V. MATERIALS AND METHODS
1. **Chemicals** - The following chemicals were obtained commercially and used without further purification: 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxy-phenyl-ethylamine (DOPAMINE), threamine, nor-leucamine, B-phenylethylamine, tyramine, seramine, cadaverine, putrescine, 3-methoxy-4-hydroxy-phenyl-ethylamine, valamine, histamine, tryptamine, leucamine, tyrosine and phenylalanine were purchased from Calbiochem, U.S.A. Catalase was obtained from Sigma Chemicals Co., U.S.A. Bovine serum albumin was purchased from Mann Research Laboratories, U.S.A., in crystalline form. Rest of the chemicals used were of A.R. grade.

2. **Plant Materials** - The plants were either grown in the garden of the department or in the wooden pots.

3. **Enzyme Preparations** -

(i) **Crude Homogenate (10 %, w/v)** - The plant material washed free from soil was accurately weighed and transferred to a chilled Potter-Elvehjem all glass homogenizer containing 0.02%
sodium phosphate buffer, pH 7.0. The homogenization was carried out for 15 minutes. The homogenate was squeezed through two layers of muslin cloth and centrifuged at 1,200 x g for 15 minutes to remove cell debris. The supernatant thus obtained was used as a source of crude enzyme.

(ii) Acetone Powder Extracts (10%, w/v) - Acetone dried powders were prepared by placing the cut plant in a chilled Waring blender, covering with 5 volumes of cold acetone (-10 °C) and blending vigorously for one minute. The resulting slurry was quickly filtered with suction through the Buchner funnel, the residue spread out on filter paper, dried at room temperature and sifted through wire mesh. The acetone dried powder thus obtained was stored in a desiccator at 4 °C.

Acetone powder of whole plant (10%, w/v) was suspended in 0.02 M sodium phosphate buffer, pH 7.0. The suspension was mechanically stirred for half an hour at 5 °C and then squeezed through two layers of muslin cloth. The extract obtained was centrifuged at 10,000 x g for 15 minutes to remove floating particles. The suspension was used as a source of enzyme.
4. Manometric Studies - Manometric experiments were carried out at 30°C in a conventional Warburg apparatus using single or double arm flasks shaken at 105 strokes per minute (89).

For oxygen uptake studies, the center well contained 0.2 ml of 20% KOH and a small roll of filter paper. For following carbon dioxide evolution and concurrent oxygen utilization, KOH was omitted from the center well and the indirect method of Warburg was used. In all the cases, unless indicated otherwise, air was used as gas phase. The rate of oxidation as well as carbon dioxide evolution has been calculated from the initial rate. One enzyme unit is defined as the amount of enzyme which is responsible for the consumption of one microliter of oxygen per hour. The specific activity has been expressed in terms of enzyme units per mg protein.

5. Estimation of Ammonia - This was estimated by the modified aeration method of Van Slyke and Cullen. In the original procedure the aerated ammonia is absorbed in standard acid and the acid back titrated with standard alkali. In the modification
described here, the ammonia is absorbed in boric acid solution and titrated directly with standard acid (90).

2 ml of incubation mixture was placed in one of the two large test tubes used in the aeration train. The test tube was connected for aeration with a second tube containing 25 ml of 2% boric acid containing bromocresol green indicator. The stopper of the tube containing the incubation mixture was removed and 5 ml of saturated potassium carbonate solution added. The stopper was placed tightly and the air current flown. The incoming air was washed by preliminary passage through a similar aeration train test tube containing 10% sulfuric acid to remove any ammonia present. After one hour of aeration, the tube containing boric acid solution was removed, the inlet tube rinsed and the contents were titrated with 0.01 N sulfuric acid until blue colour, if any, is replaced by original yellow green colour as determined by matching against a control (25 ml portion of boric acid indicator diluted with water to approximately the final volume of the titrated sample).

Boric acid solution containing bromocresol green indicator was prepared by dissolving 20 gm of boric acid in 50C
of hot water. 2 ml of 0.1% bromocresol green in alcohol was
added to the cooled boric acid solution and diluted to one
liter.

6. **Estimation of Catalase Activity** - Catalase activity was
estimated by potassium permanganate titration method (92).

At a temperature of 20°, 15 ml of 0.01 M hydrogen peroxide
in 0.01 M sodium phosphate buffer, pH 7.0, was placed in an
Erlenmeyer flask. For zero time reading, 2.0 ml of this solution
was withdrawn and added to 5 ml of 2% sulfuric acid for titration
with 0.01 N potassium permanganate solution. 0.1 ml of 1:1000
diluted catalase was pipetted into the peroxide solution which
was being swirled rapidly. Samples of 2 ml each were rapidly
withdrawn at 10, 20, 30, 40, 50 and 60 seconds and blown out
into separate flasks containing 5 ml of 2% sulfuric acid. From
the equation:

\[ K = \frac{1}{t} \log_{10} \frac{a}{(a-x)} \]

where \( a \) is the **initial** hydrogen peroxide concentration and
Fig. 2. Monomolecular velocity constants Vs time plot for calculation of Kat.f. value of catalase. See text for details.
(a - x) is the concentration at time t, k values for various time intervals were calculated and K value at zero time obtained by extrapolation (Fig. 2). Using this value for K, the Katalasefähigkeit (Kat. f.)

\[ \text{Kat. f.} = \frac{K}{\text{g. enzyme}} \]

was found to be 2,30,700.

7. **Estimation of Hydrogen Peroxide** - Hydrogen peroxide formation was tested by method of Tabor (91). The decrease in oxygen uptake in presence of initially added catalase was used as a measure of hydrogen peroxide formation.

8. **Estimation of Quinones** - The method employed was essentially the same as that of Dawson and Nelson (93). It is based on the principle that iodine set free from potassium iodide by o-benzoquinone formed during the reaction is proportional to
the amount of quinone and could be titrated with standard sodium thiosulfate solution.

To the 2 ml of incubation mixture was added, 0.5 ml of 10% sulfuric acid and 1 ml of 10% potassium iodide. The reaction mixture was allowed to stand in dark for 15 minutes. Four drops of starch were added and the blue colour was titrated against standard 0.0085 N sodium thiosulfate solution. 1 ml of 0.0085 N sodium thiosulfate is equivalent to 4.25 umoles of quinone formed during the reaction.

9. Estimation of Protein - Protein was estimated by Folin and Ciocalteu method as described by Lowry et al. (94).

To 1 ml sample containing suitable quantity of protein, 5.0 ml of alkaline copper reagent (1 ml of 0.5% copper sulfate in 1% potassium tartrate solution added to 50 ml of 2% sodium carbonate in 0.1 N NaOH solution) was added and incubated at room temperature for 15 minutes. Folin reagent (1 N) was added, mixed instantly and colour density measured after 30
Fig. 3. Standard curve for protein estimation. See text for details.
minutes in a Klett-Summerson colorimeter using red filter against the reagent blank. Crystalline bovine serum albumin solution was used as standard (Fig. 3). Under these conditions one Klett unit was found to be equal to 0.805 µg of standard protein.

10. Preparation of Carboxymethyl Cellulose Column - CM-cellulose was suspended in distilled water and packed uniformly in a column (1.2 x 15 cm) without air bubbles getting trapped. 200 ml of 1 N HCl was passed through the column and excess of acid washed with glass distilled water till the washings were free of acid. 200 ml of 1 N NaOH is then passed through the column. After removing excess of alkali with distilled water, the column was finally washed and equilibrated with 0.01 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.0.

11. Preparation of Diethylaminoethyl Cellulose Column - DEAE-cellulose was suspended in 1 N NaOH taking care to avoid air
bubbles. The pale yellow supernatant was poured off and resin washed with distilled water. The resin was packed in a column (1 x 15 cm) without air bubbles getting trapped. The column was washed with large volumes of glass distilled water till the washings were free of alkali. The chloride form of the resin was obtained by passing 200 ml of 1 M HCl through the column and washing off the excess of acid with glass distilled water. The column was then equilibrated with 0.01 M sodium phosphate buffer, pH 7.0.