Glycopeptides Obtained from Human Haptoglobin 2-1 and 2-2*

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ABSTRACT: Two major types of glycopeptides differing in amino acid composition were isolated from type 2-1 human haptoglobin. One type contained large amounts of aspartic acid, alanine, and threonine with aspartic and glutamic acids as the NH_2-terminal residues. The other type contained mainly aspartic acid and histidine, both of which were found as the NH_2-terminal groups. Both types of glycopeptides had essentially the same carbohydrate composition: 5–6 moles of hexose (galactose and mannose), 3–4 moles of *N*-acetylglucosamine, and 0–3 moles of *N*-acetylneuraminic acid, with fucose present in some units but absent in others. Molecular weights of the glycopeptides were in the range of 2000–3000, variations being probably due to the length of the peptide chain and/or to the number of *N*-acetylneuraminic acid and fucose residues.

On the basis of the size and composition of the glycopeptides isolated from the native haptoglobin of type 2-1, it is estimated that the intact molecule (mol wt 200,000) contains approximately 13 such units. The carbohydrate and amino acid composition of some haptoglobin 2-2 glycopeptides was essentially identical with that of the corresponding haptoglobin 2-1 glycopeptides, suggesting the presence of approximately 26 oligosaccharide residues/molecule (mol wt 400,000).

The haptoglobins[1] are genetically determined serum α_2-glycoproteins that exhibit the unique property of binding Hb to form a stable Hb–Hp complex. Although the nature and amounts of the carbohydrate components of the three major Hp types, Hp 1-1, Hp 2-1, and Hp 2-2, have been known for some time (Cloarec *et al.*, 1963; Schultze *et al.*, 1963) it is only recently that attempts were made to isolate and characterize glycopeptides from the Hp molecules. Cheftel *et al.* (1965) reported the isolation of oligosaccharide units from Hp 1-1, and Gerbeck *et al.* (1965) described a glycopeptide from Hp obtained from pooled plasma.

The purpose of the present study was to investigate the size, number, and composition of the oligosaccharide units of two of the major human Hp types, Hp 2-1 and Hp 2-2, and to examine the amino acids occurring in the vicinity of the carbohydrate–peptide bonds. Similar investigations carried out on thyroglobulin (Spiro, 1965), fetuin (Spiro, 1962), ovomucoid (Montgomery and Wu, 1963), ceruloplasmin (Jamieson, 1965a), transferrin (Jamieson, 1965b), and orosomucoid (Satake *et al.*, 1965), have indicated that there is considerable variation in both the number and composition of carbohydrate prosthetic units present in these glycoproteins.

The glycopeptides described in this report were obtained by Pronase digestion of the native Hp 2-1 and 2-2. Purification and fractionation of the glycopeptide mixtures were achieved through the use of gel filtration, ion-exchange chromatography, and preparative high-voltage paper electrophoresis.

Materials

Pronase B (45,000 PUK units/g) was purchased from Calbiochem, Los Angeles, Calif. DEAE-cellulose (coarse grade, 0.9 mequiv/g) was obtained from Sigma Chemical Co., St. Louis, Mo. Dowex 50-X2 (200–400 mesh, 5.2 mequiv/g) was obtained from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-25 (medium, water regain 2.5 g/g) was purchased from Pharmacia Corp., Uppsala, Sweden. Human blood was obtained from a local blood bank.

Methods

Preparation of Hp 2-1 and 2-2. The starting material for Hp isolation was fresh nonhemolyzed human blood containing EDTA or ACD as anticoagulants. Red cells and platelets were each removed separately by centrifugation at 1000 and 5000 rpm, respectively, at 4° for 30 min in a Servall anglehead rotor (GSA). Classification of sera with respect to the Hp type was

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[1] Abbreviations used: Hp, haptoglobin; Hb, hemoglobin; ACD, citrate-dextrose anticoagulant; PUK, proteolytic unit of Kaken; NANA, *N*-acetylneuraminic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

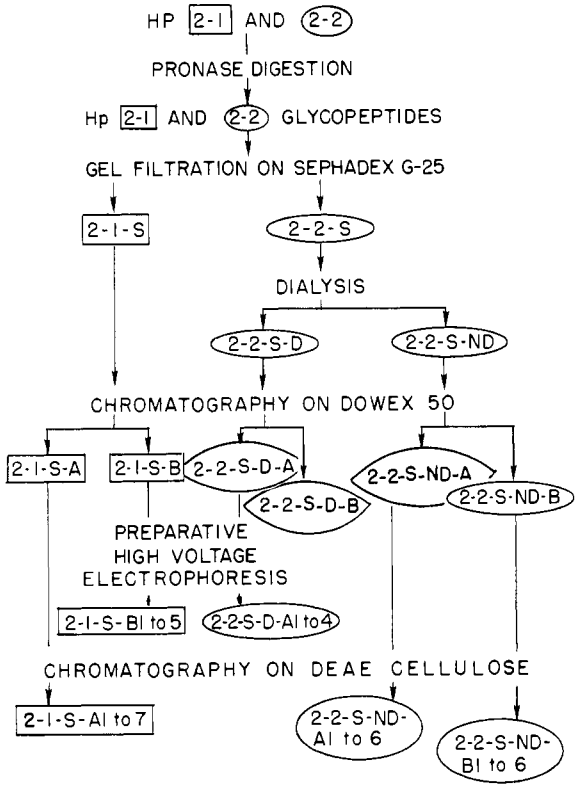

FIGURE 1: Fractionation procedure for haptoglobin 2-1 and 2-2 glycopeptides.

done by starch gel electrophoresis as described by Smithies (1959a). Hp was isolated, with minor modifications, by the ammonium sulfate fractionation procedure of Herman-Boussier *et al.* (1960), followed by chromatography of the Hp-rich fraction on DEAE-cellulose (Steinbuch and Quentin, 1961).

Electrophoresis. Homogeneity of the purified Hp 2-1 and 2-2 was determined by cellulose acetate and moving-boundary electrophoresis as described by Bezkorovainy (1965). Glycopeptide fractions were examined for homogeneity by high-voltage paper electrophoresis using a Brinkmann apparatus. Samples of approximately 50 μl, containing 0.5–1.0 mg of material, were applied in 1.0–1.5-cm streaks to Whatman No. 3 paper (34 × 40 cm) previously moistened with the buffer and electrophoresis was done at −5 to 5° for 1–2 hr at 1500–2000 v. The buffers used were pyridyl acetate, pH 3.5 (12 ml of pyridine–1000 ml of glacial acetic acid–1 l. of distilled water) and 6.0 (100 ml of pyridine–10 ml of glacial acetic acid–1 l. of distilled water). Peptides were detected by spraying with ninhydrin (0.2% in acetone) and heating for 3–5 min at 90–110° or allowing the color to develop at room temperature over a period of several hours. Glycopeptides were located with the periodate–benzidine reagent (Smith, 1960).

Determination of Sedimentation Coefficients. Sedimentation coefficients were determined in a Spinco Model E ultracentrifuge in the synthetic boundary cell at 59,780 rpm at 4–6° in 0.01 M sodium phosphate–0.093 M NaCl, pH 7.0, or 0.16 M NaCl. All sedimentation values were corrected to water at 20°.

Desalting. Glycopeptides were desalted in a Tor-Bal electrolytic desalter starting with a current of 50 ma which dropped to 10–20 ma in 20 min. The desalting procedure was usually performed twice on any given sample.

Amino-Terminal Group Analysis. Dinitrophenylation of 1–2 mg of glycopeptide was done by the method described by Fraenkel-Conrat *et al.* (1955). Quantitation was done by cutting out the spots from chromatograms, eluting with 1% $NaHCO_3$, and determining absorbances at 360 mμ.

Colorimetric Methods of Analysis. Hexoses were determined by the orcinol procedure (Winzler, 1955) using standards containing equal amounts of galactose and mannose (see under Results). Fucose was determined by the cysteine–sulfuric acid method described by Dische (1955) and glucosamine was determined as described by Winzler (1955). NANA was estimated by the Warren (1959) method. Molecular weights were estimated with TNBS reagent (Satake *et al.*, 1960). Appropriate corrections were made for the contribution by ϵ-amino groups of lysine residues. The progress of proteolytic digestions was measured by the ninhydrin reaction (Troll and Cannan, 1953) using leucine as the reference substance.

Amino Acids. Amino acids were determined with the Technicon Autoanalyzer using a microcolumn system (Moore and Stein, 1954) or with the Beckman-Spinco Model 120 amino acid analyzer using standard size columns (Piez and Morris, 1960). Samples were hydrolyzed in sealed tubes for 18 hr at 110° in 6 N HCl at concentrations of 1–2 mg/ml.

Paper Chromatography. Descending-type chromatography was done on Whatman No. 1 filter paper using the butanol–pyridine–water (6:4:3, v/v) solvent. Carbohydrates were detected by dipping in a solution of aniline phosphate (Smith, 1960), and the spots were quantitated by elution with glacial acetic acid and spectrophotometry at 400 mμ.

Results

Quality of the Starting Materials. Electrophoresis of all Hp preparations by the zone- and moving-boundary methods at pH 4.5 and 8.6 indicated the presence of only one component in each case.

Proteolytic Digestion of Hp 2-1 and Hp 2-2 with Pronase. Pronase digestion of 3.0 g of Hp 2-1 and 1.0 g of Hp 2-2 was carried out at 40° using an Hp concentration of 0.4% in 0.1 M sodium borate–0.01 M $CaCl_2$, pH 8.0, with a substrate:enzyme ratio of 20:1 and small amounts of toluene added as a preservative. Hp 2-1 was digested for a total of 43 hr with additional Pronase (substrate:enzyme ratio of 100:1) being added after 24 hr. Hp 2-2 was digested for 72 hr. In the case of Hp 2-1 it was found that 40% of the peptide bonds were cleaved after 22 hr of digestion and only a

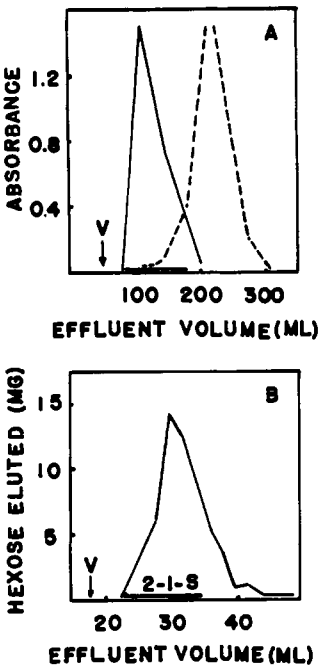

FIGURE 2: Fractionation of the Pronase digest of haptoglobin 2-1 on Sephadex G-25 (A) and rechromatography of the carbohydrate-containing material from Figure 2A on Sephadex G-25 (B). Color was developed by orcinol (———, 540 mμ), and ninhydrin (---------, 570 mμ).

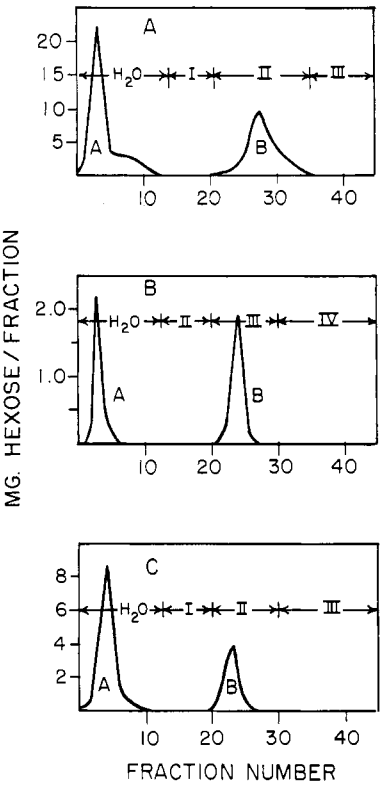

FIGURE 3: Chromatography of glycopeptides on Dowex 50 ion-exchange resin columns. Eluting solutions: I, 0.05 M NaCl; II, 0.1 M NaCl; III, 0.5 M NaCl; and IV, 1 M NaCl. (A) Fraction 2-1-S (418 mg) in 5 ml of H_2O was applied. Recovered a total of 74% of hexose present in fraction 2-1-S (180 mg in fraction 2-1-S-A and 130 mg in fraction 2-1-S-B). (B) Fraction 2-2-S-D (84 mg) in 1 ml of H_2O was applied. Recovered 66% of hexose present in fraction 2-2-S-D (28 mg each in fractions 2-2-S-D-A and 2-2-S-D-B). (C) Fraction 2-2-S-ND (107 mg) in 1 ml of H_2O was applied. Recovered 61% of hexose present in fraction 2-2-S-ND (47 mg in fraction 2-2-S-ND-A and 18 mg in fraction 2-2-S-ND-B).

slight increase in proteolysis was achieved with the additional Pronase.

Fractionation of Glycopeptide Mixtures. The procedures used to fractionate Hp digests are summarized in Figure 1. The details of each step and some properties of the fractions obtained were as follows.

A. FILTRATION OF DIGESTS ON SEPHADEX G-25. The lyophilized digests (1 g) were dissolved in 30 ml of 0.1 M pyridyl acetate buffer at pH 5.0 and subjected to gel filtration on 2 × 40 cm Sephadex G-25 columns (void volume, 50 ml) using the above buffer as column equilibrant and eluent. Fractions of 10 ml were collected at a flow rate of 20 ml/hr. The hexose-positive material appeared with the void volume, whereas the major portion of the ninhydrin-positive material was retarded (Figure 2A). The hexose-containing fractions (marked by the solid bar in Figure 2A) were lyophilized and further purified by two successive gel filtrations on Sephadex G-25 columns (0.7 × 50 cm) equilibrated with water (void volume, 18 ml). The carbohydrate-containing peak from the last Sephadex G-25 filtration step was divided into three portions accounting for 77% (fraction 2-1-S), 15, and 5%, respectively, of the hexose present in the original Hp 2-1 material (Figure 2B).

Fraction 2-1-S is extensively described in this paper, and since similar results were obtained with the remaining two fractions, their description will be omitted from this presentation. The major Hp 2-1 glycopeptide fraction which emerged with the void volume accounted for 77% of hexose present in the original Hp 2-1 substrate. Hp 2-2 yielded a corresponding glycopeptide fraction, 2-2-S, accounting for 86% of hexose present in the original Hp 2-2 substrate.

B. DIALYSIS OF FRACTION 2-2-S. Exhaustive dialysis of fraction 2-2-S against distilled water showed that 25% was diffusible (fraction 2-2-S-D) and 75% was nondiffusible (fraction 2-2-S-ND).

C. CHROMATOGRAPHY OF GLYCOPEPTIDES ON DOWEX 50 ION EXCHANGERS. Further purification of the above three fractions was achieved by chromatography on Dowex 50-X2 or -X8 columns (1 × 11 cm, H^+ form, 200–400 mesh). Fractions of 5 ml were collected at room temperature at a flow rate of 30 ml/hr. The elution patterns, sample sizes, recoveries, and eluting agents are given in Figure 3. All hexose-positive frac-

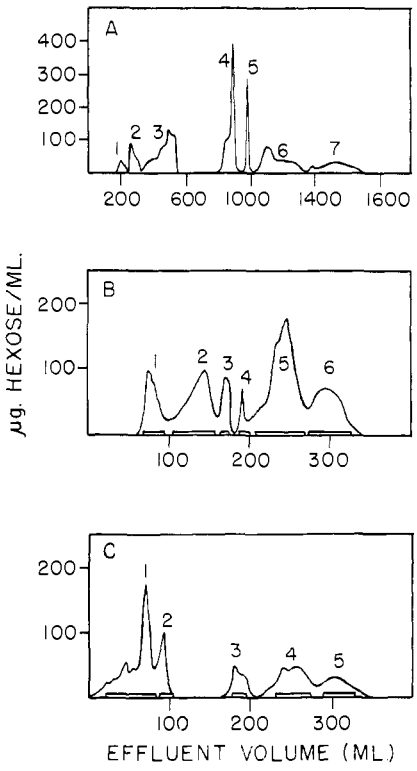

FIGURE 4: Chromatography of glycopeptide mixtures on DEAE-cellulose. (A) Fraction 2-1-S-A (200 mg) was applied to a 1.5 × 112 cm column; both mixing chamber and reservoir contained 500 ml of respective buffer (see text); 5-ml fractions were collected. The total hexose recovery was 88%, fractions 2-1-S-A3, -4, -5, and -6 yielding 11.4, 10.0, 10.5, and 5.4 mg of hexose, respectively. (B) Fraction 2-2-S-ND-A (48 mg) was applied to a 1.5 × 38 cm column; both the mixing chamber and reservoir contained 300 ml of the respective buffer; 3-ml fractions were collected with an overall weight recovery of 50%. (C) Fraction 2-2-S-ND-B (22 mg) was applied to a 1.5 × 38 cm column. Both mixing chamber and the reservoir contained 250 ml of the respective buffer; 5-ml fractions were collected. Total weight recovery was 72%.

tions obtained were electrolytically desalted and lyophilized.

The incomplete recovery of hexose noted in Figure 3 may be due to random losses on the columns or it may indicate that some additional highly charged glycopeptides failed to be eluted and remained on the resin. High-voltage paper electrophoresis at pH 3.5 and 6.0 indicated that fractions 2-1-S-A and -B, 2-2-S-D-A, and 2-2-S-ND-A and -B were free of noncarbohydrate contaminants. Trace amounts of peptides containing no carbohydrate were detected in fraction 2-2-S-D-B at both pH values.

Electrophoresis also revealed that all fractions were heterogeneous with respect to the carbohydrate-positive components. However, when NANA was

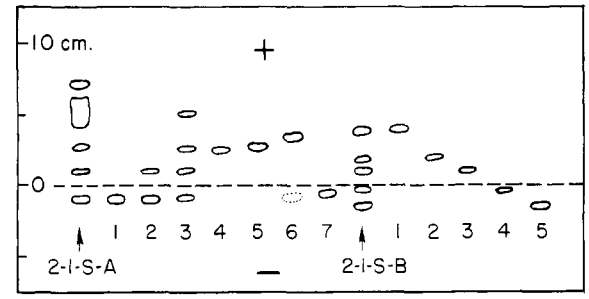

FIGURE 5: High-voltage electrophoresis of the 2-1-S-A and 2-1-S-B subfractions (see text for preparation procedures).

removed from samples of 2-1-S-A and -B by hydrolysis with 0.1 N H_2SO_4 at 80° for 30 min, prior to electrophoresis at pH 6.0, both fractions then showed only a single carbohydrate-positive spot that remained near the origin. These results suggest that the observed differences in electrophoretic mobilities, at pH 6.0, of the NANA-containing fractions were probably due to differences in the NANA content. This finding did not, however, exclude the possibility of additional variations in the other carbohydrate components of the oligosaccharide chains. For this reason, further fractionation of the Dowex 50 fractions was attempted, using DEAE-cellulose and preparative high-voltage paper electrophoresis.

D. CHROMATOGRAPHY OF GLYCOPEPTIDES ON DEAE-CELLULOSE. This procedure was carried out on fractions 2-1-S-A and 2-2-S-ND-A and -B using a linear gradient elution system of 0.0025–0.05 M, pH 7.6, sodium phosphate buffer (Spiro, 1962). Details of each particular fractionation are given in Figure 4.

A drawing of the high-voltage electropherograms of 2-1-S-A and its DEAE cellulose subfractions is shown in Figure 5. A single carbohydrate-positive component was found in fractions 2-1-S-A1, -4, -5, -6, and -7. No further work was done on A1 and A7 because of the small amounts of material present in these fractions. Fractions A2 and A3 were not examined further because of their apparent electrophoretic heterogeneity, although the heterogeneity may have been due to variations in sialic acid content.

Six subfractions were obtained from the fraction 2-2-S-ND-A (Figure 4) referred to as 2-2-S-ND-A1–6. Fractions A1, -2, -5, and -6 were subjected to high-voltage electrophoresis at pH 6.0, and all but A5 showed more than one carbohydrate-positive band.

Five fractions were obtained from 2-2-S-ND-B as shown in Figure 4. Electrophoretic examination of these fractions at pH 3.5 or 6.0 showed a single carbohydrate-positive component in every case. The amounts of material recovered in these DEAE fractions did not permit an extensive characterization of their properties and therefore these fractions were analyzed only for their carbohydrate components.

E. PREPARATIVE HIGH-VOLTAGE PAPER ELECTROPHORESIS. This technique was used to further fractionate

2-1-S-B and 2-2-S-D-A. Samples were applied to Whatman No. 3 paper (15–23 × 40 cm) previously washed with 3% aqueous acetic acid for a minimum of 24 hr and rinsed with distilled water until the washings were neutral and air dried. Preparative electrophoresis was done at pH 3.5 as described under Methods Guide strips were run on each side of the preparative section, which contained 3–6 mg of material/cm, and the glycopeptides were located by staining the guide strips with the periodate–benzidine reagent. The areas containing the glycopeptides and blanks of comparable areas were cut from the paper and eluted with 10–20 ml of distilled H_2O. The quantity of glycopeptides fractionated was usually 12–80 mg. The eluted glycopeptides were resubjected to preparative electrophoresis as needed until the desired homogeneity was attained. Figure 5 illustrates the electrophoretic patterns of the isolated glycopeptides (fractions 2-1-S-B1 to -5).

TABLE I: Ratios of N-Terminal Amino Acids Found in Some Glycopeptide Fractions after Dinitrophenylation.[a]

Fraction	DNP-Aspartic Acid: DNP-Glutamic Acid	Bis-DNP-Histidine: DNP-Aspartic Acid
2-1-S-A	3.7	—
-3[b]	5.0	—
-4	1.0	—
-5	7.0	—
-6	2.5	—
2-1-S-B3	—	1.0
-4	—	1.7
-5	—	0.6
2-2-S-ND-A5	7.0	—

[a] Values were not corrected for losses of DNP-amino acids during hydrolysis, but were corrected for differences in the extinction coefficients of bis-DNP-histidine and DNP-aspartic acid at 360 mμ. [b] This fraction also contained a trace amount of either N-terminal serine or -threonine.

The hexose recovery from each run was approximately 80%, of which 34 and 45% was accounted for by 2-1-S-B3 and -4, respectively, whereas fractions 2-1-S-B2 and -5 contained 6 and 15%, respectively. 2-1-S-B1 was identified as NANA.

Approximately 20 mg of 2-2-S-D-A was treated as described above and four fractions were obtained by this procedure. A major fraction (2-2-S-D-A1) was retained at the origin and three minor fractions were anodal to it. Fraction 2-2-S-D-A1 was eluted with water and concentrated by lyophilization (attempts to dry this material were unsuccessful). Electrophoresis of this fraction at pH 6.0 showed a major band at the origin and traces of two anodal components.

N-Terminal Studies of Glycopeptides. Several of the glycopeptide fractions obtained from DEAE-cellulose chromatography and preparative high-voltage electrophoresis were examined for N-terminal amino acids and the results obtained are shown in Table I. The values obtained were not corrected for losses of DNP-amino acids during hydrolysis but were corrected for differences in the extinction coefficients of bis-DNP-histidine and DNP-aspartic acid at 360 mμ. As can be

TABLE II: Amino Acid Composition[a] of Some Glycopeptide Fractions Isolated from Hp 2-1 and Hp 2-2.

	2-1-S								2-2-S	
	A	A4	A5	A6	B	B3	B4	B5	D-A	ND-A5
Aspartic acid	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Alanine	0.74	0.81	0.68	0.51	—	—	0.13	—	0.70	0.90
Threonine	0.61	0.63	0.58	0.60	0.11	—	0.12	—	0.56	0.74
Glutamic acid	1.1	0.60	0.27	0.68	0.36	0.31	0.42	0.36	0.78	0.33
Serine	0.53	0.32	0.27	0.55	0.41	0.36	0.23	0.37	0.38	0.39
Glycine	0.53	0.24	0.19	0.45	0.11	0.11	0.31	0.13	0.43	0.26
Proline	0.74	0.15	0.16	0.30	0.46	0.52	0.12	0.57	0.63	0.44
Valine	—	0.14	0.10	—	—	—	—	—	—	—
Leucine	0.56	0.14	0.10	0.30	—	—	—	—	0.17	—
Isoleucine	0.25	—	—	0.13	—	—	—	—	—	—
Phenylalanine	0.22	—	—	0.13	—	—	—	—	—	—
Lysine	0.23	—	—	0.21	—	—	—	—	0.12	—
Histidine	0.15	—	—	0.16	0.98	0.83	0.91	1.00	0.12	—
Total	6.57	4.03	3.35	5.02	3.43	3.13	3.24	3.43	4.89	4.06

[a] Values (in moles per mole of aspartic acid) were not corrected for destruction during hydrolysis. Amino acids for which values are not given were present in amounts less than 10% of aspartic acid.

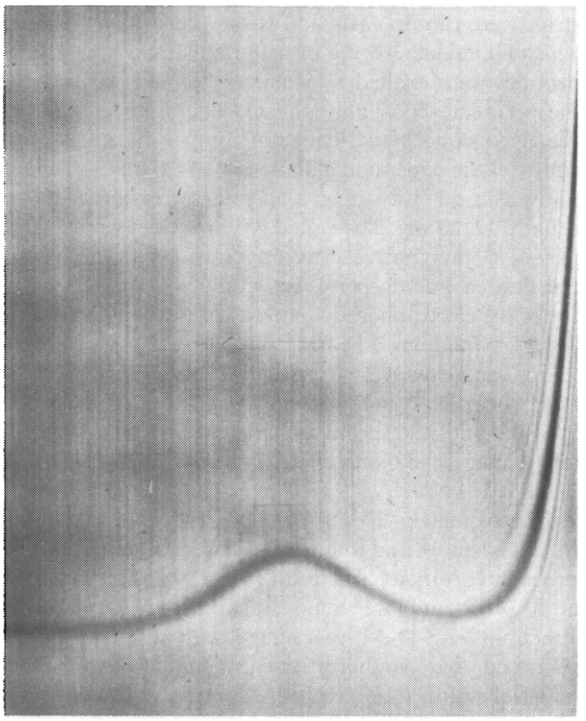

FIGURE 6: Ultracentrifugal analysis of fraction 2-2-S-ND-A5. The photograph was taken 130 min after reaching speed at a bar angle of 60°; solvent, 0.9% NaCl.

TABLE III: Sedimentation Coefficients and Molecular Weights of Some Glycopeptides.[a]

Fraction	$s_{20,w}$	Mol Wt[b]
2-1-S-A	0.83	—
-A4	0.89	3000
-A5	0.84	2900
-A6	—	2300
2-1-S-B	0.60	2200
-B3	—	1930
-B4	—	2760
-B5	—	1850
2-2-S-ND-A5	0.88	—

[a] The concentrations used in the ultracentrifuge were 0.4–0.5% for the 2-1-S-A fractions, 0.8% for 2-1-S-B, and 0.6% for 2-2-S-ND-A5. [b] Determined by the TNBS method.

seen from Table I, the 2-1-S-A peptides contained N-terminal aspartic and glutamic acids, whereas the 2-1-S-B peptides contained N-terminal aspartic acid and histidine. The finding of more than one N-terminal amino acid in each of these glycopeptide fractions is consistent with the nonstoichiometric ratios found in the amino acid analyses (Table II) and suggests the presence of similar peptides of varying chain length in each glycopeptide fraction.

Sedimentation Coefficients. The results of sedimentation velocity studies and molecular weight determinations obtained from several of the glycopeptide fractions are shown in Table III. The single symmetrical peak obtained with fraction 2-2-S-ND-A5 in the ultracentrifuge (Figure 6) is typical of the patterns obtained with these glycopeptides, and indicates that these fractions were free of components of substantially higher or lower molecular weights.

Amino Acid Composition of Glycopeptides. The amino acid composition of some of the glycopeptide fractions obtained from 2-1-S is shown in Table II. The nonstoichiometric ratios found in all fractions indicate the presence of closely related glycopeptides, which may vary somewhat in the length and/or composition of at least the peptide chain.

The principal differences in the amino acid composition found in the 2-1-S-A and 2-1-S-B fractions, namely the presence of alanine and threonine and absence of histidine in the former, and the presence of histidine but virtual absence of alanine and threonine in the latter, indicates that there are at least two different kinds of glycopeptides with respect to the amino acids in the vicinity of the peptide–carbohydrate bond in the Hp 2-1 molecule.

Carbohydrate Composition of Hp 2-1, Hp 2-2, and Their Glycopeptides. The total hexose content of Hp 2-1 and Hp 2-2 was 5.6 and 5.5%, respectively. These values are somewhat lower than those reported by Cloarec *et al.* (1963). The galactose to mannose ratios in native Hp's was determined by hydrolysis at 100° with 2 N H_2SO_4 for varying periods of time, neutralization, electrolytic desalting, concentrating, and quantitative paper chromatography. For Hp 2-1 the galactose to mannose ratios were 1.2, 0.8, 0.8, 0.8, and 1.0 after 1-, 2-, 3-, 4-, and 5-hr hydrolysis. The corresponding ratios for Hp 2-2 were 1.1, 1.0, 0.9, 0.8, and 1.0, respectively.

The carbohydrate composition of the native Hp 2-1 and Hp 2-2 and the major glycopeptide fractions obtained from them is shown in Table IV. The hexose to glucosamine ratios were found, within limits of the method, to be essentially the same in the glycopeptides and the native Hp 2-1 and Hp 2-2, indicating that these glycopeptides are probably representative of the oligosaccharide units as they exist in the intact molecules. The considerable variation in the hexose to sialic acid ratios found for the various glycopeptides may be due to losses of sialic acid during the isolation and fractionation procedures or may actually represent a true picture of the oligosaccharide units with respect to sialic acid. Furthermore, although the Hp 2-1-A and -B type glycopeptides (nonhistidine and histidine glycopeptides) differed in their amino acid composition (see Table II), their carbohydrate composition, with respect to hexose and glucosamine, was essentially the same.

TABLE IV: Carbohydrate Composition[a] (%) of Native Hp 2-1 and Hp 2-2 and Their Glycopeptide Fractions.

	Hexose	Glucosamine	NANA	Fucose	H:G[c]	H:NANA[c]
Hp 2-1	5.6	4.2	4.6	0.2	1.3	2.3
2-1-S-A	25.7	20.4	17.1		1.3	2.6
2-1-S-A4	25.8	18.9	16.4		1.5	2.7
2-1-S-A5	26.4	21.5	19.7		1.2	2.3
2-1-S-A6	23.6	13.6	23.6		1.7	1.7
2-1-S-B	29.1	21.8	8.0		1.3	6.2
2-1-S-B2[b]	1.5	1.1	0.58		1.4	2.7
2-1-S-B3	28.9	20.0	18.2		1.5	2.7
2-1-S-B4	28.4	19.3	7.8		1.3	6.3
2-1-S-B5	29.8	23.4	1.9		1.3	27.6
Hp 2-2	5.5	4.5	3.5	0.2	1.22	2.7
2-2-S-D-A1[b]	7.0	4.2	0.92	—	1.6	7.6
2-2-S-ND-A5	23.7	19.5	17.8	1.6	1.2	2.2
2-2-S-ND-B1	26.6	18.0	1.8	1.1	1.5	25.0
2-2-S-ND-B2	25.6	19.6	9.2	0.6	1.3	4.7
2-2-S-ND-B3	23.8	15.5	13.6	0.6	1.5	2.9
2-2-S-ND-B4	14.0	8.5	4.5	0.0	1.7	5.3
2-2-S-ND-B5	20.6	13.5	9.6	0.0	1.5	3.7

[a] Values were not corrected for moisture. [b] Expressed as micromoles per milliliter. [c] Molar ratios of hexose: glucosamine and hexose:NANA.

Discussion

Digestion of native Hp 2-1 and 2-2 with Pronase produced extensive proteolytic degradation without the prior removal of NANA. These results are similar to those obtained with other sialic acid containing glycoproteins subjected to Pronase digestion, such as thyroglobulin (Spiro, 1965), ceruloplasmin (Jamieson, 1965a), and transferrin (Jamieson, 1965b).

Gel filtration of the Pronase digestion mixtures on Sephadex G-25 was an effective method of removing much of the noncarbohydrate material, the glycopeptides emerging at, or near, the column volume, while the noncarbohydrate peptides were retarded. Further removal of noncarbohydrate contaminants was achieved by ion-exchange chromatography on Dowex 50, and permitted a separation of each glycopeptide fraction into essentially two subfractions. The A and B fractions of 2-1-S thus obtained (Figure 3) differed in their amino acid compositions but were similar in their hexose:glucosamine ratios.

Those glycopeptide fractions that were analyzed for amino acids all contained aspartic acid as a major amino acid, thus permitting its implication in the glycopeptide bond, as has already been found for other glycoproteins (Montgomery and Wu, 1963; Satake *et al.*, 1965; Spiro, 1962; Jamieson, 1965b; Johansen *et al.*, 1961). In addition, two types of glycopeptides, with respect to the amino acids in the vicinity of the glycopeptide bond, were found in the Hp 2-1 molecule (Table II). These two types of glycopeptides differed in that one contained large amounts of histidine with little or no alanine and threonine and the other contained substantial amounts of serine and threonine but virtually no histidine. Aspartic acid was found as the N-terminal amino acid in the nonhistidine glycopeptides together with varying amounts of N-terminal glutamic acid. Both aspartic acid and histidine were found as N-terminal amino acids in the histidine glycopeptides. Although an amino acid analysis was not done on any of the B glycopeptide fractions obtained from Hp 2-2, the amino acid composition obtained for two of the A fractions from Hp 2-2 was similar to that for the A fractions from Hp 2-1 (Table II) and for a glycopeptide fraction previously obtained from pooled Hp (Gerbeck *et al.*, 1965) by the same procedure. The possibility remains, however, that the glycopeptides isolated arose from a single sequence in the polypeptide chain, and the differences in amino acid composition (*i.e.*, histidine *vs.* the alanine–threonine peptides) are merely manifestations of variable sites of Pronase attack.

The carbohydrate composition of the various glycopeptide fractions obtained from Hp 2-1 and Hp 2-2 was similar with respect to the hexose:glucosamine ratios and essentially the same as that found in the native Hp's. These results not only indicate that the glycopeptides are representative of the oligosaccharide units existing in the intact molecules but also that the oligosaccharide units, with respect to hexose and glucosamine, are very similar in both types of Hp's. The ratios are also similar to those found in a glycopeptide isolated from pooled Hp (Gerbeck *et al.*, 1965) and to glycopeptides isolated from Hp 1-1 (Cheftel *et al.*, 1965). The variations in the hexose:NANA ratios

among the various glycopeptide fractions may have been artificially produced during the various preparative procedures or may reflect actual variations in NANA content among the various oligosaccharide units present in the Hp molecules. It may be noted through the identification of fraction 2-1-S-B1 as NANA that ketosidic hydrolysis had occurred during handling operations. The fucose determinations which were done on some of the glycopeptides from Hp 2-2 indicated that there may also be variations in the number of residues of this sugar attached to the oligosaccharide chains.

Although Hp 2-1 and Hp 2-2 are polymorphic structures, resolvable into multiple bands on starch gel electrophoresis, two basic types of subunits have nevertheless been isolated by reduction–alkylation–urea treatment and were termed the α and β chains (Shim and Bearn, 1964). The β, but not the α, chains were shown to contain the carbohydrate. Additional evidence is thus offered, in view of the similarities between Hp 2-1 and 2-2 glycopeptides, that the glycopeptide portions of the β chains of both Hp types are quite similar if not identical.

Molecular weight studies, by the TNBS method, of several of the nonhistidine glycopeptides (peak A, Figures 4A and 5) obtained from Hp 2-1 gave an average weight of approximately 2750. Molecular weights calculated on the basis of 5–6 moles of hexose, 3–4 of *N*-acetylglucosamine, 2 moles of NANA, and an average of 4 amino acid residues/mole of glycopeptide give a range of 2450–2850 which agrees well with the average of 2750 obtained by the TNBS method and with the hexose:glucosamine ratios found for these fractions. In the case of the histidine (or B) glycopeptides from Hp 2-1 the average weight by the TNBS method was 2200, and molecular weights calculated for oligosaccharide units containing 5–6 moles of hexose, 3–4 of *N*-acetylglucosamine, 0–2 moles of NANA, and an average of 3.4 amino acid residues give a range of 1800–2800 and an average of 2300, which is in good agreement with the average value of 2200 obtained by the TNBS method and agrees with the hexose:glucosamine ratios found for these fractions. Although molecular weight determinations were not done on the glycopeptide fractions from Hp 2-2, a sedimentation coefficient of 0.88 S was obtained for one fraction, 2-2-S-ND-A5, which was in the same range as those obtained for similar fractions from Hp 2-1, thus indicating that the molecular weights of the 2-2 glycopeptides are probably in the same range as those for the 2-1 glycopeptides.

We previously reported an estimated molecular weight of 10,000 for a glycopeptide isolated from pooled Hp (Gerbeck *et al.*, 1965) based on amino acid composition and fucose content. In view of the additional data now available this figure was evidently too high. Cheftel *et al.* (1965) have reported a molecular weight of 3050 $\pm$ 400 for a glycopeptide isolated from Hp 1-1 and, on the basis of carbohydrate content, calculated that the glycopeptide contains 6 moles of hexose, 4 moles of *N*-acetylglucosamine, and 2 moles of NANA.

An estimate of the number of oligosaccharide units, based on an average weight of 2200 for a unit consisting of five to six hexose residues, three to four *N*-acetylglucosamine residues, and two NANA residues and using molecular weights of 200,000 and 400,000 for Hp 2-1 and Hp 2-2 (Smithies, 1959b), respectively, and their carbohydrate content as shown in Table IV, suggests that there are approximately 13 carbohydrate prosthetic groups in the Hp 2-1 molecule and twice that number in the Hp 2-2 molecule. A more definite statement as to the exact number and composition of these prosthetic groups must certainly await more precise methods of determining the carbohydrate constituents and the molecular weights of the glycopeptides. In addition, a recent report by Cunningham *et al.* (1965) indicating heterogeneity with respect to mannose:glucosamine ratios in the aspartyl–carbohydrate oligosaccharide unit of ovalbumin, a glycoprotein with a single oligosaccharide unit, suggests that intermolecular heterogeneity of the carbohydrate prosthetic group may exist in this glycoprotein. If this is indeed the case, and such heterogeneity extends to glycoproteins other than ovalbumin, then the results obtained in such a study as we have described here may represent an average value of what may be an extremely diverse and complex type of intermolecular heterogeneity of the carbohydrate prosthetic groups of glycoproteins.

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Human Tear and Human Milk Lysozymes*

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ABSTRACT: Lysozymes from human tears and human milk were obtained in a chromatographically pure state by ion-exchange chromatography on Amberlite CG-50. The amino acid compositions were determined and the heat stability of these enzymes was studied. The tryptic digests of human milk and hen egg white lysozymes were compared with the help of a peptide AutoAnalyzer.

The present data and others previously obtained indicate the probable identity of the lysozyme of all human tissues and secretions; it has approximately 3.5 times the activity of hen egg white lysozyme.

Many studies have been devoted since 1893 to the bactericidal action of tears. They were reviewed by Thompson (1941) and more recently at the 2nd International Symposium on Lysozyme (1961). One of the most important observations was due to Fleming (1922) who indicated that tears, in common with many other agents, have a remarkable lytic activity against many saprophytes. He obtained evidence that the substance(s) concerned in the lysis was an enzyme and he gave it the name lysozyme (EC 3.2.1.17). Though many experiments concerning the biological activity were performed with the lysozyme from tears (Fleming, 1929; Thompson, 1941), its complete purification has not been achieved and no studies have been made with the enzyme obtained in a chromatographically pure state. The milk of most species contains lysozyme and human milk is a comparatively rich source of the enzyme (Bordet and Bordet, 1924; Rosenthal and Lieberman, 1931). Human milk lysozyme, the purification of which was described by Jollès and Jollès (1961), was the first entirely purified human lysozyme. This paper deals with the chromatographic purification of human tear and milk lysozymes, their amino acid composition, and some of their properties. Both lysozymes will then be compared to previously purified human lysozymes (Jollès, 1964; Jollès *et al.*, 1965; Charlemagne and Jollès, 1966) and some of the peptides obtained by tryptic digestion to those of hen egg white lysozyme.

Materials and Methods

Tears were always obtained from the same person. Because a hypersecretion causes a diminution of the concentration of lysozyme, only the first tears (0.05 ml) were collected with a pipet and frozen until used, when a sample of 1 ml was available. *Milk samples*, obtained from several mothers (1 l.), were stored at 2° during 1 night and immediately used for the preparation of lysozyme. In a few cases, the samples were kept frozen for up to 1 week before use. *Hen egg white lysozyme* was obtained from Armour, Kankakee, Ill. (lot 638040).

The lytic activity was determined by observing spectrophotometrically the extent of lysis of a suspension of dead cells of *Micrococcus lysodeikticus* (Jollès *et al.*, 1965). *The protein* content was determined qualitatively at 280 mμ with a spectrophotometer Beckman DU or with the ninhydrin reagent of Hirs *et al.* (1956) after alkaline hydrolysis. *The quantitative amino acid compositions* were determined after total hydrolysis (6 N HCl, 110°, 18 and 48 hr, sealed tubes under vacuum) with a Technicon AutoAnalyzer. Cystine was estimated as cysteic acid after performic acid oxidation

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