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THE CARBOHYDRATE UNITS OF ASIALO-OVOMUCOID: STRUCTURAL FEATURES

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ABSTRACT

The structural features of a heterogeneous glycopeptide fraction from asialo-ovomucoid have been investigated by methylation analysis of the fraction and of products obtained at each stage of its sequential degradation with exo-glycosidases. All glycopeptides in the fraction had a common core-structure β-D-Glc*p*NAc-(1→4)-[β-D-Glc*p*NAc-(1→2)]-α-D-Man*p*-(1→3)-[β-D-Glc*p*NAc-(1→4)]-[β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc→Asn. Heterogeneity in the fraction arose from variation in the amount of terminal galactose attached *via* a hexosaminyl residue to the α-D-Man*p*-(1→3) residue, and from limited variation in the number of terminal hexosaminyl groups attached to the α-D-Man*p*-(1→6) residue. One glycopeptide in the fraction contained the unusual feature of two different, triply-substituted mannosyl residues. Other structural features of the glycopeptide are discussed.

INTRODUCTION

Comparatively few investigations have been made of the structures of the carbohydrate units of ovomucoid, principally because of the difficulty of obtaining homogeneous glycopeptides. A contributory factor to this heterogeneity may be the existence in ovomucoid of several sites of glycosylation, but evidence from our work and that of others suggests that there is heterogeneity at all sites[1,2].

The most comprehensive investigation of the structure of the carbohydrate units has been that of Montreuil and his co-workers who examined the structures of oligosaccharide fragments obtained from asialo-ovomucoid glycopeptides by partial hydrolysis with acid[3] and by controlled acetolysis[4]. From their structures and from methylation analysis of the intact glycopeptide[5], they postulated an almost complete structure for the carbohydrate unit[6]. However, the proposed structure did not account for all of the carbohydrate units found in their original ovomucoid variants, and left unanswered the question of whether the other carbohydrate units present in their glycoprotein contained incomplete versions of the postulated structure or entirely different structures.

We have described[2] the preparation and purification of an asialo-ovomucoid

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and of glycopeptides therefrom. These glycopeptides, like those obtained by other workers, differed from Montreuil's glycopeptide in their carbohydrate analysis and by the fact that they were heterogeneous. Although it has not proved possible to eliminate this heterogeneity, we have determined many of the structural features present in our glycopeptide preparation. By correlation of the results obtained from methylation analysis of the various glycopeptides formed by enzymic degradation with analytical results from the glycopeptides, it has been possible to determine at which residues in the original glycopeptides heterogeneity occurred. We have also established the existence of several structural features, including the unusual feature of two different, triply-substituted mannosyl residues occurring in a single glycopeptide.

RESULTS AND DISCUSSION

Initial consideration of methylation-analysis results obtained after employing the usual acetolysis–hydrolysis procedure[7] for the release of methylated sugars showed that incompatible results were obtained for certain glycopeptides. For example, it was not possible to explain why the action of 2-acetamido-2-deoxy-β-D-glucoside 2-acetamido-2-deoxy-β-D-glucohydrolase (β-*N*-acetylglucosaminidase) on glycopeptides that apparently contained equal amounts of 2-*O*-methylmannose and 2,3,4,6-tetra-*O*-methylgalactose resulted in a glycopeptide having almost twice as much 2,4-di-*O*-methylmannose (originating from 2-*O*-methylmannose) as 2,3,4,6-tetra-*O*-methylgalactose. Results from several experiments finally revealed that acetolysis–hydrolysis did not give quantitative yields of 2-*O*-methylmannose from glycopeptides containing such a triply-substituted mannosyl residue[8]. Quantitative recoveries were obtained when a formolysis–hydrolysis procedure[9] was employed, and this method has been used for all the analyses described. This procedure causes some loss of methylated hexosamine derivatives[9], necessitating the application of a correction factor. Since, with the columns used (glass-capillary columns wall-coated with OV-1), there was some variability in molar response for hexosamine derivatives, as also observed by other workers[10,11], calculation of ratios for hexosamine derivatives was generally based on the total number of residues in the glycopeptides as determined by analysis.

Results from fractionation studies had indicated that, whatever type of fractionation was employed, each fraction consisted of a mixture of at least two glycopeptides each containing three mannose residues, with approximately half of the glycopeptides containing no galactose[2]. All ratios for the compositional analysis of the glycopeptides were therefore calculated on the basis of six mannose residues. Calculations on this basis resulted in whole-number ratios for the various methylated derivatives, and the ratios of terminal to branch-point residues supported the assumption of more than one glycopeptide. For degraded fractions from which mannose had been removed, the ratios were calculated on the basis of their galactose content or the number of mannose residues remaining in the glycopeptide.

Methylation analysis of the glycopeptides. The inidividual glycopeptide fractions A, B, and C, previously isolated by ion-exchange chromatography, which showed little difference in their compositional analysis except for their galactose content[2], showed similar patterns of distribution of their methylated sugars (Table I). Differences in the galactose ratios were smaller in the methylation analysis. In Glycopeptide A, which accounted for only 8–10% of the total glycopeptides and which had previously been found to contain the greatest amount of galactose, there was, in addition to 2,3,4,6-tetra-*O*-methylgalactose, ~0.4 unit of 2,4,6-tri-*O*-methylgalactose that may have arisen from traces of sialic acid present in the original glycoprotein being linked to O-3 of some of the galactose residues in this fraction. The proportion of certain other derivatives varied between fractions, but there was no evidence of the association of a particular structural feature with an individual fraction.

Analysis of the two glycopeptides (D and E) obtained from fragments isolated after cleavage of asialo-ovomucoid with cyanogen bromide[2] showed no structural differences (Table I), and the composition and ratios of methylated sugars were essentially similar to those obtained from a sample of unfractionated glycopeptides from the intact glycoprotein (Table III). Enzymic degradations to provide modified glycopeptides for methylation analysis were therefore carried out on a purified glycopeptide preparation that had not been subjected to further fractionation (Glycopeptide 2). Table II gives the compositional analyses of glycopeptides isolated after incubation with exo-glycosidases singly or in sequence as shown in Fig. 1, and the corresponding methylation analyses are given in Table III.

TABLE I

METHYLATION ANALYSES OF GLYCOPEPTIDE FRACTIONS

Methylated derivative[a]	*Molar ratios*[b]				
	A[c]	*B*	*C*	*D*	*E*
2,3,4,6-Gal	1.3	1.1	0.9	0.8	0.8
3,4,6-Man	0.9	0.6	0.6	1.1	1.0
2,4,6-Gal	0.4	—	—	—	—
3,6-Man	2.0	1.9	1.9	1.8	1.7
2-Man	2.0	2.0	2.0	2.0	2.0
3-Man	1.0	1.4	1.3	0.9	0.8
3,4,6-GlcNAcMe	8.4	9.1	9.4	9.1	9.1
3,6-GlcNAcMe	4.9	4.6	4.3	4.1	3.9

[a]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, *etc.*; 3,4,6-GlcNAcMe = 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucitol, *etc.* [b]Molar ratios are calculated with respect to 2 mol of 2-Man. Ratios for hexosamines are based on the total number of hexosamine residues as determined by the hexosamine content. [c]A, B, and C were fractions from a Dowex 50 column; D and E were from separate fragments of ovomucoid obtained by cleavage with cyanogen bromide[2].

TABLE II

ENZYMIC DEGRADATION OF GLYCOPEPTIDE 2

	Glycopeptide number	*Residual glycopeptide*[a]		
		Man	*Gal*	*GlcNAc*
Control	2	6.0	1.0	14.4
β-D-Galactosidase	2a	6.0	-	14.4
(*1*) β-*N*-Acetylglucosaminidase	2b	6.0	1.0	5.4
(*2*) β-D-Galactosidase	2c	6.0	-	5.4
(*1*) β-*N*-Acetylglucosaminidase	2b	6.0	1.0	5.4
(*2*) α-D-Mannosidase	2d	3.1	1.0	5.4
(*3*) β-D-Galactosidase	2e	3.1	-	5.4
(*4*) β-*N*-Acetylglucosaminidase	2f	3.1		3.7
(*5*) α-D-Mannosidase	2g	2.0	-	3.8

[a]Molar ratios are calculated from analysis of the residual glycopeptide, based on a total of six mannose residues in the control glycopeptide.

Analysis of Glycopeptide 2 indicated that two mannosyl residues substituted at O-3, O-4, and O-6 were present for every terminal galactosyl group. Since such a triply substituted mannosyl residue is commonly found attached to the internally located di-*N*-acetylchitobiose residue of asparaginyl-linked oligosaccharides in glycoproteins, this ratio indicated that, in the preparation, there were at least two glycopeptides, only one of which contained galactose. However, the presence of

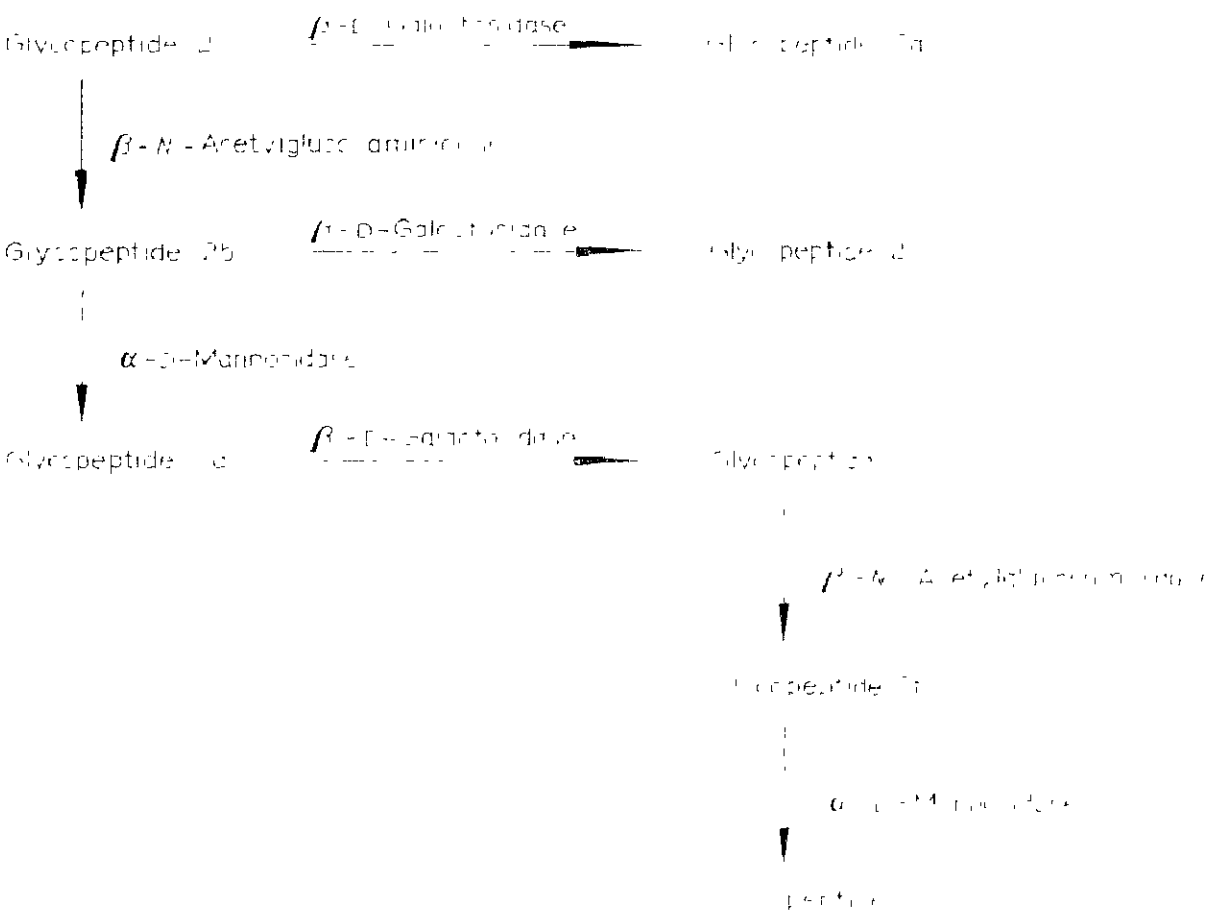


Fig. 1. Enzymic degradation of Glycopeptide 2.

TABLE III

METHYLATION ANALYSES OF ENZYMIC DEGRADATION PRODUCTS FROM GLYCOPEPTIDE 2

Methylated derivative[a]	*Molar ratios*[b]						
	2[c]	*2a*	*2b*	*2c*	*2d*	*2e*	*2f*
2,3,4,6-Gal	1.0	—	1.0*	—	1.0*	—	—
2,3,4,6-Man	—	—	3.0	3.0*	1.2	1.2*	1.9
3,4,6-Man	1.0	1.0	—	—	—	—	—
2,3,6-Man	—	—	0.7	0.7	0.8	0.7	—
2,4,6-Man	—	—	—	—	0.5	0.5	0.5
3,6-Man	2.0	2.0	0.2	0.2	0.2	0.1	—
2,4-Man	—	—	1.7	1.7	—	—	0.3
2-Man	2.0*	2.0*	0.3	0.3	0.3	0.3	—
3-Man	1.0	1.0	—	—	—	—	—
3,4,6-GlcNAcMe	9.9	10.9	0.6	1.9	0.6	1.8	—
3,6-GlcNAcMe	4.5	3.5	4.8	3.5	4.8	3.6	3.7*

[a]For key, see Table I. [b]Ratios for mannose are based on the number of residues with respect to the derivative marked *. Ratios for hexosamine are based on the total number of residues as determined by the total hexosamine content. [c]For key to numbering of fractions, see Fig. 1.

units of 2,4,6- and 2-substituted mannosyl residues in amounts equal to that of the galactose derivative suggested that the glycopeptides differed in other structural aspects. The 2,4,6-triply-substituted mannosyl residue was an unusual feature. Its identity as a 3-*O*-methylmannose derivative was confirmed by mass spectrometry after reduction with sodium borodeuteride[9]. Only terminal and 4-substituted hexosaminyl residues were present, the majority being terminally located.

Comparison of the methylation analysis results for Glycopeptide 2 with those obtained after the action of β-D-galactosidase (Glycopeptide 2a) showed that the only differences resulting from removal of galactose were an increase of one residue of terminal hexosamine and a corresponding decrease in the amount of 4-substituted hexosaminyl residues. This indicated that, in Glycopeptide 2, galactose had been terminally linked to O-4 of a hexosaminyl residue, *i.e.*, β-D-Gal*p*-(1→4)-GlcNAc-.

Action of β-*N*-acetylglucosaminidase on Glycopeptide 2 removed nine hexosamine residues to give Glycopeptide 2b. Since there was no decrease in the number of 4-substituted hexosaminyl residues, Glycopeptide 2 had no such units adjacent to terminal hexosaminyl groups. The 1.7 residues of 3,6-substituted mannose which appeared in the analysis could only have arisen from the two 3,4,6-substituted units in Glycopeptide 2, and this confirmed that each of these branched mannosyl residues had a terminal hexosaminyl group attached at O-4. Even after exhaustive digestion of Glycopeptide 2 with β-*N*-acetylglucosaminidase, 0.3 residue of 3,4,6-substituted mannose remained, suggesting that, to a limited extent, surrounding groups were hindering the action of the enzyme. Subsequent treatment with β-*N*-acetylglucosaminidase of samples that had been treated with α-D-mannosidase and β-D-galactosidase (Glycopeptide 2e) resulted in the disappearance of the remaining 3,4,6-substi-

tuted mannose and the production of a corresponding amount of 3,6-substituted mannose (Glycopeptide 2f).

Of the other four mannose residues in Glycopeptide 2b, three were in terminal positions after removal of hexosamine. One of the terminal mannosyl groups had originated from the unit corresponding to the 3,4,6-tri-*O*-methylmannose derivative found in Glycopeptide 2, indicating that this mannosyl residue originally had a single hexosaminyl group attached at O-2, *i.e.*, β-D-Glc*p*NAc-(1→2)-Man-. A second terminal mannosyl group in Glycopeptide 2b could be accounted for by the removal of two hexosaminyl residues from one of the 2,4-substituted mannosyl residues in Glycopeptide 2, which therefore contained the structure β-D-Glc*p*NAc-(1→2)-[β-D-Glc*p*NAc-(1→4)]-Man-. The remaining terminal mannose must have been produced by the removal of hexosamine from either the 2,4,6- or the second 2,4-substituted mannosyl residue in the original glycopeptide, since both of these derivatives had almost disappeared in the analysis of Glycopeptide 2b. The newly formed, 4-substituted mannosyl residue must also have originated from one or other of these substituted mannosyl residues and must have had the β-D-Gal*p*-(1→4)-GlcNAc-structure attached to it. The original glycopeptide therefore contained the structural feature β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-Man-, with the proviso that the mannosyl residue was also substituted at O-6 or at O-2 and O-6.

Treatment of Glycopeptides 2b and 2d with β-D-galactosidase gave Glycopeptides 2e and 2e, respectively (Fig. 1). As in the case of Glycopeptide 2a, the only changes in composition observed in each glycopeptide were an increase of terminal hexosamine and a decrease of 4-substituted hexosamine, confirming the existence of the structure β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-Man-.

The action of α-D-mannosidase on Glycopeptide 2b gave Glycopeptide 2d. Only three mannose residues remained, and of these one was terminally located. This residue could only have arisen as a result of the removal of two terminal mannosyl groups from one of the 3,6-substituted mannosyl residues in Glycopeptide 2b, since the 2 mol of 2,4-di-*O*-methylmannose corresponding to the latter residues were absent in the analysis of Glycopeptide 2d. This indicated that a galactose-free glycopeptide in the original fraction had been degraded to give the trisaccharide core common to almost all asparaginyl–carbohydrate units, consisting of a terminal mannosyl group β-linked to a di-*N*-acetylchitobiose residue. The existence of such a core in ovomucoid glycopeptides had been shown previously[2]. The 3-substituted mannosyl residue must have appeared as a result of the removal of the third terminal mannosyl group in Glycopeptide 2b from O-6 of the mannose core of the galactose-containing glycopeptide. This core still had attached to it a 4-substituted mannosyl residue protected from α-D-mannosidase action by the Gal-GlcNAc group. The two principal glycopeptides comprising Glycopeptide 2d therefore had the structures *A* and *B*.

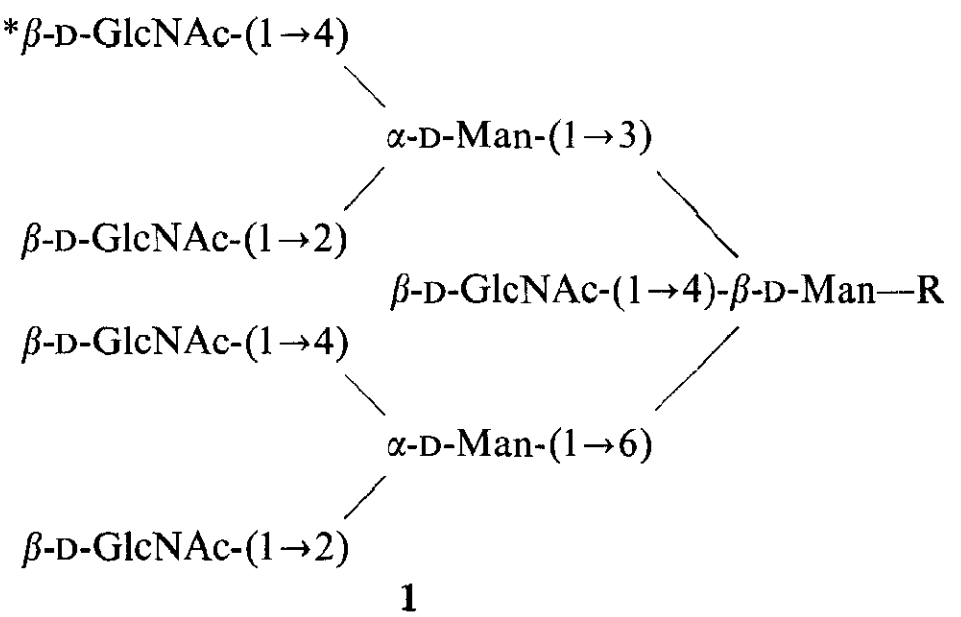
β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc → Asn (*A*)

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc →Asn (*B*)

The fact that it had not been possible at an earlier stage to remove completely the terminal hexosamine from the β-linked mannosyl residues meant that the glycopeptide mixture still contained a small proportion of glycopeptide having terminal hexosamine attached to this residue. This hexosaminyl group increased the resistance of the glycopeptide to attack by α-D-mannosidase. As well as a glycopeptide having Structure *B*, there was therefore also present a small proportion of a related glycopeptide which still contained terminal hexosaminyl and mannosyl groups. This explains the presence in the analysis of 3,4,6-substituted mannose and terminal hexosamine as well as the low ratio for the 3-substituted mannosyl residue present in the glycopeptide having Structure *B*.

Treatment of Glycopeptide 2e with β-*N*-acetylglucosaminidase gave Glycopeptide 2f. Previous removal of galactose to give Glycopeptide 2e allowed the removal of an additional hexosamine residue that had been protected by galactose. The small amount of 3,4,6-substituted mannose that had remained after treatment of Glycopeptide 2 with β-*N*-acetylglucosaminidase was now converted into the 3,6-substituted derivative, as had already occurred with the bulk of the residue (Glycopeptide 2→ Glycopeptide 2b). The only other derivatives obtained from Glycopeptide 2f were terminal and 3-substituted mannose in the ratio 1.9:0.5, together with 4-substituted hexosamine. The conversion of the 4-substituted mannosyl residue found in Glycopeptide 2e into a terminal residue after the action of β-*N*-acetylglucosaminidase, together with the continued presence in the glycopeptide of a 3-substituted mannosyl residue, confirmed that the terminal hexosaminyl group removed, which had previously been substituted by galactose, had been attached to a mannosyl residue which in turn had been attached to the core mannose by an α-(1→3) linkage. Since the 3- and 3,6-substituted mannose are derived from the same residue, this explains the apparent low recovery of 3-substituted mannose. Further treatment of Glycopeptide 2f with α-D-mannosidase resulted in a product containing only terminal mannose and 4-substituted hexosaminyl residues in the ratio 1:2, representing the core of the glycopeptide.

From consideration of the methylation-analysis data for all the glycopeptides (Table III), the structures **1–4** can be postulated for Glycopeptide 2.



1

β-D-GlcNAc-(1→2)-α-D-Man-(1→3,6)

β-D-GlcNAc-(1→4)-β-D-Man--R

*β-D-GlcNAc-(1→4)

β-D-GlcNAc-(1→2) —α-D-Man-(1→6,3)

β-D-GlcNAc-(1→6)

2

*β-D-GlcNAc-(1→4)

α-D-Man-(1→3,6)

β-D-GlcNAc-(1→2)

β-D-GlcNAc-(1→4)-β-D-Man —R

*β-D-GlcNAc-(1→4)

β-D-GlcNAc-(1→2) —α-D-Man-(1→6,3)

β-D-GlcNAc-(1→6)

3

*β-D-GlcNAc-(1→4)

α-D-Man-(1→3,6)

β-D-GlcNAc-(1→2)

β-D-GlcNAc-(1→4)-β-D-Man—R

β-D-GlcNAc-(1→2)-α-D-Man-(1→6,3)

4

R = -(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc→Asn

Results described so far indicated that Glycopeptide 2 was a mixture of either Structures **1** and **2** or **3** and **4**. Terminal galactose was β-linked to a hexosaminyl residue at one of the starred positions in one of the pair of structures, with the added proviso that the mannosyl residue to which this hexosaminyl residue was attached was in turn α-(1→3)-linked to the core mannose.

Methylation analysis of fractionated glycopeptides A, B, and C (Table I) indicated there was variation in the molar ratios of 2-substituted and 2,4,6-substituted mannose residues in the various fractions, but the sum of the molar ratios was

constant and equal to the number of mol of 3,4,6-substituted mannose. In fractions having a higher proportion of 2,4,6-substituted mannose, there was also an increase in the amount of terminal hexosamine. These facts suggested that, in the glycopeptide, 2,4,6- and 2-substituted mannose originated from the same mannosyl residue in two glycopeptides, their relative amounts depending on the degree of substitution of the residue with terminal hexosaminyl groups, and that the mannosyl residue was directly attached to the 3,4,6-substituted mannosyl residue. Taking these data into consideration, Structures **3** and **4** seemed the more probable, as variation in the relative amounts of glycopeptides having Structures **1** and **2** would have resulted in parallel variation in the amount of 2,4,6- and 2-substituted mannose, with inverse variation of the amount of 3,6-substituted mannose. This was not found in the analysis of Glycopeptides A, B, and C (Table I).

Further fractionation of the glycopeptides. Further attempts to achieve fractionation of Glycopeptide 2 were made by applying h.p.l.c. procedures to the methylated product (Fig. 2). Several of the fractions obtained were isolated, and examined by methylation analysis (Table IV). Although all of the fractions still showed evidence of heterogeneity, some additional facts regarding the structure of the glycopeptides emerged. The main effect of the procedure was a separation based on relative amounts of the structures that gave rise to 3-*O*-methylmannose and 3,4,6-tri-*O*-methylmannose (*i.e.*, 2,4,6- and 2-substituted mannose residues), since, in all fractions, the ratios of these derivatives varied inversely. The ratio of galactose was relatively constant in all fractions, which tended to rule out the possibility of galactose being linked indirectly to the 2,4,6-substituted mannosyl residue as in Structures **2** and **3**. Furthermore, Fraction A (Fig. 2), which contained the usual proportion of galactose, contained no 2,4,6-substituted mannose (Table IV). Taking previous results into consideration, the galactose residue must have been attached, *via* a hexosaminyl residue, to a doubly substituted mannosyl residue. As in the case of fractions obtained by

TABLE IV

METHYLATION ANALYSIS OF H.P.L.C. FRACTIONS[a] FROM GLYCOPEPTIDE 2

Methylated derivative[b]	*2*[c]	*Fractions*		
		A	*B*	*C*
2,3,4,6-Gal	1.0	0.8	0.8	1.0
3,4,6-Man	1.0	1.8	1.4	0.6
3,6-Man	2.0	2.0	1.8	1.8
2-Man	2.0	2.0	2.0	2.0
3-Man	1.0	—	0.4	1.4
3,4,6-GlcNAcMe	9.9	10.3	8.1	10.6
3,6-GlcNAcMe	4.5	4.0	4.0	4.6

[a]For details of fractions, see Fig. 2. [b]See Table I for key. [c]Values are expressed as molar ratios calculated with respect to 2 mol of 2-Man.

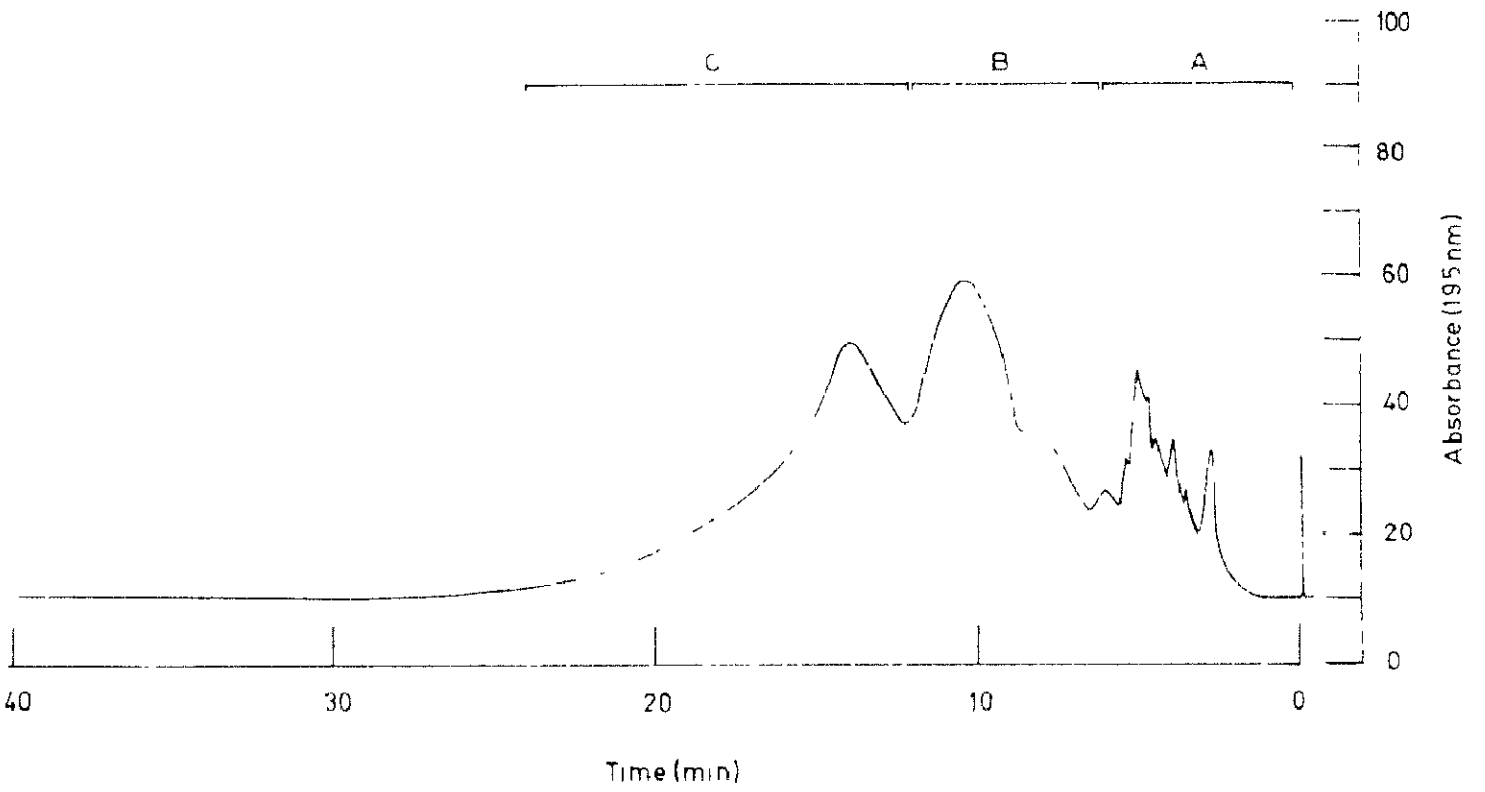


Fig. 2. Fractionation by h.p.l.c. of methylated Glycopeptide 2 on ODS-Hypersil For details, see Experimental. Fractions were combined as shown and subjected to methylation analysis (Table IV).

other procedures (Table I), the sum of the molar ratios of the 2,4,6- and the 2-substituted mannosyl derivatives was constant in each fraction, and the greater amount of terminal hexosamine in fractions containing more of the 2,4,6-substituted mannose reflected the greater degree of substitution of this residue.

Fractionation of Glycopeptide 2d was achieved on Bio-Gel P-6 (Fig. 3). Most of the fractions still showed evidence of heterogeneity on analysis (Table V), but the fraction (VI) smallest in size contained only terminal mannosyl groups and disubstituted hexosaminyl residues in the ratio 1 : 2. This confirmed that some of the original

TABLE V

METHYLATION ANALYSES OF GLYCOPEPTIDE 2d FRACTIONS[a] (BIO-GEL P-6 COLUMN)

Methylated derivative[b]	*Molar ratios*[c]						
	2d	*I*	*II*	*III*	*IV*	*V*	*VI*
2,3,4,6-Gal	1.0	1.0	1.0	1.0	1.0	1.0	—
2,3,4,6-Man	1.2	0.8	0.6	0.9	2.5	0.7	1.0
2,3,6-Man	0.8	1.3	0.9	0.6	0.6	1.1	—
2,4,6-Man	0.5	0.6	0.4	0.2	0.2	0.7	—
3,6-Man	0.2	0.2	0.2	0.2	0.2	0.1	—
2-Man	0.3	0.5	0.4	0.5	0.7	0.2	—
3,4,6-GlcNAcMe	0.6	1.0	0.9	1.0	1.3	0.3	—
3,6-GlcNAcMe	4.8	4.6	3.3	3.9	6.9	4.0	2.0

[a]For details of fractions, see Fig. 3. [b]For key, see Table I. [c]Molar ratios are calculated with respect to 1 mol of 2,3,4,6-Gal, except for Fraction VI which is calculated with respect to 1 mol of 2,3,4,6-Man.

TABLE VI

METHYLATION ANALYSES OF H.P.L.C. FRACTIONS[a] FROM GLYCOPEPTIDE 2d

Methylated derivative[b]	*Molar ratios*[c]							
	2d	*A*	*C*	*D*	*E*	*F*	*G*	*H*
2,3,4,6-Gal	1.0	1.0	1.0	1.0	1.0	0.1	—	0.1
2,3,4,6-Man	1.2	0.6	0.7	1.4	4.7	1.0	1.0	1.0
2,3,6-Man	0.8	1.1	1.0	1.1	1.3	0.2	—	0.1
2,4,6-Man	0.5	0.8	0.6	0.7	0.6	0.1	—	—
3,6-Man	0.2	—	—	—	—	—	—	—
2-Man	0.3	0.3	0.5	0.3	0.2	—	—	—
3,4,6-GlcNAcMe	0.6	—	—	0.1	0.7	—	—	—
3,6-GlcNAcMe	4.8	4.6	4.3	5.9	8.4	2.1	1.6	1.9

[a]For details of fractions, see Fig. 4. [b]For key, see Table I. [c]Molar ratios are calculated with respect to 1 mol of 2,3,4,6-Gal, except for those in Fractions F, G, and H which are calculated with respect to 1 mol of 2,3,4,6-Man.

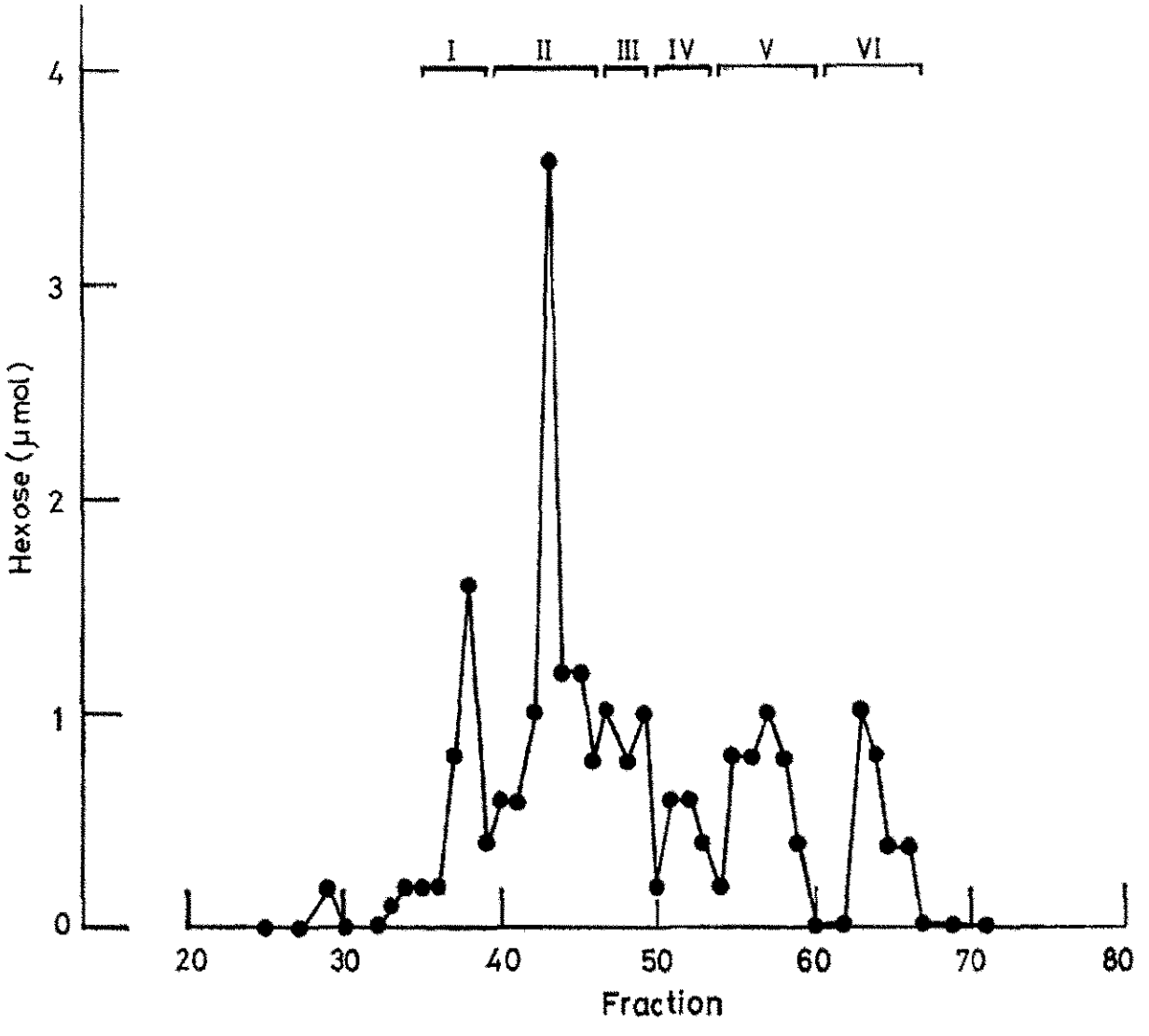


Fig. 3. Fractionation of Glycopeptide 2d on Bio-Gel P-6. For details, see Experimental. Fractions (3 mL) were combined as shown and subjected to methylation analysis (Table V).

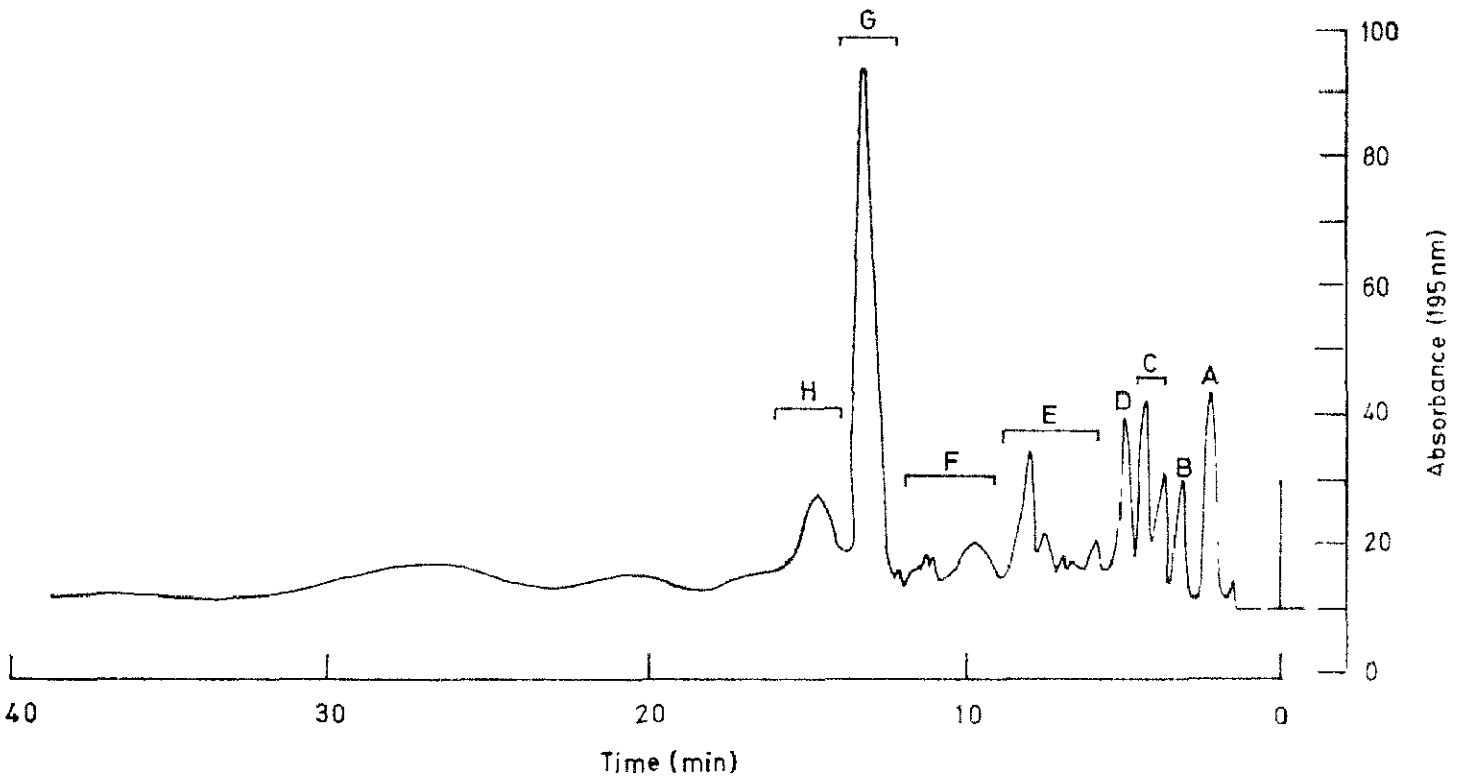
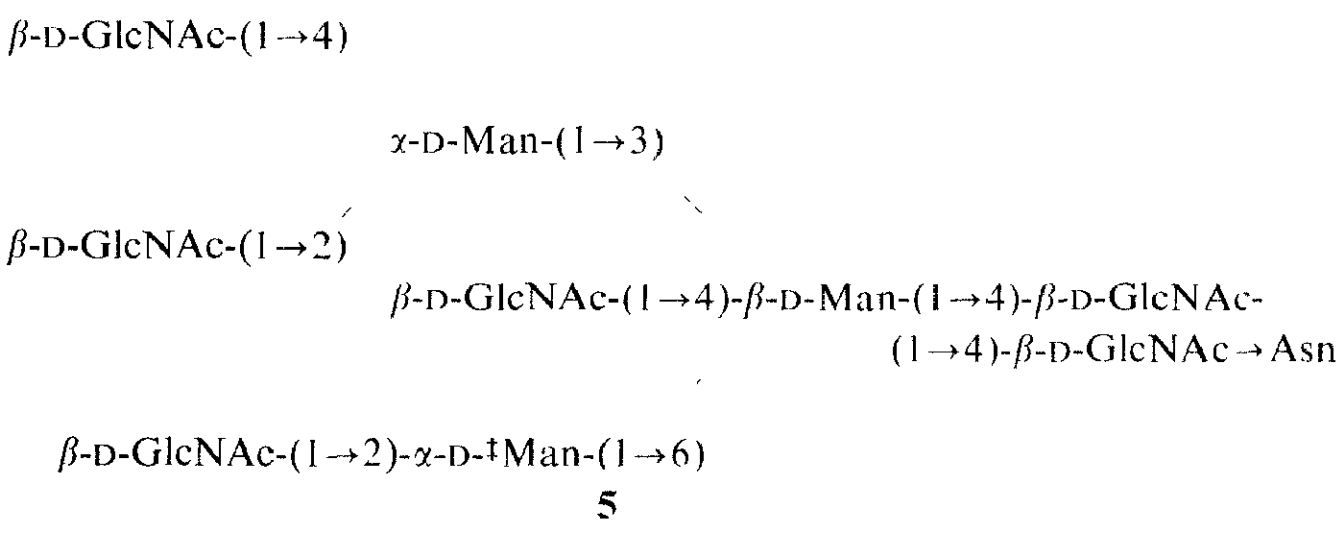


Fig. 4. Fractionation by h.p.l.c. of methylated Glycopeptide 2d on ODS-Hypersil. For details, see Experimental. Fractions were combined as shown and subjected to methylation analysis (Table VI).

glycopeptide contained no galactose and had been degraded to give Structure *A*. The remaining fractions all contained galactose and represented glycopeptides basically having Structure *B* with small amounts of other residues that had not been completely removed by enzymic degradation. Fraction IV gave higher ratios for terminal mannose and 4-substituted hexosamine and was probably contaminated with Fraction VI. These findings were confirmed when Glycopeptide 2d was methylated prior to fractionation by h.p.l.c. (Fig. 4). On analysis (Table VI), fractions containing a single terminal mannosyl group and two 4-substituted hexosaminyl residues were obtained (Fractions F, G, and H). As before, there was a fraction (E) that was essentially similar to the fractions (A–D) eluted earlier, but which appeared to be contaminated with Fraction F.



5

Taking the above results into consideration, the basic structure of the glycopeptides comprising Glycopeptide 2 was concluded to be **5**. Heterogeneity arises at

two points in the molecule. At $^{+}$GlcNAc, there is partial addition of a β-linked galactosyl group at O-4, while at ‡Man there is partial addition of β-linked hexosaminyl groups at O-2 and O-4.

The occurrence of two sites of heterogeneity in the molecule probably explains why the usual methods of glycopeptide fractionation failed to yield homogeneous products. Galactose is a frequent source of heterogeneity in glycopeptides from egg-white glycoproteins. In cases where fractional ratios for galactose are obtained, it has not generally proved possible to separate the glycopeptides into fractions with and without galactose. Difficulty in fractionating glycopeptides containing various amounts of hexosamine has been encountered with other glycopeptides. Shepherd and Montgomery[12] found that ovalbumin glycopeptides which had been fractionated and shown by several methods to be apparently homogeneous could be further fractionated on borate anion-exchange columns to give several glycopeptides that differed considerably in their mannose:hexosamine ratios. Even among the purified glycopeptides obtained by this procedure, there were some which contained 0.5 unit of terminally located galactose.

Our glycopeptide structure displays several features common to the structure proposed by Montreuil for his ovomucoid glycopeptide[6]. From consideration of the structures of the fragments obtained by him after partial hydrolysis with acid[3] and partial acetolysis[4] of his glycopeptide, the minimum size of carbohydrate unit which would yield all the fragments obtained would have a structure identical to that proposed for our basic glycopeptide, except that it would have an additional, terminal hexosaminyl group attached at O-3 of the singly substituted mannosyl residue. The final structure proposed by Montreuil was considerably larger than this minimal structure, in order to accommodate the five mannose, one galactose, and ten hexosamine residues found in the molecule. This glycopeptide was shown to be homogeneous and was obtained from two asialo-ovomucoid variants. However, as the mannose:galactose:hexosamine molar ratios for the variants[13] were $\sim$12.5:3:21.5 and $\sim$10.6:1.2:7.2, other carbohydrate units, with incomplete or totally different structures, must have been present in each glycoprotein. Indications that the glycopeptide fraction examined was probably heterogeneous can be seen in the methylation analysis[5], where there is evidence for the presence of appreciable amounts of methylated mannose derivatives that cannot be accounted for by the formula proposed.

As well as heterogeneity of natural occurrence, additional heterogeneity can arise during the structural investigations if the action of exo-glycosidases on apparently fully accessible sugars is restricted by adjacent groups in the glycopeptide. Any incomplete release of sugar adds to the heterogeneity and has a cumulative effect when sequential degradations are performed. In the case of our glycopeptides, a small amount of hexosamine attached to the core mannose persisted throughout the sequential degradation steps. There was evidence from methylation analysis and fractionation experiments that the sequential release of (1$\rightarrow$6)-linked α-mannosyl residues was also incomplete.

Because satisfactory separation of the glycopeptides has not been achieved,

it has not proved possible to assign unequivocal individual structures. The evidence described has enabled us to postulate a basic structure on which a limited number of variations arise. The structures bear a family resemblance to carbohydrate units in other egg-white glycoproteins, *e.g.*, ovotransferrin[14], and appear to be common to all sites of glycosylation in the ovomucoid molecule. The types of heterogeneity observed are becoming increasingly acknowledged as of general occurrence in glycoprotein units of mixed sugar composition. Whether this heterogeneity results from the action of endogenous glycosidases or from a lack of specificity of some of the glycosyltransferases involved in biosynthesis of the units is not known. All of the units found in asialo-ovomucoid variants may represent intermediate stages in the biosynthesis of fully sialated units. Further developments in h.p.l.c. may make it possible to achieve further fractionation but, with the possibility of heterogeneity occurring at several residues, the complete picture will certainly be complex.

EXPERIMENTAL

The preparation of the glycopeptide fractions A–E, the conditions for sequential enzymic degradation, and the methods for the determination of mannose, galactose, and hexosamine have been previously described[2].

Methylation analysis. — Glycopeptides were methylated by the procedure of Stellner *et al.*[7], and the products were hydrolysed by formolysis–hydrolysis as described by Lindberg[9], except that neutralisation was carried out with Amberlite IRA-400 (HCO_3^-) resin, and sodium borodeuteride was employed for reduction. A single application of the methylation procedure was judged sufficient to permethylate the glycopeptides, as a second application produced no variation in the ratios of the methylated sugar derivatives.

G.l.c. — This was performed with a Carlo Erba Fractovap 4160 instrument equipped with a flame-ionisation detector and an on-column injector of the Grob type[15] with secondary cooling. The column was cooled to 60 ' during injection, and then raised rapidly to 200 and maintained thereat. Separations were performed on glass-capillary columns (50 m × 0.3 mm) wall-coated with OV-1 (Phase Separations Ltd., Clwyd, Great Britain). Calculation of peak areas and ratios was carried out with a Spectra-Physics SP4100 computing integrator. Identity of compounds was confirmed by mass spectrometry (VG Micromass 16 instrument).

Liquid chromatography. — Methylated glycopeptides were fractionated using a Perkin–Elmer Series 3 liquid chromatograph with an LC-65T variable-wavelength detector and a Rheodyne Model 7105 septumless injection-valve. Elution of carbohydrate-containing peaks was monitored by measuring the absorbance of the eluate at 195 nm. To obtain sufficient material for subsequent analysis, several injections of the sample were made and the appropriate separated fractions were combined.

Deionised, redistilled water was filtered through a Millipore 0.45-μm filter before use. Acetonitrile (Rathburn Chemicals, Walkerburn, Great Britain) was

HPLC Grade S (50% transmission at 205 nm) and was filtered through a Millipore 0.5-μm filter prior to use. Both solvents were degassed before mixing, and solvent mixtures were purged with helium during pumping. Separations were carried out on a column (25 × 0.5 cm i.d.) of ODS-Hypersil (Shandon Southern Products Ltd.), and column pressures were ~5 MPa.

Glycopeptide 2 (15 mg) was methylated, and the product was dissolved in 200 μL of acetonitrile–water (43:57). Samples (15 μL) were loaded on the column and eluted at a flow rate of 1 mL/min. The major fractions (Fig. 2) were isolated and hydrolysed, and the methylated sugars identified by g.l.c. (Table IV).

Glycopeptide 2d (10 mg) was methylated, and the product was dissolved in 400 μL of acetonitrile–water (30:70). Samples (15 μL) were loaded on the column and eluted at 1 mL/min. Fractions (Fig. 4) were isolated, hydrolysed, and analysed (Table VI).

Fractionation on Bio-Gel P6. Glycopeptide 2d (19 mg) was dissolved in water (1 mL), loaded on a column (136 × 1.6 cm) of Bio-Gel P6 (200–400 mesh) equilibrated in water, and eluted at 12 mL/h. Fractions (3 mL) were monitored for hexose, and combined as shown (Fig. 3). The larger fractions were subjected to methylation analysis (Table V).

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