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Structure of Glycoproteins of Human Erythrocytes

ALKALI-STABLE OLIGOSACCHARIDES

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Studies have been made on the oligosaccharide residues of the alkali-stable carbohydrate–protein linkage of sialoglycopeptides derived from human erythrocytes. Four glycopeptides were isolated after alkaline borohydride treatment and Pronase digestion of MN-active sialoglycopeptides. The structure of one of these glycopeptides (GPIV) has been studied by sequential hydrolysis with specific glycosidases. Glycopeptide GPIV contained (per mol): 1 mol of fucose, 1 mol of sialic acid, 3 mol of galactose, 3 mol of mannose, 4 mol of acetylglucosamine, 1 mol of aspartic acid and fractional amounts of threonine, serine and glycine. The molecular weight of the glycopeptide was estimated to be 2330 by gel filtration. On the basis of glycosidase-digestion results, a tentative structure is proposed for the oligosaccharide moiety of glycopeptide GPIV.

The MN serological specificity of human erythrocytes resides in a membrane glycoprotein fraction, which can be isolated from erythrocyte stroma (de Burgh, Yu, Howe & Bovarnick, 1948; Klenk & Uhlenbruck, 1960; Kathan, Winzler & Johnson, 1961). Alternatively, sialoglycopeptides with M or N activity can be obtained by trypsin treatment of typed whole erythrocytes (Springer, Nagai & Tegtmeyer, 1966; Winzler, Harris, Pekas, Johnson & Weber, 1967; Kathan & Adamany, 1967; Thomas & Winzler, 1969*a*). The availability and ease of preparation of MN-active material therefore provides a convenient model system for the study of membrane glycoproteins and the chemical basis of serological specificity.

In previous work on blood-group-active sialoglycopeptides (Winzler *et al.* 1967; Thomas & Winzler, 1969*a*,*b*) we have shown that a portion of the carbohydrate (approx. 25%) is linked to the peptide chain by alkali-labile *O*-glycosidic bonds. Treatment of M- or N-active glycopeptides with alkaline borohydride released an identical number of oligosaccharide residues (Thomas & Winzler, 1969*b*); in each case the major product was a tetrasaccharide with the proposed structure *N*-acetylneuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[*N*-acetylneuraminyl-(2→6)]-D-*N*-acetylgalactosaminitol, although an alternative structure has also been proposed (Adamany & Kathan, 1969). It was concluded that the serological specificity could not be accounted for by differences in alkali-labile residues. The present work is concerned with the structure of the carbohydrate residues not released from glycopeptides by alkali treatment. The approach to the problem has involved treatment of MN-typed glycopeptides with alkaline borohydride, digestion of the high-molecular-weight residue with Pronase and study of the products by sequential glycosidase hydrolysis.

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EXPERIMENTAL

Materials. Sialoglycopeptides from human erythrocytes were prepared as described previously (Winzler *et al.* 1967). β-Galactosidase, α-L-fucosidase, α-mannosidase and β-*N*-acetylglucosaminidase were gifts from Dr O. P. Bahl, and β-*N*-acetylglucosaminidase was also obtained from Dr B. Weissmann. Neuraminidase was purchased from Behringwerke A.-G., Marburg/Lahn, Germany.

General methods. Neutral sugar was determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) or by g.l.c. of the alditol acetates (Lehnhardt & Winzler, 1968). Reducing sugars were determined by the Somogyi–Nelson method (Nelson, 1944). Sialic acid was determined by the thiobarbituric acid method (Warren, 1959). Acetylhexosamines were assayed by the method of Reissig, Strominger & Leloir (1955). Hexosamines and amino acids were determined with a Spinco amino acid analyser (Winzler *et al.* 1967).

Isolation of glycopeptides. Conditions for the cleavage of alkali-labile bonds and the isolation of products were as described previously (Thomas & Winzler, 1969*a*). Sialoglycopeptides (1% solution derived from MN-typed erythrocytes) were incubated in 0.1M-NaOH–0.4M-$NaBH_4$ for 26h at room temperature in sealed tubes

under N_2 in the dark. The resulting mixture was fractionated on a Sephadex G-25 column and the void-volume fractions, containing glycopeptide material, were pooled and freeze-dried. A portion (1g) of this product was incubated with Pronase (20mg) in 60ml of 0.1M-tris–HCl buffer, pH8.0, containing 1mM-NaN_3 and 2mM-$CaCl_2$, at 40°C for 24h. Additional Pronase (10mg) was added and the incubation continued for 24h. The digest was centrifuged and then samples were applied to a column (18cm×75cm) of Sephadex G-25 and the eluates monitored for protein and carbohydrate. All of the carbohydrate emerged in the void-volume fractions, which were pooled and freeze-dried. The dried material was dissolved in 0.1M-pyridine acetate buffer, pH5.6, and applied to a column (2cm×150cm) of Sephadex G-50 previously equilibrated with the same buffer. Fractions were pooled as indicated (Fig. 1) and freeze-dried. The pooled fractions B and C were purified further on Whatman 3M filter paper by descending paper chromatography for 48h in butan-1-ol–acetic acid–water (4:1:5, by vol.). The glycopeptides remained at the origin and were eluted from the dried paper with water. The glycopeptides were finally separated (Fig. 2) by repeated paper electrophoresis at 40–45V/cm for 1.5h in pyridine–acetic acid–water (1:10:289, by vol.), pH3.7, and detected with ninhydrin (Toennies & Kolb, 1951) or periodate–benzidine reagent (Gordon, Thornburg & Werum, 1956). A minimum of three electrophoretic separations was required to produce homogeneous material.

Determination of molecular weight of glycopeptide GPIV. The molecular weight of glycopeptide GPIV was estimated by the procedure of Bhatti & Clamp (1968) by using gel filtration on a Sephadex G-50 column (1.2cm×150cm), equilibrated with 0.15M-NaCl. Glucose and Blue Dextran were used in each run to permit determination of the void volume and the salt volume, and a mixture of stachyose (mol.wt. 666), a desialysed glycopeptide (mol.wt. 2297) from human orosomucoid (Wagh, Bornstein & Winzler, 1969), and a reduced oligosaccharide (mol.wt. 967) from human erythrocyte glycoprotein (Thomas & Winzler, 1969*b*) was used as a molecular-weight standard.

Glycosidase digestion. Samples of glycopeptide GPIV (0.2–0.8μM, based on aspartic acid content) were incubated with one-twentieth of their weight of the appropriate enzyme in a final volume of 400–750μl of the appropriate buffer at 37°C in the presence of toluene. The course of digestion was followed by removing samples (25–50μl) of the digest and analysing them for released reducing sugars, acetamido sugars or sialic acid. At the completion of digestion neutral sugars were also determined by g.l.c. During prolonged incubation, additional enzyme and toluene were added to the digest every 24h. In the stepwise hydrolysis with specific glycosidases, the enzyme digestion was terminated by heating at 100°C for 3min before addition of the next enzyme. Digestion with β-galactosidase and β-glucosaminidase was performed in 0.01M-sodium citrate buffer, pH4.6, and digestion with α-mannosidase and α-fucosidase was carried out in 0.01M-sodium citrate buffer, pH3.9. The pH4.6 buffer was employed for all incubations during the stepwise hydrolysis of glycopeptides.

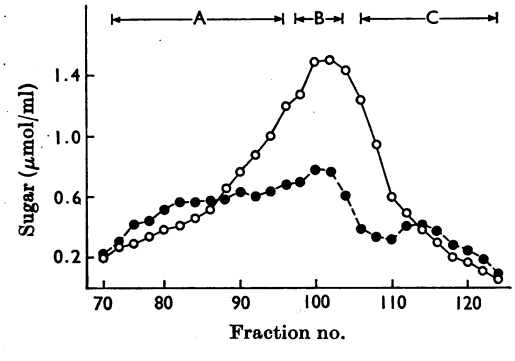

Fig. 1. Gel filtration of sialoglycopeptide mixture after alkaline borohydride treatment and Pronase digestion. The digest was applied to a column (2cm×150cm) of Sephadex G-50 and eluted with 0.1M-pyridine acetate buffer, pH5.6. Fractions (4ml) were collected. ○, Neutral sugar (Dubois *et al.* 1956); ●, sialic acid (Warren, 1959).

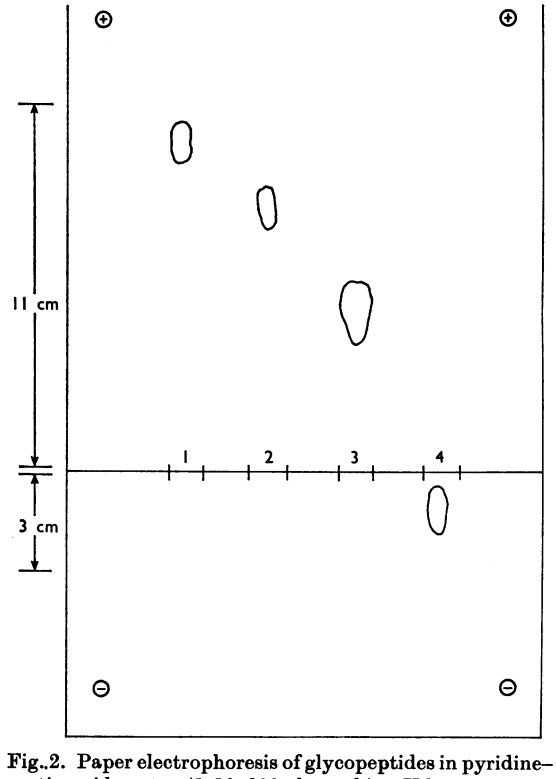

Fig. 2. Paper electrophoresis of glycopeptides in pyridine–acetic acid–water (1:10:289, by vol.), pH3.7, at 40–45 V/cm for 1.5h. 1, GPI; 2, GPII; 3, GPIII; 4, GPIV. Samples B and C (Fig. 1) were fractionated further by repeated paper electrophoresis; fraction B contained glycopeptides GPI and GPIII and fraction C contained glycopeptides GPII and GPIV as the major components.

Determination of neutral sugars by g.l.c. An internal standard of arabitol (5–25 μg) was added to portions (25–50 μl) of the enzyme digest and the mixture applied to a micro-column (1 cm × 0.25 cm) composed of equal parts of charcoal, Dowex 1 (X8; acetate form) and Dowex 50 (X8; H^+ form). The column was eluted with 5 ml of 10% (v/v) ethanol, and the eluate was evaporated to dryness under reduced pressure. The residue was assayed for neutral sugars by g.l.c. of the alditol acetates (Lehnhardt & Winzler, 1968).

Preparation of desialized glycopeptides. Glycopeptide samples (10–30 mg) were heated with 5 ml of 0.3 M-HCl at 100°C for 4 min. The solution was applied to and eluted from a column of Sephadex G-10. Fractions corresponding to the non-retarded and the retarded material were pooled separately and freeze-dried. Analysis showed that sialic acid had been completely removed from the glycopeptide; the retarded fractions contained free sialic acid and no other monosaccharides, as revealed by paper chromatography or g.l.c.

RESULTS

Table 1 shows the composition of sialoglycopeptides before and after treatment with alkaline borohydride. No further change in the composition of borohydride-treated sialoglycopeptides was observed on prolonged incubation in alkaline solution (26–72 h). After Pronase digestion of borohydride-treated sialoglycopeptides, four homogeneous glycopeptides were isolated by paper electrophoresis and designated GPI, GPII, GPIII and GPIV. No further attempt was made to define the homogeneity of these products.

Structural analysis with specific glycosidases was confined to glycopeptide GPIV. The composition of this material indicates a glycopeptide containing (per mol) 1 mol of aspartic acid, 4 mol of *N*-acetylglucosamine, 3 mol of mannose, 3 mol of galactose, 1 mol of *N*-acetylneuraminic acid and 1 mol of fucose, with fractional amounts of serine and threonine. The calculated mol.wt. of 2487 is in agreement with the value (2330) obtained by gel filtration.

Action of single glycosidases. Table 2 summarizes the effects of the glycosidases specific for the sugars present on untreated and desialized glycopeptide GPIV from MN cells. The results indicate that the glycopeptide has three terminal residues susceptible to these enzymes, namely one sialic acid residue, one fucose residue and one galactose residue.

Table 1. *Composition of sialoglycopeptides derived from MN-typed human erythrocytes*

Experimental details are given in the text.

	Content (mol/mol of aspartic acid)					
	Sialoglycopeptide	Sialoglycopeptide after borohydride treatment	Glycopeptide GPI	Glycopeptide GPII	Glycopeptide GPIII	Glycopeptide GPIV
Lysine	0.49	0.12	2.7	—	0.92	—
Histidine	0.50	0.41	—	—	0.31	—
Arginine	0.25	0.06	—	—	—	—
Aspartic acid	1.00	1.00	1.00	1.00	1.00	1.00
Threonine	2.77	0.88	1.12	0.37	1.58	0.45
Serine	2.50	0.83	0.49	0.20	0.81	0.27
Glutamic acid	0.91	0.63	0.57	0.32	0.51	0.30
Proline	0 59	0.42	—	—	1.40	—
Glycine	0.50	0.44	0.55	0.20	0.32	—
Alanine	0.74	1.25	0.95	0.40	1.44	—
Cysteine	—	—	—	—	—	—
Valine	0.67	0.50	0.30	0.13	0.26	—
Methionine	0.24	0.31	—	—	—	—
Isoleucine	0.33	0.30	—	0.12	—	—
Leucine	0.37	0.18	—	—	—	—
Tyrosine	0.22	0.21	—	—	—	—
Phenylalanine	0.03	0.03	—	—	—	—
α-Aminobutyric acid	—	0.43	—	—	0.24	0.15
N-Acetylglucosamine	1.44	1.90	15.4	9.40	13.00	3.85
N-Acetylgalactosamine	2.60	0.41	2.5	0.50	2.00	—
Sialic acid	7.80	2.40	14.4	4.40	7.00	0.90
Fucose	0.23	0.50	1.82	1.63	3.00	0.85
Mannose	0.39	1.10	10.1	4.30	8.30	2.65
Galactose	40	2.3	13.9	6.80	11.60	2.80

Desialized glycopeptide GPIV, treated with β-galactosidase, released 2 mol of galactose, indicating that the sialic acid had been linked to a galactose residue in the native material. β-*N*-Acetylglucosaminidase and an α-mannosidase had no effect upon the untreated or desialized glycopeptide GPIV.

Sequential digestion with glycosidases. Sequential digestion of glycopeptide GPIV with α-fucosidase, β-galactosidase, β-acetylglucosaminidase and α-mannosidase (Table 3) revealed the sequence of sugars in the three terminal non-reducing branches to be galactose→*N*-acetylglucosamine, fucose→galactose→*N*-acetylglucosamine and sialic acid→galactose→*N*-acetylglucosamine.

DISCUSSION

On the basis of the above findings, a tentative partial structure for glycopeptide GPIV is proposed (Fig. 3). As the structure shows, there are three non-reducing branches attached to a mannose core, with sialic acid, fucose and galactose residues at the non-reducing termini. The specificity of the α-L-fucosidase (Bahl, 1970) indicates that one penultimate galactose residue is substituted in either the 2- or the 6-position by a terminal fucose residue. The other penultimate galactose residue may be substituted on positions 2, 3, 4 or 6 with *N*-acetylneuraminic acid. The

Table 2. *Action of glycosidases on glycopeptide GPIV and desialized glycopeptide GPIV*

Experimental details are given in the text.

		Glycopeptide GPIV		Desialized GPIV	
Enzyme	Sugar content (mol/mol of aspartic acid)	Percentage released	Mol released/mol of aspartic acid (nearest integer)	Percentage released	Mol released/mol of aspartic acid (nearest integer)
Neuraminidase	*N*-Acetylneuraminic acid, 1	88	1	—	—
α-L-Fucosidase	Fucose, 1	85	1	90	1
β-Galactosidase	Galactose, 3	30	1	65	2
β-*N*-Acetylglucosaminidase	*N*-Acetylglucosamine, 4	0	0	0	0
α-Mannosidase	Mannose, 3	0	0	0	0

Table 3. *Sequential glycosidase digestion of glycopeptide GPIV and desialized glycopeptide GPIV*

Experimental details are given in the text.

			Glycopeptide GPIV		Desialized GPIV	
Expt. no.	Enzyme	Sugar released	Percentage released	Mol released/mol of aspartic acid (nearest integer)	Percentage released	Mol released/mol of aspartic acid (nearest integer)
1	First enzyme (α-L-fucosidase)	Fucose	83	1	90	1
	Second enzyme (β-galactosidase)	Galactose	60	2	86	3
	Third enzyme (β-*N*-acetylglucosaminidase)	*N*-Acetylglucosamine	43	2	65	3
	Fourth enzyme (α-mannosidase)	Mannose	15	<1	20	<1
2	First enzyme (α-L-fucosidase)	Fucose	86	1	93	1
	Second enzyme (β-galactosidase)	Galactose	55	2	79	3
	Third enzyme (α-mannosidase)	Mannose	0	0	0	0

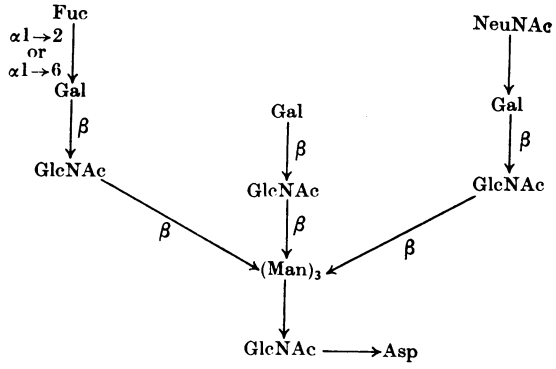

Fig. 3. Proposed structure for glycopeptide GPIV. Abbreviation: NeuNAc, *N*-acetylneuraminic acid.

third galactose residue is a non-reducing terminus. The three galactose residues are linked to *N*-acetylglucosamine, since 3mol of this sugar become susceptible to β-acetylglucosaminidase digestion on removal of galactose. Since the glycopeptide contains aspartic acid and is alkali-stable, it is probable that the fourth acetylglucosamine residue is joined by an *N*-glycosidic linkage to the amide group of asparagine (Spiro, 1969). No information is available on the sequence or linkage of mannose residues in the mannose core of the glycopeptide. Oligosaccharide sequences consisting of sialic acid or fucose→galactose→*N*-acetylglucosamine→mannose have been established for fetuin and α-acid glycoprotein (Spiro, 1969) and human chorionic gonadotrophin (Bahl, 1969) and appear to be a frequent structural feature of mammalian glycoproteins.

Similar procedures have been employed by Kornfeld & Kornfeld (1970) to isolate a glycopeptide with phytohaemagglutinin-receptor activity from human erythrocytes. The proposed structure (Fig. 3) for glycopeptide GPIV differs from that proposed by Kornfeld & Kornfeld (1970) in that it contains one additional residue each of mannose, *N*-acetylglucosamine, galactose and fucose. It should be emphasized that in both instances only a fraction of the glycopeptide material was isolated and characterized. It may be anticipated that a considerable degree of heterogeneity exists for the alkali-stable oligosaccharide residues of MN-active material.

No conclusions can be drawn from the present studies on the chemical basis of serological specificity. Treatment of M- or N-active sialoglycopeptides with alkaline borohydride results in a loss of serological specificity (R. J. Winzler, unpublished work), yet the alkali-labile oligosaccharide products are identical in each case (Thomas & Winzler, 1969*b*). Also, recent work (D. B. Thomas & R. J. Winzler, unpublished work) has shown that a glycopeptide with similar composition and electrophoretic mobility to glycopeptide GPIV can be isolated from M-typed material. Perhaps MN specificity is not a unique property of oligosaccharide residues but involves a contribution from the polypeptide sequence.

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REFERENCES

Adamany, A. M. & Kathan, R. H. (1969). *Biochem. biophys. Res. Commun.* **37**, 171.
Bahl, O. P. (1969). *J. biol. Chem.* **244**, 575.
Bahl, O. P. (1970). *J. biol. Chem.* **245**, 299.
Bhatti, T. & Clamp, J. R. (1968). *Biochim. biophys. Acta*, **170**, 206.
de Burgh, P. M., Yu, P.-C., Howe, C. & Bovarnick, M. (1948). *J. exp. Med.* **87**, 1.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Gordon, H. T., Thornburg, W. & Werum, L. N. (1956). *Analyt. Chem.* **28**, 849.
Kathan, R. H. & Adamany, A. M. (1967). *J. biol. Chem.* **242**, 1716.
Kathan, R. H., Winzler, R. J. & Johnson, C. A. (1961). *J. exp. Med.* **113**, 37.
Klenk, K. E. & Uhlenbruck, G. (1960). *Hoppe-Seyler's Z. physiol. Chem.* **319**, 151.
Kornfeld, R. & Kornfeld, S. (1970). *J. biol. Chem.* **245**, 2536.
Lehnhardt, W. F. and Winzler, R. J. (1968). *J. Chromat.* **34**, 471.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Spiro, R. G. (1969). *New Engl. J. Med.* **281**, 991.
Springer, G. F., Nagai, Y. & Tegtmeyer, H. (1966). *Biochemistry, Easton*, **5**, 3254.
Thomas, D. B. & Winzler, R. J. (1969*a*). *Biochem. biophys. Res. Commun.* **35**, 811.
Thomas, D. B. & Winzler, R. J. (1969*b*). *J. biol. Chem.* **244**, 5943.
Toennies, G. J. & Kolb, J. (1951). *Analyt. Chem.* **23**, 823.
Wagh, P. V., Bornstein, I. & Winzler, R. J. (1969). *J. biol. Chem.* **244**, 658.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A. & Weber, P. (1967). *Biochemistry, Easton*, **6**, 2195.