Appendices

APPENDIX I

ASSAY OF SUPEROXIDE DISMUTASE

(Kakkar *et al.*, 1984)

The assay of SOD is based on the inhibition of formation of NADH-phenazine methosulphate – nitroblue tetrazolium formazon. The reaction was initiated by the addition of glacial acetic acid. The colour formed at the end of the reaction was extracted into butanol layer and measured at 560nm.

REAGENTS

1. Sodium pyrophosphate buffer – 0.025M, pH 8.3
2. Phenazine methosulphate – 16μM
3. Nitroblue tetrazolium – 300μM
4. NADH – 780μM
5. Glacial acetic acid
6. n-butanol
7. Chloroform
8. Ethanol

PROCEDURE

The assay mixture contained 240μl of sodium pyrophosphate buffer, 200μl of phenazine methosulphate, 60μl of nitroblue tetrazolium and appropriately diluted homogenate in a total volume of 600μl. The reaction was started by the addition of 40μl of NADH.

After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 200μl of glacial acetic acid. The reaction mixture was stirred

vigorously and shaken with 8µl of n-butanol. The mixture was allowed to stand for 10 minutes and then centrifuged at 2000 rpm for 5 minutes. The intensity of chromogen in butanol layer was measured at 560nm against butanol as blank and the system devoid of enzyme served as control.

One unit of enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute under the assay conditions and was expressed as specific activity.

APPENDIX II

ASSAY OF CATALASE ACTIVITY

(Luck, 1974)

The UV light absorption of hydrogen peroxide can be easily measured between 230 and 250 nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity can be arrived at from this decrease.

REAGENTS

1. Phosphate buffer : 0.067M (pH 7.0)
2. Hydrogen peroxide : 30% in phosphate buffer

PROCEDURE

The homogenate of *Drosophila* was employed for the assay. Read against a control cuvette but containing the homogenate as in experimental cuvette but containing H_2O_2 free phosphate buffer.

H_2O_2-phosphate buffer (987µl) was pipetted into the experimental cuvette. To this, 10µl of homogenate was mixed with a glass / plastic rod flattened at one end. The time ΔT required for a decrease in absorbance by 0.05 was noted. This value was used for calculation. If 't' was more than 60 seconds, then repeated the measurements with a more concentrated solution of the sample.

CALCULATION

Calculated the concentration of H_2O_2 using the extinction coefficient 0.036 per ml.

APPENDIX III

ASSAY OF PEROXIDASE ACTIVITY

(Reddy *et al.*, 1995)

In the presence of the hydrogen donor pyrogallol, peroxidase converts H_2O_2 to water and oxygen. The oxidation of pyrogallol to a coloured product called purpurogalli can be followed colorimetrically.

Pyrogallol + H_2O_2 ⟶ Oxidised pyrogallol + H_2O_2 + O_2

REAGENTS

1. Pyrogallol : 0.05M in 0.1M phosphate buffer (pH 6.5)
2. 1% H_2O_2

PROCEDURE

The homogenate of the *Drosophila* was used as the source of enzyme. 750µl of 0.05M pyrogallol solution and 10µl homogenate was pipetted out in an eppendorf tube. The spectrophotometer was adjusted to read zero at 430nm. 125µl of 1% H_2O_2 was added in the test cuvette. The change in absorbance was recorded every 30 seconds upto 3 minutes.

CALCULATION

Change in absorbance / minute at 430nm = X

Volume of homogenate taken for the assay = 10µl

Change in absorbance for 10µl = X

Change in absorbance for 300µl homogenate = $\frac{X}{10} \times 300 = Y$

300 µl homogenate is obtained from 30mg of flies

Peroxidase activity in 30mg of homogenate = Y

APPENDIX IV

ESTIMATION OF ASCORBIC ACID

(Roe and Keuther, 1943)

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbic acid then reacts with 2,4-dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution, whose absorbance can be measured spectrophotometrically at 540nm.

REAGENTS

1. Trichloroacetic acid (25% and 4%)
2. Sulphuric acid (9N)
3. 2,4-dinitrophenylhydrazine reagent (2% in 9N sulphuric acid)
4. Thiourea solution (10%)
5. Sulphuric acid (85%)
6. Standard ascorbate solution: 10mg ascorbate in 100ml of 4% TCA.

PROCEDURE

50µl and 80µl aliquots were taken for the assay. The assay volumes were made up to 400µl with 4% TCA. 40 to 200µl of the working standard solution containing 4-20µg of ascorbate respectively were pipetted into clean dry test tubes. The volumes were made up to 400µl with 4% TCA.

100µl of DNPH reagent, followed by two drops of 10% thiourea solution was set as a blank. The contents of the tubes were mixed thoroughly and incubated at 37°C for 3 hours. After incubation, the orange red osazone

crystals formed were dissolved by the addition of 500µl of 85% H_2SO_4, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. After incubation for 30 minutes at room temperature, the absorbance was read at 540 nm. The content of ascorbic acid in *Drosophila* homogenate was calculated using standard values.

APPENDIX V

ESTIMATION OF TOCOPHEROL

(Varley *et al.*, 1981)

Tocopherol can be estimated using Emmerie-Engel reaction, which is based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2,2'-dipyridyl. Tocopherol and carotene are first extracted with xylene and read at 460nm to measure carotenes. A correction is made for these after adding ferric chloride and read at 520nm.

REAGENTS

1. Absolute alcohol
2. Xylene
3. 2,2'-dipyridyl (1.2g in 1 litre of n-propanol)
4. Ferric chloride (1.2g in one litre of ethanol stored in brown bottle)
5. Standard solution of D,L-α tocopherol, 10mg/L in absolute alcohol. (91mg of α-tocopherol is equivalent to 100mg of tocopherol acetate).

PROCEDURE

100µl of the homogenate of *Drosophila* was taken in 3 stoppered centrifuge tubes (test, standard and blank). 300µl of ethanol was added to the test and blank. 300µl of water was added to the standard. Then 300µl of xylene was added to all tubes, stoppered, mixed well and then centrifuged. 200µl of xylene layer was transferred into another stoppered tube, taking care not to include any protein or ethanol.

The extinction of test and standard against the blank was read at 460nm. 66µl of ferric chloride solution was added, mixed well and after exactly 15 minutes read the test and standard against the blank at 520nm. The amount of vitamin E can be calculated using the formula

$$\text{Amount of tocopherol in } \mu l = \frac{A_{520} - A_{450}}{A_{520}[\text{standard}]}$$

APPENDIX VI

ESTIMATION OF REDUCED GLUTATHIONE

(Moron *et al.*, 1979)

Reduced glutathione (GSH) is measured by its reaction with DTNB (5, 5'-dithionitrobenzoic acid), Ellman's reaction, to give a compound that absorbs at 412nm.

REAGENTS

1. 5% and 25% TCA
2. Sodium Phosphate buffer (0.2 M) pH 8.0
3. DTNB solution – 0.6 mM in 0.2M phosphate buffer
4. Standard glutathione – Dissolved 1.0mg of reduced glutathione in 100ml of 5% TCA

PROCEDURE

The volume of the aliquot was made upto 250µl with 0.2M sodium phosphate buffer (pH 8.0). 500µl of freshly prepared DTNB solution was added to the above solution and the intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 minutes.

A standard curve of GSH was prepared using concentration ranging from 2 to 10 moles of GSH in 5% TCA.