MATERIAL AND METHODS
**MATERIAL AND METHODS**

**MATERIALS**

Thirty children suffering from severe PEM and in the age group of 1 to 4 years admitted to the Department of Paediatrics, Medical College Hospital, Rohtak constituted the case material for the present study. A child was diagnosed as suffering from severe PEM if he had:

a) a weight below 60 per cent of 50th percentile of Boston Standards;

b) extreme muscle wasting;

c) no fluid retention (oedema);

d) apathy and misery; and

e) haemoglobin concentration less than 8.0 g/dl.

Care was taken to exclude cases associated with any major illness or infection. Diarrhoea, if present, was brought under control prior to the start of investigations.

Ten healthy children (weighing more than 80 per cent of the 50th percentile of Boston Standards and haemoglobin level more than 10 g/dl) of the same age group, formed the normal control group.
During the initial studies, the children were kept on a diet more or less similar to that they had been consuming at home. After the initial studies the PEM children were divided into two groups of fifteen each and were allocated to the following dietary schedules:

**Group I:**

Children were given a diet with high protein (to yield 30 per cent calories) with slightly low fat (to provide 20 per cent calories) and adequate carbohydrates (to yield 50 per cent calories). The diet was supplemented with adequate amounts of vitamins (water and fat soluble) and minerals. Total elemental iron deficit of the child was calculated according to the following formula and injected intramuscularly. The one dose did not exceed 5 mg/kg body weight per day.

Elemental iron deficit (mg) = 2.5 \times \text{Body weight (LO-Hb)}

**Group 2:**

The children of this group were maintained on a diet complete with respect to protein and calories as in group -I. However, the total elemental iron deficit
was not replenished but care was taken to supplement the diet with adequate amounts of other minerals and vitamins.

All the marasmic children were given diet to provide 150 calories per kg actual body weight/day and protein (7.0 g/kg/day). In the beginning a diet equivalent to about 50 cal/kg/day (or as much a child could tolerate) was given. The amount of diet was gradually increased so that by day 3 all the children were taking the desired amount. The experimental diets were started on 3rd or 4th day of admission (after completion of the initial studies) and continued to 14th or 15th day and then the follow up studies were conducted.

Butter, milk, skimmed milk, eggs, calcium caseinate, pulses, bread, rice/wheat, sucrose, lactose, potatoes and purified groundnut oil were used as components of the diet. Cheese and Banana were also used occasionally as dietary supplements.

The approximate composition of the above diet is given below. Dietary components, however, had to be changed occasionally depending upon the taste and
<table>
<thead>
<tr>
<th>Foodstuff</th>
<th>Amount</th>
<th>Protein (gms)</th>
<th>Fat (gms)</th>
<th>Carbohydrates (gms)</th>
<th>Calories (gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>500 ml</td>
<td>15.0</td>
<td>12.5</td>
<td>22.5</td>
<td>265</td>
</tr>
<tr>
<td>Cereals</td>
<td>50 gm</td>
<td>5.0</td>
<td>0.5</td>
<td>37.0</td>
<td>172</td>
</tr>
<tr>
<td>Pulses</td>
<td>50 g</td>
<td>11.0</td>
<td>1.3</td>
<td>30.0</td>
<td>175</td>
</tr>
<tr>
<td>Egg</td>
<td>50 g</td>
<td>6.6</td>
<td>6.6</td>
<td>-</td>
<td>87</td>
</tr>
<tr>
<td>Bananas</td>
<td>100 g</td>
<td>1.2</td>
<td>0.3</td>
<td>37.0</td>
<td>116</td>
</tr>
<tr>
<td>Sucrose</td>
<td>15 g</td>
<td>-</td>
<td>-</td>
<td>15.0</td>
<td>60</td>
</tr>
<tr>
<td>Calcium caseinate</td>
<td>30 g</td>
<td>30.0</td>
<td>-</td>
<td>-</td>
<td>120</td>
</tr>
</tbody>
</table>

**INVESTIGATIONS**

A) Marasmic (PED) Children:

The blood samples were collected within three days of admission and again after the completion of stipulated rehabilitation period (2 weeks). Each time, 10-15 ml blood was withdrawn from the scalp veins, external jugulars or femoral veins. The blood was taken into heparinized bottles and analysed for the following...
investigations on the same day.

1) Basic Haematological Characteristics:
   i) Hb.
   ii) PCV
   iii) RBC count
   iv) Peripheral blood film (PBF)
   v) Red cell indices
      a) MCV
      b) MCH
      c) MCHC
   vi) Nucleocyte count
   vii) Bone marrow smear morphology and iron grade

2) Iron and Protein Metabolism:
   i) Total plasma protein
   ii) Plasma iron
   iii) Total iron binding capacity (TIBC)
   iv) Transferrin saturation
   v) Unsaturated iron binding capacity (UIBC)

3) Erythrocyte Glutathione Metabolism:
   i) Reduced glutathione (GSH)
ii) Glutathione peroxidase (GSH-Px) 
iii) Glutathione reductase (GSSG-R) 
iv) Glucose-6-phosphate dehydrogenase (G-6-PD).

B) Normal Control Children:

The above mentioned investigations for children suffering from severe marasmus were also carried out in normal control children. However, the repeat studies after the experimental diets were not performed in these children.

METHODS

1) BasiC HeMATOLOGICAL CHArACTERISTICS

(i) Haemoglobin:

The haemoglobin was estimated by Cyanmethaemoglobin method (Drabkin and Austin, 1932). The blood is diluted in a solution containing potassium cyanide and potassium ferricyanide to convert the haemoglobin to cyanmethaemoglobin and then the absorbance of the resulting solution is measured in a photoelectric colorimeter at a wave length of 540 nm.
**Diluent (Drabkin's solution):**

It was prepared by adding 200 mg potassium ferricyanide, 50 mg potassium cyanide and 140 mg potassium dihydrogen phosphate in one liter of distilled water. The clear solution was stored in an ambered colored bottle at room temperature. The reagent was discarded if it became turbid, the pH was found to be outside 7.0 to 7.4 range or its absorbance was more than zero at 540 nm against a water blank.

**Procedure:**

20 μl of blood was added to 4.0 ml of diluent in a tube which was then stoppered and inverted several times. After being allowed to stand for 20 minutes at room temperature, its optical density (O.D.) was recorded against reagent blank at 540 nm. The optical density of cyanmethaemoglobin standard was also read in the similar way.

**Calculations:**

\[
\text{Hb (g/dl)} = \frac{O.D. \text{ Test}}{O.D. \text{ Standard}} \times \frac{\text{Conc. Standard (g/dl)}}{1000} \times d^*
\]

*d is the dilution factor (e.g., 201)
11) **Packed Cell Volume (PCV or Haematocrit value):**

Haematocrit value (literally known as blood separation) was determined by the micro-method using microhaematocrit centrifuge tubes (Dacie and Lewis, 1975).

**Procedure:**

The blood was taken in the standard heparinized microhaematocrit tubes by capillary action, leaving at least 15 mm unfilled. The tube was then sealed at the dry end and was centrifuged in the microhaematocrit centrifuge (Unipan, Type 316) for 5 min. After centrifugation the PCV was measured with the help of Micro-capillary reader (Unipan, Type 316-1).

111) **RBC Count:**

The counting of red blood cells was done by visual method using Neubauer chamber (Dacie and Lewis, 1975).

**Procedure:**

A 1:200 dilution of blood was made in formal-
citrate solution by washing 20 μl of blood into 4 ml of diluting fluid. The red cells were counted by Neubauer chamber. The red-cell diluting fluid was prepared by adding 10 ml of 40% formaldehyde and 31.3 g of trisodium citrate to 1 L of distilled water.

iv) Red Cell Indices:

The mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) have been referred to as red cell 'indices' or as 'absolute' values. These values are calculated from the results of the red-cell count, haemoglobin concentration and PCV values and have been widely used in the classification of anaemia.

a) Mean Cell Volume (MCV)

\[
\text{MCV} = \frac{\text{PCV}}{\text{RBC count(millions/mm})^3} \times 10
\]

It is expressed in femto litres (fl)

b) Mean Cell Haemoglobin (MCH)

\[
\text{MCH} = \frac{\text{Hb}(g/dl)}{\text{RBC count(millions/mm}^3)} \times 10
\]

It is expressed in picograms (pg)
c) **Mean Cell Haemoglobin Concentration (MCHC)**

\[
\frac{\text{Hb}(g/dl)}{PCV} \times 100
\]

It is expressed as g/dl

v) **Peripheral Blood Film Morphology:**

Blood film was prepared according to the method described by Dacie and Lewis (1975) and examined for the type of anaemia. Abnormality in red cell picture stems from four main causes:

a) Abnormal erythropoiesis which may be effective or ineffective;
b) inadequate Hb formation;
c) damage to, or changes affecting, the red cells after leaving the bone marrow; and
d) attempts by the bone marrow to compensate for anaemia by increased erythropoiesis.

These processes result, respectively, in the following abnormalities of the red cells:

1) increased variation in size and shape

* (anisocytosis and poikilocytosis);
ii) reduced or unequal Hb. content (hypochromasia or anisochromasia);

iii) spherocytosis, irregular contraction or fragmentation (Schistocytosis); and

iv) signs of immaturity (polychromasia, punctate basophilia and erythroblastemia).

vi) Bone Marrow Morphology and Iron Grade:

Bone marrow iron was established by Perl's modification of the technique of Prussian blue staining for the study of the reticuloendothelial iron and sideroblast count (Kumar, 1971).

Reagents:

1. Potassium Ferrocyanide (2%):

   2 gms of potassium ferrocyanide was dissolved in 100 ml of iron free or deionized water.

2. HCl (2%):

   2 ml of concentrated HCl (AR) was added to 18 ml of deionized water.

3) Prussian Blue Stain:

   Solution 1 and 2 were mixed in the ratio
of 1:1 just before staining the bone marrow smears.

4. **Neutral Red Solution (1%)**:  
   5 gms of neutral red powder was dissolved in 500 ml of deionized water.

**Procedure:**

Bone marrow aspiration was done from posterior superior iliac spine using aseptic technique and smears prepared on clean glass slides. The following procedure was adopted for the fixation of the smears.

1. The bone marrow smears were fixed by placing the slides in a Koplin's jar containing methanol for 10 minutes.
2. Methanol fixed smears were placed in an iron free Koplin's staining jar, freshly prepared Prussian blue stain was added to the jar which was then placed in water bath at 56°C for 15 minutes.
3. Slides were washed several times with deionized water and were left in it for 10-15 minutes.
4. After another washing, the slides were counter stained with neutral red for 30-45 seconds.
5. Slides were again washed with deionized water, followed by tap water and then were allowed to dry.

Slides were examined for the presence of iron particles under oil immersion. The iron particles attached to only reticuloendothelial cells were considered. Iron particles are stained as bluish particles and the free particles were ignored as artefacts. Reticular iron grading was done according to the criteria of Kumar (1971) in the following way:

**Grade**

Zero : No haemosiderin granules in whole preparation of the smear.

Traces : One or very few granules in whole preparation.

I : Fine granules in every 3 or 4 immersion field.

II : Rather more heavy granules in about every 2nd or 3rd immersion field.

III : Haemosiderin granules in every oil immersion filled in one or more cells.

IV : Massive haemosiderin deposit with clumped heavy granules.
vii) Reticulocyte Count:

Reticulocyte counts were obtained by the method described by Dacie and Lewis (1975) using new methylene blue. New methylene blue staining solution was prepared by dissolving 1.0 gm of the dye in 100 ml of citrate-saline solution (one vol. of 30 g/L sodium citrate to 4 vol. of 9.0 g/L NaCl). The mixture was filtered after the dye had dissolved.

2) Iron and Protein Metabolism

i) Total Plasma Protein:

Total protein in plasma was determined by the Biuret reaction was described by Henry et al (1957). The protein forms a coloured complex with Cu^2+ in alkaline solution. The absorption of the biuret colour complex is read at 540 nm.

Reagents:

i) Biuret reagent (Benedict's qualitative glucose reagent):
17.3 g of CuSO_4·4H_2O was dissolved in about 100 ml of hot distilled water(1). 173.0 g of sodium citrate and 100.0 g of anhydrous
\(\text{Na}_2\text{CO}_3\) was dissolved in about 800 ml of distilled water by heating\(^2\). It was cooled and solution 1 was poured into solution 2 while stirring. The reagent was adjusted to a final volume of 1 liter with distilled water.

\(11)\text{NaOH}(6\%)\):

6.0 g of anhydrous NaOH was dissolved in about 80 ml of distilled water and the final volume was made to 100 ml.

\(11i)\text{Protein Standard}(280 \text{ mg\%})\):

It was prepared from pooled clear serum containing 6.3 g/dl and diluted to the required concentration. It was then divided to small aliquots and kept in the deep freeze until use.

**Procedure:**

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (ml)</td>
<td>2.4</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>Plasma (ml)</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein standard (ml)</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>NaOH (ml)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Benedict's qualitative glucose reagent (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
The reagents were mixed and all the tubes were placed in water bath at 37°C for 10 minutes. The optical density of standard \((A_s)\) and test \((A_t)\) against the reagent blank was recorded at 540 nm.

Calculations:

\[
\text{Plasma proteins (g/dl)} = \frac{A_t}{A_s} \times 7
\]

ii) Plasma Iron:

Plasma iron was estimated by the method of Ramsay (1954, 1958) using 2,2'-dipyridyl. Ferrous iron gives a pink colour with 2,2'-dipyridyl. A solution of dipyridyl in acetic acid is added to plasma followed by a reducing agent. Proteins are removed by centrifugation after heating in boiling water bath.

Reagents:

1. 2,2'-dipyridyl (0.1%):
   100 mg of dipyridyl was dissolved in 100 ml of 3% (v/v) acetic acid.
2. Sodium Sulphite (0.1 M):
   1.26 gm of anhydrous sodium sulphite was
3. Chloroform.

4. **Stock iron standard (100 μg iron/ml):**

   It was prepared by dissolving 0.498 gm of ferrous sulphate (FeSO₄·7H₂O) in deionized water and 1 ml of concentrated H₂SO₄ was added to it. The volume was made up to one litre with distilled water.

5. **Working Iron Standard (3 μg/ml):**

   3 ml of stock iron standard was diluted to 100 ml with deionized water.

**Procedure:**

The tubes were set up in the following way:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium sulphite(ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Dipyridyl (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Plasma (ml)</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Working iron standard (ml)</td>
<td>-</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>Deionized water(ml)</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
</tr>
</tbody>
</table>
The tubes were heated in the boiling water bath for 5 minutes. After cooling the tubes, 1 ml of chloroform was added to all the tubes. The tubes were stoppered and shaken vigorously for 30 seconds. The tubes were centrifuged at 3000 rpm for 5 minutes and absorbancy of clear supernatant of standard and test was recorded at 520 nm against reagent blank.

**Calculation:**

\[
\text{Plasma iron (µg/dl) = } \frac{\text{O.D. Test}}{\text{O.D. Standard}} \times 300
\]

**iii) Total Iron-Binding Capacity (TIBC):**

TIBC was determined by Ramsay’s dipyridyl method (Ramsay, 1954, 1958).

**Reagents:**

1. **Ferric chloride solution (5 µg iron/ml) in 0.005N HCl:**

Stock ferric chloride solution was prepared by adding 145 mg of FeCl₃ per 100 ml of 0.5N HCl and diluted to 1:100 with deionized water, to obtain working ferric chloride solution.
2. Magnesium Carbonate 'light' (for adsorption)

3. Sodium Sulphite (0.2M):
   2.52 gms of anhydrous sodium sulphite was dissolved per 100 ml of dionized water.

4. 2,2'-dipyridyl (0.2%):
   200 mg of dipyridyl was added to 100 ml of 3\%(v/v) acetic acid.

5. Chloroform

6. Stock and working iron standards were prepared in the same way as described for plasma iron.

Procedure:

4.0 ml of ferric chloride solution was added after 2 ml of plasma. After 5 minutes, 400 mg of magnesium carbonate was added. It was shaken frequently and vigorously for 30 to 60 minutes and then centrifuged. 4 ml of clear supernatant was removed for the determination of iron. 1 ml each of the 0.2 M sodium sulphite and 0.2% dipyridyl solutions were added and the same procedure was adopted as described previously for plasma iron.
Calculation:

Total iron-binding capacity(μg/dl) =

O.D. Test x 450
O.D. Standard

iv) Transferrin Saturation(%)

Plasma iron x 100
TIBC(μg/dl)

v) Unsaturated iron binding capacity(UIBC):

TIBC(μg/dl) - Plasma iron(μg/dl)

3) Erythrocyte Glutathione Metabolism

1) Reduced Glutathione(GSH):

whole blood reduced glutathione(GSH) was estimated by the method as described by Kay&Murfitt(1960).
The method is based on the principle that whole lysed blood is deproteinized with TCA. It is then neutralized
with buffered NaOH. Alloxan forms a complex with GSH which has a characteristic absorption at 305 nm.

**Reagents:**

1. **TCA (10%):**
   - The solution was prepared by adding 10 gms of trichloroacetic acid to 100 ml of distilled water.

2. **Phosphate Buffer (0.24 M, pH 7.6):**
   - The phosphate buffer was prepared by mixing 0.24M Na₂HPO₄ and 0.24 M KH₂PO₄ in the ratio of 88:12 (v/v).

3. **Buffered NaOH:**
   - 0.1 ml of 1M NaOH was added to 1 ml of phosphate buffer (0.24M, pH 7.6).

4. **Alloxan Solution (1 mg/ml):**
   - 1 mg of alloxan monohydrate was dissolved in 1 ml of very dilute HCl (1 drop of concentrated HCl per 100 ml of distilled water). It was prepared freshly immediately before use and utilized within 5 minutes.
5. **GSH Standard (0.01%):**

10 mg of GSH was dissolved in 100 ml of distilled water and it was prepared freshly.

6. **Glycine (0.1 M):**

750 mg of glycine was dissolved in 100 ml of distilled water.

**Procedure:**

0.2 ml whole blood was added to 0.6 ml distilled water and it was allowed to stand for 10 minutes to ensure complete haemolysis. 0.8 ml of TCA was then added and mixed immediately with glass rod. The clear supernatant was obtained by centrifuging at 3000 rpm for 5 minutes and it was used for the estimation of GSH in the following way:
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Phosphate buffer (ml)</td>
<td>2.9</td>
<td>3.9</td>
<td>4.5</td>
<td>6.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Supernatant (ml)</td>
<td>0.4</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Buffered NaOH (ml)</td>
<td>1.2</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GSH standard (ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>Alloxan solution (ml)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
</tr>
</tbody>
</table>

After mixing the contents of the tubes they were kept in the dark for 20 minutes. The extinctions were recorded at 305 nm by UV spectrophotometer.

**Calculation:**

Reading of control against phosphate buffer blank $= E_A$

Reading of test against alloxan blank $= E_B$

Reading of standard against alloxan blank $= E_S$

$\text{mg of GSH/dl of whole blood} = \frac{40(E_B - E_A)}{E_S}$

The conc. of GSH was also calculated as mg/gHb and as $1 \times 10^{-8}$ µg/RBC.
**Processing of Red Blood Cells and Preparation of Haemolysate for Determining the Activities of Red Cell Enzymes:**

The enzyme assays were carried out on red blood cells which had been carefully freed of the other formed elements and had been washed free of plasma in 0.9% sodium chloride solution.

The whole blood was centrifuged in the cold (4°C) at approximately 2000 rpm for 5 minutes and the plasma and buffy coat were removed by aspiration from the packed red cells. The plasma was preserved for estimation of total protein, iron and total iron binding capacity. The red cell suspension was diluted (1:5) with ice-cold 0.154M sodium chloride solution and was centrifuged in the cold at 2000 rpm for 15 minutes. The supernatant and residual buffy coat, if any, were removed from the packed red cells. They were again suspended in about 5 ml of cold 0.154M NaCl solution. The suspension was centrifuged at 2000 rpm for 10 minutes and the supernatant was again removed. The washing in cold sodium chloride solution was repeated once more and the packed erythrocytes were suspended in approximately one volume of saline.
The haemolysate was prepared by addition of distilled water and it was centrifuged to remove the debris. The haemoglobin concentration of the resulting haemolysate was adjusted to 1g%. The results of enzyme assays were expressed in terms of activity per gram of haemoglobin (IU/gHb) and to the number of red cells (IU/1x10¹⁰ RBC). For latter expression, a 50% red cell suspension was used for performance of red cell counts. The assay of enzyme activity was performed within 1 hour of preparation of the haemolysate.

ii) **Glutathione Peroxidase (GSH-Px)**

The activity of GSH-Px was measured according to the method described by Hopkins and Tudhope (1973 b). Glutathione peroxidase catalyses the oxidation of GSH to GSSG by hydrogen peroxide:

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

where \(\text{R-C-O-H}\) is a peroxide and hydrogen peroxide is the most commonly used substrate for assay of this enzyme. The rate of formation of GSSG is measured by means of the glutathione reductase (GSSG-x) reaction.
GSSG\textsuperscript+ + N\textsubscript{ADPH} + H\textsuperscript+ \xrightarrow{\text{GSSG-K}} 2\text{GSH} + N\text{ADP}^+ \\

The oxidation of NADPH is followed at 340 nm.

**Reagents:**

1) **Phosphate Buffer (0.15 M, pH 7.5, 0.005 M EDTA):**
   
   It was prepared by mixing 84 ml of Na\textsubscript{2}HPO\textsubscript{4}, 16 ml of 0.15 M KH\textsubscript{2}PO\textsubscript{4} and 100 ml of distilled water. EDTA was added to the buffer in such a way so as to give a strength of 0.005M.

2) **NADPH (0.0084 M):**

   To 1 ml of distilled water, 7.0 mg of NADPH (mol.wt. 333.4) was added.

3) **GSH (0.15 M):**

   46.0 mg of GSH (mol.wt. 307.33) was dissolved in 1 ml of distilled water.

4) **Sodium azide (1.25 M):**

   The solution of sodium azide (NaN\textsubscript{3}) was prepared by adding 7.125 g of NaN\textsubscript{3} in 100 ml of distilled water.
5) Glutathione Reductase (GSSG-R, 100 e.u./ml):

It was obtained as such from Sigma chemical Company.

6) Hydrogen Peroxide (0.0022 M):

It was prepared according to the method described by Beutler (1975). The optical density of 0.9 ml of 1:10 dilution of phosphate buffer pH 7.0 was measured at 230 nm (OD₁). 0.1 ml of a 1:100 dilution of H₂O₂ (30%) solution was added to the above mentioned phosphate buffer solution and again the optical density of the resulting solution was recorded (OD₂). Since the mM extinction coefficient of H₂O₂ at 230 nm is 0.071 (Chance, 1954), the H₂O₂ concentration (C) of the 1:100 diluted peroxide solution is

\[ 141 \times (OD₂ - OD₁) \text{ mM} \]

To dilute to 0.0022 M (2.2 mM) required for the assay, 1 ml of 1:100 dilution of H₂O₂ was made to C/2.2 ml with distilled water.

Procedure:

The reagents were added to the cuvettes in
the following way:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Assay system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer(µl)</td>
<td>1390</td>
<td>1290</td>
</tr>
<tr>
<td>Haemolysate (µl)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>GSSG-R (µl)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>NaN₃ (µl)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>GSH (µl)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>NADPH (µl)</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

The reagents were allowed to equilibrate for 5 minutes at 37°C and then the reaction was initiated by the addition of 50 µl of 0.0022 M H₂O₂ to the assay system. The optical density was recorded at 1 minute interval for 5 minutes and change in optical density per minute (Δ OD/mt) was calculated.

The activity of the enzyme is defined as µ-moles of NADPH oxidized per minute under the conditions used and is expressed as the activity of GSH-Px per g of haemoglobin (IU/gHb). The activity of GSH-Px was also calculated in number of red cells (IU/1xl0¹⁰ RBC).

Calculations:

In the case of NADP+/NADPH linked enzyme
assays in which 1 mole of coenzyme is reduced or oxidized for each mole of substrate consumed. The number of enzyme units ($A$) can be calculated from the following formula:

$$A = \frac{\Delta OD/mt}{6.22} \times \frac{V_o}{V_h}$$

Where $V_c$ is the cuvette volume, $V_h$ is the volume of haemolysate added in the reaction system and OD is the change in the optical density per minute at 340 nm. The molar extinction coefficient for $\text{NADPH}/\text{NADP}^+$ at 340 nm is $6.22 \times 10^3$ M and 6.22 is the optical density of a 1 mM solution of $\text{NADPH}$.

Therefore, $\mu$ moles of $\text{NADPH}$ formed is as follows:

$$A = \frac{\Delta OD/mt}{6.22} \times \frac{1.5}{0.0005}$$

$$A = \Delta OD/mt \times 482$$

iii) Glutathione Reductase (GSSG-R):

The activity of this enzyme was measured by the method of Long and Carson (1961) as described by Brewer (1969). Glutathione reductase catalyzes the
reduction of oxidized glutathione (GSSG) by NADPH to reduced glutathione (GSH):

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

The activity of the enzyme is measured by following the oxidation of NADPH spectrophotometrically at 340 nm.

Reagents:

1. **Tris-HCl buffer (0.4M, pH 7.6):**

   It was prepared by dissolving 4.84 g of Tris (Mol. wt. 121.14) in about 80 ml of distilled water. The pH of the solution was adjusted to 7.6 with 0.4M HCl and the volume was made to 100 ml with distilled water.

2. **Oxidized glutathione (0.033M):**

   It was prepared by adding 20.2 mg of oxidized glutathione (Mol. wt. 612.0) to 1 ml of distilled water.

3. **EDTA (0.2M):**

   The solution of EDTA was prepared by adding
3.722 g of EDTA (Mol. Wt. 372.24) in 50 ml of distilled water.

4) NADPH (0.0018M):

It was prepared by dissolving 1.5 mg of NADPH (Mol. Wt. 633.4) in 1 ml of distilled water.

Procedure:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer (μl)</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>EDTA (μl)</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>GSSG (μl)</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Haemolysate (μl)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Distilled water (μl)</td>
<td>450</td>
<td>200</td>
</tr>
<tr>
<td>NADPH (μl)</td>
<td>-</td>
<td>250</td>
</tr>
</tbody>
</table>

The change in OD was measured for 20 minutes after an initial lag period of 2 minutes. The ΔOD/mt was calculated to find out the activity of GSSG-R as μ mole of NADPH oxidized/mt/g of haemoglobin. The activity was also expressed as IU/1x10^10 RBC. The enzyme activity was calculated in the same way as described for GSH-Fx.
Glucose-6-phosphate dehydrogenase (G-6-PD) catalyzes the oxidation of glucose-6-phosphate (G-6-P) to 6-phosphogluconolactone which quickly hydrolyses spontaneously to 6-phosphogluconate (6-PG).

\[ G-6-P + NADP^+ \rightarrow G-6-PD \rightarrow 6-PG + NADPH + H^+ \]

The most commonly used method for G-6-PD assay is by following the rate of reduction of NADP$^+$ to NADPH when hemolysate is incubated with glucose-6-phosphate. The erythrocytic G-6-PD activity was assayed with spectrophotometer by determining the rate of NADPH formation from the increase in extinction at 350 nm according to the method of Lohr and Waller as described by Bergmeyer (1974).

**Reagents:**

1) **Triethanolamine Buffer (50 mM, pH 7.5):**

The triethanolamine buffer was prepared by adding 0.93 g of triethanolamine hydrochloride (mol.wt. 149.19) and 0.2 g of EDTA (mol.wt. 372.24)
in 50 ml of distilled water. The pH of the resulting solution was adjusted to 7.5 with 0.01N NaOH/HCl and then it was diluted with distilled water to the final volume of 100 ml.

2) **NADP^+**:  

25 mg of NADP^+(mol.wt. 765.4) was dissolved in 1 ml of distilled water.

3) **G-6-P (40 mM)**:  

This substrate was prepared by dissolving 12.16 mg of G-6-P(mol.wt.304.1) in 1 ml of distilled water.

**Procedure:**

The reagents were added to the cuvettes in the following way:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Assay system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethanolamine Buffer(μl)</td>
<td>1425</td>
<td>1400</td>
</tr>
<tr>
<td>Haemolysate (μl)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>NADP^+ (μl)</td>
<td>-</td>
<td>25</td>
</tr>
</tbody>
</table>
The reagents were mixed and incubated for 5 minutes at 37°C. The reaction was started by the addition of 25 µl of the substrate solution.

The reagents were again mixed and optical density was read in Spectrophotometer till it reached to about 0.02. This was taken as zero minute reading and the optical density was recorded at 2 minutes interval for 10 minutes. The change in optical density per minute (ΔOD/mt) was calculated to find the activity of G-6-PD. The enzyme activity was calculated in the same way as described for GSH-Px and it was expressed as IU/gHb and IU/1x10^-10 µBc.