and not supercoiled. However, supercoiling is not a necessary condition for efficient transcription of all DNAs, and some of the most active templates for *E. coli* RNA polymerase are the linear DNAs isolated from bacteriophages T4, T5, and T7. Apparently there are two distinct classes of efficient initiation sites. The sites in one class require a structural constraint in the DNA related to supercoiling in order to function well, whereas the sites in the other class do not need this constraint. It will be of interest to learn how the sites in these two classes differ in their primary structures.

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Studies on Competitive Binding of Lectins to Human Erythrocytes†

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ABSTRACT: The binding to human group O erythrocytes of four ^{131}I-labeled lectins [*Maackia amurensis* hemagglutinins (MAM and MAH), *Ricinus communis* hemagglutinin (RCH), and *Bauhinia purpurea* hemagglutinin (BPH)] was studied in detail. Competitive binding studies permitted an analysis of the relationship between the cell receptor sites of the lectins. We conclude that MAM and RCH, or MAH and BPH, share a common oligosaccharide chain on the erythrocyte surface and bind to the same or overlapping portions of the oligosaccharide chain.

The lectins have been extensively investigated because of several peculiar biological activities. These include blood group specific hemagglutinating activity, tumor cell specific agglutinating activity, and mitogenic activity against peripheral lymphocytes (Boyd, 1963; Lis and Sharon, 1973). These activities are assumed to stem from the initial binding of the lectins to receptor sites of carbohydrate nature on the cell surface. In previous studies (Fukuda and Osawa, 1973), we have tested the antigenic receptor activity of a major glycoprotein of human group O erythrocyte membrane and suggested that the antigenic receptor sites for some lectins mostly reside in *N*-glycosidically linked oligosaccharide chains of the glycoprotein similar to the one isolated by Kornfeld and Kornfeld (1970), whereas those for the other group of lectins possibly reside in O-glycosidically linked oligosaccharide chains of the glycoprotein such as the one reported by Thomas and Winzler (1969).

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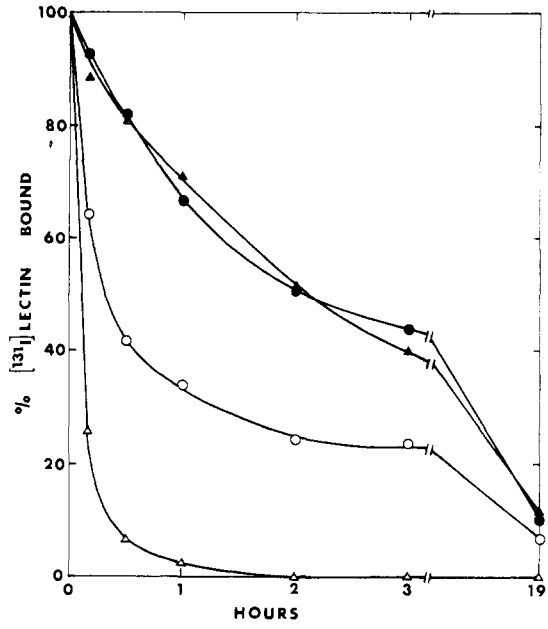

FIGURE 1: Reversal of ^{131}I-labeled lectin binding to erythrocytes. Reaction mixture containing human group O erythrocytes (6×10^7 cells) and an iodinated lectin (0.2 μg of ^{131}I-labeled MAM, 0.1 μg of ^{131}I-labeled RCH, 0.2 μg of ^{131}I-labeled MAH, or 0.7 μg of ^{131}I-labeled BPH) in a final volume of 0.2 ml were incubated for 90 min at room temperature. Then, porcine thyroglobulin (1 mg) was added to the tube containing ^{131}I-labeled MAM or ^{131}I-labeled RCH, and porcine submaxillary mucin (1 mg) was added to the tube containing ^{131}I-labeled MAH or ^{131}I-labeled BPH, and the final volume adjusted to 0.3 ml. The time course of dissociation of ^{131}I-labeled lectin was followed measuring the radioactivity bound to the cells: (●) ^{131}I-labeled MAM; (○) ^{131}I-labeled RCH; (▲) ^{131}I-labeled MAH; (△) ^{131}I-labeled BPH.

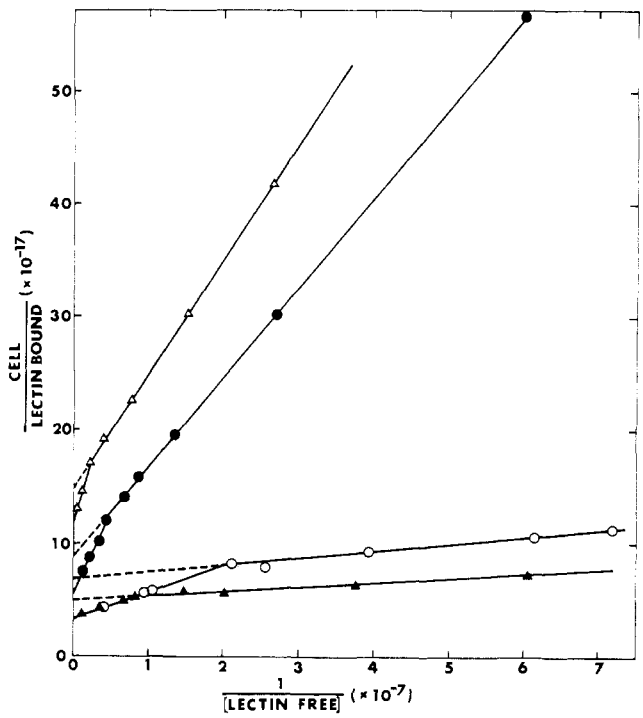

FIGURE 2: Binding of ^{131}I-labeled lectin to human group O erythrocytes. The binding reactions were performed as described in the text. The data were plotted by the method of Steck and Wallach (1965): (●) ^{131}I-labeled MAM; (○) ^{131}I-labeled RCH; (▲) ^{131}I-labeled MAH; (△) ^{131}I-labeled BPH.

Furthermore, we have recently isolated two lectins (MAM and MAH)[1] having different specificities from *Maackia amurensis* seeds and, from the results of hapten inhibition assays, we have concluded that one of them (MAM) preferentially binds to the N-glycosidically linked oligosaccharide chains and the other (MAH) mainly interacts with the O-glycosidically linked oligosaccharide chains on human erythrocyte membrane (Kawaguchi *et al.*, 1974). These conclusions should be verified by studies of the competitive binding of the lectins to the cell surface.

In this paper, we report the results of competitive binding studies of four lectins, all of which, belong to the so-called galactose-specific lectins (Mäkelä, 1957), to human group O erythrocytes.

Experimental Section

Materials. MAM and MAH were purified from *M. amurensis* seeds (purchased from F. W. Schumacher, Sandwich, Mass.) according to the method previously described (Kawaguchi *et al.*, 1974). *Ricinus communis* hemagglutinin (RCH) was prepared from commercially available *R. communis* seeds by the method of Tomita *et al.* (1972). *Bauhinia purpurea* hemagglutinin (BPH) was purified from *B. purpurea* seeds (purchased from F. W. Schumacher, Sandwich, Mass.) by the methods previously described (Irimura and Osawa, 1972). The homogeneity of these purified lectins was ascertained by ultracentrifugal analysis and electrophoresis on polyacrylamide gel. Porcine submaxillary mucin was purified from porcine submaxillary glands showing blood group H activity according to the method of Katzman and Eylar (1966). Porcine thyroglobulin was prepared by the procedure described by Ui and Tarutani (1961).

Iodination of Lectins. The purified lectins were iodinated with ^{131}I by the chloramine-T methods of Hunter (1967) using a 40-sec exposure to the chloramine-T, and the labeled lectins were freed from an excess of reagents by passage over Sephadex G-50 in the cases of MAM, MAH, and RCH, and over Bio-Gel P-60 in the case of BPH. This procedure did not affect the hemagglutinating activity of the lectins. The specific radioactivity was $0.5\text{–}6 \times 10^{10}$ cpm/μg of protein.

Preparation of Erythrocytes for Lectin Binding Studies. Human group O venous blood was withdrawn into syringes previously treated with heparin. The heparinized blood was transfered to glass cylinders, and the erythrocytes were allowed to sediment by gravity. The erythrocyte layer, after removal of leukocyte-rich plasma and buffy coat, was washed three times by centrifugation with 0.25% bovine serum albumin solution in 5 mM phosphate-buffered saline (pH 7.0), each time carefully removing the top layer of cells. The erythrocytes thus obtained were found to be free from leukocytes or cell debris.

Binding Studies. Binding reactions were carried out in silicon-coated tubes presoaked overnight with 5 mM phosphate-buffered saline–0.25% bovine serum albumin. The reaction mixture contained 6×10^6 erythrocytes and 3–500 pmol of ^{131}I-labeled lectin in a final volume of 0.3 ml of 5 mM phosphate-buffered saline–0.25% bovine serum albumin. In competitive binding studies with an unlabeled lectin, various amounts of the unlabeled lectin (up to 2000 pmol) were mixed with ^{131}I-labeled lectin just before the addition of erythrocytes. After incubation at 20° for 90 min with occasional mixing, the cells were washed twice with 3 ml of chilled phosphate-buffered saline–0.25% bovine serum albumin, and the amount of bound ^{131}I was determined in an Alloka autogamma counter.

[1] Abbreviations are: MAM, strongly mitogenic *Maackia amurensis* hemagglutinin; MAH, strongly hemagglutinating *Maackia amurensis* hemagglutinin; RCH, *Ricinus communis* hemagglutinin; BPH, *Bauhinia purpurea* hemagglutinin.

TABLE I: Binding of Various [^{131}I]Lectins to Human Group O Erythrocytes.

Lectin	K_0[a]	n[b]	n_t[c]
MAM	1.2×10^7	6.5×10^5	1.1×10^6
RCH	1.2×10^8	8.6×10^5	1.8×10^6
MAH	1.4×10^8	1.2×10^6	1.7×10^6
BPH	1.5×10^7	4.0×10^5	5.2×10^5

[a] Apparent association constant (M^{-1}) for major receptor sites. Average value of triplicate experiments. [b] Number of major receptor sites. Average value of triplicate experiments. [c] Number of total receptor sites. Average value of triplicate experiments.

When the radioactivity of the supernatant of washings was checked, the dissociation of ^{131}I during the washing process was found to be negligible. Appropriate corrections were made for nonspecific binding to the tube.

Results and Discussion

Time Course and Reversibility of Lectin Binding to Human Erythrocytes. The binding of ^{131}I-labeled lectins to human erythrocytes was found to reach a plateau after 60–90 min in the conditions described in the Experimental Section. The formation of the lectin–erythrocyte complex was found to be specifically reversed by adding to the binding mixture the corresponding hapten inhibitor as shown in Figure 1. This reversibility of the lectin binding is a necessary prerequisite for the following equilibrium studies.

Determination of Active Lectin. Since significant errors could result in the following equilibrium studies if the inactivation of lectin occurs during the process of preparation, the amount of active lectin in each preparation was determined by binding a fixed amount of ^{131}I-labeled lectin to a large excess of human erythrocytes (10^8 cells for 4 pmol of the labeled lectin). The actual amount of active lectin in the labeled preparation was calculated from the radioactivity bound to the cells. Thus, on an average, 90, 85, 82, and 72% of hemagglutinin molecules in the preparations of RCH, MAH, MAM, and BPH, respectively, were found to be active. These values were used for the correction of the concentrations of lectins in the following equilibrium studies.

Estimation of Binding Constants and Number of Receptor Sites. The binding studies of ^{131}I-labeled lectins to human erythrocytes were carried out, and the data obtained were plotted according to the method of Steck and Wallach (1965) as shown in Figure 2. It is interesting to note that the binding of the ^{131}I-labeled lectins to the erythrocytes gives biphasic lines. Since these lectins were purified by affinity chromatography and found to be homogeneous at least from the criteria of ultracentrifugation and electrophoresis, these results indicate that there exist two kinds of receptor sites, namely the one to which the lectin binds preferentially and the other to which the lectin can bind only at high concentrations, on the erythrocytes for each of these lectins. The apparent association constant for the major receptor sites (K_0) and the average number of major receptor sites (n) and of total receptor sites (n_t) per cell were calculated assuming the following molecular weights: MAM and MAH, 130,000 (Kawaguchi *et al.*, 1974); RCH, 120,000 (Tomita *et al.*, 1972); BPH, 195,000 (Irimura and Osawa, 1972). These values are listed in Table I. Since the calculation from Figure 2 of the association constant for the receptor sites, to which the lectin binds only at high concentrations, may involve significant error (Klotz and Hunston, 1971), the calculation of the association constant for the second class of receptor sites needs further investigation. As shown in Table I, the average number of major receptor sites for MAM is in close proximity to that for RCH, and these values are substantially lower than the average number of receptor sites for MAH. In view of the observations that MAM and RCH preferentially bind to N-glycosidically linked oligosaccharide chains whereas MAH binds mainly to O-glycosidically linked oligosaccharide chains on the erythrocytes (Irimura and Osawa, 1972; Kawaguchi *et al.*, 1974), these results seem to support the assumption that the O-glycosidically linked oligosaccharide chains are more abundant than the N-glycosidically linked oligosaccharide chains in the glycoprotein moiety of human erythrocyte membrane (Presant and Kornfeld, 1972; Fukuda and Osawa, 1973). Although BPH has also been assumed to bind mainly to the O-glycosidically linked oligosaccharide chains on the erythrocytes (Irimura and Osawa, 1972), the average number of major receptor sites for BPH is lower than that for MAH, possibly because of the fact that BPH has greater affinity to the incomplete oligosaccharide chains lacking terminal sialic acids whereas MAH preferentially binds to the complete oligosaccharide chains (Irimura and Osawa, 1972; Kawaguchi *et al.*, 1974).

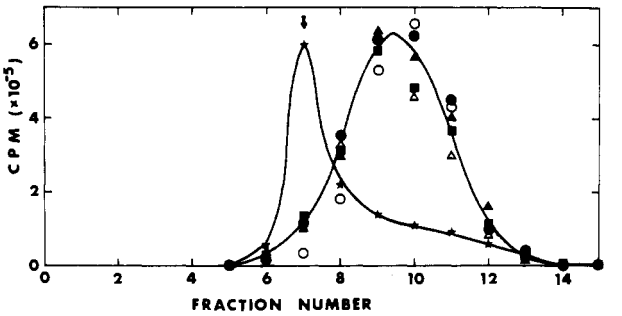

FIGURE 3: Bio-Gel P-200 gel filtration of ^{131}I-labeled MAM mixed with an unlabeled lectin. ^{131}I-labeled MAM (130 μg; 2.5×10^6 cpm) was mixed with the same amount of an unlabeled lectin [(●) MAM; (○) RCH; (▲) MAH: (△) BPH] or porcine thyroglobulin (★) or bovine serum albumin (■) in a final volume of 0.2 ml of 5 mM phosphate-buffered saline. The mixture was applied to a column of Bio-Gel P-200 (0.8 × 8.5 cm) equilibrated with the same buffer. Fractions of 0.3 ml were collected at a flow rate of 1 ml/hr at room temperature. The radioactivity of each fraction was determined. The void volume of the column is indicated by a vertical arrow.

Interaction between Lectins. The absence of direct interaction between lectins used in this study was ascertained by gel filtration on Bio-Gel P-200. Thus, when a mixture of ^{131}I-labeled MAM and another lectin was passed through a column of Bio-Gel P-200, no radioactive peak was observed in the void volume of the column as shown in Figure 3, indicating that the complex between the lectins was not formed. On the other hand, the mixture of ^{131}I-labeled MAM and its effective inhibitor, porcine thyroglobulin, gave a radioactive peak in the void volume of the column. The same results were obtained in every combination of the lectins used in this study. Furthermore, no precipitin band was formed between the lectins of this study in gel diffusion (Öuchterlony, 1967). The absence of direct interaction between these lectins thus confirmed is an important prerequisite for the following equilibrium studies.

Competitive Binding of Lectins. The effect of the presence of an unlabeled lectin in an equal concentration on the binding of ^{131}I-labeled lectin to human erythrocytes is shown in Table II. They act as inhibitors of each other's binding in any combination of lectins, and the degree of inhibition appears to depend on the relative strength of their affinity to the erythrocytes. In

TABLE II: Inhibition of Binding of Various [^{131}I]Lectins by Other Lectins.

Competing Unlabeled Lectin[c]	Binding of Labeled Lectin (% of Control)[a]			
	[^{131}I]MAM (24.0×10^{-8} M)[b]	[^{131}I]RCH (5.74×10^{-8} M)[b]	[^{131}I]MAH (5.00×10^{-8} M)[b]	[^{131}I]BPH (40.2×10^{-8} M)[b]
None	100	100	100	100
MAM	54.7	87.3	97.0	54.8
RCH	38.5	56.8	83.4	15.5
MAH	28.5	67.8	49.0	26.1
BPH	62.2	88.5	89.6	60.0

[a] Average value of duplicate experiments. [b] Concentration of iodinated lectin. [c] Concentration of competing unlabeled lectin is equal to that of iodinated lectin.

order to obtain more precise information on the competitive binding of lectins, the inhibition of the binding of ^{131}I-labeled lectin was carried out with various concentrations of an unlabeled lectin, and the number of the unlabeled inhibitor molecules (m) involved in displacing the ^{131}I-labeled lectin from the cell and K_I value of the unlabeled inhibitor were calculated by eq 11, which was derived according to Harris and Pestka (1973) as follows. We assume two mutually exclusive equilibria are present between the ^{131}I-labeled lectin (H), the unlabeled competing lectin (I), and the receptor site on human erythrocyte (R) if the iodinated lectin and the unlabeled lectin share a common receptor site. In these equilibria, the reversibility of the lectin binding and the absence of direct interaction between the lectins have been demonstrated in Figures 1 and 3. Then, eq 3 and 4 describe the equilibria, where K_0 and K_I are the association constants for the ^{131}I-labeled lectin and the unlabeled inhibitor, respectively. Thus

$$R + H \rightleftharpoons [RH] \quad (1)$$

$$R + mI \rightleftharpoons [RI_m] \quad (2)$$

$$K_0 = [RH]/[R][H] \quad (3)$$

$$K_I = [RI_m]/[I]^m[R] \quad (4)$$

$$[RI_m] = K_I[I]^m[R] \quad (5)$$

where [H] = free ^{131}I-labeled lectin concentration, [I] = [I_0] = input unlabeled lectin concentration, [R_0] = concentration of receptor (cell number $\times$ n of ^{131}I-labeled lectin), [RH] = concentration of ^{131}I-labeled lectin–receptor complex, [RI_m] = concentration of receptor–inhibitor$_m$ complex. Since

$$[R] = [R_0] - [RH] - [RI_m] \quad (6)$$

then

$$[R] = [R_0] - [RH] - K_I[I]^m[R] \quad (7)$$

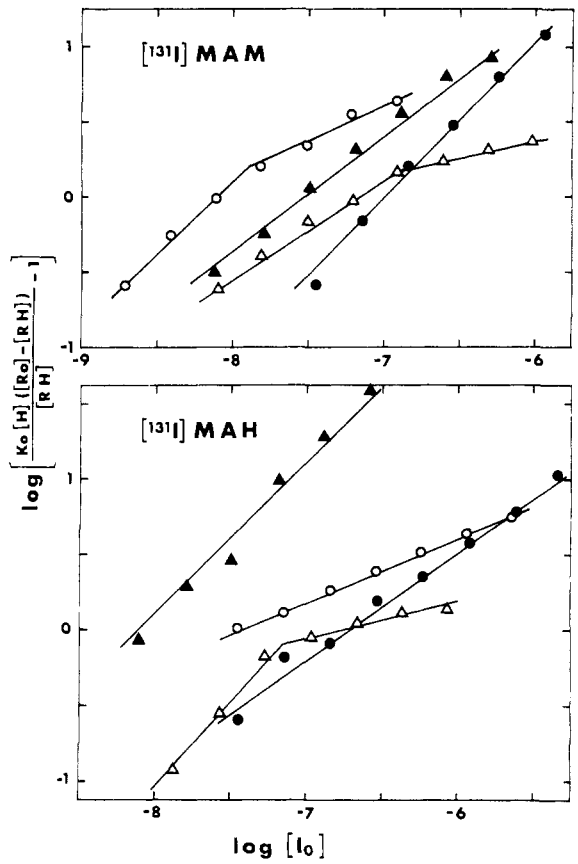

FIGURE 4: Effects of unlabeled lectins on binding of ^{131}I-labeled lectins to human group O erythrocytes and determination of K_I and m values. Binding of ^{131}I-labeled MAM (concentration: 2.0×10^{-7} M against unlabeled MAM, RCH, and BPH; 5.3×10^{-8} M against unlabeled MAH) and ^{131}I-labeled MAH (concentration: 5.0×10^{-8} M against unlabeled MAM, RCH, and MAH; 1.3×10^{-8} M against unlabeled BPH) to the erythrocytes was carried out in the presence of various concentrations of unlabeled lectins [(●) MAM; (○) RCH; (▲) MAH; (△) BPH], and log [(K_0[H]([R_0] − [RH])/[RH]) − 1] values were calculated and shown as a function of log [I_0]. K_I and m values determined are presented in Table III.

and

$$[R] = ([R_0] - [RH])/(1 + K_I[I]^m) \quad (8)$$

By substituting for R in eq 3

$$K_0 = \frac{[RH]}{\frac{[R_0] - [RH]}{1 + K_I[I]^m}[H]} \quad (9)$$

Equation 9 can be rearranged into

$$\frac{K_0[H]([R_0] - [RH])}{[RH]} - 1 = [I]^m K_I \quad (10)$$

TABLE III: K_I and m Values for Various Competing Lectins.

Competing Unlabeled Lectins	K_0 Value of Competing Lectin	K_I and m Values[a] in the Competitive Binding with			
		[^{131}I]MAM	[^{131}I]RCH	[^{131}I]MAH	[^{131}I]BPH
MAM	1.2×10^7	1.1×10^7 ($m = 1.0$)	8.0×10^6 ($m = 1.0$)	5.3×10^4 ($m = 0.70$)	2.1×10^5 ($m = 0.70$)
RCH	1.2×10^8	1.3×10^8 ($m = 1.0$)	1.4×10^8 ($m = 1.0$)	9.1×10^2 ($m = 0.40$)	2.3×10^7 ($m = 0.85$)
MAH	1.4×10^8	4.6×10^5 ($m = 0.75$)	4.9×10^3 ($m = 0.45$)	1.3×10^8 ($m = 1.0$)	1.4×10^8 ($m = 1.0$)
BPH	1.5×10^7	5.4×10^4 ($m = 0.65$)	6.4×10^5 ($m = 0.80$)	1.4×10^7 ($m = 1.0$)	1.6×10^7 ($m = 1.0$)

[a] Average values of triplicate experiments.

$$\log\left[\frac{K_0[H]([R_0]-[RH])}{[RH]}-1\right]=m\log[I]+\log K_I \quad (11)$$

Equation 11 is of the form $y = ax + b$. From the data of the competitive binding studies carried out on every combination of the four lectins, log $[(K_0[H]([R_0] - [RH])/[RH]) - 1]$ values are calculated and shown as a function of log $[I_0]$ in the examples of ^{131}I-labeled MAM and MAH in Figure 4. Thus, the slope will give m and the intercept on the ordinate will give log K_I. In some combinations, these plots give biphasic lines possibly due to the presence of two kinds of receptor sites for each lectin. The m and K_I values for the receptor sites, to which an inhibitor preferentially binds, are listed in Table III. The calculation of m and K_I values for the second class of receptor sites needs further investigations. As shown in Figure 4 and Table III, the competition between an ^{131}I-labeled lectin and the same but unlabeled lectin gave $m = 1$ and $K_I = K_0$. This indicates that, in our experimental conditions, eq 11 with the approximation $[I] = [I_0]$ is valid for the determination of m and K_I values, and is applicable to the elucidation of the relationship between the major receptor sites of two lectins. In fact, the relationships, $m = 1$ and K_I is approximately equal to K_0 of competing lectin, were also observed in the competitive binding between MAM and RCH, and between MAH and BPH. This might suggest that MAM and RCH bind to the same or at least overlapping portions most probably of the N-glycosidically linked oligosaccharide chains on the erythrocyte membrane, and MAH and BPH bind to the same or overlapping portions possibly of the O-glycosidically linked oligosaccharide chains on the cell membrane. These assumptions have been supported by the results of hapten inhibition assays using various glycopeptides as inhibitors (Kawaguchi *et al.*, 1974; T. Irimura *et al.*, unpublished results). On the other hand, the relationships, $m < 1$ and $K_I < K_0$ of competing lectin, were observed in the following other combinations: MAM–MAH, MAM–BPH, RCH–MAH, and RCH–BPH. In these combinations, the cell receptor sites are different, but they somewhat exert an inhibitory effect to each other's binding as shown in Table I and Figure 4. This could occur if binding of a lectin to its receptor sites results in a conformational change in the molecule which would in turn result in a decrease in the affinity, or in an altered specificity, of the receptor sites of another lectin. The precise feature of the inhibition in these cases requires further investigations.

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