Materials and Methods
3. MATERIALS AND METHODS

3.1 Chemicals

Taurine, epinephrine, tetraethoxypropane, amino acid standards and isoproterenol were obtained from M/s. Sigma Chemical Company, St. Louis, MO, USA. All the other chemicals used were of analytical grade.

3.2 Animals

Wistar strain male albino rats, weighing 100-120 g were used. They were housed individually in polypropylene cages under standard environmental conditions and allowed free access to food and water. The experiment was carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee.

3.3 Induction of myocardial infarction

The myocardial infarction was induced in experimental rats by injecting isoproterenol (11mg/100g body weight/day), i.p. for 2 days (Anandan et al., 2003).

3.4 Experimental protocol

Seven days after acclimatization, the rats were divided into four groups of 6 rats each and housed individually in polypropylene cages. Group I rats served as the control and were intraperitoneally injected with physiological saline for 15 days. Group II animals were intraperitoneally (i.p.) injected with taurine (100mg kg⁻¹ body wt day⁻¹, dissolved in physiological saline) for 15 days. Group III rats injected with physiological saline for 15 days and administrated isoproterenol [11mg (dissolved in physiological saline) 100g⁻¹]
body wt day⁻¹, i.p.] for 2 days for the induction of myocardial infarction. Group IV animals were injected with taurine at the above dosage for 15 days and then injected with isoproterenol [11mg 100g⁻¹ body wt day⁻¹, i. p] for 2 days.

At the end of the experimental period, i.e., 24 h after last injection of isoproterenol, the experimental animals were sacrificed, and blood was collected with and without anticoagulant for the separation of plasma and serum respectively. The heart tissue was excised immediately and washed with chilled isotonic saline. One portion of tissue was fixed in 10% buffered formalin for histopathological observations. Some amount of tissue was used for amino acid and fatty acid compositional analysis. Accurately weighed heart tissue was homogenized in ice-cold 0.1 M Tris-HCl buffer, pH 7.2 and centrifuged. The plasma, serum and tissue homogenate were used for various biochemical analyses.

3.5 Histopathological studies

The slices of the heart were fixed in 10% neutral buffered formaldehyde. Fixing prevent autolysis and putrification of tissues. Then they were dipped in different concentration of alcohol in ascending order and finally in absolute alcohol (10 min each) for removing water. They were then kept in methyl benzoate until it sank and dipped in benzene for removing alcohol. The tissues were then infiltered with molten paraffin (60-70 °C) for 1 h and 15 min. A boat was made filled with molten paraffin and the tissues were placed in it. The paraffin was then cooled until it hardened, enclosing the tissue.

Using a rotary microtome, section of 4 to 5μ paraffin infiltered tissues were made. The tissues were de-paraffinised with xylene and treated with 100%, 90% and 70% alcohol (10 min each) for removing undesirable pigment and other materials. The sections were then stained with haematoxylin and counter stained with eosin and dehydrated with
70%, 90% and 100% alcohol for 10 min each. The sections were mounted using dibutylphthalate in xylene and examined under microscope.

3.6 Diagnostic markers

3.6.1 Assay of alanine aminotransferase (EC 2.6.1.2)

The activity of alanine aminotransferase (ALT) was determined by the method of Mohur & Cook (1957).

Reagents

1. Substrate: (0.1 M phosphate buffer, pH 7.4, 0.2 M DL-alanine, 2.0 mM 2-oxoglutarate): 1.5 g dipotassium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate, 30 mg 2-oxoglutaric acid and 1.78 g DL-alanine were dissolved in distilled water. The pH was adjusted to 7.4 with 1 N NaOH and made up to 100 ml.

2. 2, 4-dinitro phenyl hydrazine (DNPH) reagent: Dissolved 200 mg of DNPH in 85 ml of concentrated hydrochloric acid and made up to a liter with distilled water.

3. Sodium hydroxide: 0.4 N

4. Standard pyruvic acid: 11.01 mg of sodium pyruvate was dissolved in 100 ml of distilled water and this was prepared freshly for the calibration curve.

Procedure

To 1.0 ml of the buffered substrate, 0.1 ml sample was added and incubated at 37 °C for 30 min. The reaction was arrested by adding 1.0 ml of DNPH and left aside for 20 min at room temperature. Color developed by the addition of 10 ml of 0.4 N NaOH was read at 540nm in a Shimadzu UV-1601 spectrophotometer against the reagent blank.

The enzyme activity was expressed as μ mol pyruvate liberated /h/l (plasma).
3.6.2 Assay of aspartate aminotransferase (EC 2.6.1.1)

The activity of aspartate aminotransferase (AST) was assayed by the method of Mohur & Cook (1957).

Reagents

1. Phosphate buffer: 0.15 M, pH 7.5.

2. Substrate: 300 mg of L-aspartic acid and 50 mg of α-ketoglutaric acid were dissolved in 20-30 ml of the phosphate buffer and added 10% sodium hydroxide to bring the pH to 7.5 and was made up to 100 ml with phosphate buffer.

3. 2, 4-dinitro phenyl hydrazine (DNPH) reagent: Dissolved 200 mg of DNPH in 85 ml of concentrated hydrochloric acid and made up to a liter with distilled water.

4. Sodium hydroxide: 0.4 N

5. Standard pyruvic acid: 11.01 mg of sodium pyruvate was dissolved in 100 ml of distilled water and this was prepared freshly for the calibration curve.

Procedure

To 1.0 ml of the buffered substrate, 0.1 ml of the sample was added and incubated for one hour at 37 °C. Then 1.0 ml of DNPH reagent was added and left for 20 min. At the end of incubation, 10 ml of 0.4 N NaOH was added and the colour developed was read at 540nm in a Shimadzu UV-1601 spectrophotometer after 10 min. The standards were also treated similarly.

The enzyme activity was expressed as µ mol pyruvate liberated /h/l (plasma).
3.6.3 Assay of lactate dehydrogenase (EC 1.1.1.27)

The lactate dehydrogenase (LDH) activity was assayed according to the method of King (1965) with slight modification. The amount of pyruvate formed in the forward reaction was measured colorimetrically.

Reagents

1. Substrate : 2.76 g of lithium lactate was dissolved in 125 ml of glycine buffer containing 75 ml of 0.1N sodium hydroxide to adjust the pH 10. This was prepared just prior to use.

2. 0.1 M Glycine buffer : 7.5 g of glycine and 5.85 g of sodium hydroxide were dissolved in one liter of distilled water.

3. Sodium hydroxide : 0.4N

4. NAD$^+$ : 5.0 mg was dissolved in 1.0 ml of distilled water. This was prepared just before use.

5. 2, 4-dinitro phenyl hydrazine (DNPH) reagent: Dissolved 200 mg of DNPH in 85 ml of concentrated hydrochloric acid and made up to a liter with distilled water.

6. Standard pyruvate solution : 11.01 mg of sodium pyruvate was dissolved in 100 ml of distilled water and this was prepared freshly for the calibration curve.

Procedure

To 1.0 ml of the buffered substrate, 0.1 ml of the sample was added and the tubes were incubated at 37 °C for 15 min. After adding 0.2 ml of NAD$^+$ solution, the incubation was continued for 30 min and then 1.0 ml of DNPH reagent was added. And the tubes
were incubated at 37 °C for 15 min. Then 7.0 ml of 0.4 N NaOH was added and the colour developed was read at 540 nm in a Shimadzu UV-1601 spectrophotometer against the reagent blank. Suitable aliquots of the standards were also treated in the same manner.

The enzyme activity was expressed as μ mol pyruvate liberated /h/l (plasma).

3.6.4 Assay of creatine phosphokinase (EC 2.7.3.2)

The assay of creatine phosphokinase (CPK) was done by the method of Okinaka et al. (1961) with slight modifications.

Reagents

1. Tris buffer : 100 mM, pH 7.4
2. Magnesium acetate solution : 25 mM
3. Creatine phosphate : 12 mM
4. Adenosine -5-diphosphate (ADP) : 4 mM
5. Cysteine : 150 mM
6. p-Chloromercuric benzoate (PCB) : 30 mM
7. Zinc sulphate solution : 50 g Zinc sulphate in 1 liter of distilled water
8. Barium hydroxide 150mM : 10 g Ba(OH)₂ was boiled in 100 ml distilled water for few min, cooled and filtered. Took 5.0 ml zinc sulphate solution in a conical flask and added 20 ml distilled water and titrated with barium hydroxide using phenolphthalein as indicator until a light rose colour end point. Adjusted the volume of barium hydroxide to 5 ml.
9. Alkali solution 60 g NaOH and 128g anhydrous sodium carbonate were dissolved in 1 liter of distilled water.
10. α-Naphthol solution : 160 mg of α-naphthol was dissolved in 10 ml of alkali solution. Freshly prepared before use. Filtered if not clear.

11. Diacetyl stock solution : 1.0 ml of diacetyl first was dissolved in small quantity of methanol and made the volume to 100 ml with distilled water. Kept in a brown bottle at 4 °C

12. Working solution of diacetyl : Diluted the stock solution into 1 to 20 with distilled water. Prepared freshly before use.

13. Standard creatine solution (6 mM) : 89.5 mg Creatine hydrate was dissolved in 100 ml distilled water. 1.0 ml of the solution was made up to 5.0 ml with distilled water.

Procedure

0.2 ml of the sample was added to the test tubes containing 0.2 ml manganese solution, 0.1 ml cysteine, and 0.25 ml creatine phosphate, mixed and incubated at 37 °C for 5 min. Added 0.25 ml ADP and incubated 37 °C for 30 min. After incubation 0.5 ml of PCB, 0.5 ml Ba(OH)₂, 0.5 ml zinc sulphate solution were added and mixed well after each addition, centrifuged at 4000 rpm for 5 min. 1.0 ml of the supernatant was taken and added 2.5 ml of α-naphthol, 0.5 ml of diluted diacetyl solution and 6.0 ml of distilled water. Incubated 37 °C for 1 hr, shaken intermittently in 15 min. Read the OD at 520nm using a Shimadzu-UV-1601 spectrophotometer. Standard and blank were also treated in the same way.

The enzyme activity was expressed as μ mol creatine liberated /h/l (plasma).
3.6.5 Assay of alkaline phosphatase (EC 3.1.3.1)

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

Reagents

1. Carbonate- bicarbonate buffer : 0.1M pH 10.0
2. Substrate : 0.01 M disodium phenyl phosphate solution.
3. Folin’s phenol reagent : This was diluted 1:2 with double distilled water before use.
4. Sodium carbonate : 15%
5. Magnesium chloride : 0.1 M
6. Standard phenol solution : A solution of distilled crystalline phenol in water, containing 50 µg/ ml was prepared.

Procedure

The incubation mixture contained the following components in a final volume of 3.0 ml - 1.5 ml of carbonate- bicarbonate buffer, 1.0 ml of substrate and 0.1 ml of magnesium chloride and requisite amount of the enzyme source (0.2 ml serum). The reaction mixture was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 1.0 ml of Folin’s phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without enzyme sources were also incubated and the enzyme source was added after the addition of Folin’s phenol reagent. The 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 min at 37 °C. The blue colour developed was read at 640 nm using a Shimadzu-UV-1601 spectrophotometer against a blank. The standards were also treated similarly.
The activity of the enzyme is expressed as μmol phenol liberated/h/l (plasma); μ mol phenol liberated/mg protein (tissue).

3.6.6 Assay of acid phosphatase (EC 3.1.3.2)

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

Reagents

1. Citrate buffer : 0.1 M pH 4.9
2. Substrate : 0.01 M Disodium phenyl phosphate solution.
3. Folin’s phenol reagent : This was diluted 1:2 with double distilled water before use.
4. Sodium carbonate : 15%
5. Standard phenol solution : A solution of distilled crystalline phenol in water, containing 50μg/ml was prepared.

Procedure

The incubation mixture contained the following components in a final volume of 3.0 ml. 1.5 ml of citrate buffer, 1.0 ml of substrate, 0.3 ml of distilled water and requisite amount of the enzyme source (0.2 ml serum). The reaction mixture was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 1.0 ml of Folin’s phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without enzyme sources were also incubated and the enzyme source was added after the addition of Folin’s phenol reagent. 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 min at 37 °C. The blue colour developed was read at 640 nm using a Shimadzu-UV-1601 spectrophotometer against a blank. The standards were also treated similarly.
The activity of the enzyme is expressed as μmoles of phenol liberated/h/l (plasma); μmol phenol liberated/mg protein (tissue).

3.6.7 Estimation of Troponin T

Troponin was estimated by Immunoassay. Electrochemiluminescence immunoassay “ECLIA” on Roche Elecsys 1010/2010 and Modular Analytics E170 (Elecsys module) immunoassay analyzers was used.

3.6.8 Estimation of Homocysteine

Homocysteine Microtiter Plate Assay package (Diazyme Laboratories) was used for the analysis. Homocysteine Microtiter Plate Assay is an EIA-like assay for the determination of tHcy in blood.

3.7 Protein, Amino acid and Glycoprotein Components.

3.7.1 Estimation of protein

The protein content in the sample was estimated by the method of Lowry et al. (1951)

Reagents

1. Alkaline copper reagent
   i. Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide solution.
   ii. Solution B: 0.5% Copper sulfate in water.
   iii. Solution C: 1% sodium potassium tartarate in water.

2. 50 ml of solution A was mixed with 0.5 ml of solution B and 1 ml of solution C just before use.

3. Folin’s phenol reagent: Diluted 1:2 with double distilled water before use.
4. Standard bovine serum albumin (BSA): dissolved 100 mg of BSA in 100 ml of distilled water in a standard flask. 10 ml of the stock was diluted to 100 ml to get a working standard of 100 μg/ml.

Procedure

Pipetted out 0.1 ml of sample and standard BSA in the range of 20-100 μg into test tubes and the total volume was made up to 1.0 ml with distilled water. The blank contained 1.0 ml of distilled water. Exactly 4.5 ml of alkaline copper reagent was added to all the tubes and left at room temperature for 10 min after which was added 0.5 ml of Folin's phenol reagent. The blue colour developed was read after 20 min at 640 nm against the reagent blank, in a Shimadzu-UV-1601 spectrophotometer.

The protein values are expressed as mg/dl (plasma); mg/g (tissue).

3.7.2 Electrophoretic separation of plasma proteins

Plasma proteins were separated by SDS-PAGE technique as described by Laemmli (1970).

It is based on the principle that, in the presence of 10% SDS and 2- mercaptoethanol, proteins dissociate into their sub units and bind large quantities of the detergent which mask the charge of the proteins and giving a constant charge to mass ratio. So that the proteins move according to their molecular weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are kept in the Tris-glycine electrode buffer. During electrophoresis, the leading ion is chloride while the trailing ion is glycine. In this experiment, 7.5% gel concentration is used for the effective separation.
Reagents

1. Tris-HCl : 0.5 M, pH 6.8
2. Tris HCl : 1.5 M, pH 8.8
3. SDS : 10%
4. Ammonium per sulphate (APS) : 10%
5. Acrylamide/Bis (2.67 % C premix) : 30%
6. Sample buffer :
   Distilled water : 3.8 ml
   Tris-HCl 0.5 M, pH 6.8 : 1 ml
   Glycerol : 0.8 ml
   10% SDS : 1.6 ml
   2-Mercapto ethanol : 0.4 ml
   1% Bromophenol blue : 0.4 ml
7. Electrode buffer :
   Tris base : 9 g
   Glycine : 43.2 g
   SDS : 3 g

These reagents are dissolved in 600 ml of distilled water.

Working solution: Dilute 100 ml from stock to 500 ml with distilled water.

1. Separating gel (7.5%)
   Distilled water : 4.05 ml
   Tris-HCl 1.5M : 2.5 ml
   10% SDS : 100 μl
Acrylamide : 3.3 ml
TEMED : 10 µl
APS 10% : 50 µl

2. Stacking gel (4%)

Distilled water : 6.1 ml
Tris-HCl 0.5M : 2.5 ml
10%SDS : 100 µl
Acrylamide : 1.33 ml
TEMED : 10 µl
APS 10% : 50 µl

Procedure

Taken 0.1 ml of the suitably diluted plasma into a microfuge tube and added 0.1 ml of the sample buffer, heated in a boiling water bath for 4 min, cooled and kept at 4 °C in refrigerator.

The separating gel was prepared without TEMED and APS. Evacuated for 15 min to remove air bubbles. Added TEMED and APS with intermittent shaking after each addition immediately transferred the solution to the electrophoretic apparatus (BIORAD-Mini-PROTEAN II cell). Added a little water on the top of the gel to level it and kept for 45 min. Prepared stacking gel in the same way. Kept the comb over the apparatus, tilted it to 45°, poured the gel slowly, and pressed the comb slowly and evenly. Kept for 45 min. and marked the wells. After removing the comb, the whole apparatus was transferred to the sandwich clamp assembly in to the inner cooling core. Rinsed the apparatus and wells with electrode buffer and filled the inner chamber of the apparatus completely and the
outer chamber to the optimum level. Injected 10 µl of the sample into the wells. The electrode lid was placed at proper position and connections were established. The power of 200V was supplied. Electrophoresis was carried out for 45 min approximately until the dye reaches the bottom. Subsequently, the gel was removed and is placed in a big petridish containing the stain, Coomassive blue. Kept for 30 min, and transferred the gel into 7% acetic acid for destaining. 7% acetic acid was changed intermittently till the gel got completely destained.

3.7.3 Extraction of glycoconjugates

100 mg of the tissue was weighed and the lipids were extracted using chloroform - methanol method by homogenization in a Potter- Elvehjem homogenizer with a Teflon pestle. The extraction was repeated thrice with fresh aliquots of the solvent mixture. The lipid extract was filtered through a fat free Whatmann No: 4 filter paper into a separating funnel. The defatted tissue in the filter paper was dried and used for the estimation of hexose and sialic acid.

3.7.3.1 Estimation of Hexose

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

Reagents

1. Sulphuric acid: Water mixture : (3:2,v/v)
2. 800 mg of orcinol dissolved in 50 ml of 1 N H₂SO₄
3. Orcinol-sulphuric acid mixture : 1.0 ml of reagent (2) was mixed with 7.5 ml of reagent (1). This mixture was prepared fresh at the time of assay.
4. **Standard hexose**: Equal quantities of galactose and mannose were dissolved in water to give a concentration of 100μg/ml.

**Procedure**

An aliquot of the delipidised sample was treated with 1.0 ml of 0.1 N NaOH. Blank contained 1.0 ml of 0.1 N NaOH. The tubes were cooled by placing in an ice-bath and 8.5 ml of orcinol-sulphuric acid mixture was added slowly and mixed well. The tubes were stoppered, incubated at 80 °C for 15 min in a water bath. Cooled and the color was allowed to develop in the dark for 25 min. The intensity was measured at 540 nm using a Shimadzu-UV-1601 spectrophotometer. Standard solutions containing 20 to 100 μg were treated similarly and hexose concentration was estimated.

The concentration of hexose was expressed as mg/g heart tissue.

### 3.7.3.2 Estimation of Sialic acid.

Sialic acid was estimated by the method of Warren (1979).

**Reagents**

1. **Sodium metaarsenite**: A 10% solution was prepared in 0.5 M sodium sulphate in 0.1 N H₂SO₄.

2. **Sodium metaperiodate**: 0.2 M solution in 9 M phosphoric acid.

3. **Thiobarbituric acid reagent**: 0.6% solution was prepared in 0.5 M sodium sulphate.

4. **Acidified butanol**: 5 ml of con HCl in 95 ml of n-butanol.
5. Standard sialic acid : 10 mg of N-acetyl neuraminic acid was dissolved in 100 ml of distilled water.

6. Working standard : 1 ml of the stock was diluted to 10 ml using distilled water.

**Procedure**

An aliquot of the delipidised sample was hydrolyzed with 0.1 N H₂SO₄ at 80 °C for 1 hour. 0.2 ml of the hydrolysate was mixed with 0.1 ml of meta periodate and the solution was kept at room temperature for 20 min. 1.0 ml of sodium meta arsenite was added and shaken well so that the yellow brown colour disappeared. 3.0 ml of thiobarbituric acid reagent was added and heated in a boiling water bath for 15 min. After cooling, 4.0 ml of acidified butanol was added, shaken well and the colour was extracted into butanol phase. The butanol phase was transferred to another set of tubes and the colour intensity was measured at 530 nm using a Shimadzu-UV-1601 spectrophotometer. The blank containing 0.2 ml of 0.1 N H₂SO₄ and standard sialic acid solutions were treated similarly.

Sialic acid is expressed as mg/g tissue.

**3.7.4 Free amino acids**

Total amino acids and free amino acids in the serum and heart tissue were determined as per the procedure of Ishida et al. (1981).

**Reagents**

1. 10% : TCA
2. HCl : 6 N
3. HCl : 0.05 M

4. Buffer A : Dissolved tri sodium citrate (58.8 g) in 21 of double distilled water, add 210 ml ethanol of 99.5%, and adjust the pH to 3.2 by adding 60% perchloric acid and make up to 3 l using double distilled water.

5. Buffer B : Dissolved tri sodium citrate, 58.8 g and boric acid, 12.4 g in double distilled water, adjust the pH to 10 by adding 4 N NaOH, and make up the volume to 11 using double distilled water.

6. Phthalaldehyde (OPA) Buffer : Dissolved 122.1 g of Na$_2$CO$_3$, 40.7 g of H$_3$BO$_3$ and 56.4 g of K$_2$SO$_4$ in double distilled water and make up the volume to 3L.

7. Phthalaldehyde solution (OPA) : Dissolved 400 mg OPA, 7 ml ethanol, 1 ml of 2-Mercaptoethanol and 2 ml of 30% w/v Brij-35 in 500 ml OPA buffer.

8. Sodium hypochlorite solution : 4% w/v Sodium hypochlorite in OPA buffer. ie., 0.3 ml Sodium hypochlorite in 100 ml OPA buffer.

**Procedure**

**Preparation of Trichloroacetic acid extract:**

Weighed the sample accurately (100mg) and extracted with 10% trichloroacetic acid by grinding in mortar. Filtered the content quantitatively through Whatman filter paper No: 1 and made up the filtrate to 1 ml. Used the TCA extract to measure free amino acids. Pipetted out about 1.0 ml TCA extract accurately and bring the pH to below 3.0 with NaOH and made up to definite volume with buffer.

Filtered the sample thus prepared again through a membrane filter of 0.45 μm and injected 20 μl of this to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin i.e., styrene di vinyl
benzene co polymer with sulphonic group. The column used was Na type i.e., ISC- 07/S 1504 Na having a length of 19 cm and diameter 5mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase of the system consists of two buffers, Buffer A and buffer B. A gradient system can be followed for the effective separation of amino acids. The oven temperature can be maintained at 60 °C. The total run was programmed for 60 min. The amino acid analysis can be done with non-switching flow method and fluorescence detection after post-column derivatization with o-phthalaldehyde. In the case of proline and hydroxyl proline, imino group is converted to amino group with hypochlorite.

Run an amino acid standard (Sigma chemical Co., St. Louis, USA) also to calculate the concentration of amino acids in the sample. Calibration of equipment using standards needs to be done before the start of analysis. The amount of each free amino acid is expressed as percentage of total amino acids.

3.8 Lipids

3.8.1 Extraction of total lipids

The total lipid content of the tissues was estimated by the method of Folch et al. (1957).

Reagents

1. Chloroform-Methanol mixture (2:1 v/v)

Procedure

A weighed amount of the tissue was subjected to lipid extraction using chloroform-methanol mixture (2:1). The extraction was repeated twice with fresh aliquot of
chloroform-methanol mixture. The lipid extracts were transferred to a separating funnel and added 20% of water into it and left overnight. Next day the lipid extracts were drained through filter paper containing anhydrous sodium sulphate and was collected in round bottom flask and was evaporated to dryness in a flash evaporator. The lipid in the round bottom flask was made up to 10 ml by using chloroform. From this 1.0 ml was taken into a pre-weighed vial and allowed to dry in warm temperature to constant weight and total lipid content were calculated from the difference in weight. Sample made up to 10 ml was used for the estimation of various lipid components viz., cholesterol, triglycerides, free fatty acids and phospholipids after evaporating the solvent in air at room temperature.

3.8.1.1 Estimation of total cholesterol

The total cholesterol present in plasma and heart was estimated according to method of Parekh & Jung (1970) with slight modifications.

Reagents

1. FeCl₃ stock solution : 10 g FeCl₃ in 100 ml acetic acid.
2. FeCl₃ - H₂SO₄ reagent : 2.0 ml of FeCl₃ stock solution was diluted to 200 ml with conc. H₂SO₄.
3. 33% KOH (w / v) : 10 g of KOH was dissolved in 20 ml distilled water.
4. Alcoholic KOH solution : 6.0 ml of 33% KOH was made up to 100 ml with distilled ethanol. This solution is prepared fresh before use.
5. Standard cholesterol solution (stock) : 1mg /ml in chloroform.
6. Working standard: 1.0 ml of the stock was diluted to 10 ml with chloroform. Concentration 100 μg/ml.

Procedure

1.0 ml of the lipid sample was taken into a 25 ml glass stoppered tube and evaporated off the chloroform. Added 5ml of freshly prepared alcoholic KOH solution. The tubes were shaken well and incubated in a water bath at 37 °C for 55 min. After cooling to room temperature, added 10 ml of petroleum ether and inverted the tubes once to mix the contents. Then added 5.0 ml of distilled water and shaken the tubes vigorously for 1 min. Take 0.5-2 ml aliquots from the supernatant (petroleum ether) into test tubes. Evaporated the petroleum ether extract under nitrogen. To each of the sample as well as the standard tubes including the blank, added 3.0 ml of glacial acetic acid followed by 0.1ml distilled water. Mixed the tubes thoroughly and added 2 ml of the FeCl₃ - H₂SO₄ reagent to the sides of the test tubes. A brown ring was formed at the interface, tap the bottom of the tubes well to effect mixing and a light colour appeared which changed to an immense purple colour, which was measured in a Shimadzu-UV spectrophotometer-1601 at 560nm.

The amount of total cholesterol was expressed as mg/dl (plasma); mg/g (heart tissue).

3.8.1.2 Estimation of triglycerides

The level of triglycerides in plasma and heart were determined by the method of Rice (1970) with slight modifications.

Reagents

1. Activated silicic acid.

2. Saponification reagent: 5.0 g of potassium hydroxide was dissolved in 60 ml distilled water and 4.0 ml isopropanol.
3. Sodium metaperiodate reagent: To 77 g of anhydrous ammonium acetate in 700 ml distilled water, added 60 ml glacial acetic acid and 650 mg of sodium metaperiodate and was dissolved and diluted to 1 litre with distilled water.

4. Acetyl acetone reagent: To 0.75 ml of acetyl acetone, 20 ml of isopropanol was added and mixed well.

5. Stock solution: 200 mg of tripalmitin was dissolved in 25 ml chloroform.

6. Working standard: 1.0 ml of the stock solution was diluted to 10 ml with chloroform. Concentration 800 μg/ml.

Procedure

0.2 ml of the lipid sample was taken into a test tube and evaporated off the chloroform, added 4.0 ml isopropanol. It was mixed well and added 0.4 g of activated silicic acid, shaken in a vortex mixer for 15 min and centrifuged at 4000 rpm for 5 min. To 2.0 ml of the supernatant and standards ranging from 20-100 μg made up to 2.0 ml with isopropanol, 0.6 ml of saponifying reagent was added and incubated at 60-70 °C for 15 min. After cooling, 1.0 ml sodium metaperiodate solution was added and mixed. To this, 5 ml acetyl acetone was added, mixed and incubated at 50 °C for 30 min. After cooling, the colour developed was read at 405 nm in a Shimadzu-UV-1601 spectrophotometer.

The value of triglyceride was expressed as mg/dl (plasma); mg/g (heart tissue).
3.8.1.3 Estimation of free fatty acids

Free fatty acid in the sample was estimated by the modified method of Horn & Menahan (1981) with colour reagent of Itaya (1977).

Reagents

1. Activated silicic acid.
2. Chloroform, heptane, methanol (CHM) : It was prepared by mixing chloroform, heptane and methanol in the ratio of 200: 150: 7(v/v).
3. Copper-triethanolamine solution : 50 ml of 0.1 M copper nitrate and 50 ml of 2 M triethanolamine were mixed with 33 g of sodium chloride. The pH of the solution was adjusted exactly to 8.1.
4. Diethyldithiocarbamate (DDC) solution : 0.1% DDC in butanol was prepared freshly.
5. Standard stock : A solution containing 2 mg per ml of palmitic acid was prepared in CHM solvent.
6. Working standard : The stock was diluted to 1:10 in CHM solvent to give a concentration of 200 µg per ml.

Procedure

To 0.1 ml of the lipid sample, 6.0 ml of CHM solvent and 200 mg of silicic acid were added. The mixture was shaken well, centrifuged at 4000 rpm for 5 min and 3.0 ml of the supernatant was taken. Standard solution in the range of 25-100 µg were taken and made up to 3.0 ml with CHM solvent. The blank contained 3.0 ml of CHM solvent. To all these tubes, 2.0 ml of copper triethanolamine solution was added and then mixed on a mechanical shaker for 10 min. The tubes were centrifuged at 4000 rpm for 5 min. 2.0 ml
of the supernatant was taken, 1.0 ml of DDC solution was added and shaken well. The
colour intensity was read immediately at 430 nm in a Shimadzu-UV-1601
spectrophotometer.

Values were expressed as mg/dl (plasma); mg/g (tissue).

3.8.1.4 Estimation of phospholipids

Phospholipid content of the sample was estimated by the method of Fiske &
Subbarow (1925) as inorganic phosphorus liberated after Bartlette’s perchloric acid
digestion (1959).

Reagents

1. Ammonium molybdate reagent : 2.5 g of ammonium molybdate was
dissolved in 100 ml of water.

2. Aminonaptho sulfonic acid (ANSA) : 0.5 g of 1,2,4 aminonapthosulfonic acid
was dissolved in 195 ml of 15% sodium metabisulfite and 50 ml of 20% sodium
sulfite was added for complete solubilisation. The solution was filtered and stored in a
brown bottle.

3. Perchloric acid

4. Stock standard solution 35.1 mg of potassium dihydrogen
phosphate was accurately weighed, dissolved and made upto 100 ml with double
distilled water to give a final concentration of 80 µg phosphorus per ml.

5. Working standard : 1 ml of the stock was diluted to 10 ml to
give a conc. of 8 µg phosphorus per ml.
Procedure

0.1 ml of the lipid sample was taken into a test tube and evaporated off chloroform. Added 0.5 ml of perchloric acid, and kept for digestion in a sand bath till the colour of the solution becomes clear. The solution was made up to 3.0 ml with double distilled water, and 1.0 ml of aliquot was taken. The tubes were made up to 4.0 ml with double distilled water. To all the tubes, 0.5 ml of ammonium molybdate reagent was added. After 10 min, added 0.5 ml of ANSA to all tubes. Aliquots of the standards and blank were carried through the same procedure. The blue colour developed was read after 20 min, at 620nm in a Shimadzu-UV-1601 spectrophotometer.

The phospholipid content of plasma was expressed as mg /dl (serum); mg/gm (tissue).

3.8.2 Lipoprotein fractionation

Addition of heparin-manganous chloride to plasma caused the precipitation of VLDL and LDL. The supernatant represented the HDL fraction. To another aliquot of plasma, addition of sodium dodecyl sulphate resulted in aggregation of VLDL. The cholesterol content of each fraction was carried out in the following manner.

Total plasma cholesterol – (HDL+LDL) cholesterol = VLDL cholesterol

3.8.2.1 Estimation of high density lipoprotein fraction

Total HDL was separated by the method of Burstein & Scholnick (1972).

Reagents

Heparin-Manganous chloride reagent: 3.167 gm of manganous chloride was added to 1.0 ml of heparin containing 20,000 units/ml. This was made up to 8.0 ml with water.
**Procedure**

0.1 ml of plasma was added to 9 μl of heparin-manganous chloride reagent and mixed well. The solution was allowed to stand at 4 °C for 30 min. The supernatant represented HDL fraction. Aliquots were taken from HDL fraction for the estimation of cholesterol.

**3.8.2.2 Estimation of low density lipoproteins**

This differential analysis was made by the method of Brustein & Scholink (1972) using sodium dodecyl sulphate.

**Reagent**

Sodium dodecyl sulphate : 10% in 0.15 M sodium chloride pH 9.0

**Procedure**

To 0.1 ml of plasma, 75 μl of sodium dodecyl sulphate solution was added, which was taken in a poly carbonate centrifuge tube. The contents were swirled briefly and packed for 2 h in a water bath at 35 °C. The contents were centrifuged in a refrigerated centrifuge at 10,000g for 30 min. VLDL got aggregated as a pellicle at the top. The supernatant was a mixture containing HDL and LDL cholesterol was estimated in 0.05 ml aliquot of the supernatant as described above.

**3.8.2.3 Estimation of Lipoprotein (a)**

Lipoprotein (a) was determined by using immunoturbidimetric test kit from DiaSys Diagnostic Systems GmbH, Germany and Photometric measurement of antigen antibody reaction.
Reagents

1. Tris buffer : 80 mM, pH 7.5
2. Antiserum N-Morpholine ethanesulfonic acid : 4 mM

Procedure

7.5 µl of sample and 7.5 µl of distilled water were taken and added 250 µl of Tris buffer. The mixture was incubated at 37 °C for 3-5 min. The absorbance of the mixture was measured at 340 nm (A1). To the above mixture 50 µl of antiserum was added and incubated for 5 min at 37 °C and read at 340 nm (A2). Standards were also treated in the same manner and the amount of Lp (a) present in the sample was calculated by comparing the sample and standard.

$$\Delta A = [(A2-A1) \text{Sample}] - [(A2-A1) \text{Blank}]$$

The values were expressed as mg/dl.

3.8.2.4 Estimation of apolipoprotein AI

Apolipoprotein AI was determined by using immunoturbidimetric test kit from DiaSys Diagnostic Systems GmbH, Germany and Photometric measurement of antigen antibody reaction.

Reagents

1. Tris buffer : 100 mM pH 7.5
2. Antiserum : Antihuman apolipoprotein AI antibody

Procedure

The estimation of apolipoprotein AI was carried out in a sophisticated semi auto analyzer by the addition of the following reaction mixtures. 250 µl of Tris buffer was
taken in a test tube and add 50 µl of antilipoprotein antibody. The reaction was started by
the addition of 3 µl of sample. The mixture was read at 560 nm in a Shimadzu-UV-1601.
Standards also treated in same manner and used for the calibration setup.

The values are expressed as mg/dl

3.8.2.5 Estimation of apolipoprotein B

Apolipoprotein B was determined by using immunoturbidimetric test kit from DiaSys
Diagnostic Systems GmbH, Germany and Photometric measurement of antigen antibody
reaction.

Reagents

1. Tris buffer : 100 mM pH 7.5
2. Antiserum : Anti human apolipoprotein B antibody

Procedure

The estimation of apolipoprotein B was carried out in a sophisticated semi auto
analyser by the addition of the following reaction mixtures. 250 µl of Tris buffer was
taken in a test tube and add 50 µl of antilipoprotein antibody. The reaction was started by
the addition of 3 µl of sample. The mixture was read at 560 nm in a Shimadzu-UV-1601.
Standards also treated in same manner and used for the calibration setup.

The values are expressed as mg/dl

3.8.3 Analysis of fatty acid composition (FAME)

Fatty acids were analyzed according to the method of AOAC (1975). Lipid content of
the tissues was estimated by the method of Folch et al. (1957). Methyl esters of fatty
acids from animal and vegetable origin having 8-24 atoms are separated and detected by
gas chromatography. Method is not applicable to epoxy, oxidized or polymerized fatty acids (Metcalfe et al., 1966).

Reagents

1. Boron trifluoride reagent
2. Methanolic sodium hydroxide solution
3. Petroleum ether
4. Sodium sulphate

Procedure

Added sample (lipid of known weight) to a flask followed by 6 ml methanolic NaOH and boiling chip. Attach condenser, and reflux until fat globules disappear (usually 5-10 min). Add 6-7 ml BF₃ solution from bulb or automatic pipette through condenser and continue boiling for 2 min. Remove heat, then condenser, and add 15 ml saturated NaCl solution. Stopper flask and shake vigorously 15 s while solution is still tepid. Transfer aqueous phase to 250 ml separator. Extract with two 30 ml portions of petroleum ether (b.p 60-80 °C). Wash combined extracts with 20 ml portions H₂O, dry over anhydrous Na₂SO₄, filter and evaporate solvent under stream of nitrogen on steam bath.

Methyl esters of the fatty acid thus obtained were separated by gas liquid chromatography (Varian CP 3800, U.S.A) equipped with a capillary column (Elite 225, 30m long and 0.25mm diameter) and a flame ionization detector in the presence of hydrogen and air. The carrier gas was nitrogen and the flow rate was 0.5ml/min the chromatograph temperature started at 150 °C and was increased 4 °C/min until a temperature of 250 °C was obtained. Fatty acids separated were identified by the comparison of retention times with those obtained by the separation of a mixture of
standard fatty acids. Measurement of peak areas and data processing were carried out by Star WS software package. Individual fatty acids were expressed as weight percentage of total fatty acids.

3.9 Lipid peroxidation and tissue antioxidant status

3.9.1 Estimation of lipid peroxides (LPO) in plasma

Serum lipid peroxide content was estimated by the method of Yagi (1976).

Reagents

1. Sulphuric acid : 0.085 N
2. Phosphotungstic acid : 10%
3. TBA reagent : Mixture of equal volumes of 0.67 % TBA aqueous solution and glacial acetic acid.
4. n-Butanol

Procedure

0.1 ml of serum was mixed with 4.0 ml of 0.085 N sulphuric acid and shake gently. To this 0.5 ml of phosphotungstic acid was added and stirred well. The contents were centrifuged for 10 min. The supernatant was discarded and the pellet was suspended in 2 ml 0.085 N sulphuric acid and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged for 10 min. The pellet obtained was suspended in 4.0 ml of distilled water and 1.0 ml of TBA reagent. The tubes were kept in a boiling water bath for one hour. After cooling 5.0 ml of n-butanol was added to each tube and the color obtained in butanol phase was read at 532 nm in a Schimadzu-UV-1601 spectrophotometer.

The serum lipid peroxide content was expressed as nmoles of malondialdehyde/ml.
3.9.2 Estimation of lipid peroxides in tissue (LPO)

Lipid peroxides content was determined by thiobarbituric acid reaction as described by Ohkawa et al. (1979).

Reagents

1. Acetic acid 20% : 20 ml of glacial acetic acid dissolved in 100 ml distilled water.
2. Thiobarbituric acid : 0.8% in 20% acetic acid.
3. Sodium dodecyl sulphate : 8.1%
4. Standard : 41.66 mg of Tetraethoxy propane (TEP) dissolved in 100 ml distilled water. 1.0 ml of above was made up to 100 ml with distilled water.

Procedure

To 0.2 ml of sample, 1.5 ml of 20% acetic acid, 0.2 ml of SDS and 1.5 ml of TBA were added. The mixture was made up to 4.0 ml with distilled water and heated in a boiling water bath for one hr. After cooling the mixture was centrifuged at 3000 g for 10 min. Supernatant was taken and absorbance was read at 532 nm in a Shimadzu-UV-1601 spectrophotometer.

The lipid peroxides content was expressed as n mol malondialdehyde/mg protein.

3.9.3 Determination of total reduced glutathione (GSH)

The total reduced glutathione was determined by the method of Ellman (1959). The method is based on the reaction of reduced glutathione with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) to give a compound that has absorbance at 412 nm.
Reagents

1. DTNB : 0.6 mM in 0.2 M Phosphate buffer pH 8.0
2. Phosphate buffer : 0.2 M, pH 8.0.
3. Trichloroacetic acid : 5%
4. Standard 61.4 mg of reduced glutathione was dissolved in 100 ml 0.02 M EDTA. 0.1 ml of this is made up to 10 ml with 0.02 M EDTA.
5. Working standard : 2.0 ml of the above was made up to 10 ml.

Procedure

0.5 ml of heart homogenates was precipitated with 0.1 ml of 5% TCA and made up the volume to 1.0 ml using distilled water. The contents were mixed well for complete precipitation of proteins and centrifuged at 4000 rpm for 15 min. To an aliquot of clear supernatant, 0.2 M phosphate buffer was added to make a final volume to 2.5 ml, added 50 μl of DTNB reagent just before reading. The absorbance was read at 412 nm against a blank containing TCA instead of sample, series of standards treated in a similar way were also run to determine the reduced glutathione content.

The amount of glutathione was expressed as nmol/g wet tissue.

3.9.4 Estimation of glutathione peroxidase (EC 1.11.1.9)

Glutathione peroxidase activity was determined by the non-enzymatic method of Paglia & Valentine et al. (1967).

Reagents

1. Phosphate buffer : 0.4 M pH 7.0
2. EDTA : 0.4 mM
3. GSH : 2 mM

4. NaN₃ : 10 mM

5. TCA : 10%

6. DTNB : 0.6 mM in 0.4 M phosphate buffer

7. H₂O₂ : 1 mM was prepared freshly from commercial 30% solution.

8. GSH standard : 61.4 mg GSH was dissolved in 100 ml distilled water. 1.0 ml of this solution was made up to 10 ml with distilled water.

9. Working standard : 2 ml of the stock was made up to 10 ml with distilled water.

**Procedure**

0.2 ml of tissue homogenate was added to a mixture containing 0.2 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide, mixed well and added 0.2 ml reduced glutathione and 0.1 ml of hydrogen peroxide. Incubated in a water bath at 37 °C for 10 min. At the end of incubation period, 0.5 ml of 10% TCA was added and centrifuged at 10000 rpm for 5 min. 1.0 ml of the supernatant was taken into a separate test tube and added 0.1 ml DTNB. Immediately read the OD at 412 nm using a Shimadzu-UV-1601 spectrophotometer.

The enzyme activity was expressed as n mol glutathione oxidized/min/mg protein.

**9.5 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 tissue homogenate was made up to 2.5 ml with water. The reaction mixture was pre-incubated at 37 °C for 5 min. 0.1 ml of GSH was added and the change in the absorbance was measured at 340 nm in a Shimadzu-UV-1601 spectrophotometer, using UV PC software package for enzyme kinetics.

The enzyme activity was expressed as μ mol CDNB conjugate formed/min/mg protein.

9.6 Assay of catalase (EC 1.11.1.6)

Catalase was assayed according to the method of Takahara et al. (1960).

Reagents

1. Phosphate buffer : 50 mM, pH 7.0
2. Hydrogen peroxide : 30 mM solution in the above buffer.

Procedure

To 2.4 ml of the phosphate buffer, 0.1 ml of the enzyme source was added and the reaction was started by the addition of 1.0 ml of H₂O₂ solution. The decrease in absorbance was measured at 240 nm using a Shimadzu-UV-1601 spectrophotometer, UV
PC Software package for enzyme kinetics. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as n moles of H$_2$O$_2$ decomposed per minute per mg protein.

3.9.7 Assay of superoxide dismutase (EC 1.15.1.1)

The superoxide dismutase was assayed according to the method of Misra & Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme.

Reagents

1. Carbonate-bicarbonate buffer : 0.1 M pH 10.0.
2. Epinephrine : 3 mM

Procedure

Taken 100 µl of sample into the cuvette and add 1.4 ml buffer and 0.5 ml epinephrine mixed well and immediately read the change in optical density at 480 nm using Shimadzu-UV-1601 spectrophotometer, UV PC Software package for enzyme kinetics.

One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

3.10 Determination of the total, protein and non-protein sulphhydryl content

Total protein and non-protein sulphhydryl contents were estimated according to the method of Sedlak & Lindsay (1968). This method is based on the development of a yellow colour when DTNB is added to compounds contained sulphhydryl groups to form 2-nitro 5- mercaptobenzoic acid.
Procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank(ml)</th>
<th>System(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total sulfhydryl content (TSH)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.2M; pH 8.2) with 2mM EDTA</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>DTNB (99 mg/25 ml methanol)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Vortexed and centrifuged at 3000 g for 10 min. Read at 412 nm. Standards (100 μg GSH/ml) were also treated in a similar way.

| **Non-protein sulfhydryl content (NPSH)** | | |
| Homogenate | - | 1.0 |
| Distilled water | 4.0 | 3.5 |
| TCA (50%) | 0.5 | 0.5 |

Centrifuged at 3000 g for 10 min. Both supernatant and pellet were saved.

| Supernatant | - | 1.0 |
| Tris-HCl buffer (0.4M; pH 8.0) with 2mM EDTA | 2.0 | 2.0 |
| DTNB (99 mg/25 ml methanol) | 50μl | 50μl |

Read at 412 nm. Standards (100 μg GSH/ml) were also treated in a similar way.

| **Protein sulfhydryl content (PSH)** | | |
| The above pellet | As such | As such |
| Tris-HCl buffer (0.2M; pH 8.2) with 2mM EDTA | 1.5 | 1.5 |
| DTNB (99 mg/25 ml methanol) | 0.1 | 0.1 |
| Methanol | 8.4 | 8.4 |

Vortexed and centrifuged at 3000 g for 10 min. Read at 412 nm. Standards (100 μg GSH/ml) were run in a similar pattern.
The total, protein and non-protein sulphydryl contents are expressed as n mol/g wet tissue.

3.11 Membrane-bound ATPases

3.11.1 Estimation of inorganic phosphorus

Inorganic Phosphorus was estimated by the method of Fiske & Subbarow (1925). The method is based on the formation of phosphomolybdic acid by the reaction between a phosphate and molybdic acid and its subsequent reduction to a dark blue phosphomolybdic acid, the intensity of which is proportional to the phosphate ion concentration.

Reagents

1. Ammonium molybdate reagent : 2.5 g of ammonium molybdate was dissolved in 100 ml of 3 N sulphuric acid.

2. Amino naphthol sulphonic acid (ANSA) : 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 solubilization. The solution was filtered and stored in a brown bottle.

3. Standard Phosphorus : 35.1 mg of potassium dihydrogen phosphate was accurately weighed, dissolved and made up to 100 ml with distilled water.

4. Working standard : 1.0 ml of the stock was diluted to 5.0 ml using distilled water.

Procedure

To suitable aliquots of the supernatant, 1.0 ml of ammonium molybdate reagent was added. 0.4 ml of ANSA was added after 10 min incubation at room temperature.
Standards and blank were also treated in the above manner. The blue colour developed was read after 20 min at 640 nm in a Shimadzu-UV-1601 Spectrophotometer.

The values were expressed as μg /mg protein.

3.11.2 Assay of Na⁺/K⁺-dependent ATPase (EC 3.6.3.9)

Na⁺/K⁺-dependent ATPase activity was measured from the amount of Pi released according to the method of Bonting (1970).

Reagents

1. Tris buffer : 184 mM, pH 7
2. Magnesium Sulphate : 50 mM
3. Potassium Chloride : 50 mM
4. Sodium Chloride : 600 mM
5. EDTA : 1.0 mM
6. ATP : 40 mM
7. TCA : 10%

Procedure

One ml of Tris-buffer and 0.2 ml each of the above assay reagents were mixed together. So the assay medium, in the final volume of 2.0 ml contained, 92 mM Tris-buffer, 5 mM magnesium sulphate, 60 mM sodium chloride, 5 mM potassium chloride, 0.1 mM EDTA and 4.0 mM ATP. After 10 min equilibration at 37 °C in an incubator, reaction was started by the addition of 0.2 ml of the enzyme solution. The assay medium was incubated for 30 min and at the end of the incubation period. The reaction was
stopped by the addition of 2 volumes of ice cold 10% TCA. The phosphorus (Pi) liberated was estimated by the method of Fiske & Subbarow (1925).

The enzyme activity was expressed as μ mol Pi liberated/min/mg protein

3.11.3 Assay of Mg\textsuperscript{2+}-dependent ATPase (EC 3.6.3.1)

Mg\textsuperscript{2+}-ATPase was assayed according to the method described by Ohinishi et al. (1982)

Reagents

1. Tris- HCl buffer : 0.375 M, pH- 7.6
2. Magnesium chloride : 0.205 M
3. ATP : 0.01 M

Procedure

Buffer 0.1ml, 0.1 ml of ATP, magnesium chloride 0.1ml and distilled water 0.1ml were taken in test tubes. 0.1ml of enzyme preparation was added. The tubes were then incubated at 37 °C for 15 min. The reaction was stopped by the addition of 1.0ml of 10% TCA. 0.1 ml of enzyme was added to the control tubes. The phosphorus (Pi) liberated was estimated by the method of Fiske & Subbarow.

The enzyme activity was expressed as μ mol Pi liberated/min/mg protein.

3.11.4 Assay of Ca\textsuperscript{2+}-dependent ATPase (EC 3.6.3.8)

Ca\textsuperscript{2+} dependent ATPase was assayed by the method of Hjerten & Pan (1983).
Reagents

1. Tris-HCl buffer : 0.125 M, pH 8.0
2. Calcium chloride : 0.05 M
3. ATP : 0.01 M
4. TCA : 10%

Procedure

Tris-HCl buffer 0.1 ml, calcium chloride 0.1 ml, ATP solution 0.1 ml and distilled water 0.1 ml were taken in test tubes. 0.1 ml of enzyme preparation was added and the tubes were incubated at 37 °C for 15 min. The reaction was arrested by the addition of 1.0 ml of 10% TCA to the incubation mixture. 0.1 ml enzyme source was added to the control tubes. The contents were centrifuged at 4000 rpm for 5 min. The supernatant was used for the estimation of inorganic phosphorous.

The enzyme activity was expressed as μ mol Pi liberated/min/ mg protein.

3.12 Estimation of minerals using Atomic Absorption Spectrophotometer

Minerals were estimated according to the method of the AOAC (1980).

Reagents

1. Nitric acid
2. Perchloric acid
3. 1& 2 in 9:4
4. Stock solution of sodium, potassium and calcium were prepared by diluting concentrated solution of 1000 mg/l (Merck).
Procedure

Samples size of 1g of heart tissue and 1 ml serum were used for the experiment. To the sample containing flask, 7ml of nitric acid and perchloric acid (9:4) mixture was added, covered with a watch glass and left at room temperature over night. The sample was then digested using a microwave digester (Milestone ETHOS PLUS lab station Closed Vessel Microwave Digestion System). The completely digested samples were allowed to cool at room temperature, filtered (glass wool) carefully transferred into a clean 50 ml volumetric standard flask and then diluted to the mark with ultra pure water (Milli Q, Millipore). The digested samples were analyzed using Varian Spectra-220 AA, Atomic Absorption Spectrophotometer equipped with a deuterium back ground corrector for the determination of minerals viz sodium, potassium and calcium.

3.13 Estimation of ATP content

ATP content was estimated by the method of Ryder (1985) using Shimadzu LC 10 AT vp, HPLC System.

Reagents

1. Perchloric acid : 0.6 M
2. KOH : 2 M
3. KOH : 1 M
4. Mobile phase : Phosphate buffer solution prepared by mixing 0.04 M KH$_2$PO$_4$ (5.4436 g/l) and 0.06 M K$_2$HPO$_4$ (10.4508 g/l) in 1:1 proportion and is filtered through 0.45 µm filter paper.

Procedure

A 0.5 g portion of the heart tissue was homogenised with 2.5 ml of chilled 0.6 M perchloric acid and centrifuged at 10,000 rpm at 4 °C for 20 min. The supernatant was
neutralised to pH 6.8 using 1 M and 2 M KOH (visualized by formation of KCl precipitate). Measured the total volume. After standing at 0 °C for 30 min, it was filtered through a syringe filter of pore size 0.45μm and was analysed by Shimadzu-LC AT vp, HPLC, using Photo Diode Array detector.

50 μl of the sample was injected into the HPLC. The method utilized a simple reverse phase (C<sub>18</sub>RP), Hypersil C18, Column (4.6mm I.Dx250mm). Phosphate buffer (0.04 M KH<sub>2</sub>PO<sub>4</sub> and 0.6 M K<sub>2</sub>HPO<sub>4</sub> in 1:1 proportion) was used as mobile phase at a flow rate of 1.5 ml/min. The peaks obtained from the sample were identified by comparing with the peak of chromatogram of the mixed standard solutions. The quantification of each nucleotide breakdown products was done by comparing the peak area of the samples with peak area of the standard corresponding to the sample.

The ATP content was expressed as n mol/g wet tissue.

3.1.4 Isolation of heart mitochondrial and lysosomal fractions

Mitochondrial and lysosomal fractions of the heart tissue were isolated by the method of Plummer (1998).

**Reagents**

<table>
<thead>
<tr>
<th>Tris HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM, pH 7.4 containing 0.25 M Sucrose and 1mM EDTA.</td>
</tr>
</tbody>
</table>

**Procedure**

Immediately after sacrifice, the heart was removed and all the blood vessels and connective tissues were trimmed off. Wash the tissue free of blood in ice-cold sucrose,
neutralised to pH 6.8 using 1 M and 2 M KOH (visualized by formation of KCl precipitate). Measured the total volume. After standing at 0 °C for 30 min, it was filtered through a syringe filter of pore size 0.45μm and was analysed by Shimadzu-LC AT vp, HPLC, using Photo Diode Array detector.

50 μl of the sample was injected in to the HPLC. The method utilized a simple reverse phase (C₁₈RP), Hypersil C₁₈, Column (4.6mm I.Dx250mm). Phosphate buffer (0.04 M KH₂PO₄ and 0.6 M K₂HPO₄ in 1:1proportion) was used as mobile phase at a flow rate of 1.5 ml/min. The peaks obtained from the sample were identified by comparing with the peak of chromatogram of the mixed standard solutions. The quantification of each nucleotide breakdown products was done by comparing the peak area of the samples with peak area of the standard corresponding to the sample.

The ATP content was expressed as n mol/g wet tissue.

14 Isolation of heart mitochondrial and lysosomal fractions

Mitochondrial and lysosomal fractions of the heart tissue were isolated by the method of Plummer (1998).

Reagents

Tris HCl : 50 mM, pH 7.4 containing 0.25 M Sucrose and 1mM EDTA.

Procedure

Immediately after sacrifice, the heart was removed and all the blood vessels and connective tissues were trimmed off. Wash the tissue free of blood in ice-cold sucrose,
lightly blot and place in a tared beaker to weigh. Cut the heart into small fragments and homogenize in buffer containing 0.25 M sucrose and 1 mM EDTA. Centrifuge the suspension in a refrigerated centrifuge.

The homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was transferred into test tubes. The pellet was dissolved in sucrose buffer. Centrifuged for 10 min at 2000 rpm. The supernatants were pooled and centrifuged for 10 min at 7400 rpm, the pellet collected represents mitochondrial fraction. The supernatant was again centrifuged for 10 min at 11400 rpm and the pellet was collected (lysosomal fraction). Each fraction should be resuspended in sucrose and the washings combined with the supernatants. This has the advantage of producing purer fractions. Carefully resuspend the mitochondrial pellet in about 2 ml of sucrose, and lysosomal pellet in 1 ml of sucrose and used as the enzyme source and store on ice until required.

3.14.1 Mitochondrial and respiratory marker enzymes

3.14.1.1 Assay of Isocitrate dehydrogenase (EC 1.1.1.42)

The enzyme activity was assayed according to the method of Bell & Baron (1960).

Reagents

1. Tris-HCl buffer : 0.1 M, pH 7.5
2. Substrate : 0.1 M trisodium DL-Isocitrate in 0.9% saline
3. Manganous chloride : 0.015 M in 0.9% saline
4. NADP : 0.001 M in 0.9% saline
5. EDTA : 5%
6. Sodium hydroxide : 0.4 N
7. 2,4 dinitrophenyl hydrazine (DNPH) : 0.001 M in 1N HCl
8. Standard Solution : 15 mg of α-ketoglutarate (potassium salt) in 50 ml of buffer.

Procedure

Buffer, 0.4 ml was taken in a test tube and 0.2 ml of substrate, 0.3 ml of manganous chloride and 0.2 ml of the mitochondrial suspension were added. A control tube was also prepared simultaneously, 0.2 ml of co-enzyme solution was added to the test tube and 0.2 ml of saline was added to the control tubes. After mixing well, both the tubes were incubated for 60 min, 1.0 ml of DNPH was added to both the tubes, followed by 0.5 ml of EDTA. The tubes were kept at room temperature for 20 min and 10 ml of 0.4 N NaOH was added to the tubes. A blank was run simultaneously. The colour intensity was measured at 390 nm in a Shimadzu- UV-1601 spectrometer. A standard curve was prepared using α-ketoglutarate.

The activity of isocitrate dehydrogenase is expressed as μ mol α-ketoglutarate liberated/ mg protein.

3.14.1.2 Assay of Succinate dehydrogenase (EC 1.3.99.1)

This enzyme activity was estimated accordingly to the method of Slater & Bonner (1952). The rate of reduction of potassium ferricyanide was measured in the presence of sufficient potassium cyanide to inhibit cytochrome oxidase by following the rate of decrease in the optical density at 420 nm.

Reagents

1. Phosphate buffer : 0.3 M pH 7.6
2. Sodium salt of ethylene diaminetetra acetate (EDTA) : 0.03 M solution.
3. Potassium cyanide : 0.03 M solution.
4. Sodium Succinate : 0.4 M solution.
5. Bovine serum albumin : 3% solution
6. Potassium ferricyanide : 0.075 M solution

Procedure

In a spectrophotometric cuvette, 1.0 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of bovine serum albumin, 0.3 ml of sodium succinate, 0.2 ml of potassium ferricyanide and 0.1 ml of potassium cyanide were added and the total volume was made up to 2.8 ml with double distilled water. The reaction was started by the addition of 0.2 ml of mitochondrial suspension. Changes in optical density at 420 nm were recorded in a Shimadzu- UV-1601 spectrophotometer using UV PC Software package for enzyme kinetics.

The activity of succinate dehydrogenase is expressed as per µ mol succinate oxidized/ mg protein.

14.1.3 Assay of Malate dehydrogenase (EC 1.1.37)

This enzyme activity was assayed by the method of Mehler et al. (1948). The activity determination is based on the measurement of the rate of oxidation of NADH in the presence of the enzyme and excess of oxaloacetate.

Reagents

1. Tris – HCl : 0.25 M, pH 7.4
2. NADH : 0.015 M
3. Oxaloacetate : 0.0076 M, pH 7.4
Procedure

To 0.3 ml of buffer 0.1 ml of NADH and 0.1 ml of oxaloacetate were added and the total volume was made to 2.9 ml with water. The reaction was started by adding 0.1 ml of mitochondrial suspension. The change in optical density was measured at 340 nm in a Shimadzu-UV-1601 spectrophotometer using UV PC Software package for enzyme kinetics.

The activity of malate dehydrogenase was expressed as μ mol NADH oxidized/mg of protein.

3.14.1.4 Assay of NADH dehydrogenase (EC 1.6.99.3)

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

Reagents

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

Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of potassium ferricyanide, 0.1 ml of NADH and 1.6 ml of distilled water in a total volume of 3.0 ml. The temperature was brought to 30 °C and NADH was added just before the addition of the sample. A suitable aliquot of mitochondrial solution was added and change in absorbance was measured at 420 nm in a Shimadzu-UV-1601 spectrophotometer, using
UV PC Software package for enzyme kinetics. A control containing all the reagents except NADH was also treated similarly.

The activity of NADH-dehydrogenase is expressed as per $\mu$ mol NADH oxidized/mg of protein.

### 3.14.1.5 Assay of $\alpha$-ketoglutarate dehydrogenase (EC 1.2.4.2)

This enzyme activity was estimated according to the method of Reed & Mukherjee (1969). It is based on the calorimetric determination of ferrocyanide produced by the decarboxylation of $\alpha$-ketoglutarate with ferricyanide as electron acceptor.

**Reagents**

1. Phosphate buffer : 0.1 M, pH 6.0
2. Thiamine pyrophosphate : 0.002 M
3. Magnesium sulphate : 0.003 M
4. $\alpha$-ketoglutarate(Potassium salt) : 0.05 M
5. Potassium ferricyanide : 0.25 M
6. TCA : 0.25 M
7. SDS (Dupanol) : 4%
8. Ferric ammonium sulphate – dupanol reagent : To 1.7 g of ferric ammonium sulphate was added 10 ml of water and filtered. To this filtrate a solution of 1.5 g of dupanol in 20 ml of water was added. Then 27 ml of 85% phosphoric acid was added and diluted to 140 ml with water.
9. Standard potassium ferrocyanide : 0.01%
Procedure

To 0.15 ml of phosphate buffer 0.1ml each of thiamine pyrophosphate, magnesium sulphate, and potassium ferrocyanide was added. The total volume made up to 1.2ml with water. Exactly 0.2 ml of the mitochondrial suspension was added to the test. A control was prepared simultaneously without the addition of mitochondria. The tubes were incubated at 30 °C for 30 min. After this period, 1.0 ml of 10%TCA was added to the control tubes. The aliquots of the supernatant after centrifugation were pipetted out into test tubes for the colour reaction. To these tubes, 0.1ml of potassium ferricyanide was added and the volume was made up to 2.4 ml with water. 1 ml of 4% dupanol and 0.5ml of ferric ammonium sulphate dupanol reagent were added and the tubes were incubated at 25 °C for 30 min. The colour intensity was measured at 540 nm in a shimadzu UV-1601 spectrophotometer. A standard potassium ferrocyanide solution in the range of 10 μg to 50 μg was run simultaneously.

The activity of α-ketoglutarate dehydrogenase is expressed as n mol potassium ferrocyanide liberated/ mg protein.

3.14.2 Mitochondrial lipid peroxidation and antioxidant status

3.14.2.1 Determination of lipid peroxides

The heart mitochondrial lipid peroxide content was determined by the thiobarbituric acid reaction as described by Ohkawa et al. (1979) [Section 3.10.2].

3.14.2.2 Assay of superoxide dismutase

The mitochondrial superoxide dismutase activity was assayed by the method of Misra Fridovich (1972). One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine autoxidation [Section 3.10.7].
3.14.2.3 Assay of catalase

The mitochondrial catalase activity was assayed by the method of Takahara, (1960). The enzyme activity was expressed as μ mol H₂O₂ consumed/min/mg of protein [Section 3.10.6].

3.14.2.4 Determination of mitochondrial GSH

GSH content in heart mitochondria was determined according to the method of Ellman (1959). The amount of glutathione is expressed as n mol/g tissue [Section 3.10.3].

3.14.2.5 Assay of glutathione peroxidase

The mitochondrial glutathione peroxidase activity was assayed by the method of Habig et al. (1974). The enzyme activity is expressed as nmol glutathione oxidized/min/mg protein. [Section 3.10.4]

3.14.2.6 Assay of glutathione-S-transferase

The mitochondrial glutathione-S-transferase activity was assayed by the method of Pagila & Valentine (1967). The enzyme activity is expressed as n mol CDNB conjugate formed/min/protein [Section 3.10.5].

3.14.3 Lysosomal marker enzymes

3.14.3.1 Assay of β- glucosidase (EC 3.2.1.21)

β- glucosidase was assayed according to the method of Conchie et al. (1967) based on the principle that β- glucosidase acts on P- nitrophenyl- β- D-glucopyranoside and liberate P-nitrophenol, which was measured at 410nm in alkaline pH.
Reagents

1. Substrate : 10 mM, p-nitro phenyl β-D-glucopyranoside in buffer
2. Phosphate buffer : 0.1 M Citrate-0.2 M phosphate, pH 4.5
3. Glycine-NaOH buffer : 0.4 M pH-10.4
4. Standard : 6 mM, p-nitrophenol
5. Working standard : 1 ml diluted to 10 ml using distilled water.

Procedure

To 0.5 ml of substrate and 0.3 ml of citrate buffer in a test tube, 0.2 ml of the enzyme solution was added, shaken gently and incubated at 37 °C for 1 h. 3 ml of glycine –NaOH buffer was added for reaction termination. Mixed and read at 410 nm using Shimadzu UV-1601 spectrophotometer.

The activity of β-glucosidase is expressed as μ mol p-nitro phenol liberated/mg protein.

3.1.4.3.2 Assay of β- galactosidase(EC 3.2.1.23)

β-galactosidase was assayed according to the method of Conchie et al. (1967) based on the principle that β-galactosidase acts on p-nitrophenyl- β-D-glucopyranoside and liberate P-nitrophenol, which was measured at 410 nm in alkaline pH.

Reagents

1. Substrate : 2 mM, p-nitro phenyl β-D-galactopyranoside in buffer
2. Phosphate buffer : 0.1 M Citrate-0.2 M phosphate, pH 4.5
3. Glycine-NaOH buffer : 0.4 M pH-10.4
4. Standard: 6 mM, p-nitrophenol
5. Working standard: 1 ml diluted to 10 ml using distilled water

**Procedure**

To 0.5 ml of substrate and 0.3 ml of citrate buffer in a test tube, 0.2 ml of the enzyme solution is added, shaken gently and incubated at 37 °C for 1 h. 3 ml of glycine–NaOH buffer is added for reaction termination. Mixed and read at 410 nm.

The activity of β-galactosidase is expressed as μmol p-nitrophenol liberated/mg protein.

### 3.14.3.3 Assay of acid phosphatase (EC 3.1.3.2)

Acid phosphatase was assayed by the method of King, (1965) using disodium phenyl phosphate as the substrate [Section 3.6.6].

**3.15 Statistical Analysis**

Results are expressed as mean ± SD. Multiple comparisons of the significant ANOVA were performed by Tukey’s multiple comparison test. A p-value <0.05 was considered as statistically significant. All data were analyzed with the aid of statistical package program SPSS 10.0 for Windows.