CHAPTER-3
MATERIAL AND METHODS
The objective of this research was to study the lipoprotein metabolism in varying degree of obesity and associated diseases in adult women of urban part of M.P. The work was carried out according to the following scheduled plan.

The present study has been carried out on 553 Volunteer subjects aged between 18 to 75 years. The Subject were selected irrespective of their caste and creed. The subjects were voluntarily presented themselves for the present study. They were from :-

• Personal relationship  • Patient attendants.
• Blood Donors.  • Clubs (Lions club, Rotary club, Agrawal Mahila Club)
• Sliming centers.  • Kitty Parties.
• Yoga Centers.  • M.Y. Hospital.

The subjects categorized in to following groups.

Control group :- The 53 subjects chosen as a control for our study. The individuals in this group were.

1. Normal, healthy, non obese, ideal height and weight, adult women.

2. The individuals were having no evidence of any infection, no history of physiological and pathological illness, Not taking any kind of treatment which likely to affect the lipid profile levels.

The subjects of control group were further divided in to three group on the basis of age.
1. Control group I (18 to 29 years) having – 18 subjects

2. Control group II (30 to 45 years) having – 18 subjects

3. Control group III (46 to 75 years) – having – 17 subjects

**Study Group:** Study group was comprising of 500 subjects. They were further divided on the basis of age and (BMI) grade of obesity.

(A) **Study Group I:** 148 Normal obese adult women of age 18 to 29 year.

1) Over Weight – 70 Subjects

2) Obese - 59 Subjects

3) Morbid obese – 19 Subjects

(B) **Study Group II:** 140 Normal obese adult women of age 30 to 45 years.

1) Over weight – 48 Subjects

2) Obese – 63 Subjects

3) Morbid obese – 29 Subjects

(C) **Study Group III:** 112 Normal obese adult women of age 46 to 75 years.

1) Over weight – 45 Subjects

2) Obese – 52 Subjects

3) Morbid obese – 15 Subjects

(D) **Study Group IV:** 100 obese adult women with obesity associated diseases.

1) Diabetes Mellitus (Type II) -33 Subjects

2) Heart diseases-37 Subjects
3) Other obesity associated Diseases which includes joint pain, arthritis, sleep-apnea, cancer, hernia etc. - 30 Subjects.

The subjects in the study group were neither compelled to participate in the survey nor subjected to any kind of risk. Patients with Renal failure were excluded.

The complete history about Name, age, height, weight, BMI (calculated by formula BMI={Wt/h}²), BP, Pulse, Dietary Habit, Marital Status, Family History, Socioeconomic Status, Tendency, Menstrual history, occupation, Habit of Smoking/Alcohol, life style, awareness and sleeping habit were taken.

The study further proceeded by collecting blood samples of subjects of the above said groups and availing them for the determination of given parameters.

The parameters mainly estimated are:-

(1) Blood glucose, Urea, Creatinine, Haemoglobin, lipid profile [Total lipid, Total cholesterol, HDL, LDL and VLDL, Triglycerides] and calcium

The review and reference studies were carried out in M.G.M Medical collage Library and Internet section Practical Study was undertaken in the Department of Biochemistry Holkar Science Collage and M.Y. Hospital.

Collection of Blood sample

About 7 ml of fasting blood sample from antecubital vein was withdrawn in a perfectly cleaned disposable syringe and about 5 ml blood transferred to a clean dry test tube and kept for 30 minutes at room temperature and 1ml blood was transferred into fluoride bulb and 1ml blood was transferred in EDTA bulb. The serum was separated by centrifugation at 3000 rpm for 10 minutes. The sample was analyzed for Urea, Creatinine, Total cholesterol, HDL-cholesterol, Triglyceride, LDL, VLDL, Total Lipid, hemoglobin and Sugar.
Estimation of Haemoglobin [God Ker]^{165}

The blood Haemoglobin was estimated by Sahli (Acid haematin) method.

Principle

When blood is added to O.I N Hydrochloric acid, Hemoglobin is converted to brown coloured acid haematin. The resulting colour after dilution is Compared with standard brown glass reference blocks of a Sahi Hemoglobinometer.

Requirements

1) Sahli hemoglobin meter
2) O.I N Hydrochloric acid
3) Distilled water
4) Pipette

Procedure

1) By using Pasteur pipette add O.I N hydrochloric acid in the tube up to the lower mark (20% Mark).

2) Draw blood up to 20 μl mark in Hb pipette. Adjust the blood column carefully without bubbles, wipe excess of the blood on the sides of the pipette by using a dry piece of cotton.

3) Transfer blood to the acid in the graduated tube, Rinse the pipette well mix the reaction mixture and allow the tube to stand for at least 10 minutes.

4) Dilute the solution with distilled water by adding few drops at a time carefully and by mixing the reaction mixture, until the colour matches with the glass plate in the comparator.
b) Sodium tungstate : 5 gm

c) 10 gm/dl sodium hydroxide : 200 ml.

d) Distilled Water : 200 ml.

Mix and boil for one hour to remove ammonia cool it to room temperature and add

e) Orthophosphoric acid : 125 ml.

Mix well and make final volume 500 ml by using distilled water.

5. Glucose Standard : 100 mg/dl (in saturated benzoic acid)

Requirements

1) Test Tube (15 x 125 mm 10 x 100 mm)

2) Folin-Wu tubes

3) Pipettes

4) Funnels

5) What man No. 44 filter papers.

6) Water Bath.

Procedure : A) Preparation of protein free filtrate, pipette in the tubes, labeled as follows:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (ml)</td>
<td>7.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Blood (ml)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>100 mg/dl glucose std. (ml)</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>2/3 N sulphuric acid (ml)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>10 gm/dl sodium tungstate (ml)</td>
<td>1.0</td>
<td>-</td>
</tr>
</tbody>
</table>
Mix thoroughly and keep at room temperature for 5 minutes. Filter the contents of test by using what man no. 44 filter paper.

B- Reduction of Cupric ions.

Pipette in the Folin-wu tubes labeled as follows

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate (ml)</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diluted Glucose Standard (ml)</td>
<td>-</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>Distilled Water (ml)</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>Alkaline Cupric Tartaret (ml)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Mix well and keep in a boiling water bath for exactly 8 minutes. Cool the tubes to room temperature.

C- Formation of Molybdenum blue.

- Add 2.0 ml of phosphomolybdic acid in all the Folin-Wu tubes, Mix well.

- Add distilled water up to 25 ml mark.

- Mix the contents thoroughly by inverting the tubes.

- Keep for about 5 minutes.

- Read the intensities of test and standard against blank at 420 mm ( violet filter).

Calculations:

\[
\text{Blood Glucose mg/dl} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 100
\]
Estimation of Urea

The serum urea is estimated by Berthelot reaction method.

The procedure is based on the Berthelot reaction. Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with Phenol in the presence of hypochlorite to form indophenol, which with alkali gives a blue coloured compound. The intensity of coloured compound can be measured at 546 nm (Green Filter). The colour of the reaction stable for 12 hours.

Requirements :

1) Test Tubes - 15 x 125 mm

2) 1.0 ml, 5.0 ml, 0.1 ml graduated pipette

3) Push button pipette (20 µl)

4) Stop watch.

Reagent Preparation :-

1) Urease Buffer : It contains urease,.1000 units dissolved in 100 ml of phosphate buffer pH 7.0.

2) Phenol reagent : 5 gm of phenol and 0.025 gm of sodium nitroprusside dissolved in 500 ml of distilled water.

3) Hypochlorite reagent : 2.5 g of sodium hydroxide and 0.21gms of sodium hypochlorite dissolved in 500 ml of distilled water.

4) Standard Urea : 20 mg/dl.
Procedure: Pipette the tube labeled as follows:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease / Buffer reagent (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Serum / Plasma (ml)</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard Urea (20mg)</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Mix well and keep at 37°C for 10 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol reagent, (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Hypochloride reagent, (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Mix well and keep at 37°C for 10 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Mix thoroughly and read optical densities of test and standards against blank at 540 nm (green filter).

O.D. test

Calculation Urea : mg/dl = \frac{\text{O.D. test}}{\text{O.D. Std.}} \times 20

Estimation of Creatinine

The serum creatinine is estimated by alkaline picrate method.

Principle

The creatinine reacts with picric acid in alkaline medium form a reddish yellow complex, intensity of which is directly proportional to the concentration of creatinine in the specimen and can be measured at 520 nm (Green Filter).

Normal Range: 0.8 to 2 mg/dl.
Requirements

1) Test Tubes : 15 x 125 mm
2) 50 ml pipette
3) 1 ml and 2 ml volumetric pipettes
4) Test Tube stand
5) Centrifuge tubes or test tubes.
6) Photometer.

Reagent Preparation

1) Picric acid :- 0.91 gm/dl
2) 10 gm/dl sodium hydroxide
3) Working creatinine standards, 1mg/dl, 5mg/dl.
4) Alkaline picrate reagent. It is prepared fresh by mixing 4 parts of reagents 1 and 1 part of reagent 2. This working reagent is stable for one day.

Procedure : Pipette in the tubes labeled as follows :-

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (ml)</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Serum (ml)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Standard 1 mg/dl (ml)</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>2/3 N Sulphuric acid (ml)</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>10g / dl Sodium Tungstate (ml)</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Centrifuge the contents in the test and get clear filtrate pipette in the tubes labeled as follows:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (ml)</td>
<td>3.0</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Filtrate ml</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dilute Std. 1mg / dl (ml)</td>
<td>-</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline Picrate Reagent (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mix and keep at room temperature for 20 minutes. Read intensities test and standard at 520 nm. (green filter) by setting bank to zone.

Calculation:

\[
\text{O.D. test} = \text{O.D. Std.} \times 20
\]

Estimation of Serum Cholesterol

The serum cholesterol is estimated by "Watson" Colorimetric method.

Principle

Cholesterol reacts with acetic anhydride in the presence of glacial acetic acid and concentrated sulphuric acid to form green coloured complex. Intensity of the colour is proportional to the cholesterol concentration and can be measured at 575 nm.

Requirements

1) Test Tubes
2) 0.2 ml pipette
3) Dispensers

4) Stop watch

5) Push Button Pipette

6) Photometer

Reagent Preparation

1) **Cholesterol reagent 1**: It is prepared by mixing 5.6 gm of 2.5 di-methylbenzene-sulphonic acid in 200 ml of glacial acetic acid and 300 ml of acetic anhydride. This reagent is Stable in an amber coloured bottle at room temperature for one year.

2) Cholesterol reagent- 2 Conc. Sulphuric acid.

3) Cholesterol Standard : 200 mg / dl.

Procedure: Dispense in the tubes labeled as follows.

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent 1 (ml)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Serum (ml)</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol Std. (ml)</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Mix well and cool at room temperature and add following reagent.

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent 2 (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mix Thoroughly, keep in the water bath at room temperature for 10 min. Read the absorbance of test and standard against blank at 575 nm. use dry glass ware.
Serum total cholesterol mg/dl. = \[ \frac{\text{O.D. test}}{\text{O.D. Std.}} \times 200 \]

**Estimation of Serum HDL-Cholesterol**

The serum HDL-Cholesterol was estimated by "Watson" Method.

**Principle**

In the presence of phosphotungstic acid and Magnesium Chloride, LDL, VLDL and Chylomicrons are precipitated. Centrifugation leaves only the HDL in supernatent. Cholesterol in the HDL fraction can be tested by the usual methods.

**Requirement**

Same for total Cholesterol determination additional requirements.

1) Centrifuge tube

2) 0.1 ml, 1.0 ml graduated pipette.

3) Centrifuge

**Reagents**

1) Cholesterol reagent 1 and

2) 2) Cholesterol reagent 2

These two reagents are the same as used in the determination of total cholesterol.

**Additional Reagents**

3) Phosphotungstic acid reagent : It is prepared by dissolving 2.25 g of phosphotungstic acid in 8.0 ml of 1 N sodium hydroxide and 42 ml of distilled water.
4) Magnesium chloride in distilled water. It is diluted to 50 ml.

5) Cholesterol Standard :- 100 mg / dl.

Procedure

Pipette in the centrifuge tubes labeled as follows :-

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ml)</td>
<td>0.5</td>
</tr>
<tr>
<td>Phosphotungestic acid reagent (ml)</td>
<td>0.05</td>
</tr>
<tr>
<td>mgcl2 reagent (ml)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Mix well and centrifuge at 3000 RPM for 20 minutes separate the supernatent by using a pasteur pipette. Now pipette in the tubes labelled as follows.

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent - 1 (ml)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Supernatent (ml)</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol Std. 100 mg / dl (ml)</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Mix Well keep in a water bath at R.T. for 5 minutes & add

<table>
<thead>
<tr>
<th>Cholesterol reagent 2 (ml)</th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mix Thoroughly, cool the tubes to room temperature by dipping in a water bath (at room temperature).

Read absorbance of test and standard against blank at 575 nm.
Calculation

O.D. Test

Serum HD. Cholesterol mg/dl =------------------ x 100
O.D. Test

Estimation of Serum Triglycerides

Enzymatic determination of Serum Triglycerides

Principle

Lipoprotein

Triglyceride + H₂O -------------> Glycerol + Fatty Acid

Lipase

Glycerol Kinase

Glycerol + ATP ------------------> Glycerol – 3 Phosphate + ADP

Glycerol Phosphate Oxidase

Glycerol–3–Phosphate+O₂ ------------------------> Dihydroacetone -
- Phosphate + H₂O.

H₂O₂ + 4 Amino-Phenazone + P. Chlorophenol ->

↓ Peroxidase

Coloured Complex. It is measured at 520 Hm (Green Filter)

Reagents

1) Buffer / Enzymes / Chromogen : It contain a) Lipoprotein lipase : 30 units
   b) Glycerol Kinase – 10 units c) Glycerol – PPhosphate oxidase 5 Units d) Peroxidase : 5 Units e) Glycerol Phosphate in 100 ml of phosphate Guffor ; Ph – 7.

2) p=Chlorophenol reagent : 30 mg/dl.

Preperation of working reagent : It is prepared fresh by mixing two part of reagent (1) and one part of reagent (2).
Procedure

Pipette in the tubes labeled as follows:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Serum (ml)</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard (ml)</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Mix well keep at 37°C for 15 minutes. Read absorbence of test and standard against blank.

Calculation

\[
\text{O.D. Test} \\
\text{Serum Triglyceride mg/dl} = \text{----------- x 100} \\
\text{O.D. Test}
\]

Estimation of serum Total Lipids

Serum Total lipids were estimated by sulpho-phospho-vaniline method.

Principle

Lipids react with vaniline in the presence of sulphuric and phosphoric acid to form a pink coloured complex.

Normal Value - 400-1000 mg/dl.

Reagents required:

1) Total lipid standard = 100 mg/dl.

2) Colour Reagent (phospho-vaniline). It is prepared by mixing.
a) 0.61 gm/dl vaniline - 350 ml

b) orthophosphoric acid - 600 ml

c) Distilled water - 50 ml.

3) Concentrated sulphuric acid (AR)

Procedure: Pipette in the tubes labeled as follows:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Solution – 1 (ml)</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>Serum (ml)</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 3 (ml)</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Mix thoroughly, plug with cotton wool, Keep in boiling water both for 10 minutes.

Then cool in a cold water bath and again pipette into dry test tubes as follows.

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>STD</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>From above solutions (ml)</td>
<td>0.10</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>Reagent-3 (ml)</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
</tr>
<tr>
<td>Colour reagent (ml)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Mix thoroughly and keep at room temperature for 15 minutes, Read absorbance of test and standard against blank in a dry cuvette at 546 nm

Calculation

\[
\text{Serum Total Lipid mg/dl} = \frac{\text{O.D. Test}}{\text{O.D. Test}} \times 100
\]
Some samples are cross checked for accuracy by auto analyser In M.Y. Hospital.

Diagnostic Test kit are based on these methods

Haemoglobin - Cynameth haemoglobin method
Urea - Enzymatic Method
Creatinine - Enzymatic method
Glucose - Glucose oxidase Method
Total Cholesterol - Enzymatic Method
HDL Cholesterol - Enzymatic Method
Triglycerids - Enzymatic Kit Method

Estimation of VLDL-Cholesterol

VLDL-Cholesterol was estimated by using formula

\[
\text{VLDL-Cholesterol mg %} = \frac{\text{Triglyceride}}{5} + \text{HDL}
\]

Estimation of LDL-Cholesterol

LDL - Cholesterol was estimated by using formula

\[
\text{LDL} = \text{Total Cholesterol} - [\text{HDL} + \text{VLDL}] \text{ mg %}.\]
PREPARATION OF RED CELL HEMOLYSATE

Red cell hemolysate was prepared from the washed Leukocyte free cells by the method of Beutler (1984). To 1 ml of blood collected in EDTA, 10 ml of 0.154 M NaCl was added and centrifuge at 3500 rpm for 10 minutes. The supernatant carrying plasma, leukocyte and other cellular components were discarded. The RBCs thus obtained were washed 3-4 times with 0.154 M NaCl. To one volume of washed red cells usually 1 ml of 0.154 M NaCl was added. To this RBCs suspension 1.8 ml of β-mercaptoethanol stabilizing solution was added. The tubes containing the hemolysate was frozen and thawed by placing the tube in a beaker at 25°C. When the hemolysate was completely thawed, it was uniformly shaken and the tubes were kept in ice bath where it was maintained at 0°C. The hemolysate prepared in this way was referred to as 1:20 hemolysate.

LIPID PEROXIDATION

Lipid peroxidation was measured in the blood hemolysate by the method of Utley et. al. (1967)⁶.

Principle

The heat-induced reaction of malonyldialdehyde (MDA) with thiobarbiturie acid (TBA) in an acid solution forms a trimethine colored substance, which is measured spectrophotometrically at 540 nm.

Reagents

1. 4% Sodium Dodecyl Sulphate (SDS): SDS (4 g) was taken with minimum quantity of distilled water and heated at 60° C for 5 minutes to dissolve it and the volume was made upto 100 ml with distilled water and kept at room temperature.

2. 20% Acetic Acid: Glacial acetic acid (20 ml) was dissolved in 0.27 M HCl and volume was made upto 100 ml. Its pH was adjusted to 3.5.
(3) 0.8% Thiobarbituric Acid (TBA): TBA (0.8 g) was dissolved in 0.1 N NaOH (50 ml). The volume was made up to 100 ml with distilled water after adjusting the pH to 7.4 and kept in freezer

Procedure
Hemolysate at 1:20 dilution was used for all determinations.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Particulars</th>
<th>Reaction blank(ml)</th>
<th>Reaction system(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>4% SDS</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>2.</td>
<td>20% Acetic acid</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>3.</td>
<td>0.8% Thiobarbituric acid</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The reaction mixtures were then heated for 30 minutes in a water bath at 85°C and then cooled. The solution thus obtained was then centrifuged at 4000 rpm for 10 minutes. The clear supernatant thus obtained was measured optically at 532 nm.

Calculation
Molar coefficient of MDA: 1.56 x 10⁻⁵
i.e. if the O.D. is 1.56 the solution will contain 1x10⁻⁵ M MDA
x O.D.=1x10⁻⁵

SUPEROXIDE DISMUTASE (SOD)
SOD was estimated according to the procedure of Marklund, S. and Marklund, G. (1974)⁷.
Principle
Pyrogallol auto-oxidizes rapidly in aqueous solution to produce a yellow color that can be read at 420 nm. The process is dependent on the presence of superoxide anions. The enzyme dismutase (SOD) inhibits the auto-oxidation of pyrogallol by catalyzing the breakdown of superoxide. The inhibition of pyrogallol oxidation by SOD is monitored at 420 nm. and the amount of enzyme producing 50% inhibition is defined as one unit of enzyme activity.

Reagent
(1) **Tris Buffer**: To prepare 100 ml of 1 M tris-HCl buffer pH 8.0 with 5mM EDTA. Tris (12.1 g) and disodium EDTA (168 mg) were placed in a beaker and dissolved in approximately 80 ml distilled water at room temperature. Concentrated HCl was added until the pH was adjusted to 8.0. The pH was adjusted to exactly 8.0 using 2 M HCl. The mixture was then carefully transferred to 100 ml volumetric flask. The final volume was made up to 100 ml.

(2) **HCl (10 mM)**: HCl (0.9 ml, Conc.) was made up to 100 ml with distilled water.

(3) **Pyrogallol**: Pyrogallol (10 mg) was dissolved in 8 ml mM HCl.

Procedure
Whole blood was washed three times with cold saline. One volume of packed cell was hemolysed with 1.5 volume of cold distilled water. Lysate (0.2) was added to ferricyanide-cyanide reagent (10 ml) for hemoglobin estimation. A chloroform-ethanol extract was prepared by adding the water hemolysate (0.5-ml) to cold distilled water (3.5 ml) followed by ethanol (1 ml) and then chloroform (0.6 ml). Samples were mixed after each addition and finally shaken for 1 minute. Tubes were mixed after each addition and finally shaken for 1 minute at 3000 rpm. Test tubes were taken and 100 μL of 1M tris HCl-5mM EDTA was pipetted in all the tubes and varying amounts of the clear supernatant extract ranging from none to 300 μL to provide 100% inhibition.
was added. Water was added to bring the volume to 980 μL. (Convenient amounts of extracts are 0 μL, 20μL, 40μL, 80μL, 100μL and 300μL.)

All test tubes were incubated at 37°C for 10 minutes. Twenty microlitre of pyrogallol solution was added to each tube. The increase in optical activity was measured on spectrophotometer at 420 nm.

Calculations:

\[
E = \frac{5.6}{0.5x\text{ml} 50\% \times 0.01x\text{Hb}} = \frac{1120}{\text{ml} 50\% \times \text{Hb}}
\]

ml 50%: The amount of extract required to inhibit pyrogallol autoxidation by 50%.

E: Enzyme activity expressed as 50% inhibitory units per gm Hb.

Hb: Hemoglobin conc. in the hemolysate in g/100 ml.

5.6: Final volume of the extract

0.5: amount of hemolysate in the extract %inhibition V 5 ml of extract is plotted as graph.

Normal value of SOD in adults –2254.8 ± 303.0 U of SOD/g Hb at 25° C.

**CATALASE**

Serum catalase was estimated by the method of L.Goth (1991)\(^8\).

**Principle**

Catalase catalyzes the breakdown of H\(_2\)O\(_2\) according to the following reactions:

\[
2 \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2 \text{H}_2\text{O}_2^+ + \text{O}_2
\]

The rate of decomposition of H\(_2\)O\(_2\) catalase is measured spectrophotometrically at 405 nm.
Reagents

(1) Sodium-Potassium Phosphate Buffer: 60 mmol/L (pH=7.4)
(2) H₂O₂ (Substrate): 65 μmol/ml H₂O₂ in 60 mmol/L Na-K-phosphate buffer.
(3) Ammonium Molybdate: 32.4 mmol/L

Procedure

Four test tubes were taken and labeled as blank-1, blank-2, blank-3 and test. To all tubes reagent were pipetted as follows:

**Blank-1:** 1.0 ml substrate, 1.0 ml molybdate and 0.2 ml serum.
**Blank-2:** 1.0 ml substrate, 1.0 ml molybdate and 0.2 ml buffer.
**Blank-3:** 1.0 ml buffer, 1.0 ml molybdate and 0.2 ml buffer.

**Test:** Serum (0.2 ml) was incubated in substrate (1.0 ml) at 37°C for 60 sec., then molybdate (1.0 ml) was added.

The enzymatic reaction was stopped with ammonium molybdate and yellow complex of molybdate and hydrogen peroxide was measured at 405 nm against blank-3.
Some samples were cross-checked for accuracy in the auto-analyzer at the M.Y hospital.

Autoanalyser Selectra E