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Immunological Studies of Phytohaemagglutinin

I. REACTION BETWEEN PHYTOHAEMAGGLUTININ AND NORMAL SERA

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Summary. Studies of the precipitation of normal human serum by the kidney bean phytohaemagglutinin, *Phaseolus vulgaris* (PHA), showed that the main serum components involved were α_2-macroglobulin, β-lipoproteins and γM immunoglobulins. Preparations of α_1-glycoprotein, orosomucoid and some γA immunoglobulins were also precipitated by PHA. Antisera prepared against the PHA–NHS precipitate recognized α_2-macroglobulin, β-lipoproteins and γM immunoglobulins, γG immunoglobulins and at least four other unidentified antigens in the β and α regions. PHA did not react with B or A, O blood group substances.

Concanavalin A, a jack bean agglutinin, precipitated the same proteins which were precipitated by *Phaseolus vulgaris* PHA but a *Dolichos biflorus* extract did not react with human serum. Incomplete chemical studies of this interaction suggested that even though the same serum proteins were precipitated by Concanavalin A and PHA, the sugar specificity, if involved, is different for both lectins.

Incomplete studies of the component or components of PHA involved in the PHA–NHS interaction suggested that three cathodally migrating proteins were recognized by both the antisera to PHA and to the precipitate formed by PHA and normal human serum.

INTRODUCTION

In studies on the antigenic properties of phytohaemagglutinin (PHA) from the red kidney bean *Phaseolus vulgaris*, it was noted that the saline extract of this bean reacted with several normal animal sera (Nakamura, Tanaka and Murakawa, 1960; Beckman, 1962; Marshall and Norins, 1965). Since the exact components have not been well characterized, the experiments reported herein were designed to identify the components of normal sera which participated in this reaction.

In recent years, there has been considerable interest in the phytohaemagglutinins because of their ability to agglutinate erythrocytes and leucocytes (Rigas and Osgood, 1955) and to induce blastogenesis in lymphocytes (Nowell, 1960) as well as other cells. On intradermal injection of the bean extract, an inflammatory reaction is induced (Schrek and Stefani, 1963; Lycette and Pearman, 1963) and in tissue culture, PHA is also reported to be toxic to macrophages (Berman and Stulberg, 1962). The possibility, therefore, exists that the structural or chemical determinants involved in the precipitation of normal serum proteins by PHA are related to one or more of these biological phenomena.

MATERIALS AND METHODS

PHA

Commercial preparations of Difco-PHA-P (Difco Laboratories, Detroit, Michigan) were used in most instances and vials were reconstituted to 5 ml with distilled water as suggested by the manufacturer unless otherwise specified. Commercial preparations of Difco-M and Wellcome PHA (Wellcome Research Laboratories, Beckenham, England) were also used. In a few experiments, the cathodally migrating peak of PHA-P obtained by zone electrophoresis (Müller-Eberhard, 1960) on Pevikon was also utilized. Most experiments were performed in phosphate buffered saline (either pH 7·2 or 8·0 with 0·15 per cent NaCl).

Sera and serum proteins

The author is indebted to Dr Robert I. Levy and Dr Donald S. Frederickson, National Institutes of Health, for a plasma from a patient with Tangier Disease and to Dr Herbert Kayden (New York University) for three abetalipoproteinaemic plasmas. Preparations of α_1 glycoprotein, orosomucoid, and a serum glycoprotein prepared by Dr. K. Schmid in 1950 were kindly supplied by Dr Calderon Howe. The author is also indebted to Dr Elliott Osserman for the sera from patients with γG, γA and γM paraproteins. Dr Ken Lloyd generously supplied the preparations of Concanavalin A, hog mucin, blood group B substance, and two preparations of ovarian cyst glycoprotein. The two preparations of ovarian cyst glycoprotein, originally prepared by Dr Don Carlson (Western Reserve University, Cleveland, Ohio) differed in that one had the sialic acid removed. The preparation of *Dolichos Biflorus*, another plant agglutinin, was obtained from Dr Kimmo Aho, Helsinki, Finland, and had been originally prepared by Dr. O Mäkelä.

Antisera

(1) *Antisera to human proteins.* Commercial antisera to as many serum proteins as possible were obtained from Hyland (Hyland Laboratories, Los Angeles, California) and Behringwerke Laboratories (Behringwerke AG., Marburg-Lahn, Germany). These antisera were produced in rabbits or goats against human α_2-macroglobulins, γM macroglobulins, β- and α-lipoproteins, β_2- and α_1-glycoproteins, orosomucoid, albumin, prealbumin, transferrin, haptoglobin, caeruloplasmin, as well as whole human serum. Rabbit antisera to complement components β_{1C} (C'_3), β_{1E} (C'_4), and 11S (C'_{1q}) were prepared in the author's laboratory.

(2) *Antisera to PHA.* Four rabbits (1C, 2C, 3C and 4C) were immunized subcutaneously at weekly intervals with equal volumes of Difco complete Freund's adjuvant and Difco PHA-P over a period of several weeks and bled after receiving the equivalent of at least one vial of Difco PHA-P.

(3) *Antisera to the precipitate of PHA and normal human sera.* The precipitate obtained by incubating 1 ml of normal human serum (NHS) and 1 ml of PHA-P overnight at 4° was washed three times in phosphate buffered saline (pH 8·0 and 0·15 per cent NaCl) and restored to original volume with Freund's adjuvant. Precipitates from two normal individuals were injected subcutaneously into two rabbits each (M9, M10 and C12, C13) and blood was obtained after a total of three weekly injections.

Immunoelectrophoresis and gel diffusion

Ouchterlony gel diffusion was performed in veronal buffer, pH 7·6, with 0·7 per cent agar or agarose and 0·33 M glycine. Immunoelectrophoresis was performed in veronal

buffer, pH 8·6, μ 0·05 with 1 or 2 per cent agar or agarose according to the method of Scheidegger (1955). Staining for protein, lipid and glycoprotein was by Amidoschwarz, Oil Red O and periodic-acid–Schiff reaction, respectively (Crowle, 1961).

Zone electrophoresis

Electrophoresis on polyvinyl chloride (Pevikon) was carried out as described by Müller-Eberhard (1960) using barbital buffer, pH 8·6, μ 0·05. Protein concentrations of the eluates were estimated by the Folin–Ciocalteu method (Lowry, Rosebrough, Farr and Randall, 1951) and various fractions were pooled and concentrated by collodion dialysis.

Column chromatography

Column chromatography in most instances was carried out on Sephadex G-200 in phosphate buffered saline.

Density gradient ultracentrifugation

Volumes of 0·1–0·5 ml of various preparations were layered on a continuous sucrose density gradient from 10 to 40 per cent sucrose in phosphate buffered saline and allowed to sediment in a Spinco Model L ultracentrifuge at 35,000 rev/min for 15 hours (Edelman, Kunkel and Franklin, 1958). Effluent fractions were collected through a pinhole in the bottom of the tube.

Sedimentation for lipoproteins was performed according to the method of Havel, Eder and Braydon (1955) and the subsequent density cuts were dialysed against phosphate buffered saline. Occasionally these fractions were concentrated two to five times by collodion dialysis.

Quantitative precipitin curves were performed according to Kabat (1961) with protein measurements performed as noted above (Lowry *et al.*, 1951).

Reduction and alkylation of the γM macroglobulins was performed as follows: 1–2 ml of the various preparations in a final concentration of 5 mg/ml were treated with 2-mercaptoethanol 1:100 v/v in 0·1 M Tris, pH 8·1, for 1–2 hours at room temperature. The preparations were dialysed against 1–2 litres Tris buffer with or without 0·02 iodoacetamide overnight at 4°. Sometimes 0·02 M iodoacetamide was added directly to the preparation.

RESULTS

The commercially available phytohaemagglutinin from *Phaseolus vulgaris* (Difco-PHA-P) formed a precipitate with all the sera thus far tested: human, monkey, rabbit, guinea-pig, rat, foetal calf and duck. The precipitate was demonstrated by fluid phase or gel diffusion. Fig. 1 shows a typical gel diffusion experiment in which PHA precipitated duck, foetal calf and human sera as well as human α_2-macroglobulin but not human fraction II γ-globulin or albumin. Most often one slightly fuzzy precipitin arc appeared with human sera but occasionally two or three separate and distinct precipitates could be demonstrated as is noted with the foetal calf serum in Fig. 1. A clear zone was often present just at the junction of the precipitates in adjacent wells. Heating the normal sera to 56° did not abolish this reaction but heating the PHA to 85° did.

Immunoelectrophoresis of human serum with PHA-P in a linear trough is shown in Fig. 3. At least three distinct fuzzy precipitates developed with mobilities in the fast γ, β and α regions. However, these three precipitates were fuzzy and appeared inconstantly

so that two other approaches were utilized. The first method employed the use of antisera made against the precipitate formed by PHA-P with normal human serum and the second approach utilized preparations of individual proteins to see if they would precipitate directly with PHA.

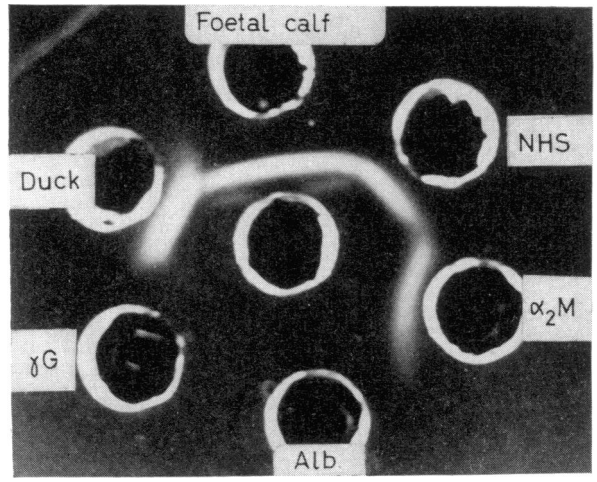

FIG. 1. Double diffusion experiment with PHA-P in the centre well and from top clockwise: foetal calf serum, normal human serum (NHS), human α_2 macroglobulin (1·3 mg/ml), human albumin (10 mg/ml), human fraction II γ-globulin (10 mg/ml), and normal duck serum.

The four antisera prepared in rabbits to the precipitate formed by the reaction of sera from two normal individuals (NHS) with PHA all recognized the following serum proteins: γG and γM immunoglobulins, α_2-macroglobulin, the β-lipoproteins and three to four other unidentified proteins in the β and α regions. These antigens were identified in

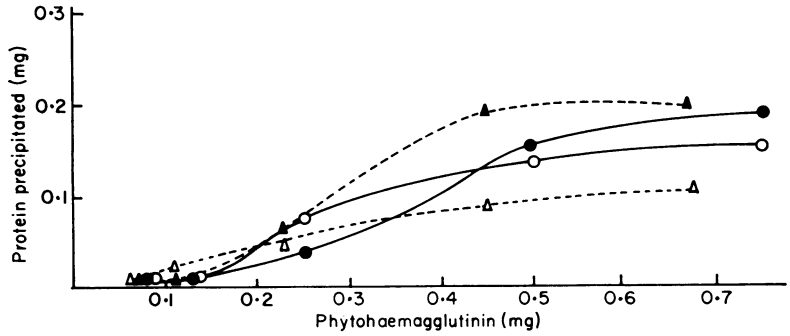

FIG. 2. Quantitative precipitin curves showing the effect of reduction and alkylation on the precipitin reaction between PHA and an isolated Waldenström macroglobulin, Rib. Increasing amounts of PHA were added to constant amounts of Rib (0·2 mg/ml) (●) and Rib treated with 0·02 M iodoacetamide (▲), 0·2 M mercaptoethanol (○), and both mercaptoethanol and iodoacetamide (△).

the following manner. A panel of commercial antisera were placed in troughs opposite the antisera produced against the PHA–NHS precipitate after immunoelectrophoresis of normal sera. Immunodiffusion was allowed to take place long enough to allow time for fusion of the precipitates which were ultimately stained with naphthalene black for protein, Oil Red O for lipid, and periodic acid–Schiff for glycoprotein identification. A

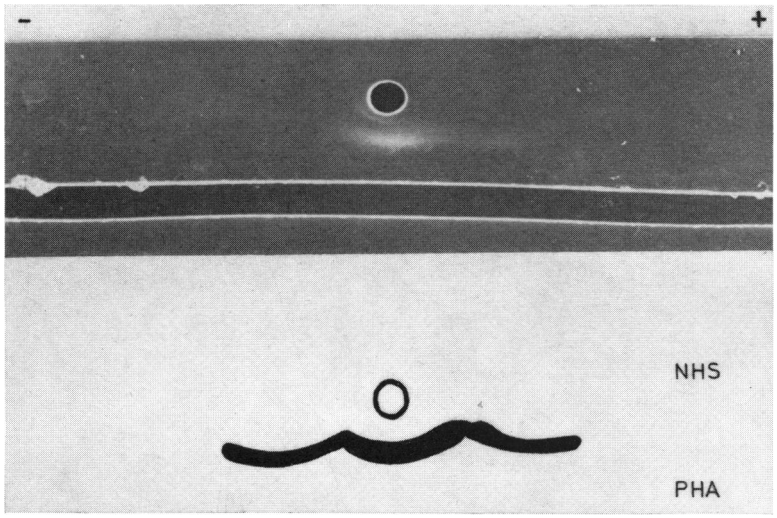

FIG. 3. Immunoelectrophoresis of normal human serum in agar developed with PHA-P in the trough. The slowest migating precipitate in the γM region did not photograph well so a drawing is included.

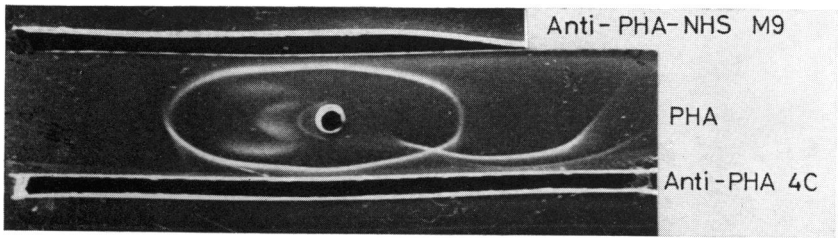

FIG. 4. Immunoelectrophoresis of PHA with antiserum M9 made against the PHA–NHS precipitate in the upper trough and an antiserum 4C to PHA in the lower trough. Three other antisera produced against the PHA–NHS precipitate gave this same pattern.

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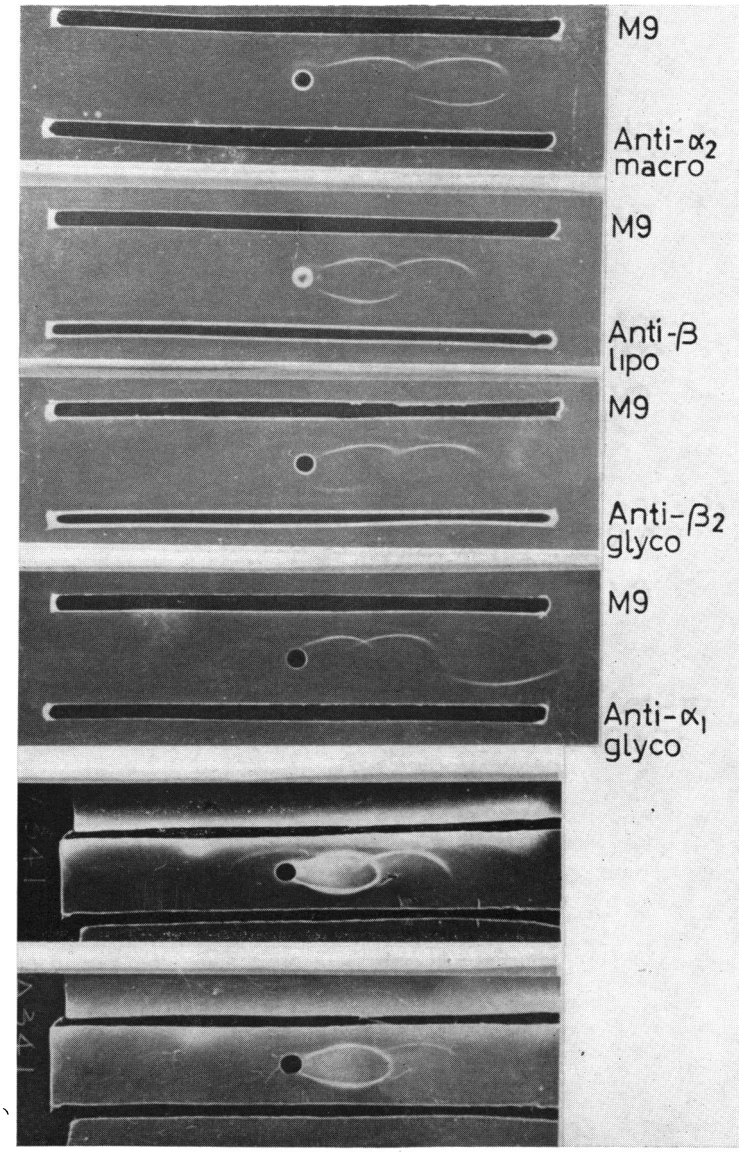

FIG. 5. Immunoelectrophoresis of normal human serum with an antiserum to the NHS-PHA precipitate (M9) placed in the upper troughs and commercial antisera to human α_2-macroglobulin, β-lipoproteins, β_2-glycoprotein and α_1 acid glycoprotein placed in the bottom troughs. The lower two slides are the second slide from the top which was washed, dried and then stained with Oil Red O (bottom slide) and naphthalene Black (second from the bottom).

typical experiment using PHA–NHS antiserum M9 is shown in Fig. 5. The following proteins were not recognized by the PHA–NHS antisera: caeruloplasmin, haptoglobulin. GC protein, transferrin, albumin, pre-albumin, the complement proteins β_{1C}, β_{1E} and the 11S globulin, and γA immunoglobulins. Negative results were also obtained with anti-β_2 and anti-α_1 glycoprotein antisera as shown in Fig. 5. Despite the panel of antisera, four serum proteins reactive with anti-PHA–NHS precipitate remained unidentified as shown in the next to the last slide from the bottom in Fig. 5. One had β mobility, two α_2 mobilities not far from α_2-macroglobulin and one was as fast as albumin. To rule out the problem of co-precipitation, especially of lipoproteins and immunoglobulins, it was considered necessary to use various pure serum protein preparations.

A panel of eleven of twelve γM pathological macroglobulins precipitated PHA in gel diffusion. They were first isolated by zone electrophoresis and then sucrose density ultracentrifugation, and varied in concentrations from 1 to 35 mg/ml. Most preparations were pure by Ouchterlony analysis and the only contaminant in a few instances was γG

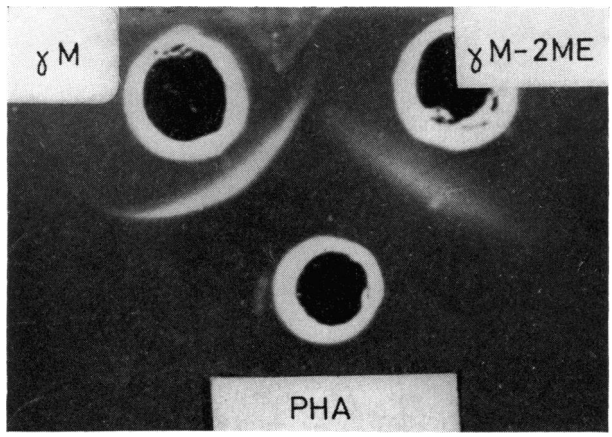

FIG. 6. Gel double diffusion experiment showing the spurring of a Waldenström γM macroglobulin, Ka, over the same preparation which had been reduced with mercaptoethanol when allowed to react with PHA.

immunoglobulin. The only macroglobulin not precipitated by PHA had a clear zone in the gel between the two reactants. Also, one macroglobulin showed spurring over the others but this heterogeneity was not studied further. L chain typing was not carried out in all instances but at least seven were type K and one type L.

This reaction between PHA and isolated macroglobulin was also studied in the fluid phase. Fig. 2 depicts the results obtained from a typical precipitin experiment, in which increasing amounts of PHA were added to a constant amount of purified macroglobulin (Rib) which also had been reduced with 0·2 M mercaptoethanol and alkylated with 0·02 M iodoacetamide. PHA precipitated the γM macroglobulin whether native or alkylated whereas reduction with or without alkylation reduced the precipitation to 55 and 80 per cent, respectively. The native γM macroglobulins spurred over the same preparation which had been reduced with mercaptoethanol when both preparations were allowed to react with either PHA or an anti-γM antiserum in gel diffusion. Fig. 6 illustrates this spurring of another isolated γM macroglobulin, Ka, over Ka which had been reduced and alkylated when PHA had been allowed to react with both preparations.

A panel of nine γG myelomas isolated only by zone electrophoresis, in varying concentrations from 1 to 15 mg/ml, did not precipitate with PHA in Ouchterlony analyses. Neither did commercially obtained fraction II γG globulin in concentrations of 0·001–100 mg/ml precipitate with PHA even though the PHA had been concentrated five times over that normally used (Fig. 1).

Five of seven γA myelomas which had been isolated first by zone electrophoresis and then Sephadex G-200 precipitated with PHA. The concentrations varied from 3 to 22 mg/ml whereas the two γA myelomas which were not precipitated by PHA were 6·9 and 7·5 mg/ml. These latter preparations were contaminated only by minimal amounts of γG immunoglobulin by Ouchterlony and immunoelectrophoretic analysis. Two of the five precipitating γAs were contaminated by an unidentified β-migrating protein on immunoelectrophoresis but had minimal amounts of γG immunoglobulins as contaminants.

TABLE 1

DOUBLE DIFFUSION EXPERIMENTS WITH VARIOUS LIPOPROTEIN SERIAL CENTRIFUGATION FRACTIONS

Lipoproteins	Density	Normal concentrated 5×		Abetalipoproteinaemia concentrated 2×		Tangier disease concentrated 2×	
		Precipitate with PHA	Oil Red O stain of precipitate	Precipitate with PHA	Oil Red O stain of precipitate	Precipitate with PHA	Oil Red O stain of precipitate
α_2-and β-lipoproteins (LDL)	<1·063*	+	+	0	0	+	+ (weak)
α_1-Lipoproteins (HDL)	1·063–1·21	+	+	0	0	0	0
Serum proteins	>1·21	+ (2–3 precipitates)	0	+	0	+	0
Serum or plasma		+ (2–3 precipitates)	+	+	0	+	0

Table 1 shows the results of precipitin experiments obtained between PHA (neat and concentrated 5×) and various lipoprotein serial centrifugation fractions. Ouchterlony plates were stained first with Oil Red O and then naphthalene Black for lipoprotein and protein identification.

* PHA also precipitated density fraction of <1·019 or α_2-lipoprotein and 1·019–1·063 or β-lipoproteins from normal individuals. These separate density fractions were not prepared for the Tangier and abetalipoprotein sera.

Table 1 summarizes the results obtained with human serum lipoproteins. The one Tangier plasma (α-lipoprotein deficiency) and four abetalipoproteinaemia plasmas all precipitated with PHA in double diffusion experiments prior to obtaining the serial centrifugation density fractions. The β-lipoproteins (LDL or density less than 1·063) and to a lesser extent the α_1-lipoproteins (HDL or density between 1·063–1·21) fractions from four normal individuals were all precipitated by PHA in double diffusion experiments. These fractions had been dialysed against phosphate buffered saline to remove the salt and were then concentrated five times. In some experiments the less than 1·019 fractions or α_2-lipoproteins were separated from the β-lipoproteins. These fractions formed weak precipitates with PHA. These precipitates, as summarized in Table 1, took both Oil Red O and naphthalene black stains although the β-lipoproteins gave a much heavier precipitate both before and after staining than the α-lipoproteins. However, neither of the serial centrifugation fractions of the abetalipoproteinaemic plasmas nor the 1·063–1·21 fraction from the Tangier plasma were precipitated by PHA. Because of limitation in starting material, these fractions had only been concentrated two times. It would appear that the plasmas from the abetalipoprotein patients were also low in high density lipoproteins,

although this is not germane to the present problem. Although these various ultracentrifuge fractions were contaminated with one or two non-lipoprotein-staining antigens in double diffusion experiments using rabbit antisera prepared against whole human sera, it was concluded that mainly β-lipoproteins (LDL) were precipitated directly by PHA.

Because certain plant haemagglutinins are known to have affinities for various sugars, it seemed important to evaluate the glycoproteins which are high in carbohydrate content. The available serum glycoprotein preparations included α_1-glycoprotein, orosomucoid and the original glycoprotein preparation of Schmid which, in concentrations of 2 mg/ml, were all precipitated by PHA. The α_1-glycoprotein was immunoelectrophoretically pure

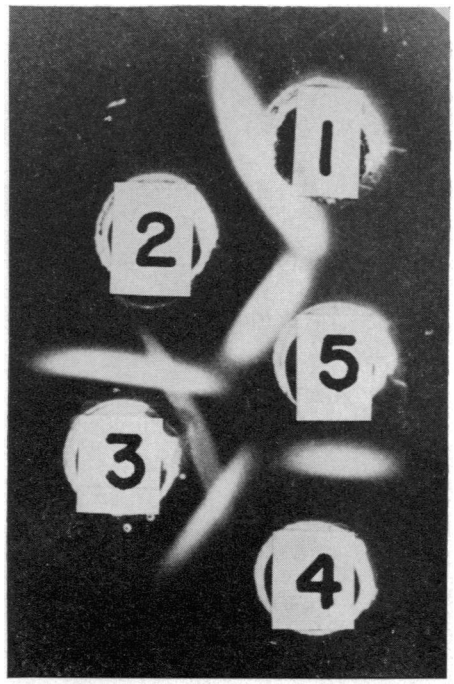

FIG. 7. Double diffusion experiments showing the precipitate formed between PHA in wells 2 and 4 and α_1-glycoprotein (2 mg/ml) in well 5. The PHA in well 2 was concentrated five times over that in well 4. Well 1 contained the antiserum against the PHA–NHS precipitate and well 3 the commercial anti-α_1 glycoprotein antiserum. The PHA-protein precipitates completely penetrated the immunological precipitates and vice-versa.

but the preparations of orosomucoid had one contaminant and the Schmid glycoprotein at least three contaminants on immunoelectrophoresis. Fig. 7 shows one of these PHA–glycoprotein precipitates. The immunological precipitate formed by the human glycoprotein and rabbit anti-glycoprotein was distinct from the PHA–glycoprotein interaction. This phenomenon was also continually observed in studying the other available serum protein preparations. Other serum glycoproteins were not examined.

Fig. 8 shows the results obtained with blood group substances. Both the neat and concentrated PHA did not react with either hog mucin (A and H substance) in concentrations of 1–7 mg/ml or B blood group substance in concentrations of 1–9 mg/ml. Another plant agglutinin, Concanavalin A, a jack bean globulin, was included as a control since previous investigations have shown it reacts with all branched carbohydrate polymers which contain multiple α-D-glucopyranosyl, α-D-mannopyranosyl or β-D-fructofuranosyl units at

non-reducing chain ends (So and Goldstein, 1967). This bean extract precipitated only the hog mucin which contains this necessary D-arabino sugar linkage. Concanavalin A also precipitated strongly with normal serum as shown in Figs. 8 and 9 (Sumner and Howell,

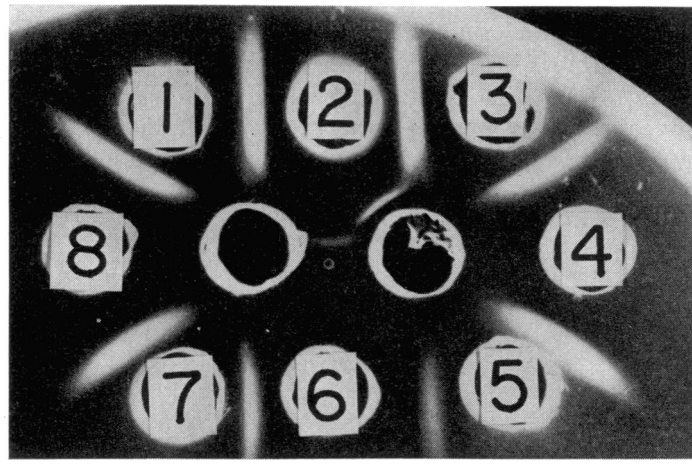

FIG. 8. Ouchterlony experiment with hog mucin (7 mg/ml) in right centre well and blood group A substance (9 mg/ml) in the left centre well. The peripheral wells contained: NHS in 1, 3 5, 7, Concanavalin A (3 mg/ml) in 2, PHA concentrated 5 times in 4, 8 and neat PHA in 6.

1936). In a brief survey using the same reagents used in the *Phaseolus vulgaris* PHA experiments, it appeared that Concanavalin A precipitated human γM, some γA immunoglobulins, α_2-macroglobulins, β-lipoproteins and to a lesser extent α-lipoproteins and Schmid glycoprotein.

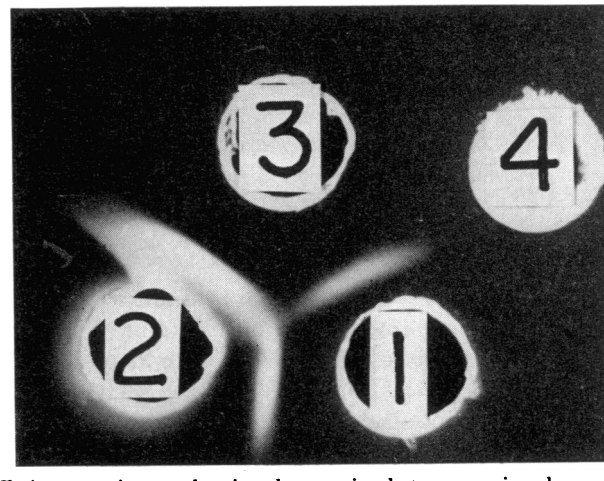

FIG. 9. Double diffusion experiments showing the reaction between various bean extracts and norma human serum (well No. 1). Wells 2, 3 and 4 contained Concanavalin A, Difco PHA-P and *Dolichos biflorus*, respectively.

Although the same serum proteins were precipitated by both bean extracts, the experiments with the blood group substances suggested that sugar specificity, if involved, must be different. Interestingly, PHA itself is precipitated by Concanavalin A (Figs. 8 and 9)

which suggested that PHA probably contains the same type of sugar linkage mentioned before. *Dolichos biflorus*, a plant agglutinin which is known to react primarily with A and A_1 blood group substances, did not precipitate normal human serum as shown in Fig. 9 even when obtained from A+ individuals. Two preparations of the same ovarian cyst glycoprotein, one of which had the sialic acid removed (1·69 mg/ml) and the other still contained sialic acid (1·91 mg/ml), were not precipitated by PHA. This suggested that sialic acid alone was probably not required for the PHA–NHS interaction.

The protein or proteins in PHA which were involved in this interaction with components of normal human serum were not fully characterized for this study. However, the following experiment suggested that either one or a combination of three cathodally migrating proteins from PHA were probably involved. As shown in Fig. 4, PHA was immunoelectrophoresed with antisera to PHA–NHS precipitate placed in the lower trough and to PHA itself in the upper trough. The only antigens in PHA recognized by both antisera were the two fuzzy cathodally migrating arcs and the longer γ-globulin-like arc which showed complete fusion. The α-migrating PHA antigens were not recognized by the anti-PHA–NHS precipitate antisera.

DISCUSSION

During the past few years, phytohaemagglutinin has been used extensively in human cytogenetical work but little attention has been paid to the protein precipitating abilities of the plant extracts. Nakamura *et al.* (1960) showed by the technique of crossing paper electrophoresis that a cathodally migrating protein from jack beans reacted with α_1-, α_2-, β-globulins and probably with γ-globulin but not with albumin. In Ouchterlony experiments this jack bean extract also precipitated human, guinea-pig, bovine, rat, sheep, hog and fowl sera as well as egg white and yolk. They also concluded that the kidney bean, *Phaseolus vulgaris*, contained less of a similar cathodally migrating substance which reacted mainly with α_2-globulin of serum. Beckman (1962) also concluded that *Phaseolus vulgaris* PHA reacted with human sera in dilutions less than 1 : 10. He noted on starch gel a decrease in slow α_2-globulin and the crossing electrophoresis experiments suggested in addition a reaction between PHA and γ-globulin.

The more recent experiments of Holland and Holland (1965) found that kidney bean PHA precipitated fifty-nine human sera as well as bovine, rabbit and rat sera. Absorption of PHA by human red cells or heating at 85° for 5 minutes destroyed this precipitating activity and decreased the haemagglutinating activity but neither technique affected lymphocyte blast formation. Precipitin reactions were also noted between PHA and human Cohn fractions II, III, IV, VI, albumin and a primary agammaglobulinaemic serum as well as pure bovine serum albumin. While preparing anti-PHA antisera, Marshall and Norins (1965) found that, after immunoelectrophoresis of the normal rabbit serum prior to immunization, a precipitate appeared in the α and β regions when PHA had been added to the trough. After immunization, a γ-globulin precipitate also appeared.

The experiments reported herein agree with most of the data just reviewed with a few exceptions. Once again the precipitin reaction between kidney bean PHA and mammalian and fowl sera was documented. The main extensions of previous information were garnered from the use of purified protein preparations and antisera which had been produced in rabbits to the precipitate formed by PHA and normal human serum (NHS). Use of the purified serum proteins showed that PHA precipitated α_2-macroglobulin, γM

macroglobulins, some γA immunoglobulins, β- and to a lesser extent α-lipoproteins, α_1-glycoprotein, Schmid acid glycoprotein, and orosomucoid. The main components recognized by all of the PHA–NHS antisera were α_2-macroglobulin and the β-lipoproteins. The antisera also recognized γM immunoglobulins, γG immunoglobulins as well as three other unidentified proteins, two α and one β. The antisera did not recognize the glycoproteins but this may have been due to the manner in which the NHS–PHA precipitate was made. The ratio of PHA to NHS has been arbitrarily chosen rather than from a precipitin curve at the point of maximum precipitation. This may have selected out the serum proteins with the greatest affinity for PHA rather than all the proteins capable of reacting with PHA. Certainly α_2-macroglobulin, β-lipoproteins and γM macroglobulins precipitated well with PHA and were the most prominent antigens recognized by the PHA–NHS antisera. It was disconcerting that four serum proteins (precipitated by PHA) remained unidentified despite use of a wide panel of commercial antisera.

The γG immunoglobulins were recognized by the PHA–NHS antisera but a variety of isolated γG myelomas and fraction II γ-globulin did not precipitate with PHA despite a wide range of concentrations of both reactants. The discrepancy between these findings and previous authors who reported that PHA reacted with γ-globulin (Nakamura *et al.*, 1960; Beckman, 1962, Holland and Holland, 1965) remains unexplained if the earlier preparations were not contaminated with γA or γM immunoglobulins. Previous investigators with one exception (Holland and Holland, 1965) agreed that PHA did not react with albumin. It should be noted that one of the unidentified antigens of the PHA–NHS antisera migrated in the same region as albumin. It is interesting that in a brief survey of the same protein preparations available for the PHA studies, Concanavalin A, another bean extract, reacted with the same proteins.

Among the most interesting problems which still remained were: (1) what substance or substances in PHA reacted with these serum proteins, (2) what was the chemical nature of the interaction, and (3) did the PHA–NHS reaction bear any relationship to one or more of the biological phenomena associated with PHA? A partial answer to the first question from as yet incomplete immunological studies suggested that one, two or possibly three proteins or aggregates thereof contained in the cathodally migrating components of PHA were capable of reacting with NHS. Purified serum proteins usually gave two to three precipitin lines with the PHA preparations. Furthermore, comparisons of antisera made against PHA and the PHA–NHS precipitate demonstrated that three antigens had been precipitated from the crude bean extract by NHS. Whether all three postulated PHA components were capable of precipitating each of the serum proteins involved needs further investigation.

Secondly, direct experiments regarding the nature of the chemical interaction have not been undertaken here. If the PHA–NHS interaction is sugar-dependent as has been demonstrated for PHA erythrocyte interaction (Borberg, Woodruff, Hirschorn, Gesner, Miescher and Silber, 1966), then sialic acid alone was not involved. Glycoprotein which contained sialic acid was not precipitated by PHA nor did PHA precipitate hog mucin which was precipitated by Concanavalin A. So and Goldstein (1967) suggested that the combining site of Concanavalin A has an essential requirement for sugars with unmodified hydroxyl groups at C3, C4 and C5 which are D-arabino configuration and also has a high degree of specificity for the α configuration at the anomeric carbon atom. Although not yet examined in this PHA–NHS system, Borberg *et al.* (1966) found that *N*-acetyl-D-galactosamine inhibited the ability of PHA to agglutinate erythrocytes.

Molecular weight and size may be important since the main proteins precipitated by PHA were γM immunoglobulins, α_2-macroglobulin and the β-lipoproteins—all with molecular weights above 500,000. Also heating PHA above 80° destroyed its ability to react with these serum proteins. Holland and Holland (1965) noted that absorption of PHA by erythrocytes or heating to 85° for 5 minutes destroyed the precipitating properties and decreased haemagglutination but the lymph growth factor was unaffected.

There is little direct evidence concerning the problem of whether the PHA–NHS reaction was related to any of the biological phenomena associated with PHA. *Phaseolus vulgaris* PHA directly agglutinated erythrocytes and leucocytes without serum. Mäkelä suggested that the erythrocyte agglutination was inhibited and not enhanced by serum (Mäkelä, 1957), whereas the blood group specific lectins in general were not inhibited by serum. Most chemical (Weber, Nordman and Grasbeck, 1967) and red cell absorption studies (Nordman, de la Chapelle and Grasbeck, 1964) noted that PHA contained leuko-agglutinins separate from haemagglutinins but serum inhibition studies were not performed.

Until recently, it was assumed that leuko-agglutination was necessary for blastogenesis. The lymphocyte transforming properties of PHA have now been dissociated from leuko-agglutination chemically (Rivera and Mueller, 1966) and by the following experiment. Lymphocytes which had been coated with an *N*-acetyl galactosamine-uronic acid polymer from *E. coli* were no longer agglutinated by PHA but were able to transform into blast cells (Borjeson, Chessin and Landy, 1967). The effect of serum was not evaluated. Recent studies of Cooperband, Green, Kennedy and Grant (1967) suggested that serum factors were probably involved in the synthesis of DNA and protein by peripheral white cell cultures. Maximum radiolabelling of protein occurred when the extracellular medium contained 5 per cent serum and maximum DNA radiolabelling occurred in cultures containing 15 per cent serum. Serum concentrations in excess of these inhibited PHA-induced synthesis of both protein and DNA. These same authors concluded that the influence of serum on PHA stimulated cultures was not an artefact since parallel results were not seen in the unstimulated cultures which did not contain PHA. It would be interesting to ascertain if one of the serum proteins which precipitated PHA was also reacting with the PHA mitogenic factor or provided a necessary co-factor for the PHA effect.

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