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Note

The determination of binding constants for binding between carbohydrate ligands and certain proteins

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For some time, we have been investigating a group of seven immunoglobulin A myeloma proteins which have binding specificity for polysaccharides containing sequences of β-D-(1→6)-linked D-galactopyranosyl residues[1–5] These globulins precipitate with larch arabinogalactan[1], gum ghatti[1], and mammalian-lung galactan[4] We have found that the fluorescence intensities of some of these proteins (J-539, M-601, X-24, and X-44) are altered upon binding with haptens, and, by utilizing this property, we have determined the binding constants (K_a values) of two of these proteins (J-539 and X-24) with (1→6)-β-D-galacto-triose and -tetraose[3] We have now found that the method is such that K_a values of 10^2 can readily be measured, this is 10^2 to 10^3 times more sensitive than the method of equilibrium dialysis[6], the foremost alternative for the determination of association constants

When proteins are irradiated at 280 nm, they fluoresce with an emission maximum at 330–350 nm This fluorescence (F) is due mainly to tryptophanyl residues, and tyrosyl residues appear to contribute very little to the phenomenon[7] Energy emitted by tryptophan in the excited state can be transferred to bound ligands by proteins in a radiationless process[8] if the ligand contains aromatic or nitro groups The quantitative change in fluorescence observed by us on binding simple carbohydrates [transparent to ultraviolet (u v) radiation] to proteins is unusual, and these changes in intensity can be rationalized as follows Any change in the micro-environment of fluorescing tryptophanyl residues will produce a change in the overall fluorescent properties of the protein, be it in the intensity or fluorescence maximum Such a change may be induced by changes in (*a*) conformation, either large or subtle, (*b*) pH, (*c*) temperature, (*d*) solvation, etc Such changes may also be brought about in certain proteins when the protein binds to small molecular moieties Thus, the intensity of lysozyme is increased upon binding oligosaccharides derived from chitin[9] Yeast enolase exhibits a similar phenomenon on binding magnesium[10]

In addition to our four anti-(1→6)-β-D-galactopyranan IgA proteins, certain myeloma immunoglobulins having antiphosphorylcholine specificity have independently been found to increase their fluorescence-intensity upon binding phos-

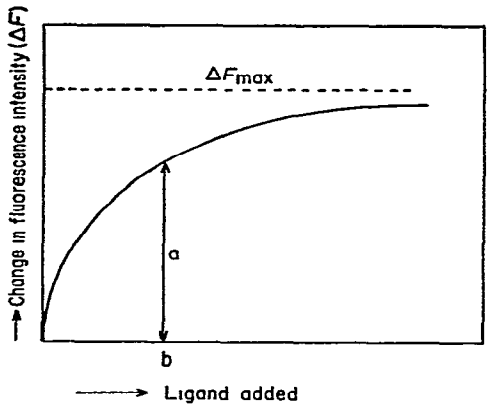

Fig 1 Typical change in fluorescence intensity when a ligand is added to a myeloma IgA

phorylcholine[11,12]. Some proteins do not show a change in fluorescence on binding with ligand, but, for those that do, the binding constant may be determined by the procedure reported here The method is well suited to carbohydrate-binding proteins, as carbohydrates do not absorb in the u v or visible spectrum, and hence cannot interfere with, or contribute to, observed fluorescence Fig 1 shows a typical curve obtained on adding various proportions of ligand to a protein The maximal fluorescence (ΔF_{max}) is obtained by adding small proportions of solid ligand to the protein until no further change occurs We assume that the relative increase in fluorescence intensity is directly proportional to the fraction of available sites occupied by ligand This assumption is valid only if all sites have the same affinity for ligand, and if the binding of ligand at one site does not affect the binding of ligand at another site Therefore, at any point *b* on the curve (see Fig 1), we can say that $\bar{v}$ (the fraction of available sites occupied by ligand) equals $a/\Delta F_{max}$ Knowing the protein concentration and the number of sites available per molecule (which may be determined by repeating the experiment at a different concentration of protein[13]), the concentration (c) of free ligand may be determined $c = [\text{ligand}] - \bar{v}[\text{total sites}]$ A Scatchard plot ($\bar{v}/c$ *versus* $\bar{v}$) of the data* will yield a line whose slope equals $-K_a$. As an example, the changes in fluorescence intensity due to the binding of methyl α-L-arabinopyranoside [methyl 5-nor(hydroxymethyl)-β-D-galactopyranoside] to the pepsin fragment (Fab') of J-539 anti-galactan IgA immunoglobulin are shown in Fig 2 and Table I, and the corresponding Scatchard plot is shown in Fig 3 The fact that a straight line is obtained is indicative of the correctness of our assumptions

The method has a limitation, in that, if no change in fluorescence intensity is observed on binding with ligand, it is useless However, from the fact that four out of seven proteins in our anti-galactan series show the phenomenon, it appears that these binding-related fluorescence changes are quite common The advantages of this method over the foremost alternative method for determination of association

*$\bar{v}/c = K_a - \bar{v}K_a$.

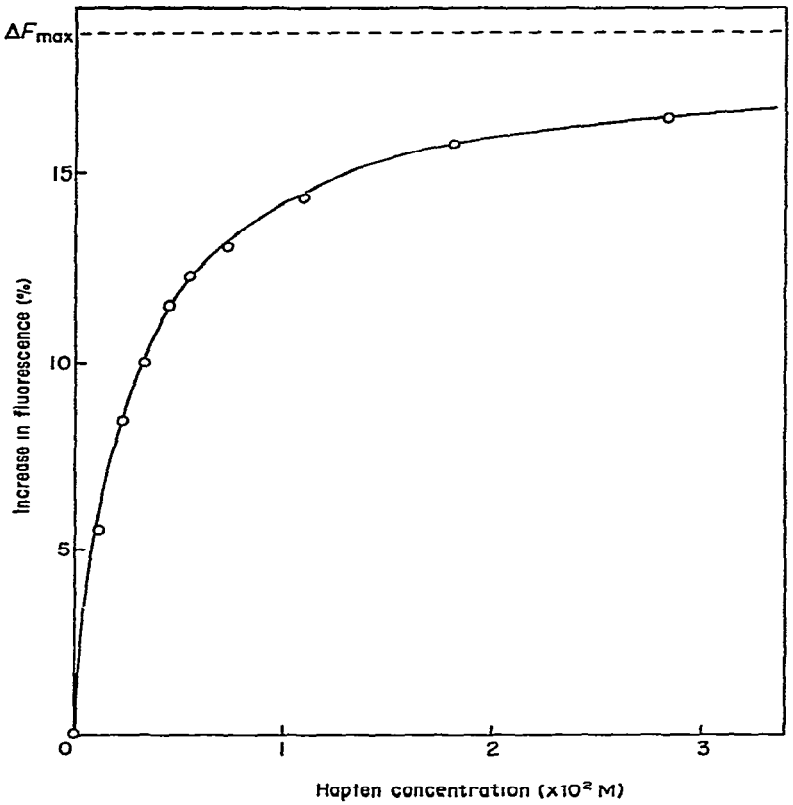

Fig 2 The change in fluorescence intensity of J-539 immunoglobulin A (Fab') upon the addition of methyl α-L-arabinopyranoside

constants (namely, equilibrium dialysis) are many (*1*) The method is simple and quick, requiring only one hour for measurement of a K_a value (*2*) It requires only small quantities of material, even for protein–ligand systems having low K_a values (For a system with a K_a as low as 10^2, the amount of protein and ligand needed are 1 nmole and 75 μmoles, respectively) (*3*) It is accurate and very reproducible (*4*) Radioactive derivatives of the ligand need not be prepared, so that haptens remain unchanged (*5*) It is versatile, there is no restriction on the K_a value measured (*6*) The K_a values of *whole* antigen with Fab' fragments of antibodies may be measured, and this has hitherto been impossible. (*7*) The protein may be of small molecular weight, and need not be nondialyzable

EXPERIMENTAL

The fluorescence titrations were performed according to the general method used for quenching-titrations[8]. A Perkin–Elmer MPF-3 fluorescence spectrophoto-

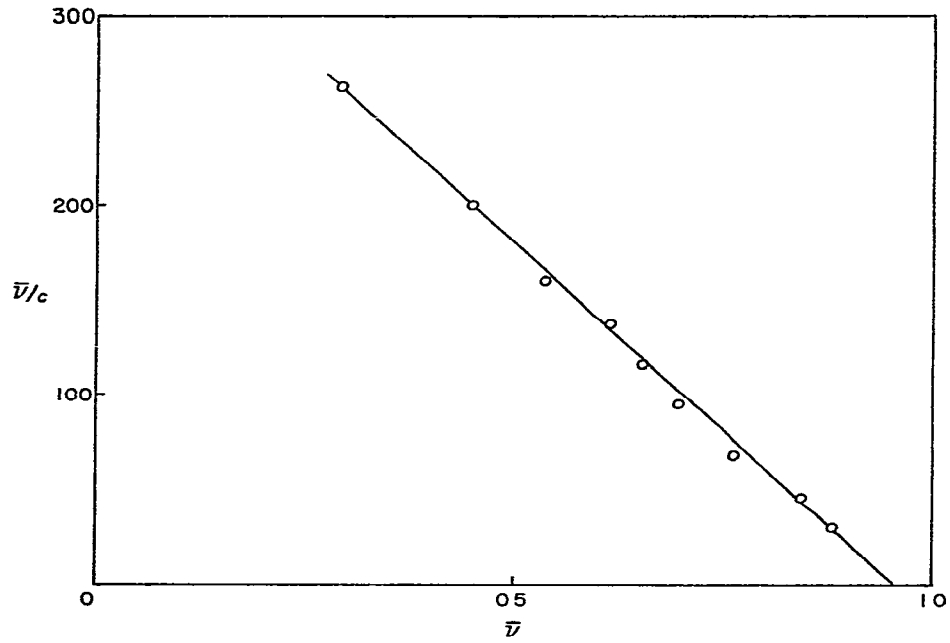

Fig 3 Scatchard plot of $\bar{\nu}/c$ *versus* $\bar{\nu}$ for immunoglobulin J-539 (Fab') and methyl α-L-arabinopyranoside (see text)

meter was used, it was fitted with a thermostated, sample-cell assembly, through which water at 25 0° was circulated For the best results, the protein solutions [having an absorbance (A) of $A_{280\,nm}$ <0 05] were centrifuged to remove suspended dust particles The protein solution (1 5 ml) was added to each of two cells (10 × 10 × 45 mm), one being used for ligand addition (test cell), and the other as the reference cell A magnetic stirring-bar (6 × 1 × 1 mm) was placed in the test cell After thermal equilibration (several minutes), the difference in the intensities of the fluorescence of the test and reference solutions at 340 nm (excitation at 295 nm, band width, 2 nm) was determined several times, and these differences were averaged The intensity of fluorescence of a buffer blank was also determined Ligand solution* was added to the test solution in small aliquots (3–45 μl) by means of a syringe microburet (model number SB2, Micro-Metric Instrument Co , Cleveland, Ohio) The mixture was stirred in the cell while ligand was added, and the cell was returned to the MPF-3 instrument, where it was left for several minutes to equilibrate The intensities of fluorescence of the test and reference solutions were then determined several times,

*Two determinations were performed for each compound For the preliminary measurement, an arbitrary ligand concentration was employed, depending on the K_a value expected—the higher the binding constant, the less concentrated the ligand solution Having thus obtained a preliminary binding-constant, the concentration of the ligand solution was so adjusted that the protein would be half-saturated after the addition of ~20 μl of ligand solution to the test cell In this way, a good distribution of points could be obtained.

TABLE I

TITRATION OF J-539 FAB′ WITH METHYL α-L-ARABINOPYRANOSIDE[a]

Total volume added (μl)	*Total volume (μl)*	*Correction factor*	*Total fluorescence*	F *(corrected)*	*ΔF (corrected)*	*ν*	*Bound ligand* (nM)	*Total ligand* (mM)	*Free ligand* (mM)	$\bar{\nu}/c$
3	1503	1 002	77 5	77 65	4 05	0 2935	209 6	1 115	1 115	263 46
6	1506	1 004	79 5	79 8	6 2	0 4493	320 9	2.225	2 224	201.96
9	1509	1 006	80 5	81 0	7 4	0 5362	383 0	3.331	3 331	160.96
12	1512	1 008	81 4	82 05	8 45	0 6123	437 4	4.433	4 433	138 15
15	1515	1 010	81 75	82 6	9 0	0 6522	465 8	5 531	5 530	117 93
20	1520	1 013	82 1	83 2	9 6	0 6957	496 9	7 349	7 349	94 66
30	1530	1 020	82 5	84 15	10 55	0 7645	546 1	10.951	10 951	69 81
50	1550	1 033	82 5	85 2	11 6	0 8406	600 4	18 018	18 017	46 66
80	1580	1 053	81 4	85 7	12 1	0 8768	626 3	28 280	28 280	31.00

[a] $F_0 = 73\ 6$, concentration of protein (C_p) = 714 nM, $\Delta F_{max} = 13\ 8$, concentration of stock solution of ligand (C_L) = 558 5 mM

and the differences averaged as before This procedure was repeated ten times, until a total volume of 0 15 ml of ligand solution had been added Small quantities of solid ligand were then added to the test solution until an increase in fluorescence no longer occurred, this point was taken as being the maximum increase in fluorescence intensity of the immunoglobulin due to the additions of ligand, *i e*, when all available sites on the protein were occupied by ligand (see Fig 2).

The increase in the intensity of fluorescence due to the addition of each aliquot of ligand was corrected for dilution of the sample (see Table I) As may be seen from Table I, the concentration of free ligand at any time is essentially identical to the amount of ligand added, due to the fact that, here, the binding constant is extremely low (see columns 9 and 10 in Table I) For higher binding-constants, the concentration of free ligand differs substantially from the total concentration of ligand The values for $\bar{\nu}/c$ were plotted *versus* $\bar{\nu}$ (see Fig 3), and association constants were determined from the slopes of these Scatchard plots by the method of least squares ($K_a = 4\ 00(\pm 0\ 03) \times 10^2$)

REFERENCES

1 M Potter, E B Mushinski, and C P J Glaudemans, *J Immunol*, 108 (1972) 295–300
2 M Potter and C P J Glaudemans, *Methods Enzymol*, 28 (1972) 388–395
3 M E Jolley, S Rudikoff, M Potter, and C P J Glaudemans, *Biochemistry*, 12 (1973) 3039–3044
4 C P J Glaudemans, M E Jolley, and M Potter, *Carbohyd Res*, 30 (1973) 409–413
5 S Rudikoff, E B Mushinski, M Potter, C P. J Glaudemans, and M E Jolley, *J Exp Med*, 138 (1973) 1095–1106
6 A H Sehon, *Methods Immunol Immunochem*, 3 (1971) 383–394
7 F W J Teale, *Biochem J*, 76 (1960) 381–388
8 J E McGuigan, *Methods Immunol Immunochem*, 3 (1971) 395–404
9 S S Lehrer and G D Fasman, *Biochem Biophys Res Commun*, 23 (1966) 133–138
10 J M Brewer and B Weber, *J Biol Chem*, 241 (1966) 2550–2557
11 R Pollet and H Edelhoch, *J Biol Chem*, 248 (1973) 5443–5447
12 R Pollet, personal communication
13 C J Halfman and T Nishida, *Biochemistry*, 11 (1972) 3493–3498