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PURIFICATION AND PROPERTIES OF ANTIBODIES HAVING SPECIFICITY FOR THE CARBOHYDRATE MOIETIES OF SYNTHETIC GLYCOCONJUGATES

JOHN H. PAZUR
Paul M. Althouse Laboratory, The Pennsylvania State University, University Park, PA 16802 (U.S.A.)
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ABSTRACT

Three types of antibodies having specificity for carbohydrate moieties of glycoconjugate antigens have been isolated from the sera of rabbits immunized with vaccines of β-D-glucosyl-bovine serum albumin, β-D-galactosyl-bovine serum albumin, or β-lactosyl-bovine serum albumin. The antibodies were isolated by affinity chromatography on adsorbents bearing appropriate carbohydrate ligands. The antibody types are specific for the β-D-glucosyl, β-D-galactosyl, or β-lactosyl groups of the synthetic glycoconjugates. The specificities have been established by data on hapten-inhibition, agar-diffusion, periodate-oxidation, and affinity-chromatography experiments. Each antibody type is of the IgG class of immunoglobulins and is of uniform molecular size, with molecular weight of 1.5×10^5. Data from gel-isoelectrofocusing experiments showed that each preparation of antibodies, although specific for identical determinant groups of the same antigen, nevertheless consisted of a set of different proteins. The anti-D-glucose antibodies consisted of five proteins, the anti-D-galactose antibodies of eleven proteins, and the anti-lactose antibodies of seventeen proteins. The suggestion has been made earlier that the members of such a set of antibodies, in which each member is induced by the same determinant group of an antigen and each member combines with this group in the precipitin reaction, be called isoantibodies.

INTRODUCTION

Antibodies having specificity for carbohydrate moieties of antigens of polysaccharide or glycoprotein structures were first detected by Avery and coworkers[1,2] in the sera of rabbits immunized with natural or synthetic antigens containing carbohydrate groups. Such anti-carbohydrate antibodies have been used extensively in studies on the nature of antibody combining-sites[3], the mechanism of antibody formation[4], and the identification of microorganisms by serological methods[5]. With the advent of affinity chromatography[6,7] it has become possible to isolate and to purify anti-carbohydrate antibodies by adsorption and elution from adsorbents with appropriate carbohydrate ligands. In the present study, three types of anti-carbo-

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hydrate antibodies have been isolated pure from sera of rabbits immunized with vaccines of synthetic glycoconjugates. The glycoconjugates contained D-glucose, D-galactose, or lactose groups and were prepared by coupling appropriate derivatives of these sugars with bovine serum albumin by procedures described previously[8].

Standard immunological methods were used to immunize a group of rabbits, initially subcutaneously and later intravenously, with vaccines of the synthetic glycoconjugates. Anti-carbohydrate antibodies were isolated from the immune sera by affinity chromatography of the sera on adsorbents bearing D-glucose, D-galactose, or lactose ligands. The antibodies having specificity for β-D-glucosyl groups were obtained from the β-D-glucosyl-Sepharose adsorbent and are called anti-D-glucose (anti-Glc) antibodies. The antibodies of the other two types exhibited specificity for β-D-galactosyl or β-lactosyl groups, were obtained from the β-D-galactosyl-Sepharose and β-lactosyl-Sepharose adsorbents, respectively, and are called anti-D-galactose (anti-Gal) antibodies and anti-lactose (anti-Lac) antibodies.

The specificities of the three types of antibodies were established by results of agar-diffusion, hapten-inhibition, and periodate-oxidation experiments. The molecular nature of the antibodies was revealed by isoelectrofocusing and ultracentrifugation. Centrifugation showed that the antibody preparations were of uniform size and molecular weight (1.5×10^5), but the isoelectrofocusing showed that each preparation of antibodies consisted of multiple protein components. The anti-Glc antibodies consisted of five isomeric proteins, the anti-Gal antibodies of eleven different isomeric proteins, and the anti-Lac antibodies of seventeen different isomeric proteins. That all of the protein components of each set reacted with the corresponding antigen was demonstrated by a coupled assay-procedure utilizing isoelectrofocusing and agar diffusion. Sets of antibodies, in which each member of the set had been induced by the identical determinant group of an antigen and in which each member reacts with the same determinant group, constitute a unique group of antibodies. Antibody preparations of this type differ from preparations in which the individual members react with different determinants of the same antigen[9,10]. In order to differentiate the two types of antibodies, the term isoantibodies has been proposed for antibodies of the first type[11].

RESULTS AND DISCUSSION

Some structural features of the three synthetic glycoconjugates are shown in the formula in Fig. 1. Two of the glycoconjugates contain monosaccharide groups, D-glucose or D-galactose, whereas the third contains a disaccharide group, lactose. The carbohydrate groups are attached first to the hydroxyl group of 9-hydroxynonanoic acid by β-glycosidic bonds and then the carboxyl group is attached by amide bonds to the amino groups of lysyl residues in bovine serum albumin. Analyses of the glycoconjugates for carbohydrate[12] showed that the glycoconjugates contained about 5% carbohydrate, and one third of the lysyl residues in the bovine serum albumin had carbohydrate moieties attached. These synthetic glycoconjugates were

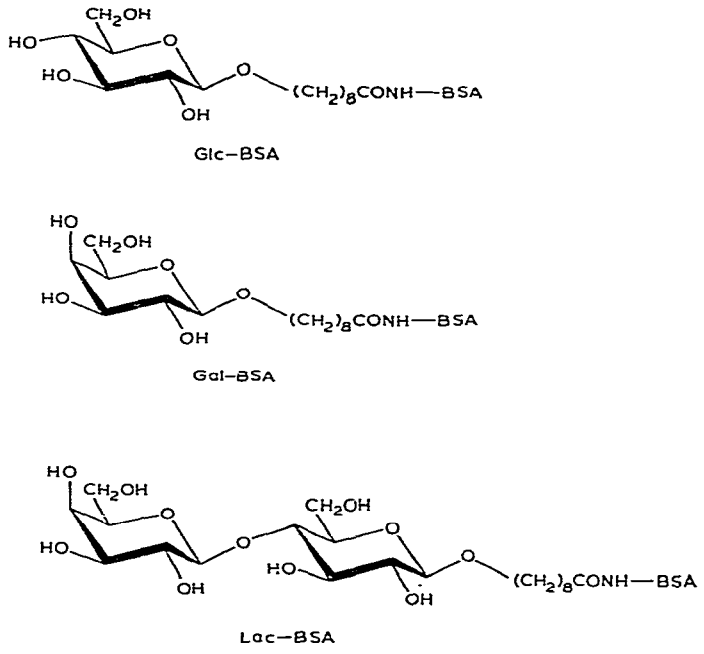

Fig. 1. Structural features of the synthetic glycoconjugates: β-D-glucosyl-bovine serum albumin (Glc-BSA), β-D-galactosyl-bovine serum albumin (Gal-BSA), and β-lactosyl-bovine serum albumin (Lac-BSA).

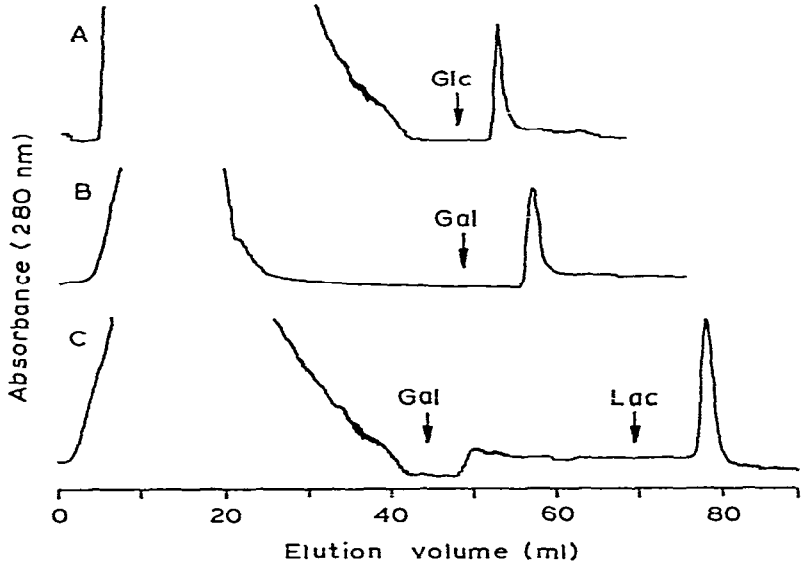

Fig. 2. Elution patterns for anti-D-glucosyl-BSA serum from D-glucosyl-Sepharose (A), for anti-D-galactosyl-BSA serum from D-galactosyl-Sepharose (B), and for anti-lactosyl-BSA serum from lactosyl-Sepharose (C). The arrows indicate the point of elution with D-glucose (Glc), D-galactose (Gal), and lactose (Lac).

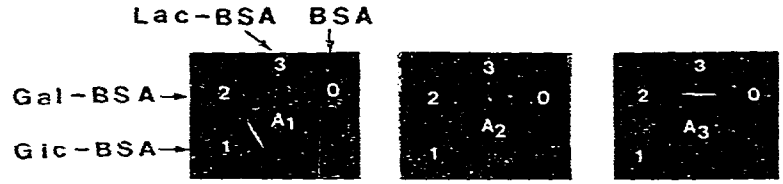

Fig. 3. Agar-diffusion patterns for the anti-carbohydrate antibodies and the synthetic glycoconjugates: anti-D-glucose antibodies (A_1), anti-D-galactose antibodies (A_2), and anti-lactose antibodies (A_3). Abbreviations as in Fig. 1.

used to immunize rabbits by the methods described in an earlier publication[13]. The antibodies in the sera from the rabbits were isolated and purified by affinity chromatography on adsorbents containing the appropriate glycosyl ligands[14]. The elution patterns for the three types of sera from adsorbents with D-glucose, D-galactose, or lactose ligands are reproduced in Fig. 2. It may be noted in Fig. 2 that u.v.-absorbing substances were obtained by elution of the columns with buffer and with solutions of carbohydrate. The first peak of u.v.-absorbing material, which was eluted with the buffer from each column, contained unadsorbed proteins. The second peak, which was eluted with the carbohydrate solutions, contained the antibodies, anti-Glc antibodies, anti-Gal antibodies, and anti-Lac antibodies. It may be seen that u.v.-absorbing substances were not eluted from the lactosyl-Sepharose with the solution of D-galactose. The antibody fractions were collected, dialyzed, and concentrated to low volume. The resulting solutions contained from 0.5–1% protein and were used in the subsequent experiments.

Fig. 3 shows results of agar-diffusion tests with the synthetic glycoconjugates (Glc-BSA, Gal-BSA, and Lac-BSA) and bovine serum albumin (BSA) with the three types of antibody preparation. It may be noted that the anti-Glc antibodies (Well A_1) reacted with the Glc-BSA (Well 1), but not with the other two glycoconjugates (Well 2 or Well 3) nor with BSA (Well 0). The anti-Gal antibodies (Well A2) reacted only with the Gal-BSA (Well 2) and the anti-Lac antibodies only with the Lac-BSA (Well 3). The latter two antibody preparations did not react with the BSA (Well 0) nor with the other two glycoconjugates. As a reaction did not occur with BSA and the antibodies, the antibodies are directed at the carbohydrate groups of the glycoconjugates and not at peptide portions of the bovine serum albumin.

In order to verify that the carbohydrate groups are essential for precipitin formation, samples of Gal-BSA and BSA were treated with periodate under standard conditions of periodate oxidation, and then the products were used in agar-diffusion tests. The agar-diffusion patterns for the native and the treated samples, with anti-BSA serum and with anti-Gal antibodies, are shown in Fig. 4. It may be noted that the Gal-BSA sample treated with periodate (Well 4) no longer yielded a precipitin band with the anti-Gal antibodies (Well A_2), but the native Gal-BSA (Well 3) gave a positive reaction. The native BSA (Well 1) and periodate-treated BSA (Well 2) also did not show a reaction with the anti-Gal antibodies. However, the three substances

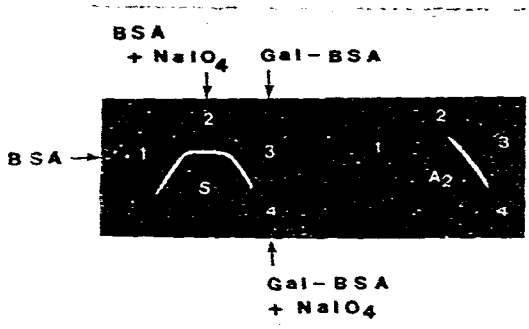

Fig. 4. Agar-diffusion patterns for anti-BSA serum (S) and anti-D-galactose antibodies (A_2) with native and periodate-treated bovine serum albumin and D-galactosyl-bovine serum albumin. Abbreviations as in Fig. 1.

(Wells 1, 2, and 4) and native Gal-BSA (Well 3) all reacted with the antibodies in anti-BSA serum (Well S). The structural groups of the synthetic glycoconjugate susceptible to periodate oxidation are the galactosyl groups. The loss of antigenicity on oxidation of the Gal-BSA results from modification of the carbohydrate groups of this substance. Therefore, the galactose groups are the immunodeterminant groups of the Gal-BSA glycoconjugate. The periodate treatment had no effect on the protein moiety of the synthetic glycoconjugate nor on the BSA, and these substances reacted with the anti-BSA serum.

Hapten-inhibition experiments were performed with the antibodies, with D-glucose, D-galactose, lactose, D-mannose, and melibiose as potential inhibitors. The results of these experiments are summarized in Table I. It may be noted in the Table that each antibody type was inhibited to the highest degree by the carbohydrate moieties that are structural units of the glycoconjugate used for immunization. Thus, D-glucose inhibited the reaction of Glc-BSA with anti-Glc antibodies, D-galactose inhibited the reaction of Gal-BSA with anti-Gal antibodies, and lactose inhibited the reaction of Lac-BSA with anti-Lac antibodies. Lactose was the most potent inhibitor of those tested and effected 96% inhibition of the precipitin reaction of the

TABLE I

PERCENT INHIBITION OF THE REACTION BETWEEN THE ANTIBODIES (50 μL) AND THE CORRESPONDING ANTIGEN (40 μg) BY CARBOHYDRATES (0.2mM)

Inhibitor	*Antibodies*		
	Anti-Glc	*Anti-Gal*	*Anti-Lac*
D-Glucose	58	6	5
D-Galactose	21	52	9
Lactose	35	19	96
D-Mannose	8	3	
Melibiose			5

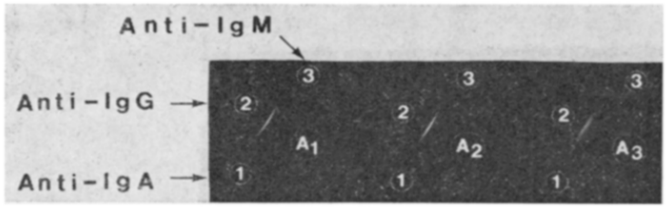

Fig. 5. Agar-diffusion patterns for the antibodies and anti-immunoglobulin sera.

Lac-BSA with anti-Lac antibodies. D-Galactose inhibited the reaction between Gal-BSA and the anti-Gal antibodies to the extent of 52%. Some inhibition of the system occurred with lactose, and this is probably because of the fact that lactose possesses terminal galactosyl groups, which may be expected to fit the combining site of the anti-Gal antibodies. D-Glucose inhibited the reaction of Glc-BSA with the anti-Glc antibodies to the extent of 58%, but lactose and D-galactose also inhibited the reaction of Glc-BSA with anti-Glc antibodies to the extent of 35 and 21%, respectively. The latter inhibitions were surprising and may indicate that the combining site of the anti-Glc antibodies is more flexible than the combining sites of the other antibodies. As a result, D-galactose and lactose can combine with the combining site of the anti-Glc antibodies and inhibition of the precipitin reaction with D-galactose and lactose does occur.

A number of other properties of the purified anti-carbohydrate antibodies have been determined. The immunoglobulin type was found by agar diffusion to be IgG for all three types of antibodies. The results of this experiment are shown in Fig. 5. It may be noted that all three types of antibodies (Wells A_1, A_2, and A_3) reacted only with the anti-IgG serum (Well 2) and not with anti-IgA serum (Well 1)

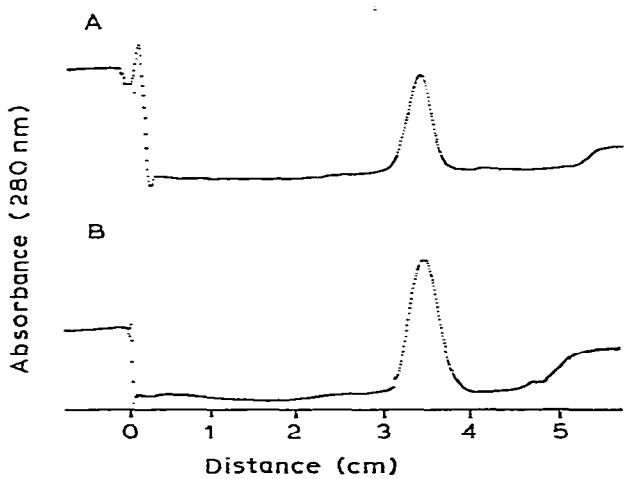

Fig. 6. Density-gradient centrifugation patterns for anti-D-galactose antibodies (A) and for D-glucose oxidase (B).

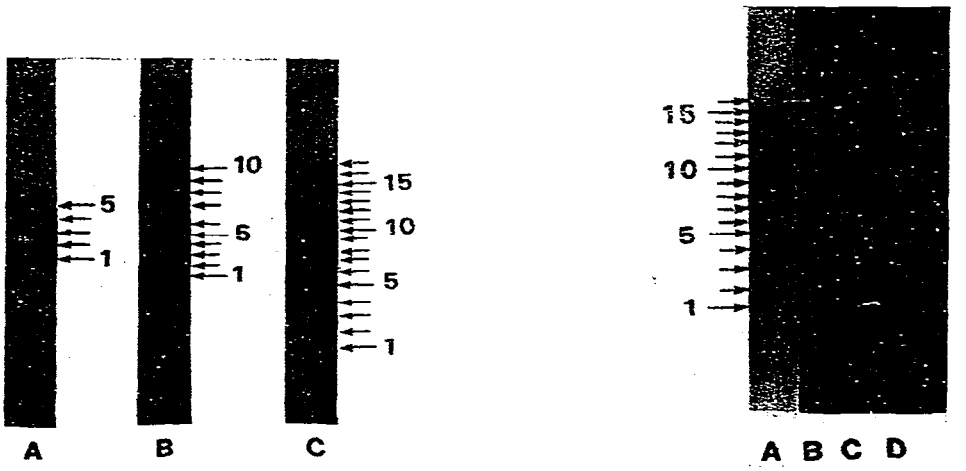

Fig. 7. Isoelectrofocusing patterns for the anti-carbohydrate antibodies: anti-D-glucose antibodies (A), anti-D-galactose antibodies (B), and anti-lactose antibodies (C).

Fig. 8. Isoelectrofocusing and agar diffusion of anti-lactose antibodies: gel stained with Coomassie Blue (A), gel not stained but embedded in agar (B), trough with lactosyl-bovine serum albumin (D), and precipitin band (C).

or anti-IgM serum (Well 3). Quite clearly, the three antibody preparations are immunoglobulins of the IgG class.

The sedimentation rates of the antibodies were determined by density-gradient ultracentrifugation[15]. The results with anti-Gal antibodies and a reference protein, D-glucose oxidase, are shown in Fig. 6. It may be seen that both the antibodies and D-glucose oxidase yielded symmetrical peaks on ultracentrifugation and that the sedimentation of the two substances occurred at approximately the same rates. It appears then that both of the preparations are composed of molecules of uniform size. Calculation from the sedimentation rates and an appropriate formula[16] yields a value of 1.5×10^5 for the molecular weight of the anti-Gal antibodies. Ultracentrifugation patterns obtained with the anti-Glc antibodies and the anti-Lac antibodies showed that the molecular weights of these antibodies were also 1.5×10^5.

Isoelectrofocusing studies on the antibody preparations yielded some surprising results. In view of the preparative methods used, it was anticipated that each antibody preparation would be homogeneous and be composed of a single molecular species. However, isoelectrofocusing experiments showed that each of the anti-carbohydrate antibody preparations was composed of several protein components. The results of isoelectrofocusing experiments are shown in Fig. 7. It may be seen that the anti-Glc antibodies (Gel A) were composed of five isomeric proteins, the anti-Gal antibodies (Gel B) of eleven different isomeric proteins, and the anti-Lac antibodies (Gel C) of seventeen different isomeric proteins.

That each protein possesses antibody activity is shown by the results of an experiment in which isoelectrofocusing was coupled with agar diffusion. Results of

one such experiment are shown in Fig. 8. In this figure, gel A shows the protein components in the anti-Lac antibody preparation, as revealed by isoelectrofocusing and protein-staining. Gel B is a duplicate gel of the antibodies that had not been stained but had been embedded in agar. Strip D is a trough containing a solution of the Lac-BSA, and area C shows the location of precipitin bands formed between antibodies in gel B and the antigen, Lac-BSA in trough D. The continuous precipitin-band corresponding to all of the protein bands in gel A shows that all of the proteins in the gel reacted with the antigen. Therefore, all of the proteins in the anti-Lac preparation are antibodies directed against the Lac-BSA. Isoelectrofocusing and agar-diffusion data on the anti-Glc antibodies and the anti-Gal antibodies showed that all protein components in these preparations were antibodies. It should be pointed out that the biosynthesis of all of the protein components in a set of antibodies is initiated by the same determinant group of the antigen and that all components combine with this determinant group to form the antigen–antibody complex. It has been proposed that such sets of antibodies be called isoantibodies[17] by analogy with the isoenzyme terminology[18]. Such antibodies are most probably the result of *de novo* synthesis of immunoglobulins[11], although the possibility of post-translational modifications of the antibodies by glycosyl transferases, transamidases, or other enzymes has not been ruled out[19].

The synthesis of multiple forms of antibodies directed against the same determinant group of an antigen has been observed earlier with natural carbohydrate antigens[11] and has now been found to occur with synthetic glycoconjugates. Isolation of the sets of anti-carbohydrate isoantibodies is now possible by affinity chromatography. Examination of sera from eight different rabbits immunized with vaccines of the synthetic glycoconjugates revealed that all of the rabbits produced isomeric forms of antibodies directed at the same carbohydrate moiety of the antigen. The occurrence of sets of isoantibodies in sera of rabbits immunized with the glycoconjugates may be of value in devising experiments for elucidating the detailed mechanisms of the biochemical pathways and genetic regulation of the synthesis of antibodies.

EXPERIMENTAL

Antigens (synthetic glycoconjugates). — The antigens used in this study were synthetic glycoconjugates prepared from derivatives of carbohydrates and bovine serum albumin (BSA). The antigens were β-D-glucosyl-bovine serum albumin (Glc-BSA), β-D-galactosyl-bovine serum albumin (Gal-BSA), and β-lactosyl-bovine serum albumin (Lac-BSA). The methods for synthesizing these substances have been described[8]. In brief, the procedures involved sequentially the preparation of the peracetylated glycosyl bromide of the sugar, reaction of this derivative with ethyl 9-hydroxynonanoate, deacetylation of the product, conversion first into the hydrazide and then into the azide, and finally coupling of the azide with the bovine serum albumin. Some of the samples of synthetic glycoconjugates and of antisera were kindly provided by Dr. D. R. Bundle, Division of Biological Sciences, National

Research Council, Ottawa, Canada, KIA OR6. The others were prepared in this laboratory. Analysis of the synthetic glycoconjugates for carbohydrate content by the phenol–sulfuric acid method[12] showed that the glycoconjugates contained ~5% carbohydrate. One third, or twenty-two, lysyl residues of the bovine serum albumin had carbohydrate groups attached.

Immunization procedures. — Vaccines of Glc-BSA, Gal-BSA, or Lac-BSA were prepared by dissolving an appropriate amount of the glycoconjugates in saline to yield a solution containing 0.2% antigen. The solutions were then mixed with an equal amount of Freund's complete adjuvant and used to immunize rabbits subcutaneously in the back of the neck at multisites[13]. For each immunization, 0.2 mL of the vaccine was used. All animals were immunized weekly for a 3-week period and then were allowed to rest for 3 weeks. The animals were then immunized further by intravenous injection of 0.4 mL of 0.1% solution of the glycoconjugate in 0.1M phosphate buffer of pH 7 in saline. The latter immunizations were continued for 3 consecutive days followed by a rest period of 4 days. The intravenous immunization was repeated for an additional 4 weeks. Blood samples were taken weekly during the latter immunization period and sera were prepared by standard methods. The serum samples were examined for antibodies by qualitative precipitin-tests and by agar diffusion.

Preparation of glycosyl-adsorbents. — Glycosyl-Sepharose adsorbents were prepared by treating the *p*-aminophenyl β-D-glycosides of glucose, galactose, or lactose with cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ 58854). The *p*-aminophenyl derivatives were obtained from Vega Fox Biochemicals, Tucson, AR 85719, and were coupled to the activated Sepharose 4B by a published procedure[20]. The procedure employed in our experiments was as follows. The glycoside (20 mg) was dissolved in 10 mL of 0.1M hydrogencarbonate buffer of pH 9 and mixed with 4 g of cyanogen bromide-activated Sepharose 4B. The resulting suspension was shaken for 24 h at 4°. The precipitate was collected on a sintered-glass filter and washed thoroughly with buffer. The washed sample was treated with 50 mL of 0.1M 2-aminoethanol at pH 9 for 2 h and then collected on the sintered-glass filter. The product was washed thoroughly with 0.02M sodium phosphate buffer of pH 7 in saline and transferred to a glass chromatography column. The derivatized Sepharose was washed further with 300 mL of phosphate buffer and stored in a cold room until used.

Isolation of anti-carbohydrate antibodies. — The affinity-chromatography procedure utilized in this laboratory in other studies[17] was employed for isolating and purifying the new anti-carbohydrate antibodies. In the procedure, the immune serum (1 mL) was placed on a glycosyl–Sepharose column and allowed to filter into the adsorbent by gravity. The column was then washed with 0.02M phosphate buffer of pH 7 in saline, and the eluates were monitored continuously for u.v.-absorbing material in a flow cell attached to a u.v. analyzer. The washing of the column was continued until unadsorbed proteins had been removed. At this point, 20 mL of 0.1M solution of the carbohydrate (D-glucose, D-galactose, or lactose) in phosphate buffer

of pH 7 in saline was introduced on the column and monitoring of the eluate for u.v.-absorbing substances was continued. The u.v.-absorbing material that eluted with the carbohydrate was collected and mixed with an equal volume of saturated ammonium sulfate. The solution was refrigerated overnight and a white precipitate was obtained. This precipitate was collected by centrifugation and then dissolved in 1 mL of 0.02M phosphate buffer of pH 7 in saline. Samples from several affinity-chromatography experiments were combined, dialyzed, and concentrated by ultrafiltration to a volume of 1 mL. Analysis of the preparation by u.v.-absorbance measurements showed that these solutions contained from 0.5–1% protein. Antibody preparations were obtained from the three types of immune serum.

Quantitative precipitin and agar-diffusion tests. — Quantitative precipitin tests were performed as described previously[21]. The amounts of antibody–antigen complex obtained in the presence or absence of various carbohydrates were measured by the protein method of Lowry and coworkers[22]. Hapten-inhibition tests were performed at optimal amount of antigen (40 μg) and constant amount of antibody preparation (50 μL), but with various concentrations of carbohydrate (0.025, 0.05, 0.1, and 0.2 mM). In the measurements, a control containing no inhibitor yielded an absorbance value of 1.2. From the absorbance values in the presence of the carbohydrate and the foregoing value, the percentage inhibition was calculated. Some of the values are recorded in Table I. Plots of all of the data yielded typical inhibition curves.

Double diffusion in agar was performed by a standard method. The plates that yielded precipitin bands in the agar-diffusion tests were photographed. In earlier work, it was found that ampholine interfered with the formation of the antigen–antibody complex in gels prepared by isoelectrofocusing. Accordingly, an assay method based on the use of isoelectrofocusing and agar diffusion was devised for detecting antibodies in poly(acrylamide) gels containing ampholine. In this method, duplicate samples of the antibody preparation were subjected to isoelectrofocusing as described in a later section. One gel of each set was then stained with Coomassie Brilliant Blue G-250 for locating the protein components and the duplicate gel was embedded in fluid agar in a petri plate. The agar was allowed to solidify for several h and antibodies in the gel were allowed to diffuse into the agar for 24 h. A trough was then cut in the agar ~2 cm from the embedded gel and a 0.2% solution of the antigen (Glc-BSA, Gal-BSA, or Lac-BAS) was placed in the trough. Diffusion of antigen and antibodies was allowed to proceed in a moist chamber for periods up to 90 h. Plates that developed precipitin bands were photographed.

Periodate oxidation. — Galactosyl-BSA (0.3 mg) was dissolved in 0.04M sodium metaperiodate (0.15 mL) and maintained in the dark for 24 h at 0°. Under these conditions, the oxidation of carbohydrate groups having hydroxyl groups on adjacent carbon atoms occurs rapidly[23]. Bovine serum albumin (0.3 mg) was also treated in the same manner. At the end of the reaction period, the periodate was decomposed by addition of 10 μL of ethylene glycol. Comparable solutions of native BSA, native Gal-BSA, and the samples treated with periodate were used in agar-diffusion tests. Anti-BSA serum (Cappell Laboratories, Cochranville, PA 19330)

and anti-Gal antibodies were used as the source of antibodies. A photograph of the agar-diffusion plate was obtained.

Isoelectrofocusing. — Isoelectrofocusing in poly(acrylamide) gels was performed by a published method[24]. Glass tubes, 100 mm long and of 6-mm inner diameter were used for supporting the gels. Prior to use, these tubes were coated internally with 1% Gelamide solution and allowed to dry in air. The tubes were then filled with 7.5% poly(acrylamide) gel containing 1.5% ampholine to a height of 9 mm and with a 5% acrylamide solution containing 2% ampholine to a height of 70 mm. The poly(acrylamide) gel was then covered with a 5% solution of sucrose and 1% solution of ampholine. The pH gradient of the ampholine solution used in these experiments was 5–7. Preliminary isoelectrofocusing was conducted for 30 min at a current of 0.7 mA/gel at 4°. The antibody samples were dissolved in 40% sucrose and 4% ampholine, and then introduced on the top of the gel. Isoelectrofocusing of the antibodies was conducted for an additional 6 h at a current of 1 mA/gel until a potential of 215 V was reached, at which point the voltage was maintained constant. At completion of the isoelectrofocusing, the gels were removed from the tubes and washed with 10% trichloroacetic acid solution for 16 h to remove the excess of reagents. The gels were stained with a solution of 0.02% Coomassie Brilliant Blue G-250 in 12.5% trichloroacetic acid. The gels were destained with 7% acetic acid in a destainer for 24 h. Gels to be used for agar-diffusion tests were not stained but only washed in 7% acetic acid for 24 h.

Ultracentrifugation. — A density-gradient centrifugation procedure described earlier[15] was used in these experiments. Samples of 0.2 mL of ~0.4% solutions of the antibodies or of reference proteins were placed carefully on sucrose density-gradients (5–40%) in separate tubes. The tubes were centrifuged in a swinging-bucket SW 65 rotor at 65,000 r.p.m. for 10 h in a Beckman L2-65 centrifuge. At the end of this time, the tubes were removed and the gradient solutions fractionated into 0.2 mL samples by means of an ISCO Density Gradient Fractionator. The u.v. absorbance of the fractions was monitored at 280 nm during fractionation. D-Glucose oxidase was used as a reference protein of molecular weight of 1.54×10^5 in the ultracentrifugation experiments[25]. The molecular weights of the antibodies were calculated from the centrifugation data and an empirical formula[16].

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