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The seed haemagglutinins of some *Phaseolus vulgaris* L. cultivars

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The haemagglutinating properties of *Phaseolus vulgaris* L. vars Canadian Wonder, Masterpiece, Mont D'or, Navy Pea and Red Kidney seed extracts against erythrocytes of the A_1A_2BO and $D(Rh_0)$ human blood groups have been investigated. Under some conditions, Navy Pea extract reacted preferentially with erythrocytes possessing the A_1 and B antigens, but the remaining extracts showed no blood group selectivity. The agglutination reactions are discussed, and it is suggested that Navy Pea seed contains a non-specific agglutinin and an anti-A_1 + B agglutinin.

THE haemagglutinin of *Phaseolus vulgaris* L. seed, first extracted by Landsteiner & Raubitschek (1908), was shown to be a mucoprotein by Renkonen (1950) and Rigas, Li & Osgood (1951). It is produced commercially in both mucoprotein and protein forms by the method of Rigas & Osgood (1955), and used for the agglutination of erythrocytes in the isolation of leucocytes from whole blood (Skoog & Beck, 1956; Seabright, 1957). It finds use as a mitogenic additive to leucocyte cultures (Nowell, 1960; Moorhead, Nowell, Mellman, Battips & Hungerford, 1960).

Renkonen (1948) discovered that seed agglutinins of some species react selectively with red cells of certain human blood groups. Although subsequent investigators (Boyd & Reguera, 1949; Cazal & Lalaurie, 1952; Krupe, 1953; Ottensooser & Silberschmidt, 1953; Makela & Makela, 1956; Makela, 1957; Bird, 1951, 1956, 1957) found further sources of "specific" phytagglutinins (lectins), some of which are used in blood group diagnosis, *P. vulgaris* L. agglutinin has been reported to be non-selective for human red cell antigens (Renkonen, 1948; Makela, 1957; Bird, 1959a). Cultivars of this species have been mentioned in connection with experiments on blood group specificity by Boyd & Reguera (1949) and Maron (1951), and it was the object of the present study to determine if the agglutinating properties of extracts of certain *P. vulgaris* seed cultivars differed.

Experimental

MATERIALS

Seeds of the following *P. vulgaris* L. cultivars were supplied by Brook, Parker & Co., Ltd., Bradford: Canadian Wonder, Masterpiece, Mont D'or, Navy Pea, Red Kidney.

First and second generation plants grown from these seeds possessed the botanical characters of *P. vulgaris* L., which are well documented (Robbins, 1917; anonymous 1920; Fawcett & Rendle, 1920; Bailey, 1949; Mclean & Ivimey-Cook, 1952; Ministry of Agriculture, 1962). Cultivars were authenticated by comparing the characters of the plant, flower, pod and seed with those recorded by Steinmetz & Arny (1932) or North & Squibbs (1953).

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Seeds of *Sophora japonica* L. and *Ulex europaeus* L. were supplied by Thompson & Morgan, Ltd., Ipswich.

METHODS

Seed extracts were titrated with human erythrocytes of the A_1A_2BO and $D(Rh_o)$ groups as follows: in 0·9% saline and 20% bovine albumin solution at 4° increments between 8° and 20° and at 2° increments between 20° and 56°. They were also examined at 20° in undiluted human AB serum. Titrations were also made at 20° in media varying from pH 4·4 to pH 9·75. The extracts were subjected to absorption, elution and sugar-inhibition tests. Controls for spontaneous agglutination were set up in each experiment and consisted of mixtures in which the seed extract was replaced by 0·9% saline.

Preparation of seed extracts, blood samples and erythrocyte suspensions. Intact seeds (50 g) were washed in 70% ethanol, soaked (16 hr) in sterile 0·9% saline (200 ml) in a Paladin Blender, and macerated for 5 min. The macerate was slowly stirred in the blender for 3 hr, centrifuged twice (715 × *g* for 30 min followed by 4,277 × *g* for 30 min) and the supernatant was clarified by membrane filtration in a sterile unit. 2 ml aliquots of the filtrate were freeze-dried in sterile, tared, wide-necked ampoules fitted with lint caps (modified from Greaves, 1946). After drying (16 hr) over P_2O_5, the weight of dry extract in each ampoule was accurately determined; ampoule necks were fused to exclude micro-organisms. Preparations were stored at −25°.

Before use, each extract was reconstituted with sterile water to produce a 5% w/v solution. Such samples from each batch were sterile when tested in aerobic and anaerobic media.

Extracts of *Sophora japonica* L. seed and *Ulex europaeus* L. seed prepared as above had comparable titres to those reported by Morgan & Watkins (1953) and Boyd & Sharpleigh (1954) respectively.

Human red cells of the following blood groups were used:

O(D+), O(D negative), A_1, A_2, B, A_1B, A_2B (all D+).

Venous blood (10 ml–15 ml) was mixed with sterile ACD* solution (2 ml) and stored at 4° for a maximum period of two weeks.

Erythrocytes were washed three times with 0·9% saline and suspended as follows:

For titration at various pH values:

pH 4·40–pH 7·95, 2% v/v in McIlvaine's Buffer (Documenta Geigy, 1962) of double-strength.

pH 8·33–pH 8·75, 2% v/v in Kolthoff's Buffer (Documenta Geigy, 1962) of double-strength.

pH 9·15–pH 9·75, 2% v/v in boric acid/sodium hydroxide buffer (Palmer, 1946) of double-strength.

*5 volumes of 2% disodium hydrogen citrate solution + 1 volume of 15% dextrose solution.

For other titrations: 5% v/v in 0·9% saline, in 20% bovine albumin solution, and in human AB serum.

For absorption and elution tests: 5% v/v in 0·9% saline and in 20% bovine albumin solution.

For inhibition tests: 5% v/v in 0·9% saline.

Titration. The method of two-fold serial dilutions was used. Equal volumes (0·05 ml) of extract dilution and erythrocyte suspension were mixed in a stoppered glass tube (50 mm × 6 mm) and allowed to stand for 2 hr in a temperature-controlled water-bath. The deposited corpuscles were re-suspended and examined for macroscopic agglutination, and, if necessary, for microscopic agglutination at ×80. The titre of an extract is the reciprocal of the greatest dilution which causes agglutination, i.e. the dilution which existed before admixture with erythrocyte suspension. pH values quoted with titres are those of the final mixtures showing agglutination.

Inhibition tests. Equal volumes (0·05 ml) of a 1/16 dilution of seed extract and 8% sugar solution were mixed, erythrocyte suspension (0·10 ml) added and the mixture allowed to stand (2 hr at 20°) before it was examined for agglutination. Controls were mixtures in which the sugar solution was replaced by 0·9% saline.

Using this method with two-fold serial dilutions of sugar solution, the minimum concentrations of D-galactose and β-lactose required to prevent agglutination by a 10% w/v extract of *Sophora japonica* L. seed were identical with those reported by Morgan & Watkins (1953).

Absorption and elution tests. For absorption, equal volumes (1 ml) of undiluted seed extract and washed packed red cells of the required group were mixed, allowed to stand overnight at 4°, and centrifuged at 2,000 rpm for 3 min. The supernatant was pipetted off and reabsorbed with half its volume of fresh, packed erythrocytes of the same group for 2 hr. The process was repeated with fresh cells of the same group until agglutination ceased to occur, after which the supernatant was tested in saline and bovine albumin media for agglutinins against red cells of other blood groups.

For elution, red corpuscles (1 ml) which had been agglutinated by seed extract (1 ml) were washed three times with 0·9% saline at room temperature and resuspended in 1 ml fresh saline. The suspension was placed in a water-bath at 56° for 15 min and the supernatant pipetted off from the cell sediment. After clarification by centrifugation (2,000 rpm for 3 min) the solution was tested for agglutinins in saline and bovine albumin media.

Results

In both saline and bovine albumin media, all extracts gave an identical variation in titre with temperature, irrespective of the phenotype of the test erythrocytes (Fig. 1). In human AB serum, Navy Pea seed extract reacted differently from the other extracts, and titres in saline, albumin and AB serum at 20° are compared in Table 1.

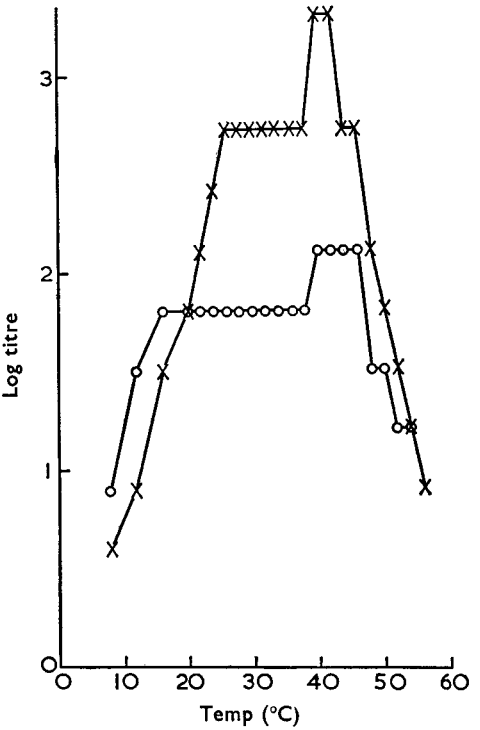

FIG. 1. Effect of temperature on the titre of *Phaseolus vulgaris* L. vars Canadian Wonder, Masterpiece, Mont D'or, Navy Pea or Red Kidney seed extract with erythrocytes of any A_1A_2BO blood group. ○ = 0·9% saline medium. × = 10% bovine albumin medium. Titres are the reciprocals of the greatest dilutions causing agglutination. Each point represents the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

TABLE 1. TITRES OF *Phaseolus vulgaris* L. SEED EXTRACTS AGAINST HUMAN ERYTHROCYTES SUSPENDED IN SALINE, BOVINE ALBUMIN AND HUMAN AB SERUM MEDIA AT 20°

Extract of	Medium	Blood group						
		O(D+)	O(D negative)	A_1(D+)	A_2(D+)	B(D+)	A_1B(D+)	A_2B(D+)
Canadian Wonder	0·9% saline	64	64	64	64	64	64	64
	10% bovine albumin	64	64	64	64	64	64	64
	human AB Serum	16	16	16	16	16	16	16
Navy Pea	0·9% saline	64	64	64	64	64	64	64
	10% bovine albumin	64	64	64	64	64	64	64
	human AB serum	16	16	64	16	32	64	32

Titres are recorded as the reciprocals of the greatest dilutions causing agglutination, and represent the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

Masterpiece, Mont D'or, and Red Kidney seed extracts reacted in the same way as Canadian Wonder seed extract.

The pH values of reconstituted extracts were: Canadian Wonder, 6·00; Masterpiece, 6·00; Mont D'or 6·10; Navy Pea, 6·10; Red Kidney, 6·00. In buffered media, four of the preparations reacted identically with erythrocytes of all A_1A_2BO groups, but between pH 6·03 and pH 7·35 the titre of Navy Pea seed extract varied with erythrocytes of different A_1A_2BO groups (Fig. 2).

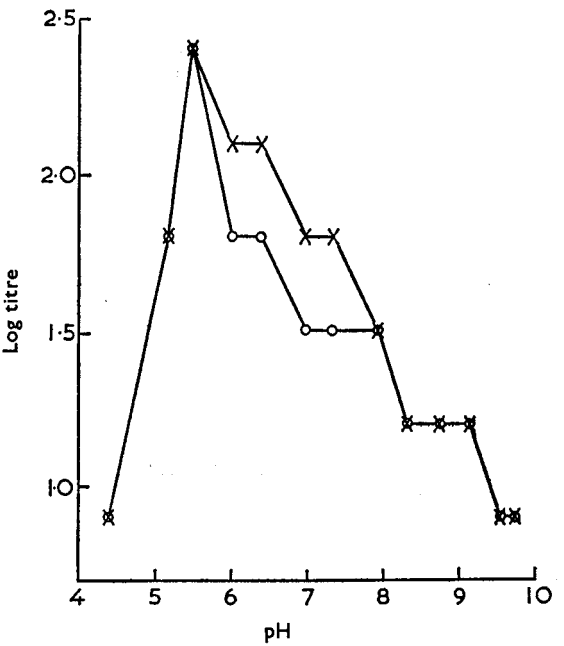

FIG. 2. Effect of pH on the titre of *Phaseolus vulgaris* L. seed extract. ○ = Canadian Wonder, Masterpiece, Mont D'or, or Red Kidney extract against erythrocytes of any A_1A_2BO blood group, or Navy Pea seed extract against O(D+), O(D negative) or A_2(D+) erythrocytes. × = Navy Pea extract against A_1, B, A_1B or A_2B (all D+) erythrocytes. Titres are the reciprocals of the greatest dilutions causing agglutination. Each point represents the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

Absorption of Canadian Wonder, Masterpiece, Mont D'or or Red Kidney seed extract with erythrocytes of any A_1A_2BO group, left supernatants devoid of haemagglutinating activity. This was also true for Navy Pea seed extract absorbed with A_1, B, A_1B, or A_2B (all D+) cells, but absorption with O(D+ and D negative) or A_2(D+) cells left a supernatant active only against cells possessing the A_1 and/or B antigen. The titres of this supernatant are recorded in Table 2.

The following 27 sugars and sugar-derivatives were tested for their capacity to inhibit agglutination: aldopentoses (D-ribose; D-arabinose; L-arabinose; D-(+)-xylose; α-L-xylose; D-lyxose), aldohexoses (D-(+)-glucose; α-D-glucosamine hydrochloride; *N*-acetyl-D-glucosamine; D-galactose; D-galactosamine hydrochloride; *N*-acetyl-D-galactosamine;

D-mannose), 6-deoxy-hexoses (L-(+)-rhamnose; D-fucose; L-fucose), ketohexoses (fructose; L-sorbose; D-tagatose), disaccharides (sucrose; maltose; β-lactose; cellobiose; D-(+)-trehalose; D-(+)-turanose), and trisaccharides (D-raffinose (pentahydrate); D-(+)-melizitose). None of these compounds inhibited agglutination.

In all experiments the cell controls behaved normally.

TABLE 2. TITRES OF SUPERNATANT FROM NAVY PEA SEED EXTRACT ABSORBED WITH O(D+), O(D NEGATIVE) OR A_2(D+) ERYTHROCYTES

Blood group	Medium	Titre
O(D+) or O(D negative) or A_2(D+)	0·9% saline	—
	10% bovine albumin	—
A_1(D+) or A_1B(D+)	saline	8
	albumin	32
B(D+)	saline	2
	albumin	16
A_2B(D+)	saline	1
	albumin	8

"—" = no reaction.
Titres are recorded as the reciprocals of the greatest dilutions causing agglutination, and represent the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

Discussion

In each experiment the extracts of Canadian Wonder, Masterpiece, Mont D'or and Red Kidney seeds reacted identically with erythrocytes of all A_1A_2BO groups.

The titres of Navy Pea seed extract with red cells of different phenotypes under various conditions are compared in Table 3, which shows that it reacted more strongly with cells possessing the A_1 and/or B antigen than with cells of groups A_2 and O, in which respect it conformed to the general pattern of the tribe Phaseoleae (Bird, 1959b) and not to the species pattern. It reacted identically with D+ and D negative erythrocytes of group O in all tests, showing that D(Rh_0) specificity was absent.

None of the extracts showed blood group selectivity in saline or bovine albumin solution at any temperature between 8° and 56°. Above 56°, agglutinin and red cells would not react. Titres in albumin were higher

TABLE 3. TITRES OF NAVY PEA SEED EXTRACT WITH HUMAN ERYTHROCYTES UNDER VARIOUS CONDITIONS

Test	Blood group					
	A_1	A_1B	B	A_2B	A_2	O
Reaction medium = human AB serum	64	64	32	32	16	16
pH 6·03–pH 6·40..	128	128	128	128	64	64
Extract absorbed with O or A_2 erythrocytes and titrated in:						
saline:	8	8	2	1	—	—
bovine albumin:	32	32	16	8	—	—

"—" = no reaction.
Titres are recorded as the reciprocals of the greatest dilutions causing agglutination, and represent the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

than those in saline between 22° and 52°, and there was a difference of 1,920 between the maximum titres in the two media (Fig. 1). The marked change in titre between 8° and 40° (Fig. 1) is of interest since Makela (1957) reported that the activity of *P. vulgaris* haemagglutinin altered little over this temperature range.

None of the 27 sugars or sugar derivatives tested had any effect on the agglutination reaction. Although the action of many phytohaemagglutinins is selectively inhibited by simple sugars, *P. vulgaris* seed extract is not unique in being unaffected by these compounds. For example, Morgan & Watkins (1953) reported that the O cell agglutinins of *Laburnum alpinum* J. Presl and *Cytisus sessilifolius* L. seeds were unaffected by 2% concentrations of twelve sugars, all of which prevented the reaction of *Lotus tetragonolobus* seed extract with group O erythrocytes.

The titre of Navy Pea seed extract from pH 6·03–pH 7·35 was higher with A_1, B, A_1B or A_2B cells than with A_2 or O cells (Fig. 2). This cannot be attributed solely to pH, since that of the undiluted extract was 6·10, and blood group selectivity did not occur in saline. It has been shown that anions have no effect on phytohaemagglutination (Makela, 1957; Liener, 1958) and so the reaction was unlikely to have been modified by the chemical natures of the buffer constituents, all of which were sodium salts. A slight turbidity was observed when Navy Pea seed extract and buffered cell suspension were mixed, and erythrocytes suspended in the buffer solutions were found on microscopical examination to be crenated, which suggests that a change in the condition of the red cell membrane and salting-out of a seed protein fraction played some part in this blood group selectivity.

The latter conclusion is supported by the results of absorption and elution tests, for whereas all haemagglutinating activity was removed from Navy Pea seed extract by absorption with A_1, B, A_1B or A_2B erythrocytes, absorption with A_2 or O erythrocytes left a supernatant reactive only with cells having the A_1 and/or B antigen. It reacted more strongly in albumin than in saline, and was of lower titre than the original extract (Table 3). Eluates from erythrocytes of each group which had been agglutinated by fresh extract contained a non-specific agglutinin. These phenomena can be explained by assuming the extract to contain a non-specific agglutinin and a separate anti$-A_1$ $+$B agglutinin.

In human AB serum, the agglutination of erythrocytes of all A_1A_2BO groups by Canadian Wonder, Masterpiece, Mont D'or and Red Kidney seed extracts was equally inhibited. Agglutination by Navy Pea seed extract of O and A_2 cells was more strongly inhibited than that of B and A_2B cells, and agglutination of A_1 and A_1B cells was not inhibited. The fact that Navy Pea seed extract differentiated between erythrocytes of different A_1A_2BO groups in AB serum may have been due to partial inhibition of the non-specific agglutinin by normal serum proteins, and partial inhibition of the anti $-A_1$ $+$B agglutinin by A and B substances.

Northrop & Liener (1959) found that a number of mucoproteins containing sialic acid prevented agglutination of papainised rabbit erythrocytes by Wax-Bean (*P. vulgaris* L.) agglutinin, and it is possible

that one or more proteins may selectively inhibit the action of Navy Pea agglutinin in such a way as to render it specific for erythrocytes of one human blood group.

Previously no importance has been attached to the cultivar of *P. vulgaris* L. seed used in the preparation of haemagglutinin, and there is no evidence to suggest that cultivars used have been systematically examined and identified. The present study has shown that the haem-agglutinating properties of seed extracts from a number of authenticated *P. vulgaris* cultivars are not identical. Since one cultivar has been shown to exhibit blood group selectivity under certain experimental conditions, it is probable that other cultivars may also do so.

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