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Lectin from *Canavalia brasiliensis* (MART.). Isolation, Characterization and Behavior during Germination

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Abstract. A lectin was isolated from *Canavalia brasiliensis* MART. seeds by combining solubility fractionation with affinity chromatography on Sephadex G-50. The lectin showed a carbohydrate specificity for D-mannose (D-glucose) binding and a requirement for Ca^{2+} and Mn^{2+}. All the hemagglutinating activity was found in the cotyledons and the presence of the lectin was followed during the first 15 days of plant germination, through the activity against rabbit erythrocytes, the presence of the "lectin peak" in Sephadex G-50 affinity chromatography, presence of the "lectin bands" in SDS-polyacrylamide gel electrophoresis and the "lectin arcs and rockets" in immunoelectrophoresis in agarose gel. On application of all these methods the lectin showed a differentiated metabolism, disappearing more slowly than the other high molecular weight proteins of the seed.

Lectins, proteins capable of agglutinating erythrocytes and other cells, are widely distributed in nature, above all among plants of several families (TOMS and WESTERN 1971, GOLDSTEIN and HAYES 1978). Although many of them are well characterized in their chemical properties and the effects they have on alien biological systems, such as erythrocytes and lymphocytes, the physiological role of plant lectins remains a subject of speculation. Over the last 20 years, several hypotheses have been formulated (for review see BOYD 1963, LIENER 1976, KAUSS 1977) but none of them is universally accepted. Although the lectins present in seeds of several species have already been well investigated, only few authors investigated their behavior during germination and early stages of plantlet development (MIALONIER *et al.* 1973, PUEPKE *et al.* 1978, ROUGÉ and PÈRE 1982). In this paper the isolation and behavior of the lectin from *Canavalia brasiliensis* MART. seeds were investigated during germination as a first step in the attempt to understand their role in the plant.

MATERIAL AND METHODS

Plant Material

Canavalia brasiliensis MART. seeds were collected in the state of Ceará, Brazil.

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Erythrocyte Samples

Rabbit erythrocyte samples were obtained from adult animals kept in an animal house.

Reagents

Sephadex G-50 was supplied by Pharmacia Fine Chemicals, N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylethylenediamine were from Eastman-Kodak Co., agarose from Sigma Chemical Co., sodium dodecyl sulphate and betamercaptoethanol from E. Merck. All the other reagents were of analytical grade.

Extraction of Protein

The proteins of dry seed flour or germinated cotyledons and axes were extracted by homogenization in a mortar with 0.15 M NaCl. After 4 h the raw extract was centrifuged 20 min at $16\,000 \times g$ at 4 °C to remove solid particles. The globulin and albumin fractions were obtained by sequential dialysis of the full extract against 0.033 M acetate buffer (pH 5.0) and water.

Hemagglutinating Activity

This was determined using rabbit blood cells as described before (MOREIRA and PERRONE 1977).

Protein Concentration

This was determined by the microbiuret method of GOA (1953) using bovine serum albumin as standard. In column effluates the absorbance at 280 nm was used to indicate protein content.

Fractional Precipitation with Ammonium Sulphate

Solid ammonium sulphate was added in small portions to the full extract to give the desired saturation. The mixtures were kept at room temperature for 4 h and then centrifuged 20 min at $16\,000 \times g$ at 4 °C. The precipitates were dissolved in water and after exhaustive dialysis against water were freeze-dried.

Affinity Chromatography on Sephadex G-50

Affinity chromatographies were carried out on Sephadex G-50 columns (1.3×19 cm) equilibrated with 1 M NaCl, containing 5 mM $CaCl_2$ and $MnCl_2$. The samples were applied in the equilibration solution and eluted with 0.1 M glucose in the same solution. The eluates were collected in 3.6 ml fractions and these were analysed for hemagglutinating activity and protein content.

Absorption Spectrum

The absorption spectrum was determined in a Varian (model UV-634S) spectrophotometer, with the sample dissolved in 0.15 M NaCl.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out with and without beta-mercaptoethanol in the protein buffer, following the method of WEBER

and OSBORN (1969) as described by XAVIER-FILHO and MOREIRA (1978). Gels were stained with 0.005%(m/v) coomassie brilliant blue in methanol-acetic acid-water (5 : 1 : 5, v/v/v).

Immunization

Adult albino rabbits were immunized with intramuscular injection of the Full Extract (10 mg) or the purified lectin P_{III} (2 mg) emulsified in Freund's adjuvant. Further booster doses of the same amounts in 0.9% NaCl were injected to increase the antibody titre. Rabbits were bled by ear puncture and the immunoserum fractionated with ammonium sulphate (33% of saturation), dialysed against water and freeze dried.

Immunoelectrophoresis in Agarose Gel

This was carried out as described by CLAUSEN (1969) in agarose (1%) gel prepared with Veronal-lactate buffer pH 8.6. After the electrophoretic run the fractions were allowed to diffuse against the anti-Full Extract or anti-P_{III} sera for 24 h and the precipitated arcs examined by diffused light or by colouring the washed plates with Ponceau Red or Light Green.

Rocket Immunoelectrophoresis

These experiments were made in agarose (1%) plates containing antisera (0.1%) prepared against the Full Extract or P_{III}, following the method described by WEEKE *et al.* (1973). The visualization of the rockets were done as above.

RESULTS

Purification of the Lectin

The seed kernel containing 32.24% of protein (N_2 basis) was extracted with 0.15 M NaCl, 97% of which were in the globulin fraction. When the Full Extract was treated with ammonium sulphate 89% of the hemagglutinating activity precipitated between 50 and 90% of saturation (Crude Lectin) as shown in Table 1. This fraction was then applied on a Sephadex G-50 column (Fig. 1) and the glucose eluted peak (P_{III}) containing all the activity, after

TABLE 1

Overall recovery of protein and hemagglutinating activity from *Canavalia brasiliensis* at various stages of purification

Fraction	Amount [g]	H.U. mg^{-1}	Total activity H.U. $\times 10^{-5}$	Extent of purification (times)
Seed flour	100	3.8	3.8	1
Full Extract	14.26	26.6	3.79	7
Albumin	0.39	30.0	0.18	7.9
Globulin	10.99	32.7	3.59	8.6
F 0/50	2.02	51.0	1.03	13.4
F 50/70	6.51	130.6	8.50	34.4
P_{III}	2.24	1 049.2	23.50	276.1

The flour was extracted with 0.15 M NaCl, centrifuged and the clear supernatant was treated with solid ammonium sulphate to saturation levels indicated. Fraction F 50/90 was further purified by affinity chromatography on Sephadex G-50.

dialysis against 0.15 M NaCl and water, was freeze-dried. In this way a purification of 276 × was achieved in relation to the whole flour. The apparent discrepancy in the recovery of P_{III} is probably due to the elimination of an "inhibitor" which is extracted with the lectin. The *C. brasiliensis* lectin can be inhibited by glucose and mannose but it is not affected by the presence

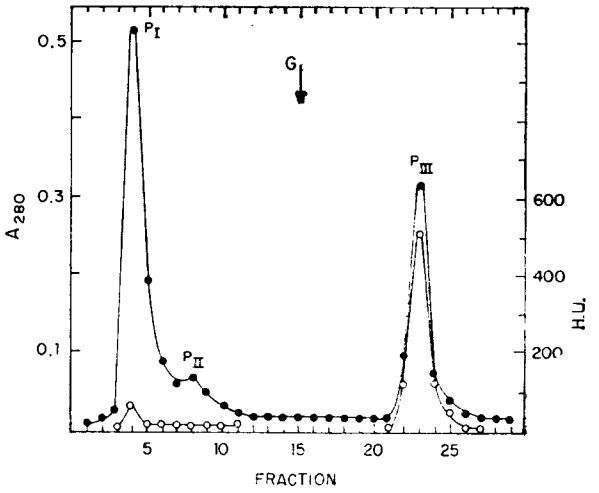

Fig. 1. Affinity chromatography of the crude lectin (F 50/90) on a Sephadex G-50 column. The sample (10 mg) was dissolved in 1 M NaCl containing 5 mM Ca^{2+} and 5 mM Mn^{2+} and applied to a column of Sephadex G-50 (1.3 × 19 cm; 25 ml column volume; 14.4 ml void volume). Flow rate was 30 ml h^{-1} and fractions of 3.6 ml were collected and read at 280 nm. Elution was performed first with the equilibration solution and changed to a solution of 0.1 M glucose/1 M NaCl/5 mM Ca^{2+}/5 mM Mn^{2+} at fraction 15 (indicated by an arrow).

of galactose. The lectin showed a dependence on divalent cation, requiring the presence of 5 mM Ca^{2+} and Mn^{2+} for activity, and presents an $E^{1\%}_{1cm}$ = = 13.52. When the Full Extract and the several protein fractions from *C. brasiliensis* seeds were electrophoresed in polyacrylamide gel in the presence of SDS and betamercaptoethanol (Fig. 2), all the active fractions presented four main bands (8 000, 13 000, 23 000, and 26 000 daltons), one of which (the 26 000-dalton band) was predominant in the pure lectin.

Germination of the Seed

The *C. brasiliensis* seeds were found to need a pretreatment with concentrated sulphuric acid in order to break the impermeability of the shell. The treated seeds were let to germinate between two sheets of filter paper in an H_2O saturated atmosphere, in the dark. The dry mass, protein content and hemagglutinating activity in the cotyledons and axes are shown in Fig. 3. The hemagglutinating activity and total protein content follow the dry mass behavior in the cotyledons. There is no detectable hemagglutinating activity in the axis.

When the Full Extracts from cotyledons and axes were applied to a Sephadex G-50 column (Fig. 4) it was noted, on the other hand, that the lectin peak (P_{III}) disappeared more slowly than the whole "reserve proteins" (P_I)

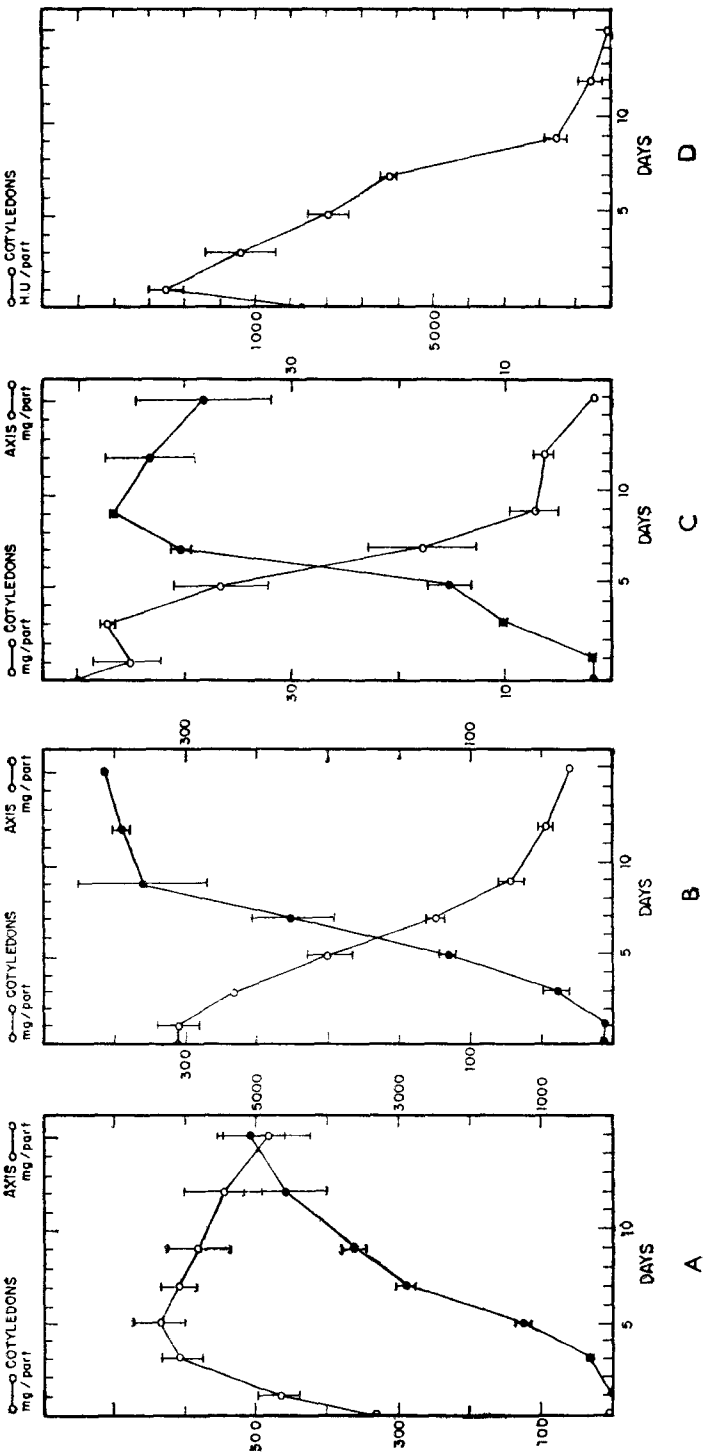

Fig. 3. Fresh matter (A), dry matter (B), total protein content (C) and hemagglutinating activity (D) of cotyledons and axes of germinating seeds of *Canavalia brasiliensis*.

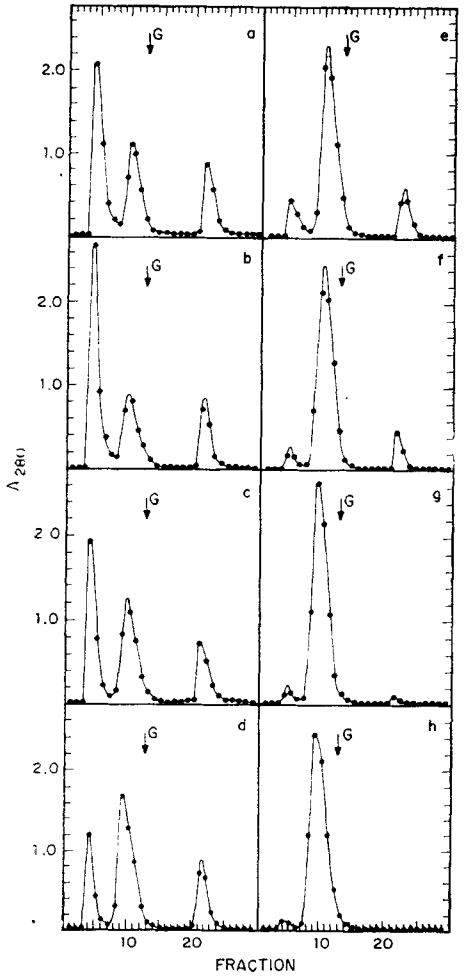

Fig. 4. Affinity chromatographies of the Full Extracts from cotyledons of germinating seeds of *Canavalia brasiliensis*. The samples (15 mg) were dissolved and the experiments were conducted as described in Fig. 1. — a: 0 day; b: 1 day; c: 3 days; d: 5 days; e: 7 days; f: 9 days; g: 12 days; h: 15 days.

indicating a differentiated mobilization. The Full Extracts obtained from germinating axes presented no retained peak, in agreement with the hemagglutinating activity data. Similar results were obtained when the same Full Extracts were submitted to SDS-polyacrylamide gel electrophoresis (Fig. 5). In these experiments, the bands corresponding to the lectin disappeared more slowly than the bands corresponding to P_I. When the Full Extracts from germinated cotyledons and axes were submitted to agarose gel immunoelectrophoresis (Fig. 6) and rocket immunoelectrophoresis (Fig. 7), the bands corresponding to P_{III} also disappeared more slowly than the P_I bands. When these experiments were made with Full Extracts obtained from germinating axes, no bands and arcs corresponding to P_{III} were found, confirming the hemagglutinating activity data.

DISCUSSION

Canavalia brasiliensis MART. and *Canavalia ensiformis* L. present many phenotypic differences and are classified as different species. In spite of this

the isolated lectin from the seeds of *C. brasiliensis* shows many similarities with Con A, although precipitating only at higher ammonium sulphate saturation. It can also be purified by affinity chromatography on Sephadex G-50, shows the same SDS-polyacrylamide gel electrophoretic subunit pattern, presents an $E^{1\%}_{1cm}$ of the same order and can be inhibited by the same sugars. Moreover, both Con A and the lectin from *C. brasiliensis* can be recognised and present an identity arc when let to diffuse against IgG raised against the *C. brasiliensis* lectin. The seeds of *C. brasiliensis* are not able to germinate without a previous scarification, their dormancy being probably due to their impermeability to water.

Although in most leguminous plants lectins can be found in all parts of the seeds — cotyledons, embryo axis and seed coat (ROUGÉ and PÈRE 1982) — the seeds of *C. brasiliensis* showed detectable amounts of lectin only in the cotyledons, during the stages investigated.

Despite the apparent parallelism between the hemagglutinating activity and the total protein (due probably to the methodology employed for determining the protein content — the microbiuret also reveals the polypeptides resulting from the breakdown of the proteins), the lectin behavior presented no parallelism with the diminishing of dry mass and other high molecular mass proteins. The lectin seems to be metabolised more slowly than the other proteins. Thus the content of Sephadex-interacting substances remains almost the same until the 5th day; the concentration of "lectin" measured by rocket immunoelectrophoresis remained almost constant until the 7th day and the SDS-polyacrylamide gel electrophoretic subunits are present until the 15th day.

Apparently, the lectin is initially broken down to an intermediate form, probably monomeric, presenting no hemagglutinating activity but a Sephadex-binding activity, being then broken down to small peptides containing the same subunits and antigenic sites as the original lectin, before disappearing at the completion of cotyledon senescence.

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Figs 2, 5, 6, 7 at the end of the issue.

BOOK REVIEW

UDE, J., KOCH, M.: DIE ZELLE. ATLAS DER ULTRASTRUKTUR. — VEB Gustav Fischer Verlag, Jena 1982. 269 S. 20 Abb., 39 Farbtafeln, 196 elektronenmikroskopische Darstellungen auf 78 Tafeln. DDR 45,— M. Ausland 55,— M.

Im letzten Jahrzehnt sind mehrere "Bilderbücher" der Ultrastruktur biologischer Objekte erschienen, wo an Hand von Rasterelektronenmikroskopaufnahmen dem Leser eine völlig neue dreidimensionale Vorstellung des Aufbaus von Organismen erschlossen worden ist. Die Verfasser des besprochenen Buches haben eine andere Variante gewählt — sie konfrontieren Aufnahmen aus dem Transmissions-Elektronenmikroskop mit sehr eindrucksvollen und anschaulichen bunten Modellen der Objekte, deren Struktur und Funktion kurz und übersichtlich beschrieben wird. Bunte Tafeln demonstrieren ebenfalls z. B. die Funktion der Atmungskette, des Zitratzyklus, der Proteinsynthese, der Photosynthese, der Mitose und Meiose, die Plastiden- und Mitochondrienvermehrung oder die chemische Zusammensetzung der Zellenbestandteile.

Das Buch ist in drei Abschnitte gegliedert. Im ersten werden kurz die Funktion und der Aufbau des Elektronenmikroskops beschrieben, im zweiten Abschnitt die Struktur der Zelle als Grundbaustein des Lebens und die Struktur und Ultrastruktur ihrer Elemente, und im dritten Abschnitt ausgewählte Zellformen wie z. B. Drüsen-, Nieren-, Lichtsinnes-, Nerven-, Muskel- und Blutzellen behandelt. In diesem Abschnitt werden Ultrastruktur und Funktion der erwähnten Zellen als Ganzes dargestellt. Im Buche werden die Prokaryonten-, Tier- und Pflanzenzellen durchgearbeitet. Jedes kleine Kapitel, z. B. über eine Organelle, schliesst mit einem eigenen Literaturverzeichnis. Der Atlas ist mit einem Sachregister ausgestattet. Die technische Ausführung ist tadellos.

Für eine eventuelle Neuausgabe des Bilderatlas wäre erwünschenswert, dass die chemische Nomenklatur an gültige Regeln angepasst wird, z. B. statt Ribulosediphosphat Ribulosebisphosphat auf S. 103 und 105.

Das vorliegende Lehrbuch der Ultrastruktur der Zelle ist vor allem für Studierende bestimmt, denen es ermöglichen soll die heute noch schwierig zu gewinnenden, eigenen Erfahrungen mit dem Elektronenmikroskopen zu ersetzen. Die lehrreichen und anschaulichen Abbildungen und Ausführungen des Buches werden jedoch auch von vielen anderen biologisch interessierten Lesern begrüsst werden.

INGRID TICHÁ (*Praha*)

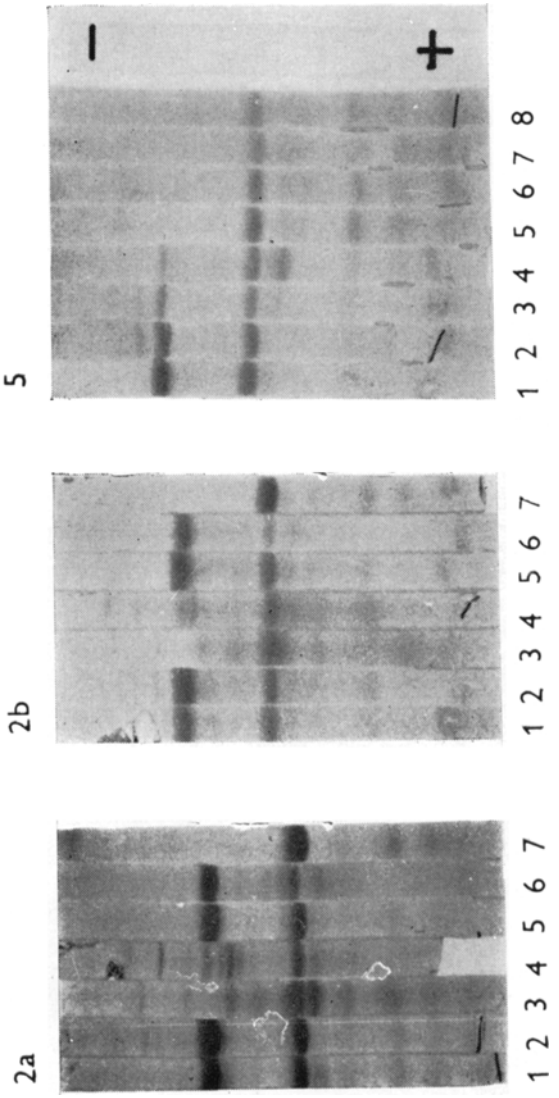

Fig. 2. Patterns of SDS-polyacrylamide gel electrophoresis of untreated a) and betamercaptoethanol treated (b) proteins from seeds of *Canavalia brasiliensis*. — 1: Full Extract; 2: Globulin; 3: Albumin; 4: Fraction F 0/50; 5: Fraction F 50/90; 6: Fraction P_I; 7: Fraction PIII.

Fig. 5. Patterns of SDS-polyacrylamide gel electrophoresis of betamercaptoethanol treated Full Extracts of cotyledons of germinating seeds from *Canavalia brasiliensis*. — 1: quiescent; 2: 1 day; 3: 3 days; 4: 5 days; 5: 7 days; 6: 9 days; 7: 12 days; 8: 15 days.

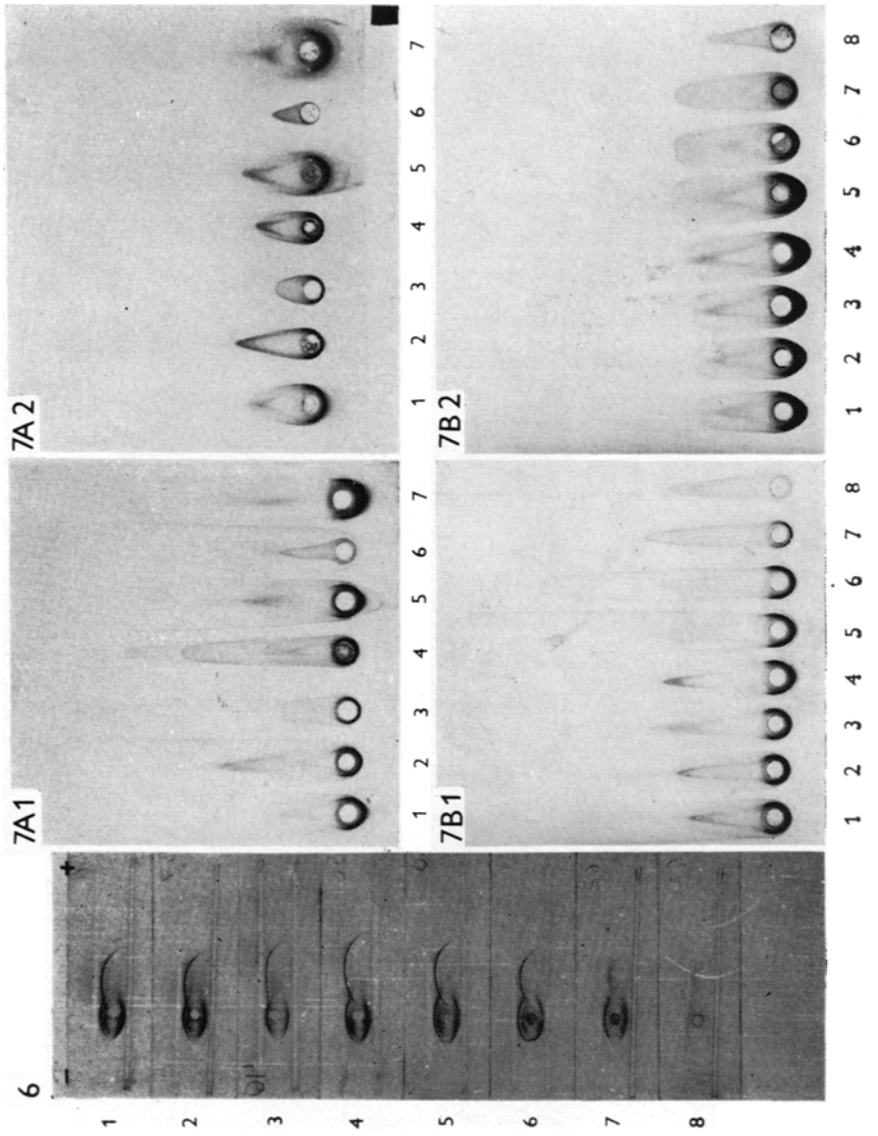

Fig. 6. Patterns of agarose immunoelectrophoresis of Full Extracts from cotyledons of germinating seeds of *Canavalia brasiliensis*. — 1: quiescent; 2: 1 day; 3: 3 days; 4: 5 days; 5: 7 days; 6: 9 days; 7: 12 days; 8: 15 days. The upperthrough contained anti-Full Extract serum, the lowerthrough contained anti-P_{III} serum.

Fig. 7 A. Patterns of rocket immunoelectrophoresis in agarose gel containing anti-Full Extract (1) and anti-P_{III} antisera (2), of proteins from quiescent seeds of *Canavalia brasiliensis*. — 1: Full Extract; 2: Globulins; 3: Albumins; 4: F 0/50; 5: F 50/70; 6: P_I; 7: P_{III}. — 7 B. Patterns of rocket immunoelectrophoresis in agarose gel containing anti-Full Extract (1) and anti-P_{III} (2) sera, of Full Extracts from cotyledons of germinating seeds of *Canavalia brasiliensis*. — 1: quiescent; 2: 1 day; 3: 3 days; 4: 5 days; 5: 7 days; 6: 9 days; 7: 12 days; 8: 15 days.