MATERIALS & METHODS
2.0 MATERIALS AND METHODS

2.1 ANIMALS

The Wistar albino rats of either sex (120-150 g) were obtained from King Institute of Preventive Medicine, Guindy, Chennai. The animals were housed in large spacious polypropylene cages and they were given food and water *ad libitum*. The atmospheric temperature in Chennai remained between 20°C to 37°C with only 5°C difference between day and night throughout the year. No special arrangements like controlled temperature and humidity were made for animals on experimentation.

2.2 ANIMAL FOOD

The commercial pelleted animal food marketed by M/s. Hindustan Lever Limited, Mumbai, India under the name "Gold Mohur rat feed" was used to feed the animals.

2.3 CHEMICALS USED

Chemicals : All Analytical grade

: Nimesulide (B.No.NMFP K) Mfg. by Dr. Reddy's Labs Ltd., Hyderabad, Andhra Pradesh, India.

: Spirulina [MCRC isolate (Murugappa Chettiar Research Centre)] processed and supplied by New Ambadi Estate, TIAM House, Chennai.
2.4 PREPARATION OF TEST DRUG

Spray dried commercial sample of *Spirulina* (MCRC - isolate) collected in the month of October and used for the study. The sample was kept in amber colour bottle and protected from direct sunlight during the study. The powder is first mixed with small quantity of water and made up the volume into desired concentration by propylene glycol I.P. This spirulina preparation in propylene glycol (SP) was used for further study. The dose of SP (1500 mg/kg/po) used in this study was taken from previous report Subramanian (1996).

2.5 TOXICITY STUDIES

2.5.1 Acute toxicity study of *Spirulina*

Albino rats, either sex of Wistar strain weighing between 120-150 g were used in this study. Rats were divided into groups of six animals each. Rats were fed orally with SP to different groups in the dose range of 500 mg to 15 g/kg b.wt. The animals were kept in a room maintained at equal light and dark cycles. Solid food and water were withdrawn 18 hrs before for oral administration of the drug. The animals were continuously observed for the first six hours for toxic symptoms and later at intervals of 24 hrs upto 72 hours.

2.5.2 Ulcerogenic Index

The animals were divided into three groups of six animals each. SP was administered daily at the dose of 250, 500 and 1500 mg/kg b.wt. for six days. The body weight changes were recorded in alternate days with
simultaneous observation for any toxic symptoms and mortality. On the 7th day the animals were sacrificed by decapitation. The stomach was removed, cut along the lesser curvature and gastric contents removed. Stomach was washed with saline and examined under the dissecting microscope (20 X) for sign of ulceration (Thuilleir et al., 1968). Simultaneously serum Alanine transaminase (ALT) and Aspartate Transaminase (AST) were also assayed.

2.6 PHARMACOLOGICAL STUDY OF SP

2.6.1 Analgesic Study (Asparicio, 1977)

The analgesic meter used in this study consist of an electrically heated hot plate which was thermostatically controlled and maintained at 56°C. There was a perplex box of dimension 30 x 20 x 20 cms over the hot plate. The top lid could be opened to place the animal on the hot plate. There is an electronic timer which could be switched on or off as desired. The rats weighing 100 ± 10g were used in this study. They were not given solid food for 24 hours prior to the experiment. The reaction time was measured as licking the forepaws or jumping as signs of discomfort. Those animals with reaction time exceeding 15 seconds or more and if the difference between the two trials was greater than 5 seconds, were excluded from the experiment. The cut off time was 30 seconds.

The hot plate was switched on and adjusted to maintain exactly 56°C. The rat was placed on the hot plate and simultaneously the electronic timer was switched on. As soon as the animal exhibited any sign of discomfort
such as licking its fore paw or jumping in an attempt to escape from the hot plate, the electronic timer was switched off and the animal was taken out. The time taken by the animal to exhibit this discomfort was noted.

The animals were divided into three groups of six animals each.

Group I - Control (propylene glycol)
Group II - SP 1500 mg/kg b.wt.
Group III - Nimesulide (5 mg/kg b.wt.)

Pretreatment reaction time was recorded twice for each animal. Thirty minutes after dosing with the drug, the reaction time was recorded by hourly intervals for 3 hours and compared with the pretreated reaction time.

2.6.2 Anti-pyretic Studies (Lassman, et al., 1977)

Wistar albino rats of either sex (100-125 g) were divided into groups of six animals each and each group received the following regimen of treatment.

Group I - Control rats (propylene glycol)
Group II - SP 1500 mg/kg b.wt.
Group III - Nimesulide (5 mg/kg b.wt.)

Yeast induced pyrexia was used to screen the drug for antipyretic activity (Vedavathy and Rao, 1991). Pyrexia was produced by injecting 2 ml of 15% suspension of dried brewer’s yeast, subcutaneously into the back of non-
fasted rats. Animals which showed a raise in body temperature of 1-2°C or more were taken for the study. Drug was administered orally after 16 hours when the temperature was at its peak.

The reduction of temperature, if any was calculated from its own in each fevered animal. The temperature measurement was made by inserting rectally a small animal (veterinary) mercury thermometer. The mercury tip being lubricated with a drop of liquid paraffin.

2.7 ANTI-INFLAMMATORY ACTIVITY OF SPIRULINA

2.7.1 Carrageenan induced Paw Oedema in Rats

Wistar albino rats of either sex (100-125 g) were divided into groups of six animals each and each group received the following regimen of treatment (Winter et al., 1962).

- **Group I**: Control rats (propylene glycol)
- **Group II**: Carrageenan injected
- **Group III**: Carrageenan + SP 1500 mg/kg b.wt.
- **Group IV**: Nimesulide (5 mg/kg b.wt.)

Animals were anesthetized with ether and injected 0.05 ml of 1% suspension of carrageenan in normal saline into the subcutaneous tissue of the hind paw of rats. The other paw was injected with 0.05 ml of propylene glycol. Since the hydration state of animals can modify the intensity of swelling, rats were fasted 24 hours before the experiment and water (1.5 ml /100 g b.wt) was orally administered twice, 20 hr and 4 hr before the injection of carrageenan.
The paw volume was measured by dipping the foot in the Mercury bath of the Plethysmograph apparatus up to the anatomic hairline - lateral malleolus. The replacement of mercury (volume displacement) produced a rise in the coloured fluid in the thin limb of the plethysmograph. Before commencing the experiment, the initial level of the coloured fluid in the thin limb was adjusted to zero. So the increase in the reading directly gave the volume of the foot.

The drugs were administered orally 1 hour after the injection of carrageenan.

Paw volume was measured on hourly interval up to four hours. The difference between the initial and final paw volume indicated the oedema volume due to inflammation. The anti-inflammatory activity of drugs were expressed as percent inhibition. Percentage inhibition was calculated by the method of Winter et al. (1962)

\[
\text{% inhibition} = \frac{V_c - V_t}{V_c} \times 100
\]

Where \(V_c\) and \(V_t\) were average oedema volume of control and treated groups respectively.

2.7.2 Induction of Adjuvant Arthritis

Adjuvant induced arthritis (AIA) has been very widely adopted in pharmacological screening programmes, being generally considered over the past decade as an appropriate model of rheumatoid arthritis (Newbould, 1963).
Adjuvant arthritis is induced in rats by injecting 0.1 ml of Freud's adjuvant subcutaneously into the hind paw of rats. The animals were grouped into five groups with six animals in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>-</th>
<th>Control animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>-</td>
<td>Adjuvant induced arthritic animals</td>
</tr>
<tr>
<td>Group II</td>
<td>-</td>
<td>Received SP 1500 mg/kg b.wt. for 28 days from the date of commencement of adjuvant induction.</td>
</tr>
<tr>
<td>Group III</td>
<td>-</td>
<td>Received SP 1500 mg/kg/day for 14 days after the first phase (14 days) of adjuvant challenge.</td>
</tr>
<tr>
<td>Group IV</td>
<td>-</td>
<td>Received SP 1500 mg/kg/day for 28 days.</td>
</tr>
</tbody>
</table>

The paw oedema volume and the body weight change were measured in alternate days during the experimental period.

2.8 COLLECTION OF SAMPLES FOR BIOCHEMICAL ESTIMATION

Rats were sacrificed on the 29th day after over night fasting, by cervical decapitation. Serum and Plasma were collected in plain and EDTA added tubes, respectively. Liver and kidney tissue samples were taken and preserved for histopathological studies. Weighed amount of tissues were homogenised in appropriate buffer for enzyme estimation and a section of tissue was fixed in 10% buffered formalin for histopathological studies.
2.9 ESTIMATION OF GLUCOSE, UREA, CREATININE AND URIC ACID

2.9.1 Glucose

Glucose was estimated by the method of Sasaki and Matsui (1972).

Reagents

1. Orthotoluidine-boric acid reagent: This reagent consisted of 2.5 g of thiourea and 2.4 g of boric acid in 100 ml of a mixture of water, acetic acid (AR) and orthotoluidine (distilled) in the ratio of 10:75:15 and kept overnight in the cold.

2. Standard: 100 mg of glucose in 0.1% benzoic acid, 10 ml of the above solution was diluted to 100 ml to give 100 µg of glucose per ml.

Procedure

To the supernatant of zero time and 30 minutes incubation form hexokinase assay 4 ml of orthotoluidine reagent was added and heated in a boiling water bath for 15 minutes along with standard solutions containing 25-100 mg/ml and a blank. The blue colour developed was read at 640 nm.
2.9.: Estimation of Bur

Blood urea nitrogen was estimated by the method of Natelson et al. (1951) by measuring the coloured complex formed with diacetyl monoxime in acidic medium.

Reagents

1. 10% Sodium tungstate
2. 2/3 N Sulphuric acid
3. Diacetyl monoxime; 2% in 2% acetic acid
4. Sulphuric acid - Phosphoric acid reagent.

40 ml of water was mixed with 150 ml of 75% phosphoric acid and then 50 ml of concentrated sulphuric acid was added slowly. Standard: 250 mg of urea was dissolved in 100 ml of water.

This solution was diluted 1 to 100 to give a solution containing 25 μg/ml which was used as working standard.

Procedure

0.1 ml of blood was mixed with 3.3 ml of water, 0.3 ml of each of sodium tungstate and sulphuric acid respectively, mixed well and centrifuged. To 1.0 ml of supernatant was mixed with 1.0 ml of water, 0.4 ml of diacetyl monoxime and 1.6 ml of sulphuric acid-phosphoric acid were added. The tubes were placed in a boiling water bath for 30 minutes and cooled. The colour
developed was read at 480 nm against blank. A series of standards were treated similarly. Blood urea nitrogen was expressed as mg/dl.

2.9.3 Estimation of Creatinine

Creatinine was estimated by the method of Slot (1965).

Reagents

1. 10% Sodium tungstate
2. 2/3 N Sulphuric acid
3. Saturated picric acid; 1.5 g of picric acid in 100 ml of distilled water.
4. Sodium hydroxide; 0.75 N.
5. Standard creatinine; 20 mg of creatinine in 100 ml of distilled water.

Procedure

1 ml of serum was mixed with 7.0 ml of water. 1 ml of sodium tungstate and 1 ml of sulphuric acid were added and centrifuged. From this 0.4 ml of supernatant was taken and mixed with 1.0 ml of sodium hydroxide and 1 ml of picric acid. The tubes were kept in a boiling water bath for 15 minutes. The colour developed was read at 470 nm using a photochem colorimeter.

Serum creatinine was expressed as mg/dl.
2.9.4 Estimation of Uric Acid

Serum uric acid was estimated by the method of Caraway (1963). Serum uric acid is expressed as mg/dl.

2.10 HAEMATOLOGICAL TESTS

2.10.1 Erythrocyte Count

Erythrocyte count was estimated by the haemocytometer method of John (1972).

Reagents

Gower’s Solution

Dissolved 12.5g of sodium sulphate and 33.3 ml of glacial acetic acid in 200 ml of distilled water.

Procedure

Venous blood was taken up to the 0.5 mark in the Thoma red cell diluting pipette and diluted up to 101 mark with diluting fluid, thus achieving a 1:200 dilution of the blood sample. The diluted sample was filled in the counting chamber and counted with the aid of the light microscope.
Calculation

\[ \text{RBC} = \text{Cells counted} \times 5 \left( \frac{1}{5} \text{Sq.Cm counted} \right) \times 10 \left( \text{depth} \right) \times 200 \left( \text{dilution factor} \right). \]

Values are expressed as cells per cubic millimeter of blood.

2.10.2 Total Leukocyte Count (WBC)

Total leukocyte count was estimated by the haemocytometer method of John (1972).

Reagents

1.5% HCl in water.

Procedure

The WBC pipette was filled to the 0.5 mark with whole blood and diluted to the 101 mark with 1.5% HCl, resulting in a 1:20 dilution of the blood sample. The haemocytometer was filled with the diluted blood and leukocytes present in the four large corner squares (1 sq.mm each) were counted.

Calculation

\[ \text{WBC (Per cu.mm)} = \frac{\text{Cells counted} \times 10 \left( \text{depth} \right) \times 20 \left( \text{dilution factor} \right)}{4 \left( \text{sq.mm counted} \right)} \]

Values are expressed as the number of leukocytes present per cubic millimeter of the blood.
2.10.3 Haemoglobin

Haemoglobin was estimated by the method of Drabkin and Austin (1932).

Reagents

1. Drabkin's reagent:
   - Sodium bicarbonate; 1.0 g
   - Potassium cyanide; 50 mg
   - Potassium ferricyanide; 2.0 mg
   - Water: 1000 ml.

2. Standard Haemoglobin: Cyanmethemoglobin standard was purchased from Span diagnostics, Surat, India.

Procedure

20 µl of blood was mixed with 4 ml of Drabkin's reagent. After 10 minutes the solution was compared with the standard and a reagent blank at 530 nm.

Values are expressed as g/dl.

2.10.4 Erythrocyte Sedimentation Rate (ESR)

ESR was estimated by the method of Westergren (1988).
2.11 ESTIMATION OF TOTAL PROTEIN

Total protein was estimated by the method of Lowry et al. (1951).

Reagents

1. Alkaline copper reagent
   Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide.
   Solution B: 0.5% copper sulphate in 1% sodium potassium tartrate.
   50 ml of solution A was mixed with 1 ml solution B just before use.

2. Folin’s phenol reagent: One volume of Folin’s reagent was diluted with two volumes of distilled water just before use.

3. Standard Bovine serum Albumin (BSA): 20 mg of BSA was dissolved in 100 ml of distilled water. Few drops of sodium hydroxide (alkali) was added to aid complete dissolution of BSA and to avoid frothing, it was allowed to stand overnight in a refrigerator.

Procedure

0.1 ml of 10% homogenate was diluted in 1.0 ml with water. From this diluted samples, 0.1 ml was made upto 1.0 ml with water. Standards were taken and made upto 1.0 ml with water. Blank contained 1.0 ml water. To all tubes 4.5 ml of alkaline copper reagent was added and kept at room temperature for 10 minutes. Then 0.5 ml of Folin’s phenol reagent was added and the colour developed was read after 20 minutes at 640 nm against a reagent blank using a photochem colorimeter.
Total protein content is expressed as mg/g of fresh or wet tissue.

Serum total protein content is expressed as g/dl.

2.12 ESTIMATION OF SERUM ALBUMIN

Total albumin in serum was estimated by the method of Reinbold (1953).

Reagents

1. Sulphate - Sulphite solution: 208g of sodium sulphate and 70g of sodium sulphite were dissolved in about 900 ml water, with constant stirring. Then 2 ml of concentration $\text{H}_2\text{SO}_4$ was added and made upto 1000 ml with distilled water.

2. Stock biuret reagent: Dissolved 45g of Rochelle Salt in about 400 ml of 0.2 N NaOH and added 15 g of CuSO$_4$ and 5 g of KI to make up to a litre with 0.2 N NaOH.

3. Biuret: Dilute 200 ml of stock reagent was diluted to a litre with 0.2 N NaOH which contains 5 gms of KI/l.

4. Tartrate - Iodide Solution: Dissolved 9g of Rochelle Salt in 0.2 N NaOH containing 5g KI/l.

5. Ether.
Procedure

a. Albumin: To 0.25 ml of plasma, 4 ml of sulphate-sulphite solution was added. Then 3 ml of ether was added, stoppered and shaken 40 times. The tubes were capped and centrifuged till a firm globulin layer was formed. After centrifugation, the tubes were tilted and 1 ml of the clear solution below the globulin layer was pipetted out into a tube and 5 ml of biuret reagent was added. The tubes were heated in a water bath at 37°C for 10 minutes. The colour developed was read at 570 nm.

b. Serum blank: 2 ml of serum sulphate-sulphite solution was added to 5 ml of tartrate iodide solution and mixed.

c. Biuret blank: 2 ml of sulphate-sulphite solution was added to 5 ml of biuret reagent.

d. Standard: 0.4 ml of standard solution was pipetted into 6 ml of sulphate-sulphite solution as above and 2 ml of this mixture was transferred into 5 ml of biuret reagent.

Total albumin in serum was expressed in terms of g/dl.
2.13 ESTIMATION OF SERUM LIPIDS

2.13.1 Cholesterol

Cholesterol content was estimated by the method of Parekh and Jung (1970).

Reagents

1. Ferric chloride - Uranyl acetate reagent: Five hundred mg of ferric chloride was dissolved in 10.0 ml of water, 3.0 ml of concentrated ammonia was added to it and centrifuged. The precipitate was washed several times with distilled water and dissolved in one litre of glacial acetic acid. 100 mg of uranyl acetate was added to the mixture and the contents were shaken well and kept over night. The reagent was stable for six months.

2. Sulphuric acid - ferrous sulphate reagent: One hundred mg of ferrous sulphate was dissolved in 100 ml of glacial acetic acid and 100 ml of sulphuric acid. After cooling to room temperature, the volume was made upto 1.0 litre with concentrated sulphuric acid. The reagent was stable for six months.

3. Cholesterol standard: Two hundred mg of cholesterol, recrystallized from ethanol was dissolved in 100 ml of chloroform. One ml of stock cholesterol was diluted to 100 ml to obtain a working standard of 20 µg of cholesterol/ml.
Procedure

0.05 ml serum was treated with 10.0 ml of ferric chloride - uranyl acetate reagent. The mixture was shaken well and allowed to stand for 5 minutes. After centrifugation, 3.0 ml of supernatant was taken for analysis. A set of standards containing 10-50 μg of cholesterol, were made upto 3.0 ml with ferric chloride - uranyl acetate reagent. Two ml of sulphuric acid - ferrous sulphate reagent was added to all the tubes and the contents were mixed well. After 20 minutes, the optical density was measured at 540 nm in a Photochem colorimeter.

Total cholesterol content in serum is expressed as mg/dl.

2.13.2 Phospholipids

Phospholipids were estimated by the method of Rouser et al. (1970) after digesting the lipid extract with perchloric acid.

Reagents

1. TCA 10% : Ten g TCA was dissolved in 100 ml distilled water.
2. Perchloric acid (AR).
3. Ammonium molybdate 3% : 3 g of ammonium molybdate was dissolved in 100 ml of distilled water.
4. Ascorbic acid 3% : 3 g of ascorbic acid was dissolved in 100 ml of distilled water.
5. Standard phosphate: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water to give a concentration of 80 µg of phosphorus/ml.

6. Working standard: A concentration of 8 µg/ml was prepared by diluting 10 ml of the stock solution to 100 ml in a standard flask.

Procedure

0.1 ml serum was diluted to 2.0 ml with distilled water and 2.0 ml of 10% TCA was added. The precipitated proteins were sedimented by centrifugation at 2000 xg for 10 minutes. The supernatant was discarded. One ml of perchloric acid was added to the residue and digested on a sand bath till the solution turned colourless. After cooling, the solution was made upto 5.0 ml with distilled water. 0.5 ml each of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 minutes. The blue colour developed was read at 620 nm in a Photochem colorimeter.

Phospholipid content is expressed as mg/dl serum after multiplication by a factor of 25 to give the phospholipid content according to Scheig and Isselbacher (1965).

2.13.3 Triglycerides

Triglycerides were estimated by the method of Rice (1970) based on the method of Van Handel (1961).
Reagents


2. Sodium chloride : Saturated in distilled water.

3. Activated silicic acid : Silicic acid was washed with 4N hydrochloric acid and then with distilled water until the pH was neutral. After drying, ether was added in sufficient amount, stirred well and the supernatant was decanted. Silicic acid was then dried at 60°C and activated at 100°C overnight prior to use.

4. Potassium hydroxide 0.4% in ethanol : Four hundred mg of potassium hydroxide was dissolved in 100 ml of ethanol.

5. Sodium meta periodate 0.1M : 2.149g of sodium meta periodate was dissolved in 100 ml of distilled water.

6. Sodium meta arsenite 0.5 M : 6.496 g sodium meta arsenite was dissolved in 100 ml of distilled water.

7. Chromotropic acid : 1.14g of chromotropic acid was dissolved in 100 ml distilled water and stored as a stock solution in a brown bottle. Before use, 10.0 ml of this solution was mixed with 45.0 ml sulphuric acid water mixture in the ratio of 2:1 (v/v).

8. Sulphuric acid 0.2N : 0.5 ml of concentrated sulphuric acid was made upto 100 ml with distilled water.
9. Tripalmitin standard: One hundred mg of tripalmitin was dissolved in 100 ml of chloroform in a 100 ml standard flask. The stock solution was diluted to 1 in 10 with chloroform. The standard solution contained 100 µg tripalmitin/ml.

Procedure

0.2 ml serum was mixed with chloroform-methanol mixture (9.8 ml) and left aside for 30 minutes before centrifugation. After centrifugation, 4.0 ml of the lipid extract was added to tubes containing 8.0 ml of saturated sodium chloride and shaken vigorously. The contents were allowed to settle for an hour and then centrifuged. The supernatant saline-methanol phase was discarded. The washed chloroform phase was filtered into a dry tube. Two hundred mg of activated silicic acid was added to chloroform phase, shaken vigorously and allowed to stand for 30 minutes. After centrifugation, to 0.5 ml supernatant, as well as standard and blank, 0.5 ml of alcoholic potassium hydroxide solution was added and the mixture was saponified in a water bath at 60-70°C for 20 minutes. 0.5 ml of 0.2 N sulphuric acid was added and kept in a boiling water bath for 10 minutes. After cooling the tubes, 0.1 ml of sodium meta periodate was added and allowed to stand for 10 minutes. The excess periodate was reduced by the addition of 0.1 ml sodium meta arsenite. Five ml chromatropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 minutes. After cooling, the colour developed was read at 540 nm in a Photochem colorimeter.

Serum triglycerides level are expressed as mg/dl plasma.
2.13.4 Free Fatty Acids

Free Fatty Acids were estimated by the method of Horn and Menahan (1981).

Reagents

2. Copper nitrate-triethanolamine solution (Cu-TEA): Fifty ml of 0.1 M copper nitrate and fifty ml of 0.2 M triethanolamine were mixed with 33.0 g of sodium chloride.
3. Colour reagent: One hundred mg of diethyl dithio carbonate was dissolved in 100 ml of n-butanol.
4. Activated Silicic acid.
5. Standard palmitic acid: Twenty mg of standard palmitic acid was made up to the mark in a 100 ml standard flask with distilled water. The working standard was prepared by diluting the stock solution from 1 to 10 ml with distilled water. The working standard contained 20 μg of palmitic acid/ml.

Procedure

An aliquot of 0.2 ml serum was mixed with 6.0 ml of chloroform: heptane: methanol solvent and was shaken vigorously. Two hundred mg of activated silicic acid was added, shaken, and left aside for 30 minutes. The
solution was then centrifuged and the supernatant was transferred to the tube containing 2.0 ml Cu-TEA reagent. Blank contained only the solvent, while standards with different concentrations were made up to a known volume with the solvent and then 2.0 ml Cu-TEA reagent was added. The contents were agitated using a mechanical shaker for 20 minutes. The mixture was separated into two phases by centrifugation. Two ml of the upper layer was mixed with 1.0 ml of the colour reagent. The colour developed was read at 430 nm in a Photochem colorimeter.

Free fatty acids concentration is expressed as mg/dl plasma.

2.13.5 Serum Lipoproteins

2.13.5.1 Fractional precipitation of lipoproteins

Lipoproteins were fractionated by a dual precipitation technique of Wilson and Spiger (1973).

Addition of heparin-manganese chloride to serum, which precipitated very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) and the high-density lipoprotein (HDL) was left in the supernatant and the content of cholesterol was measured in the fraction.

To a second aliquot of plasma, sodium dodecyl sulphate (SDS) was added which resulted in the aggregation of VLDL which flocculated on the top. The subnatant containing both HDL and LDL and the cholesterol content of this mixture was assayed.
a. Total cholesterol - subnatant from SDS (containing HDL<sub>c</sub> + LDL<sub>c</sub>) = VLDL cholesterol.

b. Subnatant from SDS-Heparin manganese chloride supernatant = LDL cholesterol.

2.13.5.2 High-density Lipoprotein (HDL)

Reagents

1. Heparin - manganese chloride reagent: 3.167g of manganese chloride was added to 1.0 ml solution of heparin containing 20,000 units. This mixture was made up to 8.0 ml with distilled water.

2. Dextran sulphate reagent: One hundred and twenty one mg of dextran sulphate (Mol. wt. 15,000) was dissolved in 10 ml of 0.89% sodium chloride.

Procedure

Two ml plasma was added to 0.18 ml heparin-manganese chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 minutes and then centrifuged at 2000 xg and maintained at 10°C for 30 minutes. The supernatant contained the HDL fraction. One ml of this was used for the estimation of cholesterol by the method of Parekh and Jung (1970) as described in section 2.13.1.
2.13.5.3 Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol assay

Reagent

1. Sodium-dodecyl sulphate (SDS): Ten percent of SDS was prepared in 0.15 M sodium chloride solution and the pH was adjusted to 9.0 with sodium hydroxide.

Procedure

Two ml of serum was added to 0.15 ml of sodium-dodecyl sulphate. The contents were mixed well and incubated at 37°C for 2 hr. The contents were centrifuged in a refrigerated centrifuge at 10000 xg for 15 minutes. The subnatant contained the HDL and LDL fractions. Cholesterol was estimated from this fraction as described in section 2.13.1.

Serum lipoprotein cholesterol is expressed as mg/dl.

2.14 ESTIMATION OF LYSOSOMAL ENZYMES IN PLASMA AND TISSUE HOMOGENATES

2.14.1 β-D-glucuronidase (EC : 3.2.1.31)

β-D-glucuronidase activity was estimated by the method of Delvin and Gianetto (1970).
Reagents

1. Acetate buffer 0.1 M, pH 4.5: 49 ml of 0.1 M sodium acetate was mixed with 51 ml of 0.1 M acetic acid.

2. Glycine buffer 0.2 M, pH 10.7: This was prepared by mixing equal volumes of 0.2 M glycine, 0.125 M sodium carbonate, and 0.1 M sodium chloride solution.

3. p-Nitrophenyl-\(\beta\)-D-glucuronide: One mg of the substrate was dissolved in 1 ml of distilled water.

4. Standard: 5 mg of p-nitrophenol was dissolved in 100 ml of distilled water.

Procedure

0.1 ml of substrate, 0.1 ml of acetate buffer, 0.1 ml of plasma (or tissue homogenates) were incubated at 37°C for an hour. The reaction was arrested by the addition of 3.3 ml of glycine buffer. Standards were also run simultaneously along with a water blank. The colour developed was read at 420 nm using a Photochem colorimeter.

The enzyme activity is expressed as \(\mu\) moles of p.nitrophenol liberated/hour/mg protein at 37°C.
2.14.2 Acid Phosphatase (EC : 3.1.3.2)

Acid phosphatase was assayed by the method of King (1965).

**Reagents**

1. Citrate Buffer 0.1 M, pH 4.8 : 2.1 g of crystalline citric acid was dissolved in water and 18.8 ml of normal sodium hydroxide was added, the volume was made upto 50 ml with distilled water. The pH was adjusted to 4.8.

2. Substrate 0.1 M : Two hundred and fifty mg of disodium phenyl phosphate was dissolved in 100 ml of distilled water.

3. Sodium Carbonate 15% : Dissolved 15g of sodium carbonate in 100 ml of distilled water.


5. Standard Phenol Solution : Ten mg of phenol was dissolved in 100 ml of distilled water.

**Procedure**

The incubation mixture containing 1.5 ml of buffer and 1.5 ml of substrate were preincubated at 37°C for 10 minutes. Then 0.1 ml of enzyme preparation was added and incubated at 37°C for 30 minutes. After incubation,
the reaction was arrested by the addition of 0.1 ml of Folin’s phenol reagent. Control without enzyme was incubated and the enzyme preparation was added after the addition of Folin’s phenol reagent. Then 1.0 ml of sodium carbonate was added. After 10 minutes, the blue colour developed was read at 640 nm using a Photochem colorimeter.

The enzyme activity is expressed as μmoles of phenol liberated/min/mg protein at 37°C.

2.14.3 β-D-Galactosidase (EC : 3.2.1.23)

The enzyme activity was assayed by the method of Rosenblit et al. (1974).

Reagents

1. Acetate buffer 0.1 M, pH 4.5 : 49 ml of 0.1 M sodium acetate was mixed with 51 ml of 0.1 M acetic acid.

2. TCA 5% : Five g of TCA was dissolved in 100 ml of distilled water.

3. Sodium hydroxide 0.1 N : Four hundred mg of NaOH was dissolved in 100 ml of distilled water.

4. Substrate: p-Nitrophenyl-β-D-galactoside : 2.37 mg of substrate was dissolved in 1.0 ml of distilled water.
Procedure

The incubation mixture containing 0.2 ml of buffer, 0.1 ml substrate and 0.1 ml of enzyme were kept at 37°C for an hour. The reaction was arrested by the addition of 0.2 ml of 5% TCA. 0.1 ml of the enzyme was added to the control tube after in addition of 5% TCA. 3.0 ml of NaOH was added to all tubes and the colour developed was read at 420 nm in a Photochem colorimeter.

The enzyme activity is expressed as μmoles of p-nitrophenol liberated/hr/mg protein at 37°C.

2.14.4 Cathepsin D (EC : 3.4.23.5)

Cathepsin D activity was estimated by the method of Sapolsky et al. (1973).

Reagents

1. Sodium acetate buffer 0.1M, pH 3.6 : 92.5 ml of 0.1 M acetic acid was mixed with 7.5 ml of 0.1 M sodium acetate solution.

2. Substrate 1.5% : 1.5g of haemoglobin was dissolved in 100 ml of sodium acetate buffer.

3. TCA 5% : Five g of TCA was dissolved in 100 ml of distilled water.

5. NaOH 5%: Five g of NaOH was dissolved in 100 ml of distilled water.

6. Alkaline copper reagent (Lowry’s reagent) was prepared as described in section 2.11.

7. Standard: A solution of tyrosine in the concentration of 10 mg in 100 ml was prepared with 0.1N HCl.

**Procedure**

0.9 ml of buffered substrate was mixed with 0.1 ml of enzyme preparation and incubated for 2 hrs at 37°C. The reaction was arrested with 1.0 ml of 5% TCA and the samples were centrifuged for 10 minutes. To the control tubes the enzyme preparation was added after the addition of TCA. To 1 ml of supernatant 1.0 ml of 5% sodium hydroxide and 4.5 ml of alkaline copper reagent were added. After 20 minutes 0.5 ml of Folin’s phenol reagent was added and the colour developed was read at 640 nm after 10 minutes. The standards were also run simultaneously.

Enzyme activity is expressed as µmoles of tyrosine released/hr/mg protein at 37°C.
2.14.5 β-N-acetyl glucosaminidase (EC : 3.2.1.30)

The activity of this lysosomal enzyme was assayed by the method of Marhur (1976).

Reagents

1. Substrate : 3.42 mg of 4-nitrophenyl-N-acetyl glucosaminide was dissolved in 1 ml of citrate buffer.

2. Citric acid - Sodium citrate buffer 0.1M, pH 4.2 : 46 ml of 0.1M sodium citrate solution was mixed with 54 ml of 0.1 M citric acid solution.

3. Glycine buffer 0.2 M, pH 10.7 : Glycine buffer was prepared by mixing equal volumes of 0.2 M glycine, 0.125 M sodium carbonate, and 0.1 M sodium chloride.

4. Standard : Five mg of p-nitrophenol in 100 ml of distilled water.

Procedure

To 0.2 ml enzyme preparation, 0.1 ml of buffered substrate was added and incubated at 37°C for 40 minutes. At the end of the incubation period, the reaction was arrested by the addition of 2.2 ml of 0.2 M glycine buffer and the colour developed was read at 420 nm using a Photochem colorimeter.
The enzyme activity is expressed as μmoles of p-nitrophenol liberated/hr/mg protein at 37°C.

2.15 ESTIMATION OF PROTEIN BOUND CARBOHYDRATES

2.15.1 Plasma Glycoproteins

The glycoproteins in plasma were precipitated, then hydrolysed and the protein bound hexose, hexosamine, and sialic acid were estimated from the hydrolysate. To 0.05 ml of plasma, 2.0 ml alcohol was added and centrifuged. The supernatant was decanted. The precipitate was hydrolysed with acid to liberate protein bound carbohydrates according to the method of Tettamanti et al. (1983) and estimated for hexose, hexosamine and sialic acid.

To the alcohol precipitate, 2.0 ml of 3N hydrochloric acid was added and the mixture was refluxed at 100°C for 4 hrs. in a test tube with suitable marble lids. The hydrolysate was neutralised with sodium hydroxide. Aliquots of the neutralised samples were taken for analysis.

2.15.2 Tissue Glycoproteins

Delipidired residues of tissues were prepared according to the method of Folch et al. (1957). The tissues were washed with saline and drained the water content. A weighed amount of tissue was homogenised with 7.0 ml of methanol in a potter Elvehjem homogeniser and filtered through a Whatman No.1 filter paper into a conical flask. The residues after filtration was scrapped and homogenized in 14 ml of chloroform. The residue was once again scrapped
from the filter paper and mixed with 10 ml of chloroform-methanol mixture (2:1 v/v) and the resulting filtrate was evaporated to dryness.

A known amount of delipidised residue of the tissues were hydrolysed with 2.0 ml of 4N HCl and 100°C for 4 hours. The hydrolysed materials was neutralised and used for the estimation of hexose and hexosamine.

2.15.2.1 Hexose

Hexose was estimated by the method of Niebes (1972).

Reagents

1. Orcinol - Sulphuric acid reagent:
   Solution A: Sixty ml of concentrated sulphuric acid was mixed with 40 ml of distilled water.
   Solution B: 1.6 g of orcinol (recrystallized from benzene) was dissolved in 100 ml of distilled water. 7.4 ml of solution A was mixed with 1 ml of solution B just before use.

2. Standard: Five mg of each of galactose and mannose were dissolved in 100 ml of distilled water (50 µg/ml).
Procedure

0.5 ml of the neutralized solution was made upto 1.0 ml with distilled water and added 8.5 ml of ice-cold orcinol reagent was added very slowly. The mixture was heated at 80°C for 15 minutes, cooled and left in the dark for 25 minutes for colour development. Then, the absorbance was read at 540 nm in a Photochem colorimeter.

Standard solutions containing 25-100 µg of hexose were treated in a similar manner.

The hexose content is expressed in plasma as mg/dl and in tissue as mg/100 mg of defatted tissue.

2.15.2.2 Hexosamine

Hexosamine was estimated by the method of Wagner (1979).

Reagent

1. Acetyl acetone reagent:
   Solution A - Trisodium phosphate 0.1M : 4.1 g of trisodium phosphate was dissolved in 25 ml of distilled water.
   Solution B - Potassium tetraborate 0.5N : 305.50 mg of potassium tetraborate was dissolved in 2.0 ml of distilled water. 3.5 ml of acetyl acetone was added to mixture of Solution A and Solution B in the ratio of 98:2 (v/v).
2. Ehrlich's reagent: Three hundred and twenty mg of p-dimethyl amino benzaldehyde was dissolved in 21 ml of isopropanol and 3.0 ml of concentrated HCl was added to it.

3. Standard galactosamine was prepared in the concentration range of 100 μg/ml in distilled water.

Procedure

0.5 ml of the neutralized sample was made up to 1.0 ml with distilled water. 0.6 ml of acetyl acetone reagent was added to all the tubes and heated in a boiling water bath for 30 minutes. After cooling 2.0 ml of Ehrilch’s reagent was added and the contents were shaken well. The pink colour developed was measured at 540 nm against the reagent blank.

Standard solution containing 10-40 μg of galactosamine were also treated in the similar manner.

The content of hexosamine is expressed in plasma as mg/dl and in tissue as mg/100 mg of defatted tissue.

2.15.2.3 Sialic Acid

The precipitated glycoproteins, as mentioned in section 2.15.1 or a known amount of delipidised residue of the tissue as mentioned in section 2.15.2 were hydrolysed with 1.0 ml of 0.1N sulphuric acid at 80°C for 60
minute to release sialic acid bound to the proteins. The solution was neutralised with sodium hydroxide.

Sialic acid was determined by the method of Warren (1959).

Reagents

1. Periodic acid 0.25 M: Fourteen g of sodium periodate was dissolved in 100 ml of 0.1 N sulphuric acid.

2. Sodium meta arsenite 4%: Four g of sodium meta arsenite was dissolved in 100 ml of 0.5 N hydrochloric acid.

3. Thiobarbituric acid: One hundred and forty four mg of thiobarbituric acid was dissolved in 20 ml of distilled water. The pH of the solution was adjusted to 9.0 ml with 6N sodium hydroxide. This reagent was prepared just before use.

4. Acidified butanol: Five ml of concentrate hydrochloric acid was added to 95 ml of n-butanol.

5. Standard: Ten mg of N-acetyl neuraminic acid was dissolved in 100 ml with distilled water.
Procedure

0.5 ml of the neutralised samples as taken along with standards (in the range of 10-50 µg). Blank contained 0.5 ml of 0.1 N sulphuric acid, 0.25 ml of periodate was added to all tubes and incubated at 37°C. After 30 minutes, 0.25 ml of arsenite solution was added to inhibit the reaction. Contents were mixed and 2.0 ml of thiobarbituric acid was added and the tubes were heated in a boiling water bath for 6 minutes. After cooling, the pink colour developed was extracted into 5.0 ml of acidified butanol phase, and was measured at 540 nm against a reagent blank.

The sialic acid content is expressed in plasma as µg/dl and in tissue as µg/100 ml of defatted tissues.

2.16 ESTIMATION OF COLLAGEN

Collagen was estimated in terms of hydroxy proline in the ankle joint cartilage of rats. For the estimation of hydroxy proline, the procedure of was followed.

Reagents

1. Copper sulphate 0.01 M : 249-98 mg of CuSO₄ was dissolved in 100 ml of distilled water.

2. Sodium hydroxide 2.5 N : Ten g of NaOH was dissolved in 100 ml of distilled water.
3. Hydrogen peroxide 6% : Five ml of hydrogen peroxide was made upto twenty ml with distilled water.

4. Sulphuric acid 3N : 8.3 ml of concentrated sulphuric acid was made upto 100 ml with distilled water.

5. Dimethyl amino benzaldehyde 5% : Five g of DMAB was dissolved in 100 ml of distilled water.

6. Standard solution : Ten mg of hydroxy proline dissolved in 100 ml of distilled water.

**Procedure**

The weighed amount of cartilage was hydrolysed with 3 ml of 6N HCl in sealed tubes for 24 hr at 110°C. The contents after hydrolysis were evaporated to dryness, decolourised with activated charcoal and made upto 3 ml with water. Aliquots were estimated for hydroxy proline. Standards with different concentration were taken. The volume was made upto 1.0 ml with distilled water. 1.0 ml each 0.05 M CuSO₄ and 2.5N NaOH were added. The tubes were then placed in a water bath at 40°C for 5 minutes and 1 ml of 6% H₂O₂ was added and kept for 10 minutes with the temperature of the water bath being increased to 80°C.
The tubes were cooled in ice and 4.0 ml of 3N \( \text{H}_2\text{SO}_4 \) was added with agitation and later 2.0 ml of 5% DMAB was added. The contents in the tubes were mixed thoroughly and placed in a water bath at 70°C for 16 min. The colour developed was read at 540 nm against a reagent blank.

The hydroxy proline was calculated and then multiplied by a constant factor 7.46 to convert percentage hydroxy proline to percentage collagen/100 mg cartilage.

2.17 ESTIMATION OF LIPID PEROXIDATION

2.17.1 Tissue Lipid Peroxidation

Tissue lipid peroxidation was measured by the method of Devasagayam and Tarachand (1987).

Reagents

1. Tris-HCl buffer 0.15 M, pH 7.4 : 1.81 g of Tris was dissolved in 90 ml of distilled water and the pH was adjusted to 7.4 with 3N HCl. The final volume made upto 100 ml with distilled water.

2. Potassium dihydrogen phosphate (\( \text{KH}_2\text{PO}_4 \)) 10 mM : One hundred and thirty six mg of \( \text{KH}_2\text{PO}_4 \) was dissolved in 100 ml of distilled water.

3. Thiobarbituric acid 1% : One g of thiobabituric acid was dissolved in 100 ml of distilled water.
4. **TCA 10%**: Ten g of TCA was dissolved in 100 ml of distilled water.

5. **Standard malondialdehyde (MDA)**: A stock (mg/ml) standard solution of malondialdehyde was prepared in distilled water. This was stored at 4°C and diluted just before use such that the working standard contained 50 nmoles/ml.

**Procedure**

The assay mixture of 2.0 ml contained 1.6 ml of Tris-HCl buffer, 0.2 ml of KH$_2$PO$_4$ (10 mM) and a suitable aliquot of the tissue homogenate. The system was incubated with shaking for 30 minutes at 37°C. Reaction was arrested by the addition of 1.0 ml of 10% TCA. 1.5 ml of 1% TBA was added to it and heated in a boiling water bath for 20 minutes. The test tubes were cooled and centrifuged at 3000 xg for 10 minutes after thoroughly mixing the content of each tube. The optical density was determined at 532 nm using a reagent blank. Standard malondialdehyde was also processed in similar method.

The values are expressed in terms of nmoles of MDA released/min/mg protein.

**2.17.2 Plasma Lipid Peroxidation**

The level of plasma lipid peroxides was determined by the method of Yogi (1976).
Reagents

1. Sulphuric acid 3N: 8.33 ml of concentrated sulphuric acid was made upto 100 ml with distilled water.

2. Phosphotungstic acid: To prepare a stock solution, 50 g of sodium tungstate (Na$_2$WO$_4$·2H$_2$O molybdate free) was dissolved in about 400 ml of distilled water. 40 ml of 85% phosphoric acid was added and refluxed gently for two hours, cooled and transferred to a 500 ml flask and made upto the mark with water. This reagent was stored in brown bottle, diluted 1 to 10 for use. This can be kept for months in a brown bottle.

3. Thiobarbituric acid (TBA) reagent: Equal volume of 0.67% thiobarbituric acid solution in water and glacial acetic acid (1:1 v/v were mixed).

4. n-Butanol.

5. Standard malondialdehyde was prepared with a concentration of 50 nmoles/ml as mentioned in section 2.17.1.

6. Sulphuric acid N/12: 0.23 ml of concentrated sulphuric acid was made upto 100 ml with distilled water.
Procedure

To 0.2 ml of plasma, 4.0 ml of 3N sulphuric acid was added, mixed well and 0.5 ml of 10% phosphotungstic acid was added. The contents were centrifuged and the supernatant was discarded. The sediment was mixed with 2.0 ml of N/12 sulphuric acid and 0.3 ml phosphotungstic acid. The mixture was centrifuged and the sediment was dissolved in 4.0 ml of distilled water and to this 1.0 ml of TBA reagent was added and the contents were heated in a boiling water bath for 60 minutes. After cooling, 5 ml of n-butanol was added and the contents were shaken vigorously. Then, it was centrifuged for 20 minutes and the supernatant was read at 535 nm in Photochem colorimeter. Standards were also processed in the same manner.

Plasma lipid peroxide values are expressed as nmoles MDA/dl plasma.

2.17.3 Erythrocyte Lipid Peroxidation

The level of erythrocyte lipid peroxide content was determined by the method of Cynamon et al. (1985).

Reagents

1. Isotonic phosphate buffered saline 0.1M, pH 7.4 : 2.3 mg of disodium hydrogen phosphate and 593 mg of sodium dihydrogen phosphate were dissolved in 81 ml of 19 ml of physiological saline (0.89% NaCl) respectively and both solutions were mixed and made upto 200 ml with saline.
2. Sodium azide 0.026%: Twenty six mg of sodium azide was dissolved in 100 ml of isotonic phosphate buffered saline.

3. TCA 40%. 40 g of TCA was dissolved in 100 ml of distilled water.

4. Thiobarbituric acid (TBA) 1%: One g of TBA was dissolved in 100 ml of distilled water.

5. Standard malondialdehyde was prepared with a concentration of 50 nmoles/ml as mentioned in section 2.17.1.

Procedure

To an aliquot of erythrocyte membrane preparation 3.8 ml of phosphate buffer and 0.2 ml of sodium azide was added and incubated at 37°C for 1 hour. After incubation, 1.0 ml of 40% TCA was added. The tubes were centrifuged and to the supernatant, 1.0 ml of 1% TBA was added. The contents were kept in a boiling water bath for 20 minutes. Then it was cooled to room temperature and read at 535 nm using a Photochem colorimeter.

The erythrocyte lipid peroxide contents are expressed as nmoles of MDA/min/mg protein.
2.18 ESTIMATION OF ANTIOXIDANTS

2.18.1 Superoxide Dismutase (EC : 1.15.1.1, SOD)

The enzyme was assayed according to the method of Marklund and Marklund (1974).

Reagents

1. Tris-HCl buffer 0.1M, pH 8.2: 1.21 g of Tris was dissolved in 90 ml of distilled water, the pH was adjusted to 8.2 with 3N HCl and the volume made up to 100 ml with distilled water.

2. Tris-HCl buffer 0.5M, pH 7.4: Six hundred and five mg of Tris was dissolved in 90 ml of distilled water, the pH was adjusted with 3N HCl and the volume made up to 100 ml with distilled water.

3. Pyrogallol solution 2 mM: 2.52 g of pyrogallol was dissolved in 10 ml of 0.05 M Tris-HCl buffer in an aluminium foil wrapped stoppered test tube.

4. Absolute ethanol (AR).

5. Chloroform (AR).
Procedure

Partially purified superoxide dismutase was prepared as described by McCord et al. (1971). To 1.0 ml of the sample, 0.25 ml of absolute ethanol and 0.15 ml of chloroform were added. After 15 minutes of shaking in a mechanical shaker, the suspension was centrifuged and the supernatant obtained constituted the enzyme extract.

The reaction mixture for autoxidation consisted of 2.0 ml of the buffer (Tris-HCl, pH 8.2), 0.5 ml 2 mM pyrogallol and 1.5 ml water. Initially, the rate of autoxidation of pyrogallol was noted at an interval of one minute for 3 minutes.

The assay mixture for the enzyme contained 2 ml of 0.1 M Tris-HCl buffer, 0.5 ml of pyrogallol, aliquots of the enzyme preparation and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol autoxidation after the addition of the enzyme was noted.

The enzyme activity is expressed in terms of units/min/mg protein in which one unit corresponds to the amount of enzyme required to bring about 50% inhibition of pyrogallol autoxidation.

2.18.2 Catalase (EC : 1.11.1.6)

The catalase activity was assayed by the method of Sinha (1972).
Reagents

1. Dichromate - acetic acid reagent : Five percent potassium dichromate in water was mixed with acetic acid in the ratio 1:3 (v/v). The solution was further diluted to 1:5 with distilled water.

2. Phosphate buffer 0.01M, pH 7.0 : One hundred and seventy three mg of disodium hydrogen phosphate and 122 mg of sodium dihydrogen phosphate were dissolved in 61 ml and 39 ml of distilled water, respectively.

3. Hydrogen peroxide 0.2 M : 2.27 ml of hydrogen peroxide was made upto 100 ml with distilled water.

Procedure

0.1 ml of the homogenate was taken to which 1.0 ml of 0.01 M phosphate buffer and 0.5 ml of hydrogen peroxide were added. The reaction was arrested by the addition of 2.0 ml dichromate - acetic acid reagent. Standard hydrogen peroxide in the range of 4 to 20 μmoles were taken and treated similarly. The tubes were heated in a boiling water bath for 10 minutes. The green colour developed was read at 570 nm in a Photochem colorimeter.

Catalase activity in tissue homogenates is expressed as μmoles of \( \text{H}_2\text{O}_2 \) consumed/minute/mg protein at 37°C.
2.18.3 Glutathione Peroxidase (EC : 1.11.1.9, GPx)

The activity of glutathione peroxidase was assayed by the method of Rotruck et al. (1973).

Reagents

1. Sodium phosphate buffer 0.32 M, pH 7.0 : 6.96 g of disodium hydrogen phosphate and 3.89 g of sodium dihydrogen phosphate were dissolved in 61 ml and 39 ml distilled water, respectively.

2. Ethylene diamine tetra acetic acid (EDTA) 0.8 mM : Two hundred and thirty three mg of EDTA was dissolved in 100 ml of distilled water.

3. Sodium azide 10 mM : One hundred and sixty five mg of sodium azide was dissolved in 100 ml of distilled water.

4. Reduced glutathione (GSH) 4 mM : One hundred and twenty three mg of GSH was dissolved in 100 ml of distilled water.

5. Hydrogen peroxide 2.5 mM : 0.03 ml of hydrogen peroxide solution was made upto 100 ml with water.

6. TCA 10% : Ten g of TCA was dissolved in 100 ml of distilled water.
7. Disodium hydrogen phosphate 0.3 M: 5.33 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.

8. 5,5’-dithio bis (2-nitrobenzoic acid) (DTNB): Forty mg of DTNB was dissolved in 100 ml of 1% Trisodium citrate.

9. Glutathione standard: Ten mg of reduced glutathione was dissolved in 100 ml of distilled water.

Procedure

0.2 ml each of EDTA, sodium azide, glutathione (reduced), hydrogen peroxide, 0.4 ml of buffer and 0.1 ml of homogenate were mixed and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged. To 0.5 ml of supernatant, 4.0 ml of disodium hydrogen phosphate and 0.5 ml of DTNB were added and the colour developed was read at 420 nm immediately using a Photochem colorimeter. Standards were also treated similarly.

Glutathione peroxidase activity is expressed as μg of glutathione utilized/minute/mg protein at 37°C.

2.18.4 Reduced Glutathione

Reduced glutathione was determined by the method of Moron et al. (1979).
Reagents

1. Phosphate solution: 5.33 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.

2. TCA 10%: Ten g of TCA was dissolved in 100 ml distilled water.

3. DTNB 0.6 mM: Four hundred mg of DTNB was dissolved in 100 ml of 1% Tri sodium citrate solution.

4. Standard: Ten mg of reduced glutathione was dissolved in 100 ml of distilled water.

Procedure

One ml of tissue homogenate was precipitated with 1.0 ml of 10% TCA. The precipitate was removed by centrifugation. To an aliquot of the supernatant was added 4.0 ml of phosphate solution and 0.5 ml of DTNB reagent. The colour developed was read at 420 nm using a Photochem colorimeter.

The amount of glutathione in tissue is expressed as μg/mg protein.

2.18.5 Total Sulphydryl Content

Sulphydryl content of the tissue was estimated by the method of Moron et al. (1979).
Reagents

1. Tris-HCl 0.2M, pH 8.2: 2.41g of Tris was dissolved in 90 ml of distilled water and the pH was adjusted to 8.2 with 3N HCl. The final volume was made upto 100 ml with distilled water. 2 mM of EDTA was added to this solution.

2. TCA 5%: Five g of TCA was dissolved in 100 ml of distilled water.

3. EDTA 2mM: Seven hundred and forty four mg of EDTA was dissolved in 100 ml of distilled water.

4. 5-5’ dithio bis (2-nitro benzoic) acid (DTNB): Ninety nine mg of DTNB was dissolved in 25 ml of methanol.

5. Standard: Ten mg of reduced glutathione was dissolved in 100 ml of distilled water.

Procedure

To 0.5 ml of homogenate 1.5 ml of 0.2 M Tris-HCl buffer, pH 8.2 containing 2mM EDTA was added followed by the addition of 0.1 ml of DTNB and 7.5 ml methanol. The contents were mixed well in a vortex mixer and then centrifuged at 3000 xg for 10 minutes. The colour developed was read at 412 nm using a reagent blank.

The total sulphhydryl content is expressed as µg/mg protein.
2.19 ESTIMATION OF ADENOSINE TRIPHOSPHATASES

2.19.1 Na⁺, K⁺-ATPase (Adenosine Triphosphatase) (EC : 3.6.1.3)

Na⁺, K⁺-ATPase was estimated by the method of Bonting (1970).

Reagents

1. Tris-HCl buffer 184 mM, pH 7.5 : 2.23 g of Tris was dissolved in 90 ml distilled water and the pH was adjusted to 7.5 with 3N HCl. Then, the final volume was made upto 100 ml with distilled water.

2. Magnesium sulphate 50 mM : Six hundred and two mg of magnesium sulphate was dissolved in 100 ml of distilled water.

3. Potassium chloride 50 mM : 372.8 mg of potassium chloride was dissolved in 100 ml of distilled water.

4. Sodium chloride 600 mM : 3.51 g sodium chloride was dissolved in 100 ml of distilled water.

5. EDTA 1.0 mM : 37.22 mg of EDTA was dissolved in 100 ml of distilled water.

6. ATP 40 mM : 220.5 mg of ATP was dissolved in 10 ml of distilled water.

7. TCA 10% : Ten g of TCA was dissolved in 100 ml of distilled water.
8. Ammonium molybdate 2.5% : 2.5 g of ammonium molybdate was dissolved in 100 ml of 3N sulphuric acid.

9. ANSA : 500 mg of Amino Napthal Sulphonic Acid was dissolved in 195 ml of 15% sodium bisulphite and 5.0 ml of 20% sodium sulphite was added to it. The solution was stored in a brown bottle.

**Procedure**

The incubation mixture contained 1.0 ml of Tris-HCl buffer, 0.2 ml each of magnesium sulphate, potassium chloride, sodium chloride, EDTA, ATP and homogenate. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of 10% TCA, mixed well and centrifuged. The supernatant along with the aliquots of standards in the range of (8-40 μg) were made up to 4.3 ml with distilled water, 0.5 ml of ammonium molybdate and 0.2 ml of ANSA were added and mixed well. The colour developed was read at 620 nm after 30 minutes against the reagent blank in a photochem colorimeter.

The enzyme activity is expressed as μmoles of phosphorus liberated/min/mg protein at 37°C.

**2.19.2 Ca²⁺-ATPase (EC : 3.6.1.3)**

The activity of Ca²⁺-ATPase was assayed according to the method of Hjerten and Pan (1983).
Reagents

1. Tris-HCl buffer 125 mM, pH 8.0: 1.54 g of Tris was dissolved in 90 ml of distilled water and the pH was adjusted to 8.0 with 3N HCl. The final volume was made up to 100 ml with water.

2. Calcium chloride 50 mM: Five hundred and fifty six mg of calcium chloride was dissolved in 100 ml of distilled water.

3. ATP 10 mM: 55.12 mg of ATP was dissolved in 10 ml of distilled water.

4. TCA 10%: Ten g of TCA was dissolved in 100 ml of distilled water.

Procedure

The incubation mixture contained 0.1 ml each of Tris-HCl buffer, calcium chloride, ATP and enzyme preparation. After incubation at 37°C for 15 minutes, the reaction was arrested by the addition of 1.0 ml 10% TCA. The amount of phosphorous liberated was estimated as described in section 2.19.1.

The enzyme activity is expressed as μmoles of phosphorous liberated/min/mg protein at 37°C.
2.19.3  \( \text{Mg}^{2+}\text{-ATPase (EC : 3.6.1.3)} \)

The activity of \( \text{Mg}^{2+}\text{-ATPase} \) was assayed by the method of Ohinishi \textit{et al.} (1982).

\textbf{Reagents}

1.  Tris-HCl buffer 375 mM, pH 7.6 : 4.54g of Tris was dissolved in 90 ml of distilled water and the pH was adjusted to 7.6 with 3N HCl. Then, the final volume was made upto 100 ml with distilled water.

2.  Magnesium chloride 25 mM : Two hundred and thirty eight mg of magnesium chloride was dissolved in 100 ml of distilled water.

3.  ATP 10 mM : 55.12 mg of ATP was dissolved in 10 ml of distilled water.

4.  TCA 10% : Ten g of TCA was dissolved in 100 ml of distilled water.

\textbf{Procedure}

The incubation mixture contained 0.1 ml each of Tris-HCl buffer, magnesium chloride, ATP and enzyme preparation. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0 ml 10% TCA. The liberated phosphorous was estimated as described in section 2.19.1.
The enzyme activity is expressed as μmoles of phosphorous liberated/min/mg protein at 37°C.

2.20 ISOLATION OF ERYTHROCYTE AND ITS MEMBRANE

Blood collected with 3.7% trisodium citrate, as anticoagulant (0.1 ml/ml), was used for erythrocyte isolation. Plasma was separated by centrifugation at 2000 xg for 20 minutes. The packed cells were washed thrice with physiological saline and the plasma, free red cells were used for the analysis. Erythrocyte membrane was isolated.

Reagents

1. Sodium chloride 0.89% : Eight hundred and ninety mg of sodium chloride was dissolved in 100 ml of distilled water.

2. Tris-HCl buffer 0.31 M, pH 7.4 : 3.76 g of Tris was dissolved in 95 ml of distilled water, the pH was adjusted with 3N HCl to 7.4. The volume was made upto 100 ml with distilled water.

3. Tris-HCl buffer 0.015 M, pH 7.2 : 102.1 mg of Tris was dissolved in 95 ml of distilled water, the pH was adjusted to 7.2 with 3N HCl. Then, the volume was made upto 100 ml with distilled water.
Procedure

Packed cells, remaining after the removal of plasma, were washed with isotonic saline to remove the buffy coat. The packed cells were then washed three times with isotonic Tris-HCl buffer, 0.31M, pH 7.4. Haemolysis was performed by pipetting out the washed red blood cell suspension into polypropylene centrifuge tubes which contained hypotonic buffered (Tris-HCl buffer 0.015M, pH 7.2). Erythrocyte ghosts were sedimented in a high speed refrigerated centrifuge at 20,000 xg for 40 minutes. The ghost sedimented at the bottom was resuspended by swirling with sufficient buffer of the same strength to reconstitute to the original volume. The ratio of the cells to washing solution was approximately 1:3 (v/v). The cell membrane was washed three times with hypotonic buffer and subsequent to haemolysis. The supernatant, after the last wash, was either pale pink or colourless.

The pellet of erythrocyte membrane was dissolved in 10 ml of Tris-HCl buffer, 0.31 M, pH 7.4. Aliquots of this were taken for the estimation of protein, membrane ATPase, and lipid peroxides.

2.20.1 Membrane ATPases

0.1 ml aliquots from the erythrocytes membrane preparation were used for the estimation of Na⁺ - K⁺, Mg²⁺ and Ca²⁺-ATPase activity as described in section 2.19.
The enzyme activity is expressed as μmoles of Pi liberated/hr/mg protein at 37°C.

2.20.2 Membrane Protein

Total protein of membrane was estimated in an aliquot of diluted membrane in Tris-HCl buffer by the method of Lowry et al. (1951) as described in section 2.7.

2.21 HISTOPATHOLOGICAL TECHNIQUE

A section of liver, kidney, heart and adrenal from all the group of animals were taken and immediately fixed in 10% formalin. Paraffin section of the fixed tissues were stained with Haematoxylin and Eosin and then examined under microscope.

2.22 STATISTICAL ANALYSIS OF DATA

All values are expressed as mean ± Standard Deviation (S.D.) for control and experimental animals. The statistical significance of values between groups were analysed using student’s t-test.