Materials and Methods
2. MATERIALS AND METHODS

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2. MATERIALS AND METHODS

2.1 AREA OF STUDY

Madras city is endemic for malaria and accounts for about 65 - 75% of new cases annually, to the malaria situation in Tamilnadu. Out of the 150 divisions of the Madras Corporation, the 55th Division and Zone X, Mylapore, are hyperendemic areas, where 90% of the malarial cases have been due to Plasmodium vivax infection and the peak transmission occurs between July and October.

The present study was carried out in the 55th Division and Zone X, Mylapore, Corporation of Madras.

2.2 SUBJECTS

2.2.1 Patients

2.2.1.1 Recurrent malaria

Only male patients (n=172), with age ranging from 14-40 years, were included for this study, if they

1. had the trophozoite and gametocyte stages of P. vivax in the peripheral blood smear.

2. were not diabetic and had not undergone any treatment prior to sampling.
3. had a single or multiple attack of malaria within a period of six months. Based on the patients clinical followup record, recurrent *P. vivax* infected patients were taken for this study and the subjects were divided into four groups:

   a. I attack - single infection  
   b. II attack - two infections  
   c. III attack - three infections  
   d. IV attack - more than three infections.

2.2.2 Control subjects

2.2.2.1 Healthy controls

Age and sex matched healthy controls who were residents of the endemic area with similar socio economic status formed the control group (n=63).

2.2.2.2 Negative controls

Patients (n=20) who had similar symptoms of malaria but reported negative for any parasites in the peripheral blood smear formed this group.

2.2.3 After treatment

A section of the infected patients (n=85) who gave fully informed consent to repeated blood sampling formed the group after treatment. Among them a section of patients were given a three day radical treatment while the other section was supplemented with vitamin E during the course of radical treatment. Blood samples were also drawn from healthy controls (n=47).
2.2.4 Therapy

Chloroquine phosphate and primaquine were administered orally to *P. vivax* infected malarial patients and the dosage was as follows:

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Chloroquine</th>
<th>Primaquine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>600 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td>Day 2</td>
<td>300 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td>Day 3</td>
<td>300 mg</td>
<td>15 mg</td>
</tr>
</tbody>
</table>

In vitamin E supplemented group, α-tocopherol acetate was given orally at a dosage level of 400 mg/day for a period of three days.

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Chloroquine</th>
<th>Primaquine</th>
<th>Vitamin-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>600 mg</td>
<td>30 mg</td>
<td>400 mg</td>
</tr>
<tr>
<td>Day 2</td>
<td>300 mg</td>
<td>30 mg</td>
<td>400 mg</td>
</tr>
<tr>
<td>Day 3</td>
<td>300 mg</td>
<td>15 mg</td>
<td>400 mg</td>
</tr>
</tbody>
</table>

The patients in the above two groups were followed for a period of five days.

2.2.5 History

A brief case history of patients attending the Malaria Clinic, Madras, were recorded containing the following information:
1. Name
2. Age and Sex
3. Permanent Address
4. Occupation
5. Type and frequency of earlier malarial infection
6. Clinical symptoms
   (i) Body temperature
   (ii) Headache, nausea, eye irritation
   (iii) Body pain, splenic palpitation
7. Other complications

2.3 SOURCES OF FINE CHEMICALS, REAGENTS AND SOLVENTS

2.3.1 Fine chemicals, reagents and solvents used during this study were obtained from the following sources:

Glucose-6-phosphate, 1,1,3,3-tetraethoxy propane-bis (diethyl acetal), bovine serum albumin, 5,5'-dithio-bis (2-nitrobenzoic acid), glutathione (GSH), acetyl phenylhydrazine, epinephrine and 2-thiobarbituric acid from Sigma Chemical Company, St. Louis, USA. Acetylthiocholine iodide and cholesterol from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England and brilliant cresyl blue from Reidel-de Haens AG, Germany.

All other chemicals, acids, bases and solvents were of the analytical grade and were obtained from Glaxo Laboratories, BDH division, Bombay, SD
α-tocopherol acetate in the form of capsules (400 mg each) were obtained from E.Merck, India Ltd.

2.4 BLOOD SamPLING AND ANALYSIS

2.4.1 Blood was drawn by slow venepuncture through a 21 gauge disposable needle and one part was transferred into a tube containing ethylene diamine tetraacetate (EDTA, 10.5 mg/7.0 ml) and the other to an empty tube and was allowed to clot. The samples were then transported on ice to the laboratory. A portion of the anticoagulated whole blood was removed and kept aside to assay haematological indices. After centrifugation at 2000 rpm at 4°C, plasma and serum were separated and stored at 4°C until further use. The buffy coat, after the removal of plasma, was aspirated and discarded.

2.4.2 Investigations carried out

2.4.2.1 Haematological indices

Parasite count, RBC count, MCV, MCH, MCHC, PCV, osmotic fragility, blood grouping, Heinz-body formation and haemoglobin.
2.4.2.2 Biochemical picture

Blood glucose, serum cholesterol, uric acid, total protein, albumin, total, conjugated and unconjugated bilirubin, alanine and aspartate aminotransferases, serum cholinesterase and RBC acetyl cholinesterase.

2.4.2.3 Peroxides and scavengers

Serum lipid peroxides, thiobarbituric acid reactive substances in RBCs, reduced glutathione and ascorbic acid in blood, total sulphhydryl content in RBC, vitamin E in serum and RBC and the circulating antioxidant protein ceruloplasmin and vitamin A in serum.

2.4.2.4 Antioxidant enzymes

Superoxide dismutase, glutathione peroxidase, catalase, glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione-S-transferase.

2.5 METHODS

2.5.1 Haemoglobin

Haemoglobin was estimated by the cyanmethaemoglobin method of Drabkin and Austin (1932). The cyanmethaemoglobin technique has been recommended as the standard method by the International Committee for Standardisation in Haematology (1965) and British Standards Institution (1966).
Principle

The haemoglobin is treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. The ferricyanide forms methaemoglobin which is converted to cyanmethaemoglobin by the cyanide.

Reagents

1. Ferricyanide-cyanide reagent: This was prepared by dissolving 200 mg potassium ferricyanide, 50 mg potassium cyanide and 140 mg potassium dihydrogen phosphate in a litre of water.

2. Cyanmethaemoglobin standard: Purchased from Span Diagnostics, Surat, India. This was kept in the dark at 4°C. It has an equivalent haemoglobin concentration of 60 mg%.

Procedure

20 μl of blood/packed cells was added to 4 ml of the reagent. For haemolysate haemoglobin estimation, 100 μl of the 1:20 diluted haemolysate was added to 5 ml of the above ferricyanide-cyanide reagent. This was allowed to stand for 15 min and was read against a reagent blank at 540 nm. The standards were diluted in ferricyanide-cyanide solution to obtain a range of concentrations in the same manner.

Blood and packed cell haemoglobin values were expressed as g/dl.
2.5.2 **Haematocrit** [Packed Cell Volume (PCV)]

PCV was estimated by the Wintrobe macromethod (Wintrobe, 1933). Haematocrit is defined as the volume of erythrocytes expressed as a percentage of the volume of the whole blood.

**Procedure**

The whole blood after adequate mixing was filled in the Wintrobe macrohaematocrit tube. The level of the blood was noted and the tubes were centrifuged at 2,500 x g for 30 min.

The result was calculated using the formula

\[
\text{Haematocrit} \, (\%) = 100 \frac{L_1}{L_2}
\]

where, \( L_1 \) = height of the red cell column in mm.

\( L_2 \) = height of the whole blood specimen in mm.

The greyish white layer of leucocytes and platelets above the erythrocyte was not included in \( L_1 \).

2.5.3 **Erythrocyte count**

Erythrocyte count was estimated by the haemocytometer method (John, 1972). The erythrocyte count is expressed as cells per cubic millimeter of blood.
Reagents

Gower's solution: 12.5 g of sodium sulphate was dissolved in 100 ml of water to which 33.3 ml of glacial acetic acid was added and the solution was made to 200 ml with distilled water.

Procedure

Venous blood after thorough mixing was taken upto the 0.5 mark in the thoma red cell diluting pipette and diluted upto 101 mark with diluting fluid, thus achieving a 1:200 dilution of the blood sample. The diluted sample was filled in the counting chamber of the Neubauer slide and counted with the aid of a light microscope.

The erythrocyte count was calculated from the following formula

\[ \text{RBC} = \frac{\text{Cells counted} \times 5}{\text{sq.cm. counted}} \times 10^x \times \text{depth} \times 200 \]

(dilution factor)

2.5.4 Erythrocyte indices

Erythrocyte indices were arrived at from the method described by Wolf et al. (1973).

Principle

The erythrocyte indice gives an accurate picture of the morphology of red cell in association with the appearance of the red cells in a fixed smear. This was calculated from the values of haemoglobin, PCV and RBC count.
The indices were obtained using the following calculations:

Mean corpuscular volume (MCV):

\[
MCV \text{ (in } \mu \text{m)} = \frac{\text{Haematocrit x 10}}{\text{erythrocyte count (millions per cu.mm)}}
\]

Mean corpuscular haemoglobin (MCH):

\[
MCH \text{ (in } \mu \text{g)} = \frac{\text{Haematocrit (g/dl) x 10}}{\text{erythrocyte count (millions per cu.mm)}}
\]

Mean corpuscular haemoglobin concentration (MCHC):

\[
MCHC \text{ (}) = \frac{\text{Haematocrit (g/dl) x 100}}{\text{Haematocrit}}
\]

2.5.5 Total leucocyte count

The total leucocyte count was estimated according to the haemocytometer method (Miale, 1972).

Principle

Blood is diluted with a fluid that lyses the non-nucleated erythrocyte precursors. If the blood smear showed nucleated erythrocytes, the cell count was corrected to the true leucocyte count according to the following formula:

\[
\text{Corrected count} = \frac{\text{Observed count}}{100 + \% \text{ nucleated erythrocytes}}
\]
Procedure

The WBC pipette was filled to the 0.5 mark with blood and diluted to the 11 mark with 1.0% HCl. This made a 1:20 dilution. The pipette was shaken for 3 min and the first two drops were discarded. The haemocytometer chamber was filled with diluted blood and left to settle for one minute.

Leucocytes present in the four large corner squares (1 sq.mm each were counted and calculated by the following formula:

\[
\text{WBC/mm}^3 = \frac{\text{Cells counted} \times 10 \text{ (depth)} \times 20 \text{ (dilution)}}{4 \text{ (sq.mm. counted)}}
\]

2.5.6 Parasite count

This was done by the method of Pinder (1973); Seshadri et al. (1983). Staining of thick and thin blood films were done by the method of Jaswant Singh and Bhattacharji (1944). Parasites per 100 leucocytes were counted in blood films and values per cubic millimeter of blood were estimated from the total leucocyte count.

2.5.7 Blood groups

Blood grouping was done by the slide technique of Weiner (1943).
Principle

Red blood cells are agglutinated if they possess the antigen, which reacts with the corresponding antibody present in the serum.

\[
\begin{align*}
\text{Unknown red blood cells} + \text{Anti-X-serum} & \quad \text{\rightarrow Agglutination-The cells are of group X} \\
& \quad \text{or} \\
& \quad \text{No agglutination-The cells are not of group X}
\end{align*}
\]

Reagents

1. Commercially available anti-A and anti-B sera was obtained from Span Diagnostics Pvt. Ltd., Surat (Code Nos.11101 and 11102).

Method

Blood obtained from finger prick was immediately mixed with the antisera using separate applicator sticks. The slide was then tilted back and forth and macroscopic and microscopic agglutination was observed. Slides which show no agglutination within two min were considered negative. With every batch of tests, samples of known A,B and O cells were included as controls.

2.5.8 Osmotic fragility

Osmotic fragility of erythrocytes was done by the method of Dacie (1960); Wolf et al. (1973).
Principle

Erythrocytes were placed in solutions of varying concentrations of sodium chloride. In hypotonic solutions, the cells took up fluid until an equilibrium was reached or the cells ruptured.

Reagents

1. 10% buffered stock saline solution: 18 g of NaCl, 2.731 g of disodium hydrogen phosphate and 0.436 g of sodium dihydrogen phosphate were dissolved in 200 ml of distilled water.

2. Working saline solution: Prepared from the stock, in the following concentrations - 0.85%, 0.70%, 0.60%, 0.52%, 0.50%, 0.48%, 0.44%, 0.42%, 0.40%, 0.38%, 0.36%, 0.32%, 0.28%, 0.16%, 0.10%.

Procedure

4 ml of working saline solution was pipetted into each tube, and 0.02 ml of blood was added and mixed gently. The tubes were incubated at room temperature for 45 min. This was followed by centrifugation at 2000 rpm for 10 min. The red cell free supernatant solution from each tube was transferred to cuvettes and optical density was read at 540 nm, against a water blank.

2.5.9 Heinz body formation: Acetylphenyl hydrazine test

Heinz body formation was carried out by the method of Bauer (1982).
Principle

Heinz bodies are small, round intraerythrocytic inclusions caused by oxidative denaturation of haemoglobin. Globin is irreversibly denatured to their Heinz bodies when the heme moiety is oxidised to methaemoglobin form (Ferrous, Fe$^{++}$ to Ferric, Fe$^{+++}$ state). Red cells containing unstable haemoglobins develop numerous small intra-erythrocytic inclusions in mature red cells when exposed to acetylphenyl hydrazine. They are stained with crystal violet.

Reagents

1. Phosphate buffer : 0.067 M, pH 7.6
   Solution A : KH$_2$PO$_4$, 0.067 M
   Solution B : Na$_2$HPO$_4$, 0.067 M

   1.3 parts of solution A and 8.7 parts of solution B were mixed to obtain 1 dl. Immediately before use, 200 mg of glucose was added to it.

2. Acetylphenylhydrazine (APH) solution : 100 mg APH in phosphate buffer, 1 dl. This solution was prepared fresh and was used within one hour.

3. Crystal violet solution : Crystal violet, 2 g in 10 ml 0.73% sodium chloride solution. The solution was shaken for 5 min and filtered. The filtrate was mixed with an equal quantity of 0.73% sodium chloride solution.
Procedure

0.1 ml of whole blood (used within 1 hour of drawing sample) was suspended in 2 ml of APH solution. The suspension was mixed immediately and aerated 2-3 times by drawing it up in to the pipette and blowing it out together with a small quantity of air. The material was incubated at 37°C for 4 hours. The mixture was aerated briefly at the completion of 2 hour and 4 hour incubation. A small drop of the mixture was placed on a cover slip after 4 hour incubation. The cover slip was inverted onto a microscopic slide on which a large drop of crystal violet solution was placed. The mixture was allowed to stand for 5-10 min. The wet preparation was then examined with the oil immersion objective.

The Heinz body count was determined as the percentage of cell that had five or more Heinz bodies by examination of 500 RBC's.

2.5.10 Heinz bodies inclusion test

Heinz bodies inclusion test was done according to the method of Bauer (1982).

Principle

Unstable haemoglobin was determined within the red cells by the use of a redox dye, such as brilliant blue and shows up as intra erythrocytic stained bodies.
Reagents

1. Brilliant cresyl blue: 1% in normal saline

Procedure

The stain was mixed before use and a small amount was filtered through Whatman no.42 filter paper. One part of stain and two parts of fresh anticoagulated blood were mixed in a test tube and incubated in a 37°C water bath and blood smears were prepared at 20 min, 1 hour and 2 hour intervals. The slides were examined for intracytoplasmic blue inclusions with oil immersion lens.

2.5.11 Blood glucose

Blood glucose was estimated in random blood samples, by the method of Dubowski (1962) modified by Sasaki and Matsui (1972).

2.5.12 Uric acid

Uric acid was estimated by the method of Caraway (1963).

Principle

In an alkaline solution, phosphotungstic acid reagent oxidises uric acid to allantoin and is itself reduced to tungsten blue.
Reagents

1. Tungstic acid reagent: 50 ml of 10% sodium tungstate was mixed with 50 ml of 2/3 N sulphuric acid and a drop of phosphoric acid was added to it. The mixture was made up to 800 ml. The reagent was stored in a brown bottle.

2. 20% sodium carbonate.

3. Stock phosphotungstic acid reagent: 50 g of sodium tungstate was dissolved in 400 ml water, 85 ml of phosphoric acid was added and the contents were refluxed for 2 hours. The mixture was made up to 500 ml. The solution was diluted 1 to 10 before use.

4. Stock uric acid: 20 ml of water was added to 60 mg of lithium carbonate and heated to 60°C. This hot solution was then mixed with 100 mg of uric acid. To this solution was added 2 ml of 4% formalin and 1 ml of 5% (V/V) acetic acid. The mixture was made up to 100 ml with water. This solution was diluted 1 to 10 before use.

Procedure

2.9 ml of tungstic acid reagent was added to 0.1 ml of serum and the mixture was centrifuged to sediment the precipitated proteins. To 2 ml supernatant, 0.6 ml of 20% sodium carbonate was added and the mixture was incubated for 10 min at 25°C. To this, 0.6 ml of phosphotungstic acid reagent was added and the mixture was incubated for 20 min. The colour developed
was measured at 700 nm. A standard curve was prepared with standard uric acid solution containing 5-25 µg.

Serum uric acid levels were expressed as mg/dl.

2.5.13 Serum bilirubin

Serum bilirubin was assessed by the method of Lathe and Ruthven (1958).

Principle

The detection and estimation of bilirubin in serum is based on the formation of purple coloured compound azobilirubin, when bilirubin reacts with the diazo-reagent. This method uses a more acid diazo-reagent and a higher dilution (1 in 35 for the conjugated and 1 in 70 for the total bilirubin).

Procedure

0.2 ml of serum was washed into 5-4 ml water and mixed. From this, 2.8 ml was pipetted into a second tube for use as the blank. To the test was added 0.7 ml of the diazo-reagent and to the blank 0.7 ml sulphanilic acid solution. The solutions were mixed and allowed to stand for 5 min after which they were read at 540 nm against water. The blank reading was subtracted from the test to give the conjugated bilirubin. To obtain the total bilirubin, 3.5 ml of methanol was added to each tube and was read again after standing for 5 min. For the standard, 0.2 ml bilirubin was added to 3.5 ml methanol. 0.7 ml of diazo-reagent was then added and, after proper mixing, 2.6 ml of water was
added and read against a water blank after 5 min. Since conjugated bilirubin has a lower extinction in water, the factor 1.05 was inserted in the calculation. The difference between total bilirubin and conjugated bilirubin gives the unconjugated bilirubin.

Serum bilirubin was expressed as mg/dl.

2.5.14 Total protein and albumin

Total protein and albumin in plasma were estimated by the method of Reinhold (1953).

Principle

Globulins are precipitated by a mixture of sodium sulphate and sulphite solution in proportions of almost 3:1, the final salt concentration after adding to the plasma being very near to 26%.

Procedure

(a) Total protein: 6 ml of sulphate-sulphite solution was pipetted into a centrifuge tube and 0.4 ml of plasma was layered on it. Mixed by inversion and 2 ml of the mixture was removed and to it 5 ml of biuret reagent was added immediately.

(b) Albumin: To the rest of the plasma, sulphate-sulphite mixture and 3 ml of ether were added, stoppered and shaken 40 times. The tubes were capped and centrifuged till a firm globulin layer was formed.
After centrifugation, the tubes were tilted and 1 ml of the clear solution below the globulin layer was pipetted and to it 5 ml of biuret reagent was added.

(c) Plasma blank: To 2 ml of plasma, sulphate-sulphite mixture and 5 ml of tartrate iodide solution were added and mixed.

(d) Biuret blank: 2 ml of sulphate-sulphite solution was added to 5 ml of biuret reagent.

(e) Standard: 0.4 ml of standard solution was pipetted into 6 ml of sulphate-sulphite solution as above and 2 ml of this mixture was transferred into 5 ml of biuret reagent.

Total protein and albumin in plasma were expressed in terms of g/dl.

2.5.15 Aspartate and alanine aminotransferases

The assay of the enzymes aspartate amino transferase, AST (also called glutamate oxaloacetate transaminase, SGOT) and alanine amino transferase, ALT (also called glutamate pyruvate transferase, SGPT) was done by the method of Reitman and Frankel (1957).

Principle

AST catalyzes the following reaction:

\[ \alpha\text{-ketoglutarate} + L\text{-aspartate} \rightarrow L\text{-glutamate} + \text{oxaloacetate} \]

ALT catalyzes the following reaction:

\[ \alpha\text{-ketoglutarate} + L\text{-alanine} \rightarrow L\text{-glutamate} + \text{pyruvate} \]
Pyruvate or oxaloacetate formed in the reaction is coupled with 2,4-dinitrophenylhydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives a brown colour in alkaline medium, which can be measured colorimetrically.

Procedure

0.1 ml serum was added to 0.5 ml buffered substrate, mixed and incubated for 60 min for AST or 30 min for ALT at 37°C in a water bath. After removing the tubes, 0.5 ml of DNPH was added and allowed to stand at room temperature for 20 min, following which 5 ml sodium hydroxide was added, mixed and read after 5 min at 540 nm. For the blank, 0.1 ml serum, 0.5 ml buffered substrate and 0.5 ml DNPH was added, mixed and completed as the test. A standard curve for AST and ALT was arrived at using the method of Karmen (1955).

(a) AST standard curve

<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>Enzyme activity (units/ml)</td>
<td>0</td>
<td>24</td>
<td>61</td>
<td>114</td>
<td>190</td>
</tr>
<tr>
<td>Buffered substrate</td>
<td>0.5</td>
<td>0.45</td>
<td>0.4</td>
<td>0.35</td>
<td>0.3</td>
</tr>
<tr>
<td>Pyruvate standard, 2 mM</td>
<td>-</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water, ml</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>DNPH, ml</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mixed and stood at room temperature for 20 min.

Sodium hydroxide, 0.4 N | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |

Mixed and stood at room temperature for 5 min and read at 540 nm against blank (Tube No.1).
(b) **ALT standard curve**

<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>Enzyme activity (units/ml)</td>
<td>0</td>
<td>28</td>
<td>57</td>
<td>97</td>
<td>150</td>
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<tr>
<td>Buffered substrate</td>
<td>0.5</td>
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<td>0.4</td>
<td>0.35</td>
<td>0.3</td>
</tr>
<tr>
<td>Pyruvate standard, 2 mM</td>
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<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water, ml</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>DNPH, ml</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mixed and stood at room temperature for 20 min.

Sodium hydroxide 0.4 N  
5.0  5.0  5.0  5.0  5.0

Mixed and stood at room temperature for 5 min and read at 540 nm against Tube No.1.

The optical density of the test was plotted on the Y-axis of the standard curve and extrapolated to the corresponding enzyme activity on X-axis.

The enzyme activities of AST and ALT were expressed as units/ml serum.

**2.5.16 Cholesterol**

Plasma cholesterol was determined by ferric chloride colour reaction by the modified method of Parekh and Jung (1970).
Reagents

1. Ferric chloride-uranyl acetate reagent: 500 mg of ferric chloride was dissolved in 10 ml of water and precipitated with 3 ml of ammonia. The precipitate was washed several times with distilled water, dissolved in glacial acetic acid and made upto one litre. To this, 100 mg of uranyl acetate was added and the contents were shaken well and left overnight. The reagent was stored in a brown bottle.

2. Sulphuric acid - ferrous sulphate reagent: 100 mg of anhydrous ferrous sulphate was partially dissolved in 100 ml of glacial acetic acid and 100 ml of concentrated sulphuric acid was added with constant stirring. After cooling to room temperature, the volume was made upto one litre with concentrated sulphuric acid.

3. Cholesterol standard: Prepared by dissolving 250 mg of cholesterol recrystallised from ethanol, in 100 ml of glacial acetic acid.

Procedure

To 50 µl of plasma, 10 ml of ferric chloride-uranyl acetate reagent was added. It was mixed well and allowed to stand for 5 min. After centrifugation, 3 ml of supernatant was taken for analysis. In the case of standards, suitable aliquots were taken and made upto 3 ml with reagent 1. Blank comprised of 3 ml of ferric chloride-uranyl acetate reagent. To all the tubes, 2 ml of sulphuric acid-ferrous sulphate reagent was added and mixed well. The optical
density was measured at 530 nm after 20 min. Plasma cholesterol was expressed as mg/dl.

### 2.5.17 Plasma lipid peroxides

Plasma lipid peroxides were estimated by the method of Ledwozyw et al. (1986).

#### Reagents

1. TCA : 1.22 mol/l in 0.6 mol/l hydrochloric acid.
2. TBA solution : 500 μg TBA in 6 ml of 1 mol/l sodium hydroxide followed by the addition of 69 ml of distilled water.
3. Chloroform
4. Standard malondialdehyde (MDA) solution : 50 μl stock solution of 1,1,3,3-tetra ethoxy propane bis (diethyl acetal) (TEP) was made to 1 ml with 0.9% sodium chloride and 30 μl of 6 N hydrochloric acid was added and made to 100 ml in distilled water. This was further diluted 1 in 50 with distilled water to obtain a concentration of 50 nmoles/ml.

#### Procedure

To 0.5 ml plasma taken in a tube, 2.5 ml TCA in hydrochloric acid was added and allowed to stand for 15 min. To this mixture, 1.5 ml of TBA solution was added and thereafter was heated in a boiling water bath. After cooling to room temperature, 4 ml of chloroform was added and the mixture was shaken vigorously for 3 min and centrifuged for 10 min at 1500 x g. The organic layer
was removed and its absorbance was measured at 535 nm. A standard curve was constructed with standard malondialdehyde containing 5-30 nmoles.

Plasma lipid peroxides were expressed as nmoles/ml plasma.

2.5.18 RBC thiobarbituric acid reactive substances (TBARS)

Erythrocyte lipid peroxidation (TBARS) was determined according to the method of Cynamon et al. (1985).

Principle

Malondialdehyde, a product of polyunsaturated fat peroxidation, was measured as the thiobarbituric acid derivative in the supernatant following incubation of erythrocytes with two different concentrations of hydrogen peroxide.

Reagents

1. Isotonic phosphate buffered saline (pH 7.4).
2. Isotonic phosphate buffered saline (pH 7.4) containing sodium azide (26 mg/100 ml buffer).
3. Hydrogen peroxide : 3% solution, prepared fresh.
4. Hydrogen peroxide : 0.75% solution, prepared fresh.
5. TCA : 40% in 0.1 mol/l sodium arsenite.
6. TBA : 1% TBA in 0.05 mol/l sodium hydroxide.
7. Standard malondialdehyde (MDA) : Prepared as in procedure 2.5.17
**Procedure**

The erythrocytes were washed well with isotonic phosphate buffered saline (pH 7.4) after removing the plasma. One aliquot of 0.2 ml packed cells was suspended in 3.8 ml of phosphate buffered saline. A second aliquot of 0.2 ml of packed cells was suspended in 3.8 ml of phosphate buffer, to which sodium azide had been added. To the azide suspended cells, 1 ml of 0.75% hydrogen peroxide, and to those without azide, 1 ml of 3% hydrogen peroxide was added. Specimen blank with no peroxide and reagent blanks were incubated at 37°C in a shaking water bath for 1 hour along with test samples.

Following incubation, 1 ml of TCA was added to all the tubes. Centrifuged and to 2 ml of the supernatant was added 0.5 ml of TBA solution. The specimens were then boiled for 15 min in a water bath. The tubes were cooled to room temperature and absorbance at 530 nm was determined. A standard absorption curve for MDA was prepared using 1,1,3,3-tetra ethoxy propane.

The % maximal TBARS release was calculated according to the following equation:

\[
\text{% maximal release (% TBARS)} = \frac{\text{TBARS release (3% H}_2\text{O}_2)}{\text{TBARS release (0.75% H}_2\text{O}_2 + \text{sodium azide})} \times 100
\]

2.5.19 **Reduced glutathione**

Glutathione was estimated by the method of Beutler *et al.* (1963).
**Principle**

Virtually all the nonprotein sulphydryl content of red cells is in the form of reduced glutathione (GSH). 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) is a disulfide compound that is readily reduced by sulphydryl compounds, forming a highly coloured yellow anion. The optical density of this yellow substance is measured at 412 nm.

**Reagents**

1. Precipitating solution: 1.67 g of glacial metaphosphoric acid, 0.2 g disodium ethylene diamine tetra acetic acid (EDTA) and 30 g of sodium chloride per 100 ml of distilled water.

2. Phosphate solution: 0.3 M disodium hydrogen phosphate solution was prepared in distilled water. Crystals developed during storage at 4°C were dissolved by heating.

3. DTNB reagent: 20 mg of DTNB per 100 ml of 1% sodium citrate solution. This reagent was stable for 13 weeks at 4°C.

**Procedure**

Two-tenth millilitre of whole blood was added to 1.8 ml of distilled water. Immediately, three millilitres of the precipitating solution was mixed with the haemolysate. After standing for 5 min, the mixture was filtered through a medium grade filter paper. Two millilitres of the filtrate was added to 8 ml phosphate solution. It was read at 412 nm against a blank prepared by adding 2 ml of 2:5 water diluted precipitating solution to 8 ml of phosphate
solution. A second optical density reading was taken within 10 min after 1 ml of DTNB reagent was added to the tubes. Suitable aliquots of the standard were also taken through a similar procedure.

Glutathione content was expressed as mg/dl of whole blood. RBC glutathione levels were estimated by the same method as described above.

2.5.20 Total sulphhydryl group (TSH)

Total sulphhydryl content in erythrocytes was estimated according to the method of Sedlack and Lindsay (1968) with a modification in the solvent extraction.

Principle

This method is based on the development of a yellow colour when 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) is added to compounds containing sulphhydryl groups to form 2-nitro 5-mercapto benzoic acid.

Reagents

1. Tris-HCl buffer, 0.2 M, pH 8.2 containing 0.02 M disodium EDTA.
2. 5,5'-dithio bis-(2-nitro benzoic acid) reagent (DTNB): 99 mg of DTNB was dissolved in 2.5 ml of absolute methanol.
4. Standard glutathione: 10 mg reduced glutathione dissolved in 100 ml distilled water.
Procedure

To 2.9 ml of buffer solution taken in a test tube, 0.02 ml packed cells and 0.1 ml DTNB were added and mixed well. To this was added 3 ml of the chloroform-isopropanol reagent and vortexed thrice at 5 minute intervals. The reaction mixture was centrifuged at 3000 x g at room temperature for 10 min and the absorbance of the clear supernatant was read at 412 nm. Standards containing 20-100 μg GSH were processed similarly.

Total sulphydryl content in RBC was expressed in terms of μg/ml of packed cells.

2.5.21 Ascorbic acid

Ascorbic acid was estimated by the method of Omaye et al. (1979).

Principle

Ascorbic acid is oxidised by copper to form dehydro ascorbic acid and diketogulonic acid. These products are treated with 2,4-dinitrophenyl hydrazine to form the derivative bis-2,4-dinitrophenyl hydrazine. This compound, in strong sulphuric acid, undergoes a rearrangement to form a product with an absorption maxima at 520 nm.

Reagents
1. Trichloroacetic acid : 5% and 10% solutions in distilled water.
2. 2,4-dinitrophenyl hydrazine/thiourea/copper (DTC) solution : 0.4 g thiourea, 0.05 g copper sulphate and 3 g 2,4-dinitrophenyl hydrazine
were added and brought to a total volume of 100 ml with 9 N sulphuric acid.


Procedure

1 ml of whole blood was added to 1 ml of ice cold 10% TCA, mixed thoroughly and centrifuged for 20 min at 3,500 x g. To 0.5 ml of the supernatant, 0.1 ml of DTC was added and incubated for 3 hr at 37°C to form the bis 2,4-dinitrophenylhydrazone. Then, 0.75 ml of ice-cold 65% sulphuric acid was added and mixed well, and the solutions were allowed to stand at room temperature for an additional 30 min and read at 520 nm. Standards were made in 5% TCA and ranged from 0-20 µg/ml.

Ascorbic acid content was expressed as mg/l whole blood.

2.5.22 Vitamin E

Vitamin E was estimated by the method of Desai (1984).

Principle

This method is based on saponification and solvent extraction of lipids, removal or destruction of interfering substances, and determination of tocopherol spectrophotometrically. A modified version of the classical Emmerie and Engle (1938) method. This method reduces ferric ions to ferrous ions in the presence of tocopherols and the formation of pink coloured complex with a
more sensitive reagent such as bathophenanthroline. Orthophosphoric acid is added as a chelating agent to reduce carotene interference by preventing its oxidation and stabilisation of colour by binding excess ferric ions, thus preventing their photochemical reduction.

**Reagents**

1. Absolute ethanol: Analytical grade ethanol redistilled in all glass apparatus after adding pellets of potassium hydroxide and crystals of potassium permanganate.

2. Hexane: Analytical grade purified by glass distillation.

3. Bathophenanthroline reagent: 0.2% solution of 4,7-diphenyl-1,10-phenanthroline in purified absolute ethanol.

4. Ferric chloride reagent: 0.001 M ferric chloride solution in purified absolute ethanol. This reagent was prepared fresh and was kept in amber coloured bottle.

5. Orthophosphoric acid reagent: 0.001 M orthophosphoric acid solution in purified absolute ethanol.

6. Vitamin E standard: α-tocopherol standards in the range of 1-10 µg per ml of purified absolute ethanol were prepared and treated in the same manner as test samples.
Procedure

a. Plasma saponification and extraction

Saponification of plasma prior to hexane extraction was necessary to facilitate tocopherol extraction and to reduce the amount of saponifiable lipid compounds from interfering with the colorimetric assay of vitamin E. 1 ml of plasma was pipetted out into a centrifuge tube to which was added 2 ml of 2% pyrogallol in purified ethanol and mixed thoroughly. The mixture was heated at 70°C for 2 min after which 0.3 ml of saturated potassium hydroxide was added and mixed again. The mixture was further incubated at 70°C for 30 min. The tubes were immediately cooled in an ice bath and 1 ml of distilled water and 4 ml of purified hexane were added. The tubes were shaken vigorously for 2 min and centrifuged at 1500 x g for 10 min to separate the phases.

b. Red blood cells saponification and extraction

2 ml of washed RBC was resuspended in isotonic phosphate buffer, pH 7.4 with 1% EDTA and was made to a final hematocrit of 50%. The saponification was carried out in glass-stoppered centrifuge tubes in the presence of 10 ml of a 2% solution of pyrogallol in purified absolute ethanol. The tubes were thoroughly mixed and placed in a 70°C water bath for 2 min, after which 0.5 ml of saturated potassium hydroxide was added to each tube and was further incubated for 30 min in the same water bath. The tubes were removed and immediately placed in an ice bath. After cooling to room temperature, 7.5 ml of distilled water and 22 ml of purified hexane were added to each tube and the extraction was carried out by shaking the stoppered tubes vigorously for 2 min. The hexane extract separated by centrifugation was used for vitamin E analysis.
c. Estimation

3 ml aliquots of hexane extract were pipetted out into suitable reaction tubes and evaporated to dryness under nitrogen. The residue was then carefully dissolved in 1 ml of purified ethanol. Tubes containing α-tocopherol standards were treated in the same way as test samples. To all the tubes, including a reagent blank, 0.2 ml of 0.2% bathophenanthroline reagent was added and the contents of the tubes were thoroughly mixed. The assay proceeded very rapidly from this point on and care was taken to reduce unnecessary exposure to direct light. 0.2 ml of ferric chloride reagent was added and the tubes were mixed by vortexing. After 1 min, 0.2 ml of orthophosphoric acid was added and the tubes were thoroughly mixed again. The absorbance of ‘blank’, ‘test’ and ‘standard’, tubes were read at 536 nm using the Uvikon 930 Spectrophotometer set to zero with purified ethanol.

Vitamin E values were expressed as μg/ml for plasma and μg/ml RBC.

2.5.23 Vitamin A

Serum vitamin A was estimated by the method of Bayfield and Cole (1974).

Reagents

1. Absolute ethanol
2. Light petroleum ether
3. Chloroform AR
4. Trichloroacetic acid AR
6. Working standard: Dilute the stock standard to 1 in 50 with light petroleum ether.

Procedure

To 1.5 ml of serum was added 1.5 ml of absolute ethanol in a stoppered centrifuge tube with constant shaking. To this, 3 ml of light petroleum ether was added and was shaken in a mechanical shaker for 2 min and then centrifuged at low speed for about 1 min. 2.5 ml of the clear supernatant was pipetted out and kept at 37°C until complete evaporation. The residue was dissolved in 2 ml of chloroform-trichloroacetic acid mixture. The absorbance was read immediately at 620 nm. Standards containing 1-7 μg of vitamin A acetate were processed similarly.

Serum vitamin A level was expressed in μg/dl.

2.5.24 Ceruloplasmin (EC 1.16.3.1)

Ceruloplasmin was assayed according to the method of Henry et al. (1960).
Principle

Ceruloplasmin (copper oxidase) catalyses the oxidation of some polyamines and its action on p-phenylene diamine was used as a measure of the amount present in serum.

Reagents

1. Acetate buffer: 0.1 M, pH 6.0, 10 ml of 0.1 M acetic acid (0.57 ml glacial acetic acid diluted with water to 100 ml) was added to 200 ml of 0.1 M sodium acetate (1.36 g CH₃ COONa·3H₂O per 100 ml). The pH was between 5.95 and 6.00.

2. Sodium azide: 0.1% in 0.1 M acetate buffer and stored in refrigerator.

3. p-phenylenediamine dihydrochloride: 0.25% in 0.1 M acetate buffer. To purify, p-phenylenediamine dihydrochloride was dissolved in a minimum of hot distilled water, decolourised with charcoal and filtered hot and allowed to crystallize. The dried crystals were kept over calcium chloride. 12.5 mg of this was dissolved in 3 ml acetate buffer and the pH was adjusted to 6 by adding 1 N NaOH dropwise. Acetate buffer was added to a final volume of 5 ml. This was kept in the dark and used before 2 hours of assay.

Procedure

2 ml of buffer was added to 1 ml of p-phenylenediamine dihydrochloride reagent for the test, and 1 ml of buffer and 1 ml p-phenylenediamine dihydrochloride reagent along with 1 ml of sodium azide
was added for the blank. The tubes were kept at 37°C for temperature equilibration and 1 ml serum was added to each tube and mixed well. The optical density of the test and blank tubes was read at 530 nm exactly at 10 min, and again after 40 min of addition of serum.

\[
\text{Ceruloplasmin units} = (A40 \text{ min} - A10 \text{ min}) \times 1000
\]

A conversion factor of 0.06 of King (1965) was used and ceruloplasmin was expressed as mg/dl.

2.5.25 Preparation of haemolysate

Packed cells remaining after the removal of plasma was diluted to approximately 1:1 v/v with 0.9% sodium chloride solution and was centrifuged in the cold at 1000 x g for 15 min. Supernatant was removed from the packed cells and again suspended in cold 0.9% NaCl solution. The suspension was centrifuged at 1000 x g for 10 min 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.

Preparation of haemolysate was carried out as described by Beutler (1984).

Reagents

1. β-mercapto ethanol-EDTA stabilising solution: This was prepared by bringing 0.25 ml of β-mercaptoethanol and 5 ml of neutralised 10% (0.27 M) EDTA to a volume of 500 ml with water. This reagent was stable only for 2 weeks at 4°C.
Procedure

0.4 ml of the above suspension was added to 3.6 ml of β-mercaptoethanol-EDTA stabilising solution and mixed well. The tube containing the haemolysate was kept into the deep freezer until completely frozen and was then thawed by placing the tube into a beaker containing water at room temperature. This freezing-thawing was repeated once again after which the haemolysate was mixed and kept at 4°C.

The haemolysate prepared in this manner was referred to as 1:20 haemolysate.

2.5.26 Superoxide dismutase (SOD) (EC 1.15.1.1)

Superoxide dismutase was assayed by the method of Misra and Fridovich (1972).

Principle

SOD catalyses the following reaction,

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \]

Superoxide, \( \text{O}_2^- \), is an intermediate in the autoxidation of epinephrine which occurs at pH 8.5. The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 provides the basis of the assay for the enzyme.
Reagents

1. Carbonate-bicarbonate buffer, pH 10.2, 0.3 M.
2. EDTA : 0.6 mM solution.
3. Epinephrine : 1.8 mM solution, prepared fresh.
4. Absolute ethanol.
5. Chloroform.

Procedure

(a) **Preparation of the enzyme**: The extraction of the enzyme was carried out according to the method of Bartosz *et al.* (1978). A suitable aliquot of the haemolysate was diluted with water. Following this, 0.25 volume of chilled ethanol and 0.15 volume of ice-cold chloroform were added. The mixture was shaken well for a few min at 4°C and then centrifuged. The supernatant was taken for the enzyme assay.

(b) **Assay**: To tubes containing 0.5 ml of the carbonate buffer and 0.5 ml of the EDTA solution, requisite amount of enzyme was added. Their final volume was made to 2.5 ml with distilled water and the reaction was initiated by the addition of 0.2 ml of epinephrine and the increase in absorbance at 480 nm was measured in a spectrophotometer. Autoxidation of epinephrine to adrenochrome was performed in a control tube without the enzyme.
SOD activity was expressed as units/min/g Hb. One unit of enzyme activity was defined as that quantity of enzyme required to give 50% inhibition of epinephrine autoxidation.

2.5.27 Catalase (Hydrogen peroxide : Hydrogen peroxide oxidoreductase, EC 1.11.1.6)

Catalase (CAT) was assayed colorimetrically by the method of Sinha (1972).

Principle

\[ 2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \]

The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. The chromic acetate is measured colorimetrically at 570-610 nm. The catalase preparation was allowed to split hydrogen peroxide for different periods of time. The reaction is stopped at different time intervals by the addition of dichromate-acetic acid mixture and the remaining hydrogen peroxide is determined colorimetrically.

Reagents

1. Phosphate buffer, pH 7.0, 0.01 M.
2. Dichromate-acetic acid reagent: This reagent was prepared by mixing a 5% solution of potassium dichromate with glacial acetic acid in the ratio 1:3. This was further diluted 1:5 with water.
3. Hydrogen peroxide : 0.2 M

Procedure

To 1 ml of the phosphate buffer taken in each of the four test tubes, 0.1 ml of the enzyme preparation was added. To this 0.5 ml of hydrogen peroxide was added. The reaction was stopped at 15, 30, 45 and 60 sec by the addition of 2 ml of the dichromate-acetic acid reagent. The tubes were boiled for 10 min, cooled and read at 610 nm. A ‘zerotime’ was also run simultaneously by adding the dichromate-acetic acid reagent before the addition of hydrogen peroxide.

Catalase activity was expressed as μmoles of hydrogen peroxide utilised/min/mg protein.

2.5.28 Glutathione peroxidase : (Glutathione : hydrogen peroxide oxidoreductase, EC 1.11.1.9)

Glutathione peroxidase was assayed according to the method of Rotruck \textit{et al.} (1973).

Reagents

1. Sodium phosphate buffer : 0.32 M, pH 7.0.
2. EDTA : 0.8 mM solution.
3. Sodium azide: 10 mM solution.
4. Reduced glutathione: 4 mM solution.
5. Hydrogen peroxide: 2.5 mM solution.
6. TCA: 5% solution.
7. Phosphate solution: 0.3 M disodium hydrogen phosphate solution was prepared. Crystals developed during storage at 4°C were dissolved by heating.
8. DTNB reagent: 20 mg 5,5' dithiobis (2-nitrobenzoic acid) per 100 ml of 1% sodium citrate. The solution is stable for 10 weeks at 4°C.
9. Reduced glutathione standard: 20 mg % solution.

Procedure

0.2 ml each of EDTA, sodium azide, glutathione and hydrogen peroxide together with a suitable volume of buffer and enzyme were mixed together to give a final concentration of 0.08 mM, 1.0 mM, 0.4 mM, 0.25 mM and 0.08 mM respectively in a total incubation volume of 2 ml. Incubation was carried out at 37°C and the reaction was terminated at one min intervals by the addition of 5% TCA.

A 'zero time' was also carried out simultaneously by adding TCA prior to the addition of the enzyme. To determine the residual glutathione content, the contents were centrifuged and to 2 ml of the supernatant added 8 ml of phosphate solution followed by 1 ml of DTNB and read immediately at 412 nm in a spectrophotometer. A blank was prepared by adding 8 ml of phosphate
solution to 2 ml of diluted precipitating solution (3 parts to 2 parts distilled water) and 1 ml of DTNB reagent.

The enzyme activity was expressed as µmoles of glutathione consumed/min/g haemoglobin.

2.5.29 Glutathione-S-transferase (GST)

GST activity was assayed by the method of Beutler (1984).

Principle

GST catalyzes the reaction of 1-chloro-2,4-dinitro benzene (CDNB) with the -SH group of glutathione.

\[
\text{CDNB} + \text{GSH} \rightarrow \text{CDNB-S-glutathione}
\]

The conjugate, CDNB-glutathione, absorbs light at 340 nm, and the activity of the enzyme was estimated by measuring the change in optical density at this wavelength.

Procedure

The following reagents were added to a cuvette with a critical volume of less than 2 ml.
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (µl)</th>
<th>System (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, 0.5 M, pH 6.5</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>CDNB in 95% ethanol, 25 mM</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}$</td>
<td>1460</td>
<td>1360</td>
</tr>
</tbody>
</table>

Incubated at $37^\circ\text{C}$ for 10 min.

GSH, 20 mM                              | 100        | 100         |

Mixed well.

1:20 haemolysate                       | -          | 100         |

The increase in optical density of the system was measured against that of blank at 340 nm at $37^\circ\text{C}$.

GST activity in red cells was expressed as IU/g Hb.

### 2.5.30 Glutathione reductase (GR)

Activity of glutathione reductase was measured by the method of Beutler (1984).

**Principle**

GR catalyzed the reduction of oxidized glutathione (GSSG) by NADPH or NADH to reduced glutathione (GSH).

$$\text{NADPH (NADH)} + \text{H}^+ + \text{GSSG} \rightarrow \text{NADP}^+ (\text{NAD})^+ + 2\text{GSH}$$

The activity of the enzyme is measured by following the oxidation of NADPH spectrophotometrically at 340 nm. GR is a flavin enzyme and it has been found that it is not fully activated by FAD in normal haemolysates.
Complete activation of apoenzyme requires the preincubation of enzyme with FAD. This is done before GSSG or NADPH is added to the reaction system.

**Procedure**

The following reagents were added to a cuvette with a critical volume of less than 2 ml.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (µl)</th>
<th>System (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl buffer 1 M, EDTA 5 mM, pH 8.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1:20 haemolysate</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>H₂O</td>
<td>1580</td>
<td>1380</td>
</tr>
<tr>
<td>FAD, 10 µm</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

Incubated at 37°C for 10 min.

GSSG, 0.033 M (neutralised) - 200

Incubated at 37°C for 10 min.

NADPH (spectrophotometrically standardised) 100 100

The decrease in optical density at 340 nm, at 37°C of the system was measured against the blank in a Uvikon 930 Spectrophotometer.

Activity of GR in red cells was expressed as IU/g Hb.

2.5.31 **Glucose-6-phosphate dehydrogenase (G6PD)**

G6PD activity was measured by the method of Beutler (1984).
Principle

G6PD catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone, which hydrolyzes spontaneously to 6-phosphogluconate (6-PGA).

\[
\text{Glucose-6-P + NADP}^+ \rightarrow 6\text{-PGA} + \text{NADPH} + H^+
\]

This assay for G6PD activity measures the rate of reduction of NADP\(^+\) to NADPH when the haemolysate is incubated with glucose-6-phosphate.

Procedure

The following reagents were added to a cuvette with a critical volume of less than 2 ml.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (μl)</th>
<th>System (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, 1 M, EDTA 5 mM, pH 8.0</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>MgCl(_2), 0.1 M</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>NADP(^+), 2 mM</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>1:20 haemolysate</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>1360</td>
<td>1160</td>
</tr>
</tbody>
</table>

Incubated at 37°C for 10 min.

G6P, 6 mM

The increase in optical density of the system was measured against that of blank at 340 nm at 37°C in Uvikon 930 Spectrophotometer.

G6PD activity in red cells was expressed as IU/g Hb.
2.5.32 Serum cholinesterase

Serum cholinesterase was assayed by the method of Wolf et al. (1973).

Principle

In this method, acetylthiocholine is used as a substrate, giving off hydrolysis products of thiocholine and acetic acid. This thiocholine is further reacted with dithiobis-(2-nitro benzoic acid) which gives off a coloured product with maximum absorbance at 405 nm.

\[
\text{Acetylthiocholine} \quad \rightarrow \quad \text{Thiocholine} + \text{Acetic acid}
\]

\[
\text{Thiocholine (sulphydryl group) + DTNB} \quad \rightarrow \quad \text{Colour}
\]

Reagents

1. Buffer : 0.05 M phosphate buffer, pH 7.2 and 0.25 mM DTNB.
2. Substrate : 0.156 M acetylthiocholine iodide solution.

Procedure

To 3 ml of buffer solution pipetted out into a cuvette, 20 μl of serum was added and mixed. This was followed by the addition of 100 μl of acetylthiocholine iodide. After proper mixing, the increase in optical density was followed for 5 min and the change in optical density per min was determined. The amount of nonenzymatic hydrolysis of acetylthiocholine was determined by running a blank with deionized water in place of serum and the resulting optical density difference was deducted from that of test.

Serum cholinesterase activity was expressed in terms of IU/l at 25°C.
2.5.33 RBC acetylcholinesterase

Activity of acetylcholinesterase was measured according to the method of Beutler (1984).

**Principle**

Acetylcholinesterase (ACHE) catalyzes the hydrolysis of acetylthiocholine to thiocholine. The rate of production of thiocholine is measured by following the reaction of thiocholine with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which produces a yellow colour, because of the formation of 5-thio-2-nitro benzoic acid. The rate of formation of the yellow anion is measured at 412 nm.

\[
\text{Acetylthiocholine iodide} \quad \rightarrow \quad \text{Thiocholine + Acetate}
\]

\[
\text{Thiocholine + DTNB} \quad \rightarrow \quad 5\text{-thio-2-nitrobenzoic acid} + \text{Oxidised thiocholine}
\]

**Procedure**

The following reagents were added to a cuvette with a critical volume of less than 2 ml.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (μl)</th>
<th>System (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl buffer, 1 M with 5 mM EDTA, pH 8.0</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>DTNB, 0.5 mM in 1% sodium citrate</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1:20 haemolysate diluted 1:10 with water</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>H₂O</td>
<td>1680</td>
<td>1580</td>
</tr>
</tbody>
</table>

Incubated at 37°C for 10 min.

Acetylthiocholine iodide 10 mM - 100
The increase in optical density was measured against that of the blank at 412 nm for 10 min. Acetylthiocholine activity in red cells was expressed as IU/g Hb.

2.5.33 Estimation of protein

Protein in the haemolysate was estimated by the method of Lowry et al. (1951).

Reagents

1. Sodium carbonate: 2% solution in 0.1 N sodium hydroxide.
2. Copper sulphate: 0.5% solution in 1.0% sodium potassium tartrate.
3. Alkaline copper solution: 50 ml of reagent 1 was mixed with 1 ml of reagent 2. This was prepared fresh.
5. Folin-Ciocalteau reagent: (Folin and Ciocalteau, 1927).

One volume of Folin-Ciocalteau reagent was diluted with two volumes of water.

Procedure

To a suitable aliquot of the haemolysate made to 2 ml with water, 5 ml of alkaline copper reagent was added. The solution was mixed well and allowed to stand at room temperature for 10 min. 0.5 ml of the diluted Folin-
Ciocalteau reagent was added and mixed. After 20 min, the colour developed was read at 660 nm.

Standard protein solution containing 50 to 200 μg bovine serum albumin were used for calibration of the standard curve.

2.6 STATISTICAL EVALUATION

All quantitative measurements were expressed as mean ± SD for the control, negative control, malaria and treated population.

(a) Statistical analysis was done using the SPSS/PC+ package on a computer. Student-Newman-Keuls test (SNK) was used to compute statistically significant differences in the above populations.

(b) One-way analysis of variance (ANOVA) was done to compare the mean levels of various parameters at different parasitaemia levels and the 'F'-ratios were computed. 'ANOVA' was also carried out to assess and compute statistical differences between individual parameters and parasite density.

(c) Pearson's correlation coefficient 'r' was arrived at to assess the degree of linear association among the different variables taken two at a time.