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SEPARATION OF ACIDIC OLIGOSACCHARIDES BY LIQUID CHROMATOGRAPHY: APPLICATION TO ANALYSIS OF SUGAR CHAINS OF GLYCOPROTEINS*†

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ABSTRACT

An ion-exchange, chromatographic system was developed to separate acidic oligosaccharides. Application of this system for the analysis of sialylated carbohydrate chains of glycoproteins was examined, and it was found that this system affords high resolution of acidic oligosaccharide isomers. (*1*) 3′-*O*-Neuraminyllactose, 6′-*O*-neuraminyllactose, and *N*-acetyl-6′-*O*-neuraminyllactosamine could be separated from bovine colostrum. (*2*) Subjection, to this system, of the oligosaccharide fraction released from bovine submaxillary mucin by treatment with alkaline borohydride resulted in good separation of the trisaccharide-alditol GlcNAc-(1→3)-[NeuAc-(2→6)]-GalNAcol and the disaccharide-alditol NeuAc- or NeuGc-(2→6)-GalNAcol. (*3*) When the serine- or threonine-linked oligosaccharide fraction obtained from human glycophorin A was subjected to this system, two monosialosylated, trisaccharide-alditol isomers, namely, NeuAc-(2→3)-Gal(1→3)-GalNAcol and Gal-(2→3)-[NeuAc-(2→6)]-GalNAcol, could be separated from each other. (*4*) The separation of so-called triantennary and biantennary, complex type of sugar chains of porcine thyroglobulin was achieved with this system.

INTRODUCTION

A number of recent studies have suggested that glycoconjugates play an important role in many biological phenomena, especially cellular-recognition processes. In order to elucidate the molecular mechanism of these recognition phenomena, elucidation of the structure of sugar chains of glycoconjugates is indispensable. Most glycoproteins were found to have acidic sugar chains containing sialic acid residues. Ion-exchange chromatography and paper electrophoresis have been widely employed for the fractionation of these acidic oligosaccharides and glycopeptides. In the past

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few years, a liquid-chromatographic technique has been employed for the separation of carbohydrates[1-10]. We had previously established a system of gel-permeation chromatography using liquid chromatography (l.c.)[11-15]. We now describe an l.c. system for the separation of acidic oligosaccharides, and also demonstrate the applicability of this system to analyses of sugar chains of such glycoproteins as porcine and bovine submaxillary mucins, human glycophorin A, and porcine thyroglobulin.

EXPERIMENTAL

Materials. — Porcine submaxillary mucin (PSM) was purified from porcine submaxillary gland showing blood-group H activity, according to the method of Katzman and Eyler[16]. Bovine colostrum was kindly supplied by Dr. T. Ishii. Bovine submaxillary mucin (BSM) and porcine thyroglobulin (PTG) were prepared as described by Tettamanti and Pigman[17] and Ui and Tarutani[18], respectively. Glycophorin A was purified from human blood-group O erythrocyte membranes by lithium diiodosalicylate–phenol extraction[19], followed by chromatography on columns of DEAE-Sephadex A-25 and Sephacryl S-200 in the presence of 0.4% Triton X-100 and 0.5% sodium dodecyl sulfate, respectively[20]. This preparation gave a single band corresponding to PAS-1 on sodium dodecyl sulfate–poly(acylamide) gel electrophoresis[21]. Neuraminidase from *Arthrobacter ureafacience* was purchased from Nakarai Chemical Co. (Kyoto, Japan). Sephadex G-50, Sephacryl S-200, and DEAE-Sephadex A-25 were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Bio-Gel P-4 was purchased from Bio-Rad Laboratories (Richmond, Calif.).

Preparation of oligosaccharides. — Serine- or threonine-linked oligosaccharides were released from PSM, BSM, and human glycophorin A by treatment in a sealed tube with 0.2M NaOH–0.4M $NaBH_4$ for 48 h at 20° in the dark[22]. The excess of borohydride was decomposed by careful addition, in an ice-bath, of 6M acetic acid, the pH being adjusted to 6.0. The oligosaccharide-alditols released were isolated by subjecting the reaction mixture to gel chromatography on a column (1.5 × 100 cm) of Sephadex G-50. Elution was performed with 50mM pyridine–25mM acetic acid, pH 5.3, and the fractions were monitored by the $NaIO_4$–resorcinol reaction[23] for sialic acid. The second peak eluted was pooled, and lyophilized.

Asparagine-linked oligosaccharides were released from PTG by hydrazinolysis as described previously[14]. After removal of hydrazine, followed by *N*-acetylation with acetic anhydride, the oligosaccharides released were isolated by gel chromatography on a column of Sephadex G-50 as already described.

An oligosaccharide fraction was prepared from bovine colostrum by the method of Kobata[24].

Ion-exchange chromatography. — A Jasco Tri-Rotor liquid chromatograph (Japan Spectroscopic Co., Ltd., Tokyo, Japan) equipped with a Model 034 gradient device (Hitachi, Tokyo, Japan) and a variable-wavelength ultraviolet spectrophotometer (Uvidec 100-II, Jasco) was employed. This system had a column (4.6 × 250 mm) of an anion-exchange resin (Hitachi Custom Resin No. 2630). Elution was

performed with a concave gradient of aqueous sodium chloride solution at a flow rate of 0.5 or 1.0 mL/min. During operation, the column was maintained at 55° by circulating warm water in a jacket. Although the absorption at 210 nm is not specific for carbohydrates, monitoring at this wavelength is the most effective method for the detection of oligosaccharides containing *N*-acetylhexosaminyl or neuraminyl residues, because loss of samples is avoidable with this method.

Gel-permeation chromatography. — In order to analyze the molecular size of desialylated oligosaccharides, gel-permeation chromatography was performed in the same liquid chromatograph (Tri-Rotor, Jasco) equipped with two columns (7.2 × 500 mm) of Bio-Gel P-4 (300–400 mesh). The columns were eluted with distilled water at a flow rate of 0.3 mL/min and maintained at 55°. The conditions for the chromatography have been described in detail[11,12]. Oligomers of D-glucose and 2-acetamido-2-deoxy-D-glucose were used as standards.

Analytical methods. — Neutral sugars, sialic acid, and 6-deoxyhexose were respectively assayed by the phenol–H_2SO_4 reaction[25], the $NaIO_4$–resorcinol reaction[23], and the thioglycolate–H_2SO_4 method[26]. The carbohydrate compositions were determined by g.l.c. on a column (0.3 × 100 cm) of 0.1% of ECNSS-M on Gas-Chrom Q (100–200 mesh), after conversion into the respective alditol acetates according to the method of Spiro[27]. Hydrolysis for this assay was conducted with 2M HCl for 2.5 h at 100°. The (formerly reducing) terminal sugar residue of the oligosaccharide-alditols was determined by the same method, except for omission of the $NaBH_4$-reduction step. Sialic acids were identified by g.l.c. on a column (0.3 × 200 cm) of Gas-Chrom Q coated with 2% of OV-1, after methanolysis followed by per(trimethylsilyl)ation according to the method of Yu and Ledeen[28].

Methylation analysis. — Oligosaccharide alditols were methylated by the method of Hakomori[29]. The permethylated products, purified on a small column of silica gel[30], were hydrolyzed with 3M HCl for 3 h at 80°, and then subjected to reduction and acetylation as described by Stellner *et al.*[31]. Alditol acetates of partially methylated sugars were analyzed by gas–liquid chromatography–mass spectrometry (Shimadzu-LKB 9000) in a column (0.3 × 200 cm) of 2% of OV-1 on Gas-Chrom Q (100–200 mesh).

RESULTS AND DISCUSSION

Separation of PSM oligosaccharides. — In order to examine the utility of this ion-exchange chromatography, an oligosaccharide fraction obtained from PSM was subjected to l.c. When NaCl-gradient elution was monitored by the absorbance at 210 nm, three sharp peaks (PSM-I, PSM-II, and PSM-III) were observed, in addition to a peak for *N*-acetylneuraminic acid pre-added to the mixture as an internal standard (see Fig. 1). All three fractions were found to contain neuraminyl residues. Phenol–H_2SO_4 assay of each fraction indicated that PSM-I and II contained neutral sugars, but that PSM-III did not. Furthermore, 6-deoxyhexose residues were shown to be present only in PSM-I. The structures of the PSM oligosaccharides had been

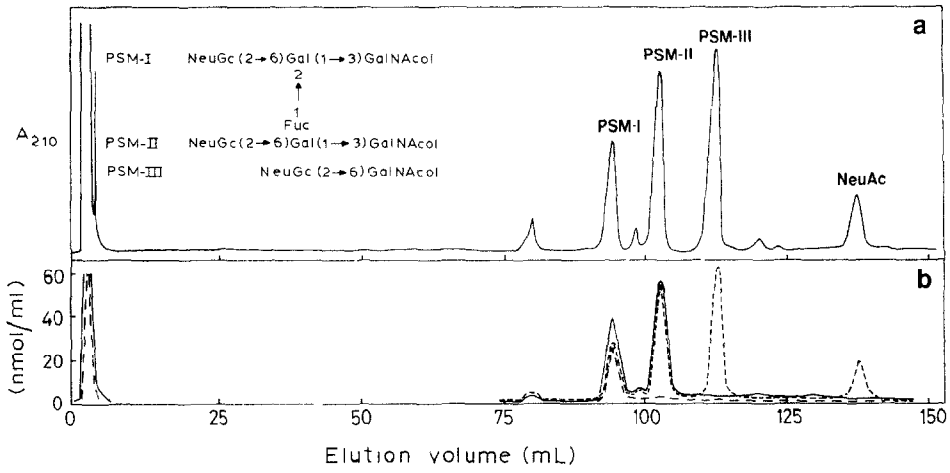

Fig. 1. Chromatogram of PSM oligosaccharide-alditols. [Experimental details are given in the text. Elution was performed with a concave gradient of aqueous sodium chloride solution at a flow rate of 0.5 mL/min. Three compartments of the gradient device were used in order to make the gradient. The first and second compartments were filled with distilled water (90 mL), and the third with 0.1M NaCl (90 mL). (a) Sugars were monitored by the absorbance at 210 nm. (b) Each 1.0-mL fraction was collected, and aliquots were analyzed for sialic acids (-----), neutral sugars (———), and 6-deoxyhexose (—·—). The structures given are according to Aminoff *et al.*[32].]

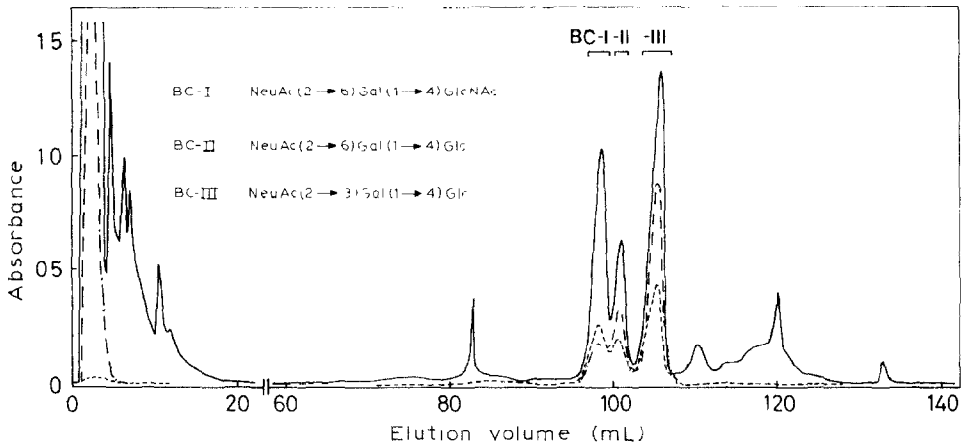

Fig. 2. Chromatogram of bovine colostrum oligosaccharides. [Experimental details are given in the text. Elution was performed with a concave gradient of sodium chloride solution at a flow rate of 0.5 mL/min. The first and second of three compartments of the gradient device were filled with distilled water (90 mL), and the third with 0.15M NaCl (90 mL). Sugars were monitored by the absorbance at 210 nm (———). Each 1.0-mL fraction was collected, and aliquots were analyzed for sialic acid (-----) and neutral sugars (—·—).]

elucidated by Aminoff *et al.*[32]. PSM-I, II, and III appeared to correspond to the following three oligosaccharide-alditols described by Aminoff *et al.*[32]: Fuc-(1→2)-[NeuGc-(2→6)]-Gal-(1→3)-GalNAcol, NeuGc-(2→6)-Gal-(1→3)-GalNAcol, and NeuGc-(2→6)-GalNAcol, respectively. Thus, it was revealed that this system could

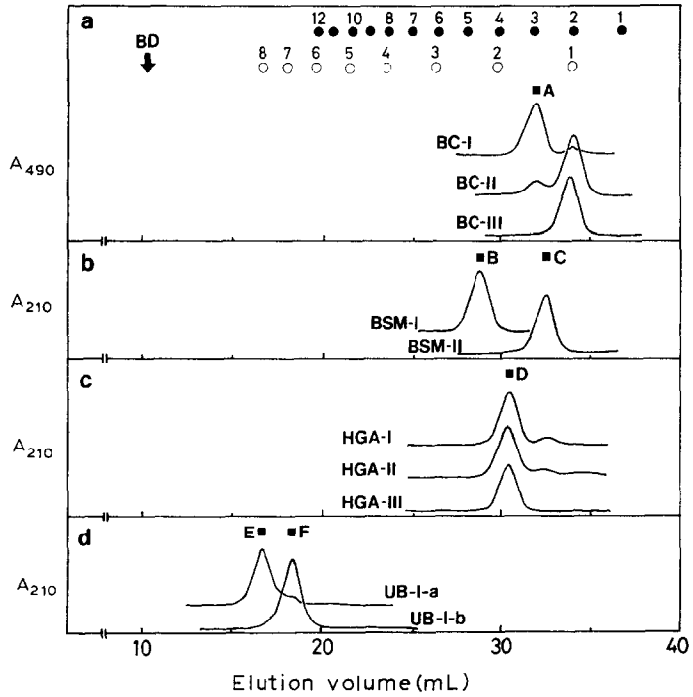

Fig. 3. Gel-permeation chromatography of desialosylated oligosaccharides on a column of Bio-Gel P-4. [Experimental details are given in the text. (a) Desialosylated oligosaccharides obtained from bovine colostrum. (b) Desialosylated oligosaccharide-alditols obtained from BSM. (c) Desialosylated oligosaccharide-alditols obtained from human glycophorin A. (d) Desialosylated UB-I oligosaccharides obtained from PTG. Sugars were monitored by the phenol–H_2SO_4 reaction (a), or the absorbance at 210 nm (b–d). A to F indicate the elution positions of authentic, standard oligosaccharides or oligosaccharide-alditols: A, Gal-GlcNAc; B, GlcNAc-GlcNAcol; C, GalNAcol; D, Gal-GalNAcol; E, Gal-GlcNAc-(Gal-GlcNAc-)Man-(Gal-GlcNAc-Man-)Man-GlcNAc-(Fuc-)GlcNAc; and F, Gal-GlcNAc-Man-(Gal-GlcNAc-Man-)Man-GlcNAc-(Fuc-)GlcNAc. Closed circles (1 to 12) and open circles (1 to 8) indicate the elution positions of oligomers of D-glucose and 2-acetamido-2-deoxy-D-glucose, respectively.]

separate monosialosylated oligosaccharide-alditols having differences in the molecular size of a single sugar residue.

Separation of bovine-milk oligosaccharides. — Secondly, we attempted to separate bovine-milk oligosaccharides. As shown in Fig. 2, three fractions (BC-I, II, and III) were detected by the absorbance at 210 nm, the phenol–H_2SO_4 reaction, and the $NaIO_4$–resorcinol reaction. From the results of colorimetric determination, BC-II and III were found to contain sialic acids and neutral sugars in the molar ratio of $\sim 1:2$, whereas BC-I was shown to contain these sugars in equimolar proportions (see Fig. 2). In all three fractions, the sialic acid was identified as *N*-acetylneuraminic acid, and the acidic oligosaccharides were converted into neutral ones by treatment with neuraminidase.

In order to determine the molecular sizes of the oligosaccharides, the desialosylated oligosaccharides thus obtained were subjected to gel-permeation chromatography

on a column of Bio-Gel P-4 (see Fig. 3a). When the sugars were monitored by the phenol–H_2SO_4 reaction for each fraction, asialo-BC-II and III gave a single peak, at a position corresponding to that of maltose, and asialo-BC-I was found to be eluted at the same elution volume as that of 2-acetamido-2-deoxy-4-*O*-*β*-D-galactosyl-D-glucose. Carbohydrate-composition analyses indicated that both asialo-BC-II and III were composed of D-galactose and D-glucose in equimolar proportions, and that asialo-BC-I consisted of equal amounts of D-galactose and 2-acetamido-2-deoxy-D-glucose.

Furthermore, methylation analysis revealed that BC-II and III were NeuAc-(2→6)-Gal-(1→4)-Glc (6′-*O*-neuraminyllactose) and NeuAc-(2→3)-Gal-(1→4)-Glc (3′-*O*-neuraminyllactose), respectively. From BC-I, 2,3,4-tri-*O*-methyl-galactitol and 1,3,5,6-tetra-*O*-methyl-2-(methylacetamido)glucitol were detected as methylated sugars in an approximately equimolar ratio, indicating that BC-I had the structure of NeuAc-(2→6)-Gal-(1→4)-GlcNAc (*N*-acetyl-6′-*O*-neuraminyllactosamine). Kuhn and Gauhe reported the existence of this oligosaccharide in bovine milk[33]. Thus, three acidic trisaccharides from bovine colostrum were well separated by this l.c. system. Particularly, the nice separation of two neuraminyllactoses (*i.e.*, 3′- and 6′-*O*-neuraminyllactose) seemed to demonstrate the effectiveness of this system.

Separation of BSM oligosaccharides. — Ion-exchange chromatography of the oligosaccharide-alditols obtained from BSM by treatment with alkaline borohydride gave two major, sialic acid-containing fractions (BSM-I and II), in addition to a few minor fractions, as shown in Fig. 4. Neither BSM-I nor II was found to contain neutral sugars (as determined by the phenol–H_2SO_4 method). The sialic acid in the BSM-I fraction was identified as *N*-acetylneuraminic acid, whereas both *N*-acetyl- and *N*-glycolylneuraminic acid were detected, in the molar ratio of ~2:1, in the BSM-II fraction. After treatment with neuraminidase, the elution profiles of asialo BSM-I and II on a column of Bio-Gel P-4 were as shown in Fig. 3b. The

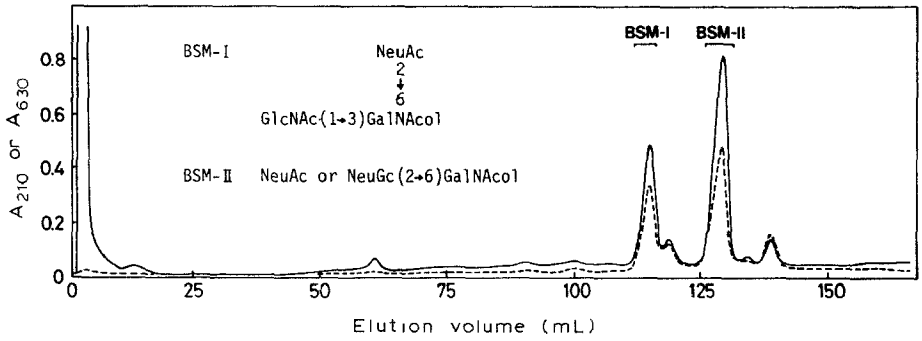

Fig. 4. Chromatogram of BSM oligosaccharide-alditols. [Experimental details are given in the text. Elution was performed with a concave gradient of sodium chloride solution at a flow rate of 1.0 mL/min. The first and second of three compartments of the gradient device were filled with distilled water (120 mL), and the third with 0.15M NaCl (120 mL). Sugars were monitored by the absorbance at 210 nm (———), and by the $NaIO_4$–resorcinol reaction (– – – – –).]

TABLE I

CARBOHYDRATE COMPOSITIONS OF DESIALOSYLATED OLIGOSACCHARIDES

Sugar	*Molar ratio*[a] *Desialosylated oligosaccharides*				
	BSM-I	*BSM-II*	*HGA-I*	*HGA-II*	*HGA-III*
D-Galactose		–	0.96	1.18	0.93
2-Acetamido-2-deoxy-D-glucose	1.04	-			
2-Acetamido-2-deoxy-D-galactitol	**1.00**	+	**1.00**	**1.00**	**1.00**

[a]Molar ratios are expressed in relation to 2-acetamido-2-deoxy-D-galactitol taken as 1.00.

elution position of asialo-BSM-II was found to be identical with that of 2-acetamido-2-deoxygalactitol.

On the other hand, asialo-BSM-I was found to be larger in size, its elution position corresponding to that of a 2-acetamido-*O*-(2-acetamido-2-deoxyhexosyl)-2-deoxyhexitol. The carbohydrate composition of asialo-BSM-I indicated that it was composed of one residue each of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactitol (see Table I). Asialo BSM-II was identified as 2-acetamido-2-deoxy-D-galactitol. Methylation analysis of BSM-I and II showed equal amounts of 2-deoxy-3,4,6-tri-*O*-methyl-2-(methylacetamido)glucitol and 2-deoxy-1,4,5-tri-*O*-methyl-2-(methylacetamido)galactitol for the former, and 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(methylacetamido)galactitol for the latter (see Table II).

Methylation of asialo-BSM-I revealed that the 2-acetamido-2-deoxy-D-galactitol that originally bore substituents at O-3 and O-6 was converted into 2-acetamido-2-deoxy-D-galactitol having only one substituent, at O-3, namely, 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(methylacetamido)galactitol, after treatment with neuraminidase. On the basis of the results, the structures of BSM-I and II were proposed to be GlcNAc-(1→3)-[NeuAc-(2→6)]GalNAcol and NeuAc(or NeuGc)-(2→6)-GalNAcol, respectively. The principal carbohydrate chain of BSM was reported by Gottschalk and Graham[34] and Murty and Horowitz[35] to be a disaccharide having the same structure as BSM-II. Furthermore, structural elucidation of BSM-I confirmed the earlier suggestion by Bertolini and Pigman[36] that a part of the BSM oligosaccharides contained 2-acetamido-2-deoxy-D-glucose.

Separation of human glycophorin A oligosaccharides. — Glycophorin A, a major sialoglycoprotein of human erythrocyte membranes, was found to have both serine(or threonine)-linked and asparagine-linked sugar chains[37]. In this study, the serine- and threonine-linked oligosaccharides of this glycoprotein were separated by the l.c. system. The structure of the major oligosaccharide was proposed by Thomas and Winzler[38] to be that of a tetrasaccharide, namely, NeuAc-(2→3)-β-D-Gal-(1→3)-[NeuAc-(2→6)]-D-GalNAcol. When the oligosaccharide mixture released

TABLE II

METHYLATION ANALYSIS OF THE OLIGOSACCHARIDE-ALDITOLS OBTAINED FROM BSM AND HUMAN GLYCOPHORIN A

Methylated alditol	*Molar ratio*[a]							
	BSM-I	*Asialo-BSM-I*	*BSM-II*	*HGA-I*	*HGA-II*	*Asialo-HGA-II*	*HGA-III*	*Asialo-HGA-III*
2,3,4,6-Tetra-*O*-methylgalactitol			—		*1.0*	*1.0*		*1.0*
2,4,6-Tri-*O*-methylgalactitol			—	*1.0*			*1.0*	
3,4,6-Tri-*O*-methyl-2-(methylacetamido)glucitol	*1.0*	*1.0*	—					
1,4,5,6-Tetra-*O*-methyl-2-(methylacetamido)galactitol[b]		1.2	—	0.9		0.9		0.7
1,3,4,5-Tetra-*O*-methyl-2-(methylacetamido)galactitol[b]			+					
1,4,5-Tri-*O*-methyl-2-(methylacetamido)galactitol[b]	1.0		—		0.8		1.0	

[a]Molar ratios normalized to 1.0 mol of the methylated sugar are italicized. [b]Values are expressed as the total of methylacetamido and acetylacetamido derivatives[40].

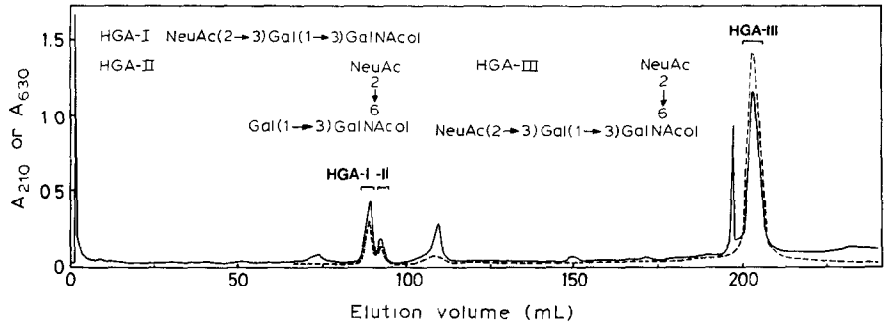

Fig. 5. Chromatogram of the oligosaccharide alditols from human glycophorin A. [Experimental details are given in the text. Elution was performed with a concave gradient of sodium chloride solution at a flow rate of 0.5 mL/min. The first and second of three compartments of the gradient device were filled with distilled water (120 mL), and the third with 0.25M NaCl (120 mL). Sugars were monitored by the absorbance at 210 nm (———) and by the $NaIO_4$–resorcinol reaction (– – – – –).]

by treatment with alkaline borohydride was subjected to the l.c. system, a few minor peaks were detected, as well as one major peak (see Fig. 5).

Structural analyses of the three oligosaccharides designated HGA-I, II, and III were conducted. The molar ratios of neuraminic acids to neutral sugars were estimated to be 1:1 for HGA-I and II, and 2:1 for HGA-III, from the results for the phenol–H_2SO_4 and $NaIO_4$–resorcinol reactions. These results, and the elution positions of these oligosaccharides, indicated that HGA-I and II were monosialylated and HGA-III was a disialylated oligosaccharide-alditol. The sialic acid in all three fractions was identified as *N*-acetylneuraminic acid, and treatment of these oligosaccharides with neuraminidase was found to convert them into neutral oligosaccharide-alditols. Gel-permeation chromatography of the desialosylated oligosaccharide-alditols revealed that their elution positions were identical, and corresponded to that of a 2-acetamido-2-deoxy-*O*-hexosylhexitol (see Fig. 3c). Furthermore, carbohydrate-composition analysis showed that all three desialosylated oligosaccharide-alditols were composed of D-galactose and 2-acetamido-2-deoxy-D-galactitol in equimolar proportions (see Table I). From the data presented, HGA-I, II, and III were found to be mono- and di-sialosylated derivatives of Gal-(1→3)-GalNAcol.

On the basis of methylation analysis (see Table II), the structures of HGA-I, II, and III were proposed to be NeuAc-(2→3)-Gal-(1→3)-GalNAcol, Gal-(1→3)-[NeuAc-(2→6)]-GalNAcol, and NeuAc-(2→3)-Gal-(1→3)-[NeuAc-(2→6)]-GalNAcol, respectively. The structure of HGA-III was identical with that reported by Thomas and Winzler[38]. Recently, Lisowska *et al.*[39] reported that three oligosaccharides were isolated from this glycoprotein by a combination of paper electrophoresis and paper chromatography, and they had the same structures as those determined in this investigation. Although our results for the structures and the yields of the three oligosaccharides are in good agreement with their report, it remains unclear whether the minor oligosaccharides (HGA-I and II) were partially degraded artifacts occurring during the isolation, or were the biosynthetic precursors of the major oligosaccharide

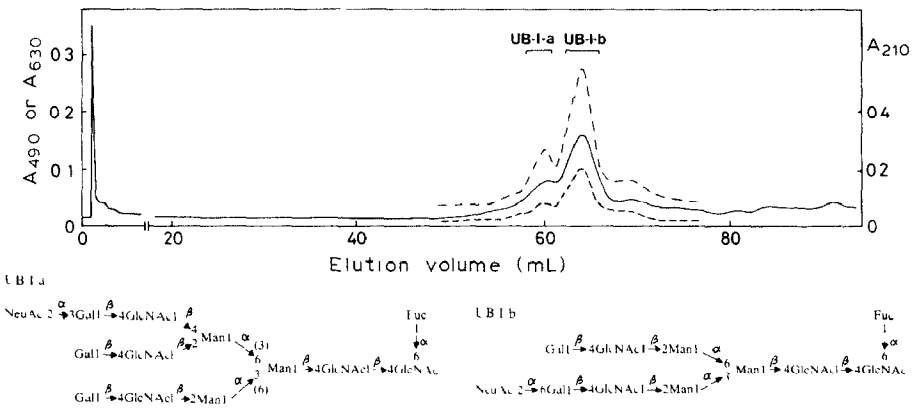

Fig. 6. Chromatogram of UB-I oligosaccharides from PTG. [Experimental details are given in the text. Elution was performed with a concave gradient of sodium chloride solution at a flow rate of 0.5 mL/min. The first and second of three compartments of the gradient device were filled with distilled water (120 mL), and the third with 0.08M NaCl (120 mL). Sugars were monitored by the absorbance at 210 nm (———). Each 1.0-mL fraction was collected, and aliquots were assayed for sialic acid (– – – –) and neutral sugars (—·—). The structures given for the dominant oligosaccharides in UB-I-a and UB-I-b fractions are according to Yamamoto *et al.*[14].]

(HGA-III). Good separation of the trisaccharide isomers, HGA-I and II, could be readily achieved with this l.c. system.

Separation of PTG oligosaccharides. — We examined the applicability of this system to the analysis of asparagine-linked, acidic oligosaccharides of porcine thyroglobulin. This glycoprotein has been shown to contain both so-called triantennary and biantennary, complex-type (unit B-type) sugar chains[14]. When the monosialosylated, complex-type oligosaccharide fraction (UB-I) was subjected to the l.c., two major peaks were observed (see Fig. 6). The first and the second peaks were respectively identified as UB-I-a and UB-I-b (reported in the previous study[14]) from the elution profiles obtained on gel-permeation chromatography after treatment with neuraminidase (see Fig. 3d). The separation of complex-type oligosaccharides having different numbers of side chains composed of 2-acetamido-2-deoxy-4-*O*-β-D-galactosyl-D-glucose was achieved, but the resolution of triantennary and biantennary complex-type oligosaccharides was not so good as expected; this may be due to the heterogeneity in the mode of linkage between NeuAc and galactosyl residues[14].

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