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Note

The binding of 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl) derivatives of D-galactose saccharides to several immunoglobulins capable of binding β-(1→6)-linked D-galactopyranans

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The use of ligand-altered tryptophanyl fluorescence of immunoglobulins[1] to study binding has been reported by this laboratory for mapping areas of hapten-interaction with several myeloma proteins[2]. Although many homogeneous, murine immunoglobulins investigated to date show this phenomenon, several do not[3]. Thus, the latter immunoglobulins would not be amenable to this sensitive method of study.

We have reported on the preparation of a number of 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl) derivatives of saccharides[4]. The synthesis of these derivatives was undertaken with the expectation that the fluorescence of the dansyl group on the saccharide would be altered by binding of the whole ligand to an immunoglobulin. Actually, as it turns out, the derivatized saccharide causes a quenching of the fluorescence of the antibody. This is not unusual[5], and it may, in fact, be possible to obtain this effect by using saccharide ligands substituted with aromatic groups simpler than a dansyl group. This quenching may be used to determine the constants of association between ligand and antibody quantitatively, and we now report our observations.

EXPERIMENTAL

Materials. — Methyl 6-*O*-dansyl-β-D-galactopyranoside (6-dansyl-Gal; **1**) and 6-*O*-(6-*O*-dansyl-β-D-galactopyranosyl)-D-galactose (6-dansyl-Gal_2; **2**) were obtained from E. Zissis[4]. 6-*O*-β-D-Galactopyranosyl-D-galactose (Gal_2; **3**) was prepared by a published method[6]. Affinity-purified, mouse-myeloma immunoglobulins J-1 and T-191 were presented by Dr. S. Rudikoff. Purified immunoglobulin J-539 and its Fab′ fragment were donated by Dr. B. N. Manjula. McPC 603 was purified[7] by affinity chromatography.

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Methods. — All of the solutions were made up in 50mM tris[2-amino-2-(hydroxymethyl)-1,3-propanediol] hydrochloride buffer, pH 7.5. Fluorescence titrations were performed as described by Jolley and Glaudemans[1]. The excitation wavelength was 280 nm, and the emission was measured at 340 nm. As the dansyl derivatives of the ligands absorb strongly at 340 nm, there was actually a continuous quenching of fluorescence with increasing amounts of ligands. The nonspecific absorption of radiation was measured by using a solution of L-tryptophan having the same initial fluorescence as the protein solution, and by adding dansylated ligand to this solution in the same increments as in the actual titration. The observed quenching of the blank was then subtracted from the observed quenching for the protein–ligand titration, in order to obtain the values for the quenching due only to bound ligand. The latter values gave the usual titration curves when ΔF_{obs} was plotted *versus* the

TABLE I

COMPETITION BETWEEN Gal_2 (3) AND 6-DANSYL-Gal_2 (2) FOR THE COMBINING SITE OF J-539 Fab'

[3] $\times 10^5$	[2] $\times 10^5$	*Theoretical*					ΔF *(observed)*
		$\bar{\nu}$ (3)	$\bar{\nu}$ (2)	ΔF (2)	ΔF (3)	*Net* ΔF *(calculated)*	
0.00	7.84	0.000	0.564	−14.72	0.00	−14.72	−15.4
6.65	7.86	0.316	0.385	−10.07	+3.24	−6.83	−7.3
13.3	7.87	0.481	0.292	−7.62	+4.94	−2.68	−2.8
19.9	7.87	0.581	0.236	−6.16	+5.97	−0.19	−0.3
33.1	7.88	0.697	0.170	−4.43	+7.16	+2.73	+2.8
46.3	7.88	0.763	0.133	−3.47	+7.84	+4.37	+4.30
66.1	7.89	0.821	0.100	−2.61	+8.43	+5.82	+5.70

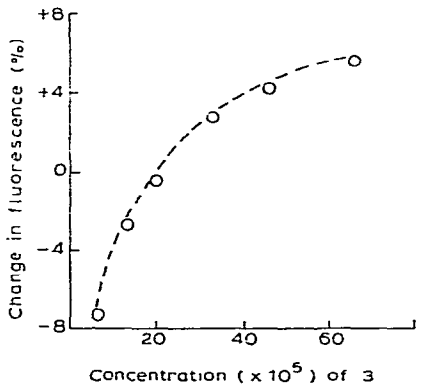

Fig. 1. A plot of the change in fluorescence (280/340 nm) of J539 Fab', in the presence of a constant proportion of **2**, as a function of the concentration of **3**. (The broken line is the theoretical curve calculated by assuming that **2** and **3** compete for the same combining region of J539 Fab'; the circles are experimentally observed values.)

concentration of free ligand, and the data were treated as already described[1]. Association constants (*Ka* values) are then obtained by the relation $\bar{\nu}/c = Ka - \bar{\nu}Ka$, where $\bar{\nu}$ is the fraction of immunoglobulin sites occupied, and c is the concentration of free ligand. As the nonspecific quenching due to unbound ligand was cumulative, it was not possible to measure the maximally obtainable change in fluorescence (ΔF_{max}) by adding a large excess of solid ligand to the protein solution. The value of ΔF_{max} was therefore obtained by plotting ΔF against $\Delta F/c$, and extrapolating to $\Delta F/c = 0$ as before[8].

Competitive binding of J-539 with 6-Dansyl-Gal$_2$ (**2**) *and Gal$_2$* (**3**). — To determine whether **2** binds to the same combining region of J-539 as **3**, a competition experiment was set up, using the immunoglobulin J-539 Fab', as described by Manjula *et al.*[9]. The results (Table I and Fig. 1) show that **2** was displaced by increasing amounts of **3**. The theoretical values for the fraction of sites saturated by each ligand were calculated according to Klotz *et al.*[10].

RESULTS AND DISCUSSION

The association constants were determined as follows. The excitation wavelength was 280 nm (the dansyl group has only a very negligible absorption at that wavelength), and the emitted (fluorescence) wavelength was 340 nm. The dansylated saccharide that binds to the immunoglobulin comes sufficiently close to the amino acid residues in the combining site of the protein to be the major cause of quenching of the emitted radiation of the tryptophan residue(s). However, in addition to that effect, there is a second quenching effect; this is so because the emitted radiation of 340 nm is also absorbed by (unbound) dansylated ligand in solution, albeit to a much lesser extent. Thus, the quenching observed is the sum of the nonspecific absorption due to *free* ligand and the specific effect due to *bound* ligand. The nonspecific effect is readily corrected for (see the Experimental Section).

TABLE II

AFFINITY CONSTANTS OF IgA'S AND IgA FRAGMENTS WITH D-GALACTOSE LIGANDS

Antigalactan immunoglobulin	*Ligand*	ΔF_{max} (%)	*Affinity constant* (Ka)
T-191	2	−30	0.87 (±0.024) × 10^5
J-1	2	−47	0.97 (±0.038) × 10^5
J-539	2	−47	1.33 (±0.05) × 10^5
J-539 Fab'	2	−49	1.64 (±0.02) × 10^5
T-191	1	−11	2.47 (±0.07) × 10^4
J-539 Fab'	3	+19	1.60 (±0.03) × 10^4

Table II shows the affinity constants (*Ka* values) for the methyl 6-*O*-dansyl-β-D-galactopyranoside (**1**) and 6-*O*-(6-*O*-dansyl-β-D-galactopyranosyl)-D-galactose (**2**) with a number of myeloma IgA's having antigalactan specificity[11]. All immuno-

globulins had been purified[1,2] by affinity chromatography. Proteins T-191 and J-1 had previously been reported to show no ligand-induced fluorescence with β-D-(1→6)-linked D-galactopyrano-oligosaccharides[2]; however, we have recently found small changes in tryptophanyl fluorescence for these two proteins upon binding to disaccharide **3** (~ +5%) and methyl β-D-galactopyranoside (~ −4%), by using the more-sensitive Perkin–Elmer MPF-3L instrument. This shows that transfer of energy for the aromatic residues near the combining region of these proteins *can* be caused by the binding of ligand, although to a smaller extent than for some of the other anti-galactan proteins already reported on.

The use of dansylated saccharides (galactose-containing) as ligands led to extensive quenching of the fluorescence of T-191 and J-1; this effect was also found for J-539, a protein that shows fluorescence changes induced by **3** (and other β-D-galactosyl ligands). It may be seen from Table II that **2** binds to J-539 Fab' with an affinity constant that is considerably larger than the constant for **3** and J-539 Fab': this prompted us to investigate whether **2** and **3** actually bind to the same combining-region of J-539. This can be done by quantitative evaluation of the degree of competition between **2** and **3** for the combining region of J-539; therefore, the fluorescence changes for J-539 Fab' in the presence of a constant amount of **2** and increasing proportions of **3** were monitored. The results of the competition experiments are shown in Table I and Fig. 1. The theoretical values for the fraction of sites saturated by **3** and **2** were calculated as follows[10].

When two ligands, A and B, compete for the same binding-site on a protein, the fraction of sites occupied by either ligand ($\bar{\nu}_A$ or $\bar{\nu}_B$) is related to the *Ka* value for A or B (K_a^A and K_a^B) and the concentration of free ligand, C_A and C_B, by the equations

$$\bar{\nu}_A = K_a^A C_A/(1 + K_a^B C_B + K_a^A C_A) \qquad (1)$$

and $$\bar{\nu}_B = K_a^B C_B/(1 + K_a^B C_B + K_a^A C_A). \qquad (2)$$

In these equations, the concentration of free ligand $C = C_{total} - C_{bound}$, or $C = C_{added} - \bar{\nu}$[sites]. In our titrations, for *Ka* values in the range of 10^4 M^{-1}, the concentration of ligand exceeds the concentration of protein by a factor of 100 or more. Therefore, the term $\bar{\nu}$[sites] is sufficiently small that $C = C_{added}$.

Hence, if K_a^A and K_a^B can be determined independently, $\bar{\nu}_A$ and $\bar{\nu}_B$ at any given concentration of A and B, when both are present simultaneously, may be calculated by equations *1* and *2*. Also, if the ΔF_{max} values for both ligands in question are known, the relative contribution of each ligand to the overall change in fluorescence may be calculated (as $\bar{\nu} = \Delta F/\Delta F_{max}$).

It is apparent from Table I that the net ΔF calculated is in very good agreement with the observed ΔF, suggesting that **3** and **2** compete for the *same* binding site on the J-539 molecule.

An explanation for the increased binding of **2** with J-539, when compared with the "homologous", **3** ligand, therefore remained to be found. Work in this laboratory

on a model of the combining region of J-539* has shown that this region is lined with a number of residues of aromatic amino acids. In addition, we have previously shown that **3** binds to J-539 with its unsubstituted 5-(hydroxymethyl) group projecting towards the solvent[2]. From that result, it is to be expected that, even a substituent as large as the dansyl group should not sterically interfere with the binding of the saccharidic portion in **2** to J-539, if that substituent is located on O-6, as is the case here. The *enhanced* binding of **2** could be due to a hydrophobic-binding contribution from the dansyl group and a nearby aromatic, amino acid residue. From the model of J-539, it appears that the aromatic substituent on Gal_2 in **2** could very well interact with tyrosine residue 101 of the H-chain, located in the binding area in J-539. to engage in hydrophobic binding. The additional binding-energy expected for the dansyl derivatives, compared to those of the unsubstituted D-galactose-ligands, would be substantial[13], and the results in Table II show this to be so. The free-energies of binding(ΔG) for J-539 with **3** and **2** are -5769 and -7156 cal, respectively, as calculated from the *Ka* values by the equation $\Delta G = -RT \ln Ka$. Thus, ~ -5.9 kJ (~ -1.4 kcal) of additional binding-energy is involved for the dansyl derivative and J-539, compared with **3** and J-539. General values for hydrophobic bonding between two aromatic residues indicate that this value is reasonable[13]. It thus appears that at least one aromatic amino acid residue in the immunoglobulin is capable of interacting with the dansylated ligand.

It is clear that values for *Ka* that are obtained for dansylated ligands and proteins by fluorescence titration should be interpreted with care, as the derivatizing group could cause additional binding. This binding may be due to a charge-transfer complex with an aromatic amino acid residue of the protein.

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*Fv, including the combining region of J-539, was constructed by using amino acid sequence-data (S. Rudikoff and D. Narayana Rao) and the X-ray coordinates of two immunoglobulins having similar hypervariable regions (C. P. J. Glaudemans, E. A. Padlan, and D. Davies).