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# SERUM GLYCOPROTEIN-TYPE SEQUENCE OF MONOSACCHARIDES IN MEMBRANE GLYCOPROTEINS OF SEMLIKI FOREST VIRUS

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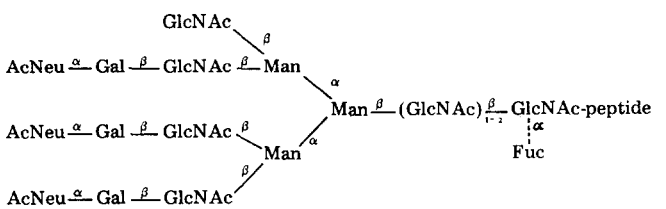
## SUMMARY

Semliki Forest virus was grown in BHK-21 cells and labelled in vivo with radioactive monosaccharides. The virus was disrupted with sodium dodecyl sulphate and the polypeptides were hydrolyzed with pronase. A mixture of type A glycopeptides (for nomenclature, see Johnson and Clamp (1971) Biochem. J. 123, 739–745) of the membrane glycoproteins $E_1$ and $E_3$ was isolated by gel filtration and subjected to sequential degradation with exo-glycosidases. The reduction in the apparent molecular weight and the cleavage of radioactive monosaccharides were monitored with gel filtration.

The results suggest that the type A oligosaccharides have similar average structures and contain at the non-reducing terminus 3.4 mol of α-D-sialic acid and 0.7 mol of α-L-fucose, followed by 3.1 mol of β-D-galactose, 4.2 mol of *N*-acetyl-β-D-glucosamine, 0.7–1.5 mol of α-D-mannose, 0.5 mol of β-D-mannose and 0.6–2.2 mol of *N*-acetyl-β-D-glucosamine attached to 1.0 mol of *N*-acetylglucosamine resistant to *N*-acetyl-β-D-glucosaminidase. This innermost monosaccharide unit, therefore, appears to be attached to the peptide. The peptides attached to this *N*-acetyl-glucosamine had an apparent molecular weight of 720±100.

We propose the following average structure, compatible with most of our data, for the type A glycopeptides of Semliki Forest virus:




Abbrevations: SF virus, Semliki Forest virus; $V_e$, elution volume in gel filtration; $V_0$, void volume in gel filtration columns.

## INTRODUCTION

Our group is studying the Semliki Forest virus (SF virus) as a model for biological membranes [1, 2]. The viral membrane consists of a host cell-derived lipid bilayer [3, 4], and projections formed by three virus-specific glycoproteins $E_1$, $E_2$ and $E_3$ (molecular weights 49000, 52000 and 10000, respectively) appearing in approximately equimolar amounts [5]. Either $E_1$ or $E_2$ or both span the lipid bilayer with a hydrophobic segment in the polypeptide sequence [6].

$E_1$ consists of 7.5%, $E_2$ of 11.5% and $E_3$ of 45.1% of carbohydrate [5]. Analysis of the pronase glycopeptides of $E_1$, $E_2$ and $E_3$ suggested that the carbohydrate exists as 1 mol of type A oligosaccharide (for nomenclature of type A and type B glycopeptides see ref. 7) in glycoproteins $E_1$ and $E_3$ ($E_1A$ and $E_3A$), $E_2$ carrying 1–2 type B oligosaccharides ($E_2B$) and unidentified oligosaccharides labelled heavily from [$^3$H]galactose [8]. The apparent molecular weights of the pronase glycopeptides are 3500 for $E_1A$, 4000 for $E_3A$, 2000 for $E_2B$ and 3100 for the unidentified glycopeptide of $E_2$ [8]. The type A glycopeptides consist of *N*-acetylglucosamine, galactose, fucose, mannose and sialic acid. $E_2B$ consists of only *N*-acetylglucosamine and mannose [8].

The present work describes the sequence and the anomeric configuration of the average type A pronase glycopeptides analyzed by means of sequential degradation with specific exo-glycosidases.

## MATERIALS AND METHODS

(1) *Growth, radioactive labelling and purification of SF virus.* SF virus was grown in a 75 cm$^2$ Falcon flask of BHK-21 cells as described earlier [8]. The infected cells were labelled for 7 h, 3 h after infection in four different ways; (1) with 250 $\mu$Ci of D-[1-$^{14}$C] glucosamine hydrochloride (52 Ci/mol); (2) with 1 mCi of D-[2-$^3$H]-mannose (2 Ci/mmol); (3) with 1 mCi of L-[1-$^3$H]fucose (2.6 Ci/mmol) and 250 $\mu$Ci of D-[1-$^{14}$C]glucosamine hydrochloride (58 Ci/mol); (4) with 1 mCi of D-[1-$^3$H]-galactose (13 Ci/mmol) and 250 $\mu$Ci of D-[1-$^{14}$C]mannose (58.7 Ci/mol). All labelled compounds were obtained from The Radiochemical Centre Ltd., Amersham, U.K. In the last two experiments the glucose concentration of the medium was reduced to one-tenth. The virus was harvested and purified from the culture fluid 10 h after infection as described [3].

(2) *Gel filtration and molecular weight estimations of the glycopeptides.* Gel filtration was carried out on a Bio-Gel P-6 (100–200 mesh, Bio-Rad) column of 0.9 × 58 cm [9]. The sample was dissolved in 300 $\mu$l of elution buffer and was eluted with 0.15 M Tris/acetate buffer, pH 7.8, containing 0.1% sodium dodecyl sulphate. Blue dextran 2000 (Pharmacia) was used in every run to determine the void volume.

The molecular weight estimations of the glycopeptides on gel filtration were carried out according to Burge and Strauss [9]. The following radiochemical standards were used to calibrate the column: lacto-*N*-difucohexaose, lacto-*N*-fucopentaose and *O*-$\beta$-D-galactopyranosyl-(1 → 3)-*O*-$\beta$-D-galactopyranosyl-(1 → 4)-D-glucose reduced with $NaB^3H_4$, $^{14}$C-acetylated type A glycopeptides of thyroglobulin ($M_r$ 4100) [8], [$^{14}$C]mannose and [$^{14}$C]glucosamine. Lacto-*N*-difucohexaose I and II, lacto-*N*-fucopentaose I (Dr. A. Gauche, Heidelberg) and the triose mentioned above were

also used in unlabelled form and analyzed by the method of Dubois et al. [10]. The unlabelled and the labelled standards had identical elution characteristics and gave a linear relationship between $\log M_r$ and $V_e/V_0$ except for the monosaccharides. The exact position of each peak was interpolated by fitting a parabola through the three highest points.

A column (1 ×63 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad) eluted with 0.02% $NaN_3$ was used to separate sodium dodecyl sulphate from pronase digests and desialylated preparations after preparative runs on Bio-Gel P-6.

(3) *Pronase digestion of SF virus.* The purified virus was disrupted with 0.1 % sodium dodecyl sulphate and the sample was diluted 10- fold with 0.1 M Tris—HCl, pH 8.0, containing 0.01 M $CaCl_2$. 50 µl of pronase (Calbiochem, B grade) as a 1 % solution in the same buffer was added three times at 0, 6 and 20 h to the sample of 1.0 ml. The digestion was carried out at 60 °C for 24 h [11]. The pronase solution was always autodigested prior to use for 2 h at 37 °C to minimize any contaminating glycosidase activity. This incubation system appeared to be equivalent with the 4 day incubation at 37 °C used before [8].

The digests were centrifuged prior to gel filtration to remove insoluble material and chromatographed on Bio-Gel P-6 which separates type A and type B glycopeptides [8]. The mixture of the type A glycopeptides was pooled, lyophilized and run through Bio-Gel P-2 to remove sodium dodecyl sulphate. At this stage the preparations labelled separately with [$^{14}$C]glucosamine and $^3$[H]mannose were pooled together. In addition to this new preparation, where the label is rather specifically in the glucosamine and mannose units, two previously described preparations were used [8]. They were double labelled with [$^3$H]galactose-[$^{14}$C]mannose and with [$^3$H]fucose-[$^{14}$C]glucosamine.

(4) *Treatment of glycopeptides with neuraminidase.* The lyophilized glycopeptides were dissolved in 200 µl of 0.1 M sodium acetate buffer, pH 5.6, containing 0.001 M $CaCl_2$ and neuraminidase from *Vibrio cholerae* (Behringwerke, EC 3.2.1.18) was added at 0 and 8 h up to the concentration of 100 units/ml and incubated at 37 °C for 24 h. The preparation was boiled for 2 min and 600 µl of 0.1 M Tris · HCl, pH 8.0 containing 0.01 M $CaCl_2$ was added. The desialylated glycopeptides were redigested with pronase.

(5) *Sequential digestions of desialylated type A glycopeptides with exo-glycosidases.* The desialylated glycopeptides were lyophilized and dissolved in 150 µl of 0.05 M sodium citrate buffer, pH 4.0. Digestions with different exo-glycosidases were performed in the following manner: (1) α-L-Fucosidase (EC 3.2.1.51) from beef kidney (Boehringer) was added to the final concentration of 0.4 unit/ml and the mixture was incubated for 10–20 h. (2) β-D-Galactosidase (EC 3.2.1.23) from Jack Bean (Dr. Y.-T.Li, New Orleans), two equal additions of the enzyme, final concentration 0.4 unit/ml, incubation time 48 h. (3) *N*-Acetyl-β-D-glucosaminidase (EC 3.2.1.50) from beef kidney (Boehringer), three equal additions of the enzyme, final concentration 2.7 units/ml, incubation time 72 h; *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) from Jack Bean (Dr. Y.-T. Li, New Orleans), three equal additions, final concentration 56 units/ml, incubation time 72 h. For the digestion of the proximal *N*-acetylglucosamine units the enzyme from beef kidney was added to the final concentration of 1.9 or 3.5 units/ml and the mixture was incubated for 48 h. (4) α-D-Mannosidase (EC 3.2.1.24) from Jack Bean (Dr. Y.-T. Li, New Orelans), three

equal additions of the enzyme, final concentration 2.1 or 5.1 units/ml, incubation time 82 or 72 h, respectively. α-D-Mannosidase from Jack Bean (Boehringer), three equal additions, final concentration 24 units/ml, incubation time 77 h. (5) β-D-Mannosidase (EC 3.2.1.25) from *Polyporus sulfureus* (Dr. Y.-T. Li, New Orleans), four equal additions, final concentration 0.07 unit/ml, incubation time 140 h. The incubations were performed at 37 °C. An aliquot was taken for gel filtration. The remaining digest was boiled for 2 min to destroy enzyme activity before the next enzyme was added. The activities of the enzyme preparations were determined using *p*-nitrophenyl-glycosides as substrates at 38 °C [12]. The $NaN_3$ present in the glycopeptide preparations after the Bio-Gel P-2 runs did not interfere with enzymatic activities. However, sodium dodecyl sulphate seems to inhibit at least neuraminidase, galactosidase and *N*-acetylglucosaminidase activity.

(6) *Acid hydrolysis of sialic acid.* The glycopeptides were incubated in 200 μl of 0.05 M $H_2SO_4$ at 80 °C for 1 h [13]. The pH was adjusted to 8.0 and a redigestion with pronase was carried out as in the desialylation procedure with neuraminidase.

(7) *Gel electrophoresis.* The glycoproteins were separated using discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate as described [8].

(8) *Scintillation counting.* Radioactivity was determined in a Wallac scintillation counter 81.0000 in plastic vials containing 10 ml xylene/Triton X-114 cocktail. Quenching was occasionally checked with an external standard. In double-label experiments the data were corrected for the crossover counts.

## RESULTS

### (1) *Glycopeptides of SF virus*

When SF virus was labelled in vivo with [1-$^{14}$C]glucosamine and subsequently digested with pronase, a mixture of glycopeptides was obtained, which chromatographed on Bio-Gel P-6 as shown in Fig. 1. The glucosamine-rich glycopeptides of type A ($M_r$ 3800) and the mannose-rich glycopeptides of type B ($M_r$ 2000) were separated from each other. They were followed by a peak consisting probably of amino acids and small peptides containing 7 % of the total radioactivity. A second experiment with another, identically labelled SF virus preparation gave almost identical results.

In contrast, our earlier experiments with glucosamine-labelled virus revealed 40 % of the radioactivity in the amino acid peak [8]. The present labelling experiments were carried out in Eagle's minimum essential medium containing the normal glucose contentration of 1.0 g/l, whereas in the previous experiments Eagle's medium with reduced glucose concentration of 0.1 g/l was used. The specificity of the glucosamine labelling in the present experiments was confirmed by polyacrylamide gel electrophoresis of the viral polypeptides; the non-glycosylated core protein of the virus [14] was only marginally labelled (data not shown), in contrast to the earlier experiments [8].

When SF virus was labelled in vivo with [2-$^{3}$H]mannose and subsequently digested with pronase, a mixture of glycopeptides was obtained, which chromatographed a Bio-Gel P-6 as shown in Fig. 1. The elution profile shows practically no amino acid peak. The labelling was in this case even more specific than with [1-$^{14}$C]-

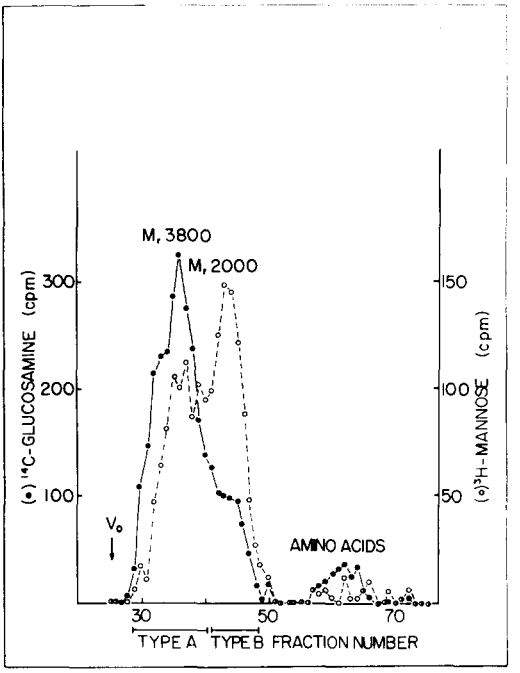



Fig. 1. SF virus labelled in vivo with radioactive monosaccharides was disrupted with sodium dodecyl sulphate and digested with pronase. The released glycopeptides were separately chromatographed on Bio-Gel P-6: ●, glycopeptides labelled with D-[1 − $^{14}$C]glucosamine; ○, glycopeptides labelled with D-[2 − $^{3}$H]mannose. $V_0$ indicates the void volume of the column.

glucosamine. In contrast, our earlier experiments with [1-$^{3}$H]mannose led to significant labelling of the amino acids during the long pulse used to label the virus [8]. The [$^{3}$H]mannose-labelled type B glycopeptides ($M_r$ 2000) emerged in the present experiment as a high peak after the peak of the type A glycopeptides ($M$. 3800). One can estimate from Fig. 1 that about 45 % of [$^{3}$H]mannose is present as type B oligosaccharides in SF virus.

The peak of the type A glycopeptides in Fig. 1 is known to be heterogeneous. Apparent molecular weights of 4000 and 3500 have been measured for $E_1$A and $E_3$A, respectively; in addition, the A peak contains the unknown oligosaccharide of $E_2$ [8]. The experiments of the present work were carried out with this mixture rather than with individual type A glycopeptides. The materials for the experiments described below were obtained by pooling fractions of the Bio-Gel P-6 chromatogram as shown in Fig. 1.

(2) *Sequential exo-glycosidase digestions of the glycopeptides*

*Neuraminidase digestion.* α-D-Neuraminidase treatment of type A glycopeptides, doubly labelled with [1-$^{14}$C]glucosamine and [1-$^{3}$H]fucose, gave two peaks (Fig. 2). The major one is doubly labelled and represents desialylated type A glycopeptides of an apparent molecular weight of 2800. Fractions 45–49 represent residual type B glycopeptides which contaminate the type A preparation. This material

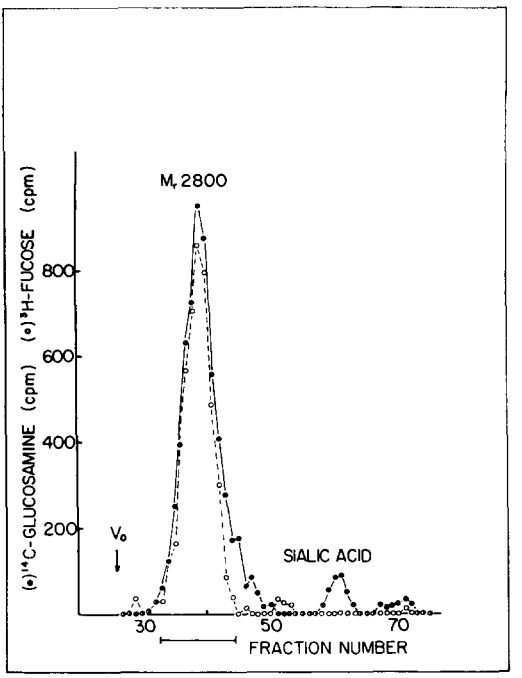



Fig. 2. Bio-Gel P-6 elution profiles of desialylated type A glycopeptides labelled with (●) D-[1 —$^{14}$C]glucosamine and (○) D-[1 —$^{3}$H]fucose. Type A glycopeptides were digested with α-D-neuraminidase. The bar extending from fraction 33 to fraction 44 shows how the material was pooled for further degradation.

contains label from [1-$^{14}$C]glucosamine but not from [1-$^{3}$H]fucose. The second peak represents sialic acid. Cleaved off from the original type A glycopeptides this peak was labelled from [1-$^{14}$C]glucosamine but not from [1-$^{3}$H]fucose, [1-$^{3}$H]-galactose or [2-$^{3}$H]mannose. The peak occupied the same position in the Bio-Gel P-6 chromatogram as pure *N*-acetylsialic acid (data not shown). The peak was also generated by mild acid hydrolysis of the glycopeptides (see below). The sialic acid peak contained 5.3 % of the total radioactivity in Fig. 2. Many cell types are known to metabolize labelled glucosamine to sialic acid [15].

The α-D-neuraminidase treatment of type A glycopeptides labelled with [2-$^{3}$H]mannose and [1-$^{14}$C]glucosamine or double labelled with [1-$^{3}$H]galactose and [1-$^{14}$C]mannose gave similar results. The apparent molecular weight of the desialylated type A glycopeptide peak was 2800 (range of nine experiments, 3050–2750). The difference of 1000 daltons in the molecular weight of the original type A glycopeptides (Fig. 1) and the desialylated glycopeptides (Fig. 2) suggests that the average type A glycopeptide contains 3.4 mol of sialic acid per mol of glycopeptide (Table III). The desialylated peak was pooled for further enzymatic degradations as indicated in Fig. 2. The completeness of neuraminidase digestion was ascertained by comparison with mild acid hydrolysis known to hydrolyze the sialic acid residues quantitatively [13]. After both methods of hydrolysis the apparent molecular weight of type A glycopeptides was 2800. Neuraminidase cleaved 5.4 % and mild acid

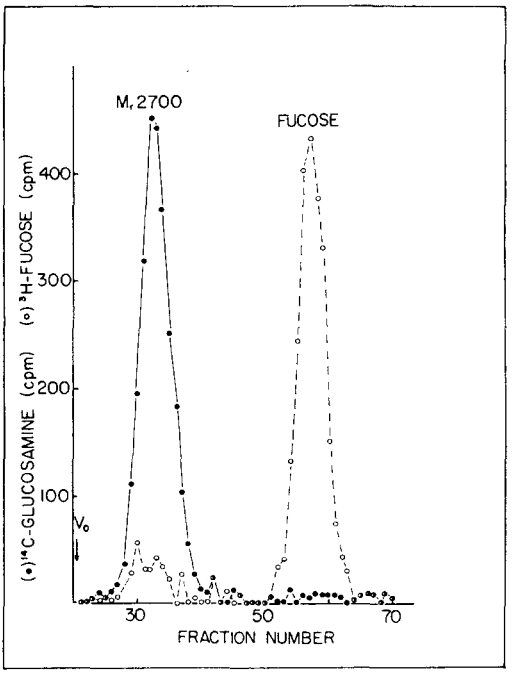



Fig. 3. Bio-Gel P-6 elution profile of type A glycopeptides digested with α-D-neuraminidase and then with α-L-fucosidase. The preparation was labelled with (●) D-[1−$^{14}$C]glucosamine and (○) L-[1−$^{3}$H]fucose.

hydrolysis cleaved 5.9 % of the $^{14}$C label. Over 90 % of the sialic acid residues is then cleaved by neuraminidase from the original glycopeptides. The mild acid hydrolysis cleaved additionally 7 % of the fucose residues (data not shown).

*Fucosidase digestion.* The elution profile of the material from the α-L-fucosidase treatment of the desialylated type A glycopeptides double labelled with [1-$^{14}$C]-glucosamine and [1-$^{3}$H]fucose is shown in Fig. 3. The type A glycopeptides have retained the glucosamine label but lost 89 % of the fucose label. The liberated α-L-fucose units chromatographed as free monosaccharide on Bio-Gel P-6. The decrease in the apparent molecular weight of the glycopeptide was about 100 daltons in two separate experiments. This is equivalent with 0.7 mol of fucose per glycopeptide (Table III).

The fucose residues are distal and freely exposed to the fucosidase even in the native type A glycopeptides. When the original type A glycopeptides, double labelled with [1-$^{14}$C]glucosamine and [1-$^{3}$H]fucose, were treated with α-L-fucosidase prior to neuraminidase treatment, 98 % of the fucose label was liberated in the form of monosaccharides (Table III). However, the hydrolysis proceeded more slowly than with the desialylated substrate. To achieve 98 % of hydrolysis both the reaction time and the enzyme concentration had to be trippled. The complete hydrolysis caused by the pure fucosidase shows that the label originally given to the host cells as [1-$^{3}$H]-fucose had largely retained its identity in the viral oligosaccharide.

TABLE I

RELEASE OF DISTAL *N*-ACETYLGLUCOSAMINE UNITS

The type A pronase glycopeptides labelled with [$^3$H]mannose and [$^{14}$C]glucosamine were predigested with α-D-neuraminidase, α-L-fucosidase and β-D-galactosidase. The preparation dissolved in 150 μl of 0.05 M sodium citrate buffer, pH 4.0, was then digested at 37 °C with three equal additions of *N*-acetyl-β-D-glucosaminidase (Boehringer) and/or *N*-acetyl-β-D-hexosaminidase (Dr. Y-T. Li, New Orleans). The digests were chromatographed on Bio-Gel P-6 for the analysis of the liberated [$^{14}$C]glucosamine label.

| Experiment | Enzyme | Final enzyme concentration (units/ml) | Incubation time (h) | Liberated *N*-acetyl-[$^{14}$C]glucosamine (%) | Apparent molecular weight of the resulting glycopeptide |
|---|---|---|---|---|---|
| 1 | beef kidney *N*-acetyl-β-D-glucosaminidase | 2.7 | 72 | 58 | 1360 |
| 2 | Jack Bean *N*-acetyl-β-D-hexosaminidase | 56.0 | 72 | 56 | 1350 |
| 3 | 1+2 | 58.7 | 144 | 57 | 1300 |

*Galactosidase digestion.* Jack Bean β-D-galactosidase liberated from the desialylated and defucosylated type A glycopeptides 96 % (range of four experiments, 94–97 %) of [1-$^3$H]galactose label as monosaccharide (Fig. 4a). The completeness of hydrolysis indicates that the galactose label had retained its identity in the viral oligosaccharides. The apparent molecular weight in the [1-$^{14}$C]glucosamine and [1-$^3$H]fucose double-labelled preparation was reduced from 2700 to 2200 (Fig. 4b) (range of five experiments, 2100–2250), which is equivalent with 3.1 mol of galactose per average glycopeptide (Table III).

Fucose is not attached to the galactose residues as β-D-galactosidase treatment of a desialylated substrate, with intact fucose residues, resulted in the loss of 95 % of the [1-$^3$H]galactose label, and gave a similar elution profile as in Fig. 4a. The molecular weight of the substrate double labelled with [1-$^{14}$C]glucosamine and [1-$^3$H]fucose was reduced from 2800 to 2300 (Table III), which is equivalent with 3.1 mol of galactose.

*Digestion of the distal N-acetylglucosamine units.* Two different virus batches were labelled separately with [1-$^{14}$C]glucosamine and with [2-$^3$H]mannose. The two preparations of type A glycopeptides were combined after pronase digestion, desialylated, defucosylated and degalactosylated. The subsequent digestion with *N*-acetyl-β-D-glucosaminidase (beef kidney) resulted in the liberation of 58 % of the *N*-acetyl-[$^{14}$C]glucosamine label (Table I), and in the reduction in the molecular weight of the glycopeptide from 2000 to 1360, equivalent with 4.2 mol of *N*-acetyl-β-glucosamine (Fig. 5, Table III). Also Jack Bean *N*-acetyl-β-D-hexosaminidase, and both enzymes together gave identical results. As elevated enzyme concentrations

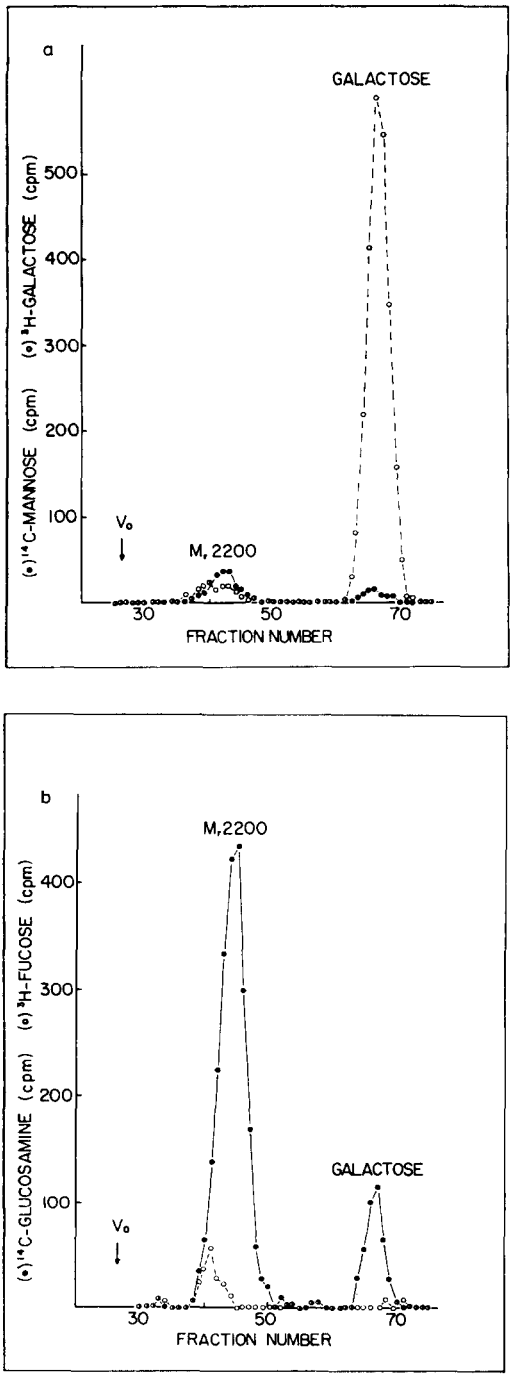



Fig. 4. Bio-Gel P-6 elution profiles of type A glycopeptides digested with β-D-galactosidase. (a) The (●) D-[1-$^{14}$C]mannose and (○) D-[1-$^{3}$H]-galactose labelled preparation had been predigested with α-D-neuraminidase and α-L-fucosidase prior to incubation with β-D-galactosidase. (b) The (●) [1-$^{14}$C]glucasamine and (○) [1-$^{3}$H]fucose labelled preparation had been predigested with α-D-neuraminidase and α-L-fucosidase. Only the substrate for β-D-galactosidase digestion was collected after a preparative run on Bio-Gel P-6. This is why the previously liberated [$^{3}$H]fucose residues are not included in this chromatogram. The liberated monosaccharide peak most probably represents [$^{14}$C]galactose which derives its label from [$^{14}$C]glucosamine.

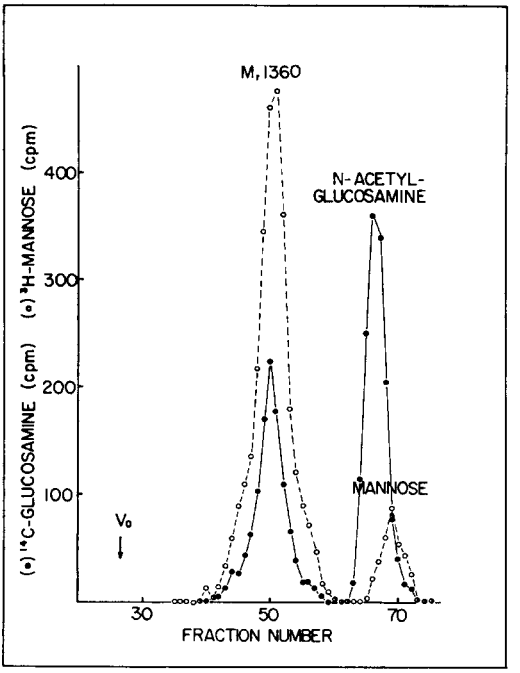



Fig. 5. Bio-Gel P-6 elution profile of type A glycopeptides digested with α-D-neuraminidase, α-L-fucosidase, β-D-galactosidase and then with *N*-acetyl-β-D-glucosaminidase. The preparation was labelled with (●) D-[1-$^{14}$C]glucosamine and (○) D-[2-$^{3}$H]mannose. About 10 % of the mannose label was released as free monosaccharide, possibly by contaminating mannosidase activity.

or prolonged reaction times did not liberate additional *N*-acetyl-β-D[$^{14}$C]glucosamine (Table I), all of the units do not seem to be susceptible for enzyme digestion at this stage. In all three enzyme digestions of Table I also 7–11 % of [$^{3}$H]mannose was liberated. It does not represent *N*-acetylglucosamine formed from [$^{3}$H]mannose as the liberated monosaccharide eluted like mannose and not like *N*-acetylglucosamine (Fig. 5). Therefore it seems that the enzyme preparations might have been contaminated with mannosidase activity.

As judged from the gel filtration data, the 42 % of *N*-acetyl-[$^{14}$C]glucosamine label remaining uncleaved would be equivalent with 3.2 mol of "resistant" *N*-acetylglucosamine units, probably located deeper in the oligosaccharide structure.

*α-Mannosidase digestion.* A sample of type A glycopeptides double labelled specifically with [2-$^{3}$H]mannose and [1-$^{14}$C]glucosamine was digested stepwise with α-D-neuraminidase, α-L-fucosidase, β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase. The unfractionated hydrolysate was then treated with Jack Bean α-D-mannosidase (Dr. Y.-T. Li, New Orleans). The incubation liberated 70 % of the mannose label as free monosaccharide (Fig. 6a). 25 % of the mannose radioactivity was found in a glycopeptide peak of 1200 daltons together with glucosamine radioactivity. Additionally, 6 % of the mannose label was found in fractions 35–44, which represents unknown material. Doubling of the α-D-mannosidase concentration did not cleave

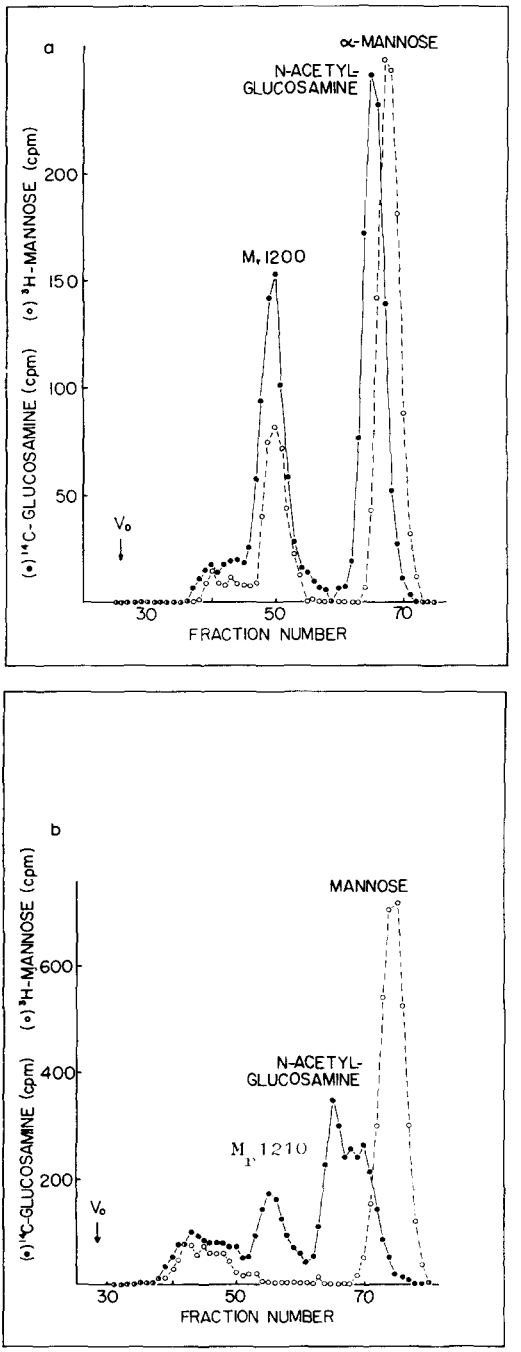



Fig. 6. Bio-Gel P-6 elution profiles of type A glycopeptides digested stepwise with α-D-neuraminidase, α-L-fucosidase, β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase and then with mannosidase. The preparation was labelled with (●) D-[1-$^{14}$C]glucosamine and (○) D-[2-$^{3}$H]mannose. (a) Digestion with α-D-mannosidase (Dr. Y.-T. Li, New Orleans). On the left side of the 1200 dalton structure same undigested unidentified material seems to be present. (b) Digestion with β-D-mannosidase after the α-D-mannosidase treatment. The unidentified material on the left of the 1210 dalton structure has retained its place on the elution profile in this digestion. The free mannose peak represents the β-mannose liberated in this experiment as well as the α-mannose liberated in the previous degradative step (a). The free *N*-acetyl-β-D-glucosamine peak has eluted anomalously in this particular run for unknown reasons. It represents units liberated in the predigestion.

additional radioactivity, 67 % was released. The glycopeptide peak had an apparent molecular weight of 1300 after this treatment.

*β-Mannosidase digestion.* The [2-$^3$H]mannose-labelled glycopeptides, which had been pretreated with α-D-neuraminidase, α-L-fucosidase, β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase and pure α-D-mannosidase ($M_r$ 1300), lost completely their mannose label during treatment with pure β-D-mannosidase (Fig. 6b).

The mannose-free glycopeptide, which was still labelled with [$^{14}$C]glucosamine had an apparent molecular weight of 1210 (Fig. 6b). The reduction in the apparent molecular weight, 90 daltons, is equivalent with 0.5 mol of β-D-mannose (Table III). The number of α-D-mannose units as judged from the ratio of α- and β-mannose label would thus be 1.5 mol (Table III). However, in gel filtration the drop in the apparent molecular weight caused by α-D-mannosidase was only 0.7 mol.

*Digestion of the proximal units of N-acetylglucosamine.* The glycopeptides labelled with [$^{14}$C]glucosamine were treated successively with α-D-neuraminidase, α-L-fucosidase, β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase and with a preparation of α-D-mannosidase (Boehringer) contaminated with β-D-mannosidase. The resulting glycopeptides ($M_r$ 1210) were finally incubated a second time with 3.5 units/ml of *N*-acetyl-β-D-glucosaminidase. The treatment liberated 37 % of the

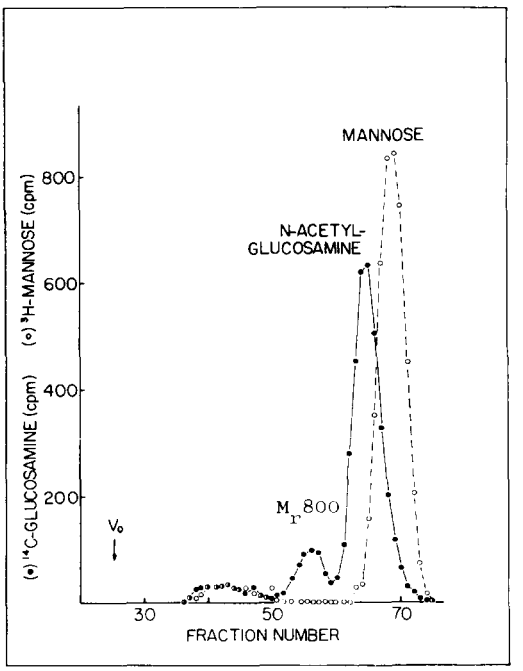
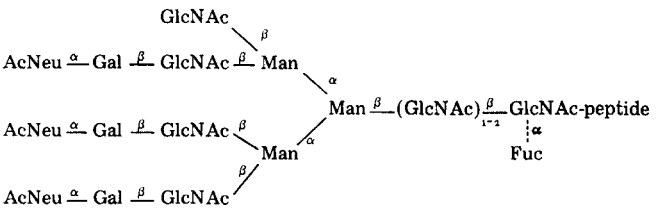



Fig. 7. Bio-Gel P-6 elution profile of type A glycopeptides digested stepwise with α-D-neuraminidase, α-L-fucosidase, β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D- and β-D-mannosidase and then once more with *N*-acetyl-β-D-glucosaminidase. The preparation was originally labelled with (●) D-[1-$^{14}$C]glucosamine and (○) D-[2-$^3$H]mannose. The free *N*-acetyl-β-D-glucosamine peak comprises of the distal residues liberated previously and additionally of about half of the proximal residues. The other half of the proximal residues have remained attached to a 800 dalton structure. The unidentified material in fractions 35–50 has retained its size even in this incubation.

[$^{14}$C]glucosamine label. The decrease of the apparent molecular weight of the glycopeptides was 410 daltons, from 1210 to 800 (Fig. 7). This is equivalent to 2.0 mol of liberated *N*-acetyl-β-D-glucosamine units (Table III). An other experiment gave a glycopeptide of 640 daltons after treatment with 1.9 units/ml of *N*-acetyl-β-D-glucosaminidase. The [$^{14}$C]glucosamine label resisting enzymatic liberation from the peptide represented 12.3 % (experiment 1) and 14.7 % (experiment 2) of the total glucosamine label in type A glycopeptides. The non-releasable *N*-acetylglucosamine units are believed to be bound directly to the peptide.

## DISCUSSION

In the present paper the sequence and the anomeric configuration of the monosaccharides in viral glycoproteins are described for the first time. The sequence appears to follow patterns familiar in the N-glycosidic type A oligosaccharides of soluble serum glycoproteins studied this far [16, 17]. Our data are compatible with the following average structure of the type A glycopeptides:




Our evidence speaking for this structure consists of sequential release of monosaccharides by highly purified exo-glycosidases from glycopeptides labelled specifically with various radioactive monosaccharides in vivo. The released radioactive monosaccharides were not identified by paper chromatography or gas-liquid chromatography because of their too small amounts. The number of released units were estimated by monitoring the decrease of the apparent molecular weight of the glycopeptides by gel filtration after each step of enzyme digestion.

The number of sialic acid, galactose and *N*-acetylglucosamine units found in the oligosaccharides are similar as the average number of these monosaccharides present in the proteins $E_1$ and $E_3$ (Table II). This suggests that approximately one oligosaccharide of type A is present in each molecule of $E_1$ and $E_3$.

The number of mannose units in the oligosaccharides remains uncertain. The chemical analysis has suggested the proteins to contain 4–5 mannose residues (Table II), but the present gel filtration experiments reveal only 1.2–2.0 mol of total mannose. This discrepancy may be related to branching of the carbohydrate chain in the oligomannose structure. The mean value of the two methods of analysis, 3 mol of mannose, is used in the proposed structure of the glycopeptides.

The average number of distal *N*-acetylglucosamine units is 4.2 mol as obtained by gel filtration. Almost the same figure is obtained from the ratio of the label in the distal and the most proximal (non-releasable) units of *N*-acetylglucosamine, which was 4.3. The present experiments provide three observations relating to the number of *N*-acetylglucosamine units in the core structure of the glycopeptide. The

TABLE II

MONOSACCHARIDE CONTENT OF $E_1$ AND $E_3$ PROTEINS AND OF THE TYPE A GLYCOPEPTIDES

| | Monosaccharides (mol/mol) | | | | | Apparent $M_r$ of the oligosaccharide |
|---|---|---|---|---|---|---|
| | Sialic acid | Galactose | Fucose | Mannose | *N*-Acetyl glucosamine | |
| Proteins $E_1$ and $E_3$[a] | 2.5 | 3.5 | 1.5 | 4.5 | 8.0 | 3800[c] |
| Average glycopeptides | 3.4 | 3.1 | 0.7 | 2.0(1.2)[b] | 6–7 | 3300[d] |

[a] Average data of $E_1$ and $E_3$ [5].

[b] The small value is based on the decrease of the apparent molecular weight of the glycopeptide; the large value on the distribution of α-mannose and β-mannose label.

[c] Calculated from the monosaccharide composition.

[d] Calculated as the difference in molecular weight between the original mixture of pronase glycopeptides and the sugar-free peptides.

figures are in the range of 1.6–3.2 mol. The largest value is obtained from the ratio of glucosamine label in the distal and the non-distal units. This figure is probably too high as it includes also the label in the unidentified material (see for instance Fig. 7). The smallest value, 1.6 mol, is obtained from the release of 37% of glucosamine label during the exhaustive digestion of the demannosylated core structure with *N*-acetylglucosaminidase. The released radioactivity represents 0.6 mol of *N*-acetylglucosamine if it is assumed that 1 mol remains peptide bound. The third value, 3.0 mol, is obtained from the decrease of the molecular weight by 2.0 mol during digestion of the demannosylated core structure with *N*-acetylglucosaminidase.

The fucose units are not attached to galactose. Possible binding sites are distal *N*-acetylglucosamine [18], or, perhaps more likely, one of the core *N*-acetylglucosamine units [19]. In Vesicular Stomatitis virus grown in BHK-cells fucose is entirely linked to the *N*-acetylglucosamine unit involved in the glycopeptide bond [15]. Also in type A oligosaccharides found on BHK-cell surface fucose is suggested to be attached to the peptide-bound *N*-acetylglucosamine unit [20].

Our previous analysis of the isolated $E_2$ protein suggested the presence of a galactose-containing oligosaccharide only marginally labelled in vivo from [$^{14}$C]-glucosamine, [$^{14}$C]mannose and [$^{3}$H]fucose. After pronase digestion the glycopeptide had an apparent molecular weight of 3100 [8]. The presence of a structure, unrelated to type A glycopeptides and containing only marginally glucosamine and mannose label, would not interfere with the results and conclusions as regards the sequence and the molar amounts of different monosaccharides in $E_1$A and $E_3$A. All degradative steps were carried out with samples labelled in the *N*-acetylglucosamine residues, and all but the last step also with mannose-labelled preparations.

It is obvious that the viral genome is too small to carry the genes for all sugar transferases needed for the oligosaccharide synthesis [21]. It has, however, seemed possible that the virus could specify one or two enzymes, which, together with cellular enzymes, would carry out the synthesis of a unique viral oligosaccharide.

TABLE III

EFFECT OF EXO-GLYCOSIDASES ON TYPE A GLYCOPEPTIDES

The number of residues is estimated from the decrease of the molecular weight of the glycopeptide.

| | Residues released (mol/mol) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sialic acid | Fucose | Galactose | *N*-Acetyl glucosamine (distal) | α-Mannose | β-Mannose | *N*-Acetyl glucosamine (proximal) |
| 1. α-D-Neuraminidase | 3.4 | | | | | | |
| 2. α-D-Neuraminidase then α-L-fucosidase | 3.4 | 0.7 | | | | | |
| 3. α-L-Fucosidase | | 0.7 | | | | | |
| 4. α-D-Neuraminidase, α-L-fusosidase then β-D-galactosidase | 3.4 | 0.7 | 3.1 | | | | |
| 5. α-D-Neuraminidase then β-D-galactosidase | 3.4 | | 3.1 | | | | |
| 6. α-D-Neuraminidase, α-L-fucosidase, β-D-galactosidase then *N*-acetyl-β-D-glucosaminidase | 3.4 | 0.7 | 3.1 | 4.2 | | | |
| 7. α-D-Neuraminidase, α-L-fucosidase, β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase then α-D-mannosidase | 3.4 | 0.7 | 3.1 | 4.2 | 1.5[a] | | |
| 8. α-D-Neuraminidase, α-L-fucosidase, β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase α-D-mannosidase then β-D-mannosidase | 3.4 | 0.7 | 3.1 | 4.2 | 1.5 | 0.5 | |
| 9. α-D-Neuraminidase, α-L-fucosidase, β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D-mannosidase, β-D-mannosidase then *N*-acetyl-β-D-glucosaminidase | 3.4 | 0.7 | 3.1 | 4.2 | 1.5 | 0.5 | 2.0(0.6)[b] |

[a] This value is based on the ratio of α-mannose and β-mannose units.

[b] The smaller value is based on the ratio of released and non-released *N*-acetylglucosamine residues.

This obviously is not the case in type A oligosaccharides of SF virus; the viral proteins and the surface proteins of the host cell [20] appear to carry oligosaccharides of similar structure. This seems to justify the use of SF virus as a probe for the glycosylation of membrane proteins within the host cell.

The hemagglutinating activity of SF virus resides in the membrane glycoprotein $E_1$ [22]. Partially [23] or completely [24] deglycosylated viruses lack this property. It should be possible to find out the exact degree of glycosylation needed for hemaglutinating activity with the arsenal of exo-glycosidases used in the present work.

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