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STRUCTURAL ANALYSIS OF THE CARBOHYDRATE MOIETIES OF GLYCOPROTEINS BY REGIOSPECIFIC DEGRADATION AND LIQUID CHROMATOGRAPHY

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ABSTRACT

The reaction sequence of hydrazinolysis, nitrosation, and reduction, followed by liquid chromatography (l.c.) has been studied as a method for the routine structural analysis of the asparagine-bound oligosaccharides of glycoproteins. Glycopeptides derived from IgM and ovalbumin by proteolysis were used as test materials. The hydrazinium sulphate-catalysed hydrazinolysis was superior to the longer uncatalysed reaction, in that there was less non-specific degradation and a higher degree of *N*-deacetylation. The nitrosation products were reduced *in situ* with sodium cyanoborohydride, and the l.c. analysis required 20 min for the fractionation of oligosaccharides up to decasaccharide. The l.c. profile is characteristic of the structure of the carbohydrate unit. The analytical l.c. column may also be used to isolate oligosaccharide fractions in quantities of several hundred micrograms.

INTRODUCTION

The elucidation of structure of the oligosaccharide units of glycoproteins is a difficult and challenging task, as there is, for carbohydrates, no sequencing method comparable to the Edman procedure for peptides. Moreover, the oligosaccharide units of glycoproteins are frequently heterogeneous[1]. Considerable improvements have been made in recent years in the methodology for the elucidation of structure of oligosaccharides[2,3], but such studies are usually carried out on homogeneous carbohydrate fractions which have been prepared in long and tedious fractionation procedures. In clinical studies, as an initial screening procedure, structural information is usually required for the total carbohydrate content of a glycoprotein so

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that comparisons can be made between normal material and that associated with disease. Our approach, therefore, has been to seek a method that can be used to analyse, and provide structural information for, the unfractionated mixture of carbohydrate components. The key features of our method are the regiospecific cleavage of certain glycosidic linkages, and product analysis by liquid chromatography (l.c.). The l.c. profile is then characteristic of the structures of the oligosaccharide units present in the original glycoproteins. The method can, of course, be used for structural studies of purified oligosaccharides.

The regiospecific cleavage of glycosidic linkages is achieved by the rearrangement which occurs when 2-amino-2-deoxy-D-glucopyranosides and 2-amino-2-deoxy-D-galactopyranosides are treated with nitrous acid[4]. Such applications of this nitrosation reaction were first suggested by Foster and Huggard[5], and there are now several examples[6]. Such hexosamines are common constituents of glycoproteins, and applications of methods involving nitrosation to the oligosaccharide units of glycoproteins have been reported[7–11].

For glycoproteins that contain asparagine-bound carbohydrate, the carbohydrate moiety may be isolated by proteolysis followed by hydrazinolysis. The latter step effects the cleavage of the glycosylamide bond between carbohydrate and protein, and also causes *N*-deacetylation of hexosamine and sialic acid residues, which is necessary for the succeeding nitrosation step. Early applications of hydrazinolysis to glycoproteins caused degradation, and a significant improvement was the introduction of hydrazinium sulphate as a catalyst[12]. Kochetkov and coworkers[13], using model glycosides, found that *N*-deacetylation of 2-acetamido-2-deoxy-D-glucopyranosides was more difficult when HO-3 was substituted. For such less-reactive amides, a longer reaction time of 10 h at 105° was required to achieve complete *N*-deacetylation. Montreuil and his collaborators, in their work on glycoproteins, used uncatalysed hydrazinolysis, the optimum conditions of 30 h at 100° being established with methyl 2-acetamido-2-deoxy-β-D-glucopyranoside[18].

RESULTS AND DISCUSSION

Two reaction conditions for hydrazinolysis have been compared using glycopeptides from ovalbumin and IgM. After removal of hydrazine and fractionation of the products on Sephadex G15 or Biogel P2, the product isolated after subsequent nitrosation and reduction had a lower content of 2-acetamido-2-deoxy-D-glucose in the 10 h/105° experiments than in the 30 h/100° experiments. To assess whether any degradation involving glycosidic cleavage had occurred during hydrazinolysis, the fractions corresponding to the monosaccharide region after gel-permeation chromatography were assayed for hexose by the phenol–sulphuric acid procedure. The weights of hexose expressed as a percentage of the weight of glycopeptide were 0.6% and 3% for the 10 h/105° and 30 h/100° experiments, respectively. All of the types of monosaccharide present in the original glycopeptide were present in this fraction, but fucose was relatively enriched, whereas galactose

TABLE I

MONOSACCHARIDE PROPORTIONS[a]

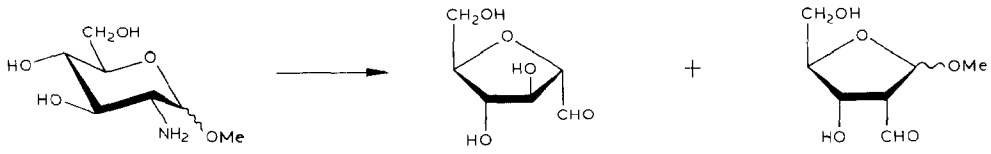
	Fuc	*Man*	*Gal*	*GlcNAc*	*AM*[b]	*NANA*[c]
IgM Glycopeptide	0.52	3	1.1	2.59	—	0.62
Degradation product	0.41	3	1.05	—	1.60	—
σ_{n-1}	0.08		0.06		0.09	

[a]Relative to 3 residues of mannose. [b]2,5-Anhydro-D-mannitol. [c]*N*-Acetylneuraminic acid (sialic acid).

and sialic acid were relatively enriched only in the product of the 30-h hydrazinolysis.

Treatment of the *N*-deacetylated glycan with sodium nitrite in dilute acetic acid gave 2,5-anhydro-D-mannose and oligosaccharides having a 2,5-anhydro-D-mannose reducing end-group, which were reduced *in situ* with sodium cyanoborohydride (an effective reducing-agent in acidic media[14]) to the corresponding 2,5-anhydro-D-mannitol. The product mixture was analysed by l.c., and the monosaccharide composition was also determined. Reproducible results were obtained, and the monosaccharide composition of the starting material and product mixtures, together with standard deviations, are given in Table I.

These results show that the conversion of 2-acetamido-2-deoxy-D-glucose into 2,5-anhydro-D-mannitol is not quantitative. Work with methyl 2-amino-2-deoxy-α- and -β-D-glucopyranosides (**1**) showed[4,15] that a side reaction occurs to give **3** in ~20% yield. The g.l.c. analysis, however, suggests that this side reaction may be less important for the oligosaccharides than for the methyl glycosides. In



1 2 3

addition, earlier work showed that the terminal residue in the hydrazinolysis product is 2-amino-2-deoxy-D-glucose hydrazone and that this is not converted quantitatively into **2**[16]. The yield of **2**, determined as 2,5-anhydro-D-mannitol, was 56% from 2-amino-2-deoxy-D-glucose hydrazone. It has also been shown[17] that 2-amino-2-deoxy-D-glucose hydrazone is partially degraded under the conditions of hydrazinolysis, to give 1-deoxy-D-fructose hydrazone.

The monosaccharide having the highest standard deviation is fucose. This is the monosaccharide most susceptible to degradation (except for 2-acetamido-2-deoxy-D-glucose as discussed above), and the monosaccharide region after gel-permeation chromatography of the hydrazinolysis product was relatively rich in fucose. The product of nitrosation of neuraminic acid has not been identified and assessed.

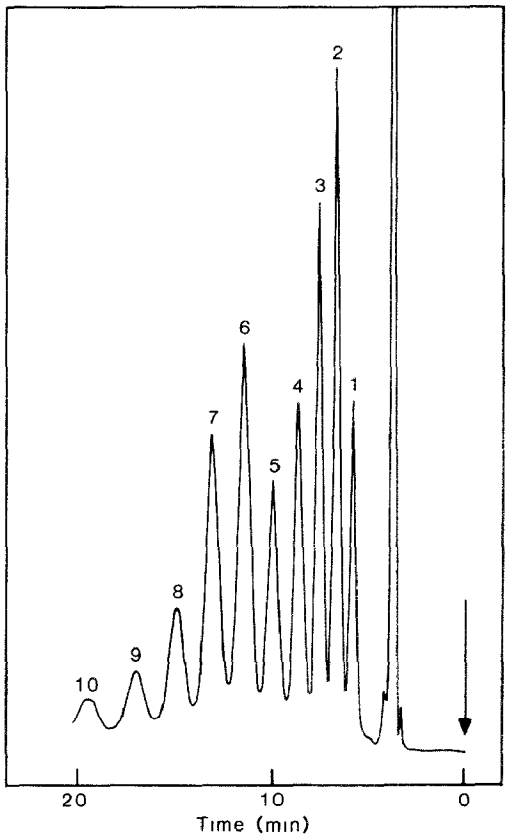


Fig. 1. L.c. analysis of a standard mixture of malto-oligosaccharides (from starch hydrolysate) on Partisil PAC. Refractive index is plotted against time. Peak numbers designate the d.p. See text for details.

A Whatman Partisil PAC column was used for the fractionation of the oligosaccharides present in the degradation product, the aqueous acetonitrile eluent being buffered with acetic acid instead of phosphate buffer[18]. The fractionation of a standard mixture of malto-oligosaccharides is illustrated in Fig. 1, and the product from IgM glycopeptide is shown in Fig. 2. The monosaccharide peak (peak 1 in Fig. 2) has the same retention time as authentic 2,5-anhydro-D-mannitol, and its identity has been confirmed by preparative l.c. followed by g.l.c. analysis. The two disaccharides (peaks 2a and 2b) have also been isolated by preparative l.c., using a solvent containing less water to increase the resolution of the constituents of low molecular weight. The major disaccharide is β-D-galactosyl-2,5-anhydro-D-mannitol, the configuration of the glycosidic linkage being established by ^{1}H-n.m.r. spectroscopy ($J_{1,2}$ 7 Hz for the D-galactosyl group). The minor disaccharide is a fucosyl-2,5-anhydro-D-mannitol, which establishes the position of the fucosyl group on the 2-acetamido-2-deoxy-D-glucosyl residue involved in the linkage to asparagine, in the complex units of IgM. The tetrasaccharide (peak 4) was similarly shown to be a trimannosyl-2,5-anhydro-D-mannitol. Both 2,5-anhydro-D-mannitol

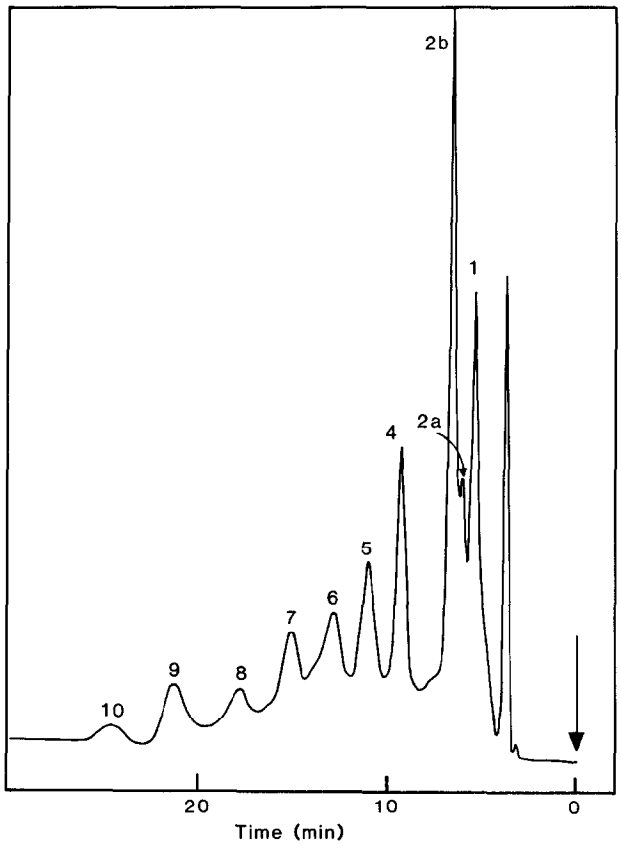
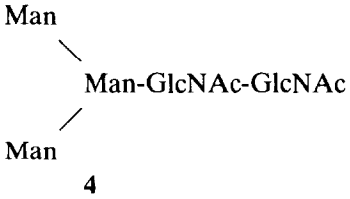


Fig. 2. L.c. analysis of the degradation products from IgM glycopeptide on Partisil PAC.

and the tetrasaccharide would be expected, in view of the common occurrence[19] of the core asparagine-bound oligosaccharide **4**. Peak 5 was shown to contain a pentasaccharide, but the degrees of polymerisation of the components in peaks 6–10 have not yet been determined.



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The major product of the nitrosation of the neuraminic acid residues is likely to be the corresponding nonulosonic acid, in which the configuration at C-5 is retained, by analogy with the reaction of methyl 4-amino-4-deoxy-D-glucopyranoside[20]. The results of Mononen *et al.*[21] are consistent with this proposal.

Oligosaccharides containing such nonulosonic acid residues would not be eluted from the l.c. column under the conditions of analysis. The foregoing results thus relate only to the neutral degradation-products. A comparison of the products from IgM glycopeptide, with and without prior removal of the sialic acid residues, showed that the products from the sialic acid-free glycopeptide contained much more D-galactosyl-2,5-anhydro-D-mannitol (peak 2b). This result is consistent with the structure published for the outer chains of the complex-type oligosaccharides (*e.g.*, NANA-Gal-GlcNAc-)[19]. When a sialic acid residue is linked to D-galactose as in NANA-Gal-GlcNAc-, the major product will be an acidic trisaccharide. Preliminary work has suggested that the acidic products can be eluted from the Whatman PAC column by using a more acidic buffer.

For preparative experiments with the analytical l.c. column, eluent containing less water is used to improve the peak separation; it is convenient to collect the mono- and di-saccharides together, and then refractionate in order to obtain the individual constituents. Up to 700 μg of an oligosaccharide has been collected in a single preparative experiment.

In choosing the conditions for the hydrazinolysis of glycopeptides, a compromise is necessary. It is not possible to effect complete *N*-deacetylation without causing some degradation. The uncatalysed reaction (30 h at 100°) is less efficient than the hydrazinium sulphate-catalysed reaction (10 h at 105°), as judged by the amount of 2-acetamido-2-deoxy-D-glucose in the final product, by the presence of additional artefact peaks in the l.c. and g.l.c. analyses of the saccharides, and by the amount of carbohydrate eluted from the Sephadex column in the monosaccharide region after hydrazinolysis. The degradation product, 1-deoxy-D-fructose hydrazone, formed from 2-amino-2-deoxy-D-glucose hydrazone, would be converted into 1-deoxy-D-mannitol and 1-deoxy-D-glucitol by nitrosation and reduction[17]. These compounds were not detected during g.l.c. analysis of the methanolysis products; 1-deoxy-D-mannitol had a similar retention time to that of α-D-mannose. For this reason, the monosaccharide ratios cited in Table I for the degradation product may be slightly in error.

EXPERIMENTAL

Glycopeptides. — A monoclonal Immunoglobuliin M (IgM; from a case of Waldenström's macroglobulinaemia) and ovalbumin were exhaustively digested with pronase. The resulting mixtures of peptides and glycopeptides were fractionated on a column of Sephadex G25, and the crude glycopeptide mixtures were purified by paper chromatography[22]. The monosaccharide contents of the glycopeptides and derivatives were determined by g.l.c. of the trimethylsilyl ethers of the methyl glycosides after methanolysis[23]. Amino acids were analysed (Beckman instrument) after hydrolysis of the glycopeptides in 6M hydrochloric acid at 105° for 24 h in a sealed tube.

Ovalbumin and IgM glycopeptides contained ~15% by weight of amino

acids, with aspartic acid preponderating. Basic amino acids were not assayed.

Hydrazinolysis. — (Preliminary experiments were carried out with ovalbumin glycopeptide.) Dry glycopeptide (5–80 mg) and hydrazinium sulphate (1.5 × the weight of glycopeptide) were placed in a hydrolysis tube fitted with a side arm and Teflon tap, and transferred to a glove box. Anhydrous hydrazine (0.2–3 mL) was added and the resulting solution was degassed by three freeze–thaw cycles over nitrogen. The tube was then kept at 105 ±0.5° for 10 h in a Berghof heating-block. Hydrazine was removed from the cooled solution *in vacuo* over conc. H_2SO_4. The glycan-amine was separated from hydrazinium sulphate, amino acid hydrazides, acetohydrazide, and 4-amino-3,5-dimethyl-1,2,4-triazole[16] by chromatography on a column (85 × 2.5 cm) of Sephadex G15 which was eluted with 0.1M acetic acid at 8.7 mL per h. Fractions were collected at intervals of 15 min, and analysed for hexose by the phenol–sulphuric acid procedure and for amines with ninhydrin. The glycan-amine was eluted in fractions 23–32, and hydrazinium sulphate in fractions 38–44. The combined carbohydrate-containing fractions were freeze-dried to give the glycan-amine, which had a weight that was 84% (σ_{n-1} ±2.7 for eight experiments) of that of the original IgM glycopeptide.

Nitrosation and reduction of the glycan-amine. — (Preliminary experiments were carried out with ovalbumin glycopeptide.) To a solution of the glycan-amine (40 mg) in water (1 mL) was added sodium nitrite (80 mg) in water (1 mL). The solution was cooled below 10° in ice, and glacial acetic acid (0.4 mL) was added in three portions during 30 min. The mixture was allowed to attain room temperature, and was purged with N_2 for 15 min. Sodium cyanoborohydride (73 mg) was then added, followed by glacial acetic acid at intervals to maintain the pH between 3 and 4. After 1 h, sodium ions were removed by addition of Amberlite IR-120 (H^+) resin (40 mL; part of the resin was added first to decompose the excess of reducing agent and, when gas evolution had ceased, the supernatant solution was passed through the rest of the resin in a column). The resin-free solution plus washings were freeze-dried, and boric acid was removed conventionally as methyl borate. In eight experiments starting with 30–70 mg of IgM glycan-amine, the yield of product was 94% (σ_{n-1} ±3) by weight. A satisfactory l.c. analysis was obtained by using a one-sixth aliquot of the product from 5 mg of IgM glycopeptide.

L.c. analysis. — A solution of the reduced degradation product in water was filtered through a Millipore 5-μm membrane and then concentrated to ~12% (w/v). Aliquots (10 μL) were analysed on a Whatman PAC 10 column (25 cm × 4.6 mm) with 70:30 acetonitrile–2.5mM acetic acid at 0.8 mL per min. An LCD refractive-index detector (Refractomonitor III) was used with thermostatting of the cell at 1° above room temperature.

Sequential nitrosation and reduction. — *(a) 2-Amino-2-deoxy-*D*-glucose hydrazone.* 2-Amino-2-deoxy-D-glucose hydrochloride (1.0 mmol) was dissolved in hydrazine hydrate (AR, 3 mL) and kept overnight. The hydrazine hydrate was then removed under reduced pressure in a vacuum desiccator over conc. H_2SO_4. A solution of the syrupy residue in water (10 mL) containing D-mannitol (100.5 mg,

0.552 mmol) was neutralised to pH 5–6 by addition of glacial acetic acid (AR). To the ice-cooled solution was added sodium nitrite (414 mg, 6 mmol) followed by glacial acetic acid (AR, 1.1 mL) in 4 portions during 1 h. The solution was removed from the ice bath, purged with N_2 for 15 min, and then treated with sodium cyanoborohydride (189 mg, 3 mmol). After 2.5 h, sodium ions were removed with Amberlite IR-120 (H^+) resin (100 mL). The mixture was filtered, and the resin was washed thoroughly with water. The filtrate was freeze-dried, and boric acid was removed from the residue with methanol in the usual way. The dried, solid residue weighed 244 mg (theoretical, 264.5 mg). G.l.c. of the trimethylsilylated residue gave a ratio of 2,5-anhydro-D-mannitol to D-mannitol of 1.015. Hence, the yield of 2,5-anhydro-D-mannitol was 56%. The noise-decoupled ^{13}C-n.m.r. spectrum (25 MHz, 30° pulse, and a pulse-repetition time of 1.6 s) of a solution of the product in D_2O contained signals for D-mannitol (73.7, 72.1, 66.0 p.p.m. from sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate) and 2,5-anhydro-D-mannitol (85.2, 79.3, 64.0 p.p.m.). Other very weak signals were also present.

(b) Methyl 2-amino-2-deoxy-β-D-glucopyranoside. The glycoside (48 mg, 0.25 mmol) and D-mannitol (51.3 mg, 0.25 mmol) were dissolved in water (2 mL). Nitrosation and subsequent reduction were effected, as in (*a*), with sodium nitrite (104 mg, 1.5 mmol), glacial acetic acid (0.15 mL), and sodium cyanoborohydride (47 mg, 0.75 mmol). The dried product weighed 97 mg. Carbohydrate analysis[23] (first step, acid-catalysed methanolysis) gave two incompletely resolved (g.l.c.) peaks (T_M 0.34, 0.35), strong peaks for 2,5-anhydro-D-mannositol (T_M 0.57) and D-mannitol (T_M 1), and weak peaks due to unidentified products. The peak-area ratio for the T_M 0.34 and 0.35 peaks/2,5-anhydro-D-mannitol was 1/7.7

The product of C-4 migration (in the nitrosation step) was shown by g.l.c.–m.s. (separate experiment) to have a retention time relative to D-mannitol of 0.34 (before methanolysis, using similar but not identical g.l.c. conditions). Methanolysis would be expected to equilibrate the pyranosides and furanosides.

The products with T_M 0.34 and 0.35 were eluted in the fucose region. However, the absence of significant peaks in this region for the degradation products from fucose-free glycopeptides implies that the side reaction involving C-4 migration in the nitrosation step is less important than for the simple methyl glycosides of 2-amino-2-deoxy-D-glucopyranose.

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