Chapter No. 4

MATERIAL AND METHODS
MATERIAL AND METHOD

The present study was carried out at the department of Biochemistry, Government Medical College, Miraj Wanless Hospital Miraj and at the Shivaji University, USIC department Kolhapur. The patients were selected from Richardson Leprosy Hospital, Miraj and from Civil Hospital Sangli. The study was conducted over a period of six years from January 1993 to 1999 July. Laboratory work was completed in Biochemistry department Government medical College, Miraj Wanless Hospital, Shivaji University Department at USIC, Kolhapur and NCL Pune.

The present study was conducted on 90 clinical established leprosy patients (male 64 and female 26). They belong to age group 11 to 60 years. Fifty healthy age matched individuals of either sex acted as control (male 30 and female 20)

Patients were included in the study after clinical diagnosis and classification according to Ridley and Jopling 1996 clinical examination was carried out by experienced leprosy workers and leprosy medical officers. Slit
skins smears were taken from all patients for determination and bacterial index.

The leprosy patients were divided into five groups as follows:

❖ Tuberculoid (TT) leprosy
❖ Borderline tuberculoid (BT) leprosy
❖ Borderline (BB) leprosy
❖ Borderline lepromatous (BL) leprosy
❖ Lepromatous (LL) leprosy

The proposed five groups system of classification was according to certain immunological features. These included the cytology of the host cell of the macrophage histocyte series, the degree of infiltration by lymphocytes and the bacterial density.

**Sample Collection**

Blood samples were collected from each patients and controls after an overnight fast at the same time in the morning in every patient. All specimens were stored at -20°C pending their analysis.
Blood sample from each patient and control was collected from anticubital vein, with all aseptic precautions using 10 ml polythene disposable syringe. 2.0 ml of the blood was dispensed in the bulb with heparin as anticoagulant and remaining 8.0 ml blood was dispensed in the plain polythene tube, for the estimation of free radicals, scavenging enzymes and trace elements. Polythene tube used to avoid glass contamination, for the estimation of trace elements.

Heparinised whole blood was used for the estimation of the enzyme activity of catalase, the Glutathione peroxidase activity superoxide dismutase and serum was used for estimation of lipid peroxide, Adenosine deaminase activity, activity of copper zinc, magnesium, manganese, selenium and vanadium. Following Biochemical investigation were carried out on sample of each subject and healthy control.

Serum lipid peroxide was estimated in Biochemistry Laboratory, General Hospital Miraj using method by K. Satoh. 98

Serum Adenosine deaminase activity was estimated in Biochemistry laboratory General Hospital Miraj using method Galanti and Gausti. 40
Trace Elements in Leprosy

Superoxide dismutase activity was measured using kit (Ransod-superoxide dismutase) manufactured by Randox laboratories ltd U.K. estimations were done at Biochemistry laboratory Wanless hospital, Miraj. \(^{66,75}\)

RBCs lysate used for estimation of catalase activity, using method Sinha A.K.1972. at Shivaji University USIC department, Kolhapur. \(^{104}\)

Glutathione peroxidase activity was measured using kit (RANSEL Glutathione peroxidase ) manufactured by Randox laboratories ltd U.K at Wanless hospital Miraj. \(^{59,83}\)

- Serum copper
- Serum zinc
- Serum manganese
- Serum magnesium
- Serum selenium
- Serum vanadium

Serum copper, zinc, manganese, magnesium, selenium and vanadium
Levels were estimated on atomic absorption spectrophotometer at USIC department Shivaji University Kolhapur and NCL Pune.

1. **Estimation Of Serum Lipid Peroxide.**

Estimation of lipid peroxide was done by method of K. Satoh.  

**Principle :-**

Serum was treated with trichloroacetic acid and precipitate was heated with thiobarbituric acid (TBA) by which coupling of lipid peroxide with TBA gives pink colored chromogen. These chromogens were extracted with n-butyl alcohol and intensity was measured an colorimeter using filter 530 mm. Values were expressed in terms of 'n' moles of malondialdehyde (MDA) per ml.

**Reagents :-**

**20% Trichloroacetic acid (TCA) :-**

Dissolved 20 gm of trichloroacetic acid is distilled water to make volume 100 ml.
**0.05 M sulphuric acid:**

Dilute 4.904 ml concentrated Sulphuric acid in distilled water to make volume 1 liter. 0.2% Thiobarbituric acid in 2 M Sodium Sulphate. Dissolve 28.4 gms of Sodium sulphate (unhydrous) in distilled water, make volume 100 ml. Weighed 200 mgs of Thiobarbituric acid dissolved in 2 M sodium sulphate solution by heating and final volume made to 100 ml with the solution.

**N-butyl alcohol:**

Available in market manufactured by Qualigens E mark etc.

**Standard MDA solution:**

164.2 mgs Malondialdehyde (1,1,3,3 tetra, ethoxypropane). Fluka AG was dissolved in one liter of distilled water that was 1mm. Solution using this, serial dilutions were made to prepare solutions of concentration ln mol to 8n mol.
**Procedure :-**

In 0.5 ml serum 2.5 ml 20% TCA was added, tube was mixed and allowed to stand for 10 minutes.

Supernatent was discarded after centrifugation and the precipitate was washed for 2 times with 0.05 M sulphuric acid.

2.5 ml 0.05 M sulphuric acid and 3.0 ml TBA in sodium sulphate was added to precipitate. Tube was heated for 30 minutes in boiling water bath.

After cooling in cold water bath 4.0 ml, n-butyl alcohol was added and shaken vigorously for extraction of chromogen in alcohol.

Colored organic phase was separated by centrifugation and absorbance was measured on colorimeter at wavelength 530 nm.

By using above procedure absorbance of standard solutions. With different concentrations were measured and graph was plotted with conc. Is ‘n’ moles of
MDA per mol. Against absorbance. From graph, values of lipid peroxide in serum were determined.

2. *Estimation Of Serum Adenosine Deaminase*

   [ The ADA activity was measured in serum by colorimetric method of Galanti Gausti ]

   *Principle:*

   a) Adenosine + H2O + ADA $\rightarrow$ Inosine + NH$_3$

   b) NH$_3$ + Clo $\rightarrow$ NH$_2$Cl + OH$^-$

   c) NH$_2$Cl + $\text{O}[-\text{OH} + \text{OH}^- \rightarrow \text{H}_2\text{N}- \text{O}^- + \text{Cl}^- + \text{H}_2\text{O}$

   d) $\text{H}_2\text{N}-\text{O}^- + \text{OH} + \frac{1}{2}\text{O}_2 \rightarrow \text{N} = \text{O}^- + \text{O}^-$

   The equilibrium of reaction is far to the right, Ammonia is determined by Berhelot's reaction. Ammonia forms an intensely blue indophenols with sodium hypochloride and phenol in alkaline solution(3). Sodium nitroprusside is the catalyst. The ammonia concentration is directly proportional to the absorbance of the indophenols. The reaction catalyzed by ADA is stopped at the end of the incubation period by the addition of the phenol nitroprusside solution.
B] Preparation of Reagents :-

All reagents were made from analytical grade chemicals using double distilled ammonia free water, Ammonia who removed by addition of little H₂SO₄ and KMnO₄ and a second distillation from a glass apparatus. This precaution is necessary if the ammonia is content of tap water is high.

All the solutions were prepared in repurified water as above.

Phosphate buffer :- (50mmol/l PH 6.5.) 4.73 gm NaH₂PO₄. H₂O and 5.62 gm. Na₂HPO₄. 12H₂O was dissolved in water and diluted to 1000 ml with boiled water.

Buffered Adenosine solution (Adenosine 21m mol/L, phosphate buffer 50 m mol/L PH 6.5) 30 ml phosphate buffer was added to 280 mg adenosine is a 50 ml volumetric flask. Diluted to 50 ml with phosphate buffer.

3) Ammonium sulphate stock solution (15m mol/L) 1-982 gm unhydrorous ammonium sulphate dissolved in water and made up to 100ml and mixed thoroughly.
Ammonium sulphate standard solution (75μmol/L) 0.5 ml ammonium sulphate stock solution (precision pipette) was diluted to 1000 ml with phosphate buffer.

Phenol/Nitroprusside solution 10gm (Phenol and 50 mg sodium Nitroprusside was dissolved in approximately 500 ml. Water and diluted to 1000 ml).

Alkaline hypochlorite solution : (NaOcl;11 m mol/L; NaOH, 125 m mol/L)

125 ml NaoH, (1 mol/L) was mixed with 16.4 ml color containing 5% (w/v) Na cl diluted to 1000 ml with water.

C] Calibration Curve for of adenosine deaminase :-

Adenosine deaminase can be standardized by calibration curve standard ammonium sulphate solution.

8 centrifuge tubes were label as S1, S2, S3, S4, S5, S6, S7.
Trace Elements in Leprosy

For standards numbers and 'B' for blank respectively following solutions are added.

<table>
<thead>
<tr>
<th>Standard Number</th>
<th>Phosphate Buffer (ml)</th>
<th>Working Std (ml)</th>
<th>μM (std ml)x75</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1.8</td>
<td>0.2</td>
<td>15</td>
</tr>
<tr>
<td>S2</td>
<td>1.6</td>
<td>0.4</td>
<td>30</td>
</tr>
<tr>
<td>S3</td>
<td>1.4</td>
<td>0.6</td>
<td>45</td>
</tr>
<tr>
<td>S4</td>
<td>1.25</td>
<td>0.75</td>
<td>56.25</td>
</tr>
<tr>
<td>S5</td>
<td>1.00</td>
<td>1.00</td>
<td>75</td>
</tr>
<tr>
<td>S6</td>
<td>0.5</td>
<td>1.5</td>
<td>111.5</td>
</tr>
<tr>
<td>S7</td>
<td>--</td>
<td>2.0</td>
<td>150.0</td>
</tr>
<tr>
<td>B</td>
<td>2.0</td>
<td>--</td>
<td>75</td>
</tr>
</tbody>
</table>

2.5 ml phenol nitroprusside solution and 2.5 ml alkaline hypochlorite were added (Total volume 7 ml) mixed and incubated at 37° C. Absorbance readings were taken in colourimeter using red filter (620 nm). A calibration curve was obtained by plotting Absorbance reading against adenosine concentration μM (or unit/L).
**D) Procedure of estimation of serum Adenosine deaminase:**

Following protocol was used. Incubation volume 1.05 ml final volume 7.05 ml. Sample blank and a reagent blank was also prepared (Fig. In table are volume in ml).

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Reagent Blank (RB)ml</th>
<th>Standard (s)ml</th>
<th>Sample Blank (SB)ml</th>
<th>Sample (T)ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>1.00</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Buffered Adenosine Solution</td>
<td>--</td>
<td>--</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium Sulphate Standard Solution</td>
<td>--</td>
<td>1.00</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Sample (serum)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.05</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.05</td>
<td>0.05</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 60 min.

| Phenol-Nitroprusside reagent | 3.0 | 3.0 | 3.0 | 3.0 |
| Serum                        | --  | --  | 0.05| --  |
| Alkaline Hypochlorite        | 3.0 | 3.0 | 3.0 | 3.0 |
Incubate at 37°C for 30 min. Absorbance was measure at 620 nm (Red filter) in 10 cm light path cavities, against distilled water.

**Calculations :-**

\[
\frac{T - SB}{S - RB} \times 50 \text{ U/L (at } 37^\circ\text{C)}
\]

i.e.  
T = sample  
SB = sample Blank  
S = std  
RB = Reagent Blank

3. **Estimation of RBC Superoxide Dismutase :-**

Estimation was done by using Kit RANSOD, procedure was followed as per literature as below, 66,75.

**Assay Principle :-**

The role of superoxide dismutase (SOD) is to accelerate the dismutation of the toxic, superoxide radical (O_2), Produced during oxidative energy processes, to hydrogen
peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl) -3-(4-nitrophenol) -5-phenyl tetrazolium chloride (I.N.T.) to form a formazan dye. The superoxide dismutase acting is then measured by the degree of inhibition of this reaction.

\[
\text{Xanthine O}_{2} \text{Uric acid} + \text{O}_{2} \\
\text{I.N.T. O}_{2} \text{Formazan dye.} \\
\text{OR} \\
\text{O}_{2} \text{SOD O}_{2} + \text{H}_{2}\text{O}_{2}
\]

**Sample Preparation:**

Use heparinized or EDTA whole blood samples. It is recommended that erythrocytes should be washed four times with 0.9% NaCl solution.

Centrifuge 0.5 ml of whole blood for 10 minutes at 3000 rpm and then aspirate off the serum then wash erythrocytes four times with 3ml of 0.9% NaCl solution centrifuging for 10 min at 3000 rpm after each wash.
The washed centrifuged erythrocytes should be made up to 2.0 ml with cold redistilled water, mixed and left the stand out +4°C for 15 minutes. The lysate is diluted with 0.91 M phosphate buffer PH 7.4, so that the % inhibition falls between 30% and 60%.

Reagent:-

<table>
<thead>
<tr>
<th>Content</th>
<th>Initial Conc. Of Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Mixed Substrate</td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.05 m mol/L</td>
</tr>
<tr>
<td>I.N.T.</td>
<td>0.025 m mol/L</td>
</tr>
<tr>
<td>2) Buffer</td>
<td></td>
</tr>
<tr>
<td>CAPS</td>
<td>50 m mol/L pH 10.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.94 m mol/L</td>
</tr>
<tr>
<td>3) Xanthine Oxidase</td>
<td>80 U/L</td>
</tr>
<tr>
<td>4) Standard</td>
<td>----</td>
</tr>
</tbody>
</table>
Preparation of solution:

Reconstitute the contents of 1 vial of mixed substrate with 20 ml of Buffer.

Buffer—Contents ready for use stable up to expiry date, when stored at +2 to 8°C.

Xanthine Oxidase: Reconstitute 1 vial of Xanthine oxidase with 10 ml. Of redistilled water stable for 2 weeks, when stored at +2 to 8°C.

Standard: Reconstitute one vial of standard with 10 ml. Of redistilled water. Subsequent dilution of this standard should be prepared with 10m M phosphate Buffer PH 7.4. It is recommended that the following dilutions are made of standard (S6) to produce a standard curve:

<table>
<thead>
<tr>
<th>Volume of Standard Solution</th>
<th>Volume of Phosphate Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>S5 5 ml of S6</td>
<td>5 ml</td>
</tr>
<tr>
<td>S4 5 ml of S5</td>
<td>5 ml</td>
</tr>
<tr>
<td>S3 5 ml of S4</td>
<td>5 ml</td>
</tr>
<tr>
<td>S2 5 ml of S3</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

S1 = Blank (Phosphate Buffer)

Stable for 2 weeks at + 2 to +8°C
Procedure:

- **Wavelength**: 505 nm
- **Cuvette**: 1 cm path length
- **Temperature**: 37°C
- **Measurement**: Against air.

Pipette into Cuvette.

<table>
<thead>
<tr>
<th></th>
<th>Reagent Blank</th>
<th>Standard</th>
<th>Diluted Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted sample</td>
<td>---</td>
<td>---</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>---</td>
<td>0.05 ml</td>
<td>---</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
<td>0.05 ml</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Mixed substrate, Mixed well</td>
<td>1.7ml</td>
<td>1.7ml</td>
<td>1.7ml</td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
</tr>
</tbody>
</table>

Mix, read initial absorbance A1 after 30 seconds and start timer simultaneously. Read final absorbance A2 after 3 minutes.

**Calculations**:

\[
\frac{A2-A1}{3} = \text{A/min of standard or sample}
\]

Reagents blanks rate = rate of inhibited = reaction = 100%
All standard rates and diluted sample rates must be converted into percentages of the blank rate, and subtracted from 100% to give a percentage inhibition.

\[
\frac{(A \text{ std/min} \times 100)}{100} - \frac{(A \text{ sample/ min} \times 100)}{100} = \% \text{ inhibition}
\]

Plot percentage inhibition for each standard against Log 10 (standard conc., in SOD units/ml).

Use percentage inhibition of sample to obtain units of SOD from standard curve.

\[
\frac{\text{SOD units / ml of whole blood}}{\text{ml form std. Curve X dilution factor}} = \frac{\text{SOD units / mg Hemoglobin}}{\text{gm Hemoglobin / ml}}
\]
In this estimation 50 fold (dilution factor = 200) dilution of lysate was done to get an inhibition between 30% and 60%.

The values were converted and expressed in SOD units/ mg of Hb.

4. Colorimetric Assay Of Catalase

Principle :-

The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of $\text{H}_2\text{O}_2$, with the formation of per chromic acid as an unstable intermediate. The chromic acetate thus produced is measured calorimetrically at 240 nm. The catalase preparation is allowed the split $\text{H}_2\text{O}_2$, for different periods of time by the addition of dichromate/acetic acid mixture and the remaining $\text{H}_2\text{O}_2$ is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.
Reagents :-

1) Dichromate / acetic acid ;

This reagent was prepared by mixing 5% solution of K$_2$Cr$_2$O$_7$ with glacial acetic acid (1:3, by volume).

2) Hydrogen Peroxide :-(0.2 M) : 30% W/V.

3) Phosphate buffer (0.01 M, PH 7.0) :-

13.79 gms of potassium monohydrogen phosphate (K$_2$HPO$_4$) and 2.69 gms of potassium dihydrogen phosphate (KH$_2$Po$_4$) was dissolved is distilled water and made the volume to 1 liter. This was then diluted 10 times with distilled water for use.

Preparation of Hemolysate :

The blood corpuscles were isolated by centrifugation. The isolated blood corpuscles were wasted with 0.9% NaCl solution. They were then based with 20 parts of cold water. The lysate was property diluted with water for the assay of its catalase content (1: 40).
**Protein Estimation** :-

Protein in haemolysate was estimated by king's method.

**Colorimetric Determination of H₂O₂ :-**

**Procedure** :

**For Standard** :

Different amounts of H₂O₂, ranging from 40 to 180 μ moles were taken in small test tubes (6.0 ml) and 2.0 ml of dichromate / acetic acid was added to each. The solution was then heated in a boiling water bath for 10 minutes. After cooling at room temperature, the optical density was measured at 620 nm in a photoelectric colorimeter. For blank 1.0 ml buffer, 0.5 ml distilled water and 2.0 ml dichromate / acetic acid was added.

**Test :-**

**For 'O' second :-**

To 1.0ml buffer, 0.1ml of diluted haemolysate sample and 0.1ml of H₂O₂ was added. The time was noted while adding H₂O₂. After 60 seconds 2ml of
dichromate / acetic acid mixture was added. Mixed and boiled for 10 minutes and after cooling at room temperature, the optical density was measured at 620 nm photoelectric colorimeter.

For blank 1.0 ml buffer. 0.2ml of distilled water and 2.0ml of dichromate / acetic acid was added.

Calculations

\[
\text{Catalase activity} = \frac{U}{\text{mg of protein}}
\]

\[
U = \mu \text{ males of } H_2O_2 \text{ minutes.}
\]

\[
U = Xx \text{ multiplication factor.}
\]

Where \( X = \text{O.D at } 0.0 \text{ sec.} - \text{O.D. at } 60 \text{ sec.} \)

Estimation of RBC Glutathione Peroxidase. 59,83

Estimation was done by using Kit RANSEL, procedure was followed as per literature as below.

\textit{UV method} :-

This method is based on that of pagila and valentine Glutathione peroxidase (GPx) catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of
Glutathione reductase (GR) and NADPH the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease absorbance at 340 nm is measured.

**Reaction Principle**

\[ 2\text{GSH} + \text{ROOH} \rightarrow \text{GSH}_2 + \text{ROH} + \text{GSSG} + \text{H}_2\text{O} \]

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + 2\text{GSH}^- \]

**Sample Preparation:**

Use heparinised whole blood, for sheep and goats dilute 0.05 ml + 3 ml diluting agent, for cattle, horses and other species including human being dilute 0.05 ml + 2 ml diluting agent.

**Reagents:**

<table>
<thead>
<tr>
<th>Contents</th>
<th>Concentrations in the test.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4) Reagent</td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>4 m mol/L</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>0.5 V/L</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.28 m mol/L</td>
</tr>
</tbody>
</table>
5) **Buffer**
   - Phosphate Buffer 0.05 m mol/ L PH 7.2
   - EDTA 4.3 m mol/L

6) **Cumene hydroperoxide** 0.18 m mol/ L

7) **Diluting Agent**

**Preparation of solutions :-**

**Reagents :-**

Reconstitute one vial of Reagent 1 with the appropriate volume of buffer.

- 6.5 ml for the 8x 6.5 ml Kit
- 10 ml for the 8 X 10 ml Kit
- 30 ml for the 8 X 30 ml Kit

Stable for 48 hrs at +4 to +8°C or 8 hrs at +15 to 25°C.

**Buffer :-**

Contents ready for use stable up to expiry date when stored at +2 to 8°C.
**Cumene hydroperoxide:**

Dilute 10 μl with 10 ml of redistilled water and mix thoroughly by shaking vigorously as the cumene is difficult to dissolve. Prepare fresh daily.

Concentrate stable up to the expiry date when stored at +2 to +8°C.

A pipette with a positive displacement and glass Capillaries, should be used to measure the cumene hydroperoxide volume.

**Diluting Agent:**

Reconstitute the contents of one vial of Diluting Agent with 200 ml of redistilled water, stable for 4 week when stored at +2 to +4°C for 3 days of +15 to +25°C

**Procedure:**

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Hg 340 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuvette</td>
<td>1 cm Light path</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Measurement</td>
<td>against air.</td>
</tr>
</tbody>
</table>
Trace Elements in Leprosy

Pipette into cuvette.

<table>
<thead>
<tr>
<th>Diluted Sample</th>
<th>Reagent Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted sample</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Reagent</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>Cumene</td>
<td>0.10 ml</td>
</tr>
</tbody>
</table>

Mix, read initial absorbance of sample and reagent blank after the minute and start timer simultaneously. Read again after 1 and 2 min. Subtract reagent blank value from that of the sample.

**Calculation:**

Glutathione Peroxidase Concentration may be calculated from the following formula.

\[
U/L \text{ of haemolysate} = 8.412 \times A_{340} \text{ nm/min.}
\]

Results where expressed by converting in units / gm of Hb.