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Studies on Thyroid Proteins. I. Isolation and Properties of a Glycopeptide from Sheep Thyroglobulin*

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ABSTRACT: A glycopeptide was isolated from a pronase hydrolysate of sheep thyroglobulin by ethanol fractionation, gel filtration, and ion-exchange chromatography. The glycopeptide contained practically no iodine. This peptide contained about 60% of the total carbohydrate of the thyroglobulin and behaved as a single component during paper electrophoresis. Ultracentrifugation indicated a molecular weight of about 2400, a value in agreement with that obtained from quantitative N-terminal amino acid analysis. Aspartic acid was found to be the sole N-terminal amino acid. The peptide contained 0.5 residue of fucose, 2 of glucosamine, 5 of hexose, and 1 of sialic acid. The peptide contained no basic and aromatic amino acids but was particularly rich in aspartic acid. It is suggested that all the carbohydrate of thyroglobulin is not present as a single prosthetic group. Aspartic acid is probably involved in the carbohydrate peptide linkage.

Thyroglobulin, an iodine-containing glycoprotein, accounts for about 70% of the protein in saline extracts of the thyroid gland (Rall *et al.*, 1960). Reports dealing with the physical properties of this protein (Edelhoch, 1960; Edelhoch and Lippoldt, 1964) and with its iodoamino acid composition (Robbins and Rall, 1960; Roche *et al.*, 1954; Shulman and Stanley, 1961) have appeared. The carbohydrate constituents of thyroglobulin have been investigated by Ujejski and Glegg (1955), Robbins (1963), and Spiro (1963). Release of several glycopeptide fractions from hog thyroglobulin by the action of pronase[1] has been reported by Spiro and Spiro (1963). The present study deals with the isolation and characterization of a major glycopeptide from sheep thyroglobulin.

Materials and Methods

Materials. Crystalline pronase and Dowex 50W-X2 (200–400 mesh) were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Fluorodinitrobenzene (FDNB)[2] and dinitrophenylamino (DNP-amino) acids were obtained from Mann Research Laboratories, Inc., New York. The purity of commercial preparations of sugars was established by paper chromatography prior to their use. Sephadex G-25 (water regain, 2.5 ± 0.2 g/g; particle size, 20–80 μ), Sephadex G-50 (water regain, 5.1 ± 0.3 g/g; particle size, 100–200 mesh), and Sephadex G-200 (water regain, 20 ± 2 g/g; particle size, 40–120 μ) were obtained from Pharmacia, Uppsala, Sweden.

Protein, Nitrogen, and Iodine. Protein and peptide were determined either by the method of Lowry *et al.* (1951) or by measuring their ultraviolet absorption at 280 mμ in a Beckman Model DU spectrophotometer. Ninhydrin analysis was carried out by the method of Matheson *et al.* (1961). Nitrogen was determined by the micro-Kjeldahl procedure (Bailey, 1962). Iodine

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[1] Pronase is a proteolytic enzyme isolated from *Streptomyces griseus.*

[2] Abbreviation used in this work: FDNB, 1-fluoro-2,4-dinitrobenzene.

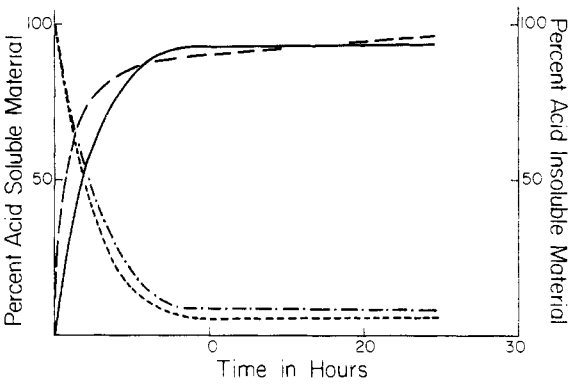

FIGURE 1: Rate of proteolysis of sheep thyroglobulin by pronase. Experimental conditions are described in the text. ———, acid-soluble hexose; ·····, acid-insoluble hexose; – – – –, ninhydrin reacting material; ·–·–·, protein.

determinations were kindly carried out by Dr. G. La Roche of Donner Laboratories, University of California, Berkeley.

Amino Acids. Five-mg portions of peptides were hydrolyzed with 1 ml of 5.7 M hydrochloric acid for 18–20 hours at 105° in evacuated sealed tubes. The hydrolysates were evaporated to dryness over NaOH *in vacuo*. The amino acid composition of the hydrolysates was then determined by the method of Moore *et al.* (1958) by the Analytica Corp. of New York, New York and Oxford Laboratories, San Mateo, Calif.

Carbohydrates. Total hexose was determined by the anthrone procedure (Südhof *et al.*, 1955) and fucose by the cysteine–sulfuric acid method of Dische and Shettles (1958). Sialic acid was estimated by the thiobarbituric acid method of Warren (1957), and hexosamine by the procedure of Rondle and Morgan (1955).

Chromatographic Analysis of Sugars. Ascending paper chromatography of sugars was done in a solvent system consisting of pyridine, ethyl acetate, acetic acid, and water (5:5:3:1). The sugars were identified by dipping the chromatograms in silver nitrate and alkali (Block *et al.*, 1958). After the excess silver nitrate was washed off the chromatograms with a 2.5% solution of sodium thiosulfate, the sugars were revealed as brown spots on a white background.

For quantitative analysis of the sugars, the chromatograms were sprayed with triphenyltetrazolium chloride reagent. The sugars appeared as pink spots on a white background. The colored complexes were eluted with a mixture consisting of methanol and acetic acid (1:1) and the absorbancy of the eluates was read at 480 mμ in the Beckman (Model DU) spectrophotometer (Wallenfels, 1950).

Electrophoresis. Electrophoresis of the peptides was performed on cellulose acetate strips in the Gelman electrophoresis unit at a potential gradient of 10 v/cm for 30 minutes. Paper electrophoresis was carried out on Whatman No. 1 filter paper strips at a potential gradient of 10 v/cm for 5–6 hours. Pyridine–acetic acid buffers of *p*H 3.5 and 6.0 were employed.

To detect the peptides, the cellulose-acetate strips were stained with a 1% solution of amido black in 40% methanol containing 10% acetic acid, whereas the filter paper strips were sprayed with 0.5% ninhydrin in acetone containing 1% pyridine. Hexosamine-containing peptides were located on paper electrophoretograms with acetylacetone and *p*-dimethylaminobenzaldehyde (Smith, 1960).

Experimental Procedures and Results

Preparation of Sheep Thyroglobulin. Sheep thyroid glands obtained from local abattoirs and freed of external connective tissue were sliced and extracted for 16 hours with 3 volumes of 0.9% NaCl at 4°. The saline extract was fractionated with ammonium sulfate after the method of Derrien *et al.* (1948). The protein fraction obtained after three ammonium sulfate fractionations was dissolved in distilled water and further purified by gel filtration at 4° through Sephadex G-200 columns, 0.1 M ammonium acetate being used as the eluent. The purified thyroglobulin was used for the isolation of glycopeptides as described further on.

Proteolysis of Sheep Thyroglobulin by Pronase. Purified thyroglobulin (100 mg) was dissolved in 2 ml of a 0.07 M Tris-HCl buffer (*p*H 8.0) that contained 0.014 M $CaCl_2$ and 0.14 M NaCl, and the mixture was incubated at 37° for 30 minutes. Crystalline pronase (2 mg) was then added, and the incubation was continued for 24 hours under a layer of toluene. The *p*H of the digest was maintained at 8.0 by repeated additions of 1 M sodium hydroxide. Periodically, 0.1-ml aliquots of the digest were withdrawn, and each was transferred to 10 volumes of 10% trichloroacetic acid. The resulting suspensions were spun at 2000 rpm for 10 minutes, the clear supernatant fractions were withdrawn, and the residues were washed twice with 0.5 ml of 10% trichloroacetic acid. The volume of each supernatant fraction along with its washings was adjusted to 2.5 ml with 10% trichloroacetic acid and analyzed for trichloroacetic acid–insoluble, ninhydrin-reacting material and hexose. The residue was dissolved in a few drops of 1 M NaOH, and the volume of the solution was adjusted to 1 ml and analyzed for protein and trichloroacetic acid–insoluble hexose.

The rate of proteolysis of thyroglobulin by pronase is shown in Figure 1. Proteolysis was rapid during the first 2 hours. The amounts of acid-soluble hexose and ninhydrin-positive materials released during digestion increased gradually with time, concomitant with a proportional decrease in the residual protein and protein-bound hexose. As much as 90% of the protein was degraded during the first 8 hours. A titrimetric study of the pronase digestion of thyroglobulin showed the same pattern of proteolysis. Alkali consumption by the digestion mixture was found to be low after 8 hours of incubation.

Isolation of Glycopeptides from Thyroglobulin. STEP 1: DIGESTION BY PRONASE. Purified sheep thyroglobulin

(1 g) was digested at 37° as described above for 24 hours with pronase (protein-to-enzyme ratio, 50:1) under a layer of toluene. The digest was clear at the start but became turbid after 8 hours, and near the end of the digestion a precipitate separated out. At the end of the incubation the digest was spun at 2000 rpm for 10 minutes and the residue was discarded. The supernatant fraction was adjusted to *p*H 4.5 and was allowed to stand at 4° for 2 hours. Traces of a precipitate that formed were removed by centrifugation. This step facilitated the separation of any undigested protein. The clear supernatant was used for step 2.

STEP 2: ETHANOL FRACTIONATION. The clear supernatant from step 1 was concentrated *in vacuo* to one-fourth its original volume. The concentrate was centrifuged and the supernatant was separated, cooled to 4°, and mixed with four volumes of cold 95% ethanol. The mixture was allowed to stand at 4° for 2 hours, and the white fluffy precipitate (AF I) that formed was collected by spinning the mixture at 1000 rpm for 5 minutes. Approximately 80% of the nitrogen remained in the supernatant whereas the glycopeptides were quantitatively recovered in the sediment. This sediment was then dissolved in 10 ml of distilled water, and traces of undissolved material were removed by centrifugation. The clear supernatant fraction was subjected to a second such alcohol fractionation. The residue finally obtained was dissolved in 5 ml of 0.1 M acetic acid, and the solution was used for the isolation of the glycopeptide fractions. This solution is designated AF II.

When the precipitate that appeared at *p*H 4.5 (see step 1) was not removed, a sticky mass formed after the addition of 2–3 volumes of ethanol and interfered with the subsequent alcohol fractionation.

Table I shows the recovery of hexose at various stages of the fractionation. The insoluble material formed during the pronase digestion of thyroglobulin and, upon adjustment of the *p*H of the digest to 4.5, contained little hexose. About 90% of the total hexose of the parent protein was recovered in fraction AF II. This material was further fractionated as described in the next paragraph.

TABLE I: Recovery of Carbohydrate during Different Stages of Isolation of Glycopeptide Fraction GP-Fr II.[a]

Fractionation Step	Sample	Total Hexose (mg)	Recovery (%)
1	Thyroglobulin	44	
	Whole pronase digest (A)	44	100
	A minus residue formed at end of digestion (B)	43	97.8
	B minus precipitate separated at *p*H 4.5	42.5	96.6
2	AF I	41.0	93.2
	AF II	40.0	90.9
3	GP-Fr II	38.0	86.4

[a] 1 g of purified thyroglobulin was digested with pronase as described in text.

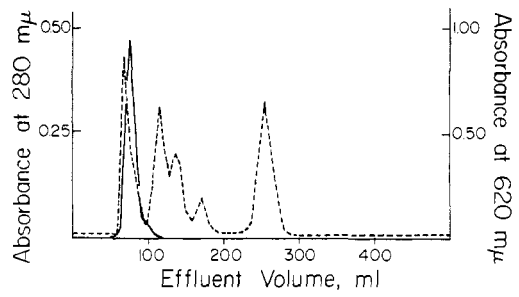

FIGURE 2: Gel filtration of glycopeptide fraction AF II (obtained after alcohol fractionation of the pronase digest of sheep thyroglobulin) on a 2 × 55-cm column of Sephadex G-25. Eluent, 0.1 M acetic acid; flow rate, 18.5 ml/hour; fraction, 5 ml/tube. ———, hexose; ---------, absorption at 280 mμ.

STEP 3: GEL FILTRATION ON SEPHADEX G-25. Fraction AF II was subjected to gel filtration on Sephadex G-25 columns, 0.1 M acetic acid being employed as eluent. For the gel filtration, the procedure described by Porath (1960) was employed. An amount of AF II corresponding to 500 mg of thyroglobulin in 5 ml of 0.1 M acetic acid was applied to a 2 × 55-cm column of Sephadex G-25 that had been equilibrated with 0.1 M acetic acid. The elution of peptides was carried out at 25° and at a flow rate of approximately 20 ml/hour. An automatic fraction collector provided with constant-volume dispensers was used to collect 3- to 5-ml fractions. The ultraviolet absorption at 280 mμ of each eluent fraction was determined. In addition, each fraction was analyzed for hexose and for ninhydrin-positive materials.

The elution pattern obtained by gel filtration of AF II on Sephadex G-25 columns is shown in Figure 2. Five peaks, all showing absorption at 280 mμ and reacting with ninhydrin, emerged from the column. The carbohydrate-containing material was present in the fractions constituting the first asymmetrical peak. Approximately 97% of the hexose applied to the column was recovered in this peak. About 98% of the nitrogen was recovered in all the peptides.

The carbohydrate-containing fractions representing the first peak were pooled and lyophilized. This fraction (GP-Fr I) was dissolved in 5 ml of 0.1 M acetic acid, and subjected to gel filtration twice as described previously. All the carbohydrate was recovered in a single peak. About 5% of the total nitrogen applied to the column was lost during this process. The carbohydrate-containing material obtained after the second gel filtration (GP-Fr II) was used for all subsequent studies. The glycopeptide fraction was separated from other peptides and salts by gel filtration.

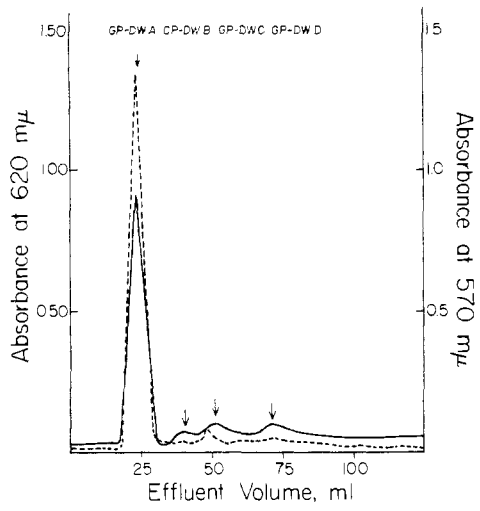

FIGURE 3: Chromatography of GP-Fr II on a 1 × 74-cm column of Dowex 50W-X2 (200–400 mesh). Eluent, 0.1 M sodium citrate buffer, *p*H 3.5; flow rate, 12.5 ml/hour; fraction, 2.5 ml/tube. ———, hexose; ---------, ninhydrin color.

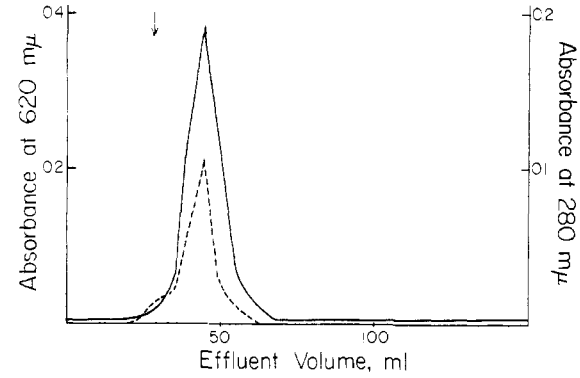

FIGURE 4: Gel filtration of the glycopeptide fraction GP-Fr II on a 2 × 30-cm column of Sephadex G-50. Eluent, 0.1 M acetic acid; flow rate, 19 ml/hour; fraction, 5 ml/tube. Arrow indicates the region where thyroglobulin is eluted from the column. ———, hexose; ---------, ninhydrin color.

STEP 4: CHROMATOGRAPHY OF GP-FR II ON DOWEX 50. GP-Fr II (25 mg) containing about 9 mg of hexose was dissolved in 2.0 ml of 0.1 M acetic acid and chromatographed on a 1 × 74-cm column of Dowex 50 (hydrogen form) equilibrated with 0.1 M sodium citrate buffer of *p*H 3.0. Elution was carried out with the same buffer at a flow rate of about 10–15 ml/hour. Fractions of 2.5 ml each were collected. Aliquots (0.05–0.1 ml) of each fraction were analyzed for hexose and for ninhydrin-positive materials.

Fractionation of GP-Fr II on Dowex 50 yielded five ninhydrin-reacting peaks, four of which contained hexoses. The elution pattern of these glycopeptide fractions from the Dowex 50 column is shown in Figure 3. These glycopeptide fractions are designated in order of their elution: GP-DW A, GP-DW B, GP-DW C, and GP-DW D (Figure 3). A major portion (about 70%) of the carbohydrate was recovered in the fraction represented by the first peptide peak, GP-DW A. Other hexose-containing peaks were diffused. The carbohydrate-containing fractions of each peak were pooled and lyophilized. The lyophilized materials were subjected to gel filtration on Sephadex G-25 as described in step 3. In all cases the glycopeptides were eluted as single components within the same effluent volume, as was the original GP-Fr II fraction. The glycopeptides obtained after gel filtration were free of salt. The yields of glycopeptides other than GP-DW A were low and hence were not studied.

Characterization of GP-Fr II and GP-DW A. GEL FILTRATION ON SEPHADEX G-50. A 2 × 30-cm column of Sephadex G-50 equilibrated with 0.1 M acetic acid was used. GP-Fr II (10 mg) containing about 3.5 mg of hexose was dissolved in 2.5 ml of 0.1 M acetic acid, and the solution was applied to the Sephadex column; elution was carried out at a flow rate of about 20 ml/hour. The effluent was collected in 5-ml fractions, and the fractions were analyzed for hexose and ninhydrin-positive materials. A typical elution pattern of GP-Fr II obtained from the Sephadex G-50 column is shown in Figure 4. GP-Fr II emerged as an asymmetrical peak. The hold-up volume of the column was determined with thyroglobulin and was found to be 26 ml.

SEDIMENTATION ANALYSES. The sedimentation coefficient of GP-Fr II was determined with the Beckman-Spinco Model E analytical ultracentrifuge in a synthetic boundary cell. The sedimentation pattern of GP-Fr II showed a symmetrical sedimenting boundary. The $s^{1\%}_{20,w}$ value was 0.65. The average molecular weight of GP-Fr II, as determined by the Archibald procedure and sedimentation equilibrium method (Schachman, 1959), was 2400.

ELECTROPHORESIS. In preliminary experiments electrophoresis of GP-Fr II on cellulose acetate strips revealed the presence of a component which stained weakly with amido black. In subsequent studies in which the precipitate formed at *p*H 4.5 (step 1) was removed during the isolation procedure, this amido black–positive band was not found.

When GP-Fr II was subjected to paper electrophoresis and the paper strips sprayed with ninhydrin, four bands were observed. Two were brownish-yellow. This indicates the presence of β-aspartyl peptides (Marks *et al.*, 1963). These two components also reacted with Ehrlich's reagent for hexosamine spray. The other two ninhydrin-positive bands were very faint. Typical paper electrophoretic patterns of GP-Fr II and GP-DW A are shown in Figure 5. At *p*H 6.0, three components had anodic mobility, while the other had cathodic mobility. At *p*H 3.5 three had cathodic mobility. GP-DW A migrated toward the cathode as a single band in both buffers.

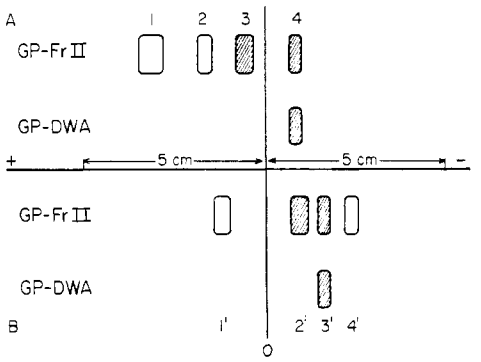

FIGURE 5: Paper electrophoresis of GP-Fr II and GP-DW A on a Whatman No. 1 filter paper at 10 v/cm for 5 hours. The spots were revealed by ninhydrin spray. The shaded spots were yellowish-brown. (A) Pyridine–acetic acid buffer, *p*H 6.0. (B) Pyridine–acetic acid buffer, *p*H 3.5.

TABLE II: N-Terminal Residue of GP-DW A.[a]

GP-DW A	N-Terminal Residue[b]	Aspartic Acid (mole/mole peptides)
Sample 1	Aspartic acid	0.68[c]
Sample 2	Aspartic acid	0.69

[a] DNP derivatives of GP-DW A were subjected to gel filtration; 5.0 mg of pure DNP peptide was hydrolyzed with 5.7 N HCl for 12 hours. The ether-soluble amino acids were separated by paper chromatography. The yellow spots were eluted with 1% $NaHCO_3$ and their absorbancy at 360 mμ was measured. [b] When DNP-aspartic acid was added to the DNP peptide and the mixture was hydrolyzed, 60% of the added DNP-aspartic acid was recovered. [c] The same value was obtained for GP-Fr II.

N-TERMINAL AMINO ACID ANALYSES. The method outlined by Fraenkel-Conrat *et al.* (1955) was followed for the preparation of DNP derivatives of GP-Fr II and GP-DW A. The glycopeptide (20 mg) was dissolved in 1 ml of 2% sodium bicarbonate, and the solution was treated with 1 ml of a 5% solution of FDNB in alcohol for 4 hours at room temperature with continuous shaking. The reaction mixture was extracted five times with 5-ml portions of ethyl ether, and then acidified. The precipitated DNP peptide was washed first with acetone and then with ether. The DNP peptide was dissolved in 2 ml of 0.1 M acetic acid and purified by gel filtration on a 1 × 67–cm column of Sephadex G-25 with 0.1 M acetic acid as eluent. The fractions were analyzed as described in step 3, except that their absorbancy at 360 mμ instead of 280 mμ was measured. All carbohydrate and a major portion of the yellow color were eluted together. Traces of yellow material obtained in later fractions were identified as dinitrophenol.

From the absorbancy of the DNP peptide dissolved in 1% sodium bicarbonate, the molecular weight of glycopeptide GP-DW A was calculated to be 2600. It is assumed that the molar extinction coefficient for a single DNP-aspartic acid residue is 15,000 (Sanger, 1949).

For N-terminal amino acid analyses 5-mg samples of the purified DNP peptide were hydrolyzed with 1 ml of 5.7 N hydrochloric acid for 12–16 hours in sealed evacuated tubes. The hydrolysate was diluted five times with distilled water and extracted with ether. The ether extract was subjected to descending paper chromatography in which the solvent system consisting of isoamyl alcohol–phenol–2 N ammonia (1:1:1) was employed. A single DNP-amino acid was observed on the chromatograms of the ether phase. This DNP-amino acid was eluted from the paper with 1% sodium carbonate, and the ultraviolet absorbancy of the eluate at 360 mμ was measured. The N-terminal residues of both GP-Fr II and GP-DW A were identified as aspartic acid. The yields of the DNP-amino acid from these fractions was 0.69 mole/mole of glycopeptide (Table II). Analysis of the aqueous phase showed no other N-terminal amino acid.

NITROGEN AND IODINE ANALYSES. Table III shows the nitrogen, iodine, and protein contents of thyroglobulin and glycopeptide fractions GP-Fr II and GP-DW A. The nitrogen contents were 14.3% for the native thyroglobulin, 6.0% for GP-Fr II, and 5.2% for GP-DW A. The protein contents of the above-mentioned samples were determined with bovine serum albumin as the standard. Compared to thyroglobulin, these glycopeptides contained considerably smaller amounts of nitrogen and groups that yielded chromogens in Lowry's procedure of protein determination (Bailey, 1962). These glycopeptides contained practically no iodine.

TABLE III: Nitrogen and Iodine Content of Sheep Thyroglobulin and Glycopeptides.

Component	Thyroglobulin (%)	GP-Fr II (%)	GP-DW A (%)
Nitrogen	14.3	6.0	5.2
Protein[a]	92.0	36.0	20.0
Iodine	0.7	0.03	0.03

[a] Determined by the method of Lowry *et al.* (1951), bovine serum albumin being used as standard.

AMINO ACID ANALYSES. The amino acid compositions of GP-Fr II and GP-DW A are shown in Table IV. GP-DW A contained fewer amino acids than did the original peptide, GP-Fr II. Arginine and lysine were

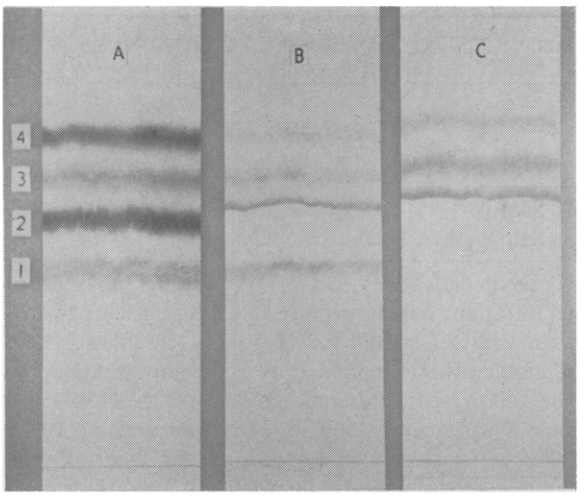

FIGURE 6: Chromatography of the sugar constituents of GP-DW A and glycopeptide fraction GP-Fr II. (A) standard, (B) GR-Fr II, (C) GP-DW A; (1) fucose, (2) galactose, (3) mannose, (4) hexosamine.

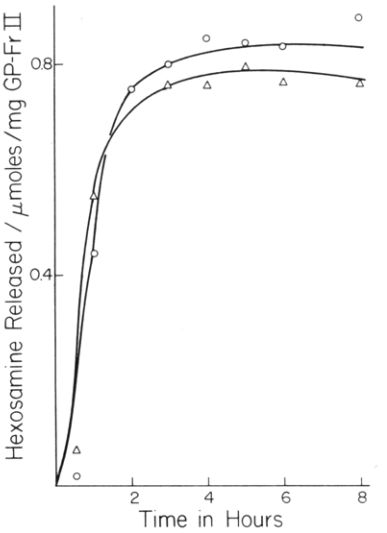

FIGURE 7: Release of hexosamine from GR-Fr II as a function of time. The hydrolysis was carried out on a 1% solution of the glycopeptide using different concentrations of hydrochloric acid at 100°. △—△, 2 N HCl; O—O, 3 N HCl.

TABLE IV: Amino Acid Composition of Glycopeptides.[a]

Amino Acid	GP-Fr II[b] (g/100 g)	GP-Fr II[b] (moles/ mole peptide)	GP-DW A[c] (g/100 g)	GP-DW A[c] (moles/ mole peptide)
Aspartic acid	4.44	0.87	4.46	0.87
Serine	2.10	0.52	2.11	0.52
Alanine	1.42	0.41	0.71	0.21
Glycine	1.11	0.38	0.87	0.30
Proline	1.18	0.27	1.18	0.27
Threonine	1.08	0.24	1.08	0.24
Glutamic acid	1.54	0.27	1.42	0.25
Valine	0.78	0.17	0.54	0.12

[a] Values not corrected for moisture. [b] Obtained by gel filtration on Sephadex G-25. This fraction also contained trace amounts of phenylalanine tyrosine, half-cystine, methionine, arginine, histidine, lysine, and leucines. [c] Obtained by chromatography of GP-Fr II on Dowex 50-X2.

present in GP-Fr II, but were not found in GP-DW A. Further, leucines, methionine, half-cystine, tyrosine, and phenylalanine were present in trace amounts in GP-Fr II, but not in GP-DW A.

CARBOHYDRATE ANALYSES. GP-Fr II and GP-DW A were hydrolyzed with 2 N sulfuric acid (1 ml/5 mg) at 100° for 2 hours. Excess acid was removed by treating the mixture with barium carbonate. The clear neutral solution was then evaporated to dryness *in vacuo* and the residue was analyzed by paper chromatography. Figure 6 shows that glucosamine, galactose, mannose, and fucose were present in both fractions. The same sugars have been reported for the parent protein (Spiro, 1963), a finding that we have confirmed.

For hexosamine analysis, the GP-Fr II samples were hydrolyzed with varying concentrations of HCl and for varying periods. The hydrolysates were neutralized with sodium carbonate and the hexosamine was estimated. The rate of release of hexosamine from GP-Fr II is shown in Figure 7. Maximum release of hexosamine from GP-Fr II was observed in 4–6 hours of hydrolysis with 3 N HCl. Hence, for quantitative estimations, all samples were hydrolyzed with 3 N HCl for 5 hours. The hydrolysates were lyophilized to remove excess acid before their colorimetric analyses.

The carbohydrate compositions of GP-Fr II and GP-DW A are given in Table V. Both glycopeptide fractions are rich in hexosamine, hexose, fucose, and sialic acid. Based on a molecular weight of 2600 for GP-DW A, it can be calculated that 1 mole of this fraction contains 5, 2, 1, and 0.5 residues of hexose, glucosamine, sialic acid, and fucose, respectively. Quantitative paper chromatography revealed a 3:2 ratio of mannose to galactose.

Discussion

Hydrolysis of thyroglobulin with pronase released most of the protein-bound hexose into an acid-soluble glycopeptide fraction (GP-Fr II). The release of glycopeptides by pronase from other glycoproteins, namely, egg albumin (Fletcher *et al.*, 1963) and ovomucoid (Montgomery and Wu, 1963), has also been observed.

The major glycopeptide fraction (GP-DW A) we obtained from thyroglobulin contained little or no

TABLE V: Carbohydrate Composition of Glycopeptides.

Preparation	Hexose (g/100 g)	Hexose (residue/mole peptide)	Hexosamine (g/100 g)	Hexosamine (residue/mole peptide)	Fucose (g/100 g)	Fucose (residue/mole peptide)	Sialic Acid (g/100 g)	Sialic Acid (residue/mole peptide)
GP-Fr II								
Lot 10	38.0	5.1	15.6	2.1	2.9	0.5	12.0	0.9
Lot 16	38.2	5.1	15.2	2.1	3.0	0.5	12.2	0.9
GP-DW A								
Lot 16	39.0	5.1	16.8	2.1	2.5	0.5	11.2	0.9
Lot 18	39.5	5.1	16.4	2.1	3.0	0.5	11.4	0.9

[a] Values not corrected for moisture.

iodine, a finding in agreement with the results of our earlier study (Tong *et al.*, 1963) in which it was shown that the complete release of ^{131}I-labeled amino acids from ^{131}I-labeled thyroprotein occurred within 6–8 hours of hydrolysis with pronase. We have observed that iodopeptides obtained by partial acid and enzyme digestions of thyroglobulin have little carbohydrate (unpublished work). These findings suggest that the iodoamino acid and carbohydrate residues in the thyroglobulin molecule are not located in the immediate vicinity of each other.

Glycopeptide fraction GP-Fr II resembles glycopeptides from other glycoproteins in being excluded from the Sephadex G-25 column (Fletcher *et al.*, 1963; Montgomery and Wu, 1963) and in its being retarded on the Sephadex G-50 column (Marks *et al.*, 1963). Our findings indicate that low and high molecular weight contaminants that could have been separated by gel filtration were not associated with GP-Fr II.

Fractionation on Dowex 50 columns separated GP-Fr II into four carbohydrate-containing peptides (Figure 3). The resolution of minor glycopeptide fractions was unsatisfactory. Only the major peak representing GP-DW A was sharp and well-defined. Similar chromatographic separations of glycopeptide mixtures from ovomucoid on Dowex 50-X2 have been reported (Montgomery and Wu, 1963; Cunningham *et al.*, 1963). The different glycopeptides obtained from GP-Fr II by chromatography on Dowex 50 may represent carbohydrate–amino acid units with different number of amino acid residues or carbohydrate composition.

Dinitrophenylation of GP-Fr II and GP-DW A yielded DNP derivatives which retained the carbohydrate-peptide linkage. Analysis of these derivatives showed that aspartic acid is the N-terminal residue. Using these DNP peptides, the molecular weight of the original peptide samples was assessed to be 2600. This value is in agreement with that obtained by ultracentrifugal analyses. A low molecular weight for the fraction GP-Fr II is also indicated by retardation of the glycopeptide during gel filtration on Sephadex G-50. Analysis of DNP derivatives of GP-DW A and GP-Fr II indicated 0.69 mole of aspartic acid per mole of the glycopeptide. This low value for aspartic acid is explained by the destruction of DNP-aspartic acid during the acid hydrolysis of the DNP peptide (Porter, 1957; Johansen *et al.*, 1961).

Amino acid analyses revealed that GP-DW A contained fewer amino acids than did GP-Fr II. The amino acids found in appreciable amounts in GP-DW A are glycine, alanine, threonine, serine, proline, aspartic acid, and glutamic acid. Glycopeptides isolated from other glycoproteins, such as ovomucoid (Montgomery and Wu, 1963) and ovalbumin (Kamiyama and Schmid, 1962), have amino acid compositions similar to GP-DW A. In all the cases aspartic acid is the major amino acid. Calculations based on a molecular weight of 2600 for GP-DW A show that a mole of this peptide contained 0.21 residue of alanine, 0.30 of glycine, 0.52 of serine, 0.24 of threonine, 0.87 of aspartic acid, and 0.25 of glutamic acid. The low yield of certain amino acids is owing to their partial destruction during hydrolysis of the peptide. It is interesting to note that GP-DW A is devoid of aromatic amino acids. This is in accordance with the ultraviolet spectra of GP-DW A which were devoid of characteristic peaks between 270 and 300 mμ. However, a 1 mg/ml solution of the peptide showed a measurable absorbancy at 280 mμ, which was employed in screening the fractions eluted from the columns used for chromatography of this peptide.

GP-DW A, having a molecular weight of 2600, contains 2 residues of glucosamine, 5 of hexose, 0.5 of fucose, and 1 of sialic acid. If the carbohydrate in thyroglobulin is present as a single moiety, the glycopeptide obtained would have a molecular weight of the order of 50,000 (assuming that about 8% of thyroglobulin, with a molecular weight of 650,000, is carbohydrate). Since the major glycopeptide isolated in the present study had a molecular weight of only 2600, it is obvious that the carbohydrate present in thyroglobulin does not exist as a single prosthetic group. In this respect thyroglobulin resembles ovomucoid, where the carbohydrate portion of the molecule seems to be organized into at least three oligosaccharide units

(Montgomery and Wu, 1963). However, from our data it is not possible to decide whether all the carbohydrate units of thyroglobulin are identical or not.

The isolation of glycopeptide GP-DW A with a low molecular weight, and with a few amino acids that account for over 60% of the carbohydrate in thyroglobulin, should prove valuable in further elucidating the protein-carbohydrate linkage and the amino acid sequence adjacent to this linkage. Treatment of GP-DW A with alkali under the conditions described by Anderson *et al.* (1964) did not result in complete destruction of serine and threonine. The fact that neither serine nor threonine were destroyed points to the absence of a linkage between the carbohydrate and hydroxyls of these amino acids of the peptide. This suggests that aspartic acid is probably the amino acid involved in the carbohydrate–amino acid linkage of the glycopeptide.

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