Materials and Methods
 CHEMICALS AND THEIR SOURCES

The fine chemicals used during the course of experiment were obtained from following sources.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Chemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cholesterol, sodium succinate, sodium deoxycholate, trisodium citrate,</td>
<td>Sigma Chemical Company, St. Louis Missouri, USA.</td>
</tr>
<tr>
<td></td>
<td>bovine serum albumin, phospholipid standard,</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Potassium α-ketoglutarate, uranyl acetate, DNPH, EDTA</td>
<td>British Drug House Ltd., Laboratory Chemicals Division, England</td>
</tr>
<tr>
<td>3.</td>
<td>Thiamine pyrophosphate, glutathione oxidised, glutathione</td>
<td>EMerck, A.G., Darmstadt, West Germany.</td>
</tr>
<tr>
<td></td>
<td>dinucleotide reduced, Adenosine diphosphate, Ammonium molybdate</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>ATP</td>
<td>Boehringer-Mannheim, Germany</td>
</tr>
<tr>
<td>6.</td>
<td>Flaxseed oil</td>
<td>Vignesh scientific consultancy, No: 38 Mambalam high road, T. Nagar, Chennai.</td>
</tr>
</tbody>
</table>

Acids, bases, solvents and salts used for the investigations were of analytical grade.
Experimental study design

Adult male albino rats of Wistar strain weighing about 120 – 150g were used for the study. The animals were obtained from Tamil Nadu University of Veterinary and Animal Sciences [TANUVAS], Madhavaram, Chennai. The animal room was well ventilated with a 12 hrs light / dark cycle throughout the experimental period. They were maintained in clean, sterile, polypropylene cages and fed with commercial pelleted rat chow (M/S Hindustan Lever Limited, Bangalore, India) and water ad libitum. All animal experiments were carried according to the guidelines of the Institutional Animals Ethics Committee (IAEC).

The rats were divided into 4 groups of 6 animals each as follows.

**Group I**  
Control rats

**Group II**  
Rats fed with high fat diet orally for 60 days (HFD rats) [Toshiharu Akiyama, 1996]

**Group III**  
Rats fed with high fat diet and administered with flaxseed oil (1g / kg b. wt / day orally) for 60 days (HFD + FO rats)

**Group IV**  
Rats administered only with flaxseed oil (1g / kg b. wt orally) for 60 days. (FO rats).

At the end of the experimental period, the rats were fasted overnight and anaesthetized with anaesthetic ether and the blood was collected through
cardiac puncture. The blood collected was used for serum separation and erythrocyte preparation. The heart, aorta and liver tissues were dissected out and washed in ice-cold saline. A portion of the tissues was homogenized in 0.1M Tris–Hcl buffer, pH 7.4 at 4°C and used for the analysis of biochemical parameters. Another portion of the heart, aorta and liver tissues were stored in formal saline and used for histological analysis.

Drug

Flaxseed oil was obtained from Vignesh Scientific Consultancy, Chennai, as a complement and used for the present investigation.

Dosage fixation

Different doses of flaxseed oil 500mg, 1000mg and 1500mg/kg body weight/day were administered orally for different time intervals of 30, 60 and 90 days to assess the effective dose and treatment duration against high fat diet induced hyperlipidemia based on the levels of plasma lipid profile and changes in body and organ weights.

ECG

Under sodium thiopentone anesthesia (30 mg/kg body weight i.p) needle electrodes were placed subcutaneously in the gently extended limbs of supine animals. ECG recording of the rat was taken under mild anesthesia using poly graph on a bipolar standard lead-2 at a sensitivity of 1 MV giving a deflection of 1 cm.
Histopathological studies

Heart, Liver and aorta from experimental and control animals were fixed in 10% neutral buffered formalin processed by standard procedure for paraffin embedding and serial sections were cut (5μ). The sections were stained with hematoxylin and eosin dyes.

Estimation of marker enzymes

Assay of Creatine kinase (ATP-Creatine phospho transferase E.C. 2.7.2.3)

Serum creatine kinase activity was determined by the method of Okinaka et al. (1961).

The reaction catalysed by this enzyme is as follows.

Creatine kinase + ATP ← ----→ Creatine phosphate + ATP

Reagents

1. Tris HCl buffer : 0.1 M pH 9.0

2. ATP : 112 mg of ATP was dissolved in 10 ml of above Tris HCl buffer.

3. Magnesium -

Cysteine reagent : 59 mg of magnesium sulfate and 15.76 mg of cysteine-HCl were dissolved in 10 ml of distilled water.
4. Creatine : 240 mM

5. Ammonium : 2.5 g of ammonium molybdate was Molybdate dissolved in 100 ml of 3N sulfuric acid.

6. TCA : 10% trichloroacetic acid

7. ANSA reagent : 0.5 g of ANSA was dissolved in 195 ml of 15% sodium metabisulphite and 5.0 ml of 20% sodium sulfite was added for complete solubilisation. This solution was filtered and stored in a brown bottle.

8. Standard phosphorus: 35.1 mg of potassium dihydrogen phosphate was accurately weighed, dissolved in 100 ml of distilled water 1.0 ml of this solution-contained 80μg of phosphorus.

Procedure

The reaction mixture contained 0.05 ml of serum, 0.1 ml of substrate, 0.1 ml of ATP solution and 0.1 ml of cysteine – hydrochloride solution.

The final volume was made upto 2.0 ml and incubated at 37°C for 30 minutes. The reaction was arrested by the addition of 1.0 ml of TCA and the contents subjected to centrifugation.

0.1 ml of the supernatant was made upto 4.3 ml with water. 1.0 ml of ammonium molybdate reagent was added incubated at room temperature for
10 minutes. 0.4 ml of ANSA was added and the color developed was read at 640 nm after 20 minutes in a Shimadzu UV spectrophotometer.

Activity of creatine kinase in serum is expressed as IU/L.

**Assay of Lactate dehydrogenase (L-Lactate; NAD Oxido-reductase E.C.1.1.1.27)**

The activity of lactate dehydrogenase (LDH) was measured by the method of Nieland (1955).

**Reagents**

1. Glycine buffer : 100 mM.
2. Buffered substrate : 2.78 g of lithium lactate was dissolved in 125 ml of glycine buffer containing 75 ml of 0.1 N sodium hydroxide solution. This was prepared just before use.
3. NAD⁺ : 20 mM
4. DNPH : 0.2% of DNPH in 1 N hydrochloric acid.
5. Sodium hydroxide : 0.4 N
6. Standard pyruvate solution : 11 mg of sodium pyruvate was dissolved in 100 ml of buffer.

**Procedure**

To a set of tubes, 1 ml of the buffered substrate and 0.1 ml of serum was added and the tubes were incubated at 37°C for 15 minutes. After adding 0.2 ml of NAD solution, the incubation was continued for another 15 minutes.
The reaction was then arrested by adding 1 ml of DNPH reagent and the tubes were incubated for further period of 15 minutes at 37°C. 0.1 ml of serum was added to blank tubes after arresting the reaction with DNPH. 7 ml of sodium hydroxide solution was added and the color developed was measured at 420 nm in a Shimadzu UV spectrophotometer. Suitable aliquots of the standard were also analyzed by the same procedure.

The enzyme activity is expressed as IU/L serum.

**Estimation of Phosphorus**

Phosphorus was estimated according to the method of Fiske and Subbarow (1925).

**Reagents**

1. Ammonium molybdate: 2.5% in 3 N sulfuric acid.

2. ANSA reagent: 0.5 g ANSA was dissolved in 195 ml of 15% sodium meta sulfite and 5 ml of 20% sodium sulfite was added for complete solubilization. This solution was filtered and stored in a brown bottle.

3. Standard phosphorus: 35.1 mg of potassium dihydrogen phosphate was accurately weighed, dissolved in 100 ml double distilled water. 1 ml of this solution contained 80 µg of phosphorus.

**Procedure**

The aliquots of supernatant and standards were taken and made upto 4.3 ml with water. 0.5 ml of ammonium molybdate and 0.2 ml of ANSA were
added and mixed. The blue color developed was read after 20 minutes at 640 nm in a Shimadzu UV spectrophotometer.

The levels of phosphorus are expressed as µ moles of Pi liberated/dl.

**Assay of Aspartate aminotransferase (Glutamate oxaloacetate transaminase, E.C. 2.6.1.1)**

Aspartate amino transaminase (AST) was assayed by the method of King (1965).

**Principle**

The reaction catalysed by aspartate amino transferase is as follows:

\[
\text{AST} \quad \text{L-Aspartate} + 2\text{-oxoglutarate} \quad \text{<------->} \quad \text{Oxaloacetate} + \text{L-glutamate}
\]

The product formed reacts with DNPH and gives a coloured complex, which can be read at 540 nm.

**Reagents**

1. Phosphate buffer : 0.1 M, pH 7.4

2. Substrate : 2.66 g DL-aspartate and 38 mg of 2-oxoglutarate were dissolved in 20.5 ml of 1.0 N sodium hydroxide with gentle heating. This was made upto 100 ml with phosphate buffer.

3. Colour reagent : 1 mM 2,4 dinitrophenyl hydrazine (DNPH) in 2N hydrochloric acid.
4. Sodium hydroxide : 0.4 N

5. Standard pyruvate : 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer 0.1 M, pH 7.4.

Procedure

In different tubes, 1 ml of the buffered substrate was added to 0.1 ml of the sample and incubated at 37°C for 1 hr. Then 1 ml of DNPH reagent was added to arrest the reaction. To the blank tubes, 0.1 ml of serum was added only after the addition of DNPH reagent. The tubes were kept aside for 15 min, then 10 ml of 0.4 N sodium hydroxide was added and read at 520 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed in serum as IU/L.

Assay of Alanine amino transferase : (Glutamate Pyruvate Transaminase E.C. 2.6.1.2)

The activity of alanine transaminase (ALT) was assayed by the method of King (1965).

Principle

The reaction catalysed by alanine amino transferase is as follows:

\[
\text{ALT} \\
\text{L-Alanine} + 2-\text{Oxoglutarate} \rightarrow \text{Pyruvate} + \text{L-glutamate}
\]

The pyruvate formed reacts with DNPH and gives a colour complex which can be read at 540 nm.
The reagents and method used were the same as those used for the assay of aspartate transaminase except for the substrate solution and the incubation time reduced to 30 min.

**Substrate**

1.78 g of DL-alanine and 38 mg of 2-oxoglutarate were dissolved in buffer. 0.5 ml in sodium hydroxide was added and the volume was made up to 100 ml with water.

The enzyme activity was expressed in serum as IU/L.

**Assay of Acid phosphatase**

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

**Reagents**

1. Acetate-acetic acid buffer; pH 4.9, 0.1M
2. Disodium phenyl phosphate solution : 0.01M
3. Folin's phenol reagent : Into a 1,500ml round bottomed flask, 100g of sodium tungstate, 25g of sodium molybdate, 700ml of water, 50ml of 85% O-phosphoric acid and 100ml of concentrated hydrochloric acid were added and refluxed for 10 hours. Then 150g of lithium sulphate, 50ml of distilled water and a few drops of bromine were added. The mixture was boiled. It was then cooled and diluted to one litre with water. The reagent was diluted 1:2 with distilled water just before use.
4. Sodium carbonate: 15%.

5. Standard phenol: 100mg of recrystallized phenol in 100 ml of water.  
   100 mg of phenol per ml was prepared and used as working standard.

Procedure

The incubation mixture of 3.0ml contains 1.5ml of buffer, 1.0ml of substrate and requisite amount of the enzyme source. The tubes were incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0ml of Folin's phenol reagent. The control tubes received the enzyme after arresting the reaction. The contents was centrifuged and to the supernatant, 1.0ml of 15% sodium carbonate was added and the mixture incubated for 10 minutes at 37°C. The colour was read at 640 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed in serum as IU/L.

Assay of Alkaline phosphatase (Ortho-phosphoric monoester phosphohydrolase) E.C. 3.1.3.1

Alkaline phosphatase (ALP) was assayed by the method of King (1965) using disodium phenyl phosphate as substrate.

Principle

Measuring the phenol liberated from disodium phenyl phosphate by the colour reaction with Folin's reagent in the presence of alkali follows the hydrolysis.
**Reagents**

1. Carbonate-bicarbonate buffer: 0.1 M, pH 10
2. Disodium phenyl phosphate solution: 0.01 M
3. Magnesium chloride solution: 0.1 M
4. Folin's - Ciocalteau reagent: A commercial preparation was diluted 1:2 with distilled water, before use.
5. Sodium carbonate solution: 15%
6. Standard phenol: 100 mg of recrystallized phenol in 100 ml of water was prepared. 100 mg of phenol/ml was then prepared by proper dilution and used as the working standard.

**Procedure**

The incubation mixture of 3 ml contains 1.5 ml of buffer, 1 ml of substrate and requisite amount of enzyme source. The reaction mixture was incubated at 37°C for 15 min. The reaction was arrested by the addition of 1 ml of Folin's phenol reagent. The control tubes received the enzyme after arresting the reaction. The contents were centrifuged and to the supernatant, 1 ml of 15% sodium carbonate solution, 1 ml of the substrate and 0.1 ml of magnesium chloride were added and the mixture incubated for 10 min at 37°C. The colour was read at 640 nm against a blank in a Shimadzu UV spectrophotometer. The standard solution of phenol of varying concentrations was also treated similarly.

The enzyme activity was expressed in serum as IU/L.
Estimation of Glucose

Glucose was estimated by the method of Sasaki et al. (1972).

Reagents

1. Trichloro acetic acid: 10%

2. O-Toluidine reagent: 12.5 g of thiourea and 12.0 g of boric acid were dissolved in 50 ml of distilled water by heating over a mild flame. 75 ml of O-toluidine (redistilled) and 375 ml of acetic acid (AR) were mixed separately. These two solutions were mixed and the total volume was made up to 500 ml with distilled water. The reagent was left overnight in the refrigerator and filtered.

3. Glucose standard: 100 mg of pure glucose was dissolved in 100 ml of distilled water containing 0.01% benzoic acid.

Procedure

To 0.1 ml of freshly drawn blood, 1.9 ml of trichloro acetic acid was added to precipitate the proteins and centrifuged. 1 ml of the supernatant was mixed with 4 ml of O-toluidine reagent and kept in a boiling water bath for 15 minutes. The greenish blue color developed was read at 640 nm in a Shimadzu UV spectrophotometer. Blank containing 2 ml of water and standards containing 20 to 40 µg of glucose were also treated similarly.

The levels are expressed as mg/dl blood.
Estimation of Urea

Urea was determined by the method of Geyer and Dabich (1971).

Reagent

1. Diacetyl monoxime - Thiosemicarbazide reagent (DAM-TSC): 36 mM diacetyl monoxime and 61.7 mM thiosemicarbazide in acetic acid.

2. Acid ferric reagent: 3.6 ml sulfuric acid, 0.12 mg ferric chloride and 38.6 ml O-phosphoric acid.

3. Trichloro acetic acid: 10%

4. Standard urea: 10 mg of urea was dissolved in 100 ml of distilled water.

Procedure

0.2 ml of blood was deproteinised with 2.8 ml of trichloro acetic acid. To 2 ml of the supernatant obtained by centrifugation, 1 ml of DAM-TSC reagent and 1.5 ml of acid ferric reagent were added and the solution was heated in a boiling water bath for 15 minutes.

Aliquots of standard urea and blank containing 2 ml water were also treated in a similar manner. After cooling, the color developed was read at 520 nm in a Shimadzu UV spectrophotometer.

The levels are expressed as mg/dl blood.
Estimation of protein

Protein content 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 sulfate in water
   Solution C : 1-% sodium potassium tartarate in water.
   50 ml of solution A was mixed with 0.5 ml of solution B and 1.0 ml of solution C just before use.

2. Folin-Ciocalteau reagent: The commercial preparation was diluted 1:2 with water before use

3. Standard bovine serum albumin: 10 mg of crystalline bovine serum albumin was dissolved in 100 ml of distilled water.

Procedure

An aliquot of the suitably diluted sample was made upto 1 ml with water. 4.5 ml of alkaline copper reagent was added to all the tubes including blank. Blank containing 1 ml of water and standard containing aliquots of bovine serum albumin were also treated similarly. After mixing, contents were left to stand for 10 minutes at room temperature. Then 0.5 ml of diluted Folin-Ciocalteau reagent was added. The blue color developed was read at 640 nm after 20 minutes in a Shimadzu UV spectrophotometer.

The levels are expressed as mg/dl in plasma.
Estimation of Uric acid

Uric acid was estimated according to the method of Caraway (1963).

Reagents

1. Coloring reagent: 50 g of molybdate free sodium tungstate was dissolved in 400 ml of distilled water. Added 40 ml of phosphoric acid and refluxed for 2 hours. A drop of bromine was added, cooled and diluted to 500 ml with water.

2. Sodium carbonate reagent: 20% aqueous solution

3. Uric acid standard: 100 mg of uric acid was dissolved in 150 ml of water containing 60 mg of lithium carbonate by heating at 60ºC. The solution was cooled to room temperature and to this 2 ml of formaldehyde (400 ml/L), diluted to about 500 ml and slightly acidified with sulfuric acid.

4. Working standard: 1 ml of the stock standard and 2 ml of 300 g/L bovine serum albumin were diluted to 10 ml with water. The working standard was prepared fresh. Albumin was added to account for the positive error induced by a co-precipitation of uric acid and proteins.

Procedure

5.4 ml of diluted tungstic acid was added to 0.6 ml of serum. The contents were mixed and centrifuged. Into the test tubes 3 ml of supernatant, standard and water (as blank) were taken. 0.6 ml of sodium
carbonate and 0.6 ml of phosphotungstic acid reagent were added, mixed and placed in a 25°C water bath for 10 minutes. The blue color developed was read at 700 nm.

The levels are expressed as mg/dl serum.

**Estimation of Creatinine**

Creatinine was estimated by the method of Slot (1971).

**Reagents**

1. Picric acid: 0.12% of picric acid in distilled water.
2. Sodium hydroxide: 3% in distilled water.
3. Alkaline picrate reagent: Equal volumes of solutions (1) and (2) were mixed before use.
4. Sodium tungstate solution: 5% in distilled water.
5. Sulfuric acid: 330 mM
6. Creatinine standard: 100 mg of creatinine was dissolved in 100 ml of 0.1-ml hydrochloric acid. Before use, this stock standard was diluted to 10 fold with water.
7. Glacial acetic acid.

**Procedure**

To 3 ml of deproteinized supernatant (0.1 ml of serum and 3.9 ml 10% TCA), added 2 ml of alkaline picrate solution. Blank containing 3 ml of water
and aliquots of standard in 3 ml of water were also treated in a similar manner. After 30 minutes the color was measured at 520 nm against the reagent blank.

The levels are expressed as mg/dl serum.

**Estimation of Homocysteine**

The axis homocysteine enzyme immunoassay by was used for the quantitative determination of total L-homocysteine in plasma.

**Principle**

Protein bound homocysteine is released to free homocysteine and enzymatically converted to S-adenosyl-homocysteine (SAM) prior to immunoassay. The enzyme is specific for the L-from of homocysteine which is the form present in blood.

The solid-phase immunoassay is based on competition between S-adenosyl-L-homocysteine hydrolase (SAH) in the sample and immobilized SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. After removal of anti SAH antibody not bound to the plate, a secondary rabbit anti- mouse antibody labelled with the enzyme horseradish peroxidase (HRP) is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance is inversely related to the concentration of total homocysteine in the sample.
## Reagents in Axis Homocysteine Kit.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>Phosphate buffer, 0.09% Sodium nitrate (NaNO₃)</td>
</tr>
<tr>
<td>Reagent B</td>
<td>Adenosine, dithiothretol, citric acid.</td>
</tr>
<tr>
<td>Reagent C</td>
<td>Bovine S-adenosyl-L-homocysteine hydrolase tris w/glycerol, methylparaphine</td>
</tr>
<tr>
<td>Reagent D</td>
<td>0.15% Merthiolate, phosphate buffer</td>
</tr>
<tr>
<td>Reagent E</td>
<td>Adenosine deaminase, phosphate buffer, BSA, 0.09% BSA 0.01 % merthiolate</td>
</tr>
<tr>
<td>Reagent F</td>
<td>Monoclonal mouse-anti-S-adenosyl-L-homosteine antibody, BSA, 0.01% merthiolate</td>
</tr>
<tr>
<td>Reagent G</td>
<td>Rabbit anti-mouse antibody enzyme conjugate, BSA, horseradish peroxidase, blue dye.</td>
</tr>
<tr>
<td>Reagent H</td>
<td>TMB⁺(Tetramethylbenzidine)</td>
</tr>
<tr>
<td>Reagent S</td>
<td>0.8 M sulphuric acid</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>Phosphate buffer, 0.01% merthiolate, Tween 20, BSA.</td>
</tr>
<tr>
<td>Calibrators</td>
<td>S.adenosyl-L-homocysteine 2-4-8-15-30 μmol/l in assay buffer</td>
</tr>
<tr>
<td>Microtitrate strips</td>
<td>Coated with BSA S-adenosyl-L-homocysteine.</td>
</tr>
</tbody>
</table>
Controls - Diluted serum samples of human origin - phosphate

Wash buffer - Phosphate buffer, 0.01%, merthiolate, Tween 20, BSA.

**Procedure**

4.5 ml Reagent A, 0.25 ml Reagent B and 0.25 ml Reagent C were mixed (sample pre-treatment solution). 25μl of calibrator / sample / control was added to 500μl of sample pretreatment solution. The mixture was incubated for 30 minutes at 18-25°C. 500μl of reagent D was added and incubated for 15 minutes at 18-25°C. Then, 500μl of reagent E was added, mixed well and incubated at 18-35°C for 5 minutes.

**Microtitre plate procedure**

25 μl of the diluted calibrator / sample / control was added into the wells of the SAH- coated microtitre strips. To each well, 200μl of reagent F was added and incubated for 30 minutes at 18–25°C. Wash was diluted with wash buffer and then 100μl of reagent G was added to each well. Incubation was carried out at 18–25°C for 20 minutes.

Another wash with wash buffer was done. 100μl of reagent H was added and incubated for 10 minutes at 18–25 °C finally, 100 μl reagent S was added to each well, and read at 450 nm within 15 minutes.

The levels of homocysteine are expressed as nmoles/ml in plasma.
Hematological Parameters

Enumeration of red blood corpuscles (RBC)

The total erythrocyte count was determined by the method of Huxtable (1990).

Reagents

1. Red blood cell diluting fluid (Hayem's fluid) : 2.5% of sodium sulphate, 0.5% of sodium chloride, 0.25% of mercuric chloride in distilled water.

Procedure

Blood was drawn exactly upto 0.5 ml mark in the RBC pipette and the Hayem's fluid was drawn immediately upto the mark. The blood was mixed thoroughly with the Hayem's fluid. It was left for 2-3 minutes for proper mixing. The Neubauer counting chamber was placed along with its cover slip in position. The capillary stem of the pipette was emptied which contains only the diluting fluid.

Charging of the counting chambers

One drop of blood was released into the groove of the Neubauer counting chamber. The cells were allowed to settle for 2-3 minutes. The counting chamber was put under the microscope and the ruled area was located. Erythrocytes were counted in the 5 squares of the counting area of 1 mm square. The number of cells in the 4 corner squares and one central square was counted.
Calculation

The total number of cells found in 5 groups of 16 squares is multiplied by 10,000 to give the number of cells in millions/mm$^3$ of blood.

Enumeration of Platelets

Platelets in blood were counted using the improved Neubauer counting chamber by the method of Dacie and Levis (1977).

Reagents

Dacies fluid

This was prepared by dissolving 5g of sodium citrate in 1 ml of 40 % formaldehyde and making upto 100 ml with distilled water. To 19 ml of this solution, 1 ml of 0.2 % brilliant cresyl blue solution was added first before use. This solution was filtered and used.

Procedure

Venous blood collected with EDTA was used for platelet count. 0.05 ml blood was diluted with 0.95 ml of dacie’s fluid and mixed well using a narrow bore Pasteur’s pipette, the counting chamber was filled with the diluted blood. The cells were allowed to settle to the bottom of the chamber for 15 minutes. To prevent from drying, the chamber was placed in a petridish, which contained a piece of wet filter paper. Using the 40 x objective with reduced condenser aperture, the platelets were counted in 1/5 sq mm 5 of
the small squares of the large center square. From this the number of platelets in cu. mm of blood was calculated as

\[
\frac{\text{Cells} \times \text{blood dilution} \times \text{chamber depth}}{\text{area of chamber counted}}
\]

Platelet count were expressed as number of cells \(10^5/\text{mm}^3\)

**Estimation of Hemoglobin**

Hemoglobin in blood was measured by the method of Drabkin and Austin (1932).

The results of the method are dilution of blood in a solution containing potassium cyanide and potassium ferricyanide during which hemoglobin is converted to cyanomethemoglobin. The absorbance of the solution was then measured at 540 nm.

**Reagents**

1. **Diluent**: Dissolved 200 mg of potassium fericyanide and 1.0 g of sodium bicarbonate in distillated water and made upto a liter. This was stored in a brown bottle.

2. **Cyanomethemoglobin standard**: This was obtained commercially and had a concentration of 16 g / dl.
Procedure

To 0.02 ml of blood was diluted with 5.0 ml of the diluent. The tube containing the solution was stoppered with rubber bung and inverted several times. After allowing to stand at room temperature for 10 minutes to ensure the completion of the reaction, the absorbance was read at 540 nm together with the standard solution of cyanometahemoglobin. Reagent blank was used to set the optical density to zero.

Blood hemoglobin was expressed as g / dl.

Determination of Haematocrit (PCV)

Packed cell volume is determined by centrifugation using Wintrobe tubes by the procedure of Samuel (1980).

0.6 ml of oxalated blood was taken and mixed thoroughly by repeated inversion and filled in Wintrobe tube to upto 100 mark and centrifuged for 30 minutes in a relative centrifugal field (RCF) of 2,000 xg. Then the volume of the packed cell was noted.

Hematocrit (PCV) is expressed as percentage.

Determination of Prothrombin time

Plasma prothrombin time was done by the Quik’s one stage method (Dacie and Lewis 1977).
Principle

A potent preparation of rabbit brain emulsion impregnated with calcium is added to citrated plasma and the clotting time estimated.

1. Thromboplastin with calcium

This was prepared from a commercially available lyophilised rabbit brain preparation. It was obtained from Bio merieux, Laboratory Reagents and Products, France.

2. Trisodium citrate – 3.18%

Procedure

1.6 ml of blood was collected into a graduated tube containing 0.4 ml of 3.18% trisodium citrate shaken and centrifuged to separate the plasma. The plasma kept at room temperature until the time of test. The test was performed within 4 hours of blood collection. 0.1 ml of plasma was delivered into the bottom of a short 5 ml test tube (75mm x10 mm) and it was placed in the water bath at 37°C. To this tube 0.2 ml of thromboplastin was added forcibly by blowing from a pipette as quick as possible, and a stop watch started simultaneously. The tube was held with its lower end submerged in water (37°C) and gently tilted back and forth until the first strand of fibrin appears. Tilting was done by gently inclining the tube from the tube from the vertical to just short of the horizontal so that the fluid content (plasma) could be observed. The stop watch was stopped at the time of appearance of the clot.
and the time noted. This test was repeated once for each specimen and the mean time was rewarded. A normal (control) plasma was included with every batch of test samples.

Prothrombin time was expressed in seconds.

**Estimation of Fibrinogen**

Fibrinogen in plasma was estimated by the method of Ratnoff and Menzie (1951) using the biuret colour reaction.

**Reagents**

1. Sodium citrate: 3.18 %
2. Calcium chloride: 0.025 M
3. Saline: 0.89 %
4. Stock biuret reagent

45 g of Rochelle salt was dissolved in about 400 ml of 0.2 N sodium hydroxide to which was added 15 g of copper sulphate stirring continuously until the solution, 5 g potassium iodide was added and made upto a litre with 0.2 N sodium hydroxide.

5. Working biuret colour solution

200 ml of the stock reagent was made upto 1 liter with 0.2N sodium hydroxide containing 5 g potassium iodide / liter.

6. Standard BSA – 6 % aqueous solution of BSA was used as the standard.
**Procedure**

1.6 ml of blood was collected into a graduate tube containing 0.4 ml of 3.18% sodium citrate, shaken and centrifuged to separate plasma. To 0.5 ml plasma, 4 ml distilled water and 3 ml calcium chloride were added mixed and the tube incubated at 37°C for 15 minutes. The tube was taken outside and centrifuged for 5 minutes. The fibrin clot stringed and it was held at the bottom of the tube. The supernatant was discarded and the fibrin clot was washed thrice with 5-ml volumes of saline. The fibrin clot in the bottom was dissolved in 5 ml of working biuret reagent by warming in boiling water bath carefully. After the biuret colour formation, the tube was removed from the water bath and 3 ml of saline was added to the tube and the optical density measured at 540 nm.

Graded concentrations of BSA standard solution ranging from 60 to 240 mg was taken. The final volume was made upto 3 ml with saline. To these tubes 5 ml of biuret reagent was added and read at 540 nm.

Fibrinogen values were expressed as mg / dl plasma.

**Isolation of erythrocyte membrane**

Erythrocyte membrane was isolated according to the method of Dodge et al. with a slight change in buffer according to Quist (1980).
Reagents

1. Tris-HCl buffer (Hypotonic) - 5mM tris and 15 mM sodium chloride in litre.
2. 0.9% sodium chloride.

Procedure

Blood was collected with EDTA as anticoagulant. Plasma was separated by centrifugation at 1500g for 15 min. The packed cells were washed well with isotonic saline solution. After washing with saline the packed cells were lysed by suspending the packed cells in the hypotonic Tris-HCl buffer for one hour. The lysed cells were centrifuged at 15,000 g for 30 minutes. The supernatant red fluid was decanted and discarded while the membrane remained at the bottom of the centrifuge tube was washed thrice with Tri-HCl buffer and the final membrane obtained was colorless to pale pink in appearance.

Membrane-Bound Enzymes

Assay of Na\(^+\)K\(^+\) adenosine triphosphatase (E.C. 3.6.1.37)

Na\(^+\)K\(^+\)-dependent ATPase activity was estimated by the method of Bonting (1970).

Reagents

1. Tris-HCl buffer : 90 mM, pH 7.5
2. MgSO\(_4\) : 500 mM
3. KCl : 50 mM
4. NaCl : 600 mM
5. EDTA : 1 mM
6. ATP : 40 mM
7. TCA : 10%

Procedure

1 ml of the buffer was taken with 0.2 ml magnesium sulphate, sodium chloride, potassium chloride, ethylene diamine tetra acetic acid and ATP solution. 0.2 ml of the membrane preparation was added for the initiation of the reaction. Contents were maintained at 37°C for 15 minutes. Adding 1 ml of trichloro acetic acid, arrested the reaction and 0.2 ml of the membrane preparation was added to the control tubes. Centrifuged and phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925) as described earlier.

Membrane protein was estimated according to the method of Lowry et al. (1951). Enzyme activity in erythrocyte membrane, heart and liver is expressed as μ moles of Pi liberated/hr/mg protein.

Assay of Ca^{2+} adenosine triphosphatase (E.C. 3.6.1.38)

Ca^{2+} ATPase activity was assayed by the method of Hjerten and Pan (1983).
Reagents

1. Tris-HCl buffer : 125 mM, pH 8.0
2. Calcium chloride : 50 mM
3. ATP : 10 mM
4. Trichloro acetic acid : 10%.

Procedure

Tris-HCl buffer 1 ml, 0.1 ml of calcium chloride, ATP, water and membrane preparation were incubated at 37°C for 15 minutes and the reaction was arrested by adding 1 ml of trichloro acetic acid. After arresting with trichloro acetic acid, 0.1 ml of membrane preparation was added to the control. Tubes were centrifuged and phosphorous content of the supernatant was estimated by the method of Fiske and Subbarow, (1925) as described earlier.

Membrane protein was estimated according to the method of Lowry et al. (1951) and the enzyme activity in erythrocyte membrane, heart and liver is expressed as μ moles of Pi liberated/hr/mg protein.

Assay of Mg^{2+} adenosine triphosphatase (E.C.3.6.1.39)

Mg^{2+} ATPase was estimated according to the method of Ohnishi et al., (1982).
Reagents

1. Tris-HCl buffer : 375 mM, pH 7.6
2. MgCl₂ : 25 mM
3. ATP : 10 mM
4. TCA : 10%

Procedure

0.1 ml of Tris-HCl buffer, magnesium chloride, ATP, water and membrane preparation was incubated at 37°C for 15 minutes. The reaction was arrested by adding 1 ml of trichloro acetic acid, 0.1 ml of membrane preparation was added to control tubes. Tubes were centrifuged and phosphorous content of the supernatant was estimated by the method of Fiske and Subbarow (1925) as described earlier.

Membrane protein was estimated according to the method of Lowry et al. (1951) and the enzyme activity in erythrocyte membrane, heart and liver is expressed as μ moles of Pi liberated/hr/mg protein.

Minerals

Analysis of Sodium, Potassium, Calcium and Magnesium

Sodium, Potassium, Calcium and Magnesium were estimated (after digestion), in Perkin-Elmer 2380 atomic absorption spectrophotometer.
Reagents

1. Concentrated nitric acid - Analar grade
2. Perchloric acid : 70%

Procedure

The wavelength, slit-setting, light source, flame type and linear working range used for sodium, potassium, calcium and magnesium are given below.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Hollow Cathode Lamp</th>
<th>Wavelength (nm)</th>
<th>Slit Setting</th>
<th>Flame type</th>
<th>Linear working range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>Na</td>
<td>589</td>
<td>0.7</td>
<td>A - Ac</td>
<td>1.0</td>
</tr>
<tr>
<td>Potassium</td>
<td>K</td>
<td>766.5</td>
<td>2.0</td>
<td>A - Ac</td>
<td>2.0</td>
</tr>
<tr>
<td>Calcium</td>
<td>Ca</td>
<td>422.7</td>
<td>0.7</td>
<td>A - Ac</td>
<td>5.0</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Mg</td>
<td>285.2</td>
<td>0.7</td>
<td>A - Ac</td>
<td>0.5</td>
</tr>
</tbody>
</table>

A-Ac - Air and Acetylene.

Estimation of Sodium and Potassium

Reagents

1. Lanthanum diluent : 0.1% (w/v) lanthanum
2. Stock Standard : Dissolve 8.183 g of dried sodium chloride in 500 ml of deionized water and make upto 1 L with the same.
3. Working Standard : The stock solution was diluted suitably to get a concentration of 1 µg/ml.
4. Stock standard for potassium: Dissolve 1.907 g of potassium chloride in 1 L of deionized water. This solution had a concentration of 1000 μg/ml.

5. Working standard: The stock solution was diluted suitably to get a concentration of 2 μg/ml.

Procedure

Serum and tissue samples were diluted 1:50 with lanthanum diluent and used directly for the estimation of potassium in serum. For the determination of sodium, an additional 1:50 dilution with deionized water was required. Standards were also analyzed along with the samples as described earlier.

The levels of sodium and potassium are expressed as mEq/g tissue.

Estimation of Calcium and Magnesium

Reagents

1. Lanthanum diluent: 0.1% (w/v) lanthanum.

2. Stock standard for calcium: To 1.249 g of primary standard calcium carbonate, 50 ml of deionized water was added. Minimum volume of hydrochloric acid was added drop wise for complete solubilization of calcium carbonate. It was then diluted to 1 L with deionized water.

3. Working standard: The stock solution was diluted suitably to get a concentration of 5 μg/ml.

4. Stock standard for magnesium: 1 g of magnesium ribbon was dissolved in a minimum volume of (1+1) HCl. This was diluted to 1 L with 1% HCl (v/v). This solution had a concentration of 1000 μg magnesium/ml.
Procedure

Serum samples were diluted 1:50 with lanthanum diluent and used directly for the estimation of calcium and magnesium in serum. Standards were also analyzed along with the samples as described earlier.

The levels of calcium and magnesium are expressed as mg/g tissue.

Estimation of Lipid peroxides

Lipid peroxide content was estimated by the method of Ohkawa et al. (1979).

Reagents

1. Sulfuric acid: 0.85 N
2. Thio barbituric acid reagent: A mixture of equal volumes of 0.67% TBA aqueous solution and glacial acetic acid.
3. Phosphotungstic acid: 10%
4. n-Butanol

Procedure

1 ml of serum was mixed with 4 ml of 0.85 N sulfuric acid and mixed gently. 0.5 ml of phosphotungstic acid was added and stirred well. The contents were centrifuged for 10 minutes. The supernatant was discarded and the sediment mixed with 2 ml of sulfuric acid and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged for 10 minutes. The sediment was suspended
in 4 ml of distilled water and 1 ml of thio barbituric acid reagent. The tubes were
kept in a boiling water bath for 1 hour. After cooling, 5 ml of butanol was added
to each tube and the color extracted in butanol phase was read at 532 nm.

The lipid peroxide content was expressed in tissues as n moles of
TBARS formed/ mg protein and in serum as n moles of TBARS formed/dl.

**Estimation of Glutathione**

Reduced glutathione was measured according to the method of

**Reagents**

1. Phosphate buffer : 200 mM, pH 8.0
2. DTNB : 0.6 mM in phosphate buffer
3. Precipitating reagent : It consisted of 417.5 mg of MPA, 50 mg of EDTA
   and 7.5 g sodium chloride in 250 ml of H₂O.
4. EDTA : 0.1%
5. Disodium hydrogen phosphate : 5.375 g in 50 ml water.
4. Standard solution : 10 mg of reduced glutathione was dissolved in
   100 ml of water.

**Procedure**

0.5 ml of blood was precipitated with 3 ml of precipitating reagent and
1.9 ml of EDTA. The contents were mixed well for complete precipitation of
protein and centrifuged. To an aliquot of 2 ml of clear supernatant, 4 ml of
disodium hydrogen phosphate and 1 ml of DTNB reagent were added. The absorbance was read at 412 nm against a blank. A series of standards treated in a similar way were also run to determine glutathione content.

The levels of glutathione are expressed as μ moles of GSSG oxidised/min /mg of Hb in erythrocytes and μ moles of GSSG oxidised/min /mg of protein in tissues.

**Assay of Superoxide dismutase (Superoxide : Superoxide oxido reductase, E.C.1.15.1.1)**

Superoxide dismutase activity was assayed by the method of Misra and Fridovich (1972).

**Reagents**

1. Carbonate - bicarbonate buffer: 300 mM, pH 10.2
2. Ethylene diamine tetra acetic acid solution: 0.6 mM
3. Epinephrine: 1.8 mM (prepared fresh)
4. Absolute ethanol
5. Chloroform.

**Procedure**

To tubes containing 0.5 ml of carbonate buffer and 0.5 ml of ethylene diamine tetra acetic acid solution, required amount of enzyme was added and the final volume was made upto 2.5 ml. The reaction was initiated by the addition of 0.4 ml of epinephrine and the increase in absorbance at 480 nm was
measured in a Shimadzu UV spectrophotometer. 50% autoxidation of epinephrine to adrenochrome was performed in a control tube without the enzyme.

The enzyme activity was expressed as 50% inhibition of epinephrine autoxidation /min/mg protein in erythrocytes and tissues.

**Assay of Catalase (Hydrogen peroxide: Hydrogen peroxide oxido reductase E.C.1.11.1.6)**

Catalase activity was assayed by the method of Sinha (1972).

**Reagents**

1. Phosphate buffer : 10 mM, pH 7.0

**Procedure**

0.05 ml of test sample was added to 1.2 ml of the phosphate buffer. To this, 1 ml of hydrogen peroxide was added to start the enzyme reaction. The decrease in absorbance was measured at 620 nm at 30 second intervals for 3 minutes. The enzyme blank was run simultaneously with 1 ml of distilled water instead of hydrogen peroxide.

Activity of catalase was expressed as n moles of H₂O₂ decomposed/min/mg protein in erythrocytes and tissues.
Assay of Glutathione peroxidase (Glutathione hydrogen peroxide oxido reductase, E.C.1.11.1.9)

Glutathione peroxidase activity was assayed by the method of Rotruck et al. (1973) with slight modifications.

Reagents

1. Sodium phosphate buffer: 400 mM, pH 7.0
2. Sodium azide: 10 mM
3. Reduced glutathione: 4 mM
4. Hydrogen peroxide: 2.5 mM
5. Trichloro acetic acid: 10%
6. Phosphate solution: 0.3 mM disodium hydrogen phosphate
7. DTNB reagent: 40 mg of DTNB in 100 ml of 1% sodium citrate

Procedure

The reaction mixture containing 0.4-ml buffer, 0.1-ml sodium azide, 0.2 ml reduced glutathione, required amount of enzyme, 0.1-ml hydrogen peroxide and water was taken to a final incubation volume 2 ml. The tubes were incubated at 30°C for 10 minutes. The reaction was terminated by the addition of 0.5-ml trichloro acetic acid. To determine the residual GSH content the supernatant was removed by centrifugation and added to 2 ml of precipitating reagent and 1 ml of DTNB reagent. The color was read at 412 nm. A blank was prepared with only sodium dihydrogen phosphate and 1 ml of DTNB reagent. Suitable aliquots of the standard were taken and treated in the same manner.
The activity was expressed as n moles of GSH oxidised/min/mg protein in erythrocytes and in tissues.

**Assay of Glutathione-S-transferase (E.C.2.5.1.18)**

Glutathione S-transferase activity was determined by the method of Habig *et al.* (1974).

**Reagents**

1. Phosphate buffer : 300 mM, pH 6.5
2. 1-chloro-2,4 dinitro benzene (CDNB) : 30 mM
3. Glutathione (reduced) : 30 mM.

**Procedure**

The reaction mixture containing 1 ml of buffer, 0.1 ml of CDNB and 0.1 ml of enzyme was made upto 3 ml with water. The reaction mixture was preincubated at 37°C for 5 minutes. 0.1 ml of GSH was added and the change in absorbance was measured at 340 nm for 3 minutes at 30 second intervals in a Shimadzu UV double beam spectrophotometer.

The enzyme activity was expressed as units/min/mg protein for erythrocytes and tissues.

**Extraction of Lipids**

The lipids were extracted by the method of Folch *et al.* (1957).
Reagents

1. Chloroform - methanol mixture : 2:1 (v/v)
2. Saline : 0.89%

Procedure

A known volume of suspension was mixed with 10 ml of chloroform-methanol mixture and homogenised. The homogenate was filtered through Whatmann filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into preweighed beakers. The upper phase was re-extracted with more of chloroform-methanol mixture and the extracts were pooled and evaporated under vacuum at room temperature. The lipid extract was redissolved in 3 ml of chloroform-methanol (2:1) mixture and aliquots were taken for the estimation of cholesterol and phospholipids.

Estimation of Cholesterol

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

Reagents

1. Ferric chloride - Uranyl acetate reagent : To 500 mg of ferric chloride was added 10 ml of water and 3 ml of concentrated ammonia solution. The precipitate formed was washed several times with distilled water
and dissolved in aldehyde free analar acetic acid. 100 mg of uranyl acetate was added and the contents were shaken well and made upto 1 L with acetic acid. It was left overnight. The reagent was stored in a brown bottle and is stable for 6 months.

2. Sulfuric acid - Ferrous sulphate reagent : 100 mg of anhydrous ferrous sulphate was dissolved in 100 ml of analar acetic acid, 100 ml of concentrated sulfuric acid was added while stirring the contents. After cooling to room temperature the volume was made upto 1 L with sulfuric acid. The reagent was stored in a brown bottle and is stable for 6 months.

3. Standard cholesterol : 100 mg of pure cholesterol was dissolved in 100 ml of aldehyde free analar acetic acid. 10 ml of this stock standard was diluted to 100 ml with acetic acid and used as working standard.

Procedure

To 0.1 ml of the serum and tissue homogenate, 2.9 ml of ferric chloride-uranyl acetate reagent was added. Then, 2 ml of sulphuric acid- ferrous sulphate reagent was added and mixed well. Blank comprised 3 ml of ferric chloride-uranyl acetate reagent and 2 ml of sulphuric acid - ferrous sulphate reagent. A calibration graph was prepared with standard cholesterol. The optical density was measured at 560 nm after 20 minutes in a Shimadzu UV spectrophotometer.

The levels are expressed as mg/dl plasma and mg/g tissue.
Estimation of Free and Ester cholesterol

Free cholesterol was precipitated by digitonin according to the method of Leffler and Mc Dougald (1963). The cholesterol digitonide formed was sedimented by centrifugation and the cholesterol in the precipitate was estimated as free cholesterol as described previously.

Reagents

1. Digitonin solution: 1% solution in 50% ethanol (v/v).
2. Isopropanol.
3. Acetone.
4. Reagents for cholesterol estimation as in serum cholesterol.

Procedure

To 0.2 ml of serum and tissue homogenate was added 1.8 ml of isopropanol, stirred in a vortex mixture and allowed to stand for 5 minutes. It was then centrifuged and to 1.0 ml of the supernatant was added 0.5 ml of 1% digitonin in 50% ethanol. After keeping the tube in a refrigerator for 30 minutes, it was centrifuged for 10 minutes. The supernatant was discarded and the precipitate was dissolved in 3.0 ml of ferric acetate-uranyl acetate reagent. 2.0 ml of sulphuric acid-ferrous sulphate reagent was then added and the pink colour developed was read at 560 nm after 20 minutes.

The esterified cholesterol was arrived at a difference between the total cholesterol and free cholesterol levels.
The level of free and ester cholesterol were expressed as mg/dl of plasma and mg/g of tissue.

**Estimation of Triglycerides**

Triglycerides were estimated by the method of Rice (1970).

**Reagents**

1. Isopropanol

2. Activated alumina: 10 g of aluminium oxide was taken in a beaker and 30 ml of distilled water was added. The suspension was stirred well and the supernatant was discarded. Washing with distilled water was repeated several times until the supernatant became clear. The water was discarded and aluminium oxide was activated in an oven at 90°C overnight. The dry powder was used.

3. Alkaline potassium hydroxide: 10 g of potassium hydroxide was dissolved in 75 ml of distilled water and 25 ml of isopropanol was added to the solution.

4. Sodium metaperiodate reagent: 7.7 g of anhydrous ammonium acetate was dissolved in 70 ml of distilled water. To this was added 6 ml of acetic acid followed by 65 mg of sodium metaperiodate. The solution was mixed well and made upto 100 ml with distilled water.

5. Acetyl acetone reagent: 0.4 ml of redistilled acetyl acetone was added to 100 ml of isopropanol and mixed well. It was stored in refrigerator.
6. Standard triglyceride solution: Stock solution was prepared by 1 ml of glycerol trioleate was dissolved and made upto 100 ml with isopropanol.

7. Working Standard: 2 ml of stock triglyceride solution was mixed with 8 ml of isopropanol.

Procedure

To 0.1 ml of sample 3.9 ml of isopropanol was added followed by 50 mg of activated alumina. It was mixed well and left for 15 minutes. It was then centrifuged and 2 ml of the supernatant was taken for analysis. 0.6 ml of alkaline potassium hydroxide was added to all the tubes. The tubes were incubated at 60°C for 10 minutes. The tubes were cooled and 1 ml of sodium metaperiodate reagent was added to the tubes followed by the addition of 0.5 ml of acetyl acetone reagent. The tubes were cooled and the color developed was read at 405 nm in a Shimadzu UV spectrophotometer against the blank.

The levels are expressed in plasma as mg/dl and mg/gm in tissues.

Estimation of Phospholipid

Phospholipids were estimated as inorganic phosphorous by the method of Fiske and Subbarow (1925) after Barlett's (1959) perchloric acid digestion.

Reagents

1. Perchloric acid
2. Ammonium molybdate : 2.5% in 5 N sulphuric acid
3. ANSA: Described earlier

4. Phosphorous standard

Procedure

0.1 ml of the sample was taken and digested with 1 ml of perchloric acid on a sand bath until the mixture became colorless. It was then made upto a known volume (3 ml) and a suitable aliquot (0.1 ml) was taken and diluted with water to 4.3 ml. Then 0.5 ml of ammonium molybdate and 0.2 ml of ANSA was added. The contents were mixed well. Blank and standards were treated similarly. The blue color developed was read after 10 minutes at 620 nm in a Shimadzu UV spectrophotometer. The phosphorous content was multiplied by a factor 25 to get total phospholipid content.

The levels are expressed as mg/dl plasma and mg/gm in tissues.

Estimation of Free Fatty Acids

Free fatty acids were measured by the method of Horn and Menahen (1981) with the color reagent of Itaya (1977).

Reagents

1. Activated silicic acid.

2. Chloroform-heptane-methanol mixture (CHM): It was prepared by mixing chloroform, heptane and methanol in the ratio of 200:150:7 (v/v/v).
3. **Cu-TEA reagent:** 50 ml of 100 mM copper nitrate and 50 ml of Triethanolamine (200 mM) were mixed with 33 g of sodium chloride.

4. **Color reagent:** 0.1% diethyl dithiocarbamate in butanol.

5. **Standard:** 2 mg/ml of palmitic acid was prepared in mixture and kept as stock. For preparing a working standard this was diluted 1:10 in CHM mixture to give a concentration of 200 μg/ml.

**Procedure**

0.1 ml of the sample was added to 5.9 ml of CHM solvent and 200 mg of activated silicic acid. The contents were shaken well and centrifuged. Standard solutions in the range of 25-100 μg were also pipetted out and made upto 6 ml with CHM. The blank comprised of 6 ml CHM only. To all these samples 2 ml of Cu-TEA reagent was added and mixed well. The tubes were centrifuged to separate the 2 phases and 2 ml of the upper phase from each tube was transferred to another set of tubes. To all these tubes were added 1 ml of color reagent and shaken well. The color intensity was measured at 430 nm in a Shimadzu UV spectrophotometer.

The levels are expressed in plasma as mg/dl and mg/gm in tissues.

**Cholesterol ester synthetase (CES) (Sterol ester acylesterase) (EC 3.1.1.13)**

Cholesterol ester synthetase was assayed by the method of Kothari (1973).
1. Acetate buffer : 0.1 M (pH 4.0)
2. Acetone : ethanol mixture: 1: 1 (v/v)
3. Acetone : ether mixture: 1:2 (v/v)
4. Glacial acetic acid : 10%
5. Digitonin in 1 ml of 50% ethanol.
6. Substrate: 15.5 – moles of pure cholesterol and 46.5 - moles of oleic acid were dissolved in 0.3 ml of ether 31 moles of sodium taurocholate and 100 mole of ammonium chloride were dissolved in 1.5 ml of acetate buffer. This was forcibly injected into the tube containing cholesterol in ether phase was evaporated.

Procedure

The incubation mixture contained 0.5 ml of acetate buffer, 0.2 ml of homogenate. Incubation was carried at 37°C for 6 hours with occasional shaking. The reaction was arrested by the addition of 5.0 ml of acetone – ethanol mixture.

The precipitated proteins were sedimented by centrifugation. 1.0 ml of digitonin was added to the supernatant, followed by two drops of 10% acetic acid. The contents were mixed well, securely closed and kept in a dark chamber for 16 hours. The cholesterol digitonide which was sedimented after centrifugation was washed twice with acetone – ether mixture and finally with ether.

The cholesterol content was expressed as nmoles of cholesterol esterified/dl/hour under incubation conditions.
**Cholesterol ester hydrolase (CEH) sterol ester acylesterase, EC 3.1.1.13)**


**Reagents**

1. Acetate buffer 0.1 M pH 4.0
2. Acetone – ethanol mixture 1:1 (v/v)
3. Acetone – ether mixture 1:2 (v/v)
4. Glacial acetic acid 10%
5. Digitonin: 5mg of digitonin in 1ml of 50% ethanol
6. Substrate: 1.54 μmole of cholesterol oleate and 3.8 μmoles of lecithin were dissolved in 0.1ml of ether 3.75 μmoles of sodium taurocholate was dissolved in 1.0 ml of acetate buffer. They were mixed together and homogenized. After evaporation of the organic solvent, the mixture was sonicated to near clarity.

**Procedure**

0.3 ml of acetate buffer was added to 0.5 ml of substrate and 0.2 ml of homogenate and mixed well. This was incubated at 37°C for 6 hours with occasional shaking. The reaction was stopped by the addition of 5.0ml of acetone - ethanol mixture. The precipitated proteins were sedimented by centrifugation at 2000 rpm.
The free cholesterol liberated from cholesterol oleate was precipitated by the addition of 1.0 ml of digitonin followed by addition of two drops of glacial acetic acid. The contents were mixed well. The tubes were securely closed and kept in dark chamber for 16 hours. The precipitated cholesterol digitonide was sedimented by centrifugation at 1000 rpm for 15 min and the upper phase was carefully decanted and acetone – ether mixture and finally with dry ether.

Ether was evaporated off and the tubes were heated in a sand bath at 110°C to 115°C for 30 min. The precipitate was then dissolved in 3.0 ml of uranyl acetate reagent and cholesterol content was estimated as described earlier.

Cholesterol ester hydrolyze activity was expressed as nmoles of cholesterol liberated /dl / hour under incubation conditions.

**Lecithin : cholesterol acyl transferase (LCAT)** (*Lecithin cholesterol acyl transferase (EC 2.3.1.43)*)

LCAT was assayed by the method of Legrand *et al.* (1979) with the modifications of Hitz *et al.* (1983).

**Reagents**

1. Dextran sulphate: 0.2 % solution in saline
2. Isopropanol
3. Acetone
4. Reagents for cholesterol estimation were prepared as given earlier

5. Substrate: A pool of extract was warmed at 56°C for 30 min to inactivate the lecithin – cholesterol acyl transferase present. The extract was then incubated at 4°C for 15 min with 0.2 % dextran sulphate (1 part of dextran sulphate was added to 20 parts of enzyme) This was followed by centrifugation for 10 min at 1750 rpm. The supernatant was used as the substrate for the enzyme assay.

6. Test extract: To 1.0 ml of enzyme was added 0.5 ml of 2% dextran sulphate. After incubation at 4°C for 15 min it was centrifuged at 1750 rpm for 10 min. The supernatant was used for the assay of enzymes.

Procedure

The incubation mixture contained 0.6 ml of the substrate and 0.6 ml of the enzyme. Then 0.4 ml of the mixture was immediately transferred to a tube containing isopropanol (1.0 ml) to arrest the reaction. This gives the amount of cholesterol present at the beginning of the experiment. Incubation at 37°C was carried out for 90 min, another 0.4 ml was pipetted out and the reaction was arrested. After centrifugation, the clear supernatant was quantitatively transferred to another set of tubes. To this was added 2.0 ml of acetone and 1.0 ml of digitonin. It was left aside for 1 hour and then centrifuged and was processed for the estimation of free cholesterol. The free cholesterol content at zero time, 90 min and 180 min were estimated as described by Parekh and Jung (1988) mentioned earlier. Control tubes were also assayed.
Lecithin: Cholesterol acyl transferase activity was expressed as nmoles of cholesterol liberated / hour /dl under incubation conditions

Lipoprotein lipase (triacylglycerol acylhydrolase, E.C.3.1.1.3)

The lipoprotein lipase was assayed by method Schmidt (1934).

Reagents

1. Triethanolamine buffer: 1.0 M (pH 8.5)
2. Deoxycholate: 10mM
3. Chloroform
4. Hexane
5. Olive oil emulsion: 50g of olive oil and 1.0g of gum arabic were mixed with 95.0 ml of saline and blended for 25 min.
6. Extraction mixture: Isopropanol, hexane and 2N sulphuric acid were mixed in the ratio of 40:10:1 (v/v)
7. Sulphuric acid: 0.1N
8. Copper reagent: 3.25 g of copper nitrate, 6.25g potassium sulphate and 17.098 g of sodium sulphate were mixed with 50.0 ml distilled water. To this solution 70 ml of triethanolamine and 0.3 ml of glacial acetic acid were added and the final volume was made upto 100 ml with water.
9. Diethylidithio carbonate: 0.1 % solution in n - butanol
10. Standard palmitic acid: It was prepared by dissolving 20.0 mg of palmitic acid in 1000 ml chloroform.
Procedure

0.5 ml of olive oil emulsion, 0.1 ml of deoxy cholate, 0.5 ml buffer and 0.5 ml of extract were incubated at 37°C for 30 minutes along with control tubes to which extract was not added. The reaction was arrested by keeping the tubes in a boiling water bath for one minute. To all the tubes 2.5 ml of distilled water were added, shaken well and centrifuged.

To 3.0 ml of supernatant, 3.0 ml of 0.1 N H₂SO₄ was added, mixed well and centrifuged. To 2.0 ml of supernatant, 0.5 ml of diethyl dithiocarbamate reagent was added. The colour developed was read at 430 nm. Aliquots of standard were also treated as mentioned above.

Lipoprotein lipase activity was expressed as nmoles of glycerol liberated /dl / min under incubation conditions.

Hydroxymethyl glutaryl – Co A reductase (HMG Co A-R) (E.C. 1.1.1.34)

Hydroxymethyl glutaryl – Co A reductase was assayed by the method of Rao and Ramakrishnan (1975).

Reagents

1. Saline–arsenate solution: 1g of sodium arsenate per liter of physiological saline.

2. Dilute perchloric acid: 50 ml of acid was diluted to one liter with distilled water.
3. Hydroxyalanine hydrochloride reagent: 2 M of solution in distilled water

4. Hydroxyalanine hydrochloride reagent for mevolanate: Equal volumes of hydroxyalanine hydrochloride reagent and water mixed just before use.

5. Hydroxyalanine reagent for HMG - Co A: Equal volumes of hydroxyalanine hydrochloride reagent and sodium hydroxide solution (4.5 mol/liter) were mixed just before.

6. Ferric chloride reagent: 5.2 g of trichloroacetic acid and 10g of ferric chloride were dissolved in 50 ml of 0.65 mol/liter HCl and diluted to 100 ml with the water.

Procedure

500 mg of fresh tissue was homogenized with 5.0 ml of saline-arsenate solution. HMG-Co A was determined by reaction with hydroxyalanine at pH 5.5 and subsequent colorimetric measurement of the resulting hydroxylamine acid by formation of complexes with ferric salts. Because mevolanate interferes in this estimation at acidic or neutral pH, alkaline hydroxyalanine was used to estimate specifically HMG-Co A only. Possible interference by coenzyme A is also minimal when readings are taken at 540 nm.

Mevolanate was estimated by reaction with same reagent, but at pH 2.1. At this pH, the lactone form of mevolanate readily reacts with hydroxyalanine to form the hydroxanate.
HMG - Co Å reductase activity was expressed as ratio of HMG- CoA to mevolanate.

Lipoprotein Fractionation

Lipoprotein were fractionated by dual precipitation technique as described by Wilson and Spiger (1973).

Separation of High Density Lipoproteins (HDL) Fractionation

Total high density lipoproteins was separated by the method of Burnstein et al. (1970).

Reagents

1. Heparin-manganese chloride reagent: 3.167 g of manganese chloride was added to 1 ml of heparin containing 20,000 units/ml. This was made upto 8 ml with water.

Procedure

To 1 ml of sample was added 0.09 ml of heparin - manganese chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 minutes and then centrifuged at 2,500 rpm for 30 minutes. The supernatant represented high density lipoproteins fraction. Aliquot was taken from HDL fraction for the estimation of cholesterol.

The levels of HDLc fractions are expressed as mg/dl in plasma.
Separation of LDL and VLDL

Reagents

1. Sodium dodecyl sulfate (SDS): 10% SDS in 150 mM sodium chloride, pH 9.0.

Procedure

To 1 ml of plasma was added 0.15 ml of sodium dodecyl sulfate. The contents were mixed well and incubated at 37°C for 2 hours. The contents were centrifuged in the refrigerated centrifuge at 10,000 rpm for 15 minutes. Very low-density lipoprotein aggregated as pellet. The supernatant contained the high-density lipoproteins and low density lipoproteins fractions. Cholesterol was estimated from this fraction.

The levels of LDLc and VLDLc fractions are expressed as mg/dl in plasma.

Fecal analysis

Collection of fecal samples

The animals were housed in cages provided with wide mesh bottom and all the excreta were collected in trays kept below. The fecal material was collected from their trays at 24 hr intervals. The collected samples were weighed, initially dried in the sun for a day and then dried to constant weight in a 60°C oven. Five days fecal samples of each animal were mixed together
and ground to a fine powder in a hand operated coffee grinder and dried at 60°C for another 24 h. The day-powered samples were stored in selected polythene bags and kept at 4°C until further analysis. Analysis were made on the samples and the values are arrived at in terms of excreta/day.

**Extraction of fecal bile acids**

Fecal bile acids were extracted by the method of Meyer (1974).

**Reagents**

1. Methanol
2. Sodium hydroxide: 1M in ethanol
3. Petroleum ether

**Procedure**

100 mg dry powered fecal sample extracted thoroughly with 100 ml of methanol. This was refluxed with 5 ml of 1M ethanolic NaOH for one hour. Cooled and added 5 ml of water and mixed. This solution was extracted with warm petroleum ether (40-60°C). Centrifugation and evaporated separated the ether phase, which contained the bile acids, to dryness. The residue was dissolved in a small volume of petroleum ether and aliquots of this were taken for the estimation of cholic acid and deoxycholic acid.
Extraction of hepatic bile acids

Bile acids were extracted from liver tissue by the method of Okishio et al. (1967).

Reagents

1. 95% ethanol containing 0.1% (V/V) of aqueous ammonia.
2. Amberlyst A-26 ion exchange resin
3. Sodium hydroxide: 1N
4. Diethyl ether
5. Hydrochloric acid

Procedure

About 250 mg liver tissue was weighed and homogenized in 10 ml of 95% ethanol. The solvent was separated by filtration through a fat free filter paper (Whatmann No.1). The residue was once again extracted with 10 ml of ethanol and finally washed with additional solvent.

The extracts were pooled and concentrated under partial vacuum and passed through Amberlyst-A-26 column was collected and mixed with 3ml of 1N NaOH and autoclaved at 15 lb/inch2 for four hours. After cooling, the solution was acidified to pH 1 with hydrochloric acid. This was then extracted with diethyl ether thoroughly. The solvent was evaporated and the residue
suspended in a small volume of ether. Aliquots of this were taken for the estimation of cholic and deoxy cholic acid.

**Estimation of deoxy cholic acid**

Deoxycholic acid was estimated by the method of Levin *et al.* (1961)

**Reagents**

1. Sulfuric acid-phosphoric acid reagent: 400 ml of 85% $\text{H}_3\text{PO}_4$ was mixed with 100 ml distilled water. Cooled this solution and mixed with 600 ml of concentrated sulfuric acid slowly.

2. Benzaldehyde solution: 1% solution of distilled benzaldehyde was prepared in the above acid mixture.

**Procedure**

Aliquots of the bile acid extract were taken and the solvents evaporated. To the residue added 4 ml of benzaldehyde and kept at 3-5°C for 120 minutes. The contents were stirred vigorously for the first 15 minutes. After 120 minutes, 4 ml of cold ethyl acetate was added and stirred well. Standard sodium deoxycholate (50-200μg) was also treated as above. After cooling the tubes, the absorbance of the color developed was read at 660 nm in a Shimadzu UV-visible spectrophotometer.
Values were expressed as mg/g in liver tissue and mg/day/rat in the case of fecal samples.

**Estimation of cholic acid**

Cholic acid was estimated by the method of Levin et al. (1961).

**Reagents**

1. Furfural solution: 0.5% 0.5 ml of freshly distilled furfural was dissolved in 100 ml of distilled water.
2. Sulfuric acid : 16N
3. Standard sodium cholate: 200μg/ml
4. Glacial acetic acid

**Procedure**

Aliquots of extracted bile acids were transferred to test tubes and evaporated the solvent. To the residue was added 6 ml of H₂SO₄ and 2 ml of furfural. Stirred well with a glass rod. Warmed at 65°C for 13 minutes and cooled to room temperature. To this solution, 5 ml glacial acetic acid was added. Standard sodium cholate (50-200μg) was also treated as above. The blue colour developed was read against a reagent blank at 640 nm in a Shimadzu UV- Vis spectrophotometer.

Values were expressed as mg/g in liver tissue and mg/day/rat in the case of fecal samples.
Estimation of neutral sterols in feces

Lipid extraction from dried powdered fecal matter was done by the method of Folch et al., (1957) as described earlier.

Reagents

1. Chloroform: methanol mixture (2:1) (V/V)
2. Reagents for cholesterol estimation (as described in section)

Procedure

About 500 mg of dry powered fecal matter was weighed. Extracted with chloroform: methanol mixture for 3 hours. The solvent was separated by centrifugation and evaporated to dryness. The residue was dissolved in a small amount of the solvent and aliquots of this were used for the estimation of neutral sterols in terms of cholesterol. The procedure for estimation of cholesterol was as described earlier.

The values were expressed as mg/day/rat.

Separation of quantification of serum LDH isoenzyme by agarose gel electrophoresis

LDH isoenzymes were separated and quantified by the method of Mckenzie et al. (1983).
Principle

The LDH isoenzyme are separated on 1% agarose gel since it has the advantage of high resolving powder and excellent separation of proteins into sharp bands. These isoenzymes were identified by numbers according to their electrophoretic mobility on agarose gel. The fastest moving component is called LDH 1 and the slowest moving component is called LDH 5. The process of the completed reactions on the electrophoretogram is illustrated as follows.

Lactate \( \text{NAD}^+ \quad \text{PMS (reduced)} \quad \text{NBT (oxidised)} \)

Pyruvate \( \text{NADH}^+ \quad \text{H}^+ \quad \text{PMS (oxidized)} \quad \text{NBT reduced formozon} \)

Violet colour

Reagents

1. 1 % Agarose gel
2. Electrophoresis buffer – Tris – glycine buffer 0.1 M, pH 8.3

Procedure

1 % agarose gel was prepared and applied immediately to the glass slide, polymerization was allowed to take place in serum samples were applied into the gel surface. After the run, the gels were removed and stained by the following method. The staining solution contained 1.0 ml of 1.0 M lithium lactate, 1.0 ml of 0.1 M sodium chloride, 1.0 ml of 5.0 mM
magnesium chloride, 2.5 ml of 0.1 (W/V) nitro blue tetrazolium (NBT), 0.25 ml of 0.1 % phenazine methosulphate (PMS), 2.5 ml of 0.5 M phosphate buffer, pH 7.5 and 10 mg of NAD$^+$ in a total volume of 10 ml, the gels were incubated with the staining solution at 37°C in dark for a suitable period. LDH isoenzymes appeared as purple bands.

**Lipoprotein analysis by agarose gel electrophoresis**

Lipoprotein pattern was analysed by the method of Mc Glashan and Pullan, (1967).

**Reagents**

1. Barbitone - acetate buffer : pH 8.6, 0.1 mole / l
2. Sodium acetate trihydrate : 6.5 g
3. Sodium barbitone : 8.87 g
4. Diethyl barbituric acid : 1.13 g/l
5. Agarose : 100 mg
6. Oil red ‘O’

To 750 mg of agarose 50 ml of water was added and boiled until solution was complete, filter, cool it at 60°C. The volume was adjusted to 50 ml with buffer. Solution was poured into the block mould. Presser slot former was placed in position to produce 4 slot 2 cm long and the block was allowed to set. Mixed slowly and constantly, 0.4 ml of Oil red ‘O’ solution was added to 0.6ml of serum, allowed to stand at least 4 h at room temperature, then warmed at
42°C in a waterbath for 10 min and mixed with 0.6 ml of agarose containing 22.5 g/l in the barbitone acetate buffer and allowed to warm at 42°C. Slot former was removed and rapidly loaded with a serum mixture by a hypodermic syringe and it is also warmed at 42°C. In the buffer compartment diluter buffer with ionic strength 80 m mole/l was filled. Current of 20 mA from a constant voltage supply for 16 h was applied. This will give a potential difference of about 1 V/cm gel. For quicker separation, a current of 60 to 80 mA for about the lipoprotein bands were assessed visually.

**Scanning electron microscopy**

Animals were sacrificed after light anesthesia with sodium pentobarbital. The aorta were dissected under an operating microscope, cut longitudinally and placed in 2.5% glutaraldehyde in tyrodes solution for at lest 5 days. The aorta were stiff and maintained their shape and dimension on dissection.

The aorta samples were stained with osmium, tetroxide dehydrated with graded ethanol series and propylene oxide and ortical point dried. The mounted and sputter coated with gold Model 5150 B sputter coater (Edwards High Vacuum Crawley U.K). Samples were examined under a (Philips SEM 505 scanning electron microscope (Phillip, Eindhoven, Netherlands). The entire endothelial surface was observed at low magnification and all areas of abnormal endothelium and lesions recorded at magnification upto 3800 X.
Statistical analysis

The results are presented as mean ± S.D. for 6 animals in each group. The statistical analysis was performed using Dunnett’s T3 multiple comparison test for all parameters. The values were considered significant at the levels of $p < 0.05$ and $p < 0.01$. The data were also analyzed by ANOVA (one way analysis of variance) using SPSS package. F values were calculated at the level of 1% significance.