Chapter 3

MATERIALS AND METHODS

Materials

Male albino rats (Sprague-Dawley strain), two months of age (weighing 130-150 g) were used for the experimental purpose. Rats (healthy and vigorous) were randomly assigned to groups in all studies. They were housed in polypropylene cages (43 \times 27 \times 15 \text{ cm}) and maintained on a normal laboratory diet (M/s Hindustan Lever Ltd., Bombay). Water was given \textit{ad libitum}.

Experimental Design

The rats were divided into five groups of six rats each. All the rats of each group were housed in polypropylene cages (three rats per cage) and were maintained on a 12-hour light-dark cycle.

Purified nicotine (Fluka AG, Chemische Fabrick CH-9470 Buschs) was diluted in physiological saline and the pH was adjusted to 7.2 with sterile 0.1 N HCl. This was administered subcutaneously to the rats of all the experimental groups once daily and five injections a week. The daily dose of nicotine for all the groups ranges from 1 to 7.5 mg/kg body weight (the dilution was done in such a way that 0.5 ml contains the required dose of
nicotine). The nicotine solution was prepared every alternate day. Also the pairfed control was given 0.5 ml physiological saline 5 days a week.

All the rats except the pairfed control were given nicotine at a dose of 1, 2.5, 5 and 7.5 mg/kg body weight. The weight of the rats was recorded every week. The experimental detail of a representative from each group is presented in Table 1.

Table 1 The daily dose and total dose of nicotine of a representative rat from each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg of nicotine/kg body weight in 0.5 ml saline</th>
<th>Total dose of nicotine administered (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Pairfed control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II - Nicotine treated</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>III - Nicotine treated</td>
<td>2.5</td>
<td>57</td>
</tr>
<tr>
<td>IV - Nicotine treated</td>
<td>5</td>
<td>114</td>
</tr>
<tr>
<td>V - Nicotine treated</td>
<td>7.5</td>
<td>170</td>
</tr>
</tbody>
</table>

At the end of 22 weeks the animals appeared very weak and most of them showed respiratory distress. So the animals were deprived of food overnight, stunned by a blow at the back and sacrificed by decapitation.

The blood for haemolysate preparation was collected in bottles containing anticoagulant whereas the blood for serum collected separately. After 30 min the blood was centrifuged at 1500 rpm for 30 min for separating serum. The serum samples were kept on ice until use. Liver and lungs were excised and transferred to ice cold containers for different estimations.
Histopathology

Upon necropsy, gross lesions and representative samples of lungs were immediately cut into pieces (5 x 7 mm size) and fixed in 10% buffered formalin which is prepared as follows:

Formalin 40% - 10 ml
Distilled water - 90 ml
NaH₂PO₄ - 350 mg
Na₂HPO₄ - 650 mg

The fixed tissues were then dehydrated by passing through an ascending series of ethyl alcohol starting from 40% to 100% with a duration of one hour each and finally two changes in 100% ethyl alcohol. Then the tissues were removed to xylol, the clearing agent. After one hour the tissue was transferred to molten paraffin wax (MP 52-55°C, E. Merc, Germany) for wax impregnation overnight.

After 12 h the wax impregnated tissues were embedded in paraffin wax. The paraffin block of tissues thus obtained were then attached to the block holder to cut serial sections of the embedded tissue by using a rotary microtome. The sections were of 4 μ thick. The ribbon of serial sections obtained in microtomy was adhered to a clean dry microslide by using Mayer's egg adhesive.

Mayer's egg adhesive

Egg white - 50 cc
Glycerin - 50 cc

Mixed well and filtered through coarse filter paper and a crystal of thymol was added to the above as a preservative.
The ribbon of serial sections attached to the microslide was kept overnight in a refrigerator.

On the next day, the microslide with serial section was dewaxed in pure xylol by dipping it in xylol for 30 min. The dewaxed slide was then rehydrated with a descending series of ethyl alcohol starting from 100% ethyl alcohol to 30% at one-hour interval and finally the slide with serial sections was used for staining.

Harris' haematoxylin (Harris, 1900) and eosin 1% aqueous was yellowish were the stains used.

Harris haematoxylin solution

- Haematoxylin: 5 g
- Ethyl alcohol: 50 ml
- Potassium or ammonium alum: 100 g
- Distilled water: 950 ml
- Mercuric oxide: 2.5 g
- Glacial acetic acid: 40 ml
- Eosin: 1% aqueous yellowish.

Haematoxylin vs eosin staining usually stains nuclei by oxidised haematoxylin (haematin) through mordant (chelate) bonds of metal such as aluminium followed by counterstaining by the xanthene dye eosin which colours the cytoplasm in different shades.

Microslide with serial sections was removed from Harris' haematoxylin after 30 min. Then washed briefly in water and differentiated in acid alcohol.
Differentiator

1% hydrochloric acid in 70% alcohol.

The slide was then removed quickly and washed well with water and did blueing for 20 sec. Again washed with water and stained with eosin solution for 30 min. The excess stain was removed by immediate washing in water and then differentiated. The microslide was then dehydrated by passing through an ascending series of alcohol, towards the end passed through two changes of 100% alcohol for one hour each. After dehydration, the slide was cleared by two changes in pure xylol with a duration of one hour each and the slide then removed from xylol, immediately mounted in DPX. The microslide was then observed under research microscope and photomicrographs of different magnifications were taken.

Preparation of Haemolysate (1:20)

Haemolysate was prepared according to the procedure of Beutler (1986). Blood was centrifuged at 2000 x g for 15 min in cold centrifuge. After centrifugation, plasma and the buffy coat were removed with a pasteur pipette. The remaining plasma and buffy coat were removed by resuspending the erythrocytes in three volumes of ice-cold 0.9% NaCl solution and centrifuged at 2000 x g for 10 min. The erythrocytes were then washed thrice with the same medium. After washing, the erythrocytes were re-suspended in an equal volume of cold 0.9% sodium chloride. About 0.2 ml of this suspension was added to 1.8 ml of β-mercaptoethanol-EDTA stabilising solution (50 μl β-mercaptoethanol, 10 ml neutralised 10% EDTA (0.27 M), made upto a volume of 1 litre) in a glass tube. Capped the tube and immersed in freezing mixture until it was completely frozen and was then thawed by placing the tube into a beaker containing water at room
temperature. This process was repeated thrice to achieve maximum lysis of the erythrocytes. The lysate was then stored at -4°C till further analysis. The haemolysate thus obtained was 1:20 dilution.

**Analytical Procedures**

In haemolysate (1:20), the following estimations, namely glutathione reductase, glutathione-S-transferase and glutathione peroxidase were carried out. The methods described by Beutler (1984 & 1986) were used for these assays.

**Glutathione reductase (GR)**

*a) Reaction*

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+ \]

*b) Reaction mixture and procedure*

<table>
<thead>
<tr>
<th></th>
<th>Blank (µl)</th>
<th>System (µl)</th>
<th>Blank (µl)</th>
<th>System (µl)</th>
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<tr>
<td>Tris-HCl, 1 M, EDTA, 5 mM, pH 8.0</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>1:20 haemolysate</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>H₂O</td>
<td>890</td>
<td>790</td>
<td>790</td>
<td>690</td>
</tr>
<tr>
<td>FAD, 10 µM</td>
<td>-</td>
<td>-</td>
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Incubate at 37°C for 10 min

<table>
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<tr>
<td>GSSG, 0.033 M (nut)</td>
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<td>100</td>
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</tbody>
</table>

Incubate at 37°C for 10 min

<table>
<thead>
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<th>System (µl)</th>
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<tbody>
<tr>
<td>NADPH₂, 2 mM</td>
<td>50</td>
<td>50</td>
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</tbody>
</table>

Measure change in OD at 340 nm
Glutathione peroxidase (GSH-Px)

a) Reaction

\[ 2\text{GSH} + R - O - O - H \xrightarrow{\text{GSH-Px}} \text{GSSG} + \text{H}_2\text{O} + R - \text{OH} \]

b) Reaction mixture and procedure

<table>
<thead>
<tr>
<th>Blank (µl)</th>
<th>System (µl)</th>
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<tbody>
<tr>
<td>100</td>
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<tr>
<td>20</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>670</td>
<td>660</td>
</tr>
</tbody>
</table>

Pre-incubate at 37°C for 10 min.

Measure change in OD at 340 nm.

Glutathione-S-transferase (GST)

a) Reaction

\[ \text{CDNB} + \text{GSH} \xrightarrow{\text{GST}} \text{CDNB-S-Glutathione} \]

b) Reaction mixture and procedure

<table>
<thead>
<tr>
<th>Blank (µl)</th>
<th>System (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>730</td>
<td>680</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 10 min.

Mix well
Measure change in OD at 340 nm.

Calculation of Results of GR/GPx/GST

The number of enzyme units/ml is defined as

\[ A = \frac{\Delta OD}{\varepsilon \times N \times V_H} \]

where \( \varepsilon \) is the millimolar extinction coefficient of the indicator substance (6.22 in the case of the NAD(P)/NAD(P)H system, \( N \), the number of molecules of indicator converted per molecule of substrate consumed, \( V_H \), the volume of haemolysate added to the cuvette in ml, and \( \Delta OD \), the change in optical density (absorbance per minute).

In a system in which the light path of the cuvette is 1 cm, the enzyme activity (E) in IU/g haemoglobin is

\[ E = 100 \times \frac{A}{Hb} \]

where \( A \) is number of enzyme units per ml and \( Hb \), the concentration of haemoglobin in g/100 ml. \( Hb \) of haemolysate was measured by the procedure recommended by Varley (1988).

For tissues such as liver and lung, homogenate was prepared as recommended by Moron et al. (1979). Protein of the homogenate was measured by using the method of Lowry et al. (1951).

Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) and Catalase were also assayed in the same haemolysate by adopting procedures developed by Paoletti and Mocali (1990) and Calibrone (1985) respectively.

Superoxide dismutase in haemolysate and in both liver and lung were assayed by the method of Francisco Paoletti and Alessandro Mocali (1990).
Reagents

1. Triethanolamine-diethanolamine (100 mM each) HCl buffer (TDB): triethanolamine (14.9 g), diethanolamine (10.5 g) and approximately 13.8 ml of concentrated HCl were dissolved made up to in 1 litre with distilled water; the final pH should be around 7.4.

2. NAD(P)H (7.5 mM): Dissolve 20 mg of either NADH or NADPH, disodium salt in 4 ml of water (amount for 100 assays).

3. EDTA-MnCl₂ (100 mM/50 mM): Prepare stock solution of 200 mM EDTA (dissolve 11.69 g EDTA-acid in 200 ml water) and adjust the pH to around 7 with 1 M NaOH and 100 mM MnCl₂ (dissolve 3.95 g MnCl₂.4H₂O in 200 ml water), combine the EDTA and MnCl₂ stock solution in a ratio of 1:1 (v/v) and adjust the pH of the mixture to pH 7 by drop-wise addition of 10 M NaOH (~0.14 ml/25 ml of reagent).

4. Mercaptoethanol (10 mM): Dilute 50 ml of concentrated thiol (14.2 M) with 71 ml of water.

Conditions of assay

Each set of assays must include its own control. The sample replaced by an equal volume of the medium used for enzyme preparations served and control. The following solutions were subsequently pipetted into the cuvette (semimicrocuvette; light path 10 mm) 0.8 ml of TDB, 40 μl NAD(P)H, 25 μl EDTA-MnCl₂ and 0.1 ml sample (or sample solvent for the control). Mixed thoroughly and read at 340 nm against air for a stable baseline recorded over a 5 min period. Within this interval, the cuvettes will equilibrate at the right temperature (if needed) and the NAD(P)H oxidase activity possibly occurring in samples could be evaluated. Then 0.1 ml mercaptoethanol was added. The final volume in the cuvette is 1.065 ml. Mixed and monitored the
decrease in absorbance for about 20 min to allow full expression of the chain length leading to NAD(P)H oxidation.

Values of ΔA340 recorded for samples with SOD activity progressively decrease depending on the amount of enzyme in the assay mixture. For the calculation, use (sample rate/control rate) × 100 = % inhibition. The data thus obtained was compared with a calibration curve with pure SOD for the conversion of percentage of inhibition to units of enzyme.

One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of NAD(P)H oxidation of the control by 50%.

Catalase

Catalase activity was measured by adopting the procedure developed by Calibrone (1985).

**Reagents**

1. Phosphate buffer 0.1 M, pH 7
   - KH₂PO₄ 544 mg
   - K₂HPO₄ 1.342 g

2. H₂O₂ (30 μl) 30 μl/10 ml buffer

**Assay**

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>20 μl or 50 μl</td>
<td>-</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

The changes in OD at 0 sec, 30 sec and 60 sec time were noted. For calculation average (T) is used.
Average $T = \frac{2y + z}{2}$

$\text{OD of 0 sec time} - \text{OD of 30 sec} = y$

$\text{OD of 0 sec time} - \text{OD of 60 sec} = z$

Therefore $T = \frac{2y + z}{2}$

**Calculation**

$$\frac{T}{0 \text{ time OD}} \times \text{Conc. of } H_2O_2 \times \frac{1000}{34} \times \frac{1}{\text{aliquot taken}} \times \frac{\text{dil. factor}}{\text{mg protein}}$$

$$= \mu \text{moles of } H_2O_2/\text{min/mg protein}.$$

In the above equation, concentration of $H_2O_2$ is 3.4 mg.

Molecular weight of $H_2O_2$ is 34

Aliquot taken 0.1 ml.

For tissues, 200 mg of tissue was homogenised in 0.1 M phosphate buffer of pH 7. Then centrifuged at 1,500 rpm for 10 min. The supernatant was used for the assays. Protein was measured by the method of Lowry *et al.* (1951).

**Thiobarbituric Acid Reactive Substances (TBARS)**

Thiobarbituric acid reactive substances (TBARS) in serum, liver and lung was estimated according to a simple procedure developed by Nichaus and Samuelson (1968).

**Reagents**

1. Tris-HCl buffer 0.1 M, pH 7.5
2. TCA-TBA-HCl reagent
   i) 15% w/v trichloroacetic acid (TCA)
   ii) 0.375% w/v thiobarbituric acid in 0.25 N hydrochloric acid.
Procedure

The tissue homogenate was prepared in 0.1 M Tris-HCl buffer (pH 7.5). 1.0 ml of the homogenate/0.5 ml serum was combined with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling the flocculent precipitate was removed by centrifugation at 1,000 rpm for 10 min. The absorbance of the sample was read at 535 nm against a blank without tissue homogenate.

Calculation

\[
\frac{OD \text{ of test} \times Vol. \text{ of homogenate} \times 100 \times 10^3}{1.56 \times 10^5 \times Vol. \text{ of extract taken}}
\]

The value is expressed in mM/100 g tissue.

It was expressed as millimoles of TBARS/100 g tissue weight.

Glutathione (GSH)

Glutathione (GSH) in the tissues was estimated by the method proposed by Patterson and Lazaroue (1965).

Reagents

1. Alloxan 0.1 M
2. Phosphate buffer 0.5 M
3. pH 7.5
4. Sodium hydroxide 0.5 M
5. Sodium hydroxide 1 N
**Procedure**

Tissues were homogenised in 0.5 M phosphate buffer (pH 7.5). The reaction mixture containing 50 μl tissue extract, 0.1 M alloxan 50 μl, 50 μl of 0.5 M phosphate buffer and 50 μl NaOH (0.5 N) was incubated at 25°C for 6 min. The reaction was stopped by the addition of 50 μl 1 N NaOH. Absorbance was noted at 305 nm in a quartz cuvette of 1 cm light path.

The GSH level was expressed as milligrams of GSH per 100 g tissue weight.

In serum, alanine transaminase (ALT) and aspartate aminotransferase (AST) were estimated by the method proposed by Mohun and Cook (1957). The procedure devised by Kind and King (1954) used for the assay of alkaline phosphatase (ALP) and acid phosphatase (ACP). γ-glutamyl transpeptidase activity was measured by the procedure recommended by SCE (1976).

The above parameters were also assayed in the liver and lung of rats. For this 500 mg of tissue was homogenised in 5 ml physiological saline in cold conditions. The homogenate was then centrifuged at 20,000 g for 20 min. The supernatant was used for the assay. Protein was measured by using the method of Lowry et al. (1951).

Serum total protein and total lipid were estimated by the methods of Lowry et al. (1951) and Frings and Anedonrrt (1970) respectively.

**Estimation of AST and ALT in Serum**

Estimation of AST and ALT in serum was done by the following procedure proposed by Mohun and Cook (1957).
Reagents

1. Buffered substrate: 100 mmol/l phosphate buffer and 2 mmol/l 2-oxoglutarate. For AST, add 100 mmol/l L-aspartic acid and for ALT 100 mmol/l of D. L. alanine. Dissolve 1.5 g K$_2$HPO$_4$, 2 g, KH$_2$PO$_4$ and 300 mg 2-oxoglutarate in 600-700 ml water. For AST add 15.7 g L-aspartate monosodium salt. For ALT add 17.8 g DL-alanine. In both cases check the pH and bring it to 7.4 using reagent 3 and make upto 1 litre with distilled water.

2. 2,4-dinitrophenyl hydrazine (DNPH): 1 mmol/l.

3. NaOH: 400 mmol/l (16 g/l)

4. Pyruvate standard 2 mmol/l: 22 mg sodium pyruvate per 100 ml (this contains 17.4 mg pyruvate).

5. Working standard: 1 in 20 dilution: 100 μmol/litre.

Procedure

Added 0.2 ml serum and 1 ml buffered substrate, mixed and incubated for 60 minutes for AST and 30 minutes for ALT at 37°C in a water bath and as control took 1 ml buffered substrate and incubated without serum. On completion of incubation, removed from the water bath and added 0.2 ml serum to control and 1 ml DNPH. Then kept for 20 minutes at room temperature. Added 10 ml NaOH and mixed well. The absorbance was noted after 5 minutes at 500-550 nm. For standard took 1 ml working standard was taken and made up to 1.2 ml with water; added DNPH and NaOH. For blank, 1.2 ml distilled water was taken and proceeded as above.
Calculation

Activity of AST in IU/L = \frac{T - C}{S - B} \times \frac{0.1 \times 1000}{0.2 \times 60}

Activity of ALT in IU/L = \frac{T - C}{S - B} \times \frac{0.1 \times 1000}{0.2 \times 30}

where 0.2 is the amount of serum used 60 and 30 are incubation periods.

Estimation of Serum Alkaline Phosphatase

Alkaline phosphatase activity was estimated by adopting the method of Kind and King (1954).

Reagents

1. Substrate: Disodium phenyl phosphate 10 mmol/l. Dissolve 2.18 g (2.541 g dihydrate) in water and make up to 1 litre. Bring quickly to boil, cool and add a little of chloroform and keep in refrigerator.
2. Buffer: Sodium carbonate-bicarbonate buffer (100 mmol/l). Dissolve 6.36 g anhydrous sodium carbonate and 3.36 g sodium bicarbonate in water and make up to one litre.
3. Buffered substrate: Mix equal volumes of substrate and buffer. This has a pH of 10.
4. Stock phenol standard: 100 mg% in 0.1 N HCl.
5. Working standard 1 mg%. Dilute stock 1-100 using 0.1 N HCl.
6. Sodium hydroxide 0.5 N (20 g/litre)
7. Sodium bicarbonate: 0.5 N (42 g/litre)
8. 4-amino antipyrine: 6 g/litre in water.
9. Potassium ferricyanide: 24 g/litre in water.
Procedure

Pipetted out 2 ml buffered substrate into each of the two tubes marked test and control and incubated for a few minutes at 37°C. Then added 0.1 ml serum to the test. Again incubated at 37°C for 15 minutes. After 15 minutes the tubes were removed from the water bath. Added 0.8 ml NaOH and 1.2 ml NaHCO₃ to both the tubes. Then added 0.1 ml serum to the control. Then added 1 ml 4-aminoantipyrine and 1 ml potassium ferricyanide to both the tubes. Read the O. D. at 520 nm. For standard, pipetted 1 ml working standard and 1.1 ml buffer. Added NaOH, NaHCO₃, 4-amino antipyrine and potassium ferricyanide as in the test and read. For blank pipetted 1 ml distilled water and added 1.1 ml buffer and proceeded as above.

Calculation

\[
\text{Alkaline phosphatase activity in IU/L} = \frac{T-C}{S} \times \frac{100}{0.1} \times 0.01 \times 7.1
\]

\[
\text{Alkaline phosphatase activity in IU/L} = \frac{T-C}{S} \times 10 \times 7.1
\]

where 0.1 ml is the volume of serum taken. 0.01 is the concentration of phenol in standard. IU is defined in the activity, which transforms 1 µmol of substrate in 1 minute under defined conditions of activity, by which 1 µmol of product is formed under defined conditions.

Determination of Acid Phosphatase

The technique is the same as that of alkaline phosphatase except that citric acid-sodium citrate buffer (pH 4.9) was used for preparing the buffered substrate. Incubation was for 1 h, and in developing the colour with
4-amino antipyrine, it was necessary to add 1.0 ml of 0.5 N NaOH and 1.0 ml 0.5 N NaHCO₃ to bring the pH to that (10.2) required for colour development.

**Gamma-glutamyl transpeptidase**

**Reagents**

1. **Buffer:** Tris 120 mmol/l, magnesium chloride 12 mmols/litre and glycylglycine 90 mmols/litre at pH 7.8 (37°C). Dissolve 14.54 g Tris, 2.44 g MgCl₂.6H₂O and 11.89 g glycylglycine in about 800 ml distilled water. Adjust the pH to 7.8 at 37°C and make to 1 litre with distilled water. It is stable for 24 h at room temperature and last for 8 weeks at 4°C.

2. **Substrate:** Dissolve 1.28 g L-γ-glutamyl-4-nitroanilide in 0.15 mols/litre hydrochloric acid and make to 100 ml with the acid. Considerable stirring may be required to dissolve the substrate. Divide into suitable volumes for one days work and store at -20°C. It is stable for several weeks. The reagent is stable for only a few hours at room temperature.

**Procedure**

Warm 100 µl serum and 1 ml buffer to 37°C. Start the reaction by adding 0.1 ml substrate, mix and monitor the reaction continuously at 405 nm in 1 cm cuvette so as to obtain the change in absorbance/minute.

**Calculation**

Serum GGT (U/litre) = \( \Delta A_{405} / \text{min} \times \frac{1000}{0.1} \times \frac{1.2}{9.9} \times \Delta A_{405} / \text{min} \times 1212 \)

where 1.2 is the final volume in the cuvette and 0.1 ml is the volume of the serum.
Lactate Dehydrogenase (LDH)

LDH activity was measured by the procedure developed by King (1959).

Reagents

1. Buffered substrate

   (a) Glycine reagent: Dissolve 7.505 g of glycine and 5.85 g of sodium chloride in about 900 ml of distilled water and make up to a litre.

   (b) Mix 125 ml of glycine buffer and 75 ml of 0.1 N sodium hydroxide. Add 4.0 g of lithium lactate/sodium lactate. Mix and adjust the pH to 10.

2. NAD solution: 10 mg of NAD is dissolved in 2.14 g/dl (0.2 M) nicotinamide solution. Stable at 2-8°C.

Procedure

1. Buffered substrate, ml 1.0
2. NAD solution, ml 0.2
3. Serum, ml 0.2

Temperature 37°C

Mix well, take the reading at 340 nm after 45 sec and then by an interval of 1, 2, 3 min. Determine the mean absorbance change/min (ΔA/min).

Calculation

\[ \text{LDH (IU)} = 9807 \times \Delta A/\text{min} \]

If the absorbance change; ΔA/min exceeds 0.100 at 340 nm, dilute serum 1:10 using normal saline (result × 10).
Estimation of Total Lipids

Measured using a calorimetric method based on sulfophosphatate devised by Frings and Dunn (1970).

Reagents

1. Conc. H₂SO₄
2. Vanillin (0.6%)
3. Phosphovanillin reagent: 200 ml of 0.6% vanillin in 800 ml of concentrated phosphoric acid.
4. Standard olive oil: Stock 1g in ethanol.
5. Working standard: 400 mg% in ethanol.

Procedure

Three tubes were labelled as test, blank and control; to these tubes 0.1 ml serum, 0.1 ml distilled water and 0.1 ml working standard respectively were taken. Then added 2 ml conc. H₂SO₄ to each tubes. Mixed well and heated in a boiling water bath for 10 minutes. Cooled and pipetted out 0.1 ml of the digested mixture from each tube and transferred into other three tubes. Then 0.1 ml conc. H₂SO₄ and 5 ml phosphovanillin reagent was added. Incubated at 37°C for 15 minutes. Read the absorbance at 540 nm.

Calculation

\[
\text{Total serum lipid in mg\%} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of std.}
\]
Determination of serum total protein (by Lowry et al., 1951)

**Reagents**

1. 2% sodium carbonate in 0.1 N sodium hydroxide (Reagent A).
2. 0.5% copper sulphate (CuSO$_4$·5H$_2$O) in 1% sodium potassium tartarate (Reagent B).
3. Alkaline copper reagent: Mixed 50 ml of A and 1 ml of B prior to use (Reagent C).
5. Protein solution (stock standard): 100 mg of bovine serum albumin in 0.1 N NaOH.
6. Working standard: Dilute 1 in 10 using 0.1 N NaOH.

**Procedure**

Pipetted out 0.2 working standard into a test tube as standard and pipetted out 0.2 ml of the sample extract in the test and with 0.2 ml of water as blank. Added 5 ml of reagent C to test and blank. Mixed well and allowed to stand for 10 min. Then added 0.5 ml of reagent D, mixed well and incubated at room temperature in the dark for 30 min. Blue colour developed was read at 660 nm. From the standard graph, the amount of protein in the sample was calculated.

**Statistical Analysis**

Statistical significance was calculated using students' 't'-test (Bennet and Franklin, 1967). The data given in the tables mean ± SEM, in each case.