Materials and Methods
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2.1 CHEMICALS

The fine chemicals used during the course of the study were purchased from the following sources:

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<th>Chemicals Provided</th>
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<tr>
<td>Sigma Chemical Company, St. Louis, MO, USA.</td>
<td>Streptozotocin, BSA, GSH, DTNB, Epinephrine, TBA, Ethidium bromide, p-nitrophenyl β-D-glucuronide, p-nitrophenyl β-N-acetyl glucosaminide, Sodium succinate, Oxaloacetate, Cytochrome-C.</td>
</tr>
<tr>
<td>Sisco Research Laboratories, Mumbai, India.</td>
<td>CDNB, NAD⁺, NADP⁺, NADPH, ATP, NED, p-nitrophenyl-β-D-galactoside, Folin-Ciocalteau reagent.</td>
</tr>
<tr>
<td>British Drug House (BDH), Pvt. Ltd., Glaxo Division, Mumbai, India.</td>
<td>EDTA, ANSA, DNPH, TEMED.</td>
</tr>
<tr>
<td>Boehringer-Mannheim Germany-India.</td>
<td>ATP, Glucostrix.</td>
</tr>
<tr>
<td>Stat Diagnostics (Linco research Inc.) Mumbai, India.</td>
<td>Insulin, C-peptide -- RIA kit, ¹²⁵I-insulin.</td>
</tr>
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Other chemicals, acids, bases, solvents and salts used for the investigations were of analytical grade (AR) and obtained from Glaxo laboratories, SRL, Mumbai, India.
2.2 EXPERIMENTAL ANIMALS

Male albino rats of the Wistar strain weighing around 160–200 g were purchased from the Tamil Nadu Veterinary and Animal Sciences University, Chennai. They were acclimatized to the animal house conditions at least for one week before carrying out any experimental work. The rats were fed ad libitum with normal pellet (Hindustan Lever Ltd., Bangalore, India.) and water. The experiments were designed and conducted in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC No. 01/034/04) for the investigation of experimental pain in conscious animals.

2.3 PLANT MATERIAL

Outermost leaves from mature, healthy, five year old plants of Aloe vera having a length of approximately 2.5 to 3 feet were collected. The plant was identified in the herbarium of the Centre for Advanced Studies in Botany, University of Madras where a specimen was deposited under accession number (CAS 1070). The leaves were washed with fresh water and thick epidermis was selectively removed and the colorless parenchyma was homogenized in a blender and centrifuged at 10,000 g to remove the fibers. The resultant supernatant was immediately lyophilized and stored at 4°C until for further use (Yagi et al., 1998).
2.3.1 Analysis of Some Important Inorganic Trace Elements in *Aloe vera* Leaf Gel

The analysis of inorganic trace elements in the lyophilized sample was determined by dry-ashing method. Briefly, dry-ashing method was adopted by placing the properly dried and ground plant sample (100 g) in a vitreosil crucible overnight in an electric muffle furnace, maintaining temperature between 410 and 440°C. Because loss of zinc may occur > 450°C and for potassium, loss may occur if the temperature is too high (> 480°C). Also, ashing will destroy all the organic materials present in the sample (Kar et al., 1999). The yield of ash from the lyophilized powder was approximately 3.25 g/100 g.

Two grams of the ash were digested with a mixture of hydrochloric acid and nitric acid in the ratio of 1:3 (Rajurkar and Damame, 1998). The digested sample was dissolved in 50 ml of distilled water and was used for the assay of trace elements through Atomic Absorption Spectroscopy (AAS - Varion 200AA) using suitable hollow cathode lamps. Sodium and potassium levels were estimated flame photometrically.

2.3.2 Preliminary Phytochemical Screeening

Known amounts of the lyophilized powder were extracted with n-hexane, benzene, ethyl acetate, chloroform, and 95% ethanol. All the solvents used were of analytical grade. The solvents were evaporated in a rotary evaporator at 40-50°C under reduced pressure.
Known amounts of individual solvent-free extracts were suspended in water to obtain the desired concentration and subjected to qualitative phytochemical screening for the detection of alkaloids, phenols, glycosides, flavonoids, tannins, proteins, aminoacids, carbohydrates, anthraquinones, saponins, sterols and triterpenoids (Harborne, 1984). The phytochemical analysis of individual solvent free extracts revealed the presence of relatively more number of active ingredients in ethanolic extract and hence ethanolic extract was used in this study. However, solvents, which showed appreciable results alone, were not presented.

2.3.2.1 Test for Phenols

(i) A small quantity of the extract was treated with 1% aqueous or alcoholic ferric chloride solution. Formation of green, purple, blue or black indicates the presence of phenol.

(ii) A small quantity of the extract was treated with aqueous mixture of 1% ferric chloride and 1% potassium ferricyanide. Appearance of green or purple or blue colour shows the presence of phenols.

2.3.2.2 Test for Alkaloids

(i) Dragendorff's test

**Dragendorff's reagent:** 8 g of Bi(NO₃)₃ 5H₂O was dissolved in 20 ml of HNO₃ and 2.72 g of potassium iodide in 50 ml of H₂O. These were mixed and allowed to stand when KNO₃ crystals out. The supernatant was decanted off and made up to 100 ml with distilled water.
**Procedure:** To 0.5 ml of the extract was added to 2 ml of HCl. To this *acidic* medium, 1 ml of Dragendorff's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

(ii) **Wagner's test**

*Wagner's reagent:* 1.2 g of iodide and 2.0 g of potassium iodide were dissolved in 5 ml of sulphuric acid and the solution was diluted to 100 ml.

**Procedure:** 10 ml of the extract was acidified by adding 1.5% v/v of HCl and a few drops of Wagner's reagent. Formation of yellow or brown precipitate confirmed the presence of alkaloid.

(iii) **Mayer's test**

*Mayer's reagent:* 1.36 g of mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water.

**Procedure:** 1.2 ml of the extract was taken in a test tube, 0.2 ml of dilute hydrochloric acid and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff coloured precipitate confirmed the presence of alkaloid.

**2.3.2.3 Test for Glycosides**

The extract was hydrolysed with HCl for few hours on a water bath and the hydrolysate was subjected to Legal's or Borntrager's test to detect the presence of glycosides.
(i) **Legal's test**

To the hydrolysate add 1 ml of pyridine and a few drops of sodium nitroprusside solutions were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

(ii) **B orntrager's test**

Hydrolysate was treated with chloroform and then the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Ammonia layer acquires pink color, shows the presence of glycosides.

### 2.3.2.4 Test for Flavonoids

(i) **Shinoda's test**

In a test tube containing 0.5 ml of extract, 5-10 drops of dil. HCl and small piece of ZnCl or magnesium were added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink colour was produced.

(ii) **Alkaline reagent test**

To 1 ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour was produced in the plant extract, which become colourless on addition of a few drops of dilute acid indicates the presence of flavonoids.
2.3.2.5 Test for Tannins

(i) Ferric chloride test

To 1-2 ml of the extract and a few drops of 5% aqueous FeCl₃ solution was added. A violet colour formation indicates the presence of tannins.

(ii) Lead acetate test

In a test tube containing about 50 ml of the extract and a few drops of 1% lead acetate was added. A yellow precipitate was formed, indicates the presence of tannins.

(iii) 5 ml of the extract was treated with 1 ml of 10% aqueous potassium dichromate solution. Formation of yellowish brown precipitate suggested the presence of tannins.

2.3.2.6 Test for Proteins and Amino Acids

(i) Ninhydrin test

1 ml of the extract was treated with few drops of Ninhydrin reagent (Triketo hydridene hydrate). Appearance of purple colour shows the presence of amino acids.

(ii) Biuret test

Equal volumes of 5% NaOH solution and 1% copper sulphate solution were added to 1 ml of the extract. Appearance of pink colour shows the presence of proteins.
2.3.2.7 Test for Carbohydrates

A small quantity of the extract was dissolved separately in 4 ml of distilled water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates.

(i) Molisch's test

Filtrate was treated with 2-3 drops of 1% alcoholic α-naphthal solution and 2 ml of Con. H₂SO₄ was added along the sides of the test tube. Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates.

2.3.2.8 Test for Anthraquinones

5 ml of the extract solution was hydrolyzed with dilute sulphuric acid and extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink colouration suggested the positive response for anthraquinones.

2.3.2.9 Test for Saponins

(i) The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 min. The formation of 1 cm layer of foam shows the presence of saponins.

(ii) 1 ml of the extract was treated with 1% lead acetate solution. Formation of white precipitate indicates the presence of saponins.
2.3.2.10 Test for Sterols

The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of sterol.

(i) Libermann Burchard test

The residue was dissolved in few drops of diluted acetic acid, 3 ml of acetic anhydride was added followed by few drops of Con. H₂SO₄. Appearance of bluish green colour shows the presence of sterol.

(ii) Salkowski test

10 mg of the extract was dissolved in 1 ml of chloroform, 1 ml of conc. H₂SO₄ was added carefully along the sides of the test tube. The red colour was produced, indicating the presence of sterols.

2.3.2.11 Test for Triterpenoids

(i) Libermann Burchard test

10 mg of the extract was dissolved in 1 ml of chloroform, 1 ml of acetic anhydride was added following the addition of 2 ml of Con. H₂SO₄. Formation of reddish violet colour indicates the presence of triterpenoids.
(ii) **Noller test**

5 mg of the extract was dissolved in 2 ml of 0.01% anhydrous stannic chloride in pure thionyl chloride. A purple colour formed then changed to deep red after few min and indicates the presence of triterpenoids.

## 2.4 ACUTE TOXICITY AND BEHAVIOURAL PATTERN STUDIES

To study any possible toxic effects and/or changes in behavioural pattern, rats were treated with graded doses of *Aloe vera* gel extract (100-1000 mg/kg b.w/rat/day) and kept under close observations for 12 hr laily for 1 week. All symptoms including changes in awareness, mood, motor activity, posture, motor co-ordination, muscle tone and reflexes were recorded for 7 days. Any mortality during the experiment and the following seven days were also recorded.

### 2.4.1 Effect of *Aloe vera* Extract on Blood Glucose Levels of Normal Fasted Rats

The effect of extract on normoglycemic rats was evaluated. A total of 4 normal rats fasted for 16 hr were divided into four equal groups. The rats of group I served as an untreated control whereas other three groups (II, III and IV) were administered the extract at a single dose of 100, 200 and 300 mg/kg body weight respectively. Blood samples were collected from the tail vein just prior to *Aloe vera* administration and at 1, 2, and 3 hr after the administration of *Aloe vera* extract, and blood glucose levels were measured.
.5 FIXATION OF OPTIMUM DOSAGE SCHEDULE

.5.1 Experimental Induction of Diabetes

The rats were fasted for 16 hr prior to induction of diabetes via intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ) 55 mg/kg b.w) in 0.1 M cold citrate buffer (pH 4.5) (Rakieten et al., 1963). Owing to its instability of streptozotocin in aqueous media, the solution is made in cold citrate buffer (pH 4.5) immediately before administration (Karunanayake et al., 1974). The remaining rats, representing the non-diabetic group were injected with buffer alone. The STZ-administered rats were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. Hyperglycaemia was confirmed one week after induction via blood glucose level measurements after a 16 hr fast. Animals with a fasting blood glucose level greater than 250 mg/dl were considered as diabetic and included in this study.

.5.2 Dosage Fixation Study

This study was conducted to ascertain the effectiveness of that dose in streptozotocin diabetic rats after a longer duration of Aloe vera extract treatment. The rats were divided into six groups consisting of six rats each. Group I (distilled water) served as normal control. Group II as the diabetic control, administered distilled water alone. The rats of group III, IV, V and VI were given different doses of Aloe vera extract (100, 200, 300 and 400 mg/kg w/rat/day) for 7, 14, and 21 days respectively. Blood samples were collected from the tail vein just prior to and on the 7, 14 and 21 days of Aloe vera
extract administration. The following parameters were measured: (a) blood glucose; (b) blood urea; (c) body weight of the rats. The dosage of *Aloe vera* gel extract, which offered maximum hypoglycemic activity (as elicited by levels of blood glucose, blood urea and body weight) against streptozotocin-induced diabetic rats, was fixed up for all the subsequent experiments. The optimum dosage for *Aloe vera* gel extract was fixed as 300 mg/kg b.w/rat/day for 21 days.

### 2.6 EFFECT OF *ALOE VER A* EXTRACT ON GLUCOSE TOLERANCE TEST IN NORMAL FASTED RATS

Fasted rats were divided into two groups of six rats in each group. Group I rats served as the control. An aqueous suspension of *Aloe vera* extract (300 mg/kg b.w) was administered orally to Group II rats. Both groups of rats were given glucose orally (2 g/kg of b.w) 30 min after administration of *Aloe vera* extract. Blood samples were collected from the tail vein just prior to *Aloe vera* administration and at 30-, 60-, 90-, and 120-min after glucose load, and blood glucose levels were measured.

### 2.7 EXPERIMENTAL DESIGN

The rats were divided into four groups comprising of ten animals in each group as follows:

- **Group I**: Control rats receiving 0.1 M citrate buffer (pH 4.5).

- **Group II**: Diabetic control rats.
Group III : Diabetic rats treated with *Aloe vera* gel extract (300 mg/kg b.w/rat/day) in aqueous solution orally for 21 days.

Group IV : Diabetic rats treated with glibenclamide (600 μg/kg b.w/rat/day) in aqueous solution orally for 21 days (Pari and Uma Maheswari, 2000).

On completion of 21 days of experimental period, the 16 h fasted rats were anaesthetized and sacrificed by cervical dislocation. Blood was collected with anticoagulant and used for the preparation of hemolysate and plasma. Blood collected without anticoagulant was used for serum separation.

2.7.1 Preparation of Hemolysate

Hemolysate was prepared according to the method of Dodge *et al.* (1963) with a change in buffer according to Quist (1980).

Reagents

1. Tris-HCl buffer (Hypotonic): 5 mM Tris and 15 mM sodium chloride in water

2. Sodium chloride: 0.9%.

Procedure

Blood was collected with EDTA as anticoagulant, plasma was separated by centrifugation at 1,500 x g for 15 min. The packed cells were washed well with isotonic saline. After washing with saline, the packed cells
were lysed by suspending them in the hypotonic Tris-HCl buffer for one hour. The lysed cells were centrifuged at 15,000 x g for 30 min. The supernatant was collected as hemolysate, which was subsequently used for the analysis of parameters.

2.7.2 Preparation of Tissue Homogenate

The liver, kidney and pancreatic tissues were excised, rinsed in ice-cold saline. Known weight of the tissues were homogenized in 0.1 M Tris-HCl buffer, pH 7.4 at 4°C, in a Potter-Elvehjem homogenizer with a Teflon-pestle at 600 rpm for 3 min. The homogenate was centrifuged at 3,000 x g for 10 min at 4°C using Sorvall-5B refrigerated centrifuge. The supernatant was collected as tissue homogenate, which was used to assay of various parameters. A portion of wet liver tissue was used for the estimation of glycogen content.

2.7.3 Histological Studies

The liver, kidney and pancreatic tissues were dissected out and washed in ice-cold saline immediately. A portion of the liver, kidney and pancreatic tissues was fixed in 10% buffered neutral formalin solution for histological studies. After fixation, tissues were embedded in paraffin, solid sections were cut at 5 μm. Liver and kidney sections were stained with haematoxylin and eosin and pancreas was stained with aldehyde fucshin. The sections were examined under light microscope and photomicrographs were taken (Gordon and Bradbury, 1990).
.8 BASIC BIOCHEMICAL PARAMETERS

.8.1 Estimation of Glucose

Blood glucose level was estimated by the method of Sasaki et al. (1972).

Reagents

1. Trichloro acetic acid (TCA): 10%

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

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

Procedure

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

The levels were expressed as mg/dl blood.
3.2 Estimation of Plasma Insulin

Plasma insulin was estimated using RIA assay kits (for rats) supplied by Linco Research Inc. (Stat Diagnostics, Mumbai).

at insulin RIA kit

kit contents

1. Assay buffer: 0.05 M phosphosaline, pH 7.4 containing 0.025 M EDTA, 0.08% sodium azide and 1% RIA grade BSA

2. Antiserum: Guinea pig anti-rat insulin serum in assay buffer

3. $^{125}$I-insulin: $^{125}$I-insulin label, HPLC purified

4. Label hydrating buffer: Assay buffer containing normal guinea pig serum as a carrier. Used to hydrate $^{125}$I rat insulin

5. Standards: Purified recombinant rat insulin in insulin standard buffer at the following concentrations: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 ng/ml

6. Quality controls 1 and 2: Purified rat insulin in assay buffer

7. Precipitating reagent: Goat anti-guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05 M phosphosaline, 0.025 M EDTA, 0.08% sodium azide.

Samples processed according to kit protocol and plasma insulin levels were determined and expressed as $\mu$U/ml.
2.8.3 Estimation of Plasma C-peptide

Plasma C-peptide was estimated using RIA assay kit (for rats) supplied by Linco Research Inc. (Stat Diagnostics, Mumbai).

Rat C-peptide RIA kit

Kit contents

1. Assay buffer: 0.05 M phosphosaline pH 7.4 containing 0.025 M EDTA, 0.08% sodium azide, and 1% RIA grade BSA.

2. Antiserum: Guinea pig anti-rat C-peptide antibody in assay buffer.

3. $^{125}$I-rat C-peptide: $^{125}$I-rat – C-peptide label, HPLC purified.

4. Label hydrating buffer: Assay buffer containing normal guinea pig serum as a carrier. Used to hydrate $^{125}$I-rat – C-peptide.

5. Standards: Purified recombinant rat C-peptide in assay buffer at the following concentrations: 25, 50, 100, 200, 400, 800, 1600 pM.


7. Precipitating reagent: Goat anti-guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05 M phosphosaline, 0.025 M EDTA, 0.08% sodium azide.

Samples processed according to kit protocol and plasma C-peptide levels were determined and expressed as pmol/ml.
2.8.4 **Estimation of Plasma Protein**

Protein was estimated by the method of Lowry *et al.* (1951).

**Reagents**

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

2. **Folin's phenol reagent (commercial reagent, 1:2 dilution)**

3. **Bovine serum albumin (BSA).**

**Procedure**

To 0.1 ml of suitably diluted plasma/homogenate/hemolysate, 0.9 ml of water and 4.5 ml of alkaline copper reagent were added and kept at room temperature for 10 min. Then 0.5 ml of Folin's reagent was added and the colour developed was read after 20 min at 640 nm.

The level was expressed as g/dl of plasma.

2.8.5 **Estimation of Blood Urea**

Urea was determined by the method of Natelson *et al.* (1951) using **diacetylmonoxime.**
Reagents

1. Diacetylmonoxime reagent: 2.0 g of diacetylmonoxime was dissolved in 100 ml of 2.0% acetic acid

2. Sulphuric acid-Phosphoric acid mixture: 25 ml of conc. H₂SO₄, 75 ml of 85% O-phosphoric acid and 70 ml of distilled water were mixed.

3. Sodium tungstate: 10% solution

4. Sulphuric acid: 0.67 N

5. Standard Urea solution: 20 mg of urea dissolved in 100 ml of distilled water.

Procedure

To 0.1 ml of plasma, 3.3 ml of water, 0.3 ml of 10% sodium tungstate and 0.3 ml of 0.67 N sulphuric acid reagents were added. The suspensions were centrifuged and to 1.0 ml of the supernatant, 1.0 ml of water, 0.4 ml of diacetylmonoxime and 2.6 ml of sulphuric acid - phosphoric acid reagents were added in that order. Standard urea (20-50 μg/ml) were also treated in a similar manner and heated in a boiling water bath for 30 min, cooled and the developed colour was measured at 480 nm in a Shimadzu UV spectrophotometer.

The values were expressed as mg/dl of plasma.
2.8.6 Estimation of Serum Uric Acid

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

Reagents

1. Phosphotungstic acid reagent: 30 g 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 hr. After cooling to room temperature, 18 g of lithium sulphate was dissolved and the solution was finally made upto 1 litre.

2. Sodium carbonate: 14%

3. Sulfuric acid: 2/3 N

4. Sodium tungstate: 10%

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 upto 100 ml with distilled water.

6. 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 by precipitating 1.0 ml of serum with 8.0 ml of water, 0.5 ml of 2/3 N sulfuric acid and 0.5 ml of 10% sodium tungstate. After centrifugation 2.0 ml of supernatant was mixed with 1.0 ml of phosphotungstic acid and 1.0 ml of 14% sodium carbonate. The blue colour
developed was read at 640 nm after 10 min. The standard curve prepared with standard uric acid was used to arrive at the serum uric acid levels.

The levels were expressed as mg/dl of serum.

2.8.7 Estimation of Serum Creatinine

Creatinine was estimated according to the method of Broad and Sirota using Jaffe’s reaction (1948).

Reagents

1. Saturated picric acid
2. Sodium hydroxide : 0.75 N
3. Sulphuric acid : 2/3 N
4. Sodium tungstate : 10%
5. Creatinine standard: 100 mg of creatinine was dissolved in 100 ml of 0.1 N hydrochloric acid. Working standard was prepared by appropriate dilution of 10 ml of the stock solution to 100 ml with 0.1 N HCl.

Procedure

A protein free filtrate was prepared by precipitating 1.0 ml of plasma 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, 0.5 ml of the clear filtrate was taken. To this 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 460 nm after 15 min. Standard and blank were also processed similarly.

The levels were expressed as mg/dl of serum.

2.8.8 Assay of Aspartate Aminotransferase (AST) (E.C. 2.6.1.1).

Aspartate aminotransferase was assayed by the method of King (1965a).

Reagents

1. Phosphate buffer: 0.15 M, pH 7.5.

2. Substrate: Dissolved 300 mg of DL-aspartate and 50 mg of α-ketoglutarate in 20-30 ml of phosphate buffer and added 10% sodium hydroxide to bring the pH to 7.5. This was made upto 100 ml with phosphate buffer.

3. Aniline-citrate reagent: Dissolved 5.0 g of citric acid in 50 ml of distilled water and to this was added an equal volume of redistilled aniline.

4. DNPH reagent: Dissolved 200 mg of 2,4-dinitrophenyl hydrazine (DNPH) in 85 ml of concentrated hydrochloric acid and made upto a litre with water.

5. Sodium hydroxide: 0.4 N solution.

6. Standard pyruvate: 12.5 mg of sodium pyruvate was dissolved in 100 ml of distilled water. 10 ml of this was diluted to 100 ml with
distilled water and was used as working standard for the standard calibration curve.

**Procedure**

To 1.0 ml of the buffered substrate, 0.1 ml of enzyme source was added and incubated for one hr at 37°C. At the end of the incubation period, 0.07 ml of aniline–citrate reagent was added and incubated for another 20 min. Then, 1.0 ml of the dinitrophenyl hydrazine reagent was added and left for 20 min. At the end of 20 min, 10 ml of 0.4 N sodium hydroxide was added and the color developed was read at 540 nm in a Shimadzu UV spectrophotometer after 10 min. The standards were also treated similarly.

The enzyme activity in serum was expressed as IU/l and in tissues as nmoles of pyruvate liberated/hr/mg protein.

2.8.9 **Assay of Alanine Aminotransferase (ALT) (E.C. 2.6.1.2)**

The activity of alanine aminotransferase was assayed by the method of King (1965a).

**Reagents**

1. Buffered substrate solution: 1.5 g of dipotassium hydrogen phosphate, 0.2 g of potassium dihydrogen phosphate, 0.03 g of α-ketoglutarate and 1.78 g of DL-alanine were dissolved in distilled water. The pH was adjusted to 7.4 with 1 N sodium hydroxide if necessary and made upto 100 ml with distilled water.
2. DNPH reagent: 20 mg of DNPH reagent in 100 ml of 1 N hydrochloric acid.

3. Sodium hydroxide: 0.4 N solution.

4. Standard pyruvate: 12.5 mg of sodium pyruvate was dissolved in 100 ml of distilled water. 10 ml of this was diluted to 100 ml with distilled water and was prepared freshly before use.

**Procedure**

To 1.0 ml of the buffered substrate, 0.1 ml of enzyme source was added and incubated at 37°C for 30 min. The reaction was arrested by adding 1.0 ml of dinitrophenyl hydrazine and left aside for 20 min at room temperature. The colour developed by the addition of 10 ml of 0.4 N sodium hydroxide was read at 540 nm in a Shimadzu UV spectrophotometer against the reagent blank.

The enzyme activity in serum was expressed as IU/l and in tissues as nmoles of pyruvate liberated/hr/mg protein.

**2.8.10 Assay of Acid Phosphatase (ACP) (E.C.3.1.3.2)**

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

**Reagents**

1. Acetate buffer: 0.1 M, pH 4.8.

2. Disodium phenyl phosphate solution: 0.01 M.

3. Folin's phenol reagent.
4. Sodium carbonate solution: 15%.

5. Standard phenol: 100 mg of recrystallised phenol in 100 ml of water, 100 µg 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 min. The reaction was arrested by the addition of 1.0 ml of Folin's phenol reagent. To the control tubes enzyme was added 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 min at 37°C and the colour was read at 640 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as K.A units/l in serum.

2.8.11 Assay of Alkaline Phosphatase (ALP) (E.C.3.1.3.1)

Alkaline phosphatase was assayed by the method as described by King (1965b).

Reagents

1. Carbonate-bicarbonate buffer : 0.1 M, pH 10.0.
2. Disodium phenyl phosphate : 0.01 M solution.
3. Magnesium chloride : 0.1 M solution.
4. Folin's phenol reagent: Diluted 1:2 (v/v) with dis H₂O.

5. Sodium carbonate: 15% solution.

6. Standard phenol solution: 100 mg of pure and crystalline phenol was dissolved in 0.1 N HCl and made upto 100 ml with the same acid. Working standard was prepared by suitable dilution of the stock.

**Procedure**

The incubation mixture of 3.0 ml contained 1.5 ml of buffer, 1.0 ml of substrate and requisite amount of the enzyme source. The tubes were incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 ml of Folin's phenol reagent. The control tubes received the enzymes after arresting the reaction. The contents were centrifuged and to the supernatant, 1.0 ml of 15% sodium carbonate, 1 ml of substrate and 0.1 ml of MgCl₂ were added and incubated for 10 min at 37°C. The colour developed was read at 640 nm in a Shimadzu UV spectrophotometer against a blank. The standard solution of phenol of varying concentrations was also treated similarly.

The enzyme activity was expressed as K.A units/l in serum.

**2.9 HEMATOLOGICAL PARAMETERS**

**2.9.1 Estimation of Hemoglobin**

Hemoglobin was estimated by the cyanmethemoglobin method of Drabkin and Austin (1932).
Reagents

1. Drabkin's reagent: This reagent contains 50 mg of potassium cyanide, 200 mg of potassium ferricyanide and 1 g of sodium bicarbonate in 1 litre of distilled water (pH 9.6). It was stored in brown bottle.


Procedure

\[ \text{Blood} \rightarrow \text{plasma} \rightarrow 0.2 \text{ ml of plasma} \]

To 0.2 ml of plasma, 5.0 ml of reagent was added, mixed well and allowed to stand for 10 min. The solution was read at 540 nm together with the standard solution of cyanmethemoglobin against a blank containing 5.0 ml of the reagent.

The values were expressed as g/dl of plasma: \[ \text{B.A.} \]

2.9.2 Estimation of Glycosylated Hemoglobin (HbA1C)

Glycosylated hemoglobin was estimated by the method Nayak and Pattabiraman (1981).

Reagents

1. Sodium chloride : 0.9%
2. Oxalic acid : 0.3 M
3. Trichloroacetic acid (TCA) : 40%
4. Thiobarbituric acid (TBA) : 0.05 M
5. Standard fructose solution : In the range of 10-40 \( \mu g \).
Procedure

0.2 ml of plasma was mixed with 1.8 ml of 0.3 M oxalic acid and the mixture was hydrolyzed for two hours, cooled and added 1.0 ml of 40% TCA. After centrifugation at 1400 g for 20 min, 1.5 ml of the supernatant was treated with 0.5 ml of 0.05 M thiobarbituric acid. Incubated at 37°C for 40 min. The colour developed was read at 443 nm. Standard fructose in the range of 10-40 μg was processed similarly.

The values were expressed as % hemoglobin in plasma.

2.9.3 Enumeration of Red Blood Corpuscles

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

Reagents

Red blood cell diluting fluid- (Hayme's fluid) - 5g of sodium sulphate, 1 g of sodium chloride, 0.5 g of mercuric chloride was dissolved in 200 ml of dis. H₂O.

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 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 the first 3-5 drops.

**Charging of the counting chambers**

One drop of diluted blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2-3 min. 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 numbers of cells found in 5 groups of 16 squares were multiplied by 10,000 to give the number of cells in millions/mm$^3$ of blood.

### 2.9.4 Enumeration of White Blood Corpuscles

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

**Reagents**

WBC diluted fluid was prepared by mixing

1. Glacial acetic acid
2. Gentian violet: 1%
**Procedure**

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

**Calculation**

The total number of cells in 4 squares were multiplied by a factor of 2500 to give the count /mm$^3$ of blood.

**2.9.5 Enumeration of Platelets**

The procedure detailed by Brecher and Cronkite (1964) was used.

**Reagent**

Platelet diluting fluid: Sodium citrate (3.8 g), formalin (0.2 ml) and Brilliant cresol blue (0.1g) were dissolved in 100 ml of distilled water and filtered before use.

**Procedure**

The dilution was done as described in RBC count. The counting chamber was charged as before and the number of cells in 5 squares was counted as RBC counting was done.

**Calculation**

The total number of cells in 5 squares were multiplied by 2000 to give the number of platelets /mm$^3$ of blood.
2.10 CARBOHYDRATE METABOLIZING ENZYMES

2.10.1 Assay of Hexokinase (E.C.2.7.1.1)

Hexokinase was assayed by the method of Branstrup et al. (1957).

Reagents

1. Glucose solution : 0.005 M
2. ATP solution : 0.72 M
3. Magnesium chloride solution : 0.05 M
4. Dipotassium hydrogen phosphate solution : 0.0125 M
5. Potassium chloride solution : 0.1 M
6. Sodium fluoride solution : 0.5 M
7. Tris-HCl buffer : 0.01 M, pH 8.0

Procedure

The reaction mixture in a total volume of 5.0 ml containing the following: 1.0 ml of glucose solution, 0.5 ml of ATP solution, 0.1 ml of magnesium chloride, 0.4 ml of dipotassium hydrogen phosphate solution, 0.4 ml of potassium chloride, 0.1 ml of sodium fluoride solution and 2.5 ml of Tris-HCl buffer (pH 8.0), were preincubated at 37°C for 5 min. The reaction was initiated by the addition of 2.0 ml of tissue homogenate. 1.0 ml aliquot of the reaction mixture was taken immediately (zero time) in to tubes containing 1.0 ml of 10% TCA. A second aliquot was removed after 30 min of incubation at 37°C. The precipitated protein was removed by centrifugation
and the residual glucose in the supernatant was estimated by the O-toluidine method of Sasaki et al. (1972). A reagent blank was run with each test. The difference between the two values gave the amount of glucose phosphorylated.

The enzyme activity was expressed as μmoles of glucose-6-phosphate formed/hr/mg protein for tissue homogenates under the incubation conditions.

2.10.2 Assay of Lactate dehydrogenase (E.C.1.1.1.27)

Lactate dehydrogenase (L-lactate; NAD-oxido reductase) was assayed according to the method of King (1959). The amount of pyruvate formed in the forward reaction was measured spectrophotometrically.

Reagents

1. 0.1 M glycine buffer: 7.5 g of glycine and 5.85 g of sodium chloride were dissolved in one litre of distilled water.

2. Buffered substrate: 2.75 g of lithium lactate was dissolved in 125 ml of glycine buffer containing 75 ml of 0.1 N sodium hydroxide solution. This was prepared just before use.

3. Sodium hydroxide solution: 0.4 N

4. NAD: 5.0 mg of NAD was dissolved in 1.0 ml of distilled water just before use.

5. 2,4-dinitrophenyl hydrazine reagent (DNPH): 200 mg of DNPH was dissolved in one litre of 1.0 N hydrochloric acid.
6. Standard pyruvate solution: 12.5 mg of sodium pyruvate was dissolved in 100 ml of buffer.

**Procedure**

To 1.0 ml of the buffered substrate, 0.1 ml of enzyme preparation 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 another 15 min. Adding 1.0 ml of DNPH reagent then arrested the reaction and the tubes were incubated for a further period of 15 min at 37°C after which 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 analyzed by the same procedure.

The activity of the enzyme was expressed as µmoles of pyruvate formed/hr/mg protein for tissues under incubation time.

**2.10.3 Assay of Glucose-6-phosphatase (E.C.3.1.3.9)**

Glucose-6-phosphatase (glucose-6-phosphate phosphorylase) was assayed according to the method of Koide and Oda (1959).

**Reagents**

1. Citrate buffer : 0.1 M, pH 6.5.
2. Substrate : 0.01 M Glucose-6-phosphate in distilled water.
3. Trichloroacetic acid: 10% solution.
Procedure

The incubation mixture in a total volume of 1.0 ml contained 0.3 ml of buffer, 0.5 ml of substrate and 0.2 ml of enzyme extract. Incubation was carried out at 37°C for 60 min. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was centrifuged and the phosphorus content of the supernatant was estimated according to the method described by Fiske and Subbarow (1925). To an aliquot of the supernatant, 4.1 ml of distilled water and 0.5 ml of ammonium molybdate were added. After 10 min 0.2 ml of ANSA was added. The tubes were shaken well, kept aside for 20 min and the blue colour developed was read at 620 nm against water blank in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as μmoles of phosphate liberated/hr/mg protein under the incubation conditions.

2.10.4 Assay of Fructose-1,6-bisphosphatase (E.C.3.1.3.11)

Fructose-1,6-bisphosphatase (Fructose 1,6-bisphosphate phosphohydrolase) was assayed by the method of Gancedo and Gancedo (1971).

Reagents

1. Tris-HCl buffer : 0.1 M, pH 7.0
2. Substrate : 0.05 M Fructose-1,6-bisphosphate.
3. Magnesium chloride : 0.1 M solution
4. Potassium chloride : 0.1 M solution
5. EDTA solution : 0.001 M solution
6. Trichloroacetic acid : 10% solution
7. Molybdic acid : 2.5% ammonium molybdate in 3 N sulphuric acid.

8. ANSA reagent: 500 mg of aminonaphthol sulphonylic acid (ANSA) was dissolved in 195 ml of 15% sodium bisulphite solution and 5 ml of 20% sodium sulphite solution was added for complete solubilisation. The solution was filtered and stored at 4°C in a brown bottle.

9. Standard Phosphorus: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water (80 µg/ml)

Procedure

The assay medium in a final volume of 2.0 ml contained 1.2 ml of buffer, 0.1 ml of substrate, 0.25 ml of magnesium chloride, 0.1 ml of potassium chloride, 0.25 ml of EDTA and 0.1 ml of the enzyme source. This mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was then centrifuged and the phosphorus content of the supernatant was estimated according to the method described by Fiske and Subbarow (1925). To an aliquot of the supernatant, 4.1 ml of distilled water and 0.5 ml of ammonium molybdate were added. After 10 min 0.2 ml of ANSA was added. The tubes were shaken well, kept aside for 20 min and the blue colour developed was read at 620 nm against water blank in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as µmoles of phosphate liberated/hr/mg of protein.
2.10.5 Assay of Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49)

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate NADP-oxido reductase) was assayed by the method of Ell and Kirkman (1961).

Reagents

1. Tris - HCl buffer : 0.5 M, pH 7.4
2. Magnesium chloride solution : 1.0 M
3. NADP⁺ solution : 0.01 M
4. Phenazine methosulphate : 0.005% in water
5. 2,6-dichlorophenol indophenol : 0.01% in water
6. Glucose-6-phosphate solution : 0.02 M

Procedure

The incubation mixture in a total volume of 5.5 ml contained: 1.0 ml of Tris-HCl buffer, 0.1 ml of magnesium chloride, 0.1 ml of NADP⁺, 0.5 ml of phenazine methosulphate, 0.4 ml of 2,6-dichlorophenol indophenol dye and requisite amount of test solution. The mixture was allowed to stand at room temperature for 10 min to permit the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 ml of glucose-6-phosphate. The optical density was read at intervals of one min for 3-5 min at 640 nm against a water blank in a Shimadzu UV spectrophotometer. The activity was obtained by multiplying the change in O.D/min by 6/17.6, which is the molar extinction co-efficient of the reduced coenzyme.

The enzyme activity was expressed as μmoles of NADPH formed/min/mg protein for tissue homogenates under the incubation conditions.
2.10.6 Estimation of Glycogen

For the estimation of glycogen, the extraction was carried out by the method of Morales et al. (1973). Glycogen was precipitated from the alkali extract of the tissues by adding 1:3 volume of 95% ethanol and a drop of 1 M ammonium acetate. The tubes were kept in a boiling water bath for 5 min. After cooling, the tubes were shaken well and placed in a freezer overnight. The precipitated glycogen was then collected by centrifugation at 3,000 x g for 40 min. The precipitate was dissolved in water, precipitated with alcohol and centrifuged again. The final precipitate was dissolved in 3.0 ml of water and heated for 5 min in a boiling water bath and different aliquots were used for the estimation of glycogen.

Procedure

Suitable aliquot of glycogen was made upto 1.0 ml with water. A set of standard glucose solutions (25-100 μg) and blank containing water alone, were set up. All the tubes were cooled in an ice-bath and 4.0 ml of anthrone reagent was added. The contents of the tubes were mixed well. All the tubes were covered with glass marbles and heated for 20 min in a boiling water bath. The tubes were cooled and the developed green colour was read at 640 nm in a Shimadzu UV spectrophotometer.

The values were expressed as mg of glucose/g wet tissue.
2.10.7 Assay of Glycogen Synthase (E.C. 2.4.1.11)

Glycogen synthase (UDP-glucose-α-1,4-glucan-α-4-glucosyl transferase) was assayed by the method of Leloir and Goldenberg (1979).

Reagents

1. Glycogen solution: 40 mg/ml.
2. 0.75 M glycine buffer, pH 8.5 containing 0.025 M EDTA.
3. Glucose-6-phosphate: 0.05M
4. Cysteine: 0.03M solution was prepared freshly.
5. Uridine diphosphate glucose: 25μM of UDPG/ml of distilled water.
6. Phosphoenol pyruvate: 0.01M in 0.14 M potassium chloride.
7. Pyruvate kinase: Freshly diluted in 0.1 M magnesium sulphate solution.
8. Dinitrophenyl hydrazine: 0.1% in 2.0 N hydrochloric acid.
9. Sodium hydroxide solution: 10 N
10. 95% ethanol.

Procedure

The following components were mixed: 0.09 ml of the glycogen, buffer, glucose-6-phosphate solution, 0.015 ml of cysteine hydrochloride solution, requisite amount of enzyme and 0.03 ml of UDPG solution. The reaction was started by the addition of UDPG and the incubation was carried out at 37°C for 5-10 min. The tubes were then heated in a boiling water bath for 60 sec. A blank in which UDPG was added after incubation was run at the same time along with UDP standards (10-60 μg).
To estimate the UDP formed in the reaction, 0.075 ml of phosphoenol pyruvate solution and 0.075 ml of pyruvate kinase were added. The tubes were incubated for 15 min at 37°C and 0.45 ml of dinitrophenyl hydrazine solution was then added. After 10 min, 0.6 ml of 10 N sodium hydroxide solution and 1.1 ml of ethanol were added, the contents were mixed well and centrifuged. The optical density of the supernatant fluid was read at 530 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as μmoles of UDP formed/hr/mg protein.

2.10.8 Assay of Glycogen Phosphorylase (E.C.2.4.1.1)

Glycogen phosphorylase (α-1,4-glucan; ortho-phosphate glucosyl transferase) was assayed by the method of Cornblath et al. (1963).

Reagents

1. Substrate: 0.05 M glucose-1-phosphate solution containing 5.7 mg glycogen per ml and 0.05 M sodium fluoride was prepared in distilled water. The pH was adjusted to 6.1 using dilute hydrochloric acid.

2. 5' AMP : 0.025 M

3. Trichloro acetic acid (TCA) : 10% solution

4. Sodium fluoride : 0.1 N

Enzyme source was diluted with ice cold 0.1 M sodium fluoride solution. Dilution was carried out just before use.
Procedure

The reaction was started by the addition of 1.0 ml of substrate, 0.1 ml of 5' AMP, 0.2 ml of sodium fluoride and 0.2 ml of enzyme. An aliquot (0.5 ml) was taken from the reaction mixture at zero time and after 10 min of incubation at 37°C, 1.0 ml of 10% TCA solution was added to arrest reaction. The contents were mixed well, centrifuged and the liberated phosphorus in the supernatant was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as μmoles of phosphate liberated/hr/mg protein.

2.11 MITOCHONDRIAL ENZYMES

2.11.1 Isolation of Mitochondria

The mitochondrial isolation procedure used was a modification of Sordahl et al. (1971). All manipulations were carried out on ice or at 4°C. The liver and kidney tissues were quickly dissected from decapitated rats into ice-cold isolation medium which consisted of 0.25 M sucrose, 1 mM Tris-HCl (pH 7.4) and 1 mM ethylene diamine tetraacetic acid (EDTA) (pH 7.2 - 7.4) and were homogenized (10% w/v) using a motorized glass - Teflon homogenizer.

The 10% homogenate was centrifuged at 700 x g for 5 min and the resultant supernatant was centrifuged at 30,000 x g for 10 min. The pellet was resuspended in isolation medium (one-half original volume) and centrifuged at 30,000 x g for 10 min. The mitochondrial pellet obtained was resuspended
in isolation medium (1ml/g tissue) to produce a suspension containing 25-40 mg mitochondrial protein/ml, which was taken for subsequent analysis.

2.11.2 Estimation of Mitochondrial Protein

The mitochondrial protein content was estimated by the method of Lowry et al. (1951) as described earlier.

2.11.3 Assay of Isocitrate dehydrogenase (E.C.1.1.1.42)

The enzyme activity was assayed according to the method of King (1965c).

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 : Sodium salt of ethylene diamine tetra acetate 5% solution in water.
6. Sodium hydroxide : 0.4 N
7. 2,4 dinitrophenyl hydrazine : 0.001 M in 1 N HCl.
8. Standard solution : 15 μg of α-ketoglutarate (potassium salt) in 50 ml of buffer.
**Procedure**

0.4 ml of the buffer 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 and 0.2 ml of saline was added to the control tubes. After mixing well, both the tubes were incubated for 60 min, 1 ml of colour reagent (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. A standard curve was prepared using α-ketoglutarate.

The enzyme activity was expressed as nmoles of α-ketoglutarate liberated/hr/mg protein.

**2.11.4 Assay of α-Ketoglutarate dehydrogenase (E.C.1.2.4.2)**

This enzyme activity was estimated according to the method of Reed and Mukherjee (1969). The assay is based on the colorimetric determination of ferrocyanide produced by the decarboxylation of α-ketoglutarate with ferricyanide as electron acceptor.

**Reagents**

1. **Phosphate buffer** : 0.1 M, pH 6.0
2. **Thiamine pyrophosphate** : 0.002M
3. **Magnesium sulphate** : 0.003 M
4. α-Ketoglutarate (Potassium salt) : 0.5 M
5. Potassium ferric cyanide : 0.25 M
6. Trichloroacetic acid (TCA) : 10%
7. Sodium dodecyl sulphate (Dupanol) : 4%
8. Ferric ammonium sulphate–dupanol reagent: 1.7 g of ferric ammonium sulphate was added with 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 8% phosphoric acid was added and diluted to 140 ml with water.
9. Standard potassium ferrocyanide: 0.01% solution.

Procedure

To 0.15 ml of phosphate buffer, 0.1 ml each of thiamine pyrophosphate, magnesium sulphate, α-ketoglutarate and potassium ferric cyanide was added. The total volume was made upto 1.2 ml with distilled water. 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 incubation, 1.0 ml of 10% TCA was added to the test and control tubes. 0.2 ml of the enzyme was now added to the control tubes. They were centrifuged and aliquots of the supernatant were pipetted out into test tubes for the colour reaction. 0.1 ml of potassium ferric cyanide was added to the tubes and the volume was made upto 2.4 ml with water. 1 ml of 4% dupanol and 0.5 ml 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. A standard potassium ferrocyanide, in the range of 10 μg to 50 μg was run simultaneously.

The enzyme activity was expressed as nmoles of potassium ferrocyanide liberated/hr/mg protein for tissues.

2.11.5 Assay of Succinate dehydrogenase (E.C.1.3.99.1)

This enzyme activity was estimated according to the method of Slater and 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 O.D. at 420 nm.

Reagents

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

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 and 0.2 ml of potassium ferricyanide were added and the total volume was made upto 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 were recorded at 15 sec interval at 420 nm for five min.

The enzyme activity was expressed as μmoles of succinate oxidized/min/mg protein.

2.11.6 Assay of Malate dehydrogenase (E.C. 1.1.1.37)

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

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 0.75 ml of phosphate buffer, 0.15 ml of NADH, 0.75 ml of oxaloacetate. The reaction was carried out at 25°C and was started by the addition of 0.2 ml mitochondrial suspension. The control tubes contained all reagents except NADH. The change in OD at 340 nm was measured for 2 min at an interval of 15 sec in Shimadzu UV spectrophotometer.

The enzyme activity was expressed as nmoles of NADH oxidized/min/mg protein.
2.11.7 Assay of NADH-dehydrogenase (E.C. 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. Potassium ferricyanide : 0.03 M
3. NADH : 0.1%

Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of potassium ferricyanide, 0.1 ml of NADH and 0.2 ml of mitochondrial suspension. The total volume was made up to 3.0 ml with water. NADH was added just before the addition of the enzyme. A control was also treated similarly without NADH. The change in OD was measured at 420 nm as function of time for 3 min at an interval of 15 sec.

The enzyme activity was expressed as μmoles of NADH oxidized/min/mg protein.

2.11.8 Assay of Cytochrome-C-oxidase (E.C.1.9.3.1)

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

1. Phosphate buffer: 0.03 M, pH 7.4
2. Cytochrome C: 0.01% in 0.03 M phosphate buffer.
3. N-phenyl-p-phenylene diamine solution: 0.2%

Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.2 ml of 0.2% N-phenyl-p-phenylene diamine, 0.1 ml of 0.01% cytochrome C and 0.5 ml of distilled water. The sample was incubated at 25°C for 5 min. 0.2 ml of the enzyme preparation was added and the change in OD was recorded at 550 nm for 5 min at an interval of 15 sec each. A control containing all reagents except cytochrome C was also processed in the same manner.

The enzyme activity was expressed as Optical density $\times 10^2$/min/mg protein.

2.12 LIPID ANALYSIS

2.12.1 Extraction of Lipids

Total lipid was extracted from the liver and kidney tissues according to the method of Folch et al. (1957).

Reagents

1. Chloroform-Methanol: 2:1 v/v
2. Saline solution: 0.89%.
**Procedure**

A known volume of suspension was mixed with 10 ml of chloroform-methanol mixture and homogenized. The homogenate was filtered through Whatmann filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into preweighed beakers. The upper phase was re-extracted with more of chloroform-methanol mixture and the extracts were pooled and evaporated to dryness. The lipids were redissolved in 1.0 ml of chloroform - methanol mixture and aliquots were used for the estimation of various lipid components namely cholesterol, triglycerides, free fatty acids and phospholipids. The aliquots were dried at room temperature to evaporate the solvent before use.

**2.12.2 Estimation of Total Cholesterol**

Plasma and tissue cholesterol content was estimated by the method of Parekh and Jung (1970).

**Reagents**

1. Ferric chloride - Uranyl acetate reagent: 500 mg of ferric chloride was dissolved in 10 ml of water, 3 ml of concentrated ammonia was added to it and centrifuged. The precipitate was washed several times with distilled water and dissolved in one litre of glacial acetic acid. 100 mg of uranyl acetate was added to the mixture and the contents
were shaken well and kept over night. The reagent was stable for six months.

2. Sulphuric acid - Ferrous sulphate reagent: 100 mg of ferrous sulphate was dissolved in 100 ml of glacial acetic acid and 100 ml of sulphuric acid. After cooling to room temperature the volume was made upto 1 litre with concentrated sulphuric acid. The reagent was stable for six months.

3. Cholesterol standard: 200 mg of cholesterol, recrystallized from ethanol was dissolved in 100 ml of chloroform.

**Procedure**

About 0.1 ml of plasma was made up to 10 ml with ferric acetate - uranyl acetate reagent. 0.1 ml of the aliquot of the total lipid extract was taken and it was evaporated to dryness. The dried extract and standards were made upto 3 ml with ferric chloride - uranyl acetate reagent. Then 2 ml of sulphuric acid - ferrous sulphate reagent was added to all the tubes and the contents were mixed well. After 20 min the colour developed was read at 540 nm using a Shimadzu UV spectrophotometer.

The level was expressed as mg/dl in plasma and mg/g in tissues.

2.12.3 *Estimation of Triglycerides (TG)*

Triacylglycerides in plasma and tissues was estimated by the method of Rice (1970).
Lipids were extracted with chloroform: methanol mixture (2:1 v/v). Phospholipids were adsorbed onto silicic acid and the triglycerides remaining in solution were saponified with alcoholic potassium hydroxide. The liberated glycerols were oxidised by periodate to formaldehyde and the excess oxidising power was destroyed by reaction with sodium arsenite. The formaldehyde formed was determined by the chromatropic colour reaction.

**Reagents**

1. Chloroform - methanol mixture (2:1 v/v).
2. Saturated sodium chloride
3. Activated silicic acid
4. Alcoholic potassium hydroxide: 400 mg of potassium hydroxide was dissolved in 100 ml of 95% ethanol.
5. Sulphuric acid: 0.2 N
6. Sodium arsenite: 0.5 M
7. Sodium metaperiodate: 0.5%
8. Chromotropic acid reagent: 1.14 g of disodium salt of chromatropic 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. Tripalmitin standard: 10 mg tripalmitin was dissolved in 100 ml of chloroform.
**Procedure**

4.0 ml of the lipid extract was added to tubes containing 8.0 ml of saturated sodium chloride and shaken vigorously. The contents were allowed to settle for one hour and then centrifuged. The supernatant saline - methanol phase was discarded. The washed chloroform phase was filtered into a dry tube. 200 mg of activated silicic acid was added to chloroform phase, shaken vigorously and allowed to stand for 30 min. After centrifugation, 0.5 ml of the supernatant as well as tripalmitin standards were evaporated to dryness. Then to the tube, standard and blank tubes, 0.5 ml of alcoholic potassium hydroxide solution was added and the mixture was saponified in a 60° - 70°C water bath for 20 min. 0.5 ml of 0.2 N sulphuric acid was added and heated in a boiling water bath for 10 min. After cooling the tubes, 0.1 ml of sodium metaperiodate was added and allowed to stand for 10 min. The excess periodate was reduced by the addition of 0.1 ml of sodium metarsenite. 5.0 ml of chromotropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 min. After cooling, 0.5 ml of thiourea solution was added. The colour developed was read at 540 nm against a blank in a Shimadzu double beam spectrophotometer.

The content of the plasma was expressed as mg/dl and tissues were expressed as mg/g of tissue.

**2.12.4 Estimation of Free Fatty Acids (FFAs)**

Free fatty acid content was estimated by the method of Hron and Menahan (1981).
Reagents

2. Copper nitrate-triethanolamine reagent (Cu-TEA)
3. Colour reagent, diethyl dithiocarbamate solution
4. Activated silicic acid
5. Standard: 20 mg of palmitic acid was dissolved in 100 ml of Chloroform: heptane: methanol mixture.

Procedure

0.2 ml of the sample (lipid extract or plasma) was mixed with 6.0 ml of chloroform: heptane: methanol solvent and was shaken vigorously. 200 mg of activated silicic acid was added, shaken and left aside for 30 min. The solution was then centrifuged and the supernatant was transferred to a tube containing 2.0 ml Cu-TEA reagent. Blank contained only the solvent, while standards with different concentrations were made upto a known volume with the solvent and then 2.0 ml of Cu-TEA reagent was added. The contents were agitated using a mechanical shaker for 20 min. The mixture was separated into two phases by centrifugation. 2.0 ml of the upper layer was mixed with 2 ml of the colour reagent and shaken well. The yellow colour developed was read at 430 nm in a Shimadzu UV spectrophotometer.

The values were expressed as mg/dl in plasma and tissues were expressed as mg/g of tissue.
2.12.5 Estimation of Phospholipids

Phospholipid was estimated by the method of Bartlette (1959) by digestion with perchloric acid and the phosphorus liberated was estimated by the method of Fiske and Subbarow (1925).

Reagents

1. Perchloric acid.

2. Molybdic acid: 2.5% ammonium molybdate in 3 N H$_2$SO$_4$.

3. Amino naphthol sulphonic acid (ANSA): 500 mg of amino naphthol sulphonic acid was dissolved in 195 ml of 15% sodium bisulphite and 5 ml of 20% sodium sulphite for complete solubilization. The solution was filtered and stored at 4°C in a brown bottle.

4. Phosphorus stock standard: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of water.

Procedure

0.1 ml of the sample (tissue lipid extract) was digested with 0.2 ml of perchloric acid over a sand bath. Digestion was continued until it was colourless. The liberated inorganic phosphorus of the digested sample was estimated.

4.3 ml of distilled water was added to the digested sample followed by 0.5 ml of ammonium molybdate. After 10 min, 0.2 ml of ANSA was added. The tubes were shaken well and kept aside for 20 min and the blue colour developed was read at 620 nm against water blank in a Shimadzu
UV spectrophotometer. Standards and blank were also treated similarly. The total phospholipids were estimated by multiplying the value of phospholipids by 25, which gave the amount of phospholipids.

The values were expressed as mg/dl in plasma and mg/g in tissues.

2.12.6 Lipoprotein Fractionation

Lipoproteins were fractionated by dual precipitation techniques (Burstein and Scholnick, 1972).

2.12.6.1 HDL-Fractionation

Reagents

1. Heparin - manganese chloride reagent: 3.167 gms of manganese chloride was added to 1.0 ml of heparin containing 20,000 units/ml. This was made upto 8.0 ml with water.

Procedure

To 1.0 ml of serum added 0.09 ml of heparin - manganese chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 min and then centrifuged at 2,500 rpm for 30 min. The supernatant represented HDL fraction. Aliquot was taken from HDL fraction for the estimation of cholesterol.
2.12.6.2 LDL - Fractionation

Reagents

1. 10% Sodium dodecylsulphate (SDS) in 0.15 M sodium chloride, pH 9.0.

Procedure

To 1.0 ml of serum added 0.15 ml of sodium dodecylsulphate. The contents were mixed well and incubated at 37°C for 2 hr. The contents were centrifuged in the refrigerated centrifuge at 10,000 rpm for 15 min. VLDL aggregated as pellet. The supernatant contained the HDL and LDL fractions. Cholesterol was estimated from this fraction.

2.12.7 Analysis of Fatty Acid Composition

Analysis of fatty acid composition was performed in gas chromatography according to the method of Morrison and Smith (1964). The lipid extract was evaporated to dryness using a water bath and subjected to saponification

Reagents

Reagent I: Saponification reagent

a) Sodium hydroxide - 45 g

b) Methanol - 150 ml

c) Deionised distilled water - 150 ml
Reagent II: Methylation reagent
   a) 6.0 N Hydrochloric acid - 325 ml
   b) Methanol - 275 ml

Reagent III: Extraction solvent
   Hexane and diethyl ether 1:1

Reagent IV: Base wash
   a) Sodium hydroxide - 10.8 g
   b) Deionised distilled water – 900 ml

Procedure

To the test tubes, 1.0 ml of reagent-I was added and vortexed for 5-10 sec and kept in a boiling water bath. After 5 min, the tubes were removed from the water bath and again vortexed for 5-10 sec. Again kept in a boiling water bath for 25 min. The tubes were removed and cooled with room temperature. To the above tubes, 2.0 ml of reagent II was added and heated at 80°C in a water bath for 10 min and removed quickly to room temperature water. The fatty acid methylesters thus formed were removed from the aqueous phase and transferred to an organic phase by liquid-liquid extraction procedure. To the above test tubes, the reagent III was added and mixed well and the aqueous lower phase was discarded. To the upper solvent phase 3.0 ml of reagent IV was added, mixed well and subjected to centrifugation and the upper solvent layer was removed and placed in a GC bottle sealed and kept in refrigerated till analysis was carried out.
The fatty acid esters thus obtained were analyzed on a Tracer 540-gas chromatograph equipped with flame ionization detector under the following conditions: Inlet temperature 200°C, detector temperature 220°C. Separating column: 2 cm long x 2 mm internal diameter, packed with 10% cilar or chromosorb W, 80/100 mesh. Fatty acids separated were identified by the comparison of a retention times with those obtained by the separation of a mixture of standard fatty acids. Electronic integrator carried out measurements of peak areas and data processing. Individual fatty acids were expressed as percentage of fatty acid/100 mg tissues.

2.13 LIPID PEROXIDATION AND ANTIOXIDANT DEFENSE SYSTEM

2.13.1 Estimation of Lipid Peroxidation (LPO)

The level of lipid peroxides was assayed by the method of Ohkawa et al. (1979).

Reagents

1. Sodium dodecyl sulfate (SDS): 8.1%
2. Acetic acid: 20%, pH 3.5.
3. Thiobarbituric acid (TBA): 0.8% in distilled water, warmed slightly.
4. n-Butanol-pyridine mixture (15:1 v/v)
5. 1,1',3,3'-tetramethoxypropane.
Procedure

To 0.2 ml of tissue homogenate or plasma, 0.2 ml of SDS, 1.5 ml of acetic acid and 1.5 ml of TBA were added. The mixture was made up to 4 ml with water and then heated in a water bath at 95\degree C for 60 min using glass ball as a condenser. After cooling, 1 ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. 1,1',3,3'-tetramethoxypropane was used as a standard.

The level was expressed as nmoles TBARS/ml in plasma and mM TBARS/100g in tissues.

2.13.2 Estimation of Hydroperoxides

Hydroperoxides was estimated by the method of Jiang et al. (1992).

Reagents

1. Methanol 90 ml
2. Sulphuric acid: 250mM
3. Butylated hydroxy toluene: 88 mg of BHT was dissolved in 250mM sulphuric acid.
4. Xylenol orange: 7.6 mg of xylenol orange was dissolved in 250mM sulphuric acid.
5. Ammonium Ion sulphate: 9.8 mg of Ammonium ion sulphate was dissolved in 250mM sulphuric acid. The above reagents were mixed and this reagent is called fox reagent.

6. Standard: 0.2M Hydrogen peroxide.

**Procedure**

1.8 ml of the fox reagent was mixed with 0.2 ml of the tissue homogenate or 0.2 ml of plasma. Then incubated for 30 min at room temperature and colour developed was read at 560nm.

The level was expressed as $10^{-5}$ mM/dl in plasma and mM/100 g tissue.

2.13.3 **Estimation of Vitamin E**

Vitamin E content was estimated by the method of Desai (1984).

**Reagents**

1. Ethanol

2. Petroleum ether

3. 4,6-diphenyl-1, 10-phenanthroline in ethanol: 0.2%

4. Ferric chloride in ethanol (fresh): 0.001 M

5. O-phosphoric acid in ethanol: 0.001 M

6. $\alpha$-Tocopherol acetate.
Procedure

To 1.0 ml of plasma, 1.0 ml of ethanol was added and thoroughly mixed. Then 3 ml of petroleum ether was added, shaken rapidly and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this 0.2 ml of baphophenanthoraline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride was added followed by 0.2 ml of O-phosphoric acid. The total volume was made up to 3 ml with ethanol. The colour developed was read at 530 nm.

The level was expressed as mg/dl of plasma.

2.13.4 Estimation of Ceruloplasmin

Plasma ceruloplasmin was estimated following the method of Ravin (1961).

Reagents

1. p-phenylene diamine hydrochloride : 0.1%
2. Acetate buffer : 0.1 M, pH 5.2
3. Sodium azide : 0.02%

Procedure

0.1 ml of fresh plasma far from hemolysis and turbidity was taken into three 15 ml test tubes, one for control and two for tests respectively. One ml of 0.02% sodium azide was added to the control tube only. Then 8.0 ml of acetate buffer was added to all the tubes, followed by the addition of 1.0 ml of
0.1% p-phenylene diamine. The solution was mixed thoroughly and placed in a water bath at 37°C for one hour. After incubation the tubes were removed and 1.0 ml of sodium azide was added to each of the tubes containing test solution. The contents were shaken well and cooled at 4°C for 30 min. The intensity of the colour developed was measured at 530 nm in Shimadzu UV spectrophotometer against a reagent blank.

The level was expressed as mg/dl of plasma.

2.13.5 Estimation of Vitamin C

The level of vitamin C was estimated by the method of Omaye et al. (1979).

Reagents

1. Trichloro acetic acid (TCA): 5%

2. DTC reagent (3g of 2,4-dinitrophenyl hydrazine, 0.4 g of thiourea and 0.05 g of copper sulphate were dissolved in 100 ml of 9 N sulphuric acid)

3. Sulphuric acid (ice cold): 65%

4. Standard ascorbic acid.

Procedure

To 0.5 ml of plasma, 0.5 ml of water and 1.0 ml of TCA were added, mixed thoroughly and centrifuged. To 1.0 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37°C for 3 hr. Then 1.5 ml of
sulphuric acid was added, mixed well and the solution was allowed to stand at room temperature for another 30 min. The colour developed was read at 520 nm.

The level was expressed as mg/dl of plasma.

2.13.6 Estimation of Reduced Glutathione (GSH)

Reduced glutathione was determined by the method of Ellman (1959).

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. Trichloro acetic acid (TCA): 5%

Procedure

0.1 ml of tissue homogenate or plasma was precipitated with 5% TCA. The contents were mixed well for complete precipitation of proteins and centrifuged. To an aliquot of clear supernatant, added 2.0 ml 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 also run to determine the glutathione content.

The level was expressed as mg/dl of plasma or mg/100g of liver and kidney or mM/100 g of pancreas.
2.13.7 Assay of Superoxide Dismutase (SOD) (E.C.1.15.1.1)

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

Reagents

1. Carbonate-bicarbonate buffer: 0.1 M, pH 10.2
2. EDTA solution: 0.6 mM
3. Epinephrine (Prepared freshly): 1.8 mM
4. Absolute ethanol
5. Chloroform.

Procedure

0.1 ml of tissue homogenate or hemolysate was added to tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of EDTA solution and 1 ml of buffer. The reaction was initiated by the addition of 0.5 ml of epinephrine and the increase in absorbance at 480 nm was measured in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as 50% inhibition of epinephrine autooxidation/min.
2.13.8 Assay of Catalase (CAT) (E.C.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 in the above buffer: 30 mM

Procedure

To 1.2 ml of phosphate buffer, 0.2 ml of the tissue homogenate or hemolysate was added and the enzyme reaction was started by the addition of 1.0 ml of $H_2O_2$ solution. The decrease in absorbance was measured at 240 nm at 30 sec intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as $\mu$moles of $H_2O_2$ decomposed/min/mg protein.

2.13.9 Assay of Glutathione Peroxidase (GPx) (EC 1.11.19)

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

1. Phosphate buffer: 0.4 M, pH 7.0
2. EDTA: 0.8 mM
3. Sodium azide: 10 mM
4. Reduced glutathione: 4 mM
5. $H_2O_2$: 2.5 mM
6. Trichloro acetic acid (TCA): 10%
7. Disodium hydrogen phosphate: 0.3 M
8. DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate)

Procedure

The reaction mixture consisted of 0.2 ml of EDTA, 0.1 ml of sodium azide, 0.1 ml of $H_2O_2$, 0.2 ml of reduced glutathione, 0.4 ml of phosphate buffer, and 0.2 ml tissue homogenate or hemolysate were incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To the supernatant 3 ml of disodium hydrogen phosphate and 1.0 ml DTNB were added and the colour developed was read at 420 nm immediately.

The enzyme activity was expressed as μmoles of glutathione oxidized/min/mg protein.
2.14 UREA CYCLE ENZYMES

2.14.1 Assay of Ornithine transcarbamoylase (E.C.2.1.3.3)

Ornithine transcarbamoylase activity in tissue was estimated by the method of Weber et al. (1972).

Reagents

1. Glycyl glycine-sodium hydroxide buffer: 0.125 M, PH 7.4
2. L-Ornithine : 0.05 M
3. Carbamoyl phosphate : 0.05 M
4. Trichloro acetic acid (TCA) : 5%

Procedure

The reaction mixture containing 0.2 ml of glycyl glycine-sodium hydroxide buffer, 0.1 ml of ornithine, 0.1 ml of carbamoyl phosphate and 0.1 ml enzyme preparation was incubated at 37°C for 10 min. After arresting the reaction by the addition of 1.0 ml of 5% TCA, the supernatant solution was used for citrulline estimation. Enzyme blanks were set up along with the test, the enzyme source being added only after the reaction was stopped.

The enzyme activity was expressed as μmoles of citrulline/hr/mg protein.
2.14.2 Assay of Argininosuccinase (E.C. 4.3.2.1)

Argininosuccinase activity was measured as the rate of urea synthesis in the presence of excess arginase according to the method of Schimke (1962).

Reagents

1. Sodium phosphate buffer : 0.05 M, pH 7.4
2. Argininosuccinate : 0.015 M
3. Arginase
4. Perchloric acid : 15%

Procedure

The medium consisted of 0.05 M sodium phosphate buffer and 0.015 M argininosuccinate, adjusted to pH 7.4 with NaOH before addition, 1 mg of arginase was added per ml of medium. Incubation was performed for 15 min in 250 µl of medium to which was added 25 µl of tissue homogenate. The reaction was stopped by the addition of 1.0 ml of 15% perchloric acid. A 1.0 ml aliquot was used for colour development. The product urea was estimated accordingly to the method of Natelson et al. (1951).

The enzyme activity was expressed as µmoles of urea formed/hr/mg protein.
2.14.3 Assay of Arginase (E.C.3.5.3.1)

Arginase activity was assayed according to the method of Herzfeld and Raper (1976).

Reagents

1. Arginine: 0.2 M, pH 9.5.
2. Glycine-sodium hydroxide buffer: 0.08 M, pH 9.5.
3. Diacetyl monoxime-thiosemicarbazide reagent (DAM-TSC): 36 mM, 61.7 mM.
4. Acid ferric reagent: This reagent contained 3.6 mM sulphuric acid, 0.12 mM ferric chloride and 38.6 mM O-phosphoric acid.
5. Trichloro acetic acid (TCA): 10%.
6. Standard urea: 10 mg of urea in 100 ml of distilled water.

Procedure

The reaction mixture containing 0.5 ml of arginine, 0.5 ml of glycine sodium hydroxide buffer and 0.05 ml of enzyme preparation was incubated at 37°C for 10 min. The reaction was stopped by the addition of 2.0 ml of 10% TCA and the supernatant obtained after centrifugation was used for urea estimation. To 0.05 ml of the supernatant, 1.0 ml of DAM-TSC solution and 1.5 ml of acid ferric reagent were added. The tubes were covered with marbles and heated for 10 min, along with the standard urea solutions in boiling water bath. After cooling, the absorbance was read at 520 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as μmoles of urea formed/hr/mg protein.
2.15 MEMBRANE BOUND PHOSPHATASES

2.15.1 Assay of Total ATPase (E.C. 3.6.1.3)

Total ATPase was assayed according to the method described by Hokins et al. (1973), which was modified from the method of Evans (1969).

Reagents

1. Imidazole-HCl buffer : 300 mM, pH 7.1.
2. NaCl : 1200 mM
3. KCl : 200 mM
4. MgCl₂ : 60 mM
5. ATP : 40 mM
6. Trichloro acetic acid (TCA): 4%

Procedure

For the assay of total ATPase activity, the incubation medium in a total volume of 1.0 ml contains the following: 0.1 ml each of buffer, sodium chloride, potassium chloride, magnesium chloride and enzyme solution. The mixture was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 2.0 ml of TCA. The suspension was centrifuged and the phosphorous content of the supernatant was estimated according to Fiske and Subbarow (1925) method.

The enzyme activity was expressed as μmoles of phosphate liberated/min/mg protein under incubation conditions.
2.15.2 Assay of Na\(^+\),K\(^+\)-ATPase (E.C. 3.6.1.37)

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

Reagents

1. Tris-HCl buffer : 90 mM, pH 7.5.
2. MgSO\(_4\) : 50 mM
3. KCl : 50 mM
4. NaCl : 600 mM
5. EDTA : 1 mM
6. ATP : 40 mM
7. Trichloro acetic acid (TCA) : 10%
8. Ammonium molybdate : 2.5% in 5 N H\(_2\)SO\(_4\)
9. ANSA

Procedure

The incubation mixture contained 1.0 ml of Tris-HCl buffer, 0.2 ml each of magnesium sulphate, potassium chloride, sodium chloride, EDTA, ATP and the homogenate. The mixture was incubated at 37\(^\circ\)C for 15 min. The reaction was arrested by the addition of 1.0 ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated according to Fiske and Subbarow (1925) method.

The enzyme activity was expressed as \(\mu\)moles of phosphate liberated/min/mg protein under incubation conditions.
2.15.3 Assay of Ca\textsuperscript{2+}-ATPase (E.C. 3.6.1.38)

The activity of Ca\textsuperscript{2+} - ATPase was assayed according to the method of Hjerten and Pan (1983).

**Reagents**

1. Tris-HCl buffer : 125 mM, pH 8.0
2. CaCl\textsubscript{2} : 50 mM
3. ATP : 10 mM
4. Trichloro acetic acid (TCA) : 10%

**Procedure**

The incubation mixture containing 0.1 ml each of Tris-HCl buffer, calcium chloride, ATP and homogenate. After incubation at 37°C for 15 min, the reaction was arrested by the addition of 1.0 ml TCA. The amount of phosphorus liberated was estimated according to the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as μmoles of phosphate liberated/min/mg protein under incubation conditions.

2.15.4 Assay of Mg\textsuperscript{2+}-ATPase (E.C.3.6.1.39)

The activity of Mg\textsuperscript{2+} - ATPase was assayed according to the method of Ohnishi *et al.* (1982).
Reagents

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

Procedure

The incubation mixture containing 0.1 ml each of Tris-HCl buffer, magnesium chloride, ATP and the homogenate. The reaction mixture was incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 ml TCA. The liberated phosphorus was estimated according to the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as μmoles of phosphate liberated/min/mg protein under incubation conditions.

2.16 GLYCOPROTEIN COMPONENTS

2.16.1 Separation of Tissue and Plasma Glycoprotein Components

The plasma glycoproteins were precipitated with alcohol. To 0.1 ml of plasma, add 2.0 ml of alcohol and centrifuged. The supernatant was decanted. The precipitate and defatted tissues were hydrolysed with acid to liberate protein bound hexose, hexosamine and sialic acid.
2.16.2 Hydrolysis of Samples for Glycoprotein Estimation

The precipitate was washed thrice with 5% TCA as mentioned by Glossman and Naville (1971). Both the alcoholic precipitate and a known amount of defatted tissue were hydrolysed with 1.0 ml of 2N HCl and 1% phosphotungstic acid at 100°C for 4 h. The hydrolysed material was neutralized with sodium hydroxide. Aliquots of these neutralized samples were used for the analysis of hexose and hexosamine. For sialic acid estimation, the samples were hydrolysed with 0.1 N \( \text{H}_2\text{SO}_4 \) for 60-90 min at 90°C. Thus the hydrolysed samples was used for the analysis of glycoprotein components.

2.16.3 Estimation of Hexose

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

Reagents

1. Orcinol - sulphuric acid reagent
   
   Reagent A: Sulphuric acid water mixture (3:2, v/v)
   
   Reagent B: 1.6 g of orcinol in 100 ml of water.
   
   Mix reagent A and B in the ratio of 15:2 (v/v)

2. Hydrochloric acid: 4 N

3. Sodium hydroxide: 4 N

4. Standard Hexose: Equal quantities (10 mg/100 ml) of galactose and mannose were dissolved in water to give a concentration of 100 µg/ml.
Procedure

0.5 ml of the neutralized sample was made upto 1 ml with water, 2 ml of orcinol-sulphuric acid reagent was added to the tubes in ice-cold condition, very slowly. The tubes were heated at 80°C for 15 min, cooled and the colour intensity was read at 540 nm after 20 min. Standard hexose in the range of 20-100 μg/ml and blank were treated in the same manner.

The values were expressed as μg/mg of dry tissues and mg/dl in plasma.

2.16.4 Estimation of Hexosamine

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

Reagents

1. Acetyl acetone reagent: 3.5% acetyl acetone was prepared in 1 M trisodium phosphate and 0.5 M potassium tetraborate mixed in the ratio of 98:2 (v/v).

2. Ehrlich's reagent: 320 mg of p-dimethylaminobenzaldehyde was dissolved in 21 ml of isopropanol and 3 ml of concentrated hydrochloric acid.

3. Hydrochloric acid: 4 N

4. Sodium hydroxide: 4 N

5. Standard hexosamine: Galactosamine hydrochloride solution containing 10 mg/100 ml was prepared.
Procedure

To 0.8 ml of the neutralized sample, 0.6 ml of acetyl acetone reagent was added. Blank contained 0.8 ml of distilled water. The tubes were heated in a boiling water bath for 30 min. The tubes were cooled and 2 ml of Ehrlich's reagent was added. The colour developed was read at 540 nm. Standard galactosamine in the range of 10-40 μg was also processed in the same manner.

The values were expressed as μg/mg of dry tissues and mg/dl in plasma.

2.16.5 Estimation of Sialic acid

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

Reagents

1. 0.2 M sodium meta periodate in 9 M phosphoric acid
2. 10% sodium arsenite in 0.5 M sodium sulphate in 0.1 N sulphuric acid.
3. 0.6% thiobarbituric acid in 0.5 M sodium sulphate
4. Acidified butanol: 100 ml of butanol containing 5 ml of concentrated hydrochloric acid.
5. Standard n-acetyl neuraminic acid solution containing 100 μg/ml, in distilled water.
**Procedure**

To 0.2 ml of the hydrolysed sample, 0.2 ml sodium metaperiodate reagent was added and kept at 37°C for 20 min. Then 0.2 ml of sodium arsenite was added and shaken well. After adding 3 ml of thiobarbituric acid, the tubes were heated in a boiling water bath for 15 min. The tubes were cooled and 5 ml of acidified butanol was added. The absorbance of the pink butanolic phase, after centrifugation was read at 540 nm. Standards and blank were processed in the same manner.

The values were expressed as µg/mg of dry tissues and mg/dl in plasma.

**2.17 LYSOSOMAL ENZYMES**

**2.17.1 Separation of Lysosomes**

The liver and kidney tissues were rinsed in ice-cold 0.25 M sucrose solution, blotted, weighed and minced. The enzyme extracts were prepared by homogenizing the tissue samples in 0.25 M sucrose solution at 4°C. A portion of this preparation was used to determine the total activity. Another portion of the homogenate was subjected to differential centrifugation and the lysosomal fraction was separated at 15,000 g for 20 min. (Wattiaux et al., 1977).

**2.17.2 Assay of β-D-Glucuronidase (EC 3.2.1.31)**

β-D-glucuronidase activity was determined by the method of Hultberg et al. (1976).
Reagents

1. Sodium phosphate buffer: 0.1 M, pH 4.5
2. p-nitrophenyl β-D-glucuronide: 0.01 M in 0.1 M sodium phosphate buffer
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 source and was made upto 1.0 ml with assay buffer, incubated at 37°C for 1 hr. The reaction was arrested by the addition of 3.0 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 Shimadzu UV spectrophotometer.

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

2.17.3 Assay of β-D-N-Acetyl glucosaminidase (EC 3.2.1.30)

The activity of β-N-Acetyl glucosaminidase was determined by the procedure of Moore and Morris (1982).
Reagents

1. Citrate buffer: 0.1 M, pH 4.5
2. Substrate: 2 mM p-nitrophenyl β-N-acetyl glucosaminide.
3. 0.2 M Glycine - NaOH buffer, pH 11.7 containing 2 M SDS
4. Standard: p-nitrophenol, 6 mM.

Procedure

Known aliquots (0.2 ml) of the enzyme source was added to 0.5 ml of incubation buffer containing 2 mM substrate (final concentration) and incubated at 37°C for 2 hr. The substrate p-nitrophenyl β-N-acetyl glucosaminide was dissolved in 0.1 M citrate buffer. At the end of the incubation period, the reaction was stopped by the addition of 4.0 ml of 0.2 M glycine NaOH buffer, pH 11.7 containing 2 M SDS and the contents were centrifuged. To the aliquots of supernatants 0.5 M NaOH was added and the absorbance was measured at 410 nm.

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

2.17.4 Estimation of β-D-Galactosidase (EC 3.2.1.23)

The activity of β-D-Galactosidase was assayed by the method of Conchie et al. (1967).
Reagents

1. 0.2 M Na$_2$HPO$_4$ – 0.1 M citric acid, pH 5.0
2. p-nitrophenyl β-D-galactoside: 5mM
3. Glycine - NaOH buffer: 0.4 M, pH 10.4
4. p-nitrophenol standard.

Procedure

The incubation mixture contained 2.0 ml of 0.2 M Na$_2$HPO$_4$, 0.1 M citric acid buffer, 0.5 ml of 5 mM p-nitrophenyl β-D-galactoside and 0.5 ml of enzyme source. Incubation was carried out for 1 hr at 37°C. The reaction was terminated by the addition of 4.0 ml of glycine - NaOH buffer. The reaction mixture was centrifuged and the absorbance of the released p-nitrophenol in the supernatant was measured at 410 nm using Shimadzu UV spectrophotometer. A standard p-nitrophenol was run simultaneously.

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

2.17.5 Assay of Cathepsin D (EC 3.4.23.50)

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

1. Sodium formate buffer : 0.2 M, pH 3.5
2. Substrate : 1.5% haemoglobin in sodium formate buffer
3. TCA : 10% solution
4. Sodium carbonate : 4% solution in 0.1 M NaOH
5. Standard tyrosine : 100 mg/ml in dilute HCl
6. Folin’s Phenol reagent : The reagent was diluted 1:2 with distilled water just before use.

Procedure

The incubation mixture 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 h. 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 min, the tubes were centrifuged at 1000 x g for 15 min. 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 and the blue colour developed was read at 670 nm in Shimadzu UV spectrophotometer. Standard containing aliquots of tyrosine and blank containing water were also treated in a similar manner.

The enzyme activity was expressed as μmoles of tyrosine released/hr/mg protein.
2.17.6 Assay of Acid Phosphatase (E.C.3.1.3.2)

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

Reagents

1. Acetate-acetic acid buffer: 0.1 M, pH 4.8
2. Disodium phenyl phosphate solution: 0.01 M
3. Folin's phenol reagent: The reagent was diluted 1:2 with distilled water just before use.
4. Sodium carbonate: 15% solution
5. Standard phenol: 100 mg of recrystallized phenol in 100 ml of water. 100 µg of phenol/ml was prepared and used as working standard.

Procedure

The incubation mixture of 3.0 ml contained 1.5 ml of buffer, 1.0 ml of substrate and 0.5 ml of the enzyme source. The tubes were incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 ml of Folin's phenol reagent. The control tubes received the enzyme after arresting the reaction. The contents were centrifuged and to the supernatant, 1.0 ml of 15% sodium carbonate was added and the mixture incubated for 15 min at 37°C. The colour was read at 640 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as µmoles of phenol liberated/min/100 mg protein.
2.18 ASSESSMENT OF DNA DAMAGE (COMET ASSAY)

DNA damage was assessed by using alkaline single cell gel electrophoresis (comet assay) according to the method of Singh *et al.* (1988).

**Reagents**

1. 100 mM Phosphate buffered saline (PBS), pH 7.4
2. 1% Low melting point agarose (LMPA)
3. 1% Normal melting point agarose (NMPA)
4. Hank's balanced salt solution (HBSS) in 0.02 M EDTA
5. Lysing solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris-HCl, pH adjusted to 10.0 with NaOH; 1% Triton X-100 and 10% DMSO were added freshly)
6. Electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13.0)
7. 0.4 M Tris - HCl, pH 7.5
8. Ethidium bromide (2 μg/ml)

**Procedure**

100 μl of 1% NMPA in phosphate buffered saline was dropped on to frosted slides, immediately covered with cover slip and kept for 10 min in a refrigerator to solidify. Then cover slips were removed and 100 μl of LMPA containing cells (100 μl of cell suspension in HBSS and 100 μl of LMPA) were added to the slides. The cover slips were replaced and the slides were kept in the refrigerator for another 10 min to solidify the LMPA. After this,
the cover slips were removed and a top layer of 100 µl of LMPA was added and the slides were again cooled for 10 min.

After removal of cover slips, the slides were immersed in cold lysing solution. The slides were kept in dark at 4°C for at least 1 hr. To prevent the occurrence of additional DNA damage, the following steps were performed under dim light. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank. The unit was filled with a freshly made electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to alkali for 20 min to allow DNA unwinding.

An electric current of 25 V and 300 mA was applied for 20 min to electrophoresis. After electrophoresis, the slides were placed horizontally, and neutralized with Tris-HCl. Finally, 50 µl of ethidium bromide was added to each slide and covered with a cover slip and analysed using a fluorescence microscope (Nikon, Japan) with a calibrated scale in ocular. Images of randomly selected 50 cells were analysed from each sample. For each cell, the length of the image (diameter of the nucleus plus migrated DNA) was measured.

2.19 INSULIN RECEPTOR STUDIES

2.19.1 Isolation of Liver Plasma Membrane

Plasma membrane isolation was performed according to the method of Neville (1968). Briefly, rats were sacrificed and transcardially perfused with saline (0.9% NaCl) until the livers were blanched. The livers were then excised, minced, and added to 4 volume (wt/vol) of 0.25 M STM (0.25 M
sucrose, 5 mM Tris, pH 7.2 and 1 mM MgCl$_2$) of buffer. The suspension was then homogenized with a 40 ml Dounce-type glass homogenizer with a loose-fitting pestle. The resulting homogenate was centrifuged at 280 x g for 5 min. The supernatant was stored and the pellet was resuspended by three strokes of the loose Dounce in half the original volume of 0.25 M STM buffer. The suspension was then centrifuged as above. The first and second supernatants were pooled and centrifuged at 1,500 x g for 10 min.

The resulting pellets were pooled and resuspended by three strokes of the loose Dounce in 1-2 ml of 0.25 M STM buffer/g liver. STM buffer (2.0 M) was added to obtain a density of 1.18 g/cm$^3$ (1.42 M) and sufficient STM buffer was added to bring the volume to approximately twice that of the original homogenate (i.e. 10% wt/vol). Aliquots of 35 ml samples were added to SW 28 centrifuge tubes and overlaid with 2-4 ml of 0.25 M STM buffer. After centrifugation for 60 min at 82,000 x g, the pellicle at the interface was collected and resuspended with a loose Dounce in sufficient 0.25 M STM buffer to obtain a density of 1.05 g/cm$^3$. The suspension was centrifuged at 1,500 x g for 10 min. The pellet, which consisted of the whole plasma membrane fraction, was resuspended in 1 ml of STM buffer/g liver.

2.19.2 Estimation of Protein

The liver plasma membrane protein content was estimated according to the method of Lowry et al. (1951).
2.19.3 Binding of $[^{125}\text{I}]-\text{Insulin}$ to Rat Liver Plasma Membrane

Radiolabelling insulin

Chemicals required

1. Chloramine–T: 10 mg/ml
2. Potassium phosphate buffer; pH 7.4.
3. Sodium metabisulphite: 10 mg/ml
4. Potassium iodide: 10 mg/ml
5. Bovine serum albumin: 0.1%
6. Bovine insulin (Sigma Chemicals Co., USA).

Insulin was iodinated by modifications of chloramine-T method (Hollenberg and Cuatrecasas, 1973). The chloramine-T method involved a 40 sec incubation of 1 mCi of Na-$[^{125}\text{I}]$ (100 mCi/ml) with 6.75 mM chloramine-T (20 μl from a fresh 10 mg/ml stock) and 4 μg of insulin in 0.1 ml of potassium metabisulphite and 34 mM potassium iodide from 10 mg/ml stocks. The reaction mixture was dialyzed overnight in distilled water containing 0.25 M phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin.

The binding of $[^{125}\text{I}]-\text{insulin}$ to rat liver plasma membrane was assayed as described by Cuatrecasas (1971) with slight modifications.

The standard binding assay was conducted at a final pH of 7.5 in 50 mM Tris-HCl (with 100 mM NaCl, 0.5 mM CaCl$_2$ and 20 mg/ml BSA) containing 100 μg of the plasma membrane protein and increasing
concentrations of $[^{125}\text{I}]-\text{insulin}$ (5-25 $\mu$g in terms of protein). This represented the total binding of the labelled insulin to the insulin receptor in the rat liver plasma membrane. Another assay was carried out as described above, but in the presence of excess (100 fold) of unlabelled insulin. This set of tubes represented the non-specific binding which is the binding that was not displaceable by excess concentrations of unlabelled insulin and all the total binding were corrected to Specific Binding by subtracting NSB from the total binding.

The binding reactions were initiated by adding the indicated amount of $[^{125}\text{I}]-\text{insulin}$ and the incubation was done at 4$^\circ$C in an ice water bath for 60 min. The reaction mixture was centrifuged at 10,000 x g for 30 min to separate the $[^{125}\text{I}]-\text{insulin}$ bound plasma membrane from the reaction mixture. The supernatant was drained and the membrane pellet was washed with the medium without suspending it. The supernatant was decanted and the precipitate remaining in the tube was counted for radioactivity in a gamma counter.

The specific binding was calculated by subtracting the non-specific binding from total binding at each concentration. The values were expressed as mean of triplicate analysis. The specific binding curves were obtained by plotting the counts per minute, versus the concentration of $[^{125}\text{I}]-\text{insulin}$ added. Scatchard analysis of the specific binding data was done. Scatchard curves were obtained by plotting the ratio of bound to free $[^{125}\text{I}]-\text{insulin}$ versus the concentration of bound $[^{125}\text{I}]-\text{insulin}$. Simple linear regression analysis of the specific binding data was done (Campbell, 1993). The
x-intercept represented $B_{\text{max}}$ i.e., the maximum binding capacity of the $[^{125}\text{I}]$-insulin to the insulin receptor present in the plasma membrane.

2.20 STATISTICAL ANALYSIS

The values are expressed as mean ± SD for six rats in each group. All other data were analysed with SPSS/10.0 student software. Hypothesis testing method included one way analysis of variance (ANOVA) followed by post hoc testing performed with least significant difference (LSD) test. The ‘p’ value of less than 0.05, 0.01, 0.001 were considered to indicate statistical significance.
REFERENCES


