III methods
III. METHODS.

III. 1 Sorghum seedlings were raised in the dark

Certified seed of Sorghum (Sorghum bicolor L.) var CSH-1 was purchased from Karnataka Seeds Corporation, Dharwad, India. The seeds were surface sterilized with chlorine water and germinated on moist vermiculite in the dark at 25°C for 72 h.

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III. 2. (Ca -Mg )-ATPase was purified from sorghum roots.

The roots were harvested in batches of 50 g on ice from the 72 h-old dark-grown seedlings and homogenized in 25 mM Tris-Mes buffer (pH 7.2) containing 3 mM EDTA and 25 mM β-ME. The homogenate was centrifuged at 3000 g for 15 min in cold and the supernatant was subjected to ammonium sulfate fractionation. The 60-80% ammonium sulfate precipitate was collected by centrifugation and dissolved in 10 ml extraction buffer and purified batch-wise on DEAE cellulose. The DEAE cellulose was equilibrated with 25 mM glycine-NaOH buffer (pH 10.0), to this the 60-80% ammonium sulfate precipitate was added and mixed. The slurry was stirred for 30 min in cold and centrifuged at 1000g for 10 min. The supernatant was collected and the DEAE cellulose pellet containing other proteins was transferred to the next pH. Similarly, the DEAE cellulose pellet was passed through the whole pH range (pH 10.0 to 4.0). The supernatant of these were collected and assayed for ATPase activity. It was observed that the ATPase activity was exhibited at pH 10.0.
where as the acid phosphatase activity was observed at pH 5.0 and 5.6. The ATPase activity eluted from the batch was concentrated by dialysing against glycerol and applied on to Sephadex G-200 column. Elution of the enzyme from Sephadex G-200 column was carried out with 25 mM Tris-Mes buffer (pH 7.2). The fractions containing ATPase activity were pooled and used for kinetic studies.

III 3 (Na–K+)–ATPase was purified from sorghum roots

Roots of sorghum were harvested from the 72 h old dark grown seedlings in batches of 100 g and collected on ice. The roots were homogenized in 25 mM Tris-Mes buffer (pH 7.2) containing 3 mM EDTA and 2.5 mM 2-mercaptoethanol. The homogenate was centrifuged at 3000 g for 15 min. The supernatant was discarded and the pellet containing the debris treated with 200 ml Tris-Mes buffer (pH 7.2) containing 0.1 % Triton x-100 and stirred in cold for 1 h and centrifuged at 3000 g for 15 min. The supernatant was subjected to 30 % ammonium sulfate precipitation. At this ammonium sulfate concentration, Triton X-100 was precipitated which carried the enzyme along with it. The detergent precipitate carrying the enzyme formed a scum after centrifugation on top of the tubes. This scum was collected and dissolved in 50 ml extraction buffer further. The purification on DEAE cellulose was carried out as described earlier. The elute of DEAE cellulose of glycine-NaOH buffer (pH 10.0) exhibited ATPase activity. This fraction was
dialyzed against glycerol (100%) and concentrated. The concentrate was applied on to a Sephadex G-200 column. (1 cm diameter x 25 cm long bed volume) and eluted with 25 mM Tris-Mes buffer (pH 7.2) The fractions showing ATPase activity were pooled and passed through Bio-bead SM-2 column (1 cm diameter x 20 cm bed volume). The elute from Bio-bead SM2 showing ATPase activity was then made 30 % with respect to ammonium sulfate and applied on to a Sepharose 4B column preequilibrated in 30 % ammonium sulfate saturation in 25 mM Tris-Mes buffer at pH 7.2. Elution of this column was made with a reverse gradient of ammonium sulfate (in Tris-Mes buffer, pH 7.2) of 30 % - 0 %. The fractions containing enzyme activity were pooled.

III 4 Lipids were extracted and purified from chicken eggs

Egg yolks were collected from 6 eggs and mixed well. To this, 150 ml of ice-cold acetone was added and allowed to stand for 15 min. The precipitate was collected by centrifugation and washed 4-5 times with ice-cold acetone until the supernatant was clear and colorless. This precipitate was then extracted with 500 ml of chloroform-methanol mixture (2:1 v/v) for 3-4 hours at room temperature. After centrifugation at 3000 g for 10 min the supernatant was collected and given a Folch wash according to Folch et al (1957). The supernatant was then transferred to a separating funnel, to this 100 ml of 88% KCl was added and shaken well before allowing it to settle. From the resultant biphasic
mixture the lower layer containing lipids was collected and evaporated to dryness under vacuum at 35-40 °C. This fraction was further purified on a sephadex G-25 column. The separation on Sephadex G-25 column was conducted according to Christie (1982). Chloroform, methanol and water in the ratio 8:4:3 were mixed and partitioned. The upper phase and lower phase were separated. Sephadex G-25 was soaked overnight in the upper phase. Column (1 cm diameter by 10 cm height) was packed with the slurry and equilibrated with lower phase. Lipid extract (200 mg) was taken in 5 ml of lower phase and applied onto the column and eluted with 25-30 ml of lower phase. This purified lipid was used in the preparation of liposomes.

III.5: Lipids were extracted and purified from sorghum seedlings

The sorghum seeds were surface sterilized with chlorine water and subsequently allowed to imbibe for 1 h either in distilled water or in solution containing methylparathion (200 ppm) or fenvalerate (10 M). The seeds were then surface washed, sown on moist filter papers in Petri dishes and allowed to germinate in the dark for 72 h at 25±3 °C. Lipids were extracted according to Bligh and Dyer (1971) in a blender with a solvent mixture consisting of chloroform and methanol (1:2 v/v). The mixture was filtered through Whatman No 1 filter paper, given Folch was further purified on Sephadex G-25 column as described earlier.

Untreated seedlings of the same age (72 h) were also
subjected to a similar extraction procedure. These lipids served as control.

Lipids were estimated according to Pande et al. (1963) using cholesterol as standard.

III. 6 Calmodulin was purified from human erythrocytes

The human erythrocyte calmodulin was prepared according to the method of Jarret and Penniston (1978) with minor modifications. The blood (500 ml) obtained from a local blood bank was washed 4 times by repeated centrifugation (3000 g) in citrate dextrose solution (2.05 % dextrose, 0.89 % sodium citrate, 0.42 % citric acid) at 4°C instead of Tris-HCl buffer as described in the original procedure. The erythrocytes were then lysed in 14 volumes of ice-cold distilled water and centrifuged at 23,000 g for 10 min. The supernatant (hemolysate) was carefully removed and mixed with 5 g of DEAE-cellulose (DE-52, whatman) stirred for 1 h and filtered. The cake obtained was packed in a column and the column was eluted step-wise with increasing concentration of NaCl (0.15, 0.3 and 0.6 M) in 10 mM citrate buffer at pH 6.5 instead of eluting with imidazole buffer according to the original procedure. The protein-containing fractions of 0.6 M NaCl elute were collected and pooled. According to the original procedure, the purification at this stage was 1,750 fold. The protein from the pooled fractions was precipitated at 50% ammonium sulfate saturation and the
precipitate was dissolved in 25mM Tris-HCl buffer (pH 7.2). This preparation was used as the erythrocyte calmodulin.

III. 7: Liposomes were prepared from purified plant and egg lipids

Liposomes were prepared according to Sila et al (1986) with minor modifications. The purified lipids were dissolved in chloroform so as to give 8 µg lipids in 100 µl of solvent. The lipids were taken in test tubes and evaporated under vacuum to dryness at 35-40°C in a water bath. A thin film of lipids was obtained at the bottom of the tube, to which the assay buffer was added, homogenized with a Dounce-type homogenizer and vortexed. The resultant turbid solution containing the multilamellar liposomes were subsequently used in enzyme assay.

III 8. Pesticide residues and degradation products were extracted from pesticide-treated seeds

III 8 a) From methylparathion-treated seeds

Methylparathion residue and degradation products were extracted and purified from sorghum seedlings according to Ambrus et al (1981) with minor modifications. Instead of diatomaceous earth, Chromosorb W (Chromatographic specialities) was used as one of the components of the column. Methylparathion-treated (200 ppm) seeds were germinated as described earlier. The residue and degradation products were extracted from 72 h.
old seedlings of sorghum. The seedlings were homogenized with acetone and filtered. The filtrate was saturated with water containing 4% of Na$_2$SO$_4$. The extract was then partitioned with 200 ml methylene chloride and filtered through anhydrous Na$_2$SO$_4$. Subsequently dried in vacuum. Final volume of the extract was made up to 2 ml in acetone and stored in dark at -20 °C. It was further purified on mixed adsorbents as per Ambrus et al. (1981).

III. 8 b) From Fenvalerate-treated seeds.

The fenvalerate residue and degradation products were extracted and purified from sorghum seedlings according to Lord et al. (1982). Fenvalerate-treated (10 μM) seeds were germinated as described earlier. The residue and degradation products were extracted from 72 h-old seedlings in a mixture of acetone and hexane (1:1 v/v) in a blender. The homogenate was shaken on a mechanical shaker for 2 h. The clear supernatant solvent layer was decanted into a separating funnel, shaken with 200 ml water. The lower phase was filtered through anhydrous Na$_2$SO$_4$, dried under vacuum and stored in 20 ml acetone at -20 °C. Residue concentration was calculated as per the original concentration of the pesticide used for the treatment.

III. 9 Pesticide residues and degradation products were extracted from pesticide-treated soil.

III. 9 a) From methylparathion-treated soil.

Methylparathion residue and degradation products were
purified from the treated soil as described by Ambrus et al (1981), with minor modifications as described earlier. The soil was treated with 20 \( \text{ug pesticide/gram of soil} \) according to Reddy & Sethunatahan (1983). The treated soil was kept moist in plastic pots for one month. After one month, soil was shaken with acetone containing 2M ammonium acetate until the soil disintegrated into small particles. The acetone layer was filtered through Whatman No. 1 filter paper in a Buchner funnel. The filtrate was saturated with water containing 4% Na\( _2\)SO\( _4 \). The extract was then partitioned with methylene chloride and filtered through anhydrous Na\( _2\)SO\( _4 \) and subsequently dried in vacuum. Before use, it was further purified on mixed adsorbents according to Ambrus et al. (1981).

As per the original pesticide concentration 0.1, 1.0 and 10.0 \( \text{ug of residue} \) was taken in test-tube, vacuum dried and used for enzyme assays.

III 9 b) From fenvalerate-treated soil:

Fenvalerate residue and degradation products were extracted and purified from soil. Soil was treated with 20 \( \text{ug fenvalerate/gram soil} \) as described earlier and the residues were extracted as described for the extraction from the treated seeds.
III 10. ATPase activity was determined by estimating the Pi released from ATP

\[ \text{ATP}_{2+} \rightarrow \text{ADP}_{2+} + \text{Pi} \]

III. 10 a) (Ca –Mg )-ATPase

ATPase activity was measured by the release of inorganic phosphate from ATP, using the method of Peterson (1978). The standard assay mixture consisted of 25 mM Tris-Mes buffer (pH 7.2), 5.0 mM ATP (sodium salt neutralized with Tris base), 0.25 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\) and was carried out with 10 \(\mu\)g of enzyme protein at 30°C. The enzyme activity was linear with time from 2 to 15 min and the initial velocity was determined from the slope of this range.

III 10 b) (Na –K )-ATPase

ATPase activity was measured by the release of inorganic phosphate from ATP, after reconstituting the enzyme according to Cocucci and Marre (1984) in liposomes prepared with 8.0 \(\mu\)g of lipids/ml of the reaction mixture. The standard assay mixture contained 25 mM Tris-Mes buffer (pH 7.2), 5.0 mM ATP (sodium salt neutralized with Tris base), 0.6 mM NaCl and 1.0 mM KCl. Optimal level of divalent cations was maintained. Ten \(\mu\)g of enzyme protein was used for enzyme assay. As described earlier, the initial velocity was determined from the slopes obtained from the time course reaction of 2 to 15 minutes.
III 11 Protein concentrations were determined by a modified Lowry's procedure

Protein concentration was determined by a modified Lowry's procedure as described by Markwell et al (1978) using bovine serum albumin as standard.

III 12 Liposome swelling was studied under isosmotic condition

Multilamellar liposomes were prepared according to Sila et al (1986), as described earlier. The osmotic swelling of liposomes was studied in a Hitachi recording spectrophotometer (model 150-20). Liposome sample prepared in 0.5 M urea containing 450 μg of lipids in 100 μl sample was added simultaneously to 3.0 ml isosmotic solution both in reference cuvette and sample holding cuvette. To start with, the absorbance levels were equalized at 450 nm and the change in absorbance was recorded for 5 minutes using time-mode scale.

The GA containing liposomes were prepared according to Pauls et al (1982). Similarly, liposomes were prepared with 1 mol% of ABA and liposome swelling was recorded.

III.13 Auxin receptor protein was identified in the (Ca$^{2+}$ -Mg$^{2+}$)-ATPase preparation by Western blot analysis.

The (Ca$^{2+}$ -Mg$^{2+}$)-ATPase purified (Section III 2) was subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli (1970) and Western blotting was carried out. The details are given in the legend to Plate 1.