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6-AMINOHEXYL GLYCOPYRANOSIDES AS LIGANDS FOR THE PREPARATION OF AFFINITY ADSORBENTS FOR THE PURIFICATION OF CARBOHYDRATE-BINDING PROTEINS*

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ABSTRACT

Syntheses are described of 6-aminohexyl and 2-aminoethyl glycopyranosides of the monosaccharides commonly found in glycoproteins and glycolipids. The ligands have been coupled to agarose, and the adsorbents so formed have been used in the isolation of the lectin from wheat-germ extracts. Various procedures for the formation of glycopyranosides from ^{14}C-labelled per-*O*-acetylglycosyl halides and 6-(trifluoroacetamido)hexanol have been examined. Silver carbonate with a trace of iodine is the best catalyst found thus far for glycosidation of simple sugars, and mercuric cyanide for that of 2-acetamido-2-deoxyaldoses. With other catalysts, higher proportions of ortho esters are obtained. The rearrangement of an ortho ester by *p*-toluenesulfonic acid gives the glycoside, a partially deacetylated glycoside, and a substantial proportion of the aglycon acetate. A simple procedure for isolation of the deacetylated glycosides by use of chromatography on Dowex-50 (pyridinium form) is described.

INTRODUCTION

The functional role of the oligosaccharide portions of glycoproteins and glycolipids in biological systems is not yet fully established, but it is evident that the oligosaccharides frequently provide "recognition sites" for the binding of complex molecules, or structures, to one another[1].

It has been shown that extracts of many plants contain proteins that can bind carbohydrates. Some of these proteins, the lectins, cause agglutination, and have been used to classify such animal cells as erythrocytes. Relatively few of the lectins have been purified to homogeneity and characterized, although the number is increasing[2].

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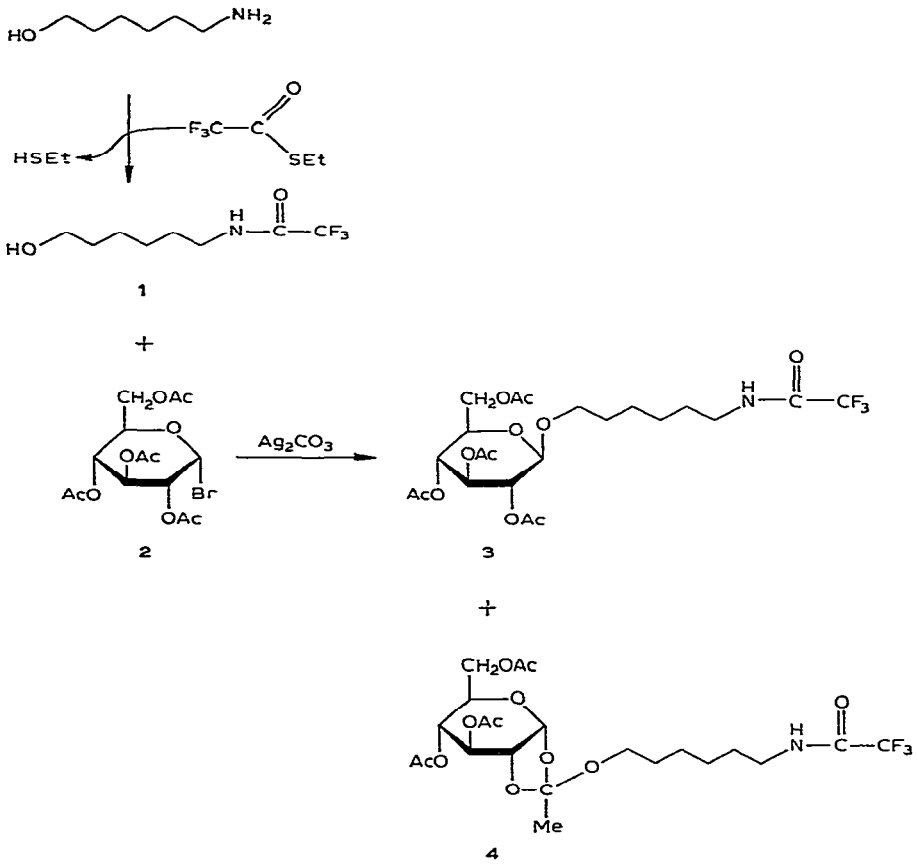
These proteins are useful reagents for the study of the role of oligosaccharides in cell-surface phenomena[3].

We now describe procedures for the synthesis of adsorbents that are useful in the isolation, from a variety of sources, of proteins that have the ability to bind to specific sugar entities. In an accompanying, paper, Weigel *et al.*[4] described the preparation of a number of more-complex ligands by use of procedures similar to those described here.

Two types of linkage are found between carbohydrates and proteins or lipids. That to asparagine is an *N*-acylglycosylamine linkage and all others are glycosidic linkages. Sharon and co-workers[5] described a convenient synthesis of *N*-acylglycosylamine derivatives, and Chipowsky and Lee[6] synthesized a number of 1-thioglycosides suitable for the preparation of adsorbents for the isolation of glycosidases, as well as for lectins and glycosyl transferases. In an earlier publication, the synthesis of 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside was described, along with its application in the purification of the galactosyl transferase from whey[7].



Scheme 1.

TABLE I

PRODUCTS FROM THE CONDENSATION OF 2,3,4,6-TETRA-*O*-ACETYL-α-D-[U-^{14}C]GLUCOPYRANOSYL BROMIDE (1 MMOL) WITH 6-(TRIFLUOROACETAMIDO)HEXANOL (2 MMOL) UNDER VARIOUS CONDITIONS

Acid acceptor; catalyst[b]	*Solvent*[c] *(20 ml)*	*Time (h)*	*Percentage composition of reaction mixture*[a]			
			Bromide	*Ortho-ester*	*Glycoside*	*Partially deacetylated glycoside*
Ag_2CO_3 (2) + I_2 (0.2)	$CHCl_3$	18	1	21	68	3
Ag_2O (2)	$CHCl_3$	26	7	29	48	9
HgO (2) + $HgBr_2$ (0.3)	$CHCl_3$	26	18	11	17	45
HgO (2) + $HgCl_2$ (0.3)	$CHCl_3$	26	8	39	38	4
$Hg(CN)_2$ (2)	$HCONMe_2$	18	<1	72		
$Hg(CN)_2$ (2)	1:1 $MeNO_2$–$CHCl_3$	30	60	30		

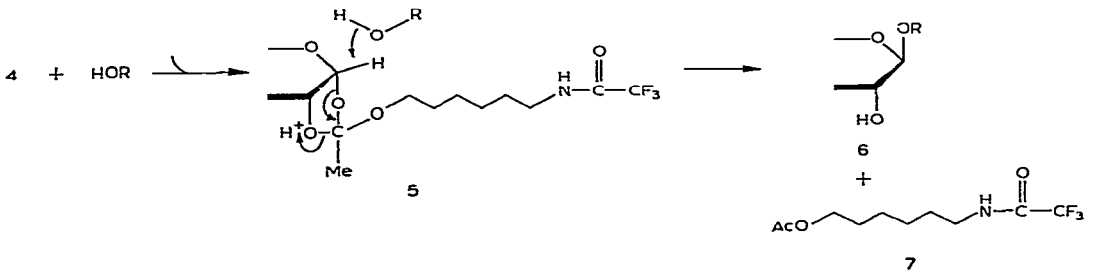
[a]Percentage composition was obtained by determining radioactivity in appropriate zones after separation by t.l.c. on Silica Gel G developed with ethyl acetate–hexane. Zones were located in relation to standard compounds. [b]Numbers in parentheses indicate mmol of reactants. [c]Dried over molecular sieves or alumina before use.

RESULTS AND DISCUSSION

Glycoside synthesis

In this paper, the synthesis of 6-aminohexyl β-D-glycopyranosides of D-fucose, D-galactose, D-glucose, D-mannose, 2-acetamido-2-deoxy-D-galactose, and D-xylose is reported, as well as that of the 2-aminoethyl β-D-glycopyranosides of D-glucose and D-galactose. The syntheses were accomplished as shown in Scheme 1.

In Table I are presented the yields of the various products from the condensation of 2,3,4,6-tetra-*O*-acetyl-α-D-[U-^{14}C]glucopyranosyl bromide (**2**) with 6-(trifluoroacetamido)hexanol (**1**), using several different catalysts for the condensation. In all cases, the simple sugars give some orthoester (**4**), and the ratio of glycoside (**3**) to orthoester is very sensitive to changes in the conditions used for the condensation step. Although data are not presented in Table I, condensations using pyridine as the solvent and the catalyst proceeded slowly, and gave only the orthoester. It should



Scheme 2.

be noted that, with D-mannose, the orthoester was obtained in high yield by using either silver carbonate or mercuric oxide–mercuric bromide as the catalyst.

In our hands, the rearrangement of the orthoesters to the glycosides by *p*-toluenesulfonic acid[8] gave partially deacetylated glycosides as the principal products[9]. Furthermore, a significant proportion of the 1-acetate (**7**) of the aglycon was produced; we have not explored this reaction extensively. It may involve attack of the free alcohol or water on the orthoester (**4**, Scheme 2), with the simultaneous formation of the glycoside having a free 2-hydroxyl group (**6**) and the 1-acetate (**7**) of the aglycon.

A simple procedure for isolating the desired, unprotected product having a free 6-amino group in the aglycon was developed in which the reaction mixture is treated with *p*-toluenesulfonic acid to form glycosides from orthoesters[8], and the whole deacetylated by using a quaternary ammonium ion-exchange resin in the hydroxide form. The products having free amino groups are then adsorbed on a sulfonic acid ion-exchange resin in the pyridinium form. Elution with a gradient of increasing concentration of pyridinium acetate separates the glycoside from 6-aminohexanol, the major contaminant. Experiments with the purified orthoesters[4] of D-[U-^{14}C]mannose indicated that they are decomposed in this sequence of reactions, as all of the radioactivity in the starting orthoester is recovered in the water eluate from the chromatographic column, indicating that the carbohydrate is not coupled to the aglycon.

The β anomer is produced in all cases examined, except for D-mannose, for which the product obtained by sequential treatment under glycosylating conditions, followed by treatment with *p*-toluenesulfonic acid and isolation by ion-exchange chromatography appears to be the α anomer. The product has ^{13}C-resonances at 100.6, 73.7, 71.6, 71.0, 68.7, and 61.9 p.p.m. downfield from tetramethylsilane, in good agreement with those observed for methyl α-D-mannopyranoside (101.9, 73.7, 71.8, 71.2, 68.0, and 62.1 p.p.m.); those of methyl β-D-mannopyranoside are[10] at 102.0, 77.3, 74.2, 71.4, 68.1, and 62.1 p.p.m.

The yields of the glycosides of various sugars are given in Table II. The best conditions for the formation of glycosides of simple sugars consist in the use of Ag_2CO_3 in ethanol-free chloroform containing a trace of iodine, at 25° By examination, by t.l.c., of the reaction mixture at intervals, the reaction was found to be complete after 8 h, but a reaction time of 18 h was generally used. Yields expressed on the basis of the acetylated glycosides refer to crystalline or chromatographically pure products. Direct isolation of the deacetylated product generally gives a greater yield, as losses incurred during the purification of the acetate are eliminated.

For the formation of 6-(trifluoracetamido)hexyl glycosides from 2-acetamido-2-deoxy-D-glucose or -galactose, the tri-*O*-acetylglycopyranosyl chlorides in the presence of mercuric cyanide in *N*,*N*-dimethylformamide give the best yield. With the related bromides from simple sugars, this catalyst gave very high yields of orthoester (as identified by t.l.c. and by p.m.r. spectroscopy). Experiments with tetra-*O*-acetyl-D-glucopyranosyl chloride, using either mercuric cyanide or silver carbonate–iodine, led to the recovery of the starting chloride in good yield after 24 h of reaction.

The synthesis of 2-aminoethyl β-D-glucopyranoside was reported by Graham and Neuberger[11], who obtained the product in 20% yield by using silver carbonate in nitromethane. The conditions used in the present study were essentially the same, as were the yields of products from both D-glucose and D-galactose when the intermediate acetate was isolated. Isolation of the glycoside after deacetylation by chromatography on Dowex-50 (pyridinium form) gave yields of ~40%.

The L-fucopyranoside was not so extensively characterized as the other pyranosides reported here. Only after deacetylation of the whole reaction-mixture was it isolated in 52% yield. The product appeared to be the expected β anomer, as the n.m.r. spectrum contained a doublet, J 9 Hz, at 4.96 p.p.m. downfield from tetramethylsilane.

Glycosyl adsorbents.

The 6-aminohexyl glycosides described couple in good yield (8–12 μmol per

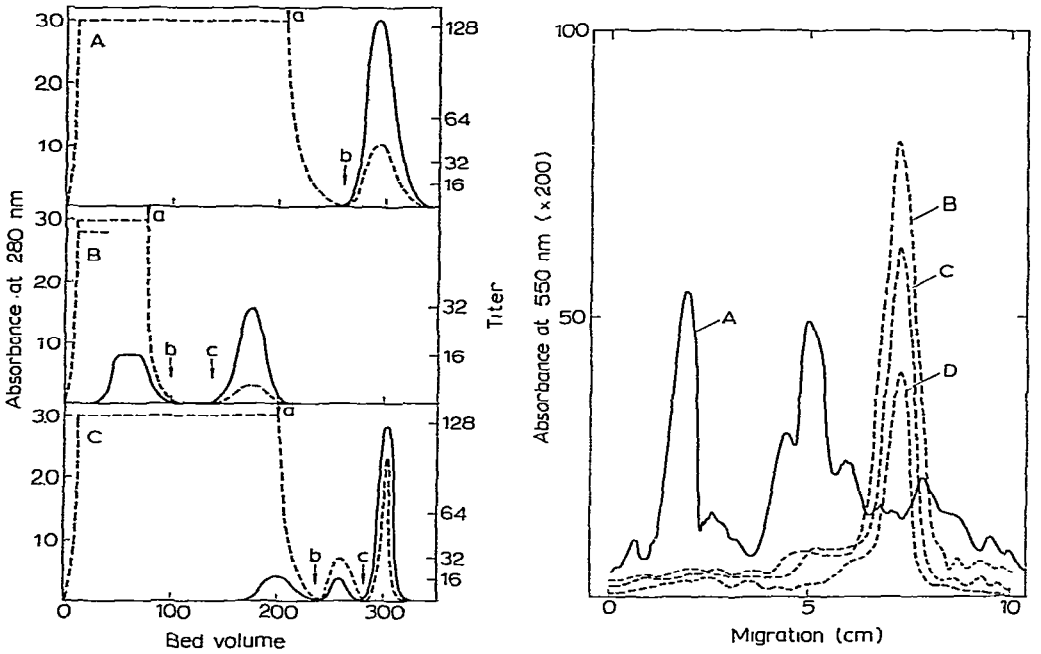


Fig. 1. Purification of wheat-germ agglutinin by affinity chromatography on 6-aminohexyl glycopyranoside derivatives of agarose. [A, 2-Acetamido-2-deoxy-D-glucopyranoside (6 μmol/ml), 4-ml bed-volume; a, elution with 0.01M sodium cacodylate buffer, pH 7.4, containing 0.15M sodium chloride; b, elution with 50mM 2-acetamido-2-deoxy-D-glucose. B, D-Galactopyranoside (5 μmol/ml), 4-ml bed-volume; a, elution with sodium cacodylate buffer, pH 7.4; b, elution with 50mM D-galactose in buffer; c, elution with 0.1M glacial acetic acid. C, 2-Acetamido-2-deoxy-D-galactopyranoside (4.5 μmol/ml), 4-ml bed-volume; a and c, as for B; b, elution with 50mM 2-acetamido-2-deoxy-D-galactose in buffer.]

Fig. 2. Scans of SDS gels of fractions from Fig. 1. [A, Wheat-germ extract. B, Fraction eluted with 50mM 2-acetamido-2-deoxy-D-glucose in Fig. 1A; 500 μg of protein was applied. C, Fraction eluted with 0.1M acetic acid in Fig. 1B, 500 μg. D, Fraction eluted with 0.1M acetic acid in Fig. 1C, 250 μg.]

ml of agarose) to agarose activated by treatment with cyanogen bromide at pH 11–12 as described by Porath[12]. The use of the 2-acetamido-2-deoxy-D-glucosyl–agarose adsorbent in the purification of a galactosyl transferase from bovine whey[7] and of wheat-germ agglutinin[13] has been described. The utility of this type of adsorbent is further shown by the application of the D-galactosyl, 2-acetamido-2-deoxy-D-galactosyl, and 2-acetamido-2-deoxy-D-glucosyl adsorbents to the purification of wheat-germ agglutinin. In Fig. 1 is shown the adsorption of agglutinating activity by three different, monosaccharidic adsorbents and its release by elution with the specific monosaccharide in acetate buffer or dilute acetic acid. The purification achieved is shown in Fig. 2 as scans of electrophoretic separations of proteins on poly(acrylamide) gels in the presence of sodium dodecyl sulfate (SDS). After separation, the proteins were stained with Coumassie Blue. Comparison of the scan of the initial extract with those of the eluted protein showed that the protein(s) adsorbed by the glycosyl–agarose adsorbents constitutes a small proportion of the protein in the extract (see Fig. 2). All of the adsorbents appear to retain the same protein, as judged by electrophoretic mobility and agglutinating ability. In addition, the protein purified by adsorption to 2-acetamido-2-deoxy-D-glucosyl–agarose can be adsorbed to the D-galactose or 2-acetamido-2-deoxy-D-galactose adsorbent. In all cases, the agglutinin appears to be identical to that described previously[13]. The agglutinin does not adsorb to agarose, even though it does adsorb, although poorly, to agarose substituted with D-galactopyranosyl groups; this may be due to steric effects or may reflect the relatively small number of (terminal) D-galactosyl groups in the unsubstituted agarose.

Similar purifications of the agglutinin present in the garden pea and in gorse have been achieved by using the D-mannosyl and L-fucosyl derivatives, respectively.

By use of a column containing 100 ml of 2-acetamido-2-deoxy-D-glucosyl-agarose, it is possible to obtain ~500 mg of wheat-germ agglutinin of high purity. The agglutinin has been coupled to agarose by using cyanogen bromide activation, and the adsorbent formed has been used to isolate the glycoproteins present in extracts of type A erythrocytes.

The glycosyl adsorbents do not appear to be significantly degraded during the separations described here. If present, glycosidases may be inhibited by low temperatures.

EXPERIMENTAL

General. — Evaporations were performed under diminished pressure at 20 to 50° in a rotary evaporator. Melting points were measured with a Fisher–Johns apparatus. Optical rotations were measured at 26° with a Zeiss PM polarimeter. Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of Iowa. ^{1}H-Nuclear magnetic resonance spectra were recorded with a Varian A-60 spectrometer, and ^{13}C-n.m.r. spectra with a Bruker WP-60 instrument equipped for Fourier transform.

Thin-layer chromatography was performed on glass plates coated with 250 μm

layers of Silica Gel G (Merck), with 1:1 (v/v) ethyl acetate–hexane as the developing solvent. Plates were heated for 30 min at 110° prior to use. Generally, components were detected by spraying with 50% sulfuric acid and heating the plates on a hot plate to charring. Ninhydrin[14] and periodate–benzidine[15] sprays were respectively used to visibilize amines and polyols. In some cases, plates were sprayed with M lithium hydroxide (to hydrolyze trifluoracetyl groups) prior to spraying with ninhydrin or 0.025% (w/v) fluorescamine in acetone. Ascending paper-chromatography was performed on Whatman No. 1 paper with 2:2:1 (v/v) 1-butanol–pyridine–water or 6:3:1 (v/v) 2-propanol–ammonia–water, and components were detected with silver nitrate, ninhydrin, or fluorescamine reagent as appropriate.

Acetylated products were isolated by chromatography on columns of silica gel G, using dichloromethane–ethyl acetate.

To facilitate quantification and isolation of products, all reactions were conducted by using ^{14}C- or ^{3}H-labelled monosaccharides having $\sim 10^6$ dpm/mmol.

Solvents were reagent grade, used without purification. Where necessary, solvents were dried over 4 A molecular sieves (Fisher) for at least 24 h before use. Before use in glycoside syntheses, chloroform was passed through a column packed with reagent-grade alumina.

6-Aminohexyl 2-acetamido-2-deoxy-β-D-galactopyranoside was prepared as previously described[7] for the *gluco* isomer.

6-(Trifluoroacetamido)hexyl β-D-glycopyranoside peracetates and 2-(trifluoroacetamido)ethyl β-D-glycopyranoside peracetates were all prepared by similar procedures. The preparation of the *galacto* isomer is given as a typical example.

A solution of tetra-*O*-acetyl-α-D-galactosyl bromide (8 mmol) in dry, ethanol-free chloroform (10 ml) was added to a well-stirred mixture of 6-(trifluoroacetamido)-hexanol (9 mmol), silver carbonate (11 mmol), iodine (80 mg), molecular sieves 4A (10 g), and dry, ethanol-free chloroform (40 ml) in a foil-wrapped reaction-vessel. After being stirred for 18 h, the suspension was filtered through Celite and the solid washed with chloroform (200 ml). The combined filtrates were washed with three 100-ml portions of water, dried (magnesium sulfate), and evaporated, to give 4.5 g of product which was fractionated by chromatography on 60 g of silica gel (60–200 mesh). Elution with 19:1 (v/v) dichloromethane–ethyl acetate, and analysis of fractions by t.l.c., allowed the isolation of 2.2 g (4.0 mmol) of gummy product that was homogeneous, and which had the characteristics reported in Table II.

6-Aminohexyl β-D-glycopyranosides from acetylated derivatives. — These products are readily obtained by slowly passing a 1% (w/v) solution of the acetylated product in 1:1 (v/v) ethyl alcohol–water over a column containing a strongly basic anion-exchange resin (OH^-; 100–200 mesh). Elution with 2 volumes of the same solvent gives a solution of the deacetylated product in quantitative yield. These products have the p.m.r. characteristics expected, and migrate as single components in two solvents in paper chromatography. They give positive reactions for amine and polyol, and contain the radioactivity added as a tracer in the starting monosaccharide. They are suitable for coupling to agarose as described later.

TABLE II

YIELDS AND PROPERTIES OF ACYLATED AMINOHEXYL AND AMINOETHYL GLYCOPYRANOSIDES ISOLATED BY CHROMATOGRAPHY ON SILICA GEL, AND YIELDS OF DEACYLATED PRODUCTS ISOLATED BY ION-EXCHANGE METHODS

Glycoside	*Catalyst*	*Reaction time (h)*	*Yield (%)*	*M.p.*[a] *(degrees)*	$[\alpha]_D^{26}$ *(degrees)*	*Elemental analysis as percentage of theoretical*			*Yield after ion exchange (%)*
						C	*H*	*N*	
6-Aminohexyl									
β-D-Glc	Ag_2CO_3 + I_2	18	68	80–81	−14[b,c]	99.4	95.0	94.5	65–75
β-D-Gal	Ag_2CO_3 + I_2	18	50	gum	+65[b,d]	99.6	101.7	111.2	65
β-D-Xyl	Ag_2CO_3 + I_2	18	26	69–70	−46[b,c]	101.0	101.0	103.0	40
β-D-GalNAc	$Hg(CN)_2$	24	42	140–141	−10.8[e,f]	100.5	100.0	100.9	—
α-D-Man	Ag_2CO_3 + I_2	18	—	—	—	—	—	—	26
α-D-Man	HgO + $HgBr_2$	18	—	—	—	—	—	—	29
β-L-Fuc	Ag_2CO_3 + I_2	18	—	—	—	—	—	—	52
2-Aminoethyl									
β-D-Glc	Ag_2CO_3 + I_2	24	13	101–102	+ 0.34[b,g]	99.5	100.0	101.0	45
β-D-Gal	Ag_2CO_3 + I_2	18	20	gum	+24.7[b,d]	101.0	105.0	95.0	42

[a]Of material recrystallized from ethyl acetate-hexane. [b]Solvent, chloroform. [c]Concentration, 1.2. [d]Concentration, 1.0. [e]Solvent, ethyl acetate. [f]Concentration, 4.0. [g]Concentration, 0.9.

Isolation of 6-aminohexyl and 2-aminoethyl β-D(or β-L)-glycopyranosides by ion-exchange methods. — The preparation of 6-aminohexyl β-L-fucopyranoside is given as a typical example. The glycoside formation from tri-*O*-acetyl-α-L-fucosyl bromide was conducted as already described for the D-*galacto* isomer. After filtration, extraction with water, and removal of solvent, the chloroform-soluble mixture of products was obtained as a gum. It was dissolved in 50% aqueous ethyl alcohol (50 ml per mmol of starting bromide), and the solution was slowly passed through a column of a strongly basic anion-exchange resin (OH^-; 100–200 mesh) (6 ml per mmol of starting material). The eluate from this column contained 72% of the input radioactivity; it was passed directly through a column of a strongly acidic cation-exchange resin in the pyridine form (100–200 mesh) (6 ml per mmol of starting material). The column was washed with 10 bed volumes of aqueous ethyl alcohol, and then eluted with a gradient of 200 ml of 0.8M pyridinium acetate mixing into 200 ml of water at the rate of 1 ml/min. The amino glycosides are eluted between ~50 and 100 ml (0.15 to 0.2M pyridinium acetate). The principal contaminant, 6-aminohexanol, is eluted at 0.30 to 0.4M pyridinium acetate.

The product thus obtained may be isolated as the acetate (salt) by combining and concentrating the fractions that contain radioactivity and that give a reaction with ninhydrin. In all cases, the products appeared to be pure (by paper chromatography) and had the expected p.m.r. spectra and specific activity relative to the starting monosaccharide. Yields are given in Table II.

Preparation of agarose adsorbents. — This was achieved by the method of Porath[12], by use of the amino glycosides described. In general, from 5 to 12 μmol of glycoside per ml of Sepharose 4-B can be incorporated by this procedure.

Wheat-germ agglutinin. — Commercial, wheat-germ meal (100 g) was extracted with 2:1 (v/v) chloroform–methyl alcohol through 10 cycles in a Soxhlet extractor, and dried. The meal was then extracted with water (500 ml) by stirring for 4 h at room temperature. The aqueous extract was filtered through a gauze pad, and the filtrate centrifuged at 8000 × *g* for 30 min at 0°. The clear, yellow supernatant liquor was dialyzed against 3 volumes of 0.01M sodium cacodylate buffer, pH 7.4, containing 0.15M sodium chloride, in a flow-dialysis system, and recentrifuged. The supernatant liquor had a titer of 16 against human, type-A erythrocytes; it was passed through a column containing 4 ml of agarose derivatized with the appropriate ligand. The eluate was examined, to determine when the agglutinating activity emerging from the column had reached a titer of 16. At this time, or after 200 bed-volumes of extract had been added, the column was washed with buffer until the eluate was free of protein (A_{280} <0.05). The adsorbed agglutinin was eluted either with 0.5M monosaccharide or with 0.1M acetic acid (see Fig. 1). Fractions eluted with acetic acid were dialyzed exhaustively against buffer before testing for agglutinin activity.

After exhaustive dialysis against water, and lyophilization, fractions were examined by electrophoresis in SDS–2-mercaptoethanol[16].

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