The Oligosaccharide Units of Rabbit Immunoglobulin G

MULTIPLE CARBOHYDRATE ATTACHMENT SITES

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(Received 15 November 1971)

The carbohydrate structure of rabbit immunoglobulin G isolated from pooled sera was investigated. Amino sugar analysis of fragments of the molecule allowed three oligosaccharides to be located at separate sites on the H-chain. The corresponding glycopeptides were isolated. The average composition of the C1-oligosaccharide was 2 glucosamine, 1 mannose and 2 galactose residues. It appeared to be present in approx. 15% of the H-chains; the carbohydrate was coupled through the amide group of asparagine in a peptide containing asparagine, glycine and threonine within the Fd fragment of the molecule. The average composition of the C2-oligosaccharide was 1 galactosamine, 1 galactose and either 1 or 2 sialic acid residues. It was present on approx. 40% of the H-chains and was attached glycosidically to the OH group of threonine in a peptide Ser-Lys-Pro-Thr-Cys-Pro-Pro-Glu-Leu in the hinge region of the molecule. The average composition of the C3-oligosaccharide was 5 glucosamine, 2 galactose, 5 mannose, 1 fucose and 1 sialic acid residue. It appeared to be present in all the H-chains and was linked through the amide group of asparagine in a peptide Gln-Gln-Phe-Asn-Ser-Thr-Ile-Arg within the Fc fragment of the molecule.

The crystallization of the Fc fragment of rabbit IgG* (Porter, 1959) allowed the first demonstration of carbohydrate in a discrete region of the IgG molecule. The finding was confirmed (Franklin, 1960), and since that time there has been discussion on whether all the carbohydrate of rabbit IgG is present at this single site (Nolan & Smith, 1962; Press, 1965) or whether there might be more than one attachment site (Fleischman *et al.*, 1963; Utsumi & Karusch, 1965; Smyth & Utsumi, 1967). With the development of a sensitive method for the measurement of aminohexose (Fanger & Smyth, 1970) and by using the method to detect carbohydrate in fragments of IgG, we have been able to locate three different oligosaccharide units in the IgG molecule. The present report describes the isolation of the glycopeptides that carry the oligosaccharides, together with a study of the nature and average amount of the monosaccharides in each carbohydrate chain. Further examination of the glycopeptides has provided information on the modes of attachment of the oligosaccharides to the polypeptide chain.

Experimental

Materials

Trypsin (TPCK, 200 units/mg), α -chymotrypsin, pepsin (2 $\times$ crystallized) and Pronase were obtained from Worthington Biochemical Corporation, Free-

*Abbreviations: IgG, immunoglobulin G; CMCys, carboxymethylcysteine.

hold, N.J., U.S.A. Papain (2 $\times$ crystallized, suspension in 0.05M-sodium acetate) was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. 2-Mercaptoethanol and *N*-ethylmorpholine were obtained from Koch-Light Ltd., Colnbrook, Bucks., U.K. Sodium borohydride was obtained from May and Baker Ltd., Dagenham, Essex, U.K., iodoacetic acid from Schuchardt, Munich, Germany, and iodo-[14 C]acetic acid (21 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. Neuraminidase (500 units/ml) was obtained from Behringwerke A.G. (Marburg, Germany).

Methods

Identification of amino sugars. The glycoprotein (3 mg) or glycopeptide (0.02 μ mol) was submitted to hydrolysis in 6M-HCl (2 ml) at 110°C for 12 h. The hydrolysate was submitted to the following procedure, which removes an artifact that chromatographs with galactosamine on the amino acid analyser (Fanger & Smyth, 1970). The acid solution was dried *in vacuo* and the residue was taken up in *N*-ethylmorpholine (2%, v/v) at pH 9. The solution (2 ml) was added to a column (5 cm $\times$ 0.9 cm) of Dowex 2 (X4) operated with 1% (v/v) *N*-ethylmorpholinium acetate (20 ml), pH 9, as eluent. The amino sugar solution was concentrated to dryness *in vacuo*, the residue was dissolved in 0.05M-sodium citrate, pH 2.2, and analysis was done at 50°C on a column (150 cm $\times$ 0.9 cm) of an amino acid analyser, by using 0.2M-citrate buffers at pH 3.25 and 4.25 (Spackman *et al.*, 1958). The

elution positions of the amino sugars were compared with the elution positions of glucosamine, galactosamine and mannosamine, first in separate analyses and then by co-chromatography of the hydrolysates with the authentic amino sugars.

The occurrence of amino sugar was taken to indicate the presence of oligosaccharide in fragments of IgG. Peptides lacking amino sugar were assumed to be devoid of all carbohydrate.

Quantitative analysis of amino sugars. The method applied to IgG and its large fragments is described in detail by Fanger & Smyth (1970); the amino sugar values given in Table 1 were calculated from the amounts of lysine (μmol) found in each amino sugar analysis, taken in conjunction with the respective amino acid compositions: IgG, Fab fragment and L-chain (Fleischman *et al.*, 1963), $F(ab')_2$ fragment (Mage & Harrison, 1966) and Fc fragment (Hill *et al.*, 1967). In glycopeptides the amino sugars were determined directly by amino acid analysis after hydrolysis in 3M-HCl at 110°C for 6h and for 24h; a correction of 10% was made for decomposition of the amino sugar during the 6h hydrolysis. The number of residues of glucosamine in the C1-oligosaccharide and in the C3-oligosaccharide was calculated relative to aspartic acid in the respective glycopeptides; the number of residues of galactosamine in the C2-oligosaccharide was calculated relative to lysine. The amino sugars were also determined in the g.l.c. analysis described below.

Quantitative analysis of neutral sugars. Mono-saccharides and sialic acid were determined as the trimethylsilyl ethers of the methyl glycosides by g.l.c. (Clamp *et al.*, 1967, 1971). Methanolysis of the glycopeptides was conducted at 80°C for 24h in 1M-HCl in dry methanol. The sugars were acetylated and allowed to react with trimethylsilyl chloride-hexamethyldisilazane before injection on to a column of SE30 on Diatoport-S, D-mannitol being added as an internal standard. *N*-Acetylglucosamine emerges as four peaks and *N*-acetylgalactosamine emerges as two peaks after elution of the neutral mono-saccharides. The values obtained for the neutral sugars were related to the amounts of glycopeptide submitted for g.l.c., those amounts being based on amino acid analysis.

Preparation of rabbit immunoglobulin G. IgG was isolated from pooled sera from New Zealand and Californian strains of rabbit (obtained from White Cloud Farm, Tring, Herts., U.K.). In the first step, 15ml batches of serum were added to a column (140cm $\times$ 2.8cm) of Sephadex G-200 operated at 4°C with 0.02M-sodium phosphate buffer, pH7.0, as eluent. Fractions (10ml) were collected. The 7S protein present in the fractions from 220 to 296ml was concentrated by vacuum dialysis, and was dialysed against 0.02M-sodium phosphate buffer, pH7. In the second step, chromatography was performed

at 4°C on a column (30cm $\times$ 2.5cm) of DEAE-cellulose with the 0.02M-sodium phosphate buffer, pH7.0, as eluent (Peterson & Sober, 1956). The IgG fractions that emerged between 69 and 320ml were combined, then the solution was concentrated by vacuum dialysis, and dialysed against 0.02M-sodium phosphate buffer, pH7.0, and the IgG was stored in the frozen state at -20°C. The yield of IgG was approx. 100mg from each 15ml batch of serum.

Isolation of L-chain. This was obtained from IgG in 0.2M-tris-HCl buffer, pH8, by reduction in 0.14M-mercaptoethanol and alkylation in 0.14M-iodoacetate. L-chain was separated from H-chain by gel filtration on a column (150cm $\times$ 2cm) of Sephadex G-100 operated with 1M-acetic acid as eluent (Fleischman *et al.*, 1963). The L-chain, which was present in the second peak of eluted protein, was concentrated by vacuum dialysis and resubmitted to gel filtration under the same conditions. The final L-chain product was identified by amino acid analysis, the results of which were in accord with the values obtained by Fleischman *et al.* (1963).

Isolation of the $F(ab')_2$ fragment of IgG. Digestion of IgG (600mg) with pepsin (enzyme/substrate ratio 1:50) was done as described by Nisonoff *et al.* (1960). The pepsin digest was submitted to gel filtration on

Fig. 1. Isolation of the $F(ab')_2$ fragment of IgG and the C3-glycopeptide

IgG (600mg) in 5ml of 0.1M-sodium acetate buffer, pH4.5, was digested with pepsin (12mg) for 36h at 37°C. The digest was added to a column (150cm $\times$ 2.5cm) of Sephadex G-100 operated at 4°C with 0.02M- NH_4HCO_3 as eluent and 5ml fractions were collected. Glycopeptide material was located by the rapid screening of fractions by mild hydrolysis and amino sugar analysis. The $F(ab')_2$ fragment emerged between 200 and 310ml. The C3-glycopeptide emerged between 560 and 680ml, indicated by the vertical broken lines. $\circ$, E_{230} ; $\bullet$, E_{280} .

Fig. 2. Separation of the Fab fragment and the hinge-region peptides

Reduced carboxymethylated $F(ab')_2$ fragment (250mg) in 5ml of 0.2M-sodium phosphate buffer, pH7.0, was digested at 37°C with papain (2.5mg) for 24h in the presence of 1.4mM-mercaptoethanol. The digest was added to a column (150cm $\times$ 2.5cm) of Sephadex G-75 with 0.02M- NH_4HCO_3 as eluent. The Fab fragment emerged between 230 and 255ml of effluent; the C2-glycopeptide and other hinge-region peptides emerged between 300 and 370ml. ●, E_{280} ; ▲, radioactivity counts/3min per 50 μ l samples of effluent fractions, measured by liquid-scintillation counting.

Sephadex G-100 (Fig. 1). The fractions containing the $F(ab')_2$ fragment were combined and were concentrated by vacuum dialysis. The $F(ab')_2$ fragment was separated from undigested IgG by further gel filtration on a column (150cm $\times$ 2cm) of Sephadex G-150 operated at 4°C with 0.02M-sodium phosphate buffer, pH7, as eluent; the $F(ab')_2$ fragment was present in the first major peak of eluted material. Final purification of $F(ab')_2$ fragment from Fc' aggregates was effected on a column (20cm $\times$ 1.5cm) of Sephadex C-25 with 0.02M-sodium acetate buffer, pH5.5, as eluent (Mage & Harrison, 1966). The $F(ab')_2$ fragment emerged between 30 and 61ml of effluent. The overall yield of $F(ab')_2$ fragment was 56%, based on the measurement of E_{280} values and amino acid composition.

Isolation of the Fab fragment of IgG. This was obtained from $F(ab')_2$ fragment by papain digestion under mild reducing conditions. $F(ab')_2$ fragment (100mg, equivalent to 2 μ mol of Fab fragment) was digested with papain (1mg) in the presence of 1.4mM-mercaptoethanol in 0.2M-sodium phosphate buffer, pH7 (5ml), for 4h. The digest was applied to a column (150cm $\times$ 2cm) of Sephadex G-75 with 0.02M-sodium phosphate buffer, pH8.0, as eluent. The Fab fragment was located in the column effluent by measurement of E_{280} ; it emerged in the fractions immediately after the exclusion volume. This pre-

paration of Fab fragment retained 83% of the glucosamine and 5% of the galactosamine of the parent $F(ab')_2$ fragment.

Isolation of the C1-glycopeptide. Fab fragment (90mg) was digested with Pronase (2mg) at pH7, 37°C for 48h. The digest was applied to a column (150cm $\times$ 2cm) of Sephadex G-25 with 0.02M-pyridinium acetate buffer, pH5, as eluent. The material containing glucosamine, located by amino sugar analysis of portions of combined effluent fractions, was excluded from the gel. The mixture of glycopeptide and peptide material was concentrated *in vacuo* and applied to Whatman no. 1 filter paper. Peptide material was separated from the glycopeptide by descending chromatography performed first with 100ml of ethanol - water - propanol - acetic acid (60:20:20:3, by vol.), then with 100ml of water (Carubelli *et al.*, 1966). The C1-glycopeptide (0.13 μ mol), which remained at the origin during the elution with organic solvent, was eluted by the water. The solution was concentrated *in vacuo* at 30°C and contaminants derived from the paper were removed by a final gel filtration on Sephadex G-25. The amino acid composition of this preparation of C1-glycopeptide is given in Table 2.

An alternative method of isolating the C1-glycopeptide involved papain digestion of the $F(ab')_2$ fragment under strong reducing conditions. The

F(ab')₂ fragment (150mg, equivalent to 3 μ mol of univalent Fab fragment) was digested with papain (1.5mg) in 7ml of 0.2M-sodium phosphate buffer, pH7, at 37°C for 24h in 0.1M-mercaptoethanol. The digest was applied to a column (150cm $\times$ 2cm) of Sephadex G-75 with 0.02M-sodium phosphate

buffer, pH7, as eluent. Glycopeptides containing glucosamine and galactosamine emerged between 300 and 400ml. The combined fractions were concentrated *in vacuo* and the salt concentration was adjusted to 1mM-sodium phosphate buffer, pH8, by passage of the solution through a short column of

Fig. 3. Isolation of hinge-region peptides lacking carbohydrate

(a) Hinge-region peptides were resolved by chromatography at 40°C on a column (50cm $\times$ 0.9cm) of Dowex 50 (X2) with 0.2M-pyridinium acetate buffer, pH3.25, as eluent. (b) Further chromatography on a column (40cm $\times$ 0.9cm) of Dowex 1 (X2) at 40°C with a gradient of 0.2M-pyridinium acetate buffer, pH5.3–pH3.25, mixer volume 200ml; $\cdots$, change of pH during chromatography; —, radioactivity. (c) and (d) Chromatography of peptide I (c) and peptide II (d) from Dowex 1 (X2) on the IR-120 column (150cm $\times$ 0.9cm) of an amino acid analyser at 50°C with 0.2M-sodium citrate buffer, pH3.25, as eluent.

Sephadex G-25. The glycopeptide mixture was applied to a column (20cm×0.9cm) of DEAE-cellulose equilibrated in 1m-sodium phosphate buffer, pH8. Stepwise elution was performed with sodium phosphate buffers, pH8, of increasing ionic strength. The C1-glycopeptide (0.35μmol) was eluted in 3m-phosphate buffer (20ml); the C2-glycopeptide (0.42μmol) was eluted in 0.1M-phosphate buffer (20ml).

Isolation of the C2-glycopeptide. A solution of F(ab')₂ fragment (250mg) in water (5ml) was adjusted to pH8 by addition of 1M-NaOH and a slow stream of N₂ was used to displace dissolved air. A dilute solution of mercaptoethanol in water, also gassed with N₂, was added to give a final concentration of mercaptoethanol of 1.4mM and reduction was allowed to proceed for 4h. The solution of reduced Fab' fragment was incubated in 2mM-iodoacetate containing 1μCi of ¹⁴C/μmol at pH8 for 1h and the solution was dialysed against 0.02M-sodium phosphate buffer, pH7.

Papain digestion of the reduced carboxymethylated F(ab')₂ fragment was done at an enzyme/substrate ratio of 1:100 in the presence of 1.4mM-mercaptoethanol, pH7, at 37°C for 24h. The digest was applied to a column of Sephadex G-75 (Fig. 2). The peptide fraction was resolved by further gel filtration on a column (150cm×2.5cm) of Sephadex G-25 with 0.02M-NH₄HCO₃ as eluent. The fractions (2ml) were monitored by measurement of extinction at 230 and 280nm and by measurement of radioactivity in a Nuclear-Chicago scintillation counter, with the solvent system of Kinard (1957). The C2-glycopeptides emerged between 204 and 220ml.

The C2-glycopeptides (0.8μmol) were separated by high-voltage electrophoresis for 2h on Whatman no. 1 paper in 1M-pyridine-acetic acid buffer, pH6.5, with a voltage gradient of 80V/cm. Two components, located by radioactive scanning, were resolved. The amino acid compositions are given in Table 2 and the yields of the more acidic and less acidic glycopeptides after elution were 0.2μmol and 0.15μmol respectively.

Digestion of the C2-glycopeptide (0.1μmol) with neuraminidase (50 units) was done at pH5.5 for 4h and the liberated neuraminic acid was determined by g.l.c.

The peptides that eluted after the C2-glycopeptides were devoid of carbohydrate, and were resolved by chromatography on Dowex 50 (X2) and Dowex 1 (X2) (Fig. 3) and sequence was assigned from the results of Edman degradation (Konigsberg, 1967). The two components were hexapeptides CMCys-Pro-Pro-Pro-Glu-Leu and [CMCys-Pro-Pro-Pro-Glu-Leu, which are interconvertible by cyclization of the *N*-terminal carboxymethylcysteine residue at mildly acidic pH (A. F. Bradbury, D. E. Massey & D. G. Smyth, unpublished work), as indicated in the formulae.

Isolation of the C3-glycopeptide. IgG (600mg) was digested at pH4.5 for 36h at 37°C with pepsin at an enzyme/substrate ratio of 1:50. The digest was applied to a column of Sephadex G-100 (Fig. 1). The fractions containing glucosamine, which emerged between 570 and 680ml, were combined and concentrated *in vacuo*. The glycopeptide mixture was submitted to further gel filtration on a column (150cm×2cm) of Sephadex G-25 with 0.02M-sodium phosphate buffer, pH7, as eluent. The glycopeptide fractions, which were excluded from the gel, were combined and

Fig. 4. Isolation of the peptide formed by β -elimination of the C2-glycopeptide

The C2-glycopeptide (2μmol) was maintained at pH13, 70°C for 1h and then was incubated in 0.1M-sodium sulphite, pH9, for 16h. The solution was added to a column (150cm×2.5cm) of Sephadex G-25 with 0.02M-NH₄HCO₃ as eluent and 6ml fractions were collected. Portions (500μl) of each fraction were dried on discs of filter paper and the radioactivity was counted in a thin-window Geiger-Müller counter.

digested with trypsin (1 mg) for 24 h at 37°C in 8 ml of 0.1 M-phosphate buffer, pH 7.5. After adjustment of the salt concentration to 5 mM-phosphate buffer, pH 8, on Sephadex G-25, the peptide solution was added to a column (5 cm × 0.9 cm) of DEAE-cellulose with 5 mM-phosphate buffer as eluent. The glycopeptide was not retarded on the column. Electrophoresis was done at 80 V/cm for 2 h at pH 3.5. A single weakly basic component was seen. A glyco-

peptide was isolated in the same manner from type I IgG, which comprises a relatively basic population of IgG molecules (Fanger & Smyth, 1972), and from anti-Lac antibody (Utsumi & Karusch, 1965). The amino acid composition of the respective C3-glycopeptides is given in Table 2.

Mode of linkage of the C1- and C3-oligosaccharides to the polypeptide chain. The C1-glycopeptide (0.2 μmol) or C3-glycopeptide (1 μmol) was allowed to react with 1 M-hydroxylamine hydrochloride in 1.8 M-NaOH for 1 h at 25°C. The reaction mixture (1 ml) was applied to a column (60 cm × 0.9 cm) of Sephadex G-25 operated with 0.02 M-phosphate buffer, pH 7, as eluent. Portions of the early, central and late fractions of the eluted peptide were submitted to acid hydrolysis (3 M-HCl, 110°C, 6 h) and the aspartic acid/glucosamine ratio was determined on the amino acid analyser. In addition, the elution positions of the glycopeptides on the column were noted before and after exposure of the peptides to hydroxylamine.

Mode of linkage of the C2-oligosaccharide to the polypeptide chain. The C2-glycopeptide (0.3 μmol) was dissolved in 0.1 M-NaOH (2 ml) and the solution was maintained at 30°C for 1 h (Carubelli *et al.*, 1966). The reaction mixture was treated with sodium sulphite (25 mg) and was kept at pH 9 for 16 h (Harbon *et al.*, 1964). Gel filtration on a column (150 cm × 2.5 cm) of Sephadex G-25 was used to

Table 1. *Amino sugar content of rabbit IgG and its component fragments*

The IgG was dialysed against 8 M-urea to remove adsorbed carbohydrate. The Fab fragment was isolated from F(ab')₂ fragment under mild reducing conditions. The Fc fragment was obtained under the conditions of papain digestion described by Porter (1959).

Component	Residues per molecule	
	Glucosamine	Galactosamine
IgG	10.4	0.88
F(ab') ₂	0.60	0.84
Fab	0.25	0.02
Fc	4.7	0.02
L-chain	0.02	—

Fig. 5. *Chain structure of rabbit IgG showing distribution of oligosaccharides*

The Fab fragment comprises the entire light (L-) chain and the heavy (H-) chain from its blocked *N*-terminus up to one of the sites of papain cleavage (Fleischman *et al.*, 1963; Givol, 1968; Smyth & Utsumi, 1967). The Fc fragment comprises the portion of the H-chain commencing at one of the sites of papain cleavage and continuing up to the C-terminus (Porter, 1959; Hill *et al.*, 1967). The F(ab')₂ fragment comprises the L-chain and the H-chain from the blocked *N*-terminus up to the leucine residue in the hinge region (Hong & Nisonoff, 1965; Smyth & Utsumi, 1967). The 'hinge region' represents the central portion of the H-chain that intervenes between the Fd section (present in the Fab fragment) and the Fc section. The C1-oligosaccharide was present on the Fd section of the H-chain in about 15% of the molecules. The C2-oligosaccharide was present in the hinge region of the H-chain in about 80% of the molecules; in some molecules it appeared to be attached principally to one H-chain and not the other (Fanger & Smyth, 1972). The C3-oligosaccharide was present on the asparagine residue at position 67 from the C-terminus in the Fc section of the H-chain; it appeared to be present in all the IgG molecules.

Table 2. *Amino acid composition of the glycopeptides of rabbit IgG*

The C1-glycopeptide was prepared by Pronase digestion of Fab fragment. A similar analysis was obtained for the C1-glycopeptide liberated by papain digestion of the F(ab')₂ fragment. The three compositions given for the C3-glycopeptide refer to experiments in which the glycopeptide was isolated from type I IgG by elution from DEAE cellulose in 5mM-sodium phosphate buffer, pH7.0, from type II IgG by elution in 0.02M-phosphate buffer, pH7.0, and from anti-Lac antibody respectively. Amino acids present in amounts of less than 0.1 residue are not included. Values in parentheses are based on the following sequences. C2: Ser-Lys-Pro-Thr-Cys-Pro-Pro-Glu-Leu (Smyth & Utsumi, 1967). C3: Gln-Gln-Phe-Asn-Ser-Thr-Ile-Arg (Hill *et al.*, 1967). The amino sugar values were obtained by amino acid analysis.

Glycopeptide ...	Residues per molecule					
	C1	C2*	C2†	C2	C3	C3
Lys	—	1.0 (1)	1.0	—	—	—
His	—	—	—	—	—	—
Arg	—	—	—	0.4 (1)	1.0	0.2
CMCys	—	+ve	+ve	—	—	—
Asp	1.0	—	—	1.0 (1)	1.0	1.1
Thr	0.5	1.0 (1)	1.0	1.1 (1)	1.0	1.1
Ser	0.8	1.0 (1)	0.9	1.3 (1)	0.9	1.2
Glu	0.5	1.1 (1)	1.0	2.1 (2)	2.2	2.0
Pro	—	3.8 (4)	3.2	—	—	—
Gly	0.9	—	—	—	—	0.3
Ala	0.4	—	—	—	—	—
Cys (half)	—	0.4 (1)	0.4	—	—	—
Val	—	—	—	—	—	0.2
Met	—	—	—	—	—	—
Ile	—	—	—	0.4 (1)	0.8	0.7
Leu	—	0.4 (1)	0.4	0.2	—	—
Tyr	—	—	—	1.0 (1)	0.9	0.9
GlcN	2.1	—	—	4.8	4.9	4.9
GalN	—	1.0	1.0	—	—	—

* Peptide showing the more negative charge on electrophoresis.

† Peptide showing the less negative charge on electrophoresis.

separate the carbohydrate-free peptide from small amounts of unchanged C2-glycopeptide (Fig. 4).

The procedure was repeated under different conditions: 48h at room temperature with the inclusion of 0.4M-NaBH₄ in the solution of C2-glycopeptide (0.5μmol) in 0.1M-NaOH. A portion of the reaction mixture was submitted to acid hydrolysis (3M-HCl, 110°C, 3h) and was examined for the presence of galactosamine and galactosaminitol by analysis on the 15cm column of the amino acid analyser with 0.4M-citrate-borate buffer, pH5.28, as eluent (Tanaka & Pigman, 1965; Weber & Winzler, 1968). A second portion of the reaction mixture was subjected to the normal conditions of acid hydrolysis and amino acid analysis.

Results

The constituent subunits and fragments of rabbit IgG, the F(ab')₂, Fab and Fc fragments, the hinge-

region peptides, and L-chain (Fig. 5), were isolated and amino sugar analysis was done. Glucosamine was the principal amino sugar of IgG; galactosamine was also present (Table 1). The majority (94%) of the glucosamine of the IgG molecule was in the Fc fragment. The remainder was present in the F(ab')₂ fragment and was retained in the Fab fragment obtained from the F(ab')₂ fragment under mild conditions of reduction and papain digestion. The L-chain was essentially devoid of amino sugar. The results allow the glucosamine of IgG to be assigned to oligosaccharides present in two different regions of the H-chain, the Fd section and the Fc section.

All the galactosamine of IgG (0.44 residue/half molecule) was present in the F(ab')₂ fragment and almost none in the Fab fragment (0.02 residue). Thus the presence of an oligosaccharide containing galactosamine was inferred in the portion of the H-chain that is present in the F(ab')₂ region and not in the Fab part. Indeed the galactosamine was found in

Table 3. *Carbohydrate composition of the C1-, C2- and C3-glycopeptides of rabbit IgG*

For details of preparation of glycopeptides see Table 2. The values were obtained by g.l.c. (Clamp *et al.*, 1967).

Glycopeptide ...	Residues per molecule				
	C1	C2	C2	C3	C3
Fucose	—	—	—	0.2	0.5
Mannose	2.1	—	—	4.1	4.1
Galactose	1.2	1.1	1.1	1.1	1.8
Glucosamine	2.0	—	—	4.6	5.0
Galactosamine	—	1.1	1.0	—	—
Neuraminic acid	—	1.9	0.8	0.5	0.6

the glycopeptide fraction derived from the hinge region (C2-glycopeptide, Table 2).

The individual glycopeptides that contain the amino sugars were isolated, and their carbohydrate compositions studied by g.l.c. (Clamp *et al.*, 1967). The C1-glycopeptide was isolated from the Fab fragment and contained asparagine, serine, glycine, threonine and glutamic acid (Table 2). More extensive digestion of the peptide with Pronase led to a decrease in the content of serine and glutamic acid. On the basis of the glucosamine content of the F(ab')₂ fragment and the finding that the C1-oligosaccharide contained an average of 2.1 residues of glucosamine/aspartic acid (Table 2), the C1-oligosaccharide was present in 15% of the H-chains. The C2-glycopeptide was isolated from a fraction derived from the hinge region of IgG. It contained 1 residue of galactosamine, 1 of galactose and either 1 or 2 residues of neuraminic acid (Table 3); in the more acidic form, only one of the two *N*-acetylneuraminic acids could be cleaved by the action of neuraminidase. On the basis of the galactosamine contents of IgG and the hinge-region glycopeptide, the C2-oligosaccharide was present in 40% of the H-chains. The C3-oligosaccharide was found in a glycopeptide derived from the Fc fragment of IgG. This peptide has been reported previously (Hill *et al.*, 1967). On the basis of the glucosamine content of IgG (5.3 residues/half molecule) and the finding that the C3-glycopeptide contained an average of 4.9 glucosamine residues/molecule (Table 2), the C3-oligosaccharide appeared in this study to be present on all the H-chains.

The mode of linkage of the C1- and C3-oligosaccharides to their respective peptides was examined by exposing the glycopeptides to hydroxylamine under conditions that might be expected to cause cleavage of linkages of the type in which the hydroxyl group of serine or threonine is involved but not amide-linked oligosaccharides. The cleavage would result in a decrease in the size of the peptide and this would affect the elution position during gel filtration on Sephadex G-25. No change in the elution position

of the C1- or C3-glycopeptide was observed after exposure of the glycopeptides to conditions of hydroxylaminolysis. Additionally the isolated hydroxylamine-treated peptides might be expected to exhibit a loss of glucosamine, if cleavage of the oligosaccharide had taken place. With the C1- and C3-glycopeptides the hydroxylamine treatment resulted in no change in the glucosamine/aspartic acid ratios; the values remained the same as in the initial glycopeptides.

The mode of attachment of the C2-oligosaccharide was investigated by submitting the C2-glycopeptide to conditions that would cause β -elimination of carbohydrate in a glycosidic linkage. After addition of sulphite to the reaction mixture, the resulting peptide was isolated (peak II, Fig. 4). The peptide was almost devoid of threonine (0.08 residue) and contained a substance which was detected with ninhydrin (0.7 residue), assumed to be either β -methylcysteic acid or *C*-sulphonylhexosamine (Weber & Winzler, 1970), which chromatographed in the position of cysteic acid. In all other respects the composition was essentially the same as that of the initial C2-glycopeptide (Table 2).

When the β -elimination was performed in the presence of reducing agent, the resulting peptide possessed an amino acid that chromatographed on the analyser in the position of α -aminobutyric acid; no galactosaminitol was seen. When *N*-acetyl-galactosamine was submitted to these conditions, galactosaminitol was formed in 53% yield.

Discussion

IgG represents a large group of proteins which possess the same overall structure (Fleischman *et al.*, 1963) but which differ individually in the amino acid sequence at certain regions of the molecule. Thus the determination of the exact amino acid or carbohydrate composition of a mixture of IgG molecules may not be significant. Carbohydrate composition is susceptible to an additional source of

variation. Heterogeneity has been seen in an oligosaccharide present in an IgA myeloma protein that has a constant amino acid sequence (Clamp *et al.*, 1968) and this form of heterogeneity may well exist in the mixture of IgG molecules from pooled sera. Further, the determination of monosaccharides is more difficult than the determination of amino acids, partly because of their ease of destruction during hydrolysis (Neuberger & Marshall, 1966). Nevertheless, the results obtained in the present study reveal unequivocally that three different oligosaccharides can be present at separate sites on the H-chain of rabbit IgG.

The exact position of attachment of the C1-oligosaccharide in the Fd section of the H-chain cannot yet be assigned because of the variations in the sequence of this region (Cebra *et al.*, 1968; Mole *et al.*, 1971); however, it was present in a peptide containing asparagine, glycine and threonine. The C2-oligosaccharide was attached to the threonine residue adjacent to the inter H-chain disulphide bridge in the hinge region. The C3-oligosaccharide was attached to an asparagine residue in the Fc section of the H-chain (position 67 commencing from the C-terminus according to Hill *et al.*, 1967).

The carbohydrate compositions of the three oligosaccharides given in Table 3 represent the average of the different forms of the respective oligosaccharides in the mixture of pooled IgG molecules, since the isolation procedures may not have resolved closely related glycopeptides. Even so, the composition of each oligosaccharide was characteristic. Moreover, the C2-oligosaccharide differed from the C1- and C3-oligosaccharides in its mode of linkage to the polypeptide chain. The attachment of the C2-oligosaccharide was through the OH group of threonine whereas the attachment of both the C3-oligosaccharide (Rosevear & Smith, 1961) and the C1-oligosaccharide was through the amide group of asparagine. It seems likely that the C2-oligosaccharide was coupled through *N*-acetylgalactosamine substituted in the 3-position. If so, β -elimination of the C2-glycopeptide in the presence of sulphite could give rise to the 3-C-sulphonylhexosamine (Weber & Winzler, 1970). On reduction, no galactosominitol would be expected and none was observed.

That the oligosaccharides of rabbit IgG are attached only at certain sites in the molecule and that two of the oligosaccharides are present in some molecules and not in others invites interesting questions on the biological functions associated with these structures.

We are indebted to Dr. J. R. Clamp (Department of Medicine, University of Bristol) for the analysis of neutral carbohydrate and neuraminic acid by g.l.c. and we thank

Dr. S. Utsumi for a gift of anti-Lac (anti- β -galactosidase) IgG. M. W. F. was in receipt of a Research Fellowship Award from Department of Health, Education and Welfare. The excellent technical assistance of Mr. D. E. Massey is gratefully acknowledged.

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