Biochem. J. (1971) **123**, 407–414
Printed in Great Britain

Isolation and Fractionation of Glycopeptides from Porcine Thyroglobulin

By MINORU FUKUDA and FUJIO EGAMI
Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo, Japan

(*Received* 12 *August* 1970)

1. Glycopeptides were isolated by gel filtration on Sephadex G-25 and Sephadex G-50 from a Pronase digest of porcine thyroglobulin. 2. Isolated glycopeptides were separated into five main fractions on a column of DEAE-Sephadex A-25. Of these fractions I to III were further purified by SE-Sephadex C-25 or DEAE-Sephadex A-25 column chromatography. Several of the purified glycopeptides were homogeneous on paper electrophoresis. 3. Based on the chemical composition and molecular weight of the fractionated glycopeptides, two distinct types of heterosaccharide chain were demonstrated. 4. One type of the heterosaccharide unit consisted of four to eight residues of mannose and two residues of glucosamine and had a molecular weight of 1000–1700. The other type of unit contained sialic acid, fucose and galactose in addition to mannose and glucosamine and had a molecular weight of about 3600. 5. Mild alkaline treatment of the glycopeptide did not result in the destruction of threonine and serine. 2-Acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine was isolated from partial acid hydrolysates.

Thyroglobulin, a major protein of the thyroid gland, is a glycoprotein and a precursor of thyroxine. In contrast with liver, which produces several plasma glycoproteins, the thyroid gland synthesizes mainly one glycoprotein, thyroglobulin. Hence, thyroglobulin is a suitable material for a study of the biosynthesis (Spiro & Spiro, 1966, 1968*a*,*b*) and the physiological role of glycoproteins, and for these studies it is essential that the structure be fully understood.

Investigations on the carbohydrate composition of thyroglobulin and on the fractionation of glycopeptides from thyroglobulin have been reported from several laboratories (Murthy, Raghupathy & Chaikoff, 1965; Spiro, 1965; Rawitch, Liao & Pierce, 1968). Spiro (1965, 1968) showed that calf thyroglobulin contained two distinct types of carbohydrate units. However, fractionation of the glycopeptides was incomplete and information concerning the carbohydrate structure of the glycopeptides has not been presented.

This paper describes the fractionation and characterization of glycopeptides with special reference to the carbohydrate structure. Several purified glycopeptides were found to be homogeneous on high-voltage paper electrophoresis and chemical studies on the carbohydrate and peptide have been carried out. The carbohydrate structure of one of the purified glycopeptides will be reported in the following paper.

MATERIALS AND METHODS

Materials. Crude thyroglobulin from porcine thyroid was kindly given by Dr Y. Kondo and Dr N. Ui of the Endocrinological Institute, Gunma University, Japan. Synthetic 2-acetamido-1-*N*-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine and a glycopeptide from orosomucoid (Yamauchi, Makino & Yamashina, 1968) were kindly given by Professor I. Yamashina of Kyoto University, Japan. Di-*N*-acetylneuraminyl lacto-*N*-tetraose and xylopentaose were given by Dr Kozeki and Dr Tsurumi of Fukushima Prefectural University, Japan, and Dr S. Fukui of Institute of Applied Microbiology, University of Tokyo, Japan, respectively. Porcine submaxillary mucin was prepared by the modified procedure of De Salegui & Plonska (1969).

Preparation of glycopeptides. Thyroglobulin was purified by ammonium sulphate fractionation as described by Ui & Tarutani (1961). The carbohydrate composition of purified thyroglobulin is shown in Table 1. Porcine thyroglobulin (5g) was digested with Pronase P in 200ml of 0.05M-sodium borate buffer, pH7.9, containing 1mM-$CaCl_2$. Initially 100mg of Pronase was added and further portions of 50mg of Pronase were added after 48 and 72h respectively. After incubation for a total period of 96h, the digest was freeze-dried, dissolved in water and centrifuged. The supernatant and washed solutions were combined and applied to a column (1.6cm×90cm) of Sephadex G-25, which was eluted with water (Fig. 1*a*). Fractions (4ml) were collected. The phenol–H_2SO_4 reaction-positive fractions were combined and were again digested with Pronase (initially 15mg of Pronase was added and after 24h 16mg was added) for 48h under the conditions described above. The digest was treated

Table 1. *Carbohydrate composition of porcine thyroglobulin*

Component	Composition (g/100g)	Composition (mol/mol)†
Fucose	0.44	23
Galactose	1.38	51
Mannose	2.47	92
Glucosamine	2.64	96
Sialic acid*	1.12	24

* Expressed as *N*-acetylneuraminic acid.
† Nearest integral values.

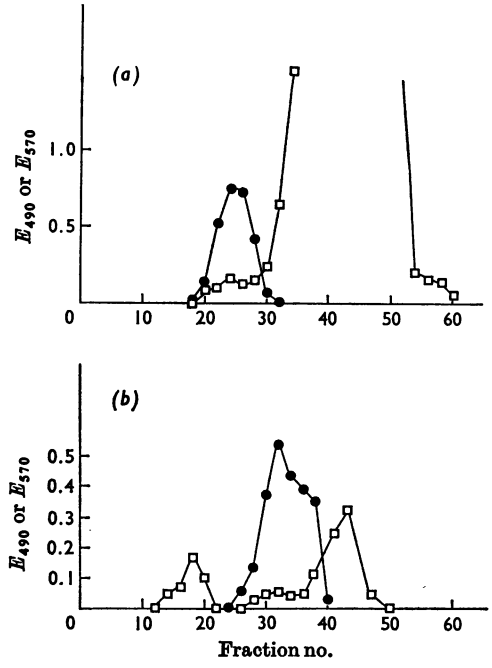

Fig. 1. (*a*) Gel filtration of the first Pronase digest of thyroglobulin on Sephadex G-25. The procedures for chromatography are described in the text. Neutral sugars and free amino groups were determined by phenol–H_2SO_4 reaction and ninhydrin reaction respectively. (*b*) Gel filtration of the second Pronase digest of thyroglobulin on Sephadex G-50. The procedures for chromatography are described in the text. A 50 μl sample of each of fractions 10–20 or a 5 μl sample of each of fractions 22–60 was used for phenol–H_2SO_4 reaction and ninhydrin reaction. ●, Phenol–H_2SO_4 reaction; □, ninhydrin reaction.

in a similar manner and subjected to gel filtration on a column (1.6 cm × 90 cm) of Sephadex G-50 and eluted with water (Fig. 1*b*). Fractions (4 ml) were collected. The first peak was Pronase, the second was glycopeptide and the last was peptide. The glycopeptides were collected by freeze-drying. They were designated unfractionated glycopeptide.

DEAE-Sephadex A-25 column chromatography of glycopeptides. A 250 mg sample of glycopeptides dissolved in 10 ml of 1 mM-sodium phosphate buffer, pH 7.0, was applied to a column (2.1 cm × 55 cm) of DEAE-Sephadex A-25 that had been equilibrated with the same buffer. The column was washed with 350 ml of the same buffer and it was developed by linear gradient elution with 600 ml of 5 mM-sodium phosphate buffer, pH 7.0, in the mixing flask and 600 ml of 20 mM-sodium phosphate buffer, pH 7.0, in the reservoir. Then the column was developed by the second linear gradient elution with 600 ml of 20 mM-sodium phosphate buffer, pH 7.0, in the mixing flask and 600 ml of 0.2 M-sodium phosphate buffer, pH 7.0, in the reservoir. Flow rate was 20 ml/h and 15 ml fractions were collected.

Chemical analysis. Hydrolysis of glycopeptides or proteins was carried out in an evacuated tube unless otherwise specified.

Determinations of neutral sugars were carried out by the phenol–H_2SO_4 procedure (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Individual neutral sugars were quantitatively determined by g.l.c.

Samples were hydrolysed in 0.5 M-H_2SO_4 on a boiling-water bath for 8 h. The hydrolysate was diluted, then a fixed amount of methyl α-D-mannopyranoside was added as an internal standard. It was passed successively through columns of Dowex 1 (X 8; 200–400 mesh; formate form) and Dowex 50 W (X 2; 200–400 mesh; H^+ form). The effluent and washings were evaporated to dryness.

The dried materials were trimethylsilylated essentially as described by Sweely, Bentley, Makita & Wells (1963), but scaled down one-twentieth. G.l.c. of trimethylsilyl derivatives was carried out with a glass column (3 mm × 200 cm, 3% SE-30 on Anakrome SD) at 162°C or with a glass column (3 mm × 200 cm, 4% SE-30 on Gas-Chrom CLH) at 170°C.

Hexosamines were determined by the borate-catalysed Morgan–Elson reaction (Strominger, Park & Thompson, 1959) after hydrolysis in 4 M-HCl at 100°C for 6 h. Glucosamine was used as a standard.

Amino acid compositions of glycopeptides were determined by using a Hitachi KLA 3B Amino Acid Analyzer. Samples were hydrolysed in constant-boiling HCl (5.7 M) at 100°C for 16 h (Yamauchi *et al.* 1968).

Bound sialic acid was determined by the modified resorcinol procedure (Cassidy, Jourdian & Roseman, 1965) with butan-1-ol instead of 3-methylbutan-1-ol for the development of colour (Svennerholm, 1957). Free sialic acid was determined by the thiobarbituric acid method (Aminoff, 1961). *N*-Acetylneuraminic acid was used as a standard. The sialic acid fraction was isolated as described by Spiro (1966). A standard sample of *N*-glycollylneuraminic acid was obtained from porcine submaxillary mucin (Hashimoto, Hashimoto & Pigman, 1964).

Molecular-weight determinations of glycopeptides by gel filtration. Molecular weights of glycopeptides were determined by gel filtration (Cunningham & Simkin, 1966; Bhatti & Clamp, 1968). Sephadex G-50 (super fine grade) equilibrated with 0.1 M-NaCl was packed into a column (1 cm × 145 cm). The glycopeptide together with Blue Dextran (0.1 mg) and standard samples were dissolved in 0.1 M-NaCl and applied to the column. It was eluted

with 0.1M-NaCl and the positions of the materials were detected by the phenol–H_2SO_4 reaction. Blue Dextran was used to determine the void volume. Xylopentaose, di-*N*-acetylneuraminyl-lacto-*N*-tetraose and a glycopeptide from human orosomucoid (mol.wt. 3300) (Yamauchi *et al.* 1968) were used as standards.

RESULTS

Chemical composition of unfractionated glycopeptide eluted from Sephadex G-50. Neutral sugars liberated by the acid hydrolysis of unfractionated glycopeptide were converted into their trimethylsilyl derivatives. On g.l.c. only peaks derived from fucose, galactose and mannose were detected.

A fixed portion of unfractionated glycopeptide was hydrolysed in 0.5M-sulphuric acid at 100°C for 4, 6, 8 and 12h respectively, and the free neutral sugar content of the hydrolysate was determined by g.l.c. The release of neutral sugars during acid hydrolysis is shown in Fig. 2. Since the maximum yield for each neutral sugar was obtained at 8–12h, 8h was selected as the optimum time.

Hexosamine was identified as glucosamine by ion-exchange chromatography with the amino acid analyser. No trace of galactosamine was found.

Identification of the sialic acid was carried out by paper chromatography with the descending solvent system butan-1-ol–acetic acid–water (3:2:1, by vol.) (Spiro, 1960). On the chromatogram, only two spots corresponding to the position of *N*-acetylneuraminic acid and *N*-glycollylneuraminic acid were detected by thiobarbituric acid spray (Warren, 1960).

The two sialic acids exhibit different molar extinction coefficients in thiobarbituric acid and resorcinol reactions (Warren, 1959). The molar ratio of the two sialic acids can therefore be obtained by a combination of the two procedures. By this means the *N*-acetylneuraminic acid/*N*-glycollylneuraminic acid molar ratio was found to be 3:2.

The chemical composition of the unfractionated glycopeptides is summarized in Table 2.

The molar proportions of the neutral sugars, amino sugar and sialic acids was almost the same as that for the intact thyroglobulin.

Fractionation of glycopeptides by DEAE-Sephadex A-25 column chromatography. On paper electrophoresis, the unfractionated glycopeptides eluted from Sephadex G-50 revealed several distinguishable fractions. Attempts to fractionate the glycopeptides were therefore carried out.

The glycopeptides were fractionated into five fractions on DEAE-Sephadex A-25 column chromatography (Fig. 3*a*). Fraction V was rechromatographed with almost the same elution system but with a smaller column (Fig. 3*b*).

Each glycopeptide fraction (fractions I–V) was freeze-dried and desalted by gel filtration on Sephadex G-25. The yield of neutral sugar in each fraction is as follows, starting from unfractionated glycopeptides containing 100mg as neutral sugar: fraction I, 27mg; fraction II, 17mg; fraction III, 8mg; fraction IV, 3.4mg; fraction V, 6.5mg; others, 18mg.

The homogeneity of the fractionated glycopeptides was examined by paper electrophoresis. Fig. 4(*a*) shows that all except fraction V were heterogeneous. It was noteworthy that there are two distinct types with respect to net charge: at pH 3.6, one moved towards the anode and the other stayed at the origin or moved a little towards the cathode. The former is separated into several spots.

Chemical composition of glycopeptides fractionated by DEAE-Sephadex A-25. The chemical composition of the fractionated glycopeptides expressed as

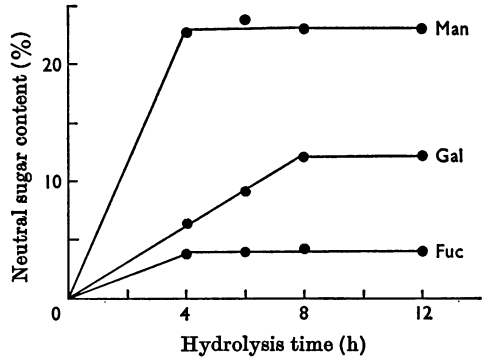

Fig. 2. Release of neutral sugars from the unfractionated glycopeptide as a function of hydrolysis time. Samples were hydrolysed in 0.5M-H_2SO_4 on a boiling-water bath for the times indicated. Hydrolysates were deionized and neutral sugar content of the hydrolysate was determined by g.l.c.

Table 2. *Composition of unfractionated glycopeptide*

Component	Composition (g/100g)	Composition (μmol/mg)
Fucose	4.1	0.25
Galactose	12.9	0.70
Mannose	23.1	1.28
Glucosamine	24.7	1.38
Sialic acid	10.5	0.34
Aspartic acid	5.8	0.44
Threonine	3.0	0.26
Serine	3.1	0.30
Glutamic acid	2.3	0.16
Proline	4.1	0.37
Glycine	0.6	0.08

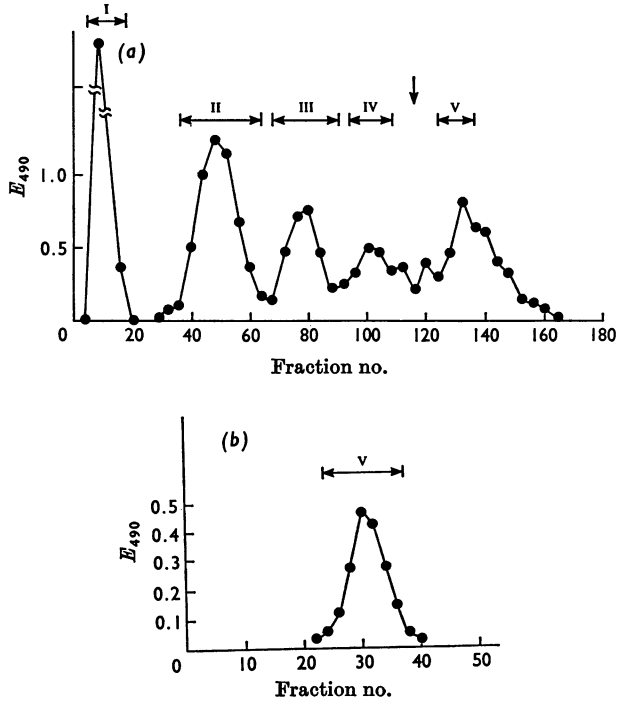

Fig. 3. (*a*) DEAE-Sephadex A-25 column chromatography of glycopeptides. A 250 mg sample of glycopeptides was applied to a column (2.1 cm × 55 cm) of DEAE-Sephadex A-25, which had been equilibrated with 1 mM-sodium phosphate buffer, pH 7.0. Chromatography was carried out by a two-step linear gradient elution and details of the procedure are described in the Materials and Methods section. Position of initiating the second linear gradient is indicated by the arrow. (*b*) Second DEAE-Sephadex A-25 column chromatography of fraction V shown in Fig. 3(*a*). The sample was applied to a column (1.2 cm × 30 cm) of DEAE-Sephadex A-25 equilibrated with 0.01 M-sodium phosphate buffer, pH 7.0. The column was developed by linear gradient elution with 150 ml of 0.01 M-sodium phosphate buffer, pH 7.0, in the mixing flask and 150 ml of 0.15 M-sodium phosphate buffer, pH 7.0, in the reservoir. Fractions (5 ml) were collected and flow rate was 10 ml/h. ●, Phenol–H_2SO_4 reaction for neutral sugars.

molar ratios to aspartic acid or fucose is given in Table 3. The carbohydrate composition of fraction I was particularly characteristic because it contains little sialic acid, fucose and galactose. Fraction III also contained little sialic acid. Because the glycopeptides other than fraction V were not homogeneous on paper electrophoresis, further fractionation of fractions I–III was carried out.

Further fractionation of fraction II by DEAE-Sephadex A-25 column chromatography. As fraction II contained sialic acid, a neutral pH was suitable for fractionation. Rechromatography on DEAE-Sephadex was then carried out. Fig. 5 shows that a very broad but single peak was present. This was divided into two fractions, which were designated fraction II-1 and fraction II-2. The recovery by chromatography was more than 80%.

Further fractionation of fraction I and fraction III by SE-Sephadex C-25 column chromatography. Since fractions I and III contained only small amounts of sialic acid, it was expected that chromatography at acid pH values would be preferred. As shown in Fig. 6, fraction I and fraction III were each separated into two fractions on SE-Sephadex C-25 column chromatography. The recovery of each chromatography was more than 90% with respect to carbohydrate. The relative yield of hexose in each fraction was as follows: fraction I-1, 38%; fraction I-2, 62%; fraction III-1, 30%; fraction III-2, 70%.

Characterization of fractionated glycopeptides. Results of high-voltage paper electrophoresis of the further-fractionated glycopeptides are given in Fig. 4(*b*). Fraction II-1 and fraction II-2, which had been fractionated on DEAE-Sephadex A-25, were still heterogeneous. However, fraction I-1, fraction III-1 and fraction III-2 were homogeneous and fraction I-2 showed only two spots, and these glycopeptides were separated by SE-Sephadex column chromatography. Therefore, fractionation on SE-

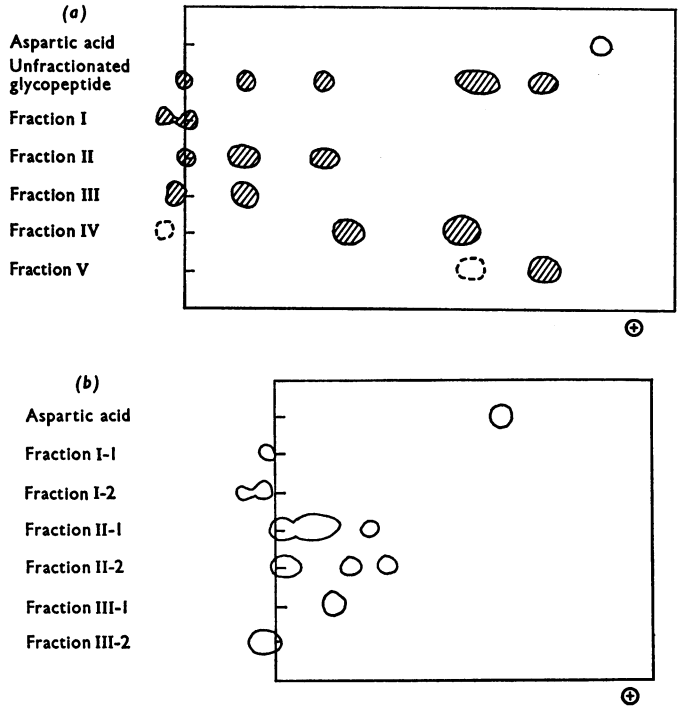

Fig. 4. Paper electrophoretograms of fractionated glycopeptides. (*a*) Electrophoretogram of fractions I–V. Electrophoresis was carried out in pyridine–acetic acid–water, pH 3.6 (1:10:89, by vol.) for 2 h at a potential gradient of 35 V/cm on Toyo no. 51 A filter paper. Glycopeptides were located by ninhydrin spray and periodate-benzidine spray (Gordon, Thornburg & Werum, 1956). The hatched spots represent glycopeptides that are visualized by both ninhydrin spray and periodate–benzidine spray. (*b*) Electrophoretogram of fractions I-1 to fraction III-2. The conditions for electrophoresis were the same as (*a*). Glycopeptides were detected by ninhydrin spray.

Table 3. *Chemical composition of fractionated glycopeptides*

The values are expressed as molar ratios to aspartic acid or fucose and blank spaces mean values less than 0.1.

	Fraction				
Component	I	II	III	IV	V
Fucose	0.2	1.0	1.0	1.0	1.0
Galactose	0.3	3.6	1.4	2.7	3.0
Mannose	6.1	4.3	5.0	3.3	2.9
Glucosamine	2.6	5.1	2.5	4.8	4.9
Sialic acid	0.1	1.8	0.6	2.5	2.4
Lysine	0.4				
Arginine		0.4			
Aspartic acid	1.0	1.8	1.3	1.9	1.4
Threonine	0.5	0.7	0.8	0.8	0.7
Serine	0.7	1.0	0.8	1.0	0.5
Glutamic acid	0.1	0.4	0.7	0.9	0.3
Proline	0.6	0.5		2.2	0.2
Glycine	0.3	0.4		0.6	0.3

Sephadex C-25 was of some use in further purification of glycopeptides.

Molecular-weight determinations of the fractionated glycopeptides were performed by gel filtration on Sephadex G-50. From the plots shown in Fig. 7, the molecular weight of each glycopeptide was estimated as follows: fraction I-1, 1000 ± 100; fraction I-2, 1700 ± 100; fraction II-1, 2200 ± 200 and 3800 ± 200; fraction III-2, 1700 ± 100; fraction V, 3800 ± 200.

Carbohydrate units of thyroglobulin. From the molecular weights and the chemical compositions, the molar ratios of the sugars and amino acids per mol of glycopeptides are shown in Table 4.

It is remarkable that fraction III-2 and fraction V, homogeneous on paper electrophoresis, gave integral values as regards carbohydrate component. In addition, the integral values for amino acids were also found in fraction III-2, although other glycopeptides showed non-integral values for the amino acids.

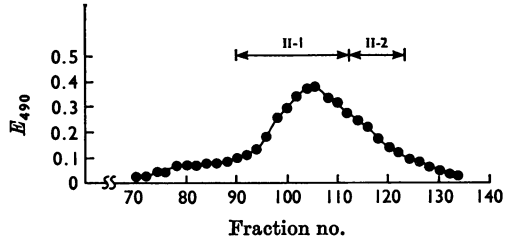

Fig. 5. Rechromatography of fraction II on DEAE-Sephadex A-25. Fraction II was applied to a column (1.4cm×30cm) of DEAE-Sephadex A-25 equilibrated with 3mM-pyridine acetate buffer, pH6.4, and washed with the same buffer. The column was eluted with 1000ml of 55mM-pyridine acetate buffer, pH6.4. Fractions (5ml) were collected and neutral sugars were measured by the extinction at 490nm after phenol–H_2SO_4 reaction.

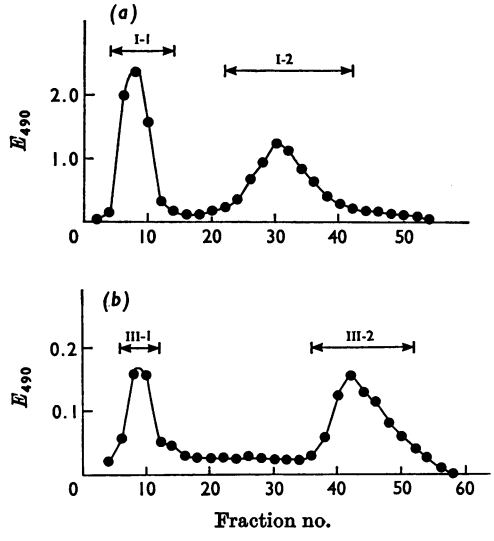

Fig. 6. SE-Sephadex C-25 column chromatography of fractions I and III. (*a*) Fraction I was applied to a column (1.2cm×40cm) of SE-Sephadex C-25 that had been equilibrated with 1mM-HCl. The column was eluted with the same solution. Fractions (5ml) were collected and neutral sugars were measured by phenol–H_2SO_4 reaction. (*b*) Fraction III was applied to a column (1.4cm×32cm) of SE-Sephadex C-25 equilibrated with 1mM-HCl solution. The column was washed with 120ml of the same solution and eluted with 150ml of 5mM-HCl. Fractions (5ml) were collected.

All of the fractionated glycopeptides had similar amino acid compositions and contained only a limited number of amino acids. Among them, aspartic acid was the only amino acid that occurred as one residue or more in each glycopeptide.

Fraction I-1 had only trace amounts of amino acids. This was probably due to cleaving of the

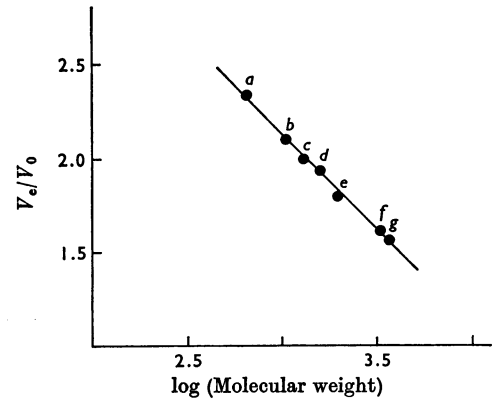

Fig. 7. Determination of molecular weight of glycopeptides by gel filtration on Sephadex G-50. *a*, Xylopentaose; *c*, di-*N*-acetylneuraminyl-lacto-*N*-tetraose; *f*, a glycopeptide from orosomucoid; *b*, fraction I-1; *d*, fraction I-2; *e* and *g*, fraction II-1; *d*, fraction III-2; *g*, fraction V. V_0, void volume; V_e, elution volume.

carbohydrate–peptide bond by Pronase. Similar results have been observed with α_1-acid glycoprotein (Eylar, 1962), fetuin (Spiro, 1962) and human thyroglobulin (Arima, Spiro & Spiro, 1970) in which the carbohydrate–peptide bonds are thought to be glycosylamine linkages.

It may be deduced from the results in Table 4 that purified glycopeptides are grouped into two types with respect to carbohydrate units. One of these is relatively simple, and contains glucosamine and mannose, and little or no other sugars. The other is a more complex heterosaccharide and contains sialic acids, fucose, and galactose in addition to glucosamine and mannose.

Nature of linkage between carbohydrate and protein. Unfractionated glycopeptide and fractionated glycopeptides contained threonine and serine in addition to aspartic acid. Recoveries of threonine and serine were quantitative after mild alkali treatment (0.5M-sodium hydroxide at 4°C for 20h), so that *O*-glycosidic linkages involving these amino acids may be absent, but it is important to emphasize that not all linkages of this general type would be split under the conditions employed (see, for example, Derevitskaya, Vatina & Kochetkov, 1967).

A substance that behaved chromatographically like 2-acetamido-1-*N*-4′-L-aspartyl-2-deoxy-β-D-glucopyranosylamine on the amino acid analyser was present in acid hydrolysates (2M-hydrochloric acid; 100°C; 20min) of the glycopeptide (Wagh, Bornstein & Winzler, 1969). The yield was 11% in terms of the total aspartic acid content.

Further, only aspartic acid occurred as one

Table 4. *Chemical composition of fractionated glycopeptides*

The values are expressed as mol/mol of glycopeptide. Blank spaces mean values less than 0.05 mol.

	Fraction						
Component	I-1	I-2	II-1	II-2	III-1	III-2	V
Fucose	0.12	0.49	1.31	1.48	1.71		1.00
Galactose	0.37	0.67	2.71	3.34	3.90		2.96
Mannose	4.04	5.00	3.44	4.55	3.54	7.90	2.93
Glucosamine	2.00	2.36	5.07	5.22	4.63	1.93	4.92
Sialic acid	0.07		1.06	1.07	1.44		2.40
Aspartic acid	0.13	1.00	1.00	1.00	1.00	1.00	1.35
Threonine		0.55	0.42	0.29	0.25	0.95	0.65
Serine	0.06	0.97	0.44	0.43	0.26	0.93	0.46
Glutamic acid			0.33	0.06		0.88	0.27
Proline		0.52	0.47				0.22

Table 5. *Carbohydrate units of porcine thyroglobulin*

	Mannose–glucosamine unit (unit A)	Hetero-saccharide unit (unit B)
Monosaccharides (mol/mol)		
Sialic acid		1–2.5
Fucose		1
Galactose		3
Mannose	4–8	3
Glucosamine	2	5
Molecular weight	1000–1700	3300
Number of units in thyroglobulin	8–11	12–13

residue or more in each glycopeptide. From these results it might be concluded that the carbohydrate moiety of thyroglobulin is linked to the protein through the reducing group of *N*-acetylglucosamine and the amide nitrogen of asparagine.

DISCUSSION

In studies on the fractionation of glycopeptides by other workers, it has been difficult to obtain a homogeneous glycopeptide. The isolation of glycopeptides which were homogeneous on high-voltage paper electrophoresis from thyroglobulin represents a notable advance. This could be accomplished by two-step linear-gradient elution on DEAE-Sephadex column chromatography and SE-Sephadex column chromatography. The combination of two column-chromatography procedures was particularly useful.

The purification and characterization of glycopeptides has provided evidence that two distinct types of carbohydrate units are present in porcine thyroglobulin, as was demonstrated earlier for the calf protein (Spiro, 1965). The two types are different in component sugars, molecular weight and behaviour on paper electrophoresis (see Table 5).

The present work also indicates that carbohydrate unit A, which contains mannose and glucosamine, is heterogeneous. It contains 2mol of glucosamine and 4–8mol of mannose/mol of glycopeptide. The composition of unit A of pig thyroglobulin is similar to the comparable type of prosthetic groups in both the human and calf hormones (Arima *et al.* 1970).

Unit A from thyroglobulin and glycopeptide from ovalbumin were similar to each other in several respects. Both glycopeptides contain only glucosamine and mannose, have molecular weights of 1000–2000 and are heterogeneous with respect to carbohydrate moiety (Johansen, Marshall & Neuberger, 1961; Cunningham, Clouse & Ford, 1963; Huang, Mayer & Montgomery, 1970).

The more complex carbohydrate unit B appears to be similar to the glycopeptides from fetuin (Spiro, 1962) and α_1-acid glycoprotein (Yamauchi *et al.* 1968).

The heterogeneity of unit B may be due in part to peripheral residues, particularly to the attachment of sialic acid and amino acids.

Since fractions II, IV, V, 30% of fraction III, and most of 'other fractions' were of unit B type, and fraction I together with 70% of fraction III were of unit A type, about 40% of the mannose in porcine thyroglobulin is present in carbohydrate unit B and this would represent about 37 residues/mol of thyroglobulin, based on the molecular weight (670000) (Edelhoch, 1960) and the mannose content (2.47%) of thyroglobulin. It may be calculated that there are 8–11 units of carbohydrate unit A and 12–13 units of carbohydrate B per molecule of pig thyroglobulin, similar to the numbers of each present in the calf protein (Spiro, 1965).

Altogether there are more than 20 different points in the peptide chain at which carbohydrate units are attached. Amino acid compositions of fractionated glycopeptides were similar to each other

and only a limited number of amino acids, particularly aspartic acid, threonine and serine, were found except in fraction IV.

The apparent existence of a small amount of galactose and fucose in fraction I-1 and fraction I-2 remains to be explained. It is not yet possible to decide whether they are due to contamination by a heterosaccharide unit or, more significantly, by intermediates between unit A and unit B.

The existence of two types as found in this study has also been observed in glycopeptides from rat liver (Li, Li & Shetlar, 1968), from liver microsomes (Miyajima, Kawasaki & Yamashina, 1969) and from coat membrane of Sindbis virus (Burge & Strauss, 1970). It may not be unreasonable to propose that the two types of polysaccharide chains as found in this study are widely distributed in glycoproteins.

The authors thank Professor A. Neuberger for the revision of the manuscript and Professor Y. Kondo for valuable discussion and support. We are obliged to Dr T. Yamakawa, Dr Y. Kaziro and Dr Y. Nagai, University of Tokyo, for the use of g.l.c. apparatus. Part of the expense of this study was defrayed by a grant from the Ministry of Education of Japan.

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