THE DISSOCIATION OF PHYTOHEMAGGLUTININ OF *PHASEOLUS VULGARIS* BY 8.0 M UREA AND THE SEPARATION OF THE MITOGENIC FROM THE ERYTHROAGGLUTINATING ACTIVITY.*

Demetrios A. Rigas and Charlotte Head

Division of Experimental Medicine and Department of Biochemistry,
University of Oregon Medical School, Portland, Oregon 97201.

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The isolation of a protein phytohemagglutinin (PPHA) from red kidney beans (*Phaseolus vulgaris*) which is homogeneous by eleven different criteria of homogeneity has been reported (1,2). This substance is a glycoprotein of about 138,000 molecular weight which contains very little, if any, cysteine and methionine and to which are bound more than one type of oligosaccharide chains containing primarily mannose and glucosamine (2,3). The molecule is dissociated by 8.0 M urea into about eight subunits which are of equal size, but which differ from each other as indicated by starch gel electrophoresis in 8.0 M urea and which reassociate upon elimination of urea (2,3,4). This glycoprotein agglutinates erythrocytes at concentrations as low as 0.1 to 0.25 µg/ml (2) and induces mitosis in normal human blood lymphocytes at concentrations as low as 0.2 µg/ml, the optimum dose for mitogenicity being 5 µg/ml (5). Both the erythroagglutinating and the mitogenic activities have been shown by adsorption of PPHA on erythrocyte stroma and subsequent elution to be properties of the same molecule, but probably of different subunits (1,2). This report presents evidence of the separation of the mitogenic from the erythroagglutinating activity by polyacrylamide gel electrophoresis of the dissociated glycoprotein, PPHA, in 8.0 M urea.

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MATERIALS AND METHODS

PPHA was prepared as previously described (2). Reagent grade urea, in water, was deionized by passing through a column of AG 501-X8(D) (Bio-Rad Laboratories, Richmond, Calif.). Electrophoresis was performed on flat 17.5 x 17.5 x 0.425 cm polyacrylamide gel slabs, prepared by a modification of Tombs' method (6). An 8% solution (w/v) of "cyanogum 41" (American Cyanamid Co., Wayne, N.J.) was made in water containing 0.1 gm % (w/v) of reagent grade ammonium persulfate. The solution was deoxygenated by evacuating with a water aspirator, 0.2 ml of TEMED (N,N,N',N'-Tetramethylethylenediamine, Matheson, Coleman and Bell, Norwood, Ohio) was added per 150 ml and the mixture was immediately poured into a tray formed on a clean glass plate to which four flat plexiglass strips of the appropriate length and thickness were attached with silicone grease. Seven 1.0 cm sample slots, 1.0 cm apart, were formed parallel to and 5.0 cm from one edge of the square, by laying on it a flat plexiglas piece holding a stainless metal blade, of razor blade thickness, with seven square teeth protruding vertically into the cyanogum. The rest of the gel surface was covered by clean glass plates to exclude atmospheric oxygen. Following gelation, the slab was equilibrated by continuous agitation for four days in an 8.0 M urea-tris-EDTA-borate buffer of pH 8.7, changing the buffer once on the third day. The composition of the buffer was 3.024 gm of trishydroxymethylaminomethane, 0.346 gm of EDTA (ethylenediaminetetracetic acid) and 1.856 gm of boric acid per liter of 8.0 M urea. The equilibrated gel was placed on a water cooled electrophoresis apparatus (Model EC 451, E-C Apparatus Corp., Philadelphia, Pa.), 10.0 µl of a 67.0 mg per ml protein solution in 8.0 M urea-tris-EDTA-borate buffer of pH 8.7, was placed in each sample slot and the slots were sealed with a layer of melted petroleum jelly. The electrode vessels were filled with borate buffer of pH 9.8, containing 18.55 gm of boric acid and 5.0 gm of NaOH per liter of 8.0 M urea. Cotton flannel wicks, soaked in gel buffer, were used to connect the gel with the electrode vessels. The gel was covered with "Saran Wrap", the upper cooling plate was placed just above the surface of the gel, and kept cold by circulating

water through the cooling plates from a constant temperature bath maintained at 5°C. Electrophoresis was run for 26 hours at 7.0 volts per cm of gel length as measured directly across 15.0 cm of gel. At the end of the run the gel was stained for protein with Amidoschwartz, or for glycoprotein by a modification of the periodic acid-Schiff reaction (7). Gels that were to be used to extract the electrophoretically separated protein bands were cut longitudinally in the direction of migration to separate the electrophoregrams of the samples before staining. Each strip was in turn similarly cut longitudinally in two halves, one of which was then cut transversely into 2.5 mm wide segments, while the other was stained and used to identify the gel segments, containing each of the bands. Segments containing the same band were pooled, homogenized with 200 ml of 0.14 M NaCl, filtered through filter paper and the clear filtrates were concentrated by ultrafiltration. The erythroagglutinating activity of each band was determined by the microscope slide technique (1) and the mitogenicity was assayed as previously described (2,5) by determining the incorporation of thymidine-2-^{14}C into the DNA of normal human blood lymphocytes. Cultures were set up containing 8 ml NCTC-109 tissue culture medium, 2 ml fresh human serum, 1000 units Potassium Penicillin G, $10x10^6$ leukocytes, 1 µc of thymidine-2-^{14}C and appropriate doses of the assayed material. The cultures were incubated for 48 hours at 37°C in a "Dubnoff" shaker under constant flow of a 20% O_2, 3% CO_2 and 77% N_2 mixture and shaking at 42 strokes per minute. The leukocytes were recovered by centrifugation and washed with 0.14 M NaCl and water-ethanol (1:5 v/v). They were then dissolved in 1 ml ammonia by heating in sealed tubes at 90°C for 1 hour, plated on stainless steel planchets and the incorporated ^{14}C was counted with a low background flow counter. The data were expressed as net counts per minute (CPM) per mg.

RESULTS

In contrast to the erythroagglutinating activity of PPHA, which is inactivated by 8.0 M urea at a moderately fast rate (2), the mitogenic activity is relatively stable. We found no decrease of the incorporation of thymidine-2-^{14}C into the DNA following an exposure of PPHA to 8.0 M urea for a week, although

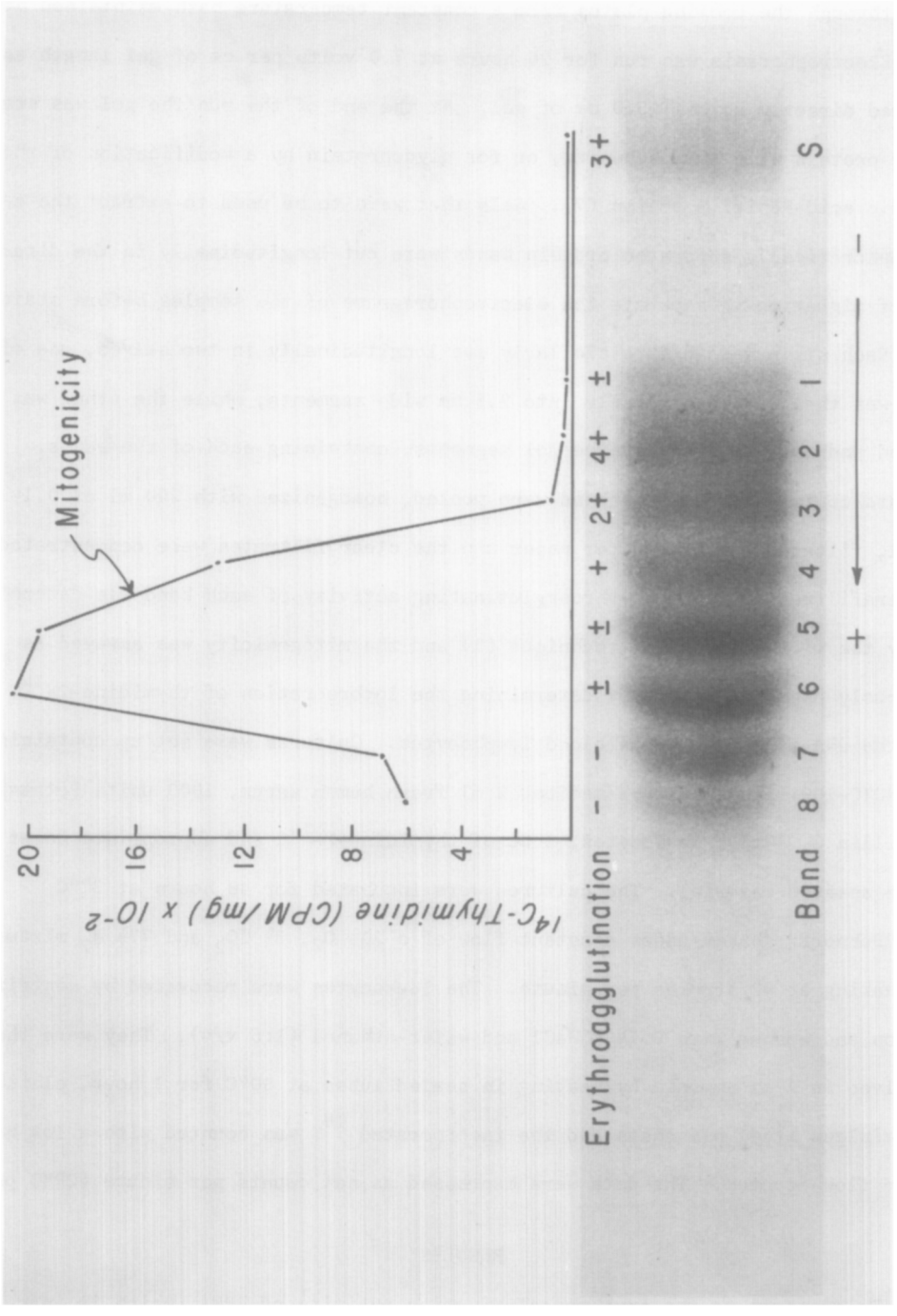

Figure 1. Polyacrylamide gel electrophoresis of PPHA in Tris-EDTA-Borate-8.0 M Urea buffer of pH 8.7 and erythroagglutination and mitogenicity of the bands.

some inactivation occured after longer exposures. This observation suggests that it may be possible to identify and separate the mitogenic and the erythroagglutinating subunits by gel electrophoresis in 8.0 M urea (2). Polyacrylamide rather than starch gel was used for electrophoresis because it can be stained for glycoprotein (7). A typical pattern obtained by electrophoresis of PPHA exposed to 8.0 M urea for 5 days and stained either with Amidoschwartz or with Schiff's reagent, is shown in Figure 1. The fact that identical patterns are obtained by both staining techniques indicates that all bands are glycoproteins. The pattern is very similar to that obtained by starch gel electrophoresis (2), in that it shows the same eight narrow bands, shown on Figure 1, but in contrast, the broad band, S, is the slowest instead of being the fastest (2). All these bands move anodically at this pH. To test the biological activities of each of the bands, they were extracted without staining, pooled and concentrated as described under methods. Although the duration of exposure to 8.0 M urea up to the time of extraction was 9 days, sufficient erythroagglutinating activity remained to permit detection. The results are shown on Figure 1 above each band and are graded from "4+" for the strongest to "-" for no detectable erythroagglutination. It can be seen that band 2 is the most potent in this regard and that erythroagglutination falls off on both sides, except in band S which is very potent. Each band was assayed for mitogenicity at three dose levels, triplicate cultures being set up at each dose. Mitogenic activity was not detected at the two lower doses in any of the bands, but at the highest dose the mitogenicity of bands 6 and 5 was high and decreased rapidly on either side. The mean incorporation of thymidine-2-^{14}C into the DNA of the triplicate cultures at the highest dose is plotted as CPM of ^{14}C per mg above the corresponding band in Figure 1. It is apparent that bands 5 and 6 have maximum mitogenicity but questionable erythroagglutinating activity. In contrast, band 2 has maximum erythroagglutinating activity but no detectable mitogenicity.

DISCUSSION

This study shows that the erythroagglutinating and the mitogenic activities of PPHA are separable and therefore on different subunits of the molecule. It is possible that the erythroagglutinating activity is confined only to band 2 and that contamination, due to imperfect technique, accounts for the erythroagglutination of the other bands. Band S is an exception and appears to be a partially inactivated, but undissociated PPHA (2). Similarly, the mitogenic activity may be present only on band 6 or band 5. This is supported by the fact that when extracted bands were re-electrophoresed they were shown to be contaminated with neighboring bands and band S dissociated into eight bands. The possibility that these bands do not represent single subunits, but only partially dissociated products, some containing more mitogenic and others more erythroagglutinating subunits, can not be excluded, although the symmetry of the dissociated peak of PPHA in the ultracentrifuge (2) does not support this view. The isolation of these bands in uncontaminated, homogeneous form, is underway in order to study their structure and biological activities. Contradictory reports of separation of the erythroagglutinating from the mitogenic activity have appeared in the literature and have been recently reviewed (8). Through chemical fractionation of extracts of red kidney beans, we obtained substances which differ from PPHA and from each other in their ratio of mitogenic to erythroagglutinating activities. However, we found no mitogenic substance that was completely devoid of erythroagglutinating activity, either by chemical fractionation, or by repeated adsorption of bean saline extracts on erythrocytes or erythrocyte stroma. Extracts that had been thus adsorbed until no erythroagglutinating activity could be demonstrated, even after concentrating them, showed no mitogenic activity when assayed over a wide range of doses. The fact that the erythroagglutinating and mitogenic activities are on separate subunits of PPHA offers a possible explanation of these observations. Thus substances of different ratios of mitogenic to erythroagglutinating activities in red kidney beans may represent combinations of mitogenic and erythroagglutinating subunits in various ratios.

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