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Studies on a Glycopeptide from Ovalbumin

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1. The structure of the carbohydrate component of the glycopeptide isolated from the proteolytic digest of ovalbumin has been investigated by chemical and enzymic methods. 2. The results are consistent with the presence of a single carbohydrate prosthetic group, linked through its reducing end group to the peptide chain. 3. Further, all the 2-amino-2-deoxy-D-glucose units appear to be in the *N*-acylated form, the phenolic hydroxyl group of tyrosine is free and the ω-carboxyl group of aspartic acid is substituted. 4. The carbohydrate component has a branched-chain structure, the two non-reducing ends being terminated by a D-mannopyranosyl and a 2-acetamido-2-deoxy-D-glucopyranosyl residue respectively. 5. The terminal D-mannopyranosyl unit is probably linked through at least one other D-mannopyranosyl residue to the remainder of the carbohydrate.

The isolation and analysis of glycopeptides after enzymic hydrolysis of ovalbumin has been described by several workers (Neuberger, 1938; Cunningham, Nuenke & Nuenke, 1957; Kaverzneva & Shmakova, 1958; Jevons, 1958; Nuenke & Cunningham, 1961; Johansen, Marshall & Neuberger, 1958, 1961; Bogdanov, Kaverzneva & Andrejeva, 1962; Lee & Montgomery, 1962; Yamashina & Makino, 1962; Clamp & Hough, 1963). The results obtained suggest that in ovalbumin there is a single oligosaccharide prosthetic group composed of five D-mannosyl and three 2-acetamido-2-deoxy-D-glucosyl residues, linked through L-asparagine to the remainder of the peptide chain. Evidence from enzymic (Marks, Marshall & Neuberger, 1962) and methylation (Bragg & Hough, 1961) studies indicates the presence of a terminal D-mannopyranosyl unit. Oxidation of ovalbumin with sodium metaperiodate destroys one unit of 2-acetamido-2-deoxy-D-glucose and three units of D-mannose (Bragg & Hough, 1961; Marks *et al.* 1962).

METHODS

Paper chromatography. The descending method (Partridge, 1948) on Whatman no. 1 filter paper was used at room temperature with the following mobile phases: (1) butan-1-ol–ethanol–water (40:11:19, by vol.); (2) butan-1-ol–acetic acid–water (12:3:5, by vol.); (3) ethyl acetate–acetic acid–water (3:1:1, by vol.); (4) butan-1-ol–pyridine–water (10:3:3, by vol.). Carbohydrates were detected with either *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) or 0·02 M-sodium metaperiodate, followed by ammoniacal silver nitrate (Richardson, 1959). Amino acids were identified by two-dimensional ascending chromatography on Whatman no. 4 filter paper (Dent, 1948), with, as the two solvent systems, phenol–water (bottom layer) in the presence of ammonia, and 2,4-lutidine–2,6-lutidine–water (11:11:10, by vol.) in the presence of diethylamine. Peptides were separated on Whatman 3MM filter paper by a two-dimensional technique (Putnam, Migita & Easley, 1962) in which descending chromatography in solvent (2) was used for 20 hr., followed by electrophoresis in the other dimension at 2000 V and 100 mA in pyridine–acetic acid–water (1:10:289, by vol.) (pH 3·6) for 1 hr. Amino acids and peptides were located with 0·5% (w/v) ninhydrin in acetone by using a dip technique.

Absorption-spectra. Ultraviolet-absorption spectra were determined with a Unicam SP. 500 spectrophotometer, and infrared-absorption spectra with a Unicam SP. 100 spectrophotometer by using both the potassium bromide disk and Nujol mull techniques.

Carbohydrate estimations. For each carbohydrate determination a calibration curve was prepared, by using a solution of D-mannose and 2-acetamido-2-deoxy-D-glucose in the same molar proportions as those present in the original glycoprotein. D-Mannose was determined by the phenol method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) or the orcinol procedure (Vasseur, 1948). 2-Amino-hexose was estimated by the Elson & Morgan (1933) method as modified by Belcher, Nutten & Sambrook (1954) or Boas (1953), after preliminary hydrolysis of the sample in 2N-HCl at 98–100° for 6 hr. and subsequent neutralization with 2 N-NaOH. A control solution was treated in exactly the same way as the unknown. 2-Acetamido-hexose was determined by the method of Morgan & Elson (1934). Reducing sugar was assayed by the Somogyi (1945) colorimetric method.

Amino acid estimations. After hydrolysis of the glycopeptide in 6-N-HCl at 105° for 22 hr. the liberated amino acids were determined (Moore & Stein, 1951) with the Beckman Spinco model MS amino acid analyser. Tyrosine

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was also estimated from the peak at 280 mμ in the ultraviolet-absorption spectrum (Goodwin & Morton, 1946).

Preparation of ovalbumin. Powdered egg albumin (British Drug Houses Ltd., Poole, Dorset) (250 g.) was stirred with distilled water (2·5 l.) for 6 hr. and then saturated (17–19°) ammonium sulphate solution (2·5 l.) was added slowly with stirring. The resultant precipitate, together with material that was originally insoluble, was separated by centrifugation (600 *g* for 1 hr.) and discarded. A mixture of saturated ammonium sulphate solution (820 ml.) and 1 N-H_2SO_4 (approx. 80 ml.) was added dropwise to the supernatant over a period of 24 hr. and the final solution adjusted to pH 4·7. The resultant precipitate was isolated by filtration, dissolved in the minimum quantity of distilled water and then dialysed against running tap water for 48 hr. The precipitation and dialysis procedures were repeated twice and the final solution was dialysed against distilled water for 24 hr. The ovalbumin was isolated by freeze-drying and dried in a vacuum desiccator over phosphoric oxide (yield, 60·4 g.) [Found: sulphated inorganic material, 0·3; N (Dumas method), 14·7; H_2O, 1·73%]. The glycoprotein appeared homogeneous on examination by paper electrophoresis at 4 v/cm. for 16 hr. in 0·1 M-sodium diethylbarbiturate–acetic acid buffer, pH 8·6, and 0·1 M-sodium acetate–HCl buffer, pH 3·6, and by chromatography on DEAE-cellulose by the method of Mandeles (1960).

Preparation of the glycopeptide. Ovalbumin (100 g.) in 0·01 N-HCl (1 l.) containing NaCl (10·5 g.) was incubated with crystalline pepsin (Sigma Chemical Co., St Louis, Mo., U.S.A.) (1 g.) at 37° for 2 weeks under a layer of toluene. At intervals, the pH of the solution was adjusted to 2·0 with 0·1 N-HCl and a further 0·5 g. of pepsin was added after 1 week. At the end of the incubation a small amount of insoluble material was removed by filtration (5·8 g.) and the solution was adjusted to pH 7·3 with 1 N-NaOH and buffered by the addition of $Na_2HPO_4,12H_2O$ (2·76 g.) and KH_2PO_4 (0·31 g.) Crystalline trypsin (Sigma Chemical Co.) (1 g.) was then added and the solution incubated at 37° for 2 weeks under a layer of toluene. The hydrolysate was brought to pH 7 with 2 N-HCl and evaporated to dryness under reduced pressure. The dried and finely powdered material was shaken with phenol–water (9:1, w/v) (5 × 100 ml.) until the extracts were no longer coloured. The residue (15·6 g.) (Found: sulphated inorganic material, > 100%) was discarded. Ethanol (1 vol.) was added to the extracts and the mixture was then kept at 4° for 12 hr., yielding a yellow precipitate [23·5 g.; D-mannosyl content (phenol method), 5·9%; 2-amino-2-deoxy-D-glucosyl content (Boas method), 3·8% (Found: N, 11·2; sulphated inorganic material, 9·9%)]. The addition of further ethanol (1 vol.) precipitated material (13·8 g.) having a D-mannosyl content of 1·10% (phenol method); no more carbohydrate-containing material could be obtained by adding acetone (2 vol.) and ether (2 vol.). The yellow precipitate obtained from the first addition of ethanol was further fractionated by chromatography on a cellulose column, as follows. Cellulose powder (Whatman, standard grade) was washed thoroughly with distilled water, followed by propan-2-ol–water (4:1, v/v) and then packed into a column (32 cm. × 3·5 cm.) as a slurry in this solvent, which was allowed to settle under gravity with gentle agitation. The material to be fractionated (15 g.) mixed with a small amount of washed cellulose powder was added to the top of the cellulose column. Propan-2-ol–water (4:1, v/v) was passed through the column until the glycopeptide material began to appear in the effluent (Molisch test), whereupon the remaining material was eluted with water [yield, 8·5 g.; D-mannosyl content (phenol method), 10·3%]. An aqueous solution of material eluted from the cellulose column (1 g.) was passed through a column (125 cm. × 2·25 cm.) of Sephadex G-25, medium grade (Pharmacia Ltd.). The carbohydrate-containing fractions (Molisch test) were pooled and evaporated to dryness [yield, 0·185 g.; D-mannosyl content (phenol method), 39·2 ± 2·2%]. This crude glycopeptide material (25 mg./chromatogram) dissolved in the minimum quantity of water was purified by descending chromatography on pre-washed Whatman 2MM filter paper in solvent (2) for 24 hr. The base-line material was eluted from the dried chromatogram with water, filtered through glass wool and evaporated to dryness under reduced pressure [yield, 20 mg.; D-mannosyl content (phenol method), 46·3 ± 1·8%].

Reducing end group. The glycopeptide (25 gm.) was added to a solution of sodium tetraborate (0·1 M) and sodium borohydride (0·075 M) (50 ml.) at pH 10·3 and kept at room temperature for 9 hr. The mixture was passed through a column (150 cm. × 2·25 cm.) of Sephadex G-25, medium grade, and the glycopeptide-containing fractions were pooled and evaporated to dryness. The dried material was hydrolysed with 4 N-HCl at 98–100° for 6 hr., evaporated to a small volume under reduced pressure and finally dried in a vacuum desiccator over phosphoric oxide and NaOH pellets. Amberlite IRA-400 (OH^- form) resin (10 g.) was added, with sufficient 0·1 M-NH_3 to cover the resin, and the mixture heated (98–100°) under reflux for 6 hr. to destroy reducing sugars. After filtration the solution was concentrated and examined by chromatography on Whatman no. 1 filter paper, with *p*-anisidine–HCl and periodate–silver nitrate spray reagents.

Periodate oxidation studies. The glycopeptide (0·25 g.) was oxidized with 0·015 M-sodium metaperiodate (100 ml.) at room temperature in the dark. A blank was run concurrently and samples were withdrawn at intervals for determination of unused periodate by thiosulphate (Neumüller & Vasseur, 1953).

Electrometric titration. The glycopeptide was accurately weighed (approx. 0·75 g.), dissolved in water (10–15 ml.) and titrated with either 0·05 N-NaOH or 0·05 N-HCl, both of which had been carefully standardized. The pH was determined after each addition (0·5 ml.) of acid or alkali with a model 23A direct-reading pH-meter (Electronic Instruments Ltd.). The concentration of H_3O^+ or HO^- ions, calculated from the dilution of the added acid or alkali, was compared with the actual concentration as determined from the pH reading and the appropriate activity coefficient. The difference between these values, corrected if necessary for any electrode effect, represents the amount of acid or alkali bound by the glycopeptide.

Enzymic hydrolysis. The glycopeptide (125 mg.) in distilled water (10 ml.), adjusted to pH 8·0 with 0·05 N-NaOH, was incubated with carboxypeptidase (Sigma Chemical Co.) (5 mg.) at room temperature. Samples (1 ml.) were removed at intervals and added to flasks containing 12 N-HCl (0·1 ml.), and the mixtures were then dried under reduced pressure over phosphoric oxide and NaOH pellets.

In both glycosidase experiments the liberated monosaccharide was estimated by using a standard curve constructed from a known solution of the sugar in the appropriate enzyme mixture. The incubations were carried out at

37° and the solutions subjected to paper chromatography at intervals to follow the monosaccharide liberation. A preparation of 2-acetamido-2-deoxy-β-D-glucopyranosidase (β-*N*-acetylglucosaminidase) from boar epididymis, kindly supplied by Dr G. A. Levvy, was made up in 0·1M-NaCl containing 0·1% of bovine serum albumin and the pH adjusted to 4·5 with 0·1 N-HCl. The glycopeptide (20 mg.) was added to this enzyme solution (10 ml.) and samples (0·5 ml.) were withdrawn at intervals for estimation of the liberated 2-acetamido-hexose.

Crude emulsin (β-glucosidase, C grade; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) (5 mg.) was added to a solution (10 ml.) of the glycopeptide (20 mg.) in 0·1 M-sodium butyrate adjusted to pH 5·5 with 1 N-HCl. Samples (0·5 ml.) were taken at intervals for the determination of 2-acetamido-hexose and total reducing sugar. The glycopeptide was incubated with β-glucosidase (as described above) for 48 hr., and then an equal volume of ethanol was added and the mixture evaporated to dryness under reduced pressure. The dried material was extracted with water, and the soluble fraction applied to a column (125 cm. × 2·25 cm.) of Sephadex G-25, medium grade. The glycopeptide-containing fraction was evaporated to dryness and then incubated with the 2-acetamido-2-deoxy-β-D-glucosidase solution as before.

Alkaline stability. Conway dishes were set up with 0·1 N-H_2SO_4 (1 ml.) in the central and 2 N-NaOH (1 ml.) in the outer compartment. Glycopeptide (approx. 100 mg.) in water (1 ml.) was added to the outer compartment and after 24 hr. at room temperature the contents of the central and outer compartments were titrated with 0·01 N-NaOH and 0·1 N-H_2SO_4 respectively.

The glycopeptide (260 mg.) was heated with 10% (w/v) $Ba(OH)_2$ solution (25 ml.) in a 250 ml. three-necked flask, fitted with a reflux condenser, under an inlet of O_2-free N_2. Samples were removed, neutralized with CO_2, filtered and dried over phosphoric oxide under reduced pressure.

RESULTS

Structural investigations on the carbohydrate units of glycoproteins are considerably facilitated by the removal of the protein with enrichment of the carbohydrate. An enzymic method was therefore investigated whereby the protein component could be progressively hydrolysed without affecting the oligosaccharide unit. A number of proteinases were employed either singly or in combination, but the highest yield of glycopeptide was obtained when ovalbumin was hydrolysed with pepsin followed by trypsin. The final enzyme digest was carefully neutralized and evaporated to dryness under reduced pressure. It was found to be important that this material should be thoroughly dried and pulverized before the phenol extraction was carried out. Extraction with phenol–water left most of the inorganic material as insoluble residue. The glycopeptides appeared to be more readily precipitated by the addition of ethanol than were ordinary peptides. The material precipitated from the phenol–water extract by the addition of ethanol (1 vol.) had a D-mannosyl content of 5·9%, and at this stage therefore the carbohydrate component had undergone a threefold enrichment as compared with the original ovalbumin. Examination by paper chromatography in solvents (2) and (4) revealed a large number of ninhydrin-positive spots with R_F values ranging from zero to 0·6, but only one periodate–silver nitrate-positive spot containing the carbohydrate (R_F 0). Although the glycopeptide did not move from the origin during paper chromatography, it had a slow rate of movement on the cellulose column with propan-2-ol–water (4:1, v/v) as the mobile phase, and was purified in this way. The D-mannosyl content of the glycopeptide fraction, finally eluted with water, had risen to 10·3%, and two-dimensional chromatography showed the presence of at least seven peptides, only one of which showed a positive test with periodate–silver nitrate. The next stage of purification took advantage of the fact that the glycopeptide, by reason of its carbohydrate content, was larger than many of the contaminating peptides, and could be separated from them by gel-filtration. After passage through the Sephadex column the crude glycopeptide material had a D-mannosyl content of approx. 40%, but still contained traces of three other peptides. However, this material was suitable for many of the structural investigations. A final purification of the glycopeptide was achieved by exploiting the fact that, whereas the glycopeptide did not move on paper chromatography in solvent (2), the contaminating peptides did. The material remaining at the base line was therefore eluted with water and appeared to be pure glycopeptide. Examination of the peptide by paper chromatography in two dimensions showed only one ninhydrin-positive spot, which also stained with periodate–silver nitrate. After acid hydrolysis of ovalbumin and the glycopeptide, D-mannose and 2-amino-2-deoxy-D-glucose were the only carbohydrates that could be detected by paper chromatography. Quantitative estimations of the carbohydrate content of ovalbumin, the crude and the pure glycopeptide are shown in Table 1. Assuming a molecular weight of 45000, ovalbumin contains five D-mannosyl units and three acetamido-hexose units. If this carbohydrate were all present in one prosthetic group, the glycopeptide having the composition shown in Tables 1 and 2 would have a molecular weight of approx. 2000. The value found by Ramsay (1949) depression-of-freezing-point micro-method was 1660 ± 300.

The amino acid and ammonia content of the glycopeptide is shown in Table 2. Application of 0·67 mg. of glycopeptide to the column yielded 0·33 μmole of aspartic acid. After hydrolysis in 6 N-hydrochloric acid at 105° for 22 hr. only approx. 50% of the amino-hexose survived, and this amino-hexose degradation may explain the higher ammonia content after more vigorous hydrolysis.

Table 1. *Carbohydrate content of ovalbumin, crude glycopeptide and purified glycopeptide*

Experimental details are given in the text.

Monosaccharide	Method of estimation	Carbohydrate content (g./100 g.) Ovalbumin	Crude glycopeptide	Purified glycopeptide
D-Mannose	Dubois *et al.* (1956)	2·16±0·49	40·2±4·2	46·3±1·8
	Vasseur (1948)	1·97±0·26	41·5±1·9	
2-Amino-2-deoxy-D-glucose	Belcher *et al.* (1954)	1·18±0·33		
	Boas (1953)	1·29±0·3	26·4±2·9	28·4±2·7

Table 2. *Amino acid and ammonia composition of the glycopeptide*

Details are given in the text. The ammonia content of the glycopeptide was estimated both after hydrolysis in 2 N-HCl at 98° for 6 hr. and 6 N-HCl at 105° for 22 hr. The results are given relative to the aspartic acid value.

	Molar proportion
Aspartic acid	1·0
Threonine	0·9
Serine	0·7
Tyrosine	0·6
Leucine	0·7
Ammonia (hydrolysis with 2 N-HCl)	1·3
Ammonia (hydrolysis with 6 N-HCl)	2·1
Tyrosine (u.v. absorption)	0·7

Table 3. *Consumption of periodate by the glycopeptide from ovalbumin*

Experimental details are given in the text.

Time of reaction (hr.)	Consumption of periodate (moles of $NaIO_4$/2000 g. of glycopeptide)	(moles of $NaIO_4$/0·7 mole of tyrosine)	(estimated molar $NaIO_4$ uptake by carbohydrate moiety)
5	3·8	0·2	3·6
29	5·4	0·3	5·1
53	5·8	0·6	5·2
101	6·2	1·1	5·1
168	7·2	2·1	5·1

The glycopeptide had $[\alpha]_D^{25}+6·65°$ (*c* 2·11 in water), and no mutarotation was observed.

The infrared-absorption spectrum showed a peak at 890 cm.$^{-1}$, which is claimed (Barker, Bourne, Stacey & Whiffen, 1954) to be characteristic of β-linked glycosides. Ester groups have a strong peak at 1750 cm.$^{-1}$, which was not present in the spectrum, indicating that this linkage is not involved in the carbohydrate–peptide bond, and this was confirmed by a negative alkaline-hydroxylamine test (Kaye & Kent, 1953; Nuenke & Cunningham, 1961).

The glycopeptide was titrated separately with acid and with alkali and the change in pH of the solutions followed. Calculation of the amount of acid, or alkali, adsorbed by the glycopeptide showed that 1·2 and 1·9 g.equiv. of H_3O^+ and HO^- ions respectively were bound/mole of glycopeptide. Within the limits of experimental error this corresponds to a free amino group and a free carboxyl group, together with the phenolic hydroxyl group of tyrosine.

The glycopeptide was relatively stable to 1 N-sodium hydroxide at room temperature. After 24 hr. no ammonia or acid groups had been liberated and chromatography showed only the glycopeptide, thus confirming the absence of an ester linkage. The glycopeptide, however, was broken down by 10% barium hydroxide acting at 100° and in the absence of oxygen, when the hexose:amino-hexose ratio changed from 1·6:1·0 (initial) to 4:1 in 4 hr.

The glycopeptide was examined for the presence of a free reducing group. This would be reduced by alkaline borohydride to the corresponding hexitol, which after hydrolysis would survive heating in an alkaline environment, whereas free hexose would be destroyed (Anderson, Andrews, & Hough, 1961). The hexitol can then be detected with the periodate–silver nitrate spray reagent. By this method it was shown that the glycopeptide contained no reducing group.

The glycopeptide (mol.wt. 2000) showed a rapid uptake of 4 moles of periodate/mole followed by slower oxidation, with a total consumption of approx. 7 moles/mole after 168 hr. The only oxidizable residues in the glycopeptide are the carbohydrate components, tyrosine and to a smaller extent the *N*-terminal amino acid. The consumption of periodate by the tyrosine component was calculated on the assumption that the uptake will resemble that of the free amino acid (Clamp & Hough, 1965). The periodate uptake of the carbohydrate unit was then estimated to be approx. 5 moles (Table 3), by subtraction of the tyrosine

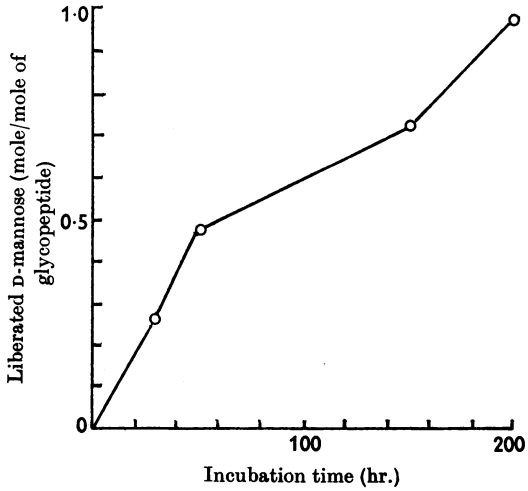

Fig. 1. Liberation of D-mannose from ovalbumin glycopeptide by emulsin. Details are given in the text.

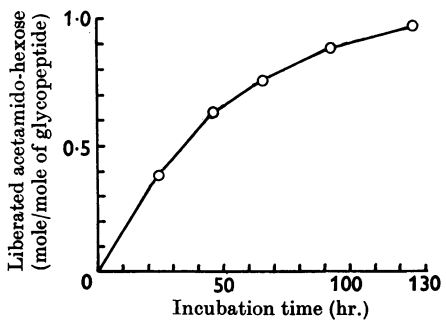

Fig. 2. Liberation of 2-acetamido-2-deoxy-D-glucose from ovalbumin glycopeptide by an enzyme preparation from boar epididymis. Details are given in the text.

values from those of the glycopeptide. The oxidized glycopeptide was isolated after treatment with periodate for 170 hr., and after hydrolysis was shown to contain: D-mannose (phenol method), 19·7 ± 1·4%; 2-amino-2-deoxy-D-glucose (Boas method), 17·5 ± 0·3%. This suggests that 2 moles of D-mannose and 2 moles of acetamido-hexose/mole remain unoxidized by periodate because of inter-unit linkages. Bragg & Hough (1961) and Fletcher, Marks, Marshall & Neuberger (1963) obtained similar results after periodate oxidation of the carbohydrate in ovalbumin.

The glycopeptide was incubated with carboxypeptidase at room temperature. Leucine was released within 1 hr. together with a trace of valine, but no further amino acids were released for 8 hr., when serine together with a smaller amount of threonine was found. The delayed release of serine and threonine that was observed is a well-recognized phenomenon when these amino acids occur as *C*-terminal units (Schmid, Bencze, Nussbaumer & Wehrmüller, 1959; Neurath & Schwert, 1950). After 24 hr. incubation the glycopeptide contained only tyrosine and aspartic acid.

Emulsin (β-glucosidase) slowly released D-mannose together with a small amount of acetamido-hexose from the glycopeptide. The amount of 2-acetamido-2-deoxy-D-glucose released was estimated, and the equivalent reducing power determined in terms of D-mannose. The total reducing power was then corrected for the liberated acetamido-hexose, with the result shown in Fig. 1. The release of D-mannose was almost linear with time, and after 200 hr. amounted to 1·0 mole/mole of glycopeptide (mol.wt. 2000).

2-Acetamido-2-deoxy-β-D-glucosidase released acetamido-hexose from the glycopeptide and no other carbohydrate could be detected by paper chromatography. The amount of acetamido-hexose liberated approached a constant value of 1 mole/mole of glycopeptide (Fig. 2). Substantially the same result was obtained after preliminary incubation of the glycopeptide with emulsin.

DISCUSSION

Ovalbumin contains 3·3% of carbohydrate composed of five units of D-mannose and three units of 2-acetamido-2-deoxy-D-glucose. From the analytical values, L-aspartic acid content and mol.wt. determination, all of this carbohydrate appears to be present in the glycopeptide produced from ovalbumin by digestion with pepsin and trypsin, indicating that ovalbumin contains a single oligosaccharide unit. Results from the treatment of the glycopeptide with carboxypeptidase, considered together with the analytical values, suggest the following sequence for the peptide moiety:

Tyr-Asp-Thr-Ser-Leu-Val

The electrometric titration results are compatible with the presence of a free phenolic hydroxyl group on tyrosine together with the *N*-terminal and *C*-terminal amino acids. The absence of additional acidic or basic groups confirms that all the amino-hexose is in the *N*-acylated form, and that the ω-carboxyl group of L-aspartic acid is substituted, probably as asparagine since 1 mole of ammonia was released/mole of glycopeptide after mild acid hydrolysis (Marks, Marshall & Neuberger, 1963). The higher ammonia content after more vigorous hydrolysis is probably derived from the amino-hexose degradation that occurs under these conditions (Clamp & Putnam, 1964).

Tyrosine and aspartic acid were the only amino acids remaining in the glycopeptide in significant amounts after carboxypeptidase treatment, and consequently the oligosaccharide moiety must be attached to one of these amino acids. Since they occur in a peptide chain, only the phenolic hydroxyl group of tyrosine and the ω-carboxylic acid group of aspartic acid remain as sites of attachment. The former, however, appears to be unsubstituted, as revealed by electrometric titration results and the ultraviolet-absorption spectrum, and so the latter probably fulfils this function. The carboxylic acid group and the carbohydrate cannot be linked directly since there was no ester peak in the infrared-absorption spectrum of the glycopeptide, which was stable to dilute alkali and gave a negative result in the Kaye & Kent (1953) test. As the glycopeptide yields approx. 1 mole of ammonia/mole after hydrolysis, the two groups may be linked as a type of glycosylamide, and Marks *et al.* (1963) have provided evidence for this linkage involving a 2-acetamido-2-deoxy-D-glucopyranosyl residue. This type of compound is hydrolysed by alkali with the progressive release of ammonia. The glycopeptide was non-reducing, which is additional evidence that the oligosaccharide moiety is glycosidically linked to aspartic acid. The glycopeptide was stable to 1 N-sodium hydroxide at room temperature but labile to 10% (w/v) barium hydroxide at 100°, which changed the hexose:amino-hexose ratio from 1·6:1·0 to 4·0:1·0. This can be explained on the basis of an initial hydrolysis of the glycosylamido linkage with subsequent degradation of the oligosaccharide from the exposed reducing group, liberating saccharinate molecules. The rate and extent of this peeling process will depend on the positions of inter-unit linkages, 4-*O*-substitution being more stable to alkali than 3-*O*-substitution, whereas multiple substitution may be associated with a termination of the degradation process (Whistler & BeMiller, 1958).

The glycopeptide was examined by the periodate oxidation technique, which was uncomplicated by problems of over-oxidation, since the glycopeptide is non-reducing. Formaldehyde was not detected (Desnuelle & Antonin, 1945) after oxidation of ovalbumin, indicating that only pyranosyl ring structures are present, and this was confirmed by the stability of the glycopeptide to mild acid hydrolysis (pH 2·0 at 100° for 6 hr.). The oligosaccharide component of the glycopeptide was found to consume 5 moles of periodate/mole after correction for the uptake by tyrosine, and a quantitative estimate revealed that three units of D-mannose and one unit of 2-acetamido-2-deoxy-D-glucose had been oxidized. The destruction of a 2-acetamido-2-deoxy-D-glucosyl residue shows that the hydroxyl groups at C-3 and C-4 are unsubstituted, so that the residue either occupies a terminal position or is linked to another unit via the 6-position (see below). The survival of two D-mannosyl residues suggests that in each of these units either the hydroxyl group at C-3 is substituted or that there is di-*O*-substitution at positions 2, 3 or 4. The liberation of formic acid during the periodate oxidation of oligosaccharides occurs when there are three free hydroxyl groups on contiguous carbon atoms, as in a terminal hexopyranosyl unit or when the hexopyranosyl unit is substituted at the 6-position. The estimation of acid released during periodate oxidation of glycopeptides is, however, unsatisfactory. Thus a glycopeptide from γ-globulin showed a steady release of acid during periodate oxidation (Rothfus & Smith, 1962; Clamp & Putnam, 1964), and Fletcher *et al.* (1963) found a similar result with ovalbumin glycopeptide. The glycopeptide was expected to release 1 equiv. of acid from the terminal D-mannopyranosyl unit, whereas it steadily released acid that after 200 hr. had reached 1·96 equiv./mole of glycopeptide. In the absence of a reducing end group and furanosides, this extra acid cannot be due to non-Malapradian oxidation of carbohydrate and therefore probably arises from the oxidation of amino acid residues.

Glycosidases are of considerable potential value as aids in the structural determination of glycopeptides derived from glycoproteins. Enzymes have not been extensively employed in this capacity, although they have been used to show the presence of D-mannose (Marks *et al.* 1962) and 2-acetamido-2-deoxy-D-glucose (Clamp & Hough, 1963) as non-reducing end groups. Emulsin has been reported (Lee, Wu & Montgomery, 1964) to contain an enzyme capable of hydrolysing the asparaginyl-carbohydrate linkage with the release of oligosaccharides. In the present study, however, only monosaccharides appeared to be released by the action of emulsin on ovalbumin glycopeptide. In addition to D-mannosidase, our emulsin preparation contained some 2-acetamido-2-deoxy-D-glucopyranosidase activity (Pigman, 1957), and to estimate the release of D-mannose from the increase in reducing power it was necessary to correct for the release of acetamido-hexose. Emulsin caused the steady release of D-mannose from the glycopeptide to the level of approx. 1 mole/mole after 200 hr. (Fig. 1), suggesting the presence of more than one enzyme-susceptible D-mannopyranosyl unit. 2-Acetamido-2-deoxy D-glucopyranosidase released actamido-hexose, which approached a constant value of 1 mole/mole (Fig. 2), and this value was not increased by prior incubation of the glycopeptide with emulsin. The glycopeptide therefore contains as non-reducing end groups both a 2-acetamido-2-deoxy-D-glucopyranosyl unit and a D-mannopyranosyl unit, and the terminal D-mannosyl residue does not appear

to be linked through an acetamido-hexose unit to the remainder of the oligosaccharide chain. The terminal D-mannosyl residue must consume 2 moles of periodate/mole, and the terminal acetamido-hexosyl unit that is oxidized consumes 1 mole/mole, thus leaving 2 moles of periodate/mole unaccounted for in the total consumption by the oligosaccharide. As two further D-mannosyl residues are oxidized, each can react with just 1 molar equivalent of periodate, showing that they are substituted at either C-2 or C-4. The oligosaccharide unit therefore can only have the two terminal monosaccharide units indicated above, and the apparent progressive release of D-mannose by emulsin must be due to the presence of at least two D-mannosyl residues sequentially linked. The possibility of four contiguous D-mannosyl units at the non-reducing end of the oligosaccharide chain was suggested by the change in the D-mannose:amino-hexose ratio from 1·6:1·0 to 4·0:1·0 on degradation with alkali, since this stripping process operates from the liberated reducing end.

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