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# Spectral Changes on Binding of Oligosaccharides to Murine Immunoglobulin A Myeloma Proteins†

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ABSTRACT: Six mouse myeloma proteins with binding activity directed against multiple $\beta(1\rightarrow6)$-linked D-galactopyranose units have been isolated from ascites on a Sepharose–bovine serum albumin–*p*-azophenyl $\beta$-D-thiogalactopyranoside column. The proteins gave single bands on immunoelectrophoresis against rabbit anti-whole mouse serum (BALB/c). The changes in fluorescence properties of the six proteins on binding with $\beta(1\rightarrow6)$-linked oligosaccharides of galactopyranose were investigated. The intensities of fluorescence of two IgA proteins, J-539 and X-24, were increased on binding with the tri- and tetrasaccharide. The fluorescence properties of three other proteins, S-10, T-191, and J-1, were not significantly affected by these oligosaccharides. The fluorescence of protein X-44 was quenched upon binding $\beta(1\rightarrow6)$-galactotriose and -tetraose. From these changes in fluorescence, binding constants for proteins J-539 and X-24 and their Fab′ fragments with the tri- and tetrasaccharide have been calculated. Strong evidence for the presence of tryptophan in or near to the active sites is presented.

Myeloma proteins[1] are homogeneous immunoglobulins produced by neoplastic plasma cells. A number of myeloma proteins have been shown to specifically bind antigens and by many immunochemical parameters to resemble homogeneous antibodies. The specificities of antigen binding myeloma proteins from mice are usually detected by screening sera against polyvalent antigens by double diffusion in agar gel. In an earlier paper (Potter *et al.*, 1972) we reported finding two IgA myeloma immunoglobulins with specificities directed against $\beta(1\rightarrow6)$-linked D-galactopyranosyl moieties. We now wish to report three more IgA myeloma immunogloublins with the same antigalactan specificity (proteins X-24, X-44, and J-1). A sixth IgA myeloma protein (J-539) previously reported (Sher and Tarikas, 1971) and shown to bind proteins substituted with $\beta$-D-galactopyranosyl units has also been investigated.

These six myeloma proteins of the IgA class are all precipitable by larchwood arabinogalactan. The general structure of this polysaccharide is fairly well known (Aspinall, 1970). It consists of backbone of $(1\rightarrow3)$-linked anhydrogalactose units, with short side chains of $\beta(1\rightarrow6)$-linked galactopyranosyl moieties. Single side groups of arabinose also occur. All immunoglobulins described here had their active sites directed toward the $\beta$-galactosyl groups. In studies to be reported elsewhere we have found that these six myeloma proteins have different idiotypic antigenic determinants indicating that they are structurally different (Rudikoff *et al.*, 1973[2]). This group of proteins therefore provides a unique set of homogeneous immunoglobulins, structurally diverse, but all directed toward an extremely simple carbohydrate determinant involving sequences of only one neutral monosaccharide moiety and only one type of linkage. We wish to describe a study of the changes in the fluorescence of these immunoglobulins following their binding with ligand. Since the inhibiting oligosaccharides[3] (Potter *et al.*, 1972) that were available were of a series $Gal_2$, $Gal_3$, and $Gal_4$, and since these oligosaccharides do not absorb in the ultraviolet (uv) spectrum, they are particularly suitable for the study of these spectral changes induced in the native protein on binding.

## Materials and Methods

The plasmacytomas which produced the myeloma proteins used in the present study arose in BALB/c mice following the intraperitoneal injection of mineral oil or pristane (Potter

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[1] Nomenclature and abbreviations are those recommended by The World Health Organization. All immunoglobulins are designated by the first letter and number of the parent tumor.

[2] Rudikoff, S., Mushinski, E. B., Potter, M., Glaudemans, C. P. J., and Jolley, M. E., manuscript in preparation.

[3] Abbreviations used are: $Gal_2$, D-galactopyranosyl-$\beta(1\rightarrow6)$-D-galactose; $Gal_3$, corresponding $\beta(1\rightarrow6)$-galactotriose; $Gal_4$, corresponding $\beta(1\rightarrow6)$-galactotetraose; APTG, *p*-aminophenyl $\beta$-D-thiogalactopyranoside.

TABLE I: Changes in the Intensity of Fluorescence Caused by the Addition of $\beta$(1→6)-Galactopyranose Oligosaccharides (35 $\mu$l, $3 \times 10^{-4}$ M) to Mouse Myeloma Immunoglobulins (0.7 ml, *Ca.* 0.3 mg/ml).[a]

| Protein | Concn (mg/ml) | % Change[c] | | |
|---|---|---|---|---|
| | | $Gal_2$ | $Gal_3$ | $Gal_4$ |
| S-10 | 0.337 | | | |
| J-1 | 0.298 | | | |
| T-191 | 0.313 | | | |
| J-539 | 0.300 | | +19.4 | +21.9 |
| X-24 | 0.333 | | +17.7 | +26.7 |
| X-44 | 0.313 | | −7.7 | −8.3 |
| T-15[b] | 0.321 | | | |
| M-603[b] | 0.298 | | | |
| M-167[b] | 0.291 | | | |

[a] A preliminary experiment using an Aminco-Bowman spectrophotofluorometer. [b] IgA immunoglobulins with anti-phosphorylcholine specificity. [c] No value indicates no change.

and Glaudenans, 1972). Plasmacytoma J-539 was kindly provided by Dr. Melvin Cohn.

*Oligosaccharides.* The oligosaccharides $Gal_2$, $Gal_3$, and $Gal_4$ were generously provided by Dr. G. O. Aspinall. $Gal_4$ was also isolated in our laboratories by extensive chromatography of the partial hydrolysate of gum ghatti (Aspinall *et al.*, 1958) as a chromatographically pure, lyophilized powder containing galactose as the only monosaccharide detectable after hydrolysis. The $R_F$ of this material on paper chromatography was identical with that of crystalline $Gal_4$ and its binding properties toward J-539 immunoglobulin were identical with those of authentic tetrasaccharide (*i.e.* fluorescence, $K_a$).

*Purification of Proteins.* A Sepharose–bovine serum albumin–*p*-azophenyl $\beta$-D-thiogalactopyranoside immunoabsorbent was prepared as described earlier (Potter and Glaudemans, 1972). APTG was diazotized and coupled to bovine serum albumin. The bovine serum albumin–azophenyl galactoside was then coupled to CNBr-activated Sepharose 2B and thoroughly washed with cold water on a Büchner funnel and stored in 0.05 M Tris-HCl, pH 7.4, containing 0.025% sodium azide.

Approximately 100 ml of ascites from tumor-bearing animals was passed through 25-ml adsorbent columns equiliberated in 0.05 M Tris-HCl, pH 7.4, at room temperature. After ascites application, columns were washed with the equilibration buffer until the $A_{280}$ of the eluate was less than 0.1. Protein was specifically eluted with a solution of *p*-nitrophenyl $\beta$-D-galactopyranoside (10 mg/ml) in equilibration buffer. The eluted protein was dialyzed against the above buffer to remove eluting hapten. The protein concentration was then determined spectrophotometrically using a molar extinction coefficient of $2.1 \times 10^5$ at 280 nm (Chesebro and Metzger, 1972).

The adsorbent was shown to bind 15–20 mg of specific protein/ml of adsorbent. Columns were equilibrated in the starting buffer or washed with 1 M acetic acid followed by re-equilibration before further use. Proteins thus purified showed single bands on immunoelectrophoresis with a rabbit anti-whole mouse serum (BALB/c). All six purified immunoglobulins had their precipitation with arabinogalactan inhibited by $Gal_2$ in agar double diffusion experiments.

*Preparation of Pepsin Fragments.* Pepsin fragments were prepared as previously described (Inbar *et al.*, 1971; Rudikoff *et al.*, 1972). Proteins were reduced with 0.01 M dithiothreitol and then alkylated with 0.022 M iodoacetamide (twice recrystallized). The resultant material was then dialyzed against 0.1 M acetate buffer, pH 4.5, and digested with pepsin at a 1:100 enzyme:protein ratio (w/w) for 4–6 hr at 37°. The pH was adjusted to 8 and the digestion mixture chromatographed on Sephadex G-100 in borate buffer. Appropriate fractions were pooled and concentrated and the borate buffer was removed by dialysis *vs.* 0.05 M Tris-HCl buffer at pH 7.4. Protein solutions were then frozen for storage.

*Fluorescence Titrations.* Flourescence titrations were performed according to the method of Pollet and Edelhoch (1973). Protein solutions of optical density 0.05 at 280 nm, in 0.05 M Tris-HCl buffer at pH 7.4, were centrifuged at 3500 rpm for 10 min in an International Size 2, Model V, centrifuge, fitted with a type 241 head, to remove suspended dust particles. An aliquot (1.5 ml) from the supernatant was added to each of two cells ($10 \times 10 \times 45$ mm) in a Perkin-Elmer MPF-3 fluorescence spectrophotometer fitted with a thermostated sample-cell assembly, through which water at 25.0° was circulated. One cell was used for ligand addition (test cell), the other being the reference cell. A magnetic stirring bar ($\sim 6 \times 1 \times 1$ mm) was added to the test cell. After thermal equilibration (several minutes) the differences in intensities of the fluorescence of the test and reference solutions at 335 nm (excitation at 280 nm) were determined several times, and these differences averaged. The intensity of fluorescence of a buffer blank was also determined. Hapten solution ($3 \times 10^{-4}$ M in the case of $Gal_4$, $5 \times 10^{-4}$ M in the case of $Gal_3$) in 0.05 M Tris-HCl buffer at pH 7.4 was added to the test solution in small aliquots (3–45 $\mu$l) by means of an Agla syringe fitted with a micrometer screw. The mixture was stirred in the cell while hapten was added, and the cell was returned to the MPF-3 where it was left for several minutes to equilibrate. The intensities of fluorescence of the test and standard solutions were then determined several times, and the differences averaged as before. This procedure was repeated ten times until a total volume of 0.15 ml of hapten solution had been added. Small quantities of solid hapten were then added to the test solution until an increase in fluorescence intensity no longer occurred. This point was taken as the maximum increase of fluorescence intensity of the immunoglobulin due to the addition of hapten, *i.e.* when all available sites on the protein were occupied by hapten.

The increase in the intensity of the fluorescence due to the addition of each aliquot of hapten was corrected for dilution of the sample. Assuming that the fraction of the available sites occupied is directly proportional to the fraction of the maximum increase in intensity of fluorescence we obtain for each point, the fraction of the available sites occupied by hapten ($\bar{\nu}$) and the free hapten concentration ($c$) may be determined. Association constants were determined from the slopes of the Scatchard plots by the method of least squares.

*Reaction of Proteins with Hg(II) Ions at pH 9.5.* A solution of Hg(II) ions in 0.2 M carbonate buffer, pH 9.8, was prepared by adding 31.8 mg of mercuric acetate to 100 ml of 0.2 M carbonate buffer at pH 9.8 and heating the mixture until the brown precipitate had dissolved (approximately 60°). The solution was cooled to room temperature (in ice) and contained 200 $\mu$g/ml of Hg(II).

Protein solutions in 0.05 M Tris-HCl buffer at pH 7.4, 0.5 ml ($\sim$1 mg/ml), were added to both the reference and sample cells ($4 \times 10 \times 42$ mm) in a Beckman Acta V spectrophotometer. To the reference cell was added 0.5 ml of 0.2 M car-

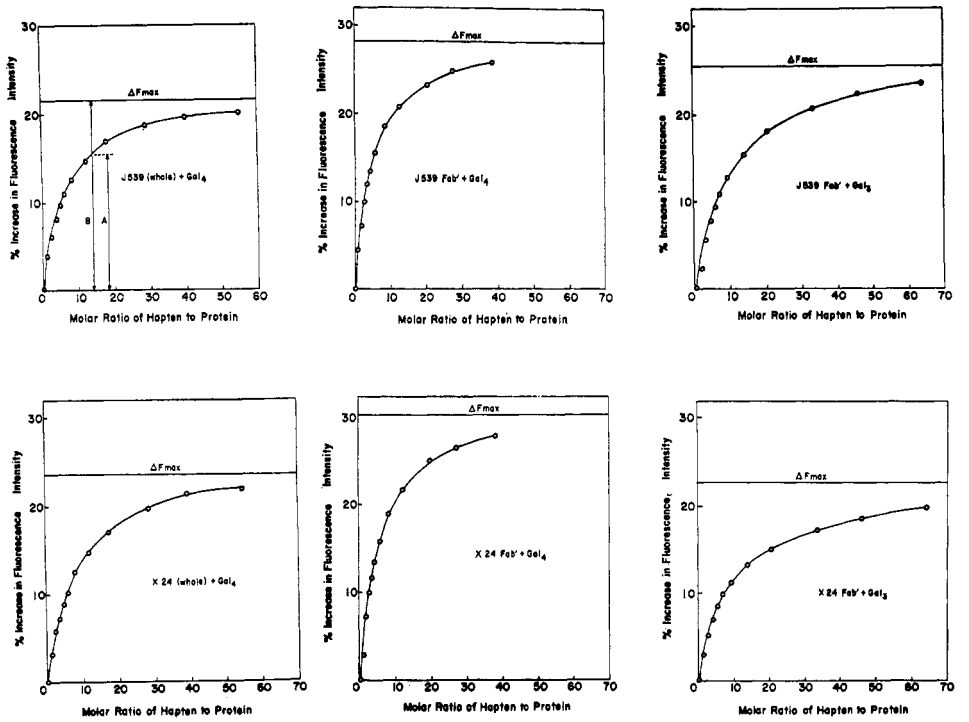


FIGURE 1: The increase in intensities of fluorescence ($\Delta F$) of J-539 and X-24 whole proteins and Fab′ fragments on titration with $Gal_3$ and $Gal_4$. For an explanation of parameters, see Results.

bonate buffer at pH 9.8 to bring the final pH of the mixture to 9.5. To the sample cell was added 0.3 ml of the same 0.2 M carbonate buffer and 0.2 ml of the Hg(II) solution while mixing thoroughly. Where hapten solution was added to the sample cell, the volume of 0.2 M carbonate buffer added was decreased correspondingly so as to keep the total volume of hapten solution plus buffer added at 0.3 ml. Precipitation of protein was followed by observing the turbidity of the sample solution at 297 nm (all measurements at room temperature). In addition, after maximum precipitation had been reached—as indicated by maximum turbidity—50 $\mu$l of $3 \times 10^{-4}$ M hapten solution was added to sample solution and the turbidity change with time was measured.

## Results

*Fluorescence Changes Associated with Binding of Hapten.* Table I shows the changes induced in the intensities of fluorescence of the six proteins by addition of excess $Gal_2$, $Gal_3$, and $Gal_4$. No effect was observed in the case of proteins S-10, T-191, and J-1 with any of the haptens. The intensities of fluorescence of J-539 and X-24 were increased significantly by the addition of $Gal_3$ and $Gal_4$, but $Gal_2$ produced no effect. Protein X-44 exhibited a decrease in its intensity of fluorescence with $Gal_3$ and $Gal_4$, while $Gal_2$ was again without effect.[4]

In addition, none of the three oligosaccharides produced changes in the fluorescence intensities of three IgA antiphosphorylcholine immunoglobulins (T-15, M-603, and M-167).

Figure 1 shows the increase in fluorescence intensities for whole proteins J-539 and X-24 and Fab′ fragments titrated with $Gal_3$ and $Gal_4$. From these results (Figure 1) the fraction of sites occupied ($\bar{\nu}$) is obtained and hence the concentration of free hapten ($c$). By plotting $\bar{\nu}/c$ *vs.* $\bar{\nu}$ the association constant $K_a$ may be obtained from the slope of the straight line.[5]

*Interaction of Mercury Ions with Myeloma Proteins in the Presence or Absence of Haptens.* From Figure 2 it can be seen that in the presence of Hg(II) ions at concentrations as low as 40 $\mu$g/ml, J-539, X-24, and X-44 readily become insoluble as evidenced by their increase in turbidity. The presence of $Gal_3$ or $Gal_4$ completely inhibits the precipitation of these proteins with Hg(II) ions. Note also that $Gal_2$ is not very efficient in protecting these three immunoglobulins. In addition, Figure 2d–f shows that the precipitates of the three immunoglobulins can be wholly or partly redissolved by the addition of $Gal_4$.

Figure 3a–c shows the difference uv spectra of the turbid suspensions obtained from the reaction of Hg(II) ions with the three immunoglobulins at pH 9.5. It can be seen that the spectra between 280 and 320 nm closely resemble those obtained by the reaction of free tryptophan with Hg(II) ions (Figure 3d) (Jolley, 1972). In addition, no such spectra were obtained from the reaction mixtures of proteins J-539, X-24, or X-44 immunoglobulins with Hg(II) in the presence of $Gal_3$ or $Gal_4$ (5 mol/mol of protein). Furthermore, proteins S-10, T-191, and J-1 were not precipitable and did not exhibit any difference uv spectra upon exposure to Hg(II) ions at pH 9.5.

## Discussion

Mouse myeloma proteins which precipitate with arabinogalactan were shown also to precipitate with bovine serum albumin–*p*-azophenyl β-D-thiogalactopyranoside. In addition, APTG was an excellent inhibitor of the immunoglobulin–arabinogalactan precipitation reactions. By coupling bovine serum albumin–*p*-azophenyl β-D-thiogalactoside to Sepharose we were thus able to specifically purify proteins with this activity by affinity chromatography. In this way six immuno-

[4] See note added in proof.

[5] $\bar{\nu} = \Delta F_{obsd}/\Delta F_{max} = A/B$ in Figure 1; $c$ = [hapten] − $\bar{\nu}$([protein]/mol wt of two-chain unit); $\bar{\nu}/c = K_a - \bar{\nu}K_a$.

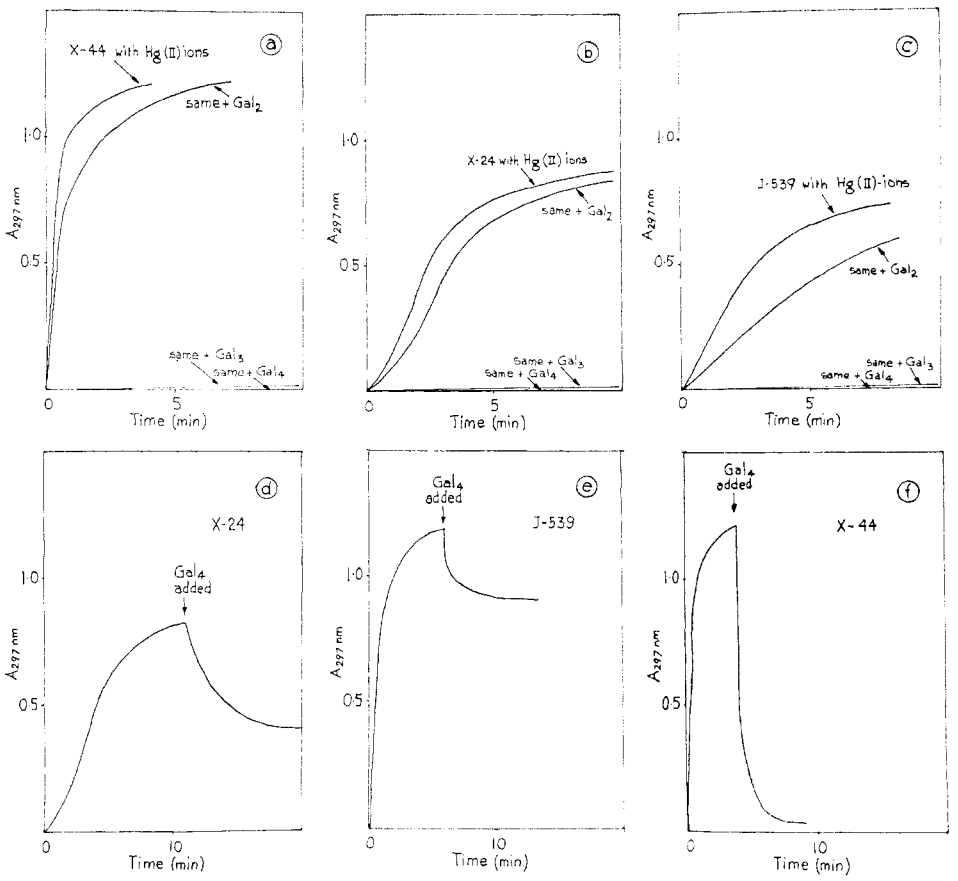


FIGURE 2: Turbidity of whole IgA solutions following addition of Hg(II) ions in the absence or presence of oligosaccharides (a–c) and clarification of suspensions by $Gal_4$ following precipitation by the addition of Hg(II) ions (d–f).

globulins were isolated. Data on the idiotypes of these proteins (to be published elsewhere) have revealed that we are indeed dealing with a set of diverse proteins.

Earlier work (Potter *et al.*, 1972) had shown that the precipitation of proteins S-10 and T-191 by arabinogalactan on agar double diffusion was increasingly inhibited in the order methyl β-D-galactoside < $Gal_2$ < $Gal_3$ < $Gal_4$. Figure 4 shows the relationship between the effectiveness of inhibition and the degree of polymerization of the oligosaccharide. As can be seen the greatest change in inhibition resulted in going from methyl β-D-galactopyranoside to $Gal_2$, and the difference between $Gal_3$ and $Gal_4$ seemed small indeed.

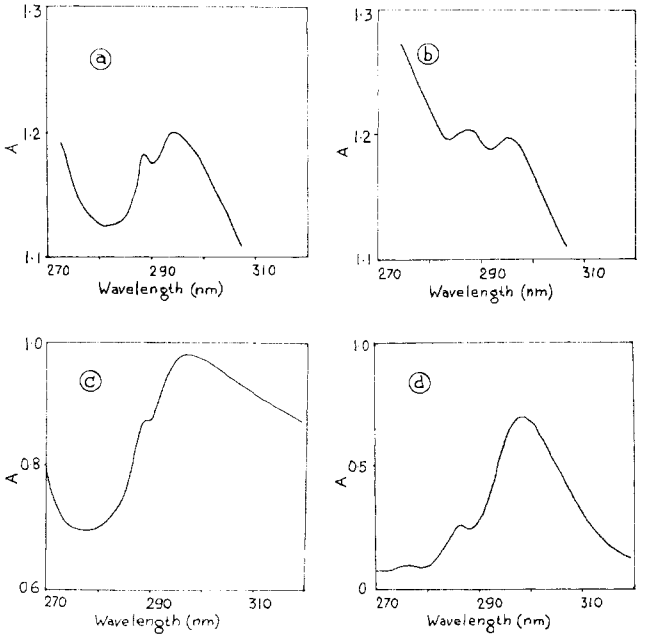


FIGURE 3: Difference spectra obtained by exposing proteins (a) J-539, (b) X-24, (c) X-44 immunoglobulins, and (d) tryptophan to Hg(II) ions at pH 9.5.

Some antibody–antigen interactions (Noble *et al.*, 1972) are known to involve changes in the native fluorescence of the antibody. Interactions of proteins with a ligand usually, but not always, involve fluorescence quenching. For example, lysozyme, upon binding with *N*-acetyl-D-glucosamine or its β(1→4)-linked oligosaccharides, exhibits an increase in fluorescence (Lehrer and Fasman, 1966). In addition, yeast enolase shows an increase in its fluorescence intensity on binding magnesium (Brewer and Weber, 1966). In both cases binding constants were derived from these phenomena. Fluorometric titrations of our immunoglobulins following the addition of ligands ($Gal_3$ and $Gal_4$) revealed that two proteins (X-24 and J-539) demonstrated increased intensity of fluorescence with $Gal_3$ and $Gal_4$. No immunoglobulin has hitherto been found to show an increase in fluorescence intensity upon binding with ligand.[6]

In addition, one immunoglobulin (X-44) showed quenching upon the addition of ligands $Gal_3$ and $Gal_4$. We assume that the relative increase in fluorescence intensity is directly propor-

[6] Pollet and Edelhoch (1973) have simultaneously with us, and independently, shown that TEPC-15 (an anti-phosphorylcholine mycloma protein) also shows an increase in fluorescence intensity upon the addition of phosphorylcholine.

TABLE II: Association Constants of Two Antigalactan Immunoglobulins and Their Fab′ Fragments as Determined by Fluorescence Data.

| | X-24 Whole | X-24 Fab′ | J-539 Whole | J-539 Fab′ |
|---|---|---|---|---|
| $Gal_3$ | | | | |
| % Increase in fluor. | Not determined | 22.7 | Not determined | 25.5 |
| $K_a \times 10^{-5}$ | | 1.75 ± 0.03 | | 1.50 ± 0.02 |
| $Gal_4$ | | | | |
| % Increase in fluor. | 23.6 | 30.2 | 21.7 | 28.3 |
| $K_a \times 10^{-5}$ | 2.66 ± 0.10 | 2.93 ± 0.05 | 3.49 ± 0.04 | 3.44 ± 0.07 |

tional to the fraction of available sites occupied by ligand. This observation can then be used to measure accurately the constant of association between ligand and protein (see Table II). This assumption is only valid if (a) all sites have the same affinity for ligand and (b) the binding of ligand at one site does not affect the binding of ligand at another site. The fact that we obtain straight lines in our $\bar{\nu}/c$ *vs.* $\bar{\nu}$ plots tends to bear out the validity of this assumption.

Since the ligand is nonabsorbing in the uv spectrum and carries neither a charge nor an aromatic group, fluorescence changes must have their origin in the protein molecule. Almost certainly these must involve a change in the microenvironment of one or more tryptophan residues.

Hg(II) ions react specifically with tryptophan residues in proteins at high pH and produce spectral changes as seen in Figure 3d (Jolley, 1972). At pH 4.5 no change is observed. Sulfhydryl groups are also reactive but show no such pH dependence and no such spectral changes. The observation of such a spectrum upon adding Hg(II) ions to proteins X-24, X-44, and J-539 indicates the presence of an accessible tryptophan residue(s) in the molecule. The absence of such a spectrum in these proteins when Hg(II) is added in the presence of ligand ($Gal_3$ and $Gal_4$) strongly suggests that this accessible tryptophan residue is actually located in or near to the active site. Furthermore, as can be seen in Figure 2, those three immunoglobulins showing the fluorescence change reported here also exhibit turbidity upon the addition of Hg(II) ions, a phenomenon that is completely inhibited by the prior addition of low molar amounts of ligands $Gal_3$ and $Gal_4$ (but not $Gal_2$). Moreover, even after turbidity has occurred the addition of $Gal_3$ or $Gal_4$ (see Figure 2) can substantially clarify the suspension in the cases of proteins X-24 and J-539, and completely clarify the suspension of protein X-44. In addition, no precipitation was observed on exposing proteins X-24, J-539, and X-44 to Hg(II) at pH 4.5, indicating that the precipitation was not due to reaction with sulfhydryl groups in the protein, but with one or more tryptophan residues alone. Finally, the observed change in fluorescence intensity upon binding $Gal_3$ and $Gal_4$ ligand is enhanced for the Fab′ fragment obtained from proteins X-24 and J-539 eliminating the $F_c$ portion as a locus for the tryptophan residue in question. From the above, it must be concluded that the tryptophan residue(s) responsible for changes in fluorescence is (are) most likely located in on near to the active site. Other workers have found evidence for tryptophan in the combining site of anti-2,4-dinitrophenyl and -2,4,6-trinitrophenyl antibodies (Little and Eisen, 1967).

The magnitude of the association constants found by us for $Gal_3$ and $Gal_4$ with X-24 and J-539 and their Fab′ fragments is between 1.5 and 3.5 $\times$ $10^5$. This is very similar to the $K_a$ found for a rabbit antipneumococcal polysaccharide IgG (of restricted heterogeneity) with an octasaccharide fragment from *Diplococcus pneumonia* S VIII (2.5 $\times$ $10^5$, Spyer and Pappenheimer, 1970). The $K_a$ is much higher than that found (Sher and Tarikas, 1971) for protein J-539 with isopropyl 1-

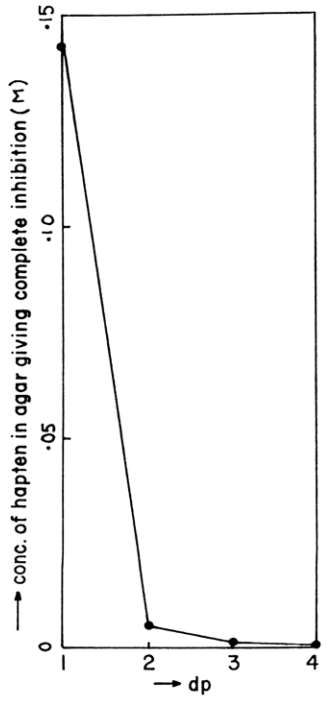


FIGURE 4: Concentration at which inhibition of S-10 and arabinogalactan precipitation is complete *vs.* degree of polymerization (dp) of inhibiting oligosaccharide.

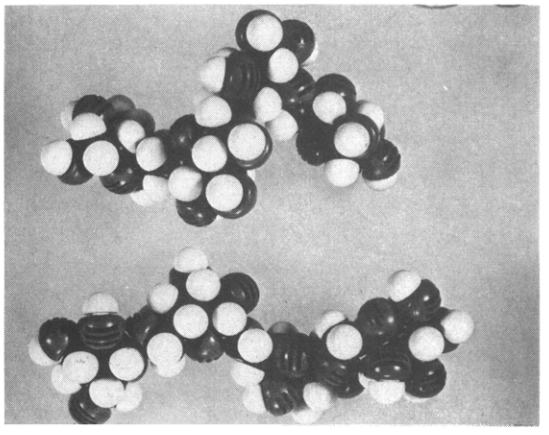

FIGURE 5: Molecular models of isomaltotetraose (bottom) and $Gal_4$ (top) showing postulated conformations.

thio-β-D-galactopyranoside ($2.2 \times 10^3$), a result which is consistent with our findings.

There seems to be little difference in binding characteristics between $Gal_3$ or $Gal_4$ with X-24 and J-539. This general leveling off after the ligand size has reached the trisaccharide stage has also been observed by others (Young *et al.*, 1971) in the case of an anti-α(1→3)-dextran IgM immunoglobulin. This result appears unlike that observed in the case of nonhomogeneous human antidextran (Kabat 1961), where maximum binding occurred at the hexasaccharide level, as indicated by inhibition studies. It is interesting to speculate on the reason for this difference. Inspection of a model of $Gal_4$ and a model of α(1→6)-linked glucotetraose (Figure 5) shows that the angle between adjacent α-(1→6)-glucosyl units needed to keep the intersaccharidic oxygen atom and ring atom from orbital overlap makes isomaltotetraose more compact than a β(1→6)-linked hexosyl oligosaccharide, such as $Gal_4$. Although this is very speculative, it may be that combining sites accommodating $Gal_4$ are of a similar size to one accommodating the isomaltohexaose ligand whose size was proposed in the pioneering work by Kabat.

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We wish to thank Ms. Elizabeth Mushinski for her technical assistance and Dr. R. Pollet for his invaluable help.

Added in Proof

We have recently found that an increase in the intensities of fluorescence of proteins J-539 and X-24 (Fab′ fragments) can be observed when high concentrations of $Gal_2$ and methyl β-D-galactopyranoside are used. We will report the quantitative data in a future communication.

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