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THE BINDING OF AROMATIC DERIVATIVES OF β-D-GALACTOPYRANOSIDES TO THE FAB' OF IMMUNOGLOBULIN J539. OBSERVATIONS ON THE NATURE OF LIGAND–ANTIBODY INTERACTIONS

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ABSTRACT

A number of D-galactopyranosides bearing aromatic substituents have been prepared, and their binding to immunoglobulin J539 (Fab') has been studied. It appears that the main contribution of the 6-*O*-aromatic moiety to binding arises from the fact that it imparts an increased hydrophobicity to the ligand, causing a decrease in its hydration (solubility) that results in a greater free-energy of binding. In the D-galactosides having an aromatic aglycon, the phenyl group appears to partake in actual interactions with the protein.

INTRODUCTION

This laboratory has reported on the binding of 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl) derivatives of D-galactose saccharides to some immunoglobulins having anti-D-galactopyranan specificity[1]. It was found that 6-*O*-(6-*O*-dansyl-β-D-galactopyranosyl)-D-galactose (6-dansyl-Gal_2) is bound to the combining area of immunoglobulin J539 (Fab') with an affinity constant which is ten times that of the parent disaccharide 6-*O*-β-D-galactopyranosyl-D-galactose (Gal_2). By examining a Kendrew model of the variable region of J539, it was concluded that the dansyl group in 6-dansyl-Gal_2 could perhaps, interact with the tyrosine unit of the heavy chain at position 101. This interaction could involve a charge-transfer complex, and it was decided to investigate this possibility more closely by examining the binding of a number of aromatic derivatives of D-galactose with immunoglobulin J539. We now report the results of this work.

EXPERIMENTAL

General. — All nitrophenyl D-galactopyranosides were purchased from ICN, K & K Laboratories, Inc. (Pharmaceuticals, Life Science Group, Plainview, N.Y.). *p*-Aminophenyl and phenyl 1-thio-β-D-galactoside were purchased from Calbiochem.

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(San Diego, Cal. 92112). *p*-Nitrophenol was obtained from Sigma Chemical Company. All glycosides commercially obtained were recrystallized from suitable solvents (absolute ethanol, methanol, or 95% ethanol) prior to use in binding studies. Immunoglobulins J539 (Fab') and HOPC-8 (Fab') were generous gifts from Dr. B. N. Manjula. Reactions were monitored by t.l.c. on silica gel GF, and components were detected under u.v. light, or by charring with hot, 10% sulfuric acid, or both. Specific rotations were measured with a Perkin–Elmer 141 polarimeter; n.m.r. spectra were recorded with a Varian HA-100 spectrometer at 100 MHz, and mass spectra with a Finnigan 10 5D mass spectrometer. Periodate oxidations were conducted by the method of Avigad[2].

Fluorescence titration. — All solutions were made up in phosphate-buffered saline (PBS; 0.01M phosphate buffer containing 0.15M NaCl), except for methyl 6-*O*-anisoyl-β-D-galactopyranoside, which has a low solubility in water and was made up in 1:1 2-methoxyethanol–PBS. Only ~50 μL of this is maximally added to 1.500 mL of the solution of protein in PBS during the titration, and a control experiment showed that it does not affect the fluorescence of the J539 solution. The association constants of Fab' J539 were determined, following the quenching, or enhancement, of antibody fluorescence, in a Perkin–Elmer MPF 3L fluorescence spectrophotometer[1,3]. The excitation wavelength was 295 nm, and the emission was measured at 340 nm, as described. Titrations were performed at 25°. As these ligands contain an aromatic nucleus, their absorption and emission properties were checked and suitably corrected for. As an example, in the case of the nitrophenyl D-galactosides (see Table I), non-specific quenching was corrected for by titrating a solution of immunoglobulin Fab'

TABLE I

BINDING OF IgA (Fab') J539 WITH D-GALACTOSE LIGANDS

Ligand	K_a (M^{-1})	$\frac{K_a}{K_a MeGal}$	$-\Delta G$ (*in kJ, with kcal in parentheses*)	ΔF_{max} (%)
Methyl β-D-galactopyranoside (MeGal))	1.37×10^3	1	18 (4.27)	+ 18
Methyl 6-*O*-benzoyl-β-D-galactopyranoside	2.89×10^3	2.10	19.89 (4.72)	− 44
Methyl 3-*O*-benzoyl-β-D-galactopyranoside				no change
Methyl 6-*O*-(*p*-methoxybenzoyl)-β-D-galactopyranoside	2.57×10^3	1.87	19.55 (4.64)	− 43
Methyl 6-*O*-(*p*-nitrobenzoyl)-β-D-galactopyranoside	?			very low change
m-Nitrophenyl β-D-galactopyranoside	1.52×10^5	110.9	29.75 (7.06)	− 43
p-Nitrophenyl β-D-galactopyranoside	1.06×10^5	77.37	28.86 (6.85)	− 33
o-Nitrophenyl β-D-galactopyranoside	4.0×10^4	29 19	26 42 (6 27)	− 26
Phenyl β-D-galactopyranoside	1.30×10^4	9.48	23.60 (5.60)	+ 23
Phenyl 1-thio-β-D-galactopyranoside	?			very low change
p-Aminophenyl 1-thio-β-D-galactopyranoside	9.35×10^3	6.82	22.79 (5.41)	− 75
p-Nitrophenyl α-D-galactopyranoside				no change
p-Nitrophenol				no change

HOPC-8 (an unrelated immunoglobulin having specificity for phosphorylcholine) under the same conditions as those used for J539 and subtracting the corresponding values. Fig. 1 shows two Scatchard plots, for *m*-nitrophenyl β-D-galactopyranoside and for methyl 6-*O*-benzoyl-β-D-galactopyranoside, both with immunoglobulin J539 (Fab')[4].

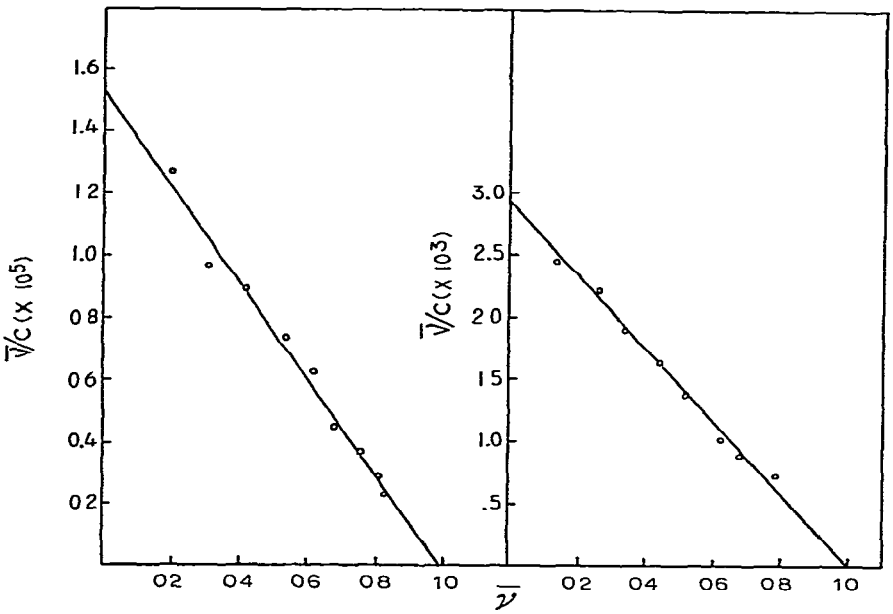

Fig. 1. Scatchard plots for the binding of J539 Fab' with *m*-nitrophenyl β-D-galactopyranoside (left) and with methyl 6-*O*-benzoyl-β-D-galactopyranoside (right). [$\bar{\nu}/C = K_a - \bar{\nu}K_a$, where $\bar{\nu}$ is the fraction of immunoglobulin sites occupied by ligand.]

Inhibition studies. — J539 ascites fluid (50 μL) was mixed with 125 μL of a solution of pneumogalactan[5] (1 mg/mL) in PBS, and with 25 μL of PBS, as a control. To separate tubes containing the same amounts of pneumogalactan, PBS, and ascites was respectively added (duplicates) methyl 6-*O*-(*p*-nitrobenzoyl)-β-D-galactopyranoside (0.85 mg, 2.48 μmol) or methyl β-D-galactopyranoside (0.48 mg, 2.47 μmol). The control precipitated 369 μg of antibody nitrogen. The 6-*O*-acylglycoside inhibited the precipitation by 35%, and the methyl D-galactoside, by 34%.

Methyl 6-O-*benzoyl-2,3,4-tri*-O-*benzyl-β-D-galactopyranoside* (**1**). — A solution of methyl 2,3,4-tri-*O*-benzyl-β-D-galactopyranoside[6] (155 mg, 0.3 mmol) in pyridine (5 mL) was cooled in an ice-bath, and benzoyl chloride (a 10% molar excess) was added, with stirring. After 2 h in the cold, the reaction flask was removed from the ice bath and kept for 18 h at room temperature. The solution was added to a saturated, aqueous solution of sodium hydrogencarbonate, and the mixture was extracted with chloroform. The extract was washed with water, dried (anhydrous sodium sulfate), and evaporated to dryness. Toluene was added to, and evaporated from, the residue (to remove residual pyridine), and the residue was crystallized from ethanol (175 mg,

99% yield); m.p. 77–79°. NH_3-Chemical-ionization, mass spectrometry (c.i.m.s.) showed a peak for *m/e* 586 ($M + NH_4^+$) for the title compound.

Methyl 6-O-*benzoyl-β-*D-*galactopyranoside* (**2**). — Compound **1** (170 mg) dissolved in 1:1 ethyl acetate–methanol was catalytically hydrogenolyzed in the presence of freshly prepared palladium black. When the reaction was complete (2 h), the catalyst was removed by filtration, and the filtrate was evaporated to a glass which was obtained crystalline from 1:1 petroleum ether–ethyl acetate (82 mg, 92% yield, m.p. 125–127°, $[\alpha]_D^{20}$ −4° (*c* 0.5, ethanol); c.i.m.s. (NH_3) showed a peak with *m/e* 316 for M + 18. The compound consumed 2.2 mol of periodate per mol.

Anal. Calc. for $C_{14}H_{18}O_7$: C, 56.07; H, 6.08. Found: C, 55.80; H, 6.14.

*Methyl 3-*O-*benzoyl-β-*D-*galactopyranoside* (**3**). — A solution of methyl β-D-galactopyranoside (2.0 g, 10.3 mmol) in pyridine (20 mL) was cooled in Dry Ice–ethanol[7], and benzoyl chloride (10.3 mmol) was slowly added. The reaction was allowed to continue for 6 h at room temperature, and the mixture was then evaporated *in vacuo* to a syrup from which pyridine was removed by azeotropic distillation with toluene *in vacuo*. The solid residue was taken up in water, insoluble material was removed by filtration, and the filtrate was evaporated to a syrup which was loaded onto a column of silica gel. Elution was achieved with 3:2 acetone–dichloromethane. Pure **3** (160 mg, 8% yield) was obtained by crystallization from 1:1 petroleum ether–ethyl acetate, m.p. 106–109°, $[\alpha]_D^{20}$ +56.7° (*c* 0.55, ethanol); c.i.m.s. (NH_3) showed a M + 18 peak of 316. The material was found to resist periodate oxidation completely. Compounds **2** and **3** are readily distinguished by t.l.c. (ethyl acetate), as **3** has a higher mobility than **2**.

Anal. Calc. for $C_{14}H_{18}O_7$: C, 56.07; H, 6.08. Found: C, 55.89; H, 6.03.

Methyl 3- and 6-O-*anisoyl-β-*D-*galactopyranoside* (**4** *and* **5**). — A solution of methyl β-D-galactopyranoside (1 g) in pyridine (20 mL) was cooled in a Dry Ice–ethanol bath, and anisoyl (*p*-methoxybenzoyl) chloride (1.1 g) in pyridine (5 mL) was added in portions during 15 min. The mixture, which was stirred for 2.5 h, was occasionally removed from the bath to prevent complete solidification. After being kept overnight at ~0°, the mixture was evaporated *in vacuo* to a residue which was extracted with acetone; the extract was concentrated, and the concentrate extracted with water. The residue (1.5 g) was loaded on the top of a column of silica gel which was eluted with 3:2 acetone–dichloromethane, to yield a mixture of two mono-esters, the faster-moving one preponderating (5:2). On treatment with methanol, the slower-moving material crystallized (needles), and, after one recrystallization from hot methanol, had m.p. 195.5–197°, $[\alpha]_D^{20}$ +3.8° (*c* 0.29, methanol). It consumed 1.86 mol of periodate per mol, showing it to be the 6-anisoate **5**.

Anal. Calc. for $C_{15}H_{20}O_8$: C, 54.87; H, 6.14. Found: C, 54.53; H, 6.05.

The filtrate from the crystallization was evaporated, and the residue yielded, from acetone–hexane, a second crop (0.12 g) of crystals consisting mainly of the more-mobile product. This mono-ester was purified by preparative chromatography on plates (1.000 mm thick) of silica gel developed with 3:2 acetone–dichloromethane, followed by recystallization from ethanol–hexane. The flat prisms of **4** (75 mg) had

m.p. 165–167°, $[\alpha]_D^{20}$ +37.0° (*c* 0.66, acetone), and resisted oxidation by periodate.

Anal. Calc. for $C_{15}H_{20}O_8$: C, 54.87; H, 6.14. Found: C, 55.06; H, 6.06.

Compound **5** could also be obtained as follows. A solution of methyl 2.3.4-tri-*O*-benzyl-β-D-galactopyranoside (200 mg) in pyridine (4 mL) was treated with anisoyl chloride (94 mL plus 1 mL of pyridine). After 6 h at room temperature, the solution was poured into cold, sodium hydrogencarbonate solution, and the resulting precipitate (730 mg) was collected by filtration, and washed with cold water. It was recrystalized twice, first from ethanol, and then from isopropyl alcohol. The resulting needles melted at 86–87° and had $[\alpha]_D^{20}$ −31.8° (*c* 1.0, chloroform).

Anal. Calc. for $C_{36}H_{38}O_8$: C, 72.22; H, 6.40. Found: C, 72.32; H, 6.47.

Catalytic hydrogenolysis of the benzyl groups in 126 mg of the foregoing compound was achieved in 1:1 ethyl acetate–methanol, using freshly prepared palladium black, to yield 65 mg of product. After one recrystallization from methanol, it showed m.p. 195.5–197°, undepressed on admixture with the compound prepared by direct acylation of methyl β-D-galactopyranoside.

Anal. Calc. for $C_{15}H_{20}O_8$: C, 54.87; H, 6.14. Found: C, 54.99; H, 6.14.

p-*Nitrobenzoylation of methyl β-D-galactopyranoside.* — A solution of methyl β-D-galactopyranoside (3 g) in pyridine (40 mL), cooled in an ethanol–Dry Ice bath, was treated with *p*-nitrobenzoyl chloride (3.7 g) in pyridine (40 mL). After 2.5 h, it was allowed to warm to room temperature, and, the following day, the mixture was evaporated to dryness *in vacuo*. The residue was taken up in warm ethanol, and crystals (1.2 g) were deposited; after recrystallization from methanol–ethyl acetate, the fine needles had m.p. 173–176°, $[\alpha]_D^{20}$ +12.5° (*c* 0.45, acetone). This compound was probably the 3,6-diester, but it was not further investigated.

Anal. Calc. for $C_{41}H_{20}N_2O_{12}$: C, 51.22; H, 4.12; N, 5.71. Found: C, 51.30; H, 4.12; N, 5.71.

The mother liquor was concentrated, and the concentrate treated with water, causing precipitation of an insoluble material, which was removed by filtration. The filtrate was evaporated to a residue which was triturated with ethyl acetate; the suspension was filtered, and the solid washed with ethyl acetate. In t.l.c. (ethyl acetate), this residue (2.6 g) showed two compounds moving substantially more slowly than the diester. From ethanol–hexane were obtained crystals that, after recrystallization from the same solvent system, weighed 0.8 g. Repeated separations, using preparative t.l.c. plates, and using three solvent systems (ethyl acetate, 8:1 dichloromethane–methanol, and 1:1 acetone–dichloromethane), finally yielded a product that was essentially pure (t.l.c.), but still had a fairly broad m.p. (153–162°), $[\alpha]_D^{20}$ +34.3° (*c* 0.68, acetone). The material was resistant to oxidation by periodate, indicating that it was methyl 3-*O*-(*p*-nitrobenzoyl)-β-D-galactopyranoside.

Anal. Calc. for $C_{14}H_{17}NO_9$: C, 48.98; H, 4.99; N, 4.08. Found: C, 48.95; H, 5.04; N, 3.94.

The other component could also be obtained pure (as judged by t.l.c.), but again, repeated chromatography did not yield material having a sharp m.p. (163–173°). The

methyl 6-*O*-(*p*-nitrobenzoyl)-β-D-galactopyranoside consumed 1.97 mol of periodate per mol.

Anal. Calc. for $C_{14}H_{17}NO_9$: C, 48.98; H, 4.99; N, 4.08. Found: C, 48.69; H, 4.88; N, 3.98.

RESULTS AND DISCUSSION

The binding of all of the ligands studied here to immunoglobulin J539 Fab' caused a significant quenching of the fluorescence of the protein, except in the case of the ligand phenyl β-D-galactopyranoside, which caused an increase in the protein fluorescence. It is assumed, as before[8], that failure of a ligand to induce a change in the protein fluorescence means that no binding takes place.

The data in Table I show that the protein only binds ligands that are β-glycosides of D-galactopyranose, *i.e.*, the protein needs to recognize the β-D-galactopyranoside moiety in order to bind the ligand. Thus, it was found that neither *p*-nitrophenyl α-D-galactopyranoside nor *p*-nitrophenol binds. The specificity of immunoglobulin J539 is for β-D-galactopyranosyl residues[9]. Why, then, do the 6-*O*-aromatically substituted β-D-galactopyranosyl residues show a *higher* affinity for the immunoglobulin combining-site compared to their unsubstituted analogs? We propose that the increased binding for the aromatic derivatives is due simply to the fact that these derivatives are more hydrophobic, and thus essentially less soluble in the aqueous phase, than their unsubstituted analogs. In an earlier paper on the binding of 6-*O*-dansyl derivatives of D-galactopyranose saccharides[1], we proposed that the dansyl group may form a charge-transfer complex with some of the aromatic amino acid residues (notably tyrosine) that occur in the combining area of the immunoglobulin[10]. Such a charge-transfer complex is, *a priori*, quite possible, but it appeared worth while to contemplate the thermodynamic aspects of the binding of ligands to proteins.

The free energy of binding, ΔG, for the ligands is derived from the association constants K_a by the equation $\Delta G = -RT\ln K_a$. The values for these free energies of binding are listed in the 3rd column in Table I. The process of binding a small, neutral ligand to a protein in aqueous solution may be regarded as starting with a hydrated ligand, stripping it of its hydration, at least partially, and then "removing" the solute from solution by "binding" it to the protein. The protein in the combining area will also be stripped of hydration, and the sum of these previously structured, water molecules will be randomly returned to the bulk solvent. The resulting increase in entropy could be the main driving-force for the binding of neutral ligands[11].

When a small molecule binds to a large molecule (such as a protein), it loses translational and rotational degrees of freedom. That loss of entropy is the major effect unfavorable to association[11]. For a molecule, such as that of a simple (oligo)-saccharide, of mol. wt. < 1000, this has been estimated to be[11] ~ 70 kJ. If the overall, favorable, free energy of binding is -18 kJ (as for methyl β-D-galactopyranoside), some process will have to yield about -88 kJ of free energy favorable to binding. The principal source of this energy could arise from the return to the solvent of surface

molecules of water, due to mutual exclusion of solvent accessibility in the contact area between ligand and protein. The contribution to the free energy of this "burial" of previously accessible surface has been estimated[12] to be ~ 10.5 kJ.nm^{-2}. By measurement, the surface area of a methyl hexopyranoside is, very approximately, 3.5 nm^2. Were this area to be enveloped by the protein surface in the combining area, the totally buried surface involved would be ~ 7–9 nm^2; that could yield some 84 kJ of free energy favorable to binding, which makes up well for the loss of rotational and translational entropy already mentioned, and would give a negative ΔG of binding.

Thus, this approximation shows that the free-energy contribution due to hydrophobic binding could be as high as —14 kJ, a major part of the total free-energy of binding for methyl β-D-galactopyranoside, which is —17.9 kJ (see Table I). Some hydrogen bonding may very well occur in addition. This description is, of course, to a large extent, qualitative; however, there is evidence that hydrophobic bonding plays a significant role[13]. For instance, the *combining area* of J539 does not display a larger number of potential, contact amino acids having a hydrophilic nature than any other surface area of the immunoglobulin F_V fragment[10]; thus, binding by hydrogen bonding to a ligand seems not to be selected for. Secondly, from Table I, it may be seen that 6-*O*-aromatically substituted glycosides show an increase in binding constants; this increase can come about in several ways. It had been reported[1] that the 6-*O*-dansyl derivative of Gal_2 shows a ten-fold increase in binding (over Gal_2) with J539 Fab', and it was suggested that the dansyl group could form a charge-transfer complex with an aromatic amino acid residue, namely, tyrosine 101H. Althought that may, indeed, be possible, we now consider this explanation to be far from unique.

The original explanation prompted us to prepare a number of differently substituted, aromatic 6-*O*-acyl derivatives of methyl β-D-galactopyranoside in which the acyl group was substituted by electron-supplying or electron-withdrawing groups. From Table I, it may be seen that 6-*O*-benzoyl- and 6-*O*-(*p*-methoxybenzoyl)-β-D-galactopyranoside have almost identical constants of association with J539 Fab'. [For two derivatized D-galactosides (methyl 6-*O*-benzoyl-β-D-galactopyranoside and *m*-nitrophenyl β-D-galactopyranoside), it was shown, by a competition experiment as described by Manjula *et al.*[14], that binding occurs in the site of J539 where binding with methyl β-D-galactopyranoside takes place. The theoretical values for the fraction of sites occupied by each ligand were calculated by the method of Klotz *et al.*[15]. The results are shown in Fig. 2.]

It is unfortunate that affinity measurements with methyl 6-*O*-(*p*-nitrobenzoyl)-β-D-galactopyranoside were not possible, as only a very small change in fluorescence of the protein could be detected. However, it was found that methyl 6-*O*-(*p*-nitrobenzoyl)-β-D-galactopyranoside and methyl β-D-galactopyranoside inhibit the precipitation of J539 immunoglobulin with pneumogalactan[5] to exactly the same extent. These results indicate that a charge-transfer complex with the 6-*O*-aromatic group is unlikely, as there is no real difference when the phenyl ring is substituted with a methoxyl or a nitro group; these derivatives show a similar increase in binding, albeit a small one. As the increase in free energy of binding in going from methyl β-D-galactopyrano-

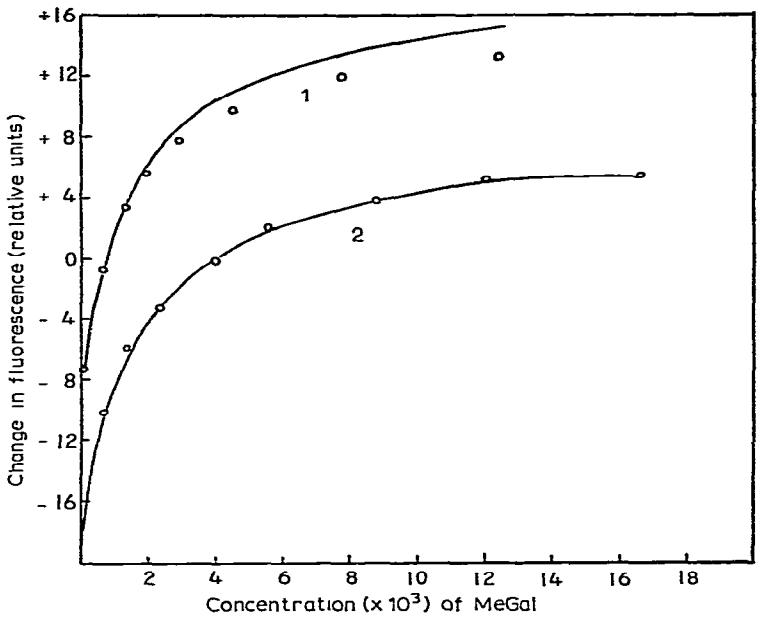

Fig. 2 Competition between (1) methyl 6-*O*-benzoyl-β-D-galactopyranoside (at a constant concentration of 3 9 mM) and (2) *m*-nitrophenyl β-D-galactopyranoside (at a constant concentration of 743 μM) in binding to J539 Fab′, as determined by fluorescence titration. (The curves are theoretical and were computed[15]. The points were determined experimentally.)

side to the corresponding 6-*O*-benzoyl and 6-*O*-(*p*-methoxybenzoyl) derivatives is in the range of 2.1 kJ (and thus small), it may well be that this is only due to a small change in the free energy of dehydration for the ligand (or removal from the solvent).

It may be recalled that D-galactose saccharides bind to J539 with little involvement of their C-5 substituent[8], and that that part of the ligand projects towards the solvent. For the removal from aqueous solution, the decrease in free energy that is associated with increased hydrophobicity of the solute removed is definite, but small. For instance, the literature[16] gives a change from 13 to 17 kJ for the free energy of hydration for the dissolution in water, on proceeding from methyl to octyl alcohol. It seems reasonable that a more hydrophobic ligand would "seek" to be removed from solution with greater ease. Thus, it appears that the increase in binding of ligands that are substituted on O-6 with aromatic groups could be mostly due to their increased hydrophobicity, and not to any charge-transfer type of binding between the ligand and tyrosyl, tryptophanyl, or histidyl residues of the protein. The larger increase in the free energy of binding previously reported[1] for 6-dansyl-Gal_2 may result from the fact that the dansyl group confers more hydrophobicity on the ligand than does the 6-*O*-benzoyl group.

Turning now to the glycosides having an aromatic aglycon (see Table I), it is, first of all, interesting that the binding affinity of phenyl β-D-galactopyranoside is essentially the same as that of Gal_2 and almost ten times that of methyl β-D-galactopyranoside; that is, there is an increase of ~5.9 kJ of binding free-energy due to the

presence of the phenyl glycosidic group. Consequently, if the aromatic substituent is the *aglycon*, its influence on the binding energy is much greater than when it is positioned as a peripheral group at O-6. Phenyl β-D-galactopyranoside has its phenyl group in the *same position* as the second D-galactose residue in Gal_2. Thus, interaction of the protein with such glycosidic substituents are much more likely[8] than with substituents at O-6. Therefore, it appears that this larger increase in binding of phenyl β-D-galactopyranoside could be due to interactions between protein and ligand that are in *addition* to simple increases in binding free-energy derived from additional hydrophobicity of the ligand.

On inspection of the model[10] of J539, it may be seen that there are three, prominent, aromatic amino acid residues in the central part of the protein, namely, at position 93 of the light chain (L93) and positions 101 and 102 of the heavy chains (H101 and H102), all of which carry a tyrosine residue. It is *a priori*, quite possible, from the molecular dimensions of the ligand and of the complementary region of the protein, that (*a*) the (aglycon) phenyl ring may be capable of entering into π–π electron interactions, or (*b*) the nitro groups of *m*-, *o*-, and *p*-nitrophenyl β-D-galactoside could possibly enter into hydrogen-bond formation with these tyrosine residues, as substitution on the ring with the nitro group significantly enhances the binding to immunoglobulin J539 (see Table I), or both. The finding that, of the three, the *o*-nitro glycoside shows the weakest binding may be rationalized by the fact that inspection of the model (see Fig. 3) of this ligand shows quite clearly that an internal

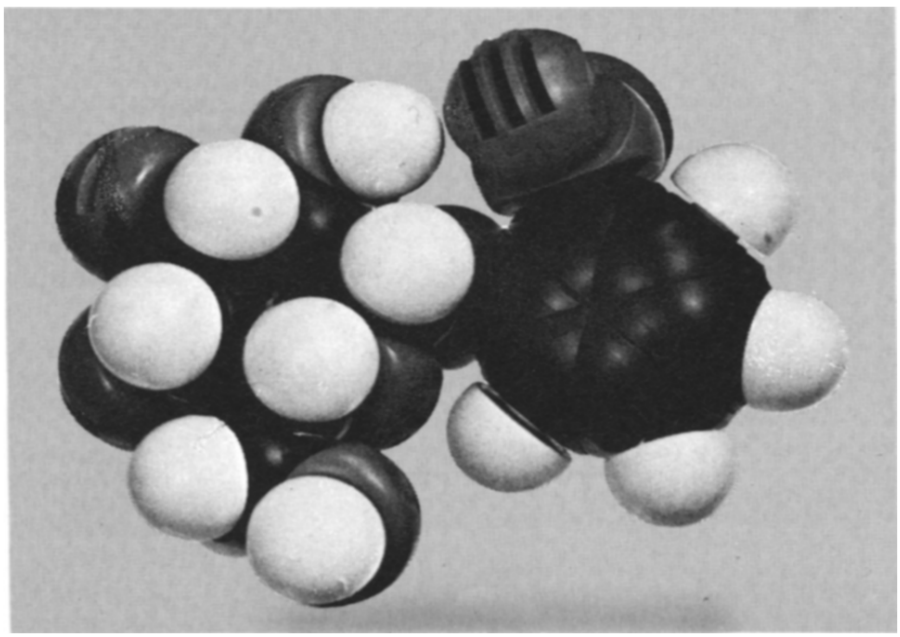

Fig. 3. Molecular model of *o*-nitrophenyl β-D-galactopyranoside. (Note the close proximity of the nitro group to the 2-hydroxyl group of the D-galactoside.)

hydrogen-bond can occur between this nitro group and the 2-hydroxyl group of the sugar, thus partially neutralizing the effect of the ring substitution.

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