Structural Requirements for the Binding of Derivatives of D-Galactose to Two Homogeneous Murine Immunoglobulins†

M. E. Jolley, C. P. J. Glaudemans,* S. Rudikoff, and M. Potter

ABSTRACT: A variety of carbohydrate derivatives have been screened for binding with two immunoglobulin A (IgA) myeloma proteins having activity directed against β(1→6)-linked multiple D-galactopyranosyl residues, using a previously described fluorescence technique. The binding constants of the derivatives which interacted with these proteins were then determined. In this way the contributions made by the specific regions of β-D-galactosyl-(1→6)-D-galactose to the total binding energy of this disaccharide could be calculated. A number of conclusions about the active sites of these two proteins have been deduced, and differences between them have been noted.

We have recently reported studies on the binding of Gal_3[1] and Gal_4 to two IgA myeloma immunoglobulins with activity directed against β(1→6)-linked multiple D-galactopyranosyl units (Jolley *et al.*, 1973). These two proteins (X-24 and J-539) are among a group of seven (J-1, X-44, T-191, S-10, M-601, X-24, and J-539), all exhibiting the same specificity. Upon binding haptens the tryptophanyl fluorescence intensities of both proteins X-24 and J-539 are increased up to maximal value, when all available sites are occupied (Jolley *et al.*, 1973; Jolley and Glaudemans, 1974; Pollet and Edelhoch, 1973). This increase in protein fluorescence provides a rapid, sensitive, and versatile method for investigating the binding properties of these two immunoglobulins with a variety of carbohydrate derivatives. The results of such a study have provided detailed information about the structural requirements in haptens for effective binding. There are differences in the modes of binding of these two proteins.

Materials and Methods

The plasmacytomas from which the myeloma proteins were obtained arose in BALB/c mice following the intraperitoneal injection of mineral oil or pristane (Potter and Glaudemans, 1972; Rudikoff *et al.*, 1973). Plasmacytoma J-539 was kindly provided by Dr. Melvin Cohn.

Oligosaccharides. The oligosaccharides Gal_3 and Gal_4 were isolated by extensive chromatography of the partial hydrolysate of gum ghatti (Aspinall *et al.*, 1958). Gal_2 was prepared by a known procedure (Freudenberg *et al.*, 1928), and its R_F value on paper chromatography was identical with an authentic sample provided by Dr. J. K. N. Jones. The synthetic material had $[\alpha]^{20}D + 25.2°$ (*c* 1.2, H_2O) (lit. $[\alpha]^{20}D + 25.1°$).

Methyl β-D-Galactopyranoside. This glycoside was obtained from Calbiochem, and was recrystallized three times from ethanol and once from methanol.

2′,3′-Epoxypropyl β-D-Galactopyranoside (EPG). Dry allyl alcohol (135 ml) was stirred at room temperature with silver carbonate (3.81 g) and powdered "Drierite" (13.5 g) for 2 hr with the exclusion of light and moisture. Acetobromogalactose (5 g) was then added and the mixture was stirred for a further 2 hr, after which the mixture was filtered through Celite and evaporated to a brown oil which was dissolved in CH_2Cl_2 (40 ml). The CH_2Cl_2 solution was washed once with distilled water, twice with 1 N HCl, three times with saturated $NaHCO_3$ solution, and then twice with distilled water. The CH_2Cl_2 layer was dried ($MgSO_4$) and evaporated, and the resulting colorless syrup dissolved in a small volume of CH_2Cl_2 and applied to a silica gel column in Et_2O–hexane (2:3). After extensive elution with this solvent system, the system was changed to Et_2O–hexane (3:2), which eluted a pure product. Appropriate fractions were pooled and concentrated to a colorless syrup (3.1 g, 66%). The allyl β-D-galactopyranoside tetraacetate thus obtained was pure by thin-layer chromatography (tlc) (R_F 0.48, EtOAc–benzene, 3:7), and gave a positive reaction to $KMnO_4$ and H_2SO_4 sprays. A solution of allyl β-D-galactopyranoside tetraacetate (3.1 g, 8.03 mmol) in CH_2Cl_2 (30 ml) was cooled in ice–H_2O. *m*-Chloroperbenzoic acid (1.52 g, 8.83 mmol) dissolved in 30 ml of CH_2Cl_2, was added while stirring. The solution was removed from the ice-water bath and was heated to reflux, the progress of the reaction being followed by tlc. Over a period of 9 hr a further 1.52 g (8.82 mmol) of *m*-chloroperbenzoic acid was added while refluxing, and after another 4.5 hr under reflux, no starting material could be detected. The solution was cooled and washed three times with 5% sodium bisulfite solution, twice with saturated $NaHCO_3$ solution, three times with distilled water, dried ($MgSO_4$), and evaporated to a colorless syrup of 2′,3′-epoxypropyl β-D-galactopyranoside tetraacetate (2.6 g, 76.5%). The product gave a single spot on tlc (R_F ~0.25, EtOAc–benzene, 3:7) which gave a negative reaction to the $KMnO_4$ spray and positive reactions to H_2SO_4 and Methyl Red–NaI sprays (the latter for epoxides). 2′,3′-Epoxypropyl β-D-galactopyranoside tetraacetate was de-O-acetylated with 0.02 M barium methoxide in the usual way (Isbell, 1930). The de-O-acetylated product, 2′,3′-epoxypropyl β-D-galactopyranoside, was twice recrystallized from ethanol: mp 125–127° (uncor) with gassing; $[\alpha]D$ −10.0° (*c* 1.44, H_2O). *Anal.* Calcd for $C_9H_{16}O_7$: C, 45.76; H,

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[1] Abbreviations used are: Gal_2, 6-*O*-β-D-galactopyranosyl-D-galactose; Gal_3, the corresponding trisaccharide; Gal_4, the corresponding tetrasaccharide; EPG, 2′,3′-epoxypropyl β-D-galactopyranoside; MEG, 2′-methoxyethyl β-D-galactopyranoside; IgA, immunoglobulin A.

[2] A solution of 5 mg of Methyl Red and 2.5 g of NaI in 50 ml of isopropyl alcohol. Epoxides yield a yellow spot on a pink background after heating the sprayed plate at 100° for 5 min.

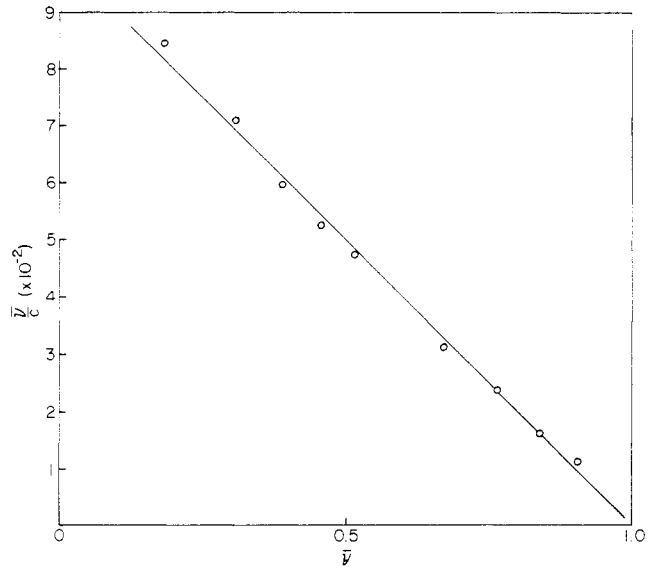


FIGURE 1: Scatchard plot for the binding of methyl β-D-galactopyranoside to the Fab′ fragment of J-539.

6.83. Found: C, 45.51; H, 6.75. The product gave a single spot on tlc (R_F ~0.37, 1-butanol–acetic acid–water, 4:1:5, upper layer), which was positive to H_2SO_4 and the Methyl Red–NaI sprays. In addition, a solution of the product in water gave a positive reaction to the $Na_2S_2O_3$–phenolphthalein test for epoxides (Ross, 1950).

2′-Methoxyethyl β-D-Galactopyranoside (MEG). Dry 2-methoxyethanol (135 ml) was stirred with silver carbonate (3.81 g) and powdered "Drierite" (13.5 g) for 1 hr in the absence of light. Acetobromogalactose (5 g) was added and the mixture stirred for 4.5 hr after which time no starting material was present (tlc). Filtration through Celite and concentration yielded a pale yellow syrup which was dissolved in CH_2Cl_2 (40 ml) and washed once with distilled water, twice with 1 N HCl, three times with saturated $NaHCO_3$ solution, and then twice with distilled water. The CH_2Cl_2 layer was dried ($MgSO_4$) and concentrated to a syrup (4.6 g, 94.0%), which gave a single spot on tlc (R_F ~0.33, EtOAc–benzene, 3:7). This was de-O-acetylated with 0.02 M barium hydroxide in the usual way (Isbell, 1930) and the product (2.6 g; 94.1%) gave a single spot on tlc (R_F ~0.61, EtOAc–HAc–H_2O, 18:7:8). The syrup was chromatographed on silica gel in EtOH–benzene (2:1) and appropriate fractions were pooled and concentrated to a colorless syrup (1.3 g). All attempts to crystallize this material failed. However, the material gave a single spot on tlc in a number of solvent systems (*n*ButOH, R_F ~0.09; *n*ButOH–HAc–H_2O, 4:1:5, upper layer, R_F ~0.36; EtOH–benzene, 2:1, R_F ~0.55; EtOAc–HAc–H_2O, 18:7:8, R_F ~0.61); $[\alpha]_D$ +8.4° (*c* 3.3, H_2O). The compound tenaciously held water (elemental analysis showing 1 mol of water). Mass spectroscopy (CI) revealed no impurities and mainly two peaks of 239 and 163 for the parent ion and the nor-2-methoxyethyl derivative.

Purification of Immunoglobulins and Their Fragments. The immunoglobulins were purified as described earlier (Potter and Glaudemans, 1972), and pepsin fragments were prepared as previously described (Inbar *et al.*, 1971; Rudikoff *et al.*, 1972).

Determination of Binding Constants. K_a values were determined by fluorescence titration, essentially as described (Jolley *et al.*, 1973; Jolley and Glaudemans, 1974). It was found that the final percentage increase in tryptophanyl fluorescence (δF_{max}) could be increased twofold by excitation at 295 nm instead of 280 nm, the emission being monitored at 340 nm. All measurements were determined at 25.0°. Compounds were initially screened for activity, *i.e.*, ability to produce an increase in fluorescence of the protein. This was done by adding the solid material to a solution of the protein (1.5 ml; OD_{280nm} 0.05) at pH 7.4 (0.05 M Tris-HCl). More solid was added until a maximum change (if any) was obtained. A preliminary K_a value was then ascertained in the usual way. There was no direct relationship in the magnitude of the maximal change in fluorescence (δF_{max}) for any hapten–protein system and the K_a of the hapten. The hapten concentration was then adjusted to give approximately 50% binding after the addition of 20 μl of hapten solution to the protein solution (1.5 ml). In this way, well-distributed points could be obtained, and a good straight-line Scatchard plot achieved (see Figure 1). The slopes and hence the accurate K_a values were determined by the method of least squares.

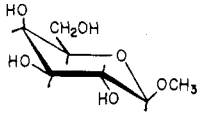
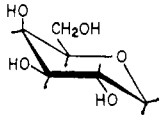
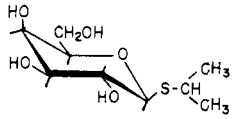
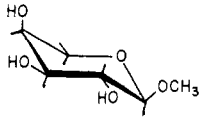
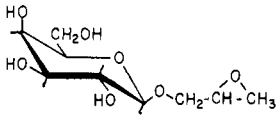
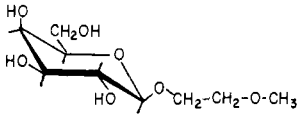
TABLE I: Maximal Changes in Tryptophan Fluorescence of Protein X-24 and J-539 Pepsin Fragments Caused by the Addition of Ligands.

Ligand	X-24 Fab′ (%)	J-539 Fab′ (%)
Gal_4	↑ 30.2	↑ 28.3
Gal_3	↑ 22.7	↑ 25.5
Gal_2	↑ 20.4	↑ 21.2
Methyl β-D-galactopyranoside	↑ 11.4	↑ 14.1
Methyl α-D-galactopyranoside	None	None
Methyl β-D-glucopyranoside	None	None
Methyl β-D-gulopyranoside	None	None
Methyl α-L-arabinopyranoside	↑ 8.4	↑ 9.4
Methyl β-L-arabinopyranoside	None	None
Methyl β-D-arabinopyranoside	None	None
Gentiobiose (Glu-β-(1→6)-Glu)	None	None
Cellobiose (Glu-β-(1→4)-Glu)	None	None
Maltose (Glu-α-(1→4)-Glu)	None	None
Sophorose (Glu-β-(1→2)-Glu)	None	None
Lactose (Gal-β-(1→4)-Glu)	None	None
N-Acetyl-D-galactosamine	None	None
1,6-Anhydro-β-D-galactopyranoside	None	None
1,5-Anhydro-D-galactitol	↑ 15.9	↑ 16.8
D-Galactose	↑ 6.2	↑ 7.5
2′,3′-Epoxypropyl β-D-galactopyranoside	↑ 12.5	↑ 12.2
Isopropyl β-D-thiogalactopyranoside	↑ 12.7	↑ 12.2
Melibiose (Gal-α-(1→6)-Glu)	None	None
2-Deoxy-D-galactose	None	None
Methyl α-D-mannopyranoside	None	None
D-Galactose oxime	None	None
Isoinositol	None	None
d,l-Inositol	None	None
meso-Inositol	None	None
α-D-Galactopyranosylinositol	None	None
1,4-Anhydro-D-galactitol	None	None

Results

Table I shows the effect by saccharides on the tryptophanyl fluorescence of the Fab′ fragments of IgA myeloma immunoglobulins X-24 and J-539. An increase in the fluorescence of these proteins was assumed to indicate binding, and any compound which showed no effect was assumed not to bind. β-Methyl glycosides of D-glucose (*i.e.*, differing from galactose only by having an equatorial hydroxyl group at C-4) and D-gulose (*i.e.*, differing from galactose only by having an axial hydroxyl group at C-3) do not bind. 2-Deoxy-D-galactose

TABLE II: The Binding Constants of a Number of Deviatives of D-Galactose with Two IgA Myeloma Proteins with Anti-galactan Specificity.

Ligand	Formula	K_a $(Fab')_{X-24}$	K_a $(Fab')_{J-539}$
Gal_4	β-D-Gal*p*-1(→6)-β-D-Gal*p* 1)$_2$→6-D-Gal*p*	2.93 (±0.05) × 10^5	3.44 (±0.07) × 10^5
Gal_3	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p*	1.75 (±0.03) × 10^5	1.50 (±0.02) × 10^5
Gal_2	β-D-Gal*p*-(1→6)-D-Gal*p*	5.75 (±0.07) × 10^3	1.14 (±0.01) × 10^4
Methyl β-D-galactopyranoside		3.77 (±0.07) × 10^2	10.01 (±0.21) × 10^2
1,5-Anhydro-D-galactopyranose		1.07 (±0.04) × 10^2	4.09 (±0.05) × 10^2
Isopropyl β-D-thiogalactopyranoside		4.71 (±0.06) × 10^2	1.06 (±0.02) × 10^3
Methyl α-L-arabinopyranoside		1.60 (±0.07) × 10^2	4.00 (±0.03) × 10^2
Epoxypropyl β-D-galactopyranoside		2.28 (±0.03) × 10^3	5.18 (±0.04) × 10^3
Methoxyethyl β-D-galactopyranoside		1.59 (±0.009) × 10^3	3.08 (±0.033) × 10^3

failed to give a significant change in fluorescence. It must be noted that galactose itself only gives a small change in the fluorescence of these proteins compared to the β-glycosides. Methyl α-L-arabinopyranoside (*i.e.*, methyl β-D-galactopyranoside lacking the hydroxymethyl group) and 1,5-anhydro-D-galactitol (*i.e.*, galactopyranose lacking the exocyclic oxygen function at C-1) bind both proteins, while methyl α-D-galactopyranoside does not. Table II shows the binding constants (K_a values) of a number of simple carbohydrate derivatives.

Discussion

All deductions in this paper are predicated on the following assumptions. (1) If a molecule does not change the intensity of the fluorescence of immunoglobulins X-24 and J-539 at 340 nm, it is assumed that it does not bind to these two proteins. Other homogeneous anti-galactan myeloma proteins (*e.g.*, T-191, S-10) studied by us that are capable of binding Gal_3 and Gal_4 do not show changed fluorescence on ligand binding. Thus J-539 and X-24 exhibit a special property, *i.e.*, a change in tryptophanyl fluorescence upon binding haptens. (2) All haptens that change the fluorescence bind in the same site on the protein. Scatchard plots for all haptens tested are strictly linear. This indicates uniform affinity of all sites for each separate hapten. In turn this indicates the probability of there being more than one site as small. (3) Corresponding parts of derivatized haptens occupy the same subsite. From our own work reported here as well as the work reported elsewhere (Kabat and Mayer, 1964), we know that in any glycoside the glycosidic moiety binds more strongly than the aglycone. If for a given glycoside a fraction of the molecules would bind in the "glycosidic subsite" and another fraction in the "aglycone subsite" the resultant observation would be a nonlinear Scatchard plot. As pointed out above, all such plots were linear. If a glycoside binds, all glycosidic moieties will in all likelihood bind in the subsite with the highest binding energy. (4) It is assumed that the binding energy of each part of a hapten is not changed by alterations elsewhere in the hapten, *i.e.*, by introducing a covalently linked addition to a hapten, a resulting change in binding energy with the immunoglobulin will be attributed to the

TABLE III: Percentage Contribution of Each Galactose Moiety to the Binding Energies of Gal_2, Gal_3, and Gal_4 with Myeloma IgA Proteins X-24 (Part A) and J-539 (Part B).

	Residue No.[a]			
	1	2	3	4
		Part A		
Gal_2	54.0	46.0		
Gal_3	38.7	33.0	28.3	
Gal_4	37.2	31.6	27.1	4.1
		Part B		
Gal_2	64.4	35.6		
Gal_3	50.5	27.9	21.6	
Gal_4	47.2	26.1	20.2	6.5

[a] Residue 1 is the nonreducing moiety.

TABLE IV: Relative Contributions by Parts of the Disaccharide Gal_2 to Its Overall Binding Energy with Protein X-24.

% Contribution to Binding Energy of Gal_2	% Contribution to Binding Energy of Gal_2 by the Indicated Portion of Hapten
100	
68.5	
54.0	14.5
58.6	9.9
	44.1
89.3	10.7
	20.8

TABLE V: Relative Contributions by Parts of the Disaccharide Gal_2 to Its Overall Binding Energy with Protein J-539.

% Contribution to Binding Energy of Gal_2	% Contribution to Binding Energy of Gal_2 by the Indicated Portion of Hapten
100	
74.1	
64.4	9.7
64.2	9.9
	54.5
91.6	8.4
	17.5

molecular moiety added. This method of the determination of binding constants (K_a) employed here offers unique advantages for the screening of a large number of carbohydrate derivatives for their binding affinities with two of our anti-β(1→6)-D-galactan myeloma immunoglobulins. The sensitivity of the method allows measurement of very low K_a's with extreme accuracy and reproducibility. It became apparent in this study that the two immunoglobulins investigated differ with regard to their active sites.

It is clear that both proteins are very specific for terminal nonreducing β-linked D-galactopyranosyl units. Since methyl β-D-glucopyranoside, methyl β-D-gulopyranoside, and 2-deoxy-D-galactose do not bind, the correct stereochemistry[3] of O-2, O-3, and O-4 in the glycosidic moiety seems to be a prerequisite for binding with either antibody. However the glycosidic C-5 hydroxymethyl group is not essential for binding, since methyl α-L-arabinopyranoside interacts with both proteins (Table II). Changing the linkages in methyl β-D-galactopyranoside from β to α results in a total loss of binding; however, the presence of a β-linked glycosidic oxygen is not essential *per se*, since 1,5-anhydro-D-galactitol binds (Table II).

Also from Table II it can be seen that at the monosaccharide level, protein J-539 is a significantly better binder of haptens than is protein X-24. It is only at the trisaccharide level that protein X-24 attains the binding ability of protein J-539. Table

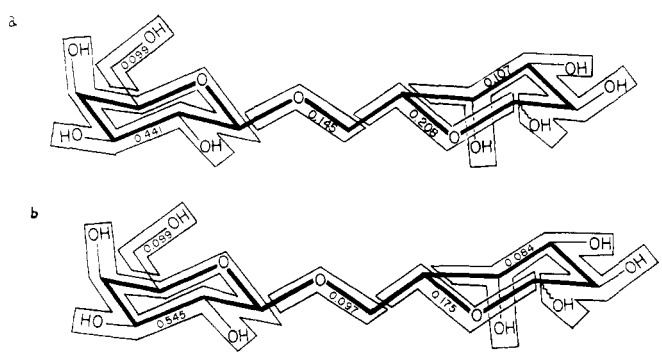


FIGURE 2: Fractional contributions made by various areas in Gal_2 to the overall binding of this disaccharide to (a) Fab' X-24, and (b) Fab' J-539.

[3] The carbon atoms in the galactose units of Figure 3 are numbered.

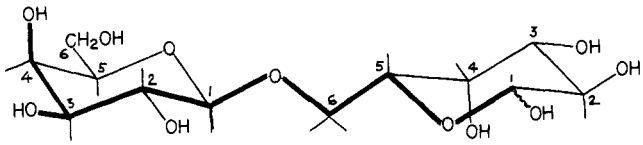


FIGURE 3: The postulated conformation of Gal_2. The dark outline shows the bonds most involved in binding to the IgA's in this study.

III shows the contributions to the total binding energy by each galactose moiety in Gal_2, Gal_3, and Gal_4. These data were obtained from the K_a values by calculation of the free energies ($\Delta G = -RT \ln K_a$). A number of observations may be made. (1) In all cases the terminal nonreducing galactosyl residue (residue 1) contributes most to the binding of each oligosaccharide. This is consistent with the concept of immunodominance for terminal nonreducing carbohydrate residues (Kabat and Mayer, 1964). (2) The terminal nonreducing galactosyl residue (residue 1) in these oligosaccharides contributes more to the binding energies in the case of immunoglobulin J-539 than in the case of immunoglobulin X-24. (3) The contributions of residues 2 and 3 to the binding of Gal_3 and Gal_4 in the case of immunoglobulin X-24 are significantly greater than in the case of immunoglobulin J-539. In both proteins residue 2 contributes more than residue 3. (4) For both proteins X-24 and J-539 residue 4 contributes little to the binding energy of Gal_4. It would appear therefore that the limiting size for the combining sites corresponds to the tetraose, in support of our earlier postulation (Jolley *et al.*, 1973).

In addition we can make some deductions with regard to the contributions made by portions of the Gal_2 molecule to its total binding energy with these two immunoglobulins. Tables IV and V show the apparent percentage contribution made by portions of haptens to the binding of Gal_2 to proteins X-24 and J-539, as determined by their relative binding energies.[4] Figure 2 shows the data pictorially. The superior binding energies of EPG and MEG (see Table II for formulae) compared to methyl β-D-galactopyranoside are presumably due to the presence of the epoxy and methoxy oxygen atoms. By model building it was determined that these oxygen atoms could take up the position of the ring oxygen atom in residue 2 of Gal_2. The increased binding energies of these two compounds over methyl β-D-galactopyranoside is presumably not due to the increased bulk of the substituent at C-1, since isopropyl β-D-thiogalactopyranoside has a binding energy only slightly greater than methyl β-D-galactopyranoside. The better binding of EPG over MEG may be due to the lesser degree of freedom of rotation of the former compound, resulting in a greater probability that the epoxy oxygen atom will be in a similar position to the one occupied by the ring oxygen of the aglycone moiety of Gal_2 at any instant in time. There is some freedom of rotation in EPG, and it may be that if this were reduced, its association constants with the two immunoglobulins would be even higher. If this were indeed so it would indicate that the remainder of residue 2 contributes even less to the binding energy of Gal_2 than these results suggest.

A number of points are noted with regard to Figure 2. (1) In the case of both proteins X-24 and J-539 the series of atoms from the exocyclic C-1 oxygen atom to the C-1 atom of residue 2 contributes significantly to the binding energy of the whole Gal_2 molecule (27.2% for protein J-539 and 35.3% for protein X-24). (2) The glycosidic C-6 hydroxyl group and its associated carbon atom contribute only approximately 10% in both cases to the total binding energy of Gal_2. (3) The second residue (excepting the ring oxygen atom and atoms C-1, C-5, and C-6) contributes little to the binding energy of Gal_2 to both immunoglobulins (8.4% for protein J-539, 10.7% for protein X-24). (4) The ring oxygen atom and hydroxyl groups (excluding the C-6 hydroxyl group) of residue 1 provide a major contribution to the binding energy of Gal_2 (54.5% for protein J-539, 44.1% for protein X-24). (5) A conformation for Gal_2 which would avoid orbital overlap between the intersaccharidic O, the O-ring (aglycone) and O-4 (aglycone) is the one depicted in Figures 2 and 3. If that conformation is correct, the region of greater binding is localized to one side of the Gal_2 molecule (see Figure 3). This would indicate that the active sites of both immunoglobulins are in the form of an extended surface area. Moreover, if the conformation pictured in Figures 2 and 3 is extended for Gal_4 (Figure 4), one can observe that residues 1, 2, and 3 all curve in the direction of the same side of the tetrasaccharide. This is the side which accounts for most of the binding energy in Gal_2. The fourth galactose residue in Gal_4 juts away rather sharply in this conformation. It may be no accident then that we find that residue 4 does not significantly improve the binding of the oligosaccharide to either immunoglobulin when added to Gal_3 to form Gal_4.

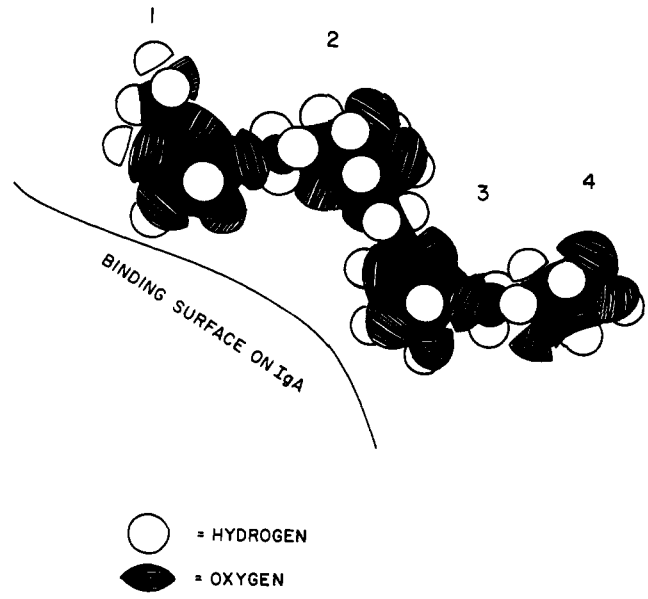


FIGURE 4: Proposed conformation of Gal_4. The suggested binding surface of the immunoglobulins is also shown.

Fluorescence titration is a unique method whereby compounds with very low and high binding constants may be investigated merely by varying the hapten concentration.

[4] Determined by comparison of structures and free-energy changes. The latter were determined from the expression $\Delta G = -RT \ln K_a$. For example, the free energy of binding (ΔG) for 1,5-anhydro-D-galactopyranose is expressed as a percentage of the binding energy of Gal_2 (Tables IV and V). This value is subtracted from the value of ΔG for the binding of methyl β-D-galactopyranoside (also expressed as a percentage of the binding energy of Gal_2, see Tables IV and V). The resulting value is the contribution made by the intersaccharidic oxygen on C-6 carbon atom of the aglycon in Gal_2 to the binding with the immunoglobulins.

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Specificity of Purified Hemagglutinin (Lectin) from *Lotus tetragonolobus*†

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ABSTRACT: The lectin from *Lotus tetragonolobus* seeds was purified by adsorption on to insoluble polyleucyl hog A + H blood-group substance and subsequent elution with L-fucose. The isolated lectin formed one line in immunodiffusion and immunoelectrophoresis against three rabbit antisera to the crude seed extract. The purified lectin showed three components on acrylamide electrophoresis under alkaline or acid conditions; these were separated by preparative isoelectric focusing having isoelectric points (IEP) of pH 5.4, 6.2, and 7.1 and corresponded to fractions B, C, and A obtained earlier on DEAE-cellulose (Kalb, A. J. (1968), *Biochim. Biophys. Acta 168,* 532). The three fractions and the original lectin are made up of noncovalently linked subunits of molecular weight of about 27,000. The purified lectin precipitated with blood-group H, Le^a and A_2 substances; it did not precipitate with blood group A_1 or B substances, with a precursor blood-group substance having I and i activities, nor with a periodate-oxidized and Smith-degraded H substance. Inhibition of precipitation with various monosaccharides and with milk and blood-group oligosaccharides indicated that the lectin is strongly specific for type 2 chains containing fucose residues on C-2 of the galactose of βDGal(1→4)DGlcNAc, with or without a second fucose on the DGlcNAc, but does not react with similarly substituted type 1 chains of structure βDGal(1→3)DGlcNAc. Inhibition data with the three fractions obtained by isoelectric focusing indicate that they have the same specificity although Kalb found them to have different association constants.

Proteins with the capacity to agglutinate red blood cells are widely dispersed in plants, vertebrates, and invertebrates (for reviews, see Kabat, 1956a, Prokop *et al.,* 1968, and Sharon and Lis, 1972). Some of these proteins, known as lectins, are highly specific in that they agglutinate erythrocytes of the human ABO or MN groups, while others agglutinate independently of these blood groups. Other lectins may precipitate various polysaccharides and glycoproteins specifically (Hammarström and Kabat, 1969; Etzler and Kabat, 1970; Lloyd and Bitoon, 1971), and have been used (Rovis *et al.,* 1973a) in structural studies on oligosaccharides isolated from various blood-group substances and of the other polysaccharides. Lectins also possess a number of other biological properties, such as stimulating resting lymphocytes to divide and form blast-like immature cells, causing agglutination of malignant cells, or of platelets, liberation of thrombin and interference with the fertilization of sea urchin eggs (*cf.* Lis and Sharon, 1973). Although many lectins have been studied as crude extracts (Mäkela, 1957), only a few have been purified, characterized, and studied immunochemically. These include concanavalin A (Agrawal and Goldstein, 1965; Lloyd *et al.,* 1969; Poretz and Goldstein, 1970), the hemagglutinins from *Dolichos biflorus* (Etzler and Kabat, 1970), *Helix pomatia* (Hammerström and Kabat, 1969), lima bean (Galbraith and Goldstein, 1972), soybean (Gordon *et al.,* 1972), wheat germ (Nagata and Burger, 1972; LeVine *et al.* 1972), and others (*cf.* Sharon and Lis, 1972).

The lectin from the seeds of *Lotus tetragonolobus* was shown to agglutinate human O(H) red blood cells (Renkonen, 1948), and the agglutination was specifically inhibited by L-fucose (Morgan and Watkins, 1953). This lectin was precipitated (Yariv *et al.,* 1967) using a trifunctional fucosyl dye, the precipitate dissolved in fucose, and the dye separated from the lectin using an ion-exchange resin. The purified hamagglutinin was shown to be composed of three types of L-fucose-binding molecules (Kalb, 1968), differing in molecular weight and binding constants.

In the present study we report the purification of the lectin from the seeds of *L. tetragonolobus* using, as an immunoadsorbent, an insoluble fucose-containing polyleucyl hog blood-group A + H substance (Kaplan and Kabat, 1966; Moreno and Kabat, 1969; Hammarström and Kabat, 1969; Etzler and Kabat, 1970; Galbraith and Goldstein, 1972), and elution with L-fucose, its characterization, and a study of the nature and specificity of its combining site. The purified lectin precipitated H, A_2, and Le^a substances, but not A_1 substances, proved to have a striking specificity for type 2 chains of blood-group substances containing fucosyl residues on C-2 of the β-

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