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Immunochemical Characterization of Polylysine Conjugates Containing Reductively Aminated Cellulose Oligosaccharides

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Neoglycoproteins prepared by direct reductive amination of cellobiose, cellotetraose and cellopentaose to polylysine were found to be effective antigens in rabbits, and the antisera were found by quantitative inhibition techniques to be predominantly hapten specific. Several analogs incorporating various structural features of the carbohydrate hapten were synthesized and examined as inhibitors of the precipitin reaction between the neoglycoproteins and homologous antisera in order to identify those structural features of the hapten important in antibody recognition. Antibodies to the reductively aminated cellobiose-polylysine conjugate were found to recognize the terminal β-glucopyranosyl residue, the *acyclic* reduced glucose residue, and the secondary ammonium linkage and methylene arm of the lysyl residue of the hapten. Antibodies to the cellotetraose-polylysine conjugate, in contrast, displayed no recognition for the secondary ammonium linkage region; they were found to recognize the non-reducing terminal β-glucopyranosyl residue, the two internal β1,4-linked glucopyranosyl residues, and the reducing end glucose residue in an *acyclic* or *cyclic* form. Inhibition studies with antisera to the cellopentaose-polylysine conjugate again established that there was no recognition of the secondary ammonium linkage region, and demonstrated that the upper limit to the size of the antibody combining site was four β1,4-linked glucopyranosyl residues.

Abbreviations: BSA, bovine serum albumin; Glc, glucose; $(Glc)_2$, $(Glc)_4$, $(Glc)_5$, compounds derived by reductive amination of cellobiose, cellotetraose and cellopentaose.

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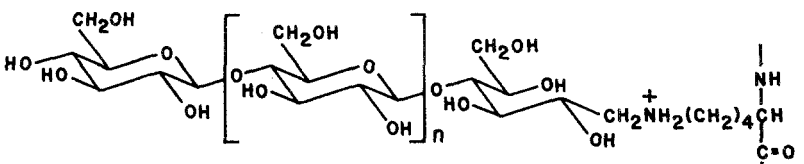
The development of chemical procedures for the covalent attachment of carbohydrates to proteins has greatly facilitated studies of carbohydrate immunochemistry and the roles carbohydrates play in various biological recognition processes. Of the many methods available for accomplishing this type of covalent modification, direct reductive amination of reducing carbohydrates with sodium cyanoborohydride is particularly simple and effective [1, 2]. Neoglycoproteins prepared by this technique have been used as immunogens [3, 4], as models for the study of lectin binding specificity [5], and as models to explore the role of carbohydrate in the clearance of glycoproteins from the circulation [6-9]. A question arises, however, with respect to the influence the unusual secondary ammonium linkage region of these conjugates has on their immunological recognition. Studies employing bovine serum albumin conjugates containing reductively aminated disaccharides have demonstrated that these conjugates are effective antigens in rabbits and that the antisera are primarily hapten-specific [3]. A detailed study of the specificity of antibodies formed to a cellobiose-BSA conjugate, however, demonstrated that the linkage region was immunodominant, i.e., antibodies recognized the terminal β-glucopyranosyl residue, the reducing terminal glucose residue only in its *acyclic* form, and the secondary ammonium linkage [3]. Recent studies employing antisera to protein conjugates containing reductively animated β1,4-linked tri-, tetra-, and penta-saccharides of *N*-acetyl-D-glucosamine revealed that some degree of recognition of the carbohydrate-protein linkage region was retained even in these larger oligosaccharide-protein conjugates, but a detailed study of linkage region involvement in the antigenicity of these conjugates was not undertaken [4].



1 a, n = 0
b, n = 2
c, n = 3

In order to explore more fully the immunochemical behavior of neoglycoproteins prepared by direct reductive amination, polylysine conjugates containing reductively aminated cellobiose, cellotetraose, and cellopentaose (**1a**, **b**, **c**, respectively) have been prepared, and antisera to these conjugates have been examined in detail in order to determine which structural features of the hapten are important in antibody recognition.

Experimental Procedures

Materials

Polylysine (poly-L-lysine hydrobromide, type VII-b, mol wt. 32 000), α-cellulose fiber, methyl β-D-glucopyranoside, maltose and hemocyanin (from *Limulus polyphemus* hemolymph) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Rabbit im-

munoglobulin G was purchased from US Biochemical Corporation. Freund's complete adjuvant was purchased from Difco Laboratories. Sodium cyanoborohydride (Alfa Inorganics) was purified as described by Borch *et al.* [10]. Cellulose oligosaccharides were obtained by the partial hydrolysis of cellulose fiber as described by Miller [11], followed by Bio-Gel P-2 (Bio-Rad, Richmond, CA, USA) column chromatography as described by Raftery *et al.* [12] for the separation of chitin oligosaccharides. Identical oligosaccharide fractions from several runs were combined and deionized by sequential treatment with Dowex 50 (H^+) and Dowex 1 (acetate), then lyophilized.

Analytical Procedures

Total carbohydrate was determined by the phenolsulfuric acid procedure [13] or by oxidation with sodium metaperiodate [14], where applicable. Reducing sugar was assayed by the dinitrosalicyclic acid method [15] or the procedure of Park and Johnson [16]. Protein concentrations were determined by the method of Lowry *et al.* [17]. Absorbances were measured using a Coleman 124D double beam spectrophotometer, and proton magnetic resonance spectra were recorded on a Varian HFT-80 spectrometer.

Immunochemical Methods

Immune response was monitored quantitatively by the precipitin reaction of serially diluted glycoconjugate with 1:2-diluted serum according to the microprecipitation technique of Marcus and Grollman [18]. Protein was quantified by the Lowry procedure with rabbit immunoglobulin G as the standard.

Immunization

Six New Zealand white female rabbits, each weighing 4-5 lbs, were immunized with the reductively aminated cellulose oligomer-polylysine conjugates complexed with succinylated hemocyanin [19], as described by Zopf *et al.* [20]. Each conjugate (1.0 mg) was dissolved in 1.0 ml of 0.15 M sodium chloride and 0.5 mg of succinylated hemocyanin was added. Subsequent chilling on ice caused a fine, white precipitate to form. This suspension was emulsified with an equal volume (1.0 ml) of Freund's complete adjuvant before injection. Injections of 0.2 ml (0.1 mg of conjugate) were administered to each of four sites, intracutaneously near the inguinal and axillary lymph nodes. Each glycoconjugate was used to immunize two rabbits. Approximately 1-3 ml of blood were collected from an ear vein prior to the initial immunization and weekly thereafter. Rabbits immunized with the cellotetraose- and cellopentaose-polylysine conjugates were boosted on days 9, 20 and 28 following the initial injection, with 0.1 ml (0.05 mg of conjugate) of emulsified antigen into each of two sites adjacent to the original immunization sites. The two sites used for boosting were alternated with each injection. On day 57 the rabbits were boosted for the last time, and on day 76, approximately 60 ml of blood was obtained from each rabbit by cardiac puncture. Rabbits immunized with the cellobiose-polylysine conjugate were boosted on days 9, 20, 28, 62, 81 and 144 after the initial injection. On day 150, approximately 60 ml of blood was collected from each rabbit by cardiac puncture. The blood from all rabbits was stored at 4°C overnight to facilitate clotting.

Cellulose Oligosaccharide - Polylysine Conjugates

Cellobiose, cellotetraose and cellopentaose were coupled to polylysine by reductive amination according to the procedure of Schwartz and Gray [2]. Each reaction mixture contained polylysine (60 mg, 1.9 μmol), oligosaccharide (~300 μmol) and sodium cyanoborohydride (150 mg, 2.4 mmol) dissolved in 5 ml (cellobiose, cellotetraose) or 10 ml (cellopentaose) of 0.2 M potassium phophate buffer, pH 8.0. The cellobiose, cellotetraose and cellopentaose reaction mixtures were incubated for 6, 20, and 16 days, respectively, at 37°C, after which time the reducing sugar content [15] of the reaction mixtures was found to decrease by ~70%. The conjugates were purified by sequential dialysis at 4°C against running water, 0.15 M sodium chloride and distilled water.

The degree of substitution of the conjugates was determined by direct analysis. Protein content was measured by the method of Lowry *et al.* [17] with polylysine (mol wt 32 000, 250 lysine residues/mol) as the standard, and total carbohydrate was determined by the phenol-sulfuric acid procedure [13] with glucose as the standard. Solutions of the conjugates were also assayed for reducing sugar [16], and the amount of reducing oligosaccharide was subtracted from the total amount of oligosaccharide as determined by the phenol-sulfuric acid assay. The cellobiose-, cellotetraose-, and cellopentaose-polylysine conjugates were found to contain, respectively, 46.3, 43.4, and 19.5 mol of reductively aminated oligosaccharide per mol of polylysine.

Inhibitors

Inhibitors containing glucose, cellobiose, cellotetraose, and cellopentaose reductively aminated to α-*N*-acetyl-L-lysine (**3**, **2a**, **4a**, and **5a**, respectively) were prepared as described by Kamicker *et al.* [3]. The products were purified by ion-exchange chromatography and gel permeation chromatography as previously reported, and were characterized by ^{1}H-NMR spectroscopy. The 1-deoxyamino derivatives of cellobiitol, cellotetraitol, and cellopentaitol (**2b**, **4b**, and **5b**, respectively) were also prepared and purified as described by Kamicker *et al.* [3], and were characterized as their *N*-acetyl derivatives [2, 3]. Oligosaccharides were converted to their alditols by reduction with excess sodium borohydride in aqueous solution at pH 10. After 18 h at room temperature, glacial acetic acid was added dropwise until hydrogen evolution had ceased and the reaction mixtures were evaporated to dryness under vacuum. The products were redissolved in a small amount of water and eluted through a column of Dowex 50 (H^+) to remove sodium ions. The eluates were evaporated to dryness, then several times from methanol/hydrochloric acid, 500/1 by vol, until boric acid was absent as measured by the turmeric assay [21].

Results

Immunochemical Properties of the Conjugates

The precipitin reactions between the reductively aminated cellulose oligosaccharide-polylysine conjugates and the homologous rabbit antisera demonstrated that all of the conjugates were effective precipitating antigens over a wide range of concentrations. In each case, the two rabbits immunized with each of the conjugates showed virtually

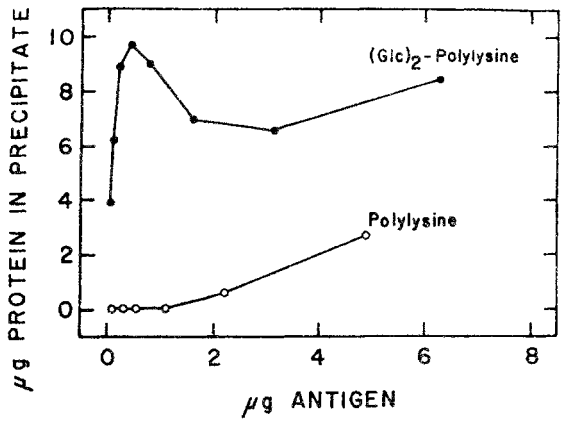


Figure 1. Quantitative precipitin reactions of immune rabbit serum with polylysine and the reductively aminated cellobiose-polylysine conjugate. Each reaction mixture contained 50 μl of two-fold-diluted serum from day 150 and the indicated amount of antigen in a total volume of 500 μl.

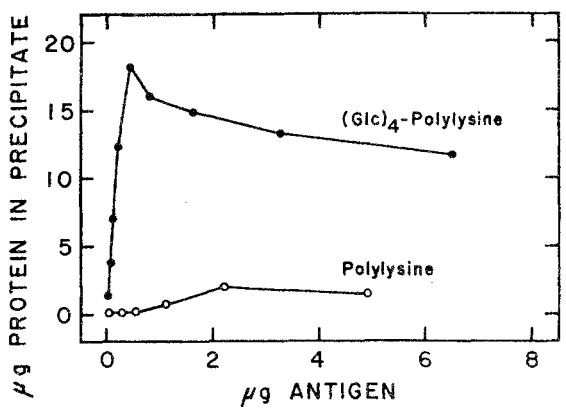


Figure 2. Quantitative precipitin reactions of immune rabbit serum with polylysine and the reductively aminated cellotetraose-polylysine conjugate. Each reaction mixture contained 50 μl of two-fold-diluted serum from day 76 and the indicated amount of antigen in a total volume of 500 μl.

identical immune responses, as measured by quantitative precipitin reactions; shown in Figs. 1-3 are precipitin reactions which are representative for each of the conjugates. In the precipitin reaction between the cellobiose-polylysine conjugate and homologous antiserum (Fig. 1), approximately 10 μg of antibody is precipitated by 0.39 μg of the conjugate at the equivalence ratio. Antibody precipitation appears to be primarily hapten specific in the region of the equivalence ratio, as essentially no precipitate is formed between polylysine and the immune serum at the antigen concentration where maximal precipitation occurred with the cellobiose-polylysine conjugate. The gradual increase in precipitate at higher concentrations of polylysine is

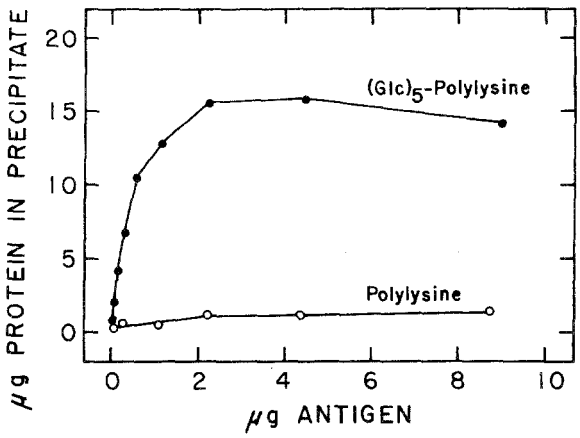


Figure 3. Quantitative precipitin reactions of immune rabbit serum with polylysine and the reductively aminated cellopentaose-polylysine conjugate. Each reaction mixture contained 50 μl of two-fold-diluted serum from day 76 and the indicated amount of antigen in a total volume of 500 μl.

possibly due to the presence of a small population of low avidity antibodies directed against the polylysine carrier protein, or the non-specific precipitation of complement proteins and other serum components by polylysine [22].

Similar results were observed in the precipitin reactions between the cellotetraose- and cellopentaose-polylysine conjugates and their homologous antisera (Figs. 2 and 3, respectively). In each case, the immune response appears to be primarily hapten specific, as evidenced by the lack of precipitate formation when the immune sera were incubated with polylysine alone. At the respective equivalence ratios, 0.41 μg of the cellotetraose-polylysine conjugate precipitated 18 μg of antibody and 2.25 μg of the cellopentaose-polylysine conjugate precipitated approximately 15 μg of antibody.

Specificity of Antibodies Formed to the Conjugates

In order to determine the structural features of the haptens to which antibodies were formed, inhibitors containing all or some of the structural features of the haptens were synthesized. Quantitative inhibition studies were conducted by preincubating the antisera with the inhibitors over a wide range of concentration, and then the amount of conjugate corresponding to the equivalence ratio of antibody and conjugate was added. The amounts of inhibitor required to decrease the amount of precipitate by 50% were determined. The reciprocals of these values, normalized in each study to the value obtained for the most effective inhibitor of neoglycoconjugate-antiserum precipitation, were calculated, and are used as an indication of the relative affinities of the inhibitors for the antibodies.

Cellobiose-polylysine Antisera

Representative curves showing the results of inhibition of precipitation between the cellobiose-polylysine conjugate and its homologous antiserum by structural analogs of the disaccharide hapten are shown in Fig. 4, and the results are summarized in Table 1.

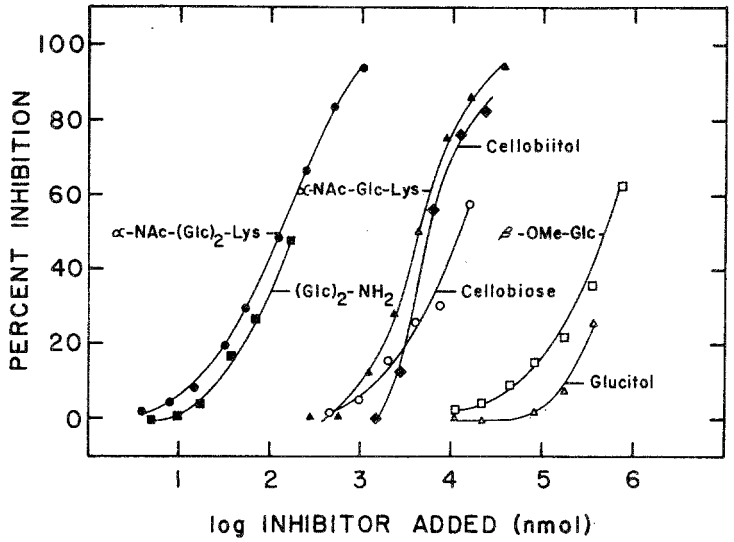
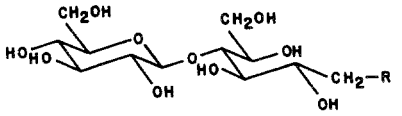


Figure 4. Inhibition of the precipitin reaction between the cellobiose-polylysine conjugate and homologous rabbit antiserum by α-*N*-acetyl-ε-*N*-1(1-deoxycellobiitol)-lysine (●), 1-deoxyaminocellobiitol (■), α-*N*-acetyl-ε-*N*-1(1-deoxyglucitol)-lysine (▲), cellobiitol (◆), cellobiose (○), methyl β-D-glucopyranoside (□) and glucitol (△). Two-fold-diluted immune rabbit serum (50 μl) and varying amounts of inhibitor were combined and diluted to a final volume of 450 μl with 0.15 M NaCl. After incubation for 2 h at 37°C, 50 μl of antigen solution (containing 0.39 μg of cellobiose-polylysine) were added, and the reaction mixtures were thoroughly mixed and incubated 48 h at 4°C. The amount of precipitin formed was determined as described in the text.

Table 1. Inhibition of the precipitin reaction between cellobiose-polylysine and homologous antiserum by structural analogs of the hapten.

Inhibitor	nmol for 50% inhibition	Relative effectiveness
α-NAc-$(Glc)_2$-lys **(2a)**	135	1.0
$(Glc)_2$-NH_2 **(2b)**	160	0.84
α-NAc-Glc-lys **(3)**	4400	0.031
Cellobiitol **(2c)**	5500	0.025
Maltitol	8600	0.016
Cellobiose	12500	0.011
β-OMe-Glc	520000	0.00026
Glc	670000	0.00020
Glucitol	$>10^6$	$<10^4$

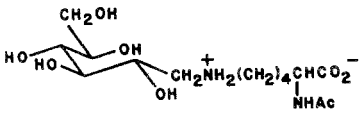
The most effective inhibitors of the precipitin reaction were α-*N*-acetyl-ε-*N*-1(1-deoxycellobiitol)-lysine [α-NAc-$(Glc)_2$-lys, **2a**] and 1-deoxyaminocellobiitol [$(Glc)_2$-NH_2, **2b**] which required 135 and 160 nmol, respectively, for 50% inhibition. Both **2a** and **2b** contain the reduced cellobiose structure and protonated ammonium linkage



2 a, R = $-\overset{+}{N}H_2(CH_2)_4\underset{NHAc}{\underset{|}{C}}HCO_2^-$

b, R = $-\overset{+}{N}H_3$

c, R = —OH



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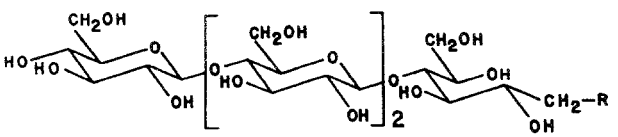
of the hapten, and in addition, **2a** contains the methylene arm of the lysine residue through which cellobiose is linked in the antigen (**1a**). Structural analogs representing different parts of the hapten were also examined as inhibitors, but none were as effective as **2a** and **2b**. α-*N*-Acetyl-ε-*N*-1(1-deoxyglucitol)-lysine [α-NAc-Glc-lys, **3**], which represents the linkage region of the antigen, was only 0.031-fold as effective as **2a**, and compounds which lacked the protonated ammonium linkage were even less effective. Cellobiitol (**2c**) was 0.025-fold as effective as **2a** and maltitol, which differs from **2c** only in the anomeric configuration of the 1,4-linked glucosyl residue, was even less effective (0.016-fold as effective as **2a**). Cellobiose, which differs from the disaccharide hapten (**1a**) in that it contains the reducing end glucose residue in the *cyclic* pyranose form rather than the acyclic form, was only 0.011-fold as effective as **2a**. Methyl β-D-glucopyranoside, glucose and glucitol were found to be completely ineffective as inhibitors at concentrations where all other compounds tested were maximally inhibitory.

Cellotetraose-polylysine Antisera

Antisera to both rabbits immunized with the cellotetraose-polylysine conjugate (**1b**) were examined by quantitative inhibition techniques, and were found to have very similar specificity. The results of studies from one of the rabbits are given in Table 2 and representative inhibition curves are shown in Fig. 5. The four tetrasaccharide analogs examined were all found to be excellent inhibitors of the precipitin reaction between



4 a, R = $-\overset{+}{N}H_2(CH_2)_4\underset{NHAc}{\underset{|}{C}}HCO_2^-$

b, R = $-\overset{+}{N}H_3$

c, R = —OH

Table 2. Inhibition of the precipitin reaction between cellotetraose-polylysine and homologous antiserum by structural analogs of the hapten.

Inhibitor	nmol for 50% inhibition	Relative effectiveness
Cellotetraose	17.0	1.0
α-NAc-$(Glc)_4$-lys **(4a)**	19.0	0.89
$(Glc)_4$-NH_2 **(4b)**	21.5	0.79
Cellotetraitol **(4c)**	22.0	0.77
Cellotriose	34.0	0.50
Cellobiose	850	0.020
Cellotriitol	1750	0.010
β-OMe-Glc	750000	2.3×10^{-5}
Glc	$>10^6$	$<10^{-5}$
Glucitol	$>10^6$	$<10^{-5}$

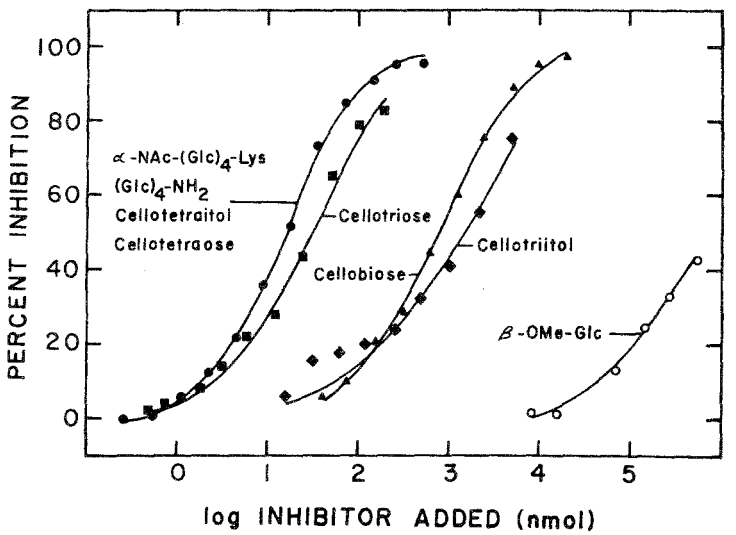


Figure 5. Inhibition of the precipitin reaction between the cellotetraose-polylysine conjugate and homologous rabbit antiserum by β-*N*-acetyl-ε-*N*-1(1-deoxycellotetraitol)-lysine, 1-deoxyaminocellotetraitol, cellotetraitol, and cellotetraose (●), cellotriose (■), cellobiose (▲), cellotriitol (◆), and methyl β-D-glucopyranoside (○). Two-fold-diluted immune rabbit serum (50 μl) and varying amounts of inhibitor were combined and diluted to a final volume of 450 μl with 0.15 M NaCl. After incubation for 2 h at 37°C, 50 μl of antigen solution (containing 0.41 μg of cellotetraose-polylysine) were added, and the reaction mixtures were thoroughly mixed and incubated 48 h at 4°C. The amount of precipitin formed was determined as described in the text.

Table 3. Inhibition of the precipitin reaction between cellopentaose-polylysine and homologous antiserum by structural analogs of the hapten.

Inhibitor	nmol for 50% inhibition	Relative effectiveness
Cellopentaose	7.4	1.0
Cellopentaitol	7.4	1.0
α-NAc-$(Glc)_5$-lys (**5a**)	8.0	0.93
Cellotetraose	8.5	0.87
$(Glc)_5$-NH_2 (**5b**)	11.5	0.64[a]
$(Glc)_4$-NH_2 (**4b**)	13.5	0.55
Cellotetraitol (**4c**)	14.2	0.52
Cellotriose	14.8	0.50
α-NAc-$(Glc)_4$-lys (**4a**)	17.0	0.44
Cellobiose	380	0.019
Cellotriitol	1100	0.0067
cellobiitol (**2c**)	2300	0.0032
α-NAc-$(Glc)_2$-lys (**2a**)	2750	0.0027
β-OMe-Glc	470000	1.6×10^{-5}
Glc	$>10^6$	$<10^{-5}$
Glucitol	$>10^6$	$<10^{-5}$

[a] The ^{1}H NMR spectrum of **5b** indicated the presence of an impurity which was not identified, therefore the validity of this value is in question.

the cellotetraose-polylysine conjugate and homologous antiserum. Cellotetraose, α-*N*-acetyl-ε-*N*-1(1-deoxycellotetraitol)-lysine [α-NAc-$(Glc)_4$-lys, **4a**], 1-deoxyaminocellotetraitol [$(Glc)_4$-NH_2, **4b**], and cellotetraitol (**4c**) gave 50% inhibition of maximal precipitation in amounts of 17.0, 19.0, 21.5 and 22.0 nmol, respectively. Cellotriose was also an effective inhibitor, requiring 34.0 nmol for 50% inhibition. The other compounds examined were much less effective as inhibitors. Cellobiose was only 0.020-fold as effective as cellotetraose, and cellotriitol was only half as effective as cellobiose (0.010-fold as effective as cellotetraose). Methyl β-D-glucopyranoside, glucose and glucitol were ineffective as inhibitors at concentrations where all other compounds tested were maximally inhibitory.

Cellopentaose-polylysine Antisera

Serum from one of the rabbits immunized with the cellopentaose-polylysine conjugate was used to determine the structural features of the pentasaccharide hapten (**1c**) important for antibody recognition. The results of these studies are given in Table 3 and representative inhibition curves are shown in Fig. 6. Cellopentaose, cellotetraose, and the three analogs derived from cellopentaose (**5a-c**) were all found to be excellent inhibitors of the precipitin reaction between conjugate **1c** and the homologous antiserum. Cellopentaose, cellopentaitol (**5c**), α-*N*-acetyl-ε-*N*-1(1-deoxycellopentaitol)-lysine [α-NAc-$(Glc)_5$-lys, **5a**], cellotetraose and 1-deoxyaminocellopentaitol [$(Glc)_5$-NH_2, **5b**] were 50% inhibitory when added in quantities of 7.4, 7.4, 8.0, 8.5 and 11.5 nmol, respectively. All five compounds possess at least four β1,4-linked D-glucopyranose residues,

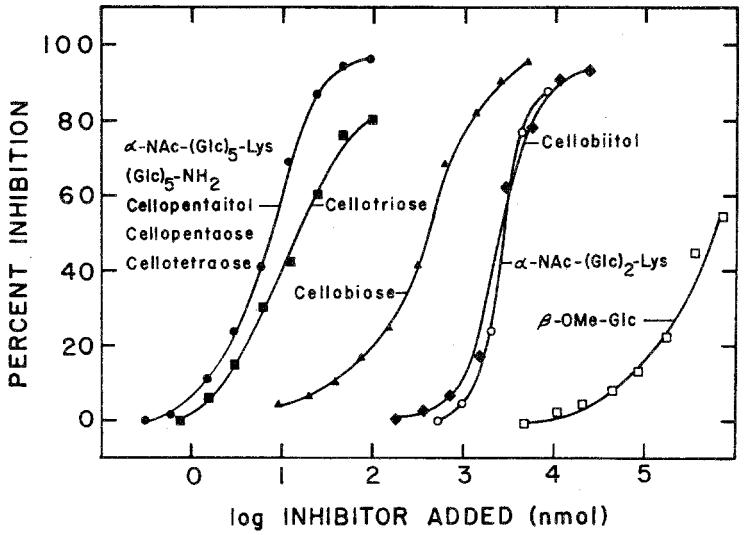
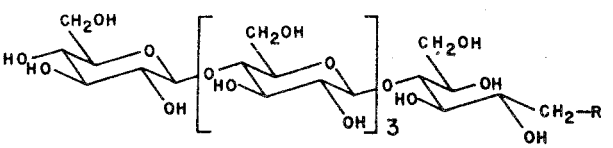


Figure 6. Inhibition of the precipitin reaction between the cellopentaose-polylysine conjugate and homologous rabbit serum by α-*N*-acetyl-ε-*N*-1(1-deoxycellopentaitol)-lysine, 1-deoxy-1-aminocellopentaitol, cellopentaitol, cellopentaose and cellotetraose (●), cellotriose (■), cellobiose (▲), cellobiitol (◆), α-*N*-acetyl-ε-*N*-1(1-deoxycellobiitol)-lysine (○) and methyl β-D-glucopyranoside (□). Two-fold-diluted immune rabbit serum (50 μl) and varying amounts of inhibitor were combined and diluted to a final volume of 450 μl with 0.15 M NaCl. After incubation of 2 h at 37°C, 50 μl of antigen solution (containing 2.25 μg of cellopentaose-polylysine) were added, and the reaction mixtures were thoroughly mixed and incubated 48 h at 4°C. The amount of precipitin formed was determined as described in the text.

and **5a-c** possess an additional acyclic reduced glucose residue. Compounds **5a** and **5b** both possess structural features of the linkage region. Compounds containing three β1,4-linked D-glucopyranose residues were also effective inhibitors of the precipitin reaction, but at slightly higher concentrations than inhibitors containing four glucopyranose residues. $(Glc)_4$-NH_2 (**4b**), cellotetraitol (**4c**), cellotriose and α-NAc-$(Glc)_4$-lys (**4a**) were 50% inhibitory when added in amounts of 13.5, 14.2, 14.8 and 17.0 nmol, respectively. Compounds containing fewer than three β1,4-linked D-glucopyranose residues were much less effective as inhibitors. Cellobiose and



5 a, R = $-\overset{+}{N}H_2(CH_2)_4\underset{NHAc}{\underset{|}{C}}HCO_2^-$

b, R = $-\overset{+}{N}H_3$

c, R = —OH

cellotriitol were only 0.019-fold and 0.0067-fold, respectively, as effective as cellopentaose, and cellobiitol (**2c**) and α-NAc-$(Glc)_2$-lys (**2a**) were even less effective (0.0032-fold and 0.0027-fold, respectively, relative to cellopentaose). Methyl β-D-glucopyranoside, glucose and glucitol were ineffective as inhibitors except at extremely high concentrations.

Discussion

Polylysine conjugates containing reductively aminated cellobiose, cellotetraose and cellopentaose are effective antigens in rabbits, and the antibodies which are formed are predominantly specific for the hapten. The detailed structural features of the haptens important for antibody recognition were established by quantitative hapten inhibition experiments, employing inhibitors containing various structural features of the carbohydrate hapten and the linkage region to the carrier protein.

Antibodies raised against the cellobiose-polylysine conjugate recognize four distinct structural regions of the hapten: the terminal β-D-glucopyranosyl residue, the acyclic reduced glucose residue, the protonated ammonium linkage, and the methylene arm of the lysine residue to which cellobiose is linked in the antigen. The lysine residue plays only a minor role in determining the specificity of the antibody combining region, as $(Glc)_2$-NH_2 (**2b**) is only slightly poorer as an inhibitor than α-NAc-$(Glc)_2$-lys (**2a**). The absence of the secondary ammonium linkage, however, in an otherwise identical analog drastically decreased inhibitory effectiveness; cellobiitol (**2c**) was approximately 34-fold less effective than **2b**, which demonstrates the immunodominance of the linkage region in antibodies raised against reductively aminated cellobiose-polylysine conjugates. In support of this conclusion, α-NAc-Glc-lys (**3**), which contains the structural features of the linkage region, was a relatively effective inhibitor. The internal, acyclic glucose residue also is important in determining antibody specificity, as cellobiitol (**2c**) is more than twice as effective as cellobiose as an inhibitor. The nonreducing terminal glucosyl residue forms the fourth structural region of the hapten for recognition. It is preferentially recognized in its β-pyranose form as cellobiitol is a 2-fold better inhibitor than maltitol, but it is not recognized on its own; i.e., glucose and methyl β-D-glucopyranoside are extremely poor inhibitors of the precipitin reaction, being only 0.01-fold as effective as cellobiitol.

These results are in good agreement with those reached from an identical study employing a bovine serum albumin conjugate containing reductively aminated cellobiose [3]. The one interesting difference is the decreased effectiveness of α-NAc-Glc-lys (**3**) as an inhibitor of the precipitin reaction in the cellobiose-bovine serum albumin system (0.000038-fold relative to **2a**) relative to the cellobiose-polylysine system (0.031-fold relative to **2a**). Although the origin of this 800-fold difference is not know with certainty, it probably arises due to differences in the microenvironments of the haptens in the respective carrier proteins. In the bovine serum albumin conjugate the lysyl residues through which the hapten is linked are in widely differing environments, due to the presence of different neighboring amino acids and to the effects of tertiary structure, while in the polylysine conjugate, the lysyl residues are in identical environments.

The results obtained from quantitative inhibition studies employing the reductively aminated cellobiose-polylysine conjugate are also in good agreement with the results

of studies using disaccharide-protein conjugates prepared by other procedures. In general, these studies have shown that there is antibody recognition of the non-reducing terminal sugar of the disaccharide and the configuration of its anomeric linkage, the reducing end monosaccharide residue in a *cyclic* or *acyclic* form depending upon the method of coupling, and the linkage region to the protein carrier [23-27].

Quantitative inhibition studies designed to determine the specificity of the precipitin reaction between the cellotetraose-polylysine conjugate and homologous antiserum demonstrated that all four tetrasaccharide derivatives [cellotetraose, α-NAc-$(Glc)_4$-lys, $(Glc)_4$-NH_2, and cellotetraitol] were approximately equally effective as inhibitors of conjugate-antiserum precipitation, thus demonstrating that the secondary ammonium linkage region is not an immunodominant feature of the cellotetraose hapten. Cellotriose was only two-fold poorer than cellotetraose as an inhibitor; thus, the absence of the fourth β1,4-linked glucose residue decreases the binding effectiveness only two-fold. Inhibitors which did not contain three intact glucopyranosyl residues were much less effective. Cellobiose was only 0.02-fold as effective as cellotetraose and cellotriitol was even less effective than cellobiose as an inhibitor. The monosaccharide analogs were ineffective as inhibitors, as expected, as each possesses only one structural feature in common with the tetrasaccharide.

Thus, the antibody combining region of the anti-cellotetraose-polylysine antibody can be envisioned to contain four subsites. The first three subsites from the non-reducing end of the saccharide have a strict requirement for β1,4-linked glucopyranosyl residues, while the structural requirements for binding in the fourth subsite are much less demanding. The fourth subsite can accomodate a *cyclic* or *acyclic* glucose residue, either with or without the protonated amine group or reductively aminated lysine residue. The immunodominance of the secondary ammonium linkage that was observed with the cellobiose-polylysine conjugate is therefore lost when the size of the oligosaccharide hapten is increased to the tetrasaccharide.

Similar observations have been made in studies utilizing tetrasaccharide conjugates prepared by other coupling methods. Zopf *et al.* [28] examined the specificity of rabbit antibodies to the conjugate edestin-N=N-C_6H_4-CH_2CH_2NH-(1-deoxymannotetraitol), prepared by reductive amination of mannotetraose to β-(*p*-aminophenyl)-ethylamine and subsequent diazonium coupling, and observed that they were specific for carbohydrate chains containing three mannopyranosyl residues with the correct linkages. In contrast to the results obtained with the cellotetraose-polylysine conjugate, however, binding to the antibody was further enhanced if the inhibitor contained a fourth *acyclic* mannose residue; i.e., the reduced tetrasaccharide and its reductively aminated β-(*p*-aminophenyl)-ethylamine derivative were 11-fold and 47-fold, respectively, more effective inhibitors of the precipitin reaction between the conjugate and homologous antiserum than was the intact tetrasaccharide. The same technique was used to prepare a protein conjugate of another tetrasaccharide, lacto-*N*-tetraose ($LcOse_4$), and hapten inhibition experiments with the homologous antiserum again demonstrated preferential binding of the reduced oligosaccharide; i.e., lacto-*N*-tetraitol and its reductively aminated β-(*p*-aminophenyl)-ethylamine derivative were 833-fold and 1250-fold better inhibitors than lacto-*N*-tetraose.

Inhibition studies using antisera to the reductively aminated cellotetraose-polylysine conjugate demonstrated that the immunodominance of the linkage region is lost as the

size of the attached oligosaccharide hapten is increased from a disaccharide to a tetrasaccharide, but from these studies, no conclusion can be drawn as to the dimensions of the antibody combining site for cellulose oligosaccharides. For this reason, a similar study was performed using antisera to a reductively aminated cellopentaose-polylysine conjugate. The most effective inhibitors of the precipitin reaction between conjugate **1c** and its homologous antiserum (Fig. 3, Table 3) were the five analogs containing at least four contiguous β1,4-linked glucopyranose residues, i.e., cellopentaose, cellotetraose, cellopentaitol (**5c**), α-NAc-$(Glc)_5$-lys (**5a**), and $(Glc)_5$-NH_2 (**5b**). Those pentasaccharide analogs containing one or more structural features of the linkage region (**5a, b, c**) were no more effective as inhibitors than was cellopentaose, demonstrating that the carbohydrate-protein linkage no longer plays a significant role in determining the specificity of these antibodies. These analogs (**5a, b, c**) were not signficantly poorer as inhibitors than cellopentaose, however, indicating that the linkage region residue did not interfere with binding to the antibody. Analogs which contain three contiguous β1,4-linked glucopyranose residues, i.e., cellotriose, cellotetraitol, α-NAc-$(Glc)_4$-lys (**4a**) and $(Glc)_4$-NH_2 (**4b**), were approximately equally effective as inhibitors and were only two-fold poorer than analogs containing four contiguous β1,4-linked glucopyranose residues. Analogs containing fewer than three β1,4-linked glucopyranose residues were ineffective as inhibitors.

The combining region of antibodies to the reductively aminated cellopentaose-polylysine hapten can therefore also be divided into four subsites; the first three subsites have a strict requirements for β1,4-linked glucopyranosyl residues, but the binding requirements for the fourth subsite are not as stringent. Glucopyranose residues are preferentially bound in this subsite, but acyclic glucose residues are also tolerated. The anti-cellopentaose-polylysine antibody shows virtually the same specificity of binding as does the anti-cellotetraose-polylysine antibody. These results, and the fact that cellopentaose is at most only slightly more effective than cellotetraose as an inhibitor of precipitation between the cellopentaose-polylysine conjugate and its homologous antiserum, demonstrate that the upper limit to the size of the rabbit antibody combining site for cellulose oligomers is four β1,4-linked glucopyranose residues.

In conclusion, the unusual secondary ammonium linkage formed in the direct reductive amination of cellulose oligosaccharides and polylysine does not retain its immunodominance in conjugates prepared with tetrasaccharide or pentasaccharide haptens. Although these results cannot be generalized for conjugates containing other oligosaccharide haptens, they serve to demonstrate that the direct reductive amination procedure warrants consideration as a means to prepare immunogens containing oligosaccharide haptens where highly specific anti-carbohydrate antibodies are desired.

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