic acid-chloroform mixture, followed by 0.05ml of potassium iodide solution were quickly added. The test tubes were stoppered and the contents were mixed. The samples were placed in dark at room temperature exactly for 5 minutes, and 3ml of 0.5% cadmium acetate solution was added. The solution was mixed and centrifuged at 1000 X g for 10 minutes. The absorbance of the upper phase was determined at 353nm against a reagent blank containing the complete assay mixture except the lipid.

Standardization of the above reaction was done using cumene hydroperoxide as the peroxide standard at a molar extinction coefficient of 1.73 X 10^4 per molar solution.

The values are expressed as nM of HYPX formed/mg of protein.

3.3.1.3 Conjugated diene (CJDN)

The procedure given by Recknagel and Ghoshal (1966) was used for the assay of conjugated dienes.

Reagents

1. *Chloroform-methanol solvent mixture (2:1)*

   2 parts of chloroform and 1 part of methanol were mixed together
2. *Cyclohexane*
3. *Standard Octadecandinoic acid methyl ester (OCME)*

   0.294ml of OCME was mixed up to 100ml with cyclohexane.

Procedure

1ml of tissue homogenate was extracted with 5ml of hot chloroform-methanol solvent mixture. It was added with 2.5ml of chloroform-methanol mixture followed by 1.25ml of distilled water. The resulting biphasic system was mixed and centrifuged at 1000 X g for 5 minutes. The recovered chloroform layer was evaporated, the recovered lipid residue was dissolved in 1.5ml of cyclohexane, and the absorbance was determined at 233 nm, against a cyclohexane blank. The approximate amount of conjugated diene produced was calculated using a molar extinction coefficient of 2.52 X 10^4 per molar solution.

The values are expressed as nM of CJDN formed/mg protein.

3.3.2 Assay of Endogenous Antioxidants

3.3.2.1 Superoxide dismutase (SOD) (Superoxide: Superoxide oxido reductase, EC-1.15.1.1)

Superoxide dismutase was estimated using the method developed by Misra and Fridovich (1972).

Reagents

1. *Carbonate buffer (0.05M, pH 10.2)*

   16.8gm of sodium bicarbonate and 22gm of sodium carbonate was dissolved in 500ml of distilled water and the volume was made up to 1000ml with distilled water.

2. *Ethylenediaminetetra acetic acid (EDTA) solution (0.49M)*

   1.82gm of EDTA was dissolved in 200ml of distilled water and the final volume was adjusted to 1000ml with distilled water.

3. *Epinephrine solution (3mM)*

   0.99gm of epinephrine bitartarate was dissolved in 100ml of distilled water and the final volume was adjusted to 1000ml with distilled water.

4. *Superoxide Dismutase standard (SOD) (100 U/L)*

   1mg (1000 U/mg) of SOD from bovine liver was dissolved in 100ml of carbonate buffer.

Procedure

0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol, and 0.15ml of ice-cold chloroform were added. The mixture was mixed well using cyclo mixer for 5 minute and centrifuged at 2000 rpm. To 0.05ml of supernatant, 1.5ml of carbonate buffer and 0.5ml of EDTA solution was added. The reaction was initiated by the addition of 0.4ml of epinephrine and the change in optical density/minute was measured at 480nm against reagent blank.

SOD activity was expressed as units/mg of protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken the enzyme unit (M Kat Units/mg protein). Calibration curve was prepared by using 10-125 units of SOD.

3.3.2.2 Catalase (CAT) (Hydrogen peroxide: Hydrogen peroxide Oxido reductase, EC-1.11.1.6)

The procedure described by Sinha (1972) was used for the assay of catalase.

Reagents

1. *Hydrogen peroxide solution (0.2M)*

   2.26ml of (30% w/v) hydrogen peroxide was diluted up to 100ml of distilled water.

2. *Dichromate acetic acid reagent*

   5% w/v of potassium dichromate was prepared in dilute acetic acid (1:3 v/v in distilled water).

3. *Phosphate buffer (0.01M, pH 7.0)*

   a) Potassium dihydrogen orthophosphate (0.01M)

      1.361gm of potassium dihydrogen orthophosphate was dissolved in 200ml of distilled water and the final volume was adjusted to 1000ml with distilled water.

   b) Sodium hydroxide (0.01M)

      0.4gm of sodium hydroxide was dissolved in 300ml of carbon dioxide free water and the final volume was adjusted to 1000ml with carbon dioxide free water.

   50ml of potassium dihydrogen orthophosphate solution was placed in a 200ml volumetric flask. To this 29.1ml of sodium hydroxide solution was added and the final volume was made up to 200ml with distilled water.

4. *Catalase Standard (65000 U/mg protein; 1mg protein/ml)*

   Crystalline beef-liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01M phosphate buffer, pH 7.0, to give a final concentration of 1.0mg protein/ml. Before assay, it was diluted with distilled water to obtain 1000 U/ml.

Procedure

1ml of phosphate buffer was taken in each of the four test tubes and 0.1ml of the tissue homogenate was added. To this, 0.5ml of hydrogen peroxide was added. The reaction was stopped at 15, 30, 45 and 60 seconds by the addition of 2ml of the dichromate-acetic acid reagent. The contents were boiled for 10 minutes, cooled and absorbance read at 570nm against sample blank. A zero time set was also run simultaneously by adding the dichromate acetic acid reagent before the addition of hydrogen peroxide.

Catalase activity was expressed as μmoles of H_2O_2 consumed/minute/mg of protein (Kat f).

3.3.2.3 Reduced Glutathione (GSH)

Reduced glutathione was determined as described by Moran et al. (1979).

Reagents

1. *Trichloroacetic acid (10% w/v)*

   Accurately weighed 10gm trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was adjusted to 100ml with distilled water.

2. *Phosphate buffer (0.2M, pH 8.0)*

   0.2M sodium phosphate was prepared by dissolving 30.2gm sodium phosphate in 600ml of distilled water, the pH was adjusted to 8.0 with 0.2M sodium hydroxide solution and the final volume was adjusted up to 1000ml with distilled water.

3. *DTNB reagent (0.6 mM)*

   60mg of 5, 5'-dithiobis (2-nitro benzoic acid) was dissolved in 50ml of buffer and the final volume was adjusted to 100ml with 0.2M sodium phosphate (pH 8.0).

4. *Standard glutathione*

   10mg of reduced glutathione was dissolved in 60ml of distilled water and the final volume was made up to 100ml with distilled water.

Procedure

To 1ml of the tissue homogenate, 1ml of 10% TCA was added. The precipitated fraction was centrifuged and to the supernatant, 2ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The color developed was read at 412nm against reagent blank.

The amount of glutathione was expressed as µg of GSH/mg protein.

3.3.3 Assay of Gluconeogenic Enzyme

3.3.3.1 Glucose-6-phosphate dehydrogenase (G-6-PD) (Glucose-6-phosphate phosphohydrolase, EC-3.1.3.9)

Liver Glucose-6-phosphate dehydrogenase was estimated following the method described by Varley et al. (1991).

Reagents

1. *Nicotinamide Adenine Diphosphate (NADP) solution (0.01M)*

   8.33mg of NADP was dissolved per ml of distilled water.
2. *Glucose-6-phosphate solution*

   9.42mg of glucose-6-phosphate was dissolved per ml of distilled water.
3. *Triethanolamine buffer (50mM, pH 7.6)*

   7.45g triethanolamine was dissolved in sufficient quantity of distilled water to produce 1000ml containing 1.861 (5mM) of disodium edetate (disodium EDTA).

Procedure

To a test-tube 2ml triethanolamine buffer (pH 7.6), 0.1ml NADP and 1ml liver homogenate were added mixed well and allowed to stand for 5 minutes. To this, 50µl of glucose-6-phosphate was added. After a period of 2 minutes the absorbance was read at 340nm for every five minutes against a blank prepared without G-6-P and NADP.

The Liver G-6-PD was expressed in Units / Litre. ΔE_{340}/min was converted to U/L by multiplying with 500.

3.3.4 Assay of Membrane Bound Enzymes and Phosphorous

3.3.4.1 Sodium-potassium dependent adenosine triphosphatase (Na^+-K^+-ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Na^+-K^+-ATPase was assayed according to the method described by Bonting (1970).

Reagents

1. *Tris-hydrochloride buffer (92mM, pH 7.5)*

   11.13gm of Tris-hydrochloride was dissolved in 900ml of distilled water. The pH of solution was adjusted to 7.5 with 2M hydrochloric acid and the volume was made up to 1000ml with distilled water.
2. *Magnesium sulphate solution (5mM)*

   1.232gm of magnesium sulphate was dissolved and made up to 1000ml with distilled water.
3. *Potassium chloride solution (5mM)*

   0.372gm of potassium chloride was dissolved in 400ml of distilled water and the final volume was made up to 1000ml with distilled water.
4. *Sodium chloride solution (60mM)*

   3.231gm of sodium chloride was dissolved in 400ml of distilled water and the final volume was made up to 1000ml with distilled water.
5. *EDTA solution (0.1mM)*

   0.372gm of EDTA was dissolved in 200ml of distilled water and the final volume was made up to 1000ml with distilled water.
6. *Adenosine triphosphate (ATP) solution (40mM)*

   0.220gm of ATP was dissolved in 4ml of 0.1N sodium hydroxide and the final volume was made up to 10ml with distilled water.
7. *Trichloroacetic acid (TCA) (10% w/v)*

   10gm of trichloroacetic acid was dissolved in 40ml of distilled water and the final volume was made up to 100ml with distilled water.

Procedure

1.0ml of tris-hydrochloride buffer and 0.2ml each of magnesium sulphate, sodium chloride, potassium chloride, EDTA, ATP were added to test tube containing the homogenate. The mixture was incubated at 36°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated as described in 3.5.5.4.

The enzyme activity was expressed as mM of inorganic phosphorous liberated/mg of protein/minute.

3.3.4.2 Calcium dependent adenosine triphosphatase (Ca^{++}-ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Ca^{++}-ATPase was estimated according to the method described by Hjerken and Pan (1983).

Reagents

1. *Tris hydrochloride buffer (125mM, pH 7.5)*

   15.12gm of Tris-hydrochloride was dissolved in 900ml of distilled water. The pH of solution was adjusted to 7.5 with 2M hydrochloric acid and the volume was made up to 1000ml with distilled water.
2. *Calcium chloride solution (50mM)*

   5.55gm of calcium chloride was dissolved in 400ml of distilled water and the final volume was made up to 1000ml with distilled water.
3. *Adenosine triphosphate solution (ATP) (10mM)*

   0.551gm of ATP was dissolved in 40ml of distilled water and the final volume was made up to 100ml with distilled water.
4. *Trichloroacetic acid (TCA) (10% w/v)*

   10gm of trichloroacetic acid was dissolved in 40ml of distilled water and the final volume was made up to 100ml with distilled water.

Procedure

The incubation mixture contained 0.1ml each of Tris-hydrochloride buffer, calcium chloride, ATP and homogenate in a test-tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0ml of TCA (10%). The amount of phosphorous liberated was estimated as described in section 3.5.5.4. The enzyme activity was expressed as mM of inorganic phosphorous liberated/mg protein/minute.

3.3.4.3 Magnesium dependent adenosine triphosphatase (Mg^{++}-ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Mg^{++}-ATPase was assayed by the method described by Ohinishi et al., (1982).

Reagent

1. *Tris-hydrochloride buffer (374mM, pH 7.6)*

   45.24gm Tris-hydrochloride was dissolved in 900ml of distilled water. The pH of the solution was adjusted to 7.6 with 2M hydrochloric acid and finally the volume was made up to 1000ml with distilled water.

5. *Magnesium chloride solution (25mM)*

   6.16gm of magnesium chloride was dissolved in 400ml of distilled water and the final volume was made up to 1000ml with distilled water.

6. *Adenosine triphosphate solution (ATP) (10mM)*

   0.551gm of ATP was dissolved in 40ml of distilled water and the final volume was made up to 100ml with distilled water.

7. *Trichloroacetic acid (TCA) (10% w/v)*

   10gm of trichloroacetic acid was dissolved in 40ml of distilled water and the final volume was made up to 100ml with distilled water.

Procedure

The incubation mixture contained 0.1ml each of Tris-hydrochloride buffer, magnesium chloride, ATP and homogenate in test-tube. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0ml of TCA (10%). The liberated phosphorous was estimated as described in section 3.5.5.4.

The enzyme activity was expressed as mM of inorganic phosphorous liberated /mg of protein/minute.

3.3.4.4 Determination of Inorganic phosphorous (Pi)

The inorganic phosphorous was estimated by the method described by Fiske and Subbarow (1925).

Reagents

1. *Ammonium molybdate reagent (2.5% w/v)*

   2.5gm of ammonium molybdate was dissolved up to 100ml with 3M sulphuric acid.

2. *1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent*

   0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite.

   15gm of sodium metabisulphite was dissolved in 100ml of distilled water, and the solution was used as a vehicle for ANSA reagent.

   Accurately weighed 20gm of sodium sulphite was dissolved in 100ml of distilled water.

   250mg of 1-amino-2-naphthol, 4-sulphonic acid was dissolved in 97.5ml of 15% sodium metabisulphite and 2.5ml of 20% sodium sulphite, mixed well and stored at room temperature.

3. *Standard Phosphorous*

   35.1mg of potassium dihydrogen orthophosphate was dissolved in 100ml of distilled water, containing 80mg of phosphorous/ml.

Procedure

1ml of the supernatant was taken and the volume was made up to 5.0ml with distilled water. To this, 1.0ml of 2.5% ammonium molybdate reagent and 0.5ml of ANSA-I reagent was added. The color developed within 20 minutes was read using blank containing water instead of sample at 620nm. A standard graph was prepared taking different concentrations of standard phosphorous (4-20μg).

The values are expressed as μM of phosphorous liberated/mg of protein/minute.

3.3.5 Estimation of Protein

3.3.5.1 Total protein

The method of Lowry et al. (1951) was used for the estimation of protein.

Reagents

1. *Sodium hydroxide (0.1M)*

   4gm of sodium hydroxide was dissolved in 400ml of carbon dioxide free water and the final volume was adjusted to 1000ml with carbon dioxide free distilled water.

2. *Lowry C reagent*

   a) Copper sulphate in 1% sodium potassium tartarate (1% w/v)

   0.5gm of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1gm of sodium potassium tartarate in 100ml distilled water).

   b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v)

   2gm of sodium carbonate was dissolved in 100ml of 0.1M sodium hydroxide.

   2ml of solution (a) was mixed with 100ml of solution (b) just before use.

3. *Standard Protein (Bovine serum albumin)*

   20mg of bovine serum albumin was dissolved in 80ml of water and few drops of sodium hydroxide was added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100ml with distilled water and stored overnight in a refrigerator.

Procedure

Diluted membrane fraction aliquots (0.1ml) were taken in test-tubes. To this, 0.8ml of 0.1M sodium hydroxide and 5ml of Lowry C reagent was added and the solution was allowed to stand. After 15 minutes, 0.5ml of 1N Cio-Calteu reagent (Folin's phenol reagent) was added and the contents were mixed well on vortex mixer. Color developed was measured at 640nm against reagent blank containing distilled water instead of sample. Different concentrations (20-200μg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph.

The values are expressed as mg of protein/gm of wet, fresh tissue (mg / gm).

3.3.6 Extraction and Estimation of Lipids

The procedure of Folch et al. (1955) as modified by Suzuki (1965), was used for the extraction of lipids from the organs. Suitable quantity (1g) of tissue was homogenised with total 20ml of cold chloroform-methanol (2:1 v/v) mixture in the Potter Elvehjem homogeniser with Teflon pestle. The contents were filtered through Whatman filter paper. The residue was re-extracted twice with small volumes of chloroform-methanol (2:1 v/v) mixture and then filtered again. The filtrates were pooled together and 0.3ml of glass distilled water was added, mixed well by vortexing and allowed to stand for phase separation at room temperature. The upper layer was removed with pasture pipette and the lower phase was washed with 0.4ml of Folch's pure upper phase solvent (Consists of chloroform-methanol-water in a proportion of 3:48:47) and centrifuged at 3000rpm for 10 minutes. The upper phase was removed. The lower phase was used for the estimation of cholesterol, triglyceride and phospholipid.

3.3.6.1 Cholesterol (CHOL)

Cholesterol in acetic acid gives a red color with ferric chloride and sulphuric acid. The estimation of total cholesterol was carried out by Zlatkis, Zak and Boyle's (1953) method.

Reagents

1. *Ferric chloride solution*

   A 0.05% w/v solution of Ferric chloride, ($FeCl_3.6\ H_2O$) was prepared in glacial acetic acid (aldehyde free).
2. *Concentrated sulphuric acid*
3. *Glacial acetic acid*
4. *Stock solution of cholesterol*

   Accurately weighed 100mg of cholesterol was dissolved in 100ml of glacial acetic acid. This stock solution was then used for the preparation of working standard solution.

Procedure

Suitable aliquots of the samples (0.5ml) were transferred accurately into series of 10ml volumetric flasks. To this, 4ml of ferric chloride solution and 4ml of sulphuric acid were added. The solution were made up to 10ml with glacial acetic acid, flasks were closed tightly with lid and mixed by repeated inversion. Then the lid was loosened and the flasks were left half-open for 20 to 30 minutes. The color developed was read against a reagent blank at 550 nm.

The values are expressed as mg of cholesterol / gm of wet, fresh tissue (mg / gm).

3.3.6.2 Triglycerides (TGLY)

Triglycerides was estimated by the method described by Foster and Dunn (1973) based on the method of Fletcher (1968).

Reagents

1. *Isopropanol*
2. *Alumina (neutral)*
3. *Potassium hydroxide (1M)*

   56.11gm of potassium hydroxide was dissolved in 1000ml of distilled water.
4. *Sodium meta periodate solution*

   77gm of anhydrous ammonium acetate was dissolved in about 700ml of distilled water. To this solution, 6ml of glacial acetic acid and 65mg of sodium meta periodate were added and the volume was made up to 1000ml with distilled water.
5. *Acetyl acetone reagent*

   7.5ml of acetyl acetone was dissolved in 200ml of isopropanol and the volume was made up to 1000ml with distilled water.
6. *Trieoline standard (10mM/L)*

   0.885ml of trieolein (glycerol trioleate) was dissolved in 60ml of isopropanol and the final volume was made up to 100ml with isopropanol.

Procedure

Aliquots of the lipid extract (0.2ml) were taken in duplicates in test-tubes. Lipid extract, 4ml of isopropanol and 400mg of alumina were mixed by vortexing for 15 minutes, centrifuged and 2ml of supernatant was transferred into separate test-tubes. 0.6ml of potassium hydroxide was added to the supernatants and the mixture was incubated at 60-70°C for 1 minute. After cooling, 1ml sodium meta periodate solution was added and mixed in vortex mixer. 0.5ml acetylacetone reagent was added to the vortexed mixture, stoppered and incubated at 50°C for 30 minutes. It was allowed to cool and the absorbance was read at 405nm against a reagent blank. A calibration curve of triolein standard (10 mM/L) was prepared in isopropanol in the range of 0-4 mM/L. The values were multiplied by 350 to obtain the triglyceride content. The values are expressed as mg of triglyceride / gm of wet, fresh tissue (mg / gm).

3.3.6.3 Phospholipid (PLIPI)

Phospholipids were estimated by the method described by Stewert (1980).

Reagents

1. *Ammonium ferrothiocyanate solution (0.1M)*

   27.03gm of ferric chloride hexahydrate and 30.4gm of ammonium thiocyanate was dissolved in distilled water, and the volume was made up to 1000ml with distilled water.
2. *Anhydrous sodium sulphate*
3. *Standard Phospholipid (lecithin) (0.1mg/ml)*

   Stock solution

   50mg of egg lecithin was dissolved in 10ml of chloroform. 2ml of this solution was diluted to 10ml with chloroform.

   Working standard

   1.0ml of diluted stock solution was further diluted to 10ml to give the final concentration of 0.1mg/ml and used for the preparation of calibration curve.

Procedure

0.5ml of lipid extract was mixed thoroughly in a test-tube with 2.0ml of ammonium ferrothiocyanate solution and 2.5ml of chloroform. The contents of tubes were vortexed vigorously using Cyclo Mixer for 5 minutes and centrifuged at 1000 rpm for 15 minutes. The lower layer was removed by using syringe with long needle and retained in another test-tube containing a pinch of anhydrous sodium sulphate. The absorbance of solutions was noted at 485nm using a reagent blank.

The values are for phospholipids expressed as mg of phospholipid / gm of wet, fresh tissue (mg / gm).

3.4 SERUM ESTIMATIONS

3.4.1 Alanine aminotransferase (GPT) (L-alanin: L-alanin; 2-oxoglutarate amino transferase, EC-2.6.1.2)

Serum glutamate pyruvate transaminase (GPT) or Alanine aminotransferase (ALT) was assayed by the method described by Bergemeyer and Bernt (1974) based on the method of Reitman and Frankel (1957).

Reagents

1. *Buffer substrate*

   1.78gm of alanine and 30mg of 2-oxoglutarate were dissolved in 20ml of phosphate buffer. To this 0.5ml sodium hydroxide (1N) was added and the final volume was made up to 100ml with phosphate buffer.

2. *Dinitropheyl hydrazine (DNPH) reagent*

   200mg of accurately weighed 2,4-dinitrophenyl hydrazine was dissolved in 1000ml of 1N hydrochloric acid.

3. *Phosphate buffer (0.1M, pH 7.8)*

a) Potassium dihydrogen orthophosphate solution (0.1M)

   13.609gm of potassium dihydrogen orthophosphate was dissolved in 1000ml of distilled water.

b) Sodium hydroxide solution (0.1M)

   4gm of sodium hydroxide was dissolved in 1000ml of carbon dioxide free water.

   50ml of 0.1M potassium dihydrogen orthophosphate was placed in a 200ml volumetric flask. To this 44.5ml of 0.1M sodium hydroxide was added and the final volume was made up to 200ml with distilled water.

4. *Hydrochloric acid solution (0.1N)*

   85ml of hydrochloric acid was diluted to 1000ml with distilled water and stored below 30°C.

5. *Sodium hydroxide solution (1N)*

   40gm of sodium hydroxide was dissolved in 1000ml of carbon dioxide free water.

6. *Standard Sodium pyruvate solution*

Stock solution

200mg of sodium pyruvate was dissolved in 100ml phosphate buffer.

Working solution

10ml of stock solution was diluted to 100ml with phosphate buffer.

Procedure

0.2ml of serum was mixed with 1ml of buffer substrate and the tubes were incubated for 30 minutes at 37°C in a water bath. To the tubes, 1ml of DNPH reagent was added and the tubes were kept at room temperature for 20 minutes. Then 5ml of 1N sodium hydroxide was added and the color developed was read at 540nm against reagent blank. A calibration curve was prepared by taking different concentrations of sodium pyruvate (0-150 U/L).

The enzyme activity was expressed as µmoles of pyruvate liberated/mg protein/minute (Units / Litre).

3.4.2 Aspartate aminotransferase (GOT) (L-aspartate: L-aspartate; 2-oxoglutarate aminotransferase, EC-2.6.1.1)

Serum glutamate oxaloacetate transaminase (GOT) or aspartate aminotransferase (AST) was estimated by the method described by Bergemeyer and Bernt (1974) based on the method of Reitman and Frankel (1957).

Reagents

1. *Buffer substrate (pH 7.4)*

   2.66gm of aspartic acid and 30mg of 2-oxoglutarate were dissolved in 20.5ml of 1N sodium hydroxide, adjusted to pH 7.4 and the final volume was made up to 100ml with phosphate buffer.

2. *Sodium hydroxide (1N)*

   40gm of sodium hydroxide was dissolved in 300ml of carbon dioxide free water and the final volume was made up to 1000ml with carbon dioxide free water.

3. *Dinitrophenyl hydrazine (DNPH) reagent*

   200mg of accurately weighed 2,4-dinitrophenyl hydrazine was dissolved in 400ml of 1N hydrochloric acid and the final volume was made upto 1000ml with the same.

4. Standard Sodium pyruvate solution

   Stock solution

   200mg sodium pyruvate was dissolved in 60ml of phosphate buffer and the final volume was made upto 100ml with phosphate buffer.

   Working solution

   10ml of stock solution was diluted to 100ml with phosphate buffer.

Procedure

0.2ml of serum was mixed with 1ml of buffer substrate and the tubes were incubated for 60 minutes at 37°C in the water bath. To the tubes, 1ml of DNPH reagent was added and the tubes were kept at room temperature for 30 minutes. Then 5ml of 0.4N sodium hydroxide was added and the color developed was read at 540nm against reagent blank. A calibration curve was prepared by taking different concentrations of sodium pyruvate (0-190 U/L).

The enzyme activity was expressed as µM of pyruvate liberated/mg of protein/minute (Units / Litre).

3.4.3 Alkaline phosphatase (Alkp) (Orthophosphoric acid monoester phosphohydrolase, EC-3.1.3.1)

Alkaline phosphatase assay was performed as reported by King (1965).

Reagents

1. *Substrate (Disodium phenyl phosphate, 0.1M, pH 10)*

   2.18gm of disodium phenyl phosphate was dissolved in 1 liter of water and boiled quickly. It was cooled immediately and preserved with a little chloroform (4ml/L), at 4°C.

2. *Carbonate-bicarbonate buffer (0.1M, pH 10)*

   6.3gm of anhydrous sodium carbonate and 3.36gm of sodium bicarbonate was dissolved in 1000ml of distilled water and stored at 4°C.

3. *Magnesium chloride (0.1M)*

   20.333gm of magnesium chloride was dissolved in 1000ml of distilled water.

4. *Sodium carbonate (1.5% w/v)*

   1.5gm of sodium carbonate was dissolved in 100ml of distilled water.

5. *Folin's phenol reagent*

   One volume of Folin's phenol reagent was diluted with two volumes of distilled water.

6. *Standard Phenol solution (1mg/ml)*

   Stock solution

   1gm of pure crystalline phenol was dissolved in 10ml of distilled water and was stored at 4°C in amber bottle.

   Working phenol

   1ml of stock solution of phenol was diluted to 100ml with distilled water, preserved with few drops of chloroform and kept at 4°C in amber bottle.

Procedure

1ml of substrate was pre-incubated with 1.5ml of carbonate-bicarbonate buffer and 0.1ml of magnesium chloride at 37°C for 10 minutes. To this reaction mixture, 0.1ml of serum was added and incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1ml of Folin's phenol reagent and centrifuged. To the supernatant, 1ml of sodium carbonate was added. After 10 minutes, the blue color developed was read at 640nm against reagent blank.

Phenol (1mg/ml) was used as the standard for the preparation of calibration curve.

The enzyme activity was expressed as μM of phenol liberated/mg of protein under incubation conditions (KA Units / Litre).

3.4.4 Acid phosphatase (Acidp) (Ortho-phosphoric acid monoester phosphohydrolase, EC-3.1.3.2)

Acid phosphatase was estimated by the method described by King (1965).

Reagents

1. *Substrate (Disodium phenyl phosphate, 0.1M, pH 10)*

   2.18gm of disodium phenyl phosphate was dissolved in a 1 liter of water and boiled quickly. It was cooled immediately and preserved with a little chloroform (4ml/L) stored at 4°C.
2. *Citrate buffer (0.1M, pH 4.3)*

   21gm of citric acid monohydrate was dissolved in 500ml of distilled water placed in a 1liter volumetric flask. To it, 180ml of 1N sodium hydroxide and 100ml of 0.01N hydrochloric acid was added. The pH was adjusted to 4.3 with acid or alkali, diluted to 1000ml with distilled water and preserved with few drops of chloroform at 4°C.
3. *Sodium carbonate (15 % w/v)*

   15gm of sodium carbonate was dissolved in 100ml of distilled water.
4. *Folin's phenol reagent*

   One volume of Folin's phenol reagent was diluted with two volumes of distilled water.
5. *Standard Phenol solution (1mg/ml)*

   Stock solution

   1gm crystalline phenol was dissolved in distilled water and kept at 4°C in amber colored bottle.

   Working phenol solution

   1ml of stock solution of phenol was diluted to 100ml with distilled water, preserved with few drops of chloroform and kept at 4°C in amber colored bottle.

Procedure

1ml of substrate was pre-incubated with 1.5ml of citrate buffer for 10 minutes. To this reaction mixture, 0.1ml of serum was added and incubated at 37°C for 30 minutes. After incubation, the reaction was arrested by the addition of 1ml of Folin's phenol reagent. Simultaneously, the control without substrate was incubated and the serum was added after the addition of Folin's phenol reagent. 1ml of sodium carbonate was added. After 10 minutes the blue color developed was read at 640nm against reagent blank. Phenol solution (1mg/ml) was used as the standard.

The enzyme activity was expressed as μM of phenol liberated/mg of protein under incubation conditions (KA Units / Litre).

3.4.5 Lactate dehydrogenase (LDH) (L-lactate; NAD$^+$ oxido reductase, EC-1.1.1.27)

Lactate dehydrogenase was estimated according to the method described by Gerhardt et al. (1974) as given by Gowenlock (1988).

Reagents

1. *Tris (56mM)-EDTA (5.6mM) buffer (pH 7.4)*

   6.8g of Tris (hydroxymethyl)-aminomethane and 2.1g of EDTA disodium salt dihydrate was dissolved in approximately 500ml distilled water. The pH was adjusted to 7.4, at 37°C, with 1.0mM hydrochloric acid and the volume was made to 1000ml with distilled water and stored at 4°C in tightly capped dark bottle.

2. *Reduced Nicotinamide Adenine Dinucleotide (NADH) (0.17mM)*

   13mg of NADH-disodium salt trihydrate was dissolved in 90ml of Tris (56mM)-EDTA (5.6mM) buffer, pH 7.4, and mixed well.

3. *Sodium pyruvate solution (13.5mM)*

   149mg of sodium pyruvate was dissolved in 100ml of distilled water, and stored at 4°C in a tightly capped bottle and used within 20 days.

Procedure

To the test tubes, 2.0ml of NADH solution and 50μl of serum was taken, mixed and incubated at 37°C for 10 minutes. To this 0.2ml of sodium pyruvate solution prewarmed to 37°C, was mixed and the rate of change of absorbance for 1 minute was monitored at 340nm. The final calculated value of absorbance after 45 seconds was multiplied by 7143. The values are expressed as Units / decilitre of serum.

3.4.6 Total Bilirubin (TBIL)

The estimation of serum Bilirubin was done by the method of Malloy and Evelyn (1988) as given by Goel (1988).

Reagents

1. *Concentrated hydrochloric acid.*
2. *Normal Saline (0.9% w/v sodium chloride solution).*
3. *Methanol*
4. *Diazo reagent*
   - I. Diazo A (Van den Berge solution A)

     0.5gm of sulfanilic acid was dissolved in 60ml of conc. hydrochloric acid and final volume was adjusted to 1000ml with distilled water.
   - II. Diazo B (Van den Berge solution B)
     - a. Stock solution (20% w/v sodium nitrite solution)

       20gm of sodium nitrite was dissolved in 100ml of distilled water.
     - b. Working solution (2% w/v sodium nitrite solution)

       10ml of stock solution was diluted to 100ml with distilled water.
   - III. Working Diazo reagent

     The solution was prepared freshly at each time from the stock solutions by mixing 0.3 parts (0.9ml) of Diazo (B) and 10 parts (30ml) of Diazo (A) solutions.
5. *Diazo blank solution*

   60ml of concentrated hydrochloric acid was diluted up to 1000ml with distilled water.
6. *Standard Bilirubin solution*

   20mg of bilirubin was dissolved and made up to 100ml with chloroform.

For determination of bilirubin, the serum was diluted to 1:10 with normal saline.

Procedure

Three test tubes were taken in test-tube stand and marked as TD (direct bilirubin), TT (total bilirubin) and B (blank).

To each of these tubes, 1ml of diluted serum was placed. 1ml of diazo blank was added to tube marked B and mixed well. 1ml of diazo reagent was added to each of the tubes marked TT and TD and mixed well. Exactly after 1 minute, after the addition of diazo reagent, the absorbance of the colored solution in tube TD was taken at 560nm using solution of the test tube marked B as blank. The mixing of diazo reagent was done in such a way that each sample was reacted only for 1 minute. 6ml of methanol was added to tube TT and mixed gently. It was then allowed to stand for 30 minutes and absorbance of the solution was taken at 540nm using the solution of the tube B as blank. Calibration curve was prepared by using suitable aliquots of standard bilirubin instead of serum.

The values are expressed as total bilirubin mg / decilitre of serum.

3.4.7 Total proteins

Total proteins were estimated as described in section 3.5.6.1. 0.1ml of serum was used instead of homogenate.

3.4.8 Estimation of Lipids

3.4.8.1 Cholesterol

Total Cholesterol was estimated as described in section 3.5.7.1. 0.1ml of serum was used instead of homogenate.

3.4.8.2 Triglycerides

Triglycerides were estimated as described in section 3.5.7.2. 0.1ml of serum was used instead of homogenate.

3.4.8.3 Phospholipids

Phospholipids were estimated as described in section 3.5.7.3. 0.1ml of serum was used instead of homogenate.

3.5 DETERMINATION OF LEVELS OF METALS IN TISSUES

The metal (lead and cadmium) levels of the tissues were estimated by using Perkin Elmer-2380 Atomic Absorption Spectrophotometer by nitric acid digestion method (Greenberg et al., 1992).

All the apparatus were made lead free before use, by rinsing them with concentrated nitric acid and then with de-ionized water for several times.

The suitable aliquots of homogenates were placed in a beaker. To this, 5ml of concentrated nitric acid was added. It was brought to boil slowly and evaporated on a hot plate to the lowest possible volume (about 3ml) before precipitation. The heating and addition of concentrated nitric acid was continued till the tissue was digested and the solution formed thus, became clear with a light greenish yellow color. After completion of digestion, the solution was allowed to cool and filtered in 50ml volumetric flask through Whatman filter paper No. 1. The walls of beaker were washed with distilled water and filtered. Then the final volume was adjusted to 50ml with distilled water. Samples prepared in this way were subjected to Atomic Absorption Spectrophotometry for estimation of lead and cadmium.

The amount of metal was expressed as µg/gm of tissues.

3.6 HISTOPATHOLOGICAL STUDIES

After 30 days of several treatments, the rats were sacrificed and liver, lung, heart and kidney excised, blotted free of blood and tissue fluids and preserved in 5% v/v formalin solution. After a week, the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embeded in paraffin wax. Sections of 5 µ thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The hematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were observed under Olympus BX40 Photomicroscope and photographed.

3.7 STATISTICAL ANALYSIS OF DATA

The values expressed as Mean ± SEM (n = 6) was calculated for each group to determine the significance of inter group differences. Each parameter was analysed separately by using one-way analysis of variance (ANOVA) with Huynh Feldt Epsilon modification of degrees of freedom to correct the departure from difference. To find the differences between the groups, Student's t-test was used. A 'p' value of <0.05 was considered to be significant.