The Enzymic Degradation of Ovalbumin and its Glycopeptides

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1. Ovalbumin glycopeptides, freed from all amino acids other than aspartic acid and a small proportion of leucine by repeated digestion with Pronase, were hydrolysed by 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase (glycoaspartamidase) to the corresponding oligosaccharides. The glycoaspartamidase did not attack ovalbumin itself. 2. Ovalbumin, with mannose/hexosamine ratio 5:4, lost 1.5 moles of *N*-acetylglucosamine and more than 2 moles of mannose after incubation with α -mannosidase and β -*N*-acetylglucosaminidase respectively. 3. In ovalbumin glycopeptides with approximate mannose/hexosamine ratios 5:3 and 5:4, one and two *N*-acetylglucosamine residues respectively were accessible to the action of β -*N*-acetylglucosaminidase. 4. A mixture of α -mannosidase and β -*N*-acetylglucosaminidase, acting on an ovalbumin glycopeptide with mannose/hexosamine ratio 5:3.7, removed nearly 4 moles of mannose and 1.5 moles of *N*-acetylglucosamine. 5. α -Mannosidase removed about 1.5 moles of mannose from the ovalbumin oligosaccharide with mannose/hexosamine ratio approx. 5:3. The subsequent action of β -*N*-acetylglucosaminidase liberated less than 1 mole of *N*-acetylglucosamine and made at least 1 mole further of mannose accessible to α -mannosidase action. 6. It is concluded that the carbohydrate moiety of ovalbumin is linked through a glycosyl group to asparagine. In a molecule with mannose/hexosamine ratio 5:4, there are two β -*N*-acetylglucosamine residues linked together in a terminal position, followed by α -mannose. There is also present a side chain containing two α -mannose units.

The recent purification from various sources of the enzyme 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase (Makino, Kojima & Yamashina, 1966; Mahadevan & Tappel, 1967; Makino, Kojima, Ohgushi & Yamashina, 1968; Conchie & Strachan, 1969), which attacks the glycosylamine linkage between β -*N*-acetylglucosamine and aspartic acid in glycopeptides, has introduced the possibility of obtaining intact oligosaccharides from such glycopeptides. Further, the existence of purified glycosidase preparations such as jack-bean-meal α -mannosidase (Li, 1967; Snaith & Levvy, 1968a) and boar epididymal β -*N*-acetylglucosaminidase (Findlay & Levvy, 1960) makes feasible the partial or complete degradation of the oligosaccharides or glycopeptides by enzymic means alone.

The glycopeptides obtained from ovalbumin by proteolytic digestion are particularly suitable for such a study, since ovalbumin contains only one oligosaccharide unit per molecule, linked to aspartic acid via *N*-acetylglucosamine, and this unit contains only the two sugars mannose and *N*-acetylglucosamine. It has been shown that a range of glycopeptides can be obtained from ovalbumin, differing in the ratio of their mannose and hexosamine contents (Levy, Conchie & Hay, 1966).

This heterogeneity gives additional interest to the study of the carbohydrate moiety in ovalbumin glycopeptides, and an investigation has been made of the enzymic degradation products obtained by the action of α -mannosidase and β -*N*-acetylglucosaminidase (sequentially or in combination) on the glycopeptides themselves or on the oligosaccharides obtained by the action of 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase (glycoaspartamidase). The glycopeptides were first submitted to exhaustive Pronase digestion, to leave aspartic acid as virtually the only amino acid residue.

There have been preliminary reports of the action of the glycoaspartamidase on ovalbumin glycopeptide (Makino *et al.* 1968; Plummer, Tarentino & Maley, 1968), and also of the action of α -mannosidase (Li, 1966; Muramatsu & Egami, 1967; Snaith & Levvy, 1968b) and β -*N*-acetylglucosaminidase (Clamp & Hough, 1963; Kaufman & Marshall, 1964).

MATERIALS AND METHODS

Materials

We are indebted to Dr Sybil M. Snaith for samples of purified α -mannosidase from jack-bean meal and from the limpet *Patella vulgata*. The preparations liberated no

N-acetylglucosamine under the conditions of our experiments.

Ovalbumin and glycopeptides from ovalbumin were prepared as described by Levvy *et al.* (1966).

Pronase was obtained from Cambrian Chemicals Ltd., Croydon, and before use it was purified by acetone fractionation as described by Nomoto & Narahashi (1959).

β -*N*-Acetylglucosaminidase was prepared from boar epididymis as described by Findlay & Levvy (1960). The preparation was taken to stage 6(b). It liberated no mannose under the conditions of our experiments.

1-Aspartamido- β -*N*-acetylglucosamine amidohydrolase was purified from rat liver as described by Conchie & Strachan (1969).

Enzyme assays

α -Mannosidase. The hydrolysis of 6mm-*p*-nitrophenyl α -D-mannoside in 0.125M-acetic acid-NaOH buffer, pH 5, in the presence of 0.01% bovine serum albumin, was measured as described by Levvy & Conchie (1966). One unit in the text is equal to 1 μ g. of *p*-nitrophenol liberated by the enzyme preparation in 1 hr. at 38°.

β -*N*-Acetylglucosaminidase. The hydrolysis of 5mm-*p*-nitrophenyl β -*N*-acetylglucosaminide in 0.05M-citric acid-NaOH buffer, pH 4.4, in the presence of 0.1M-NaCl and 0.01% bovine serum albumin was measured as described by Levvy & Conchie (1966). One unit in the text is equal to 1 μ g. of *p*-nitrophenol liberated by the enzyme preparation in 1 hr. at 38°.

1-Aspartamido- β -*N*-acetylglucosamine amidohydrolase. The hydrolysis of 2.8mm-2-acetamido-1-L- β -aspartamido-1,2-dideoxy- β -D-glucose (1-aspartamido- β -*N*-acetylglucosamine) in 0.05M-Na₂HPO₄-KH₂PO₄ buffer, pH 7.0, was measured as described by Conchie & Strachan (1969). One unit in the text is equal to 1 μ mole of *N*-acetylglucosamine liberated by the enzyme preparation in 1 hr. at 38°.

General methods

Reducing-sugar determinations. Reducing sugars were measured by the method of Park & Johnson (1949), except that the FeNH₄(SO₄)₂ solution was made up in 0.075M-H₂SO₄ instead of 0.025M-H₂SO₄.

Mannose determinations. Mannose was determined by the modification described by François, Marshall & Neuberger (1962) of the orcinol-H₂SO₄ method of Winzler (1955).

Hexosamine determinations. Determination of glucosamine and of *N*-acetylglucosamine was carried out by the method of Levvy & McAllan (1959), except that the *p*-dimethylaminobenzaldehyde was purified and the borate buffer was prepared as described by Conchie & Strachan (1969).

For determination of hexosamine in ovalbumin and in glycopeptides and oligosaccharides from ovalbumin, hydrolysis was carried out with 4M-HCl for 4 hr. at 100° in stoppered tubes. With ovalbumin, the hydrolysate was passed through Dowex 50 and eluted as described by Boas (1953) before determination of glucosamine by the above method.

Amino acid analyses. These were done on the various fractions with the Technicon AutoAnalyzer. Hydrolysis of the fractions was carried out in 6M-HCl for 24 hr. at 100°.

Enzyme hydrolyses. In early experiments, precautions were taken against bacterial contamination, including incubation under toluene. When purified enzyme preparations were used, however, such special precautions were found to be unnecessary and no bacterial contamination was observed.

EXPERIMENTAL

Pronase digestion of ovalbumin glycopeptides

A series of glycopeptides with various mannose/hexosamine ratios had already been separated after a single digestion of ovalbumin with Pronase (Levy *et al.* 1966). These glycopeptides were attacked by the glycoaspartamidase, but the action of the enzyme was slow and only about one-quarter of the total aspartic acid was removed from the glycopeptide preparations. To decrease the amino acid content of the glycopeptides, further digestion with Pronase was carried out.

The glycopeptide (250mg.) was dissolved in a solution containing Pronase (15mg.) and calcium chloride (23.8mg.). The solution was adjusted to pH 8.4 and made up with water to 10ml. The mixture was incubated at 38° for about 66 hr., concentrated *in vacuo* at 40°, and fractionated on a column of Sephadex G-25 (130 cm. $\times$ 2 cm.); elution was carried out with 0.1M-acetic acid and fractions (5ml.) were collected. The mannose-containing fractions were combined and concentrated by evaporation *in vacuo*. A second Pronase digestion was carried out on the product and the mixture was again fractionated on Sephadex G-25. The combined mannose-containing fractions were concentrated *in vacuo* to about 1.5 ml. and the product was precipitated by addition of ethanol to give a concentration of 90% (v/v).

The resulting product, after drying *in vacuo* and

Table 1. Removal of amino acids from ovalbumin glycopeptide

Incubations with Pronase and with 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase were carried out as described in the text. Amino acid analyses were done as described in the Materials and Methods section. Results are expressed as μ mole of amino acid/mg. of glycopeptide.

Amino acid	Initial composition	After Pronase incubation	After amidase incubation
Aspartic acid	0.52	0.52	0.039
Leucine	0.36	0.11	0.017
Glycine	0.21	0.026	0.006
Serine	0.17	0.023	0.007
Glutamic acid	0.12	0.022	0.011
Alanine	0.16	0.022	0.003
Threonine	0.39	Nil	Nil
Valine	0.13	Nil	Nil

analysis for amino acids, was found to contain only aspartic acid as a major component, with a smaller proportion of leucine and negligible amounts of the other amino acids. An amino acid analysis of a typical glycopeptide before and after this double Pronase digestion is given in Table 1. Pronase produced no change in mannose/hexosamine ratios. The trace of galactose sometimes detected in our earlier glycopeptide preparations (Levy *et al.* 1966) was not present in the products from exhaustive Pronase digestion.

Exhaustive digestion of the glycopeptides with Pronase was found to be more effective in removing amino acids than reaction of the glycopeptide with benzyloxycarbonyl chloride before treatment with carboxypeptidase, as described by Fletcher, Marks, Marshall & Neuberger (1963). Glycopeptide preparations after this treatment still contained appreciable amounts of threonine as well as leucine.

Action of 1-aspartamido- β -N-acetylglucosamine amidohydrolase on ovalbumin glycopeptides

Purified 1-aspartamido- β -N-acetylglucosamine amidohydrolase had no action on ovalbumin, and, as mentioned above, the extent of its action on ovalbumin glycopeptides that contained several amino acids was limited. For this reason, glycopeptides that had been subjected to the exhaustive Pronase digestion were employed and the glycoaspartamidase removed almost all the aspartic acid and leucine.

The glycopeptide (200 mg.) was incubated at 38° with glycoaspartamidase (9.2 units) in 0.05M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.95, the total volume of the incubation mixture being 2 ml. Reducing sugar was measured at intervals and the incubation was stopped when the value became constant (at about 65 hr.). The incubation mixture was passed through a Sephadex G-25 column (60 cm. $\times$ 1.3 cm.), elution being carried out with 0.1M-acetic acid. The mannose-containing fractions were combined and concentrated *in vacuo* to 1–2 ml. and precipitated with ethanol at a concentration of 90%. The product, after drying *in vacuo*, was analysed for amino acid content. An amino acid analysis of the product from a typical glycopeptide after treatment with glycoaspartamidase is given in Table 1. The products in every case were practically pure oligosaccharides and had essentially the same mannose/hexosamine ratios as the original glycopeptides.

Action of glycosidases on ovalbumin

β -N-Acetylglucosaminidase. A sample of recrystallized ovalbumin, containing 31.1 μ moles of mannose and 25.7 μ moles of N-acetylglucosamine

Table 2. *Action of β -N-acetylglucosaminidase on ovalbumin*

For details of experiment see the text. The ovalbumin sample had an original mannose/hexosamine ratio 5:4.12 and contained 25.7 μ moles of N-acetylglucosamine. Hexosamine released was measured by the method of Levy & McAllan (1959).

Period of incubation (hr.)	N-Acetylglucosamine liberated (μ moles)	Hexosamine liberated (moles/5 moles of mannose)
24	6.92	1.12
48	8.00	1.29
72	8.73	1.40
96	8.78	1.41
144	9.03	1.45

(mannose/hexosamine ratio 5:4.12), was incubated with boar epididymal β -N-acetylglucosaminidase (12.4×10^6 units) in the presence of 0.1M-sodium chloride and 0.01% albumin. The solution was adjusted to pH 4.5 with 0.1M-hydrochloric acid and the total volume was 30.5 ml. At intervals, samples (2 ml.) were removed, 10% (w/v) trichloroacetic acid (0.5 ml.) was added, and after standing for 10 min. at 0° the mixture was centrifuged at 1500g for 15 min. The supernatant was adjusted to pH 5–7 with 0.1M-sodium hydroxide and determinations of N-acetylglucosamine were carried out on this solution.

After incubation for 72 hr., about 35% of the original hexosamine content had been released from the ovalbumin sample by the enzyme (Table 2) and there was no further release of amino sugar on prolonging the incubation. Enzyme assays were also done at intervals. The enzyme was fairly stable and after 96 hr. retained about one-half of its original activity.

α -Mannosidase. A sample of ovalbumin containing 40.5 μ moles of mannose and 31.6 μ moles of N-acetylglucosamine (mannose/hexosamine ratio 5:3.9) was incubated with jack-bean-meal α -mannosidase (4.1×10^5 units) in 0.25M-acetic acid-sodium hydroxide buffer, pH 5.0, in the presence of 0.1M-sodium chloride, 0.01% albumin and 1 mM-zinc sulphate (Snaith & Levy, 1968a), the volume of the incubation mixture being 10 ml. Samples were removed at intervals and reducing-sugar determinations were carried out. After 116 hr., when reducing-sugar values were constant, 15% (w/v) trichloroacetic acid (5 ml.) was added and the precipitated glycoprotein and enzyme were removed by centrifugation at 1500g for 15 min.

Analysis of the mannose content of the supernatant by the orcinol method showed that 18.2 μ moles (45%) of the original mannose content of the ovalbumin had been released by the enzyme.

Analysis of the precipitated material confirmed that 54% of the original mannose content remained. At the end of the incubation, the enzyme had 75% of its original activity.

Action of glycosidases on ovalbumin glycopeptides

β -N-Acetylglucosaminidase. Two glycopeptides, A and B, obtained after a single digestion of ovalbumin with Pronase (Levy *et al.* 1966), were each incubated with boar epididymal β -N-acetylglucosaminidase (3.9×10^6 units) in the presence of 0.1M-sodium chloride and 0.01% albumin. The incubation mixtures were adjusted to pH 4.5 and the total volume in each case was 26ml. The sample of glycopeptide A (36mg.), with mannose/hexosamine ratio 5:4.24, contained 70.5 μ moles of mannose and 59.8 μ moles of *N*-acetylglucosamine and the sample of glycopeptide B (39.4mg.), with mannose/hexosamine ratio 5:2.63, contained 111 μ moles of mannose and 58.3 μ moles of *N*-acetylglucosamine. Samples of each incubation mixture were removed and analysed for *N*-acetylglucosamine (Table 3).

After 72hr. there was no further liberation of *N*-acetylglucosamine from either glycopeptide, and the enzyme had released about 47% and 29% of the original *N*-acetylglucosamine content of glycopeptides A and B respectively. After 96hr. the activity of the enzyme had fallen to about one-quarter of its original value.

α -Mannosidase and β -N-Acetylglucosaminidase. For the following experiments a sample of ovalbumin glycopeptide (C) that had been exhaustively digested with Pronase (see above) was used. Its mean mannose/hexosamine ratio was intermediate

between the values for glycopeptides A and B above. The results are summarized in Table 4.

The glycopeptide (20mg.), containing 52 μ moles of mannose and 38 μ moles of *N*-acetylglucosamine (mannose/hexosamine ratio 5:3.7), was incubated in 0.1M-acetic acid-sodium hydroxide buffer, pH 4.5, with a mixture of jack-bean-meal α -mannosidase (2×10^5 units) and boar epididymal β -N-acetylglucosaminidase (1.25×10^6 units) in the presence of 0.1M-sodium chloride, 0.01% albumin and 1mM-zinc sulphate. The total volume of the incubation mixture was 10ml. Samples were removed at intervals and reducing-sugar determinations made. When liberation of reducing sugar had ceased (after 260hr.), the incubation mixture was passed through a charcoal-Celite column (6cm. $\times$ 1cm.) prepared as described by Spiro (1966). The column was eluted with aq. 4% (v/v) ethanol until no further reducing material was eluted (90ml.).

Analysis of the 4%-ethanol eluate for the individual sugars showed it to contain 28.0 μ moles of mannose and 10.3 μ moles of *N*-acetylglucosamine, corresponding to the liberation of 54% and 27% of the original mannose and *N*-acetylglucosamine respectively.

The residual glycopeptide was obtained by elution of the charcoal-Celite column with aq. 50% (v/v) ethanol until all the mannose-containing material had been eluted (200ml.). The eluate was evaporated to dryness *in vacuo* at 40° and the residue was subjected to a second incubation with a fresh mixture of the enzymes. After 180hr., liberation of reducing sugar had ceased, and the incubation mixture was fractionated on a charcoal-Celite

Table 3. *Action of β -N-Acetylglucosaminidase on ovalbumin glycopeptides*

For details of experiment see the text. Glycopeptide A had mannose/hexosamine ratio 5:4.24 and contained 59.8 μ moles of *N*-acetylglucosamine. Glycopeptide B had mannose/hexosamine ratio of 5:2.63 and contained 58.3 μ moles of *N*-acetylglucosamine. Hexosamine was determined as described in Table 2.

	Period of incubation (hr.)	<i>N</i> -Acetylglucosamine (μ moles)	Hexosamine (moles/5 moles of mannose)
Glycopeptide A	24	21.80	1.60
	48	25.40	1.87
	72	28.85	2.12
	96	28.10	2.07
Glycopeptide B	24	14.00	0.64
	48	15.65	0.71
	72	17.55	0.80
	96	17.55	0.80

Table 4. *Liberation of mannose and *N*-acetylglucosamine from ovalbumin glycopeptide*

For details of experiment see the text. The first and second incubations were with jack-bean-meal α -mannosidase, and the third was with limpet α -mannosidase. The total amount of each sugar released by the mixture of enzymes at each stage is expressed as a percentage of the amount in the glycopeptide at the start. Mannose was determined by the orcinol reaction and hexosamine as described by Levy & McAllan (1959). The mannose/hexosamine ratio in the glycopeptide at the start was 5:3.7.

Incubation	Total sugar released			
	(% of original)		(moles/5 moles of mannose)	
	Mannose	<i>N</i> -Acetylglucosamine	Mannose	Hexosamine
First	55	27	2.75	1.01
Second	65	33	3.25	1.24
Third	75	40	3.75	1.50

column as before. Analysis of the 4%-ethanol eluate showed that 24% of the residual mannose and 8% of the residual *N*-acetylglucosamine in the glycopeptide had been released.

The residual glycopeptide was again obtained by elution with 50% ethanol and, after recovery, was subjected to a third incubation with fresh enzymes. On this occasion, limpet α -mannosidase (17250 units) and boar epididymal β -*N*-acetylglucosaminidase (6.6×10^5 units) were used in an incubation mixture of 5 ml. After 192 hr. hydrolysis was terminated and the free sugars were separated as before on a charcoal-Celite column. Analysis of the 4%-ethanol eluate indicated that 30% of the residual mannose and 11% of the residual *N*-acetylglucosamine had been released.

Sequential degradation of an oligosaccharide from ovalbumin

An oligosaccharide, obtained by the action of glycoaspartamidase on an ovalbumin glycopeptide, was incubated with jack-bean-meal α -mannosidase and boar epididymal β -*N*-acetylglucosaminidase alternately, the product of the reaction being analysed at each step (Table 5).

(1) *Action of α -mannosidase.* The oligosaccharide (19.75 mg.), containing 58 μ moles of mannose and 36.9 μ moles of *N*-acetylglucosamine (mannose/hexosamine ratio 5:3.18), was incubated with jack-bean-meal α -mannosidase (2.26×10^5 units) in 0.1 M-acetic acid-sodium hydroxide buffer, pH 5.0, containing 0.1 M-sodium chloride, 0.01% albumin and 1 mM-zinc sulphate. The total incubation

volume was 20 ml. After 53 hr., further liberation of sugar had ceased, as shown by the constant reducing-sugar value, and the incubation mixture was passed through a charcoal-Celite column (6 cm. $\times$ 1 cm.) and elution of mannose with 4% ethanol carried out to completion (100 ml.). Analysis of the eluate by the orcinol reaction showed that 29% of the mannose in the oligosaccharide had been released by the enzyme.

(2) *Action of β -*N*-acetylglucosaminidase.* The residual oligosaccharide, obtained by elution of the column with 20% ethanol and evaporation of the eluate to dryness *in vacuo* at 40°, was incubated with boar epididymal β -*N*-acetylglucosaminidase (2.5×10^6 units) in 0.1 M-acetic acid-sodium hydroxide buffer, pH 4.5, containing 0.1 M-sodium chloride and 0.01% albumin. The total volume of the solution was 20 ml. After 100 hr. the reducing sugar in the mixture had reached a constant value, and the mixture was fractionated on a charcoal-Celite column as described above. The 4%-ethanol eluate contained 21% of the original *N*-acetylglucosamine content of the oligosaccharide.

(3) *Action of α -mannosidase.* After isolation of the residual oligosaccharide by elution with 20% ethanol, a further incubation for 116 hr. with jack-bean-meal α -mannosidase (4×10^5 units in 20 ml. of incubation mixture) and fractionation on a charcoal-Celite column resulted in the appearance in the 4%-ethanol eluate of 36% of the mannose in the residual oligosaccharide.

DISCUSSION

The production of oligosaccharides with the same mannose/hexosamine ratios as the original glycopeptides from ovalbumin through the action of rat liver glycoaspartamidase provides further biochemical support for the correctness of the structure of the aspartic acid-*N*-acetylglucosamine linkage in ovalbumin first proposed by Neuberger and his co-workers (Marks, Marshall & Neuberger, 1963).

It was observed by Makino *et al.* (1968) that their glycoaspartamidase from pig serum would not attack glycopeptides prepared from α_1 acid glycoprotein that still contained other amino acids besides aspartic acid. Nor would the enzyme attack glycopeptides of the structure Thr-Asn-NAcGlc and Asn-NAcGlc-Thr, where NAcGlc represents *N*-acetylglucosamine. They therefore deduced that both the α -amino and α -carboxyl groups had to be free before hydrolysis by the aspartamidase could occur.

In our own experiments it was observed that the action of the glycoaspartamidase was slow and incomplete when ovalbumin glycopeptides that still contained several amino acids were used as substrates: this could be due to heterogeneity in the

Table 5. *Sequential liberation of sugars from ovalbumin oligosaccharide*

For details of experiment see the text. The cumulative total for mannose is expressed as a percentage of the amount in the oligosaccharide at the start. The individual sugars were determined as described in Table 4. The mannose/hexosamine ratio in the oligosaccharide at the start was 5:3.18.

	Total sugar released			
	(% of original)		(moles/5 moles of mannose)	
	Mannose	<i>N</i> -Acetylglucosamine	Mannose	Hexosamine
α -Mannosidase	29		1.45	
β - <i>N</i> -Acetylglucosaminidase		21		0.67
α -Mannosidase	55		2.75	

amino acid portion of the glycopeptide preparation. When the amino acid content of the glycopeptide had been decreased by further Pronase digestion until the only amino acids present were aspartic acid and leucine, in the molar ratio 1:0.2, then the aspartamido- β -*N*-acetylglucosamine linkage in the glycopeptides was almost completely split by the enzyme. Either the specificity of our glycoaspartamidase is not so strict as that claimed for the pig serum enzyme of Makino *et al.* (1968) or our preparation contained a peptidase, which removed leucine and allowed the amidase to act.

Ovalbumin was attacked by both α -mannosidase and β -*N*-acetylglucosaminidase, but not by the glycoaspartamidase. With our samples of ovalbumin, which had mannose/hexosamine ratio 5:4, 1.5 moles of *N*-acetylglucosamine and more than 2 moles of mannose were released by the appropriate individual enzymes.

The results obtained when β -*N*-acetylglucosaminidase was incubated with two ovalbumin glycopeptides of different composition indicated that in the glycopeptide with mannose/hexosamine ratio approaching 5:4 there were two hexosamine residues easily accessible to the enzyme, but in the glycopeptide with mannose/hexosamine ratio approx. 5:3 only one such residue was susceptible to attack. It is evident that the extra hexosamine residue in the first glycopeptide was terminal.

When an ovalbumin glycopeptide with a mannose/hexosamine ratio of 5:3.7 was subjected to exhaustive hydrolysis by a mixture of α -mannosidase and β -*N*-acetylglucosaminidase, 1.5 moles of *N*-acetylglucosamine and nearly 4 moles of mannose were liberated.

When α -mannosidase and β -*N*-acetylglucosaminidase acted sequentially on an oligosaccharide of mannose/hexosamine ratio approx. 5:3, the results suggested that one hexosamine and 1–2 mannose residues were terminal. After removal of hexosamine, at least one more mannose residue became accessible to enzymic hydrolysis. In all, however, one mannose unit less was removed than from the glycopeptide acted on by the mixed glycosidases. Unless the explanation lies in heterogeneity of structure with respect to mannose, we can only suggest that the difference between the oligosaccharide and the glycopeptide in susceptibility to α -mannosidase is due to a steric effect.

The experiments with the exoglycosidases have not yet reached a stage at which definite conclusions may be drawn as to the structure of the carbohydrate moiety in ovalbumin. It is possible, however, considering all the results together (Table 6), to conclude that the extra hexosamine residue in compounds with hexose/hexosamine 5:4 ratio is linked to the single terminal *N*-acetylglucosamine residue in compounds with ratio 5:3. The terminal

Table 6. *Summary of glycosidase experiments*

Values are given to the nearest mole or 0.5 mole.

Substrate	Mannose/ hexosamine ratio	Sugar liberated (moles/5 moles of mannose)	
		α -Mannosidase	β - <i>N</i> -Acetylglucosaminidase
Ovalbumin	5:4	2	—
Ovalbumin	5:4	—	1.5
Glycopeptide A	5:4	—	2
Glycopeptide C	5:4	4	1.5
Glycopeptide B	5:3	—	1
Oligosaccharide	5:3	1.5	—
Oligosaccharide	5:3	3	1

hexosamine(s) prevents enzymic hydrolysis of probably two mannose units, and the molecule may also contain a side chain of two mannose units. These conclusions are not entirely consistent with any of the structures that have been previously put forward, mainly on chemical grounds, for the glycopeptide with ratio 5:3 (Clamp & Hough, 1963, 1965; Montgomery, Wu & Lee, 1965; Makino & Yamashina, 1966), but they do support the assignment of the α -configuration to mannose and the β -configuration to *N*-acetylglucosamine. For purposes of comparison, the structure suggested by Clamp & Hough (1963) for a glycopeptide with ratio 5:3 is shown.

It is not impossible that the heterogeneity of the ovalbumin glycopeptide with respect to its hexosamine content extends to mannose and that an ordered pattern of synthesis *in vivo* leads to a product in which the value 5 for mannose content, like the earlier accepted value of 3 for hexosamine, is a statistical mean. The possibility must also be entertained that the action of our glycosidase preparations is governed, quantitatively at least, not only by the nature of the glycoside bond, but also by its point of attachment.

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