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THE CARBOHYDRATE UNITS OF ASIALO-OVOMUCOID: THEIR HETEROGENEITY AND ENZYMIC DEGRADATION

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ABSTRACT

An ovomucoid variant free from sialic acid has been prepared in a pure state by ion-exchange chromatography on DEAE-cellulose. The purified glycoprotein contained 10–11 residues of mannose, 2–3 residues of galactose, and 21 residues of 2-acetamido-2-deoxyglucose. Glycopeptides have been prepared by exhaustive digestion with Pronase followed by ion-exchange chromatography on Dowex 50 (X2) resin. Three fractions were obtained, all with similar contents of mannose and hexosamine but with various contents of galactose. The sugar–aspartic acid ratios indicated that all of the fractions were heterogeneous, the major fraction having mannose–galactose–hexosamine–aspartic acid ratios of 2.6:0.5:5.8:1.0. Cleavage of asialo-ovomucoid with cyanogen bromide and proteolytic digestion of the isolated fragments gave two heterogeneous glycopeptide fractions of similar composition. Both asialo-ovomucoid and the principal glycopeptide fraction were degraded with β-D-galactosidase, α-D-mannosidase, and β-*N*-acetylglucosaminidase singly and in sequence. Removal of much of the carbohydrate from asialo-ovomucoid had no appreciable effect on its anti-tryptic activity. By sequential degradation of the glycopeptide, a pentasaccharide core α-D-Man-[α-D-Man]-β-D-Man-β-D-GlcNAc-β-D-GlcNAc-Asn was obtained. Other structural features revealed by enzymic degradation are discussed.

INTRODUCTION

Ovomucoid, a glycoprotein of egg-white, has been known since 1894, but it was not until 1947 that a satisfactory method for its isolation relatively free from other egg-white glycoproteins was devised[1]. Several alternative methods for the isolation and purification of ovomucoid fractions have since been published[2–6]. Most of these products have similar physical properties and amino acid composition, but vary considerably in their carbohydrate composition. The glycoprotein has a molecular weight of ~28,000 and contains 25% of carbohydrate distributed among several units, the exact number of which may vary from one preparation to another. The units, composed of mannose, galactose, and 2-acetamido-2-deoxyglucose, are attached to the polypeptide chain by an asparaginyl–2-acetamido-2-deoxyglucose linkage.

Most preparations of ovomucoid vary in their content of sialic acid, which is principally, but not entirely, responsible for the electrophoretic heterogeneity observed in the glycoprotein. Even ovomucoid variants which contain no sialic acid can show considerable variation in their total content of neutral carbohydrate.

Ovomucoid has aroused interest as a powerful inhibitor of trypsin activity[1], and the protein component has been investigated to determine which structural features are associated with this property[7,8]. Little is known of the effect on the inhibitory activity of removing carbohydrate.

We now describe the isolation and characterisation of an asialo-ovomucoid from egg-white, the preparation of glycopeptides therefrom and their fractionation, and structural features deduced from the action of exo-glycosidases on the glycopeptides. Further structural studies are described in the following paper. Since the exo-glycosidases were also able to remove the appropriate carbohydrate residues from the intact glycoprotein, the effect of such removal on the anti-tryptic activity was determined.

RESULTS

Fractionation of ovomucoid. Chromatography of crude ovomucoid on DEAE-cellulose gave three fractions (Fig. 1*a*). Fraction II contained the major part of the carbohydrate, and was less rich in sialic acid than Fraction III (Table I). Analysis at various points on the elution peak of Fraction II indicated that it contained a large

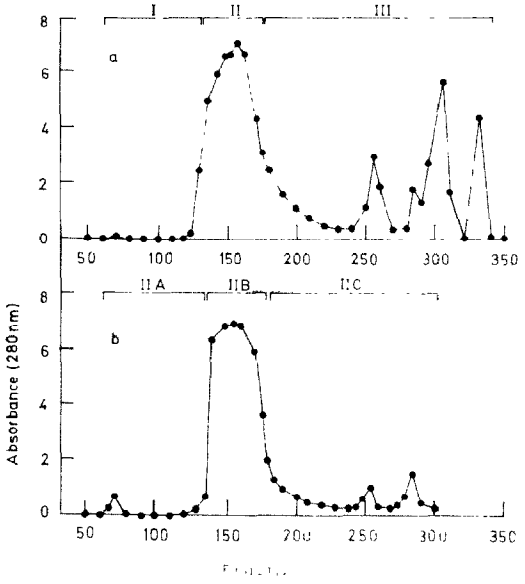


Fig. 1. Chromatography of ovomucoid on DEAE-cellulose. For details, see Experimental. Fractions (16 mL) were combined as indicated. (*a*) Fractionation of crude ovomucoid; (*b*) refractionation of Fraction II

component with no sialic acid but having the same hexosamine–hexose ratio as the total fraction. A similar detailed analysis of Fraction III showed that each of sub-fractions contained sialic acid. Fraction II was re-chromatographed on DEAE-cellulose (Fig. 1*b*). The main component (IIB) was essentially devoid of sialic acid, but had the same hexosamine–hexose ratio as the original Fraction II. All later experiments were restricted to Fraction IIB, which contained 10–11 residues of mannose, 2–3 residues of galactose, and 21 residues of 2-acetamido-2-deoxyglucose (Table II).

Fractions II and IIB were subjected to electrophoresis on polyacrylamide gel at pH 4.5, 6.5, and 9.5. Fraction II contained several components, but Fraction IIB showed only one major band at each pH value, with slight traces of a minor component at pH 9.5. When gel electrophoresis was carried out in the presence of sodium dodecyl-sulphate, the mobility of Fraction IIB was consistent with a molecular weight of 29,000 $\pm$500. With the exception of the minor fraction I, the fractions had similar anti-tryptic activities (Table I). No fractions showed anti-chymotryptic activity.

Action of exo-glycosidases on ovomucoid IIB. In contrast to the experience of some workers, it proved possible to remove peripherally situated sugars from ovomucoid by the action of exo-glycosidases (Table III). All of the galactose could be

TABLE I

CARBOHYDRATE COMPOSITION OF OVOMUCOID FRACTIONS

Fraction	*Yield (%)*	*Hexose (g/100g)*	*GlcNAc (g/100g)*	*NANA (g/100g)*	*Hexosamine/ Hexose*[a]	*Antitryptic activity (mg)*
Crude ovomucoid	—	9.10	16.50	0.48	1.47	0.674
I	1.0	2.56	2.76	0.06	0.88	1.015
II	75.6	9.09	16.15	0.34	1.40	0.542
III	17.7	7.85	10.40	0.68	1.08	0.633
IIB	85.7	9.10	16.55	0.09	1.47	0.516

[a]Ratios are molar.

TABLE II

MOLAR CARBOHYDRATE COMPOSITION OF MAJOR OVOMUCOID FRACTIONS

Fraction	*Man*	*Gal*	*GlcNAc*	*Man/Gal*	*GlcNAc/Man*	*GlcNAc/Gal*
	(mol/mol of ovomucoid)					
II	10.8	3.0	20.0	3.63	1.85	6.67
III	8.2	2.8	12.9	2.94	1.57	4.60
IIB	10.5	2.4	21.1	4.52	2.01	8.79

TABLE III

ACTION OF GLYCOSIDASES ON OVOMUCOID

	Sugar released (%)			*Trypsin inhibition (% of control)*
	Gal	*Man*	*GlcNAc*	
α-D-Mannosidase		0	--	100
β-*N*-Acetylglucosaminidase	--	--	56 (12)[a]	97
β-D-Galactosidase	96 (2.4)	--	--	100
β-*N*-Acetylglucosaminidase	-	-	56 (12)	95
α-D-Mannosidase	--	44 (4.6)	56 (12)	97
β-*N*-Acetylglucosaminidase	--	-	56 (12)	97
β-D-Galactosidase	100 (2.4)	--	56 (12)	95
β-*N*-Acetylglucosaminidase	100 (2.4)	-	71 (15)	90

[a]Figures in brackets are the number of residues removed. The original glycoprotein contained 10.5 mannose, 2.4 galactose, and 21.1 hexosamine residues.

TABLE IV

COMPOSITION[a] OF GLYCOPEPTIDE FRACTIONS

	Mixed glycopeptides	*Fractions*			*P1*	*P2*
		A[b]	*B*	*C*		
Aspartic acid	1.0	1.0	1.0	1.0	1.0	1.0
Threonine	0.3	0.3	0.3	0.3	0.3	0.3
Proline	0.3			0.3		
Glycine	0.2	0.2		0.1	0.3	0.1
Phenylalanine	0.2		0.1	0.4		
Half-cystine						0.1
Mannose	2.9	4.4	3.1	2.6	5.7	3.0
Galactose	0.6	1.6	0.8	0.5		
Hexosamine	6.0	10.1	5.9	5.8	8.7	5.9
Man/Gal	4.8	2.8	3.9	5.2		
Hexosamine/Man	2.1	2.3	1.9	2.2		
Yield (%)		10	33	56	10	68

[a]Mol per mol of aspartic acid. [b]Fractions A, B, and C were obtained by elution of a column of Dowex 50 (X2) with a sodium acetate gradient (Fig. 2), P1 and P2 by elution of a column of Dowex 50 (X2) with a water and pyridine formate gradient (Fig. 3).

removed from the intact glycoprotein, indicating that none was internally located. The mannose residues, on the other hand, were all internally located and could only be removed after removal of some of the hexosamine residues. More than half of the hexosamine residues were peripheral, and results indicated that preliminary removal of galactose rendered more hexosamine residues liable to attack by 2-acetamido-2-deoxy-β-D-glucoside 2-acetamido-2-deoxy-β-D-glucohydrolase (β-*N*-acetyl-

glucosaminidase). Removal of the sugars had little effect on the trypsin inhibitory activity of the glycoprotein (Table III).

Fractionation of glycopeptides. In the ovomucoid molecule, there are 25–32 residues of aspartic acid[4,8] of which only a few (2–5) carry carbohydrate units. In order to relate sugar ratios to glycosylated aspartic acid residues, it was therefore necessary to digest the glycoprotein exhaustively with Pronase in order to ensure removal of all non-glycosylated aspartic acid residues. After digestion, the mixed glycopeptides contained aspartic acid as the major amino acid component, with threonine, proline, glycine, and phenylalanine present in much smaller amounts (Table IV). There was therefore a preponderance of glycopeptides having aspartic acid as the sole amino acid component.

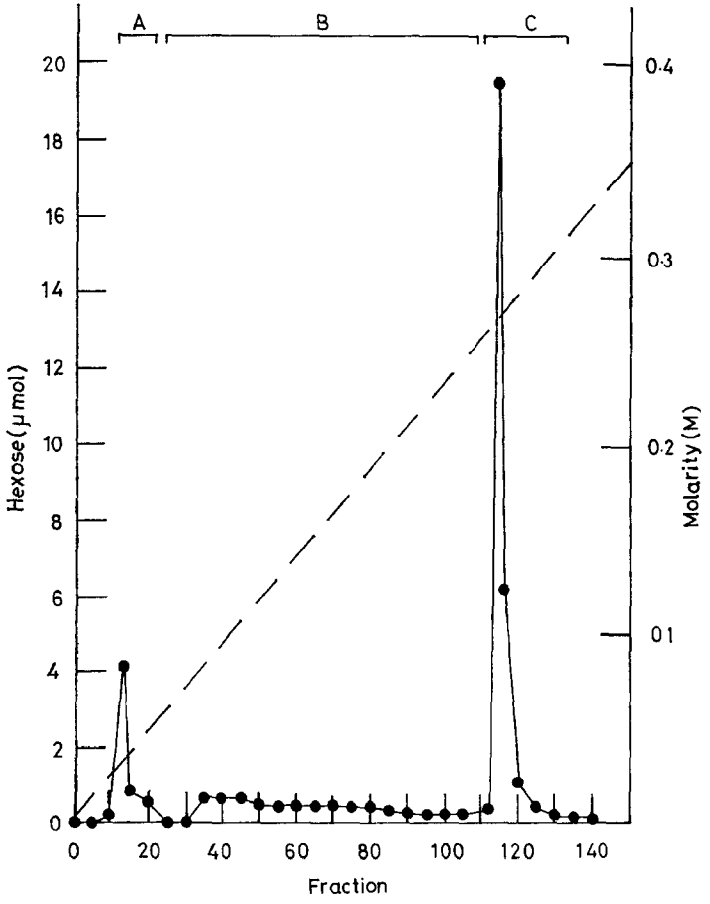


Fig. 2. Fractionation of asialo-ovomucoid glycopeptides on Dowex 50 (X2) resin. Fractions (4 mL) were eluted with a gradient of sodium acetate buffer (pH 2.6) (see Experimental), combined as indicated, and purified by refractionation on Dowex 50 (X2) resin and by gel filtration on Sephadex G-25.

TABLE V

MOLECULAR WEIGHTS OF GLYCOPEPTIDES

Glycopeptide[a]	*Molecular weight*[b]		*Calculated molecular weight*[c]
	Sephadex G-25	*Sephadex G-50*	
A	2190 200	2000 200	3196
C	2130	2000	1937
P1	2300	2140	2870
P2	2160	2040	1915

[a]For key, see Table IV. [b]For description of method, see Experimental; the mean of three determinations. [c]Calculated from compositional analysis, Table IV.

Fractionation of the mixed glycopeptides on Dowex 50 resin (gradient elution with sodium acetate buffer, pH 2.6) gave three fractions (Fig. 2). The first fraction (A) was always present in small quantity, accounting for 10% or less of the total glycopeptides. The elution pattern of Fraction B indicated considerable heterogeneity, whereas that of Fraction C, which comprised the major part of the glycopeptide mixture, showed less evidence of heterogeneity. Each of the three glycopeptide fractions, even after re-chromatography on Dowex 50, had carbohydrate–aspartic acid ratios which indicated that the fractions were heterogeneous (Table IV). Much of this heterogeneity appeared to be due to variation in the galactose content of the fractions, since their mannose–hexosamine ratios were similar.

Further analysis of Fraction A produced conflicting results. The mannose and hexosamine contents of A were similar to those of B and C, suggesting that each fraction contained carbohydrate units of similar size. However, the aspartic acid content of A was much lower than that of B and C, so calculations of carbohydrate ratios related to one residue of aspartic acid indicated a much larger carbohydrate unit in A. Molecular weight determinations, based on exclusion patterns from calibrated columns of Sephadex G-25 and G-50, were made on A and C (Table V). These glycopeptides had similar molecular weights, and the values indicated carbohydrate units of a size similar to that calculated from the analysis for C. A possible explanation for this discrepancy might be that a slight loss of aspartic acid had occurred from the original, mixed glycopeptides due to traces of glycoaspartamidase or endo-glucosaminidase in the Pronase preparation. Such trace activity might only become significant during exhaustive proteolytic digestion. The resulting small amount of oligosaccharide could then have co-chromatographed with the small fraction of glycopeptide eluted from the Dowex column by the buffer of lowest ionic strength.

Fractionation of the mixed glycopeptides was also carried out on Dowex 50, using gradient elution with a pyridine-formic acid buffer (pH 3.5) (Fig. 3). The procedure was similar to that used by Montreuil to obtain his homogeneous glycopeptide fraction[9]. When water was used as eluant prior to application of the buffer,

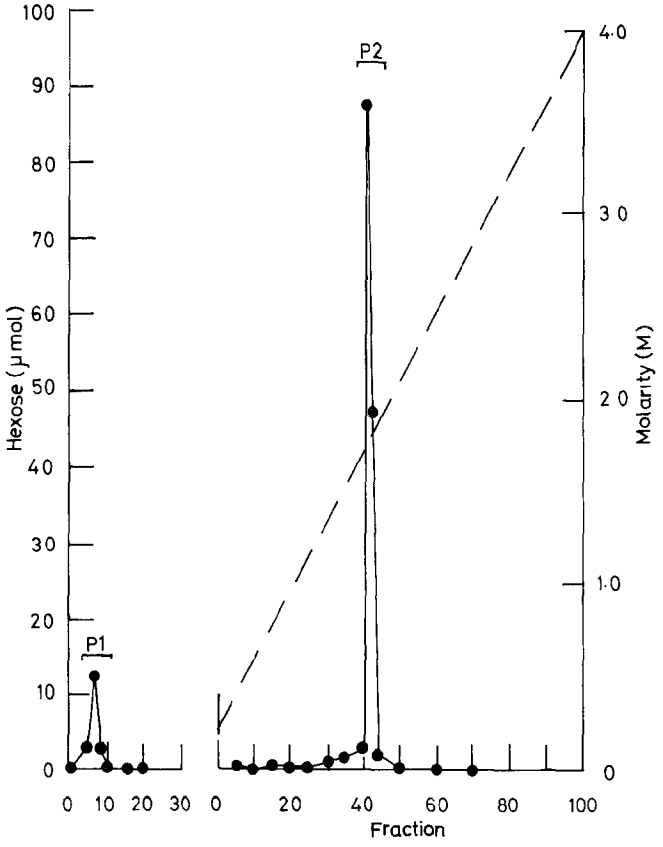


Fig. 3. Fractionation of asialo-ovomucoid glycopeptides on Dowex 50 (X2) resin (H^+ form). Fractions (10 mL) were eluted with (*a*) water to give combined Fraction P1, and (*b*) a gradient of pyridine formate buffer (see Experimental) to give Fraction P2. Combined fractions were purified by gel filtration on Sephadex G-25.

a small fraction (P1), accounting for 11% of the total glycopeptides, was obtained. This fraction had a low content of aspartic acid and contained very small amounts of other amino acids. The ratios of carbohydrates–aspartic acid were similar to those found in Fraction A (Table IV). The bulk of the glycopeptides (68%) was eluted with the buffer as a single fraction (P2) which had a carbohydrate and amino acid analysis similar to that of C. The molecular weights of P1 and P2 (Table V) were similar to those determined for A and C.

Peptide sequence of Fraction C. The only glycopeptide fraction with appreciable amounts of amino acids other than aspartic acid was C. When this fraction was assayed for *N*-terminal amino acids by the dansylation technique, it was found that aspartic acid, phenylalanine (Phe), and threonine (Thr) were in terminal positions. After an Edman degradation, Phe and Thr were released. No phenylthiohydantoin (PTH) derivative of aspartic acid was observed, but analysis of the residual glycopeptide showed that 60% of the original aspartic acid had been released, and presum-

ably was attached to carbohydrate because the hexose content of the residue showed a similar fall in value. Dansylation of this residual glycopeptide indicated proline (Pro) as the main *N*-terminal amino acid, with traces of Thr. A second Edman degradation removed both Pro and Thr, and analysis of the residue confirmed this as well as showing that there had been no loss of aspartic acid. Third and fourth Edman degradations gave no further release of amino acids as PTH derivatives. From these results, it appeared that the major part of C consisted of Asn-(Carbohydrate), with smaller amounts of Thr.Asn-(Carbohydrate) and Phe.Pro.Asn-(Carbohydrate) also present.

Glycopeptides from CNBr fragments. Other glycopeptides were obtained from individual fragments of ovomucoid after its cleavage with cyanogen bromide. After cleavage, two fragments (II and III) as well as unchanged ovomucoid (I) were obtained (Fig. 4). Each fragment was digested with Pronase and the products were fractionated on Dowex 50 resin with a gradient of sodium acetate buffer as eluant (Figs. 5*a* and 5*b*). A single, major carbohydrate-containing glycopeptide was obtained from each fraction and analysis (Table VI) showed them to be of similar composition. The molar ratios obtained suggested that heterogeneity probably occurred at all sites of glycosylation

Action of exo-glycosidases on Fraction C. The action of purified exo-glycosidases acting singly and in sequence on Fraction C is shown in Table VII. Consideration of the molecular weights and the results of analysis led to the conclusion that Fraction C comprised two glycopeptides each containing three mannose residues but only one containing galactose. Residues removed by glycosidase action were therefore calculated on the basis of an initial content of six mannose residues.

There were no terminally located mannosyl residues in Fraction C, but galactose was in a terminal position. All of the galactose in Fractions A and B, which had relatively greater contents of galactose, was removed equally easily and was therefore

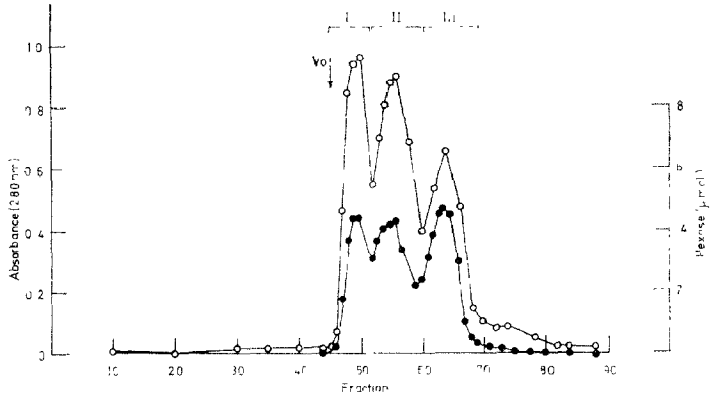


Fig. 4. Fractionation on Sephadex G-25 of fragments produced by cleavage of asialo-ovomucoid with cyanogen bromide. Fractions (4.5 mL) were monitored for absorbance at 280 nm (- -○- -) and hexose (—●—), and combined as indicated. V_0 is the void volume of the column, determined with Blue Dextran

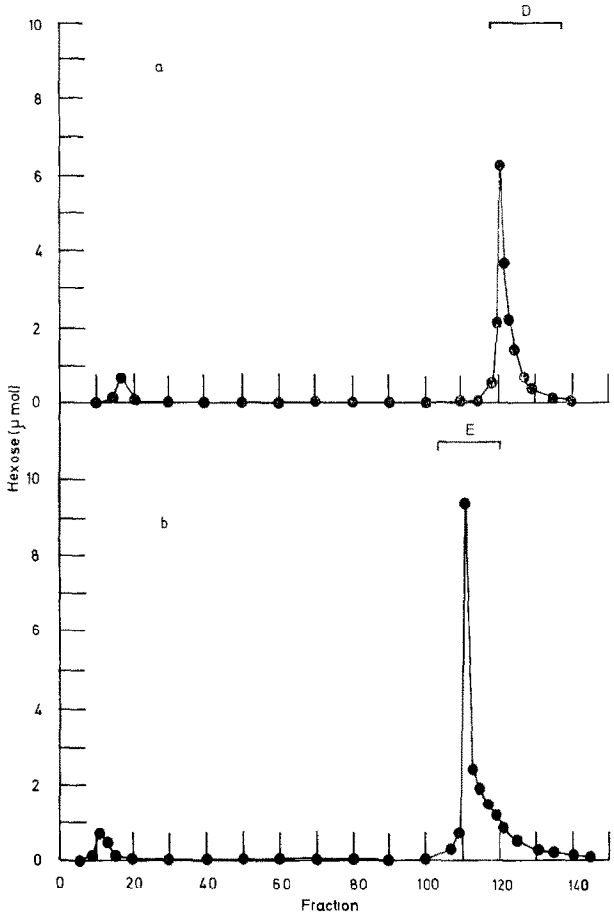


Fig. 5. Fractionation on Dowex 50 (X2) resin of glycopeptides from CNBr-cleavage fragments of asialo-ovomucoid: (*a*) Fraction II and (*b*) Fraction III. Fractions (4.5 mL) were eluted with a gradient of sodium acetate buffer (see Experimental), combined as shown, and purified by gel filtration on Sephadex G-25.

terminal or linked to a terminal galactose residue. In C, nine of the 13–14 hexosaminyl residues were either terminal or adjacent to terminal hexosaminyl groups and could be removed directly by β-*N*-acetylglucosaminidase. This loss of nine hexosamine residues permitted the release of three mannose residues. Removal of galactose from the original glycopeptides did not allow the release of any mannose, but permitted the release of an additional hexosamine residue, indicating that galactose had been β-linked to a hexosaminyl residue. When this latter residue was removed, a further mannose residue could be released, indicating that the Gal-GlcNAc group had been β-linked to an unsubstituted α-mannosyl residue. No further hexosamine could be released after removal of this mannose residue, nor could the remaining two mannose residues be removed by α-D-mannosidase. Analysis of the residual glycopeptide, as

TABLE VI

COMPOSITION OF GLYCOPEPTIDES FROM PRONASE DIGESTION OF CNBr FRAGMENTS

	Molar ratios[a]			*Molar ratios*	
	D	*E*		*D*	*E*
Aspartic acid	1.0	1.0	Mannose	3.4	3.3
Threonine	0.6	0.3	Galactose	0.6	0.6
Proline		0.5	Hexosamine	6.5	6.6
Glycine	0.4	0.1	Man/Gal	5.7	5.5
Alanine	0.1	0.1	Hexosamine/Man	1.9	2.0
Half-cystine	0.3	0.1			

[a]Molar ratios calculated with respect to 1 mol of aspartic acid.

TABLE VII

ACTION OF GLYCOSIDASES ON GLYCOPEPTIDE C

Enzyme	*Residual glycopeptide composition*[a]		
	Man	*Gal*	*GlcNAc*
Control	6.0	1.2	13.3
α-D-Mannosidase	6.0	1.2	13.3
β-D-Galactosidase	6.0	0.1	13.3
β-*N*-Acetylglucosaminidase	6.0	1.2	4.6
α-D-Mannosidase	3.2	1.2	4.6
β-*N*-Acetylglucosaminidase	3.2	1.2	4.6
β-D-Galactosidase	6.0	0.2	13.3
α-D-Mannosidase	6.0	0.2	13.3
β-*N*-Acetylglucosaminidase	6.0	0.2	3.5
α-D-Mannosidase	2.8	0.2	3.5

[a]Ratios for sugars in residual glycopeptide were calculated on the basis of six mannose residues in the original control, since there were two glycopeptides present (see Results).

well as the results from methylation analysis (reported in the following paper), suggested that the basic core common to each glycopeptide of the original fraction contained a β-linked mannosyl residue attached *via* a di-*N*-acetylchitobiose residue to asparagine.

When carrying out digestion of the glycopeptides with β-*N*-acetylglucosaminidase from jack-bean meal, it was observed that, for a given concentration of enzyme, there was a concentration of glycopeptide above which no release of hexosamine was obtained (Table VIII). When solutions containing glycopeptide in concentrations exceeding this value were tested for enzyme activity with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as substrate, little or no enzyme

TABLE VIII

EFFECT OF GLYCOPEPTIDE CONCENTRATION ON RELEASE OF HEXOSAMINE BY β-*N*-ACETYLGLUCOSAMINIDASE[a]

Wt. of glycopeptide (mg)	*Hexosamine content (μmol)*	*Hexosamine released (% of total)*		
		24 h	*48 h*	*120 h*
0.423	1.07	51	53	53
0.846	2.14	31	59	65
1.693	4.28	18	39	59
2.539	6.42	9	22	44
3.385	8.56	0	0	0
4.485	11.35	0	0	0

[a]Each incubation solution contained 3.2 units of β-*N*-acetylglucosaminidase in a final incubation volume of 150 μL (see Experimental for details).

activity was observed. In the presence of lower concentrations of glycopeptide, the enzyme activity over the period of incubation, as measured with the synthetic substrate, differed little from that observed in a control incubation containing no glycopeptide.

DISCUSSION

Investigations on the structure of the carbohydrate units of ovomucoid have always been greatly frustrated by the difficulty of obtaining homogeneous glycopeptides. This stems from the extreme variability of ovomucoid preparations. The most characteristic feature of early investigations was the considerable variation in the sialic acid content of different samples, depending on the method of preparation[8]. More recently, ovomucoid has been separated by electrophoresis[6] and by ion-exchange chromatography[4] into several fractions, all of which appear to have similar amino acid composition, but which vary in the proportions of some of their carbohydrate components. Both techniques gave a preponderance of ovomucoid variants free from sialic acid. This also proved to be the case for our preparation, where precautions were taken to avoid acid conditions that might cause removal of sialic acid. All asialo-ovomucoids prepared have had a small but definite proportion of galactose. The fact that sialo-ovomucoids have a higher proportion of galactose may indicate that these variants represent molecules in the biosynthetic pathway which have been processed more completely. The smaller variation in carbohydrate composition of asialo-ovomucoid, together with its preponderance in ovomucoid preparations, makes it the most suitable fraction for structural investigations.

Heterogeneity is of general occurrence in ovomucoids from a variety of birds[10]. This finding supports the idea of the existence of multiple forms of ovomucoid, rather than that of a single form mixed with non-ovomucoid contaminants. Moreover, the fact that heterogeneity has been found in ovomucoid from the eggs of

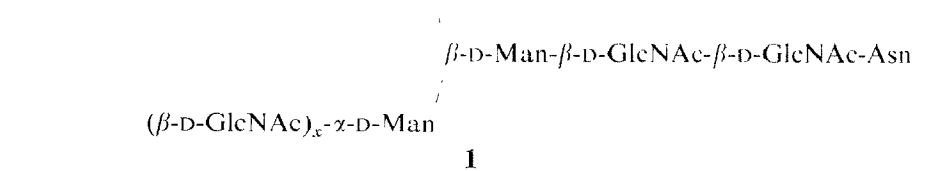
individual hens[10] is evidence that heterogeneity does not arise from mixtures of eggs with different phenotypes.

It is increasingly accepted that, for the carbohydrate units of all glycoproteins, heterogeneity is the rule rather than the exception. Recently, it has been shown that ovomucoid glycopeptides isolated from different sites of glycosylation have similar carbohydrate composition and show heterogeneity[11]. Similar results were obtained when our preparation was cleaved with cyanogen bromide, and glycopeptides from isolated fragments were examined. Even structural heterogeneity can occur at single glycosylation sites. A recent study of sixteen glycopeptides from α_1-acid glycoprotein concluded that carbohydrate units of different structure must have arisen from the same glycosylation site[12]. Ovalbumin carbohydrate units, which originated from a single glycosylation site, also showed considerable heterogeneity[13,14], and even ovalbumin glycopeptides that appeared homogeneous by ion-exchange chromatography, electrophoresis, and ultracentrifugation were heterogeneous[15].

None of the fractionation procedures described herein has resulted in the isolation of glycopeptides having sugar ratios that are whole numbers with respect to aspartic acid. The glycopeptides had similar contents of mannose and 2-acetamido-2-deoxyglucose, but showed variation in their contents of galactose. This lack of whole number ratios, particularly for galactose, is characteristic of almost all ovomucoid glycopeptides isolated so far. It is not satisfactory to relate the sugar ratios to galactose, since some glycopeptides almost certainly have no galactose. Moreover, since it is the carbohydrate present in much the smallest amount, small errors in its assay will produce considerable variation in the ratios of mannose and hexosamine.

Because of the impossibility of obtaining homogeneous products, our investigations of structure were performed on glycopeptides in their heterogeneous state. It was hoped that the action of glycosidases might result, at some stage of the degradation, in the production of a homogeneous fraction that would provide an indication as to the sites of heterogeneity. However, the large number of hexosamine residues which were found to be peripherally located in the glycopeptide made it clear that such degradative studies would have to be allied to methylation analysis before the finer details of the glycopeptide structure could be elucidated.

While the results described do not permit the assignment of any definitive structures, they allow certain features to be noted. If Fraction C contains two glycopeptides, only one of which contains galactose, then each has the pentasaccharide core common to most asparaginyl–carbohydrate glycopeptides, and galactose and hexosamine are in the peripheral locations as shown in **1**.



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The results leave undecided the question as to whether, in cases where x is greater than 1, the hexosaminyl residues are linked to each other or to different positions of the α-linked mannosyl residues. They also leave open the possibility that there are terminal hexosaminyl groups attached to O-4 of the β-linked mannosyl residues (as is the case for ovalbumin glycopeptides) and to the hexosaminyl residue to which the terminal galactosyl group is attached. These points have been elucidated by methylation analysis of the various degraded glycopeptides (see following paper).

EXPERIMENTAL

General methods. — Hexose was determined by the phenol–sulphuric acid method[16]. Mannose and galactose were determined, after hydrolysis with 2M HCl for 3 h, as their alditol acetates by g.l.c.[17]. These hydrolysis conditions gave maximum yields of mannose and galactose. For g.l.c., a glass column (1 m × 0.2 cm) of 5% of SP 2340 on Gas Chrom Q (100–200 mesh) was used. This column, when programmed from 210–240° at 2°/min with an initial post-injection period of 3 min, gave satisfactory separation of mannose and galactose derivatives together with reasonably rapid removal of the hexosamine derivative from the column. Hexosamine, reducing sugar, and amino acid determinations were carried out as described previously[18]. Determinations of hexosamine after hydrolysis with *p*-toluenesulphonic acid[19] gave results identical to those obtained by our standard procedure. Protein determinations were carried out by the method of Lowry *et al.*[20], with bovine albumin as standard. Pronase (Cambrian Chemicals Ltd.) was purified by acetone fractionation[21] before use, and had no detectable β-D-galactosidase, α-D-mannosidase, or β-*N*-acetylglucosaminidase activities.

Sialic acid determination. — Sialic acid was determined, after release by hydrolysis in 0.05M H_2SO_4 at 80° for 1 h, by a modification of the method of Warren[21]. Sodium sulphate was omitted from the sodium arsenite and thiobarbituric acid solutions. Following colour development, 2-ethoxyethanol (2.7 mL) was added to the cooled reaction mixture (4.3 mL). After mixing, the absorbance at 545 nm was measured. A standard curve was prepared with *N*-acetylneuraminic acid. The intensity of colour produced was 16% lower than that obtained after extraction with cyclohexanone (7 mL) as in the original procedure. Since other carbohydrates may interfere in this method, especially when the carbohydrate–*N*-acetylneuraminic acid ratio is >5:1, acid hydrolysates were first passed through a column of Dowex 1 (X10) resin as described by Whitehouse and Zilliken[23]. The fraction eluted with the buffer was lyophilised, the dried material was dissolved in water (3 mL), and the sialic acid content was determined.

Trypsin inhibition. — Tryptic activity was measured as described by Laskowski[24], using Hammerstein casein (British Drug Houses Ltd.) as substrate and bovine trypsin (Miles Laboratories Ltd.). Anti-tryptic activity of the ovomucoid fractions was expressed as the weight required to inhibit completely 1 mg of trypsin. Chymotrypsin inhibition was measured as described by Erlanger *et al.*[25].

Polyacrylamide gel electrophoresis. — Polyacrylamide gel electrophoresis was carried out according to the method described by Toombs and Akroyd[26], with a Shandon analytical polyacrylamide gel electrophoresis apparatus. Optimum resolution was obtained with 7.5% gels. The gel buffers were 0.4M Tris-glycine (pH 9.5), 0.4M Tris-H_3PO_4 (pH 6.5), and 0.23M glycine–acetic acid (pH 4.5). Electrophoresis was carried out at a constant current of 5 mA per gel for the duration of the run (25–110 min). Gels were stained with 0.25% Coomassie Brilliant Blue R250 in methanol–water–acetic acid (5:5:1); destaining was performed with the same solvent, the final washing being with 7% acetic acid.

For determinations of molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate, the method of Weber and Osborn[27] was followed, using a 10% gel concentration and cytochrome *c* (ex horse heart, Koch-Light Laboratories Ltd.) as internal marker. Electrophoresis was carried out at a constant current of 6 mA per gel for 4 h.

Determination of molecular weight by gel chromatography. — A column (91 × 1.6 cm) of Sephadex G-25 was prepared and equilibrated with 0.1M acetic acid at 20°. The column was standardised with sucrose (mol. wt. 342), raffinose (504), stachyose (666), ovalbumin glycopeptide E (1528), and fetuin glycopeptide (2380). Cytochrome *c* (mol. wt. 21,000) was used to determine the void volume, V_0. Each sample (2 mg) was dissolved in 0.1M acetic acid (0.5 mL) and applied to the column. The column was eluted with 0.1M acetic acid at 10 mL per h, and 1-mL fractions were collected, and monitored for carbohydrate by the phenol–sulphuric acid method and for cytochrome *c* by absorbance at 418 nm. A calibration curve for the column was obtained by plotting V_e/V_0 *versus* $\log_{10}$ M for each of the standards. Determinations were repeated using a calibrated column (90 × 1.6 cm) of Sephadex G-50 prepared and equilibrated with 0.1M acetic acid.

Peptide sequencing. — Amino acid sequencing of the peptide moiety of Glycopeptide C was performed by the direct Edman technique, as described by Blömback *et al.*[28]. The phenylthiohydantoins were identified by comparison of their t.l.c. mobilities (silica gel) with those of standards, using (*1*) chloroform–methanol (9:1), and (*2*) chloroform–methanol–acetic acid (70:30:2). *N*-Terminal amino acids were identified by the dansylation technique described by Woods and Wang[29]. Chromatography was performed on 5 × 5 cm polyamide sheets with the following solvents: 1st vector, water–90% formic acid (200:3); 2nd vector, toluene–acetic acid (9:1). Elution in the second vector was repeated with butyl acetate–methanol–acetic acid (20:1:1) for improved separation.

Purification of exo-glycosidases. — All assays of enzyme activity were performed at 37°. One unit of activity is defined as that which causes cleavage of 1 μmol of substrate/min under the appropriate assay conditions. Activities were determined as previously described[30] with the appropriate nitrophenyl glycopyranoside as substrate.

(*a*) *2-Acetamido-2-deoxy-β-*D*-glucoside 2-acetamido-2-deoxy-β-*D*-glucohydrolase (β-N-acetylglucosaminidase, EC 3.2.1.30).* This enzyme was prepared from boar

epididymis as described by Findlay and Levvy[31]. Further purification was necessary to remove traces of α-D-mannosidase and β-D-galactosidase activity. The preparation was dialysed against 0.05M sodium phosphate buffer (pH 6.5) and loaded on a column (27 × 1.6 cm) of Sephadex CM-25 equilibrated in the same buffer. Elution was carried out with (*1*) 0.05M sodium phosphate buffer (pH 6.5) and (*2*) the same buffer containing 0.1M sodium chloride. The fraction in the latter eluant, which contained the major part of the β-*N*-acetylglucosaminidase activity, was washed on an Amicon PM 10 filter with phosphate buffer to remove sodium chloride, concentrated, and refractionated on the same Sephadex column with eluant (*2*). After washing and concentration, the preparation was further fractionated with acetone, the fraction precipitating between 20 and 35% saturation limits being taken. The precipitate was dissolved in 0.05M sodium citrate buffer (pH 5.0). The preparation contained no detectable β-D-galactosidase or α-D-mannosidase activity, and had β-*N*-acetylglucosaminidase activity of 57 units per mg of protein.

(*b*) *β-D-Galactoside galactohydrolase* (*β-D-galactosidase, EC 3.2.1.23*). β-D-Galactosidase in jack-bean meal is closely associated with α-D-mannosidase and β-*N*-acetylglucosaminidase, and had to be extensively purified to remove traces of these enzymes. An acetone-dried powder was prepared from jack-bean meal and extracted as described by Snaith and Levvy[32]. The extract, after freezing and thawing, was centrifuged and the supernatant subjected to pH adjustments and heat treatment as described by Li and Li[33]. The precipitate obtained between the fractionation limits of 40 and 60% saturation with ammonium sulphate was dissolved in, and dialysed against, 0.05M sodium phosphate buffer (pH 7.0). The enzyme solution was loaded on a column (53 × 1.6 cm) of DEAE-Sephadex A-50 equilibrated in 0.05M sodium phosphate (pH 7.0), and elution was carried out with the same buffer. β-D-Galactosidase was not adsorbed and was collected. The β-D-galactosidase-containing fraction was recycled twice, after which it was free from α-D-mannosidase and β-*N*-acetylglucosaminidase. The fraction was concentrated by ultrafiltration, using an Amicon stirred cell with a PM 10 membrane. The activity of the preparation was 1.0 unit/mg of protein.

(*c*) *α-D-Mannoside mannohydrolase* (*α-D-mannosidase, EC 3.2.1.24*). α-D-Mannosidase was prepared from jack-bean meal as described by Snaith and Levvy[32]. The product still contained a small proportion of β-D-galactosidase. The purified enzyme was heated with pyridine at 37° for 20 min (2 mL of pyridine per 10 mL of enzyme solution). After cooling and centrifugation, the supernatant solution was brought to 60% saturation with acetone and allowed to stand for 15 min at 0°. The precipitate was collected, and dissolved in 0.05M sodium acetate buffer (pH 5.0) containing 0.1M sodium chloride and 0.1mM zinc sulphate. The above treatment, while decreasing the specific activity of the original, purified α-D-mannosidase to about one-third, effectively removed all traces of β-D-galactosidase. The final preparation had an α-D-mannosidase activity of 13 units/ mg of protein.

Preparation of asialo-ovomucoid. — Ovomucoid was prepared, using the method of Lineweaver and Murray[1], from the separated egg-white of unfertilised

eggs (Honneger White strain), collected within 18 h of laying. At each precipitation stage, care was taken to exclude extraneous material by collecting precipitates as soon as separation appeared to be complete, rejecting cloudy supernatant-solutions. The yield was 0.7 g from 100 mL of egg-white.

Purification of the crude ovomucoid preparation was accomplished by fractionation on DEAE-cellulose. A column (61 × 5.3 cm) of DEAE-cellulose DE-52 (Whatman Biochemicals Ltd.) was equilibrated in mM sodium phosphate buffer (pH 7.0). Ovomucoid (9.5 g) was dissolved in water (30 mL) and dialysed against 2 L of the same buffer for 24 h at room temperature with four changes of buffer. The dialysed solution (60 mL) was loaded on the column, and elution was carried out with the buffer at a flow rate of 65 mL/h, the eluate being continuously monitored for absorbance at 253 nm. Fractions (16 mL) were collected and combined as shown (Fig. 1).

After the first peak had been obtained (Fig. 1*a*, Fraction I), elution was continued with 0.05M sodium phosphate buffer (pH 7.0) until fractions corresponding to a second peak had been eluted (Fraction II), after which, elution was continued with a linear gradient consisting of equal volumes of M sodium sulphate and 0.05M sodium phosphate buffer (pH 7.0) in the reservoir and mixing chamber, respectively (Fraction III). All combined fractions were dialysed against distilled water, lyophilised, and analysed. Fraction II was refractionated by the same procedure (Fig. 1*b*). Fraction IIB was taken as asialo-ovomucoid for further study.

Preparation of glycopeptides. — Glycopeptides were prepared from asialo-ovomucoid by exhaustive digestion with purified Pronase. Asialo-ovomucoid (2.5 g) was incubated at 37° with purified Pronase (50 mg) in the presence of 10mM calcium chloride in a volume of 125 mL, the pH of the incubation solution being maintained at 8.0. Toluene was added to inhibit bacterial growth. The rate and extent of proteolysis was followed by measurement of released α-amino groups with a ninhydrin assay[34]. After 72 h, when no further release of α-amino groups occurred, incubation was terminated by adjusting the solution to pH 4.5 with acetic acid, and the solution was concentrated to 10 mL *in vacuo* at 40°. Glycopeptides were precipitated by the addition of ethanol, to give a concentration of 80%, and, after storage for 24 h at 0°, the precipitate was collected, dissolved in 0.1M acetic acid (5 mL), and separated from the bulk of peptide material by gel filtration on a column (35 × 2.6 cm) of Sephadex G-25 equilibrated in 0.1M acetic acid. The hexose-containing fractions were combined and lyophilised. To obtain maximum proteolysis, it was necessary to perform three incubations of 72 h, the glycopeptides being isolated after each incubation. The yield of final product was 0.6 g and this contained ~80% of the original carbohydrate content.

Fractionation of glycopeptides. — The glycopeptides (0.1 g) were fractionated by elution from a column (150 × 2 cm) of Dowex 50 (X2) resin equilibrated in sodium acetate–acetic acid buffer (pH 2.6) and mM in Na^+. Elution was with a linear gradient produced from equal volumes (300 mL) of sodium acetate-acetic acid buffers mM and 350mM in Na^+ in the mixing chamber and reservoir, respectively. The flow rate

was maintained at 25 mL/h. Fractions (4 mL) were monitored for hexose and combined as shown in Fig. 2. Combined fractions were purified by one or more refractionations on the column of Dowex 50. The fractions were concentrated *in vacuo*, desalted by gel filtration with Sephadex G-25, and lyophilised.

A further sample of mixed glycopeptides (150 mg) was loaded on a column (150 × 2 cm) of Dowex 50 (X2) resin (H^+ form) which had been well washed with water before use. Elution was carried out with (*a*) water (1 L) and (*b*) a gradient produced by equal volumes (500 mL) of 0.2M pyridine formate buffer (8 mL of pyridine, 12.5 mL of formic acid, 479.5 mL of water) and 4M pyridine formate buffer (160 mL of pyridine, 250 mL of formic acid, 90 mL of water) in the mixing chamber and reservoir, respectively. The flow rate was maintained at 30 mL/h. Fractions (10 mL) were collected, monitored for hexose, and combined as shown (Fig. 3). The fractions were evaporated *in vacuo* with addition of water, purified by gel filtration on Sephadex G-25, and lyophilised.

Glycopeptides from CNBr-cleavage fragments. — Ovomucoid (150 mg) in 70% aqueous formic acid (15 mL) was treated with cyanogen bromide (150 mg), as described by Beeley[7]. The resulting fragments were separated on a column (125 × 2.6 cm) of Sephadex G-25 equilibrated in 4M acetic acid. Elution was at a flow rate of 20 mL/h. Fractions (4.5 mL) were collected, and their hexose content and absorbance at 280 nm were determined. Fractions II and III (Fig. 4) were combined as shown and, after lyophilisation, were refractionated on the Sephadex column. Three further samples of ovomucoid were treated, and Fractions II and III from each sample were combined to give total yields of 108 and 103 mg, respectively.

Fractions II and III were each digested for 48 h with Pronase, as described for ovomucoid. The digests were fractionated on a column (35 × 2.6 cm) of Sephadex G-25 equilibrated in 0.1M acetic acid. Elution was at a flow rate of 25 mL/h and 4.5-mL fractions were collected. The hexose-containing fractions were combined, concentrated, digested with Pronase for a further 48 h, and again separated from released peptides on Sephadex G-25. The glycopeptide mixture was loaded on a column (150 × 2 cm) of Dowex 50 (X2) resin and eluted with sodium acetate buffers as described above (Figs. 5*a* and 5*b*). Only one major carbohydrate-containing fraction was obtained from each of the original Fractions II and III. Analysis of the fractions is given in Table VI.

Action of glycosidases on ovomucoid and glycopeptides. — All incubations were carried out at 37° in an atmosphere of toluene to prevent bacterial contamination. In most experiments, the substrates were subjected to further incubations with the enzyme in order to ensure that the limit of action of the enzyme had been reached.

(*a*) *β-N-Acetylglucosaminidase.* Ovomucoid (150 mg containing 88.4 μmol of hexosamine) was incubated with enzyme (80 units) in 0.05M sodium citrate buffer (pH 4.5) containing 0.1M NaCl and 0.01% of bovine plasma albumin, the total volume of the incubation mixture being 5 mL. The release of hexosamine[18] and the activity of the enzyme were measured at intervals. After 48 h, the glycoprotein was separated from released hexosamine on a column of Bio-Gel P-6; after combination of fractions

and lyophilisation, the glycoprotein was subjected to two further incubations with enzyme. T.l.c. of the monosaccharide fraction with ethyl acetate–pyridine–water (10:4:3) showed that the only sugar released was 2-acetamido-2-deoxyglucose. Analysis of the degraded glycoprotein confirmed that the mannose and galactose contents remained constant. In the case of Glycopeptide C, 254 mg (containing 621 μmol of hexosamine) was incubated as above with 270 units of enzyme in 25 mL of solution.

(*b*) α-D-*Mannosidase*. Ovomucoid (50 mg containing 18.1 μmol of mannose) was incubated with α-D-mannosidase (20 units) in 0.25M sodium acetate buffer (pH 4.5) containing mM zinc sulphate in an incubation volume of 4 mL. The enzyme activity and release of reducing sugar[18] were monitored at intervals. After 48 h, the glycoprotein was isolated as described above and subjected to a further incubation with 20 units of enzyme for 48 h. In the case of the glycopeptide, 124 mg (containing 140 μmol of mannose) was incubated with 40 units of enzyme in 10 mL under the same conditions.

(*c*) β-D-*Galactosidase*. Ovomucoid (24.3 mg containing 2.21 μmol of galactose) was incubated with 0.6 unit of β-D-galactosidase in 0.125M sodium acetate buffer (pH 4.2) containing 0.01% of bovine plasma albumin, the final incubation volume being 0.5 mL. Enzyme activity and release of reducing sugar[18] were monitored at intervals. After 48 h, the glycoprotein was isolated as described above and subjected to a further incubation with enzyme. For the glycopeptide, 31 mg (containing 6.3 μmol of galactose) was incubated with 1 unit of enzyme in 1.5 mL under the same conditions.

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