Studies on Glycopeptides Derived from Acidic Glycoproteins of Guinea-Pig Serum

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1. Fraction I, a fraction containing acidic glycoproteins, isolated from guinea-pig serum, was digested with Pronase after removal of sialic acid and a major and a minor glycopeptide fraction were isolated by chromatography with Sephadex G-25 and G-50. 2. The major fraction was examined by various methods and shown to contain several glycopeptides. Estimates of molecular weight of the glycopeptide fractions were obtained. Although some variation appeared to occur, the glycopeptides were not grossly heterogeneous with respect to size. An average prosthetic group was estimated to contain about 15 sugar residues. 3. Aspartic acid was the principal amino acid present in the fractions and in all subfractions of the major fraction investigated. Where examined, ammonia was liberated on acid hydrolysis in approximately equimolar amounts to the aspartic acid present. The carbohydrate composition of the fractions was also determined. 4. The glycopeptides showed relatively little degradation in alkaline solution. 5. These results suggest that an *N*-acylglycosylamine bond involving aspartic acid forms the major type of linkage between carbohydrate and polypeptide. The isolation of a compound with the composition and chromatographic properties of 2-acetamido-1-(*L*- β -aspartamido)-1,2-dideoxy- β -D-glucose supports this view, and indicates that *N*-acetylglucosamine is the sugar involved in at least many linkages. 6. Fraction I contains some glycoproteins that are susceptible to Pronase and one or more others that resist digestion before the removal of sialic acid. A brief examination revealed some similarities between prosthetic groups derived from both kinds of glycoprotein.

The present work forms part of investigations on the characteristics and biosynthesis of components of an α -globulin fraction containing acidic glycoproteins, fraction I, isolated from guinea-pig serum. In previous work, the physical and immunological characteristics of fraction I and the chemical composition of its carbohydrate prosthetic groups were studied (Simkin, Skinner & Seshadri, 1964b), and some information was obtained on the structure of the prosthetic groups (Simkin, Seshadri & Skinner, 1964a). Fraction I is composed in large part of molecules with closely similar properties, and other components present show some similarities to the major part of the material.

The aims of the present study were to investigate the size of the carbohydrate groups and the nature of the bonds linking them to polypeptide. This work should provide some information on the heterogeneity of the carbohydrate groups and thus show whether the general similarity of the glyco-

proteins is reflected in their prosthetic groups. Glycopeptides were prepared by digestion of fraction I with the protease, Pronase, followed by fractionation by chromatography on Sephadex and by other techniques. Estimates for molecular weight were obtained from results with Sephadex G-25 and, with one glycopeptide fraction, by analysis in the ultracentrifuge. Apart from analysis of glycopeptides for amino acid and sugar constituents, the yield of ammonia on mild acid hydrolysis was determined because *N*-acylglycosylamine bonds known to join carbohydrate to polypeptide in some glycoproteins split with the production of ammonia under such conditions (see, e.g., Grant & Simkin, 1965). The effect of alkali on two glycopeptide fractions was studied since the various carbohydrate-polypeptide bonds known to occur in glycoproteins differ markedly in their stability to alkali (e.g. Grant & Simkin, 1965). The products of partial acid hydrolysis of two fractions were examined since this approach can lead to the isolation of a fragment containing only a single

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amino acid and sugar residue, such as AADG* (e.g. Marshall & Neuberger, 1964; for other references see Grant & Simkin, 1965).

EXPERIMENTAL

Fraction I. This was prepared as described by Simkin *et al.* (1964b).

Preparation of glycopeptide fractions

Chromatography was carried out at 2° throughout. Fractionations were followed by measurement of total hexose (see below); where appropriate, measurement of ultraviolet extinction (usually at 230 m μ) and the ninhydrin reaction were used as supplementary methods (see the Results section).

Fractions A₁ and A₂ and subfractions. Fraction I (200 mg.) was dissolved at 6 mg./ml. in 100 mm-tris-55 mM-HCl buffer, pH 8.0, containing CaCl₂ (10 mM), and 1.5% of its weight of Pronase (grade B; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., batch no. 502117; hexose content by orcinol method about 2%) was added. The mixture was incubated at 37° for 24 hr. in the presence of toluene. Pronase equal to one-half of that initially used was added and incubation continued for 24 hr. After centrifuging, the digest was concentrated *in vacuo* to 10 ml. and applied to a column (4.2 cm. $\times$ 50 cm.) of Sephadex G-25 (coarse grade). The column was eluted with 10 mM-tris buffer, pH 8.0, at about 40 ml./hr. Appropriate fractions (see the Results section) were pooled and concentrated *in vacuo*. Pronase equal to the first addition made above was added, and the concentrations of tris buffer and CaCl₂ were adjusted to those used for the first digestion; the total volume was 30% of that used for the latter. The mixture was incubated for 24 hr. at 37°. It was concentrated *in vacuo* to 5 ml., applied to a column (2 cm. $\times$ 65 cm.) of Sephadex G-50 (medium grade), and elution carried out with 100 mM-tris buffer, pH 8.0, at about 10 ml./hr. Fractions corresponding to non-retarded material and fraction A₂ (see the Results section) were pooled and concentrated *in vacuo* to 2.0–2.5 ml. They were desalted on a column (1.5 cm. $\times$ 60 cm.) of Sephadex G-25 (medium grade), elution (about 15 ml./hr.) being with 5 mM-pyridine-1.35 mM-acetic acid buffer, pH 5.6. Appropriate fractions were pooled and freeze-dried. For the preparation of fraction A₁, non-retarded material (35 mg.) was heated at 2 mg./ml. with 25 mM-H₂SO₄ at 80° for 60 min. to remove sialic acid (see the Results section). The hydrolysate was dialysed against water at 2° for 20 hr. and the non-diffusible material freeze-dried. Digestion with Pronase was carried out as above, except that incubation after the second addition of Pronase was for 48 hr. The digest was fractionated on a column (2.1 cm. $\times$ 65 cm.) of Sephadex G-25 (coarse grade) and suitable fractions were reincubated with Pronase as described above. Fractionation with Sephadex G-50 and desalting with Sephadex G-25 were then carried out as before.

A second preparation of fraction A₂ was made in a similar way from 80 mg. of fraction I, with volumes etc. suitably adjusted.

* Abbreviation: AADG, 2-acetamido-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose.

Fraction A_{2a} was prepared by dissolving fraction A₂ (3.3 mg. of total hexose) in 2.3 ml. of 200 mM-N-ethylmorpholine-35 mM-acetic acid buffer, pH 8.4, containing 0.3 mg. of leucine aminopeptidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.); 0.025 mM-MgCl₂ was present (cf. Ambler, 1963). Incubation was for 24 hr. at 37°. The mixture was freeze-dried and glycopeptide reisolated by application to a column (1.5 cm. $\times$ 32 cm.) of Sephadex G-50 (medium grade) and elution with 5 mM-pyridine-acetate buffer, pH 5.6. Fraction A_{2b} was prepared by streaking fraction A_{2a} (0.6 mg. of hexose) on Whatman no. 3MM paper and electrophoresis with 600 mM-pyridine-42 mM-acetic acid buffer, pH 6.4, for 3 hr. at about 30 v/cm. Glycopeptides were located on guide strips by staining with ninhydrin (Fletcher, Marks, Marshall & Neuberger, 1963) or periodate-benzidine (Montgomery & Wu, 1963). The main band detected was cut out (together with a paper blank) and eluted (Dent, 1947) with 5 mM-pyridine-acetate buffer, pH 5.6.

Fractions B₁ and B₂ and subfractions. Sialic acid was removed from fraction I as described above. The treated material (160 mg.) was incubated with Pronase as indicated for the preparation of fraction A₂, and this was followed by fractionation on a similar column of Sephadex G-25 (coarse grade). The glycopeptide material was, however, refractionated after concentration on a column (2 cm. $\times$ 65 cm.) of Sephadex G-25 (coarse grade) before further treatment with Pronase to ensure complete separation from low-molecular-weight material and equilibration with 10 mM-tris buffer. After further incubation with Pronase as before, the digest was applied to a column (2 cm. $\times$ 65 cm.) of Sephadex G-50 (fine-bead grade). Elution (24 ml./hr.) was with 100 mM-tris buffer, pH 8.0, and fractions corresponding to fractions B₁ and B₂ (see the Results section) were pooled, concentrated and desalted as described above. A second fractionation for material to be studied by partial acid hydrolysis was begun with 86 mg. of material, with volumes etc. adjusted appropriately.

For the preparation of fraction B_{2a}, fraction B₂ (4.1 mg. of hexose) was incubated with 0.5 mg. of leucine aminopeptidase as indicated above for fraction A_{2a}; the total volume was 5.5 ml. After 24 hr. at 37°, a further 0.25 mg. of enzyme was added and incubation continued for 12 hr. The mixture was freeze-dried and glycopeptide reisolated by chromatography on a column (1.5 cm. $\times$ 60 cm.) of Sephadex G-50 (fine-bead grade) with 5 mM-pyridine-acetate buffer, pH 5.6, as eluent. Fraction B_{2b} was isolated from fraction B_{2a} (0.9 mg. of hexose) by paper electrophoresis as for fraction A_{2b}.

Ovalbumin. Egg albumin (1.1 g.) (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was digested with Pronase and the digest fractionated with ethanol as described by Fletcher *et al.* (1963). The ethanol-precipitated material was fractionated on a column (4.2 cm. $\times$ 50 cm.) of Sephadex G-25 (coarse grade) with 100 mM-acetic acid as eluent. Appropriate fractions were pooled, concentrated and further chromatographed on a column (1.5 cm. $\times$ 57 cm.) of Sephadex G-25 (medium grade) with 5 mM-pyridine-acetate buffer, pH 5.6, as eluent.

Ovomucoid. The starting material (500 mg. of trypsin inhibitor; Sigma Chemical Co., St Louis, Mo., U.S.A.) was first fractionated by a method based on that of Chatterjee & Montgomery (1962). It was applied to a column (3 cm. $\times$ 29 cm.) of DEAE-cellulose (prepared by the method of

Peterson & Sober, 1956) equilibrated with 3.05 mM- Na_2HPO_4 –1.95 mM- NaH_2PO_4 buffer, pH 7.0. After washing with 25 ml. of 5 mM-buffer, the column was eluted with a linear gradient prepared by mixing 125 ml. volumes of 50 mM-phosphate buffer, pH 7.0, without and with Na_2SO_4 (500 mM). The main peak found, which was eluted about 140 ml. after connexion to the gradient, was dialysed against water and freeze-dried. Digestion of 400 mg. of material with Pronase and fractionation with Sephadex G-25 (coarse grade) were carried out as for the preparation of fraction A_2 . After further digestion, fractionation with Sephadex G-50 was as for the preparation of fractions B_1 and B_2 . The major hexose-containing peak was desalted as usual.

Examination of glycopeptide fractions

Molecular weight. (1) Glycopeptide samples (about 0.15–0.35 mg. of hexose; 0.2 ml.) were applied to a column (1.1 cm. $\times$ 115 cm.) of Sephadex G-25 (medium grade; water regain 2.4 g./g.). Elution at 2° was with 5 mM-pyridine-acetate buffer, pH 5.6, or 100 mM-acetic acid at 4.5 ml./hr. Fractions (0.45 ml.) were collected and analysed for total hexose. Bovine serum albumin (Armour Pharmaceutical Co. Ltd.) and glucose (0.5 mg. each) were added as reference compounds to all samples examined. Isomaltopentaose and isomaltohexaose used as other reference compounds were obtained from Dr J. R. Turvey (University College of North Wales, Bangor). (2) Fraction B_{2a} and the ovomucoid glycopeptide fraction in solution (about 2.5 and 3 mg. of hexose/ml. respectively) in 43 mM-sodium acetate–57 mM-acetic acid buffer, pH 4.5, were centrifuged at 27 690 rev./min. for 4 days at 20° in a Spinco model E ultracentrifuge fitted with Rayleigh interference optics. A 12 mm. interference cell was used; the column height was 4–5 mm.

Paper electrophoresis. The conditions used were similar to those given above for preparative separations. Fractions A_2 and A_{2a} were also examined with 124 mM-pyridine–70 mM-acetic acid buffer, pH 5.3.

Treatment with alkali. Glycopeptide (e.g. fraction B_2 , 0.25 mg. of hexose) was kept in 0.3 ml. of 0.5 N-NaOH for 24 hr. at 2° and then reisolated by chromatography on a column (1.5 cm. $\times$ 35 cm.) of Sephadex G-25 (medium grade) with 5 mM-pyridine-acetate buffer, pH 5.6, as eluent.

Partial acid hydrolysis. Fractions B_1 and B_2 (1.7 and 2 mg. of hexose respectively) were heated with 0.2 ml. of 2 N-HCl for 12 min. at 100°. The hydrolysates were evaporated to dryness in a rotary evaporator. The residue from fraction B_1 was dissolved in 0.2 ml. of 100 mM-pyridine–300 mM-acetic acid–370 mM-formic acid buffer, pH 3.05 (cf. Micheel, Tanaka & Römer, 1964), and a 0.05 ml. sample chromatographed on a column (0.6 cm. $\times$ 100 cm.) of Aminex MS Fraction B resin (no. 48730; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) with the same buffer as eluent. The residue from fraction B_2 was dissolved in 0.15 ml. of 50 mM-sodium citrate–50 mM-NaCl–75 mM-HCl buffer, pH 2.87, containing 5.5% (v/v) of methanol, and a 0.025 ml. sample chromatographed on the 0.6 cm. Chromobead A column of a Technicon amino acid analyser with the same buffer as eluent. The effluent from each column was connected to the analytical system of a Technicon amino acid analyser. The remainder of each hydrolysate was later chromatographed in a similar way to the respective analytical fractionation and 0.75 ml. fractions were collected. The positions of the peaks of eluted material

were determined by comparison with the initial fractionation, and appropriate fractions were pooled and evaporated to dryness. The elution volumes of synthetic AADG and 2-L- β -aspartamido-2-deoxy-D-glucose (the latter was applied only to the Chromobead column) were determined in separate experiments; the samples were provided by Dr R. D. Marshall (St Mary's Hospital Medical School, London). Of the material that corresponded in elution volume to AADG, all of that derived from fraction B_1 and one-quarter of that from fraction B_2 were heated with 0.2 ml. of 4 N-HCl for 3 hr. at 100°, and amino acid compositions determined as described below. The remaining material from fraction B_2 was analysed for ammonia as indicated below. Other pooled fractions from the columns were heated with 0.5 ml. of 6 N-HCl at 100° for 12 hr. before the determination of amino acid content.

Chemical analyses

Total hexose. This was determined by the method of Winzler (1955) scaled down to a total volume of 0.85 ml. Extinction at 540 μm (or occasionally 510 μm) was determined with a Unicam SP.600 spectrophotometer fitted with 2 cm. light-path micro-cells. An equimolar mixture of galactose and mannose was used as standard.

Individual sugars and protein. These were measured as described by Simkin *et al.* (1964b), except that ethyl acetate–pyridine–water (10:4:3, by vol.) was used as solvent in the paper-chromatographic separation of galactose and mannose, and sialic acid was estimated only by the thiobarbituric acid method.

Ninhydrin reaction. The procedure of Yemm & Cocking (1955) was used, often scaled down and used in conjunction with micro-cells.

Amino acid composition. This was determined with a Technicon amino acid analyser. Unless otherwise stated, hydrolysis was with 6 N-HCl at 110° for 24 hr. Hydrolysates were prepared *in vacuo* and evaporated etc. as described by Moore & Stein (1963), humin being removed by filtration.

Ammonia. Samples (about 0.35 mg. of hexose) were hydrolysed with 0.5 ml. of 2 N-HCl at 100° for 3 hr. (sialic acid was removed from fraction A_2 before analysis by hydrolysis with 25 mM- H_2SO_4 at 85° for 60 min., followed by chromatography on Sephadex G-25). Hydrolysates were evaporated to dryness in a rotary evaporator. The residues were dissolved in 0.6 ml. of water, and 0.5 ml. of solution was transferred to a no. 2 micro-diffusion unit (Conway, 1962). The ammonia liberated in 5 hr. at 2° after the addition of 0.5 ml. of saturated K_2CO_3 (cf. Tracey, 1952) was trapped in 0.3 ml. of 50 mM- H_2SO_4 . The acid was made up to 2.0 ml. with water, and 0.5 ml. samples were analysed by the ninhydrin method. Ammonium sulphate was used as standard. Acid-distilled water was used throughout. Among controls usually included were test samples that had not been subjected to hydrolysis with HCl.

RESULTS

Preparation of glycopeptides. When fraction I was digested with Pronase and the digest applied to Sephadex G-25, almost all of the hexose applied was eluted at the void volume and was well

Fig. 1. Chromatography on Sephadex G-50 of a Pronase digest of hexose-containing material isolated from fraction I by means of Sephadex G-25 after initial treatment with Pronase; the eluent was 100 mM-tris buffer, pH 8.0. —, E_{540} , orcinol reaction; ----, E_{570} , ninhydrin reaction; ····, E_{230} . The fractions indicated were pooled and taken as non-retarded material (N) and glycopeptide fraction A_2 .

separated from smaller amino acid-containing material; there was also a slightly retarded very small hexose-positive peak. The principal hexose-containing peak was redigested with Pronase and applied to Sephadex G-50 (Fig. 1). Two hexose-containing peaks were found; the first was eluted at the void volume and showed a high extinction at 230μ , and the second had V_e/V_0 1.8 (V_e , elution volume; V_0 , void volume). The first peak contained about 40% of the hexose eluted. Fractions were pooled as indicated in Fig. 1 and desalted on Sephadex G-25; the hexose recovered after desalting was 85% of that present in the fraction I originally taken. Fraction A_2 was eluted as a single hexose-containing peak (V_e/V_0 1.1) that showed no obvious heterogeneity.

The material that was not retarded on Sephadex G-50 was also eluted near to the void volume on application to a column (1.5 cm. $\times$ 61 cm.) of Sephadex G-75 and elution with *N*-propionic acid. Fraction I itself shows little or no retardation on Sephadex G-75. This and other results suggested that no more than limited proteolysis of this material could have taken place. For example, most of the material recovered from Sephadex G-75 travelled with a mobility similar to the main band of fraction I on starch-gel electrophoresis (pH 9 buffer system; Simkin *et al.* 1964b). Some serum glycoproteins, such as human orosomucoid, show little proteolysis until sialic acid is removed (e.g. Winzler, 1960). It was concluded that a similar

Fig. 2. Chromatography on Sephadex G-50 of a Pronase digest of hexose-containing material isolated from sialic acid-free fraction I by means of Sephadex G-25 after initial treatment with Pronase; the eluent was 100 mM-tris buffer, pH 8.0. —, E_{540} , orcinol reaction; ----, E_{570} , ninhydrin reaction; ····, E_{230} . The fractions indicated were pooled and taken as glycopeptide fractions B_1 and B_2 .

situation obtained with the non-retarded material from the results of control experiments in which it was found that, when the non-retarded material was heated at 80° for 60 min. with either water or 25 mM-sulphuric acid before proteolysis, treatment with acid (which removes most or all sialic acid) gave a much greater increase in susceptibility to Pronase than heating with water. The major part of the non-retarded material was thus hydrolysed with dilute acid and then digested with Pronase and fractionated by procedures similar to those used for the preparation of fraction A_2 . On application to Sephadex G-25, almost all of the hexose was eluted as a single peak (V_e/V_0 1.19). After further digestion and application to Sephadex G-50, there were two hexose-positive peaks, a small one (V_e/V_0 1.7), which was discarded, and a large peak (V_e/V_0 2.2), which was taken as fraction A_1 . This was desalted on Sephadex G-25 and yielded a single hexose-containing peak (V_e/V_0 1.25) with no obvious heterogeneity.

Because of the variation in susceptibility to Pronase found, and in an effort to obtain a greater degree of proteolysis, for further work fraction I was heated with dilute sulphuric acid before treatment with Pronase. On application of the initial Pronase digest to Sephadex G-25, all of the hexose-containing material was eluted as a peak (V_e/V_0 1.2) with a small trail. After redigestion and application to Sephadex G-50 (Fig. 2), two hexose-containing peaks (V_e/V_0 1.50 and 1.77) were found. The major peak showed a shoulder at V_e/V_0 1.66.

Table 1. V_e/V_0 values for glycopeptide fractions and oligosaccharides

Results were obtained by chromatography on a column (1.1 cm. $\times$ 115 cm.) of Sephadex G-25 with 100 mM-acetic acid as eluent. V_0 was determined with bovine serum albumin. The molecular weights of fractions B₁ and B_{2a} and the ovomucoid fraction were calculated from V_e/V_0 -log(molecular weight) data (see the text), and that of the ovalbumin fraction was calculated from chemical composition. The values shown in parentheses were obtained with 5 mM-pyridine-acetate buffer, pH 5.6, as eluent.

Sample	V_e/V_0		Molecular weight
Fraction B ₁	1.17	(1.12)	3070
Fraction B _{2a}	1.22*, 1.26	(1.16, 1.24)	2700, 2450
Ovomucoid fraction	1.20, 1.24	(1.17, 1.20)	2840, 2580
Ovalbumin fraction	1.37	(1.31)	1880
Isomaltohexaose	1.63	(1.61)	990
Isomaltopentaose	1.68	(1.66)	828
Raffinose	1.93	(1.93)	504

* Apparent as shoulder on peak.

Fractions were pooled as indicated in Fig. 2. Fraction B₁ contained 20–25% of the hexose present in fraction B₂. Both fractions were desalted on Sephadex G-25 and no further fractionation was achieved under the conditions used; fractions B₁ and B₂ showed V_e/V_0 1.10 and 1.15 respectively. The final recovery in terms of hexose was about 80%.

Subfractionation and molecular-weight studies. The two major glycopeptide fractions isolated, fractions A₂ and B₂, and the materials obtained from them by treatment with leucine aminopeptidase (fractions A_{2a} and B_{2a} respectively) were examined by paper electrophoresis. The patterns obtained from fractions A₂ and A_{2a} at pH 5.3 and from fraction A_{2a} at pH 6.4 were essentially similar: one large, one medium and three minor bands were present, all of which stained with ninhydrin and gave a positive reaction for carbohydrate with periodate-benzidine. The two larger bands gave a brown-purple colour with ninhydrin. Fractions B₂ and B_{2a} showed similar patterns at pH 6.4: one major and two minor bands were found, all of which stained with both reagents. Fractions B₁ and B_{2a} were examined by chromatography on a column of Sephadex G-25 longer than those used for preparative work to gain further information on heterogeneity and to obtain an estimate of molecular weight by comparison with several oligosaccharides and with glycopeptides isolated from ovalbumin and ovomucoid. Both 5 mM-pyridine-acetate buffer, pH 5.6, and 100 mM-acetic acid were used as eluents. Table 1 shows that there was a tendency for compounds to be eluted slightly more slowly with pyridine-acetate than with acetic acid. In addition, fraction B_{2a} showed a more obvious separation into two peaks with the former (Fig. 3), in contrast with the result obtained with acetic acid where heterogeneity was apparent

Fig. 3. Chromatography of glycopeptide fractions B₁ (.....) and B_{2a} (—) on a column (1.1 cm. $\times$ 115 cm.) of Sephadex G-25 with 5 mM-pyridine-acetate buffer, pH 5.6, as eluent. E_{540} , orcinol reaction.

only as a shoulder on the ascending side of the peak found. Fraction B₁ showed a single broad peak with either eluent (e.g. Fig. 3). The ovomucoid glycopeptide fraction showed two incompletely resolved peaks of similar size. The slight retardation of material in pyridine-acetate buffer as compared with acetic acid and the greater resolution of fraction B_{2a} may be due to the occurrence in addition to simple gel filtration of adsorption resulting from the use of an eluent of low ionic strength (e.g. Determann, 1964; see also the Discussion section). Estimates for molecular weight were therefore calculated from data obtained with acetic acid. From published work on the composition of the prosthetic group of ovalbumin (e.g. Fletcher *et al.* 1963) and the amino acid composition of the fraction prepared, the average molecular weight of the ovalbumin fraction was calculated to be 1880. When the V_e/V_0 values for the ovalbumin glycopeptide fraction and the oligosaccharides were plotted against log (molecular weight) (e.g. Andrews,

1964, 1965; Whitaker, 1963), the points lay close to a straight line, and values for the molecular weights of glycopeptides present in fractions B₁ and B_{2a} and in the ovomucoid preparation were calculated by use of this line (Table 1). Examination of glycopeptides with a similar column of Sephadex G-50 was also attempted, but with acetic acid as eluent poor separation of the various fractions was obtained (cf. Neuberger & Papkoff, 1963). Fraction B_{2a} and the ovomucoid fraction were also examined in the analytical ultracentrifuge by an equilibrium sedimentation method. With each a straight line was obtained on plotting log of concentration (expressed in Rayleigh fringes) against the square of radial distance. A value for molecular weight was obtained from the slope of each line, assuming a partial specific volume of 0.664 ml./g. as found by Spiro (1962) for glycopeptides from fetuin. The values obtained were 2100 for fraction B_{2a} and 2500 for the ovomucoid fraction.

Chemical analysis of glycopeptides. Analysis of glycopeptides from fraction I (Table 2) showed that in all instances aspartic acid was the principal amino acid present. This was also true of subfractions of

fraction B_{2a} examined in addition to fraction B_{2b} cited in the Table, namely the two minor fractions obtained on electrophoresis (see above) and the two fractions separated by chromatography on Sephadex G-25 with pyridine-acetate buffer as eluent (Fig. 3). The latter two fractions showed relatively minor differences in amino acid composition except for glutamic acid: the subfraction that was eluted more rapidly had a molar content of glutamic acid relative to aspartic acid of 0.31 as compared with 0.05 for the slower material. The main subfractions (A_{2b} and B_{2b}) isolated from fractions A_{2a} and B_{2a} respectively by electrophoresis also had a relatively low content of glutamic acid (Table 2). Both of these subfractions accounted for about 50% of the hexose of the material taken for electrophoresis (not corrected for losses). Treatment of fractions A₂ and B₂ with leucine aminopeptidase (see fractions A_{2a} and B_{2a}, Table 2), in an effort to remove any *N*-terminal amino acids not involved in linkage with carbohydrate, decreased the content of several minor amino acids somewhat. Table 2 shows that, with all fractions examined, ammonia was liberated on mild acid hydrolysis in amounts that in molar terms are

Table 2. *Composition of glycopeptide fractions isolated from fraction I*

Values are in molar terms in relation to aspartic acid. Values for threonine and serine have been corrected for destruction (Moore & Stein, 1963) from results with fraction B₂ that was hydrolysed for 41 hr. in addition to the customary 24 hr. period. Ammonia was determined after hydrolysis with 2N-HCl for 3 hr. Fraction B_{2(OH)} refers to sample treated with alkali (see the text). 'Trace' represents a value of 0.03 or less.

Fraction ...	A ₁	A ₂	A _{2a}	A _{2b}	B ₁	B ₂	B _{2(OH)}	B _{2a} *	B _{2b}
Aspartic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Threonine	0.30	0.53	0.47	0.47	0.51	0.42	0.38	0.38	0.44
Serine	0.17	0.48	0.42	0.37	0.34	0.25	0.22	0.22	0.25
Glutamic acid	0.15	0.29	0.19	0.08	0.33	0.17	0.22	0.15	Trace
Proline	0.14	0.32	0.34	Trace	0.33	0.27	0.26	0.21	0.18
Glycine	0.19	0.34	0.34	0.26	0.36	0.34	0.27	0.25	0.16
Alanine	0.09	0.18	0.15	0.13	0.18	0.12	0.14	0.09	0.09
Valine	0.04	0.12	0.08	0.13	0.12	0.07	0.08	Trace	0.04
Cystine (half)	—	Trace	—	—	0.13	Trace	Trace	Trace	—
Methionine	—	—	—	—	—	—	—	—	—
Isoleucine	Trace	0.06	Trace	Trace	0.04	Trace	Trace	Trace	—
Leucine	0.06	0.15	0.04	0.07	0.10	0.04	0.09	Trace	Trace
Tyrosine	—	Trace	—	—	0.04	Trace	Trace	—	—
Phenylalanine	0.15	0.10	0.05	0.09	0.11	0.10	0.14	0.10	0.08
Lysine	Trace	0.08	Trace	Trace	0.09	Trace	0.08	Trace	Trace
Histidine	—	Trace	Trace	—	0.05	Trace	Trace	Trace	—
Arginine	—	0.05	Trace	—	Trace	—	—	—	—
Ammonia	—	1.2	—	—	1.3	1.0	—	1.2	1.1
Total hexose†	4.8	5.6	5.5	—	5.6	6.0	5.8	6.0	—
Galactose	—	2.7	—	—	2.7	—	—	2.9	—
Mannose	—	2.9	—	—	2.9	—	—	3.1	—
Hexosamine	—	4.0	—	—	3.3	—	—	5.0	—

* Mean of duplicate values except for total hexose.

† Orcinol method.

equal to or slightly greater than the aspartic acid present.

Though the content of total hexose in relation to aspartic acid was determined in most instances, only three fractions were analysed for individual sugars (Table 2). Mannose and galactose were present in approximately equimolar amounts in each fraction as in fraction I (see below). The hexosamine/galactose molar ratio 1.47 for fraction A₂ was similar to that found for fraction I, but the ratios for fractions B₁ and B_{2a} were 1.23 and 1.74 respectively (the mean molar proportions of sugars obtained by analysis of four samples of fraction I in the present work were: galactose, 1.00; mannose, 1.05; hexosamine, 1.50; these values are close to those of Simkin *et al.* 1964b). Fraction A₂ contained a slightly lower sialic acid/galactose ratio than fraction I (see also the Discussion section).

Treatment with alkali and partial acid hydrolysis. To test their stability towards alkali, fractions B₁ and B₂ were kept in 0.5N-sodium hydroxide at 2° for 24hr. (cf. Anderson *et al.* 1964) and then reisolated and analysed. Table 2 shows that there was very little degradation of fraction B₂ and that the aspartic acid/total hexose ratio was not altered significantly. A similar result was obtained with material that was analysed without reisolation of glycopeptide. The degradation in alkali of threonine and serine in the minor fraction B₁ was somewhat greater (results not shown in Table 2), about one-third of the total threonine plus serine disappearing; the aspartic acid/total hexose ratio was changed only slightly.

Fractions B₁ and B₂ were subjected to partial acid hydrolysis (2N-hydrochloric acid at 100° for 12min.; cf. Marshall & Neuberger, 1964) and the

products examined by ion-exchange chromatography. Fig. 4 shows results obtained on fractionation of the products of fraction B₂ on the column of a Technicon amino acid analyser (cf. Marks, Marshall & Neuberger, 1963). A number of peaks were present that were eluted faster than aspartic acid; some other small peaks due to substances other than free amino acids and with a slower mobility than aspartic acid were also found. One of the faster peaks had an elution volume similar to that of synthetic AADG and showed the high E_{440}/E_{570} ratio characteristic of that compound. Its elution volume was smaller than that of synthetic 2-L- β -aspartamido-2-deoxy-D-glucose (cf. Marks *et al.* 1963). The material in question was isolated and yielded aspartic acid, glucosamine and ammonia on hydrolysis in the molar proportions 1.00:1.18:0.94; no other ninhydrin-positive substances were detected. The amount of aspartic acid present represented about 10% of that in the fraction B₂ taken for partial hydrolysis. About 50% of synthetic AADG broke down under the conditions used. The other peaks with small elution volumes appeared to represent glycopeptides that had suffered relatively little degradation. A partial hydrolysate of fraction B₁ was chromatographed on a column of somewhat lower resolution than that used for fraction B₂, and again a peak with an elution volume and E_{440}/E_{570} ratio similar to those of AADG was detected. This peak yielded mainly aspartic acid and glucosamine on hydrolysis. The presence of small amounts of other amino acids may have resulted from incomplete separation from other products of hydrolysis.

DISCUSSION

The results obtained on chromatography on Sephadex of the glycopeptides derived from fraction I by either fractionation scheme show that the prosthetic groups of the components of fraction I are not grossly heterogeneous with respect to size. Some relatively small differences in size do appear to occur; e.g., fraction B₁ is eluted somewhat more rapidly than fraction B_{2a} (e.g. Figs. 2 and 3). The difference in hexosamine/hexose ratio between these fractions may indicate that the prosthetic groups represented by them are somewhat different.

The methods used for the estimation of molecular weight are subject to some uncertainties. Results with Sephadex may be complicated by ionic interaction with the gel (e.g. Determann, 1964), especially with eluents of low ionic strength. The separation of fraction B_{2a} into two peaks was more marked at lower ionic strength (Table 1 and Fig. 3), and the relative content of glutamic acid in the two peaks was as expected if the gel were

Fig. 4. Chromatography of a partial acid hydrolysate of glycopeptide fraction B₂ on the column of a Technicon amino acid analyser followed by reaction of the effluent with ninhydrin; —, E_{570} ; ····, E_{440} . Only aspartic acid (Asp) and substances of smaller elution volume than it are shown. The material marked A had an elution volume similar to that of AADG.

negatively charged. Another difficulty lies in accurate calibration, since proteins and polysaccharides of similar molecular weight are eluted at different volumes on Sephadex (e.g. Andrews, 1964, 1965; Whitaker, 1963). In an attempt to overcome this, glycopeptides from two well-investigated glycoproteins, ovalbumin and ovomucoid, were used as reference compounds, and, since the glycopeptides are composed predominantly of carbohydrate, three oligosaccharides were used as additional standards. The values obtained for molecular weight of glycopeptides from ovomucoid (Table 1) were close to what would be expected from earlier work (e.g. Montgomery & Wu, 1963; Neuberger & Papkoff, 1963). The value obtained for the principal peak of fraction B_{2a} under comparable conditions was 2450. A glycopeptide with the composition found for fraction B_{2a} and which contained one molecule of aspartic acid/molecule would have a molecular weight of 2250. The results obtained by ultracentrifuge analysis were somewhat lower than were found by the use of Sephadex. An uncertainty is introduced into this method by the use of an assumed partial specific volume (see above).

To obtain true values for the size of the prosthetic groups, correction must be made (except for fraction A₂) for sialic acid removed before proteolysis. For example, a corrected value of about 3200 is obtained for a glycopeptide with the composition of fraction B_{2a} and a molecular weight as isolated of 2400. For simplicity, the content of sialic acid in fraction I has been taken for this calculation (see, however, below). Taking the range of molecular weights of glycopeptides found in the present work together with data reported by Simkin *et al.* (1964b), there will be on average three or four prosthetic groups/molecule of glycoprotein.

The findings that aspartic acid is the principal amino acid present in all glycopeptide fractions examined, that ammonia is liberated on hydrolysis in approximately equimolar quantities to the aspartic acid present, even when little glutamic acid is found (fraction B_{2b}; Table 2), and that most of the glycopeptides are not degraded by alkali suggest that the predominant linkage between carbohydrate and polypeptide is of the *N*-acylglycosylamine type and that aspartic acid is very probably the amino acid involved in the linkage. Evidence supporting this view is provided by the isolation of a compound with the composition, elution volume and characteristic reaction with ninhydrin of AADG. The yield of aspartic acid in the form of AADG, when corrected for breakdown, suggests that at least an appreciable number of carbohydrate-polypeptide linkages must involve the union of the β -carbon atom of aspartic acid with the reducing group of *N*-acetylglucosamine by

means of an *N*-acylglycosylamine bond. Although there appeared to be some destruction of threonine and serine when the minor fraction B₁ was treated with alkali, it was calculated that this represented the breakage of not more than 5% of the carbohydrate-polypeptide linkages of fraction I as a whole.

Some differences in carbohydrate composition between the glycopeptide fractions B₁ and B_{2a} were found with regard to the hexosamine/hexose ratio. The composition of fraction A₂ was rather similar to that of fraction I, except that the sialic acid/total hexose ratio (0.34) was lower than that found for fraction I (0.39). Though the carbohydrate composition of fraction A₁ was not determined, the glycoprotein fraction from which the glycopeptides were derived was analysed. The sialic acid/total hexose ratio (0.58) was higher than that of fraction I, and this may account for the resistance of this glycoprotein fraction to proteolysis before the removal of sialic acid. The hexosamine/total hexose ratio (0.63) was somewhat lower than in fraction I (0.73), but the total carbohydrate content was somewhat higher, there being 11.5g. of total hexose/100g. of polypeptide in contrast with 9.6g./100g. for fraction I.

Though it is clear that identity does not exist throughout, the results of the present study, which relate to glycopeptides that represent a very large proportion of the carbohydrate of fraction I, show that there is a significant degree of similarity between at least most prosthetic groups. Further, similarity still exists whether the prosthetic groups are present in glycoproteins sensitive or resistant to Pronase in their isolated state. Thus the general similarities already reported in the physical properties (Simkin *et al.* 1964b) and in the structure of the prosthetic groups (Simkin *et al.* 1964a) of most of the molecules present in fraction I can be extended to include size of prosthetic groups and nature of the bonds linking them to polypeptide.

The results obtained with respect to size of prosthetic groups, complement of amino acids present in glycopeptides and nature of carbohydrate-polypeptide bonds show a general similarity to findings with comparable serum glycoproteins from other sources and certain other glycoproteins (e.g. Grant & Simkin, 1965).

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