The Oligosaccharide Units of Rabbit Immunoglobulin G

ASYMMETRIC ATTACHMENT OF C2-OLIGOSACCHARIDE

By M. W. FANGER and D. G. SMYTH

National Institute for Medical Research, Mill Hill, London N.W.7, U.K.

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Two populations of immunoglobulin G (IgG) molecules were isolated from pooled rabbit serum. The first was almost devoid of galactosamine and was obtained in a yield of 7% of the total IgG; the second contained on average a single residue of galactosamine per molecule and was obtained in a yield of 30% of the total IgG. The galactosamine, which is present solely in the C2-oligosaccharide, appeared to be present on one H-chain and not the other in the four-chain structure. Evidence was obtained by the isolation of a glycopeptide linked through a disulphide bridge to a second peptide of the same sequence; the oligosaccharide attached to the first peptide was absent from the complementary peptide. Further evidence was obtained by degradation and analysis of the 5S IgG fragment, which comprises an intact half-molecule coupled through a disulphide bridge to the Fc fragment derived from the opposing half molecule (Goodman, 1965); only the intact H-chain carried the C2-oligosaccharide.

With the finding that a preparation of rabbit IgG* contained 0.4 residue of galactosamine per γ -chain (H-chain) and that all the galactosamine was present in the C2-oligosaccharide (Smyth & Utsumi, 1967), it became clear that some IgG molecules possess the C2-oligosaccharide whereas others lack it. This is consistent with reports that rabbit IgG exhibits heterogeneity with respect to its carbohydrate composition (Blacklow & Marshall, 1968). We have extended Blacklow & Marshall's (1968) study and report here a separation of rabbit IgG into two populations of molecules. 'Type I' was almost devoid of galactosamine; 'type II' contained on average a single residue of galactosamine per molecule. In the type II molecules, it was not clear whether the C2-oligosaccharide was attached to both H-chains or to one H-chain only in the four-chain structure. The results presented here support the view that the C2-oligosaccharide is attached asymmetrically.

Materials and Methods

Enzymes

Pepsin was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Papain and Pronase were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

Gel filtration and ion exchangers

The various grades of gels and the cation exchanger Sephadex C-25 were obtained from Pharmacia,

* Abbreviation: IgG, immunoglobulin G.

Uppsala, Sweden. Dowex 2 (X4) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and Amberlite IR-120 from Evans Electrochemical Ltd., Harlow, Essex, U.K. Whatman DEAE- and CM-cellulose were purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K.

All chemicals used were of AnalaR grade obtained from British Drug Houses Ltd., unless otherwise stated. *N*-Ethylmorpholine, α -D-glucosamine hydrochloride, D-galactosamine hydrochloride and DL-lysine were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; iodo[14 C]acetic acid and sodium [14 C]cyanate were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Descending paper chromatography

Purification of glycopeptides was done on Whatman no. 1 paper. Elution was performed initially with the solvent mixture (propan-1-ol-ethanol-water-acetic acid, 7:20:7:1, by vol.) of Kamiyama & Schmidt (1962). The contaminating peptides were eluted in the organic solvent whereas the glycopeptides remained on the paper. The paper was dried in a stream of air and the glycopeptides were recovered in approx. 85% yield by elution with water.

Analysis of amino sugar content of IgG and its fragments

A method was employed for the determination of glucosamine and galactosamine at the level of 5 nmol (Fanger & Smyth, 1970). As decomposition of carbohydrate occurs under the normal conditions of acid

hydrolysis employed for amino acid analysis (Johansen *et al.*, 1960; discussed by Neuberger & Marshall, 1966), protein samples were hydrolysed under mild conditions in 3M-HCl at 110°C for a series of time-intervals and the amount of amino sugar remaining was determined on the amino acid analyser. The amount initially present in the protein was estimated by extrapolation from the values obtained. Additionally, it was found that some of the products of partial hydrolysis of a protein may interfere with the chromatography of galactosamine. The hydrolysates were therefore passed through a column of Dowex 2 anion-exchange resin before the final analysis on the amino acid analyser.

Glucosamine and galactosamine were not retained on the IR-120 column of the analyser at pH2.2, and their elution was a function of the ionic strength and not the pH of the eluate. An improved resolution of glucosamine from galactosamine was achieved by application of the hydrolysates to the column in 0.05M-sodium citrate at pH2.2 instead of the 0.2M-sodium citrate normally employed in amino acid analysis. Glucosamine and galactosamine were determined on the analyser with the aid of a high-sensitivity flow cell, and lysine in the same analysis was determined with a standard-sensitivity flow cell. Amino sugar content was calculated relative to lysine by assuming 63 lysine residues/molecule of IgG (Fleischman *et al.*, 1963) and 17 lysine residues/univalent Fab' fragment (Mage & Harrison, 1966).

The measurement of amino sugar values in a glycopeptide is considered more precise than the measurement of amino sugar in a glycoprotein. The rate of decomposition during hydrolysis is less susceptible to variation. The values obtained for the fragments of IgG, however, were found to agree adequately with the measured amino sugar values obtained on the whole molecule.

Preparative methods

Rabbit IgG. This was isolated from pooled serum (500ml) derived from Californian and New Zealand strain rabbits (White Cloud Farm, Tring, Herts., U.K.). The crude IgG was precipitated three times in 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and after dialysis against 0.02M-sodium phosphate buffer, pH7, was added to and eluted from a DEAE-cellulose column (30cm $\times$ 3.5cm) equilibrated with the 0.02M-phosphate (Peterson & Sober, 1956). The combined IgG fractions were dialysed overnight at 4°C against 5mM-sodium phosphate buffer, pH7. The IgG solution, clarified by centrifugation, was used for subfractionation into type I and type II IgG or was stored at -20°C.

Isolation of type I IgG and type II IgG. The solution of IgG (200mg) was applied to a column (30cm $\times$ 3.5cm) of DEAE-cellulose washed with 1M-sodium phosphate buffer, pH7, and equilibrated in 5mM-

sodium phosphate at the same pH. Elution was done at room temperature with the 5mM-phosphate buffer and at 210ml the eluent was changed to 0.03M-sodium phosphate. The elution pattern is shown in Fig. 1.

The two fractions (A and B) were concentrated by vacuum dialysis and the buffer was changed to 5mM-sodium phosphate (pH 6.0) by gel filtration on a column (20cm $\times$ 2cm) of Sephadex G-25. The first fraction, containing 48mg of IgG, was subjected to chromatography on a column (40cm $\times$ 2.8cm) of CM-cellulose by using a linear concentration and pH gradient from 0.01M-sodium phosphate (pH6) to 0.5M-sodium phosphate at pH8, with a mixing vessel of 250ml capacity; the main fraction emerged between 320ml and 420ml of effluent (Fig. 2) and contained 23mg of IgG. The second fraction from the DEAE-cellulose column, containing 130mg of IgG, was rechromatographed on a column (30cm $\times$ 2.5cm) of CM-cellulose with a gradient of increasing ionic strength from 0.01M- to 0.5M-sodium phosphate at pH6.0, again with the 250ml mixer; the main fraction emerged between 510ml and 625ml and contained 72mg of IgG. These fractions had amino sugar compositions almost identical with the respective precursor fractions from DEAE-cellulose. The two protein fractions obtained from CM-cellulose were finally submitted to gel filtration on a

Fig. 1. Fractionation of rabbit IgG by chromatography on DEAE-cellulose

Rabbit IgG (200mg) obtained by chromatography on DEAE-cellulose in 0.02M-sodium phosphate (Peterson & Sober, 1956) was subdivided into two fractions by chromatography with 5mM-sodium phosphate (pH7) as eluent and then with 0.03M-sodium phosphate. The arrows in the Figure indicate the points at which 0.03M- and 0.5M-phosphate eluents were applied. Fraction C represents the small amount of IgG that remained on the resin in 0.03M-phosphate; fractions A and B were purified further.

Fig. 2. Chromatography of IgG fraction A on CM-cellulose

Rabbit IgG (48 mg) eluted from DEAE-cellulose in 5 mM-sodium phosphate, pH 7, was chromatographed on CM-cellulose as described in the text. The ionic strength of the eluent was determined at intervals by measurement of conductivity. ●, E_{280} ; ○, ionic strength. The fraction comprising type I IgG is indicated.

column (150 cm × 2.5 cm) of Sephadex G-200 in 0.02 M-sodium phosphate (pH 7) and the 7S components (termed 'type I' IgG and 'type II' IgG respectively) were analysed for amino sugar content (Table 1). The final yield of type I IgG was 7% and of type II IgG was 30% of the total IgG. The calculation of yield was based on measurements of E_{280} , assuming a value of 1.4 for a 1 mg/ml solution of IgG.

Isolation of the $F(ab')_2$ fragments from type I IgG and type II IgG. The IgG (30 mg) was digested for 24 h with 2% (w/w) pepsin at pH 4.4 and 37°C (Mandy *et al.*, 1961) and the respective $F(ab')_2$ fragments were separated from residual IgG and from small peptides on a column (150 cm × 2.5 cm) of Sephadex G-100 with 0.02 M- NH_4HCO_3 as eluent (Utsumi & Karusch, 1965). To ensure removal of the small amounts of Fc' fragment, the $F(ab')_2$ fragment preparations were passed through a column (30 cm × 0.9 cm) of Sephadex C-25 eluted with 0.02 M-sodium acetate, pH 5.5, as described by Mage & Harrison (1966). The purified $F(ab')_2$ fragments, derived from type I IgG and type II IgG, were analysed for amino sugar content (Table 1). The overall procedure ensured that the glucosamine in each of the final $F(ab')_2$ fragment preparations was an intrinsic component of the respective $F(ab')_2$ fragment and was not due to contamination by the glycopeptide that carries the C3-oligosaccharide, derived from the Fc region of the molecule. The yield of type I $F(ab')_2$ fragment was 45% and of type II $F(ab')_2$ fragment was 70% of the initial IgG, based on the measurement of E_{280} values.

Isolation of the 'Goodman' 5S intermediate formed during limited digestion of IgG by papain. IgG (508 mg) was dissolved in 0.2 M-sodium phosphate buffer (6.6 ml), pH 7, and EDTA (5.3 mg) was added.

Papain (5 mg), first reduced in 1.4 mM-mercaptoethanol for 1 h at pH 8 and desalted on Sephadex G-25 in 0.02 M-acetic acid, was added to the IgG solution and incubation was done for 30 min at pH 7. The reaction mixture was cooled to 4°C and applied to a column (150 cm × 2 cm) of Sephadex G-150 with 0.02 M-sodium phosphate buffer, pH 7, as eluent. The principal component obtained was a 7S protein, which was concentrated by vacuum dialysis, and then reduced in 3 mM-mercaptoethanol at pH 8 and 30°C for 1 h and alkylated at pH 8 for 3 h in 3 mM-iodoacetic acid containing 10 μ Ci of iodo[14 C]acetate. The reaction mixture was fractionated on the Sephadex G-150 column. The yield of 5S component represented 25% of the total IgG used, based on E_{280} values. The measured sedimentation coefficient of the final preparation in 0.15 M-NaCl at 20°C was 5.2S.

Separation of IgG half molecules and Fc fragment derived from the 'Goodman' 5S molecule. The radioactively labelled 5S-component (90 mg) was dialysed against 0.025 M-NaCl acidified to pH 2.4 with HCl and was added to a column (150 cm × 2.4 cm) of Sephadex G-100 eluted with the acid NaCl solution (Fig. 3). The first fraction from the Sephadex G-100 column appeared to be a mixture of half molecules and aggregates, as its amino acid composition was the same as that of the initial IgG. This fraction was concentrated by vacuum dialysis and was dialysed against 0.1 M- $NaHCO_3$, pH 9.5. The solution was clarified by centrifugation and submitted to gel filtration on a column (150 cm × 2 cm) of Sephadex G-100 at pH 9.5. The first material emerging from this column had a sedimentation coefficient of 7.2S and appeared to contain whole IgG molecules formed by reassociation of half molecules.

The second fraction from the gel filtration at acid pH was concentrated and again submitted to gel

filtration at pH 2.4. The protein emerged as a single symmetrical peak and its identity with fragment Fc was confirmed by amino acid analysis.

Amino sugar analysis was done on the 5S molecule obtained by limited papain digestion of IgG, on the Fc fragment dissociated from the 5S molecule, on

the half-IgG molecule derived from the 5S molecule, and on the 7S molecule formed by reassociation of the half molecules (Table 2).

Isolation of a 'hinge' peptide containing the H-H-chain disulphide bridge and the C2-oligosaccharide.

Table 1. Amino sugar content of IgG fractions separated by chromatography on DEAE- and CM-cellulose and of the $F(ab')_2$ fragments obtained from these fractions

The majority of the glucosamine in IgG is derived from the C3-oligosaccharide in the Fc region of the molecule. The glucosamine in the $F(ab')_2$ fragment of type II IgG is derived from the C1-oligosaccharide, which contains on average 2.1 residues of glucosamine/carbohydrate chain and is present on approx. 15% of the H-chains in pooled IgG; the galactosamine in the $F(ab')_2$ fragment of type II IgG is derived from the C2-oligosaccharide, which contains 1 residue of galactosamine/carbohydrate chain and is present on approx. 50% of the H-chains (Fanger & Smyth, 1972).

	Residues per half-molecule	
	Glucosamine	Galactosamine
Type I IgG	5.2	0.07
Type II IgG	5.3	0.52
$F(ab')_2$ I	0.08	0.06
$F(ab')_2$ II	0.34	0.46

Type II IgG (230 mg) was digested with pepsin (12 mg) in 0.01 M-HCl (5 ml) at pH 2.0, 37°C for 60 h; the pH of the solution was readjusted at intervals during the digestion. The solution was neutralized, then was centrifuged to remove a small amount of insoluble material and was submitted to gel filtration on a column (150 cm $\times$ 2 cm) of Sephadex G-75 with 0.02 M-NH₄HCO₃ as eluent (Fig. 4). The second peak of eluted material, which contained substantially all the galactosamine of the initial IgG, was concentrated *in vacuo* and then was desalted on Sephadex G-25. After adjustment of the pH of the solution to 3.0 with 1 M-HCl, the peptide mixture was applied to an Amberlite IR-120 column (8 cm $\times$ 0.9 cm) equilibrated with 0.2 M-sodium citrate buffer, pH 3.25. The galactosamine-containing material remained on the column at pH 3.25 and was eluted in 0.4 M-sodium citrate buffer, pH 5.28. After adjustment of the salt concentration to 0.02 M-sodium phosphate by gel filtration at pH 7, the glycopeptide (2 ml) was digested with Pronase (2.1 mg) for 24 h at pH 7, 37°C. The digest was added to a column (150 cm $\times$ 2.5 cm) of Sephadex G-50 operated with 0.02 M-NH₄HCO₃ as eluent. The glycopeptide, located by measurement of E_{215} and by amino sugar analysis of combined frac-

Fig. 3. Dissociation of the 5S molecule obtained by limited papain digestion of IgG

Reduced [¹⁴C]carboxymethylated 5S IgG fragment (90 mg) was allowed to disaggregate in 0.025 M-NaCl, pH 2.4, and the products were separated by gel filtration on Sephadex G-100, as described in the text. ○, Radioactivity (c.p.m.); ●, E_{280} .

Table 2. Amino sugar content of the Goodman 5S component and of its subunits

For experimental details see the text.

	Residues per molecule	
	Glucosamine	Galactosamine
5S component	10.2	1.1
Fc fragment (Fig. 3, second peak)	4.8	0.2
Half-IgG molecule (Fig. 3, first peak)	5.1	1.1
Reconstituted 7S molecule	10.3	2.3

tions, emerged at 285ml. Final purification was performed by descending paper chromatography, as described in the Materials and Methods section. In a second preparation, the hinge peptide was obtained from fragment F(ab')₂. The results and conclusions were analogous to those obtained in the preparation from IgG.

Labelling of the disulphide-containing hinge peptide and separation of the reduced peptides. The galactosamine-containing hinge peptide (0.4 mg) was labelled by reaction with 20 μ Ci of sodium[¹⁴C]cyanate (0.01 M) at pH 7.0, 37°C for 2 h. The reaction mixture was added to a column (150 cm $\times$ 2.5 cm) of Sephadex G-25 with 0.02 M-NH₄HCO₃ as eluent. The glycopeptide was excluded; a portion was hydrolysed and analysed and the remainder was reduced at 30°C in 0.14 M-mercaptoethanol at pH 8 for 1 h and then was alkylated in 0.14 M-iodoacetic acid for 1 h. The reaction mixture was fractionated on the same Sephadex G-25 column (Fig. 5). The compositions of the disulphide peptide and the two peptides released from it by reduction are given in Table 3. The two peptides contained 80% of the radioactivity of the initial hinge peptide.

The hinge peptide was also reduced in 0.01 M-mercaptoethanol and the released SH groups were labelled with 0.01 M-iodoacetate containing 10 μ Ci of ¹⁴C. A similar pattern was obtained to that shown in Fig. 5.

Results and Discussion

In rabbit IgG galactosamine occurs solely in the C2-oligosaccharide, which is attached through a threonine residue in the 'hinge region' of the molecule (Smyth & Utsumi, 1967). Hence amino sugar analysis provides an index of the presence or absence of the C2-oligosaccharide in IgG and its fragments. On this basis, two populations of IgG molecules were recognized when pooled rabbit IgG was submitted to chromatography on DEAE-cellulose and CM-cellulose. Type I IgG was almost devoid of galactosamine whereas type II IgG contained on average a single residue of galactosamine/molecule (Table 1). A re-

Fig. 4. Gel filtration of IgG fragments liberated by pepsin at pH 2.0

Rabbit IgG was digested with 5% (w/v) pepsin in 0.01 M-HCl, pH 2.0, for 60 h and the products were fractionated by gel filtration on Sephadex G-75, as described in the text. The galactosamine-containing fragments of the molecule were present in the fraction (indicated by the vertical dashed lines) emerging between 190 ml and 250 ml of effluent.

port that the A11 allotypic variant of rabbit IgG possesses methionine instead of threonine in the hinge region (Prah et al, 1970) is consistent with the finding that some IgG molecules lack the C2-oligosaccharide whereas others carry it. The side chain of methionine, of course, cannot participate in a glycosidic linkage.

Type I IgG and type II IgG both contained approx. 10 residues of glucosamine per molecule (Table 1), almost all of which is accounted for in the C3-carbohydrate attached to the Fc section of the H-chain (Porter, 1959). Since the C3-oligosaccharide has been reported to contain an average of five residues of glucosamine (Fanger & Smyth, 1972) and

Fig. 5. Gel filtration of the C2-oligosaccharide-containing H-H-linked hinge peptide and the peptides released from it on reduction

The reduction and alkylation procedures applied to the disulphide peptide are described in the text. Samples of the effluent fractions (4ml) were counted for radioactivity on a Nuclear-Chicago flow-type counter. ●, Elution pattern exhibited by the ^{14}C -labelled disulphide peptide on Sephadex G-25; ○, elution pattern of the ^{14}C -labelled reduction products.

both type I and type II IgG contain approx. five residues of glucosamine/half-molecule (Table 1), it is concluded that the C3-oligosaccharide is present on essentially all the H-chains in the molecules that we have examined.

It was decided to investigate the disposition of the galactosamine in the type II IgG molecule. It was already known that the glycopeptide that carries the C2-oligosaccharide (Fig. 6) is resistant to digestion by papain, whereas the corresponding peptide lacking the C2 oligosaccharide is readily cleaved between the threonine and cystine residues (Smyth & Utsumi, 1967). The hinge peptide forms a part of the relatively

small region of the IgG molecule that is susceptible to attack by papain (Givol, 1968), so it was plausible that the same pattern of papain cleavage might exist in the intact IgG molecule. Indeed, a limited digestion of IgG with papain gives rise to a 5S component comprising an intact 'upper' half molecule coupled to the Fc fragment only of the 'lower' half molecule (Goodman, 1965). That the asymmetric 5S molecule is formed at all implies that one H-chain is more resistant than the other to digestion by papain. The difference might be related to the presence of the C2-oligosaccharide on one chain and not the other. To investigate this possibility we isolated the 5S molecule and examined its component parts.

The complete 5S molecule contained 1.1 residues of galactosamine and approx. 10 residues of glucosamine (Table 2), a result consistent with the possibility that the intact H-chain carries the C2-oligosaccharide whereas the Fc section of the complementary H-chain does not. The 'upper' half molecule of the 5S component, which contains the intact H-chain, showed on analysis 1.1 residues of galactosamine whereas the Fc section of the H-chain from the 'lower' half molecule showed less than 0.2 residue of galactosamine.

The Fc fragment of IgG released under mild conditions of papain digestion includes the cysteine of the H-H disulphide bridge (Delaney & Hill, 1968; Hong & Nisonoff, 1965) and it would be expected also to include the C2-oligosaccharide, since papain is unable to cleave between the cysteine and the adjacent glyothreonine residue (Smyth & Utsumi, 1967). The finding that very little galactosamine was in the Fc chain isolated from the 5S molecule suggests that this Fc chain must have originated principally from an H-chain lacking the C2-oligosaccharide. It appears therefore that in releasing the 5S fragment from 7S IgG papain cleaves an H-chain lacking the C2-oligosaccharide more readily than it cleaves an H-chain bearing the C2-oligosaccharide. These results support the hypothesis that the 5S component is derived from an IgG molecule that is asymmetric with respect to the C2-oligosaccharide attachment. It must be noted that the 5S fragment was isolated in only 25% yield and the conclusions based on its analysis are relevant to this proportion of molecules; however, milder conditions of papain digestion have been reported to give a 60% yield of the 5S intermediate (Nelson, 1964).

After prolonged digestion of IgG by papain the Fab and Fc fragments are liberated in high yield (Porter, 1959), which shows that H-chains carrying the C2-oligosaccharide do eventually undergo cleavage. Thus the influence of the C2-oligosaccharide on the papain digestion appears to reside in its ability to confer a degree of stability on the H-chain that carries it. It may be relevant that a classification of rabbit IgG molecules based on their sensitivity to

Table 3. *Amino acid composition of the C2-oligosaccharide-containing hinge peptide and the peptides released from it on reduction*

The recovery of carboxymethylcysteine (CMCys) was variable, particularly in hydrolysates of peptide containing carbohydrate. The numbers in brackets represent the expected number of residues of each amino acid. On the basis of the amount of the hinge peptide submitted to reduction, the yield of the constituent peptides was 95 and 65 % respectively.

	Composition of hinge peptide (residues/molecule)		
	Before reduction	After reduction	
		1st peptide	2nd peptide
Thr	2.1 (2)	1.1 (1)	1.0 (1)
Ser	2.0 (2)	1.1 (1)	1.1 (1)
Glu	2.0 (2)	1.1 (1)	1.1 (1)
Pro	7.3 (8)	3.6 (4)	3.1 (4)
Gly	0.2	0.2	
Ala	0.1	0.1	
GalN	1.1 (1)	1.0 (1)	
Lys	1.8 (2)	0.9 (1)	0.9 (1)
CMCys	+ve	+ve	+ve

Fig. 6. *Amino acid sequence of the C2-glycopeptide*

digestion by papain has been proposed (Jefferis & Stanworth, 1967).

More definite evidence was sought to demonstrate an asymmetric attachment of the C2-oligosaccharide and this was obtained by isolation of a C2-containing fragment of IgG located at the C-terminus of fragment F(ab')₂. To obtain this peptide with an intact disulphide bridge (Fig. 8), conditions were selected which minimized disulphide exchange. IgG, and in other experiments fragment F(ab')₂, was exhaustively digested with pepsin at low pH values and after extensive purification a disulphide-containing peptide was obtained, which possessed galactosamine and no glucosamine (Table 3); the overall yield was 14%. The composition of the disulphide peptide allowed its identification within the structure of the hinge region (Smyth & Utsumi, 1967; Cebra *et al.*, 1968). Reduction of the peptide led to the isolation of two components (Fig. 5) that had the same amino acid composition. The larger peptide contained one residue of galactosamine whereas the smaller was devoid of galactosamine. The larger peptide was recovered quantitatively from the disulphide peptide; the smaller peptide was obtained in 65% yield.

The two peptides appear to be derived from the

complementary H-chains of the four-chain IgG molecule because they were initially linked through a disulphide bridge in the precursor peptide. Moreover, it is this disulphide that is selectively reduced when half-molecules are formed from IgG (Hong & Nisonoff, 1965; Smyth & Utsumi, 1967). On this evidence the cystine residue in the sequence Cys-Pro-Pro-Pro-Glu can be identified as the one that forms the H-H disulphide bridge. The finding that the C2-carbohydrate was present on one half and absent from the complementary half of the disulphide-linked hinge peptide is good evidence in support of the view that in some molecules the C2-oligosaccharide is attached asymmetrically.

It should be emphasized that the overall yield of the disulphide-containing glycopeptide was less than 15% of the total IgG and the conclusions drawn about the asymmetric attachment of carbohydrate must be limited to this proportion of the total IgG. It remains to be seen whether all the C2-oligosaccharides are attached in this manner or whether the type II IgG mixture contains molecules that totally lack the C2-oligosaccharide (type I) as well as others with the C2-oligosaccharide attached to both H-chains. The present experiments gave no evidence of the latter form.

The finding of an asymmetric structure in a multi-chain molecule raises questions about biosynthesis and function. In the present case, it may be that during biosynthesis the enzymic attachment of the C2-oligosaccharide to the second H-chain is prevented sterically by the presence of C2-oligosaccharide on

Fig. 7. Relationship between the various subunits and fragments of rabbit IgG

The 7S molecule comprises two H-chains and two L-chains. The Fab fragment contains one L-chain and an H-chain from the blocked *N*-terminus up to the sites of papain cleavage; the Fc fragment contains the remaining portion of the H-chain. The F(ab')₂ fragment contains two L-chains and the portion of two H-chains from the blocked *N*-terminus up to the sites of pepsin cleavage. The Goodman 5S molecule is enclosed within the dashed lines.

Fig. 8. Primary structure of the hinge peptide with asymmetric attachment of the C2-oligosaccharide

the first, particularly in view of the restraints imposed by the neighbouring inter-chain disulphide bridge. It would be of interest, however, if this unusual structure should prove critical to the expression of a definite biological activity such as cellular attachment, antigenicity or antibody affinity.

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