MATERIALS
AND
METHODS
A. MATERIALS

1. Biological Materials

The sensitive plant *Mimosa pudica* Linn, *Desmodium gyrans* DC. also known as telegraphic plant and insensitive plant *Mimosa rubricaulis* Lamk. were collected from the experimental field of Bose Institute. Goat brain was purchased from the local slaughter house and collected into ice immediately after decapitation. Rabbit of body weight 1-1.5 Kg was obtained from local supplier.

2. Chemicals and Radiochemicals

All ribonucleotides and deoxyribonucleotides, iodoacetamide, p-hydroxymercuribenzoate, oligomycin, DCCD, reduced glutathione, NAD\(^+\), colcemid, coomassie brilliant blue R250 were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. L(+) cysteine was purchased from E. Merck, Germany. Colchicine was the product of Aldrich Chemical Co. Inc., U.S.A.

DEAE-sephadex A25 and Biogel P20 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE 52), DEAE-cellulose impregnated papers (DE 81) and glass fiber filters (GF/C) were purchased from Whatman Ltd., England.
Acrylamide, N, N'-methylene-bis-acrylamide and N, N, N', N'-tetramethylethlenediamine were supplied by Eastman Kodak, Rochester, N.Y., U.S.A. All other chemicals were of analytical grade obtained from BDH, Glaxo Laboratories, India.

$^{32}$P-orthophosphoric acid was obtained from the Isotope Division, Bhaba Atomic Research Centre, Bombay, India. Both tritiated colchicine (ring C, methoxy-$^3$H) and tritiated colcemid (ring C, methoxy-$^3$H) were products of New England Nuclear Corporation, U.S.A.

3. Fluor Substances

2, 5-Diphenyl oxazole (POPOP) and 1, 4-bis (2, 4-methyl-5-phenyl oxazolyl) benzene (POPOP) were obtained from Bhaba Atomic Research Centre, Bombay, India.

B. METHODS

I. Description of Response Recorder

Response Recorder was invented by Sir J.C. Bose (1928) to record the movements in plants under stimulation. The tip of the responding plant organ is attached by means of a fine thread to one end of the delicately balanced lever (a) (Fig. 1), which is pivoted on jewel bearings. The other end of the lever carries a small weight (b) by which the string is kept taut. The movement can be magnified according to requirement by using a
Fig. 1 Response Recorder.Inset: representation of functioning of lever.
single or double lever arrangement. From the middle of
the lever and at right angles to it, there extends a
fine glass tube with a curved end (c) serving as the
pen. The tip of this pen just touches a smoked glass
plate (d) on which the record is made. This glass plate
is moved at a uniform rate regulated by a clockwork in
a direction perpendicular to the tip of the recording
pen. The pen (c) is not allowed to remain in continuous
contact with the smoked glass plate as in that case the
pressure would be enough to modify or arrest the move-
ment. This difficulty has been removed by making an
interrupted instead of a permanent contact, thus giving
rise to a series of dots in the record instead of a
continuous line.

In the resulting dotted curve produced, the
horizontal distance between two consecutive dots
represent the time interval between successive plate
oscillations, and the vertical component represents the
magnitude of the magnified response.

With the oscillating plate, the time interval
between successive dots can be varied between 1-20 sec
and even longer, and is suitable for recording the res-
ponses of very sluggish plants. For recording very rapid
rates of response, as in the excitable Mimosa, it is
necessary to measure intervals of time as low as between
0.1-0.001 sec. For this purpose the principle of forced
oscillation is used, in which instead of oscillating the smoked plate the pen is made to oscillate at a known frequency by an electromagnet, whose primary current is made to interrupt at a definite rate by a vibrating reed of adjustable frequency. Bose called the arrangement as Resonant Recorder.

The principle of the Resonant Recorder is based on sympathetic vibration. If the strings of two violins are exactly tuned, then a note sounded on one will cause the other to vibrate in sympathy. In the same manner the pen(c) is tuned to vibrate a hundred times in a second by sounding a note which causes an air vibration of one hundred times per second, the pen will vibrate in sympathy. It will no longer remain in continuous contact with the recording plate, but will deliver a succession of taps a hundred times a second. The record will, therefore, consist of a series of dots, the distance between one dot and the next representing an interval of one-hundredth part of a second.

II. Preparative Procedures

1. Preparation of $[\gamma^{-32}\text{P}]\text{ATP}$

The method of preparation of $[\gamma^{-32}\text{P}]\text{ATP}$ was a modification of that originally described by Glynn and Chappell (1964). The method involves the exchange
reaction between inorganic phosphate and the terminal phosphate group (γ-phosphate) of ATP that occurs in the presence of phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and other ingredients.

\[
\text{3-Phosphoglycerate} + \text{ATP} \rightleftharpoons \text{1, 3-Diphosphoglycerate} + \text{ADP}
\]

\[
\text{1, 3-Diphosphoglycerate} + \text{Glyceraldehyde-3-phosphate dehydrogenase} \rightleftharpoons \text{3-Phosphoglycerolenzyme} + \text{Inorganic phosphate}.
\]

The final reaction mixture contained 5 mCi carrier free \(^{32}\text{P}O_4\) (neutralized with solid Tris-base), 1 ml cofactor solution containing 2 mM \(\text{Na}_2\text{EDTA}\), 2.5 mM \(\text{Na}_2\text{ATP}\), 0.5 mM \(\text{Na}_2\text{ADP}\), 2.5 mM 3-phosphoglyceric acid and 0.1 mM NAD\(^+\), 0.5 ml of 0.5 M Tris-\(\text{HCl}\) (pH 8.1), 2 µl of 2-mercaptoethanol, 10 µg of phosphoglycerate kinase, 30 µg of glyceraldehyde-3-phosphate dehydrogenase and 0.1 ml of 0.05 M MgCl\(_2\). The reaction mixture was incubated at 25° for 90 min. The reaction was terminated by exposing the incubation mixture in boiling water bath for 5 min. \(^{\gamma-32P}\)ATP formed was separated from other ingredients following the method of Schendel and Wells (1973). The final reaction mixture was diluted to 25 ml with distilled water. It was then passed through 2 ml packed volume of DEAE-sephadex A25 column previously equilibrated with
0.01 M NH₄HCO₃. The column was washed with the same solution and eluted thrice with 10 ml 0.1 M NH₄HCO₃, 20 ml each of 0.23 M NH₄HCO₃ and 0.4 M NH₄HCO₃. Fractions of 2.5 ml were collected. The fractions eluting at 0.4 M NH₄HCO₃ contained the [γ-³²P]ATP. Pooled ATP fractions (7.5 ml) were then allowed to be adsorbed by 50 mg of purified norit A (charcoal) to remove NH₄HCO₃, incubated at 30° for 60 min, centrifuged and the radioactivity was eluted from the precipitated charcoal with 1% NH₄OH in 50% ethanol. The eluate was dried in vacuum desiccator and finally dissolved in 1.5 ml distilled water.

The purity of the product was tested by paper chromatography in the solvent system containing isopropanol : NH₄OH : H₂O : 7 : l : 2 using standard ATP as the marker. The slight possibility of labelling of the phosphate group in α and β position was ascertained as follows. An aliquot of the solution containing labelled ATP was hydrolyzed with 1 M NaOH to AMP by incubation at 37° for 4 hr. AMP formed was separated by paper chromatography in the above solvent system using pure AMP as reference. The AMP spot was eluted, concentrated and rechromatographed on paper in another solvent system (isobutyric acid : NH₃ : water : 66 : 1 : 33). The eluted material had only negligible radioactivity. Similar procedure involving hydrolysis with 1 M NaOH for 30 min
yielded a mixture of AMP and ADP. The ADP spot purified as above showed negligible radioactivity.

2. Isolation and purification of nucleoside triphosphatase from *M. pudica* L.

Fresh *M. pudica* L. leaves were selected for the purpose of isolating the enzyme. The leaves were washed, minced with scissors and then partially homogenized in mortar-pestle. To partially homogenized leaves 20 mM Tris-HCl (pH 7.0) buffer (W/V = 1:3) was added and completely homogenized using sorvall omnimixer for three times (operating 60 secs each time). The homogenate which was highly viscous, passed through two layers of cheese cloth. The filtrate was centrifuged at 10,000 g for 25 min. The supernatant was collected and designated as crude extract.

Ammonium sulphate fractionation

The crude extract was then subjected to 85% saturation by gradual addition of solid (NH$_4$)$_2$SO$_4$ with constant stirring. The pH of the suspension was adjusted to 7.0 by adding requisite volume of 1 M NaOH and then kept for 6-8 hrs in ice for complete precipitation of protein. The precipitate was collected by centrifugation at 10,000 g for 20 min and the supernatant was discarded. The pelleted protein was suspended in 20 mM Tris-HCl (pH 7.0) and dialysed overnight against the same buffer.
The dialysate was centrifuged at 10,000 g for 30 min. The soluble protein was used for DEAE-cellulose column chromatography.

**DEAE-cellulose column chromatography**

The soluble protein thus obtained was layered on a DEAE-cellulose column (1.7 x 5.3 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.0). The column was then washed with the same buffer to remove nonspecific surface adhered protein from the column and eluted stepwise with different concentrations of KCl solution in 20 mM Tris-HCl (pH 7.0) until the bulk of the protein had been eluted. Fractions of 2 ml were collected in LKB fraction collector. The fractions were scanned for their absorbance at 220 nm and then assayed for enzyme activity, by incubating suitable aliquots from each fraction with substrate and cofactor at 37°. Most of the work was done with ATP as substrate. The enzyme activity was located in 0.25 M KCl eluted fractions (Fig. 2). Fractions containing enzyme activity were pooled together and dialyzed against the said buffer to remove KCl and the dialysate was then concentrated by lyophilisation. This enzyme preparation was used for subsequent experiments unless otherwise mentioned. However, for some experiments the enzyme was further purified.
DEAE-cellulose column chromatography of ammonium sulphate fraction of M. pudica L. leaf extract. 19.2 mg protein was applied to the DEAE-cellulose column (1.7 x 5.3 cm) equilibrated with 20 ml Tris-HCl buffer (pH 7.0). The surface adhered protein was removed by the same buffer. The elution was done stepwise with the same buffer containing 0.1 M, 0.25 M and 0.4 M KCl respectively, at a flow rate of 1 ml/2 min. Fractions of 2 ml were collected and assayed for ATPase (---). Protein (-----) was measured by absorbance at 220 nM.

Fig. 2 DEAE-cellulose column chromatography of ammonium sulphate fraction of M. pudica L. leaf extract. 19.2 mg protein was applied to the DEAE-cellulose column (1.7 x 5.3 cm) equilibrated with 20 ml Tris-HCl buffer (pH 7.0). The surface adhered protein was removed by the same buffer. The elution was done stepwise with the same buffer containing 0.1 M, 0.25 M and 0.4 M KCl respectively, at a flow rate of 1 ml/2 min. Fractions of 2 ml were collected and assayed for ATPase (---). Protein (-----) was measured by absorbance at 220 nM.
Polyacrylamide gel electrophoresis

The DEAE-cellulose column purified protein was further purified by preparative polyacrylamide gel electrophoresis using the method described by Davis (1964) with a little modification. For this DEAE-cellulose purified protein (50-100 μg) was subjected to polyacrylamide gel electrophoresis at pH 8.3 using 7.5% polyacrylamide gel. On completion of the run, the gels were sliced (2 mm thick) and the slices were suspended in 20 mM Tris-HCl (pH 7.0), kept in ice with occasional stirring (or gels were homogenized and kept in ice overnight. The homogenized fractions were centrifuged at 2,000 g for 10 min). The fractions were then assayed for ATPase activity using suitable aliquots from the supernatant of each fraction. Supernatant containing enzyme activity was pooled, dialyzed and concentrated by lyophilisation.

All operations were carried out at 0-4°.

3. Buffers used in detection of colchicine binding protein.

PM buffer - 10 mM potassium phosphate (pH 7.0), 10 mM MgCl₂.

PMG buffer - 0.1 mM GTP in PM buffer.

SPMG buffer - 0.25 M sucrose in PMG buffer.
4. **Isolation of goat brain tubulin**

Tubulin was purified from goat brain using ion exchange chromatography in PMG buffer as described by Weisenberg et al. (1968) with a modification in that DEAE-cellulose (Whatman DE 52) was used instead of DEAE-sephadex. The active fractions eluting from the DE 52 column, as judged by $[^3H]$colchicine binding assay, were pooled and concentrated by overnight dialysis against 10 volumes of 1 M sucrose or 8 M glycerol in PMG. The concentrated protein was then stored in small aliquots at -20°.

5. **Isolation of colchicine binding protein from *M. pudica* L.**

Soft and fresh leaves of *M. pudica* L. were washed. They were first minced with scissors then partially homogenized in mortar-pestle. To partially homogenized leaves SPMG buffer ($W/V = 1:2$) was poured and completely homogenized using sorvall omnimixer for 3 times (operating 60 secs each time). The homogenate was strained through two layers of cheese cloth. The filtrate was spun at 10,000 g for 25 min. The pellet was discarded and the supernatant was then subjected to 85% saturation by gradual addition of solid ammonium sulphate to it with constant stirring, kept for 3-4 hrs in ice for complete precipitation of protein. The precipitate was
collected by centrifugation at 10,000 g for 20 min and the supernatant was discarded. The pellet was dissolved in a minimum volume of PMG buffer and dialyzed in 20 volumes of same buffer with two changes for 90 min.

The dialysate was loaded to a DEAE-cellulose (Whatman DE 52) column (1.3 x 7.5 cm) previously equilibrated with PMG buffer (pH 7.0). The column was eluted with PMG buffer and then with 0.2 M KCl in the same buffer until the bulk of the protein had been eluted. Fractions of 2 ml were collected in LKB fraction collector. Fractions were scanned for their absorbance at 220 nm and then assayed for [3H]colchicine binding activity, by incubating suitable aliquots from each fraction with [3H]colchicine at 37°. The enzyme activity was located in PMG eluted fractions (Fig. 3). The fractions containing colchicine binding activity were pooled and concentrated by lyophilisation.

6. Preparation of myosin from rabbit muscle

Preparation of muscle:

Rabbit of body weight (1-1.5 Kg) was made unconscious with chloroform. Skin was removed around the hind leg to bare the muscle. White muscle was removed from the back with a sharp knife. Muscle was kept in ice containing phosphate buffer (pH 6.8).
Fig. 3  DEAE-cellulose column chromatography of ammonium sulphate fraction of *M. pudica* L. leaf extract. 22 mg protein was applied to a DE 52 column (1.3 x 7.5 cm) equilibrated with PMG buffer (pH 7.0). The elution was done stepwise with PMG buffer and 0.2 M KCl in PMG buffer. Fractions of 2 ml were collected, absorbance at 220 nm of the fractions was scanned (•) and colchicine binding activity (○) was determined with [3H]colchicine for 60 min at 37°C.
Solutions required:

1) 0.05 M phosphate buffer (pH 6.8), 1 mM EDTA.
2) Guba straub solution - 44.7 gms KCl, 200 ml 1 M KH$_2$PO$_4$, 100 ml 1 M K$_2$HPO$_4$; volume made up to 2000 ml with water.
3) 1 mM EDTA, pH 7.0.
4) 2.5 M KCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.0.
5) 1 M MgCl$_2$.
6) 1 M K$_2$PO$_4$, pH 7.0.
7) Saturated ammonium sulphate pH 7.0, 1 mM EDTA.

Procedure

All fats and connective tissues were removed and ground in cold. To the muscle 5 volumes of 0.05 M phosphate, 1 mM EDTA pH 6.8 (5 ml/gm muscle) was added, stirred well and centrifuged for 10 minutes and the supernatant containing blood proteins was removed.

To the pellet 3 volumes of Guba Straub solution containing 2 mM ATP and 1 mM EDTA pH 7.0 was added and extracted for 12 min. Then equal volume of 1 mM EDTA pH 7.0 was added and centrifuged. The supernatant was diluted to 6.5 to 7 times by 1 mM EDTA, pH 6.5-7.0 (6.5-7.0 ml EDTA solution/ml supernatant) and stirred well. Precipitate was allowed to settle overnight in cold room and collected it by centrifugation.

To the pellet 2.5 M KCl, 1.0 mM EDTA, 10 mM Tris-HCl pH 6.5-7.0 (20 ml/100 gm of muscle) buffer was
added and the pellet was dissolved using a loose pestle hand held homogenizer in ice bucket. Enough 10 mM Tris-HCl, 1 mM EDTA pH 7.0 was added to bring the total volume of the solution to the number of grams of muscle used. Then 1 M MgCl$_2$ and 1 M KPi pH 7.0 were added to bring the final concentration to 5 mM and by adding dilute tris base during the addition of KPi with stirring the pH of the suspension was adjusted to 7.0. This was then centrifuged at 80,000 g for 45 min and the supernatant was collected.

To the supernatant cold saturated ammonium sulphate solution was added to make the concentration of ammonium sulphate to 38%. Solution was then kept in ice for 30 min and then centrifuged at 10,000 g for 20 min. Supernatant was collected. To this supernatant more cold saturated ammonium sulphate solution was added to bring the saturation to 50%. It was then kept in ice for 30 min and centrifuged at 10,000 g for 20 min. The myosin pellet was collected. Myosin could be preserved as ammonium sulphate pellet in deepfreeze along with some supernatant.

To use myosin, freezeed ammonium sulphate pellet was allowed to thaw in ice bucket and dialyzed against 0.5 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0 with changes. Dialyzed protein was used within 7 days.
7. Isolation of actin like and myosin like proteins from *M. pudica* L.

For the preparation of actin like protein from *M. pudica* L. method of Hartwig and Stossel (1975) with a little modification was used. Fresh leaves of *M. pudica* L. were washed. They were first minced with scissors then partially homogenized in mortar-pestle. To partially homogenized leaflets 2 volumes of homogenizing buffer (0.34 M sucrose, 10 mM DTT, 0.5 mM ATP, 1 mM EDTA, 20 mM Tris-HCl buffer, pH 7.0) was poured and homogenized using sorvall omnimixer for 3 times (operating 60 secs each time). The homogenate was passed through two layers of cheese cloth. The filtrate was spun at 10,000 g for 25 min. Pellet was discarded and the supernatant was centrifuged at 100,000 g for 1 hr in Beckman L5-50 ultracentrifuge yielding a clear supernatant and a pellet (designated as P1). The pH of the supernatant was adjusted to about 7.4 with 0.1 M NaOH.

The supernatant was warmed to 25° made 75 mM in KCl and 2 mM in MgCl₂ and stirred with a magnetic stirrer for 1.5-2.0 hrs. It was then centrifuged at 10,000 g for 40 min at 4°. The precipitate (designated as P2) was washed once with ice cold 75 mM KCl in 10 mM Tris-HCl, pH 7.0 solution. The supernatant fluid was kept for isolation of myosin like protein. The pellet was suspended in 0.6 M KCl, 20 mM Tris-HCl buffer, pH 7.0 and
then centrifuged for 3 hr at 80,000 g in Beckman L5-50 ultracentrifuge. The pellet was dissolved in 2 mM MgCl$_2$, 10 mM Tris-HCl, pH 8.0 and assayed for actin-like activity.

The supernatant kept for myosin like protein isolation was subjected to 85% saturation by gradual addition of solid (NH$_4$)$_2$SO$_4$ with constant stirring. It was then kept for 6-8 hr in ice for complete precipitation of the protein. The precipitate was collected by centrifugation at 10,000 g for 20 min. The pellet was suspended and dialyzed against the buffer (0.5 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0). The dialyzed fraction was assayed for myosin-like activity.

III. Analytical Methods

1. Estimation of protein:

Two methods were employed for protein estimation. a) Protein estimation was carried out according to William E. Groves et al. (1968) as described below.

Protein samples were taken in cuvettes of 1 cm light path. The absorbance at 224 nm and 233 nm was monitored using appropriate blank in a Beckman Du-2 spectrophotometer. Difference in absorbance was calculated. The concentration of protein was determined by comparing the difference of absorbance, thus obtained,
to a standard curve using bovine serum albumin.

b) Protein estimation was also carried out following the method of Lowry et al. (1951) as described below.

**Reagents:**

- **Solution A:** 3% Sodium carbonate in 0.1 M NaOH
- **Solution B:** 4% Na-K-tartarate
- **Solution C:** 2% CuSO₄
- **Folin Ciocalteau reagent:** Adjusted to 1 M in HCl
- **Protein reagent:** Solution A, B and C were mixed at a ratio of 10:0.1:0.1 (V/V/V).

An aliquot of protein sample was diluted to 0.25 ml with distilled water. To this 2.5 ml of freshly prepared protein reagent was added, mixed well, kept at room temperature for 10 min. Then 0.25 ml of Folin Ciocalteau reagent was added and mixed well immediately. This was kept at room temperature for 30 min and the blue colour thus developed was measured by taking absorbance at 660 nm in a Beckman Du-2 spectrophotometer against a blank containing no protein. Protein concentration was determined using BSA as standard protein.

2. **Estimation of inorganic phosphorus**

Inorganic phosphorus was estimated according to the method of Chen et al. (1956). An aliquot containing inorganic phosphate was diluted to 2 ml and 2 ml of
phosphate reagent containing 1 volume of 6 N H$_2$SO$_4$, 1 volume of 2.5% (W/V) ammonium molybdate, 1 volume of 10% (W/V) freshly prepared ascorbic acid and 2 volumes of distilled water was added and mixed well. The mixture was then incubated at 37° for 60 min and the colour developed was measured at 820 nm. The phosphate content was determined by comparing with the standard curve using known amount of KH$_2$PO$_4$. The appropriate blank was prepared and necessary corrections were made.

3. **Estimation of total sugar**

Total sugar was estimated following the method of Michel Dubois et al. (1956). An aliquot of samples containing sugar was diluted to 1 ml with distilled water. 25 μl of 80% (W/V) phenol was added and then 2.5 ml of concentrated sulphuric acid was added rapidly (5-10 sec) and mixed well. The tube was allowed to stand for 10 min, then shaken well and placed in a water bath at 25-30° for 10-20 min. Yellow-orange colour thus developed was measured at 480 nm against proper blank. The concentration of sugar was determined with reference to a standard curve using L-arabinose.

4. **Chromatographic techniques**

a) **DEAE-cellulose column chromatography**

DEAE-cellulose (Whatman DE 52) was suspended in 5 volumes of 0.5 M HCl for 30 min, filtered in a sintered
glass funnel and washed with distilled water until the pH became neutral. The cake was again suspended in 5 volumes of 0.5 M NaOH for 30 min and washed with distilled water until the pH was about 8.0. The cake was then suspended in and rinsed for several times with the desired buffer in which column was to be equilibrated. The slurry was then poured into the column. The column washing was tested for pH so that the pH of the washing was identical to that of the buffer used at 4°.

b) DEAE-sephadex A25 column chromatography

DEAE-sephadex A25 was gently stirred with 0.3 M NaOH for 20 min and washed with distilled water to remove the alkali. The cake was then stirred with 0.3 M HCl for 20 min and similarly washed with distilled water until the pH was about 6.0. The cake was then suspended in 0.1 M Tris-HCl buffer (pH 8.5) overnight after which the suspension was adjusted to desired pH. Finally the slurry was kept in 0.01 M of the desired buffer containing 0.02% sodium azide to avoid fungal contamination.

c) Biogel P10 gel filtration

Biogel P10 dry beads were suspended in distilled water and kept overnight (or 24 hr) at room temperature. The fine materials were rejected by decantation. After complete swelling of the gel beads, the slurry was suspended in the required buffer at 4° and slowly poured into the
column, so that the gel particles settled uniformly to form a homogeneous bed.

5. **Polyacrylamide disc gel electrophoresis of proteins under non-denaturing conditions.**

Disc electrophoresis in polyacrylamide gel was performed according to the method of Davis (1964) with little modifications.

**Reagents**

Solution A: Acrylamide, 30 g and N, N'-methylene-bis-acrylamide, 0.8 g in 100 ml water.

Solution B: Acrylamide, 5 g and N, N'-methylene-bis-acrylamide, 1.25 g in 100 ml water.

Solution C: Tris base, 18.15 g, 1 M HCl 24.0 ml and TEMED 0.4 ml, volume made up to 100 ml with water.

Solution D: Tris base, 2.16 g, TEMED 0.1-0.2 ml, 1 M HCl 13 ml; volume made up to 100 ml with water.

Solution E: Riboflavin 0.002% in 2 M sucrose.

Ammonium persulfate: 0.3% solution.

Stock buffer: Tris base 3 g, glycine 14.4 g in 500 ml water, the final pH being 8.3.

**Procedure**

The lower or running gel was prepared by mixing equal volumes of solution A, C and ammonium persulfate
(different gel concentrations with respect to acrylamide were prepared by varying the solution A to the mixture keeping the volume ratio same). 1.75 ml portion of this gel mixture was poured into the electrophoresis tubes (8 cm long with an internal diameter 5.5 mm) and carefully overlaid with a thin layer of water. After polymerization for 30 min the water layer was removed by soaking with tissue paper wicks and 0.2 ml portions of the spacer (upper) gel was poured into the tubes and overlaid with water as above. The spacer gel was prepared by mixing 2 volumes of solution B, 1 volumes of solution D and 1 volume of solution E. The spacer gel was photopolymerized for about 40 min with the help of fluorescent lamp. After the gels were set and the top water layers were removed, the tubes were filled with electrode buffer and placed in the electrophoretic tank. Protein solution 5-10 μg for analytical purpose (activity staining) and 50-100 μg for preparative purpose (protein purification) was mixed with a little sucrose or glycerol and a trace of bromophenol blue as tracking dye and then carefully layered over the spacer gel. The electrophoresis was carried out in cold room (2-4°C) for about 2.5 hr with the anode at the bottom at a current of 2 mA/tube initially, which was increased to 3 mA/tube when the dye front reached the top of the lower gel. The electrode buffer was made by diluting the stock buffer
in the volume ratio of 1:2 with distilled water. After the run, the gels were removed from the tubes by rimming with water from a hypodermic needle.

The enzyme activity in gel was located by two methods:

a) **Localization of activity by gel slicing**

The gels were removed from the tubes after electrophoresis at 2-4°C, they were placed in a gel slicer one at a time and then sliced (2 mm thick). Each slice was collected in different tubes. The slices were suspended in 20 mM Tris-HCl (pH 7.0) buffer, kept in ice with occasional stirring (or gels were homogenized and kept in ice overnight. The homogenized fractions were centrifuged at 2,000 g for 10 min). The fractions were then assayed for ATPase activity using suitable aliquots from the supernatant of each fraction. The enzyme activity was plotted against gel slice number. The activity peak corresponds to the mobility of nucleoside triphosphatase enzyme in gel.

b) **Localization of activity by activity staining**

Activity staining in gel according to the method of Banerjee and Racker (1977) was followed as described below:

The gels were removed from the tubes after electrophoresis at 2-4°C, they were then incubated at 37°C
for 30-60 min depending on the activity of the enzyme in a reaction mixture containing 10 mM Tris-HCl (pH 8.0), 3 mM ATP (or other nucleotides where mentioned), 5 mM MgCl₂ and 50 mM CaCl₂. Gels were then washed with distilled water until the background was clear. The white band formed due to calcium phosphate indicated the position of the enzyme.

6. **Amino acid analysis of proteins**

For amino acid analysis, the dual column methodology of Spackman et al. (1958) was followed. A weighed sample of the lyophilized protein was placed into a heavy walled pyrex test tube, the tube had been previously washed with chromic acid, distilled water and 1 M HCL in the sequence mentioned and the residual HCL was removed in an air oven at 110°. The protein in the tube was suspended in 6 M HCL. A section of the tube, about 3 cm from the top, was constricted (oxygen flame) to about 1 mm bore. The tube was then inserted in a bath of liquid oxygen. When the sample was frozen, the tube was connected to vacuum pump and sealed off with a pinpoint oxygen flame. The hydrolysis was conducted in the sealed tube at 110 ± 1° for 24 hr. After the hydrolysis, the acid was removed by desiccation in presence of NaOH flakes. The dried sample was dissolved in citrate buffer (pH 2.2) and chromatographed in Beckman Multichrom amino acid analyzer, Model 4255, using resin M82 for neutral
and acidic amino acids (long column, pH 3.19 and 4.21) and resin M17 for basic amino acids (short column, pH 5.28). A calibration run using equimolar amounts of the different amino acids was performed before the analysis of the sample.

7. Enzymatic assays

Nucleoside triphosphatase activity could be measured in two ways.

a) Colourimetric assay

The reaction mixture contained 100 mM sodium acetate - acetic acid buffer (pH 5.0), 3 mM MnCl₂, 3 mM ATP (or other nucleotides where mentioned) and enzyme (1.5-5 µg, depending on the activity of the preparation) in a total volume of 0.5 ml. It was then incubated at 37° for 30 min. The reaction was arrested with 0.5 ml cold 10% TCA. The phosphate liberated was measured by the method of Chen et al. as was described before (III.2). The substrate blanks were subtracted to calculate enzyme activities.

1.0 A₈₂₀ = 0.16 µmole Inorganic phosphate (Pi).

b) Radioactive assay

In this assay [γ⁻³²P]ATP was used as substrate and followed the same method as described in case of colourimetric assay. But in this case, after arresting
the reaction by TCA, \([\gamma^{32P}]\) liberated was measured as described by Crane and Lipmann (1953). To the above reaction mixture 1 ml 10% norit A charcoal suspension was added and uniformly suspended in the solution by stirring. Then the charcoal was sedimented at 2,000 g for 5 min. Aliquots were taken from the supernatant to take counts of \([^{32P}]\) in 2 ml water.

Unit of enzyme: One unit of enzyme is that amount of it which produces 1.0 µmole of Pi per 30 min incubation at 37°.

3. Binding assays

a) Colchicine binding assay

One of the convenient means for detection of colchicine binding activity is by retention of the tubulin-colchicine complex on filter paper impregnated with DEAE-cellulose (DE 81 filter paper) according to the method of Weisenberg et al. (1968). Recently Banerjee and Bhattacharyya (1979) used GF/C filter disc (Whatman) for detection of tubulin colcemid binding complex. For detection of colchicine binding activity in this system (M. pudica L.) we used GF/C filter disc which has the following advantage over DE 81 filter paper: (a) better flow rate during washing, (b) much less quenching of count using same scintillation flour, (c) costs less.
M. pudica L. protein sample was mixed with \[^{3}H\]colchicine in appropriate concentrations, in cold (0\(^{\circ}\)) and volume of reaction mixture made upto 250 \(\mu l\) with PMG buffer. The reaction mixture was then incubated at 37\(^{\circ}\) for the required period and then cooled rapidly to 0\(^{\circ}\). Assay was done after the incubation was over. The assay method involves equilibration of GF/C filter disc with cold PM buffer by passing 1 ml of it under mild suction. Then 1 ml of PM buffer containing colchicine (10\(^{-5}\)M) was applied to the filter paper to which first 100 \(\mu l\) incubation mixture and then 4 ml PM buffer containing colchicine (10\(^{-5}\)M) was added all at 0-2\(^{\circ}\). This was drained off slowly under mild vacuum. The GF/C filter disc was then washed thrice with 3 ml cold (2\(^{\circ}\)) PM buffer each time. The filter papers were then air dried and counts were taken in 5 ml of toluene based flour in liquid scintillation counter (LS-100, Beckman). Two GF/C filter discs were used for each assay. All the assays were performed in duplicate and mean count was taken.

b) Colcemid binding assay

Colcemid binding assay was performed according to the method of Banerjee and Bhattacharyya (1979) with little modification. Whatman GF/C filter discs was equilibrated with 1 ml cold (2\(^{\circ}\)) PM buffer by suction. Then 1 ml of PM buffer was applied to the filter paper to
which an aliquot (100 μl) of the sample (immediately after incubation with [³H]colcemid at 37° for the required period) was applied and then 1 ml PM buffer at 0-2° was added. This drained off slowly under mild vacuum. The GF/C filter disc was then washed thrice with 3 ml portions of cold PM buffer by mild suction. The filter papers were then dried and counts were taken in 5 ml of toluene based flour. Two GF/C filter discs were used for each assay. All the assays were performed in duplicate and mean count was taken.

9. Radioactivity measurements

³²P-labelled samples were subjected to Cerenkov counting in a Beckman LS-100 Liquid Scintillation Spectrometer. ³H-labelled samples were in general collected on filter discs. The discs were dried at room temperature and were counted in a Beckman LS-100 Liquid Scintillation Spectrometer (with 40% ³H-counting efficiency), using 5 ml of toluene based flour containing 0.5% PPO and 0.005% POP.

10. Spectrophotometric measurements

Absorbance of samples in ultraviolet and visible range was measured with a Beckman Du-2 Spectrophotometer using cuvettes of 1 cm light path.
REFERENCES


