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Purification and properties of a plant Agglutinin

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BOSTON UNIVERSITY

GRADUATE SCHOOL

Thesis

PURIFICATION AND PROPERTIES OF A PLANT AGGLUTININ

by

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(B.S., Grove City College, 1954)

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My sincere thanks to Dr. Boyd for his endless guidance in this research. It was indeed a privilege to study under him.

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INTRODUCTION

The purpose of the work reported in this thesis was to study the properties of the hemagglutinin in the seeds of *Bauhinia purpurea alba*; to attempt to purify it; and to find out if it could be of practical use in blood typing. Another objective was to try and find any facts that would lead to a better understanding of the chemical constituents of this lectin (10).

It had been previously discovered by Mäkelä (37) and Boyd and McMaster (16) that the seeds of certain species of *Bauhinia* contain a hemagglutinin with anti-N specificity.

Possible rewards for the study of these plant extracts are:

a. The possibility of finding cheap plant sources for blood grouping reagents suitable for use in the determination of blood groups in man (17).

b. The discovery of reactive proteins which could be obtained in pure form and upon which the effects of deliberate chemical modification could be studied (17).

c. The isolation of a pure protein having the properties of a hemagglutinin, which could then be investigated chemically. Eventually, one might hope to work out the amino acid sequence, and determine what portions of the molecule are responsible for the specificity (13).

With these and other ideas resulting from discussion with Dr. Boyd, plus consultation of the

literature, the writer tried to work out, what he thought to be, the most promising approach to this problem.

SURVEY OF THE LITERATURE

The knowledge that certain plant substances would agglutinate blood goes back before the turn of the century. Stillmark (42) working with ricin found that it would agglutinate erythrocytes of certain species. With the discovery of bacteria and their antigenic properties, plant agglutinins were somewhat lost from sight. There was still some work with plant agglutinins, but there was none that would distinguish one individual of a species from another individual of the same species.

The first mention of any specific plant agglutinins was made in 1947 by Boyd in his Fundamentals of Immunology (14). This referred to the discovery, by Boyd in late 1945, that a lima bean would selectively agglutinate A erythrocytes strongest, B erythrocytes much weaker, and O erythrocytes not at all. Renkonen (41) in 1948 published a study on agglutinins in *Leguminoseae* plants. In this study Renkonen found six specific agglutinins, one of which, *Vicia cracca*, showed a titer of 1:640 with A_1 and A_1B erythrocytes. Early the following year Boyd and Reguera (17) tested 262 varieties of plant extracts. Of these 25 varieties showed some specificity. Also in this paper (17) the authors have a note on 62 varieties of lima beans studied; and of these, 30 showed strong specificity for group A erythrocytes. Also at this time Koulumies (30),

a student of Renkonen, made a detail study of _Vicia cracca_. In 1950 Boyd (12) conducted a study on Egyptian plant extracts. In 1951 Bird (4) found _Dolichos biflorus_ to be completely specific for A_1 erythrocytes. The same year Elo, Estola, and Malmstrom (26) working with mushroom extracts found that 36 out of the 139 species they studied would agglutinate erythrocytes. _Marasmium oreades_ showed a very long titer with B erythrocytes. In 1952 Krüpe and Braun (34) studied 300 varieties of plant extracts. In this same year Cazal and Lalaurie (23) conducted a study on 420 species of _Leguminoseae_. Of these they found 9 to have specificity toward certain human erythrocytes. In 1953 Ottensooser and Silberschmidt (39) studied extracts from four species of _Vicia_. They found _Vicia graminea_ would agglutinate N and MN erythrocytes to a titer of 64. This was the first discovery of a plant extract that was specific for N and MN erythrocytes. The next test on plant extracts was carried out by Bachrach, Gurevitch, and Zaitchek (1). These authors tested 27 Israeli plant extracts, of which 8 showed titers not exceeding 16. These authors found that _Tetragonolobus palaestinus_ shows a titer of 8 with O erythrocytes; and _Crotalaria aegyptiaca_, the strongest plant extract, was found to have a titer of 640 with A_1 and A_1B erythrocytes. Finally in 1957 Mäkelä (37) made the largest examination of plant extracts. This author

worked with 1,408 Leguminoseae plant extracts, some of which had previously been tested by other investigators. Of these, he found the following specific plant extracts: 41 anti-H, 11 anti-A, 8 anti-A+B, 1 anti-B, and 8 anti-N.

The use of these plant agglutinins or lectins, as Boyd (10) proposes to call specific plant agglutinins, is recommended by some authors. I am sure that a large number are used and preference differs with various laboratories. Bird (8) suggests that Phaseolus lunatus could be used as an anti-A reagent because of its close correlation to human anti-A. In this same paper Bird recommends that Dolichos biflorus be used as an anti-A_1 because of its weak reaction with A_2B erythrocytes. Krüpe (33) has also recommended the use of certain plant lectins. Boyd and Shapleigh (18) state that by using Dolichos biflorus, a strong anti-A_1, and Ulex europeus, an anti-H, one can easily distinguish between A_1, A_2, A_1B, and A_2B erythrocytes. Also Boyd and Shapleigh (19) have shown that by the use of Ulex europeus, an anti-H, secretors and non-secretors of any blood group can be distinguished. Levine (36) states that he feels that Vicia graminea extracts can be used as regular typing sera.

There has been considerable research on the chemical make-up of plant extracts. Bird (4) froze an extract of Dolichos biflorus for two months and found that at the end of this time the extract hemolized all erythrocytes it came in contact with. Bird (3) working with Glycine soja, a

nonspecific extract, states that if the extract was absorbed with O erythrocytes, it would show only A and B specificity; and that if now absorbed with A erythrocytes, it would show only B specificity. The temperature also has an effect on the extract. It would show nonspecific agglutination only at 4°C.; while after absorption with O erythrocytes, the extract showed agglutination for A and B erythrocytes at 4°C. and 37°C. Bird (6) also made a complete study on the plant extract from Calpurnia aurea. It is an anti-A+B. He could absorb out the activity with either the A or B erythrocytes, so he states that it is a single agglutinin that gives this reaction. Boyd and Shapleigh (20) have been able to show that lectins are specific precipitens. They have shown this with Sieva and Dolichos biflorus. Boyd and Shapleigh (21) state that they feel that the lectins have shown that there is close similarity between the different human blood antigens. Normally we get a strong reaction with human antigens and almost no cross reactions, yet this does not mean necessarily great chemical differences. They believe that the lectins have shown that these antigens have a close chemical composition. Boyd, Shapleigh, and McMaster (22) working with the lectin from lima beans give a purification method which they used to produce a lectin that was about 30% reactive protein. In this same paper the authors give the ultracentrifugal results and electrophoretic determination of this lectin. Also a quantitative study of the

precipitation curve of the lectin is given and compared with human anti-A. The lectin agrees quite well with the value previously found for human anti-A.

In 1952 Watkins and Morgan (43) discovered a very interesting fact -- that they could inhibit anti-H agglutinin from eel sera by simple sugars. It was found by them that L-fucose and closely related sugars could cause this inhibition. Watkins and Morgan (44) found that an enzyme from Trichomonas foetus would inactivate the H sites on erythrocytes and possibly the M and N sites also. In this paper they also found the enzyme could be inhibited by the addition of L-fucose and closely related sugars. Feinberg and Morgan (27) working with the enzyme from Trichomonas foetus extracted a polysaccharide that contained fucose and other monosaccharides. Morgan and Watkins (38) tried to inhibit certain plant extracts with simple sugars. With Lotus tetragonolobus, an anti-H, L-fucose and closely related sugars were able to inhibit this lectin. The same sugars showed inhibition activity against eel sera, but in some cases there was a difference in the strength of the sugar needed to inhibit the lectin and eel sera. They also used Sophora japonica, which agglutinates A and B erythrocytes more strongly than O erythrocytes. With this plant extract it was found that N-acetyl-galactosamine was the best inhibitor. D-galactose and melibiose would also inhibit. Lactose was found to inhibit against B erythrocytes.

They used Vicia cracca, which showed greater affinity for A_1 and A_2 erythrocytes than any other. This was inhibited by N-acetyl-galactosamine. Lima bean extracts were also used, and it was found that N-acetyl-galactosamine gave only slight inhibition. Finally, they tested Laburum alpinum and Cytisus sessilifolius, both of which showed a greater affinity for O and A_2 erythrocytes than any other. The simple sugars showed no inhibition at all.

Krüpe (32) has worked with the complete and incomplete agglutinins of plant extract. He feels that in saline solution the sugars are not able to inhibit the incomplete agglutination, because they are not in the right state. He also feels that the incomplete may be caused by a change in the protein structure.

Bird (2) has reacted certain plant extracts with a variety of animals. He found that Phaseolus lunatus was specific for human A erythrocytes. Vicia faba would react with all human types plus the erythrocytes of guinea pigs and rabbits, and that Dolichos lablab would react with all these, plus pigeon and chicken erythrocytes. Mäkelä (37) found that some Bauhinia extracts would react with guinea pig and rabbit erythrocytes.

Bird (7), with the aid of lectins, tried to prove that the T receptor was a modification of one of the blood antigens. He was unable to show this, so he now feels that it may be something entirely different.

METHODS

The seeds of Bauhinia purpurea alba are ground in an electric coffee grinder set at "drip", then 8 times (by weight) of 0.9% saline is added to the powder. This is kept overnight at 4°C. The following morning, this is pressed, by hand, through 3 layers of cheese cloth and centrifuged at 4°C., 3,500 r.p.m. for 10 minutes. The supernate is then placed in the cold bath until its temperature reaches -3°C., the temperature of the cold bath. Cold ethyl alcohol is added until the concentration of alcohol is 5%. This mixture is allowed to stand for 1½ hours in the cold bath. At the end of this time, the mixture is centrifuged at -3°C. The supernate is kept and returned to the cold bath. The alcohol concentration is now brought up to 10%. After standing overnight in the cold bath, this mixture is again centrifuged at -3°C.; but this time the precipitate is kept. To the precipitate an equal volume of 0.9% saline is added, and the mixture is let stand overnight. The agglutinin dissolves in the saline; and when the mixture is centrifuged at 4°C., a clear supernate is obtained.

The supernate, which from now on the writer will call the lectin, was tested against fresh erythrocytes in the following way. One drop of extract and 1 drop of a 2% suspension of erythrocytes is placed in an "Rh" tube

(70 x 6 mm). The tube is shaken and placed in the refrigerator (0°C.) for 1 hour. After this time, the tubes were again shaken and then centrifuged at 1,500 r.p.m. for 1 minute. The readings of the tubes were made as follows: 4, one large clump; 3, one large clump with small ones; 2, all medium-size clumps; 1, small clumps; and negative, no clumping at all as seen in a low magnifying mirror. Table 1 shows a typical result with this extract.

TABLE 1

TYPICAL AGGLUTINATION OF ERYTHROCYTES BY LECTIN

Cells	Dilution of Lectin							
	ST	2	4	8	16	32	64	128
M	-	-	-	-	-	-	-	-
MN	3	2	2	2	1	1	1	-
N	4	4	4	4	3½	3	1	½

Inhibition tests were also used in this work. This is a test to see if a substance (a sugar) added to another substance (lectin) will diminish its ability to agglutinate cells. In some cases, the reaction with a specific type of erythrocytes is affected. These tests were not done in the usual way as described, e.g., by Boyd (11); instead it was found preferable to add erythrocytes, lectin, and inhibiting substance all at the same time. The mixture was placed in the refrigerator for

1 hour, and the reactions read the same as an ordinary agglutination test.

Lyophilizing was used to remove water from the solution with minimum effect on the substance. This was done, as usual, by freezing the liquid in a delivery flask, attaching the flask to a condenser flask coated with dry ice and ethyl alcohol and applying a vacuum. The water sublimes into the condenser, where it freezes and remains. This leaves only the non-volatile substances behind in the delivery flask. After admitting air, the delivery flask is removed and the solid taken from the flask.

The extract was dialyzed as follows. The extract was placed in cellophane tubing and immersed in water or saline. Small molecules such as electrolytes and sugars pass through the membrane because of the difference in concentration on the two sides. This leaves only large molecules behind.

FINDINGS AND CONCLUSIONS

The first interest in the lectin was to check its agglutinating ability for N specificity after having been subjected to the purification method. Some preliminary runs were made, in which it was found that the erythrocytes should be washed before they are used. Then the lectin was tried against 90 random blood samples. These samples were also tested in the regular way with anti-M and N absorbed rabbit sera. (See Table 2).

In one case the lectin gave a negative reaction with the blood, while the anti-N of the rabbit gave a positive reaction. Since no reason can be shown for this difference, it is thought by the writer that this lectin could not be used at the present time as a typing serum.

Nevertheless, the lectin showed a high degree of specificity, so an attempt was made to find out something about its chemical composition. The method of purification was altered to see if a more specific agglutinin could be produced. The writer tried altering the pH, but this was found to give even poorer results; thus, the extract was left at its regular pH of 5.8. Also, ammonium sulfate was used in place of ethyl alcohol, but this was found to make the extract nonspecific. The extract also became nonspecific after dialysis. The dialyzing was carried out in three ways. The first method was to pass

TABLE 2

THE TYPING OF 90 RANDOM BLOOD SAMPLES BY BAUHINIA PURPUREA ALBA AND RABBIT ANTI-N

No.	Rabbit Anti-N	Lectin	No.	Rabbit Anti-N	Lectin
1	3	3	46	3	1½
2	2	2½	47	3	2½
3	-	-	48	-	-
4	-	-	49	4	2
5	-	-	50	3	3
6	3	2	51	3	2
7	3	2	52	4	3½
8	3½	4	53	4	2
9	-	-	54	3	2
10	-	-	55	3	4
11	-	-	56	4	3
12	3	4	57	4	3
13	4	3	58	-	-
14	4	3½	59	4	4
15	4	4	60	4	3
16	4	3	61	3	2½
17	3	3	62	-	-
18	3	1½	63	4	4
19	3	2	64	-	-
20	4	4	65	4	4
21	3	1½	66	4	4
22	-	-	67	4	4
23	-	-	68	4	-
24	4	4	69	4	4
25	4	3	70	-	-
26	-	-	71	4	4
27	4	3	72	4	2
28	3	3	73	4	4
29	-	-	74	4	4
30	3	3	75	4	3
31	4	2	76	-	-
*32	3	-	77	4	4
33	3	3	78	4	3
34	4	4	79	4	4
35	-	-	80	-	-
36	-	-	81	-	-
37	4	3	82	-	-
38	4	4	83	4	3
39	3	2	84	4	4
40	4	3½	85	4	3½
41	-	-	86	4	3
42	4	4	87	4	4
43	-	-	88	4	4
44	4	4	89	4	4
45	-	-	90	-	-

*Lectin and rabbit serum disagreed.

the crude extract through the cheese cloth and then place it in a cellophane tube. This tube was then placed in a 10 qt. pail and running tap water was passed over it for 18 hours. The second method was to place 250 cc. of the crude extract in the cellophane tube, which was then placed in a 10 qt. pail filled with 0.9% saline. The pail and contents were placed in the refrigerator (4°C.) for 18 hours. The third method was to place 250 cc. of the crude extract in the cellophane tube and place the tube in a 10 qt. pail containing distilled water. The pail and contents were then placed in the refrigerator (4°C.). The substance left in the cellophane tube, in all three methods, was removed and tested for its agglutination activity. In all three cases this substance, which from now on will be called Y, was found to be nonspecific. From these findings it is easy to see that at least two parts are necessary for the extract to be specific, one of which was of protein size, that is, it would not pass through the dialyzing membrane, and one that was much smaller in size because it would pass through the membrane. The seed's brown covering was removed before it was purified, but this had no effect on the extract.

Before investigating the above idea, the extract was treated with formalin (40). One cc. of formalin was added to 9 cc. of extract, and this was left stand at room temperature for 24, 48, and 72 hours. A control was

also run. The extract was tested at the end of the times stated, but it was found that all the activity was lost at the end of the first day. The results are shown in Table 3.

TABLE 3

THE EFFECT OF FORMALIN ON LECTIN

Erythrocytes	Dilution of Lectin				
Formalin treated	ST	2	4	8	16
M	-	-	-	-	-
MN	-	-	-	-	-
N	-	-	-	-	-
Control					
M	-	-	-	-	-
MN	2	3	-	-	-
N	2	3	2	-	-

The reaction of formaldehyde with proteins is not thoroughly understood. The chemical reacts with the free amino group of amino acid, and one would think that it would also do this with proteins. The formaldehyde may still react with the amino carbon atom of proteins and also take place in reactions with other parts of the protein. Hewitt (28) has shown that the reaction of formaldehyde and protein is slower and irreversible. Thus, he feels that the formaldehyde may possibly react at different sites on the protein. Hewitt also states that

it is possible that formaldehyde may cause the combination of two molecules at their active site, thus destroying all activity. Eaton (25) has shown the optical rotation of the protein molecule is not altered by formaldehyde; thus the combination may not effect the optically active carbon atom. We can therefore say little about the effect of this on the extract. Since all activity is lost, it is also possible that the protein may have been denatured.

The erythrocytes were treated with ficin (11) and then reacted with the lectin. The results are shown in Table 4.

TABLE 4

THE EFFECT OF FICIN ON ERYTHROCYTES

Cells	Dilution of Lectin								
	ST	2	4	8	16	32	64	128	256
M	4	4	4	4	4	4	3	2	-
MN	4	4	4	4	4	4	2	1	-
N	4	4	4	4	4	4	$3\frac{1}{2}$	3	$\frac{1}{2}$

It is possible that the ficin removed something from the cell, thus producing another site with which the lectin reacted. This may be true, because not only did it react with M erythrocytes, but also titered out further than usual with N and MN erythrocytes.

The writer then tried inhibition of the lectin with sugars. The following sugars were used: sorbitol, methyl-D-glucoside, melibiose, galactose, N-acetyl-glucosamine, galactosamine HCl, raffinose, and glucosamine. The sugars showed no inhibition effect on the lectin at all. See Table 5.

TABLE 5

THE INHIBITION EFFECT OF THE FOLLOWING SUGARS ON LECTIN

Cells	Dilution of Sugars							
	ST	2	4	8	16	32	64	128
	Sorbitol							
M	-	-	-	-	-	-	-	-
MN	3	3	3	3	3	2½	3	3
N	4	4	4	4	4	4	4	3½
	Methyl-D-Glucoside							
M	-	-	-	-	-	-	-	-
MN	s2	s2½	s2½	3	3	3	3	2
N	4	4	4	4	4	4	3	3
	Melibiose							
M	s-	s-	s-	s-	s-	s-	s-	s-
MN	s-	½	2	2	2	1½	1	½
N	s-	s-	3½	3½	4	4	4	4

s - stuck
h - hemolized

TABLE 5 - Continued

Cells	Dilutions of Sugars							
	ST	2	4	8	16	32	64	128
	Galactose							
M	-	-	-	-	-	-	-	-
MN	s-	s-	s-	s2	1	2	2½	1
N	s½	3½	3½	4	4	4	4	4
	N-Acetyl-Glucosamine							
M	-	-	-	-	-	-	-	-
MN	3½	3½	3½	3½	2½	3½	3½	2
N	4	4	3½	4	4	4	4	4
	Galactosamine HCl							
M	h	h	h	-	-	-	-	-
MN	h	3½	3½	3½	3½	3½	2	1
N	h	4	4	4	4	3½	3	3
	Raffinose							
M	-	-	-	-	-	-	-	-
MN	2	3½	3½	3	3	3	3	3
N	4	4	4	4	4	4	4	4
	Glucosamine							
M	-	-	-	-	-	-	-	-
MN	3	3	4	3	3	3	2½	3
N	4	4	4	4	4	4	4	4

s - stuck
h - hemolized

A small portion of the lectin was frozen on October 4, 1957. This was kept frozen until January 20, 1958. Table 6 shows the results. The lectin lost some of its strength in freezing but still was specific.

TABLE 6

FREEZING OF LECTIN

Cells	Dilution of Lectin							
	ST	2	4	8	16	32	64	128
	Lectin Before Being Frozen							
M MN N	- 3 4	- 2 4	- 2 4	- 2 4	- 1 3½	- 1 3	- 1 1	- - ½
	Lectin After Freezing							
M MN N	- 2 4	- 3 3	- ½ 2	- - 1½	- - -	- - -	- - -	- - -

It was stated earlier, after dialyzing the crude extract, the substance remaining in the cellophane tube was nonspecific. It was then thought that by dialyzing against distilled water at $4^{\circ}C.$, as in Method 3 stated earlier, the material that passed through the membrane could be recovered. The dialyzing was carried out in this manner and the liquid was lyophilized. After lyophilizing, a light brown substance remained which showed

a great affinity for water. When a small amount of water was added, a thick syrup was produced. The lyophilized material was then added back to the non-dialyzable substance, Y. The results are shown in Table 7. From this table I am sure it can be seen that X, the substance that passed through the membrane, makes the extract specific again for N and MN erythrocytes.

This X was then tested with Benedict's reagent and gave a positive test for a reducing sugar. It was also tested with Barfoed's reagent and gave a negative reaction. This suggested that there was little monosaccharide present. The indole test (24) for glucosamine and galactosamine gave a positive color suggesting the presence of at least one, if not both.

Since X inhibits the non-dialyzable substance, Y, and shows the presence of carbohydrates or carbohydrate-like substances, Y was tested against the same sugars as was the lectin. The results are listed in Table 8. The sugars melibiose, raffinose, and galactose all have the ability to inhibit the reaction of Y with M erythrocytes.

It was not clear why the inhibition tests could be run with or without an incubation period. To investigate this problem, the inhibition tests were run as follows: Y and sugar were placed in tubes and incubated at 37°C. for 1 hour, and then the erythrocytes were added to the tubes, and the tubes were placed in the refrigerator

TABLE 7

THE INHIBITION OF Y BY X

Cells	Dilution of X														
	ST	2	4	8	16	32	64	128	256	512	1024	2048	4096	8182	16364
M	-	-	-	-	-	-	-	-	-	1	1	2½	2	3	3½
MN	-	-	-	-	-	-	-	-	2	3½	3½	3½	3½	3	3½
N	-	-	-	-	½	1	2½	3½	4	4	4	4	4	4	4

TABLE 8

THE INHIBITION EFFECT OF THE FOLLOWING SUGARS ON Y

Cells	Dilution of Sugars							
	ST	2	4	8	16	32	64	128
	Sorbitol							
M	2	3½	4	4	4	3	4	4
MN	3½	3½	3½	3½	3½	3½	3	3
N	4	4	4	4	4	4	4	4
	Methyl-D-Glucoside							
M	1	3	4	4	4	4	4	4
MN	4	4	4	4	4	4	4	4
N	4	4	4	4	4	4	4	4
	Melibiose							
M	-	-	-	-	-	-	-	-
MN	-	-	-	½	1	2	2½	3
N	-	-	½	1	2	3½	3½	4
	Galactose							
M	-	-	-	-	-	-	-	½
MN	s-	s-	s-	s-	s1	1	2	3
N	s-	s-	s1	2	2½	3	3½	3½
	N-Acetyl-Glucosamine							
M	3	4	4	4	4	4	4	4
MN	3	4	4	4	4	4	4	4
N	3	4	4	4	4	4	4	3½

s - stuck
h - hemolized

TABLE 8 - Continued

Cells	Dilution of Sugars							
	ST	2	4	8	16	32	64	128
	Raffinose							
M	-	-	-	-	-	-	-	-
MN	2	3½	3½	3	3	3	3	3
N	1	1½	2	2	2	3½	4	4
	Glucosamine							
M	4	4	4	4	3½	4	3	3
MN	4	4	4	4	4	4	4	4
N	3½	4	4	4	4	4	4	4
	Galactosamine HCl							
M	h	h	h	1	1	2½	3½	3½
MN	h	3	3½	4	3	3	4	3
N	h	4	4	4	4	4	4	4

s - stuck
h - hemolized

at 0°C. for 1 hour and then read as usual. The results are shown in Table 9-a. In another series Y, sugar, and erythrocytes were all placed in the same tube and then the tubes were placed in the refrigerator at 0°C. and at the end of one hour read. See Table 9-b. Finally, the erythrocytes and sugars alone were placed in tubes for an hour at 37°C.; and after this time, Y was added to

the tubes. These tubes were then placed in the refrigerator at $0^{\circ}C.$ for 1 hour and read as usual. See Table 9-c. The erythrocytes, Y, and sugars for all of the above tests were from the same supply.

The results shown in this table are difficult to explain. As can be seen, the inhibition differs with the method used, with Method b showing the best inhibition. This agrees with Krüpe, as cited in Mäkelä (37). He states that if one has a solution of lectin and agglutinated erythrocytes and then adds the appropriate sugar to these, the clumps of erythrocytes will break up, thus showing that the lectin has left the erythrocytes and combined with the sugar. This then would explain why Method b is better than Methods a or c. The incubation of the erythrocytes and sugar, as in Method c, reduces the strength of the agglutination for N and MN erythrocytes. This could possibly be explained by some sort of chemical combination between the erythrocytes and sugar, thus inhibiting the sites where Y would combine with N and MN erythrocytes. Method a, the usual inhibition method, shows almost the same results as b; but because Method b takes less time, it was preferred by the writer.

These <u>Bauhinia purpurea alba</u> extracts have shown some very interesting reactions. The most important of these is that of Y and the inhibition of the sugars

TABLE 9

INHIBITION TESTS

Cells	Dilution of Sugars				
	3	9	27	81	243

TABLE 9-a

	Melibiose				
M	-	-	-	½	3
MN	-	2	3	3	3
N	-	3	3½	4	4
	Raffinose				
M	-	-	-	½	3
MN	-	2	3	3	3
N	2	4	4	4	4

TABLE 9-b

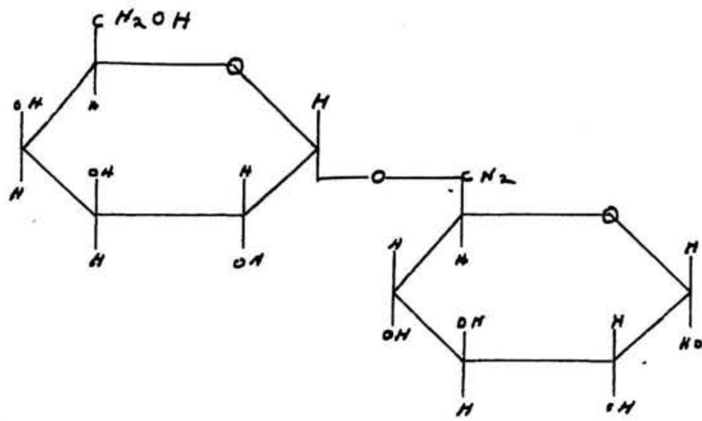
	Melibiose				
M	-	-	-	-	½
MN	-	2	2	3	4
N	-	3½	3½	3	4
	Raffinose				
M	-	-	-	½	½
MN	½	1	1	1½	3
N	1	3½	4	4	4

TABLE 9 - Continued

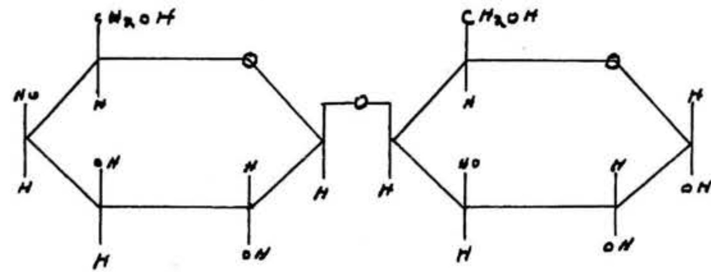
Cells	Dilution of Sugars				
	3	9	27	81	243
TABLE 9-c					
	Melibiose				
M MN N	- - ½	- - ½	1 - 1	- ½ 3	- 2 3
	Raffinose				
M MN N	- - 1	- 1½ 1	- 1 3	½ 1 1	½ 2½ 1

galactose, melibiose, lactose (13), and raffinose. Of these sugars the terminal carbohydrate is galactose, and in all cases the next to terminal carbohydrate is glucose. The linkage between these two carbohydrates is not the same in all cases. Melibiose and raffinose have a 1-6 alpha linkage between their monosaccharide, while lactose has a 1-4 beta linkage between monosaccharides. Raffinose, a trisaccharide, also has a 1-2 alpha linkage between glucose and fructose. (See Figure 1.)

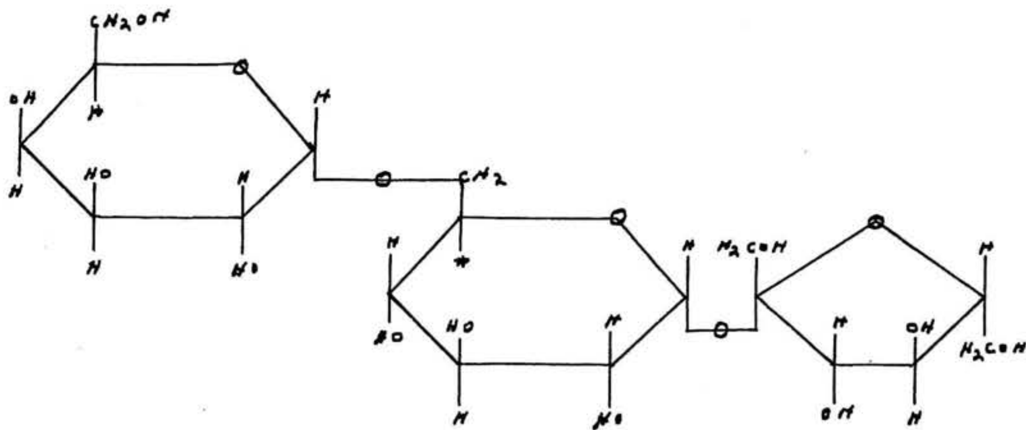
FIGURE 1



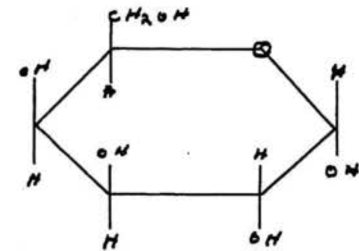
MELIBIOSE



LACTOSE



RAFFINOSE



GALACTOSE

Other important work done in Dr. Boyd's laboratory, but not by the writer, showed that glucose does not inhibit as well as galactose and that fructose does not inhibit at all. In addition, lactose is not as good an inhibitor as melibiose and raffinose. Another fact is that stachyose, a tetrasaccharide, has no inhibition effect.

Landsteiner (35) has shown with heptans that the terminal molecule has the greatest effect on inhibition, the next to the terminal molecule the next greatest effect, and so on down the chain of molecules.

The writer feels that the carbohydrate series most important for the inhibition is alpha-D-galactose-1,6-alpha glucose or melibiose.

Morgan and Watkins (38) have shown that fucose would inhibit anti-H, that N-acetyl-D-galactosamine would inhibit anti-A, and D-galactose would inhibit anti-B substance. Kabat and Keslowitz (29) have shown that next to the terminal carbohydrate in B substance is probably N-acetyl-glucosamine linked by a 1-6 linkage to alpha-D-galactose. From the work of these four authors, most workers feel that N-acetyl-galactosamine is the terminal carbohydrate of A substance; that alpha-D-galactose-1,6-N-acetyl-glucosamine are the first two sugar molecules of B substance; and L-fucose is the terminal carbohydrate of H substance.

With the results given here with the plant extracts of Bauhinia purpurea alba, the writer was tempted to state that alpha-D-galactose-1,6-alpha-glucose is the end disaccharide of M substance. However, this does not seem to be so. X was added to unadsorbed rabbit sera, both anti-M and anti-N. The results showed a weak tendency to inhibit the anti-N rabbit sera but no effect on the anti-M sera. Then the different inhibiting sugars were run against adsorbed rabbit anti-M and N. The results showed no inhibition effect. If melibiose or some closely related sugar were the terminal disaccharide of M substance, then there is no reason that it would not inhibit rabbit sera.

Morgan and Watkins (38) have shown that Sophora japonica, which is an anti-A+B, can also be inhibited by galactose and melibiose for both A and B erythrocytes; and lactose will inhibit against B erythrocytes. Thus, this is nearly the same results that the writer has shown with extracts from Bauhinia purpurea alba, yet the latter shows N specificity.

The human blood stream possesses a strong antigen and there is little cross reacting between different blood types and antigens. This does not necessarily mean that the different blood substances are not closely related chemically. As Boyd and Shapleigh (21) have stated, they feel there is a close chemical correlation between blood

group substances. Thus, the writer feels that it is possible that the terminal disaccharide of the B and M blood group substances could be the same or so closely related that the plant extract cannot distinguish between the two and thus can be inhibited by the same disaccharides. The antibodies produced in rabbit sera may be more specific and thus will not be inhibited by closely related disaccharides. Boyd (13) feels that *Sophora japonica* and the dialyzed Y of *Bauhinia purpurea alba* react with some receptor common to all human cells and closely related to the A and B substance, and thus can be inhibited by the same sugars.

The results given here, especially those that deal with the chemical nature of the inhibiting substances, are suggestive. Although the writer feels that no conclusive results have as yet been established, more investigation along the lines described here may well succeed in establishing the chemical nature of the antigen of the M blood group.

APPENDIX

Since cross reactions between blood of different species is of interest, the writer tested both the lectin and Y against 4 rabbits, 5 guinea pigs, 5 mice, and 2 dogs. As can be seen in Table 10, the rabbit and guinea pig erythrocytes all agglutinated by both lectin and Y. Of the 5 mice, the erythrocytes of only 1 mouse were agglutinated. The dogs' erythrocytes were all agglutinated by Y, but one did not react with lectin.

TABLE 10

THE REACTION OF DIFFERENT SPECIES WITH LECTIN AND Y FROM BAUHINIA PURPUREA ALBA

Animal	Number	Reaction with Undiluted Substance	
		Lectin	Y
Rabbit	1	3	4
	2	4	4
	3	4	4
	4	4	4
Guinea pig	1	3	3
	2	2	2
	3	1	3
	4	2	1
	5	1	3
Mouse	1	-	-
	2	-	-
	3	s-2	s-2
	4	-	-
	5	-	-
Dog	1	-	3
	2	3	3

s - stuck

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ABSTRACT

This study involved work with extracts of the seeds of Bauhinia purpurea alba, in which an N specific lectin was found by Mäkelä (37) and Boyd and McMaster (16). There were two main purposes in mind. First, to see if the lectin could be of practical use as a typing sera; and secondly, to get some insight into its chemical make-up.

After working out a purification method and a method for checking the agglutinating power of the lectin, the lectin was tested against several small samples. The writer then tested this lectin against 90 random blood samples. The lectin disagreed with rabbit anti-N in one case -- the lectin typed one MN as an M. There is as yet no known reason for this difference, so it would seem that one could not use the lectin as a typing sera at the present time.

The lectin was treated with formaldehyde and lost all its activity as a result.

Erythrocytes were treated with an enzyme, ficin, and were then reacted with the lectin. The results showed an increase in the titer of the lectin, and the specificity of the lectin was lost.

The freezing of a sample of the lectin for four months resulted in the loss of some of its activity. It remained specific, however.

Upon dialyzing the crude extract against saline or water, the substances (Y) that remained behind in the membrane became nonspecific. This showed that there were at least two parts to the extract, one of large molecular weight because it would not pass through the membrane, and the other smaller in size because it would pass through the membrane. The substance that passed through the membrane was called X. By dialyzing the crude extract against distilled water and lyophilizing the distilled water, a substance was found which gave the reaction of a sugar and also seemed to have either or both glucosamine and galactosamine present.

Since X showed the presence of reducing sugars and could be shown to inhibit Y against the M site on erythrocytes (see Table 7), it was thought that some sugars should be tested against Y to see if any of them would be able to inhibit Y. The same sugars were also tested against the lectin (Table 5). It was found that none of the sugars had any effect on the lectin; but raffinose, melibiose, and galactose all were found to inhibit Y (Table 8). It has also been shown by other workers (13) that lactose will inhibit Y.

The inhibition of the sugars against lectin and Y were run in three ways. The first was the usual inhibition method where the inhibiting substance (sugar, in this case) is placed with the substance to be inhibited (Y) and

incubated for one hour. Then the appropriate erythrocyte was added. The second method was to place erythrocytes, Y, and sugar all in the same tube at the same time. The third was to incubate sugar and erythrocytes together and then add Y. The results are given in Table 9. It was found by the writer that Method 2 was the best. This agrees with Krüpe as cited in Mäkelä (37) who states that if an appropriate sugar is added to erythrocytes that are agglutinated by a lectin, the lectin will leave the erythrocytes and pick up the sugar. This is shown by the breaking up of the clumps of erythrocytes.

The sugars that would inhibit Y were tried against adsorbed rabbit sera, both anti-M and N. The sugars showed no effect against the rabbit sera. The writer feels that the lectin may not be as specific as the rabbit sera; thus, these inhibiting sugars may only be closely related to the true configuration of the M site.