CHAPTER II

MATERIALS AND METHODS

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Chemicals :

Ethyleneglycol-bis-(β -aminoethyl ether) -N,N,N',N'-tetraacetic acid (EGTA), glucose-6-phosphate dehydrogenase, phenazine methosulfate (PMS), reactive blue-sepharose Cl-6B, DEAE-sephadex (A 25-120), bovine brain calmodulin, trifluoperazine (TFP), calmidazolium (R_{24571}), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7), diltiazem, flunarizine, 5-hydroxytryptamine (5-HT), cycloheximide (CH), chloramphenicol, actinomycin D, 1,3-diaminopropane, putrescine, cadaverine, spermidine, spermine, agmatine, creatine, creatinine, guanidino butyric acid, guanidino acetic acid were obtained from Sigma Chem. Co., USA.

Verapamil was from Rallies India Ltd., Bombay. $LaCl_3$ was from BDH Laboratory. Other chemicals used were obtained from the local market. All chemicals were of analytical grade.

Plant material and light treatment :

Pea (Pisum sativum L.) seeds were purchased from local market, soaked in water for 8 h and then grown in moist vermiculite at 24°C in dark. The seedlings were exposed to white (W), red (R) and far-red (FR) lights as specified in the results section. All manipulations in "dark" were performed under green safe light obtained by wrapping ten green cellophane sheets to a 40W fluorescent tube. R light was obtained by filtering the light from four 160 W fluorescent lamps through two blue cellophane sheets snadwiched between red acrylic sheets (Acros,India). FR light was obtained by filtering the light from four 160W

tungsten lamps through two blue, one red and one yellow cellophane sheet sandwiched between transparent acrylic sheets. A 1 cm layer of water in a transparent tray was kept between the light source and filter to avoid heating. White light was obtained from four 40 W fluorescent tubes. Fluorescent tubes, lamps and tungsten lamps were obtained from Phillips India Limited. Fluence rates of R (λmax $\sim$ 660 nm), FR (λ max $>$ 700 nm), white light and green safe lights were 50,40,250 and 1 $\mu W/cm^2$.

Preparation of homogenate :

At specific periods, after light treatment, the terminal buds from the seedlings were excised and a homogenate was prepared by grinding 0.5 g fresh weight of tissue with 5 ml of 0.1 M Tris-HCl buffer (pH 7.0) in a chilled mortar and pestle at 4°C. Crude homogenate was used for enzyme assays.

Enzyme assays :

Glutamine Synthetase (GS) :

GS was routinely assayed as transferase activity according to the method of Trush (1964). In some experiments GS was also assayed as synthetase according to the method of Elliott (1953).

The assay system for transferase consisted of acetate buffer, pH 5.5, 100 μmol; L-glutamine, 10 μmol; hydroxylamine-HCl, 20 μmol; $MnCl_2$, 10 μmol; Na_2AsO_4, 10 μmol; ATP, 2.5 μmol and 0.5 ml of enzyme extract in a total volume of 5 ml. The assay system for synthetase consisted of Tris-HCl buffer, pH 8.0; 200 μmol; Na-glutamate, 1.25 mmol;

hydroxylamine-HCl, 300 μmol; $MgSO_4$, 250 μmol; ATP, 30 μmol and 0.5 ml enzyme extract in a total volume of 5 ml.

After incubation at 37°C for 1 h the reaction was terminated by the addition of 1ml of $FeCl_3$ reagent (A mixture of equal volume of 10% $FeCl_3$ in 0.2N HCl, 24% TCA and 6N HCl). In control tubes glutamine/ glutamate was added after termination of the reaction. The γ-glutamyl hydroxamate formed was measured at 540 nm.

NAD kinase :

NAD kinase activity was assayed according to the method of Anderson and Cormier (1978). The assay system consisted of Tris-HCl buffer (pH 8.0), 100 μmol; NAD, 8 μmol; ATP, 12 μmol; $CaCl_2$, 4 μmol; $MgCl_2$, 40 μmol and 0.5 ml enzyme extract in a total volume of 4 ml. After incubation for 1 h at 37°C, the reaction was terminated by keeping the tubes in boiling water bath for 3 min. In control tubes, NAD was added after the termination of the reaction. Precipitated proteins were removed by centrifugation.

NADP formed was estimated in the supernatant according to the method of Muto and Miyachi (1977) using glucose-6-phosphate dehydrogenase. The assay system for glucose-6-phosphate dehydrogenase contained Tris-HCl buffer (pH 7.5), 100 μmol; 2,6-dichlorophenol indophenol, 0.1 μmol; phenazine methosulfate, 65 nmol, glucose-6-phosphate, 2 μmol, 0.1 unit of glucose-6-phosphate dehydrogenase and an aliquot of supernatant containing NADP in a total volume of 3 ml. The reaction was started by the addition of enzyme and the rate of decrease in absorbance at 660 nm was recorded. The NADP content was calculated using calibration

curve prepared with authentic NADP sample.

The assay conditions used were found to give optimum activity. A "Katal" unit of enzyme is defined as the amount of enzyme required to produce 1 mol of product per sec.

All the data reported in the thesis are a mean of 3 independent sets of experiments.

Preparation of crude calmodulin :

Crude calmodulin from terminal buds treated with different lights was prepared by homogenizing 0.5 g of tissue in 10 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EGTA. The homogenate was heated at 90°C in a water bath for 4 min. The tubes were then rapidly cooled in ice and centrifuged at 12,000 g for 15 min. The supernatant thus obtained was used as crude calmodulin preparation.

Determination of calmodulin content :

Calmodulin content of the tissue was estimated by its ability to activate the calmodulin - dependent calmodulin - deficient NAD kinase using pure bovine brain calmodulin as standard.

Preparation of calmodulin - deficient NAD kinase :

Pea terminal buds from 8-day old light grown seedlings were harvested and a 30% homogenate was prepared in 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM EGTA. The homogenate was filtered through two layers of cheese cloth and then centrifuged at 12,000 g for 30 min. 12 ml of supernatant was added to 7 g of reactive blue-sepharose

CL-6B previously equilibrated with the homogenizing medium and was stirred for 1 h at 4°C. It was then packed in a column and the column was washed with 600 ml of homogenizing medium. The enzyme adsorbed on blue-sepharose was eluted by the homogenizing medium containing 0.4 M KCl and the enzyme activity was assayed both with or without addition of crude calmodulin. The enzyme was then dialyzed for 15 h against 25 mM Tris-HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM EGTA. The dialysed enzyme was passed through DEAE-sephadex column (bed volume 10 ml) which was preequilibrated with the homogenizing medium. The NAD kinase did not bind to DEAE-sephadex. The unadsorbed fractions containing enzyme were pooled together. The pooled enzyme fraction was completely free from calmodulin.