A. MATERIALS:

1. Proteins:

   Bovine serum albumin (lot no. 100F - 0249), ovalbumin (lot no. 105C-8022), chymotrypsinogen A (lot no. 40F - 8050), cytochrome c (lot no. 09C - 0088) and trypsin type II (lot no. T - 8128) were purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A. Salt fractionated goat IgG was a kind gift from Ms. Zoya Galzie of this laboratory. Soybean agglutinin was obtained from Sisco Research Laboratories, India and was used without further purification.

2. Sugars:

   Glucose, lactose and sucrose were purchased from BDH, Bombay, India. Galactose was purchased from LOBA CHEMIE, Bombay, India. N-Acetylgalactosamine (lot no. 22F - 5043), galactosamine (lot no. 86F - 0340), methyl α-galactoside (lot no. 74F - 5611), methyl β-galactoside (lot no. 56F - 5624), melibiose (lot no. 78F - 0014) and p-nitrophenyl α-galactoside (lot no. N-0877) were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A.

3. Chromatographic media:

   Sepharose 6B and Sephadex G-150 were purchased from
Sigma Chemical Company, St. Louis., Mo., U.S.A. Divinyl sulfone (98% v/v) was obtained from MERCK-Schuchardt, Germany. Blue Dextran 2000 was purchased from Pharmacia Fine Chemicals, Uppsala.

4. Reagents used in sodium dodecyl sulphate polyacrylamide gel electrophoresis:

Reagents used in SDS-polyacrylamide gel electrophoresis were acrylamide and ammonium persulphate from Merck, Dramstadt, Germany; N-N'-methylene bisacrylamide from Reanal Budapest, Hungary; N, N, N', N'-tetramethyl-ethylenediamine from Ferak, Berlin, West Germany; sodium dodecyl sulphate, glycerol, methanol, acetic acid and chloroform from BDH, Bombay, India; bromophenol blue, dichlorodimethyl silane and 2-mercaptoethanol from BDH, Poole, England; coomassie brilliant blue R-250 (lot no. B - 0630) and tris (hydroxymethyl) amino methane (lot no. T -1378) from Sigma Chemical Company, St. Louis, Mo., U.S.A.

5. Miscellaneous reagents:

Sodium azide was from Fluka, Switzerland. N-Acetyl-neuraminic acid (lot no. A 2501) was purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A. Phenol, sodium arsenate, thiobarbituric acid, cyclohexanone and sodium metaperiodate were from BDH, Bombay, India. All other
reagents were of analytical grade. *Phaseolus mungo* seeds were obtained from the local market.

Glass distilled water was used throughout these studies.

B. METHODS:

1. Measurement of pH:
   
   Elico digital pH meter, model L1-122, was used for pH measurements in conjunction with Elico combined electrode at room temperature. The pH meter was standardized with 0.05 M potassium hydrogen pthalate buffer, pH 4.0, in the acidic range and with 0.01 M sodium tetraborate buffer, pH 9.2, in the basic range.

2. Determination of protein concentration:

   Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard protein. To 1 ml of protein solution, 5 ml of copper reagent was added. After 10 minutes, 1 ml of Folin phenol reagent was mixed and the solution was allowed to stand at room temperature for 30 minutes. The colour intensity was read at 700 nm against an appropriate blank on AIMIL photochem-8 colorimeter. A calibration
curve between optical density and protein concentration was obtained and found to fit the equation:

\[(O.\text{D.})_{700\text{nm}} = 1.4 \text{ (Protein, mg)} + 0.048 \quad (1)\]

3. Hemagglutinating activity of lectin:

Hemagglutinating activity of lectin was detected using trypsinized rabbit erythrocytes. Rabbit blood was collected in the presence of 2.8% EDTA in normal saline and centrifuged at 1000 rpm for 20 minutes. The supernatant was discarded and the pellet of red blood cells was washed three times with 0.01 M sodium phosphate buffered saline, pH 7.8 (PBS). For trypsinization, 10 mg of trypsin was incubated with 1 ml of packed cells in PBS, for 10 minutes at 37°C. Then the trypsinized cells were washed thrice with PBS and used in the agglutination experiment.

Hemagglutinating activity of *Phaseolus mungo* lectin was measured against trypsinized rabbit erythrocytes by counting the cells on a hemocytometer. Typically, in 1 ml of incubation mixture, 88 µg of lectin in 0.01 M Tris-HCl buffer, pH 7.4 containing 0.25 M NaCl (Tris-HCl buffer) was incubated with \(1 \times 10^6\) trypsinized rabbit erythrocytes for 30 minutes at room temperature (25°C). The number of unagglutinated cells were counted by hemocytometer and
percent hemagglutination calculated.

4. Optical measurements:

In the ultraviolet region, light absorption measurements were performed on Cecil UV-spectrophotometer model CE-202 or on Cecil double beam spectrophotometer, model CE-594 using silica cells of 1 cm path length. Fluorescence measurements were carried out on Shimadzu spectrofluorometer, model RF-540. The slit width was 10 nm.

5. Preparation of affinity gel:

Following Fornstedt and Porath (1975), 50 ml of Sepharose 6B gel was washed with distilled water and then with 0.5 M carbonate buffer, pH 11.0; the washed gel was then incubated with 5 ml of divinyl sulfone for 70 minutes with continuous stirring. The activated gel slurry was washed extensively with distilled water. Fifty millilitres of 20% (w/v) galactose solution in 0.5 M carbonate buffer, pH 10.0 was added to the gel slurry and the coupling reaction allowed to take place overnight at room temperature (33°C). The affinity gel was washed extensively with distilled water and then with 0.5 M bicarbonate buffer, pH 8.5 and finally treated with 1 ml of 2-mercaptoethanol for 2 hours. The galactosyl Sepharose 6B gel was washed with distilled water before use.
6. Gel chromatography:

Gel chromatography was performed on a Sephadex G-150 column (1.78 x 99 cm) which was packed by the method of Ansari and Salahuddin (1973). The radius of the column was determined to be 0.89 cm. The column was packed with Sephadex G-150 gel and equilibrated with Tris-HCl buffer. The packing of the column was checked by passing Blue Dextran (see Fig. 3). The pattern of the elution profile showed uniform packing of the column. The void volume, $V_0$, of the column, determined from the elution profile of Blue Dextran was found to be 78 ml. The inner volume, $V_i$, of the column was determined by passing galactose solution through the column for which the elution volume was found to be 230 ml (see Fig. 4). The value for distribution coefficient, $K_d$, for galactose is unity. With these values, the inner volume, $V_i$, of the column was calculated with the help of equation:

$$V_i = \frac{V_e - V_0}{K_d}$$

which was found to be 152 ml.

The total volume, $V_t$, of the column was calculated to be 246 ml.
Fig. 3. Elution profile of Blue Dextran on Sephadex G-150 column.

About 10 mg of the sample was applied to the column (1.78 x 99 cm) equilibrated with Tris-HCl buffer. Fractions of 3 ml were collected at a flow rate of 20 ml/hr. The colour intensity was read at 610 nm.
Fig. 4. Elution profile of galactose on Sephadex G-150 column.

About 0.9% galactose solution was applied to the column (1.78 x 99 cm) equilibrated with Tris-HCl buffer. Fractions of 5 ml were collected at a flow rate of 20 ml/hr and monitored by the method of Dubois et al. (1956).
7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (1969) in 0.1 M sodium phosphate buffer, pH 7.0 containing 0.1% SDS. The glass tubes (0.5 x 9 cm) previously washed with detergent and chromic acid, were siliconized with 7% dichlorodimethyl silane in chloroform. The tubes were mounted vertically in a stand with their lower ends closed with rubber stoppers. A solution containing 7.5% acrylamide (w/v), 0.2% (w/v) N,N'-methylene bisacrylamide, 0.01% (v/v) N,N',N'-tetramethylethylenediamine (TEMED), 0.04% (w/v) ammonium persulphate and 0.1% SDS (w/v) in 0.1 M sodium phosphate buffer, pH 7.0 was prepared. This gel solution was poured in each gel tube and to this a few drops of water were layered.

For the preparation of sample, the protein was dialyzed against 0.01 M sodium phosphate buffer, pH 7.0 containing 1% SDS. Then the sample was heated for 30 minutes in a boiling water bath. After cooling the sample, few drops of glycerol containing 0.1% (w/v) bromophenol blue were added. The sample was reduced by adding 0.1 M 2-mercaptoethanol. About 45 μg of protein was applied on the top of the gel and electrophoresis was carried out for 6-7
hours at 6 mA current flow per tube. The gels were removed from the gel tubes with the help of a long needle attached to a syringe. The gels were stained with 0.2% coomassie brilliant blue R-250 dye in 25% (v/v) methanol and 10% (v/v) acetic acid and destained mechanically with 10% (v/v) acetic acid.

8. Determination of carbohydrate content:

(a) Neutral carbohydrate

The neutral carbohydrate content was determined by the method of Dubois et al. (1956) using galactose as standard.

To 1 ml of protein solution, 1 ml of 2% (w/v) phenol solution was added. Then 5 ml of concentrated sulphuric acid was mixed with it and the solution was allowed to cool at room temperature for 20 minutes. The colour intensity was measured at 490 nm against an appropriate blank prepared similarly with bovine serum albumin.

A calibration curve between carbohydrate concentration in milligram and absorbance at 490 nm was obtained by the method of least squares. The linear curve (Fig. 5) fits the equation:

\[(O.D.)_{490\text{nm}} = 6.26 \text{ (Galactose, mg)} + 0.08 \] (3)
Fig. 5. Calibration curve for the estimation of neutral carbohydrate by the method of Dubois et al. (1956) using D-galactose as standard. The straight line drawn by the method of least squares fits the equation:

\[(\text{O.D.})_{490\text{nm}} = 6.26 \times \text{(Galactose, mg)} + 0.08\]
(b) Sialic acid:

The sialic acid content of *Phaseolus mungo* lectin was determined by the method of Warren (1959) using N-acetyl neuraminic acid as standard.

The protein was heated in 0.1 M sulphuric acid for 45 minutes at 80°C in a water bath to release sialic acid. For the estimation of sialic acid, 0.5 ml of solution was mixed with 0.1 ml of 0.2 M sodium metaperiodate in 9 M orthophosphoric acid and kept at room temperature for 20 minutes. Then 1 ml of 10% (w/v) sodium arsenate in a solution of 0.5 M sodium sulphate and 0.1M sulphuric acid, was added with vigorous shaking until the yellow colour which appeared momentarily, disappeared. This was followed by the addition of 3 ml of 0.6% (w/v) thiobarbituric acid in 0.5 M sodium sulphate. The mixture was heated in boiling water bath for 15 minutes. After cooling, equal volume of cyclohexanone was added and centrifuged at 2000 rpm for 15 minutes. The colour intensity of cyclohexanone layer was read at 550 nm against an appropriate blank. The solution prepared with bovine serum albumin was taken as a control.

A calibration curve between sialic acid concentration in microgram and absorbance at 550 nm was obtained by the method of least squares. The linear curve (Fig. 6) fits
Fig. 6. Calibration curve for the estimation of sialic acid by the method of Warren (1959) using sialic acid as standard. The straight line drawn by the method of least squares fits the equation:

\[(\text{O.D.})_{590\text{nm}} = 0.038 \times (\text{sialic acid, } \mu\text{g}) + 0.015\]
the equation:

\[(\text{O.D.})_{550\text{nm}} = 0.038 \times (\text{Sialic acid, } \mu\text{g}) + 0.015\] (4)

**9. Isolation of lectin from Phaseolus mungo seeds:**

For isolation of lectin, Phaseolus mungo seeds (50 g) were soaked in 250 ml of 0.01 M Tris-HCl buffer, pH 7.4 containing 0.25 M NaCl, for overnight at room temperature and kept in freezer for 15 minutes. The seeds were then homogenized in a blender for 5 minutes and the suspension was filtered through a muslin cloth. The pH of the solution was lowered to 4.0 by addition of 0.3 M acetic acid and it was kept overnight at 8°C. The precipitate was removed by centrifugation at 6000 rpm for 30 minutes. The clear supernatant was extensively dialysed against operating Tris-HCl buffer for purification of lectin by affinity chromatography.

**10. Affinity chromatography:**

Galactosyl Sepharose 6B gel was packed into a glass column (4 x 8.8 cm) and was subsequently equilibrated with the operating Tris-HCl buffer. Thirty millilitres of seed homogenate containing 153 mg protein was applied to the column keeping the flow rate at 5 ml/hr. The column was washed with the operating buffer. The bound protein was specifically eluted with 0.25 M galactose in operating
Tris-HCl buffer. The lectin activity was measured by hemagglutination assay in presence and absence of galactose.

11. **Effect of temperature on hemagglutinating activity of lectin:**

Effect of temperature on the *Phaseolus mungo* lectin was observed by hemagglutination assay. Affinity purified lectin (88 µg) was exposed to a temperature range of 10°C to 70°C in an incubator for 40 minutes and subsequently incubated overnight at room temperature. The residual hemagglutinating activity of lectin was determined against trypsinized rabbit erythrocytes.

12. **Effect of pH on hemagglutinating activity of lectin:**

The effect of pH on the hemagglutinating activity of *Phaseolus mungo* lectin was studied at different pH values in the pH range 4 to 8 which was maintained by 0.01 M citrate phosphate buffer (pH 4 to 8) containing 0.25 M NaCl. First, the affinity purified lectin was dialysed extensively against the citrate phosphate buffer of different pH (4-8) and then incubated with trypsinized rabbit erythrocytes (1 x 10^6 cells) for 30 minutes. The activity of lectin at different pH was determined by hemagglutination assay as described earlier.
13. Effect of ionic strength on hemagglutinating activity of lectin:

The effect of ionic strength on hemagglutinating activity of Phaseolus mungo lectin was investigated in 0.01 M citrate phosphate buffer, pH 6.0 containing different concentration of NaCl (0.15 - 0.9 M). The cells and lectin were exposed to desired ionic strength for 30 minutes and the hemagglutinating activity determined as described earlier.

14. Carbohydrate binding specificity:

The hemagglutinating activity of lectin was determined in presence and absence of increasing concentrations of ten sugars. The purified mungo lectin (88 μg) in Tris-HCl buffer was incubated with different concentrations of sugar for 30 minutes at room temperature. To this incubation mixture, 0.1 ml of trypsinized rabbit erythrocyte (1 x 10⁶ cells) suspension was added and kept at room temperature for 30 minutes. After 30 minutes, the number of unagglutinated cells were counted and percent hemagglutination was determined as described earlier.

Similarly, hemagglutination assay was performed with soybean agglutinin. The lectin (40 μg) in 0.01 M Tris-HCl buffer, pH 7.4 containing 0.25 M NaCl and 0.002 M each of CaCl₂ and MnCl₂ was incubated with different concentra-
tions of sugar for 30 minutes at room temperature. To this incubation mixture, 0.1 ml of rabbit erythrocytes containing $1 \times 10^6$ cells were added and kept at room temperature for 30 minutes. The number of unagglutinated cells were counted and percent hemagglutination determined.