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Carbohydrates in Protein

7. THE NATURE OF THE CARBOHYDRATE IN OVOMUCOID*

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The study of covalently linked carbohydrate in various glycoproteins has shown that the oligosaccharide portion of the protein may be present either as a single moiety or in the form of several smaller units. An example of the former is ovalbumin, which has been extensively studied in recent years by a number of groups of investigators (Jevons, 1958; Johansen, Marshall & Neuberger, 1960, 1961; Nuenke & Cunningham, 1961; Kaverzneva & Bogdanov, 1961; Lee & Montgomery, 1962). This glycoprotein contains its carbohydrate as a single unit which is linked to an aspartic acid residue in the peptide chain. In the submaxillary-gland mucoprotein, however, the carbohydrate appears to consist of a large number of disaccharide units each individually linked to the protein (Gottschalk & Graham, 1959; Graham & Gottschalk, 1960).

In the present paper we report experiments on the nature of the carbohydrate in ovomucoid, the latter being the antitryptic factor found in hen's-egg white (Lineweaver & Murray, 1947). This glycoprotein has a molecular weight of 28 800 (Lineweaver & Murray, 1947; Fredericq & Deutsch, 1949) and contains about 25% of carbohydrate, which has been identified as consisting of glucosamine, mannose and galactose (Sørensen, 1934*a*, *b*; Stacey & Woolley, 1940, 1942; Dixon, 1955; Gottschalk & Ada, 1956; Bragg & Hough, 1961).

In the present work ovomucoid was digested with the proteolytic enzyme from *Streptomyces griseus* (Pronase) and a fraction consisting largely of carbohydrate was isolated. This fraction was studied by procedures that, it was hoped, would indicate whether the carbohydrate existed as a single prosthetic group or was composed of several smaller units. A preliminary description of the procedure for preparing the carbohydrate-rich fraction has been published (Marks, Marshall, Neuberger & Papkoff, 1962).

* Part 6: Marks, Marshall & Neuberger (1963).

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EXPERIMENTAL

Preparation of ovomucoid. Egg white was diluted with an equal volume of water, solid ammonium sulphate was added to give 50% saturation, and the pH was adjusted to 7·0. After removal of the resultant precipitate by centrifuging, the pH was adjusted to 4·6, which led to the precipitation of the ovalbumin fraction (Warner, 1954). The mixture was centrifuged, and solid ammonium sulphate was added to the supernatant fluid to give 90% saturation. The fraction that was precipitated consisted largely of conalbumin and ovomucoid. Final purification was achieved by chromatography on carboxymethylcellulose and diethylaminoethylcellulose as described by Rhodes, Azari & Feeney (1958) and Rhodes, Bennett & Feeney (1960). The purified ovomucoid fraction was concentrated by precipitation with ammonium sulphate, extensively dialysed against water and freeze-dried.

When examined by free-boundary electrophoresis in a Perkin–Elmer electrophoresis apparatus the preparation migrated essentially as a single component in sodium veronal buffer, pH 8·5 and *I* 0·1. Antitryptic activity was assessed by the ability to inhibit crystalline trypsin, casein being used as the substrate. Within the limits of experimental error, the ovomucoid preparation completely inhibited the activity of an equal weight of trypsin.

Determination of sugars. Hexose was determined by the orcinol–H_2SO_4 procedure of Winzler (1955) as modified by François, Marshall & Neuberger (1962). Mannose was used in all experiments as a standard. Glucosamine was determined by the Rondle & Morgan (1955) procedure after hydrolysis of the glycoprotein and glycopeptide fractions in 4N-HCl at 100° for 4 hr. as described by Johansen *et al.* (1960) and Marshall & Neuberger (1960).

Gel-filtration. Sephadex G-25 and Sephadex G-50 (Pharmacia, Uppsala) were used for the gel-filtration experiments, which were carried out as described by Porath (1960). Sephadex G-25 had a water regain of 2·5 g./g. of dry gel and Sephadex G-50 a water regain of 5·7 g./g. of dry gel. Before use, the fines were removed by several decantations. Two column volumes of developing solution, in these experiments 0·1N-acetic acid, were passed through the packed column before use.

Proteolytic digestion. The pH of a solution containing 200 mg. of ovomucoid and 4 mg. of Pronase-P (Kaken Chemical Co., Tokyo) in 10 ml. of 15 mM-$CaCl_2$ was adjusted to 8·5 with methylamine followed by incubation at 38°. The pH was adjusted to 8·5 periodically during the first 6 hr. of digestion after which time there was little change. Small samples were taken at various intervals and allowed to react with the ninhydrin reagent of Moore & Stein (1948). After 24 hr. of digestion the ninhydrin colour was almost constant and the proteolysis apparently complete. The ninhydrin colour at the end of 24 hr. of incubation was 11 times that of a zero-time sample. The digest was then made 0·1N with respect to acetic acid and fractionated on a Sephadex G-25 column to obtain the carbohydrate-rich fraction. In the experiment shown in Fig. 1, 200 mg. of digested ovomucoid in 10 ml. were applied to a column (2 cm. × 130 cm.) of Sephadex G-25 equilibrated with 0·1N-acetic acid, which was also used to develop the column. All the material containing carbohydrate, as shown by the orcinol reaction, emerged from the column slightly retarded and well separated from lower-molecular-weight materials. Tubes 14–18 were pooled and freeze-dried, giving a yield of 46 mg. This material, the 'glycopeptide fraction', was used for the characterization and fractionation studies described below.

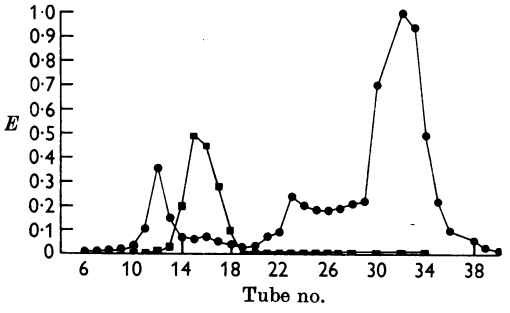

Fig. 1. Gel-filtration of 200 mg. of ovomucoid digested with Pronase on a column (2 cm. × 130 cm.) of Sephadex G-25 in 0·1N-acetic acid. Fractions of 10 ml./tube were collected. ●, Extinction at 280 mμ; ■, extinction at 505 mμ (hexose as determined by the orcinol–H_2SO_4 reaction on 0·2 ml. samples).

RESULTS

Sugar analysis, spectral studies and amino acid analysis. Determinations by the orcinol–sulphuric acid procedure showed that the glycopeptide fraction contained 26·7% of hexose (i.e. non-aminohexose) in contrast with a value of 8·4% for the ovomucoid used, mannose being used as standard. In addition, nitrogen analysis by the Kjeldahl procedure showed a reduction from 11·8% for native ovomucoid to 5·5% for the glycopeptide fraction. Analysis of the ultraviolet-absorption spectrum in the range 210–350 mμ indicated the complete absence of tyrosine and tryptophan. Hydrolysis of the glycopeptide fraction in 5·7N-hydrochloric acid at 110° for 16 hr. in a sealed tube followed by amino acid analysis in the Technicon Autoanalyser indicated the following number of amino acids per 16 residues of hexose: aspartic acid, 5·0; threonine, 3·8; serine, 1·3; glutamic acid, 0·7; proline, 1·1; glycine, 1·5; cysteine, 1·5; phenylalanine, 1·1. Alanine was also present but not sufficiently separated from the glucosamine peak to be determined. In addition, traces of valine, isoleucine and leucine were observed.

Gel-filtration on Sephadex G-50. The fact that the glycopeptide fraction emerges from a Sephadex G-25 column slightly retarded (Fig. 1) suggested that the molecular size might be near the lower limit of exclusion from penetration into the gel granules (i.e. 2000–3000). When chromatographed on Sephadex G-50 in 0·1N-acetic acid, the glycopeptide fraction emerged considerably retarded from the column. Fig. 2 demonstrates that a mixture of ovomucoid, the glycopeptide fraction and mannose can be effectively resolved on a Sephadex G-50 column. Further, a sample of the pure glycopeptide obtained from ovalbumin (Marks *et al.* 1962) emerges from the column with

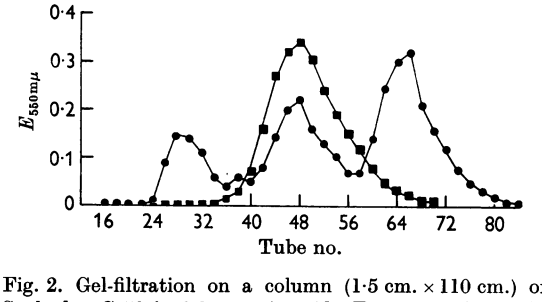

Fig. 2. Gel-filtration on a column (1·5 cm. × 110 cm.) of Sephadex G-50 in 0·1N-acetic acid. Fractions of 2·8 ml./tube were collected. Samples of 0·5 ml. were taken for the orcinol–H_2SO_4 reaction. ■, 10 mg. of ovalbumin glycopeptide; ●, the peaks from left to right are due to 30 mg. of ovomucoid, 15 mg. of ovomucoid glycopeptide fraction and 5 mg. of mannose respectively.

about the same degree of retardation as the ovomucoid glycopeptide fraction, suggesting a similar order of molecular size for the latter. The molecular weight of the ovalbumin glycopeptide, based on its constituents, should be 1500–1600.

Thin-layer dialysis. Craig, King & Stracher (1957) have shown that dialysis techniques can be used, not only to study the homogeneity of a material, but also to obtain an approximate estimate of its molecular size. By using an apparatus similar to that described by Craig *et al.* (1957), the rate of diffusion of the glycopeptide fraction during dialysis was compared with that of the glycopeptide from ovalbumin. Visking tubing (8/32 in.) was employed and 0·1 N-acetic acid used as the solvent. Fig. 3 shows that the rate of diffusion of the ovalbumin glycopeptide is a linear function of time, as expected for an homogeneous material. The behaviour of the glycopeptide fraction from ovomucoid, however, as indicated by the non-linearity of the curve, indicates a mixture of several components. It was found that 50 % of the ovalbumin glycopeptide escaped in 265 min., in contrast with 335 min. for 50 % of the ovomucoid glycopeptide fraction. Under these conditions no detectable ovomucoid had diffused in 6 hr.

Dinitrophenylation. The glycopeptide fraction from ovomucoid was treated with 1-fluoro-2,4-dinitrobenzene essentially by the methods described by Sanger (1945) and Levy (1955). To a 2 % (w/v) solution of glycopeptide in 5 % (w/v) sodium hydrogen carbonate 2 vol. of 5 % (v/v) 1-fluoro-2,4-dinitrobenzene in ethanol was added and the mixture was shaken at room temperature for 4 hr. The mixture was extracted twice with several volumes of ether, acidified and then applied to a column (1·5 cm. × 50 cm.) of Sephadex G-25 equilibrated with 0·1 N-acetic acid. In this way the DNP-glycopeptide fraction, which is very water-soluble, was displaced into 0·1 N-acetic acid and freed from salt, excess of 1-fluoro-2,4-dinitrobenzene and its reaction products. The column eluate containing the DNP-glycopeptide fraction was freeze-dried to obtain the salt-free material.

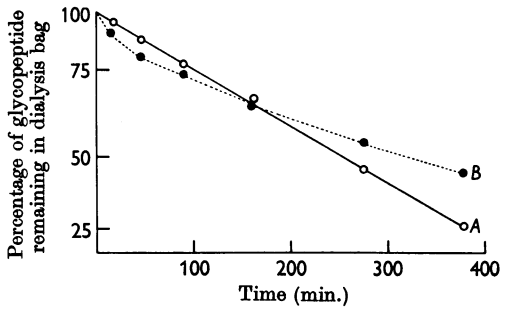

Fig. 3. Rate of diffusion during dialysis of the ovalbumin glycopeptide (*A*) and the ovomucoid glycopeptide fraction (*B*). The starting solution contained 7 mg. of glycopeptide/ml. of 0·1 N-acetic acid. Visking tubing ($\frac{8}{32}$ in.) was used. The sample volume:membrane area ratio was approx. 1 ml.:30 cm.^{2}.

The absorption spectrum of a solution (0·11 mg./ml.) of DNP-glycopeptide in 1 % (w/v) sodium hydrogen carbonate was examined in the range 230–440 mμ. The absorption spectrum observed was that of a typical DNP-peptide (Sanger, 1945) with a maximum at 350 mμ. At this wavelength the solution had an extinction of 0·622. If the molar extinction coefficient is assumed to be 16 000 (Sanger, 1945), it can be calculated that there is approximately 1 DNP-amino acid residue/2800 g. of DNP-glycopeptide.

Fractionation of the 2,4-dinitrophenylglycopeptides. The gel-filtration, dialysis and dinitrophenylation experiments indicated that the glycopeptide fraction had an average molecular weight considerably less than 10 000, the value that would be expected if the carbohydrate existed as a single moiety. An appreciably lower average molecular weight would require the presence of more than one glycopeptide unit. The homogeneity of the DNP-glycopeptide fraction was therefore investigated. The presence of 1–2 residues of cystine in this material suggested the desirability of rupturing the disulphide bonds as the initial step. Therefore, 100 mg. of DNP-glycopeptide was dissolved in 5 ml. of preformed performic acid (9 vol. of formic acid plus 1 vol. of 30 % hydrogen peroxide) and kept at 0° for 1 hr. The solution was chromatographed on a column (2 cm. × 130 cm.) of Sephadex G-25 equilibrated with 0·1 N-acetic acid. Elution with the same solvent yielded two fractions, one (fraction A) emerging almost unretarded, and the other (fraction B) considerably retarded. The contents of the tubes containing fraction A were pooled and applied to a column (0·9 cm. × 20 cm.) of Amberlite IRC-50 equilibrated with 0·1 N-acetic acid. A considerable amount of material was adsorbed on the resin and was eluted with 100 % acetic acid (fraction C). The unadsorbed fraction from the Amberlite IRC-50 column was then applied to a column (2·7 cm. × 24 cm.) of diethylaminoethylcellulose equilibrated with 0·1 N-acetic acid. Development with 0·1 N-acetic acid resulted in the elution after about 10 hold-up volumes of a fraction containing DNP-carbohydrate (fraction D). A final fraction (fraction E) was obtained by washing the diethylaminoethylcellulose column with water, followed by 0·1 N-ammonia. Fractions B–E were subsequently freeze-dried and together accounted for about 80 % of the starting material.

End-group and amino acid analyses on fractions B–E were performed qualitatively by the techniques described by Levy (1955). After hydrolysis

Table 1. *Amino acids present in glycopeptide fractions from ovomucoid*

Experimental details are given in the text. +, Present; −, not present.

Amino acid	Glycopeptide fraction B	Glycopeptide fraction C	Glycopeptide fraction D	Glycopeptide fraction E
Aspartic acid	−	+	+	+
Serine	+	+	−	−
Threonine	+	+	+*	+
Alanine	+	+	Trace	+
Cysteic acid	+*	+	−	+
Proline	−	+	−	−
Phenylalanine	−	+	−	−
Glycine	−	+	−	−
Valine	Trace	−	−	−
Glutamic acid	−	−	Trace	+*

* *N*-Terminal residue.

Table 2. *Hexose and hexosamine content in glycopeptide fractions from ovomucoid*

Experimental details are given in the text. Results are given as means ± S.E. with the numbers of experiments in parentheses.

Glycopeptide fraction	Hexose content (moles/mole of DNP-amino acid residue)	Glucosamine content (moles/mole of DNP-amino acid residue)
B	0·7 ± 0·2 (3)	0·0 (2)
C	3·9 ± 0·2 (3)	7·3 ± 0·2 (3)
D	4·5 ± 0·1 (3)	8·0 ± 0·3 (3)
E	5·8 ± 0·2 (4)	8·1 ± 0·4 (3)

in 5·7 N-hydrochloric acid at 110° for 16 hr., ether-soluble DNP-amino acid could be detected in only fractions D and E. In fraction D, DNP-threonine was identified as well as a small amount of DNP-glutamic acid. Fraction E showed only DNP-glutamic acid. In the aqueous acidic solution after ether extraction, DNP-cysteic acid was found in fraction B. No DNP-amino acid could be detected in the hydrolysate of fraction C, suggesting the possibility of DNP-glycine or DNP-proline, known to be extremely unstable under these conditions of hydrolysis (Sanger, 1945). DNP-proline was excluded by spectrophotometric analysis, in so far as DNP-proline has an adsorption maximum at 375 mμ and fraction C has a maximum at 350 mμ. All the hydrolysates, after ether extraction, were examined chromatographically for the presence of amino acids and the results are summarized in Table 1.

The hexose and glucosamine contents of each of the fractions was related to the amount of DNP-amino acid residue present, determined spectrophotometrically. The results (summarized in Table 2) indicate that fraction B, liberated by oxidation with performic acid, is not a glycopeptide, the small amount of hexose present probably being due to contamination. Fractions C, D and E, however, each contain both hexose and hexosamine. The variation in the hexose content of fractions C, D and E (3·9, 4·5 and 5·8 residues/mole of DNP-amino acid respectively) suggests that the three glycopeptides are not identical. To the nearest integer, 15 residues of hexose and 23 residues of glucosamine are accounted for, in good agreement with the number present in the native protein (Marshall & Neuberger, 1960).

DISCUSSION

The results of our experiments strongly suggest that the carbohydrate of ovomucoid is not present as a single large prosthetic group but as several smaller units, each individually linked to the protein. If the carbohydrate were present as a single moiety a molecular weight of about 7500 would be expected from the sugar content alone; and with the associated amino acids, as shown after digestion with Pronase and separation on Sephadex G-25, a molecular weight closer to 10 000–11 000 could be expected. The results of chromatography on both Sephadex G-25 and Sephadex G-50, the dialysis experiments and the dinitrophenylation studies argue against the concept of a single prosthetic group. Indeed, the fact that the crude DNP-glycopeptide could be separated into fractions with different carbohydrate and amino acid contents strongly argues against the possibility of a single large prosthetic group. These results are in contrast with those of Hartley & Jevons (1962), who interpreted their findings as indicating that the carbohydrate in the protein is present as a single large prosthetic group although the possibility of the presence of two or more smaller groups 'all similar in composition' was not dismissed.

Tanaka (1961*a*), on the other hand, has reported experiments in which he digested ovomucoid with trypsin and obtained by dialysis glycopeptides that had different carbohydrate contents. In a preliminary experiment, we have digested performic acid-oxidized ovomucoid with trypsin and

chromatographed the digest on a Sephadex G-50 column. The complex pattern that was observed with respect to distribution of the carbohydrate suggested the presence of at least three poorly resolved glycopeptides.

Although no definite conclusion may be drawn from our experiments about which particular amino acid is linked to the carbohydrate, a few points can be made. Of the amino acids that have often been considered as possible points of bonding between carbohydrate and protein, namely aspartic acid, threonine and serine, the first is present in all three glycopeptide fractions, as is also threonine (Table 1). Serine, on the other hand, occurs in only one of the glycopeptides. When the DNP-glycopeptides were treated with carboxypeptidase under the conditions described by Harris, Li, Condliffe & Pon (1954), some threonine was liberated from all three peptide fractions. Threonine, therefore, is not likely to be involved in the carbohydrate–peptide linkage; however, this evidence is not conclusive as we have not shown that all the threonine is liberated by the enzyme treatment. That at least one of the carbohydrate moieties is linked by means of glucosamine to aspartic acid is indicated by the results of Tanaka (1961*b*).

SUMMARY

1. Ovomucoid prepared from the egg white of hen's eggs was digested with the proteolytic enzyme Pronase, obtained from *Streptomyces griseus*, and the glycopeptides were isolated by chromatography in Sephadex G-25.

2. The glycopeptide fraction, when chromatographed on Sephadex G-50, was considerably retarded, suggesting a molecular weight much less than 10 000. The glycopeptide from ovalbumin (mol.wt. about 1500) behaved on the Sephadex column in a fashion similar to that of the ovomucoid glycopeptide.

3. The glycopeptide fraction was readily diffusible and, from the characteristics of its rate of diffusion during dialysis, appears to be a mixture. The rate of diffusion was comparable with that of the ovalbumin glycopeptide.

4. Dinitrophenylation of the glycopeptide fraction indicated that there was approximately 1 DNP-amino acid residue/2800 g. of glycopeptide fraction.

5. The DNP-glycopeptide fraction could be separated into three fractions containing different amounts of hexose, hexosamine and amino acids.

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