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Isolation, Characterization, and Biological Activities of Five Mitogens from Pokeweed†

Myron J. Waxdal

ABSTRACT: Salt extracts of *Phytolacca americana,* pokeweed or pigeon berry, have been fractionated by a new procedure to yield five separate proteins, designated Pa-1 through Pa-5. These proteins have distinct physicochemical properties, different biological activities, and appear at different times of the year. Pa-1 is the most potent hemagglutinin, a potent mitogen, and differs considerably from the other proteins in amino acid composition and in that it appears to be a polymer of 22,000 mol wt subunits. The four other proteins appear to be monomers and are unusual in that half-cystine, Asx, Glx, and glycine account for about 60% of their amino acid residues. The predominant proteins, Pa-2 and Pa-4, contain 25 and 20 disulfide bonds, respectively, bind to the same receptors on erythrocytes, and are both mitogenic for murine spleen cells. However, Pa-2 is a hemagglutinin while Pa-4 is not. Pa-3 is a minor component resembling Pa-2. Pa-5 is also a minor component during most of the year and resembles Pa-4. All of these proteins are mitogenic over an unusually wide range of protein concentrations.

A variety of plant proteins have proven to be useful tools for the study of cell surfaces and the induction of mitosis in lymphoid cells (Sharon and Lis, 1972). Many of these proteins have been detected by their ability to induce mitosis or cause hemagglutination, but only a few have been purified or described in detail. One of the most potent plant mitogens has been isolated from *Phytolacca americana* (pokeweed or pigeon berry). The juice of berries from this plant has been found to cause mitosis of lymphoid cells *in vivo* after ingestion or systemic exposure (Barker *et al.,* 1966) and extracts of other portions of the plant were also found to induce mitosis *in vivo* and *in vitro* (Farnes *et al.,* 1964; Barker *et al.,* 1965).

Extracts of pokeweed have been fractionated previously and a single protein with both hemagglutinating and mitotic activities has been isolated (Borjeson *et al.,* 1966; Reisfeld *et al.,* 1967; Chessin *et al.,* 1966; Douglas *et al.,* 1967). In contrast to other soluble mitogens, such as concanavalin A and phytohemagglutinin which are mitogenic for only the T (thymus dependent) class of lymphocytes (Greaves and Roitt, 1968; Janossy and Greaves, 1971), extracts of *Phytolacca americana* have been reported to be mitogenic for both thymus-dependent (T) and thymus-independent (B) lymphocytes (Janossy and Greaves, 1971, 1972; Stockman *et al.,* 1971; Greaves and Bauminger, 1972; Greaves *et al.,* 1972).

In view of the biological importance of these activities, extracts of pokeweed roots have been fractionated by other methods and not one, but five proteins with different mitogenic activities have been found. This paper describes the isolation, characterization, and biological activities of these five proteins. It is evident from these and other studies (Sharon and Lis, 1972) that a single plant species may contain a variety of lectins with both large and small differences in their physicochemical properties and biological activities.

Materials and Methods

Pokeweed roots were collected periodically throughout the year from a single group of plants in Montgomery County, Md. Roots were also harvested from other sites in Montgomery County and in Westchester County, N. Y.

Ground roots were extracted with phosphate-buffered saline[1] (PBS, 310 mosm, pH 7.4) and the extract was dialyzed

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[1] Abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSS, Hanks balanced salt solution.

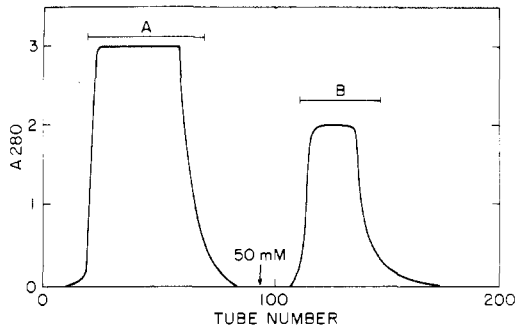

FIGURE 1: Fractionation of 1 l. of dialyzed salt extract of *Phytolacca americana* roots on hydroxylapatite. Column dimension, 5 × 30 cm; volume per tube 10 ml. The initial eluent was 5 mM potassium phosphate (pH 7.8). The arrow indicates a change in eluent to 50 mM potassium phosphate (pH 7.8).

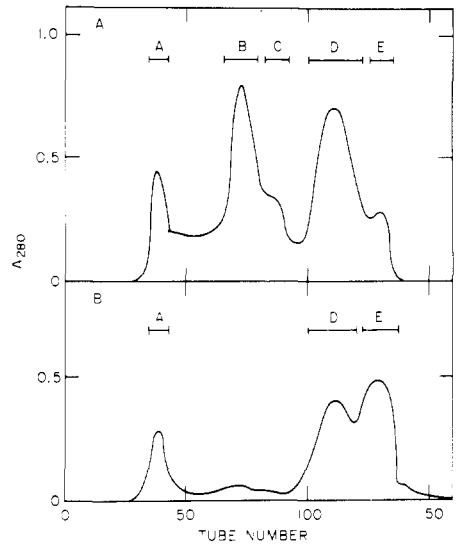

FIGURE 2: Fractionation of 50 mg of pool B (Figure 1) on Sephadex G-75 in 1 M propionic acid. Column dimensions, 2.5 × 90 cm; volume per tube, 3 ml. (A) Roots harvested in late autumn; (B) roots harvested in winter.

against water. The clear supernatant was decanted from a brown precipitate and was found to contain the hemagglutinating and mitogenic activities. This solution was chromatographed on hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories) in 5 mM potassium phosphate (pH 7.5). The major portion of the dissolved material was not retained on the column. After the effluent absorbancy at 280 nm had returned to base line, a single fraction was eluted with 50 mM potassium phosphate. This material contained all of the hemagglutinating and mitogenic activity. If the ground root is extracted with 5 mM potassium phosphate (pH 7.5) or with water (instead of PBS), material is extracted which markedly slows or stops the flow rate of the subsequent hydroxylapatite column. Extraction with PBS eliminates the extraction of much of the material which would be soluble only at low salt concentrations and extensive dialysis against water then precipitates much of the material soluble only at higher salt concentrations. The five proteins isolated are soluble at either of the salt concentrations used.

Gel Filtration. Sephadex G-75 and G-200 were equilibrated with PBS, 1 M propionic acid, or 0.1% NH_4OH and poured into 2.5 × 90-cm columns. Samples (50–100 mg) were dissolved in 2–5 ml of the appropriate buffer, applied to the column, and eluted with the same buffer. The effluent was monitored at 280 nm.

Amino Acid Analysis. Samples for amino acid analysis were hydrolyzed in metal-free 6 N HCl at 110° *in vacuo* for 24, 48, or 72 hr. The analyses were performed according to the technique of Spackman *et al.* (1958) as previously described (Edelman *et al.*, 1968). Methionine and half-cystine values were determined on unalkylated samples by the performic acid oxidation method of Moore (1963). Half-cystine values were also determined as [^{14}C]CM-cystine by amino acid analysis and by scintillation counting after complete reduction and alkylation with [1-^{14}C]iodoacetamide under the conditions described by Waxdal *et al.* (1968). Tryptophan values were estimated by the spectrophotometric method of Goodwin and Morton (1964).

Analysis of NH_2- and COOH-Terminal Residues. Qualitative identification of NH_2-terminal amino acid residues was performed by the dansyl method of Gray (1967). The dansyl-amino acids were separated by two-dimensional thin-layer chromatography on polyamide plates (Woods and Wang, 1967; Gottlieb *et al.*, 1970). Quantitative identification of the NH_2-terminal and COOH-terminal residues was performed by digestion with leucine aminopeptidase and with carboxypeptidase A and B (Worthington, Freehold, N. J.) as previously described (Gottlieb *et al.*, 1968).

Carbohydrate Analysis. Assays for neutral carbohydrate were performed on each of the five proteins by the phenol–sulfuric acid method (Ashwell, 1966). Some variation in the carbohydrate content of the proteins was observed from one preparation to another. This was attributed to bound carbohydrate or to leaching from the Sephadex columns. Consistent minimal values were obtained after subjecting the proteins to gel filtration in 0.1% NH_4OH and these are reported in Table II.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis in sodium dodecyl sulfate was carried out by the method of Weber and Osborn (1969) and by the method of Laemmli (1970).

Molecular Weight Determination. A Spinco Model E ultracentrifuge equipped with interference optics and automatic temperature control was used to determine the molecular weights of the proteins. The high-speed sedimentation equilibrium experiments were performed in PBS by the method of Yphantis (1964). Uncertainty in molecular weight due to errors in the determination of the concentration distribution in the cell was estimated to be about 5%. Uncertainty in the calculated partial specific volumes (McMeekin *et al.*, 1949) may introduce further errors of about 5%.

Extinction coefficients were determined on 1% solutions of Pa-2 and Pa-4 in H_2O. The path length was 10 mm and the absorbancy at 280 nm was measured with a spectrophotometer.

Iodination with ^{125}I. Iodinated proteins were prepared by the method of Greenwood *et al.* (1963) using chloramine-T. The iodinated proteins were dialyzed extensively against phosphate-buffered saline (PBS) to remove unbound iodine. The specific radioactivity of each protein was determined by counting the γ radiation and performing amino acid analysis on aliquots. The specific activity of Pa-2 and Pa-4 was 1.5×10^{-8} and 1.3×10^{-8} cpm/molecule, respectively.

Binding Studies. The number of protein molecules bound to human erythrocytes and murine splenocytes was determined using ^{125}I-labeled proteins; 100-μl samples, containing known concentrations of ^{125}I-labeled protein, were added to 10^6 or 5×10^8 fresh cells in 100 μl of PBS, which in some assays was also 5% in bovine serum albumin, and the solutions were mixed in a 12 × 125 mm test tube. After 45-min incubation at 0° the samples were diluted with 5 ml of PBS and centrifuged and the supernatant was discarded. This washing procedure was re-

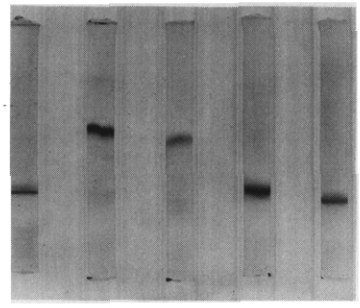

FIGURE 3: Polyacrylamide gel electrophoresis in SDS by the method of Laemmli (1970). The samples in order are the rechromatographed pools A, B, C, D, and E from Figure 2A. Migration is from top to bottom.

peated three times and the cells were transferred to a new tube. The cells were resuspended in 1 ml of PBS and counted for ^{125}I. All experiments were carried out in duplicate and the arithmetic average was used in the calculations. All experimental points were within ±10% of the mean.

Hemagglutination Assays. The protein to be assayed was dissolved in PBS (1 mg/ml) and twofold serial dilutions were made; 25-μl aliquots of PBS or a solution of the inhibiting protein were added to 25-μl aliquots of the serial dilutions in 500-μl plastic cups. A 25-μl suspension of human erythrocytes, or murine splenocytes (1×10^8 cells/ml), in PBS was added to each cup. The resulting 75-μl suspension was mixed well and allowed to settle at room temperature for 3 hr. Agglutination titres were determined by eye.

Cell Preparation. ERYTHROCYTES. Human blood was obtained from volunteers by venipuncture, using EDTA as the anticoagulant. The blood was mixed with an equal volume of PBS and the cells were spun down. This washing procedure was repeated twice. The cells were counted with a haemocytometer and diluted to 1×10^8 cells/ml in PBS.

SPLEEN CELLS. BALB/c mice were sacrificed by cervical dislocation and the spleens removed sterilely. The spleens were then forced through a sieve into Hanks balanced salt solution (BSS). The clumps were allowed to settle and the supernatant was transferred to a sterile plastic centrifuge tube. For mitotic studies the cells were washed two times with Hanks BSS and finally with RPMI-1640 (GIBCO). The cells were counted and diluted to 4×10^6 cells/ml with RPMI-1640. For the agglutination and binding studies the spleen cells were washed with Hanks BSS, 0.83% NH_4Cl to lyse the erythrocytes (Boyle, 1968), and Hanks BSS and diluted to 1×10^8 cells/ml in PBS.

Mitogenic Assay. The culture medium was composed of 100 ml of RPMI-1640, 7.5 ml of heated fetal calf serum, 1 ml of 3% glutamine, and 1 ml of a solution containing 10,000 units of penicillin and 10 mg of streptomycin; 1 ml of this media, containing a known amount of the lectin, was added to sterile 12 × 75 mm plastic snaptop culture tubes (Falcon). In a series of control experiments, bovine serum albumin was used in place of the pokeweed lectins; 0.5 ml of the cell suspension (2×10^6 cells) was added and the cultures were incubated at 37° under 5% CO_2. After 48 hr 0.1 ml of RPMI-1640 containing 1 μCi of [^{3}H]thymidine (specific activity 1.9 Ci/mmol), was added and the cells were cultured 24 hr longer. The cells were harvested using a Millipore filter (Robbins *et al.*, 1972) and washed with PBS, 10% trichloroacetic acid, and ethanol. The filters were placed in Aquasol (New England Nuclear) and the radioactivity was determined with a scintillation counter. All assays were performed in triplicate and the arithmetic average value was used for each data point. As measured by trypan blue exclusion, about one-fourth of the cell population died during the first 24 hr of culture, the cell number then remained relatively constant up to 72 hr.

TABLE I: Molecular Weights.

Protein	Molecular Weight	
	SDS Gel	Ultracentrifuge
Pa-1	22,000 ± 3300	
Pa-2	31,000 ± 4600	28,000 ± 2800
Pa-3	25,000 ● 3700	
Pa-4	21,000 ± 3200	21,000 ± 2100
Pa-5	19,000 ± 2900	

Results

The hemagglutinating and mitogenic activities present in the extract of pokeweed root were recovered in fraction B from preliminary fractionation on hydroxylapatite (Figure 1). This material was dialyzed against water, lyophilized, redissolved in 1 M propionic acid or PBS, and subjected to gel filtration on Sephadex G-75.

The eluent profile varied considerably with the time of year at which the roots were harvested. Figure 2A and B shows the profiles obtained with roots harvested in the early autumn and winter, respectively. The five fractions shown in Figure 2A were pooled and lyophilized; with the exception of fraction A they were resubjected to gel filtration under the same conditions, or in phosphate-buffered saline, to yield single peaks. Fraction A was composed of several proteins, most of which were insoluble in 0.1% NH_4OH. Extraction with this solvent resulted in the separation of a mitogenic protein from this mixture, which was purified by gel filtration on Sephadex G-200 in the same solvent.

The bulk of the material isolated from pokeweed roots (1 kg) was contained in fractions B (290 mg) and D (277 mg). The remaining recovered material was found in peaks A (184 mg), C(19 mg), and E (48 mg). The amount of material in each fraction varied markedly with the season of the year in which the roots were harvested.

Polyacrylamide gel electrophoresis in SDS (Figure 3) of the material isolated by gel filtration of fractions A–E (Figure 2A) showed single bands indicating that each fraction contained a single protein. These proteins were designated Pa-1 through Pa-5, respectively, from the initials of *Phytolacca americana* and the order of their elution from the Sephadex column. The gel electrophoresis shown in Figure 3 were performed according to Laemmli (1970) in order to determine the presence or absence of contaminants or multiple polypeptide chains. The approximate molecular weights (Table I) of these proteins were determined by the method of Weber and Osborn (1969) which gave more reproducible results, but does not show contaminants as readily as the Laemmli procedure. The same molecular weights were obtained with or without prior reduction and alkylation of the proteins. More accurate estimations of the molecular weight of the two major components Pa-2 and Pa-4 (fractions B and D, Figure 2A) were made by high-speed equilibrium sedimentation in phosphate-buffered saline (Table I).

The amino acid composition and carbohydrate content of each of these five proteins are given in Table II. Because of the large number of half-cystine residues in four of these proteins,

TABLE II: Amino Acid Compositions.[a]

Amino Acid	Pa-1	Pa-2	Pa-3	Pa-4	Pa-5
Asx	22.4	33.3	31.6	21.7	21.6
Thr[b]	12.1	10.5	12.5	8.0	7.0
Ser[b]	16.9	19.9	15.9	14.3	11.1
Glx	21.6	34.4	32.3	27.3	26.6
Pro	9.6	10.6	6.3	4.8	8.2
Cys/2[e]	7.8	49.7	42.3	38.9	29.9
Gly	21.8	41.9	36.3	35.1	25.6
Ala	15.2	6.9	5.7	5.2	4.6
Val[c]	17.0	11.2	11.6	6.3	2.2
Met[d]	3.0	0	0	1.7	2.1
Ile[c]	9.3	1.1	1.4	0.4	0.4
Leu	14.5	8.5	8.8	7.0	4.6
Tyr	8.0	5.5	5.8	9.2	11.5
Phe	6.8	7.2	6.5	3.1	2.7
His	3.9	7.5	7.1	4.3	3.5
Lys	11.7	7.0	6.9	7.6	8.3
Arg	5.3	12.3	6.7	7.5	10.7
Trp[f]		8.1		7.8	
CHO[g]	1.8	8.4	12.5	5.1	2.9

[a] Values are expressed as residues/mole, based on the molecular weights given in Table I. [b] Extrapolated to 0 time. [c] 72-hr hydrolysate. [d] Determined as methionine sulfone. [e] See Table III. [f] Determined by the method of Goodwin and Morton (1964). [g] Total residues of neutral carbohydrate expressed as galactose equivalents (Ashwell, 1966).

and because it is frequently difficult to obtain accurate values for this residue by acid hydrolysis, this amino acid was determined by four methods: acid hydrolysis of the native proteins, of the performic acid oxidized proteins, of the reduced and alkylated proteins, and by incorporation of [^{14}C]iodoacetamide after reduction in guanidine-HCl. The results of these four methods are given in Table III and agree within 0.8 residue for Pa-1 and within 1.3 residues for Pa-2 through Pa-5. The major proteins, Pa-2 and Pa-4, contained 50 and 39 residues of half-cystine. In the absence of reducing agents, iodoacetamide was not incorporated into Pa-2 and Pa-4 in 6 M guanidine hydrochloride, suggesting that there are no accessible free sulfhydryl groups in either protein and that there are 25 disulfide bonds in each molecule of Pa-2 and 19–20 in Pa-4.

The extinction coefficients of Pa-2 and Pa-4 were determined in H_2O at 280 nm using a 10-mm path length. Values of 21.4 and 21.1 were calculated for 1% solutions of Pa-2 and Pa-4. These results are in accord with the amino acid compositions of the two proteins.

Both Pa-2 and Pa-4, as isolated by this procedure, were resistant to digestion with trypsin, chymotrypsin, pepsin, leucine aminopeptidase, carboxypeptidase A, and carboxypeptidase B. After complete reduction and alkylation both proteins were insoluble at neutral pH values and remained resistant to these enzymes, except that they could be digested with pepsin in 5% formic acid. Further attempts to determine the amino terminal residue were performed by the dansyl method and none of the five proteins yielded a free amino terminal residue.

TABLE III: Half-Cystine Residues.

Method	Protein				
	Pa-1	Pa-2	Pa-3	Pa-4	Pa-5
Acid hydrolysis	7.0	48.6	41.3	37.6	29.7
Oxidation	7.8	49.7	42.3	38.8	29.9
CM-Cys					
Hydrolysis	7.7	49.4	42.0	38.6	
Radioactivity		49.7		38.9	

TABLE IV: Biological Activities.

Sample	Hemagglutination[a] (ng/ml)	Mitogenesis[b] (μg/ml)
Extract	41,000[c]	
Pa-1	150	10–100
Pa-2	310	1–100
Pa-3	1,250	10–100
Pa-4	>166,000	50–1000
Pa-5	2,500	50–500

[a] Lowest protein concentration producing hemagglutination. [b] Concentration range for optimal mitogenic stimulation. [c] Total protein content of saline extract of *P. americana* root.

The hemagglutination titers (Table IV) of these five proteins were determined using human blood of type A, B, AB, and O. Several blood samples were used, including Rh+ and Rh−, for each blood type. No significant differences in hemagglutination titer were observed. Pa-1 was the most potent hemagglutinin and Pa-5 was the weakest. Pa-4 did not give a consistent result even at high concentrations (170,000 ng/ml) and therefore was not considered to be an agglutinin for human erythrocytes. Pa-4 inhibited hemagglutination by Pa-2 and Pa-3, but did not effect hemagglutination by Pa-1. In the presence of 25 μg/ml of Pa-4, the end point for agglutination by Pa-2 increased from 310 to 20,500 ng/ml. To investigate the inhibition phenomenon further, binding studies were carried out using ^{125}I-labeled Pa-2 and Pa-4 (Figure 4). The results indicate that Pa-2 and Pa-4 bind to the erythrocyte with equal avidity and to the same number of receptors. In order to determine whether or not the two proteins bound to the same sites, competitive binding stud-

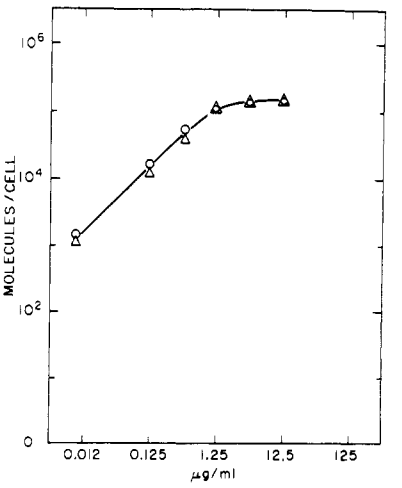

FIGURE 4: The binding of ^{125}I-labeled proteins to human erythrocytes as a function of protein concentration. Cells (10^6 and 5×10^8) were incubated with the lectin at 0° for 45 min. The cells were washed and counted as described in the text (Pa-2, ○; Pa-4, △).

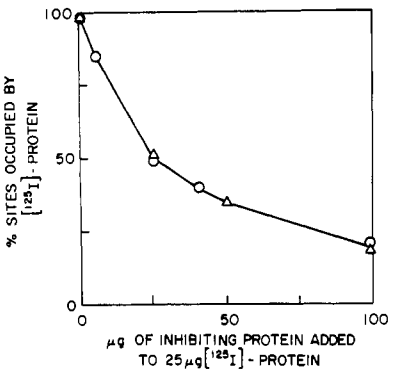

FIGURE 5: The competitive binding of Pa-2 and Pa-4 to human erythrocytes; 25 μg of [^{125}I]protein was mixed with the indicated amount of the inhibiting protein and the assay performed as described in the text. The binding of [^{125}I]Pa-2 in the presence of Pa-4 (○); the binding of [^{125}I]Pa-4 in the presence of Pa-2 (△).

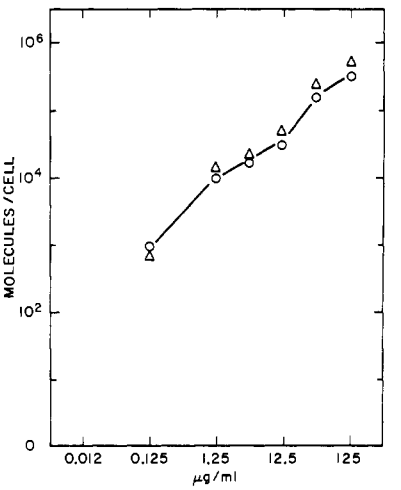

FIGURE 6: The binding of ^{125}I-labeled proteins to murine splenocytes as a function of protein concentration. Cells (10^6 and 5×10^8) were incubated with the lectin at 0° for 45 min. The cells were washed and counted as described in the text (Pa-2, ○; Pa-4, △).

ies between Pa-2 and Pa-4 were performed (Figure 5). In each experiment 25 μg of ^{125}I-labeled protein was used; this amount was at least ten times that required to occupy 100% of the sites for the protein in the absence of the competing protein (Figure 4). Varying amounts of the competing protein were mixed with the labeled protein and the binding assays performed as described above. The results (Figure 5) clearly indicate that Pa-2 and Pa-4 compete for binding sites on the erythrocyte.

Reflecting their hemagglutination activities, Pa-2 agglutinated murine splenocytes down to a concentration of 310 ng/ml and Pa-4 did not cause agglutination. In the presence of 166 μg/ml of Pa-4, the end point of agglutination by Pa-2 rose from 310 to 83,000 ng/ml, again indicating competitive binding to the cell. The number of molecules of Pa-2 and Pa-4 which could bind to murine splenocytes were also estimated using [^{125}I]proteins (Figure 6). Although saturation of the receptors was not achieved, the results show that Pa-2 and Pa-4 bind to similar numbers of sites on the splenocyte and with similar avidity.

The mitogenic activity of the five proteins for murine splenocytes was determined over a wide concentration range (Table IV, Figure 7). Up to 1000 μg/ml of added bovine serum albumin had no effect on the uptake of [^{3}H]thymidine by cells in the control cultures. The most potent mitogens (Pa-1, Pa-2, and Pa-3) showed broad maxima of cell stimulation over a concentration range from less than 5 to 100 μg/ml (Figure 7). Pa-4 and Pa-5 showed maximum mitogenic activity between 50 and 500 μg/ml but the extent of stimulation was less than that caused by Pa-1, Pa-2, or Pa-3.

Discussion

The purification procedure developed in the present study isolates a larger number of mitogenic proteins than previous methods and also avoids the need for the centrifugation, heat coagulation, ethanol or trichloroacetic acid precipitation, and preparative polyacrylamide electrophoresis steps used previously (Borjeson *et al.*, 1966; Reisfeld *et al.*, 1967).

The combination of data on molecular weight, amino acid composition, carbohydrate content, and biological activities indicate that each of these mitogens is a separate protein. A comparison of properties indicated that Pa-2 is similar or identical with the previously isolated pokeweed mitogen (Borjeson *et al.*, 1966; Reisfeld *et al.*, 1967).

The results from gel filtration with different eluents and polyacrylamide gel electrophoresis in SDS suggests that Pa-1 exists in aqueous solution as a polymer of subunits with molecular weights of 22,000, which are not linked by disulfide bonds.

A comparison of the molecular weights obtained from the SDS gels and the ultracentrifuge in aqueous solution (Table I) suggests that Pa-2 and Pa-4 both exist as monomers; the state of aggregation of Pa-3 and Pa-5 is less well defined, but they also appear to be monomers. Pa-1 is distinctive among these proteins in that it contains only 3.4% half-cystine. The amino acid compositions of the other four proteins are unusual in that each contains about 18% half-cystine. No free sulfhydryl groups were detected in Pa-2 and Pa-4, suggesting that all of these residues are involved in disulfide bridges, approximately 25 for each molecule of Pa-2 and 19 or 20 for Pa-4.

The amino acid compositions of Pa-2, Pa-3, Pa-4, and Pa-5 are similar to each other and in addition to half-cystine have large amounts of Asx, Glx, and Gly; these four amino acids account for 60% of the residues in each protein. In contrast, these four amino acids account for only 32% of Pa-1. The smaller differences between the compositions (Table II) of Pa-2, Pa-3, Pa-4, and Pa-5 are important in determining the possible relationships between these proteins, because naturally occurring fragments of another mitogen, concanavalin A, have been re-

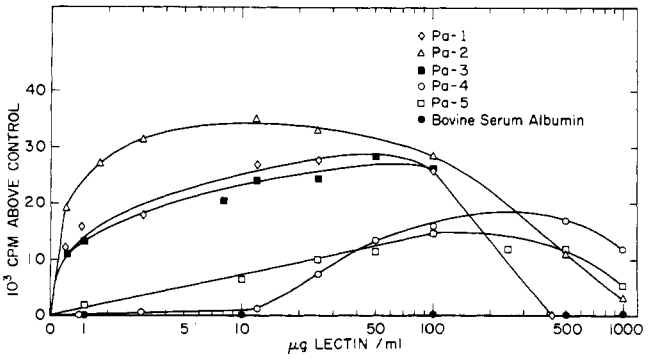

FIGURE 7: The incorporation of [^{3}H]thymidine into murine splenocytes as a function of the mitogenic protein concentration. The incorporation is expressed as the cpm above the control (about 4000 cpm). A second control containing various amounts of bovine serum albumin in place of the mitogens is shown. All data points are the arithmetic average of triplicate cultures from several experiments.

ported (Wang *et al.*, 1971). The presence of more tyrosine in Pa-4 than in Pa-2 and the presence of methionine in Pa-4 which is absent from Pa-2 indicates that Pa-4 is not a fragment of the larger Pa-2. The presence of more proline, tyrosine, and arginine in Pa-5 indicates that it is not a fragment of Pa-4, and the presence of methionine further indicates that it is not a fragment of Pa-2 nor of Pa-3. From their amino acid compositions, the possibility that Pa-3 is a fragment of Pa-2 cannot be excluded; however, Pa-3 contains more carbohydrate (Table II) than Pa-2, suggesting that it is not such a fragment.

A comparison of biological activities (Table IV) indicates that three of the proteins (Pa-1–Pa-3) are potent mitogens and hemagglutinins. Pa-5 is a weak mitogen and hemagglutinin. Pa-4 is also a weak mitogen, but is not a hemagglutinin under these conditions and blocks agglutination by Pa-2 and Pa-3. This suggests that Pa-2, Pa-3, and Pa-4 bind the same sites on cell surfaces. In contrast, hemagglutination by Pa-1 is not blocked by Pa-4, suggesting that Pa-1 binds to a different site. The polymeric state of Pa-1 may correlate with the observation that it is the most potent hemagglutinin.

Pa-2 and Pa-4 are the two predominant mitogens in pokeweed roots. The binding studies indicate that at saturation of the binding sites, there are about 2×10^5 receptors per erythrocyte for Pa-2 and Pa-4 (Figure 4) and show that at equal concentrations equal amounts of these two proteins are bound. Along with the competitive binding studies (Figure 5), these data strongly suggest that Pa-2 and Pa-4 bind to the erythrocyte with the same avidity and to the same receptors, or to closely adjacent receptors.

Unlike exclusive T cell mitogens the pokeweed mitogens are active over a wide concentration range. Pa-2 is mitogenic from below 0.5 μg/ml to over 500 μg/ml, a range of three orders of magnitude. The other pokeweed mitogens described herein are also active over large concentration ranges. Concanavalin A (Janossy and Greaves, 1972; Stobo *et al.*, 1972) and phytohemagglutinin (Janossy and Greaves, 1971), for example, have much narrower dose response curves. In contrast, the B cell mitogen lipopolysaccharide (Peavy *et al.*, 1970) also has a broad dose-response curve and hence may act in a fashion similar to the pokeweed mitogens. Even though the physicochemical properties of Pa-1 and Pa-3 were different (Tables I–III) their dose-response curves are similar (Figure 7). These two mitogens reached half-maximal activity at about 1 μg/ml and were fully active to 100 μg/ml. Pa-2 showed half-maximal activity below 0.5 μg/ml and reached maximal activity at about 5 μg/ml. Janossy and Greaves (1971) have reported a similar maximum for the activity of the lectin corresponding to Pa-2. However, Reisfeld *et al.*, (1967), using human peripheral lymphocytes, found maximal stimulation to begin below 1 μg/ml.

The possibility that the mitogenic activities of Pa-4 and Pa-5 are due to low level contamination (*ca.* 1%) by one or more of the more potent mitogens cannot be excluded. Although the present studies do not shed light on this point, contamination seems unlikely because the shape of the dose-response curves are not consistent with a constant percentage of contamination by one of the other mitogens.

The mechanism of stimulation by plant mitogens remains unknown and the possibility that there are several mechanisms cannot be excluded. The present studies make available a number of new proteins in reasonably pure state for studies on mitogenesis in both T and B cells (Waxdal, 1974, unpublished data). Because several of the pokeweed mitogens exist as monomers, it may be possible to test whether cross-linking of several identical or nonidentical receptors on the cell surface is necessary for lymphocyte stimulation.

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Use of Benzoylated Cellulose Columns for the Isolation of Poly(adenylic acid) Containing RNA and Other Polynucleotides with Little Secondary Structure†

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ABSTRACT: Chromatography on benzoylated cellulose columns was found to be a simple and efficient procedure for the isolation of polynucleotides which contain extended nucleotide sequences free of secondary structure. Included among the polynucleotides which could be selectively bound to the benzoylated cellulose were poly(A), poly(U), poly(A)-containing RNA, formaldehyde-treated ribosomal RNA, and denatured DNA. Various conditions of adsorption and elution of polynucleotides were investigated; these indicated that the mechanism of binding is a stacking interaction between the polynucleotide bases and the benzoyl groups of the benzoylated cellulose. Using this procedure, milligram quantities of poly(A)-containing RNA were isolated from the cytoplasmic and nuclear fractions of Ehrlich ascites cells. These RNAs were analyzed by zonal centrifugation and nucleotide composition, using both ultraviolet adsorption and [^{32}P]orthophosphate labeling for a comparative analysis, and shown to be free from detectable contamination with ribosomal RNA.

Recently, a number of procedures have been developed for the isolation of poly(A)-containing RNAs from eukaryotes. These procedures can be classified into two main groups, depending upon the principle used for the isolation. One group utilizes for the isolation hydrogen bonding between the poly(A) moiety of the RNA and poly(U) or poly(dT) immobilized on glass filter membranes, cellulose, or Sepharose (Edmonds and Caramela, 1969; Kates, 1970; Adesnik *et al.,* 1972; Sheldon *et al.,* 1972). The second group of procedures is characterized by the firm binding at high salt concentrations of these RNAs to a variety of substances including methylated albumin (Asano, 1965; Ellem, 1966; Roberts and Quinlivan, 1969), polystyrene (Lim *et al.,* 1969; Lim and Canellakis, 1970), filter membranes (Lee *et al.,* 1971; Brawerman *et al.,* 1972), and cellulose (Sullivan and Roberts, 1971, 1973; Kitos *et al.,* 1972). The binding of poly(A)-containing RNA to filter membranes and cellulose appears to require the presence of polyaromatic lignins in the cellulose (Sullivan and Roberts, 1973; DeLarco and Guroff, 1973). This suggested a common aromatic ring stacking mechanism of binding for these latter procedures (Kitos, 1973), and prompted this investigation of benzoylated cellulose as a possible reagent with high capacity and flexibility for the isolation of poly(A)-containing RNA.

Experimental Section

Materials. Poly(A), poly(U), and poly(C) were purchased from Miles Laboratories. Poly(A)·poly(U) was prepared by mixing equal molar mixtures of poly(A) and poly(U), 50 μg/ml in 1 mM magnesium acetate; molar extinction coefficients at 260 nm of 9400 for poly(A) and 8900 for poly(U) were assumed. STE buffer was 0.3 M NaCl–0.1 M Tris·HCl (pH 7.0)–0.001 M EDTA; STE/10 buffer was STE diluted 1:10 with water. The ribosomal RNA (rRNA[1]) used in Table I was cytoplasmic RNA precipitated with 1.5 M NaCl and passed through a benzoylated cellulose column in 1 mM magnesium acetate to remove poly(A)-containing RNA. Heterogeneous cytoplasmic RNA (hcRNA) and heterogeneous nuclear RNA (hnRNA) are defined operationally in this paper as those RNAs from the corresponding cellular fractions which were synthesized in the presence of 0.04 μg/ml of actinomycin D (Perry, 1962; Roberts and Newman, 1966; Penman *et al.,* 1968) and could be precipitated with 1.5 M NaCl (for a review of heterogeneous RNAs see Darnell, 1968 and Darnell *et al.,* 1973); the hcRNA and hnRNA in Table I were poly(A)-containing hRNAs isolated from benzoylated cellulose columns and rerun to determine binding capacity. Formaldehyde-treated rRNA was prepared by dissolving rRNA in STE/10 (100 μg/ml), making the solution 1 M in formaldehyde and heating the solution at 60° for 10 min; the RNA was precipitated by adjusting the NaCl concentration to 0.1 M, mixing the solution with an equal volume of isopropyl alcohol, and placing the solution at −20° overnight. Denatured DNA was prepared by dissolving calf thymus DNA (Calbiochem) in distilled water, heating the solution to 90° followed by quick cooling, and adjusting the solution to 1 mM in magnesium acetate. Mengovirus RNA was prepared as before (Sullivan and Roberts, 1973).

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[1] Abbreviations used are: rRNA, ribosomal RNA; hcRNA, heterogeneous cytoplasmic RNA; hnRNA, heterogeneous nuclear RNA; nRNA, nuclear RNA; cRNA, cytoplasmic RNA; STE buffer, 0.3 M NaCl-0.1 M Tris-HCl (pH 7.0)-0.001 M EDTA; STE/10 buffer, STE diluted 1:10 with water.