CHAPTER II
MATERIALS AND METHODS

ANIMALS : Healthy, adult male albino rats (*Rattus norvegicus*) of Charles Foster strain and colony bred adult male albino mice (*Mus musculus*) of Swiss inbred strain were used for the experiments. The rats weighed about 200-250 gram whereas the mice weighed about 25-30 gram. These animals were supplied by the National Institute Of Occupational Health (NIOH), Ahmedabad, India. The animals were housed in an air conditioned animal house at a temperature of 26±2°C and exposed to 12-14 hours of daylight. They were maintained on a standard chow and water *ad libitum*. A maximum number of 5 animals per cage was maintained. Animals under different groups of experiments were caged separately.

The different treatments given were as follows:

(A) Administration of alcoholic *Carica papaya* seed extract in rats:

The alcoholic seed extract of *Carica papaya* was prepared according to the WHO protocol CG-04 (WHO, 1983a). The extract was used for making up the required dose by dissolving it in double distilled water.

**DOSAGE:** 5mg/kg.b.wt/rat/day of the extract was fed orally by using a feeding metal canula. The feeding was done at the same time of the day, daily.
DURATION OF THE TREATMENT. The treatment was given for 60 days according to the WHO protocol - MB 50 (WHO, 1983b).

The experimental animals were grouped as follows:

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
<th>NO OF ANIMALS USED</th>
<th>DAY OF AUTOPSY</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>10</td>
<td>Sacrificed along with treated animals.</td>
</tr>
<tr>
<td>II</td>
<td>C. papaya seed extract treated 60 days</td>
<td>25</td>
<td>61st day.</td>
</tr>
</tbody>
</table>

(B) Administration of alcoholic Azadirachta indica seed extract in mice:

Preparation of extract: During summer, fully ripened fruits of Azadirachta indica were collected and the seeds were removed and pooled. The pulp of the fruit sticking to the seeds was removed as far as possible and finally the seeds were allowed to shade dry after which they were ground in a grinder. About 50 gram of the powder was taken in a conical flask and was covered with sufficient volume of 95% ethanol. A glass funnel was placed over the mouth of the flask to serve as a condensing surface. The material was boiled for 30-60 minutes, cooled and filtered with Whatman filter paper No.1. The marc after filtration was boiled again with approximately the same quantity of alcohol, this time for 1 hour, cooled and filtered. The filtrates from the first and second extractions were pooled and evaporated to dryness. The extract obtained was used for making up the required dose by dissolving it in double distilled water.
This method of extraction is according to WHO protocol CG-04, (WHO, 1983a).

Dosage: 350 mg/kg b.wt/mouse/day of the extract was fed orally at the same time of the day, daily.

Duration of treatment: The treatment was given for 15, 30 and 60 days to different groups of mice as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals used</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control</td>
<td>40</td>
<td>Sacrificed in batches of 10 along with respective treatments.</td>
</tr>
<tr>
<td>II.</td>
<td>15 days</td>
<td>18</td>
<td>16th day.</td>
</tr>
<tr>
<td>III.</td>
<td>30 days</td>
<td>18</td>
<td>31st day.</td>
</tr>
<tr>
<td>IV.</td>
<td>60 days</td>
<td>10</td>
<td>61st day.</td>
</tr>
<tr>
<td>V.</td>
<td>Treatment as in group IV &amp; withdrawal for 60 days</td>
<td>10</td>
<td>61st day.</td>
</tr>
</tbody>
</table>

LD 50 STUDIES: In the treatment of alcoholic Azadirachta indica seed extract in mice, the LD 50 (24 hours) test was carried out according to WHO Protocol MB-100 (WHO, 1983c). Groups of 8 animals were used for this study. The extract was dissolved in distilled water and 500 mg/kg b.wt./mouse was administered orally using a feeding canula. The dose was increased till 50% mortality was obtained within 24 hours.
Preparation of injection: The androgen antagonist Cyproterone acetate (Schering A.G. Berlin) was made up in a vehicle of benzyl benzoate and castor oil (1:25) according to the method of Walsh and Gittes (1970).

Dosage: Cyproterone acetate was injected intramuscularly into the rats, in the thigh region, at a dosage of 5 mg/rat/day for 7, 15 and 30 days.

The experimental animals were grouped as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals used</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control</td>
<td>12</td>
<td>Sacrificed in batches along with treated.</td>
</tr>
<tr>
<td>II.</td>
<td>Cyproterone acetate (CA) treated for 7 days.</td>
<td>10</td>
<td>8th day</td>
</tr>
<tr>
<td>III.</td>
<td>CA treated for 15 days.</td>
<td>20</td>
<td>16th day</td>
</tr>
<tr>
<td>IV.</td>
<td>Vehicle (benzyl benzoate: Castor oil; 1:25) treated for 30 days.</td>
<td>5</td>
<td>31st day</td>
</tr>
<tr>
<td>V.</td>
<td>CA treated for 30 days.</td>
<td>20</td>
<td>31st day</td>
</tr>
<tr>
<td>VI.</td>
<td>CA treated (30 days) + withdrawal for 60 days.</td>
<td>10</td>
<td>61st day</td>
</tr>
</tbody>
</table>

(C) Administration of Cyproterone acetate, intramuscularly, in rats:

Preparation of injection: The androgen antagonist Cyproterone acetate (Schering A.G. Berlin) was made up in a vehicle of benzyl benzoate and castor oil (1:25) according to the method of Walsh and Gittes (1970).

Dosage: Cyproterone acetate was injected intramuscularly into the rats, in the thigh region, at a dosage of 5 mg/rat/day for 7, 15 and 30 days.

The experimental animals were grouped as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals used</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control</td>
<td>12</td>
<td>Sacrificed in batches along with treated.</td>
</tr>
<tr>
<td>II.</td>
<td>Cyproterone acetate (CA) treated for 7 days.</td>
<td>10</td>
<td>8th day</td>
</tr>
<tr>
<td>III.</td>
<td>CA treated for 15 days.</td>
<td>20</td>
<td>16th day</td>
</tr>
<tr>
<td>IV.</td>
<td>Vehicle (benzyl benzoate: Castor oil; 1:25) treated for 30 days.</td>
<td>5</td>
<td>31st day</td>
</tr>
<tr>
<td>V.</td>
<td>CA treated for 30 days.</td>
<td>20</td>
<td>31st day</td>
</tr>
<tr>
<td>VI.</td>
<td>CA treated (30 days) + withdrawal for 60 days.</td>
<td>10</td>
<td>61st day</td>
</tr>
</tbody>
</table>
(D) Effects of Cyproterone acetate (CA) treatment by a single microdose injection bilaterally into the vas deferens of male rats:

Preparation of injection: Cyproterone acetate (Schering A.G. Berlin) was made up in a vehicle of benzyl benzoate and castor oil (1:25) as mentioned above.

Dosage and mode of treatment: Normal, intact, adult male rats were operated under light ether anesthesia and sterile conditions. A small median incision was made on the scrotal region and the vasa deferentia were exposed. A single microdose of Cyproterone acetate (50 μg/50 μl/rat) was injected with a tuberculin syringe fitted with a fine needle (26 gauge) in the retrograde direction in the distal vas deferens of each side, according to the method of Freeman and Coffey (1973 a,b).

The experimental animals were grouped as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals used</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control</td>
<td>20</td>
<td>Sacrificed in batches along with treated.</td>
</tr>
<tr>
<td>II.</td>
<td>30 days after cyproterone acetate (CA) vasal injection.</td>
<td>15</td>
<td>31st day</td>
</tr>
<tr>
<td>III.</td>
<td>60 days after CA vasal injection.</td>
<td>15</td>
<td>61st day</td>
</tr>
</tbody>
</table>

35
Administration of a combination of Testosterone propionate (TP) + Diethylstilbesterol (DES) in male rats by single microdose injection bilaterally into the vas deferens:

Preparation of injection: A combination of Testosterone propionate (TP) and Diethylstilbesterol (DES) at a 1:1 ratio was made up in groundnut oil.

Dosage and mode of treatment: The combination of TP + DES was injected bilaterally into the distal vas deferens, of the experimental animals in the same way as described before at a dosage of 50 μg/50 μl/rat.

The experimental animals were grouped as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals used</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control</td>
<td>10</td>
<td>Sacrificed in batches along with treated.</td>
</tr>
<tr>
<td>II.</td>
<td>TP + DES</td>
<td>12</td>
<td>31st day</td>
</tr>
<tr>
<td></td>
<td>30 days after single vasal injection.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III.</td>
<td>TP + DES</td>
<td>12</td>
<td>61st day</td>
</tr>
<tr>
<td></td>
<td>60 days after single vasal injection.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
After the last day of the respective treatment, the body weights of each animal was recorded and were then sacrificed. Blood was collected in sterile tubes. Liver, kidney and adrenal of both the sides of each animal were excised from the surrounding tissue and blotted free of blood. The tissues were weighed on a torsion balance.

**PARAMETERS STUDIED**: The parameters studied were as follows:

(i) **Whole body and organ weights**: After the treatment, the animals were weighed and autopsied. The liver, kidney and adrenal were excised and weighed to the nearest milligram on a torsion balance.

(ii) **Serum collection**: Serum was extracted from the blood following the method of Henry (1964).

Three times as much blood as the amount of serum required was drawn and allowed to clot spontaneously in a centrifuge tube at room temperature. After about half an hour the blood clot was carefully detached from the sides of the test tube with a glass rod. Centrifuged the test-tubes immediately at 1000 rpm for 5 minutes and pipetted off the clear supernatant - serum. The sera could be stored for up to 4 hours at room temperature and for 24 hours at 4°C. If longer storage is required, it is advisable to freeze the samples (Richterich, 1968).

The serum could be diluted with physiological saline and used for the various biochemical parameters.
(iii) **Biochemical parameters**:

(a) **Serum protein**:

The levels of serum protein were estimated by the method of Gornall et al. (1949). Four ml of Biuret reagent (1.5 g copper sulphate, 6.0 g sodium potassium tartarate and 10% NaOH was made upto 1 litre) was added to the sample tubes containing 1.0 ml of serum. The blank tube was run with 1.0 ml of saline instead of serum. The tubes were incubated at room temperature for 30 minutes and the optical density of the resultant colour intensity was recorded at 540 nm on a spectronic 106 colorimeter. The protein concentration was assayed using the following regression formula:

\[
X = 18.7 \ Y + 0.077
\]

where \(X\) = concentration of protein in sample

\(Y\) = optical density of sample

The protein concentration was expressed as mg/ml serum.

(b) **Serum cholesterol**:

Level of serum cholesterol was estimated by the method of Pearson et al. (1953) using paratoluene sulphonic acid as the colouring reagent. Cholesterol reacts at room temperature with acetic anhydride and concentrated sulphuric acid forming an intense red-brown complex.
To the test tube containing 5 ml of colouring reagent (4 gram of paratoluene sulphonlic acid dissolved in a mixture of glacial acetic acid and acetic anhydride, 40:60 ml), 0.2 ml of serum was added. To the blank tube, the serum was substituted by 0.2 ml of glacial acetic acid. The standard tube contained 0.1 ml of cholesterol (100 μg/0.1 ml) and 0.1 ml of glacial acetic acid, instead of the serum. The contents were mixed and 1 ml of concentrated sulphuric acid was added to each tube and the colour was allowed to develop. Thereafter the optical density (OD) was read at 620 nm on a spectronic 106 colorimeter. The cholesterol concentration was estimated by the following formula:

\[
\frac{\text{Sample O.D.} - \text{Blank O.D.}}{\text{Standard O.D.} - \text{Blank O.D.}} \times \frac{\text{Standard conc.}}{1000} \times 5 \times 100
\]

Concentration of cholesterol was expressed as mg/ml serum.

(c) **Determination of the activity of serum glutamate oxaloacetate transaminase (SGOT) and glutamate pyruvate transaminase (SGPT):**

The determination of Serum Glutamate Oxaloacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase (SGPT) was carried out by the method of Reitman and Frankel (1957).

The serum was allowed to act in a buffered solution on ketoglutarate and aspartate (GOT), and on ketoglutarate and alanine (GPT), respectively and the quantity of oxaloacetate and pyruvate so formed was measured. The reaction products were
measured photometrically as the corresponding 2,4-dinitrophenyl hydrazones in an alkaline medium. As the α-ketoglutarate present in the sample also forms a hydrazone, the measurements were conducted in the 500-550 nm range where the absorbances of different hydrazones differ maximally.

(c - 1) **Serum glutamate oxaloacetate transaminase (SGOT)**

E.C. 2.6.1.1.

One ml. of buffer substrate (0.1 M phosphate buffer, pH 7.4; 0.1 M L-aspartate, 0.002 M α-ketoglutarate) was pipetted out in the sample as well as the blank tubes which were kept for 5 minutes in a water bath at 37°C. Then 0.2 ml serum was added to the sample tubes, mixed thoroughly and incubated at 37°C for exactly 60 minutes. Then 1 ml of the colouring reagent (0.001 M 2,4-dinitrophenyl hydrazine in 1N HCl) was added which was followed by 0.2 ml. serum in the blank tubes. The tubes were allowed to stand at room temperature for exactly 20 minutes. Then 10 ml of 0.4 N Sodium hydroxide (16 gram NaOH in 1000 ml distilled water) was added and mixed thoroughly and allowed to stand for 5 minutes. The absorbance of the sample was measured against the blank at 545 nm on a systronic spectronic 106 colorimeter.

(c - 2) **Serum glutamate pyruvate transaminase (SGPT)** E.C. 2.6.1.2.

The method is same as that of SGOT but instead of L-aspartate, alanine was used as the substrate (0.1 M phosphate buffer, pH 7.4; 0.2 M DL-alanine; 0.002 M α-ketoglutarate) and
the tubes were incubated exactly for 30 minutes after the addition of serum.

The SGOT and SGPT concentrations were determined from the measured absorbance by reading off calibration as follows:

<table>
<thead>
<tr>
<th>Absorbance</th>
<th>GPT (mU / ml)</th>
<th>GOT (mU / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0.04</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>0.06</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>0.08</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>0.10</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>0.12</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>0.14</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>0.16</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>0.18</td>
<td>31</td>
<td>41</td>
</tr>
<tr>
<td>0.20</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>0.22</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>0.24</td>
<td>45</td>
<td>72</td>
</tr>
<tr>
<td>0.26</td>
<td>50</td>
<td>86</td>
</tr>
<tr>
<td>0.28</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>0.34</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>0.36</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>0.38</td>
<td>93</td>
<td></td>
</tr>
</tbody>
</table>

The values were expressed as mU/ml.

(d) **Liver Glycogen**:

The concentration of glycogen in liver was estimated by the method of Seifter et al. (1950). A known amount of tissue was digested in 2 ml of 30% potassium hydroxide (30 gram KOH in 100 ml distilled water) in a boiling water bath. After digestion, the tubes were cooled and 3 ml of 95% ethanol was added and the contents were boiled gently in a water bath, cooled and centrifuged for 15 minutes at 3000 rpm. The supernatant was discarded and the process was repeated twice. Finally the
supernatant was decanted and fresh ethanol was added and kept for 30 minutes at 20°C for the precipitation of glucose. After that the tubes were centrifuged, the supernatant was discarded and the tubes were kept inverted in a beaker for 10-15 minutes. Then the precipitates were dissolved in a known amount (5 ml) of distilled water. This was used as the sample.

4 ml of 0.2 % anthrone (in 95 % sulphuric acid) was added to each 1 ml of sample tube kept in an ice bath. The blank contained 1 ml of distilled water instead of the sample and the standard tube had 1 ml of standard glucose solution (40 μg/ml). The contents were mixed and boiled in a water bath for 10 minutes. The tubes were cooled in an ice bath and the percentage transmission was read at 620 nm on a spectronic 106 colorimeter. The concentration of glycogen was estimated by the formula:

\[
\frac{\% \text{ Transmission of sample}}{\% \text{ Transmission of standard}} \times \frac{\text{Concentration of standard}}{\text{Tissue weight}} \times \frac{\text{Conversion factor}}{100}
\]

where conversion factor = 1.11

The concentration of glycogen was expressed as μg/100 mg tissue weight.

(e) Total ascorbic acid in the liver and adrenal:

Levels of total ascorbic acid in liver and adrenal were estimated by the method of Roe and Kuether (1943). Total ascorbic acid was oxidised to dehydro ascorbic acid by NORIT reagent in the presence of tri-chloro-acetic acid. This couples with 2,4-dinitrophenyl hydrazine to yield a red coloured complex by the action of sulphuric acid which is measured colorimetrically.
A known amount of tissue was homogenised in 10 ml of NORIT reagent (prepared by dissolving 2 gram activated charcoal in 100 ml of 6% tri-chloro-acetic acid. The mixture was shaken well and allowed to stand for 15 minutes and then filtered through Whatman filter paper No. 42). To 4 ml of the homogenate, 1 ml of 2,4-dinitrophenyl hydrazine reagent (2 gram in 100 ml 9N sulphuric acid) was added and then 1 drop of 10% thiourea (10 gram thiourea in 100 ml of 50% ethanol) was added in order to activate the reaction. In the blank tube, 4 ml of 6% tri-chloro-acetic acid (6 gram TCA in 100 ml distilled water) was taken instead of the homogenate and in the standard tube, 4 ml of ascorbic acid solution (50 mg of ascorbic acid dissolved in 50 ml of 6% tri-chloro-acetic acid. One ml of this solution was diluted upto 100 ml with 4% tri-chloro-acetic acid. This contained 10 μg ascorbic acid per 1 ml). The contents of the tubes were mixed well and were kept in boiling water for 15 minutes and thereafter were cooled in an ice-bath. Then 5 ml of 85% sulphuric acid was added along the sides of the tubes, kept in an ice-bath. After that, the tubes were allowed to stand for 30 minutes and the optical density was measured at 540 nm against blank on a spectronic 106 colorimeter. The concentration of ascorbic acid was calculated by the formula:

\[
\text{Sample O.D.} \times \frac{\text{Concentration of standard}}{\text{Standard O.D.}} \times \frac{\text{Dilution}}{\text{Aliquot volume}} \times 1000 \times \text{Tissue Wt. in mg.}
\]

The concentrations were expressed as mg/gram fresh tissue weight.
(f) **Kidney protein**: Protein levels in the kidney were estimated by the method of Gornall et al. (1949). Known amount of tissue was homogenised in 5 ml distilled water. To 1 ml of homogenate, 4 ml of Biuret reagent was added, mixed and kept at room temperature for 30 minutes. The optical density of the resultant colour was read at 540 nm on a spectronic 106 colorimeter. The calculations were done by the formula:

\[
\text{Concentration of O.D.} \ \frac{\text{obtained from Std. graph}}{\text{Tissue weight in mg.}} \times \frac{\text{Dilution}}{\text{Aliquot volume}} \times 100
\]

Concentration of kidney protein was expressed as mg/100 mg fresh tissue weight.

(iv) **Haematological parameters**: 

Erythrocyte and Leucocyte counts: The RBC and WBC counts were carried out according to the standard method by using the Neubauer's chamber.

The RBC count was expressed as millions/mm³ blood and the WBC count was expressed as thousand per mm³.

Haemoglobin content: Haemoglobin content was measured according to the standard method using a haemometer. The haemoglobin content was expressed as Gm %.

(v) **Histological studies**: Histological studies of kidney, adrenal, liver and lung were carried out by using the standard technique of Haematoxylin - eosin staining. After the autopsy of
the experimental animals, the tissues were excised and cleared off fat and connective tissue and were immediately fixed in alcoholic Bouin's fixative. The tissues were transferred to 70% alcohol after 16-18 hours of fixation. A pinch of lithium carbonate was added to remove excess yellow stain from the tissues. After 2-3 changes in 70% alcohol, the tissues were dehydrated through ascending grades of alcohol upto 100%. The tissues were then cleared in xylene and embedded in paraffin wax and sectioned at 4-5μ thickness on a rotary microtome. These sections were stained with haematoxylin and eosin and mounted in DPX.

(vi) **Histocytometric studies**: Histocytometric measurements (μm) were taken using an ocular eye-piece and a micrometer scale. A minimum of 25-30 readings were recorded for each tissue. All the data were subjected to statistical analysis using Student's 't' test and the level of significance was calculated.

(vii) **Flamephotometry of Na⁺ AND K⁺levels in the kidney**: The Na⁺ and K⁺ contents in the kidney were estimated using the Systronics Flamephotometer, Digital Unit type 125, according to the method of Dean (1960).

Each sample of kidney (200 mg) was digested in 5 ml of nitric acid and heated in a long necked flask till complete evaporation occurred. This was followed by digestion in 5 ml each of perchloric acid, concentrated hydrochloric acid and then distilled water. In all the steps, the sample was heated in a fume chamber till complete evaporation. Finally, the residue was
dissolved in 3 ml distilled water. This 3 ml of sample was used for the estimation of the Na\(^+\) and K\(^+\) levels. The samples could be diluted with distilled water, if required.

The sample for analysis was sprayed as a fine mist into a non-luminous flame which becomes coloured according to the characteristic emission of the element. A very narrow band of wavelength corresponding to the element being analysed was selected by a light filter and allowed to fall on a photodetector, which measures the concentration of the element and is displayed on a digital display.

Standard solutions of NaCl (1 ppm - 10 ppm) and KCl (1 ppm - 10 ppm) were used for determination of Na\(^+\) and K\(^+\) levels, respectively.

Calculations were done by plotting the graphs of meter readings of working standard solutions against the Na\(^+\) and K\(^+\) concentrations respectively. It was repeated as many times necessary until all points fell very nearly on a straight line in a standard curve.

The concentrations of Na\(^+\) and K\(^+\) were expressed as mg/100 mg tissue weight.

For all biochemical assays, a minimum of 5 replicates were done and the data subjected to statistical analysis using the Student's 't' test.
Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) of serum:

Serum protein separation was carried out according to the modified method of Laemmli (1970) as described in the SIGMA Technical Bulletin No. MWS-877 L (1982).

The materials required for the electrophoresis were prepared from Analar chemicals.

A. SEPARATING GEL BUFFER

Prepare solution by combining:

Tris buffer GR .................. 36.3 g

N,N,N',N' - Tetramethyl - ethylenediamine GR................ 0.23 ml.

Dissolve and dilute to approximately 90 ml with double distilled water. Adjust pH 8.9 at 25°C with conc. HCl. Dilute with water to a final volume of 100 ml.

Solution is stable for at least 2 weeks when stored in refrigerator at 0-5°C.

B. STACKING GEL BUFFER:

Prepare solution by combining:

Tris buffer GR .................. 5.98 g

N,N,N',N' - Tetramethyl - ethylenediamine GR................ 0.46 ml.

Dissolve and dilute to 80 ml. distilled water.

Adjust to pH 6.7 at 25°C with conc. HCl.

Dilute with water to a final volume of 100 ml.

Solution is stable for at least 2 weeks when stored in refrigerator at 0-5°C.
C. SEPARATING GEL SOLUTION:

Prepare solution by combining:

Acrylamide........................ 28.0 g.

N,N' - Methylene bis-acrylamide GR................... 0.74 g.

Dissolve and dilute with water to a final volume of 100 ml.

Remove insoluble material by filtration. The solution is stable for at least one month when stored in a dark bottle in refrigerator at 0-5°C.

D. STACKING GEL SOLUTION:

Prepare solution by combining:

Acrylamide........................ 10.0 g.

N,N' - Methylene bis-acrylamide GR................... 2.5 g.

Dissolve and dilute with water to a final volume of 100 ml.

Remove insoluble material by filtration. Solution is stable for at least 1 month when stored in a dark bottle in refrigerator at 0-5°C.

E. SDS SOLUTION

Prepare solution by combining:

Sodium dodecyl sulfate
(Sodium lauryl sulfate)...................0.21 g.

Dissolve and dilute with water to a final volume of 100 ml. Solution is stable for at least 2 weeks when stored at 25°C.

(Note : Solution may become cloudy at temperatures below 20°C but clarity may be restored by warming to 25-30°C and mixing).
F. **2 X SAMPLE BUFFER :**

Tris buffer GR......................... 1.51 g.
Glycerol ............................. 20.0 ml.

Dissolve with 35 ml. of water.

Adjust pH to 6.75 with concentrated HCl, then add:

SDS................................... 4.0 g.
2 - Mercaptoethanol ............. 10.0 ml.
Bromophenol Blue ...................... 0.002 g.

Dilute with water to a final volume of 100 ml.

Solution is stable for at least one month when stored frozen at -20°C.

G. **ELECTRODE BUFFER :**

Tris buffer GR....................... 6.05 g.
Glycine ................................ 28.8 g.
SDS ................................ 2.0 g.

Dissolve and dilute with water to a final volume of 2.0 litres. Final pH should be about 8.3.

H. **FIXATIVE SOLUTION**

Prepare solution by combining:

Methanol ....................... 400 ml.
Glacial acetic acid .......... 70 ml
Distilled water................. 530 ml.

I. **STAINING REAGENT**

Prepare solution by dissolving:

Coomassie Brilliant Blue R 250... 1.25 g.

Dissolve in 500 ml. of Reagent H.

Store tightly capped at room temperature. This reagent is stable for several months.
PREPARATION OF SAMPLE:

The unknown proteins may be prepared by diluting an appropriate amount (0.05 - 1.0 ml) of protein solution at 2mg/ml with an equal volume of Reagent F. This should be incubated at 37° C for 2 hours prior to electrophoresis.

PREPARATION OF ELECTROPHORESIS GELS:

The gel tubes, having an inner diameter of 0.5 cm and long enough to hold a 8 cm gel, were used by capping them at one end and keeping it as the lower end.

Gels having a pore size of 9.5% were prepared, for carrying out the electrophoresis of serum protein, as follows:

(1) Preparation of lower/separating gel:

3 ml Reagent A (Separating Gel Buffer)
8.4 ml Reagent C (Separating Gel Solution).
11.5 ml Reagent E (SDS Solution) containing 17 mg Ammonium persulphate, freshly prepared.
1.36 ml Distilled water.

2) Mix the solution thoroughly.
3) Carefully dispense 1.6 ml of this solution into each gel tube taking care to avoid trapping air bubbles.
4) Before the gel hardens, carefully layer about 0.05 ml distilled water on top of each gel without disturbing the surface in order to get a flat surface and not a meniscus. Allow to polymerize at room temperature.
5) Once polymerization has occurred, which should take above 15 minutes, decant off the water and rinse the top of the gels with a few drops of water, 3 times.
6) Then prepare the stacking/upper gel, by mixing the following:

- 1 ml Reagent B (Stacking Gel Buffer)
- 2 ml Reagent D (Stacking Gel Solution)
- 4 ml Reagent E (SDS Solution)
- 1 ml Distilled water containing 8 mg. of ammonium persulphate, freshly prepared.

7) Rinse the polymerized lower gels with few drops of stacking gel solution (Reagent D). Remove as much of the stacking gel solution from the tube as possible.

8) Apply 0.20 ml of the freshly prepared stacking/upper gel on the lower gel.

9) Before the gels polymerize, carefully layer about 0.05 ml distilled water on top of each gel without disturbing the surface.

10) Once polymerization has occurred which should take about 15 minutes and will be indicated by the gels becoming white and opaque, rinse the top of the gels with a few drops of water. Then as much as possible, the water should be removed from the tubes.

After that, the caps of the gel tubes at the lower ends should be removed and the tubes were fixed in the electrophoretic chamber designed in our laboratory in order to carry out the electrophoresis.
ELECTROPHORESIS:

1) The lower chamber of the electrophoresis tank was filled with Reagent G (Electrode buffer) so that about 2 cm of lower part of the tubes were dipping into the buffer.

2) 20µl of the serum sample was layered on top of the stacking gel.

3) Then the upper chamber was filled up with the electrode buffer.

After that, electrophoresis was carried out at a constant current of 1.5 milliamperes/gel, with the positive electrode in the lower chamber, until the marker dye (Bromophenol Blue) was one cm from the lower end (anodic end) of the gel tube. Then the power supply was switched off and the gel tubes removed from the chamber. The gels were removed from the tubes by squirting water from a syringe between the gel and the tube wall and rotating the tubes at the same time.

FIXATION AND STAINING OF GELS:

1) The gels were immediately immersed in Reagent H (fixative solution) for at least 10 hours with several changes of the fixative.

2) Then the gels were stained in Reagent I (staining reagent) for at least 3 hours. Overnight staining is preferred.
DESTAINING OF GELS:

The gels were destained by diffusion with several changes of Reagent H until the bands were obtained and rest of the gel portion was clear. Gels may then be transferred to 7% glacial acetic acid for storage, scanning or measurements. The gels will swell somewhat in acetic acid. Extended destaining with Reagent H will lead to decolourization of some protein bands.

(ix) Chromosome preparation of bone marrow cells:

Chromosome preparations of the experimental animals were carried out using the standard air drying technique.

The experimental animals were injected with 0.2 ml/100 gram body weight of colchicine (0.2%) and kept for 2-2 1/2 hours after which they were autopsied. The femur bones of both sides were excised and cleaned off from attached muscles and immediately the bone marrow was flushed out into hypotonic solution (0.56% KCl in distilled water) and kept for 20-25 minutes at 37°C, preferably in a water bath. This was followed by centrifugation at 500 rpm for 5 minutes. The supernatant was discarded and about 5-7 ml of freshly prepared, chilled fixative (acetic acid : methanol, 1:3) was added and kept for 30 minutes. After that, the tubes were again centrifuged and the supernatant removed. Five ml of fixative was added and the cells were resuspended by shaking the tubes. The tubes were allowed to stand for 3-5 minutes and then the procedure of centrifugation was repeated once again. Finally, the tubes were centrifuged, supernatant discarded and 1 ml of fresh fixative was added and
the cells were resuspended by shaking the tubes. With a long Pasteur pipette, 2-3 drops of the cell suspension were dropped from about 14-16 inches height on a clean, wet microslide which were kept in a beaker containing chilled distilled water. Blow on each slide to assist in spreading and drying. After the slides were dried completely, they were stained in 2% Giemsa and after drying, the stained slides were examined under a microscope to study the chromosome plates.