3. Materials and Methods

3.1. Materials

3.1.1. Chemicals

Glucose-6-phosphate and alanine dehydrogenase were purchased from Sigma Chemical Company (St. Louis, MO, USA). Bovine serum albumin (BSA), 5, 5'-dithiobistirinitrobenzoic acid (DTNB), Folin-Ciocalteau reagent, tris, disodium ethylene tetra acetate (EDTA), oxidized glutathione (GSSG), reduced glutathione (GSH), adenosine-5'-triphosphate (ATP), nicotinamide adenine dinucleotide phosphate, reduced nicotinamide adenine dinucleotide phosphate (NADP and NADPH) and S-acetylthiocholine iodide were purchased from SRL, Mumbai, India. 5-Sulphosalicylic acid dihydrate and 2-thiobarbituric acid (TBA) were purchased from Spectrochem, Mumbai, India. 1-Chloro-2, 4-dinitrobenzene and L-alanine were purchased from Loba Cheme, Mumbai, India. Other chemicals used throughout the investigation were of analytical grade.

3.1.2. Animals and Diets

Male growing rats of Wistar strain weighing 100-120g were used for the present study. The animals were kept in a well ventilated room with 12 hrs. day-light cycle. The animals were accustomed with this condition for 7 days with adequate amount of food containing protein (casein) 18%, carbohydrate (amylum) 71%, fat (groundnut oil) 7%, salt mixture 4% and adequate amount of vitamins mixture as reported elsewhere (Chatterjee et al., 1976; Chatterjee et al., 1984). The composition of the salt mixture used was as described by Hawk & Oser (1931). The composition was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium-citrate, 4H₂O</td>
<td>308.2 g</td>
</tr>
<tr>
<td>Ca (H₂PO₄)₂, H₂O</td>
<td>112.8 g</td>
</tr>
<tr>
<td>K₂H PO₄</td>
<td>218.7 g</td>
</tr>
<tr>
<td>KCl</td>
<td>124.7 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>77.0 g</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>68.5 g</td>
</tr>
<tr>
<td>3 Mg CO₃ Mg (OH)₂ 3H₂O</td>
<td>35.1 g</td>
</tr>
<tr>
<td>Mg SO₄ anhydrous</td>
<td>38.3 g</td>
</tr>
</tbody>
</table>
The following fat soluble vitamins were added per 100g of the diet:

- Codliver oil concentrated - 0.2mg (Adexolin, Glaxo, Mumbai, India)
- α-tocopherol acetate - 3.0mg (Viteolin, Glaxo, Mumbai, India)
- Acetomenaphthone - 0.2mg (Kapalin, Glaxo, Mumbai, India)

This composition of fat-soluble vitamins has also been employed by several workers (Chatterjee et al., 1969; Chatterjee et al., 1970; Chatterjee, 1972; Chatterjee & Roy, 1973; Sengupta & Chatterjee, 1978; Chatterjee et al., 1984).

Water-soluble vitamins together with inositol and choline chloride were added according to Berg (1967) per 100g of the diet as follows:

- Vitamin B₁₂ - 0.005 mg
- Biotin - 0.06 mg
- Thiamin hydrochloride - 1.0 mg
- Folic acid - 1.1 mg
- para-Amino benzoic acid - 2.0 mg
- Riboflavin - 2.0 mg
- Nicotinic acid - 4.0 mg
- Calcium pantothenate - 10.0 mg
- Inositol - 80.0 mg
- Choline chloride - 100.0 mg

Then the animals were divided into four groups of equal average body weight. The animals of half of the groups were continued with diet containing 18% protein.
(casein), while those of the remaining groups were maintained on the diet containing 6% protein (casein), and 83% carbohydrate (amylum). The 18% protein was used as it was considered as an adequate (normal) dietary protein level which was used on earlier occasions (Chatterjee et al., 1984; Ghosh et al., 1992). The 6% protein was used as an inadequate dietary protein level (protein inadequacy) to study the influence of dietary protein inadequacy. This level of dietary protein was also employed on earlier occasions (Ghosh et al., 1992).

### 3.1.3. Heat Exposure

After maintaining for three weeks on experimental diets, rats of experimental groups were exposed to heat stress. From one week before the onset of heat exposure, body weight, food intake and rectal temperature had been recorded on every alternate day till the termination of the heat-exposure period. Rats were exposed to heat stress in a well maintained climatic chamber.

To study the effect of supplementation of ascorbic acid, one of the nutritional antioxidants, it was supplemented to rats from one week before the beginning of heat exposure to increase the antioxidant reserves of the rats, and supplementation was continued till the day before their sacrifice. Ascorbic acid was supplemented to the rats at a dose of 20mg per 100g of body weight intraperitoneally. Effective thermal stress was determined by varying the duration of heat exposure and keeping the exposure temperature constant and vice versa. In both conditions rectal temperature of each rat was recorded at regular time intervals and also after the termination of heat-exposure in each day to know the pattern and degree of heat stress imposed on the rats. Following these approaches, temperature of 43°C with 2 hrs. duration per day for 15 successive days and 43°C temperature with 3 hrs. duration in one day were considered optimum to produce the effect of chronic and acute heat exposure, respectively. Rats were exposed to heat between 2 and 6 p.m. in each day to avoid the diurnal variation of temperature. To maintain the uniformity in the heat stress induced, no experiment was performed in the months of summer (April to June) and winter (December to February). The entire study was carried out with several sets of experiments involving different groups of rats and keeping all the above conditions identical.
3.1.4. Tissue Collection

At the end of experimental period rats were kept fasting for 18 hrs. and then sacrificed by cervical dislocation. Blood was collected immediately from the hepatic vein with a heparinized syringe and kept in polypropylene vials at 4°C, taking proper care to prevent any chance of haemolysis. To obtain erythrocytes, heparinized blood was centrifuged (1000×g at 4°C for 10 mins.). Plasma was collected and stored in deep freeze. The buffy layer was removed completely by aspiration. The erythrocytes were washed three times with 20mM Tris-buffered-saline solution. The washed and packed erythrocytes were used for the preparation of ghost membrane and haemolysate for enzyme assay, estimation of reduced glutathione (GSH) and ascorbic acid content, assay of alanine and thiobarbituric acid reactive substances, and the extraction of membrane lipids.

3.2. Methods Employed

3.2.1. Measurement of Rectal Temperature

Rectal temperature of each rat of both the treated and control groups was measured with the help of a clinical thermometer. Proper care was taken to avoid faulty measurement due to varied depth of penetration, defication and accumulated feces in the rectum at the time of temperature study. In case of treated rats rectal temperature was measured twice a day – before and at the end of heat exposure period.

3.2.2. Determination of Haematocrit Value

Rats were sacrificed by cervical dislocation and blood was collected from hepatic vein in EDTA-containing polypropylene vials. The whole blood was centrifuged at 3000 r.p.m. (2300×g) for 30 mins. at 4°C in Wintrobe's haematocrit tube to determine haematocrit value.

3.2.3. Estimation of Haemoglobin (Hb)

Haemoglobin was estimated following the method as described by Evelyn and Malloy (1938) using Drabkin reagent. One hundred microlitre of saline washed packed red cell was mixed with 0.4ml of triton X-100 solution. It was centrifuged and
0.1ml of supernatant was taken in a cuvette. To it 0.9ml of Drabkin reagent was added, followed by mixing and the absorbance was then read at 546nm. The calculation was done using a standard curve prepared with commercial Hb.

3.2.4. Preparation of Erythrocyte Membrane

Fresh blood obtained from the rat was used to prepare ghost membrane following the procedure as developed by Marchesi and Pallade (1967). The packed red cells were washed thrice in 130mM NaCl and 20mM Tris-HCl (pH 7.4) mixture and recovered by centrifugation at 2500 r.p.m. for 15mins. Saline-washed red cells were lysed in 5mM Tris-HCl (pH 7.4) buffer containing 1mM EDTA and kept at 4°C for 15 mins. Thirty nine volumes of haemolysing fluid were added to 1 volume of packed red cell, followed by centrifugation at 25,000xg for 30 mins. at 4°C. The supernatant was discarded and the membrane settled down was resuspended in the same medium and centrifuged again. The same procedure was repeated for 3 to 4 times until the membrane became milky white. Finally, the membrane was suspended in 50mM Tris-HCl buffer (pH 7.4) containing 1mM EDTA and kept frozen. All operations were carried out in cold.

3.2.5. Estimation of Membrane Protein

The protein was estimated by modified Lowry method (Shakir et al., 1994). A 50% Folin-Ciocalteau reagent (v/v) was prepared freshly with H₂O. The alkaline CuSO₄ solution was prepared with addition of equal volume of solution I (1.85mM Na₂CO₃ and 98.10mM NaOH) and solution II (0.39mM CuSO₄, 5H₂O and 0.70mM KNa C₄H₄O₆, 4H₂O). It was prepared few hours before the estimation of protein. The membrane suspension (20µl) was added to 1ml of alkaline CuSO₄ solution and the mixture was incubated at 37°C for 5 mins. Then to it 0.1ml of Folin-Ciocalteau reagent (1:1 dilute) and 1.08ml of H₂O were added followed by mixing. The mixture was incubated again at 37°C for 5 mins. The absorbance was read at 500nm and the protein content was calculated using a standard curve prepared with aliquots of BSA-solution having known concentration.

3.2.6. Estimation of Ascorbic Acid

Ascorbic acid was estimated in plasma and erythrocytes following the method of Roe and Kuether (1943). Three millilitres of 20% TCA was added to 1ml of plasma
or 0.5ml packed red cell suspended in 0.5ml H\textsubscript{2}O. The mixture was vortexed, centrifuged and the supernatant was brominated. After debrominization by passing water-saturated air, 1ml of supernatant was treated with 0.2ml of 2% DNPH and incubated at 37\degree C for 3 hrs. The mixture was cooled in ice followed by addition of 0.8ml of 85\% H\textsubscript{2}SO\textsubscript{4}. It was mixed and allowed to attain the room temperature. The absorbance was then read at 540nm and the result was computed using the standard curve prepared with aliquots of ascorbic acid solution having known concentration.

### 3.2.7. Extraction of Membrane Lipid

The lipid was extracted from erythrocyte membrane by the method as described by Rose and Oklander (1965). The procedure was as follows: To 0.1ml of membrane suspension 4.9ml of cold isopropanol was added. It was vortexed and kept in ice for 15mins. Then to it 3ml of cold chloroform was added. It was vortexed again and kept in ice for 15 mins. It was allowed to stand at room temperature for 1 hr. with frequent vortexing. The supernatant was collected after centrifugation. Five millilitres of supernatant was evaporated to dryness with N\textsubscript{2} to get the lipid.

### 3.2.8. Estimation of Membrane Cholesterol

To extract the membrane cholesterol 0.1ml of membrane suspension was mixed with 2.9ml of ether-acetone (1:1) mixture and the mixture was warmed at 70\degree C for 10 mins. It was centrifuged and 2ml of supernatant was taken for drying with N\textsubscript{2}. The cholesterol was estimated according to the method as described by Zlatkis et al. (1953). The residue was mixed with 2.5ml of 0.03\% FeCl\textsubscript{3} (anhydrous) solution and vortexed several times with 10 mins. intervals. To it 1.5ml ice-cold conc. H\textsubscript{2}SO\textsubscript{4} was added followed by mixing. It was then allowed to attain the room temperature and the absorbance was read at 540nm. The result was then computed using the standard curve prepared with aliquots of cholesterol solution having known concentration.

### 3.2.9. Estimation of Membrane Phospholipid

The lipid was extracted from the membrane with ether-alcohol (1:3) mixture. To 0.1ml of membrane suspension 5.9ml of ether-alcohol mixture was added. It was vortexed and incubated at 37\degree C for 12 hrs. Following centrifugation, 5ml of supernatant was taken for drying. The residue was digested with 0.5ml of 10(N)
H$_2$SO$_4$ and 15μl conc. HNO$_3$ under a low flame of a burner until it became colourless. To it 2ml of distilled water, 0.25ml of 1% ascorbic acid solution and 0.25ml of 1% ammonium molybdate solution were added and mixed. The absorbance was read at 640nm just after 30 mins. of addition of ammonium molybdate. The total procedure was based on the method as developed by Youngburg and Youngburg (1930).

3.2.10. Determination of Iodine Number of Membrane Lipids

Iodine number was determined by the method as described by Plummer (1992). Extraction of lipid was made from packed red cells following the method as described by Rose and Oklander (1965) without any modification. An aliquot of extract was taken in a conical flask and evaporated to dryness with N$_2$ to get the lipid. To the residue 1ml chloroform, 1ml ICl (0.2M), 1ml 10% KI and 2ml H$_2$O were added and it was mixed well. The mixture was then titrated with 0.1M Na$_2$S$_2$O$_3$, 5H$_2$O using 0.5ml of 1% starch solution as an indicator.

3.2.11. Estimation of Reduced Glutathione (GSH)

Reduced glutathione was estimated by the method as adopted by Owens and Belcher (1965) with slight modification as follows: 0.1ml saline-washed packed red cell was lysed by the addition of 0.1 ml of 6.6% CH$_3$COOH (v/v). It was then vortexed and deproteinized with addition of 0.4ml of 10% cold sulfosalicylic acid containing 1mM EDTA. The contents were vortexed and allowed to stand for 15mins. at 4°C and then centrifuged. The protein-free supernatant (0.1ml) was added to 0.9ml of 0.1M DTNB solution made in 0.1M phosphate buffer (pH 8.0). It was then allowed to stand for 15 mins. and the absorbance was read at 412nm with DTNB solution as blank. The absorbance values were compared with the standard curve prepared with aliquots of GSH solution having known concentration to compute the GSH content.

3.2.12. Estimation of Total Membrane Thiol (–SH) Groups

A modification of Ellman’s procedure was used to quantitate the total membrane –SH groups (Ellman et al., 1961). The membrane suspension (0.1ml) was mixed with 0.8ml of incubation medium containing 300mM Na$_2$HPO$_4$, 8M urea and 2% sodium dodecyl sulphate (SDS). Then 0.1 ml of 0.02% DTNB in 1% sodium citrate was added to both sample and reagent blank. The absorbance was read at
412nm 10 mins. after addition of DTNB. The total -SH group content was calculated by referring the absorbance value to a standard curve of GSH.

3.2.13. Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

The lipid peroxidation in erythrocytes was determined by the thiobarbituric acid (TBA) test using the method as adopted by Stokes and Dormandy (1971) with slight modification. To 0.2ml of packed erythrocytes, 0.8ml of 0.1M phosphate buffer (pH 7.4) was added. It was deproteinized with addition of 0.5ml of 30% TCA. The contents were vortexed and allowed to stand in ice for 2 hrs. After centrifugation, 1ml of supernatant was mixed with 75µl of 0.1M EDTA and 0.25 ml 1% TBA made in 0.5M NaOH, followed by warming in boiling water bath for 15 mins. It was cooled and the absorbance was read at 532nm against a reagent blank.

In case of erythrocyte membrane the level of lipid peroxidation was measured adopting the method as described by Buege and Aust (1978).

3.2.14. Determination of Conjugated Diene Formation in Membrane Lipid

The conjugated diene was measured according to the procedure as described by Buege and Aust (1978). One millilitre of membrane suspension was mixed with 5ml of chloroform-methanol (2:1) mixture and the mixture was left for 15 mins. with frequent vortexing. It was then centrifuged at 100×g for 5 mins. to separate the mixture into two phases. The upper layer was removed by suction and 3ml of the lower chloroform layer was taken and put to dryness in a water bath at 40°C under a stream of N₂; the residue was dissolved with 1.5ml of cyclohexane and the absorbance was read at 233nm against a cyclohexane blank.

3.2.15. Estimation of Alanine Content in Erythrocytes

Alanine content of erythrocyte was measured enzymatically following the method as described by Davies and Goldberg (1987). One ml of cold 1.6M PCA was added to 3ml of chilled saline-washed cell suspension and it was kept in ice for 10min with frequent vortexing. The mixture was centrifuged at 500×g for 10 mins. to get the sample supernatant.
To 1ml sample supernatant 200μl of 2M KOH and 800μl of 0.5M. Tris-HCl buffer (pH 9.0) were added and it was then kept in ice for 2 hrs. during which the perchlorate precipitated the alanine present in each sample supernatant. The mixture containing 500μl of the supernatant containing alanine, 500μl of 0.8M Tris-buffer (pH 9.0) containing 0.04M EDTA, 500μl of 6.6 hydrazine hydrate solution, 100μl of 20mM NAD⁺ and 100μl of 20 mg/ml of alanine dehydrogenase (30 units/mg) was incubated at 37°C for 60mins. Following termination of incubation, the alanine content was determined fluorometrically by the reduction of NAD⁺ to NADH catalyzed by alanine dehydrogenase that was measured at excitation wavelength of 340nm and emission wavelength of 450nm. The results were then computed by using a standard curve prepared with alanine solution having varied amounts ranging from 0-50 μmoles.

3.2.16. Assay of Acetylcholine Esterase (AchE) (EC 3.1.1.8) Activity of Erythrocyte Membrane

The activity of AchE was assayed by the method as described by Ellman et al. (1961), with slight modification as follows: To 280μl of 0.1M phosphate buffer (pH 7.4), 20μl of membrane suspension was added, followed by mixing and warming in incubator at 37°C for 5 mins. To start the reaction, 0.1ml of 3mM S-butylthiocholine iodide was added to it. After incubating for 30 mins. at 37°C, the reaction was stopped with addition of 0.1ml of 15% cooled PCA (v/v) and centrifuged to get the supernatant. A definite amount (0.1ml) of supernatant was added to 0.9ml of 0.25mM DTNB prepared in 0.1M phosphate buffer (pH 8.0) and the absorbance was read at 412nm 10 mins after the addition of DTNB against a reagent blank. The absorbance values were compared with a standard curve prepared with GSH solution having known concentration to compute the – SH group liberation by AchE.

3.2.17. Assay of ATPases (EC 3.6.7.3) Activities of Erythrocyte Membrane

The activities of ATPases were assayed according to the method as developed by Ronner et al. (1977). During assay of three ATPases (Na⁺-K⁺, Mg²⁺ and Ca²⁺-Mg²-ATPases), the total volume of reaction mixture, amount of HEPES buffer (300mM, pH 7.4) and membrane suspension were kept constant. The mixtures were preincubated for 5 mins. in a water bath at 37°C and the reaction was started with
addition of 0.1ml of 10mM ATP solution, and incubated for 30mins. at 37°C with constant shaking. The reaction was stopped by the addition of 50% ice-cold TCA solution and kept cooled until the liberated Pi was estimated. Heat-treated membrane suspension (at 80°C for 5 mins.) was used as control so that ATP blank could be determined. All assays were carried out in duplicate. Ouabain (1%) and EGTA (1%) were used as blockers for Na⁺-K⁺-ATPase, and Ca²⁺-Mg²⁺-ATPase respectively, and 330mM NaCl, 1200mM KCl, 50mM MgCl₂ and 1mM CaCl₂ were used for the preparation of incubation medium.

3.2.18. Assay of Catalase (EC 1.11.1.6) Activity

The catalase activity was assayed in both haemolysate and erythrocyte membrane. The membrane suspension was dissolved in 1% triton X-100 solution and used as sample. The substrate was made by adding 0.1ml of 30% H₂O₂ (v/v) to a solution of 0.05M phosphate buffer (pH 7.0) to obtain the absorbance at 240nm in the range of 0.520 to 0.550. The activity of catalase was assayed by following the rate of decomposition of H₂O₂ as described by Aebi (1989) and the activity of enzyme was expressed as μmoles of H₂O₂ degraded/mg haemoglobin/min. in case of erythrocyte haemolysate or μmoles of H₂O₂ broken down/mg membrane protein/min. in case of erythrocyte membrane.

3.2.19. Assay of Superoxide Dismutase (SOD) (EC 1.15.1.1) Activity

The assay of SOD activity was carried out by following the inhibition of pyrogallol autooxidation rate by SOD as described by Marklund and Marklund (1974). A definite amount (0.1ml) of 20mM pyrogallol solution was added to 0.9ml of 0.05M phosphate buffer (pH 7.8) taken in an 1ml cuvette and read at 420nm at every 30 secs. for a period of 2 mins. to determine the rate of autooxidation of pyrogallol. After addition of haemolysate as sample the rate of autooxidation of pyrogallol was measured in the same way to determine the enzyme activity. In this case the rate of autooxidation was decreased due to presence of SOD in the haemolysate. The activity of the enzyme was determined and expressed in unit “U”, assuming that activity of enzyme as one unit (U) which inhibits autooxidation of pyrogallol by 50%.
3.2.20. Assay of Glutathione Peroxidase (GPx) (EC 1.11.1.19) Activity

The freshly prepared erythrocyte lysate was treated with Drabkin reagent to remove haemoglobin as described by Paglia and Valentine (1967) and assayed for the activity of glutathione peroxidase by coupling to the glutathione reductase reaction according to the method of Flohe and Gunzler (1984). To 0.6ml of 0.1M phosphate buffer (pH 7.0) 0.1ml of haemolysate was added. To that mixture 75μl of 1:250 diluted GR (2.4 U/ml) in 0.1M phosphate buffer (pH 7.0), 75μl of 1.5mM NADPH in 0.1% NaHCO₃ and 10ml of NaN₃ (1.125mM) were added. It was mixed and incubated at 37°C for 10 mins.

After the addition of 75μl of 1.5mM H₂O₂, prepared from a 30% H₂O₂ (v/v) in phosphate buffer (pH 7.0), the decrease in absorbance was read at 340nm at every 30 secs. for a period of 5 mins. Nonenzymatic consumption of NADPH and GSH was studied by substituting β-mercapto-ethanol-EDTA stabilizing solution for the haemolysate, keeping all other reagents same. This was taken into account while computing the enzyme activity. The activity of enzyme was expressed in terms of μmoles of NADPH oxidized/g haemoglobin/min. under assay condition.

3.2.21. Assay of Glutathione Reductase (GR) (EC 1.6.2.4) Activity

The freshly prepared haemolysate stabilized in 0.7mM β-mercapto-ethanol was used for the assay of GR activity according to the method of Beutler (1984). The mixture containing 50μl of 1M. Tris-HCl buffer (pH 8.0), 10μl of haemolysate, 100μl of 0.033M oxidized glutathione (GSSG) and 790μl of H₂O₂ was incubated at 37°C for 10 mins. The rate of decrease in absorbance was measured at 340nm after addition of 50μl of 2mM NADPH solution. The activity of enzyme was expressed in terms of μmoles of NADPH oxidized/g haemoglobin/min under assay condition.

3.2.22. Assay of Glucose-6-phosphate Dehydrogenase (G6PD) (EC 1.1.1.49) Activity

The reduction of NADP to NADPH was measured at 340nm to assay the enzyme activity as described by Deutsch (1983). The mixture containing 550μl of 0.1M Tris-HCl (pH 8.0), 200μl of 0.1M MgCl₂, 100μl of 2mM NADP and 50μl of
haemolysate (1:20 in triton X-100) was incubated at 37°C for 10 mins. Then 100μl of 8mM glucose-6-phosphate was added and the increase in absorbance was measured at 340nm. The activity of enzyme was expressed in terms of μmoles of NADP reduced /g haemoglobin/min.

3.2.23. Statistical Analysis

The data obtained from each experiment described above (N<30) were subjected to statistical analysis. The level of significance of the observed changes between the control and treated groups of animals was calculated according to two-tail student’s ‘t’-test and the probability of chance of occurrence (p) was determined according to the Table (Level of significance for two-tail ‘t’-test) of Fisher and Yates (1974). Differences were considered significant at p<0.05.