Partial Deglycosylation of Blood-Group-Specific Glycoproteins

Alastair S. R. DONALD

Division of Immunochemical Genetics, M.R.C. Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.

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The time course for the partial deglycosylation of blood-group-specific glycoproteins from human ovarian-cyst fluids with 0.25 M-H₂SO₄/acetic acid and 6 M-HCl in methanol was studied. Either reagent readily removed about 80% of the carbohydrate from the glycoproteins to leave non-diffusible glycopeptides that contain *N*-acetylgalactosamine as the predominant sugar. Some changes in amino acid distribution were observed during the deglycosylation, which were attributed to an accelerated break-up of the non-glycosylated regions of the parent glycoprotein. The *N*-acetylgalactosaminyl-peptides isolated were judged to be polydisperse by gel filtration, and ion-exchange chromatography divided the glycopeptide population into several fractions with differing amino acid compositions. A *Lumbricus terrestris* hexosaminidase preparation was successful in removing almost all the remaining sugar from the glycopeptides, but caused further rupture of the peptide. When a per-*O*-acetylated glycoprotein was treated with the H₂SO₄/acetic acid reagent the glycopeptide contained, in addition to *N*-acetylgalactosamine, about 50% of the sialic acid present in the parent glycoprotein, indicating that most of this sugar is located near the peptide end of the carbohydrate chains.

Although much evidence has accumulated about the heterogeneous nature of the carbohydrate chains of the ovarian-cyst blood-group-specific glycoproteins, and considerable knowledge has been gained about the structure and biosynthesis of the sugar groupings responsible for the blood-group specificities of these macromolecules (for a review see Watkins, 1972), little is known of the peptide moiety. The amino acid compositions of a number of preparations have been published (e.g. see Pusztai & Morgan, 1963) and show a predominance of the amino acids threonine, serine and proline. The large losses of threonine and serine that occur on alkaline treatment of the glycoproteins are thought to be the result of a β -elimination reaction, and indicate the presence of *O*-glycosidic bonds between carbohydrate and these hydroxy amino acids (Anderson *et al.*, 1963; Adams, 1965; Kabat *et al.*, 1965). The results of Pronase-digestion experiments suggest that the peptide consists of some domains rich in threonine and serine, which are almost totally glycosylated, and other non-glycosylated domains, which contain a much higher proportion of the acidic and basic amino acids (Donald, 1973). Further progress in structural studies on the peptide moiety has been hindered by the difficulty of obtaining sugar-free peptide. The partial deglycosylation of the glycoproteins by using H₂SO₄ in acetic acid was des-

cribed in a preliminary communication (Donald *et al.*, 1969). The products of this reaction were glycopeptides whose predominant sugar component was *N*-acetylgalactosamine, and their isolation confirmed the results of alkaline-borohydride degradation (Kabat *et al.*, 1965; Iyer & Carlson, 1971), implicating *N*-acetylgalactosamine as the linkage sugar. The glycopeptides were used as starting materials for the isolation and characterization of a series of di- to hepta-peptide fragments (Goodwin & Watkins, 1974). The present paper gives a more detailed account of the preparation and properties of the *N*-acetylgalactosaminyl-peptides.

Experimental

Materials

The blood-group-specific glycoproteins were provided by Professor W. T. J. Morgan of the M.R.C. Clinical Research Centre, Harrow, Middx., U.K. The glycoproteins were prepared from human ovarian-cyst fluids by the phenol-extraction procedure (see Morgan, 1967); the blood-group substances specifically mentioned in the text were: (1) substance H 500 PI/WI/Pr/0–60% ethanol, (2) substance A 584 PI/WS/40–47% ethanol, (3) substance Le^a 610 PI/WS/43–50% ethanol and (4) substance Le^a 603 PI/WS/0–45% ethanol [PI, phenol-

insoluble; WS, water-soluble; WI, water-insoluble; Pr, Pronase-digested; as described by Donald (1973)]. The ethanol percentages refer to the concentration (v/v) range over which the substance was precipitated.

Per-*O*-acetylated substance Le^a 610 was prepared by a procedure essentially that of Slomiany & Meyer (1973). The glycoprotein (1 g) was dissolved in formamide (20 ml), and 60 ml of acetic anhydride/pyridine (2:1, v/v) was added. The mixture was stirred at room temperature (approx. 25°C) for 48 h and was then transferred, together with acetone washings, to 18/32 Visking tubing. The dialysis bags were filled to only half their capacity. After exhaustive dialysis the non-diffusible material was centrifuged (27 000 *g* for 15 min) and the residue was suspended in a little water and freeze-dried (yield 1.4 g, acetyl content 34.1%).

The sources of the enzymes, sugars, chromatographic media and other chemicals have already been given (Donald, 1973).

Methods

Most of the general methods are as described in Donald (1973). Anhydrous methanol was prepared by refluxing AnalaR-grade methanol (1 litre) with Mg turnings (5 g) for 1 h and then distilling. The 6M-HCl/methanol was prepared by passing dry HCl gas into cooled methanol until the required molarity was reached.

Analyses

Amino acids, hexosamines and hexosaminotols were determined on an amino acid analyser as previously described (Donald, 1973). Values given for peptide content were obtained by summation of the values found for the individual amino acids. Methyl 2-amino-2-deoxygalactopyranosides were analysed by using the standard hexosamine procedure. Acetyl groups were determined by g.l.c. (Radhakrishnamurthy *et al.*, 1968). Colorimetric analysis for fucose (Gibbons, 1955) and galactose (Dubois *et al.*, 1956) were sometimes used where stated. Otherwise these sugars were analysed by g.l.c. as follows. The samples (10–100 µg of sugar) and mannitol (10 µg) internal standard were hydrolysed with 2M-HCl for 2 h at 100°C. The hydrolysates were passed through columns (0.4 cm internal diam.) containing Zeo-Karb 225 (H⁺ form; X8; 200–400 mesh; 0.5 cm height) overlaid with De-Acidite FFIP (formate form; X4; 52–100 mesh; 2.5 cm height). The columns were washed with 1% (v/v) formic acid in 10% (v/v) ethanol, and the eluates were evaporated to dryness. After further overnight drying under vacuum with P₂O₅, the residues were trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane (10:2:1, by vol.) for 30 min. The derivatives were analysed on a Pye series 104

model 64 gas chromatograph with columns (150 cm × 0.4 cm) of 3% SE30 on Gas-Chrom Q operated with a temperature programme rising from 110°C to 180°C at 6°C/min. For analysis of samples that might have additionally contained alditols, controls without mannitol were included to establish the absence of galactitol, which co-chromatographs with mannitol.

Sialic acid was determined by the thiobarbituric acid method (Warren, 1959) after hydrolysis of the sample for 1 h at 80°C with 0.05 M-H₂SO₄, or as methoxyneuraminic acid on an amino acid analyser by the procedure of Liu (1972).

Partial deglycosylation with H₂SO₄/acetic acid

Small-scale experiments to determine a suitable time for the reaction were performed as follows. Samples (20–25 mg) of the glycoprotein were accurately weighed into screw-capped tubes (100 mm × 14 mm) and collected at the bottoms of the tubes by repeated tapping. The samples were re-dried under vacuum, and 0.25 M-H₂SO₄ in acetic acid (2 ml) was added; the tubes were then capped and placed in a water bath at 60°C. At intervals between 1 h and 30 h tubes were removed, cooled in tap water, and water (1 ml) was added. The contents of the tubes were then transferred, together with aqueous washings, to 8/32 Visking tubing. Sometimes a sticky solid resulted when the reaction mixture was diluted with water. This was easily transferred to the dialysis tube by holding it by suction to the tip of a Pasteur pipette. The samples were first dialysed thoroughly against water, then against 0.02 M-NaOH (3 litres, 2 h with stirring at room temperature) to remove *O*-acetyl groups; this was followed by dialysis against 0.1% acetic acid and then a final exhaustive dialysis against water. The non-diffusible residues were then made up to accurately known volumes with water. Samples for the zero time were prepared by adding water (2 ml) to the glycoprotein and following all the stages described above.

For large-scale preparation of the glycopeptides, the glycoprotein (5 g) was mixed with 0.25 M-H₂SO₄ in acetic acid (500 ml) that had been preheated to 60°C. The reaction vessel was then stoppered and placed in a water bath at 60°C for 6 h, during which time it was periodically shaken. The mixture was then cooled, poured into ice/water (500 ml) and the product dialysed exhaustively. The non-diffusible material was made 0.02 M with respect to NaOH. Any insoluble material present dissolved at this stage. After 1 h the solution was adjusted to approx. pH 3 with acetic acid, and any precipitate that formed was removed by centrifugation (27 000 *g* for 1 h at 3°C). Both the supernatant and residue were dialysed exhaustively and the non-diffusible materials were dried from the frozen state.

Partial deglycosylation with HCl/methanol

Samples (20–25 mg) of glycoprotein were prepared as described for the H_2SO_4 /acetic acid treatment. Freshly prepared 6 M-HCl in anhydrous methanol (1 ml) was added, and the tubes were capped and left in the dark at room temperature. At intervals over 12 days tubes were opened and the contents and aqueous washings were added to 1 M- NH_4HCO_3 (6.5 ml). Acetic acid was then added to adjust the pH to about 4–5. The solutions were then dialysed exhaustively; the non-diffusible materials were evaporated to dryness and then made up to a known volume with water. The zero-time sample was prepared as described above, but water was substituted for the HCl/methanol and NH_4HCO_3 solution.

For larger-scale preparations the glycoprotein (1 g) was placed in a screw-capped bottle together with freshly prepared 6 M-HCl in anhydrous methanol (50 ml). The bottle was left in the dark for 3 days at room temperature. A sticky solid formed, which was separated from the supernatant by decantation and washed with methanol (3×20 ml). The combined supernatant and washings were evaporated to dryness and thrice re-evaporated after the addition of methanol (100 ml each time). The residue was dissolved in water, and the resulting solution was added to the sticky solid in the reaction vessel; the solid dissolved readily. The solution was then dialysed exhaustively against water and freeze-dried. Sometimes methyl ester groupings were removed by treating the glycopeptide with 0.05 M-NaOH for 1 h, neutralizing the solution with acetic acid and recovering the glycopeptide by dialysis and freeze-drying.

To estimate the extent of possible hexosamine de-N-acetylation occurring during the HCl/methanol treatment, N-acetylgalactosamine (10 mg) was dissolved in 6 M-HCl/methanol (5 ml). Samples (25 μ l) were withdrawn at intervals and examined on the amino acid analyser for methyl 2-amino-2-deoxy-galactopyranosides.

Alkaline-borohydride treatment

The glycopeptide (1 mg) was treated with 0.05 M-NaOH in 1 M- NaBH_4 (1 ml) for 16 h at 50°C (Iyer & Carlson, 1971). The sample was then placed in an ice/water bath and cautiously acidified with acetic acid. Na^+ ions were removed by passing the solution through a column (5 cm $\times$ 0.4 cm internal diam.) of Zeo-Karb 225 (H^+ form; X8; 100–200 mesh). The column was washed with water (3×3 ml), and the combined eluates were evaporated to dryness. The boric acid was removed by repeatedly evaporating the residue with methanol (2 ml). Finally the residue was dissolved in water (1 ml) and samples were hydrolysed before carbohydrate analysis.

Coupling of a glycopeptide to Sepharose

An affinity absorbent for the isolation of hexosaminidases was prepared by coupling a glycopeptide to Sepharose. CNBr-activated Sepharose 4B was prepared by the procedure of March *et al.* (1974). A glycopeptide (690 mg), prepared by HCl/methanol treatment of Le^a substance 603, was coupled to the gel (500 ml) by the method of these authors. The product contained 0.35 mg of N-acetylgalactosamine/ml of gel.

Isolation of a hexosaminidase fraction from earthworms

The citrate buffers used for the enzyme purification were (i) 1.66 M-sodium citrate adjusted to pH 4.0 with HCl and (ii) 0.5 M-sodium citrate adjusted to pH 6.5 with HCl.

Earthworms (*Lumbricus terrestris* 100 g), collected from lawns during darkness, were macerated for 2 min in a Waring blender with the pH 4.0 sodium citrate buffer (500 ml). The stirred mixture was held at pH 4.0 for 1 h by the periodic addition of 1 M-HCl and then centrifuged (18000 g for 1 h at 2°C). The supernatant was then loaded on to a column (13 cm $\times$ 6 cm internal diam.) of the glycopeptide-Sepharose, previously equilibrated with the above sodium citrate buffer, and the column was eluted with the same buffer (500 ml) and then washed with 0.5 M-sodium citrate, pH 6.5. Hexosaminidase activity, as measured with *p*-nitrophenyl α - and β -2-acetamido-2-deoxy-glucopyranosides and -galactopyranosides as substrates, emerged from the column at the buffer change front and was entirely contained within 150 ml of eluate. The active fraction was dialysed against 0.05 M-sodium citrate, pH 4.9.

The enzyme preparation contained α - and β -N-acetylglucosaminidase and α - and β -N-acetylgalactosaminidase activities, together with some weak α -galactosidase activity. It was found that, after repeated use of the affinity column over a period of more than a year, the N-acetylgalactosamine content of the gel was virtually unchanged, indicating that little or no cleavage of the N-acetylgalactosamine to threonine or serine bond had occurred.

Enzymic deglycosylation of the glycopeptide

The glycopeptide (25 mg), prepared by HCl/methanol treatment of substance H 500, was dissolved in the hexosaminidase solution (10 ml), and 0.4 M- MgCl_2 solution (0.5 ml) was added. The mixture was incubated for 7 days at 47.5°C, then heated at 100°C for 10 min, cooled, diluted to 50 ml with water and the pH adjusted to 7.5 with solid NaHCO_3 . The solution was then passed through a column (3 cm $\times$ 1 cm internal diam.) of Whatman DE-52 DEAE-cellulose, and the column was washed with water and the eluates were evaporated to dryness. Experiments with a heat-inactivated enzyme

and untreated glycopeptide established that enzyme protein is completely removed by the ion-exchanger under these conditions, whereas the glycopeptide passes straight through.

The residue was redissolved in water and chromatographed on a column (90 cm $\times$ 1.5 cm internal diam.) of Sephadex G-25 (medium grade), equilibrated and eluted with water. Fractions were pooled as indicated on Fig. 6.

Results

Partial deglycosylation with H_2SO_4 /acetic acid

The blood-group-specific glycoproteins used for this study are those isolated from human ovarian-cyst fluids by the phenol extraction procedure (see Morgan, 1967). These glycoproteins undergo partial deglycosylation when treated with 0.25 M- H_2SO_4 in acetic acid to leave non-diffusible *O*-acetylated glycopeptides. The time course for this reaction was followed by analysis of the de-*O*-acetylated non-diffusible residues obtained after various times of treatment. The recovery of the individual sugars and of peptide for substance Le^a 610 are shown in Fig. 1. More than 98% of the fucose was lost from the non-diffusible fraction after 2 h of treatment. The loss of *N*-acetylglucosamine was almost as rapid (98% lost after 6 h), and the galactose loss was a little slower (93% lost after 6 h). Amino acids and *N*-acetylgalactosamine were removed much more slowly; only 20% or so was lost after 6 h. Large losses (75–80%) of these components from the non-diffusible fraction did, however, occur after 30 h. The losses of

amino acids and *N*-acetylgalactosamine were roughly equal throughout the course of the reaction.

The sialic acid recovery curve was different from that of any of the other components. There was an initial rapid fall during the first hour of the reaction, comparable with that observed for fucose, followed by a much lower rate of loss, comparable with that of *N*-acetylgalactosamine and peptide. Similarly shaped curves were obtained when sialic acid was measured either by the thiobarbituric acid assay (Warren, 1959) or by the amino acid analyser method (Liu, 1972). The retention of some sialic acid in the glycopeptide fraction was unexpected. If the glycoprotein was per-*O*-acetylated before the reaction with H_2SO_4 /acetic acid the recovery curve shown in Fig. 2 was obtained. The loss of all the components was somewhat slower than observed for the non-acetylated glycoprotein, but the effect was most marked on the sialic acid residues. After 6 h more than 50% of this sugar was still present in the non-diffusible fraction.

Other substances with blood-group-A (two examples), –B and –H activities (non-*O*-acetylated) were found to give curves similar to those shown in Fig. 1. The only exception was the expected accelerated loss of *N*-acetylgalactosamine from the blood-group-A substances. This is consistent with the early removal of that portion of the *N*-acetylgalactosamine which is located at the ends of the carbohydrate chains furthest removed from the peptide, where it forms part of the blood-group-A determinant (see Watkins, 1972). The only other manner in which the behaviour of the glycoproteins differed during the reaction

Fig. 1. *Partial deglycosylation with H_2SO_4 /acetic acid*

Glycoprotein Le^a 610 was treated with H_2SO_4 /acetic acid as described in the text. The non-diffusible glycopeptides isolated were analysed and the percentage recovery of each component is shown as a function of time of treatment.

Fig. 2. Partial deglycosylation of an *O*-acetylated glycoprotein with H_2SO_4 /acetic acid
Per-*O*-acetylated substance Le^a 610 was treated with H_2SO_4 /acetic acid as described in the text. The recoveries of the various components in the non-diffusible glycopeptides obtained were determined at different times.

Fig. 3. Changes in the proportions of some amino acids on H_2SO_4 /acetic acid treatment of substance A 584 and of Pronase-treated substance H 500

The Figure shows the variation with time of the proportions of the acidic (●) and hydroxy (○) amino acids in the non-diffusible glycopeptides obtained by H_2SO_4 /acetic acid treatment of (a) substance A 584 and (b) Pronase-treated substance H 500.

was the way in which the amino acid distribution of the non-diffusible materials changed. In general, the proportion of threonine, serine and proline rose, and that of the acidic and basic amino acids fell with increasing reaction time. This was most noticeable

for glycoproteins, such as substance A 584, that had a high content of the charged amino acids. The changes in the relative proportions of the hydroxy and acidic amino acids that occurred on H_2SO_4 /acetic acid treatment of substance A 584 are shown in Fig. 3(a). This type of glycoprotein possesses a high proportion of the non-glycosylated protein, which is rich in the charged amino acids (Donald, 1973). The results suggest that this non-glycosylated protein is undergoing a more rapid fragmentation to diffusible species than is the threonine- and serine-rich glycosylated region. Consistent with this, Pronase-treated substance H 500, in which the non-glycosylated protein has been enzymically removed (Donald, 1973), showed a much smaller change in amino acid distribution on H_2SO_4 /acetic acid treatment (Fig. 3b).

On the basis of the results summarized in Fig. 1, a reaction time of 6 h was judged to be suitable for the preparation of glycopeptides with a high ratio of *N*-acetylgalactosamine to other sugars. At this time 95–100% of fucose and *N*-acetylglucosamine are removed, together with 90–95% of the galactose. The residue contains about 80% of the original *N*-acetylgalactosamine and peptide and approx. 20% of the sialic acid. After a large-scale treatment of the glycoproteins for 6 h with H_2SO_4 /acetic acid the glycopeptides were recovered by dialysis, de-*O*-acetylated with mild alkali and re-dialysed. The compositions of two glycoproteins and their derived glycopeptides are shown in Table 1.

The glycoprotein A 584 gave a water-insoluble glycopeptide as well as the more usual water-soluble one. The water-insoluble material had a higher content of the charged amino acids than did the water-

Table 1. *Analytical values for the glycopeptides and the glycoprotein starting materials*

The values given refer to glycoprotein A 584 and Pronase-treated glycoprotein H 500 and to the glycopeptides prepared from them with H₂SO₄/acetic acid and/or HCl/methanol, as described in the text.

Substance A 584							Pronase-treated substance H 500		
Yield* ...	Glycoprotein —	H ₂ SO ₄ / acetic acid water-soluble glycopeptide 38	H ₂ SO ₄ / acetic acid water-insoluble glycopeptide 9	Glycoprotein —	H ₂ SO ₄ / acetic acid glycopeptide 35	HCl/methanol glycopeptide 31			
		Content (individual amino acids expressed as residues/100 residues of amino acids)							
Asp	7.0	5.9	9.5	1.0	0.6	0.5			
Thr	16.1	18.8	10.2	37.0	37.7	40.6			
Ser	10.6	13.7	8.3	23.6	24.4	24.2			
Glu	8.7	8.1	11.4	2.1	1.4	1.7			
Pro	9.5	12.5	7.8	14.9	13.9	12.9			
Gly	6.1	5.7	6.3	3.8	3.5	3.4			
Ala	8.7	8.6	7.9	7.4	7.7	8.1			
½Cys	3.0	0.6	2.2	0	0	0			
Val	6.4	5.1	5.8	3.6	2.8	2.4			
Met	0.3	0.5	1.1	0.1	0.2	0			
Ile	2.8	2.3	2.6	1.9	1.8	2.0			
Leu	5.9	4.6	8.3	1.3	1.0	0.9			
Tyr	2.1	1.5	3.2	0.1	0.1	0.1			
Phe	3.0	2.3	4.5	0.3	0.2	0.3			
His	2.3	2.2	1.9	0.9	2.1	0.7			
Lys	4.1	4.0	4.7	1.0	1.1	0.7			
Arg	3.4	3.5	3.9	1.1	1.3	1.1			
Total amino acids (%)	32.2	56.0	69.0	15.8	43.7	44.1			
Sialic acid (%)	6.3	2.1	0.7	4.0	1.9	0			
Fuc (%)	10.2†	0.3	0.6	18.4†	0.5	0.1			
Gal (%)	26.4†	4.2	2.9	31.5†	5.7	0.4			
GlcNAc (%)	11.4	1.4	1.4	22.2	1.6	7.8			
GalNAc (%)	10.9	24.7	7.3	20.9	42.5	52.0			

* As g of glycopeptide/100 g of starting material.

† Determined colorimetrically as indicated in the Experimental section.

soluble. This production of a water-insoluble glycopeptide has been found to be typical of glycoproteins that are themselves only partially soluble. The glycopeptide obtained from Pronase-treated substance H 500 was readily water-soluble. Amino acids and *N*-acetylgalactosamine together account for 86% of this glycopeptide. Galactose (6%) was the next largest component, followed by sialic acid (1.9%).

Partial deglycosylation with HCl/methanol

Methanolysis of blood-group substances is required as a preliminary step in the analysis of the sugar methyl glycosides as trimethylsilyl ethers. In the early stages of this procedure it was observed that some material remains insoluble in the HCl/methanol, and analysis of this residue revealed it to consist largely of *N*-acetylgalactosamine and peptide. An investigation showed that 0.1M-HCl in methanol at 100°C causes rupture of the *N*-acetyl-

galactosamine-peptide bond, whereas the use of 6M-HCl/methanol at ambient temperature favours retention of this linkage. The time course of the latter reaction was studied. The glycopeptides isolated after various times of treatment of Pronase-digested substance H 500 were recovered by dialysis and analysed; the recoveries of the components with time are shown in Fig. 4. After 3 days less than 2% of the original sialic acid, fucose and galactose were recovered; *N*-acetylglucosamine was lost more slowly (10% recovered), whereas *N*-acetylgalactosamine and peptide were largely retained (about 80% recovery after 3 days). The *N*-acetylgalactosamine/(threonine + serine) ratio remained constant to within ±4% (maximum deviation) throughout the course of the reaction. The amino acid distributions of the recovered glycopeptides have been found to vary with time in the same way as did the H₂SO₄/acetic acid products. Experiments in which *N*-acetylgalactosamine was subjected to the

Fig. 4. Partial deglycosylation of Pronase-treated substance H 500 with HCl/methanol

Pronase-treated substance H 500 was treated with 6 M-HCl/methanol as described in the text. The percentage recoveries of the components in the non-diffusible glycopeptides isolated are shown as a function of time of treatment.

HCl/methanol treatment established that after 6 days less than 1% of the sugar was converted into methyl 2-amino-2-deoxygalactopyranosides, i.e. virtually no de-*N*-acetylation had occurred.

For the large-scale preparations of glycopeptides, the glycoproteins were treated for 3 days with 6 M-HCl/methanol. The analytical results for the glycopeptide prepared from Pronase-treated substance H 500 are shown in Table 1. Compared with that prepared by using H₂SO₄/acetic acid, it contained rather more *N*-acetylglucosamine and less galactose. Glycopeptides prepared by the HCl/methanol method contain some *O*-methyl ester groupings, which are readily removed by mild alkaline treatment.

Properties of the glycopeptides

Glycopeptides prepared by the H₂SO₄/acetic acid method tended to be pale brown. Decolorization was easily effected by passing an aqueous solution through a short column of DEAE-cellulose and recovering the glycopeptides by elution with 0.5 M-NaCl and dialysis. Glycopeptides prepared with HCl/methanol were colourless.

Chromatography on a column of Sephacryl S-200 indicated that glycopeptides prepared by either method were polydisperse (see Fig. 5). Further indication of the heterogeneity of the glycopeptides comes from the results of DEAE-cellulose chromatography. When the glycopeptide prepared from substance A 584 by the H₂SO₄/acetic acid method was loaded on to a column of DE-52 DEAE-cellulose and eluted stepwise with increasing concentrations of NaCl, it was divided up into a number of fractions (Fig. 6). Almost all the *N*-acetylgalactos-

Fig. 5. Elution profiles of the glycopeptides from Pronase-digested substance H 500 on Sephacryl S-200

The glycopeptides prepared by both H₂SO₄/acetic acid (—) and HCl/methanol (---) treatment of Pronase-digested substance H 500 were chromatographed on a column (90 cm × 2.5 cm internal diam.) of Sephacryl S-200. The column was equilibrated and eluted with 0.15 M-NaCl. Fractions (5 ml) were collected and the absorbance at 220 nm was determined. The upper curve has been offset upwards by 0.2 A₂₂₀ unit.

amine was recovered from the first four peaks to emerge from the column. The materials giving these peaks were found to differ slightly in amino acid composition, but all were considerably enriched in threonine, serine and proline when compared with the glycopeptide before DEAE-cellulose chromatography. End-group analysis of glycopeptides prepared by the H₂SO₄/acetic acid method by the dansyl chloride procedure (Gray, 1967) have indicated the presence of several *N*-terminal amino acids (Goodwin & Watkins, 1974).

When the glycopeptides obtained from substance H 500 by either the H₂SO₄/acetic acid or the

Fig. 6. Chromatography of a glycopeptide from substance A 584 on DEAE-cellulose

The water-soluble glycopeptide (100mg) prepared from substance A 584 by using H_2SO_4 /acetic acid was dissolved in 0.01M-NaCl and loaded on to a column (30cm $\times$ 1cm internal diam.) of Whatman DE-52 DEAE-cellulose equilibrated with 0.01M-NaCl. The column was eluted stepwise with increasing concentrations of NaCl as indicated by the arrows, and then with 10% acetic acid. Fractions (6ml) were collected and their absorbance at 220 nm (—) or 280 nm (----) was measured. The fractions were pooled as indicated by the bars.

HCl/methanol procedure were subjected to the alkaline-borohydride conditions described by Iyer & Carlson (1971), no fucitol, galactitol or *N*-acetylglucosaminitol was found among the reaction products. Glycopeptides prepared by the HCl/methanol method showed a greater conversion of *N*-acetylglucosamine into *N*-acetylglucosaminitol (84%) than did the glycopeptide prepared by using H_2SO_4 /acetic acid (72%). The values for recovered *N*-acetylglucosamine were 4 and 7% respectively. These results are consistent with the involvement of the large majority of the *N*-acetylglucosamine residues in *O*-glycosidic linkages to threonine and serine.

The removal of most of the *N*-acetylglucosamine from the glycopeptides with an α -specific *N*-acetylglucosaminidase from *Trichomonas foetus* has been reported (Donald *et al.*, 1969). However, a hexosaminidase preparation obtained from *Lumbricus terrestris* by affinity chromatography on a column of glycopeptide-Sepharose 4B has proved to be more effective for glycopeptide deglycosylation. The glycopeptide prepared from substance H 500 by using HCl/methanol was treated for 7 days with the enzyme preparation; enzyme protein was removed on a column of DEAE-cellulose, and the product

Table 2. Enzymic removal of sugars from the *N*-acetylglucosaminyl-peptides

The values are the percentage recoveries of the components in the fractions from the Sephadex G-25 column.

Fraction ...	A	B	C
Amino acid	31.0	36.3	25.6
GlcNAc	12.0	3.8	39.1
GalNAc	3.7	1.0	88.5

was chromatographed on a column of Sephadex G-25 (Fig. 7). The materials emerging at and just after the void volume of the column (fractions A and B) were free of unbound *N*-acetylhexosamine. They contained 67% of the peptide and 5% of the *N*-acetylglucosamine originally present in the glycopeptide (Table 2). However, fraction C also contained some peptide (26% recovery), and it is apparent that the glycosidase treatment is causing further break-up of the peptide backbone. Presumably trace amounts of proteolytic enzymes are able to attack the peptide once the shielding *N*-acetylglucosamine residues are removed.

The glycopeptides inhibited the agglutination of

Fig. 7. Gel filtration of the hexosaminidase digest of a glycopeptide

The digest, prepared as described in the text, was applied to a column (90 cm $\times$ 1.5 cm internal diam.) of Sephadex G-25 (medium grade), equilibrated and eluted with water. Fractions (1 ml) were collected. O, Absorbance at 220 nm; ●, absorbance at 585 nm after assay of 50 μ l samples for free *N*-acetylgalactosamine by the procedure of Reissig *et al.* (1955). The fractions were pooled as indicated by the bars.

blood-group-Tn erythrocytes (see Race & Sanger, 1975; Springer & Desai, 1974) by the Tn-specific lectin from the plant *Salvia sclarea*, and immunization of rabbit gave antibodies specific for α -linked *N*-acetylgalactosamine.

Discussion

The original aim of the work described in the present paper was the deglycosylation of blood-group-specific glycoproteins. The results presented show that almost sugar-free peptide can be produced by a combination of partial chemical deglycosylation and enzymic removal of the remaining *N*-acetylgalactosamine. However, the gel-filtration patterns indicate that both stages of this procedure lead to some breakdown of the peptide moiety. No molecular-weight determinations were performed on the glycopeptides. However, the elution profiles of these materials indicate a molecular weight that is considerably higher than the 1.2×10^4 value (Donald *et al.*, 1969) given for a glycopeptide prepared by using a longer (24 h) treatment with H_2SO_4 /acetic acid. The work of Hill *et al.* (1977), in which the complete removal of *N*-acetylgalactosamine from asialo-ovine submaxillary mucin was reported, suggests that the use of proteinase inhibitors might have prevented the peptide degradation that was observed

during the hexosaminidase treatment of the glycopeptides. It is possible that the recently introduced HF technique (Mort & Lampert, 1977) may provide a more satisfactory method for deglycosylating these glycoproteins.

Nevertheless the isolation and properties of the glycopeptides have provided useful information about the structure of the glycoproteins. Firstly, the isolation of glycopeptides in which *N*-acetylgalactosamine is virtually the only sugar present gives direct evidence that *N*-acetylgalactosamine is the sugar involved in the carbohydrate-peptide linkage, thus confirming the indirect evidence obtained from alkaline-borohydride reduction (Kabat *et al.*, 1965; Iyer & Carlson, 1971; Donald, 1973). Until recently it had been assumed that *N*-acetylgalactosamine occupied only this position, except in blood-group-A-active substances, in which *N*-acetylgalactosamine also occurs as part of the blood-group-A determinant at the non-reducing ends of the carbohydrate chains (see Watkins, 1972). However, Maisonrouge-McAuliffe & Kabat (1976) isolated small amounts of the disaccharide *N*-acetylgalactosamine- α -(1 $\rightarrow$ 3)-*N*-acetylgalactosaminitol from an ovarian-cyst substance with blood-group-B, -I and -i activities, which suggests that some of the *N*-acetylgalactosamine in the glycopeptide might be involved in *N*-acetylgalactosamine-*N*-acetylgalactosamine structures. However, since less than 7% of this sugar in glycopeptides prepared from substance H 500 survived alkaline-borohydride treatment, and as part of that which remains is probably bound up in resistant *O*-glycosidic linkages (Derevitskaya *et al.*, 1967; Wakabayashi & Pigman, 1974), only a very small amount of *N*-acetylgalactosamine could originally have been present as *N*-acetylgalactosamine-*N*-acetylgalactosamine structures. It appears likely therefore that the vast majority of the *N*-acetylgalactosamine residues in the glycopeptides are singly linked to threonine and serine residues in the peptide backbone. This must be true also for the parent glycoprotein, since the HCl/methanol treatment of substance H 500 scarcely changed the *N*-acetylgalactosamine/(threonine + serine) ratio. The nearly equal losses of *N*-acetylgalactosamine and hydroxy amino acids observed after alkaline-borohydride treatment of other, non-blood-group-A-active, glycoproteins (Donald, 1973) also suggests that the number of *N*-acetylgalactosamine-*N*-acetylgalactosamine structures is relatively small. Thus it is still a valid approximation (for non-blood-group-A-active materials) to use the *N*-acetylgalactosamine/(threonine + serine) ratio as a measure of the extent of glycosylation (Donald, 1973) and the *N*-acetylgalactosamine/(total sugar) ratio as an indication of carbohydrate chain length.

Glycopeptides prepared by using H_2SO_4 /acetic acid were slightly different in carbohydrate com-

position from those prepared by the HCl/methanol procedure, and this difference is probably due to the reagents having different linkage specificities. As well as the *N*-acetylgalactosamine- α -(1 $\rightarrow$ 3)-*N*-acetylgalactosamine structure discussed above, two other structures involving the peptide-linked *N*-acetylgalactosamine have been established, namely *N*-acetylglucosamine- β -(1 $\rightarrow$ 6)-*N*-acetylgalactosamine and galactose- β -(1 $\rightarrow$ 3)-*N*-acetylgalactosamine (see Watkins, 1972). The higher *N*-acetylglucosamine content of the glycopeptides prepared by the HCl/methanol procedure is consistent with incomplete scission of *N*-acetylglucosamine- β -(1 $\rightarrow$ 6)-*N*-acetylgalactosamine; bonds between *N*-acetylhexosamine residues are difficult to cleave with HCl/methanol (Siddiqui & Hakomori, 1971). However, (1 $\rightarrow$ 6)-linkages are more labile than (1 $\rightarrow$ 3)-linkages under acetolysis conditions (see Stewart *et al.*, 1968), so the higher galactose value of the glycopeptides prepared by the H₂SO₄/acetic acid method probably results from survival of some of the galactose- β -(1 $\rightarrow$ 3)-*N*-acetylgalactosamine structures.

The acetolysis-like conditions of the H₂SO₄/acetic acid method are probably also responsible for the presence of sialic acid in glycopeptides prepared by this technique; linkages involving sialic acid partly survive acetolysis (see Marshall & Neuberger, 1972), but are more labile to methanolysis or aqueous acid hydrolysis. However, the finding that just over 50% of the original sialic acid was retained in the glycopeptide prepared by the H₂SO₄/acetic acid treatment of a per-*O*-acetylated Le^a substance was unexpected. The glycopeptide had the sugar recoveries and molar proportions shown in Table 3. It is not possible to deduce to which sugar the sialic acid was attached, since there is enough of each of galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine for it to be linked to any of them. However, the results clearly indicate that most of the sialic acid in this glycoprotein is located near the peptide

moiety. The sialic acid released from an Le^a substance by the action of neuraminidase has been shown to be *N*-acetylneuraminic acid (Pusztai & Morgan, 1961). However, the presence of other sialic acids in ovarian-cyst blood-group substances cannot at this stage be excluded. Glycoproteins of other specificities usually have a lower sialic acid content than have Le^a substances (Pusztai & Morgan, 1961). However, all the glycoproteins studied gave sialic acid recovery curves similar to those shown in Fig. 1, and probably all have a substantial proportion of their sialic acid located near the peptide. Such a position for this sugar is supported by the isolation of a series of di- to tetra-saccharides after alkaline-borohydride treatment of a sialic acid-rich (29%) blood-group-inactive glycoprotein (Lehman & Yaron, 1974; see also Carlson, 1977). These oligosaccharides contained sialic acid linked to *N*-acetylgalactosaminitol and to the disaccharides galactose-*N*-acetylgalactosaminitol and *N*-acetylglucosamine-*N*-acetylgalactosaminitol; the position and anomeric nature of the linkages were unspecified. It is unclear whether the sialic acid in the glycoproteins described here was evenly distributed between long and short carbohydrate chains, or was located only on short oligosaccharides of the type described above. It is believed that the addition of a sialic acid residue to a growing carbohydrate chain inhibits the action of the glycosyltransferases responsible for adding the other component sugars of the glycoprotein, and thus terminates biosynthesis (Schachter *et al.*, 1971). Roberts (1974) has reported that the sialic acid in human bronchial glycoprotein, which is probably structurally similar to the ovarian-cyst substances, is linked to C-3 of galactose residues at the non-reducing ends of the carbohydrate chains. No indication was given as to the length of these sialic acid-containing chains.

The shortening of long carbohydrate chains to units of predominantly one sugar each not only allows identification of the linkage sugar, but also provides a substrate on which a specific exo-glycosidase may be used to determine the anomeric nature of the carbohydrate-peptide bond. The methods described in the present paper should find similar uses in structural studies on other epithelial glycoproteins. Although these methods caused some rupture of the peptide backbone of the ovarian-cyst substances, it should nevertheless be possible to obtain structurally important evidence by the isolation and characterization of homogeneous glycopeptides from the reaction products.

Table 3. Sugar recoveries and molar ratios for the glycopeptide obtained from a per-*O*-acetylated glycoprotein

The values given refer to the glycopeptide prepared from per-*O*-acetylated substance Le^a 610 by using H₂SO₄/acetic acid as described in the text.

	Recovery of component (%)	Molar proportions
Sialic acid	53	0.26
Fuc	3	0.03
Gal	20	0.88
GlcNAc	10	0.24
GalNAc	82	1.00
Total amino acids	82	—

References

- Adams, J. B. (1965) *Biochem. J.* **97**, 345–352
- Anderson, B., Hoffman, P. & Meyer, K. (1963) *Biochim. Biophys. Acta* **74**, 309–311

- Carlson, D. M. (1977) in *Mucus in Health and Disease* (Elstein, M. & Parke, D. V., eds.), pp. 251–273, Plenum Press, New York and London
- Derevitskaya, V. A., Vafina, M. G. & Kochetkov, N. K. (1967) *Carbohydr. Res.* **3**, 377–388
- Donald, A. S. R. (1973) *Biochim. Biophys. Acta* **317**, 420–436
- Donald, A. S. R., Creeth, J. M., Morgan, W. T. J. & Watkins, W. M. (1969) *Biochem. J.* **115**, 125–127
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Gibbons, M. N. (1955) *Analyst (London)* **80**, 268–276
- Goodwin, S. D. & Watkins, W. M. (1974) *Eur. J. Biochem.* **47**, 371–382
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–145
- Hill, H. D., Reynolds, J. A. & Hill, R. L. (1977) *J. Biol. Chem.* **252**, 3791–3798
- Iyer, R. N. & Carlson, D. M. (1971) *Arch. Biochem. Biophys.* **142**, 101–105
- Kabat, E. A., Bassat, E. W., Pryzwansky, K., Lloyd, K. O., Kaplan, M. E. & Layug, E. J. (1965) *Biochemistry* **4**, 1632–1638
- Lehman, E. D. & Yaron, A. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 1497
- Liu, T. Y. (1972) *Methods Enzymol.* **28**, 48–54
- Maisonrouge-McAuliffe, F. & Kabat, E. A. (1976) *Arch. Biochem. Biophys.* **175**, 90–113
- March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152
- Marshall, R. D. & Neuberger, A. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A. ed.), pp. 322–380, Elsevier, Amsterdam
- Morgan, W. T. J. (1967) *Methods Immunol. Immunochem.* **1**, 75–81
- Mort, A. J. & Lampert, D. T. A. (1977) *Anal. Biochem.* **82**, 289–309
- Pusztai, A. & Morgan, W. T. J. (1961) *Biochem. J.* **78**, 135–146
- Pusztai, A. & Morgan, W. T. J. (1963) *Biochem. J.* **88**, 546–555
- Race, R. R. & Sanger, R. (1975) *Blood Groups in Man*, 6th edn., pp. 488–491, Blackwell, Oxford
- Radhakrishnamurthy, B., Dalferes, E. R. & Berenson, G. S. (1968) *Anal. Biochem.* **26**, 61–67
- Reissig, J. L., Strominger, J. L. & Leloir, L. E. (1955) *J. Biol. Chem.* **217**, 959–966
- Roberts, G. P. (1974) *Eur. J. Biochem.* **50**, 265–280
- Schachter, H., McGuire, E. J. & Roseman, S. (1971) *J. Biol. Chem.* **246**, 5321–5328
- Siddiqui, B. & Hakomori, S. (1971) *J. Biol. Chem.* **246**, 5766–5769
- Slomiany, B. L. & Meyer, K. (1973) *J. Biol. Chem.* **248**, 2290–2295
- Springer, G. F. & Desai, P. R. (1974) *Biochem. Biophys. Res. Commun.* **61**, 470–475
- Stewart, T. S., Menderschausen, P. B. & Ballou, C. E. (1968) *Biochemistry* **7**, 1843–1854
- Wakabayashi, K. & Pigman, W. (1974) *Carbohydr. Res.* **35**, 3–14
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
- Watkins, W. M. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A. ed.), pp. 830–891, Elsevier, Amsterdam