Glycosidase analysis of large acidic-type glycopeptides from viral and cellular membrane glycoproteins

Evidence for a common oligomannosyl core with branch sugar heterogeneity

Lawrence A. HUNT

Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY 40292, U.S.A.

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The asparagine-linked oligosaccharides of the complex acidic-type from [^3H]mannose-, [^3H]glucosamine- or [^3H]galactose-labelled membrane glycoproteins of BHK21 cells and Rous-sarcoma virus were analysed by gel filtration combined with extensive digestion with endo- and exo-glycosidases from bacterial and eukaryotic sources. The neutral products from the digestion with a mixture of exoglycosidases and endo- β -*N*-acetylglucosaminidase D from *Diplococcus pneumoniae* included a series of [^3H]mannose- and [^3H]glucosamine-labelled neutral oligosaccharides that were all converted by digestion with eukaryotic β -*N*-acetylglucosaminidases into free *N*-acetylglucosamine and a small oligomannosyl core containing two α -linked mannose residues and a third mannose residue β -linked to *N*-acetylglucosamine. These studies suggested that the complex acidic-type oligosaccharides from cellular and viral membrane glycoproteins contained a common oligomannosyl core region ($\text{Man}_2^{\alpha}\text{-Man}_1^{\beta}\text{-GlcNAc}_2$), with heterogeneity in the number and/or linkage of outer branch *N*-acetylglucosamine residues resulting in partial resistance to β -*N*-acetylglucosaminidase from a bacterial source.

The asparagine-linked oligosaccharides of viral and cellular membrane glycoproteins are of two major types: complex acidic [$(\text{NeuAc} \pm \text{Gal-GlcNAc})_n\text{-Man}_3\text{GlcNAc}_2(\pm \text{Fuc})\text{-Asn}$] and mannose-rich neutral ($\text{Man}_{5-9}\text{GlcNAc}_2\text{-Asn}$) (Kornfeld & Kornfeld, 1980). Both types are derived from a common precursor oligosaccharide ($\text{Glc}_{2-3}\text{Man}_9\text{GlcNAc}_2$) that is pre-assembled on lipid and transferred *en bloc* to the appropriate asparagine sites on nascent polypeptides (Robbins *et al.*, 1977; Hunt *et al.*, 1978; Li *et al.*, 1978; Tabas *et al.*, 1978). Endo- β -*N*-acetylglucosaminidases from bacterial sources [endoglycosidase H from *Streptomyces plicatus* (Tarentino *et al.*, 1978), endoglycosidase D from *Diplococcus pneumoniae* (Muramatsu, 1978) and endoglycosidases C_I and C_{II} from *Clostridium perfringens* (Kobata, 1978)] have been indispensable tools in distinguishing between the two major types of asparaginyl oligosaccharides and in analysing the oligomannosyl core structures of glycoproteins from radioactive-sugar-labelled virus and cells.

Previous studies with the Pronase-digested glyco-

peptides from membrane glycoproteins of uninfected BHK21 cells (Hunt, 1979) and Rous sarcoma virus (Hunt *et al.*, 1979) suggested possible heterogeneity in the oligomannosyl cores derived from extensive endoglycosidase-D and bacterial-exoglycosidase digestions of the acidic-type oligosaccharides ($\text{Man}_3\text{GlcNAc}_1$ and larger $\text{Man}_5\text{-GlcNAc}_1$ -size oligosaccharides), in addition to the size heterogeneity observed in the products of endoglycosidase-H-digested neutral-type glycopeptides ($\text{Man}_{5-9}\text{GlcNAc}_1$). The oligomannosyl core structures of acidic-type glycopeptides from the same cells and virus have been re-examined in order to determine whether structures with more than three mannose residues, such as the hybrid-type oligosaccharides of ovalbumin (Yamashita *et al.*, 1978), or incomplete exoglycosidase removal of outer branch sugars ($\text{Gal} \pm \text{GlcNAc}$) as observed with fetuin glycopeptides (Baenziger & Fiete, 1979), were responsible for the heterogeneity. The present studies, utilizing glycopeptides radiolabelled with one of several different sugars and both eukaryotic and bacterial glycosidases, supported the latter poss-

ibility, and suggested a common three-mannose-residue core for all of the acidic-type asparaginyl-oligosaccharides.

Materials and methods

Cells, virus and radiolabelling

BHK21 cells (a continuous cell line derived from the kidneys of baby Syrian hamsters) were grown in monolayer culture in minimal essential medium (GIBCO Laboratories) supplemented with 5% (v/v) of foetal bovine serum. Confluent cell cultures in 75 cm² culture flasks were radiolabelled for 24 h at 37°C in medium containing 2.5% foetal bovine serum, one-third the normal amount of glucose and one of the following radioactive precursors at a concentration of 50 µCi/ml: D-[2-³H]mannose (14 Ci/mmol; New England Nuclear), D-[6-³H]-glucosamine (15 Ci/mmol; Amersham International) or D-[1-³H]galactose (22 Ci/mmol; Amersham International) (Hunt, 1979). Chicken-embryo fibroblasts that were infected and transformed by Rous sarcoma virus (Prague C strain) were obtained from Dr. Stephen Wright (Veterans Administration Medical Center, Salt Lake City, UT, U.S.A.), and grown to confluence in 75 cm² culture flasks (Hunt *et al.*, 1979, 1981). Cultures were radiolabelled in medium similar to that used for the BHK21 cultures, supplemented with 1% (v/v) dimethyl sulphoxide, as described previously (Hunt *et al.*, 1979, 1981). After labelling had continued for 7–8 h at 37°C, the medium was harvested; then fresh medium lacking radioactive sugars was added, and the medium was harvested after an additional 14–16 h at 37°C. The two harvests of culture medium were combined, and radiolabelled virus was purified from clarified medium by sucrose-density-gradient ultracentrifugation as previously described (Hunt *et al.*, 1979).

Preparation of viral and cellular membrane glycoproteins and glycopeptides

Membrane pellets were prepared from clarified BHK21-cell homogenates (Hunt & Summers, 1976) by centrifugation at 20000 g for 10 min. Membrane proteins were solubilized by resuspending the pellet in 1 mM-EDTA/10 mM-Tris/HCl buffer, pH 7.4, containing 1% Nonidet P40 (Shell Chemical Co.) for 15 min at room temperature, followed by re-centrifugation to pellet unsolubilized membrane material. Radiolabelled glycoprotein was then extracted from the detergent-solubilized cell-membrane protein or detergent-treated Rous sarcoma virus with 2 vol. of butan-1-ol (Hunt & Summers, 1976), followed by extensive digestion of the extracted protein with Pronase (Calbiochem) (Hunt & Summers, 1976).

Glycosidase digestions of glycopeptides

Glycopeptides were desalted on a column of Sephadex G-15/G-50 before glycosidase digestions (Hunt & Summers, 1976). Glycopeptides were digested with exoglycosidases and endoglycosidases as described previously (Etchison *et al.*, 1977; Hunt *et al.*, 1978). The mixture of exoglycosidases and endoglycosidase D from *Diplococcus pneumoniae* was obtained from Dr. James Etchison (University of California, Davis, CA, U.S.A.). This partially purified mixture of endo- and exo-glycosidases was prepared by (NH₄)₂SO₄ precipitation of the bacterial culture filtrate and gel filtration on Sephadex G-200 (Kobata & Takasaki, 1978), and contained the following enzymic activities: neuraminidase, β -galactosidase, β -N-acetylglucosaminidase, endo- β -N-acetylglucosaminidase D, endo- α -N-acetylgalactosaminidase and endo- β -galactosidase. The enzyme mixture did not contain detectable α -fucosidase or α -mannosidase activities. The same enzyme mixture under identical conditions completely removes the 'branch' sugars (NeuAc-Gal-GlcNAc) from diantennary acidic-type glycopeptides, and also releases a neutral oligomannosyl core (Man₃GlcNAc₁) from the N-acetylglucosamine ($\pm$ fucose)-asparaginyl peptide, as previously shown for the glycopeptides from purified vesicular-stomatitis virus or Sindbis virus and the corresponding virus-infected-cell glycopeptides (Hunt, 1980b, 1981). Jack-bean α -mannosidase and β -N-acetylglucosaminidase and *Clostridium perfringens* neuraminidase (fraction IX) were purchased from Sigma Chemical Co., and bovine kidney β -N-acetylglucosaminidase was from Boehringer Mannheim. With the digestion conditions used in the present studies, the jack-bean α -mannosidase preparation from Sigma Chemical Co. lacked detectable β -mannosidase activity or other exoglycosidase activities (neuraminidase, galactosidase and glucosaminidase). These digestion conditions were sufficient to digest [³H]mannose-labelled large neutral oligosaccharides (Man₆GlcNAc₁) completely to free mannose and Man ^{β} GlcNAc, with a ratio of radiolabel of approx. 8:1 for Man/Man ^{β} GlcNAc (Hunt, 1980a). A preparation of jack-bean α -mannosidase purchased from Boehringer Mannheim also contained contaminating β -mannosidase activity that could release [³H]mannose from the Man ^{β} GlcNAc disaccharide (L. A. Hunt, unpublished work). After exoglycosidase and endoglycosidase-D digestions, the neutral oligomannosyl cores were separated from residual glycopeptides and released sialic acid (both of which would be radiolabelled in [³H]-glucosamine-labelled samples) by passing the digests through a column of Dowex AG1-X2 (formate form) (Robertson *et al.*, 1978).

Gel-filtration analysis

Radiolabelled glycopeptides and oligosaccharides were analysed by gel filtration through columns (120 cm $\times$ 1.5 cm) of Bio-Gel P-4 (–400 mesh; Bio-Rad Laboratories) along with various unlabelled and ^{14}C -labelled gel-filtration markers (Hunt & Summers, 1976; Hunt *et al.*, 1978). These internal standards were not utilized as molecular-weight standards, but were useful in comparing the elution profiles of the same sample on different columns, or different samples run separately on the same column. Before the running of unknown ^3H -labelled samples, each column was calibrated for endoglycosidase-D and -H digestion products by determining the elution position of [^3H]mannose-labelled neutral oligomannosyl cores ($\text{Man}_n\text{GlcNAc}_1$, $n = 3\text{--}9$) of known composition relative to the internal standards. The oligomannosyl cores included: $\text{Man}_3\text{GlcNAc}_1$, $\text{Man}_4\text{GlcNAc}_1$ and $\text{Man}_5\text{GlcNAc}_1$ from mature glycoprotein of vesicular-stomatitis virus released from wild-type and lectin-resistant mutant Chinese-hamster ovary cells (Robertson *et al.*, 1978; Hunt, 1980b), $\text{Man}_6\text{GlcNAc}_1$ and $\text{Man}_7\text{GlcNAc}_1$ from pulse-labelled precursor glycopeptides derived from the lectin-resistant cells (Hunt, 1980a), mixtures of $\text{Man}_{5-9}\text{GlcNAc}_1$ from neutral-type glycopeptides of Rous sarcoma virus (Hunt *et al.*, 1979, 1981) and Sindbis virus (Hunt, 1981), and $\text{Man}_1\text{GlcNAc}_1$ from extensive α -mannosidase digestions of one or more of the above samples.

Results

[^3H]Sugar-labelled BHK21-cell membrane glycopeptides

To elucidate better the compositional or structural heterogeneity in the oligomannosyl core regions of the cell-membrane glycoproteins, three separate BHK21-cell cultures were radiolabelled with [^3H]mannose, [^3H]glucosamine or [^3H]galactose. Sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of detergent-solubilized membrane proteins indicated that radiolabel from all three sugars was incorporated into a heterogeneous array of glycoprotein species with molecular weights in the range 30 000–200 000 (results not shown). Pronase-digested glycopeptides from these three different [^3H]sugar-labelled membrane protein samples were analysed by gel filtration on a column of Bio-Gel P-4 (Fig. 1). Large glycopeptides eluted in the void volume or shortly thereafter (fractions 30–50) were observed in all three samples, and these glycopeptides corresponded in elution position to the complex acidic-type structures previously described for [^3H]mannose-labelled BHK21-cell glycopeptides (Hunt,

1979). An additional peak of smaller glycopeptides (fractions 50–70) was also observed in major amounts in the [^3H]mannose-labelled sample (Fig. 1a) and in smaller amounts in the [^3H]glucosamine-labelled sample (Fig. 1b), and these glycopeptides were previously shown to contain endoglycosidase-H-sensitive neutral-type oligosaccharides ($\text{Man}_{5-9}\text{GlcNAc}_2\text{-Asn}$) (Hunt, 1979).

The [^3H]mannose- and [^3H]glucosamine-labelled products from the combined endoglycosidase-D and bacterial-exoglycosidase digestion of the large glycopeptides included several larger neutral oligosaccharides (peaks II to IV) in addition to a $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharide (peak I in Figs. 1a and 1b). The proportions of radiolabel in the four peaks (I:II:III:IV) were 1.0:0.6:0.3:0.1 for the [^3H]mannose-labelled sample (Fig. 1a) and 1.0:1.1:1.1:0.8 for the [^3H]glucosamine-labelled sample (Fig. 1b). The neutral products labelled with [^3H]glucosamine (Fig. 1b) also included a major peak in the position of free *N*-acetylglucosamine (fractions 103–107; approx. 59% of the total radioactivity in Dowex neutral products) and another unidentified peak eluted just ahead of the *N*-acetylglucosamine peak (fractions 98–101; approx. 8% of total radioactivity in Dowex neutral products).

In contrast with the [^3H]mannose- and [^3H]glucosamine-labelled samples, the only glycosidase digestion products from the [^3H]galactose-labelled glycopeptides (Fig. 1c) were a major peak eluted in the position of free galactose and a minor peak eluted in the same position as the unidentified [^3H]glucosamine-labelled peak. This unidentified peak was eluted in the same position as a hexose-*N*-acetylhexosamine disaccharide ($\text{Man}^2\text{GlcNAc}$), but was not sensitive to jack-bean β -mannosidase.

Jack-bean-exoglycosidase digestion of neutral core structures from BHK21-cell acidic-type glycopeptides

The mixture of neutral digestion products from the [^3H]glucosamine-labelled sample (Fig. 1b) was further analysed by gel filtration after additional digestion with either jack-bean β -*N*-acetylglucosaminidase (Fig. 2a) or jack-bean α -mannosidase (Fig. 2b). The glucosaminidase digestion converted essentially all of the radiolabelled material in peaks II, III and IV into lower-molecular-weight material (Fig. 2a). There was an apparent increase in radiolabel in both free *N*-acetylglucosamine [from 59% of total radioactivity in Fig. 1(b) to 71% of total radioactivity in Fig. 2(a)] and the $\text{Man}_3\text{GlcNAc}_1$ -size peak-I oligosaccharides (from 8% to 15% of total radioactivity) after this additional exoglycosidase digestion. The same result was obtained with β -*N*-acetylglucosaminidase from bovine kidney (results not shown).

Fig. 1. Gel filtration of untreated and glycosidase-treated glycopeptides from [^3H]sugar-labelled BHK21-cell membrane glycoproteins

Glycopeptides were analysed by preparative Bio-Gel P-4 chromatography (O—O, left-hand scale), and the higher-molecular-weight glycopeptide fractions indicated by the broken brackets were pooled and further digested with the mixture of bacterial exoglycosidases and endoglycosidase D. (a) [2- ^3H]Mannose-labelled glycopeptides; (b) [6- ^3H]glucosamine-labelled glycopeptides; (c) [1- ^3H]galactose-labelled glycopeptides. The products of this digestion were re-analysed on the same column (●—●, right-hand scale), with the profiles aligned with that of the original untreated glycopeptides by alignment of the peak elution positions of the four internal standards (from left to right) Blue Dextran, [^{14}C]acetylated ovalbumin glycopeptide (Etchison *et al.*, 1977), stachyose and mannose (indicated by vertical arrows). Roman numerals I to IV correspond to neutral oligosaccharide products of increas-

Fig. 2. Gel filtration of exoglycosidase-treated neutral products from the large BHK21-cell glycopeptides labelled with [^3H]glucosamine

Samples of the neutral products of bacterial-exoglycosidase and endoglycosidase-D digestion shown in Fig. 1(b) [including peak-I-IV oligosaccharides and the smaller-molecular-weight products; approx. 11×10^3 c.p.m. total in Fig. 1(b) profile] were further digested with either jack-bean β -N-acetylglucosaminidase (a; approx. 4×10^3 c.p.m. total) or jack-bean α -mannosidase (b; approx. 9×10^3 c.p.m. total) and re-analysed on the same Bio-Gel P-4 column with the same internal standards. The small vertical arrows and Roman numerals indicate the original elution positions of the untreated peak I-IV oligosaccharides.

ing size, with peak I eluted in the position of a $\text{Man}_5\text{GlcNAc}_1$ oligosaccharide. The profile of [^3H]glucosamine-labelled products of glycosidase digestion (b, ●—●) represents only the neutral fraction from a Dowex column (approx. 65% of total radiolabel). The profiles of [^3H]mannose-labelled and [^3H]galactose-labelled products represent the total products, but were essentially identical with profiles (not shown) of Dowex neutral fractions that contained greater than 90% and 95% of the total radiolabel for the [^3H]mannose-labelled and [^3H]galactose-labelled samples respectively.

After digestion with jack-bean α -mannosidase, the labelled oligosaccharides in peaks III and IV were unaffected, whereas the radiolabel in peak II was shifted to a slightly-smaller-size oligosaccharide (Fig. 2*b*). The radiolabel in peak I was completely eliminated by this treatment, with a relative increase observed for radiolabel in the $\text{Man}_1\text{GlcNAc}_1$ -size peak [from 8% of total radioactivity in Fig. 1(*b*) to 13% of total radioactivity in Fig. 2(*b*)].

These α -mannosidase digestion studies were repeated with separated peak-I and peak-II oligosaccharides labelled with [^3H]mannose (Fig. 3). The radiolabel from peak-I oligosaccharides was eluted in two smaller peaks equivalent to the expected positions of $\text{Man}_2\text{GlcNAc}$ and free mannose, with a ratio of radiolabel of 1.0:2.0 (Fig. 3*a*). After the corresponding α -mannosidase digestion of the peak-II oligosaccharides, the major peak was eluted several fractions later than undigested peak II, and approximately one-quarter of the radiolabel was eluted with free mannose (Fig. 3*b*).

Glycosidase analysis of virus glycopeptides and oligosaccharides

Because a similar oligomannosyl core heterogeneity was previously observed for Rous-sarcoma-virus glycopeptides (Hunt *et al.*, 1979, 1981), [^3H]glucosamine-labelled virus glycopeptides were subjected to the same digestion with the *Diplococcus pneumoniae* glycosidase mixture, and the neutral fraction was resolved into individual products by preparative gel filtration (Fig. 4). Before the glycosidase digestion, the Pronase-digested glycopeptides were eluted in a single broad peak of higher-molecular-weight material, and were deficient in the smaller neutral-type glycopeptides compared with the corresponding [^3H]glucosamine-labelled BHK21-cell glycopeptides (Fig. 1*b*) and [^3H]mannose-labelled Rous-sarcoma-virus glycopeptides (Hunt *et al.*, 1979, 1981). The profile of the Dowex neutral fraction from the glycosidase-digested virus glycopeptides contained the same products observed in the corresponding BHK21-cell sample in Fig. 1 (peaks I–IV, $\text{Man}_1\text{GlcNAc}_1$ -size peak, free *N*-acetylglucosamine) and, in addition, higher-molecular-weight material (fractions 62–68 in Fig. 4) that was eluted in the same position as a major peak of partially digested but endoglycosidase-resistant glycopeptides in the Dowex acidic fraction (profile not shown). The peak-II oligosaccharides (fractions 93–98, Fig. 4) were eluted approximately one-and-one-half fractions ahead of an authentic [^{14}C]glucosamine-labelled $\text{Man}_5\text{GlcNAc}_1$ oligosaccharide included as an internal standard.

Individual peaks from the preparative-gel-filtration profile in Fig. 4 were further analysed after digestion with jack-bean β -*N*-acetylglucosaminidase

Fig. 3. Gel filtration of α -mannosidase-treated oligosaccharides I and II from [^3H]mannose-labelled BHK21-cell membrane glycopeptides

Peak-I and peak-II structures obtained from a preparative-gel-filtration profile similar to that shown in Fig. 1(*a*) were subjected to jack-bean- α -mannosidase digestion, and then re-analysed on a Bio-Gel P-4 column as described in the legend to Fig. 2, with peak-I oligosaccharides shown in (*a*) and peak II oligosaccharides in (*b*).

or α -mannosidase (Fig. 5). Whereas the peak-I oligosaccharides were resistant to β -*N*-acetylglucosaminidase (results not shown), almost all of the radiolabelled material in peaks II–IV was converted into material in two smaller peaks eluted in the positions of $\text{Man}_3\text{GlcNAc}_1$ and free *N*-acetylglucosamine (Figs. 5*a*–5*c*). The peak of undigested oligosaccharide in the peak-II sample (Fig. 5*a*) represented 13% of the total radioactivity in the profile and was co-eluted with the $\text{Man}_3\text{GlcNAc}_1$ internal standard rather than the original peak II. The residual undigested oligosaccharides in the peak-IV sample (Fig. 5*c*) also represented approx. 13% of the total radioactivity in the profile.

The ratio of radiolabel in free *N*-acetylglucosamine to that in $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharides in the jack-bean- β -*N*-acetylglucosaminidase digestion products increased along with the size of the original neutral oligomannosyl core:

Fig. 4. Gel filtration of [^3H]glucosamine-labelled Rous-sarcoma-virus glycopeptides before and after glycosidase treatment

The conditions of gel filtration in this Figure and in Fig. 5 were similar to those in Figs. 1–3 except that a ^{14}C -labelled neutral-type glycopeptide (left broken arrow) and $\text{Man}_5\text{GlcNAc}_1$ oligosaccharide (right broken arrow) from Chinese-hamster ovary cells (Hunt, 1980a) were used along with the three unlabelled internal standards instead of the [^{14}C]acetylated ovalbumin glycopeptide. The analytical-gel-filtration profile of untreated glycopeptides (O—O, left-hand scale) was aligned with the preparative-gel-filtration profile (only 0.1 ml of 1 ml fractions were assayed for radioactivity) of the Dowex neutral fraction after digestion with the mixture of *Diplococcus pneumoniae* glycosidases (●—●, right-hand scale). The brackets indicate the fractions pooled for further analysis of individual peaks of oligosaccharides (I–IV) and incompletely digested glycopeptides (fractions 63–68).

0.0:0.1:1.0 for peak I, 1.5:1.0 for peak II, 2.3:1.0 for peak III, and 3.9:1.0 for peak IV.

The results of α -mannosidase digestion of [^3H]glucosamine-labelled peak-I and peak-II viral oligosaccharides (Figs. 5d and 5e) were analogous to those with the [^3H]mannose- or [^3H]glucosamine-labelled BHK21-cell oligosaccharides (Figs. 2 and 3): the [^3H]glucosamine-labelled peak-I material was completely digested to a $\text{Man}_1\text{GlcNAc}_1$ -size product, and the [^3H]glucosamine-labelled peak-II material was shifted in elution position to that of a slightly-smaller-size oligosaccharide. The minor amount of radiolabel in the $\text{Man}_1\text{GlcNAc}_1$ -size peak in the digestion of peak-II material (Fig. 5e) was attributed to the α -mannosidase digestion of the previously mentioned $\text{Man}_5\text{GlcNAc}_1$ -size oligosaccharides present in small amounts in peak II.

Fig. 5(f) shows the gel-filtration profile obtained from the jack-bean- β -N-acetylglucosaminidase digestion of the partially digested [^3H]glucosamine-labelled glycopeptides from the Dowex acidic and neutral fractions (Fig. 4, fractions 62–68) of the original endoglycosidase-D and bacterial-exoglycosidase digestion. Most of the radiolabelled material was converted into free N-acetylglucosamine and smaller glycopeptides.

Discussion

The previously observed heterogeneity in the exoglycosidase and endoglycosidase-D-released core oligosaccharides of complex acidic-type glycopeptides from the membrane proteins of BHK21 cells (Hunt, 1979) and Rous sarcoma virus (Hunt *et al.*, 1979, 1981) was shown in the present studies to be the presence of bacterial-exoglycosidase-resistant N-acetylglucosamine (presumably zero to three residues for peak-I–IV structures) at the non-reducing termini of a common inner core structure ($\text{Man}_2\alpha\text{Man}\beta\text{GlcNAc}$). These results were consistent with the common three-mannose-residue inner core ($\text{Man}\alpha 1\rightarrow 3(\text{Man}\alpha 1\rightarrow 6)\text{Man}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 4\text{GlcNAc-Asn}$) previously demonstrated in detailed studies of acidic-type asparaginyl oligosaccharides from a number of serum proteins (Bayard & Roux, 1975) or a mixture of calf thymocyte membrane glycoproteins (Yoshima *et al.*, 1980).

Because the results of these glycosidase and gel-filtration studies were apparently identical whether the source of radiolabelled oligosaccharides was BHK21-cell glycopeptides or Rous-sarcoma-virus glycopeptides, the results from the two samples are combined in the following summary of the

Fig. 5. Gel filtration of exoglycosidase-treated peak-I-IV oligosaccharides from [^3H]glucosamine-labelled Rous-sarcoma-virus glycopeptides

Individual peaks of neutral oligosaccharides I-IV (a-e) or incompletely digested glycopeptides (f) obtained from the preparative-gel-filtration profile shown in Fig. 4 were further digested with either jack-bean β -N-acetylglucosaminidase or jack-bean α -mannosidase, and then analysed by gel filtration. The bracketed and broken arrows indicate the original elution positions of the peak-I-IV oligosaccharides or partially digested and endoglycosidase-D-resistant glycopeptides. (a) β -N-Acetylglucosaminidase digestion of peak-II oligosaccharides; (b) β -N-acetylglucosaminidase digestion of peak-III oligosaccharides; (c) β -N-acetylglucosaminidase digestion of peak-IV oligosaccharides; (d) α -mannosidase digestion of peak-I oligosaccharides; (e) α -mannosidase digestion of peak-II oligosaccharides; (f) β -N-acetylglucosaminidase digestion of partially digested and endoglycosidase-resistant glycopeptides (GP).

individual characteristics of each of the four neutral oligosaccharide species. The specific radiolabelling patterns indicated that all four species contained mannose and *N*-acetylglucosamine, but not galactose. The size, complete digestion by α -mannosidase to free mannose and a mannose-*N*-acetylglucosamine disaccharide (2:1 ratio for [^3H]mannose-labelled species; Fig. 3) and resistance to digestion with jack-bean β -*N*-acetylglucosaminidase were all consistent with a $(\text{Man})_2 \xrightarrow{\alpha} \text{Man} \xrightarrow{\beta} \text{GlcNAc}$ structure for the peak I oligosaccharide species.

In contrast, the partial sensitivity of the peak-II oligosaccharides to α -mannosidase and their digestion by the eukaryotic β -*N*-acetylglucosaminidases to free *N*-acetylglucosamine and a $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharide were suggestive of a three-mannose-residue core containing an additional β -linked *N*-acetylglucosamine residue at a non-reducing terminus, with one of the α -linked mannose residues in a terminal position. Kobata *et al.* (1978) have reported that an *N*-acetylglucosamine residue behaves as two hexose units in Bio-Gel-P-4-gel filtration, so that the elution of the peak-II species slightly ahead of the $\text{Man}_3\text{GlcNAc}_1$ internal standard (Fig. 4) was consistent with a $\text{GlcNAc} \xrightarrow{\beta} (\text{Man}_3\text{GlcNAc})$ structure. The ratio of radiolabel in free *N*-acetylglucosamine to that in $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharides (1.5:1.0; Fig. 5a) in the jack-bean- β -*N*-acetylglucosaminidase digestion products of the [^3H]glucosamine-labelled peak-II oligosaccharides was higher than expected for a $\text{GlcNAc}_1(\text{Man}_3\text{GlcNAc})$ structure, and suggested that the labelling of the inner chitobiose-derived *N*-acetylglucosamine residue and the outer branch *N*-acetylglucosamine residue was non-uniform. Peak-II oligosaccharides from the viral glycopeptides also contained minor amounts of β -*N*-acetylglucosaminidase-resistant $\text{Man}_3\text{GlcNAc}_1$ -size structures (Fig. 5a) that may have resulted from the endoglycosidase-D digestion of small neutral-type glycopeptides (Hunt *et al.*, 1979, 1981) or endoglycosidase-D-plus-exoglycosidase digestion of hybrid-type glycopeptides containing five-mannose-residue cores (Hunt *et al.*, 1981; Hunt & Wright, 1981).

The jack-bean- β -*N*-acetylglucosaminidase digestion of the peak-III and peak-IV neutral oligosaccharides indicated that these were also $\text{GlcNAc}_n \xrightarrow{\beta} (\text{Man}_3\text{GlcNAc})$ structures, and the gel-filtration properties of these species were most consistent with oligosaccharides containing two and three *N*-acetylglucosamine residues respectively in terminal non-reducing positions. The ratios of [^3H]glucosamine label in β -*N*-acetylglucosaminidase-released *N*-acetylglucosamine to that in the $\text{Man}_3\text{GlcNAc}_1$ -size core (2.3:1.0 rather than the expected 2.0:1.0 for peak III; 3.9:1.0 rather than

the expected 3.0:1.0 for peak IV; Figs. 5b and 5c) also suggested non-uniform labelling of the innermost and branch *N*-acetylglucosamine residues. The α -mannosidase-resistance of peak-III and peak-IV oligosaccharides was consistent with either the absence of a terminal α -linked mannose residue, or alternatively the presence of a terminal α -linked mannose residue that was not susceptible to the jack-bean α -mannosidase because of apparent steric hindrance, as has been previously reported for certain partially digested acidic-type oligosaccharides (Yoshima *et al.*, 1980).

The products of the *Diplococcus pneumoniae*-glycosidase digestion of the viral glycopeptides also included partially digested glycopeptides with β -linked *N*-acetylglucosamine residues at non-reducing termini, as demonstrated by the release of free *N*-acetylglucosamine during the digestion with jack-bean β -*N*-acetylglucosaminidase (Fig. 5f). The presence of these *N*-acetylglucosamine residues may have specifically prevented the cleavage of these glycopeptides by endoglycosidase D.

The inability of bacterial β -*N*-acetylglucosaminidases to remove certain *N*-acetylglucosamine residues that could be removed by eukaryotic enzymes has been reported previously in analyses of the triantennary acidic-type glycopeptides of fetuin (Baenziger & Fiete, 1979) and the tri- and tetra-antennary acidic-type structures of calf thymocyte membrane glycoproteins (Yoshima *et al.*, 1980). The detailed substrate specificity of purified β -*N*-acetylglucosaminidase from *Diplococcus pneumoniae* has been elucidated by Yamashita *et al.* (1981): the enzyme cannot cleave $\text{GlcNAc}\beta 1 \rightarrow 4\text{Man}$ and $\text{GlcNAc}\beta 1 \rightarrow 6\text{Man}$ linkages, although it readily hydrolyses $\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}$ linkages unless the mannose residue is also substituted at the 6-position with a second *N*-acetylglucosamine residue [$\text{GlcNAc}\beta 1 \rightarrow 6(\text{GlcNAc}\beta 1 \rightarrow 2)\text{Man}$]. The results of these three experimental studies suggest that tri- and/or tetra-antennary acidic-type glycopeptides with additional $\beta 1 \rightarrow 4$ - and/or $\beta 1 \rightarrow 6$ -linked *N*-acetylglucosamine may have been the source of the larger (peaks II–IV) oligomannosyl core products, and smaller, di-antennary, glycopeptides containing only $\beta 1 \rightarrow 2$ -linked outer branch *N*-acetylglucosamine may have been the source of the peak-I $\text{Man}_3\text{GlcNAc}_1$ oligosaccharides.

The inability of *Diplococcus pneumoniae* β -*N*-acetylglucosaminidase to remove all of the outer branch β -*N*-acetylglucosamine residues from the more highly branched (presumably tri- and/or tetra-antennary) acidic-type oligosaccharides observed in the present studies is apparently not a property of all bacterial β -*N*-acetylhexosaminidases: a mixture of glycosidases from *Clostridium perfringens* [containing neuraminidase, β -galactosidase, β -*N*-acetylglucosaminidase and endo-

β -N-acetylglucosaminidases C_I and C_{II} (Kobata, 1978)] was able to digest the acidic-type glycopeptides from Rous sarcoma virus completely to a Man₃GlcNAc₁-size peak-I oligomannosyl core (L. A. Hunt, unpublished work).

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