Materials and Methods
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Male adult albino rats of Wistar strain weighing between 100-120 g were procured from "Tamil Nadu Veterinary and Animal Sciences University", Chennai, Tamil Nadu, India. The animal house was well-ventilated and the animals had 12 ± 1 h day and night rhythm throughout the experimental period. The animals were housed in large spacious polypropylene cages and they were given food and water ad libitum. During the course of the experiment, the temperature remained between 27°C and 37°C.

2.1.1.1 Diet

The animals received a balanced commercially available pelleted rat Feed (Gold Mohur, M/s Hindustan Lever Ltd, Mumbai, India) and water ad libitum. The feed contained Protein (21%), Lipids (5%), Crude Fibre (4%), ash (8%), Calcium (1%), phosphorus (0.6%), Nitrogen Free extract (55%) and Metabolizable energy of 3600 KCal/kg. Guidelines for breeding and Experiments on Animals, 1998 defined by the Ministry of Social Justice and Empowerment, Government of India was followed (IAEC Number 07/015/02).
2.2 INDUCTION OF FIBROSARCOMA

Fibrosarcoma was induced in Wistar strain male albino rats by subcutaneous implantation of millipore filter disc impregnated with a 5% suspension of 20-methylcholanthrene in paraffin oil (Nagarajan and Sankaran, 1973). Tumours which appear in about 4 weeks after implantation were highly localised and were maintained by serial transplantation. The tumour was minced and suspended in normal saline. A suspension of about $1 \times 10^6$ cells in 0.5 ml of saline was injected subcutaneously in to the thigh. The transplanted tumour became palpable in 4 to 6 days time and measurable on day 9th and then steadily grew up to the end of the second week after which necrosis set in and the animal eventually died in about 4 weeks.

2.2.1 Plant Source

The plant *Indigofera aspalathioides* were obtained, identified and authenticated from chief botanist, Tamil Nadu Aromatic Medicinal plants corporation Limited (TAMPCOL), Government Arignar Anna Siddha medical college and Hospital campus, Arumbakkam, Chennai, Tamil Nadu, India. The aerial parts of the plant (Leaves, Seeds, Stem) was used for this study.

2.2.2 Aqueous Extraction of the Plant *Indigofera aspalathioides*

1 kg of shade dried coarsely powdered aerial parts of the plant material was charged is an aspiration bottle and allowed to soak in water
for 48 hrs at room temperature. The extract was filtered and concentrated on a water bath and dried in a vacuum.

### 2.2.3 Tumour Weight

Tumour weight was estimated according to the method of Geran et al. (1972). The resultant solid tumour was considered to be prolate ellipsoid with one long axis and two short axis. The two short axis were measured with vernier caliper. The tumour weight was calculated by multiplying the length of the tumour with the square of the width and dividing the product by two.

\[
\text{Tumour weight (g)} = \frac{\text{Length (cm)} \times \text{width (cm}^2)}{2}
\]

Tumour weight obtained from vernier caliper measurements and the actual weight measurement of the tumour were found to be nearly the same.

**Indigofera aspalathiodes**

Vahl ex DC. Prodr. 2

(1825) 231

**Family**: Papilionaceae

**Indian Names**

**Tamil**: Sivanarvembu, Iraivanvembu

**Malayalam**: Mahali

**Sanskrit**: Ratakohomba
Indigofera aspalathoides
2.3 EXPERIMENTAL DESIGN

The rats were divided into four groups with six animals in each group and were given dose regimen as given below.

**Group I**: Control animals  
(Normal Saline 0.9%)

**Group II**: Fibrosarcoma bearing animals

**Group III**: Fibrosarcoma induced animals treated with the aqueous extract of *Indigofera aspalathoides* at a dose of 250 mg/kg.bw for 30 days.

**Group IV**: Animals administered with the aqueous extract of *Indigofera aspalathoides* at a dose of 250 mg/kg.bw for 30 days.

2.4 METHODS

2.4.1 Collection of Samples

The animals were sacrificed by cervical decapitation at the end of the experimental period and blood was collected to separate serum for biochemical analysis. The liver and kidney were dissected out and known weight of liver and kidney tissue were homogenized in 0.1M Tris-HCl buffer (pH 7.4). Animals were starved overnight before sacrifice.

The following biochemical parameters were carried out in the serum, liver and kidney samples.
2.4.2 Estimation of Urea, Uric Acid and Creatinine

1 ml of plasma was diluted with 7 ml of distilled water. 1 ml of sodium tungstate and 1 ml of 2/3N H₂SO₄ were added and centrifuged at 3000 g for 15 minutes. The supernatant were taken for the estimation of urea, uric acid and creatinine.

2.4.3 Estimation of Urea (Natelson et al., 1951)

Reagents

1. Diacetylmonoxirne (DAM): 2% solution in 2% acetic acid.
2. Sulphuric acid - phosphoric acid reagent: Added 150 ml of 85% phosphoric acid to 140 ml of water, mixed and 50 ml of concentrated H₂SO₄ was added slowly.
3. Stock standard: Stock urea standard was prepared containing 2.5 mg/ml.
4. Working standard: Working standards was aimed at by 1 in 100 dilution (1/100).

To 0.5 ml of supernatant, 3.0 ml of DAM and 1.0 ml of acid mixture were added and kept in a boiling water bath for 30 minutes. The tubes were cooled and colour developed was read at 480 nm. Standards were treated similarly. The urea values were expressed as mg/dl.
2.4.4 Estimation of Uric Acid (Caraway, 1963)

Reagents

1. Phosphotungstic acid: To prepare a stock solution 50 ml of 10% sodium tungstate was dissolved in 400 ml of water. 40 ml of 85% phosphotungstic acid was refluxed gently for 2 h, cooled and transferred to a 500 ml flask and made up to the mark with water. This reagent was stored in a brown bottle, diluted 1/10 before use. This can be kept for months in a brown bottle.

2. Sodium carbonate: 10%

3. Stock standard: 100 mg of uric acid and 6 mg of lithium carbonate were dissolved in 50 ml of water. This was heated at 60°C to dissolve the uric acid completely. After cooling, the solution was finally made up to 100 ml with water.

4. Working Standard: 1.0 ml of stock standard was diluted with 10 ml of distilled water.

Procedure

2 ml of supernatant was added in a test tube followed by 0.6 ml of phosphotungstic acid and 0.6 ml of sodium carbonate. 3.0 ml of water as a blank. The blank and standards treated similar manner. The colour developed was read at 640 nm. The uric acid values were expressed as mg/dl.
2.4.5 Estimation of Creatinine (Owen et al., 1954)

Reagents

1. Picric acid : 0.04 M
2. Sodium hydroxide : 0.75 N
3. Stock standard (mg/ml) : 1.0 g of creatinine was dissolved in 0.1 N HCl.
4. Working Standard : Prepared from the stock by diluting 1.0 ml of stock solution to 100 ml with water.

Procedure

To 4 ml supernatant, 1.0 ml of picric acid and 1.0 ml of sodium hydroxide were added and a reagent blank were treated similarly. The tubes were shaken gently with the addition of each reagent. After 15 minutes, the colour developed was read at 540 nm. The creatinine values were expressed as mg/dl.

2.4.6 Total Protein (Lowry et al., 1951)

Reagents

1. Lowry's reagent

Solution A : 2% sodium carbonate in 0.1 N Sodium hydroxide
Solution B : 0.5% copper sulphate in 1% Sodium potassium tartrate.
Solution C : 50 ml of solution ‘A’ and 1 ml of solution ‘B’
Folin - Cio calteau regent: Diluted 1:2 water before use.

Standard: Bovine Serum albumin (BSA) containing 20 mg in 100 ml was prepared.

Procedure

0.1 ml of the sample was made up to 1.0 ml with distilled water, 5 ml of Lowry’s reagent was added, shaken well and allowed to stand for 10 minutes. 0.5 ml of Folin - Cocalteau reagent was added, shaken well, and kept at room temperature for 20 min. Standard and blanks were prepared similarly to read along with sample were treated in the similar manner. The intensity of blue colour developed was read at 620 nm. The total protein content was expressed as mg/g of wet tissue.

2.4.7 Estimation of Nucleic Acids

2.4.7.1 Extraction of nucleic acids (Schneider, 1957)

Known amount of the tissue were homogenized in 5.0 ml of ice-cold distilled water using a Potter - Elvehjem homogenizer with a teflon pestle, 5.0 ml of 5% trichloroacetic acid was added to the homogenate and this was kept in ice-for 30 minutes to allow complete precipitation of proteins. The mixture was centrifuged and the precipitate obtained was washed thrice with ice-cold 10% TCA. Then it was treated with 95% ethanol to remove lipids. The final precipitate was heated at 90°C for 15 minutes, which facilitated the quantitative separation of nucleic acids from protein. The supernatant after centrifugation was used for the estimation of DNA and RNA.
2.4.7.2 Deoxy ribonucleic acid (DNA) (Burton, 1956)

Reagents

1. Diphenylamine reagent: 1.5 g of diphenylamine was dissolved in 100 ml of redistilled acetic acid and 1.5 ml of concentrated H₂SO₄ was added. The reagent was stored at 4°C in dark before use. 0.1 ml of aqueous acetaldehyde (0.16%) was mixed with every 20 ml of the reagent.

2. Stock standard: Highly polymerized Calf-thymus DNA was dissolved in 5 mM NaOH to give a concentration of 0.4 mg/ml.

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

Procedure

A known volume of the nucleic acid extract was made upto 3.0 ml with 1N perchloric acid. This was mixed with 2.0 ml of diphenylamine reagent. A reagent blank and standards were also carried out. This was kept in a boiling water bath for 10 minutes and the blue colour developed was read at 640 nm in a spectrophotometer. The values are expressed as mg/g wet tissue.
2.4.7.3 Ribonucleic acid (RNA) (Rawal et al., 1977)

Reagents

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

2. Standard: This was prepared by dissolving 2.0 mg of yeast RNA in 600 ml of 5% TCA.

Procedure

Aliquots of nucleic acid extract were made up to 2.0 ml with 5% TCA. To this 3.0 ml of orcinol - Ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 minutes. Reagent blank and standards were also treated in the same way. The tubes were cooled and the colour developed was measured at 640 nm using a spectrophotometer. The values are expressed as mg/g wet tissue.

2.4.8 Estimation of Marker Enzymes

2.4.8.1 Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (Balasubramanian et al., 1983)

Reagents

1. Carbonate - bicarbonate buffer : 0.1 M, pH 10.0
2. Disodium phenylphosphate : 0.1M
3. Magnesium Chloride : 0.1M
4. Sodium Carbonate : 15%
5. Folin's Phenol reagent :
6. Standard : 100 mg of phenol was dissolved in 100 ml of distilled water.

Procedure

The incubation mixture containing 1.5 ml of buffer, 1.0 ml of substrate and 0.1 ml of magnesium chloride were preincubated at 37°C for 10 minutes. Then 0.1 ml of the homogenate was added and incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of folin's Phenol reagent and centrifuged at 5000 g for 10 minutes. Control without enzyme was also incubated and the homogenate was added after the addition of folin's phenol reagent. To the supernatant 1.0 ml of sodium carbonate was added. After 10 minutes the blue colour developed was read at 640 nm using a spectrophotometer. The enzyme activity was expressed as μmoles of phenol liberated/mg protein/min.

2.4.8.2 Acid phosphatase (Orthophosphoric - Monoester phosphohydrolase, EC 3.1.3.2 (Balasubramanian et al., 1983))

Reagents

1. Citrate buffer : 0.1M, pH 4.3
2. Disodium phenylphosphate : 0.1M
3. Sodium carbonate : 15%
4. Folin's phenol reagent

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

**Procedure**

The incubation mixture containing 1.5 ml of buffer and 1.0 ml of substrate were preincubated at 37°C for 10 minutes. Then 0.1 ml of homogenate was added and incubated at 37°C for 30 minutes. After incubation, the reaction was arrested by the addition of 1.0 ml of Folin's phenol reagent and centrifuged at 5000 g for 5 min. Control without enzyme was incubated and the homogenate was added after the addition of Folin's phenol reagent. To the supernatant, 1.0 ml of sodium carbonate was added. After 10 minutes the blue colour developed was read at 640 nm using a photochem colorimeter. The enzyme activity was expressed as μmoles of phenol liberated/mg protein/min.

**2.4.8.3 Aspartate aminotransferase (L-Aspartate: 2-oxoglutarate amino-transferase, EC 2.6.1.1) (King, 1965a)**

**Reagents**

1. Phosphate buffer: 0.1 M (pH 7.5)

2. Substrate: 1.33 g of aspartic acid and 15 mg of 2-oxoglutarate were dissolved in 20.5 ml of IN sodium hydroxide and made up to 100 ml with buffer.
3. **2,4-dinitrophenyl hydrazine (DNPH):** 0.02% of DNPH in hydrochloric acid.

4. **Sodium hydroxide:** 0.4N

5. **Standard:** 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contains 1 mole of pyruvate in 1 ml.

**Procedure**

1 ml of substrate was incubated at 37°C for 10 minutes. Then 0.2 ml of enzyme was added and the mixture was incubated at 37°C for one hour. To the control tubes, the enzyme was added after the reaction and it was arrested by the addition of 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 30 min. Then 5.0 ml of sodium hydroxide was added. A set of pyruvate solution standard were also treated in a similar manner. The colour developed was read at 540 nm using a photochem colorimeter. The enzyme activity was expressed as μmoles of pyruvate liberated/mg protein/min.

2.4.8.4 **Alanine aminotransferase (L-Alanine: 2-Oxoglutamate aminotransferase, EC 2.6.1.2) (King, 1965b)**

**Reagents**

1. **Phosphate buffer:** 0.1M, pH 7.5

2. **Substrate:** 1.78 g of DL-alanine and 30 mg of 2-oxoglutarate were dissolved in 20 ml of buffer, 0.5 ml of IN sodium hydroxide was added and made upto 100 ml with distilled water.
2.4-dinitrophenyl hydrazine (DNPH): 0.02% of DNPH in hydrochloric acid.

Sodium hydroxide: 0.4N

Standards: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 mole of pyruvate/ml.

Procedure

1.0 ml of substrate was incubated at 37°C for 10 minutes. after 10 minutes 0.2 ml of enzyme solution was added. The tubes were incubated at 37°C for 30 minutes. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 20 minutes. Then 5.0 ml of 0.4 N Sodium hydroxide was added and then the colour developed was read at 540 nm using a photochem colorimeter. The enzyme activity was expressed as μmoles of pyruvate liberated mg protein/min.

2.4.8.5 Assay of 5'-Nucleotidase (EC.3.1.3.5) (Luly et al., 1972)

Reagents

1. Tris - HCl buffer 184 mM, pH 7.5: 2.23 g Tris was dissolved in 100 ml of deionised water and pH was adjusted to 7.5 with HCl.

2. Magnesium sulphate 50 mM: 616.2 mg of MgSO₄ was dissolved in 50 ml of deionised water.

3. Potassium chloride 650 mM: 1.211g of KCl was dissolved in 25 ml of deionised water.
EDTA 1mM: 37.23 mg of EDTA was dissolved in 100 ml of deionised water.

TCA 10%

Substrate: 5'-Adenosine monophosphate 40 mM was prepared by dissolving 69.4 mg in 5.0 ml of deionised water.

Procedure

The reaction mixture contained 1.0 ml of Tris-HCl buffer and 0.1 ml each of magnesium sulphate, KCl, EDTA, substrate and water. The reaction was initiated by the addition of 0.2 ml serum or tissue homogenate and incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 2.0 ml of 10% TCA and centrifuged.

The enzyme activity was expressed as nmole of phosphorus liberated/mg protein/min.

2.5 LYSOSOMAL ENZYMES

2.5.1 Assay of N-acetyl β-D-glucosaminidase (EC, 3.2.1.30) (Marhun, 1976)

Reagents

Citric acid - sodium citrate buffer 0.1 M (pH 4.2): 46 ml of 0.1 M sodium citrate solution was mixed with 54 ml of 0.1 M citric acid solution.
Glycine buffer 0.2 M (pH 10.7): Glycine buffer was prepared by mixing equal volume of 0.2 M glycine, 0.125 M sodium carbonate and 0.1 M sodium chloride.

Substrate: 3.42 mg 4-nitrophenyl-N-acetyl-glucosaminide was dissolved in 1.0 ml of citrate buffer.

Standard: 5 ml of p-nitrophenol in 100 ml of distilled water.

**Procedure**

The incubation mixture contained 0.5 ml of substrate and incubated at 37°C for 40 minutes. The reaction was terminated by the addition of 2.2 ml of 0.2 M glycine buffer. The colour developed was measured at 420 nm in a spectrophotometer. A standard p-nitrophenol was run simultaneously. The N-acetyl β-D-glucosaminidase activity was expressed as n moles of p-nitrophenol liberated/h.

**2.5.2 Assay of β-D-glucuronidase (EC 3.2.1.31) (Kawai and Anno, 1971)**

**Reagents**

1. Acetate buffer - 0.1 M pH 5.0
2. Glycine buffer - pH 10.7
   This was prepared by mixing equal volume of 0.2 M glycine, 0.125 M Sodium carbonate and 0.1M sodium chloride
3. Substrate: 1 mg of p-nitrophenyl-β-D-glucuronide was dissolved in distilled water.
Standard: 5 mg of p-nitrophenol was dissolved in 100 ml of distilled water.

Procedure

0.05 ml of substrate, 0.05 ml of acetate buffer, 0.3 ml of homogenate were incubated at 37°C for one hour. The reaction was arrested by the addition of 3.9 ml of glycine buffer. Standards were also run simultaneously along with a water blank. The colour developed was read at 420 nm. The enzyme activity was expressed as μmoles of p-nitrophenol liberated/mg protein/h.

2.6 ESTIMATION OF GLYCOPROTEINS

A known volume of serum was mixed with 2.0 ml of alcohol, left for 20 minutes and centrifuged. To the precipitate, 3.0 ml of 3N HCl was added and hydrolysed for 4 hours at 100°C in a water bath for the estimation of hexose and hexosamine. Similarly, a known amount of delipidised residues of tissues, prepared according to the method of Floch et al., 1951.

2.6.1 Hexose (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
Regent A and B were mixed in the ratio of 15:2 (v/v) just before use.

Hexose standard solution:
50 mg of galactose and 50 mg Mannose were dissolved in 100 ml distilled water. This solution was diluted to 1:10 proportion which gave a concentration of hexose 100 μg/ml.

Procedure

To 0.5 ml of neutralised sample, 7.0 ml of orcinol reagent was added very slowly, by placing the tubes in ice water bath. The contents were mixed well and the tubes were heated at 80°C for 15 minutes. Then the colour developed in dark, after cooling the colour was read at 540 nm using a spectrophotometer. Standard solution containing 25-100 μg of hexose and blank containing 0.5 ml of water instead of neutralised sample was also treated in a similar manner. The hexose content was expressed as mg/dl for serum and mg/g of defatted tissue for liver and kidney.

2.6.2 Hexosamine (Wagner, 1979)

Reagents

1 Acetyl acetone reagent 3.5%:
Reagent A: Trisodium phosphate 1M-38g of trisodium phosphate was dissolved in 100 ml of water.
Reagent B: Potassium tetraborate : 0.5M-1.9g of potassium tetraborate was dissolved in 10 ml of water. 3.5 ml of
acetylacetone was added to the mixture containing reagent A and B in the ratio of 98:2 (v/v)

2 Ehrlich's reagent: 320 mg of p-dimethyl aminobenzaldehyde was dissolved in 21 ml of isopropanol and 3.0 ml of concentrated HCl was added to it.

3 Standard: 10 mg of galactosamine was dissolved in 100 ml of water which gave a concentration of 100 μg/ml.

Procedure

0.5 ml of the neutralised sample was made upto 1.0 ml with water. Standard galactosamine was also made upto 1.0 ml. Blank comprised of 1.0 ml water, 0.6 ml of acetyl acetone reagent was added to all the tubes and heated in a boiling water bath for 30 minutes. After cooling, 2.0 ml of Ehrlich's reagent was added and the contents were shaken well. The pink colour developed was read at 540 nm against a blank using a spectrophotometer. Hexosamine content was expressed as mg/dl for serum and mg/g of defatted tissue of liver and kidney.

2.6.3 Sialic Acid (Warren, 1969)

A known volume of serum was mixed with 2.0 ml alcohol, kept for 20 minutes and centrifuged. To the precipitate, 1.0 ml of 0.1 N H₂SO₄ was added and hydrolysed for one hour at 80°C. Similarly, a known amount of delipidised residues of the tissues were hydrolysed with 1.0 ml of 0.1N H₂SO₄ at 80°C for one hour. The hydrolysed materials were neutralised and used for the estimation of sialic acid.
Reagents

1. Periodate 0.25 M: 53.4 mg of Sodium Periodate was dissolved in 100 ml of 0.1N H₂SO₄.

2. Sodium arsenite 4%: 4g of sodium arsenite was dissolved in 100 ml of 0.5 N HCl.

3. Thiobarbituric acid (TBA) 0.1 M: 144 mg of TBA was dissolved in 10 ml of water. The pH was adjusted to 9.0 with 6N NaOH and the reagent was prepared freshly.

4. Acidified butanol: 95 ml of n-butanol was mixed with 5.0 ml of HCl.

5. Standards: 10 mg of N-acetyl neuraminic acid was dissolved in 100 ml of distilled water which has a concentration of 100 μg/ml.

Procedure

To 0.5 ml of neutralised sample, 0.25 ml of periodate was added and incubated at 37°C for 30 minutes. After incubation the reaction was arrested by the addition of 0.25 ml of arsenite. The tubes were shaken well, 2.0 ml of TBA was added and the tubes were heated in a boiling water bath for 6 minutes. After cooling, 5.0 ml of acidified butanol was added and the butanol phase was separated after shaking well. The absorbance was read at 540 nm against a blank treated similarly using a spectrophotometer. Standard (N-acetyl neuraminic acid) was also treated similarly. Sialic acid content was expressed as mg/dl for serum and mg/g of defatted tissue for liver and kidney.
2.7 ESTIMATION OF SODIUM AND POTASSIUM

Sodium and potassium were determined on a diluted aliquot of sample solution by using flame photometry.

Standard: 2.2919 g of Na₂SO₄ and 1.85 g of K₂SO₄ was weighed accurately and dissolved in 200 ml standard flask using deionised water. This solution contains 2000 ppm each of sodium and potassium.

Working Standard: The above stock solutions were diluted to give the concentration of 10 ppm to 50 ppm and were used as working standards.

Procedure

A known concentration of sodium and potassium solutions were used as standards. Deionised water was used to set zero. The fluid analysis is sprayed as a fine mist into a non-ruminous flame, which becomes coloured according to the characteristic emission of the sodium and potassium present in the fluid. The flame is simultaneously monitored by both the sodium and potassium channels.

A dilution correction was made for the tissue, and serum sodium and potassium concentrations with the known standard concentrations of sodium and potassium.
2.7.1 Estimation of Calcium

Calcium was estimated by atomic absorption spectrometry (Perkin-Elmer model 2380).

Standards: 1.249 g of dried calcium carbonate in 25 ml of 1N HCl and diluted to 1 litre to give a standard containing 500 g/ml calcium.

Light source: Hollow Cathode lamp
Lamp Current: 3.0 mA
Flame Type: Air-acetylene

<table>
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<th>Wave length nm</th>
<th>Slit width nm</th>
<th>Working range g/ml</th>
<th>Sensitivity g/ml</th>
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<tbody>
<tr>
<td>422.7</td>
<td>4 (1.4nm)</td>
<td>1-5</td>
<td>0.08</td>
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</tbody>
</table>

2.7.2 Estimation of Magnesium

Magnesium was estimated by atomic absorption spectrometry (Perkin-Elmer Model 2380).

Standard: 1 g of magnesium metal (ribbon or turning) in 50 ml of 5N HCl and diluted to 1 litre with deionised water to give a standard containing 1000 μg/ml magnesium.

Light source: Hollow Cathode lamp
Lamp Current: 3.0 mA
Flame type: Air-acetylene
Adenosine triphosphatases catalyse the conversion of adenosine triphosphate into adenosine diphosphate. During the conversion, one molecule of phosphorus is liberated. The inorganic phosphorus is estimated according to the method of Fiske and Subbarow (1925). The proteins were precipitated with Trichloroacetic acid. The free filtrate reacts with acid molybdate solution to form phospho molybdic acid which is reduced by the addition of 1-amino 2-naphthol-4-sulphuric acid (ANSA) to produce blue colour. The intensity of the colour is proportional to the amount of phosphorus present.

### Wave length
- **nm**: 285.2
- **Slit width (nm)**: 4 (1.4nm)
- **Working range g/ml**: 1-5
- **Sensitivity g/ml**: 0.08

#### 2.7.4 Isolation of Erythrocyte and its Membrane

Blood collected with 3.7% trisodium citrate, as anticoagulant (1 ml), was used for erythrocyte isolation. Plasma was separated by centrifugation of 2000 g for 20 minutes. The packed cells were washed thrice with physiological saline and the plasma free red cells was used for the analysis. Erythrocyte membrane was isolated according to the method of Dodge et al. (1963) with a change in buffer, according to Quist (1980).
Reagents

1. Sodium chloride: 0.89%
2. Tris-HCl buffer: 0.31 M, pH 7.4
3. Tris-HCl buffer: 0.15 M, pH 7.2

Procedure

Packed cells, remaining after the removal of plasma were washed with isotonic saline to remove the buffy coat. The packed cells were then washed three times with isotonic Tris-HCl buffer 0.31M, pH 7.4. Hemolysis was performed by pipetting out the washed red blood cell suspension into polypropylene centrifuge tubes which contained hypotonic buffer (Tris-HCl buffer 0.01M, pH 7.2). Erythrocyte ghosts were sedimented in a high speed refrigerated centrifuge at 20,000 g for 40 minutes.

The ghost sedimented at the bottom was resuspended by swirling with sufficient buffer of the same strength to reconstitute to the original volume. The ratio of the cells to washing solution was approximately 1:3 (v/v). The cell membrane was washed thrice with hypotonic buffer and subsequently the hemolysis. The supernatant, after the last wash, was either pale pink or colourless. The pellet of erythrocyte membrane was dissolved in 10ml of Tris-HCl buffer, 0.31 M, pH 7.4. Aliquots of this was taken for the estimation of various biochemical parameters.
2.7.5 Na⁺/K⁺-ATPase (ATP : Phosphohydrolase, EC 3.6.1.3) (Bonting, 1970)

Reagents

<table>
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<tr>
<td>2</td>
<td>MgSO₄            :</td>
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<td>2.5%</td>
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<td>9</td>
<td>ANSA</td>
<td></td>
</tr>
</tbody>
</table>

Procedure

The incubation mixture contained, 1 ml of Tris-HCl buffer, 0.2 ml each of magnesium sulphate, potassium chloride, sodium chloride, EDTA, ATP and the enzyme sample. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1 ml of 10% TCA. Mixed well and centrifuged. The colour developed was read at 620 nm after 20 min against the reagent blank using a spectrophotometer. The enzyme activity was expressed as μ moles of inorganic phosphorus liberated/mg protein/min.
2.7.6 Mg\(^{2+}\) ATPase (ATP : Phosphohydrolase, EC 3.6.1.3) (Ohnishi et al., 1962)

Reagents

1. Tris - HCl buffer : 375 mM, pH 7.6
2. MgCl\(_2\) : 25 mM
3. ATP : 10 mM
4. TCA : 10%

Procedure

The incubation mixture contained, 0.1 ml each of Tris-HCl buffer, Magnesium chloride, ATP and enzyme sample. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1 ml TCA. The liberated phosphorus was estimated as described by Fiske and Subbarow (1925). The enzyme activity was expressed as μmoles of inorganic phosphorus liberated/mg protein/min.

2.7.7 Ca\(^{2+}\) - ATPase (ATP : Phosphohydrolase, EC 3.6.1.3) (Hjerton and Pan, 1983)

Reagents

1. Tris - HCl buffer : 125 mM, H 8.0
2. CaCl\(_2\) : 50 mM
3. ATP : 10 mM
4. TCA : 10%
Procedure

The incubation mixture contained, 0.1 ml each of Tris - HCl buffer, Calcium chloride, ATP and enzyme sample. After incubation at 37°C for 15 minutes, the reaction was arrested by the addition of 1 ml TCA. The amount of phosphorus liberated was estimated and described by Fiske and Subbarow (1925). The enzyme activity was expressed as μ moles of inorganic phosphorus liberated/mg protein/min.

2.8 BLOOD GLUCOSE (Sasaki et al., 1972)

Reagents

1. Ortho - toluidine boric acid reagent:
   The reagent consisted of 2.5 g of thiourea and 2.4 g of boric acid in 100 ml of a mixture of water, acetic acid and ortho-toluidine (freshly distilled) in the ratio of 10:75:15 (v/v)
2. TCA : 10%
3. Standard: 100 mg glucose (anhydrous) was dissolved in 100 ml of 0.1% benzoic acid. 10 ml of this solution was diluted to 100 ml to give a concentration of 100 μg/ml.

Procedure

To 0.05 ml of blood, 1.0 ml of 10% TCA was added and the solution was centrifuged. To the supernatant 4.0 ml of Orthotoluidine reagent was added. Standard solution in the range of 25-100 μg of glucose were also treated with orthotoluidine reagent along with a blank. The
tubes were heated in a boiling water bath for 15 minutes. The colour developed was read at 640 nm. Blood glucose values were expressed as mg/dl.

2.9 ESTIMATION OF GLYCOGEN

Glycogen was estimated by the method of Morales et al. (1973).

Reagents

1. 30% potassium hydroxide
2. 95% ethanol and 60% ethanol
3. 2N sulphuric acid

Procedure

The tissue was taken out rapidly from the animals and the excess blood removed by blotting between folds or filter paper and immediately put into a weigh stoppered test tube containing 30%. Potassium hydroxide and weighed again. The amount of alkali was then adjusted to get 2 ml per g of liver.

The tissue was digested in a boiling water bath for one and a half hour and cooled in ice cold water. Two volumes of 95% ethanol were then added and the mixture heated to boiling, spurting was avoided.
It was left to stand overnight in the cold, centrifuged and the precipitate was dissolved in 5-10 ml of warm water. The glycogen was reprecipitated with 2 volumes of 95% ethanol.

The precipitate was centrifuged and washed several times with 60% ethanol 2 ml of 2N sulphuric acid per g of liver was added and hydrolysed in a boiling water bath for 3 to 4 hours. The solution was neutralised with sodium hydroxide using phenol red as indicator, then made up to the volume and filtered. Glucose was determined in the aliquot. The factor 0.93 was used to convert glucose to glycogen. The values are expressed as mg/g tissue.

2.10 ESTIMATION OF GLUCONEOGENIC ENZYMES

2.10.1 Glucose-6-phosphatase (Glucose-6-phosphate : Phosphohydrolase) (EC 3.1.3.9) (King, 1965a)

Reagents

1. Citrate buffer : 0.1M, pH 6.5
2. Substrate : Glucose - 6 - Phosphate, 0.01 M
3. TCA : 10%
4. Ammonium molybdate
5. ANSA

Procedure

The incubation mixture in a total volume of 1.0 ml contained 0.3 ml of buffer, 0.5 ml substrate and 0.2 ml of the enzyme sample.
Incubation was carried out at 37°C for 60 minutes. The reaction was arrested by the addition of 1 ml of TCA and centrifuged. The phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity was expressed as n moles of inorganic phosphorus liberated/mg protein/min.

2.10.2 Fructose-1,6-diphosphatase (Fructose-1,6-diphosphate : Phosphohydrolase, EC 3.1.3.11) (Gancedo and Gancedo, 1971)

Reagents

1. Tris - HCl buffer : 0.1 M, pH 7.0
2. Substrate, Fructose-1,6-diphosphate : 0.05M
3. MgCl₂ : 0.1 M
4. KCl : 0.1 M
5. EDTA : 0.001 M
6. TCA : 10%
7. Ammonium molybdate
8. ANSA

Procedure

The assay medium in a final volume of 2 ml containing 1.2 ml of buffer, 0.1 ml of substrate, 0.5 ml of magnesium chloride, 0.1 ml of potassium chloride, 0.25 ml of EDTA and 0.1 ml of enzyme. The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by
the addition of 1 ml of TCA. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity was expressed as nmoles of inorganic phosphorus released/mg protein/min.

2.11 ESTIMATION OF LIPID PEROXIDATION (LPO) AND ENZYMIC ANTIOXIDANTS

2.11.1 Lipid Peroxidation (LPO)

Tissue lipid peroxidation was estimated by the method of Hogberg et al., (1974). Ferrous sulphate and ascorbate included lipid peroxidation was carried out by the method of Devasagayam and Tarachand (1987).

Reagents

1. Tris-HCl buffer : 0.3M, pH 7.5
2. TCA 10%
3. Sodium pyrophosphate : 2 mM
4. Tihobarbituric acid (TBA) : 1.5%
5. Ferrous sulphate : 10 mM
6. Ascorbate : 0.1 mM
7. Stock Standard : A 50 mM Solution was prepared from 1,1,3,3-tetraethoxy propane.
8. Working Standard : 1.0 ml of the stock was diluted to 10 ml to get a working standard containing 50 nmoles/ml.
Procedure

The basal lipid peroxidation system consisted of 1 ml of 0.3 M Tris-HCl buffer, 0.2 ml sodium pyrophosphate and 0.2 ml of diluted tissue homogenate. The inducer system contained 0.2 ml Ferrous sulphate, 0.2 ml of ascorbate (as an inducer), 0.2 ml of sodium pyrophosphate and 0.2 ml of diluted tissue homogenate. The volume was made upto 2.0 ml with water. The tubes were incubated at 37°C with constant shaking for 20 minutes. The reaction was stopped by the addition of 1.0 ml of 10% TCA. The tubes were shaken well and 1.5 ml TBA reagent was added and were heated at 90°C for 20 minutes. The tubes were centrifuged and the colour developed in the supernatant was read at 530 nm. Standards (1.5 nmoles) were taken in 2.0 ml volumes and were processed as above along with a blank containing 2.0 ml water. The basal and inducers added lipid peroxidation in the experimental setup was compared with respective controls. Levels of lipid peroxidation were expressed as TBARS formed/mg protein.

2.11.2 Peroxide Induced Lipid Peroxidation

Peroxide induced tissue lipid peroxidation was assayed by the method of Devasagayam and Tarachand (1987).

Reagents

1. Tris-HCl buffer : 0.2M, pH 7.5
2. Hydrogen peroxide : 7.5 mM
3. TCA 10%
4. Sodium pyrophosphate : 2 mM
5. TBA : 1.5%

Procedure

The peroxidation system consisted of 1.4 ml of buffer, 0.2 ml of hydrogen peroxide and 0.2 ml of homogenate. The volume was made upto 2.0 ml with water. The tubes were incubated at 37°C with water. The tubes were incubated at 37°C with constant shaking for 20 minutes, the reaction was stoppered by the addition of 1.0 ml of 1.0% TCA. Melondialdihyde (MDA) was then estimated by the TBA reagent. The MDA content of the samples after peroxide stress was expressed as nmoles of MDA formed/mg protein.

2.11.3 Enzymic Antioxidants

2.11.3.1 Catalase (Hydrogen-peroxide : Hydrogen-peroxide oxidoreductase, EC 1.11.1.6) (Sinha, 1972)

Reagents

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

2. Phosphate buffer : 0.01M, pH 7.0
3. Hydrogen peroxide : 0.2M
**Procedure**

0.1 ml of the homogenate was taken to which 1.0 ml of phosphate buffer and 0.5 ml of hydrogen peroxide were added. The reaction was arrested by the addition of 2 ml of dichromate acetic acid reagent. Standard hydrogen peroxide in the range of 4-20 μm were taken and treated similarly. The tubes were heated in a boiling water bath for 10 minutes. The green colour developed was read at 570 nm in a spectrophotometer. The enzyme catalase activity was expressed as μmoles of H₂O₂ utilized/mg protein/min.

2.11.3.2 Superoxide dismutase (superoxide : superoxide oxidoreductase, EC 1.15.1.1) (Marklund and Marlund, 1974)

**Reagents**

1. Tris-HCl buffer : 0.1M, pH 8.2
2. Tris-HCl buffer : 0.06M, pH 7.9
3. Pyrogallol stock solution : 2 mM in 0.05M Tris-HCl buffer
4. Absolute ethanol
5. Chloroform

**Procedure**

Partially purified superoxide dismutase was prepared as described by McCord *et al.* (1971). To 1 ml of the sample, 0.25 ml of absolute ethanol and 0.15 ml of chloroform were added. After 15 minutes of shaking in a mechanical shaker, the suspension was centrifuged, and the supernatant
obtained constituted the enzyme extract. The reaction mixture for auto-oxidation consisted of 2 ml of the buffer (Tris-Hcl, pH 8.2), 0.5 ml (2 mM) pyrogallol and 1.5 ml water. Initially, the rate of auto-oxidation of pyrogallol was noted at an interval of one minute for 3 minutes. The assay mixture for the enzyme contained 2 ml of 0.1 m Tris-Hcl buffer, 0.5 ml of pyrogallol aliquots of the enzyme preparation and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted. The enzyme activity was expressed in terms of units/mg protein in which one unit corresponds to the amount of enzyme required to inhibit the auto-oxidation reaction by 50%.

2.11.3.3 Glutathione peroxidase (Glutathione : Hydrogen peroxide oxidoreductase, EC 1.11.1.9) (Rotruck et al., 1973)

Reagents

1. Sodium phosphate buffer : 0.32M, pH 7.0
2. Ethylene diamine tetra acetate (EDTA) : 0.8 mM
3. Sodium azide : 10 mM
4. Reduced glutathione : 4 mM
5. Hydrogen peroxide : 2.5 mM
6. Trichloroacetic acid : 10%
7. Disodium hydrogen phosphate : 0.3 M
8. 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)
9. Standard : 10 mg reduced glutathione was dissolved in 100 ml of distilled water.
**Procedure**

0.2 ml each of EDTA, sodium azide, glutathione (reduced), hydrogen peroxide, 0.4 ml of buffer and 0.1 ml of homogenate were mixed and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 g. To 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 1 ml of DTNB were added and the colour developed was read at 420 nm. The standards were also treated similarly. Glutathione peroxide activity was expressed as μg of glutathione utilized/mg protein/min.

**2.12 ESTIMATION OF NON-ENZYMIC ANTIOXIDANTS**

**2.12.1 Ascorbic Acid (Vitamin C) (Omaye et al., 1971)**

**Reagents**

1. TCA : 10%
2. 2,4-Dinitrophenyl hydrazine - thiourea-copper sulphate reagent (DTC) : 0.4g thiourea, 0.05g copper sulphate and 3.0 g of 2,4-dinitrophenyl hydrazine were dissolved in 100 ml of 9N sulphuric acid.
3. Sulphuric acid : 65% (v/v)
4. Standard : 50 mg of ascorbic acid was dissolved in 100 ml of 4% oxalic acid.
Procedure

To 0.5 ml homogenate, 0.5 ml of distilled water and 1 ml of 10% TCA were added, mixed thoroughly and centrifuged for 20 minutes. To 1 ml of the supernatant 0.2 ml of DTC reagent was mixed and incubated at 37°C for 3 h. Then 1.5 ml of 65% sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30 minutes. The colour developed was read at 520 nm using a spectrophotometer. Standards were also treated similarly. The ascorbic acid content was expressed as mg/g of wet tissue.

2.12.2 α-Tocopherol (Vitamin E) (Desai, 1984)

Reagents

1. Absolute ethanol
2. Petroleum ether
3. Bathophenanthroline : 0.2%
4. Ferric chloride : 0.001 M
5. Ortho-phosphoric acid : 0.001 M
6. Standard : 1 mg of α-tocopherol was dissolved in 100 ml of absolute ethanol in a standard flask.

Procedure

To 1 ml of tissue homogenate taken in a glass stoppered centrifuge tube, 1 ml of redistilled ethanol was added and thoroughly
mixed. Then 3 ml of petroleum ether was added and shaken rapidly in a mechanical shaker for 3 minutes. The tubes were centrifuged and 2 ml of ether layer was transferred to another tube and evaporated to dryness. The residue was carefully redissolved in 3 ml of absolute ethanol. 0.2 ml of 0.2% bathophenanthroline reagent was added and mixed. The tubes were protected from exposure to direct light and the assay was carried out radially from this point, 0.2 ml of Ferric chloride reagent was added and mixed in a vortex mixer. After one minute, 0.2 ml of orthophosphoric acid reagent was added and shaken well. Tubes containing α-tocopherol standards were made up to 3 ml with ethanol and were treated in a similar manner along with a blank containing 3 ml of ethanol. α-tocopherol level was expressed as mg/g of wet tissue.

2.12.3 Reduced glutathione (Moron et al., 1979)

Reagents

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

2. TCA : 10%

3. DTNB : 0.6 mM

4. Standard : 10 mg of reduced glutathione was dissolved in 100 ml of distilled water.
**Procedure**

1 ml of tissue homogenate was precipitated with 1 ml of 10% TCA. The precipitate was removed by centrifugation. To an aliquot of the supernatant 4.0 ml of phosphate solution was added and 0.5 ml of DTNB reagent. The colour developed was read at 420 nm using a spectrophotometer. The amount of glutathione in tissues was expressed as µg of GSH/mg protein.

2.12.4 Non-protein thiols (Sedlak and Lindsay, 1968)

To 1 ml of the homogenate, 4 ml of distilled water and 1 ml of 5% TCA solution were added. The contents were centrifuged at 3000g for 10 minutes. To 2 ml of the supernatant, 4 ml of 0.2M Tris-HCl buffer at pH 8.2 containing 0.2M EDTA and 0.1 ml of DTNB were added and centrifuged at 3000g for 10 minutes and the colour developed was read at 410 nm, against a reagent blank. Standards were also treated in a similar manner (100 µg/ml of reduced GSH). The non-protein sulphydryl content was expressed as µg of GSH/mg protein.

2.12.5 Serum ceruloplasmin (Ravin, 1961)

**Reagents**

1. Acetate buffer : 0.4M, pH 5.5
2. Sodium azide : 0.5%
3. Para-phenylene diamine hydrochloride (PPD) : 0.05% in acetate buffer
Procedure

0.1 ml enzyme solution was pipetted out into a test tube. To the control tube, 1 ml sodium azide was added. Then, 8 ml of acetate buffer was added to both the control and test followed by 1 ml of p-phenylene diamine hydrochloride. The tubes were shaken and incubated at 37°C for 1 h, after which 1 ml sodium azide was added to the test. The tubes were shaken well and cooled at 4°C for 30 minutes. The colour developed was read at 530 nm. Ceruloplasmin level was expressed as mg/dl serum.

2.13 ESTIMATION OF LIPIDS

2.13.1 Extraction of Lipids (Folch et al., 1951)

Procedure

The tissues were washed with saline and dried with a filter paper. A weighed amount of tissue was homogenised with 7 ml of methanol in a Potter Elvehjem homogenizer and filtered through a Whatman No.1 filter paper in to a conical flask. The residue after filtration was scraped and homogenised in 14 ml of chloroform. The residue was once again scraped from the filter paper and homogenised with 7 ml of chloroform : methanol mixture (2:1 v/v) and the resulting filtrate was evaporated to dryness.

2.13.2 Folch Wash

The dried lipid residue, after evaporation was dissolved in 5 ml of chloroform-methanol mixture. The redissolved lipid extract was mixed with 1.0 ml of 0.1M KCl and the contents were shaken well. The upper
aqueous phase containing gangliosides and other water soluble compounds were separated. The lower phase containing neutral and phospholipids in chloroform, was again washed three times with 2 ml of Folch's reagent and then upper aqueous phase was aspirated. The lower chloroform phase was made upto a known volume and 0.1 ml aliquots were taken for the analysis of total cholesterol, phospholipids and free fatty acids.

### 2.13.3 Total cholesterol (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. 1.0 ml of stock cholesterol was diluted to 100 ml to obtain a working standard of 20 μg of cholesterol/ml.
**Procedure**

0.1 ml of the aliquots was taken and it was evaporated to dryness. The dried extract and standards were made up to 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 was mixed well. After 20 minutes the colour developed was read at 540 nm using a spectrophotometer. Total cholesterol was expressed as mg/dl for serum and mg/g of wet tissue for liver and kidney.

### 2.13.4 Phospholipids

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

**Reagents**

1. Ammonium molybdate : 3%
2. Ascorbic acid : 3%
3. Perchloric acid : 70%
4. Standard : 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of dissolved water to give a concentration of 80 µg of phosphorus/ml.

**Procedure**

0.1 ml of dried lipid extract was dissolved in 1 ml of perchloric acid and kept in a sand bath till the solution become colourless. After
cooling, the solution was made up to 5 ml with distilled water. To all the tubes, 0.5 ml of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 minutes. The blue colour developed was read at 710 nm using a spectrophotometer. Phospholipid content was expressed as mg/dl for serum and mg/g of wet tissue for liver and kidney.

2.13.5 Free fatty acids (Hron and Menahan, 1981)

Reagents

2. Copper nitrate : Triethanolamine reagent (Cu-TEA).
3. Colour reagent : Diethyldithio carbamate solution
4. Activated sialic acid
5. Standard 20 mg of palmitic acid was dissolved in 100 ml of distilled water.

Procedure

0.2 ml of lipid extract was mixed with 6.0 ml of chloroform : heptane : methanol solvent and was shaken vigorously. 200 mg of activated sialic acid was added, shaken and left aside for 30 minutes. The solution was then centrifuged and the supernatant was transferred to a tube containing 2 ml Cu-TEA reagent. The contents were agitated using a mechanical shaker for 20 minutes. The mixture was separated into two
phases by centrifugation. 2 ml of the upper layer was mixed with 1 ml of the colour reagent and shaken well. The yellow colour developed was read at 430 nm in a spectrophotometer. Free fatty acid level was expressed as mg/dl for serum and mg/g of wet tissue for liver and kidney.

2.14 PREPARATION OF LIVER MICROSOMES

The liver microsomes were separated according to the method of Boyd and Burka (1978) with slight modification by Kamath and Narayan (1972).

Reagents

1. Homogenizing buffer: Tris-HCl buffer 50 mM (pH 7.4) containing 0.15 M KCl: 605.5 mg of Tris and 1.15 g of KCl were dissolved in 100 ml of water and the pH was adjusted to 7.4 with HCl.

2. Calcium chloride: 8 mM: 117.6 mg of calcium chloride was dissolved in 100 ml of distilled water.

Procedure

The tissue was homogenized. Then the tubes were covered with parafilm and mixed gently by inversion. Then the homogenate was centrifuged at 10,000g for 30 minutes. The floating lipid layer on the top of the supernatant was discarded. Calcium chloride was added to the post mitochondrial supernatant and centrifuged at 15,000g for 15 minutes. The pellet was suspended in buffer, homogenized and made upto a known
The microsomal suspension was used for the analysis of biotransformation enzymes.

2.14.1 Estimation of cytochrome $P_{450}$ (Omura and Sato, 1964)

Reagents

1. Potassium phosphate buffer 0.2M, pH 7.4
2. Sodium dithionate
3. Carbon monoxide gas: carbon monoxide can be generated freshly by the action of concentrated sulphuric acid on formic acid and purified by passing through KOH-dithionite solution. (Since Co is a highly poisonous gas, necessary precaution should be followed during the preparation).

Procedure

To the mixture containing 1.0 ml of buffer and 0.1 ml of tissue homogenate 2 g of solid sodium dithionite was added. The cuvette containing the sample and blank (containing buffer) were scanned between 450 to 490 am to attain a clear bage line. Then carbon monoxide was gently bubbled approximately for 1 minute and the sample were read from 450 and 490 nm. The difference in absorbance spectrum between 450 nm and 490 nm can then be used for the calculation of cytochrome $P_{450}$ activity. The cytochrome $P_{450}$ was expressed an $n$ moles/mg protein.
2.14.2 Estimation of cytochrome b₅ (Omura and Sato, 1964)

Reagents

1. Phosphate buffer : 0.2M pH 7.4 : 2.88 g of disodium hydrogen phosphate and 456 mg of sodium dihydrogen phosphate were dissolved in 100 ml of water.

2. NADH 2% (w/v) : 200 mg of NADH was dissolved in 1 ml of water.

Procedure

The mixture containing 0.1 ml buffer and 0.5 ml enzyme was read in a spectrophotometer at 450 nm and 590 nm against a buffer blank. After the addition of 0.025 ml of NADH again the absorbance was read at 450 and 590 nm. The difference in the absorption spectrum which is the Cytochrome b₅ content and result was expressed as n moles/mg microsomal protein.

2.14.3 Microsomal Protein

The protein content of microsomes in the tissues were estimated according to the method of Lowry et al., (1951). The protein content was expressed as mg microsomes/g wet tissue.
2.15 BIOTRANSFORMATION ENZYMES

2.15.1 Glutathione reductase (NADPH Oxidised Glutathione: Glutathione Oxidoreductase, EC 1.6.4.2) (Staal et al., 1969)

Reagents

1. Sodium phosphate buffer : 0.3 M, pH 6.8
2. EDTA : 250 mM
3. Glutathione oxidised (GSSG) : 12.5 mM
4. Nicotinamide adenine dinucleotide phosphate reduced (NADPH) : 3 mM

Procedure

The reaction mixture containing 1 ml of phosphate buffer, 0.5 ml EDTA, 0.5 ml GSSG and 0.2 ml of NADPH was made upto 3 ml with distilled water. After the addition of 0.1 ml of tissue homogenate, the change in optical density at 340 nm was monitored for 2 minutes at 30 seconds intervals. The enzyme activity was expressed as n moles of NADPH oxidised/mg protein/min.

2.15.2 Aniline Hydroxylase

The aniline hydroxylase activity was assayed by the method of Imai et al. (1966) as modified by Brien and Rahimtula (1978).
Reagents

1. Tris HCl buffer 0.1 M pH 7.5: 1.21 g of Tris was dissolved in 100 ml of water and pH was adjusted to 7.5 with HCl.
2. Cumene hydroperoxide 1.5 mM (at 4°C): 0.228 mg of cumene hydroperoxide was dissolved in 1 ml of water.
3. Aniline hydrochloride 10 mM: 129.6 mg of aniline hydrochloride was dissolved in 10 ml of water.
4. TCA 70%
5. Sodium carbonate 1 M: 1.06 g of sodium carbonate was dissolved in 10 ml of water.
6. Phenol reagent 2%: 2 g of phenol was dissolved in 100 ml of 0.5 M NaOH.

Procedure

1.7 ml of Tris buffer was mixed with 0.1 ml of aniline hydrochloride and 0.1 ml of enzyme. After 1 minute, 0.1 ml of cumene hydroperoxide was added. The tubes were shaken for 2 minutes, and the reaction was arrested by adding 0.3 ml of TCA. The enzyme was added to the controls and centrifuged. To 0.1 ml of supernatant 1.0 ml of Na₂CO₃ and 1.0 ml of phenol reagent were added. The blue colour developed after 30 minutes was read at 640 nm using spectrophotometer. The enzyme activity is expressed as n moles of p-aminophenol formed/mg microsomal protein/min.
2.15.3 NADPH - Cytochrome C reductase (EC 1.6.2.4) (Philips and Langdon, 1962)

Reagents

1. Phosphate buffer 0.33 M pH 7.6: 5.11 g of disodium hydrogen phosphate and 514.7 mg of sodium dihydrogen phosphate were dissolved in 100 ml of water.

2. NADPH 4.2 x 10^{-6} M: 0.35 mg of NADPH was dissolved in 10 ml of water.

3. Potassium cyanide (1 x 10^{-3} M): 3.292 mg of KCN was dissolved in 10 ml of water.

4. Cytochrome C (5 x 10^{-5} M): 0.06 mg of cytochrome C was dissolved in 100 ml water.

Procedure

The tubes containing 2.5 ml buffer, 0.2 ml KCN, 0.1 ml cytochrome 'C' and 0.1 ml enzyme were closed with parafilm and mixed gently by inversion. After 3 minutes, 0.1 ml of NADPH was added. The change in optical density was recorded at 30 seconds intervals for 3 minutes against buffer blank at 550 nm in double beam spectrophotometer. The enzyme activity was expressed as nmoles of cytochrome C oxidised/mg protein/min.
2.15.4 Glutathione-S-transferase (1-chloro 2,4-dinitrobenzene : Reduced Glutathione Transferase, EC 2.5.1.18) (Habig et al., 1974)

Reagents

1. Phosphate buffer : 0.5M, pH 6.5
2. 1-chloro 2,4-dinitrobenzene (CDNB) in 95% ethanol : 30 mM.
3. Reduced glutathione : 30 mM.

Procedure

To 1 ml of buffer, 0.1 ml of homogenate, 1.7 ml of water and 0.1 ml of CDNB were added and incubated at 37°C for 15 minutes. After incubation, 0.1 ml of reduced glutathione was added. The increase in optical density was measured against that of the blank at 340 nm. The enzyme activity was expressed as μmol of CDNB conjugated/mg protein/min.

2.15.5 UDP-glucuronyl transferase

The UDP glucuronyl transferase was estimated by the method of Issalbacher et al. (1962) modified by Hollman and Touster, (1962).

Reagents

1. Tris-HCl buffer 1M, pH 7.4
   12.11 g of Tris was dissolved in 100 ml of water and pH was adjusted to 7.4 with HCl.
**Triton X-100 (0.25% v/v)**

**Magnesium chloride 50 mM**

47.5 mg of MgCl₂ was dissolved in 10 ml of water.

**p-nitrophenol 5 mM:** 0.84 mg of p-nitrophenol was dissolved in 1 ml of water.

**UDP-glucuronic acid 30 mM:** 19.38 mg of UDP-glucuronic acid was dissolved in 1 ml of water.

**TCA 5%**

**NaOH 2 M.**

**Procedure**

The incubation mixture containing 0.5 ml buffer, 0.2 ml Triton X-100, 0.05 ml MgCl₂, 0.05 ml p-nitrophenol, 0.18 ml water and 0.1 ml enzyme was incubated at 37°C for 2 min. Then 0.1 ml of UDP-glucuronic acid was added. Then 0.1 ml aliquot of this mixture was arrested at 0, 10 and 15 minutes with TCA and centrifuged. To 1 ml of the supernatant 0.25 ml of NaOH was added and read at 450 nm. The enzyme activity was expressed as units/mg protein.

**2.16 TISSUE PROCESSING FOR HISTOLOGICAL STUDIES**

Immediately after sacrifice, the animals were autopsied. Their liver and kidney were rapidly excised, serially sectioned with a razor blade and macroscopically examined. Microscopic samples from liver were taken from right portion of the median lobe since this portion of the rat liver has been found to be a site of more apparent histological lesions (Kalengavi
and Desmet, 1975a). The tissues were fixed in 10% formal saline. Consecutive sections were stained with haematoxylin and eosin (H and E).

2.17 STATISTICAL ANALYSIS

Data are presented as the mean ± standard deviation (SD). One way analysis of variance (ANOVA) was done and the F. ratio was computed to detect the significant changes between the groups. The Tukey’s multiple comparison method was used to compare the means of different groups and the significance was denoted by ‘P’ value. All these analysis were carried out in personal computer using statistical package for social science version 7.5.