CHAPTER III

MATERIALS AND METHODS
CHEMICALS

Human ceruloplasmin type X, glutathione reductase (E.C. 1.6.4.2.) type IV from Bakers' Yeast, Superoxide dismutase (E.C. 1.15.1.1) from bovine erythrocytes, 5,5' - dithiobis 2-nitrobenzoic acid, 2, 3 -dihydroxy benzoic acid and bovine serum albumin (fraction V) were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Catalase (E.C. 1.11.1.6) from bovine liver, cumene hydroperoxide and alpha-tocopherol were obtained from Fluka AG, Buchs, Switzerland. Diethyldithiocarbamate was obtained from BDH Chemicals Ltd. Poole, England. L-ascorbic acid and riboflavin were from E.Merck, AG Darmstadt, Germany. Reduced glutathione and horse radish peroxidase were procured from CSIR centre for Biochemicals, New Delhi. Oxidised glutathione, NADPH and 2-deoxy ribose were obtained from SISCO Research Laboratories, Bombay. All other chemicals used were commercial products of highest analytical grade available in India.
METHODS :

1. **In vitro ascorbate autoxidation** :

   The rate of ascorbate autoxidation was determined by following the procedure described by Mishra and Kovachich (1). The assay mixture contained 0.2 mg L-ascorbic acid (1.137 μ moles) unless otherwise stated, in 5 mM potassium phosphate buffer containing 0.15 M NaCl, pH 7.4, in a final volume of 10 ml. The reaction mixture was incubated at 37°C in uncorked test tubes in a water bath. Two ml aliquots at given time intervals were withdrawn from the incubation mixture and the absorbancy at 265 nm was measured spectrophotometrically for determining the amount of unoxidised ascorbic acid remaining in the sample. Suitable controls were always run to account for any other absorption at 265 nm. The ascorbate concentration employed was within the physiological limits as present in human plasma. Stock solutions of ascorbic acid were prepared fresh each time.

   Double glass distilled water was used for preparing the reagents and buffer solutions. Transition metal ions were present largely as a result of trace contaminants in the buffer salts. Since trace metals were desired in these experiments, no further attempts were made to decrease the level of trace metals in the buffer solutions. These experiments may therefore be taken to represent standard laboratory conditions.

   The effect of addition of plasma, tissue homogenates or other substances on the rate of ascorbate autoxidation was studied by incorporating each of these in the same buffer. Alpha-tocopherol was added to the
incubation mixture in 0.05 ml ethanol and mixed immediately. Tables and Figures give the final concentrations of the added substances in the incubation mixture.

In order to assess the specificity of the absorption (265 nm) method for the assay of unoxidised ascorbic acid in the incubation mixture, 2, 6-dichlorophenol indophenol titration method was followed (2). Suitable aliquots of the incubation mixture, at specified time intervals were used for titration against 1 ml of standardised 2, 6-dichlorophenol indophenol dye solution (1 ml = 0.02 mg of reduced ascorbic acid) in the presence of 1 ml of 5% metaphosphoric acid solution. The red coloured dye in acid medium becomes colourless on reduction.

2. Determination of total ascorbic acid (reduced and dehydro):

The total ascorbic acid at different time intervals of incubation was determined by following the method of Roe and Kuether (3). To 0.5 ml of the incubation mixture (described under method 1), 2 ml of freshly prepared 6% metaphosphoric acid was added and mixed well on a vortex mixer and centrifuged for 10 min; 1.2 ml of the clear supernatant was added to 0.4 ml of 2, 4-dinitrophenyl hydrazine-thiourea-copper sulphate reagent, mixed well and incubated at 37°C for 3 hrs, chilled for 10 min in an ice bath, mixed with 2 ml of cold 12M H₂SO₄ and the O D measured at 520 nm. A standard curve was prepared using fresh solutions of ascorbic acid and following the same assay procedure.
3. **Preparations of samples and RBC stroma:**

Blood was collected from healthy human volunteers by venous puncture into heparinised syringes; plasma and erythrocytes were separated by centrifugation. The erythrocytes were washed 3 times with cold 10 mM potassium phosphate buffer containing 0.15M NaCl, pH 7.4. Erythrocyte hemolysate was prepared by mixing 0.2 ml of packed cells with 4.8 ml of distilled water. The erythrocyte stroma was prepared according to Dodge *et al* (4) with slight modifications. The washed cells were lysed by mixing them with 3 volumes of cold distilled water and centrifuged at 4°C at 20,000 x g for 20 min. The ghost pellet was repeatedly washed with water and centrifuged as above, till the supernatant became clear. The membrane pellet was finally suspended as a fine suspension in the cold saline phosphate buffer (10 mM, pH 7.4) in the required dilution.

4. **Effect of pH on ascorbate autoxidation:**

This was studied at pH values ranging from 6 to 8.2 using different 5mM potassium phosphate buffers containing 0.15 M NaCl. (Note: In all other experiments of ascorbate autoxidation studies, the maintenance of pH to 7.4 was strictly adhered to).

5. **Plasma dialysis:**

Dialysis of plasma was performed using normal human plasma diluted 100 fold with saline phosphate buffer (5mM, pH 7.4) against the same buffer in the cold for 12 hrs. The dialysis tubing used had a 25,000 M.W. cut off point.
6. Heat treatment of plasma:

Plasma was diluted 100 fold with the saline phosphate buffer (5 mM, pH 7.4) and heated at 60°C for 20 min in a water bath.

7. Separation of albumin from human plasma:

This was done by the method of Reinhold (5). To 6 ml. of sodium sulphate-sulphite mixture (27.8%) taken in a stoppered centrifuge tube, was added 0.4 ml human plasma. Inverted to mix and 4 ml of diethyl ether was added, stoppered and shaken gently (40 times) for 20 sec using a movement of the arm of about 15 inches, and centrifuged for 5 min. The clear solution (albumin), after separating the globulin layer, was used for ascorbate autoxidation studies after proper dilution with the saline phosphate buffer (5mM, pH 7.4). Since very high dilutions were required, the final pH of the diluted solutions were still 7.4.

8. Estimation of protein:

Protein estimations in all the samples were carried out by the method of Lowry et al. (6). To 2 ml of the protein solution (range 40-400 μg), 4 ml of alkaline copper reagent was added. After 10 min, 0.4 ml of IN Folin reagent was added. The resulting colour was measured after 30 min in Spectronic 20 spectrophotometer at 540 nm.

9. Hydrogen peroxide estimation:

The $H_2O_2$ content in the incubation mixture (from the assay system used for ascorbate autoxidation studies, method 1) was determined by the procedure of Thurman et al. (7) with certain modifications. At given time intervals, 2 ml aliquots of the incubation mixture were pipetted into
Fig. 2: Standard curve for $\text{H}_2\text{O}_2$
Fig. 3: Standard curve for 2, 3 dihydroxybenzoate.
a test tube and 2 ml of ice cold 20% TCA, 0.8 ml of 10mM ferrous ammonium sulphate and 4 ml of 2.5M potassium thiocyanate were added. The absorbance of the red ferricthiocyanate complex was determined at 480 nm and was compared with a standard curve prepared with fresh dilutions of H$_2$O$_2$. This assay was linear under these conditions over a concentration range of 10 to 120 μM H$_2$O$_2$ (Figure 2).

10. Assay of hydroxyl radicals:

This was carried out as given by Halliwell and Gutteridge (8) with certain modifications. The reaction mixture contained in a final volume of 10 ml, 5 mM potassium phosphate buffer in 0.15M NaCl (pH 7.4), 2 mM sodium salicylate and 113.7 μM ascorbate. Following incubation at 37°C for 3 hrs, the reaction was terminated by the addition of 0.4 ml of ConcHCl. The hydroxylated products of salicylate were extracted in ether after the addition of 2.5 gm of NaCl. Six ml of the ether layer was removed and evaporated to dryness at 40°C. The hydroxylated products in the dry residue were dissolved in 1 ml of ice cold deionised double distilled water and added in order, 0.5 ml of 10% TCA in 0.5 M HCl, 1ml of 10% sodium tungstate and 1ml of 0.5% sodium nitrite. After 5 min, 2ml of 1M KOH was added and the absorbance at 510nm was measured (Note: 1M KOH was used instead of 0.5M KOH in order to increase the stability of the final pink colour). A standard curve was constructed using 2, 3-dihydroxy benzoate solutions carried through the same extraction and assay procedure. The assay was linear under the conditions followed over a concentration range of 25-300 n moles (Figure 3).
11. Deoxy ribose degradation as measured by TBA reactivity:

The method of Halliwell and Gutteridge (9) was followed to determine the damage to 2-deoxyribose during ascorbate autoxidation by the TBA reaction with certain modifications. The assay mixture in a total volume of 2.2 ml contained the following reagents at the final concentrations stated (or as shown in appropriate figures and tables); 2-deoxyribose (2.5 mM), ascorbic acid (113.7 μM), potassium phosphate buffer (10 mM, pH 7.4) and NaCl (63 mM). Addition of other substances were done after incorporating them in the 10 mM phosphate buffer. The tubes were incubated at 37°C for 30 min, with appropriate blanks without ascorbic acid. At the end, 2 ml of TBA (1% in 0.05 M NaOH) was added followed by 2ml of 2.8% TCA. The tubes were heated for 10 min at 100°C in a water bath, cooled and the absorbance read at 532 nm.

In duplicate experiments, the amount of ascorbic acid remaining unoxidised in the incubation mixture was determined by measuring the absorbancy at 265 nm. (Note: Stock solutions of 2-deoxyribose, ascorbic acid, EDTA, Fe salts were prepared just before use, throughout the course of the study).

ANIMAL EXPERIMENTS:

I. Maintenance and Care:

Albino rats (Wistar strain) of the Pharmacology Department of Kasturba Medical College, Manipal were used throughout the present study.
The rats were housed under hygienic conditions in plastic cages. They were maintained on a synthetic pellet diet (supplied by Shaw Wallace and Co., Madras) and water *ad libitum*. The pellet diet has been nutritionally assayed by Ounch and Thomas (10) and has been reported to be comparable with a synthetic balance diet. According to the manufacturers, the diet has the following composition.

<table>
<thead>
<tr>
<th>Content</th>
<th>Percentage</th>
</tr>
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<tbody>
<tr>
<td>Protein</td>
<td>24.0</td>
</tr>
<tr>
<td>Fat</td>
<td>5.0</td>
</tr>
<tr>
<td>Fibre</td>
<td>6.0</td>
</tr>
<tr>
<td>Acid soluble ash</td>
<td>3.0</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.6</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.3</td>
</tr>
</tbody>
</table>

and adequate amounts of carbohydrates, minerals and vitamins. Animals were given soaked bengal gram and leafy vegetables twice a week. The rats reared on the pellet diet attained 150-200 gm body wt at 9-12 weeks of age and were used for most of the studies.

II. **Preparation of experimental animal models**:

Diethyldithiocarbamate treatment to rats was followed according to the procedure of Bartoli *et al* (11).

A. Two groups of male rats of body wt 150-200 gms maintained on pellet diet were selected. The first group of 6 rats received a single dose of 0.5 ml
of 0.9% saline i.p. which served as control. The second group of 30 rats were injected i.p. with 1.2 gm/kg of DDC (Sigma Chemical Company, St. Louis, MO, USA) in 0.5 ml saline.

Rats which received saline alone were sacrificed exactly 1 hr after the injection. This group of rats were considered as control group.

The DDC treated rats in batches of 6 each were sacrificed successively at intervals of 1, 6, 12, 24 and 48 hr after the injection.

B. Feeding with vitamin E and vitamin A, and DDC treatment:

Three groups of male albino rats of body weight 150-200 gm were selected.

Group I consisting of 30 rats were given orally 0.5 ml of ground-nut oil (procured from local market) each day for 7 days.

Group II and III consisting of 30 rats each were fed orally 10 mgs of alpha-tocopherol (Fluka AG, Buchs, Switzerland) in 0.5 ml groundnut oil and 1000 µg of retinol (Indian Drugs and Pharmaceuticals) in 0.5 ml of groundnut oil respectively, on each day for 7 days.

Five animals each from groundnut oil fed, alpha-tocopherol fed and retinol fed were injected i.p. with 0.5 ml of saline and were sacrificed exactly 1 hr after the injection. These animals served as control for the respective groups.
The rest of the animals were given DDC (1.2 gm/kg) i.p. in saline. A batch of 5 rats from each of the groups I, II and III were sacrificed successively at 1, 6, 12, 24 and 48 hr after the injection.

(Note : None of the experimental animals died during the course of the experiments).

III. Mode of Sacrifice :

Rats were sacrificed at the same time of the day under light ether anaesthesia. They were bled by cardiac puncture and blood was collected using heparin rinsed syringes. Liver, kidney and brain were quickly excised off, washed in sequence with cold saline, saline phosphate buffer and blotted and stored in the freezer. Estimations were completed within 48 hrs of sacrifice.

IV. Preparation of tissue homogenates :

Kidney, brain and liver homogenates (10% W/V) were prepared in 10mM potassium phosphate containing 0.15M NaCl, pH 7.4, in the cold using a mechanical homogenizer. The unbroken cells and cellular debris were removed by centrifugation at 700xg for 10 min using a Remi'C-24 refrigerated centrifuge. The supernatant thus obtained was termed as homogenate and stored at 4°C until used. Lipid peroxidation studies were performed almost immediately after preparing the homogenate.

V. Preparation of erythrocyte suspensions :

The erythrocyte suspension was prepared according to the method of Kartha and Krishnamurthy (12). The heparinised blood was centrifuged.
Plasma and buffy coat were carefully removed and the separated cells were washed 3 times with 10mM potassium phosphate buffer (pH 7.4) containing 0.15M NaCl. A 20% cell suspension (V/V) was then made in the same buffer and stored at 4°C in the refrigerator.

VI. Studies with rat tissue homogenates, plasma, erythrocyte suspensions and erythrocyte hemolysate:

1. \( \text{H}_2\text{O}_2 \) induced hemolysis of red blood cells:

   The method followed was that of Kartha and Krishnamurthy (12). One ml of 20% cell suspension was added to 5 ml of phosphate buffer (10mM, pH 7.4) containing 0.15M NaCl in a 50ml Erlenmeyer flask followed by 1.5ml of \( \text{H}_2\text{O}_2 \) to give a final concentration of 0.44M \( \text{H}_2\text{O}_2 \). In the controls, \( \text{H}_2\text{O}_2 \) was replaced by an equal volume of saline phosphate buffer. The flasks were stoppered and incubated at 37°C with gentle shaking once in every 15 min. Aliquots of 0.5 ml each were withdrawn at the required time intervals and transferred to 4.5 ml of 10mM saline phosphate buffer (pH 7.4), mixed gently, centrifuged and the O D of the supernatant was measured at 520 nm. Total hemolysis was determined after complete lysis of 0.5 ml of the incubation mixture with 4.5 ml of distilled water and the hemolysis in the test was expressed as percent of the total hemolysis.

2. \( \text{H}_2\text{O}_2 \) induced lipid peroxidation of red blood cells:

   This was performed by the method of George (13). The incubation mixture containing red blood cells, \( \text{H}_2\text{O}_2 \) and saline phosphate buffer was prepared in an exactly similar way as that described above for \( \text{H}_2\text{O}_2 \) induced
hemolysis. One ml of the incubation mixture was withdrawn at specified
time intervals and added to 1.5 ml of cold 20% TCA, mixed and centrifuged.
Two ml of the supernatant was mixed with an equal volume of 0.67% TBA
and kept in a boiling water bath for 10 min, cooled and the O D of the
pink chromogen determined at 535 nm. The amount of TBA-reactive
material was expressed in terms of nmoles of malondialdehyde (MDA)/ml
RBC taking the molar extinction coefficient of MDA as 1.56 x 10\(^5\) (14).

[Note : Commercial 2-thiobarbituric acid was routinely purified as stated
below : The 0.67% aqueous solution of TBA (100 ml) was mixed with
5-10 gm of neutral alumina, allowed to stand for about 8 hrs and filtered
through Whatman No. 1 filter paper. The clear filtrate thus obtained was
used].

3. Lipid peroxidation of tissue homogenates :

The method followed was that of Kartha and Krishnamurthy (15).
Five ml of 10% (W/V) homogenate (freshly prepared) was incubated in 50ml
stoppered Erlenmeyer flask at 37°C. One ml aliquots at specified time
intervals were added to 1.5 ml of cold 20% TCA and centrifuged. Two ml
of the supernatant was mixed with 2 ml of 0.67% TBA and kept in a
boiling water bath for 10 min. After cooling, the pink chromogen was
measured at 535 nm and the O D values were expressed as nmoles of
MDA/gm wet tissue. (as in the case of H\(_2\)O\(_2\) induced lipid peroxidation
of RBC).
4. Copper induced lipid peroxidation of tissue homogenates:

This was performed by including Cu^{++} ions in saline phosphate buffer to give a final concentration of 80 μM. The rest of the procedures followed were similar to those described above.

5. Serum lipid peroxide content:

Serum lipid peroxides were determined following the thiobarbituric acid method modified for serum, by Satoh (16). This modified method is reported to be more specific, being free from interference by sialic acid, and gives true value of peroxides. To 0.5ml of serum, 2.5ml of 20% TCA was added and allowed to stand for 10 min at room temperature, centrifuged at 3000 r.p.m. for 10 min. Discarded the supernatant. The precipitate was washed with 2ml of 0.05M H_2SO_4 and resuspended in 2.5 ml of 0.05M H_2SO_4. That was followed by the addition of 3ml of 0.2% TBA in 2M sodium sulphite. The coupling of TBA with lipid peroxide was carried out by boiling it in a water bath for 10 min. After cooling, the TBA pigment was extracted with 4ml of n-butanol with shaking and the organic layer separated by centrifugation. The absorbance of the butanol layer was measured at 535nm and the O D values were expressed as nmoles of MDA/ml serum.

6. Determination of conjugated dienes:

The presence of conjugated dienes in tissues or tissue fluids is also a measure of lipid peroxidation. Their levels in the tissues/fluids were measured by the method of Bacon et al (17). Lipids were extracted from 0.5 ml of the sample (serum or tissue homogenates) with 5 ml of chloroform-
methanol mixture (2:1). One ml of the supernatant (equivalent to lipids of 0.1 ml serum or 10mg wet tissue) was evaporated to dryness under a stream of oxygen-free nitrogen. The chloroform free lipid was then redissolved in cyclohexane (spectrophotometric grade) and the absorbance at 230nm was recorded against a cyclohexane blank in Spectronic 21 spectrophotometer.

The O D obtained was taken as the measure of conjugated diene content.

7. Assay of superoxide dismutase (E.C. 1.15.1.1)

Illumination of riboflavin in the presence of $O_2$ and electron donor like methionine or EDTA generates superoxide anions and this has been used as the basis of assay of SOD. The reduction of Nitroblue tetrazolium (NBT) by $O_2$ was followed at 560nm using the Bauch & Lomb spectrophotometer. The procedure adopted was that of Beauchamp and Fridovich (18).

The reaction mixture contained $5.6 \times 10^{-5}$ M NBT, $1.17 \times 10^{-6}$ M riboflavin, $1 \times 10^{-2}$ M methionine in 0.05M potassium phosphate buffer, pH 7.8, with suitably diluted erythrocyte hemolysate or tissue homogenate in a total volume of 3ml. Illumination of the solution was carried out in an aluminium foil lined box fitted with a 15W fluorescent lamp. The solutions taken in 10ml beakers were kept in the box and illuminated for exactly 10min. Controls without the enzyme source were always included. The absorbancy was measured at 560nm. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT
by 50% under the specified conditions. The values were expressed as units/mg protein of tissue homogenate or as units/ml of hemolysate.

8. Preparation of hemolysate for the assay of SOD activity:

This was done by the method of McCord and Fridovich (19). To 1ml of the packed erythrocytes (washed with 0.9% NaCl) was added 1ml of deionised water to lyse the cells followed by 0.5 ml of ethanol and 0.3ml of chloroform. Mixed and allowed to stand for 15 min. It was diluted by adding 0.2 ml water and centrifuged at 4°C. The supernatant contained SOD activity and was used for the assay of SOD.

9. Preparation of tissue homogenates for the assay of SOD activity:

Tissues from the experimental rats were extirpated, washed with saline thoroughly, blotted and homogenised (10% W/V) in 10mM potassium phosphate buffer, pH 7.8, in the cold. The unbroken cells and cell debris were removed by centrifugation at 700xg for 10 min in a refrigerated centrifuge. The supernatant obtained was used for the measurement of SOD activity.

10. Assay of glutathione peroxidase (GSHpx) activity:

The enzyme activity was measured by the method of Paglia and Valentine (20) as modified by Lawrence and Burk (21). The reaction mixture consisted of 50mM potassium phosphate buffer (pH 7.0), 1mM EDTA, 1mM NaN₃, 0.2mM NADPH, 1 enzyme unit/ml glutathione reductase, 1mM GSH, 1.5mM cumene hydroperoxide (for total GSHpx) or 0.25mM H₂O₂ (for selenoenzyme) in a total volume of 2ml. All ingredients except the enzyme
source and the peroxide were combined at the beginning of the experiment. To 1.6 ml of the above mixture, 0.2ml enzyme source (tissue homogenate or hemolysate) was added and the mixture was allowed to incubate at room temperature for 5 min before the initiation of the reaction by the addition of 0.2ml of peroxide solution. The oxidation of NADPH was followed at room temperature using Spectronic 21 spectrophotometer for the next 5 min at 340 nm. Blank reactions with enzyme source replaced by distilled water were subtracted from each assay.

Enzyme activity was expressed as nmoles of NADPH oxidised/min/mg protein, by taking the molar absorption of NADPH as 6,220 (22). Se-independent GSHpx was obtained by subtracting Se-dependent enzyme activity from the total GSHpx.

11. Preparation of hemolysate for GSHpx and glutathione reductase:

To 0.2 ml of saline washed erythrocytes, 4.8ml of distilled water was added and mixed. The supernatant obtained after centrifugation was used immediately.

12. Preparation of tissue homogenates for GSHpx and glutathione reductase:

Tissues from the rats were quickly excised off and were perfused transcardially with normal saline. The samples were weighed while frozen and homogenised (10% W/V) in 0.1M potassium phosphate buffer, pH 7.4, in the cold. The tissue extracts were centrifuged at 700xg for 10 min in a refrigerated centrifuge and the supernatant was used for enzymatic assays for peroxidase and reductase.
13. **Assay of glutathione reductase activity:**

The activity of glutathione reductase was determined by the procedure of Horn and Burns as given by Bergmeyer (23). The cuvette contained 1.6ml of 0.067M potassium phosphate buffer, pH 6.6, 0.12ml of 0.06% NADPH, 0.12ml of 1.15% G-S-S-G, 0.1ml of the enzyme source and water in a final volume of 2ml. Mixed well and recorded the decrease in OD at 340 nm for 3-5 min in Spectronic 21 spectrophotometer. For each series of measurements, controls were done containing water instead of G-S-S-G. Under the conditions of the assay, NADPH dependent glutathione reductase activity was linear with time. The enzyme activity was expressed as nmoles of NADPH oxidised/min/mg protein.

14. **Estimation of reduced glutathione content:**

The GSH content of erythrocytes and tissue homogenates (10% W/V in 10mM potassium phosphate buffer, pH 7.4) was determined as described by the improved method of Beutler et al (24). To 0.2ml of the packed erythrocytes or tissue homogenates, was added 1.8ml of distilled water and 3ml of the precipitating solution (1.67 gm of glacial metaphosphoric acid, 0.2gm of EDTA and 3.0gm of NaCl per 100ml of distilled water). After mixing, the solution was allowed to stand for 5 min and filtered. Two ml of the filtrate was added to 8ml of phosphate solution (0.3M Na₂HPO₄ in distilled water) followed by 1ml of DTNB solution (0.04% 5, 5' dithiobis 2-nitrobenzoic acid). The OD was measured at 412nm. Blank was prepared by substituting the sample with water and following the entire procedure as for test. A
standard curve was calibrated with freshly prepared GSH solution from which the sample values were calculated and expressed as mgs of GSH/gm of tissue protein or as mgs of GSH/dl of erythrocytes.

15. Determination of ceruloplasmin content:

The method followed was essentially that of Henry et al. (25). To the blank, 1 ml of sodium azide reagent (0.1% in 0.1M acetate buffer, pH 6.0), 1 ml of acetate buffer (0.1M, pH 6.0) and 1 ml of PPD reagent (0.25% p-phenylene diamine in 0.1M acetate buffer) were added. To the test, 2 ml of the acetate buffer and 1 ml of PPD reagent were added. The tubes were incubated at 37°C for 5 min in the dark. Then 0.1 ml serum (or tissue homogenate, 10% W/V in 10mM potassium phosphate buffer, pH 7.4) was added to both test as well as blank and mixed. The absorbances of the test against blank at 530 nm were read 10 min and again 40 min after the addition of the sample. The formula used for the calculation of the results is given below. Mgs of ceruloplasmin/dl serum

\[ \text{Mgs of ceruloplasmin/dl serum} = A_{40 \text{ min}} - A_{10 \text{ min}} \times 1000 \times 0.06. \]

In the case of brain and liver homogenates, the values were expressed as mgs of ceruloplasmin/gm protein.

16. Ascorbate autoxidation studies in the presence of tissue homogenates or plasma:

The rate of ascorbate autoxidation in the presence of plasma or tissue homogenates was studied by including these substances equivalent to
140 μg protein, in the 10ml incubation mixture containing 1.137 μ moles of ascorbate. Other details of the experiment are given in the beginning of this section.

Statistical Analysis:

All the data are expressed as Mean ± S.D. In the animal experiments, the significance of difference was assessed by the Student's 't' test as per the "standard connotation".


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