Carbohydrate Research, 151 (1986) 89–103
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION OF OLIGOSACCHARIDES HAVING A FREE REDUCING-END FROM GLYCOPEPTIDE SOURCES*†

BRAD BENDIAK‡,
Department of Biochemistry, Research Institute, The Hospital for Sick Children, Toronto, Ontario M5G 1X8 (Canada)

AND DALE A. CUMMING
Department of Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8 (Canada)

(Received August 14th, 1985; accepted for publication in revised form, October 25th, 1985)

ABSTRACT

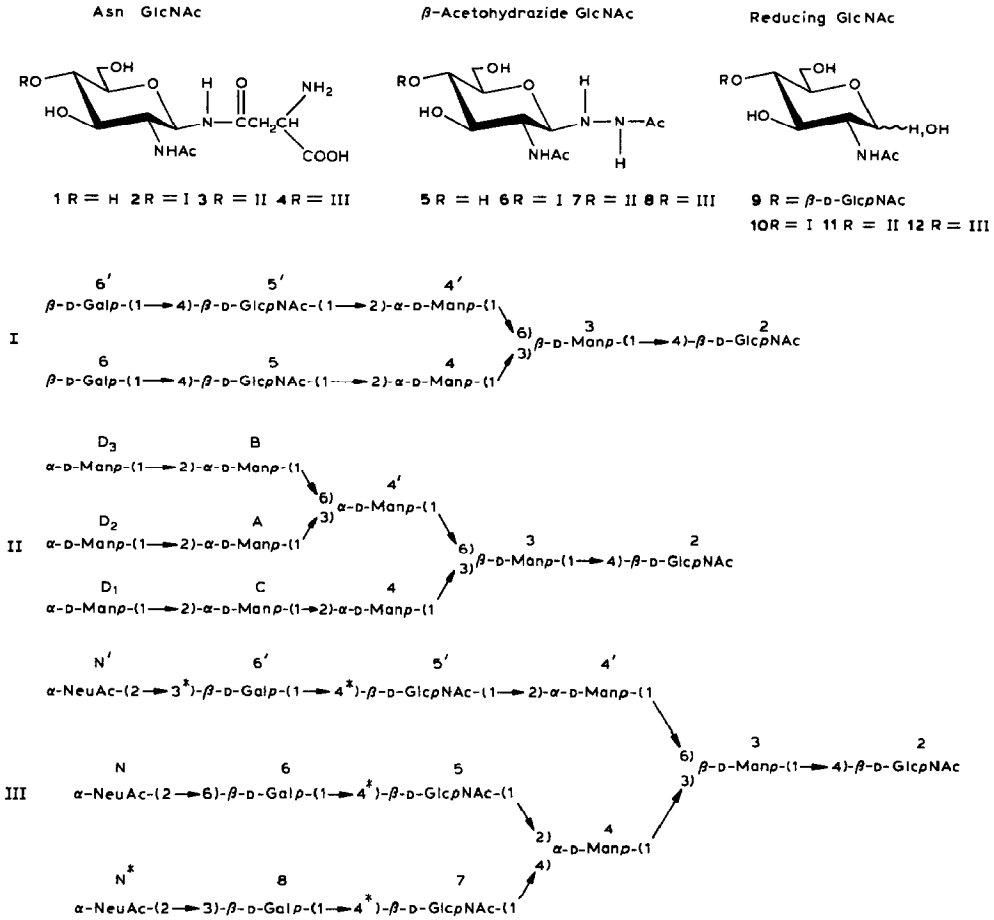
A hydrazinolysis–*N*-reacetylation procedure, modified by the inclusion of a mild acid-hydrolysis step after *N*-acetylation, was used to prepare, in overall yields of 60–70%, pure oligosaccharides containing a reducing D-GlcNAc residue from glycopeptide sources. Three types of asparagine-linked glycopeptides were treated: a high-mannose type, a complex-type not containing sialic acid, and a complex-type containing sialic acid, linked both α-(2→3) and α-(2→6) to β-D-Gal*p* residues. After the hydrazinolysis–*N*-reacetylation procedure, there was often contamination of the reducing oligosaccharides with glycopeptide that remained intact through the procedure, as well as minor oligosaccharide products, altered in the nature of the residue at the reducing end. Oligosaccharides having a reducing D-GlcNAc residue were purified by standard liquid chromatography and high-pressure liquid chromatography (l.c.) 360-MHz ^{1}H-n.m.r. was valuable in establishing common structural reporter signals which enabled major products to be identified at stages during the production of free reducing oligosaccharides, and their purity to be assessed.

INTRODUCTION

Hydrazinolysis–*N*-reacetylation of glycoproteins and glycopeptides has become a popular method for isolation of asparagine-linked oligosaccharides. Since the original work by Yosizawa *et al.*[1], a number of investigators have used the procedure for identification of oligosaccharide structures from a wide variety of glycoproteins or glycoprotein mixtures[2–5]. However, various investigations[6–9] have consistently reported heterogeneity in the derivatives present at the reducing terminus of oligosaccharides released from glycopeptides by the conventional

*Dedicated to Roger W. Jeanloz.
†Part II of the series Hydrazinolysis–*N*-Reacetylation of Glycopeptides and Glycoproteins.
‡To whom correspondence should be addressed.

0008-6215/86/$ 03.50



Scheme I. Structures of the carbohydrates referred to in this paper. Individual sugar residues of oligosaccharides are denoted above each residue, using the general notation system of Vliegenthart *et al.*[18], except that residue 1 (the asparagine-linked D-GlcpNAc residue in their glycopeptide structures) is referred to as Asn GlcNAc, β-acetohydrazide GlcNAc, or reducing GlcNAc, denoting the structures shown. In glycopeptides **2** and **3**, asparagine was the major amino acid present, there being negligible amounts of other amino acids observable by n.m.r. spectroscopy. In glycopeptide **4**, there were additional amino acids attached at either or both the amino or carboxyl group of the asparagine residue. Linkages marked with an asterisk (structures **4, 8,** and **12**) indicate heterogeneity at that linkage; the linkage of the predominant species is indicated.

hydrazinolysis–*N*-reacetylation procedure[2]. It became apparent during our investigations that the nature of the series of chemical reactions occurring at the reducing end of oligosaccharides during the procedure was not clearly understood, and we sought to define these reactions in order to maximize yields of oligosaccharides containing a free reducing GlcNAc residue. Using 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine **1** (Scheme I) as a model compound, we identified the major product after hydrazinolysis–*N*-reacetylation as the 1-β-glycosyl-2-*N*-acetylhydrazine ("β-acetohydrazide") derivative **5**, and verified its

structure by chemical synthesis[9]. Notably, this derivative is hydrolyzed under mildly acidic conditions, and we have defined the mildest conditions necessary for its quantitative hydrolysis to 2-acetamido-2-deoxy-D-glucose. Tang and Williams[10] have also recently reported the synthesis of **5**, and have noted its hydrolysis upon exposure to acidic ion-exchange resins.

In this paper, we describe a detailed study of the modified hydrazinolysis–*N*-reacetylation procedure as applied to naturally occurring glycopeptides. Three glycopeptides (**2, 3,** and **4**) were used, representative of the major classes typically present in glycoproteins. As observed with the model compound, these studies revealed that the glycopeptides were initially converted into β-acetohydrazide derivatives at the reducing end. Reducing oligosaccharide arose only upon exposure of the β-acetohydrazide derivatives to mildly acidic conditions. Of particular importance is the application of the mild acid treatment in the isolation of reducing oligosaccharides containing sialic acid, in which the acidic conditions must be carefully controlled to prevent cleavage of the acid-labile α-sialoside linkage. The results demonstrate that the hydrazinolysis–*N*-reacetylation–mild acid hydrolysis procedure, followed by standard and high-pressure liquid chromatographic (l.c.) separations, can be utilized to prepare pure reducing oligosaccharides from asparagine-linked glycopeptides. The procedure offers a simple route to preparative quantities of pure oligosaccharides for use in chemical syntheses.

RESULTS AND DISCUSSION

The 360-MHz ^{1}H-n.m.r. spectra of the starting glycopeptides **2** and **3** are shown in Figs. 1a and 2a, respectively. Signals in the structural reporter and *N*-acetyl regions of the spectra (Table I) clearly indicate the molecules to have the structures shown, in agreement with the published structures[11,12]. Glycopeptide **4**, the asparagine-linked triantennary glycopeptide of fetuin, contained the core branching-pattern described by Nilsson *et al.*[13]. However, examination of the spectrum (Fig. 3a) revealed[14] heterogeneity at the linkages marked with asterisks in Scheme I. The nature of this heterogeneity is currently under investigation and is not pertinent to the study of the reactions occurring at the reducing terminus of the oligosaccharide during the hydrazinolysis–*N*-reacetylation procedure.

Treatment of glycopeptide **2** with anhydrous hydrazine, followed by *N*-acetylation, resulted in a product similar in molecular weight to the original glycopeptide, as determined by their elution profiles on Sephadex G-15 (Fig. 4). This result indicated that glycosidic linkages remained uncleaved during the hydrazinolysis step, a result which verifies other reports[1–5], and was confirmed by ^{1}H-n.m.r. (see later). Glycopeptides **3** and **4** showed similar behavior on Sephadex G-15 both before and after hydrazinolysis–*N*-reacetylation (data not shown); these glycopeptides and resultant products all migrated as single peaks near the void volume of the column.

Examination of the products at this stage by ^{1}H-n.m.r. (Figs. 1b and 2b,

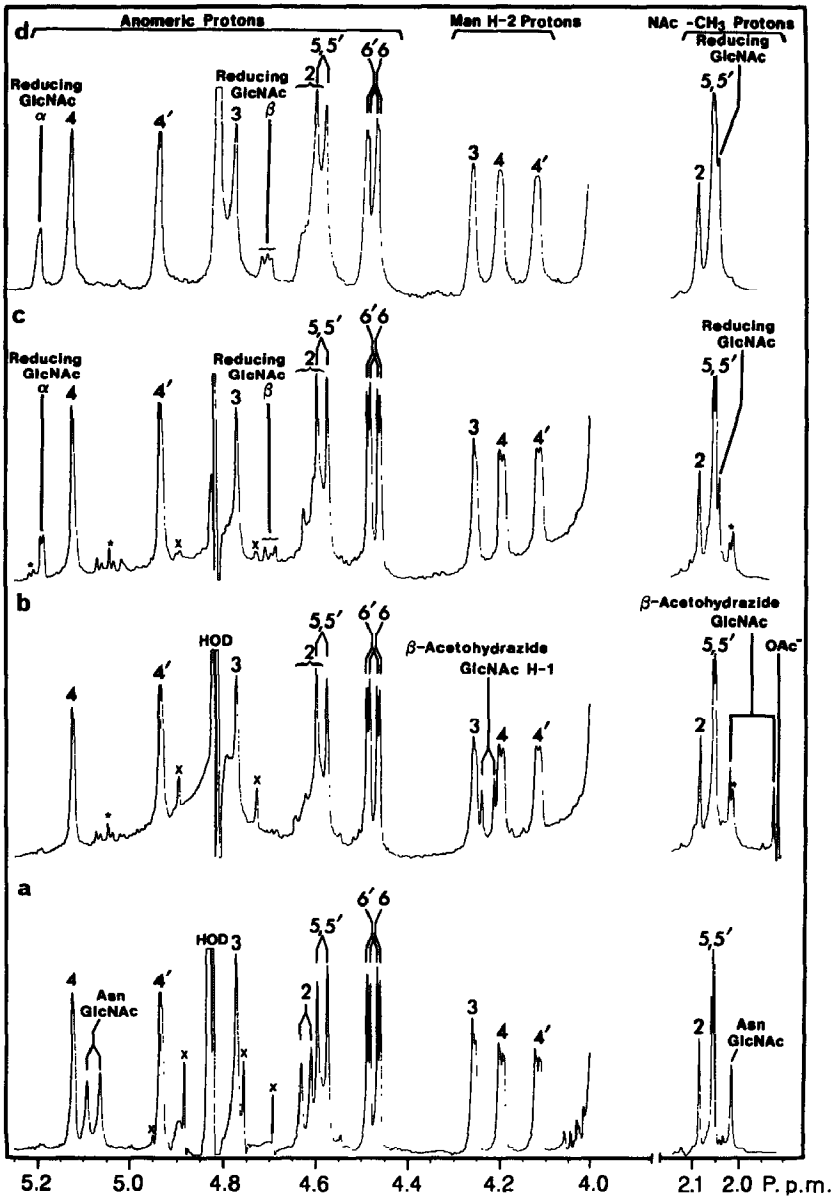


Fig. 1. Structural reporter regions of 360-MHz ^{1}H-n.m.r. spectra of the products obtained during sequential stages of preparation of the oligosaccharide **10**: (*a*) The original glycopeptide **2** from human fibrinogen; (*b*) Product after hydrazinolysis–*N*-reacetylation; (*c*) Product after mild acid hydrolysis; (*d*) Pure oligosaccharide **10** after chromatography on Bio-Gel P4 and l.c. Assignments are indicated. Signals of minor products are marked with asterisks; spinning sidebands are marked by ×. Resonances in the *N*-acetyl region are shown at approximately one-sixth to one-tenth intensity.

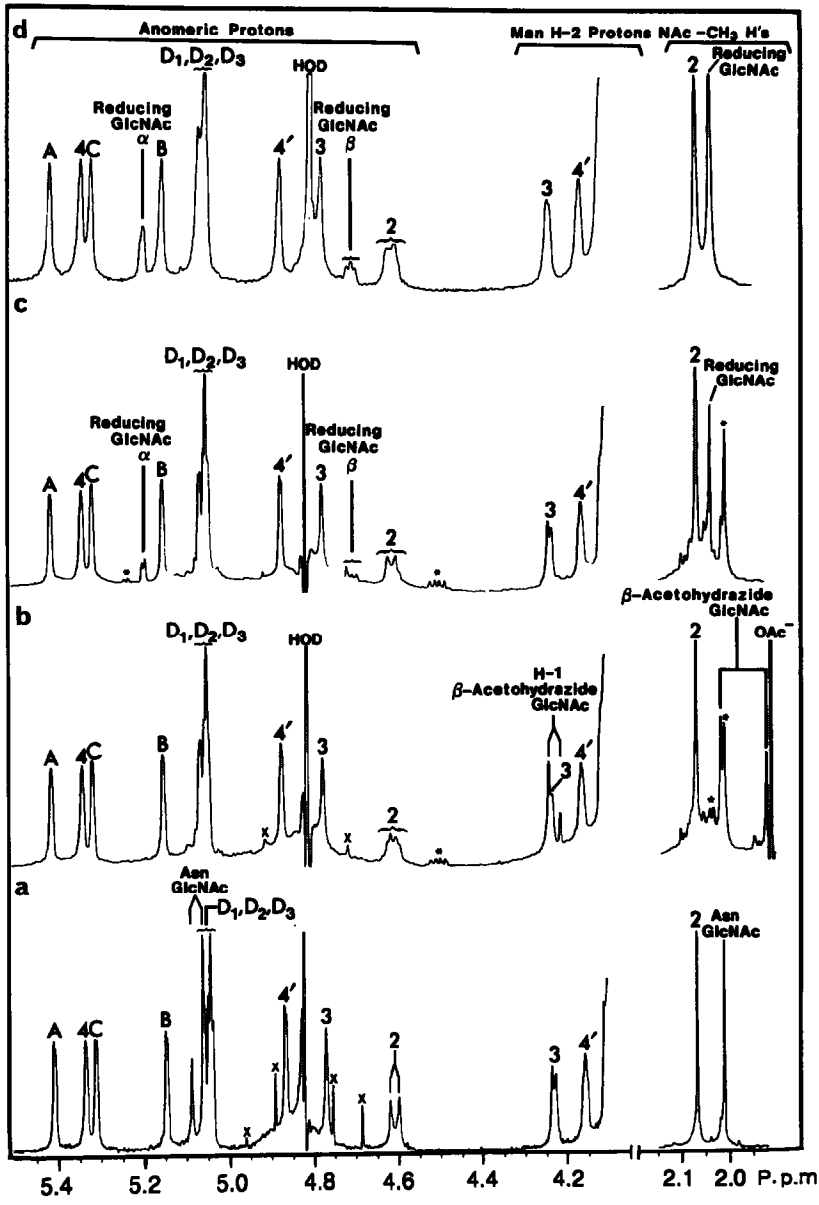


Fig. 2. Structural reporter regions of 360-MHz ^{1}H-n.m.r. spectra of products at sequential stages of preparation of the high-mannose oligosaccharide **11**: (*a*) The glycopeptide **3** from soybean agglutinin; (*b*) Product after hydrazinolysis–*N*-reacetylation; (*c*) Product after mild acid hydrolysis; (*d*) Pure oligosaccharide **11** after chromatography on Bio-Gel P4 and l.c. Assignments are indicated. Signals of minor products are marked with asterisks; spinning sidebands are marked by ×. Resonances in the *N*-acetyl region are shown at approximately one-half intensity.

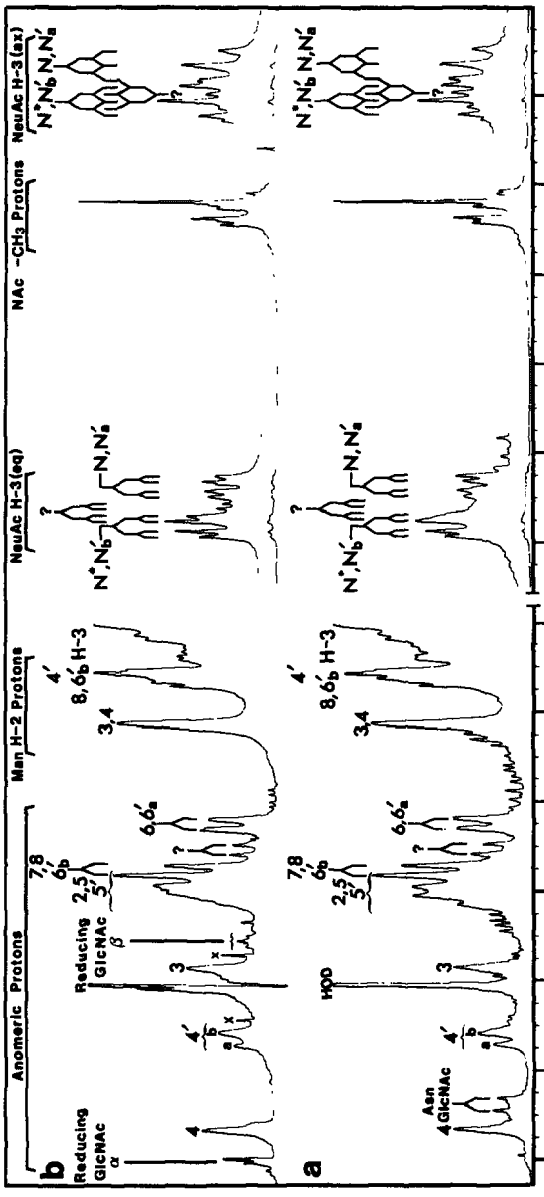


Fig. 3. (*a*) Structural reporter regions of the 360-MHz ^{1}H-n.m.r. spectrum of the asparagine-linked glycopeptide **4** obtained from the glycoprotein fetuin; (*b*) Spectrum of the product obtained after hydrazinolysis–*N*-reacetylation, and mild acid hydrolysis of **4**. Assignments are indicated. Resonances in the *N*-acetyl region are shown at approximately one-tenth intensity. Because of heterogeneity in the linkage (2→3 and 2→6) of the α-NeuAc residue to the β-D-Gal*p* residue on the branch linked to the 6 position of the β-D-Man*p* residue, two assignments for each monosaccharide on this branch are reported, marked with the subscripts *a* and *b*, denoting a terminal NeuAc α-(2→6) or α-(2→3) linkage, respectively.

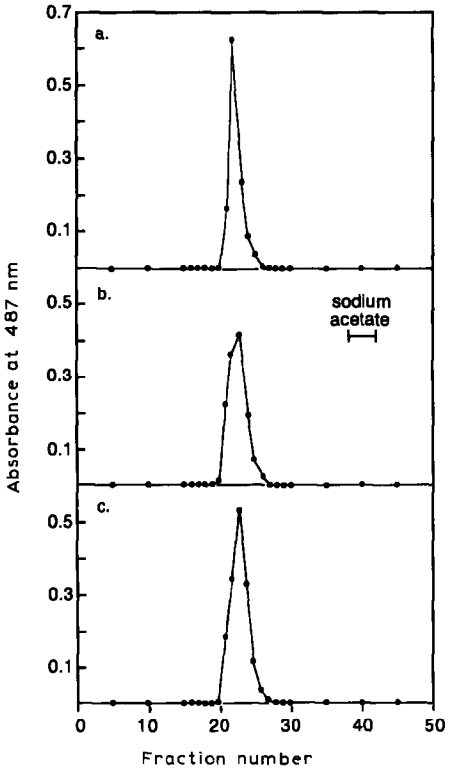


Fig. 4. (*a*) Sephadex G-15 profile of the fibrinogen glycopeptide **2**, and profiles of the products obtained after (*b*) hydrazinolysis–*N*-reacetylation of **2**, and (*c*) after mild acid hydrolysis of the products in (*b*). Hexose was monitored by the phenol–sulfuric acid test[21], and sodium acetate was detected with ferric chloride, as described in the Experimental section.

Table I) showed that, in all cases, the β-acetohydrazide derivatives (**6**, **7**, and **8**) were the major component. This conclusion is based on the appearance of the characteristic signals of the H-1 proton of the β-acetohydrazide GlcNAc (δ 4.218, *J* 9.4 Hz) and the acetyl group of the acetohydrazide (δ 1.918, which is resolved from acetate, δ 1.907). These assignments were made based on those of the model compound, **5** (Table I, and ref. 9). At this stage in the procedure, a negligible amount of oligosaccharide was present that contained a free reducing 2-acetamido-2-deoxy-D-glucose residue. N.m.r. signals corresponding to the H-1 of the asparagine-linked GlcNAc were often absent at this stage. However, in some cases, intact glycopeptide was evident (Fig. 1b, H-1 of GlcNAcAsn marked with an asterisk). Although unusual, the amount of uncleaved glycopeptide could be as high as 30%. All signals corresponding to H-1 and H-2 of D-Man*p*, H-1 of branch β-D-Glc*p*NAc and β-D-Gal*p*, as well as H-3 protons of NeuAc were not affected by the presence of the β-acetohydrazide moiety (Figs. 1b, 2b, and Table I). The only signal altered significantly was that of the H-1 of β-D-Glc*p*NAc residue 2, which broadened. After *N*-acetylation, the complete removal of sodium acetate from the

TABLE I

CHEMICAL SHIFTS OF STRUCTURAL REPORTER GROUPS OF COMPOUNDS **2–12**

Reporter group	*Residue*	*Compound*										
		2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
H-1	Asn-GlcNAc	5.073	5.068	5.076								
	β-Acetohydrazide-GlcNAc				4.227	4.219	4.218	4.218				
	Reducing GlcNAc (α)								5.188	5.185	5.185	5.194
	Reducing GlcNAc (β)								4.695	4.695	4.694	4.703
	2	4.616	4.603	4.61[a]		4.61[a]	4.60[a]	4.60[a]	4.593[b] 4.584	4.61[c]	4.60[c]	4.60[c]
	3	4.765	4.764	4.764		4.764	4.764	4.764		4.764	4.765	4.764
	4	5.116	5.332	5.125		5.117	5.332	5.126		5.116	5.332	5.126
	4′	4.928	4.861	4.905[d] 4.933		4.928	4.864	4.908[d] 4.935		4.927	4.865	4.908[d] 4.936
	5	4.579		4.59[a]		4.580		4.59[a]		4.579		4.59[a]
	5′	4.579		4.57[a,d] 4.59		4.580		4.57[a,d] 4.59		4.579		4.57[a,d] 4.59
	6	4.465		4.444		4.466		4.444		4.464		4.444
	6′	4.471		4.547[d] 4.444		4.471		4.547[d] 4.444		4.470		4.547[d] 4.444
	7			4.547				4.547				4.547
	8			4.547				4.547				4.547
	A		5.403				5.403				5.404	
	B		5.140				5.142				5.142	
	C		5.306				5.308				5.308	

	D_1		5.040				5.042				5.043	
	D_2		5.053				5.054				5.053	
	D_3		5.034				5.036				5.035	
H-2	3	4.250	4.227	4.219		4.251	4.227	4.218		4.251	4.227	4.218
	4	4.192	4.10[a]	4.219		4.192	4.10[a]	4.218		4.192	4.10[a]	4.218
	4′	4.114	4.152	4.12[a]		4.113	4.154	4.12[a]		4.113	4.153	4.12[a]
H-3a	N			1.722				1.722				1.721
	N′			1.802[d]				1.802[d]				1.801[d]
				1.722				1.722				1.721
	N*			1.802				1.802				1.801
H-3e	N			2.668				2.666				2.666
	N′			2.756[d]				2.756[d]				2.755[d]
				2.668				2.666				2.666
	N*			2.756				2.756				2.755
NAc(CH_3)	Asn-GlcNAc	2.010	2.007	2.007								
	β-Acetohydrazide-GlcNAc				2.020	2.014	2.012	2.014				
	Acetohydrazide-Ac				1.923	1.918	1.918	1.919				
	Reducing GlcNAc								2.037	2.036	2.037	2.036
	2	2.079	2.062	2.079		2.078	2.063	2.079	2.068	2.079	2.067	2.080
	5	2.051		2.066		2.050		2.066		2.049		2.066
	5′	2.046		2.042[d]		2.046		2.042[d]		2.045		2.044[d]
				2.066				2.066				2.066
	7			2.073				2.072				2.072
	NeuAc's			2.029				2.028				2.029

[a]Chemical shifts could not be assigned to three decimal places because of the complexity of the spectrum in that region. [b]Shift of H-1 of the β-D-Glc*p*NAc residue 2 in the α compound. Its shift in the β compound is shown beneath. [c]Average of two unresolved shifts, arising from the effect of the reducing (α,β)-D-Glc*p*NAc on the shift of the H-1 of β-D-Glc*p*NAc residue 2. [d]Assignments of the major component. Shifts of the minor component are shown beneath that of the major one.

oligosaccharide is important, so that the defined acidic conditions in the next step are not altered by the buffering capacity of the acetate anion. Sephadex G-15 proved to be the best gel available for this purpose, providing optimal separation between oligosaccharides of the size used in this report and sodium acetate. The use of Bio-Gel P-2 or P-4 resulted in the presence of significant amounts of sodium acetate in the oligosaccharide fractions, as observed in n.m.r. spectra. Using Sephadex G-15, there were only traces of acetate in the sample observed by n.m.r., an amount insufficient to affect the subsequent hydrolysis step, in which the concentration of acid was at least 100-fold in excess of that of acetate.

After chromatography on Sephadex G-15, fractions containing carbohydrate were concentrated and subjected to mild acid hydrolysis, under the conditions described in the Experimental section. After neutralizing the solution, the oligosaccharides were re-chromatographed on the Sephadex G-15 column. The profile of the carbohydrate product **10** is shown in Fig. 4c; the oligosaccharide was very similar in molecular weight to **6** (before acid treatment), and the glycosidic linkages of the molecule were stable to these acidic conditions, as confirmed by ^{1}H-n.m.r. spectroscopy (see later). This was expected, as treatment of asparagine-linked oligosaccharides with 0.05M H_2SO_4 at much higher temperatures (80°) has been used for removing sialic acids from the molecules, while leaving the remaining linkages intact[15]. The ^{1}H-n.m.r. spectrum of the product at this stage (Fig. 1c) revealed the presence of the free reducing oligosaccharide; new signals, corresponding to the α and β H-1 resonances of the reducing GlcNAc of **10** were observed. Signals corresponding to the acetohydrazide derivative **6** had disappeared. Other signals, arising from remaining unreacted glycopeptide and minor products, were present at low intensities.

After mild acid hydrolysis, the high-mannose structure **11** migrated as a single peak near the void volume on Sephadex G-15, virtually identical to the compound **7** before hydrolysis (data not shown). The ^{1}H-n.m.r. spectrum of the product at this stage (Fig. 2c) showed the presence of the α,β signals of H-1 of the reducing GlcNAc of **11**, and the absence of any signals corresponding to the GlcNAc β-acetohydrazide of **7**. Again, low-intensity signals from small amounts of unreacted glycopeptide and minor products were present.

For oligosaccharides containing *N*-acetylneuraminic acid, slightly milder conditions for cleavage of the β-acetohydrazide were used, because of the susceptibility of the α-sialoside linkage to acid hydrolysis. In control experiments, glycopeptide **4** was exposed to 0.05M H_2SO_4 for 1 h at various temperatures, and the release of NeuAc was quantitated by the thiobarbituric acid assay of Warren[16]. Values were compared to hydrolyses performed at 80°, taken as 100%. At 40°, 9% of the NeuAc was released, and at 35°, 3% of the NeuAc was released. Hydrolysis at 35° cleaves at least 95% of the β-acetohydrazide derivative, as determined previously[9]. Therefore, 35° is optimal, resulting in maximal hydrolysis of the β-acetohydrazide, with minimal hydrolysis of NeuAc from the molecule. The product of mild acid hydrolysis of the sialylated structure **8** eluted as a single peak on Sephadex

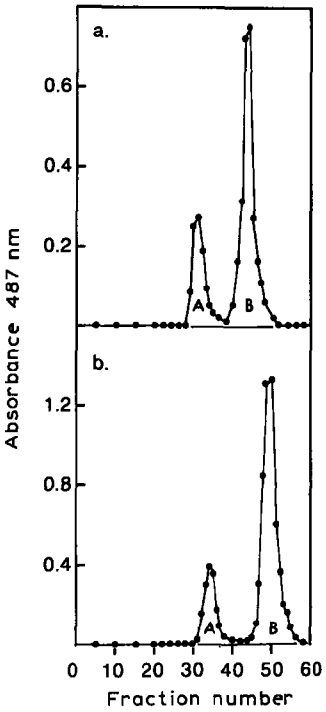


Fig. 5. Separation of the products of hydrazinolysis–*N*-reacetylation–mild acid hydrolysis of (*a*) glycopeptide **2** and (*b*) glycopeptide **3** on a column of Bio-Gel P4. Hexose was monitored by the phenol–sulfuric acid test[21]. Fractions comprising peaks A and B in both elution profiles were pooled separately for examination by n.m.r. spectroscopy.

G-15 near the void volume (data not shown), virtually identical to the elution position of the oligosaccharide before hydrolysis. A ^{1}H-n.m.r. spectrum of the product **12** after hydrolysis (Fig. 3b) showed the presence of the α,β signals of the reducing GlcNAc residue. The H-3 axial and equatorial signals of NeuAc residues remained essentially unchanged, indicating that hydrolysis at 35° does not remove enough NeuAc to be measured by n.m.r. Because of heterogeneity in the carbohydrate structure of **12**, further fractionation was not performed.

Reducing oligosaccharides **10** and **11** were purified further on a column of Bio-Gel P4 (Fig. 5). Preparations of either oligosaccharide showed 2 peaks. Peak A, which eluted well ahead on the column, was found in all runs to be *N*-acetylated glycopeptide by ^{1}H-n.m.r. (data not shown). Peak B (Figs. 5a and 5b) contained the reducing oligosaccharides **10** and **11**, as well as minor side-products. The explanation for such a wide separation of the compounds is that, when run in water at low ionic strength ($\leq$ 20mM), such negatively charged molecules as these *N*-acetylated glycopeptides are partially excluded from the gel, by virtue of residual carboxyl groups on the matrix. This effect has been described by the manufacturer[17].

Further purification of the structures **10** and **11** was achieved by l.c. (Fig. 6)

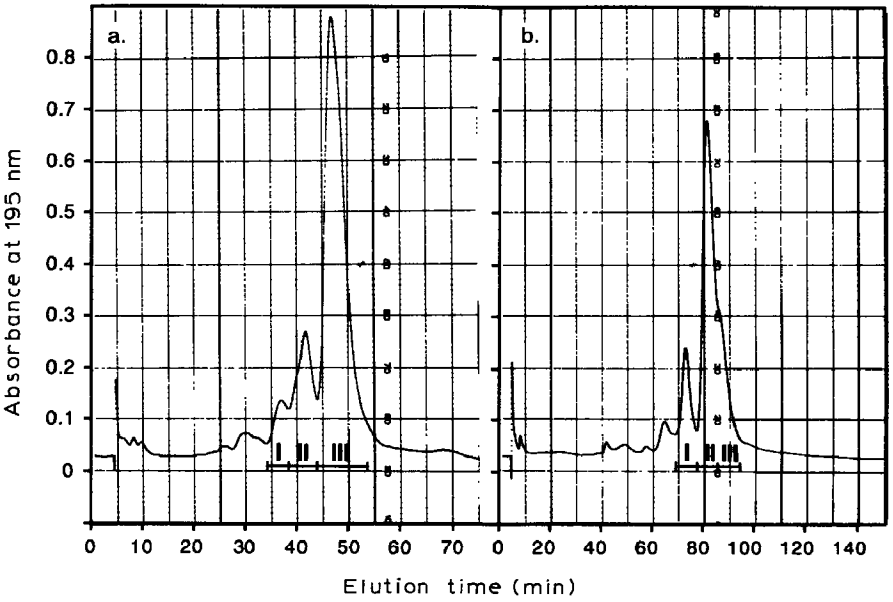


Fig. 6. High-pressure liquid chromatography (l.c.) of (*a*) oligosaccharides present in peak B (Fig. 5a), and (*b*) oligosaccharides present in peak B (Fig. 5b). Conditions are described in the Experimental section. Compounds were detected by absorbance at 195 nm; material in peaks was pooled into fractions marked under the elution profiles.

of the material present in peak B (Figs. 5a and 5b). More than one peak was always observed in the elution profiles. The major peaks (Fig. 6a, III and Fig. 6b, II) were pure **10** and **11**, respectively. Overall yields of the oligosaccharides ranged from 60–70%. The 360-MHz ^{1}H-n.m.r. spectra of these compounds are shown in Figs. 1d (**10**) and 2d (**11**). A comparison with the spectral parameters of 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucose **9** (Table I) aids in the interpretation of signals unique to this class of oligosaccharides. The signal arising from H-1 of the α anomer of the reducing Glc*p*NAc is observed near 5.19 and exhibits a small coupling constant (≤2.5 Hz). This chemical shift is diagnostic for these oligosaccharides; it is observed slightly upfield as compared to similar oligosaccharides that contain a β-D-Man*p* residue linked (1→4) to just one reducing GlcNAc[18]. The H-1 signal of the β anomer has a chemical shift near 4.7, and exhibits a second-order multiplet pattern through virtual coupling. In addition, the H-1 signal of the nonreducing β-D-Glc*p*NAc of **9** (Table I) is divided into two doublets of differing intensity. This is attributable to the effects of the two anomers of the reducing D-Glc*p*NAc on the chemical shift of H-1 of the nonreducing sugar, and is also observed in the larger oligosaccharides (Figs. 1d, 2d). Anomerization does not affect the chemical shifts of any of the H-1 or H-2 signals of D-Man*p*, or any of the H-1 shifts of branch β-D-Glc*p*NAc or β-D-Gal*p*, or H-3 shifts of NeuAc. This is in contrast to similar oligosaccharides containing just one α,β-Glc*p*NAc residue at the reducing end, which exhibit anomerization effects on the H-1 signals

of a number of residues of the oligosaccharides[18]. Another important feature of the spectrum of **9** is that the chemical shift of the *N*-acetyl resonance of the reducing GlcNAc is not affected by anomerization, or, at least, any shift difference is not resolvable at 360 MHz. This was also observed in the larger oligosaccharides, the *N*-acetyl resonance of the reducing GlcNAc appearing at δ 2.036 as a single peak. The *N*-acetyl region is particularily valuable in assessing the purity of the oligosaccharides at various stages of the purification (Figs. 1 and 2), as pure products contain just one acetyl resonance per *N*-acetylglucosamine. Based on the 360-MHz ^{1}H-n.m.r. spectra, the oligosaccharides **10** and **11** are estimated to be of >95% purity; a higher estimate is not possible because of the relative insensitivity of n.m.r. in detecting very minor impurities.

Minor products, observed as peaks of lower intensity in l.c. profiles (Fig. 6), did not contain a reducing D-GlcNAc residue, although n.m.r. spectra of these compounds revealed the presence of all other H-1 resonances, including the signal due to the β-D-Glc*p*NAc residue 2. This indicates that side reactions are limited to the terminal reducing sugar during the hydrazinolysis–*N*-reacetylation procedure. The nature of these side products was not determined for these particular oligosaccharides, but has been the topic of some previous studies[7–9].

To examine the primary structures of oligosaccharides present in complex mixtures of *N*-linked glycoproteins, the goal of such chemical treatments as hydrazinolysis–*N*-reacetylation is to produce oligosaccharides homogenous at the reducing end, thereby enabling fractionation to be performed solely on the nature of the carbohydrate moiety. As additional heterogeneity at the reducing terminus of oligosaccharides is generated during hydrazinolysis, the procedure falls short of the desired goal. Therefore, although the procedure can be utilized to prepare pure reducing oligosaccharides from glycopeptides containing a single carbohydrate structure, the problems encountered in isolation of pure oligosaccharides from mixtures become increasingly difficult as the complexity of the mixture increases.

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded with a Nicolet NT-360 spectrometer located at the Toronto Biomedical N.M.R. Centre. All spectra were recorded using D_2O as the solvent under experimental conditions described previously[9]. Chemical shifts were measured relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) (indirectly through acetone at 2.225 p.p.m.).

Materials. — Glycopeptides **2, 3,** and **4** were prepared by exhaustive pronase digestions of human fibrinogen, soybean agglutinin, and bovine fetuin, respectively, and isolated by procedures published previously[11,19,20]. *N*-Acetylneuraminic acid was removed from the fibrinogen glycopeptide by mild acid hydrolysis[15], and after neutralizing with NaOH, free NeuAc was largely removed by gel filtration on a column (2.5 × 95 cm) of Bio-Gel P-6 (200–400 mesh) eluted with water. A trace of free NeuAc was present, as observed by n.m.r. The disaccharide **9** was purchased from Calbiochem.

Procedures. — Hydrazinolysis–*N*-reacetylation of glycopeptides was performed as described previously[9], using 2–3 μmol. of glycopeptide/mL of anhydrous hydrazine. During *N*-acetylation, the pH of the solution gradually decreases as acetic anhydride is hydrolyzed to acetic acid. Therefore, solid $NaHCO_3$ was added to bring the solution to neutrality. At this stage, the solution was *not* passed through Dowex 50 (H^+) as described in ref. 9. Sodium acetate was removed by gel filtration on a column (1.6 × 90 cm) of Sephadex G-15 washed with at least 10 column volumes of water immediately before use, and eluted with water. Fractions (3.3 mL) were collected, and aliquots (20–50 μL) were first analyzed for hexose by a small-scale phenol–sulfuric acid test[21]. The region of the profile expected to contain sodium acetate was monitored by a qualitative test: to a 0.2-mL aliquot was added 10 μL of M ferric chloride. In the presence of relatively high (>100mM) concentrations of sodium acetate, the color changes from light yellow to red. Hexose-containing fractions were pooled and lyophilized. A portion was exchanged with D_2O for n.m.r. analysis. The remainder was dissolved in 0.1M HCl or 0.05M H_2SO_4 at a concentration of 1 μmol/mL, and incubated in a water bath for 1 h at 40°. For oligosaccharides containing NeuAc, incubation was carried out for 1 h at 35°. Solutions were made neutral with solid $NaHCO_3$, concentrated about 10-fold by rotary evaporation, and loaded again on the same Sephadex G-15 column as already described, eluting with water. Hexose-containing fractions were pooled, rotary evaporated to 1–2 mL, and a portion was examined by 360 MHz ^{1}H-n.m.r. spectroscopy.

In preparations of oligosaccharides **10** and **11**, the samples were then loaded on a column (117 × 1.4 cm) of Bio-Gel P4 (200–400 mesh), and eluted with water. Fractions (2 mL) were collected, and aliquots analyzed for hexose as already described. Fractions from the two hexose-containing peaks were pooled, and n.m.r. spectra were recorded at this stage. Peak A (Fig. 5a and 5b) contained primarily the *N*-acetylated, asparagine-linked starting compounds (**2** and **3**) and no attempt was made to subfractionate this peak. Peak B (Fig. 5a and 5b) contained reducing oligosaccharide which was ~85% pure, as judged by n.m.r. spectroscopy. Fractions of each peak B were pooled, rotary evaporated to dryness, and taken up in water at a concentration of ~10 μmol/mL. Further separation was achieved by l.c. on a column (25 × 0.46 cm) of Alltech NH2 (5-μm particle size), using a precolumn of 37–53 μm silica gel (Whatman). Acetonitrile–water (62:38, v/v) was used for isocratic elution, utilizing LKB 2150/2152 pumps and control unit. Detection was performed by monitoring the absorbance at 195 nm with an LKB 2151 variable-wavelength recorder. A flow rate of 0.7 mL/min, with resultant pressures ranging from 130–160 bar was typically used. About 200 nmol of oligosaccharide was injected per run; peaks were accumulated from a number of runs, pooled, and rotary evaporated to near-dryness. Because of contaminating materials which either were derived from the solvent, or resulted from column deterioration, samples were again chromatographed on the same Sephadex G-15 column as described previously. Hexose-containing fractions, which eluted as before, were pooled, lyophilized, and exchanged in D_2O to record n.m.r. spectra.

ACKNOWLEDGMENTS

The authors thank J. Orr and S. Allen for their help in preparation of glycopeptides, and Drs. A. Grey, J. P. Carver, and H. Schachter for helpful discussions. Research was supported by the MRC of Canada and the Terry Fox Special Initiatives Fund (Grant to Drs. J. P. Carver and H. Schachter).

REFERENCES

1 Z. YOSIZAWA, T. SATO, AND K. SCHMID, *Biochim. Biophys. Acta,* 121 (1966) 417–420.
2 S. TAKASAKI, T. MIZUOCHI, AND A. KOBATA, *Methods Enzymol.,* 83 (1982) 263–268.
3 M. FUKUDA, T. KONDO, AND T. OSAWA, *J. Biochem. (Tokyo),* 80 (1976) 1223–1232.
4 J. PAZ-PARENTE, G. STRECKER, Y. LEROY, J. MONTREUIL, B. FOURNET, H. VAN HALBEEK, L. DORLAND, AND J. F. G. VLIEGENTHART, *FEBS Lett.,* 152 (1983) 145–152.
5 S. J. MELLIS AND J. U. BAENZIGER, *J. Biol. Chem.,* 258 (1983) 11546–11556.
6 M. S. SAEED AND J. M. WILLIAMS, *Carbohydr. Res.,* 84 (1980) 83–94.
7 P. W. TANG AND J. M. WILLIAMS, *Carbohydr. Res.,* 121 (1983) 89–97.
8 J. MICHALSKI, J. PETER-KATALINIC, H. EGGE, J. PAZ-PARENTE, J. MONTREUIL, AND G. STRECKER, *Carbohydr. Res.,* 134 (1984) 177–189.
9 B. BENDIAK AND D. A. CUMMING, *Carbohydr. Res.,* 144 (1985) 1–12.
10 P. W. TANG AND J. M. WILLIAMS, *Carbohydr. Res.,* 136 (1985) 259–271.
11 R. R. TOWNSEND, E. HILLIKER, Y. LI, R. A. LAINE, W. R. BELL, AND Y. C. LEE, *J. Biol. Chem.,* 257 (1982) 9704–9710.
12 L. DORLAND, H. VAN HALBEEK, J. F. G. VLIEGENTHART, H. LIS, AND N. SHARON, *J. Biol. Chem.,* 256 (1981) 7708–7711.
13 B. NILSSON, N. E. NORDEN, AND S. SVENSSON, *J. Biol. Chem.,* 254 (1979) 4545–4553.
14 D. A. CUMMING, B. BENDIAK, AND J. P. CARVER, unpublished results.
15 R. G. SPIRO, *Methods Enzymol.,* 8 (1966) 1–26.
16 L. WARREN, *J. Biol. Chem.,* 234 (1959) 1971–1975.
17 *Gel Chromatography,* Bio-Rad Laboratories Publications, Richmond, California, 1975.
18 J. F. G. VLIEGENTHART, L. DORLAND, AND H. VAN HALBEEK, *Adv. Carbohydr. Chem. Biochem.,* 41 (1983) 209–374.
19 R. G. SPIRO AND V. D. BHOYROO, *J. Biol. Chem.,* 249 (1974) 5704–5717.
20 H. LIS AND N. SHARON, *J. Biol. Chem.,* 253 (1978) 3468–3476.
21 A. KOBATA, *Methods Enzymol.,* 28 (1972) 262–271.