STUDIES ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES IN VITRO

I. Biological and Physicochemical Properties of the Pokeweed Mitogen

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Plant lectins with blood group specificity for mammalian red cells were first described more than 50 years ago (1). As part of this body of knowledge it is of special interest that in 1908 Landsteiner and Raubitschek (2) demonstrated the presence of a panhemagglutinin in saline extracts of the red kidney bean, *Phaseolus vulgaris*. In succeeding decades an increasing number of plant lectins were identified. However, it was only in 1960 that the lectin from *Phaseolus vulgaris*, phytohemagglutinin (PHA) was recognized as being distinctive by virtue of its possessing the additional properties of leukagglutination (3, 4) and mitogenic activities for human peripheral blood lymphocytes (5–10). For a time it appeared that among an extensive spectrum of plant lectins PHA might be unique in this regard. However, Farnes *et al.* (11) have recently reported that extracts of *Phytolacca americana*, pokeweed (PWM), although apparently lacking hemagglutinating and leukagglutinating activity induced transformation of human lymphocytes in vitro. In this laboratory, further studies of the properties of the pokeweed extracts disclosed that this material did in fact possess hemagglutinating and leukagglutinating activities; moreover, it was ascertained that the properties of pokeweed differed from PHA in a number of important respects.

In this report we will present data on the biological and chemical properties of the pokeweed mitogen and in the subsequent papers, consider the unique cytologic, biochemical, and fine structural features of the PWM-transformed cells (12, 13).

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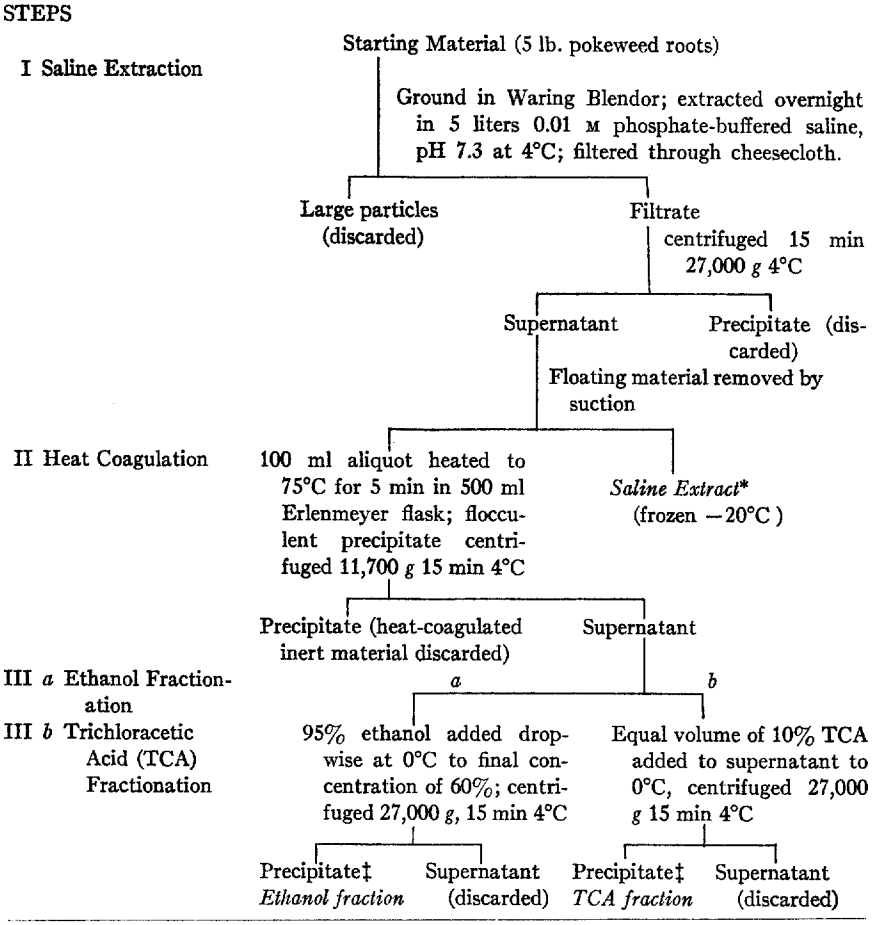
Materials and Methods

Preparation of Pokeweed Mitogen (PWM) Fractions.—Roots from the plant *Phytolacca americana*, pokeweed growing in the vicinity of Bethesda, were harvested in early fall. A

TABLE I
Fractionation Scheme for Pokeweed Mitogen



* Stored at −20°C for at least 2 months without demonstrable loss in mitogenic activity.
‡ Dried first over absolute ethanol, then over anhydrous ether.

diagrammatic representation of the procedure for preparing the various fractions is shown in Table I.

Calcium Phosphate (Hydroxylapatite) Column Chromatography.—The method of Main et al. (14) was used in preparation of the calcium phosphate. Precipitated calcium phosphate was boiled with calcium hydroxide and then allowed to sediment. To obtain an acceptable flow rate, only the crystals sedimenting during the first 10 min were used. A column (47 × 255

mm) was packed with the calcium phosphate crystals in 0.005 M phosphate buffer at pH 7.5, and washed with this same buffer for 24 hr prior to the addition of the sample.

The TCA fraction was dissolved in distilled water, centrifuged at 27,000 *g*, and the supernatant dialyzed for 24 hr against frequent changes of buffer. 300 ml of the dissolved TCA fraction (0.780 OD_{280} units/ml) was applied to the column. 10 ml fractions were collected at a flow rate of 70 ml/hr by stepwise elution with pH 7.5 phosphate buffer of the following molarities: 0.005 M (1000 ml), 0.05 M (1200 ml), and 0.5 M (1000 ml) and the relative protein concentration in each tube was estimated by measuring the absorption at 280 mμ. The eluted fractions were combined and dialyzed for 48 hr against frequent changes of distilled water and then lyophilized.

Polyacrylamide Disc Electrophoresis.—Disc electrophoresis on polyacrylamide gels was performed according to the procedure described by Reisfeld et al. (15) using a modified buffer system (16) without urea. Electrophoresis was carried out for 55 min in a 7.5% acrylamide gel at a constant current of 2.5 ma/tube. Protein bands were localized by fixing the gel in 5% TCA or by staining with amido black.

For the isolation and characterization of specific bands, duplicate gels, electrophoresed in parallel, were fixed with TCA. The R_f value for each band was determined from the relative position of the buffer front marked with bromphenol blue to that of the TCA-precipitated protein bands. Having determined the R_f value for each band, duplicate gels, not fixed with TCA, were then cut into 1 mm sections, and the material from each section eluted in buffer and tested in hemagglutination, leukagglutination, and mitogen assays.

Phytohemagglutinin (PHA).—The PHA used throughout this study was prepared according to the method of Börjeson et al. (17). For use in hemagglutination, leukagglutination, and mitogen assays PHA was dissolved in 0.01 M phosphate-buffered saline at pH 7.3.

Hemagglutination Assays.—

Test cells: Fresh human group A_1, A_2, B, and O red cells collected in ACD, were washed three times with 0.01 M phosphate-buffered saline at pH 7.3 and resuspended in the same buffer. A final 2% red cell suspension was used in all hemagglutination assays.

Saline titration: Serial twofold dilutions of 0.2 ml of phytomitogen were mixed with an equal volume of 4% red cell suspension, incubated for 2 hr at 37°C, and examined microscopically for agglutination.

Dextran titration: A 4% red cell suspension was made up in Dextran (6% in saline, Abbott Laboratories, North Chicago, Illinois). Reaction mixtures were prepared and read as in saline agglutination titration.

Titration with enzyme-treated cells: Trypsinized human red cells were prepared according to the method of Morton and Pickles (18), and ficin-treated red cells were prepared according to the method of Wiener and Katz (19). The final reaction mixture containing 0.2 ml of enzyme-treated red cells and 0.2 ml of phytomitogen was incubated at 37°C for 2 hr and read microscopically.

Mercaptoethanol Treatment of the Phytomitogens.—Equal volumes of phytomitogens and 0.4 M mercaptoethanol in phosphate-buffered saline at pH 7.3 were incubated overnight at 4°C. The reaction mixture was then dialyzed in the cold for 24 hr against frequent changes of 0.01 M phosphate-buffered saline at pH 7.3.

Periodate Treatment of the Phytomitogens.—Equal volumes of phytomitogen and 0.1 M sodium metaperiodate were incubated in the dark overnight at 4°C. The reaction mixture was then dialyzed in the cold against frequent changes of 0.01 M phosphate-buffered saline at pH 7.3.

Leukagglutination Assay.—Leukocyte suspensions for agglutination tests were prepared and examined according to the method of van Rood and van Leeuwen (20) with modifications as described by Chessin et al. (21).

Antisera to Pokeweed Mitogen (PWM) and Phytohemagglutinin (PHA).—Antisera to

PWM and PHA were prepared in rabbits. Adult New Zealand White rabbits each weighing 3 to 5 kg were injected subcutaneously in six sites on the back with 1.0 ml of complete Freund's adjuvant (85% Bayol F, 15% Arlacel A and 2.0 mg dried *Mycobacterium butyricum* H 37 Rv) emulsified with saline containing 2.0 mg of either crude PWM saline extract or the ethanol fraction of PHA (17). The animals were given bimonthly booster injections of 1.0 ml of incomplete Freund's adjuvant emulsified with 2 mg of mitogen.

Immunodiffusion and Immunoelectrophoresis.—Double diffusion studies were performed according to the method of Ouchterlony (22). Microimmunoelectrophoresis was carried out according to the technique of Grabar and Williams (23), in an 0.1 M veronal buffer pH 8.4 at 5 v/cm for a period of 45 min. Antibody diffusion was allowed to continue for 24 to 48 hr at room temperature. Slides were washed free of unreacted protein, dried, and stained with light green (24).

Lymphocyte Cultures for Mitogen Assay. Cultures were prepared from human peripheral blood lymphocytes separated on glass wool columns. Blood drawn from normal volunteers into Fenwal packs containing heparin was transferred to glass cylinders and the red cells were allowed to sediment by gravity at 37°C. The leukocyte-rich plasma was then passed through a glass wool column (100 ml syringe prepacked with 3 g of Pyrex glass wool filtering fiber 3950, Corning Glass Works, Corning, New York) and the eluate centrifuged for 10 min at 250 *g*. The cell pellet was washed once with Dulbecco's phosphate-buffered saline and the lymphocytes were counted and diluted in 85% Eagle's Spinner MEM (25) containing 0.02 mmole/ml fresh L-glutamine penicillin 100 units/ml, streptomycin 100 μg/ml and either 15% heat-inactivated calf serum or 15% autologous serum.

For all mitogen assays a standard inoculum of 3×10^6 lymphocytes in 5 ml of growth medium was incubated at 37°C for 72 hr in 16 × 125 mm screw cap disposable glass tubes (Demuth Glass Works, Inc., Parkersburg, West Virginia). The morphological and biochemical characteristics of freshly harvested lymphocytes from glass wool columns will be described in greater detail in the following paper (12).

Assay Methods.—Lymphocyte transformation was determined by the following methods; radioactivity assay of thymidine-H^3 incorporated into extracted DNA, enumeration of the per cent labeled cells in Giemsa-stained radioautographs and enumeration of the per cent transformed cells by phase and light microscopy.

Radioactivity assays were performed on lymphocyte cultures grown continuously in the presence of 1 μc thymidine-H^3/ml (TdR-H^3, specific activity, 1.9 c/mmole, Schwartz Bio-Research, Inc., Orangeburg, New York). After the appropriate incubation time, the cells were washed 3 times with 0.01 M phosphate-buffered saline at pH 7.3 centrifuged at 250 *g*, and then frozen at −20°C. After freezing and thawing, the disrupted cells were digested with 100 μg papain in a 1 ml solution of 0.05 M phosphate buffer at pH 7.0 containing 5 mM EDTA, 5 mM cysteine hydrochloride. The cell lysate was digested for 4 hr at 65 C in a Dubinoff water bath shaker. After digestion the suspension was adjusted to approximately pH 8 with 1 N sodium hydroxide. DNA was precipitated by addition of 2 ml of 1% cetyltrimethylammonium-bromide (CTAB). The tubes were then placed at room temperature overnight so that maximal DNA precipitation could take place (26). The precipitate, a water insoluble CTAB-nucleic acid complex, was centrifuged at 2000 *g* for 45 min, dissolved in a 1:1 mixture of CTAB and 1-propanol and added to Bray's solution (27), for counting in a liquid scintillation spectrometer (Packard 4000 Tri-Carb).

RESULTS

Properties of Different Pokeweed Fractions.—

Crude saline extract: The crude saline extract of PWM possesses the ability to agglutinate human adult and cord A, B, and O red blood cells, to agglutinate white blood cells and to transform human peripheral blood lymphocytes.

The hemagglutinin of PWM saline extract differs from PHA and most plant lectins (1) in that hemagglutination by PWM takes place optimally in the presence of high molecular weight substances; e.g., 6% dextran, PVP (polyvinylpyrolidone) or heterologous serum rather than in saline. Hemagglutination will also take place with PWM after pretreatment of the red cells with either trypsin or ficin. The PWM hemagglutinin is inhibited by 0.003 M EDTA, whereas similar concentrations of EDTA do not inhibit the human isoagglutinins. The leukagglutinin of PWM similarly is weak in the presence of saline, strong in the

TABLE II

Properties of PWM Fractions

Fraction	μg N/mg dry weight substance*	Agglutinin		Mitogen	
		End point for hemagglutination‡	Leukagglutination	End point for maximal TdR -H^3 incorporation‡	Per cent TdR-H^3 incorporation§ (transformation index)
Saline extract (step I)	99	2.5	++	n.d.‖	22
Heat coagulated saline extract (step II)	79	2.5	+++	n.d.	29
Ethanol precipitate (step III *a*)	—	3.1	+++	0.25	35
TCA precipitate (step III *b*)	118	2.5	+++	0.25	60
0.05 M late moving fraction (hydroxylapatite column)	69	150	±	2.5	56
0.05 M front moving fraction (hydroxylapatite column)	120	0.6	++	<0.25	56

* Kjeldahl determination.

‡ Expressed as μg dry weight of substance.

§ Transformation index = counts per minute (PWM)/counts per minute (PHA).

‖ Not determined.

presence of dextran or serum, and is inhibited by EDTA. Both hemagglutination and leukagglutination by the crude saline extract can occur using as little as 2.5μg of material. Heating the crude saline extract to 75°C precipitated much of the inert protein with a resultant albeit slight increase in mitogenic activity. The maximum transformation rate obtained with the crude saline extract (step I) was 22% while that of the heat-coagulated saline extract (step II) was 29% (Table II).

TCA and ethanol-precipitated fractions of the heat-coagulated saline extract: Precipitation of the heat-coagulated saline extract with 10% trichloracetic acid (TCA) gave almost a threefold increase in the transformation index as compared to the saline extract, whereas ethanol fractionation resulted in only a small increase in mitogenic activity (Table II). On the other hand, hemagglutinating activity was not increased by step II (heat coagulation),

step III *a* (ethanol fractionation) or step III *b* (TCA fractionation) (Table II). Maximum transformation rate by both these fractions was achieved with 0.25 μg of lyophilized substance. Absorption of the TCA fraction with either intact human red cells or stroma resulted in a loss of the hemagglutinating activity without any appreciable loss in leukagglutinating or mitogenic activities, while absorption with human leukocytes resulted in loss of all three activities.

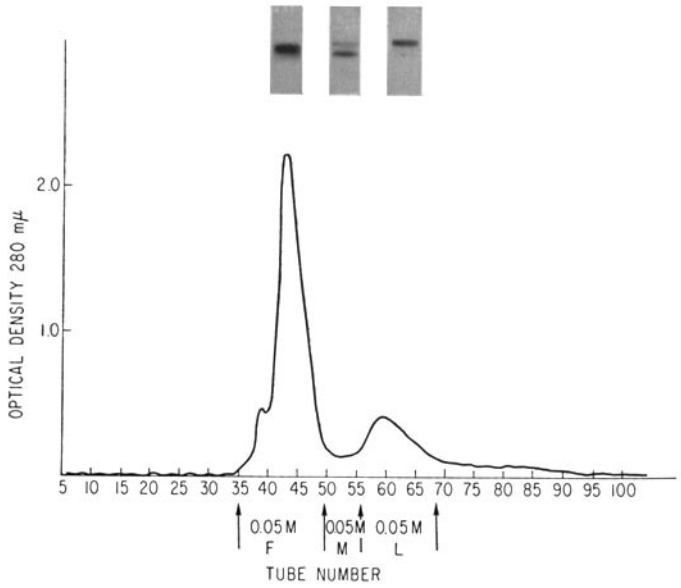


FIG. 1. Chromatographic pattern of the PWM (TCA fraction) eluted with 0.05 M phosphate buffer at pH 7.5. Superimposed is the electrophoretic pattern of the three different eluates (0.05 M (F), 0.05 M (M), and 0.05 M (L)) as seen after analytical separation on polyacrylamide gels. The prominent band (R_f 0.43) seen with the front moving fraction (F) is also seen to a lesser extent in middle (M), and late (L) moving fractions.

Fractions from column chromatography and polyacrylamide disc electrophoresis: Stepwise elution of the TCA-precipitated fraction from the calci um phosphate column with 0.005, 0.05, and 0.5 M phosphate buffer at pH 7.5 resulted in four peaks (OD_{280}). The 0.05 M eluate contained all three biological activities (Fig. 1) with more than 95% of all hemagglutinating, leukagglutinating, and mitogenic activities present in the front moving fraction (F), whereas the 0.05 M late moving fraction (L) contained less than 5% of the biological activity. There was no activity present in either the 0.005 or 0.5 M eluates.

Analytical separation of the various PWM fractions on polyacrylamide disc electrophoresis, as well as isolation of the bands and reelectrophoresis of gel slices revealed that the mitogen, hemagglutinin, and leukagglutinin were inti-

mately associated with a prominent homogeneous band with an R_f value of 0.43 (Fig. 2). All three activities were found to a lesser extent in the middle fraction and least in the late fraction (Table II). This correlated well with the prominence of the active band in the different fractions. The ultraviolet absorption spectrum of PWM (0.05 M front fraction) resembles that of PHA (Fig. 3). Maximum absorption was seen at 280 mμ. A secondary peak present at 290 mμ suggested that this substance was rich in tryptophane. Biologically inactive protein fractions from both PHA and PWM did not contain the peak at 290 mμ.

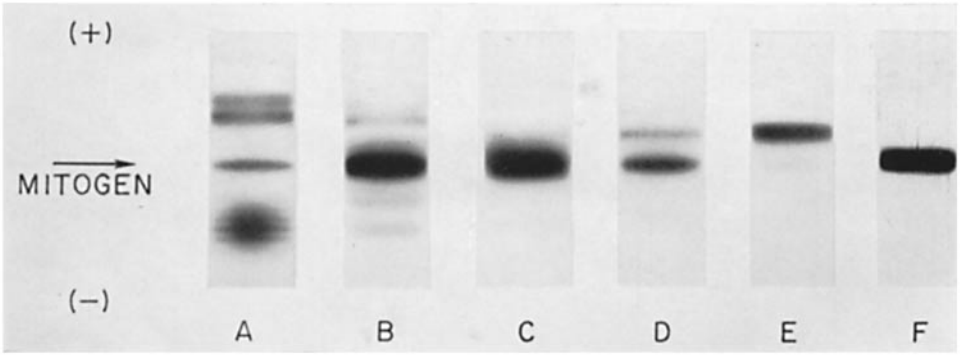


FIG. 2. Analytical separation of various PWM fractions on polyacrylamide disc electrophoresis. To each analytical gel 100 μg (nitrogen equivalent) of sample was applied. Electrophoresis was carried out for 55 min in a 7% gel at a constant current of 2.5 ma/tube. The gels were stained with amido black.

A, ethanol fraction; B, TCA fraction; C, D, and E, the fractions eluted with 0.05 M phosphate buffer pH 7.5 from the calcium phosphate column. C, represents the front moving (F) fraction; D, the middle moving (M) fraction; and E, the late moving (L) fraction; F, isolation and reelectrophoresis of the major component shown in B. This component with an R_f value of 0.43 contained all three biological activities as seen in A to F.

Double Diffusion and Immunoelectrophoresis Studies.—Double diffusion studies with rabbit anti-PHA against the crude pokeweed saline extract and PHA revealed a single line of partial identity (Fig. 4, L). On the other hand, rabbit antipokeweed serum did not give a precipitation line against the purified PHA (Fig. 4, M).

Immunoelectrophoresis performed with the crude saline extract of PWM and rabbit antipokeweed serum gave four lines with our saline preparation (Fig. 4, G) and eight lines with another preparation (Grand Island Biologicals, Grand Island, New York) (Fig. 4, F). There were as many as three cathodal and five anodal lines seen with the latter preparation.

During successive stages of purification, immunoelectrophoretic and double diffusion analysis with the rabbit anti-PWM serum was performed with each fraction as shown in Fig. 4, A to K. It is shown that there is a loss of the cross-

reactivity with PHA (as represented by the cathodal lines) at an early stage of purification (heat coagulation, step II) (Fig. 4, F to I). The most active mitogen, the 0.05 M front moving fraction gave a single precipitation line on immunoelectrophoresis and immunodiffusion (Fig. 4, C to K).

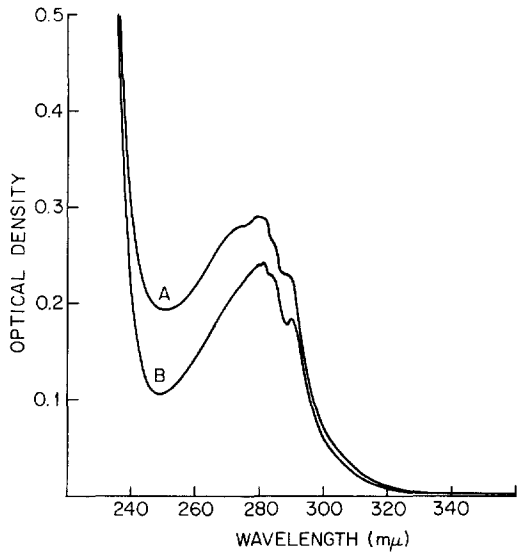


FIG. 3. Ultraviolet absorption spectra of PWM and PHA. 200 μg/ml of the purified PHA (17) and the 0.05 M front moving PWM fraction were dissolved in 0.01 M phosphate-buffered saline pH 7.3. The ultraviolet absorption spectra were determined in a Zeiss PMQ II spectrophotometer using a 1 cm silica cell. Curve A shows the spectrum for purified PWM (0.05 M front moving fraction (F)) with peaks at 280 and 290 mμ. Curve B shows the spectrum for purified PHA with peaks at the same wavelengths.

DISCUSSION

A study of the biological and chemical properties of pokeweed mitogen (PWM) revealed that it possessed several significant features which distinguished it from PHA. These features are summarized in Table III.

PWM agglutinated red cells optimally in the presence of high molecular weight substances or after pretreatment of red cells with proteolytic enzymes. It is of interest that the conditions optimal for PWM hemagglutination are similar to those described for the human incomplete isoagglutinins or 7S antibodies, while the conditions for PHA hemagglutination are similar to those described for the complete isoagglutinins or 19S antibodies (28). The difference in the hemagglutination pattern seen with these two mitogens may be related to the valency and the size of the molecules as has been postulated for the human isoagglutinins (29).

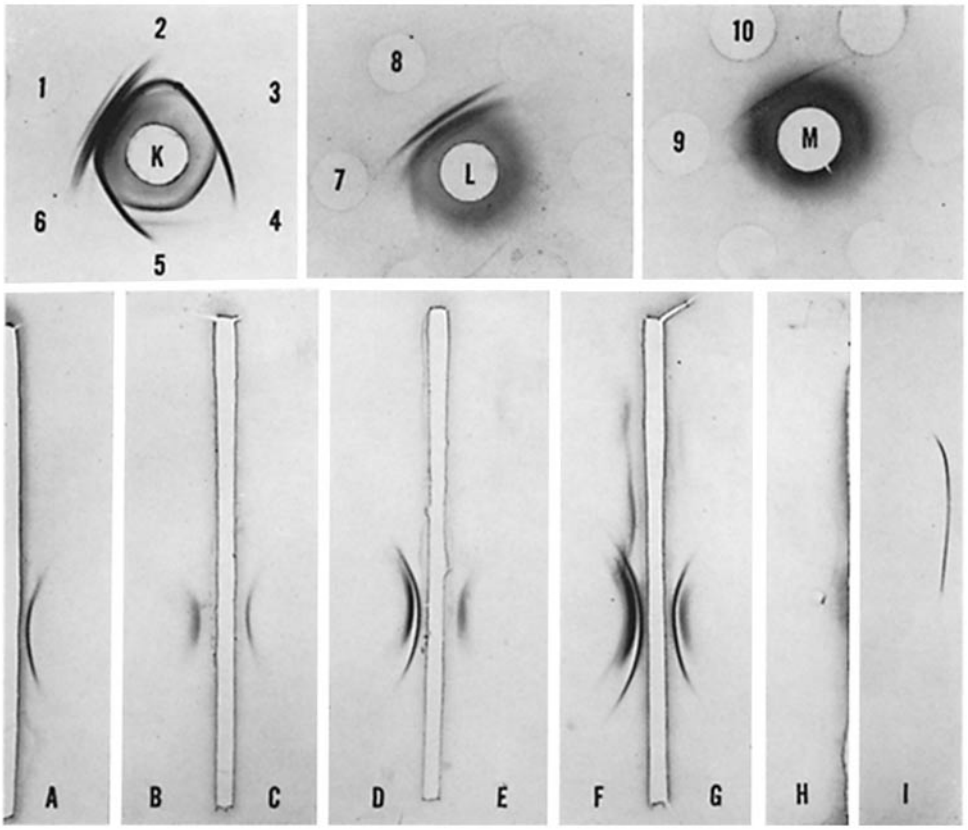


FIG. 4. Immunoprecipitin studies. A to I, electrophoresis was performed in 2% Noble Agar (Difco Laboratories, Inc., Detroit) in a 0.01 M veronal buffer, pH 8.4 at 5 v/cm for a period of 45 min. Antibody diffusion was allowed to continue for 48 hr.

In A to G troughs contained anti-PWM. Antigen wells contained the following: A, 0.5 M PWM fraction; B, 0.05 M PWM late (L) fraction; C, 0.05 M PWM front (F) fraction; D, PWM heat-coagulated fraction (step II); E, PWM TCA fraction (step III_b); F, PWM saline extract (Grand Island Biologicals); and G, PWM saline extract (step I).

Troughs H and I contained anti-PHA. Antigen wells contained the following: H, saline extract (step I); and I, purified PHA.

K to M, double diffusion studies. K, center well, anti-PWM. Well 1, PWM saline extract (step I); Well 2, PWM TCA fraction (step III_b); Well 3, PWM 0.05 M front (F) fraction; Well 4, PWM 0.05 M late (L) fraction; Well 5, PWM 0.5 M fraction; and Well 6, PWM 0.05 M front (F) fraction.

L, center well, PWM saline extract (step I). Well 7, Anti-PHA; and Well 8, Anti-PWM.

M, center well, purified PHA. Well 9, Anti-PWM; Well 10, Anti-PHA.

TABLE III

Summary of the Biological and Physicochemical Properties of Phytohemagglutinin and Pokeweed Mitogen

Material	PHA (Phaseolus vulgaris)	PWM (Phytolacca americana)
Source	Bean	Root
Biological properties		
1. Hemagglutinin	Complete panhemagglutinin	Incomplete panhemagglutinin
2. Leukagglutinin	Complete	Incomplete
a. Agglutination in saline	a. Agglutination in the presence of high mol. wt. substances (dextran, serum, PVP)	
b. Agglutination inhibition in EDTA	b. Same as PHA	
c. Leukagglutination unnecessary for transformation	c. Same as PHA	
3. Mitogen activity		
a. Transformation, %	80–90	50–60
b. Amount of substances necessary for stimulation of 3×10^6 lymphocytes, μg	1	0.1
Physicochemical properties		
1. Heat stability	Stable 80°C for 5 min, destroyed 100°C for 5 min	Same as PHA
2. Periodate, mercaptoethanol treatment	Resistant	Same as PHA
3. Protein content	138 μg N/mg	120 μg N/mg
4. UV absorption	Peaks at 280 and 290	Same as PHA
5. Activity eluted from calcium phosphate chromatography	0.5 M phosphate, pH 7.5	0.05 M phosphate, pH 7.5
6. Migration in electrical field		
a. Agar elecrophoresis, pH 8.6	Cathodal	Anodal
b. Polyacrylamide disc electrophoresis pH 9.3	Two bands, R_f 0.1–0.15	Single band R_f 0.43

During fractionation of the pokeweed mitogen it was observed that both the hemagglutinating and leukagglutinating activities were intimately associated with the mitogenic activity. Absorption of either purified mitogen, with intact red cells or stroma resulted in loss of the hemagglutinating activity without any significant alteration in the leukagglutinating or mitogenic activity while absorption with leukocytes resulted in loss of both the leukagglutinin and mitogen. These data indicated that the hemagglutinin and leukagglutinin were not only dissociable but that the receptor sites for these agglutinins might be distinctly different. Further, the active moieties associated with the leukagglutinin and mitogen could not be separated by absorption. This latter finding has led some workers (3, 4) to conclude that leukagglutination is a necessary step in lymphocyte transformation. In studies with staphylococcal filtrate (30, 31) and anti-allotype sera (31), in which leukagglutination is not demonstrable, lymphocyte transformation occurs. The fact that the transformation of lymphocytes by PHA and PWM still occurs following coating of lymphocytes with the Vi antigen, a suppressant of leukagglutination (32), further supports the view that leukagglutination is not a necessary event in lymphocyte transformation.

Although absorption of both mitogens with leukocytes removes both leukagglutinating and mitogenic activities, the relationship between the leukagglutinin and mitogen is not yet defined. From the biological data it would seem likely that the macromolecular structure of PWM has at least two active moieties, one the hemagglutinin, and the other the leukagglutinin and mitogen. Furthermore, these biological activities were not inactivated by treatment with periodate or 2-mercaptoethanol, suggesting that neither the splitting of carbon-carbon bonds in hexoses nor the reduction of possible disulfide linkages alters any of the three biological activities under the stated conditions.

The electrophoretic and chromatographic data indicate that the pokeweed mitogen has a different mobility and charge properties from PHA. This may be due to a difference in molecular weight, shape or size of the molecule, as suggested from both the hemagglutinating properties and polyacrylamide disc electrophoresis.

Analytical separation of the 0.05 M front moving fraction (F) eluted from calcium phosphate column revealed that all three biological activities were present in a single component which was homogeneous in polyacrylamide disc electrophoresis. This same band gave a single line on immunoelectrophoresis. Although these data suggest homogeneity of the active fraction, it does not exclude the possibility that this fraction is made up of active subunits. Rigas et al. (33) in studying a purified phytohemagglutinin has reported that subunits derived from IRC 50 chromatography possess both hemagglutinating and mitogenic activities in different ratios, suggesting that these activities are present on the same molecule.

Apart from the finding that the 0.05 M front fraction possessed all three bio-

logical activities, mitogenic assays revealed that PWM gave a transformation rate of 60% of PHA. In the subsequent papers we will demonstrate that the pokeweed mitogen is capable of stimulating a qualitatively distinct transformation with the expression of biochemical, histochemical, and fine structural features resembling early antibody-forming cells (12, 13).

SUMMARY

The saline extract from the roots of *Phytolacca americana* (pokeweed) possesses three biological properties; hemagglutinin, leukagglutinin, and mitogen. Fractionation and further purification on calcium phosphate column chromatography revealed that the biologically active substance was eluted in the front moving fraction with 0.05 M phosphate buffer pH 7.5. Analytical separation on polyacrylamide gels in disc electrophoresis yielded a single homogeneous band with an R_f value of 0.43 containing all three biological activities. This fraction had an ultraviolet absorption spectrum similar to PHA, was stable to both periodate and mercaptoethanol treatment and gave a single band in double diffusion and immunoelectrophoretic analysis against the antibody prepared to the crude PWM saline extract.

Absorption studies with red cells or stroma revealed that the hemagglutinin could be selectively removed without significantly altering the mitogen, whereas absorption with leukocytes resulted in loss of both the mitogenic and leukagglutinating activities.

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