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THE PREPARATION OF CARBOHYDRATE–PROTEIN CONJUGATES: CYANURIC TRICHLORIDE COUPLING OF 2-AMINOETHYL GLYCOSIDES, AND MIXED-ANHYDRIDE COUPLING OF 8-CARBOXYOCTYL GLYCOSIDES TO BOVINE SERUM ALBUMIN*†

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ABSTRACT

Preparation of the following glycosides is described: 2-aminoethyl β-D-glycosides of (A) 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranose, (B) 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranose (*N*,*N*′-diacetylchitobiose pentaacetate), (C) 4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranose (cellobiose heptaacetate); 8-carboxyoctyl glycosides of (D) cellobiose, and (E) *N*,*N*′-diacetylchitobiose. Conjugates were prepared from (A), (B), and (C) by coupling to bovine serum albumin by cyanuric trichloride and subsequent deacetylation; (D) and (E) were coupled to bovine serum albumin by the mixed-anhydride reaction. Conjugates (A) and (B) were insoluble; conjugates (C), (D), and (E) functioned as artificial antigens and gave rise to precipitating antibodies in rabbits. Specificities of the antisera were determined by inhibition studies.

INTRODUCTION

The preparation of carbohydrate–protein conjugates may be achieved in several ways. The methods used most extensively have involved the synthesis of a saccharide derivative containing an aromatic amino group that can be coupled to a carrier protein by diazotization[1–4]. Other techniques are the preparation of serine glycosides that can then be coupled to protein by the Leuchs type of anhydride[5], and the generation of aldonic acid end-groups that can be coupled to protein by a mixed-anhydride reaction[6]. More recently, Lemieux, Bundle, and Baker reported the synthesis of blood-group antigens in which the oligosaccharide determinants were coupled to bovine serum albumin by way of an 8′-(azidocarbonyloctyl) aglycon[7].

*Dedicated to the memory of Professor J. K. N. Jones, F.R.S.

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A previous report from this laboratory described the use of cyanuric trichloride (2,4,6-trichloro-*s*-triazine) to couple carbohydrates to amino acids as a model system for the preparation of carbohydrate–protein conjugates[8]. The present paper reports the preparation of some new carbohydrate derivatives that are suitable for coupling to proteins. One series of derivatives involves preparation of *O*-glycosides of ethanolamine, thus providing a free amino group for coupling by the cyanuric trichloride procedure. The other series contained the same carbonyloctyl aglycon as used by Lemieux *et al.*[7], but coupling was achieved by the mixed-anhydride reaction[6,9] rather than through the azidocarbonyl derivative. As additional evidence that coupling had occurred as predicted, the carbohydrate–protein conjugates were tested for antigenicity in rabbits and the specificities of antisera obtained were checked by inhibition studies.

EXPERIMENTAL

General methods. — Melting points are uncorrected and were determined on a Kofler hot-stage microscope. Optical rotations were measured with a Perkin–Elmer 141 polarimeter and are equilibrium values unless stated otherwise. Infrared spectra were recorded with a Perkin–Elmer Infracord spectrophotometer using Nujol mulls of the compounds. P.m.r. spectra were recorded on a Varian A-60A or Anaspect EM360 n.m.r. spectrometer with tetramethylsilane as a standard (internal or external as noted). Quantitative analyses of amino sugars and amino acids were obtained by ion-exchange chromatography with a Model 1200 Spinco amino acid analyzer[10]. Hexoses were estimated by the phenol–sulfuric acid method[11]. T.l.c. was performed on glass plates coated with Silica Gel F-254 (containing a fluorescent indicator) with the following solvent mixtures (v/v): (A) 9:1 chloroform–ethanol, (B) 3:2 ethyl acetate–light petroleum (40–60°). To detect components on thin-layer chromatograms, the plates were examined under ultraviolet light and were also sprayed with 5% sulphuric acid in ethanol and then charred at 110°.

2-Aminoethyl glycosides

N-2-(Benzyloxycarbonyl)aminoethanol (**1**). — Benzylchloroformate (10 ml) was added dropwise with stirring to a solution of ethanolamine (2 ml) in water (5 ml) at 0°. Sodium carbonate (5 g) was added in small portions over 1 h and the mixture was then stirred at for 18 h room temperature (24°). The mixture was extracted with chloroform and the extract was washed with water, dried (sodium sulfate), and evaporated to yield a crystalline residue. Recrystallization from ether–light petroleum (40–60°) gave compound **1** (8.3 g), m.p. 59–60°; ν_{max} 3310 (OH, NH), 1690 (CONH), and 1545 cm^{-1} (NH).

Anal. Calc. for $C_{10}H_{13}NO_3$: C, 61.52; H, 6.71; N, 7.18. Found: C, 61.41; H, 6.65; N, 7.11.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (**3**). — A mixture of compound **1** (2.0 g), 2-acetamido-3,4,6-tri-*O*-

acetyl-2-deoxy-α-D-glucopyranosyl chloride[12] (**2**, 4.5 g), cadmium carbonate (2.5 g), and Drierite (5.0 g) in 1:1 toluene–nitromethane (100 ml) was stirred and heated under reflux until examination by t.l.c. (solvent A) indicated complete reaction of **2** (~0.5 h). The inorganic residue was removed by filtration and washed with chloroform (2 × 75 ml). The combined filtrate and washings were evaporated to dryness and the residue was crystallized from ethanol to yield compound **3** (3.7 g), m.p. 170–172°, $[\alpha]_D^{24}$ −15.0° (*c* 1.0, chloroform); ν_{max} 3365–3320 (NH), 1740 (OAc), 1695, 1655 (CONH), 1550, and 1528 cm^{-1} (NH).

Anal. Calc. for $C_{24}H_{32}N_2O_{11}$: C, 54.96; H, 6.11; N, 5.34. Found: C, 54.73; H, 6.02; N, 5.11.

Deacetylation of a small portion of **3** by sodium methoxide yielded 2-(benzyloxycarbonyl)aminoethyl 2-acetamido-2-deoxy-β-D-glucopyranoside, a new glycoside of 2-acetamido-2-deoxy-D-glucose, m.p. 188–190°, $[\alpha]_D^{24}$ −21.5° (*c* 1.0, chloroform); ν_{max} 3180–3350 (OH, NH), 1690–1655 (CONH), and 1525–1550 cm^{-1} (NH); n.m.r. (D_2O): τ 2.46 (5 H singlet, aryl), 4.81 (2 H singlet, benzylic protons), 5.38 (1 H doublet, $J_{1,2}$ 6.0 Hz, H-1), 7.93 (3 H singlet, N-Ac).

Anal. Calc. for $C_{18}H_{26}N_2O_8$: C, 54.26; H, 6.58; N, 7.03. Found: C, 53.94; H, 6.44; N, 7.24.

2-Aminoethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (**4**). — The *N*-benzyloxycarbonyl derivative **3** (2.5 g), in 95% ethanol (40 ml), was stirred with palladium black (500 mg) for 18 h under hydrogen at atmospheric pressure. The catalyst was removed by filtration and the filtrate was evaporated to dryness under diminished pressure. The residue was crystallized from acetone to give compound **4** (1.46 g), m.p. 225–227°, $[\alpha]_D^{24}$ −8.5° (*c* 1.0, ethanol); ν_{max} 3350–3250 (NH), 1745 (OAc), 1660 (CONH), 1550, and 1530 cm^{-1} (NH).

Anal. Calc. for $C_{16}H_{26}N_2O_9$: C, 49.22; H, 6.71; N, 7.18. Found: C, 49.18; H, 6.58; N, 6.73.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**6**). — A mixture of compound **1** (0.7 g), 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[13] (**5**, 3.5 g), cadmium carbonate (1.2 g), and Drierite (4 g) in 1:1 toluene–nitromethane (80 ml) was stirred and heated under reflux until t.l.c. (solvent A) showed complete reaction of **5** (~2.5 h). The inorganic residue was removed by filtration and washed with chloroform (2 × 100 ml). The combined filtrate and washings were evaporated under diminished pressure to yield a syrup. Purification of this residue by preparative t.l.c. (solvent A) and crystallization from ethanol gave compound **6** (1.28 g), m.p. 236–238°, $[\alpha]_D^{24}$ −29.5° (*c* 0.5, chloroform); ν_{max} 3300 (NH), 1748 (OAc), 1695, 1660 (CONH), and 1545 cm^{-1} (NH).

Anal. Calc. for $C_{36}H_{49}N_3O_{18}$: C, 53.27; H, 6.08; N, 5.18. Found: C, 53.10; H, 6.02; N, 5.04.

2-Aminoethyl 2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**7**). — Compound **6**

(1.1 g) in 95% aqueous methanol (75 ml) was stirred with palladium black (200 mg) for 18 h under hydrogen at atmospheric pressure. The catalyst was removed by filtration, and evaporation of the filtrate left a residue that was crystallized from acetone to yield compound **7** (765 mg), m.p. 175° (decomp.), $[\alpha]_D^{24}$ −16.8° (*c* 1.0, ethanol); ν_{max} 3325–3250 (NH), 1745 (OAc), 1665 (CONH), and 1550–1530 cm^{-1} (NH).

Anal. Calc. for $C_{28}H_{43}N_3O_{16}$: C, 49.63; H, 6.40; N, 6.20. Found: C, 49.31; H, 6.43; N, 5.76.

2-(Benzyloxycarbonyl)aminoethyl 4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**9**). — A mixture of compound **1** (0.5 g), 4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl bromide[14] (**8**, 2.5 g), cadmium carbonate (0.8 g), and Drierite (3 g), in 1:1 toluene–nitromethane (60 ml) was stirred and heated under reflux until examination by t.l.c. (solvent B) indicated complete reaction of **8** (~0.75 h). The ionorganic residue was removed by filtration and washed with chloroform (2 × 75 ml). The combined filtrate and washings were evaporated to a syrup. Purification of this residue by preparative t.l.c. yielded the title compound (**9**, 1.53 g) that was crystallized from ether; m.p. 106–107°, $[\alpha]_D^{24}$ −19.0° (*c* 1.0, chloroform); ν_{max} 3350 (NH), 1750 (OAc), 1700 (CONH), and 1530 cm^{-1} (NH).

Anal. Calc. for $C_{36}H_{47}NO_{20}$: C, 53.14; H, 5.78; N, 1.62. Found: C, 53.10; H, 5.62; N, 1.41.

2-Aminoethyl 4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**10**). — The benzyloxycarbonyl derivative (**9**, 1.0 g) was hydrogenated in 95% ethanol over palladium black (200 mg) as described for compound **7**. The product was crystallized from ether to give the title compound (**10**, 760 mg), m.p. 152–154°, $[\alpha]_D^{24}$ −8.6° (*c* 1.0, ethanol); ν_{max} 3380 (NH), 1750 (OAc), and 1550 cm^{-1} (NH).

Anal. Calc. for $C_{28}H_{41}NO_{18}$: C, 49.48; H, 6.04; N, 2.06. Found: C, 49.33; H, 5.97; N, 1.94.

8-(Methoxycarbonyl)octyl glycosides

8-(Methoxycarbonyl)octyl 4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**12**). — A mixture of 8-(methoxycarbonyl)octanol[15] (**11**, 0.5 ml), 4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl bromide[14] (**8**, 2.0 g), cadmium carbonate (0.6 g), and Drierite (3 g) in toluene–nitromethane (1:1, 50 ml) was stirred and heated under reflux until examination by t.l.c. (solvent B) indicated complete reaction of **8** (~1 h). The inorganic residue was removed by filtration, and washed with chloroform (2 × 75 ml), and the combined filtrate and washings were evaporated to dryness. The syrupy residue was purified by preparative t.l.c. (solvent B), and crystallized from ether to give the title compound (**12**, 0.85 g), m.p. 92–94°, $[\alpha]_D^{24}$ −14.3° (*c* 1.0, chloroform).

Anal. Calc. for $C_{36}H_{54}O_{20}$: C, 53.59; H, 6.75. Found: C, 53.41; H, 6.82.

8-(Methoxycarbonyl)octyl 4-O-β-D-glucopyranosyl-β-D-glucopyranoside (**13**). — Compound (**12**, 800 mg) was deacetylated in methanol (20 ml) by sodium methoxide (0.1M, 25 ml). The solution was deionized by Amberlite IR-120 (H^+) resin and evaporated to dryness to give **13** as a syrup, $[\alpha]_D^{24}$ −10.7° (*c* 1.0, methanol); ν_{max} 1745 cm^{-1} (CO_2Me); p.m.r. (D_2O, external tetramethylsilane) τ 5.49 and 5.52 (1H doublets, $J_{1,2}$ 7.5 Hz, H-1 and H-1'), 8.71 (3H singlet, CO_2Me).

8-Carboxyoctyl 4-O-β-D-glucopyranosyl-β-D-glucopyranoside (**14**). — A solution of the methyl ester (**13**, 400 mg) in 0.1M sodium hydroxide (10 ml) was kept for 2 h at 24°. The solution was deionized with Amberlite IR-120 (H^+) resin and freeze-dried to yield **14** (374 mg) as a white powder that had $[\alpha]_D^{24}$ −10.4° (*c* 1.0, water); ν_{max} 1720 cm^{-1} (CO_2H).

8-(Methoxycarbonyl)octyl 2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**15**). — A mixture of 8-(methoxycarbonyl)octanol[15] (**11**, 0.5 ml), 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[13] (**5**, 1.8 g), cadmium carbonate (0.5 g), and Drierite (3.0 g) in toluene–nitromethane (1:1, 50 ml) was stirred and heated under reflux until examination by t.l.c. (solvent A) indicated complete reaction of **5** (~3 h). Chloroform (150 ml) was added, the inorganic solids were removed by filtration, and the filtrate was washed with saturated aqueous sodium hydrogencarbonate (75 ml) and with water (75 ml). Evaporation of the organic solvents left a syrup that crystallized from methanol to give **15** (730 mg), m.p. 216–218°, $[\alpha]_D^{24}$ −26.2° (*c* 1.0, methanol).

Anal. Calc. for $C_{36}H_{56}N_2O_{18}$: C, 53.71; H, 7.02; N, 3.48. Found: C, 53.62; H, 7.13; N, 3.29.

8-(Methoxycarbonyl)octyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**16**). — Deacetylation of compound **15** (700 mg), as described for compound **12**, yielded compound **16** (412 mg) that crystallized from water, m.p. 270° (decomp.), $[\alpha]_D^{24}$ −19.2° (*c* 1.0, water); ν_{max} 1745 (CO_2CH_3), 1660 (CONH), and 1545 cm^{-1} (NH); p.m.r. (D_2O, external tetramethylsilane): τ 5.37 and 5.40 (1H doublets, $J_{1,2}$ 6.5 Hz, H-1 and H-1'), 7.83 (3H singlet, COMe), 7.87 (3H singlet, COMe), 8.60 (3H singlet, CO_2Me).

Anal. Calc. for $C_{26}H_{46}N_2O_{13}$: C, 52.53; H, 7.80; N, 4.71. Found: C, 52.18; H, 7.46; N, 4.54.

8-Carboxyoctyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**17**). — The ester **16** (390 mg) was saponified as described for preparation of compound **14** to yield **17** (360 mg) as a powder having $[\alpha]_D^{24}$ −18° (*c* 1.0, water) and ν_{max} 1720 cm^{-1} (CO_2H).

Coupling to bovine serum albumin

(A) *By cyanuric trichloride (2,4,6-trichloro-s-triazine).* — Compounds, **4**, **7**, and **10** were coupled separately to bovine serum albumin (BSA) by the same procedure[8]. The compound (500 mg) was dissolved in water (15 ml) and the solution was cooled to 0°. To the cooled, stirred solution was added a solution of cyanuric trichloride

(230 mg) in 1,4-dioxane (10 ml). The mixture was stirred for 1 h at 0° during which time the pH was maintained at 7.0 by the slow addition of 10% aqueous sodium hydrogencarbonate. A solution of bovine serum albumin (1.5 g) in 0.8% aqueous sodium chloride (25 ml) was then added and the mixture was stirred for 1 h at 45°. Products of low molecular weight were removed by dialysis against distilled water for 35 h and the non-dialyzable material was recovered by freeze-drying. This product was suspended in anhydrous methanol (125 ml) and the suspension was saturated at 0° with dry ammonia. After being kept for 3 days at 0°, the mixture was evaporated to dryness under diminished pressure at 40°. The deacetylated residue was suspended in water, dialyzed against distilled water for 3 days, and then freeze-dried to yield the conjugates A, B, and C (see Scheme 1). Portions of A, B, and C were analyzed for amino acids, amino sugars, and hexose (Table I). The amounts of carbohydrate incorporated corresponded to the drop in lysine contents, and indicated that A, B, and C contained 19, 19, and 24 moles of carbohydrate determinant/mole BSA respectively, coupled through the ε-amino group of lysine.

TABLE I

ANALYSES OF CARBOHYDRATE–BSA CONJUGATES

Conjugate	L-*Lysine*[a]		*2-Amino-2-deoxy*-D-*Glucose*[a,b]		D-*Glucose*[c]	
	%	*mol./mol.* BSA	%	*mol./mol.* BSA	%	*mol./mol.* BSA
A	8.5	51	10.5	19	—	—
B	8.7	50	5.6	19	—	—
C	7.3	44	trace	—	10.3	24
D	11.7	68	trace	—	7.7	18
E	11.3	66	8.5	15	—	—
BSA	12.9	68	0.1	—	—	—

[a]Hydrolysis, 16 h, 100°, 6M hydrochloric acid. [b]Corrected for losses during hydrolysis. [c]Phenol–sulfuric acid determination[11] directly on conjugate.

(B) *By mixed anhydride.* — Compounds **14** and **17** were coupled separately to bovine serum albumin by the same procedure[6]. In each case, BSA (500 mg) was dissolved in water (10 ml) and cold *N,N*-dimethylformamide (6 ml) was added. The pH of the solution was adjusted to 11.9 with 0.1M sodium hydroxide and the solution was kept cold in an ice bath. The compound (**14** or **17**) (200 mg) was dissolved in cold *N,N*-dimethylformamide (6 ml) containing tributylamine (0.12 ml) and cold isobutyl chloroformate (0.10 ml) was added. This mixture was stirred in an ice bath for 15 min before a second addition of tributylamine (0.10 ml), and was then added immediately to the BSA solution. The pH of the mixture was adjusted to 11.9 with 0.1M sodium hydroxide and the mixture was then stirred for 18 h at 24°. Material of low molecular weight was removed by dialysis for four days against distilled water. The soluble, non-dialyzable material was recovered by freeze-drying, and was then passed through a

column of Biogel P-6. Analysis of hydrolyzates indicated that D and E (Scheme 1) contained 18 and 15 moles of carbohydrate determinant/mole BSA, respectively (see Table I).

Antigenicity tests. — The carbohydrate–protein conjugate (5 mg) was dissolved or suspended in 0.15M saline (2.5 ml) and emulsified with Freund's complete adjuvant (2.5 ml). Male New Zealand rabbits were given weekly injections; 100 µl in the toe pad and 300 µl subcutaneously for 3 consecutive weeks. Trial bleedings were made 6 days after the final injection and if the antibody titre was not adequate the rabbits were rested for 6 weeks and the injection schedule was repeated.

Quantitative precipitin tests. — Quantitative precipitin tests and hapten-inhibition studies were done by standard procedures[16]. Inhibition studies were performed with ratios of antigen and antisera that gave maximum precipitation.

RESULTS AND DISCUSSION

The preparation of the conjugates is summarized in Scheme 1. This involved the synthesis of a series of *O*-β-D-glycosides in which the aglycon possessed a terminal grouping that could be coupled to a carrier protein. The syntheses were achieved by reaction of appropriate alcohols with acetylated glycosyl halides of the carbohydrates[17]. In one series of compounds, the alcohol was 2-aminoethanol with the amino group protected as the *N*-benzyloxycarbonyl derivative. The resulting derivatives of 2-amino-2-deoxy-D-glucose (3), chitobiose (6), and cellobiose (9) were then

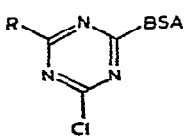
2-Aminoethyl glycosides: R-O-CH_2CH_2-NHR′

R = 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl (3, 4)
R = penta-*O*-acetyl-*N*,*N*′-diacetyl-β-D-chitobiosyl (6, 7)
R = hepta-*O*-acetyl-β-D-cellobiosyl (9, 10)
R′ = benzyloxycarbonyl (3, 6, 9)
R′ = H (4, 7, 10)

8-Carboxyoctyl glycosides: R-O-$[CH_2]_8$-C(=O)-OR′

R = hepta-*O*-acetyl-β-D-cellobiosyl (12)
R = penta-*O*-acetyl-*N*,*N*-diacetyl-β-D-chitobiosyl (15)
R = β-D-cellobiosyl (13, 14)
R = *N*,*N*-diacetyl-β-D-chitobiosyl (16, 17)
R′ = CH_3 (12, 13, 15, 16)
R′ = H (14, 17)

Conjugates:



R′-BSA

	R		*R′*
A	(4, deacetylated)	D	(14)
B	(7, deacetylated)	E	(17)
C	(10, deacetylated)		

Scheme 1.

hydrogenated to remove the *N*-benzyloxycarbonyl groups and provide the free amines (**4**, **7**, and **10**) for conjugation with the carrier protein. In the other series of compounds, the alcohol was 8-carboxyoctanol with the carboxyl group protected as the methyl ester. Reaction of this alcohol with acetylated glycosyl halides of cellobiose and chitobiose gave the glycosides **12** and **15**, respectively. Deacetylation gave the methyl esters **13** and **16**, which were then fully saponified to the free carboxylic acids **14** and **17**, suitable for conjugation with protein. To the best of our knowledge, none of these derivatives have been reported previously.

The 2-aminoethyl glycoside acetates (**4**, **7**, and **10**) were coupled to bovine serum albumin by cyanuric trichloride using the two-step, temperature-controlled procedure[8]. The coupled products were deacetylated to give the conjugates A, B, and C containing respectively 2-amino-2-deoxy-D-glucose, chitobiose, and cellobiose in the amounts of 19, 19, and 24 mol./mol. BSA. When attempts were made to couple deacetylated **4**, **7**, and **10**, the resulting products were completely insoluble. The 8-carboxyoctyl glycosides (**14** and **17**) were coupled to bovine serum albumin by the mixed-anhydride procedure[6] to yield conjugates D and E containing respectively cellobiose and chitobiose in the amounts of 18 and 15 mol./mol. BSA.

The two coupling strategies were used for comparative purposes. It was of interest to see if the model studies of cyanuric trichloride as a coupling agent[8] could indeed be extended to a polymer system, and if there were any major differences between the two series of conjugates that contained the same determinants, but different bridging groups. The amounts of carbohydrate determinants in the conjugates were much the same in each series (15–24 mol./mol BSA). These degrees of substitution were lower than those obtained by Lemieux *et al.* using azido coupling (30–38 mol./mol. BSA)[7], higher than those given by phenyl isothiocyanate coupling (4 mol./mol. BSA)[18], and about the same as those obtained by diazo coupling of *p*-aminophenyl glycosides (14–17 mol./mol. of BSA)[19].

Antisera from rabbits that had been immunized with the five conjugates were examined by immunodiffusion in agarose gel, and quantitative precipitin tests were conducted on those that gave a positive response. Conjugates A and B did not generate precipitating antibody in rabbits, probably because of the insolubility of those products. Another explanation might have been that the triazine compound[20] or the amino sugar determinants were metabolized. However, the positive responses given by conjugates C and D indicated that both of these groups remained stable. The introduction of the hydrophobic triazine ring in A, B, and C seemed to cause greater insolubility than the octyl group in D and E; the latter two products were readily solubilized. Conjugate C, while sufficiently soluble (~0.3 mg/ml) to produce an antigenic response in rabbits, could not be dissolved in amounts sufficient for quantitative precipitin tests. Thus, the cellobiose-specific antibody in antiserum C was determined by precipitation with conjugate D that contained the same determinant. Fig. 1 shows the results of quantitative precipitin tests on the three antisera. Typical precipitin curves were obtained and each antiserum contained antibodies to the carrier protein (BSA) in addition to those directed towards the carbohydrate

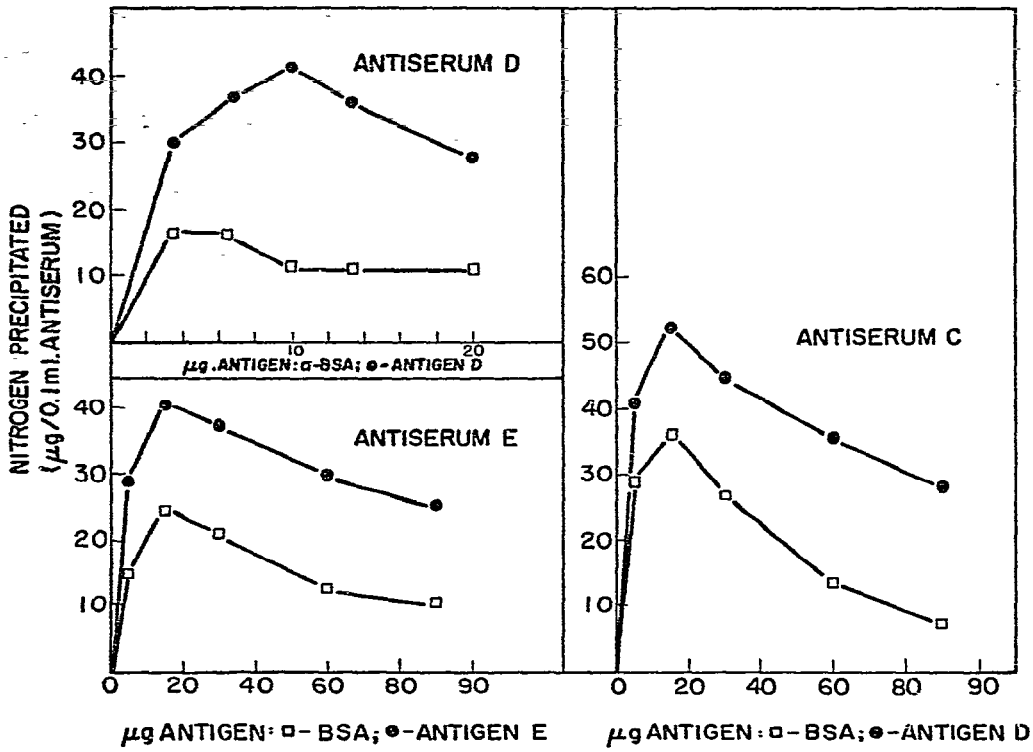


Fig. 1. Quantitative precipitin curves for carbohydrate–protein conjugates and their antisera.

TABLE II

INHIBITION OF PRECIPITIN REACTIONS

Inhibitor	*μM required for 50% inhibition*[a]		
	1	2	3
Cellobiose [β-D-Glc*p*-(1→4)-D-Glc*p*]	0.8	0.6	3.8
Maltose [α-D-Glc*p*-(1→4)-D-Glc*p*]	2.0	1.0	15.3
Gentobiose [β-D-Glc*p*-(1→6)-D-Glc*p*]	2.7	0.8	7.5
Sophorose [β-D-Glc*p*-(1→2)-D-Glc*p*]	4.1	0.7	15.3
Chitobiose [β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc]	4.8	16.5	0.4
Kojibiose [α-D-Glc*p*-(1→2)-D-Glc*p*]	5.0	0.7	7.5
Melibiose [α-D-Gal*p*-(1→6)-D-Glc*p*]	8.1	7.0	∞

[a] 1, Antiserum C–antigen D (cellobiose determinant); 2, antiserum D–antigen D (cellobiose determinant); 3, antiserum E–antigen E (chitobiose determinant).

determinant. The specificities of each antiserum for its carbohydrate determinant were tested by inhibition of the precipitin reaction with a series of disaccharides. The results, given in Table II, showed that, for each of the three antisera, the best inhibitor was the disaccharide that corresponded to the determinant in the artificial antigen. Antisera C and E showed a greater degree of specificity than antiserum D, in which several disaccharides of D-glucose were almost as good inhibitors as the determinant cellobiose. However, comparisons of titres and specificities of antisera from different individuals or species are always uncertain because of the genetic variability of the immune response.

The present results show that carbohydrate–protein conjugates can be prepared successfully by the two methods: (a) cyanuric trichloride coupling of 2-aminoethyl glycosides, (b) mixed-anhydride coupling of 8-carboxyoctyl glycosides. Both procedures involve the initial preparation of a glycoside and subsequent removal of protective groups from the aglycon prior to coupling, and both procedures retain the complete structural integrity of the carbohydrate. The degrees of substitution are similar by both procedures and occur through the ε-amino groups of lysine residues. The low solubility of the conjugates prepared by cyanuric trichloride coupling would seem to limit the application of this procedure for the preparation of artificial antigens. It is possible that the solubility properties of antigens prepared by that procedure could be improved by variation of the carrier protein or by a lower degree of substitution. On the other hand, the insolubility of the products linked by cyanuric trichloride could make this a preferred procedure for the preparation of absorbents for affinity chromatography.

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REFERENCES

1 O. T. Avery and W. F. Goebel, *J. Exp. Med.*, 50 (1929) 533–550.
2 P. Q. Allen, I. J. Goldstein, and R. N. Iyer, *Biochemistry*, 6 (1967) 3029–3036.
3 O. Lüderitz, A. M. Staub, and O. Westphal, *Bacteriol. Rev.*, 30 (1966) 192–255.
4 K. Himmelspach, O. Westphal, and B. Teichmann, *Eur. J. Immunol.*, 1 (1971) 106–112.
5 E. Rüde, M. Meyer-Delius, and M.-L. Gundelach, *Eur. J. Immunol.*, 1 (1971) 113–123.
6 Y. Arakatsu, G. Ashwell, and E. A. Kabat, *J. Immunol.*, 97 (1966) 858–866.
7 R. U. Lemieux, D. R. Bundle, and D. A. Baker, *J. Am. Chem. Soc.*, 97 (1975) 4076–4083.
8 A. S. Chaudhari and C. T. Bishop, *Can. J. Chem.*, 50 (1972) 1987–1991.
9 B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.*, 228 (1957) 713–727.
10 M. Yaguchi and M. B. Perry, *Can. J. Biochem.*, 48 (1970) 386–388.
11 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
12 D. Horton, *Org. Syn.*, 46 (1966) 1–5.
13 M. Spinola and R. W. Jeanloz, *J. Biol. Chem.*, 345 (1970) 4158–4162.
14 G. Zemplén, *Ber.*, 53 (1920) 996–1006.
15 N. M. Yoon, C. S. Pak, H. C. Brown, S. Krishnamurthy, and T. P. Stocky, *J. Org. Chem.*, 38 (1973) 2786–2792.

16 E. A. KABAT AND M. M. MAYER, *Experimental Immunochemistry*, 2nd ed., Charles C. Thomas, Springfield, Ill., U. S. A., 1961.
17 W. KOENIGS AND E. KNORR, *Ber.*, 34 (1901) 957–981.
18 A. A. LINDBERG, L. T. ROSENBERG, A. LJUNGGREN, P. J. GAREGG, S. SVENSSON, AND N. H. WALLIN, *Infect. Immun.*, 10 (1974) 541–545.
19 I. J. GOLDSTEIN AND R. N. IYER, *Biochim. Biophys. Acta*, 121 (1966) 197–200.
20 R. H. SHIMABUKURO, G. L. LAMOUREUX, D. S. FREAN, AND J. E. BAKKE, in A. S. TAHORI (Ed.), *Pesticide Terminal Residues*, Butterworths, London, 1971.