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A STUDY OF THE SPECIFICITY OF *Bandeiraea simplicifolia* LECTIN I BY COMPETITIVE-BINDING ASSAY WITH BLOOD-GROUP SUBSTANCES AND WITH BLOOD-GROUP A AND B ACTIVE AND OTHER OLIGOSACCHARIDES*†

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ABSTRACT‡

The specificity of *Bandeiraea simplicifolia* lectin I (BS I) has been studied by competitive-binding assays (CBA) using tritium-labeled human B and hog A substances Blood-group B substances isolated from horse gastric mucosae and from human ovarian-cyst fluids were much better inhibitors of binding of tritiated blood-group B substance to insoluble BS I–Sepharose 2B than were human blood-group A substances from saliva and ovarian-cyst fluid A and B active blood-group substances showed the same range of potency in inhibiting binding of tritium-labeled hog A substance to BS I–Sepharose 2B

CBA with BS I–Sepharose 2B, labeled human blood-group B substance, and human blood-group A and B active oligosaccharides separated the haptens into two groups differing in slope Group 1, containing methyl α-D-GalNAc*p*, D-GalNAc*p*, and an A active pentasaccharide AR_L 0 52, with 3, 19, and 25 nmol respectively needed for 50% inhibition of binding, has a lower slope than group 2, which contains α-D-GalNAc*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol and *p*-nitrophenyl α-D-GalNAc*p*, with 3 nmol of each required for 50% inhibition of binding, as well as ten glycosides with terminal, nonreducing, α-linked D-Gal*p* The most potent inhibitors of this group were *p*-nitrophenyl α-D-Gal*p*, α-D-Gal*p*-(1→3)-D-Gal*p*, α-D-Gal*p*-(1→6)-D-Glc*p*, and methyl α-D-Gal*p*, with 5, 7 4, 9 6, and 11 nmol respectively needed to inhibit binding by 50% The difference in slopes was explainable in terms of a recent finding that BS I exists as a mixture of five isolectins composed of two

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‡Abbreviations used BS I, *Bandeiraea simplicifolia* lectin I, CBA, competitive-binding assay, cpm counts per minute, Fuc*p*, 6-deoxygalactopyranose, Gal*p*, galactopyranose, GalNAc*p*, 2-acetamido-2-deoxygalactopyranose, Glc*p*, glucopyranose, GlcNAc*p*, 2-acetamido-2-deoxyglucopyranose, HGM, hog gastric mucin, PBS, phosphate-buffered saline (0 01M PO_4, 0 15M NaCl, pH 7 1), R, 3-hexenetetrol(s)

subunits having different specificities, subunit A is most specific for α-linked, terminal, nonreducing D-GalNAc*p*, but it also reacts with α-linked, terminal, nonreducing D-Gal*p*, whereas subunit B tends to be more specific for terminal, nonreducing, α-linked D-Gal*p*

INTRODUCTION

A number of carbohydrate-binding proteins from plants and from certain invertebrates specifically agglutinate erythrocytes of the ABO or MN groups [1,2], and precipitate with water-soluble, blood-group substances The specificities of their combining sites have been defined by immunochemical methods, including quantitative, precipitin inhibition and hemagglutination-inhibition assays[2–10] Lectins having such defined specificities have proved useful in structural studies of carbohydrate-containing polymers from rat stomach and duodenum[11], mammalian blood-group substances[12], canine secretory alloantigens[13] and human meconium[14] lectins have also been used to elucidate structures of oligosaccharides isolated from horse gastric-mucosa[15] and human ovarian-cyst[16,17] glycoproteins

Lectins covalently coupled to an insoluble matrix can be used to isolate blood-group-related glycoproteins from complex mixtures[11,18], elution with a specific hapten will release the glycoprotein from the adsorbent Other glycoproteins have been purified by these methods (for a review, see ref 19)

Bandeiraea simplicifolia seed extracts contain two lectins, one of which, BS I agglutinates B and AB erythrocytes very strongly[7,20] and A_1 erythrocytes weakly[7] and a second that does not agglutinate A B or O erythrocytes but is specific[21,22] for terminal nonreducing D-GlcNAc*p* The B-specific lectin has been purified to homogeneity by affinity chromatography on Bio-Gel–melibionate[7], it is a glycoprotein of molecular weight 114 000, with four subunits The lectin precipitates[7] polysaccharides and glycoproteins containing terminal, nonreducing, α-linked D-GalNAc*p* or D-Gal*p* Glycosides of α-D-Gal*p*, namely, methyl α-D-Gal*p*, melibiose [α-D-Gal*p*-(1→6)-D-Glc*p*] and *p*-nitrophenyl α-D-Gal*p*, were the best inhibitors, as determined by inhibition of precipitation[7] As only carbohydrates of low molecular weight and simple linear oligosaccharides were used the site could be more complex

In the present study, competitive-binding assays with carbohydrates and oligosaccharides, some derived from blood-group B or A active glycoproteins, were used to determine the specificity of the BS I combining-site Surprisingly, the results show [^{3}H]human blood-group B substance to be inhibited best in binding to BS I by some glycosides having terminal, nonreducing α-D-GalNAc*p*, as well as by those having nonreducing α-D-Gal*p* The lectin is unusual, in that the oligosaccharides studied fell into two groups, having different slopes in the competitive-binding assay One group contained the most active inhibitors, including *p*-nitrophenyl α-D-GalNAc*p* (**5**) and α-D-GalNAc*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol (**4**) and ten oligosaccharides having terminal, nonreducing α-D-Gal*p* The second group, having a lower slope in competitive binding contained three compounds, one of these, methyl α-D-GalNAc*p*

Methyl α-D-GalNAc*p*

1

D-GalNAc*p*

2

α-L-Fuc*p*
1
↓
2
α-D-GalNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→6)-R

3 (AR_L 0 52)

α-D-GalNAc*p*-(1→3)-2-acetamido-2-deoxy-D-galac

4 (Tij R_L 1 37)

p-Nitrophenyl α-D-GalNAc*p*

5

(**1**), despite its lower slope, had an activity in the same range as the most active compounds in the first group The two others, D-GalNAc*p* (**2**) and an A active pentasaccharide, AR_L 0 52 (**3**), were less active, and had a binding affinity similar to that of methyl α-D-GalNAc*p* (**1**), as reflected by the slopes of their inhibition lines By quantitative inhibition assay Hayes and Goldstein[7] had also found that methyl α-D-GalNAc*p* (**1**) gives a line different from those of the other oligosaccharides tested The glycosides having terminal, α-linked D-Gal*p* were potent inhibitors and all fell into the first group, with high binding-affinity for the BS I site Branched oligosaccharides larger than disaccharides and the linear trisaccharide raffinose showed lessened inhibitory activity In conjunction with the recent findings of Murphy and Goldstein[3 5] that BS I is a mixture of two isolectins, A and B existing in five forms (A_4 A_3B, A_2B_2, AB_3, and B_4), these results suggest that, when two different types of lines are obtained in competitive-binding assays, or in precipitin-inhibition assays it should be considered that a mixture of lectins is being dealt with

MATERIALS AND METHODS

Bandeiraea simplicifolia lectin I (BS I) was a gift from Dr Irwin J Goldstein Its purification, and physical and immunochemical properties, have been described[7 23] The lectin was coupled to cyanogen bromide-activated Sepharose 2B in the presence of melibiose to protect the site[18 24 25]

The tritium-labeled Beach phenol-insoluble (B active) and HGM GalNAc eluate (A active) blood-group substances were prepared by labeling free amino groups of the polypeptide backbone with [^{3}H]acetic anhydride[10 25] The labeled products were isolated by affinity chromatography on BS I–Sepharose and *Dolichos biflorus*–Sepharose 2B, respectively

Blood-group substances* used in CBA were from human ovarian-cysts or saliva, from hog-gastric mucin, and from horse stomach-linings, prepared and described in articles from this laboratory[26–30]

Monosaccharides were obtained from Nutritional Biochemical Corp and Schwartz/Mann Laboratories The blood-group oligosaccharides used were those described previously[17 28]

Competitive-binding assays were performed[10 25] by adding a volume of a 1 10 aqueous suspension of BS I–Sepharose (25 μg of BS I per mL of Sepharose 2B) sufficient to bind 50% of the ~2000 cpm of labeled blood-group substance to a mixture of labeled blood-group substance and unlabeled substance or hapten The tubes were mixed by constant rotation for 16 h at 4° Separation of bound from free label was achieved by repeated centrifugation, and a portion of the supernatant liquor was counted for tritium

The data are expressed graphically as percent inhibition (of binding of labeled antigen) *versus* nanograms of blood-group substance, or nanomoles of hapten added The formula used to compute percent inhibition was as follows

$$1 - \left(\frac{\text{total cpm added} - \text{cpm in supernatant liquor with inhibitor}}{\text{total cpm added} - \text{cpm in supernatant liquor without inhibitor}} \right) \times 100$$

All determinations were set up in duplicate, analyses generally did not differ by more than ± 5 percent Competitive-binding data in the Figures give the combined results of three experiments with each substance

RESULTS

Competitive-binding assays with hog, horse, and human blood-group substances — Competition assays were performed with BS I–Sepharose 2B and labeled Beach phenol-insoluble or labeled HGM GalNAc eluate and various blood-group substances Fig 1(a–f) shows the curves of competition between the B-active [^{3}H]Beach phenol-insoluble melibiose eluate and blood-group A and B active substances The horse fractions in Fig 1(a–c) show decreasing inhibitory activity as more ethanol is needed to precipitate them from phenol, this is consistent with findings of a similar decrease in their activity to inhibit binding of [^{3}H]Beach phenol-insoluble melibiose eluate to insolubilized human anti-B serum[25] and to precipitate with human anti-B serum[29] This is not the case with the human substance Tij (Fig 1d), the 10% 2X fraction being more active than the phenol-insoluble fraction, which is as active as the 20% from 2nd 10% fraction, both requiring 1300–1500 ng for inhibition of binding by 50% compared to 880 ng for Tij 10% 2X, Tij 20% 2X is least active, with 4,400 μg being required CBA with insolubilized human anti-B[25] showed the Tij phenol-insoluble and 10% 2X fraction to be similar in activity, whereas the 20% from 2nd 10% and 20% 2X had decreasing inhibitory activity The most B-active substances

*The symbols in Figs 1 and 2 refer to the fractional-precipitation scheme used in isolating the various blood-group substances[29]

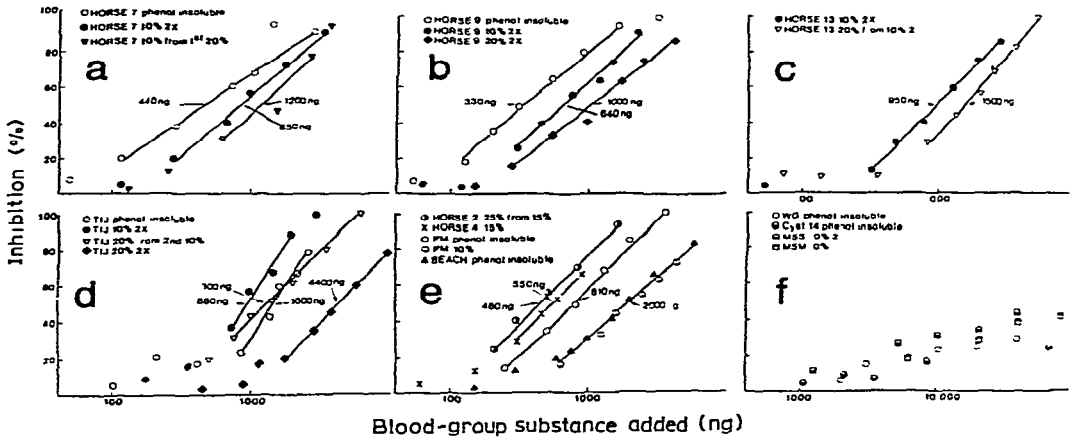


Fig 1 Competitive-binding assays of (a–e) various horse and human B substances and (f) human A substances with BS I–Sepharose and B-active [^{3}H]Beach phenol-insoluble melibiose eluate (The numbers and arrows indicate quantity of substance giving 50% inhibition of binding)

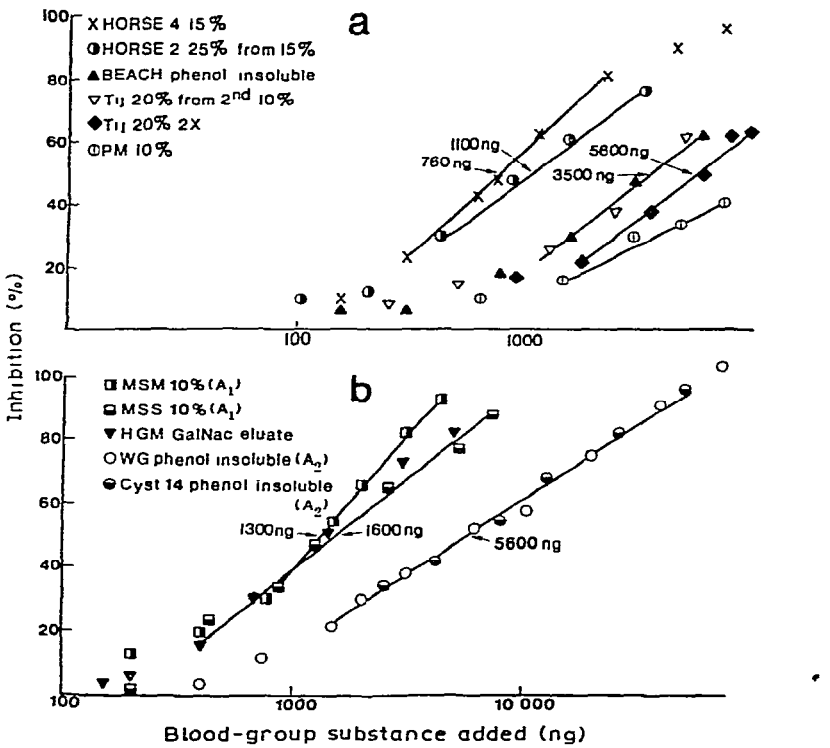


Fig 2 Competitive-binding assays of (a) horse and human B substances, and (b) hog and human A substances with BS I–Sepharose and A-active [^{3}H]HGM GalNAc eluate (The numbers and arrows indicate quantity of substance giving 50% inhibition of binding)

were Horse 9 phenol-insoluble, Horse 7 phenol-insoluble, Horse 2 25% from 15%, and Horse 4 15%, as 330, 440, 460, and 550 ng respectively gave 50% inhibition, this is in agreement with quantitative precipitin data[29 31], in that these horse fractions were of equal potency in precipitating human anti-B PM phenol-insoluble is almost two and one-half times as active as the 10% precipitate from phenol, 810 and 2000 ng respectively giving 50% inhibition Beach phenol-insoluble is as active as PM 10% The order of activity of these three substances by quantitative precipitation with human anti-B[32] is like that found here, *i e*, PM phenol-insoluble precipitates about one-fifth more AbN at equivalence than either Beach phenol-insoluble or PM 10%, which are nearly equal in precipitating ability Various A_1 and A_2 blood-group substances were poor inhibitors, the four tested were estimated by extrapolation to require over 40,000 ng for 50% inhibition

Competition between various A and B active blood-group substances and the A-active [^{3}H]HGM GalNAc eluate is shown in Figs 2a and 2b The order of B inhibitors, from most to least active, was the same as those in Fig 1, except for PM

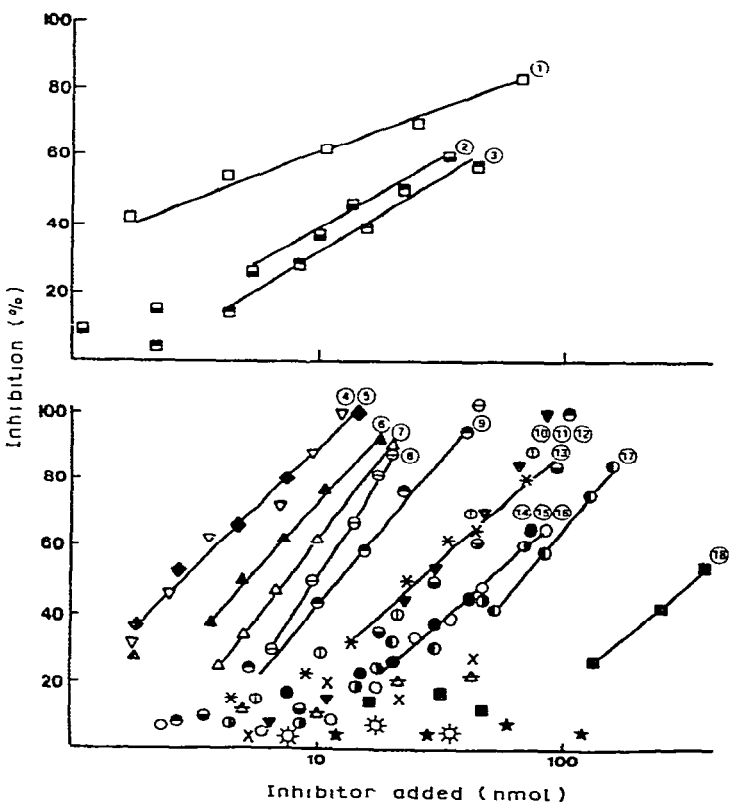


Fig 3 Competitive-binding assays of monosaccharides and oligosaccharides with BS I–Sepharose and B-active [^{3}H]Beach phenol-insoluble melibiose eluate (For symbol and number designation refer to Table I)

10%, which was less active than Beach phenol-insoluble (see Fig 2a), but as active in Fig 1e Horse 4 15% and Horse 2 25% from 15% were the best inhibitors, 760 and 1100 ng being required, respectively Beach phenol-insoluble and Tij 20% from 2nd 10% are equally potent, 3,500 ng giving 50% inhibition Tij 20% 2X, 5,600 ng for inhibition of binding by 40%, has about two-thirds the activity of Beach phenol-insoluble and Tij 20% from 2nd 10% The A_1 and A_2 substances tested differ in the amount required to inhibit binding by 50%, almost four times as much A_2 as A_1 substance (by weight) is needed The ratio at 50 percent inhibition of inhibiting power of A_2 to A_1, ~5600 1500, or 3 7 1, is ~40 percent of that found with *Dolichos*–Sepharose 2B and the [^{3}H]HGM GalNAc eluate by[33] CBA

Competitive-binding assays with mono- and oligo-saccharides — The results of hapten inhibition of binding of labeled B active blood-group substance to BS I–Sepharose are shown in Figs 3a and 3b Table I gives the symbols used in Fig 3, as well as the amounts of hapten added to inhibit binding by 50% The numbers refer to the inhibitors falling on a particular line Fig 3b differs from Fig 3a, in that the inhibition lines in Fig 3b have a steeper slope The steeper slope is an indication that the haptens in Fig 3b combine relatively more strongly[34] than those in Fig 3a Compounds 1, 2, and 3 in Fig 3a and Table I will be referred to as group 1, while those in Fig 3b with the higher binding-affinity will be group 2

Three of the four haptens having terminal, nonreducing, α-linked D-GalNAc*p* were the best inhibitors of binding These include one member of group 1, methyl α-D-GalNAc*p* (**1**) at 4 nmol for 50% inhibition, and two of group 2, *p*-nitrophenyl α-D-GalNAc*p* (**5**) and α-D-GalNAc*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol (Tij R_L 1 37) (**4**), which are equally active, 3 nmol giving 50% inhibition The other two inhibitors in group 1, D-GalNAc*p* (**2**) and AR_L 0 52 (**3**) are one-fifth and one-sixth as active as methyl α-D-GalNAc*p* (**1**), the group 2 inhibitor *p*-nitrophenyl α-D-GalNAc*p* (**6**) at 5 0 nmol for 50% inhibition, is 60 percent as active as *p*-nitrophenyl α-D-GalNAc*p* (**5**) Two disaccharides having D-Gal*p* linked α-(1→3) to D-Gal*p*, compound 7, or α-(1→6) to D-Glc*p* compound **8**, were almost equal in inhibiting power at 7 4 and 9 6 nmol, respectively Methyl α-D-Gal*p* (**9**) required 11 nmol for 50% inhibition,

p-Nitrophenyl α-D-Gal*p* (6) α-D-Gal*p*-(1→3)-D-Gal*p* (7) α-D-Gal*p*-(1→6)-D-Glc*p* (8, melibiose) Methyl α-D-Gal*p* (9)

indicating that the methyl group in the α position contributes less to the binding than the α-linked *p*-nitrophenyl group, or a second sugar linked α-(1→3) to D-Gal*p* or α-(1→6) to D-Glc*p* However, replacement of the hydroxyl group by an acetamido group on C-2 of methyl α-D-Gal*p*, shifting the compound from group 2 to group 1, increases inhibitory activity by a factor of 2 5 at 50 percent inhibition of binding, but decreases the binding affinity, as evidenced by the lower slope In Fig 3a, compound **1** and D-GalNAc*p* (**2**), although a slightly better inhibitor than D-Gal*p* (**11**) at 50% inhibition, 19 and 26 nmol, respectively, has a lower slope, indicating lowered

TABLE I

INHIBITION OF [^{3}H]BEACH PHENOL-INSOLUBLE MELIBIOSE ELUATE BINDING TO BS I–SEPHAROSE BY VARIOUS MONO- AND OLIGO-SACCHARIDES (SEE FIG 3)

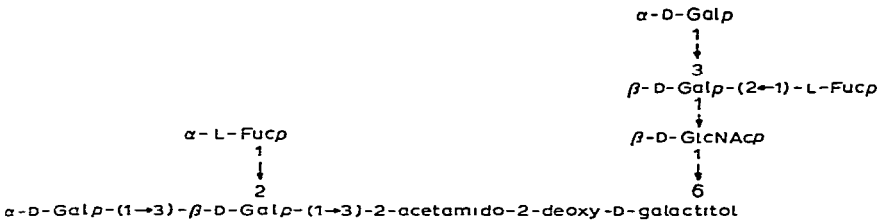
Symbol	*Number*	*Amount for 50% inhibition (nmol)*
Group 1		
□	1	3 0
▬	2	19 0
▬	3	25 0
Group 2		
◆	4	3 0
▽	5	3 0
▲	6	5 0
△	7	7 4
⊖	8	9 6
◓	9	11 0
✳	10	26 0
◐	11	26 0
▼	12	26 0
◒	13	26 0
●	14	52 0
○	15	52 0
◑	16	52 0
◐	17	68 0
■	18	320 0
★	19	inactive at 120 nmol
☼	20	inactive at 35 nmol
×	21	27% at 45 nmol
⏢	22	22% at 45 nmol
◇	23	12% at 13 nmol

α-D-Galp-(1→6)-α-D-Glcp-(1↔2)-β-D-Fruf

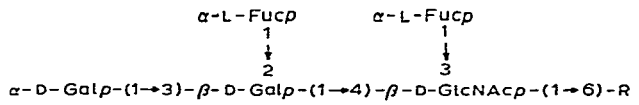
10 (raffinose)

D-Galp

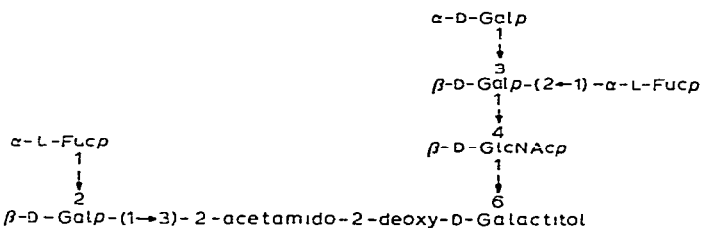
11



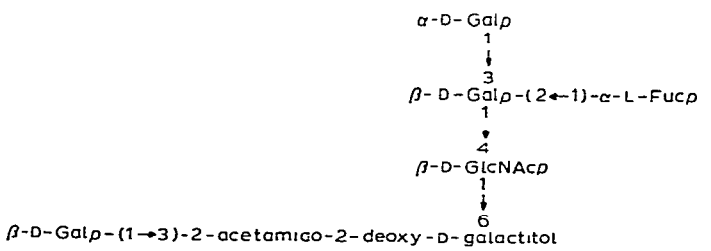
12 (Tij R_{IM5} 0 36)



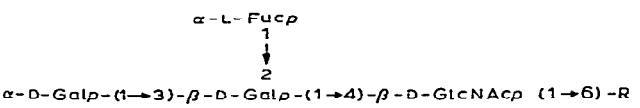
13 (BR_{IM5} 1 2)



14 (Tij R_{IM5} 0 92)



15 (Tij R_{IM5} 0 90)



16 (BR_L 0 44)

binding-affinity for BS I In Fig 3b, raffinose (**10**), D-Gal*p* (**11**), Tij R_{IM5} 0 36 (**12**), and BR_{IM5} 1 2 (**13**), all cluster about a value of 26 nmol Also, compound **3**, AR_L 0 52, required a similar amount for 50% inhibition, but had a lower slope BR_{IM5} 1 2 (**13**) is a difucosyl-substituted, type 2 chain B determinant The corresponding B active compound, BR_L 0 44 (**16**), a monofucosyl-substituted type 2 determinant is half as active. 52 nmol, as AR_L 0 52 (**3**) and BR_{IM5} 1 2 (**13**) Thus, all branched compounds larger than a disaccharide and the linear trisaccharide raffinose show lowered activity in binding BS I Tij R_{IM5} 0 36 (**12**), has two monofucosyl-substituted B determinants, and each may be able to bind to BS I, as compounds **14** and **15**, with only one monofucosyl-substituted type 2B determinant β-(1→6)-linked to 2-acetamido-2-deoxy-D-galactitol (*N*-acetyl-D-galactosaminitol), are half as active as compound **12** These findings are similar to those with antibodies by precipitin-inhibition assay[15 17] and[25] CBA

Methyl β-D-Gal*p* (**17**) is one-sixth as effective, at 68 nmol, as the corresponding α anomer All other β-linked D-Gal*p* compounds were poor inhibitors The non B active oligosaccharide. Tij R_L 1 32 (**18**), was the poorest of the inhibitors tested, 320 nmol being needed for 50% inhibition Tij R_L 1 68b (**19**) was inactive at 120 nmol, the highest amount tested

Methyl β-D-Gal*p*

17

α-L-Fuc*p*
1
↓
2
β-D-Glc*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol

18 (Tij R_L 32)

β-D-Gal*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol

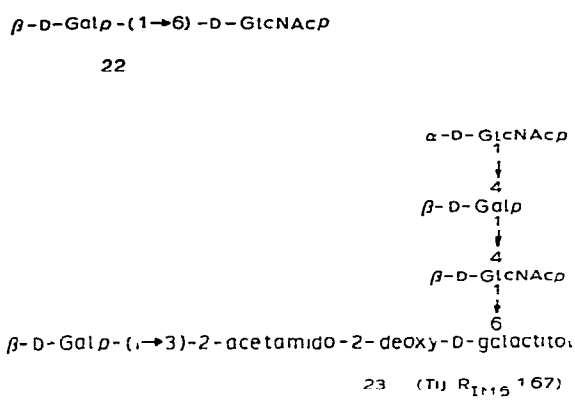
19

β-D-GlcNAc*p*
1
↓
6
β-D-Gal*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol

20 (Tij R_L 0 82)

β-D-Gal*p*-(1→6)-D-Gal*p*

21



DISCUSSION

In an earlier study[7] of inhibition of precipitation of BS I with guar gum methyl α-D-GalNAc*p* was found to give an inhibition curve of slope entirely different from that of oligosaccharides and glycosides of D-Gal*p* The present study confirms and extends this finding, competitive-binding assays clearly show two groups of inhibitors having different slopes Group 1, containing methyl α-D-GalNAc*p* D-GalNAc*p*, and AR_L 0 52 (compounds **1**, **2**, and **3**), has a lower slope than group 2 which contains α-D-GalNAc*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol and *p*-nitrophenyl α-D-GalNAc*p*, compounds **4** and **5**, as well as ten glycosides and oligosaccharides of D-Gal*p* Although the slopes of the most active compounds in group 2 are greater than those of group 1, the most active compounds in each group fall into the same range with respect to inhibiting power That substances fell into two groups with different slopes is surprising for a homogeneous lectin Attempts to account for this on the basis of a single site, by inspection of molecular models, were unsuccessful

The recent findings[35] of Murphy and Goldstein, that BS I is a mixture of two subunits, A and B, and may exist in five forms, A_4, A_3B, A_2B_2, AB_3, and B_4, like PHA[36] and lactic dehydrogenase[37], and that these subunits differ in specificity, provide an explanation for the results Thus, a partially separated mixture containing AB_3 and B_4 was shown to be relatively highly specific for α-linked D-Gal*p*, with methyl α-D-GalNAc*p* (**1**) being ~1/90th as potent as methyl α-D-Gal*p*, and, with purified A_4, methyl α-D-GalNAc*p* (**1**) was 20 times as active as[35] methyl α-D-Gal*p* (**9**) The finding of two different slopes with BS I, a mixture of five isolectins, now becomes understandable Compounds of group 1 would mainly inhibit the A subunit, whereas those of group 2 (having terminal D-Gal*p*) would inhibit the B subunit most effectively, but also inhibit the A subunit The only two compounds of group 2 having terminal, nonreducing D-GalNAc*p* are *p*-nitrophenyl α-D-GalNAc*p* (**2**) and γ-D-GalNAc*p*-(1→3)-2-acetamido-2-deoxy-D-galactitol (**4**), with *p*-nitrophenyl α-D-GalNAc*p* (**5**), the additional hydrophobic group enhances reactivity with the D-Gal*p* specific B subunit, and, evidently, the D-GalNAc*p*-(1→3)-2-acetamido-2-deoxy-D-

galactitol contributes in a similar way These compounds have not been studied[35] with the separated A_4 and the mixture of AB_3 and B_4 isolectins

The presence of five isolectins, and the relatively greater specificity of the B chain for D-Gal*p*, also account for the poor ability of A (see Fig 1f) as compared with B substances to displace the labeled B substance (see Fig 1a–e), indeed, with A substances, it was not possible to reach 50 percent inhibition, even with 40 000 μg, whereas even the least active B substance gave 80 percent inhibition with much lower amounts A and B substances are of comparable potency in displacing labeled A substance (see Fig 2), which would react mainly with the A subunits of the insoluble lectin

Although it is evident that, if a lectin is known to be built of two subunits of different specificities, and if these have been separated, the A_4 and B_4 isolectins would be used for competitive-binding or oligosaccharide-inhibition assays, nevertheless, this study shows that, when such assays with oligosaccharides indicate two types of binding, mixtures of two chains having different specificities may be present and further fractionation should be attempted The specificity and nature of the sites on BS I should be pursued by immunochemical methods with pure A_4 and B_4

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