BINDING PROPERTIES OF IMMUNOGLOBULIN COMBINING SITES SPECIFIC FOR TERMINAL OR NONTERMINAL ANTIGENIC DETERMINANTS IN DEXTRAN*

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Since the discovery by Landsteiner (1, 2) that low molecular weight haptens would react with antihapten antibody to inhibit precipitation by azoprotein antigens, and of Marrack and Smith (3) that binding of haptens to antibody could be demonstrated by equilibrium dialysis, such interactions have been used for identifying antigenic determinants and for establishing specificities and sizes of antibody combining sites (cf. 4, 5).

Specificities for carbohydrate antigens often appear to be directed toward the structure and linkage of terminal nonreducing sugars. This was first recognized by Goebel, Avery, and Babers (6), who found that the structure of the terminal nonreducing sugar was of predominant importance in determining cross-reactions with rabbit antisera to various disaccharides conjugated to horse serum globulin. Later, Karush (7) showed that lactoside-specific rabbit antibodies, produced by immunization with a lactosyl-azoprotein conjugate, had the major portion of their binding energies directed against the terminal β-linked galactose. With glycoproteins and polysaccharides there are many examples of terminal antigenic determinants. These include the A, B, H, and Lewis specificities of human blood group substances (8, 9), terminal glucuronic acid residues which are involved in the specificity of Type II pneumoccous polysaccharide (10–13 cf. 4, 5, 14), and terminal β-*N*-acetylglucosamine for Group A (15) and α-*N*-acetylgalactosamine for Group C (16) specificities in streptococcal polysaccharides (cf. 17). Certain determinants in teichoic acids (17–21 cf. 22) and in the somatic antigens of *Salmonella* (23, 24) and *Shigella* (25) are also of this type.

Antigenic determinants which do not require a terminal end group have also been recognized. Polysaccharides such as the type III and VIII pneumococcal polysaccharides which were shown to be linear (26) and thus to have one nonreducing end per milecule had long been known to precipitate with homologous antisera and also to cross-react (cf. 4). The determinant of the Type III polysaccharide (S III) was shown to consist of three repeating units of the disaccharide, cellobiuronic acid (27). Moreover, Heidelberger and

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Rebers (28) attributed the cross-reactivity between anti-S IV sera and S II to (1 → 3)-linked L-rhamnose units which occur at intervals along linear sugar chains. Nonterminal determinants are important in *Shigella* (25) and *Salmonella* (24), examples being determinants 3 and 15 of group E *Salmonella* which involve repeating [β D Gal(1→6)D Man-(1→4)L Rham(1→3)] sequences (29). In addition, certain specificities for teichoic acids (30 cf. 22) are of this type. It has been suggested that steric factors play a role in the proper exposure of certain terminal (31–33) and nonterminal (34) sugar determinants.

Dextrans are branched polymers of α-linked D glucopyranosyl units and their relatively simple structures have made them especially useful in immunochemical studies of homopolysaccharide antigens. Specificities involving $\alpha(1 \rightarrow 6)$- and $\alpha(1 \rightarrow 2)$-, $\alpha(1 \rightarrow 3)$-, $\alpha(1 \rightarrow 4)$-linked D glucopyranosyl units have been described (35-43). The reactions of $\alpha(1 \rightarrow 6)$-specific antidextrans have been thought to occur at the nonreducing ends of glucose chains with the terminal $\alpha(1 \rightarrow 6)$-linked D glucopyranosyl residue being immunodominant. The evidence for this has come from the structure of these polysaccharides which have several nonreducing ends but only one reducing end, and from inhibition of precipitation studies with isomaltose oligosaccharides from which it appeared that a majority of the binding energy was contributed by the terminal nonreducing glucose (44, 45, cf. 4, 5). In addition, rabbits which had been immunized with bovine serum albumin (BSA)[1] conjugates of isomaltotrionic acid (IM3-CONH-BSA) (46), and isomaltohexaonic acid (IM6-CONH-BSA) (47), produced antibodies whose quantitative precipitin reactions with dextrans were similar to those of $\alpha(1 \rightarrow 6)$-specific antidextrans. However, the best inhibitor for certain rabbit sera to IM3-CONH-BSA was larger than the trisaccharide which suggested as one possibility that specificities for nonterminal antigenic determinants such as (-*O*-(1 → 6)-D Glc-α-*O*-) sequences might exist (46). Moreover, Richter (48) has shown that rabbit antibodies to a dextran-protein conjugate made with a dextran fragment of mol wt 4,400 could precipitate a synthetic linear dextran (49, 50) which implied that these antibodies were capable of reacting with nonterminal glucosyl residues. Concanavalin A, which is thought to react at terminal nonreducing ends of dextran chains, does not precipitate with synthetic linear dextran (51). These findings raise two questions of fundamental importance: (*a*) Are antidextrans and antibodies against isomaltosyl oligosaccharides coupled to protein, mixtures of antibodies with specificities directed toward terminal nonreducing as well as nonterminal oligosaccharide sequences? (*b*) Can antibodies against terminal determinants cross-react at nonterminal locations along the dextran chain?

The existence of several homogeneous BALB/c mouse myeloma proteins with specificities for dextrans (52–55), fructosans (54–56), and galactans (57–59) provides a very useful approach to these questions. Three dextran-reactive myeloma globulins, W3129, W3434 and QUPC 52, have been shown (55) to have $\alpha(1 \rightarrow 6)$ specificities. Proteins W3129 and W3434 differed slightly in specificity (55) and idiotype (60), but both showed maximum complementarity to isomaltopentaose (IM5) while protein QUPC 52 showed maximum binding for isomaltohexaose (IM6) and was not related idiotypically to W3129.

We have now determined the binding energies for the interactions of proteins W3129 and QUPC 52 with each member of the isomaltose series and with methyl αDglucoside. These results indicate the protein W3129 has a nonreducing terminal specificity for $\alpha(1 \rightarrow 6)$ chains of dextran while protein QUPC 52 has nonterminal specificity. This was confirmed by precipitin reactions with a synthetic linear dextran which failed to precipitate protein W3129 but precipitated QUPC 52. The correlation between the binding properties of these myeloma proteins and their precipitin reactions with the synthetic dextran (49, 50) provides further evidence that this dextran reacts immunochemically as a

completely linear molecule. In addition, quantitative precipitin studies with linear and branched dextrans favor the concept that some human antidextran molecules also react only at terminal nonreducing ends while others can react at nonterminal locations along linear dextran chains. Moreover, rabbit antisera against IM3 or IM6 coupled to BSA[1] contain fractions of molecules which precipitate linear dextran and thus must be capable of nonterminal reactions with dextran chains. These findings provide a fundamental insight into the immunochemistry of dextran and probably other polysaccharide antigens.

Materials and Methods

Human and Rabbit Antisera and Mouse Myeloma Proteins. Human antidextrans are listed in Table I along with the dextrans employed for immunization. Serum 1 $D_{54\text{-}60}$ was a pool obtained by plasmapheresis of subject 1 seven times over a period of 2 mo, and was taken 18½ years after immunization with dextran B1255. Rabbit antisera against IM3-CONH-BSA (46) and IM6-CONH-BSA (47) were those studied previously. BALB/c mouse IgA myeloma proteins W3129, QUPC 52, W3082, and UPC 61 have been described (55) and were from serum or ascites fluid generously provided by Doctors Melvin Cohn and Martin Weigert (The Salk Institute, San Diego, Calif.), and Dr. Michael Potter of NIH.

Purification of Human Antidextrans and Mouse Myeloma Proteins. Purification of antidextrans and myeloma proteins was by a standard batch-wise immunoadsorption procedure (63-65) using Sephadex G75 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) for human antidextrans and proteins W3129 and QUPC 52 and levan gel (66, 68, 69) for human antilevans and proteins W3082 and UPC 61. After adsorption of protein, the insoluble gels were washed with 0.01 M phosphate-buffered saline, pH 7.2, 0.02% sodium azide (PBS) until supernates gave negligible absorption at 280 nm. Hapten elution was at 37°C for 1 h and proteins were freed of hapten by at least two passes through columns of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) (68, 69) and concentrated by ultrafiltration with collodion bags (Schleicher & Schuell, Inc., Keene, N. H.) or above a Diaflow UM-10 membrane (Amicon Corporation, Lexington, Mass.).

Antidextrans from 3,726 ml of 1 $D_{54\text{-}60}$ which contained 15.1 mg antidextran nitrogen (N) were adsorbed on to 922 mg Sephadex G75 and eluted with IM3 and IM6 in a batch-wise modification of the procedure described by Harisdangkul and Kabat (68). The packed Sephadex G75 was eluted first with 270 mg of IM3 in 15 ml PBS. This eluate (1 $D_{54\text{-}60}$ IM3El 1) was concentrated and the ultrafiltrate containing most of the IM3 was used for the second IM3 elution and after this, the process was repeated a third time. The Sephadex was then washed with PBS and the washings were combined with the second and third IM3 eluates to give 1 $D_{54\text{-}60}$ IM3El 2-3. Then 202 mg IM3 in 15 ml PBS were added to the packed Sephadex and this eluate plus the next two were combined to give 1 $D_{54\text{-}60}$ IM3El 4-6. The washed Sephadex was then extracted six times as above starting with 90 mg IM6 in 15 ml PBS and these were pooled to give 1 $D_{54\text{-}60}$ IM6El 1-6. The total recovery from all elutions was 10.2 mg antidextran N (68%) of which 1 $D_{54\text{-}60}$ IM3El 1, El 2-3, El 4-6, and IM6El 1-6 accounted for 52, 21, 8, and 19% respectively. After removal of antidextrans, human antilevans were purified from 1 $D_{54\text{-}60}$ sera by elution from levan gel with 0.1 M acetate buffer, pH 3.7 (66, 68).

Dextran- and fructosan-specific mouse myeloma proteins were eluted with methyl αDglucoside and sucrose respectively, which had previously been dialyzed to remove polysaccharides (70) and recrystallized from ethanol. With protein W3129, 45.5 mg of myeloma N was adsorbed to 4 g of Sephadex G75, and eluted three times with about 200 mg methyl α D glucoside per mg of myeloma N; the final recovery was 36 mg W3129 N (79%). With protein QUPC 52, 92 mg of myeloma N was adsorbed to 6.3 g of Sephadex, washed, and eluted 10 times with methyl αDglucoside at about 420 mg methyl αDglucoside per mg of myeloma N; the final recovery was only 26 mg QUPC 52 N (28%). The G75 was then eluted repeatedly in the cold (30 min per elution) with 0.1 M glycine HCl buffers ranging from pH 3.0 to pH 1.8 and eluates were neutralized immediately after collection. The

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; IM2, isomaltose; IM3, isomaltotriose; IM4, isomaltotetraose; IM5, isomaltopentaose; IM6, isomaltohexaose; IM7, isomaltoheptaose; PBS, phosphate-buffered saline.

TABLE I
Human Antidextrans

Antidextran	Immunizing dextran	Linkages			Reference
		$\alpha(1 \rightarrow 6)$	$\alpha(1 \rightarrow 3)$-like	$\alpha(1 \rightarrow 4)$-like	
		%	%	%	
$1D_{54\text{-}60}$	B1255 (Native)	86	0	14	4, 5, 36, 37, 45, 61, 63, 65, 66, 67, 68
$20D_{10}$	OP 155 (Clinical)	88			4, 5, 36, 44, 45, 61, 63, 64, 65, 66, 67
$30D_3$	OP 163 (Clinical)	88			4, 5, 35, 36, 37, 44, 45, 61, 66, 67
$116D_1$	S-5-A-1.0 (Clinical)	86	10	4	4, 37
$176D_4$	NRC Fr. 4 (Clinical)	96	4	0	4, 5, 45, 62, 64
$219D_3$	APC-54 (Clinical)				62

final yields were: 2.4 mg N at pH 3.0 (one elution) 11.8 mg N at pH at pH 2.7 to 2.4 (four elutions), 13.7 mg N at pH 2.0 (three elutions), and 4.6 mg N at pH 1.8 (three elutions) giving a total recovery in the low pH elutions of 32.5 mg QUPC 52 N (35%). All eluates were between 90 and 95% reactive with Sephadex G75. With proteins W3082 and UPC 61, 1.2 mg myeloma N was adsorbed onto 65 mg levan gel and eluted twice with 300 mg sucrose per mg myeloma N which removed virtually all adsorbed protein.

Dextrans. Native dextran B512 and clinical dextran N-150N have been described (61 cf. 4). Prof. Conrad Schuerch, Chemistry Department, State University College of Forestry, Syracuse, N. Y., kindly provided dextran D3 which by chemical methods appears to be a completely linear polymer of α (1 $\rightarrow$ 6)-linked D glucopyranosyl units having a mol wt by viscosity of 36,500 (49, 50). Enzymatic degradation experiments indicate that synthetic dextran contains 1–2% of structural flaws (71).

Qligosaccharides. The isomaltose oligosaccharides, methyl αDglucopyranoside, the β (2 $\rightarrow$ 1)-linked series of fructose oligosaccharides and sucrose were those described (55). Methyl α-isomaltoside and methyl α-isomaltotrioside (72) were provided by Dr. Allene Jeanes and have been studied previously (4, 65).

Tritium-labeled isomaltoheptitol ([^{3}H]IM7-OH) was prepared by overnight reduction at 4°C of 90 μmol IM7 with 25 μmol [^{3}H] sodium borohydride (383 mCi/nmol), and the reaction was stopped by adding small amounts of 25% acetic acid. The partially reduced and tritiated IM7 was then completely reduced with a 20-fold molar excess of $NaBH_4$. After 2 days at 4°C, excess $NaBH_4$ was destroyed and the [^{3}H]IM7-OH was purified by ethanol elution from a charcoal-celite column, followed by preparative paper chromatography with a propanol-ethanol-water (6:1:3) solvent system and final chromatography on a Bio-Gel P-2 column (minus 400 mesh, 70 $\times$ 1.9 cm) (73).

Immunochemical Methods. Quantitative precipitin and inhibition assays were done by a microtechnique (4) and total N in the washed precipitates was measured by the ninhydrin method (74). The separation of IgA monomer and polymer fractions of protein QUPC 52 and quantitative immunoadsorption experiments with dextran-reactive proteins using Sephadex G75 were like those described for protein W3129 (55).

Isoelectric Focusing. Analytical isoelectric focusing in a thin layer of 5% polyacrylamide gel (3% cross-linked) containing 2% carrier ampholytes (ampholine pH 3.5–10) was performed with an LKB 2117 Multiphor unit according to the directions of the manufacturer (LKB-Producter AB, Sweden). Gels were fixed overnight in 12% trichloroacetic acid then rinsed in several changes of water to remove carrier ampholytes before staining with 0.02% Coomassie Blue in methanol-acetic acid-water (45:9:46) and destaining with a solution of methanol (25%) and acetic acid (10%). Preparative

isoelectric focusing of 1 D_{54-60} IM3El 2–3 (1.6 mg N) was done in a 110 ml LKB column at 4°C using 3% carrier ampholytes (pH 3.5–10) in a sucrose gradient as described by the manufacturer. Electrofocusing was for 3 days with a final potential of 700 V.

Equilibrium Dialysis. Equilibrium dialysis and displacement experiments were carried out in lucite microdialysis chambers (75) at 25°C ± 0.1° and PBS was the buffer in all experiments. Dialysis tubing was boiled in 2 M Na_2CO_3 and rinsed thoroughly before use. Control experiments (4) established that there was no detectable binding of the ligand [^{3}H]IM7-OH to the dialysis membrane, to normal human γ-globulin (4.1 mg/ml) or to the levan-reactive IgA mouse myeloma protein Y5476 (14 mg/ml) (55). Experiments were done with equal volumes (50 or 75 μl) on each side of the membrane and dialysis cells were allowed to reach equilibrium over a 72-h period of continuous mixing; however, displacement experiments with protein QUPC 52 were given an extra day because of the high concentrations of competitors employed. Control experiments indicated that equilibrium was probably attained in less than 24 h. Samples (20 μl) for radioactivity measurements were taken from the hapten side only (76) and counted in duplicate or triplicate. The [^{3}H]IM7-OH gave about 2.9 × 10^6 cpm/μmol in Bray's solution (77) and 3.5 × 10^6 cpm/μmol in Insta-Gel (Packard Instrument Co., Downers Grove, Ill.). In displacement experiments, nonlabeled competitors did not cause any detectable quenching of counts except for the highest amounts of methyl α D glucoside employed with protein QUPC 52 and this occurred only in Insta-Gel and was corrected for. The free concentration of competitor was calculated by the method of Nisonoff and Pressman (78).

Fluorescence Titrations. Fluorescence titrations were performed with square quartz semimicro cuvettes (5 mm light path, 0.6 ml capacity) and an Aminco-Bowman spectrophotofluorometer equipped with a cell jacket thermostated at 25°C (68). Haptens were added in 5–20 μl volumes to 250 μl portions of protein solutions having optical densities of about 0.18 (1 cm light path) at 280 nm. None of the haptens absorbed in the 280 nm region and the excitation wave length was set between 280 and 285 nm depending on the protein and emission was measured between 345 and 350 nm. Values for Q_{max} were determined at the end of each titration by averaging the results from two additions of a concentrated ligand solution, each resulting in a greater than 97% saturation of sites. All titrations were done against a reference cell containing the solution being titrated and in this way it was possible to detect and correct for instrument drift. It was also necessary to plan experiments so that each addition of hapten resulted in at least a 3% quenching of fluorescence after correction for dilution.

Results

Isoelectric Focusing of Human Antibodies. Purified antidextrans from 1 D_{54-60} were 90–95% reactive with Sephadex G75 and analytical isoelectric focusing revealed a restriction in heterogeneity as compared with whole γ-globulin from the same individual. The patterns for antidextrans eluted with IM3 (Fig. 1 A,IM3El 1, IM3El 2–3, and IM3El 4–6) were similar with three major bands near pH 7.0 and several less prominent bands between pH 6.0 and pH 8.5. The antidextrans eluted with IM6 (Fig. 1 A, IM6El 1–6) were qualitatively like the IM3 eluates, but the three major bands near pH 7.0 were less obvious while those between pH 7.0 and pH 8.0 accounted for a greater proportion of the total protein. Purified antilevan from 1 D_{54-60} (Fig. 1 A, L) and antibodies to blood group A substance from 1 D_{33-34} (Fig. 1 A, A) also were restricted in heterogeneity and visibly different from each other and from the 1 D_{54-60} antidextrans. The anti-A antibodies had relatively low isoelectric points. A pH 3.5 to pH 10 gradient was used for the preparative separation of 1 D_{54-60} IM3El 2–3 into seven fractions (Fig. 1 B and C) which were studied later.

Equilibrium Dialysis of Mouse Myeloma Proteins and Human Antidextrans. Purified BALB/c mouse myeloma proteins W3129 and QUPC 52 were approximately 95% reactive with Sephadex G75, had two binding sites for [^{3}H]IM7-OH per IgA monomer, and gave linear Scatchard plots indicating homogeneous bind-

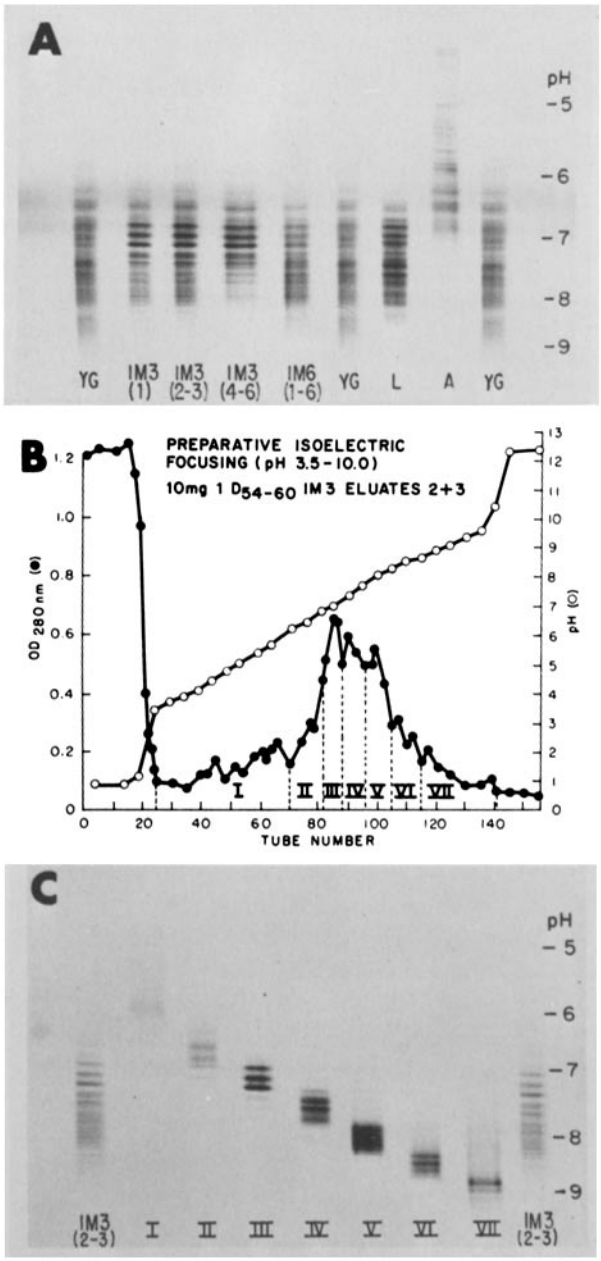

FIG. 1. A. Analytical isoelectric focusing of γ-globulin (γG) and purified antibodies from subject 1. Antidextrans from 1 D_{54-60} were those eluted from Sephadex G75 by sequential extraction with IM3 (IM3 eluate 1, IM3 eluate 2-3, and IM3 eluate 4-6) then IM6 (IM6 eluate 1-6). The antilevan (L) from 1 D_{54-60} and anti-blood group A antibodies (A) from 1 D_{33-34} γG fraction of GalNAc eluate (79) are shown. Approximately 5.2 μg N of all samples were applied to the gel. (B) Preparative isoelectric focusing of 10 mg 1 D_{54-60} IM3 eluate 2-3 showing how fractions I to VII were collected. (C) Analytical isoelectric focusing of 1 D_{54-60} IM3 eluate 2-3 and fractions I to VII from B. Approximately 3.3 μg N of all samples were applied to the gel.

ing (Fig. 2 A), as did the heterogeneity indices of 1.0 from Sips plots (not shown) for protein QUPC 52 and 0.9 for W3129. Both proteins were mixtures of monomeric and polymeric IgA, and the homogeneous binding shows that polymerization of IgA monomers does not alter the binding constants toward monovalent haptens. The binding constant of protein W3129 (K_o = 1.0 × 10^5 M^{-1}) was about 10-fold greater than that of protein QUPC 52 (K_o = 8.4 × 10^3 M^{-1}).

As expected from their isoelectric focusing patterns (Fig. 1 A), binding of [^{3}H]IM7-OH by human antidextrans 1 D_{54-60} IM3El 1 and 1 D_{54-60} IM6El 1–6 was heterogeneous as revealed by nonlinear Scatchard plots (Fig. 2 B). From Sips

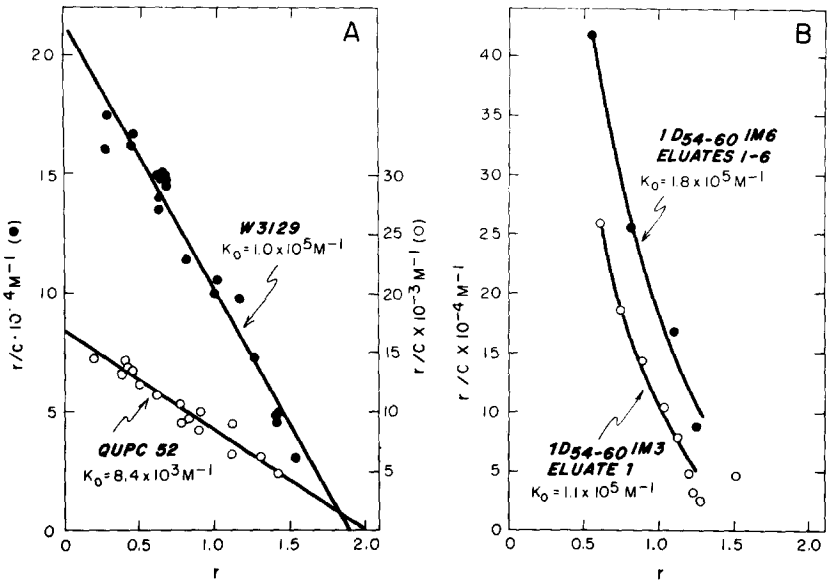

FIG. 2. Scatchard plots of equilibrium dialysis data at 25°C with [^{3}H]isomalthoheptitol. (A) Protein W3129 (●) at 10.5 mg/ml, and protein QUPC 52 (○) at 20.6 mg/ml. (B) Purified human antidextrans, 1 D_{54-60} IM3 eluate 1 (○) at 6.68 mg/ml, and 1 D_{54-60} IM6 eluates 1–6 (●) at 2.56 mg/ml. Calculations were done with a mol wt of 150,000 for myeloma proteins and human antidextrans.

analyses (not shown) heterogeneity indexes of 0.6 were calculated for both the IM3 and IM6 antibody. Association constants for 1 D_{54-60} IM3El 1 (K_o = 1.1 × 10^5 M^{-1}) and 1 D_{54-60} IM6El 1–6 (K_o = 1.8 × 10^5 M^{-1}) differed slightly and were similar to the value obtained for myeloma protein W3129.

Equilibrium Dialysis Displacement Studies with Myeloma Proteins W3129 and QUPC 52. Association constants for isomaltose oligosaccharides and methyl α-D-glucoside were determined by measuring the abilities of unlabeled oligosaccharides to displace [^{3}H]IM7-OH from the binding sites of proteins W3129 and QUPC 52. For both proteins, displacement curves with IM6 and IM7 were the same as self displacement curves calculated (78) for [^{3}H]IM7-OH and, thus, the association constants for IM6 and IM7 with proteins W3129 and QUPC 52 are 1.0 × 10^5 M^{-1} and 8.4 × 10^3 M^{-1} respectively. With each protein, a

displacement curve was calculated for a hypothetical competitor with $K_o = 1\ M^{-1}$ and thus a $\Delta F° = 0$ cal/mol ($\Delta F° = -2.303\ RT \log K$), and this serves as a useful reference as the distance between it and the displacement curve of a given competitor is directly proportional to the $\Delta F°$ for the competitor.

With protein W3129 (Fig. 3 A and Table II) over half of the binding energy for IM6 and IM7 is directed against methyl αDglucoside or IM2 which are similar. These are much less active than IM3 or IM4 which are equal and are bound almost as firmly as longer oligosaccharides. Isomaltopentaose is most efficient at displacing [^{3}H]IM7-OH and even somewhat more active than IM6 or IM7.

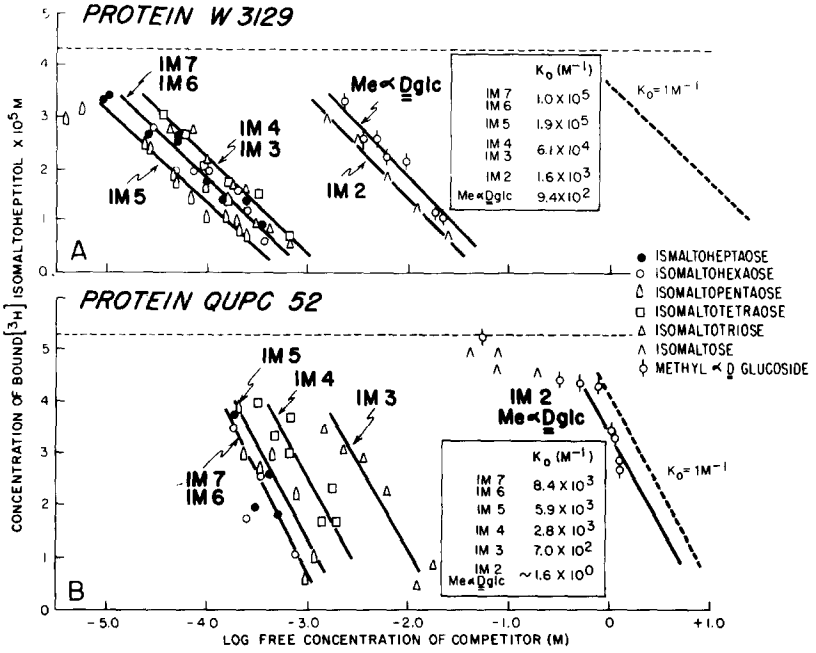

FIG. 3. Equilibrium dialysis displacement at 25°C of [^{3}H]isomaltoheptitol by unlabeled isomaltose oligosaccharides and methyl αDglucoside. (A) Protein W3129 (10.5 mg/ml) with a concentration of [^{3}H]isomaltoheptitol giving $r = 0.62$ in the absence of competitor. (B) Protein QUPC 52 (20.6 mg/ml) with a concentration of [^{3}H]isomaltoheptitol giving $r = 0.39$ in the absence of competitor. The dashed displacement curve corresponds to a hypothetical competitor with $K_o = 1\ M^{-1}$. The dashed horizontal line signified the concentration of bound [^{3}H]isomaltoheptitol in the absence of competitor.

Thus, while maximum complementarity is for IM5, 91% of the total binding energy is directed toward IM3 and 56% is for methyl αDglucoside.

The pattern of displacement reactions for protein QUPC 52 (Fig. 3 B and Table II) were strikingly different than those of myeloma W3129. Most significant were the findings that displacement by methyl αDglucoside and IM2, which could not be studied at higher concentrations than those shown in Fig. 3 B, were close to the hypothetical displacement curve having $K_o = 1\ M^{-1}$, and these competitors were bound at QUPC 52 sites with only about 5% of the total binding energy for IM6 or IM7 (Table II). By contrast, IM3 was much more active and 72% of the total binding energy was directed against the trisaccharide. There were increases

TABLE II A

Binding Properties of Mouse Myeloma Proteins and Human Antidextrans

Oligosaccharide	Protein W3129									Protein QUPC 52						1 $D_{54\text{-}60}$ IM3 E1 1: Precipitin inhibition (Fig. 5 A)			1 $D_{54\text{-}60}$ IM6 E1 1–6: Precipitin inhibition (Fig. 5 B)		
	Equilibrium dialysis (Fig. 3 A)			Fluorescence titrations (Fig. 4 A)			Precipitin inhibition (55, Fig. 5 C)			Equilibrium dialysis (Fig. 3 B)			Precipitin inhibition (55, Fig. 5 D)								
	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding
	M^{-1}		%	M^{-1}		%	M^{-1}		%	M^{-1}		%	M^{-1}		%	M^{-1}		%	M^{-1}		%
IM7-IM6	1.0×10^5	−6,800	95	1.7×10^5	−7,110	100	$1.7\times10^{5*}$	−7,110	100	8.4×10^3	−5,340	100	8.4×10^{3}‡	−5,340	100	1.1×10^{5}‡	−6,860	100	1.8×10^{5}‡	−7,150	100
IM5	1.9×10^5	−7,180	100	1.7×10^5	−7,110	100	1.7×10^5	−7,110	100	5.9×10^3	−5,130	96	4.0×10^3	−4,900	92						
IM4	6.1×10^4	−6,510	91	7.5×10^4	−6,630	93	8.5×10^4	−6,700	94	2.8×10^3	−4,690	88	1.3×10^3	−4,250	80						
IM3	6.1×10^4	−6,510	91	7.5×10^4	−6,630	93	8.4×10^4	−6,700	94	7.0×10^2	−3,870	72	3.4×10^2	−3,440	64	1.1×10^4	−5,470	80	4.0×10^3	−4,900	69
IM2	1.6×10^3	−4,360	61	1.4×10^3	−4,280	60	8.0×10^2	−3,960	56	1.6×10^0	−280	5				2.3×10^3	−4,570	67	9.0×10^2	−4,020	56
Methyl α D glucoside	9.4×10^2	−4,040	56	1.4×10^3	−4,280	60	8.0×10^2	−3,960	56	1.6×10^0	−280	5	0.4×10^0	+580	<0	1.2×10^1	−1,450	21	1.0×10^1	−1,370	19

* From fluorescence titrations. ‡ From equilibrium dialysis.

TABLE II B

Binding Properties of Mouse Myeloma Proteins and Human Antidextrans

Oligosaccharide	Proteins W3082 and UPC 61: Fluorescence titrations (Fig. 4 B)			Protein W3082: Precipitin inhibition (55)			Protein UPC 61: Precipitin inhibition (55)		
	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding
	M^{-1}		%	M^{-1}		%	M^{-1}		%
3 F1G	3.6×10^5	−7,560	100	$3.6\times10^{5*}$	−7,560	100	$3.6\times10^{5*}$	−7,560	100
2 F1G	3.6×10^5	−7,560	100	1.9×10^5	−7,180	95	2.5×10^5	−7,340	97
2 F1G reduced				2.2×10^4	−5,910	78	4.5×10^4	−6,330	84
1 F1G				2.6×10^2	−3,280	43	8.1×10^2	−3,960	52
Sucrose	6.3×10^2	−3,810	50	2.6×10^2	−3,280	43	5.0×10^2	−3,670	49
Fructose				9.0×10^0	−1,280	17	1.6×10^1	−1,640	22

Oligosaccharide	Protein X-24: Fluorescence titrations (58, 59)			Protein J539: Fluorescence titrations (58, 59)		
	K_o	Δ F° cal	Contribution to total binding	K_o	Δ F° cal	Contribution to total binding
	M^{-1}		%	M^{-1}		%
β(1 → 6) galactotetraose	2.93×10^6	−7,430	100	3.44×10^5	−7,530	100
β(1 → 6) galactotriose	1.75×10^6	−7,130	96	1.50×10^5	−7,040	93
β(1 → 6) galactobiose	5.75×10^3	−5,110	69	1.14×10^4	−5,520	73
Methyl βDgalactoside	3.77×10^2	−3,500	47	10.0×10^2	−4,080	54

1F1G βDfructofuranosyl(2 → 6)Dglucopyranose; 2F1G βDfructofuranosyl(2 → 1)βDfructofuranosyl(2 → 6)Dglucopyranose; 3F1G βDfructofuranosyl-(2 → 1)βDfructofuranosyl(2 → 6)Dglucopyranose.

* From fluorescence titrations.

in binding energy between IM3 and IM4 and from IM4 to IM5. IM5 seemed to be slightly less active than IM6 or IM7 but this difference, although known to exist from inhibition studies (55) is so small that it probably is within experimental error in the present data. Thus, maximum complementarity is for the hexasaccharide, and while 72% of the total binding energy is for IM3, almost none of this can function in the binding of IM2 or methyl α D glucoside.

Fluorescence Studies with Mouse Myeloma Proteins and Human Antidextrans. The binding of isomaltose oligosaccharides and methyl α D glucoside by protein W3129 was associated with a quenching of the protein's fluorescence and Q_{max} values from several titrations varied from 16 to 20% with IM3 through IM7 and from 20 to 23% with IM2 and methyl α D glucoside. The binding data (Fig. 4 A) from fluorescence titrations are in good agreement with results from equilibrium dialysis displacement and from quantitative precipitin inhibition assays (Table II). However, one difference was that IM5, IM6, and IM7 gave similar association constants in fluorescence titrations while in equilibrium dialysis displacement IM6 and IM7 were less active than IM5. Both methods show that maximum binding occurs with IM5 and gave similar K_o values for its binding by protein W3129. Titrations with monomer and polymer fractions (not shown) gave the same results as with unfractionated W3129 and supported the previous conclusion that polymerization of IgA subunits does not effect binding of a monovalent hapten. Compounds other than isomaltose oligosaccharides also

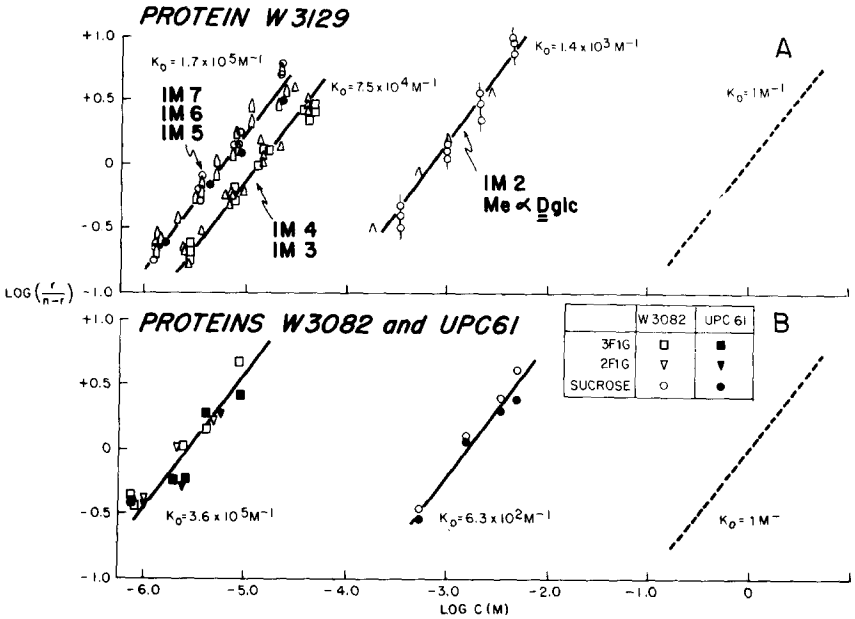

FIG. 4. (A) Sips plots of data from fluorescence titrations at 25°C of dextran-specific myeloma protein W3129 with isomaltose oligosaccharides and methyl αDglucoside (symbols as in Fig. 3) and of (B) fructosan-specific myeloma proteins W3082 and UPC 61 with β (2 → 1)-linked fructose oligosaccharides (2F1G and 3F1G) and with sucrose. All proteins were at 20 μg N/ml, and the excitation and emission wave lengths with W3129 were 280 and 345 nm respectively, and 285 and 345 nm respectively with W3082 and UPC 61.

quenched the fluorescence of protein W3129, but with these it was often not possible to reach Q_{max}. By using Q_{max} obtained from adding IM5, association constants were estimated for the α (1 → 2), α (1 → 3), and α (1 → 4)-linked glucose disaccharides, kojibiose, nigerose, and maltose, respectively, as being between 10^2 M^{-1} and 10^3 M^{-1}, and were lower than those of IM2 and methyl αDglucoside. Methyl βDglucoside, methyl αDglucoside, methyl αDmannoside, trehalose (αD-glucopyranosyl αDglucopyranoside) and glucose all had K_o values less than 5×10^1 M^{-1}. These findings emphasize the specificity which the W3129 site has for isomaltosyl structures. The binding of isomaltose oligosaccharides by protein QUPC 52 was not associated with a change in fluorescence.

The fructosan-specific myeloma proteins W3082 and UPC 61 (55) were indistinguishable and had Q_{max} values from 6 to 9% with the tetrasaccharide 3F1G, 5 to 7% with the trisaccharide 2F1G, and 10 to 13% with sucrose. Both proteins had association constants of 3.6×10^5 M^{-1} with 3F1G and 2F1G and 6.3×10^2 M^{-1} with sucrose (Fig. 4 B and Table II). Thus, 50% of the total binding energy was directed against the nonreducing, β-linked, fructosyl unit of sucrose which represents the immunodominant group. The oligosaccharides 2F1G and 3F1G present two and three fructosyl units respectively in β (2 → 1) linkage plus an additional β linkage and were similar, although earlier studies (55) showed that 3F1G was slightly more active than 2F1G (Table II). In the fluorescence data this small difference would be expected to be within experimental error.

Purified human antidextrans were then assayed for changes in fluorescence associated with the binding of IM5 and IM7 and fluorescence enhancement was found with all fractions tested. Q_{max} values for 1 $D_{54\text{-}60}$ IM3El 1 and El 2-3 were between 9 and 11%; however, with 1 $D_{54\text{-}60}$ IM6El 1-6, Q_{max} was only 4% and thus differed significantly from the IM3 eluates. Fractions II through VI from isoelectric focusing of 1 $D_{54\text{-}60}$ IM3El 2-3 (Fig. 1 B and 1 C) were then assayed and Q_{max} values for fluorescence enhancement were 6, 5, 8, 17, and 17% respectively. Thus, there was a significant difference between fractions II, III, and IV compared with fractions V and VI.

Quantitative Inhibition Assays with Myeloma Proteins and Human Antidextrans. The binding of oligosaccharides by human antidextrans and the mouse myeloma proteins was compared further by quantitative inhibition assays. The dashed inhibition curves in Fig. 5 correspond to a hypothetical inhibitor with K_o = 1 M^{-1} (ΔF° = 0 cal/mol) and their positions were calculated from binding constants obtained from equilibrium dialysis (cf. Fig. 2). With the IM3 and IM6 eluates of 1 $D_{54\text{-}60}$ (Fig. 5 A and B, respectively), approximately 20% of the binding energy for IM7 is for methyl α D glucoside while the corresponding values for the myeloma proteins are 56% for W3129 (Fig. 5 C) and close to 0% for QUPC 52 (Fig. 5 D). Large amounts of methyl αDglucoside (> 10% wt/vol) were required to inhibit precipitation of the human antidextrans and some inhibition could have been nonspecific. Indeed, the relatively steep slopes of these inhibition curves and the finding that fructose gave inhibition in the same range (Fig. 5 A and B) suggest that methyl αDglucoside may actually be bound by the human antidextrans with less than 20% of the total binding energy. Isomaltose was a much more effective inhibitor of the human antidextrans than was methyl αDglucoside and the disaccharide was bound by the IM3 and IM6 eluates with

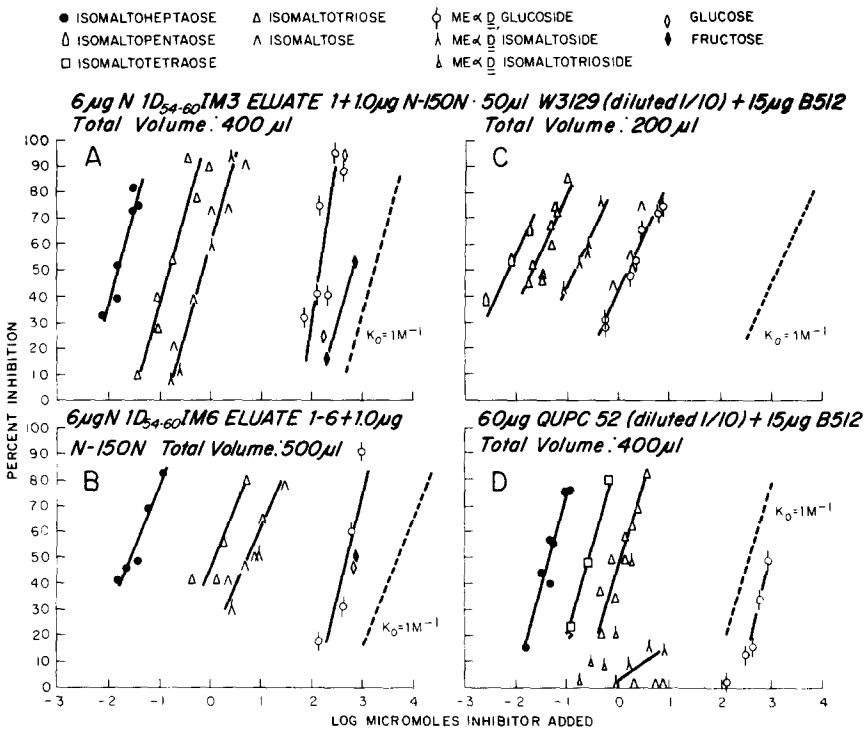

FIG. 5. Inhibition by various oligosaccharides of precipitation of human antidextrans 1 D_{54-60} IM3 eluate 1 (A) and 1 D_{54-60} IM6 eluate 1-6 (B) and mouse myeloma proteins W3129 (C), and QUPC 52 (D) by dextran.

67 and 56% of their total binding energies, respectively. By contrast, IM2 and methyl αDglucoside were similar with proteins W3129 and QUPC 52. Methyl α-IM2 was then assayed to determine if closing the reducing glucose in IM2 could increase its molar inhibitory power to that of IM3. With the human antidextrans, methyl α-IM2 and IM2 gave the same amount of inhibition on a molar basis, but with protein W3129, methyl α-IM2 was intermediate between IM2 and IM3. It was not possible to use large enough amounts of methyl α-IM2 to reach 50% inhibition with protein QUPC 52 but the results indicated that methyl α-IM2 was much less active than IM3. The binding of IM3 contributed 80% of the total binding energy with the 1 D_{54-60} IM3 antidextrans, 69% with the 1 D_{54-60} IM6 antidextrans, 94% with protein W3129, and 64% with protein QUPC 52. Methyl α-IM3 and IM3 had similar molar inhibitory powers with protein QUPC 52 and both were less active than IM4. With the myeloma proteins the conclusions from inhibition studies were in good agreement with those from equilibrium dialysis and fluorescence titrations (Table II). Although the IM3 and IM6 eluates of 1 D_{54-60} are heterogeneous, it seems likely that these antibodies differ from either myeloma protein in that the greatest increase in binding occurs between methyl αDglucoside and IM2.

Quantitative Precipitin Studies with Branched and Linear Dextrans. Quantitative precipitin reactions for proteins W3129 and QUPC 52 with branched dextrans B512 (mol wt $> 10^6$) and N-150N (mol wt $\simeq$ 60,000) and with linear

dextran D3 (mol wt ≃ 36,500) were then determined. Protein W3129, containing monomeric and polymeric IgA (Fig. 6, I *a*) and a polymer-rich fraction of W3129 (Fig. 6, I *b*) reacted well with the branched dextrans but did not precipitate with linear dextran D3. Moreover, quantitative inhibition assays indicated that dextran D3 and IM5 were similar on a molar basis, in their abilities to inhibit precipitation of W3129 by native dextran B512. Protein QUPC 52 containing monomeric and polymeric IgA precipitated best with dextran B512 and less but equally with the low molecular weight branched dextran, N-150N and linear dextran D3 (Fig. 6, I *c*). After separation of QUPC 52 into polymeric and monomeric forms, the polymer-rich fraction precipitated almost as well with dextrans N-150N and D3 as with native dextran B512 (Fig. 6, I *d*). The monomer-rich fraction of QUPC 52 gave significant precipitation with native

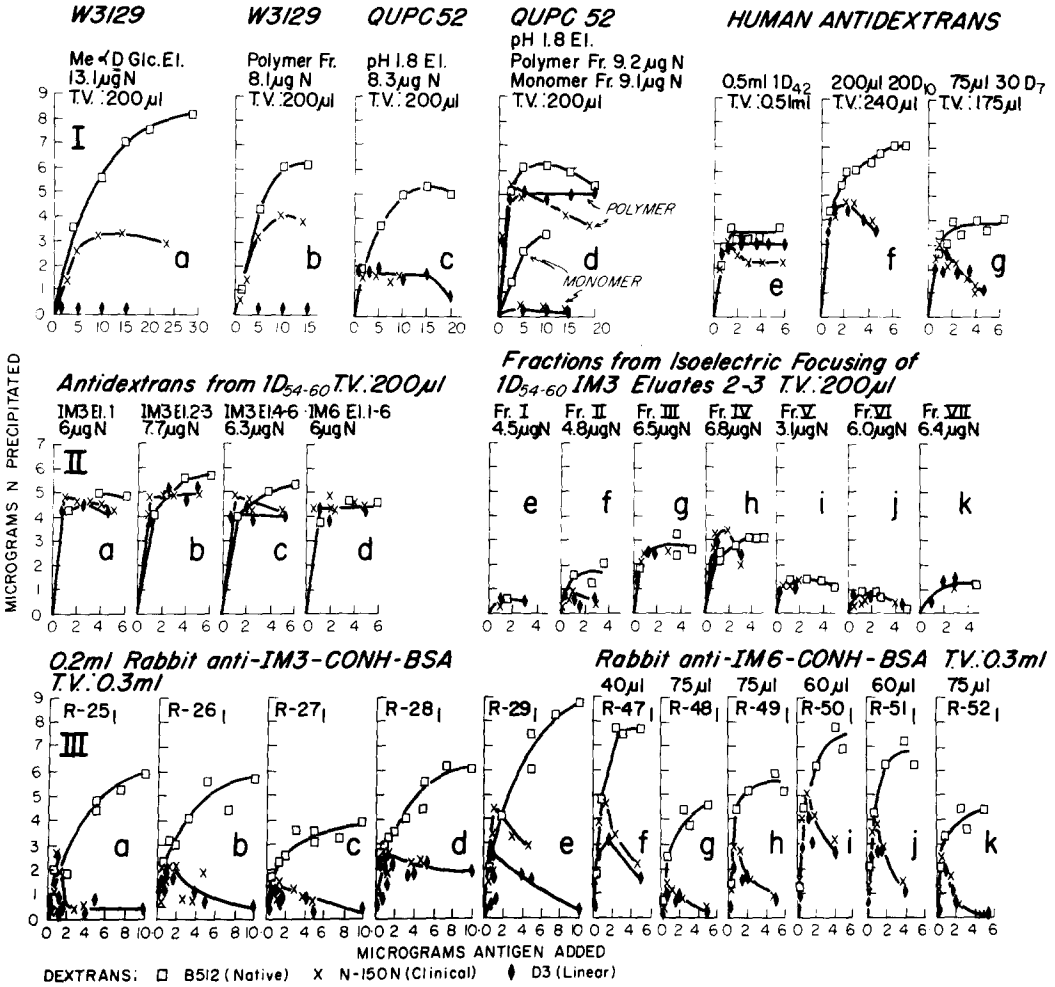

FIG. 6. Quantitative precipitin curves with native, clinical and linear dextrans and mouse myeloma proteins (I *a* to *d*), human antidextrans (I *e* to *g*), purified antidextrans from 1 D_{54-60} (II *a* to *d*), fractions from isoelectric focusing of 1 D_{54-60} IM3 eluate 2-3 (II *e* to *k*), and rabbit antisera against IM3-CONH-BSA (III *a* to *e*) and IM6-CONH-BSA (III *f* to *k*).

dextran B512 but almost no precipitation with dextrans N-150N and D3, respectively (Fig. 6, I *d*). Thus, with protein QUPC 52, as was shown for protein W3129 (55), the decreased precipitation of myeloma protein by clinical as compared with native dextran results from the monovalent reaction of IgA monomers with dextran molecules. Monovalent binding of monomers inhibits precipitation of polymeric IgA more so with low molecular weight dextrans than with high molecular weight dextrans which contain a greater number of antigenic determinants per molecule (37, 61 cf. 4).

With individual human antidextrans the fraction of antibody precipitated by linear dextran D3 compared with that precipitated by native dextran B512 was at least 90% for 1 D_{42} (Fig. 6, I *e*), 66% for 20 D_{10} (Fig. 6, I *f*), 71% for 30 D_7 (Fig. 6, I *g*), and 73, 80, and 48% for 116 D_1, 176 D_4, and 219 D_3 respectively (not shown). In addition 20 D_{10} serum, after absorption with linear dextran D_3, could still precipitate branched dextran B512. With the IM3 and IM6 eluted antidextrans from 1 $D_{54\text{-}60}$ (Fig. 6, II *a* to *d*), the data suggest that the IM3 eluates but not the IM6 eluates precipitate somewhat better with B512 than with D3 although the differences are slight. The fractions from isoelectric focusing of 1 $D_{54\text{-}60}$ IM3 eluates 2–3 were also studied (Fig. 6, II *e* to *k*), and with the possible exception of Fr. II, all fractions precipitated the same with dextrans B512 and D3. Unfractionated 1 $D_{54\text{-}60}$ IM3El 2–3 (Fig. 6, II *b*) was 88% precipitable with dextran B512 while the fractions from isoelectric focusing were less than 50% precipitable. No attempt was made to determine the activity of recombined fractions. Thus, the antidextrans from subject 1 react almost equally with linear and branched dextran, but with subjects 20, 30, 116, 176, and 219 there are clear differences in the amounts of antibody precipitated by linear dextran D3 and native dextran B512.

Five rabbit antisera (R-25_1 to R-29_1, Fig. 6, III *a* to *e*) against IM3-CONH-BSA were studied and the fractions of antibody precipitated by dextran D3 compared with dextran B512 were 38, 33, 37, 38, and 28% respectively. With six rabbit antisera against IM6-CONH-BSA (R-47_1 to R-52_1, Fig. 6, III *f* to *k*) the corresponding fractions of antibody precipitated by dextran D3 were 39, 28, 44, 59, 44, and 52% respectively. Clinical dextran N-150N and linear dextran D3 reacted in a similar fashion with the various rabbit antisera.

Discussion

The present findings demonstrate a new parameter in the immunochemistry of homopolysaccharide antigens. Binding data for myeloma proteins W3129 and QUPC 52 show clearly that protein W3129 reacts only at terminal nonreducing ends of dextran chains, and that protein QUPC 52 can react at nonterminal locations along linear α $(1 \rightarrow 6)$-linked dextran chains. An understanding of how these different reactivities manifest themselves in immunochemical data should aid greatly in understanding the immunochemistry of dextrans. It strongly supports the concept that antidextrans induced by immunization are generally mixtures of molecules, some with terminal and others with nonterminal specificities. This principle may apply to antibodies against other homopolysaccharide antigens including levans, and may hold equally well with heteropolysaccharides.

Previous precipitin inhibition data (55) suggested that myeloma proteins W3129 and QUPC 52 differed in the extents of their complementary regions for isomaltose oligosaccharides, with combining sites for five and six glucose residues respectively. These interpretations were based on the assumptions that terminal nonreducing ends of isomaltose oligosaccharides were immunodominant for both proteins, and that both had binding constants for IM6 of about 10^5 M^{-1} (ΔF° = −7,000 cal/mol). The present results show these assumptions are correct for protein W3129 but not for protein QUPC 52. With protein W3129 equilibrium dialysis and fluorescence titrations establish that maximum complementarity is for IM5 and that 55–60% of the total binding energy is for the terminal nonreducing glucose as represented by methyl α-D-glucoside (Table II). Isomaltose and methyl α D-glucoside were equally active on a molar basis, although previous results (55) had indicated that the inhibitory power of IM2 was intermediate between methyl α-D-glucoside and IM3. The present findings (Table II) are correct as earlier data (55) with IM2 were obtained with a sample found to contain a small amount of IM3 as contaminant. Equilibrium dialysis experiments with protein QUPC 52 showed that its binding constant for IM7 and IM6 is only 8.4×10^3 M^{-1} and both dialysis displacement experiments and quantitative precipitin inhibition data (Table II) indicate that about 70% of the total binding energy is for IM3 but almost none is for IM2 or methyl α-D-glucoside. Thus, the terminal nonreducing glucose is not the immunodominant structure. These findings suggest a terminal nonreducing specificity for protein W3129 and a nonterminal specificity for protein QUPC 52. Quantitative precipitin reactions with the polymer fractions of both proteins (Fig. 6, I *b* and I *d*) and linear dextran D3 strongly support this surprising difference in reactivity. The high binding affinity of protein W3129 for terminal nonreducing ends of isomaltose oligosaccharides along with its inability to precipitate linear dextran D3 serves as an essential control and establishes that linear dextran D3 reacts immunochemically as a population of molecules each having a single nonreducing end.

The above findings and those with concanavalin A (51) make it almost certain that precipitation of linear dextran is a valid assay for antidextrans which can react with α (1 → 6)-linked nonterminal determinants. For heterogeneous antidextrans composed of populations with terminal and nonterminal specificities, linear dextran molecules would be cross-linked by antibodies having nonterminal specificities, and would provide an insoluble matrix for bringing down some antibody with terminal specificity. Thus, the difference in antibody N precipitated from an antiserum by a branched dextran vs. a linear dextran should reflect the presence of antibodies with terminal specificities for dextran chains, but it would not provide a quantitative estimate.

From the above reasoning, quantitative precipitin reactions indicate that human antidextrans from different individuals can differ in their relative contents of antibodies having terminal and nonterminal specificities. The antidextrans from subject 1 appear to contain the largest fraction of molecules with nonterminal specificity as linear dextran D3 and branched native dextran B512 precipitate about the same amount of antibody N (Fig. 6, I *e*, II *a* to II *k*). Likewise, the antidextrans from subjects 20 (Fig. 6, I *f*) and 219 seem to have large fractions of molecules with terminal specificities as the differences in

precipitation by branched native dextran compared to linear dextran are the greatest. Rabbits immunized with IM3 or IM6 coupled to BSA also make antibodies which precipitate linear dextran (Fig. 6, III *a* to III *k*); however, there is a relatively great difference in the amounts of antibody precipitated by dextran B512 and linear dextran D3 indicating that these sera contain sizeable fractions of antibody having nonreducing terminal specificity for the isomaltosyl chains. The finding that a fraction of antibodies induced by immunization with IM3-CONH-BSA could react at nonterminal locations along dextran chains suggests as a possibility that nonreducing terminal determinants might trigger certain clones which synthesize antibodies having sites with high binding affinities for terminal glucosyl residues, but which can also cross-react at nonterminal locations along linear dextran chains. This seems reasonable as the only distinguishing feature between terminal and nonterminal nonreducing glucosyl residues would be the hydrogen atom on the number six hydroxyl group of the terminal nonreducing glucose. This poses the question of why certain combining sites, like that of protein W3129, react only with terminal determinants and do not cross-react at nonterminal locations. The most obvious explanation would be to imagine a combining site with terminal specificity as having binding energy for isomaltosyl structures distributed in such a manner that the terminal nonreducing glucose residue is held in a fixed location within a partially enclosed space. This model would attribute terminal specificity to the shape of the combining site and perhaps also to specific binding energy for unique structural features of the terminal nonreducing sugar residue. A site with nonterminal reactivity can be imagined as a groove or depression with binding energy distributed in such a way that the terminal end of the determinant is not held within a space into which only it can fit; sites with a relatively linear groove or depression would be expected to bind less strongly as is observed for QUPC 52. The existence of rabbit anti-IM3-CONH-BSA antibodies which precipitate linear dextran D3 might also be accounted for if structures as short as [α DGlc-(1 $\rightarrow$ 6) α DGlc-$OCH_2(CHOH)_4$-CONH-] are capable of presenting antigenic determinants with immunodominant groups which do not involve the entire terminal nonreducing glucosyl residue.

An unexpected finding with the human antidextrans (Fig. 6, I *e* to *g*) and rabbit antisera against IM3 and IM6 coupled to BSA (Fig. 6, III *a* to *k*) was that clinical dextran N-150N reacted more like linear dextran D3 than did native dextran B512 which has more branches. By contrast, clinical dextran N-150N but not linear dextran D3 was precipitated by protein W3129 (Fig. 6, I *a* and I *b*). An explanation for this apparent discrepancy emerges from the structures of these dextrans. Clinical dextran N-150N has a mol wt of about 60,000 and comes from mild acid hydrolysis of native dextran B512 which has a molecular weight in the millions (61, cf. 4). Both dextrans have about 4% branching due to α (1 $\rightarrow$ 3) linkages. The lengths of side chains for dextran B512 have been studied (80) and about 40% are single glucosyl residues in α (1 $\rightarrow$ 3) linkage to the α (1 $\rightarrow$ 6) linked backbone, while at least 45% are only two glucosyl residues long [αDGlc (1 $\rightarrow$ 6) αDGlc (1 $\rightarrow$ 3)-]. These short chains may not function as antigenic determinants for combining sites with terminal specificity for five or six glucosyl residues in α (1 $\rightarrow$ 6) linkage. A maximum of 15% of the side chains are thought to be longer

than two glucoses and in the present discussion these will be considered as nonreducing terminal determinants. Thus, an average molecule of dextran B512 with mol wt of 10×10^6 would contain 60,000 glucosyl residues, 2,400 branch points, and about 360 side chains which could function as terminal determinants. Assuming that clinical dextran has about the same side chain distribution as dextran B512, then an average molecule of dextran N-150N (mol wt $\simeq$ 60,000) would be composed of 400 glucosyl residues, 16 branch points with a maximum of but two or three which could function as terminal determinants. Thus, dextran N-150N would have an abundance of nonterminal determinants but would seem to be deficient in terminal determinants. Moreover, antibodies with terminal specificity could be blocked from reacting with dextran N-150N by antibodies having nonterminal specificity but which react near the termini; the reverse would not be true. Accordingly, the quantitative precipitin reactions of heterogeneous antibodies with clinical dextran N-150N would appear to be more like those of linear dextran than those of a branched native dextran and the findings (61, 62, 67) that antidextrans from different individuals differ in the percentages of antibody precipitated by low molecular weight compared with native dextrans would appear to be related to their contents of antibodies with terminal specificities. Moreover, a fraction of antibody from Type II antipneumococcal serum cross-reacts with dextrans and, as with human antidextrans, the cross-reactive fraction gives reduced precipitation with low molecular weight dextrans (11). This suggests that cross-reactions between Type II antipneumococcal sera and dextran might occur at both terminal and nonterminal locations. Finally, the conclusion (48) that rabbit antisera against a B512 dextran of mol wt 4,400 lacked antibodies with terminal specificities may require modification as it was based only on the similarity of reactions between a linear dextran and a clinical dextran (mol wt $\simeq$ 40,000). With a homogeneous myeloma protein like W3129 which has 50 to 60% of its binding energy directed against the terminal nonreducing glucose, the limited branching of N-150N proved adequate for precipitation.

It is significant that the IM3 and IM6 eluates of antidextran 20 D_3 differed in the fraction of total antibody N which was precipitated by low molecular weight dextrans (63). With 20 D_3-IM3Ab, dextran fraction NRC 1 (mol wt $\simeq$ 10,600) precipitated about half the antibody N which was precipitable with dextran fraction NRC 7 (mol wt $\simeq$ 225,000), but with 20 D_3-IM6 Ab the values were nearly the same. Thus, in addition to separating antidextrans into populations which differ in the extent of their complementary regions for isomaltose oligosaccharides (36, 63, 64, 65, 68, cf. 4, 5), sequential elution with IM3 and IM6 might also tend to fractionate antidextrans into populations which are enriched with antibodies having terminal and nonterminal specificities respectively. Indeed, with mixtures of antibody populations having binding properties similar to those of proteins W3129 or QUPC 52 (Table II), IM3 would be expected preferentially to elute molecules like W3129 from Sephadex G75 and leave molecules like QUPC 52 for elution with IM6.

Antigenic determinants for α (1 $\rightarrow$ 6)-specific human antidextrans can involve as many as six and perhaps seven glucosyl residues and the nonreducing end has been thought to be immunodominant, thus making the greatest individual contribution to binding and specificity (36, 39, 44, 45 cf. 4, 5). The combining site

of protein W3129 is of this type and has the terminal nonreducing α-linked glucosyl residue as its immunodominant group. This would be bound at one end of the combining site, and IM5, which is the shortest oligosaccharide showing maximum binding (Table II), serves to measure the extent of the complementary region. The immunodominant group for protein QUPC 52 is not expressed by structures smaller than IM3 and could require contributions from a nonterminal residue, portions of both adjacent pyranose rings and the adjoining α (1 $\rightarrow$ 6) linkages. Moreover, for combining sites with nonterminal specificity the subsite which binds the immunodominant group could occur at one end of the groove or in the middle. Isomaltose oligosaccharides larger than IM3 but smaller than the entire antigenic determinant, which could form the immunodominant group in more than one way, would be expected to occupy positions within the site which result in maximum binding energies. Thus, the findings that IM5 is slightly less active than IM6 which has the same binding constant as IM7 (Table II) suggest that maximum complementarity exists for five or six glucosyl residues; however, parts of both terminal nonreducing and reducing glucoses may not participate in binding. If the QUPC 52 site were smaller there would be many ways for haptens to enter as the chain got longer and hence the increment of increase in binding activity should not decrease progressively with increasing chain length. The immunodominant group for the heterogeneous antidextrans from subject 1 appears to involve two adjacent glucosyl residues and the adjoining α (1 $\rightarrow$ 6) linkage. This interpretation is favored by the findings that these antidextrans have mainly nonterminal specificity and that the ΔF° for IM2 is approximately $-4{,}000$ to $-4{,}500$ cal/mol while methyl αDglucoside binds with only about $-1{,}400$ cal/mol (Table II). Moreover, as pointed out earlier, the estimated binding energy for methyl α Dglucoside may be excessive. Thus, different structures in a chain of α (1 $\rightarrow$ 6)-linked glucosyl units can be recognized as the immunodominant group and some appear to require contributions from more than a single residue.

The present findings aid in the interpretation of earlier quantitative precipitin reactions with various dextrans (55). Protein W3129 reacted equally with dextrans rich in α (1 $\rightarrow$ 6) linkages and with those having high contents of α (1 $\rightarrow$ 4)-like and α (1 $\rightarrow$ 6) linkages while dextrans with high contents of α (1 $\rightarrow$ 3)-like and α (1 $\rightarrow$ 6) linkages were somewhat less efficient at precipitating. Protein QUPC 52 reacted best with the α (1 $\rightarrow$ 6)-linked dextrans but poorly with those having relatively high contents of α (1 $\rightarrow$ 4)-like and α (1 $\rightarrow$ 3)-like linkages in addition to α (1 $\rightarrow$ 6) linkages. The greater reactivity of protein W3129 over protein QUPC 52 with dextrans high in non α (1 $\rightarrow$ 6) linkages would be accounted for if many branches initiated by non α (1 $\rightarrow$6) linkages were shorter than three α (1 $\rightarrow$ 6)-linked glucosyl residues. These probably could provide determinants for protein W3129 but with protein QUPC 52 short branches would decrease the reactivity of the internal α (1 $\rightarrow$ 6)-linked backbone. This is illustrated with dextran B1299-S-3, which contains 50% α (1 $\rightarrow$ 4)-like linkages as determined from periodate oxidation (81) and which was also found to precipitate better than expected with various α (1 $\rightarrow$ 6)-specific human antidextrans (37 cf. 4). Methylation data (82) with this dextran indicated a high degree of branching by α (1 $\rightarrow$ 2) linkages with one-third of the αDglucopyranosyl residues existing at branch points, one-third as nonreducing end groups, and the remainder as nonterminal residues in glycosidic linkage through carbons 1 and 6 (26%) or 1 and 3 (7%). Protein W3129, which requires a single terminal nonreducing α-linked glucosyl residue for its immunodominant

group, precipitates as well with dextran B1299-S-3 as with branched dextrans high in α (1 $\rightarrow$ 6) linkages (55). The α (1 $\rightarrow$ 6)-specific antidextrans from subject 1, for which two glucosyl residues are thought to form the immunodominant structure, give quantitative precipitin reactions with dextran B1299-S-3 which are intermediate between those of dextrans containing greater than 70% α (1 $\rightarrow$ 6) linkages and that of dextran B1355-S-4 which has a high content of α (1 $\rightarrow$ 3)-like linkages and reacts poorly (37 cf. 4). Protein QUPC 52, which requires three glucosyl residues to form its immunodominant group, reacts poorly and the same with dextrans B1299-S-3 and B1355-S-4 (55). Thus, the abilities of these proteins to precipitate the highly branched dextran B1299-S-3 are correlated with the number of glucosyl residues needed to form the respective immunodominant structures. This suggests that the sites of protein W3129, the human antidextrans, and possibly protein QUPC 52 are able to bind dextran molecules at branches that form the proper immunodominant structures but which may be shorter than entire antigenic determinants of five or six sugar residues. Studies with α (1 $\rightarrow$ 6)-specific human antidextrans also have identified dextran B742L-R with 81% α (1 $\rightarrow$ 6) linkages, as behaving anomalously in that it failed to precipitate as well as other dextrans having greater than 70% α (1 $\rightarrow$ 6) linkages (37 cf. 4). This was suggested to result from short branches occurring near nonreducing termini (37, 42 cf. 4). The findings that dextran B742L-R displayed less than expected reactivity with myeloma proteins W3129 and QUPC 52 (55) would require that short branches of perhaps single glucosyl residues also occur along interior α (1 $\rightarrow$ 6)-linked chains. An alternative possibility would be the occurrence of interior α (1 $\rightarrow$ 4)-like linkages which do not form branch points but instead interrupt α (1 $\rightarrow$ 6)-linked chains so as to limit nonterminal binding without increasing the number of terminal nonreducing determinants. Similarly, with both myeloma proteins (55) and various α (1 $\rightarrow$ 6)-specific human antidextrans (37 cf. 4) the low reactivity of dextran B1355-S-4 could result in part from internal α (1 $\rightarrow$ 3)-linked residues which are not involved in branching (83).

Certain data with fructosan-reactive myeloma proteins (54, 55) and human antilevans (55, 84) are consistent with terminal and nonterminal specificities for levan chains. Proteins W3082 and UPC 61 which are probably identical and different from protein J606 (54, 55, 60) all have been shown to have β (2 $\rightarrow$ 1) specificity but are thought to cross-react at the nonreducing ends of β (2 $\rightarrow$ 6)-linked fructosyl chains in levan (55). The two identical proteins have at least 50% of their binding energy for terminal nonreducing β-linked fructose as shown from fluorescence titrations with sucrose (Table II). This estimate might have been higher if methyl βD-fructoside were available as the nonreducing glucose in sucrose may have interfered with binding. Also, all three proteins give virtually no precipitation with rye grass levan (54, 55) which is composed of about 25–30 fructose residues (85) joined almost entirely by β (2 $\rightarrow$ 6) linkages (85, 86) and would thus be nearly linear. Proteins UPC 10 and Y5476 are thought to have β (2 $\rightarrow$ 6) specificity because of their reactions with levans and not with inulin which is β (2 $\rightarrow$ 1)-linked (55). These proteins precipitate well with rye grass levan and this would be most easily accounted for if they react at nonterminal locations on the β (2 $\rightarrow$ 6)-linked chain. A fraction of the antilevan from subject 1 also precipitates with rye grass levan (55, 84) and this could result from precipitation of antilevans with nonterminal specificity leaving most of the antibodies with terminal specificity in the supernate. Large amounts (100 μmol) of sucrose (55), which would be expected to present a nonreducing β-linked fructose, do not inhibit precipitation by the β (2 $\rightarrow$ 6)-specific proteins which suggests that the immunodominant structures require a contribution from more than a single fructosyl residue.

Myeloma proteins which can be considered as having terminal specificities for α (1 $\rightarrow$ 6)-linked dextran chains (W3129), β (2 $\rightarrow$ 1)-linked fructosan chains (W3082 and UPC 61) (55) and β (1 $\rightarrow$ 6)-linked galactan chains (X-24 and J539)

(58, 59), all have binding constants slightly greater than 10^5 M^{-1} and are similar in the way binding energy is directed against successive sugar residues in their corresponding antigenic determinants (Table II). Especially striking is the finding that each protein has about −4,000 cal/mol for its terminal nonreducing sugar residue properly linked. Protein QUPC 52 which has a much lower binding constant than the other myeloma proteins and has nonterminal specificity also has about −4,000 cal/mol for IM3, the smallest oligosaccharide tested that could form the immunodominant structure. The significance of these similarities in binding is unclear but they could result from common mechanisms for the binding by myeloma proteins and antibodies as well as from architectural similarities among combining sites specific for various oligosaccharide determinants.

Crystallographic studies on the combining sites of myeloma proteins or antibodies specific for nonterminal and for terminal carbohydrate determinants, the architecture of the two types of combining sites, and the relative contributions of the shapes of the sites and of the contacting residues to the binding would contribute materially to our knowledge of antibody structure and specificity. The finding of myeloma antibodies with homogeneous binding sites of one or the other of these two kinds of sites supports the concept that they represent monoclonal proteins selected from a large population of lymphoid cells each synthesizing a specific antibody. It would be of considerable importance to develop methods for separating these two kinds of antibody and studying their isoelectric spectra and heterogeneity.

Summary

Binding constants of the dextran-reactive BALB/c mouse IgA myeloma proteins W3129 and QUPC 52 have been determined for each member of the isomaltose series of oligosaccharides and for methyl αDglucoside. Protein W3129 has maximum complementarity for isomaltopentaose (IM5) ($\Delta F°$ = −7,180 cal/mol) with 55–60% of the total binding energy directed against methyl αDglucoside. Protein QUPC 52 gives maximum binding with isomaltohexaose (IM6) ($\Delta F°$ = −5,340 cal/mol) and has about 70% of its total binding energy for isomaltotriose (IM3), but at most only 5% for isomaltose (IM2) or methyl αDglucoside. Protein W3129 precipitates with branched dextrans high in α (1 → 6) linkages and reacts with but does not precipitate a synthetic α (1 → 6)-linked linear dextran. Protein QUPC 52 precipitates both branched and linear dextrans. Thus, the immunodominant group for protein W3129 is mimicked by methyl αDglucoside and this protein reacts exclusively at the terminal nonreducing ends of α (1 → 6)-linked dextran chains. Protein QUPC 52 has an immunodominant group which is expressed by IM3 but not smaller oligosaccharides and this protein can react at nonterminal locations along α (1 → 6)-linked dextran chains. Precipitation of linear dextran seems to be a valid although not quantitative assay for antidextrans with nonterminal specificity. Quantitative precipitin reactions with branched and linear dextrans suggest that α (1 → 6)-specific human antidextrans are mixtures of molecules having terminal and nonterminal specificities and that the fraction of each type can vary among individuals. Rabbit antisera against IM3 or IM6 coupled to bovine serum albumin also

appear to contain antibodies with nonterminal specificity for dextran chains although a large fraction has terminal specificity. Low molecular weight clinical dextran N-150N ($\simeq$ 60,000) reacted more like linear dextran than like its parent native-branched dextran B512. This is thought to result from an abundance of nonterminal determinants in clinical dextran N-150N but a very small number of functional terminal determinants per molecule. An appreciation of terminal and nonterminal specificities and of the different immunodominant structures in isomaltosyl chains has proven to be of great value in understanding the immunochemical reactions of dextrans. Moreover, certain previous findings with fructosan-reactive mouse myeloma proteins and human antilevans (55, 84) also suggest terminal and nonterminal specificities for levan chains.

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