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## CHEMICALS AND THEIR SOURCES

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Chemical Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Menhaden fish oil, DL Isoproterenol hydrochloride, Sodium succinate, Rotenone, Oxaloacetate, Hexokinase, Glucose-6-phosphate dehydrogenase, Sodium deoxycholate, Cytochrome C, Trisodium isocitrate, Bovine serum albumin, Phospholipid standard, Sodium dodecyl sulphate, L-cysteine hydrochloride, Calfthymus DNA, p-nitrophenyl-β-D-glucoronide, p-nitrophenol, p-nitrophenyl 2-acetamido 2-deoxy-β-D-glucopyranoside, Tripalmitin, chromotropic acid, Tris(hydroxy methyl amino methane).</td>
<td>Sigma Chemical Company, St. Louis Missouri, USA.</td>
</tr>
<tr>
<td>2.</td>
<td>Potassium α-Ketoglutarate, uranylacetate, DNP, EDTA, lithium lactate, silicic acid, Orcinol, acetyl acetone, Diethyl ammonium diethyl dithiocarbamate</td>
<td>British Drug house Ltd., Laboratory Chemical Division, England.</td>
</tr>
<tr>
<td>3.</td>
<td>Thiamine pyrophosphate, glutathione</td>
<td>E.Mark, A.G.Darmstadt, West Germany.</td>
</tr>
<tr>
<td>5.</td>
<td>Adenosine triphosphate, L-Aspartic acid, DL-alanine, 1 amino-2-naphthol-4-sulphonic acid, diacetyl monoxime</td>
<td>Sisco Research Laboratories Ltd. Bombay, India.</td>
</tr>
</tbody>
</table>
Male Wistar rats weighing 100-150 gms were housed six per cage at 27 ± 2°C with constant 55% humidity, on a 12 hr light/dark cycle. The animals were fed with diet obtained from the Hindustan Lever. The rats were fed with feed and water ad libitum and were divided into 4 groups. Group I animals served as control, Group II animals were given subcutaneous injection of isoproterenol hydrochloride (IPH) (60 mg/kg body weight) dissolved in 0.1 ml of normal saline, at an interval of 24 hrs for two days. Group III animals were given fish oil (FO) 0.05 ml orally by gastric intubation for 45 days. Group IV animals received both fish oil and isoproterenol (at the end of fish oil treatment i.e., on 45th day) at the above mentioned dosages.

12 hours after the second injection of IPH, the animals were sacrificed by cervical decapitation method. Blood was collected in ice cold containers without any anticoagulant and the serum was separated. Immediately after the sacrifice, the rats were dissected, heart was removed and washed in ice cold saline. About 100 mg of the tissue was weighed and homogenised in chilled 0.1 M Tris-HCl buffer in potter - Elvejhem Teflon homogenizer. The homogenate was used for the assay of various biochemical parameters.

3.1 HISTOPATHOLOGICAL STUDIES

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

Under sodium thiopentone anaesthesia (30 mg/kg body weight IP) needle electrodes were placed subcutaneously in the gently extended limbs of the supine animals. ECG recordings of the rat were taken under mild anaesthesia using polygraph on a bipolar standard lead-2 at a sensitivity of 1 mv giving a deflection of 1 cm.

3.3 HAEMATOLOGICAL STUDIES
3.3.1 Enumeration of red blood corpuscles

The total erythrocyte count was determined accurately by diluting a measured quantity of blood with a fluid isotonic solution by the method of Huxtable (1990).

Reagents

Red Blood cell dilution fluid - (Hayem’s fluid) 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200 ml of distilled water.

Procedure

Blood was sucked exactly upto the 0.5 ml mark in the RBC pipette and the diluting fluid was drawn immediately upto the mark and the blood mixed thoroughly with the diluting fluid. It was left for 2-3 min for proper mixing. The Neubauer counting chamber was placed along with its cover glass in
position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding first 3-5 drops.

**Charging of the counting chambers**

One drop of dilute blood was released into the groove of the Neubauer counting chamber. It was left for cells 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.

**3.3.2 Estimation of haemoglobin**

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

**Reagents**

1. Drabkin’s reagent: Dissolved 200 mg potassium ferricyanide, 50 mg potassium cyanide and 1.0g sodium bicarbonate in water and made up to a litre. The reagent had a pale yellow colour and a pH of 9.6
2. Cyanometemoglobin standard: 16g/dl
Procedure

0.02 ml of blood was diluted with 5.0 ml of the reagent. The diluted blood was mixed well and allowed to stand for 10 minutes to ensure the completion of the reaction. The solution was read at 540 nm together with the standard solution of cyanomethemoglobin.

Blood haemoglobin levels were expressed as g/dl.

3.3.3 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 upto 100 mark and centrifuged for 30 minutes in a relative centrifugal field (RCF) of 2,000 to 2,300 x g. Then the volume of the packed cell was noted.

Haematocrit (PCV) is expressed as percentage.

3.3.4 Platelet count

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

Dacie's fluid

This was prepared by dissolving 5.0g of sodium citrate and 1 ml of 40% formaldehyde and making up to 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. 0.05ml blood was diluted with 0.95 ml of Dacie’s fluid and mixed well using a narrow bore pasteur 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 40x objective with reduced condenser aperture, the platelets were counted in 1/5 sq.mm-5 of the small squares of the large centre square. From this the number of platelets in cu.mm of blood was calculated as

$$\text{Cells} \times \text{blood dilution} \times \text{chamber depth}$$

$$\text{Area of chamber counted}$$

Platelet count is expressed as number of cells $10^5$/mm$^3$
3.3.5 Determination of erythrocyte sedimentation rate

Erythrocyte sedimentation rate was determined using Westergren’s method.

0.4 ml of 3.0% sodium citrate was taken in a tube. 2 ml of venous blood was withdrawn in a dry sterile syringe and exactly 1.6 ml of blood was placed in the tube containing sodium citrate solution. The tube was inverted 2 to 3 times to mix the blood with the citrate solution. The Westergren’s ESR tube was filled to exactly the zero mark and placed in the stand. The tube was held firmly at an exactly vertical position. The reading was taken.

Erythrocyte sedimentation rate in blood is expressed as mm/hour.

3.3.6 Estimation of fibrinogen

Plasma fibrinogen is estimated by the method of Reinhold (1953).

Reagents

1. Sulphate sulphite solution 27.8%. Weigh out 208 g of sodium sulphate (anhydrous) and 70 g of sodium sulphite (anhydrous) and dissolve with stirring in about 900 ml of water to which 2 ml of conc. $\text{H}_2\text{SO}_4$ has been added in a 2 litre beater. Transfer to a litre volumetric flask and make to the mark with water. The pH should be above 7.0 keep it in an incubator at 37°C in a stoppered bottle.
2. Stock biuret reagent of weichselbaum dissolve 45 gms of Rochelle salt in about 400 ml of 0.2 N NaOH and add 15 g of copper sulphate (small crystals of CuSO₄, 5H₂O) stirring continuously until solution is complete. Add 5 g of potassium iodide and make upto a litre with 0.2 N sodium hydroxide.

3. Biuret solution for use. Dilute 200 ml of the stock reagent to a litre with 0.2 N sodium hydroxide which contains 5 g of potassium iodide per litre.

4. Tartarate-iodide solution. Dissolve 9 g of Rochelle salt in 0.2 N sodium hydroxide containing 5 g of potassium iodide per titre.

5. Ether (analar grade)


**Procedure**

Mix 0.5 ml of plasma, 14 ml of distilled water, and 0.5 ml of 2.5% calcium chloride solution in a small beaker, place a fine glass rod in the liquid, and allow to stand in an incubator at 37°C until a clot is formed. Thirty minutes is usually sufficient but it may be necessary to leave it longer, even overnight, before it is concluded a clot is not forming. Then rotate the rod to collect the clot on it. Press the rod against the side of the beaker to squeeze out any solution and to compress the clot. Take care to pick up on the rod any small pieces of clot which may have become detached. Dry by pressing carefully against a piece of filter paper. Then transfer the clot to the tube in
which the digestion is to be carried out. Dissolve the clot prepared in 3.5 ml of the biuret reagent. After the biuret colour formation, 3 ml of saline was added and the OD measured at 540 nm.

**Biuret blank:** Add 2 ml of the sulphate sulphite solution to the biuret reagent.

**Standard:** Pipette 0.4 ml of the standard serum into 6.0 ml of sulphate-sulphite solution and transfer 2 ml of the mixture to 5 ml of the biuret reagent in a test tube.

**Standard serum blank**

Add 2 ml of the sulphate sulphite mixture to 5 ml of tartarate iodide solution and mix. This should be put whenever the serum is opalescent or abnormally pigmented. Otherwise this step can be omitted shake the test tube and place the tubes in a water bath at 37°C for 10 minutes. Allow to cool for 5 minutes at room temperature then read the absorbance at 540 to 560 nm using a yellow-green filter. Read them against biuret blank.

**Calculation**

\[
\frac{\text{Reading of unknown} - \text{Reading of biuret blank}}{\text{Reading of std.} - \text{Reading of std. serum blank}} \times \text{Conc. of standard}
\]

Fibrinogen values were expressed as mg/dl plasma.
3.3.7 Determination of bleeding time

Bleeding time was found using the Caocci (1978) method.

The bleeding time is the time required for the blood to stop flowing from a deep cut. The tail is rubbed to ensure adequate circulation and then a clean deep puncture was made. The time when the first drop of blood appeared was noted. For every thirty seconds the blood was blotted without touching the skin until the bleeding stops. The interval between the appearance of the first drop and the removal of the last drop represents the bleeding time.

Bleeding time is expressed in seconds.

3.3.8 Determination of clotting time

The clotting time was noted using the Sabraze's Capillary tube method (Samuel, 1980).

Three capillary tubes 10 cm in length with a diameter of 0.8 to 1.2mm was taken. The tail was cleaned and a deep puncture was made. The first two drops of blood was discarded. The third drop of blood was secured and the time was noted when the tubes were filled with the blood by capillary attraction. Tubes were placed on the table. At the end of 30 seconds about one centimeter length of tubing was broken and the clotting time was recorded as the interval from the time the blood appeared on the skin until the fibrin thread bridges the broken ends when they have been separated by a distance of 5 mm of more. The time was noted. The second and third tubes was used to check the
results observed with the first tube. The time between the appearance of the blood and filling the tubes and the appearance of the fibrin is the coagulation time.

Clotting time is expressed in seconds.

3.3.9 Enumeration of white blood corpuscles

WBC dilution fluid or Turk's fluid was used as the diluent which can destroy RBC's. The procedure of Raghuramulu et al., (1983) was followed.

Reagents

WBC diluting fluid was prepared by mixing

1. Glacial acetic acid
2. Gentian Violet 1%
3. Water 95 ml.

Procedure

The method of counting is similarly to RBC counting except that the count is made in 4 large (1 mm) cover squares of the Neubauer counting chamber.

Calculation

The total number of cells in 4 square is multiplied by a factor of 2,500 to give the count/mm$^3$ of blood.
3.3.10 Determination of differential leucocyte count

The film was prepared by Leishman’s method.

Leishman’s Method

A Romanowsky type stain designed to differentiate leucocyte was used.

Reagents

Leishman’s stain powder - 0.15g
Methyl Alcohol, Acetone free - 100 ml.

Procedure

0.15g of Leishman’s stain powder was taken in a glass mortar and few ml of methyl alcohol was added. The stain was ground to dissolve completely. The stain was then transferred to a 100 ml measuring cylinder. Few ml of methyl alcohol was added into the mortar and ground. The same was repeated till the Leishman’s stain powder is completely dissolved. The volume was made upto 100 ml with methyl alcohol. The stain was poured into a clean dry bottle and closed well and used after two to three weeks.

The blood films was placed in a level positing and the dry blood film was covered with the stain, which should be evenly distributed over the entire slide. At the end of one minute, the quantity of buffer solution or distilled water was doubled carefully and mixed with the stain by means of the clean
pipette. The film was allowed to stain for 7 or 8 minutes and the excess stain was removed by washing with the distilled water for 2 minutes. The water was then washed off with fresh distilled water. The film was dried in air. When the film was dry it was then examined microscopically.

3.4 ASSAY OF CARDIAC MARKERS

3.4.1 Enzyme - immunological test for the quantitative determination of troponin-T (Tn-T) in vitro Cat No. 1556428

Principle: Elisa / 1-step sandwich assay using streptavidin technology.

Reagents

Sol. 1: Buffer for incubation - phosphate buffer 40 mmol/l: pH 7.0 monoclonal (mouse) anti-T antibodies (biotinylated) 1.5 µg/ml.

Sol. 2: Anti-Tn-T-POD conjugate (monoclonal mouse) POD ≥ 10 U/ml (lyophilisate) → It is dissolved in 1.5 ml dist. water.

1a incubating solution: Prepare the quantity required by mixing solutions 1 and 2 in the relationship 100+1 atleast one hour before starting the determination. Avoid the formation of foam.

Standards: Tn-T in human serum lyophilisate for 1 x 2.5 ml 3a, 3b, 3c, 3d, 3e (standards) + 1 x 2.5 ml 3d (for recalibration).

Carefully dissolve the contents of each bottle in 2.5 ml of distilled water. Let stand closed to reconstitute for at least 15 minutes and then carefully stir. The
occurrence of slight opalescence does not impair the assay's performance. The solutions are stable for shows at 20-25°C.

Control I & II - lyophilisate for 4.0 ml. Tn-T in human serum. Carefully dissolved the contents in 4.0 ml distilled water and allow to stand closed for at least 15 minutes to reconstitute. Carefully mix. Stable for 8 hrs at 20-25°C.

Enzymun-Test substrate/buffer - phosphate/citrate 100 mmol/l, pH 4.4 H₂O₂ (as sodium perborate) 3.2 mmol/l.
Chromogen - ABTS - 1.9 mmol/l.

Substrate - chromogen solution: Add the chromogen to substrate buffer and dissolve completely at least 1 hour before use. Store protected from light.

Plastic tubes coated with streptavidin with binding capacity of approximately 14 ng biotin per tube only should be used for Troponin-T Enzymum-test determination.

**Procedure**

Take the required no. of streptavidin tubes for the standards, controls and the test samples. Add 200 µl of the sample to the test streptavidin tube and then add 1 ml of the enzyme buffer. For the standards and controls add only the enzyme buffer (1a sol). Incubate for 30 minutes at room temperature. After 30 minutes wash twice thoroughly with distilled water and remove water droplets completely. Add 1 ml of chromogen and incubate for 15 mts. at room
temperature. Then mix thoroughly and take the reading in a spectrophotometer at wavelength of 422 nm.

Plot a curve with absorbance on the x axis and concentration on y axis. Troponin-T values are expressed as ng/ml.

3.4.2 Separation and quantification of serum LDH isoenzymes by agarose gel electrophoresis

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

Principle

The LDH isoenzymes are separated on 1% agarose gel since it has the advantage of high resolving power and excellent separation of proteins into sharp bands. The isoenzymes are identified by numbers according to their electrophoretic mobility on agarose gel. The fastest moving component is called LDH₁ and the slowest moving component is called LDH₅. The process of the coupled reactions on the electrophoretogram is illustrated as follows:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate → NAD⁺</td>
<td>PMS (reduced)</td>
</tr>
<tr>
<td>Pyruvate → NADH + H⁺</td>
<td>PMS oxidized</td>
</tr>
</tbody>
</table>
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 serum samples were applied into a well in 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) nitroblue 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. The gels were washed with 7.5% acetic acid, preserved in 5% acetic acid and scanned using a densitometer.

3.4.3 PROTEIN

Total proteins in serum was estimated by the method of Lowry et al., (1951).
**Reagents**

1. Lowry's reagent (alkaline copper reagent)
   
   Solution A: 2% sodium carbonate in 0.1N sodium hydroxide solution
   
   Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate.
   
   50 ml of solution A was mixed with 1.0 ml of solution B just before use.

2. Folin's-phenol reagent
   
   This reagent was prepared according to the method of Folin and Ciocalteau (1927) and diluted 1:2 with water just before use.

3. Standard protein solution
   
   100 mg of bovine serum albumin was dissolved in 100 ml of distilled water with the aid of small amounts of alkali.
   
   The above stock solution was diluted 1 in 10 to give a working standard containing 100 µg bovine serum albumin/ml.

**Procedure**

0.1 ml of serum was diluted to 10 ml with water and 0.1 ml aliquot was taken from this. The volume was made upto 1.0 ml with water and 4.5 ml of Lowry's reagent added, mixed well and allowed to stand at room
temperature for 20 minutes. Standards containing bovine serum albumin in the range of 10-100 µg and a blank containing 1.0 ml water were treated similarly. 0.5 ml of Folin's-phenol reagent was then added and the blue colour developed was read at 640 nm after 10 minutes.

Serum protein levels are expressed as gm/dl.

3.4.4 Aspartate transaminase (L-aspartate : 2-oxoglutarate amino transferase, E.C.2.6.1.1.)

This enzyme was assayed according to the method of Bergmeyer and Bernt (1974).

The reaction catalysed by this enzyme is as follows.

\[
\text{GOT} \\
\text{L-Aspartate} + 2 \text{ oxoglutarate} \xrightarrow{\text{-----}} \text{Oxaloacetate} + \text{L-glutamate}.
\]

REAGENTS

1. Buffered Substrate

Dissolved 15 gm dipotassium hydrogen phosphate, 2.0 gm potassium dihydrogen phosphate and 300 mg 2-oxoglutarate in 700 to 800 ml distilled water and 13.2 gm L-aspartic acid was added. The pH of the solution was adjusted to 7.4 with sodium hydroxide and made upto a litre with water.
2. **2,4-Dinitro Phenyl hydrazine (DNPH)**

1 mmol (200 mg)/l in 1 mol/l hydrochloric acid.

3. **Sodium hydroxide solution, 400 m mol (16g/l)**

4. **Pyruvate solution, 2 m mol/l (22 mg sodium pyruvate/100 ml)**

**Procedure**

0.2 ml of serum was added to 1.0 ml of buffered substrate, mixed well and incubated for 60 minutes in a water bath. 1.0 ml of DNPH was added and allowed to stand for 20 minutes at room temperature, 10 ml of 0.4 N sodium hydroxide solution was added and the colour developed was read at 530 nm. Blank contained 1.0 ml buffered substrate and 1.0 ml DNPH solution with 10 ml of 0.4 N sodium hydroxide solution.

The values are expressed as IU/l of serum.

**3.4.5 Alanine transaminase (L-alanine : 2 Oxalo glutarate amino transferase, E.C.2.6.1.2)**

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

The reaction catalysed by this enzyme is as follows.

\[
\text{L-Alanine} + 2 \text{ oxaloglutarate} \quad \overset{\text{GPT}}{\longrightarrow} \quad \text{Pyruvate} + \text{glutamate.}
\]
Reagents

1. **Buffered Substrate**

   15 gm of dipotassium hydrogen phosphate, 2.0 gm potassium dihydrogen phosphate and 300 mg 2 - oxaloglutarate were dissolved in 700 to 800 ml distilled water. Then 17.8 gm of DL-alanine was added. The pH of the solution was adjusted to 7.4 with sodium hydroxide and made upto a litre with water.

2. **2,4 - Dinitrophenyl hydrazine (DNPH)**

   1 mmol (200 mg)/l in 1 mol/l hydrochloricacid

3. **Sodium hydroxide solution, 400 m mol (16g/l) (4N)**

4. **Pyruvate solution, 2 mmol/l (22 mg sodium pyruvate/100 ml)**

Procedure

0.2 ml of serum was added to 1.0 ml of buffered substrate, mixed well and incubated for 30 minutes in a water bath. After cooling 1.0 ml of DNPH was added and allowed to stand at room temperature for 20 minutes. Then 10 ml of sodium hydroxide was added, mixed well and after 5 minutes the colour developed was read at 530 nm in a spectrophotometer. Blank contained 1.0 ml of buffered substrate, 1.0 ml of DNPH and 10 ml of 0.4 N sodium hydroxide as in the test.
The enzyme activity was expressed as I.U/l of serum.

3.4.6 Assay of creatine kinase

(ATP -Creatine phospho transferase E.C.2.7.2.3).

The reaction catalysed by this enzyme is as follows:

CPK
Creatine + ATP  $\leftrightarrow$ creatine phosphate + ATP.

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

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 sulphate and 15.76 mg of cysteine - Hcl were dissolved in 10 ml of distilled water

4. Creatine  240 mM

5. Ammonium molybdate  2.5 g of ammonium molybdate was dissolved in 100 ml of 3 N Sulphuric 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 sulphite 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 80 μg 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 cystein-hydrochloride solution. The final volume was made up to 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 up to 4.3 ml with water. 1.0 ml of ammonium molybdate reagent was added and 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 min in a Shimadzu UV spectrophotometer.

Activity of creatine kinase in serum is expressed as IU/litre and tissue is expressed as micromoles of phosphorus liberated/min/mg protein.
3.4.7 Assay of lactate dehydrogenase

(L-lactate NAD - Oxidoreductase E.C.1.1.1.27)

The enzyme activity was assayed according to the method of King J (1965). The enzyme catalyses the reaction

\[
\text{LDH} \\
\text{Pyruvate + NADH + H}^+ \rightleftharpoons \text{lactate + NAD}^+ 
\]

Reagents

1. Glycine buffer
   - 7.5 g of glycine and 5.85 g of (0.1 M) sodium chloride was dissolved in one litre of distilled water.

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. The pH was adjusted to 10.0.

3. 2,4-Dinitrophenyl hydrazine reagent
   - 200 mg of DNPH was dissolved one litre of 1.0 N HCl (DNPH)

4. NAD
   - 5 mg/ml

5. Standard pyruvate
   - 11.0 mg of sodium pyruvate was dissolved in 100 ml of buffer.

Procedure

To a set of tubes, 1.0 ml of 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 1.0 ml of DNPH reagent and then 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. 7.0 ml of 0.4 N sodium hydroxide solution was added and the colour developed was measured at 420 nm in a shimadzu UV spectrophotometer. Suitable aliquots of the standards were also analysed by the same procedure.

The enzyme activity is expressed as U/litre, for serum and as nanomoles of pyruvate liberated/min/mg of protein for tissues.

3.5 GENERAL BIOCHEMICAL PARAMETERS

3.5.1 Serum uric acid

Uric acid was estimated in serum according to the procedure of Caraway (1963).

Reagents

1. Phosphotungstic acid reagent

30 gm of sodium tungstate was dissolved in 300 ml of water. 32 ml of 85% O-Phosphoric acid was added and refluxed under a low flame for 2 hrs. After cooling to room temperature, 18 gm of lithium sulphate was dissolved and the solution was finally made upto 1 litre.

2. 14% - sodium carbonate

3. 2/3 N - Sulphuric acid
4. 10% - Sodium tungstate

5. Stock uric acid standard

100 mg of uric acid and 60 mg of lithium carbonate were taken in about 50 ml of water. This was heated to about 80°C to dissolve the uric acid completely. After cooling, the solution was finally made up to 100 ml with distilled water.

**Working standard**

1.0 ml of the stock standard was diluted to 10 ml to get the working standard which contained 100 µg uric acid/ml.

**Procedure**

A protein free filtrate was prepared as in serum creatinine, 2.0 ml of the supernatant was mixed with 1.0 ml of phosphotungstic acid and 1.0 ml of 14% sodium carbonate. The blue color developed was read at 640 nm after 10 minutes. A standard curve prepared with standard uric acid in the range of 10-50 µg was used to arrive at the serum uric acid levels.

Uric acid values are expressed as mg/dl of serum.

**3.5.2 Blood Glucose**

This was estimated using O-toluidine according to the method of Dubowski (1962) and modified by Sasaki and Matsui (1972).
Reagents

1. 10% trichloroacetic acid (TCA)
2. O-Toluidine-boric acid reagent

This contained 2.4 gm boric acid and 2.5 gm thiourea in 100 ml of a solution containing water, glacial acetic acid and freshly distilled orthotoluidine in the ratio of 10:75:15.

3. Standard glucose

Stock standard glucose was prepared by dissolving 100 mg of D-glucose in 100 ml of 0.1% benzoic acid. 10 ml of this solution was diluted to 100 ml to get a working standard solution containing 100 µg of glucose/ml.

Procedure

0.1 ml of blood was mixed with 3.0 ml of 10% TCA and the precipitated proteins are sedimented. To 1.0 ml of the protein free supernatant taken in a test tube, 4.0 ml of O-toluidine reagent was added. Standard solutions containing 25-100 µg of glucose in 1.0 ml volume and a blank containing 1.0 ml water were also treated in a similar manner. The tubes were heated in a boiling water bath for 8 minutes. The blue colour developed was read at 640 nm after cooling.

Blood glucose values are expressed as mg/dl.
3.5.3 Serum creatinine

This was estimated according to the method of Broad and Sirota (1948) using Jaffe's colour reaction.

Reagents

1. Saturated picric acid
2. 0.75 N - Sodium hydroxide
3. 2/3 N - Sulphur acid
4. 10% - Sodium tungstate
5. Stock standard for creatinine

100 mg of creatinine was dissolved and made upto 100 ml using 0.1 N hydrochloric acid.

6. Working Standard

1.0 ml of the stock standard was diluted to 100 ml using distilled water. This contained 10 µg creatinine/ml.

Procedure

A protein free filtrate was prepared by precipitating 1.0 ml of serum with 8.0 ml of water, 0.5 ml of 2/3 N sulphuric acid and 0.5 ml of 10% sodium tungstate. After centrifugation, 5.0 ml of the clear filtrate was taken. To it was added 1.5 ml of saturated picric acid solution and 1.5 ml of 0.75 N sodium
hydroxide. The colour intensity was measured at 470 nm after 15 minutes. Standards and blanks were processed similarly.

Serum creatinine levels are expressed as mg/dl.

3.5.4 Blood Urea

The method employed was that of Natelson et al. (1951) by measuring the optical density of the coloured complex formed with diacetyl monoxime in the protein free filtrate of the whole blood.

Reagents

1. 10% sodium tungstate
2. 2/3 N Sulphuric acid
3. 2% solution of diacetyl monoxime in 2% glacial acetic acid

2.0 gm diacetyl monoxime was added to 60 ml of water containing 2.0 ml of glacial acetic acid. With slight warming, it was dissolved and made upto 100 ml with water.

4. Sulphuric acid - phosphoric acid reagent.

To 140 ml of H₂O, 150 ml of 85% O-phosphoric acid was added, mixed well and then 50 ml of concentrated sulphuric acid was added slowly.
5. **Stock solution of urea**

Dissolved 250 mg of urea in 100 ml of water.

6. **Working standard**

The stock was diluted 1 to 100 to give a solution containing 25 μg of urea/ml.

**Procedure**

0.1 ml of blood was diluted with 3.3 ml of water, 0.3 ml of 10% sodium tungstate and 0.3 ml of 2/3 N sulphuric acid. Mixed well and centrifuged. To 1.0 ml of supernatant taken a test tube, 1.0 ml of water, 0.4 ml of diacetyl monoxime and 1.6 ml of sulphuric acid - phosphoric acid mixture was added. The tubes were heated in a boiling water bath for 30 minutes and the colour intensity was read against a water blank at 480 nm. At the same time, the standard containing 5-25 μg of urea were also developed for comparison.

Blood urea levels are expressed as mg/dl.

3.5.5 **Tissue protein**

Protein content in heart tissue was estimated by the method of Lowry *et al.*, (1951). A known amount (100 mg) of tissue was homogenised in a Potter-Elvejhem homogeniser with a Teflon pestle in 0.1 M Tris-HCl buffer, pH 7.5. From the crude homogenate 0.1 ml aliquots were taken and the protein content was estimated as described in section 3.4.3.

Tissue protein content are expressed as mg/gm of fresh tissue.
3.5.6 Extraction of nucleic acids

The nucleic acids were extracted by the method of Schneider (1957).

Reagents

1. 10% TCA
2. 5% TCA
3. Absolute ethanol

Known amount (100 mg) of the tissues were homogenized in 5.0 ml of ice-cold distilled water using a Potter-Elvehjem homogenizer with a teflon-pestle. 5.0 ml of 5% TCA was added to the homogenate and this was kept in ice for 30 min, to allow complete precipitation of proteins and nucleic acids. The contents were centrifuged and the precipitate obtained was washed thrice with 1.0 ml of ice-cold 10% TCA. Then it was treated with 3.0 ml of 95% ethanol to remove lipids. The supernatant was decanted and then subjected to centrifugation. The final precipitate was dissolved in 5.0 ml of 5% TCA and kept in a water bath maintained at 90°C for 15 min with occasional shaking which facilitated the quantitative separation of nucleic acids from protein. The supernatant after centrifugation was used for the estimation of DNA and RNA.

3.5.7 Estimation of DNA

DNA was estimated according to the method of Burton (1956).
Reagents

1. Diphenylamine reagent: 1.5 g diphenylamine was dissolved in 100 ml of redistilled acetic acid and 1.5 ml of concentrated sulphuric acid was added. The reagent was stored at 4°C in dark. Before use 0.1 ml of aqueous acetaldehyde (0.16%) was mixed with every 20 ml of the reagent.

2. Stock standard: Highly polymerized calf-thymus DNA solution was dissolved in 5 mM sodium hydroxide to give a concentration of 0.4 mg/ml. (0.4 mg of standard DNA in 1 ml 50 mM NaOH).

3. Working standard: This was prepared by mixing 2.0 ml of the stock solution with an equal volume of 1N perchloric acid and was heated at 70°C for 15 min. This contained 200 μg of DNA/ml.

Procedure

1.0 ml of the nucleic acid extract was made upto 3.0 ml with 1N perchloric acid. This was mixed with 2.0 ml of diphenylamine reagent. A reagent blank and standards were also carried out concurrently. This was kept in a boiling water bath for 10 min. and the blue colour developed was read at 640 nm in a Shimadzu spectrophotometer.

The values are expressed as mg/g wet tissue.

3.5.8 Estimation of RNA

RNA was estimated according to the method of Rawal et al., (1977).
Reagents

1. Orcinol - Ferric chloride reagent: 1 g of orcinol was dissolved in 100 ml of concentrated hydrochloric acid containing 0.5 g of ferric chloride. This reagent was prepared freshly.

2. Standard RNA solution: This was prepared by dissolving 20 mg of yeast RNA in 100 ml of 5% TCA.

Procedure

Aliquots of nucleic acid extract were made up to 2.0 ml with 5% TCA. To this 3.0 ml of orcinol-ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 min. Reagent blank and standard RNA solutions were also treated in the same way. The tubes were cooled and the colour developed was measured at 640 nm in a Shimadzu spectrophotometer.

The values are expressed as mg/g wet tissue.

3.6 ASSAY OF MEMBRANE BOUND ATPASES

3.6.1 Assay of sodium, potassium dependent adenosine triphosphate (EC 3.6.1.3)

This enzyme was assayed according to the method of Bonting (1970) in heart tissue.
Reagents

1. Tris-HCl buffer : 0.09M, pH 7.5
2. Magnesium sulphate : 0.05 M
3. Potassium chloride : 0.05 M
4. Sodium chloride : 0.6 M
5. EDTA : 0.001 M
6. ATP solution : 0.04 M
7. Trichloroacetic acid : 10%
8. Ammonium molybdate : 2.5% in 5 N sulphuric acid
9. Amino Napthol Sulphonic Acid (ANSA) : This reagent was prepared as described in section.
10. Phosphorous standard : 80µ gms/ml solution was prepared.

Procedure

To 1.0 ml of the buffer was added, 0.2 ml of each of potassium chloride, magnesium sulphate, sodium chloride, EDTA and ATP solution. The 0.2 ml of the enzyme preparation was added. The contents were incubated at 37°C for 15 minutes. At the end of the incubation period the reaction was arrested by the addition of 1.0 ml of 10% TCA and 0.2 ml of the enzyme was added to the control tubes. The tubes were centrifuged and the phosphorous content of the supernatant was estimated according to Fiske and Subbarow's method.

The enzyme activity was expressed as µ moles of phosphorous liberated/min/mg protein.
3.6.2 Assay of Calcium - dependent Adenosine triphosphatase (EC 3.6.1.3)

Calcium - dependent ATPase was assayed according to the method of Hjerten and Pan (1963).

Reagents

1. Tris-HCl buffer : 0.125M, pH 8.0
2. Calcium chloride : 0.05M
3. ATP : 0.01M

Procedure

Tris-HCl buffer 1.0 ml, calcium chloride 0.1 ml, ATP solution 0.1 ml and distilled water 0.1 ml were taken in two sets of test tubes labelled ‘test’ and ‘control’ and 0.1 ml of enzyme preparation was added to ‘test’ tubes. The tubes were incubated at 37°C for 15 minutes. Then 1.0 ml of 10% TCA was added to arrest the reaction. 0.1 ml of enzyme was then added to the ‘control’ tubes. The tubes were centrifuged. The supernatant was used for the estimation of phosphorous liberated as described in the previous procedure.

The enzyme activity was expressed as μmoles of phosphorous liberated/min/mg protein.
3.6.3 \( \text{Mg}^{2+} \)-ATPase - (\( \text{Mg}^{2+} \)-dependent adenosine triphosphatase, E.C. 3.6.1.3)

This enzyme was assayed by the method of Ohnishi et al., (1982) in heart.

**Reagents**

1. 375 mM Tris-HCl buffer, pH 7.4
2. 25 mM Magnesium chloride
3. 10 mM ATP.

**Procedure**

The incubation mixture consisted of 0.1 ml of Tris buffer, 0.1 ml magnesium chloride, 0.1 ml of ATP solution and 0.1 ml of water. After equilibrating the tubes at 37°C, the reaction was initiated by the addition of 0.1 ml of enzyme solution. The assay medium was further incubated at 37°C for 15 minutes at the end of which the reaction was arrested by the addition of 0.5 ml of ice cold 10% TCA. The liberated phosphorus was estimated according to the method of Fiske and Subbarow (1925).

The TCA supernatant along with aliquots of standard were made upto 5.0 ml with water. 1.0 ml of ammonium molybdate followed by 0.5 ml of ANSA were added and mixed. The blue colour developed was read at 620 nm after 20 minutes against a reagent blank.
Enzyme activity of Mg$^{2+}$-ATPase in the tissue is expressed as μ moles of phosphorus liberated/mg protein/hour.

3.7 ESTIMATION OF ELEMENTS (CA, MG, NA, K) IN HEART TISSUE AND SERUM

Calcium, magnesium, sodium and potassium were estimated in serum and tissues after digestion in Perkin-Elmer 2380 atomic absorption spectrophotometer. Serum samples were diluted 1:50 with lanthanum diluent and used directly for the estimation of potassium, calcium and magnesium in serum. For the estimation of sodium an additional 1:50 dilution with deionized water was required.

For the estimation of Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$ in tissue, 500 mg of accurately weighed tissue was placed in a kjeldalhs flask, 2.5 ml of deionized water and 1.0 ml of 1 : 1 mixture of cone nitric acid and perchloricacid were added. The samples were digested on a sand bath till the solution became clear and the digest was made upto 10 ml with deionized water with thorough mixing.
The wavelength, slit setting, light source, flame type and linear working range used for calcium, magnesium, sodium and potassium are given below:

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Wavelength nm</th>
<th>Slit setting nm</th>
<th>Light source</th>
<th>Flame type</th>
<th>Linear working range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>589.0</td>
<td>0.7</td>
<td>Hollow cathode</td>
<td>Air acetylene flame</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td></td>
<td>589.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
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<td>2.0</td>
<td>Hollow cathode</td>
<td>Air acetylene flame</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>Calcium</td>
<td>422.7</td>
<td>0.7</td>
<td>Hollow cathode</td>
<td>Air acetylene flame</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Magnesium</td>
<td>285.2</td>
<td>0.7</td>
<td>Hollow cathode</td>
<td>Air acetylene flame</td>
<td>0.5 µg/ml</td>
</tr>
</tbody>
</table>

3.8 ESTIMATION OF LIPID PEROXIDES AND ANTIOXIDANT STATUS

3.8.1 Lipid peroxidation estimation

Lipid peroxide content in tissue was determined by thio barbituric acid reaction as described by Okhawa (1979).

Reagents

1. Acetic acid : 20% solution, pH 3.5
2. Sodium dodecyl sulphate : 8.1% solution
3. TBA 0.8% solution

4. n-Butanol-pyridine mixture: 15:1 (V/V)

5. Standard solution

16 mg of 1,1,3,3' tetramethoxy propane was accurately weighed and dissolved in 100 ml of distilled water. Further dilution was made so that the working standard solution contained 100 n moles per ml.

Procedure

To 0.2 ml of the heart tissue homogenate, 1.5 ml of 20% acetic acid, 0.2 ml of SDS and 1.5 ml of TBA were added. The mixture was made upto 4 ml with distilled water and heated for 60 minutes at 95°C. After cooling the contents to room temperature 4.0 ml of butanol-pyridine mixture was added and shaken well. After centrifugation at 400 rpm for 10 minutes the organic layer was taken and its absorbance was read at 532 nm.

The lipid peroxide content was expressed as nanomoles of TBA reactants/100 mg protein.

3.8.2 Lipid peroxide estimation in serum

Lipid peroxide levels in serum was determined by the method of Yagi (1976) using the thiobarbituric acid reaction.

In this method, the estimation of water soluble substances that react with TBA was attained by precipitating lipid peroxides along with serum proteins in a phosphotungstic acid system. The interference from sialic acid
and bilirubin both of which react with TBA, was arrived by performing the TBA reaction in an acetic acid solution (Yagi 1982).

**Reagents**

1. Sulphuric acid N/12
2. Phosphotungstic acid 10%
3. Thio barbituric acid reagent: Equal volumes of 0.67% thio barbituric acid aqueous solution and glacial acetic acid were mixed.
4. n-Butanol
5. Standard malandialdehyde (MDA)

**Procedure**

To 0.2 ml of plasma 4 ml of N/12 sulphuric acid was added, mixed well and 0.5 ml of 10% phospho tungstic acid was added. The contents were centrifuged and the supernatant discarded. The sediment was mixed with 2.0 ml of N/12 sulphuric acid and 0.3 ml of phosphotungstic acid. The mixture was centrifuged and the sediment was dissolved in 4.0 ml of distilled water and 1.0 ml of TBA reagent 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, centrifuged for 20 minutes and the supernatant read at 515 nm. Standard graph was obtained taking 2-10 n moles of tetraethoxy propane mixed with TBA reagent and processed in the same manner.

MDA values are expressed as n moles/dL of serum.
3.8.3 Estimation of Total Reduced Glutathione (GSH)

The method is based on the procedure described by Moron et al. (1979).

Reagents

1. Phosphate buffer : 0.2 M, pH 8.0
2. DTNB : 0.6 mM in phosphate buffer, pH 8.0
3. Trichloroacetic acid : 5% solution.

Procedure

0.5 ml of the sample (tissue homogenate) was precipitated with 5% TCA. The contents were mixed well for complete precipitation of protein and centrifuged. To an aliquot of clear supernatant, were added 2.0 ml of DTNB reagent and 0.2 M phosphate buffer to a final volume of 4.0 ml. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar way were also run to determine the glutathione content.

The amount of glutathione was expressed as nanomoles/100 mg protein.

3.8.4 Estimation of serum iron

Serum iron content was estimated by the method of Ramsay (1958).
Reagents

1. 2-2 - dipyr-idyl 0.1% in 3% acetic acid.
2. Sodium sulphite 0.1 M Dissolved in 1.26 g of anhydrous sulphite or 2.52g of sodium sulphite in water and made upto 100 ml. This reagent was prepared freshly.

3. Chloroform

4. Standard iron solution Dissolved 498 mg of ferrous sulphate in water, added 1 ml of concentrated sulphuric acid and made upto one litre. This contains 100 microgram iron per ml.

5. Working Standard 3 ml of the stock solution was diluted to 100 ml with water to obtain a solution containing 3 mg/ml

Procedure

Equal volumes of serum, 0.1 M sodium sulphite and dipyr-idyl reagent were mixed in glass stoppered centrifuge tube. The tubes were heated in a boiling water bath for 5 minutes. The contents were cooled and 12 ml of Chloroform was added in each tube. The tubes were stoppered and mixed vigorously for 30 secs and centrifuged for 5 minutes at 1000 rpm. The color intensity was measured at 520 nm. Blank contained ferrous sulphate were also treated similarly.

The values were expressed as mg/dl of serum.
3.8.5 Estimation of ceruloplasmin activity

Ceruloplasmin is a copper oxidase which can catalyse the oxidation of some polyamines and its action on p-phenylene diamine was used as a measure of the amount present in serum. It is estimated by the method of Reinhold (1953).

Reagents

1. P-phenylene diamine hydrochloride : 0.5%
2. Acetate buffer : 0.4 M, pH 5.5
3. Sodium azide : 0.5 % solution.

Procedure

0.1 ml of fresh serum (free from haemolysis and turbidity) was taken into three 15 ml test tubes, one for control and two for test. 1.0 ml of 0.5 percent sodium azide was added to the control. Then added 8.0 ml of acetate buffer to each tube, followed by 1.0 ml of the P-phenylene diamine. The solutions were mixed and placed in a water bath at 37°C for one hour. After the incubation, the tubes were removed and added 1.0 ml of sodium azide to each of the tubes. The color intensity was measured at 580 nm against the reagent blank.

The optical density read in this way is used as a measure of the serum copper oxidase activity.
3.8.6 Assay of Superoxide dismutase (Superoxide : Superoxide reductase, EC 1.15.1.1)

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

Reagents

1. Carbonate - bicarbonate buffer : 0.3 M, pH 10.2
2. EDTA solution : 0.6 mM
3. Epinephrine : 1.8 mM (Prepared fresh)

Procedure

To tubes containing 0.5 ml of the carbonate buffer and 0.5 ml of EDTA solution, required amount of enzyme was added, 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 UV spectrophotometer. 50% autooxidation of epinephrine to adrenochrome was performed in a control tube without the enzyme.

The enzyme activity was expressed as Units/min/100 mg protein.

3.8.7 Assay of catalase (Hydrogen peroxide : Hydrogen peroxide oxido reductase; EC 1.11.1.6)

Catalase activity was assayed by the method of Claiborne (1985).
Catalase activity was determined by monitoring the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide. The difference in extinction/unit time is a measure of the catalase activity.

**Reagents**

1. Phosphate buffer: 50 mM, pH 7.0
2. Hydrogen peroxide: 30 mM in phosphate buffer.

**Procedure**

The reaction mixture containing 2.0 ml of suitably diluted sample and 1.0 ml of phosphate buffer was kept in the reference cuvette. 0.2 ml of the enzyme was taken in a sample or test cuvette and 1.0 ml of diluted H₂O₂ was added and decrease in absorbance was measured at 240 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as nanomoles of H₂O₂ decomposed/min/100 mg protein.

### 3.8.8 Assay of glutathione-S-transferase (EC.2.5.1.18)

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

1. Phosphate buffer : 0.3 M, pH 6.5
2. 1-chloro, 2,4-dinitrobenzene (CDNB) : 30 mM
3. Reduced glutathione (GSH) : 30 mM

Procedure

The reaction mixture containing 1.0 ml of buffer, 0.1 ml of CDNB and 0.1 ml of enzyme homogenate was made upto 3.0 ml with H₂O. 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.

The enzyme activity was expressed as nanomoles of CDNB conjugated/min/100 mg protein.

3.8.9 Assay of glutathione peroxidase (Glutathione : Hydrogen peroxide oxidoreductase EC.1.11.1.19)

Glutathione peroxidase was assayed according to the method of Rotruck et al., (1973) with some modification.

Reagents

1. Sodium phosphate buffer : 0.4 M, pH 7.0
2. Sodium azide : 10 mM
3. Reduced glutathione : 4 mM
4. Hydrogen peroxide : 2.5 mM
5. Trichloro acetic acid : 10% solution
6. Phosphate solution : 0.3 mM disodium hydrogen phosphate
7. DTNB reagent : 40 mg of 5,5' dithiobis (2-nitrobenzoic acid)/100 ml of 1% sodium citrate
8. Reduced glutathione standard : 20 mg % solution.

Procedure

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 were taken to a final incubation volume 2 ml. The tubes were incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 0.5 ml TCA. To determine the residual GSH content the supernatant was removed by centrifugation and added to 3 ml of disodium hydrogen phosphate and 1 ml of DTNB reagent. The colour 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 activities were expressed as nanomoles of GSH oxidised/min/100 mg protein.
3.9 ESTIMATION OF LIPIDS IN SERUM AND HEART TISSUE

3.9.1 Estimation of cholesterol

Cholesterol was estimated by the method of Parekh and Jung (1970) using the ferric sulphate colour reaction in the presence of uranyl acetate.

Reagents

1. Ferric-Uranyl Acetate Reagent

10 ml of water and 30 ml of concentrated ammonia were added to 500mg of crystalline ferric chloride. The precipitate was washed several times with distilled water to get rid of ammonia and was dissolved in cholesterol grade glacial acetic acid and made upto one litre with acetic acid. 100 mg of uranyl acetate was then added to it and the contents were well shaken and left overnight. The reagent was stored in a brown bottle.

2. Sulphuric Acid-Ferrous Sulphate Reagent

To 100 ml of cholesterol grade glacial acetic acid, 100 mg of anhydrous ferrous sulphate was added and shaken well. To this 100 ml of concentrated sulphuric acid was added. After cooling, the volume was made upto 1 litre with concentrated sulphuric acid.
3. **Cholesterol Standard**

Reagent grade cholesterol was recrystallized from ethanol. The stock standard was prepared by dissolving 200 mg of cholesterol in 100 ml of cholesterol grade acetic acid.

**Procedure**

10 ml of ferric-uranyl acetate reagent was added to 0.1 ml of extracted lipid, mixed well, allowed to stand for 5 min. and centrifuged. 3.0 ml of the supernatant was taken for analysis. Similarly, 0.1 ml of standard cholesterol was mixed and 3.0 ml aliquots were taken. Blank tube contained 3.0 ml ferric-uranyl acetate reagent. 2.0 ml sulphuric acid-ferrous sulphate reagent was added to all tubes and mixed well. The colour intensity was measured at 560 nm after 20 min. in a Shimadzu spectrophotometer.

The values are expressed as mg/g tissue.

3.9.2 **Estimation of free and ester cholesterol**

Free cholesterol was precipitated by digitonin according to the method of Leffler and McDougald (1963). The cholesterol digitonide formed was sedimented by centrifugation and the cholesterol in the precipitate was estimated as free cholesterol as described previously (section 3.9.2).
Reagents

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

Procedure

To 0.2 ml of serum 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 from the difference between the total cholesterol and free cholesterol levels.

The level of free and ester cholesterol were expressed as mg/dl of plasma.
3.9.3 Estimation of phospholipids

Serum phospholipids were estimated according to the method of Rouser et al., 1970 by digestion with perchloric acid and estimating the inorganic phosphorous liberated.

Reagents

1. 10% trichloroacetic acid (TCA)
2. Perchloric acid (Analar grade)
3. 3% ammonium molybdate
4. 3% Ascorbic acid
5. Standard phosphorous solution
   Stock solution of potassium dihydrogen phosphate containing 80 μg of phosphorous/ml was prepared and diluted 1 to 10 for use.

Procedure

0.2 ml of serum was taken into a test tube containing 1.8 ml of water and 1.5 ml of 10% TCA. Mixed well and centrifuged for 15 minutes to get a clear supernatant. The supernatant was discarded. To the residue was added 1.0 ml of perchloric acid, digested on a sand bath till solution becomes colourless. After cooling the tubes, the solutions were made up to 5.0 ml with water. Added 0.5 ml of Ammonium molybdate followed by 0.5 ml of Ammonium molybdate followed by 0.5 ml of ascorbic acid. Mixed thoroughly and then left in a boiling water bath for exactly 6 minutes. The blue colour developed was
measured immediately at 710 nm standard phosphate solutions and blank (dist. water) were also mixed with reagents in the same order. 0.8 ml of perchloric acid was added before the addition of ammonium molybdate and ascorbic acid.

Serum phospholipids are expressed as mg/dl.

3.9.4 Estimation of triglycerides

Plasma triglycerides were estimated according to the method of Rice (1970). Triglycerides were extracted with chloroform: methanol solvent mixture and the phospholipids were removed by adsorption on silicic acid. The resultant lipid was saponified and the glycerol oxidized to formaldehyde was measured by its colour reaction with chromatropic acid reagent.

Reagents

1. Chloroform-methanol mixture (2:1 v/v)
2. Sodium chloride - saturated
3. Activated silicic acid
4. Alcoholic potassium hydroxide: 400 mg of KOH was dissolved in 100 ml of 95% ethanol.
5. Sulphuric acid - 0.2 N
6. Sodium metaperiodate - 0.1 M
7. Sodium arsenite - 0.5 M
8. Chronotropic acid reagent.

1.14g of disodium salt of chromotropic acid was dissolved in 100 ml of distilled water. Then 450 ml of dilute acid solution (prepared by mixing concentrated sulphuric acid and distilled water in the ratio 2:1 v/v) was added and stirred well.

9. 7% thiourea solution

10. Tripalmitin standard: 10 mg tripalmitin was dissolved in 100 ml of Chloroform.

Procedure

To 0.2 ml of serum was added 9.8 ml of chloroform methanol mixture, shaken well and left at room temperature for about 30 minutes with occasional shaking. The solution was centrifuged and the supernatant was decanted into a tube. 4.0 ml aliquots of this supernatant in duplicate, were transferred to another set of tubes. To each tube was added 8.0 ml of super saturated saline and the tubes were shaken vigorously. This was centrifuged and the upper aqueous layer was aspirated. The lower chloroform phase was filtered into another tube and to this was added 250 mg of activated silicic acid. This was centrifuged and aliquots of the chloroform layer were pipetted into tubes and dried. Standard tripalmitin solution containing 25-100 µg were dried along with the test samples. The test samples, standard tubes and blank were saponified as given below.

To all the tubes were added 0.5 ml of alcoholic potassium hydroxide solution and saponified by refluxing at 60°C for 20 minutes. The mixture was
neutralized with 0.5 ml of 0.2 N sulphuric acid and the excess alcohol was removed by evaporation in a boiling water bath. 0.1 ml of sodium metaperiodate was added and left at room temperature for 10 minutes, after which 0.1 ml of sodium meta arsenite was added. 10 minutes later, 5.0 ml of chromotropic acid was added to the mixture and the tubes were heated in a boiling water bath for 30 minutes. After cooling 0.5 ml of thiourea solution was added and the colour intensity was measured at 540 nm.

Serum triglyceride levels are expressed as mg/dl.

3.9.5 Estimation of free fatty acids

Free fatty acids were measured by the method of Horn and Menahe (1981) with the colour 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 0.1M copper nitrate and 50ml of Triethanolamine (0.2M) were mixed with 33 gm of sodium chloride.

4. Colour reagent: 0.1% diethyl dithiocarbamate in butanol was prepared.

5. Standard: A solution containing 2 mg/ml of palmitic acid was prepared in chloroform-heptane-methanol mixture and kept as stock. For
preparing a working standard this was diluted 1:10 in chloroform - heptane-methanol mixture to give a concentration of 200μg/ml.

Procedure

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

Free fatty acids were expressed as nanomoles/mg protein.

3.9.6 Serum lipoproteins

Fractional precipitation of Lipoproteins

Lipoproteins were fractionated by a dual precipitation technique (Hilson and Spiger, 1973).

Addition of heparin-manganese chloride to plasma caused precipitation of VLDL and LDL. The supernatant obtained after sedimentation represented the HDL fraction and cholesterol was measured in this fraction.
HDL is expressed as HDL cholesterol (HDLC) in mg/dl plasma.

In an aliquot of plasma, sodium dodecyl sulphate (SDS) was added which resulted in the aggregation of VLDL which flocculates on the top. The subnatant contained both HDL and LDL, and cholesterol content of this mixture was assayed (Burstein and Scholnick, 1972).

A third aliquot of plasma was assayed for total cholesterol. The cholesterol content of each fraction was arrived at in the following manner.

Total cholesterol - Subnatant from SDS (containing HDL + LDL) = VLDL cholesterol.

Subnatant from SDS - Heparin - Mn²⁺ supernatant (HDLc) = LDL cholesterol.

Reagents

1. Heparin-manganese chloride reagent

3.167gm of manganese chloride was added to 1.0ml solution of heparin containing 20,000 units. This mixture was made upto 8.0ml with distilled water.

2. Dextran sulphate

121mg of dextran sulphate (Mol. Wt. 15,000) was dissolved in 10ml of 0.89% saline.
Procedure

2.0ml of plasma was added to 0.18ml of heparin-manganese chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 minutes and then centrifuged in a refrigerated centrifuge at 2,500 rpm maintained at 10°C for 30 minutes. The supernatant contained the HDL fraction. It was taken for the estimation of cholesterol as described in 3.9.1

3.9.7 LDL and VLDL

Reagents

1. Sodium dodecyl sulphate (SDS) - 10% solution in 0.15 M.
2. Sodium chloride, pH 9.0

Procedure

2.0ml of plasma was added to 0.15ml of sodium dodecyl sulphate. The contents were mixed well and incubated at 37°C for 2 hours. The contents were centrifuged in a refrigerated centrifuge at 10,000 rpm for 15 mins. VLDL aggregated as a pellicle at the top. The supernatant contained the HDL and LDL fractions. Cholesterol was estimated in this fraction as described in section 3.9.1.
3.9.8 Identification and quantification of fatty acids by gas chromatography

Fatty acids in tissue phospholipids were quantified after extraction with hexane/isopropanol (3:2), separation of the phospholipids by thin layer chromatography and transmethylation in methanolic hydrochloride. The resulting methylesters were quantified by capillary gas chromatography.

Principle

Fatty acids are obtained from lipids by saponification (hydrolysis with alkali). The fatty acids are easily identified by GAs chromatography as methyl esters. The methyl esters have lower boiling point and would become volatile than fatty acids as such. The chemical conversion of lipids into fatty acid methyl esters is as follows:

1. Saponification

\[
\text{CHO}.\text{COR}_2 + 3 \text{KOH} \rightarrow \text{CHOH} + R_2\text{COOK}
\]

\[
\text{CH}_2\text{O}.\text{COR}_3 \rightarrow \text{CH}_2\text{OH} + R_3\text{COOK}
\]

triglyceride

glycerol

k salts of fatty acids

2. Acidification

\[
\text{RCOOK} + \text{HCl} \rightarrow \text{R COOH} + \text{KCl}
\]

K salt of fatty acid

free fatty acid.
3. **Methyl ester conversion**

\[ \text{RCOOH} + \text{CH}_3\text{OH} \xrightarrow{\text{H}^+} \text{R-CO-CH}_3 + \text{O} \]

free acid (non volatile)  \hspace{1cm} \text{methyl ester} (volatile)

**Reagents**

1. **Saponification reagent**

   Dissolve 45g of NaOH in 300 ml of methanol water mixture (1:1) (v/v).

2. **Methylation reagent**

   Prepare by mixing 325 ml of 6N HCl with 275ml CH\textsubscript{3}OH.

3. **Extraction solvent**

   Mix 200 ml of hexane with 200 ml of anhydrous diethyl ether.

4. **Base wash**

   Dissolve 10.8g of NaOH in 900 ml distilled water.

**Procedure**

1. Prepare lipid sample as described in isolation of lipids.

2. To a known volume of the lipid sample (100 \( \mu \text{l} \)) add 1 ml of saponification reagent and tightly close the tube with teflon lined screw cap.
3. Vortex and boil the contents for 30 min. (bubbling in the tube during saponification indicates leakage).

4. To it add 2ml of methylation reagent and mix thoroughly

5. Boil the tube in a water bath at 80°C for 20 min.

6. Cool it to room temperature and add 1.25 ml of extraction solvent. Tightly close the tube and rotate for 10 min. end-over-end.

7. Discard the aqueous lower phase.

8. Add 3 ml of base wash to the tube. Rotate for 5 min. end over end by tightly closing the tube.

9. Transfer 2/3 of the organic extract (upper phase) to a GC vial.

10. Run the GC in the following conditions

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<th>Setting</th>
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</thead>
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<tr>
<td>Injection port temp.</td>
<td>200°C</td>
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<tr>
<td>Detector temp.</td>
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<td>Nitrogen</td>
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Integrator setting

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<td>Attenuation</td>
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<tr>
<td>Chart speed</td>
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<tr>
<td>Peak width</td>
</tr>
</tbody>
</table>
Thrush 4
Chart speed 0.5, time 6
Peak width 0.64, time 5.

Result

Run methyl esters of standard fatty acids (C8:0-C24:20). From the retention time (RT) find out the fatty acid of the unknown sample and quantify them by using peak area. The peaks are reliably identified by pure standards and published retention times representing > 98% of total peak area. Retention times used to identify the eicosapentaenoic acid and docosahexaenoic acids were 44.83 and 48.94 min respectively.

3.9.9 Extraction of lipids from tissues

Lipids were extracted according to the method of Folch et al., (1957) from heart tissue.

The tissue were washed with ice-cold saline and dried between the folds of a filter paper. A weighed amount of tissue (500 mg) was mixed well and homogenised in 7.0 ml of methanol in a Potter-Elvejhem homogeniser with a Teflon pestle. It was then filtered through a Whatman No.1 filter paper into a previously weighed side arm flask. The residue on the filter paper, remaining after filtration, was scrapped and homogenised in 14 ml of chloroform-methanol mixture (2:1, v/v) and filtered as before. The defatted tissue was scrapped once again from the filter paper and homogenised with 10 ml of
chloroform. The pooled extract in the side-arm flask was then adjusted to chloroform-methanol (2:1, v/v) and evaporated to dryness under vacuum and the flask weighed. The lipid was redissolved in 50 ml of chloroform-methanol (2:1, v/v).

To the lipid extract, 1.0 ml of 0.1 N potassium chloride solution was added and shaken well. The upper phase contained gangliosides and other water-soluble components and were aspirated. The lower lipid-containing layer was washed thrice with Folch's upper phase 0.1 N potassium chloride: methanol: chloroform (10: 10:1, v/v). The upper phase was further concentrated and aliquots were taken for the analysis of cholesterol, triglycerides, phospholipids and free fatty acids.

3.9.10 Tissue Cholesterol

0.2 ml of the lipid extract was taken, it was evaporated and the cholesterol content was estimated as described in section .... Tissue cholesterol levels are expressed as mg/gm of fresh tissue.

3.9.11 Tissue ester and free cholesterol

To 0.2 ml of the lipid extract was added 0.5 ml of 1% digitonin in 50% ethanol. After keeping the tubes in a refrigerator for 30 minutes it was centrifuged for 10 minutes. The supernatant was decanted and the precipitate was washed twice with acetone. The second wash was decanted and the
precipitate was used for the estimation of free cholesterol as described in section 3.9.2.

Tissue free and ester cholesterol levels are expressed as mg/gm of fresh tissue.

### 3.9.12 Tissue phospholipids

0.2 ml of the lipid extract was taken and digested with 1.0 ml of 70% perchloric acid. The phosphorous content of the lipid was estimated by the method of Rouser et al., (1970) as described in section 3.9.3.

Tissue phospholipid levels are expressed as mg/gm of tissue.

### 3.9.13 Tissue triglycerides

0.2 ml of the total lipid extract was taken in a 1.0 ml of chloroform methanol mixture (2:1, v/v) to which 200 mg of activated silicic acid was added and mixed well to remove the phospholipids. From this an aliquot was taken for ethanolic potassium hydroxide saponification as described in section 3.9.4.

Tissue triglyceride levels are expressed as mg/gm of fresh tissue.

### 3.9.14 Tissue free fatty acids

0.2 ml of the lipid extract was taken in 6.0 ml of chloroform-heptane-methanol (200:150:7, v/v) and estimated for free fatty acids by the method of Hron and Menahan (1981) as described in section 3.9.5.
3.10 ASSAY OF MITOCHONDRIAL ENZYMES IN HEART

3.10.1 Isolation of mitochondria

Mitochondria was isolated by the method of Johnson and Lardy (1967).

Principle

The selected tissue was disrupted by homogenisation in ice-cold isotonic sucrose. Differential centrifugation was then employed to separate the mitochondria from cell debris, red blood cells, nuclei, microsomes and soluble components.

Reagents

0.25 M sucrose.

Procedure

Heart was homogenised in ice-cold sucrose (0.25M). This homogenate was centrifuged at 600xg for 10 minutes. The supernatant fraction was decanted and saved. The pellet containing cell debris and tissue fragments were discarded. The supernatant was then centrifuged in a refrigerated Beckman J2-21 centrifuge at 10,000xg for 5 minutes. This pellet was taken as mitochondria and the supernatant was discarded. The mitochondrial pellet was suspended in 0.25M sucrose containing 10mM Tris-HCl (pH 7.4) and 1mM EDTA to a known volume (2ml).
3.10.2 Protein estimation

Protein was estimated by the method of Lowry et al. (1951) and modified according to Spach et al. (1979).

Reagents

1. 5% solution in 0.01M NaOH.
2. Alkaline Copper reagent
3. Folin’s phenol-reagent

Procedure

The mitochondrial pellet was homogenised and appropriate dilution was carried out. The protein was solubilised in 1ml of 5% (w/v) sodium deoxycholate for 30 minutes. It was made up to a known volume and 4.5 ml of alkaline copper tartarate reagent was added and left for 1 hour. 0.5ml of Folin’s phenol reagent was added and mixed well. The blue colour developed was read after twenty minutes at 620nm in Shimadzu UV spectrophotometer.

The protein content of mitochondria was expressed as mg/gm tissue.
3.10.3 Isocitrate dehydrogenase (threo-DS-isocitrate NADP oxido reductase decarboxlating, EC 1.1.1.42)

The activity of Isocitrate dehydrogenase was assayed by the method of Bell and Baron (1960).

Reagents

1. Tris-HCl buffer: 0.1 M, pH 7.5
2. NaCl: 0.15 M
3. Trisodium citrate: 0.9 M in 0.15 M NaCl
4. Manganese Chloride: 0.015M
5. NADP+: 0.001 M solution in 0.15 M NaCl (fresh)
6. EDTA: 0.5%
7. Colouring reagent: 0.001M 2,4 dinitrophenyl hydrazine in 1.0 N hydrochloric acid.
8. Sodium hydroxide: 0.4 N
9. Standard: α-ketoglutaric acid in buffer

Procedure

The incubation mixture contained 0.4 ml of buffer, 0.2 ml of substrate, 0.2 ml of manganese chloride, 0.2 ml of NADP+ and required amount of enzyme. The NADP+ was replaced by 0.2 ml of saline in tubes labelled as 'control'. A suitable aliquot of enzyme preparation was added and mixed well. The tubes were then incubated at 37°C for 60 minutes. At the end of the
incubation period, 1 ml of the colouring reagent was added to arrest the reaction, followed by 0.5 ml of EDTA. The contents of the tubes were mixed well and allowed to stand for 20 minutes at room temperature and 10 ml of 0.4 N sodium hydroxide was added and the colour intensity was read at 420 nm after 10 minutes in a spectrophotometer. A calibration curve was established with α-ketoglutaric acid as standard.

The activity of the enzyme was expressed as nanomoles of α-ketoglutarate formed/hr/mg ptn.

3.10.4 α-Ketoglutarate dehydrogenase (2-oxoglutarate lipoate oxidoreductase, EC 1.2.4.2)

The activity of α-Ketoglutarate dehydrogenase was assayed by the method of Reed and Mukerjee (1969).

Reagents

1. Potassium phosphate buffer : 1M, pH 6.0
2. Potassium α-ketoglutarate : 0.5 M
3. Thiamine pyrophosphate: 0.002 M
4. Magnesium sulphate: 0.003 M
5. Potassium ferricyanide: 0.25 M
6. TCA: 10% solution
7. Duponol: 4% solution
8. Standard potassium ferrocyanide
9. Ferric ammonium sulphate-Duponol reagent: 1.7 gm of ferric ammonium sulphate was dissolved in 10 ml of distilled water. The suspension was filtered, and 20 ml of the solution containing 1.5 g of duponol in water was added to the filtrate. The above solution was mixed with 27 ml of 85% Ortho phosphoric acid and diluted with water to a final volume of 140 ml.

Procedure

The incubation mixture contained 0.15 ml of phosphate buffer, 0.1 ml of thiamine pyrophosphate, 0.1 ml of magnesium sulphate, 0.1 ml of potassium $\alpha$-keto glutarate and 0.1 ml of potassium ferricyanide and water to a final volume of 1.4 ml. A suitable aliquot of the enzyme preparation was added to tubes labelled 'test', while it was, replaced by water in tubes labelled 'control'. The mixture was then incubated at 30°C for 30 minutes. At the end of this period the reaction was terminated by the addition of 1 ml of 10% TCA.

The enzyme preparation was added to the control tubes after TCA was added. The sample was centrifuged and to 1 ml of supernatant 1 ml of 10% TCA, 1.5 ml of water, 1 ml of 4% duponol and 0.5 ml of ferric ammonium sulphate duponal reagent were added. It was then allowed to stand at room temperature for 30 minutes. The colour intensity was measured at 540 nm in a spectrophotometer. A calibration curve was prepared with potassium ferrocyanide as standard.
The activity of the enzyme was expressed as nanomoles of ferrocyanide formed/hour/mg protein.

3.10.5 Succinate dehydrogenase (Succinate: (acceptor) oxido reductase EC 1.3.99.1)

The activity of succinate dehydrogenase was assayed according to the method of Slater and Bonner (1952).

Principle

The rate of reduction of potassium ferricyanide was measured in the presence of sufficient potassium cyanide to inhibit cytochrome oxidase activity by following the rate of decrease of optical density at 455 nm.

Reagents

1. Phosphate buffer : 0.3 M, pH 7.6
2. EDTA : 0.03 M
3. Sodium cyanide : 0.03 M
4. Sodium succinate : 0.4 M
5. Bovine serum albumin : 3% solution
6. Potassium ferricyanide : 0.075 M
Procedure

The spectrophotometric cuvette contained the following: 1 ml of phosphate buffer, 0.1 ml of BSA, 0.1 ml of sodium cyanide, 0.3 ml of sodium succinate, 0.2 ml of potassium ferricyanide and 2 ml of water. The contents of the cuvette were mixed and brought to a temperature of 25°C. The reference cuvette contained water only. The control contained all reagents except sodium succinate. At zero time 0.2 ml of enzyme preparation was added to the test cuvette and the control cuvette and the extinction at 455 nm was followed as a function of time for a total period of 5 minutes in a Shimadzu UV spectrophotometer.

The activity of the enzyme was expressed as nanomoles of succinate oxidised/min/mg protein using the extinction factor of 0.485.

3.10.6 Malate dehydrogenase (L.Malate: NAD: Oxidoreductase, EC 1.1.1.37)

The activity of malate dehydrogenase was assayed by the method of Mehler et al. (1948).

Principle

The determination of the enzyme activity is based on the measurement of the rate of oxidation of NADH (decrease in optical density at 340 nm) in the presence of enzyme and excess of oxaloacetate.
Reagents

1. Potassium phosphate buffer : 0.25 M, pH 7.4
2. Oxaloacetate : 0.0076 M
3. NADH : 0.005 M

Procedure

The reaction mixture contained the following reagents and enzyme in a total volume of 3 ml. 75 micromoles of phosphate buffer, 0.15 micromoles of NADH and 0.76 micromoles of oxaloacetate and 0.2 ml of enzyme preparation. The reaction was carried out at 25°C and was started by the addition of enzyme preparation. The control tubes contained all reagents except NADH. The change in optical density at 340 nm was measured for 2 minutes at intervals of 15 seconds in a Shimadzu UV spectrophotometer.

The activity of the enzyme was expressed as nanomoles of NADH oxidised/minute/mg protein using the extinction coefficient of NADH as 6.22 x 10^6 cm² mole⁻¹.

3.10.7 Lipid peroxide estimation

Lipid peroxide content in heart mitochondria was determined by thiobarbituric acid reaction as described by Okhawa et al. (1979) as described earlier in section 3.8.1.
3.10.8 NADH Dehydrogenase: (NADH (acceptor) oxido reductase, EC 1.6.99.3)

The enzyme activity was assayed according to the method of Minakami et al. (1962).

Reagents

1. Phosphate buffer : 0.1M, pH 7.4
2. NADH : 0.1% solution
3. Potassium ferricyanide : 0.03 M

Procedure

The reaction mixture contained 110 micromoles of phosphate buffer, pH 7.4, and 0.45 micromoles of NADH and 10 micromoles of potassium ferricyanide in a total volume of 3 ml. The temperature was brought to 30°C and NADH was added just before the addition of the enzyme. A suitable aliquot of the enzyme was added at zero time and the change in optical density at 420 nm was measured as a function of time for 3 minutes at intervals of 15 seconds in a spectrophotometer.

The activity of the enzyme was expressed as nanomoles of NADH oxidised/min/mg protein using the factor of 0.485.
3.10.9 Cytochrome - C-oxidase - (Cytochrome - C: Oxygen Oxido reductase, EC, 1.9.3.1)

The activity of cytochrome - C - oxidase was assayed by the method of Pearl et al. (1963).

Principle

The enzyme activity was determined utilising the accumulation of free radical formed by the enzymatic univalent oxidation of a stable nontoxic substrate, N-phenyl-p-phenylene diamine.

Reagents

1. Phosphate buffer : 0.03 M, pH 7.4
2. Cytochrome C : 0.01 percent
3. N-Phenyl-p-phenylene diamine : 0.2 percent solution

Procedure

The reaction mixture contained 0.1 ml of phosphate buffer, pH 7.4, 0.04 ml of 0.2% N-phenyl-p-phenylene diamine, 0.02 ml of 0.01% cytochrome C and 0.02 ml of water. The sample was incubated at 25°C for 5 minutes. 0.2 ml of the enzyme preparation was added and the change in optical density was recorded at 550 nm for 5 minutes at intervals of 15 seconds each. A control containing all reagents except cytochrome C was also processed in the same manner.

The enzyme activity was expressed as nmoles/min/mg protein.
3.10.10 Assay of phospholipase (Phosphatide acyl hydrolase 
EC. 3.1.1.4)

Phospholipase activity was determined by the method of Gatt and 

Reagents

1. Ovolecithin
2. Glycyl glycine - NaOH buffer: 0.1M, pH 7.3
3. Sodium deoxy cholate : Weigh 50mg in a 20ml volumetric flask. 
   Dissolve in 5ml of water. Make upto volume just before use.
4. Dole's mixture: It was prepared by mixing isopropanol: heptane and 
   1N H₂SO₄ in the ratio of 40:10:1 (v/v/v).
5. Chloroform - methanol 2:1 (v/v)
6. Hexane
7. Cu - TEA reagent - 50 ml of 0.1M copper nitrate and 50ml of 
   triethanolamine (0.2M) were mixed with 33 gm of sodium chloride.
8. Diethyl dithiocarbamate: 0.1% diethyl dithiocarbamate in n-butanol.

Procedure

An aliquot of ovolecithin stock containing 15mg of the substrate was 
transferred to a glass stoppered tube. The chloroform was evaporated and the 
lipid was emulsified in 7.5ml of buffer. 6.0 ml of sodium deoxy cholate was 
added, mixed vigorously and immersed in a shaking water bath at 37°C. After
temperature equilibration, aliquots were withdrawn at appropriate time intervals (i.e. 0, 5, 10, 20 and 60 minutes) and added 5ml of Dole's mixture followed by the addition of 4ml of chloroform - methanol, 3ml of hexane and 2ml of water. The tubes were centrifuged and 2ml of aliquot was taken. 1ml of Cu-TEA reagent and 2ml of chloroform was added and recentrifuged. 2ml of aliquots was taken for analysis and added 0.5ml of diethyl dithiocarbamate. The colour intensity was measured at 430nm.

The enzyme activity was expressed as nanomoles of free fatty acid liberated/min/100 mg protein.

3.10.11 Oxidative Phosphorylation

The oxidation of sodium succinate was followed by an oxygen electrode according to the method of Katyare et al. (1971).

Reagents

1. Potassium phosphate buffer : 16 mM, pH 7.4
2. Sodium chloride : 38 mM
3. Potassium chloride : 40 mM
4. Potassium fluoride : 12 mM
5. Magnesium Chloride : 6 mM
6. Sodium Succinate : 10 mM
7. Rotenone : 1 μM
8. ADP : 0.2 μM
Procedure

The reaction medium used contained 16 mM phosphate buffer, 38 mM sodium chloride, 40 mM potassium chloride, 12 mM potassium fluoride, 6 mM magnesium chloride, 10 mM sodium succinate and about 4-6 mg of mitochondrial protein in a total volume of 2 ml. Rotenone was added and the rate of oxidation of succinate was studied for 3 minutes by the nanomoles of oxygen that is consumed. Small aliquots of ADP (20 µl) were added and the rate of oxidation was studied. ADP/O ratio and respiratory control ratio (RCR) were calculated as described by Estabrook.

3.10.12 Estimation of lactate

The plasma lactic acid content was estimated by the method of Barker and summerson (1941).

Reagents

1. Trichloroacetic acid : 10%
2. Calcium hydroxide powder
3. Copper sulphate solution : 4%
4. Copper sulphate solution : 20%
5. P-hydroxy diphenyl reagent in 0.5% sodium hydroxide : 1.5% p-hydroxy diphenyl solution
6. Concentrated sulphuric acid.
7. Standard lactate solution: 21.3 mg of lithium lactate was dissolved in 100 ml of distilled water containing 6.1 ml concentrated sulphuric acid.

Procedure

To 2.0 ml of deproteinized supernatant obtained after centrifugation, 1.0 ml of 20% copper sulphate was added and diluted to 10 ml with water. Aliquots of standard lactate and blank were also treated in a similar manner.

To each tube 1.0 g of powdered calcium hydroxide was added with repeated stirring for half an hour. After centrifugation, to 1.0 ml of aliquots was added 0.05 ml of copper sulphate and 6.0 ml of sulphuric acid and heated for 5 minutes in boiling water bath and cooled. Then 0.1 ml of p-hydroxy diphenyl reagent was added. The tubes were left at room temperature for 30 minutes. Finally the tubes were placed in a boiling water bath for exactly 90 seconds, removed and cooled in cold water. Then the colour developed was read at 540 nm in a Shimadzu UV spectrophotometer.

The values are expressed as mg/dl blood.

3.10.13 Mitochondrial calcium

Mitochondrial calcium was estimated as described in section 3.7.
3.10.14 Estimation of ATP

Mitochondrial ATP concentration was measured by using the hexokinase-glucose 6 phosphate dehydrogenase couple and following NADP+ reduction fluorimetrically by the method of Williamson and Corkey (1969).

Principle

Hexokinase catalyses the phosphorylation of glucose by ATP in the presence of Mg$^{2+}$ according to the equation (a).

\[ \text{Mg}^{2+} \quad \text{Glucose} + \text{ATP} \quad \longrightarrow \quad \text{glucose-6-phosphate} + \text{ADP} \quad \text{(a)} \]

Glucose-6-phosphate dehydrogenase catalyses the oxidation of glucose-6-phosphate by NADP according to the equation (b).

\[ \text{Glucose-6-phosphate} + \text{NADP} \quad \longrightarrow \quad 6\text{-phosphoglucuronate} + \text{NADPH} + \text{H}^+ \text{(b)} \]

The increase in fluorescence or optical density accompanying the conversion gives a quantitative measure of ATP.

Reagents

1. Triethanolamine buffer : 50 mM
2. Magnesium chloride : 10 mM
3. EDTA : 5 mM
4. Glucose : 1 M
5. NADP+ : 10 mg/ml
6. Hexokinase : 2 mg/ml
7. Glucose-6-phosphate dehydrogenase : 0.2 mg/ml

Procedure

The incubation mixture contained 2 ml of buffer pH 7.4, 10 μl of 10 mM magnesium chloride, 10 μl of 5 mM EDTA, 10μl of glucose, 10 μl of NADP⁺ 5 μl of glucose 6-phosphate dehydrogenase and mixed thoroughly and the fluorescence was recorded. Then, 5 μl of hexokinase, 10 μl of ATP was added and the increase in fluorescence at 340 nm was recorded.

The ATP content was expressed as nanomoles of ATP/mg protein.

3.11 ASSAY OF LYSOSOMAL ENZYMES IN SERUM, HEART AND LYSOSOMAL FRACTION OF MYOCARDIAL TISSUE

3.11.1 Lysosomal fraction was isolated by the method of Wattiaux et al. (1977)

Fresh heart tissue was homogenised in 0.25 M sucrose solution. The homogenate was filtered and centrifuged at 3000xg for 10 minutes in a refrigerated Beckman J2-21 centrifuge. The pellet was removed, rehomogenised and resuspended as before. The supernatants were combined and centrifuged again at 15,000xg for 20 minutes.

The lysosome pellet was suspended in 1.15% KCl homogenised and used for the estimation of enzymes.
3.11.2 Lysosomal enzymes

β-D-Glucuronidase (β-D glucuronide glucuronohydrolase EC 3.2.1.31)

β-D-glucuronidase activity was determined by the method of Hultberg et al.

Principle

β-D-glucuronidase hydrolyses p-nitrophenyl β-D-glucuronide to glucuronic acid and paranitrophenol. The latter is measured spectrophotometrically at alkaline pH.

Reagents

1. Sodium acetate buffer : 0.1 M, pH 4.5
2. p-nitrophenyl β-D-glucuronide : 0.01 M
3. Glycine - NaOH buffer : 0.4 M, pH 10.6
4. Standard : p-nitrophenol, 6 mM

Procedure

The assay mixture contained 0.5 ml of freshly prepared substrate in sodium phosphate buffer, 0.3 ml of enzyme and made upto 1 ml with assay buffer, incubated at 37°C for 1 hr. The reaction was arrested by the addition of 3 ml of glycine-NaOH buffer. The enzyme was added to the controls after the addition of glycine buffer. The absorbance was measured at 410 nm using a Schimadzu UV spectrophotometer.
The enzyme activity was expressed as \( \mu \text{M of p-nitrophenol liberated/hr/100 mg protein.} \)

### 3.11.3 Assay of \( \beta \)-D-N-acetylglucosaminidase (2-acetamido 2-deoxy-\( \beta \)-D-glucoside acetamido deoxy glucohydrolase EC 3.2.1.31)

\( \beta \)-D-N-acetyl glucosaminidase activity was assayed according to the method of Moore and Moris (1982).

#### Principle

The enzyme hydrolyses p-nitrophenyl \( \beta \)-D-N-acetyl glucosaminide to N-acetyl glucosamine and paranitrophenol and the latter is measured spectrophotometrically at 410 nm.

#### Reagents

1. Citrate-Phosphate buffer: pH 4.5 (60 mM sodium citrate and 0.1 M \( \text{Na}_2\text{HPO}_4 \) in \( \text{H}_2\text{O} \)).
2. Substrate: p-nitrophenyl \( \beta \)-D-N-acetyl glucosaminide 4 mM.
3. Glycine: \( \text{NaOH} \) buffer, 0.2 M, pH 11.7.
4. Standard: p-nitrophenol, 6 mM.

#### Procedure

The reaction mixture contained 0.5 ml of citrate-phosphate buffer, 0.5 ml of freshly prepared substrate and 0.12 ml of enzyme solution. The
mixture was incubated at 37°C for 1 hr. The reaction was stopped by the addition of 3 ml of glycine - NaOH buffer, mixed well and centrifuged. The supernatant was transferred into the spectrophotometric cuvette and the absorbance of p-nitrophenol was measured at 410 nm.

The enzyme activity was expressed as µmoles of p-nitrophenol liberated/hr/100 mg protein.

3.11.4 Acid phosphatase (Ortho-phosphoric mono ester hydrolase, EC 3.1.3.1)

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

Reagents

1. Acetate buffer: 0.1M, pH 4.8.
2. Disodium phenyl phosphate solution: 0.01 M.
3. Folin’s Coicalteau reagent (Folin’s phenol reagent): It was diluted 1:2 prior to use.
4. Sodium carbonate: 15% solution.
5. Standard Phenol : 100 mg of recrystallised phenol in 100 ml of water, 100 microgram of phenol/ml was prepared by proper dilution and used as working standard.
Procedure

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

The enzyme activity was expressed as μ moles of phenol liberated/min/100 mg ptn.

3.11.5 Cathepsin - D: (Acid Proteinase) (EC 3.4.23.50)

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

Principle

\[
\text{Haemoglobin} \xrightarrow{\text{Cathepsin D}} \text{TCA soluble products} \xrightarrow{\text{H}_2\text{O}}
\]
Reagents

1. Buffer : Sodium formate - 0.2 M, pH 3.5
2. Substrate : 1.5% Haemoglobin in sodium formate buffer, 0.2 M, pH 3.5
3. TCA : 10% solution
4. Sodium carbonate : 4.0% solution in 0.1 M NaOH
5. Standard Tyrosine : 100 mg/ml in dilute HCl.

Procedure

The incubation mixture in a final volume of 2.0 ml; contained 0.8 ml of buffer, 1.0 ml of substrate and 0.2 ml of enzyme homogenate. The tubes were incubated at 37°C for 2 hours. The enzyme reaction was arrested by the addition of 2.0 ml of 10% TCA. The control tubes received the enzyme after arresting the reaction.

After 30 minutes, the tubes were centrifuged at 1000 x g for 15 minutes. 2.5 ml of sodium carbonate in NaOH was added to the supernatant and mixed well. Then, 0.5 ml of Folin’s phenol reagent was added and the contents were immediately mixed. Standard containing aliquots of tyrosine and blank containing water were also treated in a similar manner. The blue colour developed was read at 670 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as μ moles of tyrosine released/hr/100 mg protein.