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THE CARBOHYDRATE UNITS OF OVALBUMIN: COMPLETE STRUCTURES OF THREE GLYCOPEPTIDES

JAMES CONCHIE AND IAN STRACHAN

Department of Carbohydrate Biochemistry, Rowett Research Institute, Bucksburn, Aberdeen (Great Britain)

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ABSTRACT

Three glycopeptides, obtained in quantity from ovalbumin by exhaustive digestion with Pronase and purified by ion-exchange chromatography and gel filtration, had mannose–2-acetamido-2-deoxyglucose–aspartic acid ratios of 5:4:1, 6:2:1, and 5:2:1. The structures of the glycopeptides have been investigated by sequential digestion with purified exo-glycosidases, Smith degradation, and selective acetolysis, and by methylation analysis of the glycopeptides and their degradation products. The resulting data indicated the structures to be α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-α-D-Man*p*-(1→6)-[β-D-GlcNAc*p*-(1→4)]-[β-D-GlcNAc*p*-(1→2)-α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-β-D-GlcNAc*p*→Asn, α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-β-D-GlcNAc*p*→Asn, and α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-β-D-GlcNAc*p*→Asn. The glycopeptides had a common-core structure consisting of five mannose and two hexosamine residues, but the two larger glycopeptides were not homologous.

INTRODUCTION

Ovalbumin is one of the most extensively studied glycoproteins as regards the structure of its carbohydrate moiety. It was the first glycoprotein in which the existence of the 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glycopyranosylamine linkage was demonstrated[1–4], and one of the first in which microheterogeneity of the carbohydrate unit was clearly shown[5–7]. This heterogeneity, and the consequent difficulty of obtaining homogeneous glycopeptides, has made detailed structural studies extremely difficult, and has invalidated some of the earlier observations that were made on what have since been demonstrated to be mixtures of glycopeptides.

Recently, it has been shown that the asparagine residue in ovalbumin glycopeptides is linked to a di-*N*-acetylchitobiose unit to which is attached a β-linked D-mannosyl residue[8–10], the mannose being attached to the hexosamine residue located furthest from asparagine[11,12]. In spite of the elucidation of this core structure,

results from enzymic and chemical degradation of individual glycopeptides did not permit the assignment of unequivocal structures to the various glycopeptides.

By applying methylation analysis, not only to purified glycopeptides, but also to many of the products isolated at various stages of enzymic and chemical degradation of these glycopeptides, we have succeeded in elucidating the complete structures of the three principal glycopeptides obtained from our sample of ovalbumin.

While this work was in progress, studies[13] on the specificity of an endo-β-*N*-acetylglucosaminidase (endo-2-acetamido-2-deoxy-β-D-glucosidase) from *Diplococcus pneumoniae* resulted in the determination of the structures of two glycopeptides from ovalbumin with mannose–hexosamine ratios of 6:2 and 5:2. The structures described are identical to those obtained by us for two of our glycopeptides.

RESULTS

Fractionation of ovalbumin glycopeptides. — The glycopeptides from ovalbumin, after fractionation (Fig. 1) and purification, all contained aspartic acid as the sole amino acid. Their hexose and hexosamine contents are given in Table I. Mannose was the only hexose present in Glycopeptides **4–6**, but Glycopeptides **1–3** also contained small proportions of galactose (13, 9, and 6%, respectively, of the total hexose content). Further purification did not alter the amount of galactose present, and it seemed probable that galactose formed an integral part of these glycopeptides, which together accounted for 22% of the total Asn-carbohydrates and were not examined in detail.

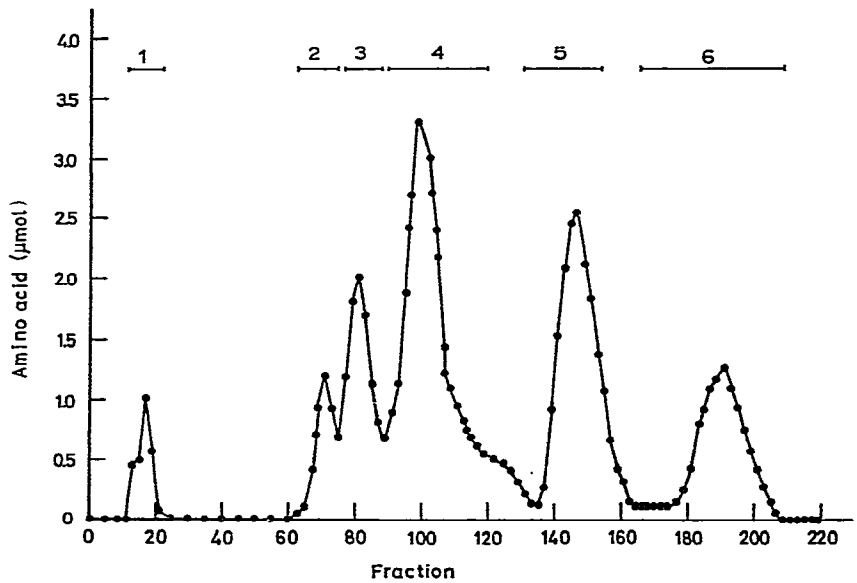


Fig. 1. Fractionation of ovalbumin glycopeptides on Dowex 50 (X2) resin. For conditions, see Experimental section. Fractions (14 ml) were combined as shown, and purified by refractionation on Dowex 50 (X2) and gel filtration on Bio-Gel P4.

TABLE I

FRACTIONATION OF GLYCOPEPTIDES FROM OVALBUMIN[a]

Fraction	*Mannose*[b]	*Hexosamine*[b]	*Yield* (%)
1	8.2	5.1	3.8
2	5.6	4.0	5.7
3	5.8	4.5	12.5
4	5.0	4.0	38.5
5	5.9	1.9	23.5
6	5.2	1.8	14.5

[a]For details of fractionation, see Experimental section. [b]Results are expressed as mol/mol of aspartic acid.

TABLE II

SEQUENTIAL ENZYMIC DEGRADATION[a] OF GLYCOPEPTIDES 5 AND 6

	Residual glycopeptide[b]			
	5		6	
	Man	*GlcNAc*	*Man*	*GlcNAc*
Control	5.9	1.9	5.2	1.8
(1) α-D-Mannosidase	1.2	1.9	1.3	1.8
(2) β-D-Mannosidase	0.1	1.9	0.1	1.8
(3) β-*N*-Acetylglucosaminidase	0.1	0.9	0.1	0.8

[a]Techniques are described in the Experimental section. [b]Values are expressed as mol of sugar/mol of glycopeptide.

TABLE III

PRODUCTS FROM SMITH DEGRADATIONS[a] OF GLYCOPEPTIDES 5 AND 6

	Residual glycopeptide[b]			
	5		6	
	Man	*GlcNAc*	*Man*	*GlcNAc*
Control	5.9	1.9	5.2	1.8
First degradation	2.1	1.8	1.9	1.7
Second degradation	0.1	1.7	0.1	1.7

[a]Techniques are described in the Experimental section. [b]Values are expressed as mol of sugar/mol of glycopeptide.

Sequential enzymic degradation of Glycopeptides **5** *and* **6**. — There was no liberation of 2-acetamido-2-deoxyglucose when the glycopeptides were incubated with β-*N*-acetylglucosaminidase, indicating that neither had hexosamine residues located in a terminal position. Incubation with α-D-mannosidase released 4.7 mannose residues from **5**, and 3.9 residues from **6** (Table II). No hexosamine could be released from the residual glycopeptides by β-*N*-acetylglucosaminidase, and the remaining mannosyl unit in each glycopeptide could only be removed by incubation with β-D-mannosidase. After removal of this final mannose residue, the glycopeptides were susceptible to attack by β-*N*-acetylglucosaminidase, and one hexosamine residue was removed from each glycopeptide (Table II). The resulting glycopeptides were cleaved with 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine amidohydrolase to give 2-acetamido-2-deoxy-D-glucose and aspartic acid.

Smith degradation of Glycopeptides **5** *and* **6**. — The isolation of glycopeptides having a mannose–hexosamine ratio of 2:2 from **5** and **6** after Smith degradation (periodate oxidation followed by reduction with borohydride and acid hydrolysis; Table III) indicated that **5** and **6** had a common-core structure. The two residual mannosyl units must have been internally situated in the original glycopeptides and substituted either singly at C-3 or doubly at C-2 and C-3, C-2 and C-4, C-3 and C-4, or C-3 and C-6. A second Smith-degradation of the residual glycopeptides resulted in the removal of all the mannose from the glycopeptides. Since the two mannosyl units were linked together, as shown by the results of methylation analysis (see below),

TABLE IV

SEPARATION AND IDENTIFICATION OF ACETATES[a] OF PARTIALLY *O*-METHYLATED D-MANNITOLS AND 2-DEOXY-2-*N*-METHYLACETAMIDO-D-GLUCITOLS

Methylated derivative[b]	*Retention time*[c] (T)	*Primary fragments* (m/e)
2,3,4,6-Man	1.0	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Man	1.78	43, 45, 87, 99, 101, 117, 129, 161, 233
3,4,6-Man	2.14	43, 45, 87, 101, 129, 161, 189
2,3,4-Man	2.49	43, 87, 99, 101, 117, 129, 161, 189
2,6-Man	3.02	43, 45, 87, 117, 129
3,6-Man	3.45	43, 45, 87, 99, 113, 129, 189, 233
2,4-Man	3.65	43, 87, 117, 129, 189, 233
3,4-Man	3.81	43, 87, 99, 129, 189
2-Man	4.15	43, 117, 139
3,4,6-GlcNAcMe	4.47	43, 45, 74, 116, 129, 142, 145. 158. 202, 205
3,6-GlcNAcMe	5.55	43, 74, 87, 116, 142, 158, 233

[a]On 2% of OV-210; conditions are described in the Experimental section. [b]2,3,4,6-Man = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol *etc.*; 3,4,6-GlcNAcMe = 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucitol *etc.* [c]Retention times (*T*) are relative to that of 2,3,4,6-Man. The molar responses used in methylation analysis with the above column were: 2,3,4,6-Man, 1.0; 2,4,6-Man, 3,4,6-Man, and 2,3,4-Man, 0.9; 2,6-Man and 2,4-Man, 0.8; 2-Man, 0.7; 3,4,6-GlcNAcMe, 0.8; 3,6-GlcNAcMe, 0.8.

TABLE V

METHYLATION ANALYSES OF GLYCOPEPTIDES **5** AND **6** AND OF THEIR DEGRADATION PRODUCTS[a]

Methylated derivative[b]	*Molar ratios*					
	5	**5a**	**5b**	**6**	**6a**	**6b**
2,3,4,6-Man	3.0	1.1	0.9	3.1	1.2	0.9
3,4,6-Man	1.0	—	—	—	—	—
2,4,6-Man	—	—	—	—	—	—
2,3,4-Man	—	—	1.1	—	—	1.0
2,4-Man	1.9	—	—	2.0	—	—
3,6-GlcNAcMe	1.8	1.8	1.8	1.8	1.8	1.8

[a]**5a** and **6a** were obtained after degradation of **5** and **6** with α-D-mannosidase, and **5b** and **6b** after one Smith degradation of **5** and **6**. [b]See Table IV for key and molar responses.

removal of both by Smith degradation proved that the inner, β-linked, D-mannosyl residue had not been substituted at C-3.

Methylation analysis of Glycopeptides **5** *and* **6** *and their degradation products.* — The glycopeptides were subjected to sequential methylation, acetolysis/hydrolysis, reduction, and acetylation. The resulting mixtures of methylated mannitol acetates and 2-deoxy-2-*N*-methylacetamidoglucitol acetates were separated and identified by g.l.c.–m.s. (Tables IV and V).

Both **5** and **6** gave 3 mol. (molecular equivalents) of 2,3,4,6-tetra-*O*-methylmannose, indicating that both glycopeptides contained three mannosyl units situated in terminal positions. In each glycopeptide, the two hexosamine residues, which had already been shown by enzymic-degradation experiments to be located next to the asparagine residue, must have been linked at C-1 and C-4. There was no evidence of any monomethyl-2-deoxy-2-*N*-methylacetamidoglucose, so neither hexosamine residue formed a branch point in the glycopeptides. Each glycopeptide gave 2 mol. of 2,4-di-*O*-methylmannose, indicating the presence of two doubly substituted mannosyl residues. The presence of 1 mol. of 3,4,6-tri-*O*-methylmannose in the products of methylation from **5** showed that there had been an α-Man*p*-(1→2)-α-Man*p* group in this glycopeptide.

Since only one mannosyl residue was linked directly to the di-*N*-acetylchitobiose unit, the arrangement of sugars necessary to fulfil the requirements of the methylation-analysis results for both glycopeptides must be

α-D-Man*p*→α-D-Man*p*→β-D-Man*p*→β-D-GlcNAc*p*→β-D-GlcNAc*p*→Asn,
↑ ↑
α-D-Man*p* α-D-Man*p*

with the position of the sixth mannosyl residue in Glycopeptide **5** as yet undecided. The isolation of glycopeptides having a mannose–hexosamine ratio of 2:2 after a single Smith-degradation had already indicated that there were doubly substituted mannosyl residues linked together, and a second treatment had shown that they were

not joined by an α-(1→3) linkage. The isolation of 2,3,4-tri-*O*-methylmannose from the products of methylation of Glycopeptides **5b** and **6b** (Table V) confirmed that the doubly substituted mannosyl residues in the original glycopeptides were joined by an α-(1→6) linkage.

Correlation of all the results enables the definitive structure for Glycopeptide **6** to be written as:

α-D-Man*p*-(1→6)-α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-β-D-GlcNAc*p*→Asn.
3 3
↑ ↑
1 1
α-D-Man*p* α-D-Man*p*

In **5**, five of the mannose residues must be arranged as in **6**, but there are three possible positions of attachment of the additional mannosyl unit, each of which would be compatible with the results so far described.

Selective acetolysis of Glycopeptides **5** *and* **6**. — Acetolysis under carefully controlled conditions selectively cleaves (1→6) linkages in hexose-containing di- and oligo-saccharides[14,15]. Little is known of the stabilities of GlcNAc→GlcNAc, GlcNAc→Asn, GlcNAc→Man, or Man→GlcNAc linkages under the same conditions. When **5** and **6** were subjected to controlled acetolysis and the breakdown products fractionated on Bio-Gel P2, each glycopeptide gave three major mannose-containing fragments as well as other minor products (Figs. 2 and 3). The fractions corresponding to Peaks *A*, *B*, and *C* were combined, and purified by refractionation on the Bio-Gel P2 column. Fraction *A* from both glycopeptides was reducing and contained hexosamine, whereas Fractions *B* and *C* were reducing and contained no hexosamine. After reduction with borohydride, both Fractions *B* showed a 50% fall in hexose content, indicating that the original fractions had consisted of a mannose disaccharide.

Methylation analysis of the freeze-dried fractions (Table VI) indicated the following structures for the fragments from Glycopeptide **6**: *A*, Man*p*-(1→3)-Man*p*-(1→4)-GlcNAc; *B*, Man*p*-(1→3)-Man; *C*, Man; and from Glycopeptide **5**: *A*, Man*p*-(1→2)-Man*p*-(1→3)-Man*p*-(1→4)-GlcNAc; *B*, Man*p*-(1→3)-Man; *C*, Man.

TABLE VI

METHYLATION ANALYSES[a] OF FRAGMENTS FROM ACETOLYSIS OF GLYCOPEPTIDES **5** AND **6**

Methylated derivative[b]	*Fractions*[c]			
	5		**6**	
	A	B	A	B
2,3,4,6-Man	1.0	1.0	1.0	1.0
2,4,6-Man	0.9	0.9	0.7	1.0
3,4,6-Man	0.9	—	—	—
3,6-GlcNAcMe	1.0	—	0.9	—

[a]Details of procedure are given in the Experimental section. [b]See Table IV for key and molar responses. [c]For details of fractions, see Figs. 2 and 3. Values are expressed as molar ratios.

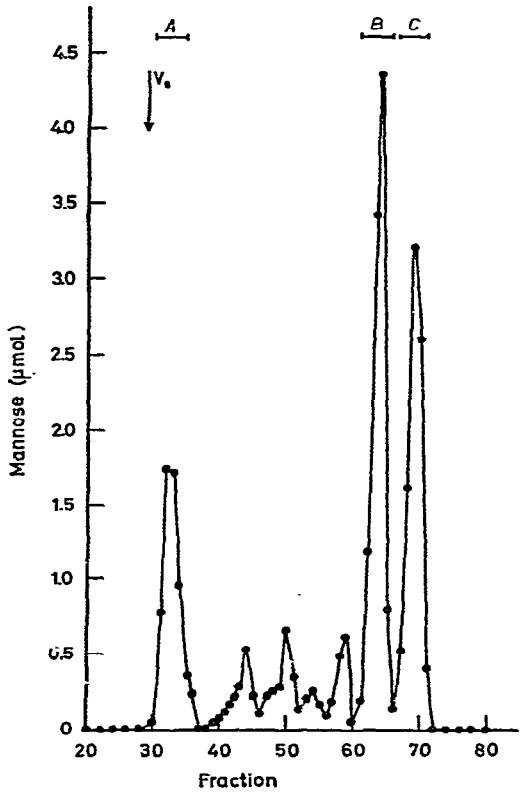


Fig. 2. Separation by gel filtration on Bio-Gel P2 of the products from the controlled acetolysis of Glycopeptide 5. For details, see Experimental section. Fractions (3 ml) were combined as shown and, after freeze-drying, subjected to methylation analysis (Table VI). V_0 is the void volume of the column, determined with Blue Dextran.

Considering the fragments from **6** in relation to the already determined structure of the glycopeptide, it is apparent that the greatest amount of cleavage has been at the (1→6) linkages of the glycopeptide, with an additional cleavage of the GlcNAc-(1→4)-GlcNAc linkage. The structure of **6** is thus confirmed, while that of **5** is:

α-D-Man*p*-(1→6)-α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-β-D-GlcNAc*p*→Asn.

3 3
↑ ↑
1 1
α-D-Man*p* α-D-Man*p*
2
↑
1
α-D-Man*p*

Enzymic degradation of Glycopeptide **4**. — Two of the four hexosamine residues in **4** could be removed by incubation with β-*N*-acetylglucosaminidase. Only one of the

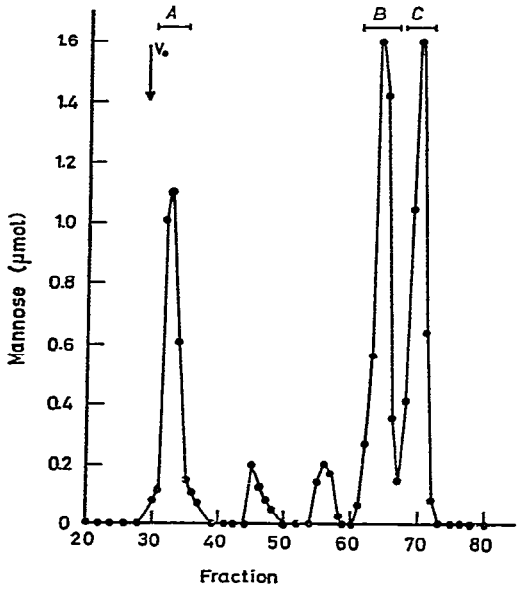


Fig. 3. Gel filtration on Bio-Gel P2 of products from the controlled acetolysis of Glycopeptide 6. For details, see Experimental section. Fractions (3 ml) were combined as shown and, after freeze-drying, subjected to methylation analysis (Table VI). V_o is the void volume of the column, determined with Blue Dextran.

five mannose residues could be removed by α-D-mannosidase preparations from jack-bean meal and from limpet. Although methylation analysis indicated that two mannosyl units were located in terminal positions (see below), it was not possible to remove the second terminal mannosyl unit by prolonged incubation with either α- or β-D-mannosidase. When the glycopeptide produced by the action of β-*N*-acetyl-glucosaminidase, with a mannose–hexosamine ratio of 5:2, was incubated with α-D-mannosidase, four mannose residues were removed (Table VII). Treatment of this residual glycopeptide with limpet β-D-mannosidase removed the remaining mannosyl unit, and subsequent incubation with β-*N*-acetylglucosaminidase released

TABLE VII

SEQUENTIAL ENZYMIC DEGRADATION[a] OF GLYCOPEPTIDE 4

	Residual glycopeptide[b]	
	Man	*GlcNAc*
Control	5.0	4.0
(1) β-*N*-Acetylglucosaminidase	5.0	2.0
(2) α-D-Mannosidase	1.2	2.0
(3) β-D-Mannosidase	0.1	2.0
(4) β-*N*-Acetylglucosaminidase	—	1.1

[a]For details, see Experimental section. [b]Values are expressed as mol of sugar/mol of glycopeptide.

one hexosamine unit (Table VII). The remaining hexosamine residue could be released by incubation with 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine amidohydrolase.

Smith degradation of Glycopeptide **4**. — Smith degradation of glycopeptide **4** gave a glycopeptide containing two mannose and two hexosamine residues (Table VIII). When the procedures were repeated on this residual glycopeptide, both of the mannose residues were removed.

Methylation analysis of Glycopeptide **4** *and its degradation products*. — Methylation analysis was carried out on **4** and on several glycopeptides arising from the enzymic degradation of **4** (Tables IV and IX). As in the case of **5** and **6**, there was no monomethyl-2-deoxy-2-*N*-methylacetamidoglucose in the products of methylation; the g.l.c. programme was continued for a period sufficiently long for its presence to be observed. Its absence meant that neither of the internally situated hexosaminyl

TABLE VIII

PRODUCTS FROM SMITH DEGRADATIONS[a] OF GLYCOPEPTIDE **4**

	Residual glycopeptide[b]	
	Man	*GlcNAc*
Control	5.0	4.0
First degradation	1.9	1.7
Second degradation	0.1	1.8

[a]Details are given in the Experimental section. [b]Values are expressed as mol of sugar/mol of glycopeptide.

TABLE IX

METHYLATION ANALYSES[a] OF GLYCOPEPTIDE **4** AND OF PRODUCTS FROM ITS ENYZMIC DEGRADATION

Methylated derivative[b]	*Glycopeptide*[c]			
	4	**4a**	**4b**	**4c**
2,3,4,6-Man	1.9	2.9	0.8	0.9
2,4,6-Man	—	—	0.8	—
3,4,6-Man	1.0	—	1.0	—
2,4-Man	0.9	1.9	—	—
2-Man	1.0	—	1.0	—
3,4,6-GlcNAcMe	2.0	—	2.2	—
3,6-GlcNAcMe	2.0	2.0	2.1	2.0

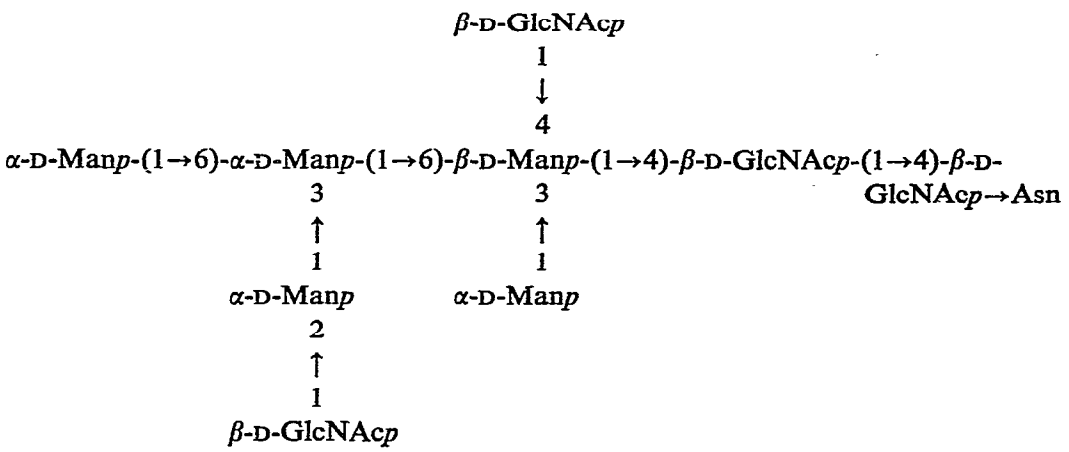
[a]Details are given in the Experimental section. [b]See Table IV for key and molar responses. [c]**4a** was obtained after action of β-*N*-acetylglucosaminidase on **4**, **4b** after action of α-D-mannosidase on **4**, and **4c** after action of β-*N*-acetylglucosaminidase and α-D-mannosidase in sequence on **4**. Values are expressed as molar ratios.

residues in **4** formed a branch point. The presence of 2 mol. of 2,3,4,6-tetra-*O*-methylmannose in the products from **4** indicated that two mannose residues were located in terminal nonreducing positions in the glycopeptide, while the analysis of the **4b** products (Table IX), which still contained 1 mol. of 2,3,4,6-tetra-*O*-methylmannose, confirmed that one terminally situated mannose residue remained, even after the prolonged action of α-D-mannosidase.

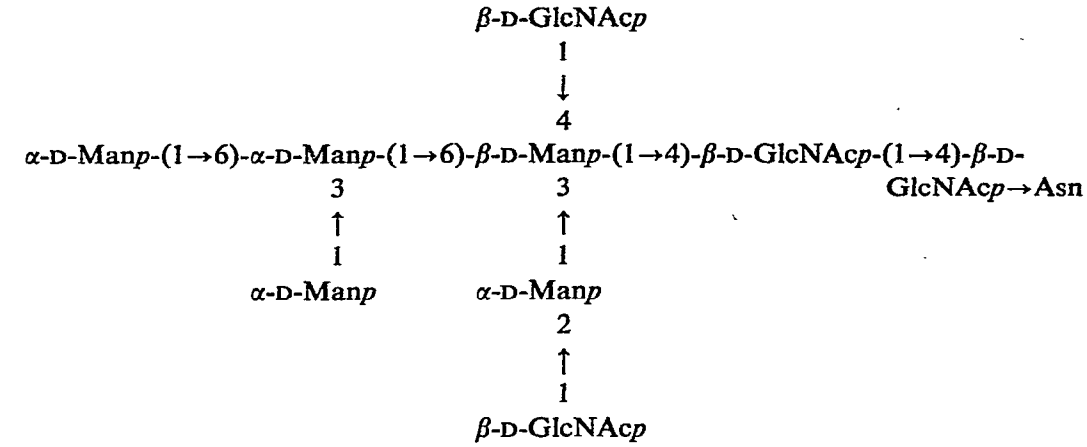
Glycopeptide **4a**, obtained after the action of β-*N*-acetylglucosaminidase, had a mannose–hexosamine ratio of 5:2 and gave the same methylated sugars, in the same proportions, as were obtained after methylation of Glycopeptide **6** (Table IX). This result strongly indicated that the greater part of the structure of **4** was the same as that of **6**. When the analysis of **4a** was compared with that of **4**, the absence of 1 mol. of 2-*O*-methylmannose and a corresponding increase in 2,4-di-*O*-methylmannose indicated that one of the terminal, nonreducing hexosamine residues in **4** had been linked to O-4 of the triply substituted mannosyl residue. The appearance of an additional 1 mol. of 2,3,4,6-tetra-*O*-methylmannose, coupled with the absence of a corresponding amount of 3,4,6-tri-*O*-methylmannose, meant that the second terminal hexosaminyl unit was attached by a β-(1→2) linkage to a singly substituted mannosyl residue.

In **4b**, the glycopeptide obtained after the action of α-D-mannosidase, there was, as expected, 1 mol. less of 2,3,4,6-tetra-*O*-methylmannose than in **4**. The absence of 2,4-di-*O*-methylmannose, coupled with the appearance of 1 mol. of 2,4,6-tri-*O*-methylmannose, indicated that the terminal, nonreducing mannosyl unit removed by α-D-mannosidase had been linked in **4** to O-6 of a doubly substituted mannosyl residue. Analysis of **4c** confirmed that the core of the glycopeptide was identical to that of **5** and **6**.

Considering all the results described, there remain two possible structures (*A* and *B*) for Glycopeptide **4**.



Structure *A*



Structure *B*

Selective acetolysis of Glycopeptides **4** *and* **4c**. — When **4** was acetolysed under the same conditions employed for **5** and **6**, a series of fractions was obtained from the Bio-Gel P2 column (Fig. 4). In the fraction which appeared in a position corresponding to that occupied by the mannose disaccharide in previous experiments (Fraction *C*), there was free hexosamine as well as a mannose derivative; hexosamine has[16] the retention volume of a hexose disaccharide on columns of Bio-Gel. The presence of free hexosamine in the fragments produced by acetolysis indicated that at least some GlcNAc→Man linkages were being cleaved, although methylation analysis had shown that these were not (1→6) linkages. Examination of Fraction *A* (Fig. 4) by methylation analysis did not give whole molar ratios of mannose derivatives (Table X), and suggested that the fraction was a mixture of two oligosaccharides, each of which had lost a different, terminal, nonreducing hexosaminyl residue. There had also been

TABLE X

METHYLATION ANALYSES[a] OF FRAGMENTS FROM ACETOLYSIS OF GLYCOPEPTIDES **4** AND **4c**

Methylated derivative[b]	*Glycopeptide*				
	4			**4c**[c]	
	A	B	C	A	B
2,3,4,6-Man	0.4	—	1.0	1.0	1.0
2,4,6-Man	0.3	—	0.8	0.8	1.0
3,4,6-Man	0.5	1.0	—	—	—
2,6-Man	0.2	0.9	—	—	—
3,4,6-GlcNAcMe	0.8	2.0	—	—	—
3,6-GlcNAcMe	1.0	0.1	—	1.0	—

[a]Details are given in the Experimental section. [b]See Table IV for key and molar responses. [c]**4c** was obtained after action of β-*N*-acetylglucosaminidase on **4**.

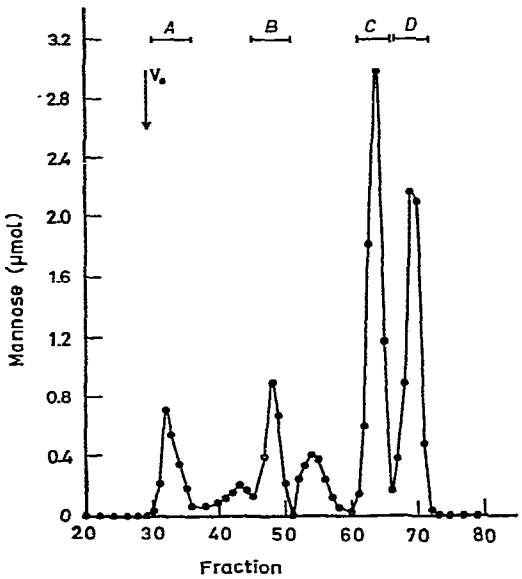


Fig. 4. Gel filtration on Bio-Gel P2 of products from the controlled acetolysis of Glycopeptide 4. For details, see Experimental section. Fractions (3 ml) were combined as shown and, after freeze-drying, subjected to methylation analysis (Table X). V_0 is the void volume of the column, determined with Blue Dextran.

cleavage of the -GlcNAc→GlcNAc linkage. However, the presence of ~1 mol. of 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamidoglucose, together with 0.5 mol. of 3,4,6-tri-*O*-methylmannose, indicated that Structure *B* was correct for **4**, as Structure *A* would not have yielded 3,4,6-tri-*O*-methylmannose from its largest acetolysis fragment.

Attempts were made to eliminate or diminish cleavage of the terminal hexosaminyl linkages by reducing the period of acetolysis from 16 h to 5 h. This resulted in a somewhat higher yield of Fraction *A*, while the yield of Fraction *B* remained constant. There was no indication of the presence of any larger glycopeptides having a hexosamine content higher than that of these fractions.

Further evidence on structure was obtained from methylation analysis of the second major fraction, Fraction *B* (Table X). No 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamidoglucose was formed, so cleavage of the -β-Man-(1→4)-GlcNAc linkage had occurred. This was confirmed by the fact that the hexose content fell to 50% of the original value when the fraction was reduced with borohydride, indicating that one of the two mannose residues in the original fraction had a reducing group. On the other hand, the presence of 2 mol. of 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamidoglucose and the absence of 2,3,4,6-tetra-*O*-methylmannose showed that both terminal glucosaminyl residues were present in this fraction, which thus had the structure GlcNAc*p*-(1→2)-Man*p*-(1→3)-[GlcNAc*p*-(1→4)]-Man.

A fraction with this structure could only arise from acetolysis of a glycopeptide having Structure *B*. Confirmation that Structure *B* was correct was obtained by

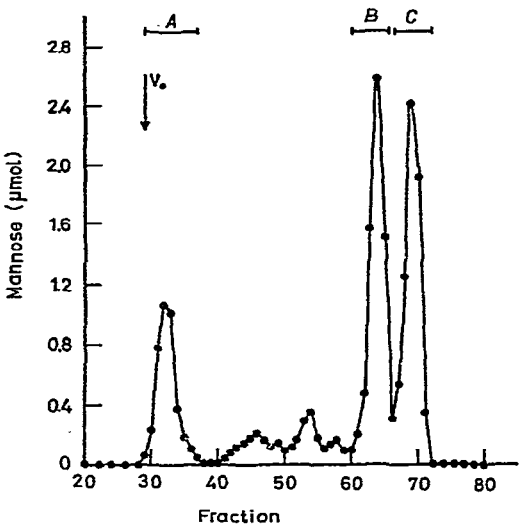


Fig. 5. Gel filtration on Bio-Gel P2 of products of controlled acetolysis of Glycopeptide **4c**. For details, see Experimental section. Fractions (3 ml) were combined as shown and, after freeze-drying, subjected to methylation analysis (Table X). V_o is the void volume of the column, determined with Blue Dextran.

methylation analysis of Fraction *C* (Table X). This fraction had the structure Man*p*-(1→3)-Man. Fraction *D* (Fig. 4) consisted of mannose.

Acetolysis of **4c**, obtained from **4** by the action of *β*-*N*-acetylglucosaminidase, gave three major fractions (Fig. 5). On methylation analysis, these fractions gave the same methylated sugars, in the same proportions, as found for Glycopeptide **6**, providing further evidence that **4**, **5**, and **6** had a common structure for five of their mannose and two of their hexosamine residues.

DISCUSSION

The principal drawback in structural studies of the glycopeptides from ovalbumin has been the considerable degree of microheterogeneity which exists in the carbohydrate unit, and the consequent difficulty of obtaining pure samples of each glycopeptide. From our results and those of other workers, it appears that, even when essentially similar separation procedures are used, different specimens of ovalbumin give somewhat different mixtures of glycopeptides, although all samples contain considerable amounts of glycopeptides having mannose–hexosamine–aspartic acid ratios of 6:2:1 and 5:2:1. Even in the case of 6:2 glycopeptides, there may well be variations in structure, as our results and those of Tai *et al.*[13] do not completely agree with those of other workers[7,17].

A further point of difference was observed in the analysis of those glycopeptides eluted first from the Dowex resin and present in relatively small proportions. There were at least three of these glycopeptides, and each contained 6–13% of galactose

which was not removed on refractionation of the glycopeptides. Other workers have also reported the presence of galactose in ovalbumin even after extensive purification[17]. Whether the galactose is part of the ovalbumin carbohydrate units or part of a small proportion of contaminating glycoprotein remains uncertain. The hexose–hexosamine ratios in the galactose-containing glycopeptides are not very different from those for the other glycopeptides, and our preliminary methylation analysis results suggest they have similar structures, with the galactose residue in a terminal position.

In experiments involving incubation of glycopeptides with exo-glycosidases, the possible concomitant action of 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine amidohydrolase has always to be considered, as we have observed that partially purified glycosidase preparations from such sources as limpet, jack-bean meal, and boar epididymis contain this enzyme. In highly purified preparations, however, the activity is negligible under the incubation conditions used. A further aspect which merits attention is the possible action of an endo-β-*N*-acetylglucosaminidase which can cleave the di-*N*-acetylchitobiose linkage in some glycopeptides. This enzyme occurs in *Streptomyces griseus*[19], which is the source of commercial Pronase. The absence of reducing oligosaccharides in our products of Pronase digestion confirmed the absence of endo-β-*N*-acetylglucosaminidase and 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine amidohydrolase activities in our purified Pronase preparation under the conditions of the experiments.

Recent structural studies on purified ovalbumin glycopeptides have concentrated on those glycopeptides having a low hexosamine content (6:2 and 5:2 glycopeptides), with a view to identifying the core of these compounds. While it was conclusively demonstrated[11,12] that, in such glycopeptides, the core was β-Man*p*-(1→4)-β-GlcNAc*p*-(1→4)-β-GlcNAc*p*→Asn, it remained uncertain whether another branch containing mannose residues was attached to one or other of the hexosamine residues. Earlier studies appeared to indicate the existence of such a branch[7,20].

In the three glycopeptides we have studied, there is no branch on either hexosaminyl residue, although branching occurs at the β-linked D-mannosyl residue. This core of five sugar residues α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-β-D-GlcNAc*p*→Asn is common to glycopeptides from several sources, e.g., taka amylase[21] and human serotransferrin[22].

Selective acetolysis, followed by methylation analysis of the isolated fragments, has proved to be of considerable value in enabling a decision to be made between alternative structures for the glycopeptides. Although there is a clear distinction between the stabilities of (1→6) and other mannosyl–mannose linkages, we have found that the presence of terminal hexosaminyl units in a glycopeptide considerably modifies the specificity of the method. As well as cleavage of all Man-(1→6)-Man linkages, there was partial cleavage of Man-(1→4)-GlcNAc, GlcNAc-(1→4)-Man, GlcNAc-(1→2)-Man, and GlcNAc-(1→4)-GlcNAc linkages. Similar cleavages have been observed when glycopeptides from human serotransferrin are acetolysed under somewhat different conditions (1–4 days at 20°)[22].

An unexpected feature of the enzymic degradation of Glycopeptide **4** was the

inability of α-D-mannosidase preparations from jack-bean meal or limpet to remove one of the two terminal mannosyl units from the intact glycopeptide. Indication that this inability to cleave the α-(1→3) linkage was due to steric factors involving one or both of the terminal hexosaminyl units was given by the fact that removal of these residues enabled four of the five mannose residues to be removed by α-D-mannosidase. A similar observation has been made by Raschke and Ballou[23], who found that the presence of a hexosaminyl unit at a branch point of a mannose tetrasaccharide prevented the cleavage of an adjacent (1→3)-α-D-mannosyl linkage by α-D-mannosidase preparations from various sources.

Although the complete structure of Glycopeptide **6** is common to all three glycopeptides examined, Glycopeptides **4** and **5** do not represent different stages in the biosynthesis of a single "complete" molecule, as the additional (1→2)-α-D-mannosyl unit in **5** has been replaced in **4** by a (1→2)-β-hexosaminyl unit. It appears that after completion of **6** there is the possibility of heterogeneity, perhaps due to some lack of specificity in the glycosyl transferases This may explain variations in results obtained by different laboratories. In our sample of ovalbumin, there appeared to be smaller proportions of glycopeptides having high mannose and hexosamine contents than has been found elsewhere.

EXPERIMENTAL

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[24], *p*-nitrophenyl α-D-mannopyranoside[25], and phenyl β-D-mannopyranoside[26] were prepared by the literature procedures. 2-Acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine was kindly donated by Professor L. Hough, Queen Elizabeth College, London. Ovalbumin was prepared from fertile eggs (Sterling White Link) by the method of Kekwick and Cannan[27], and was crystallized five times from sodium sulphate. The product was dialyzed against distilled water and freeze-dried.

General methods. — Mannose was determined by the phenol–sulphuric acid method[28], with mannose as standard. Galactose and mannose were determined by g.l.c. as their alditol acetates[29]. Hexosamine, reducing sugar, and amino acid determinations were carried out as described previously[30]. Protein determinations were carried out according to the method of Lowry *et al.*[31], with bovine albumin as standard. Aspartic acid in glycopeptide fractions was monitored by the ninhydrin procedure of Rosen[32]. Pronase (Cambrian Chemicals Ltd., Croydon) was purified before use by acetone fractionation[33].

Methylation. — Glycopeptides (1–5 mg) were permethylated by the method of Hakomori[34], as described by Lindberg[35], except that methylation was continued for 2 h. To ensure quantitative yields of methylated 2-deoxy-2-*N*-methylacetamidohexitol acetates as well as of methylated hexitol acetates, the permethylated glycopeptides were subjected to acetolysis with 0.25M sulphuric acid in 95% acetic acid followed by aqueous hydrolysis, reduction, and acetylation as described by Stellner *et al.*[36]. A single application of the methylation procedure was judged sufficient to permethylate

the glycopeptides, as a second application to several glycopeptides showed no variation in the ratio of the methylated sugar derivatives.

Gas–liquid chromatography. — G.l.c. (Perkin–Elmer chromatograph, Model F30, flame ionization) was performed with nitrogen as carrier gas (30 ml/min). Methylated alditol acetates were separated on glass columns (180 × 0.3 cm) containing (*a*) 3% of ECNSS-M, (*b*) 1% of OV-225, and (*c*) 2% of OV-210, all on Gas Chrom Q (100–120 mesh) as support. For identification purposes, separations were performed on columns (*a*) and (*b*) at 170° (for neutral sugar derivatives) and 190° (for amino sugar derivatives). For quantitative evaluation of mixtures of partially methylated derivatives, columns (*a*) and (*c*) were programmed from 150–180° at 1°/min, with an initial post-injection period of 30 min, and column (*b*) was programmed from 160–200° at 1°/min, with a post-injection period of 10 min. Peak areas were measured with a Hewlett Packard integrator, Model 3370B. Columns (*a*) and (*b*) were unsatisfactory for the complete resolution of mixtures of 2,4,6- and 3,4,6-tri-*O*-methyl-, and of 2,4- and 3,4-di-*O*-methyl-, mannitol acetates. Column (*c*), as well as giving good resolution of these derivatives (Table IV), gave a larger molar response with methylated 2-deoxy-2-*N*-methylacetamidoglucitol acetates than did columns (*a*) and (*b*). Methylated mannitol acetates were identified by comparison of their relative retention times on columns (*a*) and (*b*) with those of standards, where available, and with the data of Lindberg[35]. Confirmation of the identity of methylated mannitol and 2-deoxy-2-*N*-methylacetamidoglucitol acetates was made by comparison of their mass spectra with published data[36–38]. Mass spectra were obtained with a GEC-AEI MS 902 double-focussing mass spectrometer coupled to a Pye Series 104 temperature-programmed gas chromatograph with a Biemann–Watson molecular separator. The mass-spectral data were processed on an IBM 1130 computer.

Smith degradation. — Before oxidation with periodate, the glycopeptides were selectively *N*-acetylated[39] in order to protect the amino group of asparagine from oxidation. The glycopeptide (10–20 mg) was dissolved in water (2 ml) to which was added the acetylating reagent (0.2 ml) and saturated, aqueous sodium hydrogen carbonate (0.2 ml). The mixture was allowed to stand at room temperature for 30 min, deionized, and then treated with 0.05M sodium metaperiodate (10 ml) in 0.05M sodium acetate buffer (pH 4.6) at 4° in the dark. The reaction was followed[40] by spectrophotometric readings at 260 nm. After 24–48 h, the excess of periodate was reduced by the addition of 1 : 1 (v/v) ethylene glycol–water (1.0 ml). The oxidized glycopeptide was isolated by elution with water from a column (24 × 1.6 cm) of Bio-Gel P2; fractions were assayed for mannose and hexosamine. The combined glycopeptide fractions were freeze-dried, and reduced with 0.15M sodium borohydride in 0.3M potassium borate buffer (pH 8.0, 10 ml) for 18 h at 4°. The excess of borohydride was decomposed by the addition of acetic acid, and the solution was adjusted to pH 1.0 with 2M sulphuric acid. After 24 h at room temperature, the solution was neutralized with 6M sodium hydroxide and evaporated to dryness, and the residue was desalted by gel filtration through the Bio-Gel P2 column. The fraction containing ninhydrin-reacting material was freeze-dried and analyzed for mannose, hexosamine,

and aspartic acid. For sequential Smith-degradations, the product was subjected to a repeat of the above procedure.

Controlled acetolysis. — Experiments involving the selective acetolysis of (1→6) linkages were based on the method of Stewart *et al.*[14]. The glycopeptide (10–15 mg) was suspended in 1:1 (v/v) acetic anhydride–pyridine (1 ml) and heated at 100°. The suspension was sonicated for 1-min periods at intervals of 1 h until the glycopeptide had completely dissolved (~3 h). Acetylation was continued for a further 5 h, after which the solution was evaporated to dryness on a rotary evaporator, toluene being added to assist removal of acetylation reagents. To the dry, acetylated glycopeptide was added the acetolysis agent (acetic anhydride–acetic acid–sulphuric acid 10:10:1, v/v; 1 ml), and acetolysis was allowed to proceed for 16 h at 37°. The reaction was stopped by the addition of pyridine (0.4 ml) to the cooled solution, which was then evaporated on a rotary evaporator, toluene being added to assist evaporation. The residue was dissolved in water (5 ml) and extracted twice with chloroform (10 ml). The combined extracts were washed with water (10 ml), dried ($MgSO_4$), and evaporated; traces of water remaining were removed by azeotropic distillation with benzene. The residue was deacetylated by treatment with 0.05M methanolic sodium methoxide (1 ml) at room temperature for 30 min. Deacetylation was terminated by the addition of ethyl acetate (1 ml), after which the solution was evaporated to dryness. The acetolysis products were fractionated by dissolving the residue in water (2 ml) and passing the solution through a column (140 × 1.7 cm) of Bio-Gel P2 (–400 mesh) with water as eluent, the rate of flow being maintained at 12 ml/h. Fractions (3 ml) were assayed for mannose and, where appropriate, for hexosamine. Fractions corresponding to the principal mannose-containing peaks were combined, freeze-dried, assayed for mannose and hexosamine, and subjected to methylation analysis.

Enzyme preparations. — All assays of enzyme activity were performed at 37°. One unit of activity is defined as that which causes the cleavage of 1 μmol of substrate per min under the appropriate assay conditions.

2-Acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine amidohydrolase (1-L-β-aspartamido-2-acetamido-1,2-dideoxy-β-D-glucose amidohydrolase EC 3.5.1.26) was prepared from rat liver, and assayed by using 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine as substrate[41]. The enzyme had an activity of 0.13 unit per mg of protein.

2-Acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase (β-*N*-acetylglucosaminidase, EC 3.2.1.30) was prepared from boar epididymis as described by Findlay and Levvy[42]. The enzyme was assayed by using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as substrate[25], and had an activity of 330 units per mg of protein. The enzyme preparation did not liberate mannose from glycopeptides under the conditions of the experiment.

α-D-Mannoside mannohydrolase (α-mannosidase, EC 3.2.1.24) preparations from limpet (*Patella vulgata*)[24] and from jack-bean meal[43] were kindly donated by Dr. S. M. Snaith. The enzymes were assayed by using *p*-nitrophenyl α-D-manno-

pyranoside as substrate[25], and had activities of 43 and 13 units, respectively, per mg of protein. Both preparations were free from β-mannosidase and β-*N*-acetylglucosaminidase activities.

β-D-Mannoside mannohydrolase (β-mannosidase, EC 3.2.1.25) was prepared from the visceral hump of the limpet (*Patella vulgata*), a known source of the enzyme[44]. Details of the purification and properties of this enzyme will be published elsewhere. The enzyme was assayed with phenyl β-D-mannopyranoside as substrate, by using a modification of the assay for phenyl glycosidases described by Asp[45]. The enzyme was incubated for 1 h at 37° in 0.125M sodium acetate buffer (pH 4.5) containing 0.1M sodium chloride, 0.01% of albumin, and 5mM phenyl β-D-mannopyranoside in a total volume of 0.4 ml. The reaction was stopped by the addition of 1 ml of M Tris buffer (pH 8.5) containing 1.3mM 4-aminophenazone, followed by the addition of 0.25 ml of potassium ferricyanide solution (4 mg/ml). After allowing 10 min at room temperature for full colour development, the absorbance at 508 nm was read on a spectrophotometer. The enzyme had an activity of 0.8 unit per mg of protein and was free from α-mannosidase and β-*N*-acetylglucosaminidase activities.

Enzymic degradation of glycopeptides. — All incubations were carried out at 37°, and an atmosphere of toluene was maintained to prevent bacterial contamination. In most experiments, the glycopeptide product was subjected to a further incubation with the same enzyme, in order to ensure that the limit of action of the enzyme had been reached.

(*a*) *2-Acetamido-1-*N-(*4-*L-*aspartyl*)*-2-deoxy-*β-D-*glucopyranosylamine amidohydrolase.* The glycopeptides (containing 10–15 μmol of hexosamine) were incubated with the enzyme (0.3 unit) in 0.05M Na_2HPO_4–KH_2PO_4 buffer (pH 7.0), the total volume of the incubation mixture being 400 μl. Reducing-sugar estimations were done at intervals, and incubations were stopped by heating at 100° for 3 min when values became constant (at ~48 h). Hexosamine was then determined as 2-acetamido-2-deoxyglucose.

(*b*) α-D-*Mannosidase.* The glycopeptides (containing 20–50 μmol of mannose) were incubated in 0.1M sodium acetate buffer (pH 4.5) with jack-bean meal α-D-mannosidase (6–10 units) in the presence of 0.1M sodium chloride, 0.01% of albumin, and mM zinc sulphate, the total volume of the incubation mixture being 400 μl. Samples were removed at intervals for reducing-sugar determinations. When liberation of reducing sugar had ceased (~48 h), the reaction was stopped by heating at 100° for 3 min, and the incubation mixture was loaded on a column (26 × 1.6 cm) of Bio-Gel P2 and eluted with water. Fractions were monitored for hexose[28] and for amino acid[32]. Ninhydrin-reacting fractions were combined, freeze-dried, and assayed for hexose, hexosamine, and aspartic acid. For experiments using limpet α-D-mannosidase, the glycopeptides were incubated with 3.5 units of enzyme in 0.1M sodium acetate buffer (pH 3.5).

(*c*) β-D-*Mannosidase.* The glycopeptides (containing 6–8 μmol of mannose) were incubated in 0.125M sodium acetate buffer (pH 4.5; containing 0.1M sodium chloride and 0.01% of albumin) with limpet β-D-mannosidase (0.1 unit). The total

volume of the incubation mixture was 400 μl. Reducing-sugar determinations were made at intervals. When liberation of reducing sugar had ceased (~24 h), the incubation was stopped, and the solution was loaded on a column (24 × 1.6 cm) of Bio-Gel P2 and eluted with water. Fractions were assayed for amino acid[32], and the ninhydrin-reacting fractions were combined, freeze-dried, and analyzed.

(*d*) *β-N-Acetylglucosaminidase.* The glycopeptides (containing 15–30 μmol of hexosamine) were incubated in 0.05M sodium citrate buffer (pH 4.0; containing 0.1M sodium chloride and 0.01% of albumin) with boar epididymal *β-N*-acetylglucosaminidase (9 units). The total volume of the incubation mixture was 2 ml. At intervals, samples were assayed for 2-acetamido-2-deoxyglucose; when liberation had ceased (~24 h), the incubation was stopped, and the mixture was loaded on a column (140 × 1.6 cm) of Sephadex G25 and eluted with 0.1M acetic acid. Fractions were assayed for mannose and 2-acetamido-2-deoxyglucose. The mannose-containing fractions were combined and freeze-dried, and the product was analyzed for mannose, 2-acetamido-2-deoxyglucose, and aspartic acid. With the small glycopeptides obtained after several sequences of enzymic degradation, a column of Bio-Gel P2 was used for separation of the residual glycopeptides.

Preparation of glycopeptides. — Glycopeptides were prepared by exhaustive digestion of ovalbumin with purified Pronase. Ovalbumin (30 g) was incubated at 37° with purified Pronase (600 mg) in the presence of 15mM calcium chloride in a volume of 200 ml, the pH of the incubation mixture being maintained at 8.5. Digestion was carried out for 24 h, the digest was concentrated *in vacuo* and precipitated with 80% ethanol, and the glycopeptides were separated from the bulk of peptide material by gel filtration with Sephadex G25. The material from three such digests was combined and subjected to three further digestions (each for 48 h) with 50 mg of Pronase in the presence of 15mM calcium chloride in a volume of 25 ml, the glycopeptides being separated after each digestion. From 90 g of ovalbumin, a total of 2.3–2.5 g of mixed glycopeptides was obtained. Analysis of the mixed glycopeptides gave the following values (mol/mol of aspartic acid): hexose, 5.65; hexosamine, 3.59; leucine, 0.44; threonine, 0.12; serine, 0.02. Analysis by g.l.c. showed that 2–3% of the hexose was present as galactose.

The glycopeptides (0.6 g) were fractionated by elution from a column (150 × 2 cm) of Dowex 50 (X2) resin with mM sodium acetate buffer (pH 2.6), according to the method of Huang *et al.*[7]. Fractionation was improved by maintaining the temperature of the column at 4° and by adopting a slow pumping rate (30 ml/h). A total of 400 mg of individual fractions was eluted with mM sodium acetate buffer (Table I). These fractions all contained aspartic acid as the sole amino acid. The remainder of the glycopeptides were eluted with 0.3M sodium acetate buffer (pH 2.6), and contained carbohydrate attached to a peptide or peptides containing leucine, threonine, and serine, as well as aspartic acid. Individual fractions were further purified by one or more refractionations on the Dowex-50 column, followed by gel filtration on a column (145 × 1.8 cm) of Bio-Gel P4. The purified fractions were freeze-dried and assayed for hexose, hexosamine, and aspartic acid. None of the individual fractions had any

reducing power, confirming the absence of any products arising from possible 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine amidohydrolase or endo-β-*N*-acetylglucosaminidase action of the Pronase preparation.

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