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Immunochemical Studies on Methyl α- and Methyl β-Glucosaminide-Azoprotein Conjugates[1]

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Conjugates consisting of methyl 2(*p*-azobenzamido)2-deoxy-α-D-glucopyranoside and methyl 2(*p*-azobenzamido)-2-β-D-glucopyranoside coupled to bovine serum albumin were used as synthetic antigens. Rabbits immunized with artificial antigen provided specific anti-methyl α-glucosaminide or anti-methyl β-glucosaminide anti-hapten sera. Quantitative hapten inhibition was employed to elucidate the specificity of anti-hapten combining sites and to evaluate some structural and configurational features contributing to the immunochemical interaction with the antibody. It is shown that the configuration at the anomeric carbon atom makes a major contribution to the immunochemical specificity of the anti-hapten antibodies against α- and β-methyl glycosides.

Artificial antigens possessing *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine as an immunodeterminant sugar have been employed previously (1–3). Synthetic antigens used in earlier studies to prepare anti-hexosaminide sera have consisted of *N*-acetyl-D-hexosamine linked through the anomeric carbon to carrier protein by means of an azophenyl moiety. Although antisera prepared against an azophenyl β-*N*-acetyl-glucosaminide conjugate were shown to have a specificity directed against the introduced β-*N*-acetyl-glucosaminide hapten group (1), the structural and configurational features involved in the binding of sugar ligands to the anti-hexosaminide combining site were not explored or evaluated.

In the present study, methyl 2-amino-2-deoxy-α-D-glucopyranoside and methyl 2-amino-2-deoxy-β-D-glucopyranoside coupled to bovine serum albumin by means of a *p*-azobenzamido group at the C-2 position were used as artificial antigens. The structural formula of the methyl 2-(*p*-azobenzamido)-2-deoxy-β-D-glycopyranoside haptenic group is shown in Fig. 1. Rabbits immunized with synthetic conjugates provided anti-methyl α-glucosaminide and anti-methyl β-glucosaminide sera which were used to evaluate structural and configurational features contributing to immunochemical interaction with anti-hapten. Use of both α- and β-isomeric methyl glucosaminides as haptens linked through their 2-amino position permitted an examination of the role of the anomeric configuration of the glucosaminide bond in immunochemical behavior.

MATERIALS AND METHODS

Antisera. Rabbits were immunized with methyl α-glucosaminide or methyl β-glucosaminide–bovine serum albumin (BSA)[3] conjugates incorporated into complete Freund's adjuvant as described in earlier studies (4–6). Antisera were rendered specific for the introduced hapten by serial adsorption with whole calf serum to remove antibodies to carrier protein. Antibodies not involving the sugar moiety but possessing a specificity directed against the aglycon portion of the introduced hapten were removed by precipitation with *p*-hydroxyphenylazo-BSA (4).

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[3] Abbreviations used: BSA, bovine serum albumin; SXIV, type XIV pneumococcal capsular polysaccharide; Ab, antibody.

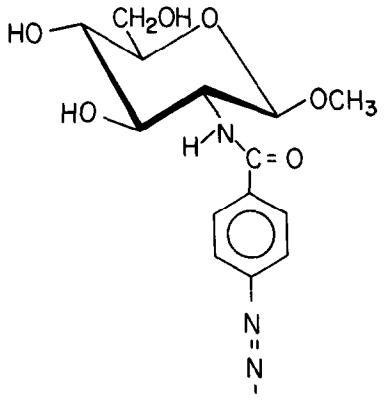

FIG. 1. Structural formula of the methyl 2-(*p*-azobenzamido)-2-deoxy-β-D-glucopyranoside haptenic grouping.

Antigens. Methyl 2-(*p*-aminobenzamide)-2-deoxy-α-D-glucopyranoside and its isomeric β-anomer, methyl 2-(*p*-aminobenzamido)2-deoxy-β-D-glucopyranoside (7) were diazotized and coupled to BSA (8) to preprepare *p*-azobenzamido-glucosaminide conjugates. The number of sugar residues introduced per mole of carrier BSA, determined spectrophotometrically (8), was estimated to be 19 and 20 residues, respectively, for the synthetic *p*-azobenzamido conjugates of methyl α-glucosaminide and methyl β-glucosaminide. Preparation of glycosyl-phenylazo-BSA antigens possessing α-D-glucosyl, β-D-glucosyl, β-sophorosyl, β-laminaribiosyl, β-cellobiosyl, β-gentiobiosyl, β-D-galactosyl, and β-*N*-acetyl-D-glucosaminyl sugar residues have been previously described (4, 5, 8).

Hog gastric mucin purchased from Nutritional Biochemical Corporation was purified by phenol extraction (9). Acid hydrolyzed mucin was prepared by treating hog gastric mucin at pH 1.5, for 2 h at 100°C. Type XIV pneumococcal capsular polysaccharide (SXIV) was isolated from bacterial culture filtrates by fractional ethanol precipitation (10).

Hapten inhibitors. The synthesis and characterization of methyl 2-(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside, methyl 2-(*p*-aminobenzamido)-2-deoxy-β-D-glucopyranoside, methyl 2-(*p*-aminobenzamido)-2-α-D-galactopyranoside, methyl 2-(*p*-nitrobenzamido)-2-deoxy-α-D-glucopyranoside, methyl 2-(*p*-nitrobenzamide)-2-deoxy-α-D-glucopyranoside, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside have been previously described in detail by Buss and Goldstein (7). Phenyl β-D-glucoside, *N*-acetyl-D-glucosamine, methyl α-D-glucoside, and D-glucose were purchased from Pfanstiehl Laboratories. Methyl β-D-glucoside was obtained from Mann Research Laboratories. *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide was purchased from Sigma Chemical Company.

Quantitative precipitin and hapten inhibition analysis. Quantitative precipitin curves were obtained with 0.5-ml aliquots of rabbit anti-conjugate in a total reaction volume of 1.5 ml. Antibody nitrogen (Ab N) precipitable by methyl α-glucosaminide or methyl β-glucosaminide conjugate was determined by micro-Kjeldahl analysis (10).

Haptens were assayed for their ability to inhibit precipitation of 11 μg of Ab N from 75 μl of carrier adsorbed anti-methyl α-glucosaminide serum R4α or 8 μg of Ab N from 100 μl of anti-methyl β-glucosaminide serum R1β. With both sera, 1 μg of conjugate N was used to provide an equivalent amount of antigen for maximum precipitation of Ab. All assay tubes were adjusted to a final total volume of 400 μl with 0.01 M phosphate buffer (pH 7.5) containing 0.15 NaCl. Hapten inhibition assays were carried out as previously described for antipanoside and anti-laminaribioside (4, 5). The relative inhibitory ability of various sugars was estimated by comparing the micromolar concentration of ligand required for 40% inhibition with that of methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside or its β-anomeric isomer.

Immunodiffusion and immunoelectrophoresis. Double diffusion in agar gel and immunoelectrophoresis were carried out essentially as described in earlier studies (5) except that gel used for immunodiffusion was buffered at pH 7.5 with millimolar phosphate.

RESULTS

Antisera obtained from rabbits after a single course of immunization with methyl α-glucosaminide or methyl β-glucosaminide conjugate were examined by immunodiffusion before and after adsorption with whole calf serum and *p*-hydroxyphenylazo-BSA. All antisera gave an intense band of precipitation with native carrier protein (BSA) which could be eliminated by adsorption with calf serum. When carrier adsorbed antisera were diffused against the *p*-azophenyl-BSA conjugates of α-D-glucoside, β-D-glucoside, β-D-galactoside, β-D-galactoside, β-sophoroside, β-laminaribioside, and β-cellobioside a faint band of precipitation was produced by each, which showed complete fusion with one another. This faint band was, however, "spurred over" by an intense band of precipitation produced by homologous antigen. Absorption with *p*-hydroxyphenylazo-BSA completely removed such cross-reactivity.

Following absorption with *p*-hydroxy-

phenylazo-BSA, anti-methyl α-glucosaminide and anti-methyl β-glucosaminide sera showed a reactivity in immunodiffusion confined to the conjugate used for immunization and failed to show any cross-precipitation with test antigen differing only in the anomeric configuration of its methyl glucosaminide haptenic grouping. Type XIV pneumococcal capsular polysaccharide, hog gastric mucin, and mild acid-treated hog gastric mucin also failed to show reactivity with any anti-methyl α or anti-methyl β-glucosaminide sera tested.

Immunoelectrophoresis, employing methyl α- or β-glucosaminide–BSA as antigen to localize specific antibody and goat anti-rabbit serum to locate rabbit serum proteins, showed anti-conjugate to be confined entirely to the γ_2 globulin region.

Quantitative precipitin curves obtained with four carrier adsorbed rabbit anti-methyl α-glucosaminide and four anti-methyl β-glucosaminide sera are shown in Fig. 2. The maximum amount of antibody precipitated by homologous antigen from 0.5 ml of anti-conjugate varied from 34 to 75 μg of Ab N for anti-methyl α-glucosaminide and 39 to 47 μg of Ab N for anti-methyl β-glucosaminide sera.

The overall combining site specificity of anti-conjugate as well as an evaluation of the relative contribution of various portions of the methyl glucosaminide structure to interaction with antibody was examined by hapten inhibition. Quantitative hapten inhibition data for anti-methyl α-glucosaminide and anti-methyl β-glucosaminide sera are shown in Fig. 3.

Of the haptens examined, methyl 2-(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside and methyl 2-(*p*-nitrobenzamido)-2-deoxy-α-D-glucopyranoside were the most effective inhibitors of anti-methyl α-glucosaminide serum R4α, only 2.5×10^{-2} μmol of each, giving 40% inhibition. Similarly, methyl 2-(*p*-aminobenzamide)-2-deoxy-β-D-glucopyranoside and methyl 2-*p*-nitrobenzamido)-2-deoxy-β-D-glucopyranoside were found to be the most potent inhibitors of anti-methyl β-glucosaminide serum R1β, since 2.6×10^{-2} μmol of each gave 40% inhibition. An antibody combin-

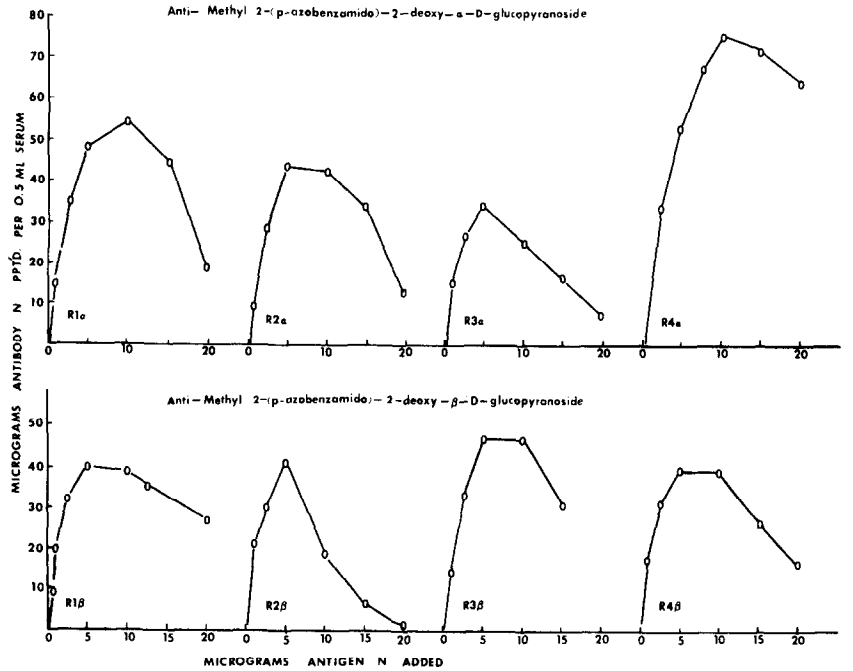

FIG. 2. Quantitative precipitin curves obtained with carrier adsorbed rabbit anti-methyl α-glucosaminide sera R1α, R2α, R3α, R4α, (upper graphs) and rabbit anti-methyl β-glucosaminide sera R1β, R2β, R3β, R4β (lower graphs).

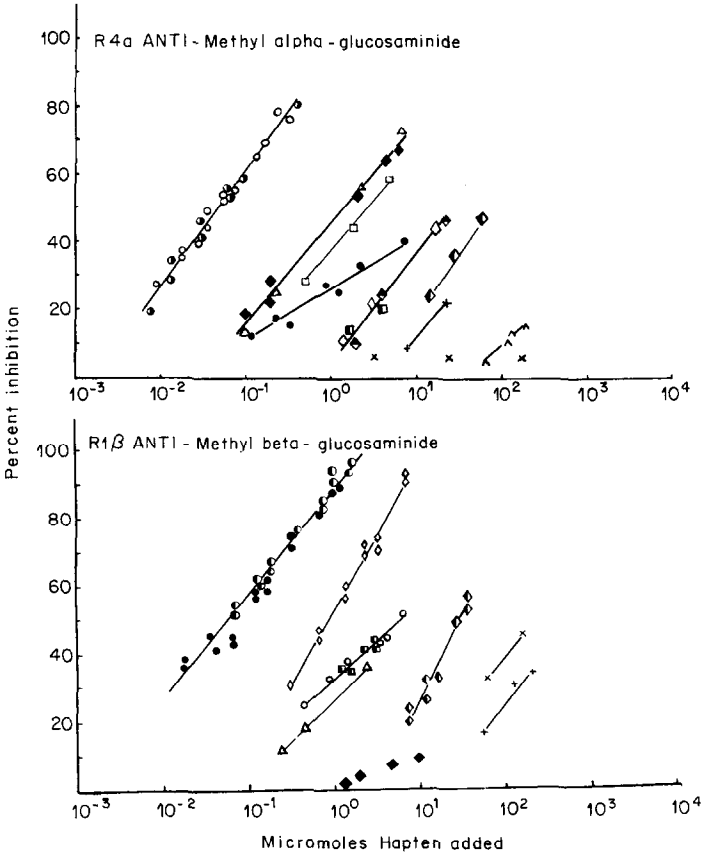

FIG. 3. Inhibition by various sugars of anti-methyl α-glucosaminide precipitation from antiserum R4α and anti-methyl β-glucosaminide antiserum R1β. ○, methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside; ◑, methyl 2(*p*-nitrobenzamido)-2-deoxy-α-D-glucopyranoside; ●, methyl 2(*p*-aminobenzamido)-2-deoxy-β-D-glucopyranoside; ◐, methyl 2(*p*-nitrobenzamido)-2-deoxy-β-D-glucopyranoside; □, methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-galactopyranoside; △, 2(*p*-nitrobenzamido)-2-deoxy-D-glucose; ◆, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside; ◇, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside; ◈, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside; ◈, 2-acetamido-2-deoxy-D-glucose; +, methyl α-D-glucopyranoside; ^, D-glucose; x, methyl β-D-glucopyranoside; ◧, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide.

ing site specificity involving the configuration of the anomeric methyl group is evident from a comparison of the ability of α, β isomeric haptens to inhibit precipitation. The α-glycoside, methyl 2-(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside is 300 times more effective in inhibiting antiserum R4α than its β-anomer, which required a 7.5 μM concentration to give 40% inhibition (Table I). When used to inhibit antiserum R1β, however, methyl 2-(*p*-aminobenzamido)-2-deoxy-β-D-glucopyranoside was 100 times more effective as an inhibitor than its α-anomer.

Anti-hapten specificity directed against the methyl α-D-glucosaminide or methyl β-D-glucosaminide moiety is also apparent from a comparison of the inhibition obtained with methyl 2-acetamido-2-deoxy-2-α-D-glucopyranoside, methyl β-N-acetyl-D-glucosamine, and *N*-acetyl-D-glucosamine. When used to inhibit antiserum R4α, methyl α-*N*-acetyl-D-glucosaminide was 22 times more effective than methyl β-*N*-acetyl-D-glucosaminide and 42 times more effective than *N*-acetyl-D-glucosamine (Table I). When assayed with antiserum R1β, however, methyl β-*N*-acetyl-D-glucosa-

TABLE I

RELATIVE ABILITY OF VARIOUS SUGARS TO INHIBIT ANTI-METHYL α- AND ANTI-METHYL β-GLUCOSAMINIDE ANTIBODY

Sugar ligand tested	Anti-methyl α-glucoaminide serum R4α		Anti-methyl β-glucosaminide serum R1β	
	Concentration giving 40% inhibition (μM)	Relative inhibitory ability[a]	Concentration giving 40% inhibition (μM)	Relative inhibitory ability
methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside	2.5×10^{-2}	1	2.5	100
methyl 2(*p*-nitrobenzamido)-2-deoxy-α-D-glucopyranoside	2.5×10^{-2}	1	–	–
methyl 2(*p*-aminobenzamido)-2-deoxy-β-D-glucopyranoside	7.5	300	2.6×10^{-2}	1
methyl 2(*p*-nitrobenzamido)-2-deoxy-β-D-glucopyranoside	–	–	2.6×10^{-2}	1
methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-galactopyranoside	1.2	48	–	–
2(*p*-nitrobenzamido-2-deoxy-D-glucose	0.68	27	–	–
methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	0.68	27	⩾10	⩾384
methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	15	700	0.54	21
methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	15	700	–	–
2-acetamido-2-deoxy-D-glucose	29	1080	22	846
methyl α-D-glycopyranoside	(20)[b]	(2957)	(180)	(15,000)
D-glucose	(300)	(42,857)	–	–
methyl β-D-glucopyranoside	(>300)	(>42,857)	(50)	(4,166)
p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside	–	–	2.5	100

[a] Relative inhibitory ability = (μM test ligand required for 40% inhibition)/(μM methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside giving 40% inhibition serum R4α, corresponding β-anomer used with serum R1β).

[b] Values in parentheses were determined at the 30% inhibition level.

minide was at least 18-fold more effective than methyl α-*N*-acetyl-D-glucosaminide and 40-fold more effective than *N*-acetyl-D-glucosamine.

A contribution of binding provided by the aromatic aglycon portion of hapten structure is indicated by the finding that substitution of the *N*-acetyl group of methyl *N*-acetyl-D-glucosaminide by a benzamido group to give methyl 2(*p*-aminobenzamido)-2-deoxy-D-glucopyranoside results in a 27- to 21-fold increase in the inhibitory potency of α- and β-anomers with their respective antisera. Similarly, substitution of the *N*-acetyl group by a *p*-nitrobenzamido group to give *p*-nitrobenzamido-2-deoxy-D-glucose results in a 42-fold increase in ability to inhibit anti-methyl α-glucosaminide serum R4α (Table I).

DISCUSSION

Immunization of rabbits with synthetic antigen consisting of methyl α- or methyl β-glucosaminide haptenic groups attached to BSA through an azobenzamido moiety at carbon atom 2 (Fig. 1) leads to the production of specific anti-hapten antibodies. Precipitin curves obtained with carrier absorbed antiserum produced by each of four rabbits injected with methyl 2-(*p*-azobenzamido)-2-deoxy-α-D-glucopyranoside-BSA or methyl 2-(*p*-azobenzamido)-2-deoxy-β-D-glucopyranoside-BSA are shown in Fig. 2.

In immunoelectrophoresis, both anti-methyl α- and anti-methyl β-glucosaminide antibodies migrate slowly with the mobility of γ_2 globulin. Antihapten sera absorbed with *p*-hydroxyphenylazo-BSA were highly specific for homologous anti-

gen. In immunodiffusion such antisera showed no cross-reactivity with BSA coupled to the isomeric methyl glucosaminide anomer nor did they precipitate with *p*-azophenyl-2-acetamido-2-deoxy-β-D-glucoside–BSA conjugate. Similarly, SXIV and hog gastric mucin, naturally occurring polysaccharides which contain *N*-acetyl-D-glucosamine, showed no cross-precipitation with anti-hapten.

Quantitative inhibition data (Fig. 3) establish a combining site for anti-hapten directed mainly against the methyl 2-deoxy-α-D-glucopyranoside or methyl 2-deoxy-β-D-glucopyranoside sugar group but includes in addition the benzamido aglycon moiety. This is shown by a comparison of the efficiency of inhibition of precipitation by various ligands which establishes the following inhibitory order for antiserum R4α: methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside > methyl 2-acetamido-2-deoxy-α-D-glucopyranoside = 2(*p*-nitrobenzamido)-2-deoxy-D-glucose > methyl 2(*p*-aminobenzamido-2-deoxy-α-D-galactopyranoside > methyl 2 (*p*-aminobenzamido)-2-deoxy-β-D-glucopyranoside > 2-acetamido-2-deoxy-D-glucose. With anti-methyl β-glucosaminide serum (R1β), the inhibitory order obtained was: methyl 2(*p*-aminobenzamido)-2-deoxy-β-D-glucopyranoside > methyl 2-acetamido-2-deoxy-β-D-glucopyranoside > *p*-nitrophenyl β-D-glucosaminide = methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucosaminide.

Comparison of the relative molar ability of epimeric sugars to inhibit precipitation (Table I) permits an evaluation of the contribution of the configuration of carbon atoms C-1 and C-4 to immunochemical behavior. That a major contribution to anti-hapten binding is associated with the anomeric configuration of the glycosidic linkage at C-1 is shown by large difference in inhibitory ability shown by (α, β) anomers. Inversion in C-1 configuration of methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside results in a 300-fold decrease in ability to inhibit anti-methyl α-glucosaminide and a 100-fold increase in ability to inhibit anti-methyl β-glucosaminide.

Similarly, inversion of the OH group at C-4 from an equatorial to an axial position, converting methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-glucopyranoside into its C-4 epimer, methyl 2(*p*-aminobenzamido)-2-deoxy-α-D-galactopyranoside, results in a 48-fold decrease in inhibitory ability. Although inversion in C-1 or C-4 configuration each results in decreased interaction with anti-methyl α-glucosaminide, the magnitude of the decrease is 6-fold greater for the anomeric C-1 position.

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