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Fluorescence polarization as a tool to study lectin-sugar interaction

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Abstract. The binding of *Ricinus communis* agglutinin and *Abrus* agglutinin to 4-methylumbelliferyl β-D-galactopyranoside was studied by equilibrium dialysis, fluorescence quenching and fluorescence polarization. The number of binding sites and the association constant value obtained by fluorescence polarization for both *Ricinus communis* agglutinin and *Abrus* agglutinin are in close agreement with those obtained by the other methods. This indicates the potential of ligand-fluorescence polarization measurements in the investigation of lectin-sugar interactions.

Keywords. Fluorescence polarization; lectin; sugar binding.

Introduction

Lectins, the carbohydrate binding proteins have proved to be useful tools for probing structural features of cell-surface glycoconjugates (Lis and Sharon, 1973; Rapin and Burger, 1974; Goldstein and Hayes, 1978). The binding of lectins to the carbohydrate moiety of cell membrane receptors is a prelude to a wide range of biological effects of lectins on cells (Cuatrecasas and Tell, 1973; Sharon, 1976). Fluorescently labelled sugars have been used for investigation of the binding parameters of certain lectins such as concanavalin A and Wheat Germ Agglutinin (Dean and Homer, 1973; Privat *et al.,* 1974; Loontiens *et al.,* 1977). On the other hand,lectins such as that from *Pisum sativum* (pea) and *Ricinus communis* (castor-bean), which do not show appreciable ligand fluorescence quenching or enhancement could not be investigated by utilizing these sugars.

During our investigation of 4-methylumbelliferyl β-D-galactopyranoside (MeUmb-Gal$_p$) binding to *R. communis* agglutinin (RCA$_1$) and *Abrus* agglutinin by fluorescence spectroscopy, no appreciable quenching or enhancement of ligand fluorescence was observed in the case of RCA$_1$, whereas it was 30% quenched by *Abrus* agglutinin (Khan *et al.,* 1980, 1981).

Fluorescence polarization of a molecule depends on the molecular size, geometry and rigidity (Weber 1952; Steiner, 1954; Haber and Bennett, 1962; Kierszenbaum *et al.,* 1969). It was anticipated that the interaction of MeUmb-Gal$_p$ with

Abbreviations used: RCA$_1$, *Ricinus communis* agglutinin; MeUmB-Gal$_p$, 4-methylumbelliferyl β-D-galactopyranoside; MeUmb-Glu $_p$, 4-methylumbelliferyl β-D-glucopyranoside.

RCA_1 and *Abrus* agglutinin would lead to alterations in polarization of its fluorescence. Therefore, changes in fluorescence polarization of the sugar on its binding to RCA_1 and *Abrus* agglutinin appeared to be an ideal approach to characterize this interaction. A marked change in the polarization of MeUmb-Gal_p fluorescence was observed on binding with RCA_1 and *Abrus* agglutinin. The number of binding sites obtained by fluorescence polarization was two for both the lectins. The association constant value for RCA_1 (K_a=2.9×10^4 M^{-1}) and *Abrus* agglutinin (K_a = 1.42×10^4 M^{-1}) obtained by polarization compares well with those obtained by equilibrium dialysis and other methods.

Materials and methods

4-Methylumbelliferyl β-D-galactopyranoside and 4-methylumbelliferyl β-D-glucopyranoside (MeUmb-Glu_p) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. The concentration of the fluorescent sugar solutions was determined at 318 nm using ε = 1.36×10^4 M^{-1} cm^{-1} (Loontiens *et al.,* 1977). Lactose was obtained from British Drug House, Poole, Dorset, UK, and all other chemicals used were of analytical grade.

Lectins

Ricinus communis agglutinin(RCA_1) and *Abrus* agglutinin were purified by affinity chromatography on cross-linked guar gum (Appukuttan *et al.,* 1978) and arabinogalactan (Mazumdar and Surolia, 1978, 1979) respectively. Concentration of RCA_1 and *Abrus* agglutinin solutions was determined using
$A_{280}^{1\%,1\,cm}$ = 14 and $A_{280}^{1\%,1\,cm}$ = 14.6 (Olsnes *et al.,* 1974) respectively.

Equilibrium dialysis

A fixed concentration of RCA_1 (50 μM) or *Abrus* agglutinin (23 μM) was placed in one compartment of the dialysis cell and varying concentrations of MeUmb-Gal_p (5-150 μM) in the other compartment. After equilibration at 25° C for 24 h, the concentration of the sugar was determined spectrophotometrically in the protein free compartment and the data analysed according to Scatchard (1949).

Fluorescence titration

A fixed amount (1 ml) of MeUmb-Gal_p, (3 μM) was titrated with a 192 μM solution of RCA_1 (Khan *et al.,* 1980) or 229 μM solution of *Abrus* agglutinin (Khan *et al.,* 1981) at 25° C. The fluorescence spectra were recorded in a Perkin-Elmer MPF-44A ratio-recording spectrofluorimeter. Excitations were done at 318 nm and the emission spectra recorded above 330 nm, with a slit of 5 nm for both the monochromaters.

Fluorescence-polarization measurement

Fluorescence-polarization measurements were made on the above spectrofluorimeter equipped with polarizing accessory. Excitation and emission wavelengths were 318 nm and 375 nm respectively. The changes in the fluorescence polarization of a 2.2 μM MeUmb-Gal_p solution on titration against increasing amounts of 192 μM RCA_1 solution (Khan *et al.,* 1980) and a 2 μM MeUmb-Gal_p solution titrated against increasing amounts of 229 μM *Abrus* agglutinin solution *(Khanetal.,*

1981) was recorded. In another set of experiments the concentration of RCA_1 (20 μM) and *Abrus* agglutinin (53.8 μM) was fixed and the polarization of various concentrations of MeUmb-Gal_pwere recorded respectively.

Results

The equilibrium dialysis data, when plotted according to Scatchard (1949) as in figure 1 A and B were consistent with two binding sites per molecule of M_r 120,000 and 130,000 for RCA_1 and *Abrus* agglutinin respectively. The association constant obtaind by equilibrium dialysis for RCA_1 and *Abrus* agglutinin were K_a= 2.6×10^4 M^{-1} and K_a = 1.62×10^4 M^{-1} at 25°C, respectively.

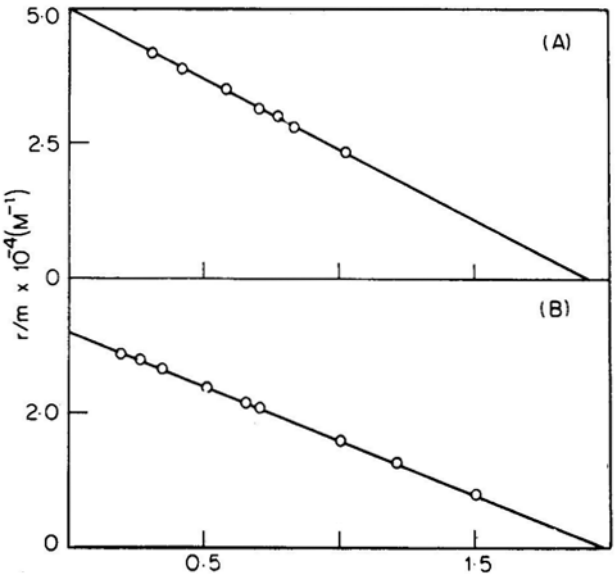

Figure 1. A. Scatchard plot for the binding of MeUmb-Gal_p to *Abrus* agglutinin. **B**. Scatchard plot for the binding of MeUmb-Gal_p,to RCA_1 Determined by equilibrium dialysis at pH 7.2, 25° C.

The binding was monitored at 318 nm for MeUmb-Gal_p in the protein-free compartment; γ is the ratio of bound ligand to total protein concentration and *m* is the free MeUmb-Gal_p concentration at equilibrium. From the slope K_a value was calculated. Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl.

The fluorescence of MeUmb-Gal_p even in the presence of large excess of RCA_1 shows an enhancement of only 2.5%, indicating that there is virtually no change in the polar environment of the sugar when it is bound to the lectin. The fluorescence of MeUmb-Gal_p when bound to *Abrus* agglutinin was 30% quenched and was inhibited by lactose, whereas MeUmb-Glu_p fluorescence was not quenched at all. From the quenching titration the association constant of the sugar for the lectin was estimated by the relationship given earlier (Loontiens *et al.,* 1977; Khan *et al.,* 1981).

$$\log\left(\frac{F_0-F}{F-F_\alpha}\right) = \log K_a + \log\left(|P|_t-|M|_t\,\frac{(F_0-F)}{F_0}\right)$$

A plot of log $(F_0—F)/(F— F_a)$ against log p, where p is the free protein concentration given by the log term within the parentheses; assuming 1:1 stoichiometry for the binding, with an equivalent weight of 65,000 for *Abrus* agglutinin gave an association constant 1.51×10^4 M^{-1} at 25° C (figure 2A, B).

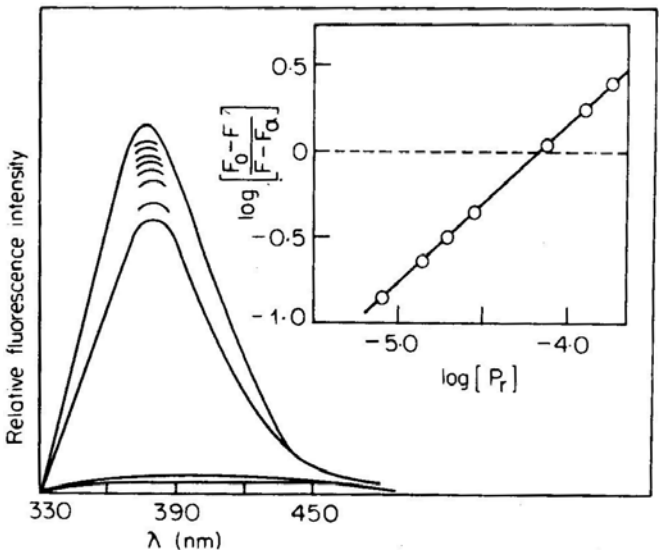

Figure 2. Quenching of the fluorescence of MeUmb-Gal$_p$ by *Abrus* agglutinin.

Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl at 25°C. The fluorescence spectra were recorded in the absence (top curve) and after addition of several aliquots of 229 µM. *Abrus* agglutinin to 1 ml of 3 µM solution of MeUmb-Gal$_p$ (30 µl, 60 µl, 90 µl, 120 µl, 320 µl, 530 µl respectively). After each addition of the agglutinin, part of the spectrum was recorded, and the full spectrum is given for the final addition. After correction for dilution, the value of the association constant was determined graphically (inset).

Fluorescence polarization

Polarization, p, of a molecule is expressed quantitatively in terms of intensities (I), polarized either parallel ($\parallel$) or perpendicular ($\perp$) to the incident electric field:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad (1)$$

For a randomly oriented molecule in a rigid medium, the maximum value of p observed with linearly polarized light is 0.5; if the molecules are subject to rotary Brownian motion, the molecular rotation taking place between the time of absorption and emission may be expected to result in values of p between 0.5 and zero. The extent of this rotation is a function of molecular dimensions and structure, solvent and temperature. Low-molecular-weight compounds will give rise to a depolarized fluorescence and thus, for MeUmb-Gal$_p$, the p value is 0.07 at 25° C.

If p, p_B and p_F represent the polarization of the sample, bound sugar and free sugar respectively, the molar fraction of bound sugar (f_B) is given by:

$$f_B = \frac{p-p_F}{p_B-p_F} \quad , \tag{2}$$

from which the molar fraction of free sugar (f_F) can be calculated as:

$$f_F = 1 - f_B = \frac{p_B-p}{p_B-p_F} \tag{3}$$

This will yield

$$\frac{f_B}{f_F} = \frac{p-p_F}{p_B-p} \quad , \tag{4}$$

provided the quantum yields of bound and free sugar are the same. If not,

$$\frac{f_B}{f_F} = \frac{Q_F}{Q_B} \cdot \frac{p-p_F}{p_B-p} \quad , \tag{5}$$

where Q_F and Q_B are the quantum yields of free and bound sugar respectively. The Scatchard equation can be written as:

$$\frac{f_B\,[P]}{f_F} = \frac{n}{K_D} - \frac{f_B[M]}{K_D[P]} \quad , \tag{6}$$

where $[M]$ and $[P]$ are the concentration of the sugar and protein respectively; n is the number of sugar molecules bound per molecule of protein and K_D is the dissociation constant. Thus a plot of f_BP/f_F against f_B $[M]$ / $[P]$ yields a straight line with intercepts of n/K_D and n on the y and x axes respectively. Of the parameters employed p_F and Q_F are the values for free sugar, whereas p_B and Q_B have to be determined. The values of polarization of fluorescence intensity, when the probe is completely bound to the lectin, will give p_B and Q_B . In case where complete binding cannot be achieved experimentally, p_B and Q_B are obtained by an extrapolation as described for antigen-antibody interaction (Dandliker *et al.,* 1978). If the equilibrium values *of* p, plotted against concentration $[M]_t$ are extrapolated to zero concentration, where $[M]_t$ is that total molar concentration of the ligand in both free and bound forms; p approaches a limit, p'. The values of p' for different lectin concentrations plotted against (p' — p_F) divided by lectin concentration in any arbitrary convenient unit, p, gives p_B as the intercept of a straight line:

$$p' = p_B - Q_F \frac{(p'-p_F)}{Q_{Bp}} \tag{7}$$

A similar relationship can facilitate the determination of Q_B :

$$Q' = Q_B + \frac{Q_F-Q'}{p} \tag{8}$$

The changes in the fluorescence polarization of MeUmb-Gal$_p$ on titration of a fixed concentration of RCA$_1$, or *Abrus* agglutinin by various concentration of MeUmb-Gal$_p$ were subjected to Scatchard analysis according to eqn. 6 and the data plotted are shown in figure 3 A and B. At 25°C the value of K_a is, 2.94×10^4 M^{-1} for RCA$_1$ and for *Abrus* agglutinin the K_a is 1.42×10^4 M^{-1}. The number of binding sites for both the lectins is approximately equal to two.

The changes in fluorescence polarization of a 2.2 μM solution of MeUmb-Gal$_p$ on titration with RCA$_1$ (192 μM) is shown in figure 4 A. An increase in ligand fluorescence polarization with increasing RCA$_1$ concentration is observed, reaching a

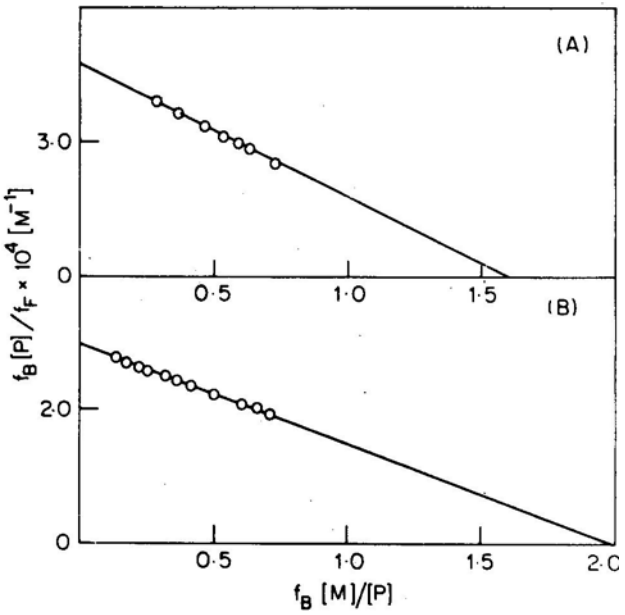

Figure 3. A. Scatchard plot for the binding of MeUmb-Gal$_p$ to RCA$_1$ determined by fluorescence polarization. **B**. Scatchard plot for the binding of MeUmb-Gal$_p$ to *Abrus* agglutinin determined by fluorescence polarization.

RCA$_1$ (20 μM) was titrated with various concentrations of MeUmb-Gal$_p$ (A).

Abrus agglutinin (54 μM) was titrated with various concentrations of MeUmb-Gal$_p$. f_B *and* f_F are the molar fraction of bound sugar and free sugar respectively. [*M*] and [*P*] are the concentration of the sugar and lectin respectively. From the slope, the K_a value was calculated. Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl (B).

saturation value at 20 μM-RCA$_1$. A complete reversal of the polarization was observed on addition of lactose to lectin fluorescent sugar solution. The changes in fluorescence polarization of a 2.0 μM solution of MeUmb-Gal$_p$ on titration with *Abrus* agglutinin (229 μM) is shown in figure 4 B. An enhancement in the polarization of the ligand fluorescence was also observed with increasing concentration of *Abrus* agglutinin, and it was reversed on addition of lactose.

From the polarization data of titration of a fixed concentration of MeUmb-Gal$_p$ by various concentration of RCA$_1$ or *Abrus* agglutinin, the association constant can also be obtained by a modified form of the relationship given earlier(Chipman *et al.,*1967).

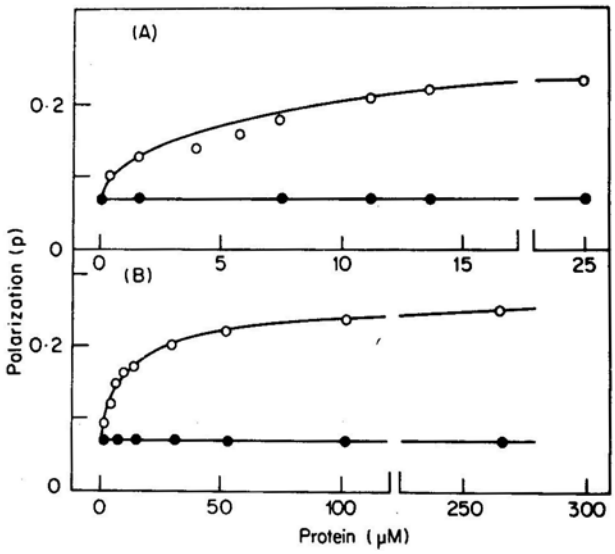

Figure 4. Changes in fluorescence polarization of MeUmb-Gal$_p$ at different concentration of RCA$_1$ and *Abrus* agglutinin.

A. A 2.2 μM solution of MeUmb-Gal$_p$ was titrated with portions of 192 μM RCA$_1$ (O). Titration curve of 2.2 μM solution MeUmb-Gal$_p$ with 192 μM RCA$_1$ solution preincubated with lactose (●).

B. A 2.0 μM solution of MeUmb-Gal$_p$ was titrated with portions of 229 μM *Abrus* agglutinin (O). Titration curve of 2.0 μM MeUmb-GaJ$_p$ with 229 μM *Abrus* agglutinin preincubated with 0.1 M lactose (●). Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl at 25°C.

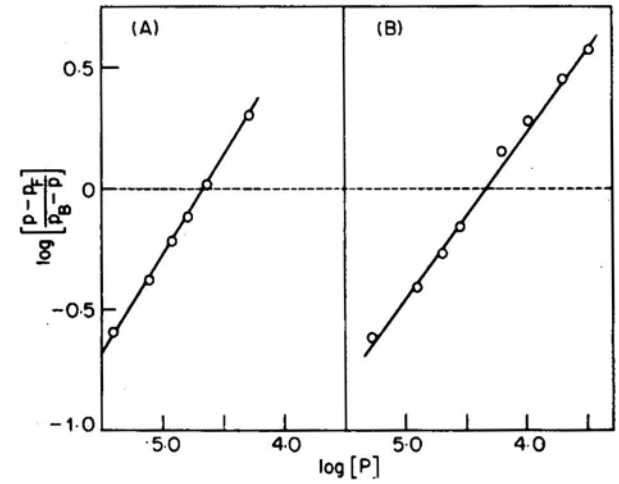

Figure 5. Sample plot of fluorescence polarization data of MeUmb-Gal$_p$ with various concentration of RCA$_1$ (A) and *Abrus* agglutinin (B).

p, p_B and p_F are the relative polarizations of sugar sample, bound sugar and free sugar respectively. [P] is the free protein concentration. From the intercept on the x-axis, K_a was calculated. Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl.

$$\log\left(\frac{p-p_F}{p_B-p}\right) = \log K + \log\left([P]_t-[M]_t\,\frac{(p-p_F)}{p}\right)$$

The log term within the bracket represents the free protein concentration denoted by log P in figure 5 A and B. A plot of log $(p - p_F) / (p_B - p)$ against log P assuming 1:1 stoichiometry for ligand-protein interaction for 60,000 M_r RCA_1 or 65,000 M_r *Ahrus* agglutinin gave straight line (figure 5 A and B). The slope of the plots was unity, which proved that the assumed stoichiometry was correct. The intercept on the x-axis gave the value of $K_a = 4.2\times10^4$ M^{-1} for RCA_1 and $K_a = 2.14\times10^4$ M^{-1} for *Abrus* agglutinin.

Discussion

The fluorescence of MeUmb-Gal_p was not quenched nor appreciably enhanced on binding to RCA_1, whereas on binding to *Abrus* agglutinin it was quenched by 30%. The reversal of the quenching in case of *Abrus* agglutinin by lactose and no quenching of MeUmb-Glu_p fluorescence indicates that the quenching is due to sugar specific binding only. The total lack of quenching of MeUmb-Gal_p fluorescence by RCA_1 indicates a polar environment around the sugar binding site of RCA_1 (Delmotte *et al,* 1975), whereas 30% quenching by *Abrus* agglutinin indicates a less polar environment around its binding site (van Landschoot *et al.,* 1977).

A marked change in the polarization of MeUmb-Gal_p fluorescence was observed on binding to RCA_1 or *Abrus* agglutinin. This increase in polarization was utilized for determining the equilibirum parameters for these interactions. The number of binding sites obtained by fluorescence polarization was two for both RCA_1 and *Abrus* agglutinin and this is in agreement with the values obtained by equilibrium dialysis and fluorescence quenching. The associaton constant values obtained for RCA_1 and *Abrus* agglutinin for MeUmb-Gal_p are consistent with those obtained by equilibrium dialysis, fluorescence quenching and with those reported in literature for other sugars (van Wauve *et al.,* 1973; Podder *et al.,* 1974, 1978; Olsnes, 1978). The Scatchard plots of fluorescence polarisation and equilibrium dialysis are linear in fractional and high saturation ranges, indicating the absence of additional low-affinity sites and lack of interaction between the two identical sites. This is consistent with several studies reported in literature for binding of other lectins to simple carbohydrates and their aromatic glycosides (Hassing and Goldstein, 1970; van Wauve *et ah,* 1973; Podder *et al.,* 1974; van Landschoot *et al.,* 1977). The consistency in the number of binding sites and association constant values obtained for RCA_1 and *Abrus* agglutinin by equilibium dialysis, fluorescence quenching and polarization, demonstrates the validity of the fluorescence polarization studies.

The observed alterations in the polarization of ligand fluorescence, that occurs on binding of the fluorescent sugar to the lectins is advantageous, because it provides a direct and rapid measure of the ratio of bound/free sugar. The small amount of material required for these studies is an added advantage. In summary, the present work demonstrates that the fluorescence-polarization measurements can be used for the determination of (a) the number of binding sites, (b) the degree of heterogeneity of the sites and (c) K_a values for the binding of proteins to their ligands. Fluorescence polarization will thus prove a valuable tool in elucidating the

mode of interaction of sugars, especially fluorescently labelled sugars, with several other lectins that do not markedly influence the emission of ligand fluorescence.

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