Biochem. J. (1968) **110**, 385
Printed in Great Britain

Studies on the Glycopeptides Isolated from the Urinary Protein in Heavy-Chain Disease

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(*Received* 12 *June* 1968)

The urinary protein excreted in heavy-chain disease was separated by ion-exchange chromatography into two broad fractions designated Cra-1 and Cra-2. For a dimeric molecular weight of approx. 51000, Cra-1 contained 3–4 residues of 6-deoxy-L-galactose (L-fucose), 10 of D-mannose, 5–6 of D-galactose, 12 of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) and 4–5 of *N*-acetylneuraminic acid (sialic acid), whereas the corresponding values for Cra-2 were 2, 10, 7, 12 and 7. Cra-2 contained in addition 1 residue of 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine). Cra-1 contained an average of four oligosaccharide units, two of which contained 1 residue of 6-deoxy-L-galactose, 3 of D-mannose, 1 of D-galactose and 3 of 2-acetamido-2-deoxy-D-glucose, whereas the other two units contained the same proportions of 6-deoxy-L-galactose, D-mannose and 2-acetamido-2-deoxy-D-glucose but 2 residues of D-galactose and 2 of *N*-acetylneuraminic acid. Cra-2 also contained an average of four oligosaccharide units, but the range of glycopeptides was much wider, containing 0–1 residue of 6-deoxy-L-galactose, 2–3 of D-mannose, 2–3 of D-galactose, 2–3 of 2-acetamido-2-deoxy-D-glucose and 1–3 of *N*-acetylneuraminic acid. Possible reasons for this heterogeneity are discussed. Glycopeptides were also isolated from Cra-2 that contained 1 residue of D-mannose, 2 of D-galactose, 1 of 2-acetamido-2-deoxy-D-galactose and 0–3 of *N*-acetylneuraminic acid.

Human immunoglobulins have been shown (Edelman & Poulik, 1961) to consist of two types of polypeptide chain, namely light chain, which is common to all classes of immunoglobulins, and the class-specific heavy polypeptide chain (Chaplin, Cohen & Press, 1965). Human IgG* contains (Heremans, 1959; Rosevear & Smith, 1961; Clamp & Putnam, 1964) approx. 3% of carbohydrate, which appears to be associated with the *C*-terminal portion (Fc fragment) of the heavy chain (Franklin, 1960; Chaplin *et al.* 1965; Hill, Delaney, Fellows & Lebowitz, 1966). Bence-Jones proteins, which are analogous to light chains, are often found in excessive amounts in the urine of patients with malignancies of the plasma cell. The corresponding situation in which excessive amounts of heavy chain or fragments therefrom appear in the urine in disease is much less common and was only reported recently (Franklin, Meltzer, Guggenheim & Lowenstein, 1963; Franklin, Lowenstein, Bigelow & Meltzer, 1964; Franklin, 1964). Ultracentrifugal analysis of heavy-chain fragment Cra (Franklin, 1964) indicated a dimeric molecular weight of 51000. This fragment has been shown to be antigenically and structurally related to the Fc fragment of IgG, and contains the antigenic determinants associated with the γG1 subclass (Bernier, Ballieux, Tominaga & Putnam, 1967). Cra heavy-chain fragment has been reported (Franklin, 1964) to contain 21% of carbohydrate, which is a much greater proportion than that found in the Fc fragments isolated from IgG (Clamp & Putnam, 1964), though IgG subclasses may vary in their carbohydrate content (P. Weston & J. R. Clamp, unpublished work).

The isolated glycopeptides were investigated to determine the number and composition of the oligosaccharide units in Cra protein. In addition, as the abnormal cells in this disease appear to show a disorder of protein biosynthesis in that only a fragment of the carbohydrate-containing polypeptide chain is excreted, the glycopeptides were investigated for features that might indicate a corresponding disorganization of biosynthesis of the oligosaccharide units.

*Abbreviations: IgG and IgA, immunoglobulins G and A respectively.

MATERIALS

Pronase (B grade) was obtained from Calbiochem Ltd. (London, W. 1). All other reagents and chemicals were AnalaR grade where available and were supplied by

British Drug Houses Ltd., Poole, Dorset. Sephadex resins were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden, and Whatman DEAE-cellulose by H. Reeve Angel and Co. Ltd., London, E.C. 4.

METHODS

Preparation of pure heavy-chain fragment (Cra). The 1·8M-$(NH_4)_2SO_4$ precipitate (5g.) obtained from the urine of patient Cra (Franklin, 1964) was dissolved in and dialysed against 0·02M-tris–HCl buffer, pH 7·4, for 48hr. at 4°. The solution was then applied to a column (41cm.× 4·3cm. diam.) of DEAE-cellulose (Whatman DE32) and a linear gradient to the limiting buffer, 0·02M-tris–HCl–0·5M-NaCl, pH 7·4, was started. The effluent was monitored at 280mμ and the peak containing Cra protein (Fig. 1) was identified by immunoelectrophoresis and divided into two fractions (Cra-1 and Cra-2), which were dialysed against water and freeze-dried. The purity of the proteins was checked by starch-gel electrophoresis and immunoelectrophoresis (Dawson & Clamp, 1968).

Preparation and isolation of glycopeptide material. The protein fractions Cra-1 (1·4g.) and Cra-2 (0·9g.) were separately digested with Pronase (25mg.) in 0·02M-$CaCl_2$, pH 8·0 (50ml.). The pH was maintained at 8·0 with 0·02N-NaOH, and after 24hr. a second addition of Pronase (25mg.) was made. The digestion was continued for a further 48hr. and the mixture was then centrifuged and the supernatant freeze-dried. The dried material was extracted with water (10ml.) and applied to a column (140cm.× 3·2cm. diam.) of Sephadex G-25 (fine grade). Fractions (5ml.) were collected and the total hexose content of a sample (0·1ml.) from each tube was determined by the method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). The hexose-containing fractions were pooled, freeze-dried and subjected to further incubation with Pronase for 24hr. as described above. The digest was concentrated by freeze-drying and exclusion chromatography was then performed on a column (140cm.× 2·2cm. diam.) of Sephadex G-25 (fine grade). The effluent was monitored for hexose as before and the fractions from Cra-1 were pooled as shown in Fig. 2, and those from Cra-2 were pooled as shown in Fig. 3. A two-dimensional 'map' (Clamp & Putnam, 1964) of the Sephadex fractions showed the presence of small amounts of contaminating peptides. A final purification of the glycopeptide material was therefore carried out on pre-washed Whatman 3MM filter paper (57cm.× 46cm.; 25mg. of glycopeptide/chromatogram) by descending chromatography for 48hr. in butan-1-ol–acetic acid–water (12:3:5, by vol.), followed, after drying, by elution of the base-line material with water (Dawson & Clamp, 1968). The glycopeptides were then isolated according to their electrophoretic mobilities: in this procedure, the glycopeptide material was applied to pre-washed Whatman 3MM filter paper (57cm.× 46cm.; 25mg. of glycopeptide/electrophoretogram) and subjected to electrophoresis for 3hr. at 2kv and 100mA in pyridine–acetic acid–water (1:10:289, by vol.), pH 3·6. The areas corresponding to ninhydrin-positive spots were eluted from the paper with water, pooled with corresponding material from duplicated or triplicated electrophoretic runs, and freeze-dried. The purity of the material was checked by electrophoresis.

Detection and determination of monosaccharides. Monosaccharides were determined by gas–liquid chromatography as the trimethylsilyl ethers of their methyl glycosides,

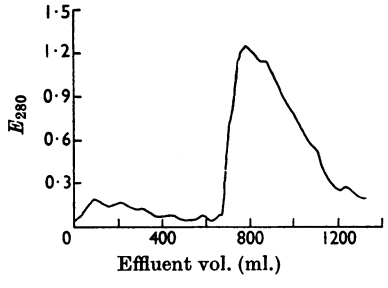


Fig. 1. Elution diagram of Cra protein on a column of DEAE-cellulose (Whatman DE32) equilibrated with the starting buffer, 0·02M-tris–HCl buffer, pH 7·4. After 500ml. had been collected a linear gradient to the limiting buffer, 0·02M-tris–HCl–0·5M-NaCl, pH 7·4, was started. The material eluted between 650ml. and 850ml. was pooled and designated Cra-1, and that eluted between 850 and 1200ml. was pooled and designated Cra-2.

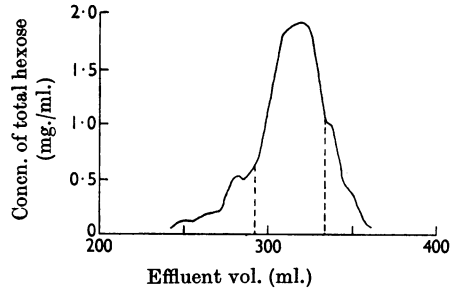


Fig. 2. Chromatography on a column of Sephadex G-25 of a Pronase digest of Cra-1. Samples from each tube were monitored for hexose content by the method of Dubois *et al.* (1956). Three pools were made, as shown by the broken lines.

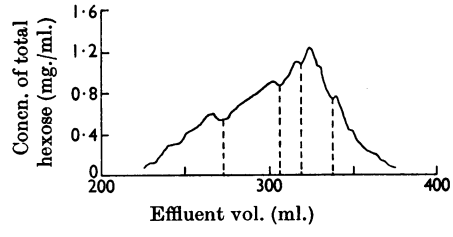


Fig. 3. Chromatography on a column of Sephadex G-25 of a Pronase digest of Cra-2. Samples from each tube were monitored for hexose content by the method of Dubois *et al.* (1956). Five pools were made, as shown by the broken lines.

with D-mannitol as internal standard (Clamp, Dawson & Hough, 1967).

Periodate oxidation procedure. Glycopeptide material (0·4 μmole) was dissolved in 15mM-$NaIO_4$ (1·0ml.) and kept in the dark at 20°. Samples (0·05ml.) were removed at intervals and diluted to 10ml., and their extinction was read at 223mμ (Dixon & Lipkin, 1954). The standard curve was constructed by plotting the E_{223} of a solution of $NaIO_3$ and $NaIO_4$ against the molarity of $NaIO_4$. When the reduction of periodate had ceased, $NaBH_4$ (1·0mg.) was added to reduce both the resultant aldehyde groups and the excess of periodate (J. R. Clamp, G. Dawson & R. B. Nichols, unpublished work). D-Mannitol (0·4 μmole) was then added and the solution was dried in a vacuum desiccator over P_2O_5. The surviving monosaccharide residues were determined by gas–liquid chromatography.

Amino acid analyses. Amino acid analyses were kindly performed by Dr B. Pickering of the Department of Pharmacology, University of Bristol.

RESULTS

The two fractions Cra-1 and Cra-2 (Fig. 1) obtained by ion-exchange chromatography of the urinary protein appeared to be free from contaminating proteins by starch-gel electrophoresis and immunoelectrophoresis, and showed a significant difference in their content of 6-deoxy-L-galactose, D-galactose and sialic acid. Thus Cra-1 for a dimeric molecular weight of 51000 contained 3–4 residues of 6-deoxy-L-galactose, 10 of D-mannose, 5–6 of D-galactose, 12 of 2-acetamido-2-deoxy-D-glucose and 4–5 of *N*-acetylneuraminic acid, whereas the corresponding values for Cra-2 were 2, 10, 7, 12 and 7. Cra-2 contained in addition 1 residue of 2-acetamido-2-deoxy-D-galactose.

The Pronase digest of Cra-1 was subjected to exclusion chromatography, and the hexose-containing effluent was separated into three fractions (Fig. 2), whereas Cra-2 after similar treatment gave a broader hexose profile, which was divided into five fractions (Fig. 3). The major fractions were Sephadex fraction 2 from Cra-1 (Table 1) and Sephadex fractions 1 and 2 from Cra-2 (Table 2), and these were used for more detailed studies.

High-voltage electrophoresis revealed a different pattern of glycopeptides in the major Sephadex fractions (Fig. 4). Cra-1 Sephadex fraction 2 contained 11 glycopeptides, though only two of these (nos. 2 and 5) accounted for over two-thirds of the total material (Table 3). Cra-2 Sephadex fractions 1 and 2 both contained nine glycopeptides

Table 1. *Carbohydrate content of Cra-1 and derived glycopeptide material*

Experimental details are given in the text. The results for the urinary heavy-chain fragment are expressed as residues of monosaccharide/dimeric molecule of molecular weight 51000, and those for the derived glycopeptide material as moles/2 moles of D-galactose.

	Carbohydrate composition			
	Cra-1	Sephadex G-25 fraction	1	2	3
Monosaccharide	(mean ± S.D.)	Yield (g./100g. of total hexose) ...	26	60	14
6-Deoxy-L-galactose	3·4 ± 0·4		0·8	1·0	1·0
D-Mannose	10·1 ± 1·5		2·9	3·9	4·7
D-Galactose	5·6 ± 0·3		2·0	2·0	2·0
2-Acetamido-2-deoxy-D-glucose	12·0 ± 1·0		3·5	4·4	4·9
N-Acetylneuraminic acid	4·5 ± 0·0		2·7	2·1	1·9

Table 2. *Carbohydrate analyses of Cra-2 and derived glycopeptide material*

Experimental details are given in the text. The results for the urinary heavy-chain fragment are expressed as residues of monosaccharide/dimeric molecule of molecular weight 51000, and those for the derived glycopeptide material as moles/2 moles of D-galactose.

	Carbohydrate composition						
	Cra-2	Sephadex G-25 fraction	1	2	3	4	5
Monosaccharide	(mean ± S.D.)	Yield (g./100g. of total hexose) ...	21	33	14	18	14
6-Deoxy-L-galactose	2·2 ± 0·1		0·2	0·5	0·7	0·7	0·9
D-Mannose	10·2 ± 0·7		1·4	2·4	2·9	3·4	4·2
D-Galactose	7·1 ± 0·3		2·0	2·0	2·0	2·0	2·0
2-Acetamido-2-deoxy-D-glucose	11·8 ± 0·5		2·1	3·1	4·3	4·2	4·1
2-Acetamido-2-deoxy-D-galactose	1·1 ± 0·2		0·1	0·1	0	0·3	0·5
N-Acetylneuraminic acid	7·1 ± 0·5		2·4	2·9	2·9	2·0	2·0

Table 3. *Composition of glycopeptides derived from Cra-1 Sephadex fraction 2*

Experimental details are given in the text. The results are expressed as moles of monosaccharide/2 moles of D-galactose, with the exception of those for glycopeptides that were analysed for amino acids, namely nos. 2 and 5, which are expressed as moles/mole of aspartic acid. The amino acid chromatogram of glycopeptide no. 2 showed the presence of serine and threonine (+), but these were not calculated. The glycopeptides are numbered according to their increasing anodic mobility (Fig. 4).

	Composition										
Glycopeptide no.	1	2	3	4	5	6	7	8	9	10	11
Yield (%)	4	39	4	2	28	2	3	2	6	8	2
Component											
6-Deoxy-L-galactose	0·9	0·7	0·7	0·9	0·8	0·7	0·9	1·1	1·0	0·9	0·7
D-Mannose	3·3	3·0	2·9	3·0	2·9	2·5	3·2	3·3	3·4	3·2	3·0
D-Galactose	2·0	1·1	1·0	2·0	2·0	2·0	2·0	2·0	2·0	2·0	2·0
2-Acetamido-2-deoxy-D-glucose	3·6	2·9	2·9	3·9	3·2	2·6	3·7	4·2	3·9	2·8	3·1
N-Acetylneuraminic acid	0	0	0	0	2·0	2·5	1·9	2·4	2·0	2·0	2·1
Aspartic acid		1·0			1·0						
Serine		+			0·5						
Threonine		+			0·5						

Table 4. *Carbohydrate composition of glycopeptides isolated from Cra-2 Sephadex fraction 1*

Experimental details are given in the text. Results are expressed as residues of monosaccharide/2 moles of D-galactose. The glycopeptides are numbered according to their increasing anodic mobility (Fig. 4).

	Carbohydrate composition								
Glycopeptide no.	1	2	3	4	5	6	7	8	9
Yield (%)	Trace	Trace	2	Trace	20	8	20	38	12
Monosaccharide									
6-Deoxy-L-galactose			0		0·2	0·4	0·3	0·2	0·2
D-Mannose			0·8		1·7	2·0	1·9	1·5	1·0
D-Galactose			2·0		2·0	2·0	2·0	2·0	2·0
2-Acetamido-2-deoxy-D-glucose			0		1·7	2·9	3·1	2·5	2·6
2-Acetamido-2-deoxy-D-galactose			1·2		0	0	0	0	0
N-Acetylneuraminic acid			3·5		1·3	1·6	2·0	2·6	2·5

Table 5. *Carbohydrate composition of glycopeptides isolated from Cra-2 Sephadex fraction 2*

Experimental details are given in the text. Results are expressed as residues of monosaccharide/2 moles of D-galactose, apart from those for glycopeptide no. 8, which are expressed as moles/mole of aspartic acid. The glycopeptides are numbered according to their increasing anodic mobility (Fig. 4).

	Composition								
Glycopeptide no.	1	2	3	4	5	6	7	8	9
Yield (%)	1	2	2	22	9	13	12	28	11
Component									
6-Deoxy-L-galactose	0	0·6	0	0·8	0·4	0·5	0·6	0·2	0·3
D-Mannose	0·8	2·3	1·0	3·0	2·1	2·3	2·6	2·5	1·9
D-Galactose	2·0	2·0	2·0	2·0	2·0	2·0	2·0	2·8	2·0
2-Acetamido-2-deoxy-D-glucose	0	2·1	0	3·1	2·4	2·7	2·8	3·2	3·0
2-Acetamido-2-deoxy-D-galactose	1·0	0	1·2	0	0	0	0	0	0
N-Acetylneuraminic acid	0	1·0	2·6	2·0	2·1	2·4	2·5	3·3	2·9
Aspartic acid								1·0	
Serine								0·3	
Threonine								0·6	

(Fig. 4). In fraction 1 glycopeptides nos. 5, 7 and 8 accounted for 78% of the total material (Table 4) and in fraction 2 glycopeptides nos. 4 and 8 accounted for 50% of the total material (Table 5). The major fraction after Cra-2 Sephadex fractions 1 and 2 was fraction 4, which contained similar glycopeptides to Cra-1 Sephadex fraction 2 (Fig. 4).

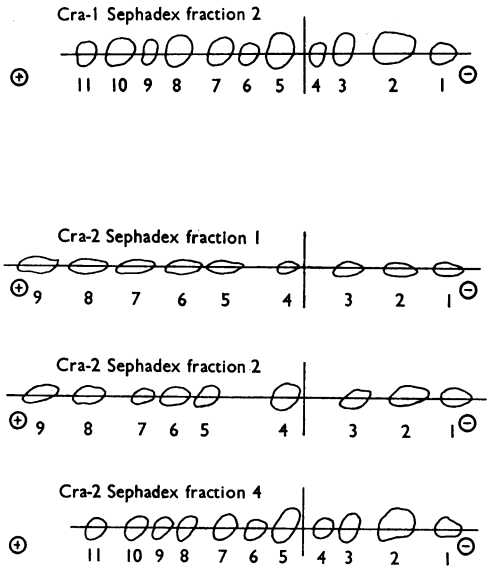


Fig. 4. High-voltage-electrophoretic separation of the glycopeptides present in Sephadex G-25 fraction 2 from Cra-1 and fractions 1, 2 and 4 from Cra-2 obtained after Pronase digestion of the proteins. Glycopeptide material was applied to Whatman 3MM filter paper and subjected to electrophoresis for 3hr. at 2kv and 100mA in pyridine–acetic acid–water (1:10:289, by vol.), pH3·6.

Glycopeptide no. 5 from Cra-1 Sephadex fraction 2 rapidly reduced approx. 10 moles of periodate/mole of aspartic acid (Fig. 5), after which analysis showed that all the 6-deoxy-L-galactose, D-galactose and sialic acid had been oxidized but that only 2 of the 3 residues of D-mannose and less than 1 residue of 2-acetamido-2-deoxy-D-glucose were destroyed (Table 6). Glycopeptide no. 2 rapidly reduced approx. 6 moles of periodate/mole of aspartic acid (Fig. 5), and analysis showed a similar result to glycopeptide no. 5 in that all the 6-deoxy-L-galactose and D-galactose had been oxidized together with 2 of the 3 residues of D-mannose and 1 residue of 2-acetamido-2-deoxy-D-glucose (Table 6).

Cra-2 fractions contained glycopeptides in which the only acetamidohexose was 2-acetamido-2-deoxy-D-galactose. Glycopeptide no. 8 from Cra-2 Sephadex fraction 1 contained more sialic acid than D-galactose and had a low content of D-mannose. Periodate oxidation destroyed all the 6-deoxy-L-galactose and sialic acid and 1 residue of 2-acetamido-2-deoxy-D-glucose, but none of the D-mannose or D-galactose (Table 6).

DISCUSSION

Little is known at present about the mode of biosynthesis of the oligosaccharide units of glycoproteins. Export proteins appear to be synthesized on membrane-attached polyribosomes (Campbell, Serck-Hanssen & Lowe, 1965), and in immunoglobulin-producing cells (De Petris, 1967) these are of two different sizes corresponding to the synthesis of light chains and of heavy chains (Scharff & Uhr,

Table 6. *Carbohydrate composition of glycopeptides before and after oxidation with periodate*

Experimental details are given in the text. The glycopeptides were oxidized with 15mM-$NaIO_4$ at 20° in the dark and subsequently reduced with $NaBH_4$. The results before (*a*) and after (*b*) oxidation with periodate are expressed as moles/mole of aspartic acid.

	Composition					
	Cra-1 Sephadex fraction 2				Cra-2 Sephadex fraction 1	
Glycopeptide no. ...	2		5		8	
	a	*b*	*a*	*b*	*a*	*b*
Monosaccharide						
6-Deoxy-L-galactose	0·7	0	0·8	0	0·2	0
D-Mannose	3·0	1·0	2·9	1·0	1·5	1·8
D-Galactose	1·1	0	2·0	0	2·0	2·0
2-Acetamido-2-deoxy-D-glucose	2·9	1·8	3·2	2·8	2·5	1·4
N-Acetylneuraminic acid	0	0	2·0	0	2·6	0

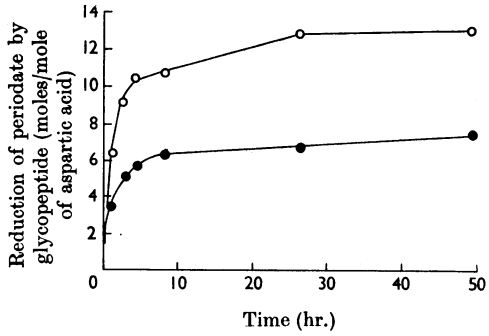
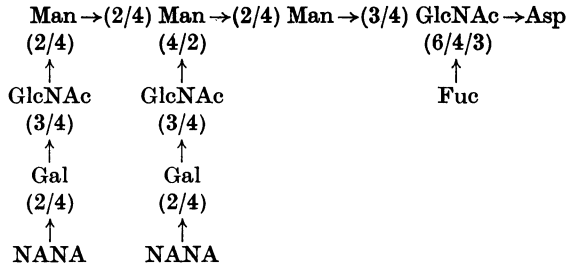


Fig. 5. Reduction of 15mM-$NaIO_4$ at 20° in the dark by Cra-1 Sephadex fraction 2 glycopeptides nos. 2 (●) and 5 (○).

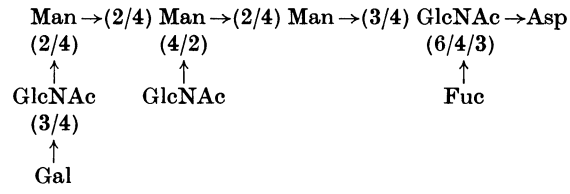
1965; Shapiro, Scharff, Maizel & Uhr, 1966). Light chains may combine with and effect the release of heavy chains from ribosomes (Askonas & Williamson, 1966). The assembled immunoglobulin molecules are found in the cisternae of the endoplasmic reticulum, where they form the principal, if not the sole, protein component (Swenson & Kern, 1967*a*). In liver glycoproteins the carbohydrate is added after the polypeptide is synthesized (Sarcione, Bohne & Leahy, 1964; Richmond, 1965) and this process is associated with the endoplasmic reticulum (Molnar, Robinson & Winzler, 1965). A similar process appears to occur during immunoglobulin synthesis, in which the addition of carbohydrate is associated with the intracellular transit of the protein, the first carbohydrate residues being added soon after the synthesis of the protein and the last just before its secretion (Swenson & Kern, 1968). The release of immunoglobulin from the cisternae of the endoplasmic reticulum (Swenson & Kern, 1967*a*) may not occur until the oligosaccharide moiety is added, since only the cytoplasmic-protein fraction contains appreciable carbohydrate (Swenson & Kern, 1967*b*). Cells in which protein synthesis is deranged might therefore show some disorganization of biosynthesis of the associated oligosaccharide units. In heavy-chain disease the abnormal cells appear to be synthesizing an incomplete protein, and a heavy-chain fragment (Cra protein) analogous to the Fc fragment of IgG1 is present in the blood and urine (Franklin, 1964; Franklin *et al.* 1964). The amount of carbohydrate present in Cra protein is considerably higher than that present in Fc fragment of normal human pooled IgG (Clamp & Putnam, 1964). However, the carbohydrate content may vary between IgG molecules, and an IgG myeloma globulin has been found (P. Weston & J. R. Clamp, unpublished work) that contains approximately double the average carbohydrate content of pooled IgG.

Ion-exchange chromatography of the Cra protein gave a broad peak, which was divided into two fractions, Cra-1 and Cra-2. These had similar contents of D-mannose and 2-acetamido-2-deoxy-D-glucose, but showed significant differences in their contents of 6-deoxy-L-galactose, D-galactose and *N*-acetylneuraminic acid. The difference in sialic acid content may account to some extent for the separation on ion-exchange chromatography. Cra-2 contained 2-acetamido-2-deoxy-D-galactose, which was not present in Cra-1. This acetamidohexose has not previously been found in human IgG, though it has been found in rabbit IgG (Smyth & Utsumi, 1967) and human IgA (Dawson & Clamp, 1968). The observed ratios of the *N*-acetylneuraminic acid to D-galactose are higher in the Sephadex fractions than in the original protein. Thus in Table 1 the ratio in Cra-1 is 0·8:1·0, whereas the value for the pooled Sephadex fractions is 1·1:1·0. In Table 2 the ratio for Cra-2 is 1·0:1·0 whereas that for the pooled Sephadex fractions is 1·2:1·0. However, the analytical results for *N*-acetylneuraminic acid are less reliable than those of the other monosaccharides and may show a standard deviation of 25% (Clamp *et al.* 1967). The results of the carbohydrate analyses of the proteins in Tables 1 and 2 are the means of five determinations, whereas the analyses of the Sephadex fractions, which were carried out as a screening procedure, are each the result of a single estimation.

A large number of glycopeptides were isolated from Cra-1 and Cra-2, and this degree of heterogeneity is greater than that found in an IgA myeloma globulin (Dawson & Clamp, 1968). Thus Cra-1 yielded 11 glycopeptides of which nos. 2 and 5 were the major components. Glycopeptides nos. 2 and 5 both contained, relative to 1 mole of aspartic acid, approx. 1 residue of 6-deoxy-L-galactose, 3 of D-mannose and 3 of 2-acetamido-2-deoxy-D-glucose, but the D-galactose and *N*-acetylneuraminic acid content differed, glycopeptide no. 5 containing 2 residues of D-galactose and 2 of *N*-acetylneuraminic acid, and glycopeptide no. 2 containing 1 residue of D-galactose and no sialic acid. After periodate oxidation of glycopeptide no. 5, only 1 residue of D-mannose and 3 residues of 2-acetamido-2-deoxy-D-glucose remained relative to 1 residue of aspartic acid. The neutral hexoses that were oxidized must be terminal, substituted at C-6 or monosubstituted at C-2 or C-4. The D-mannose residue that survived must have been either substituted at C-3 or di-*O*-substituted at C-2, C-3 or C-4. As no acetamidohexose was oxidized, none could have been in a terminal position. These results are similar to those obtained with glycopeptides from an IgA



(I) NANA, *N*-acetylneuraminic acid; GlcNAc, 2-acetamide-2-deoxy-D-glucose; Fuc, 6-deoxy-L-galactose (fucose).



(II) GlcNAc, 2-acetamide-2-deoxy-D-glucose; Fuc, 6-deoxy-L-galactose (fucose).

myeloma globulin for which a tentative structure (I) was proposed by Dawson & Clamp (1968).

After periodate oxidation, the carbohydrate content of glycopeptide no. 2, on the other hand, decreased to 1 residue of D-mannose and 2 residues of 2-acetamido-2-deoxy-D-glucose relative to an aspartic acid content of 1 residue. These results are consistent with the tentative structure shown (II).

Structure (I) would theoretically reduce 10 moles of periodate, whereas structure (II) would theoretically reduce 7 moles, both of which are almost identical with the observed values. Glycopeptide no. 2 could give rise to glycopeptide no. 5 by the addition of a D-galactose residue and 2 residues of sialic acid. Cra-1 also contained small amounts of another type of oligosaccharide unit, as shown by glycopeptides nos. 4, 7, 8 and 9, in which the molar proportions of 6-deoxy-L-galactose, D-mannose, D-galactose and 2-acetamido-2-deoxy-D-glucose were approx. 1:3:2:4. Such a unit could arise by the addition of 2-acetamido-2-deoxy-D-glucose to the first D-mannose residue of structure (I).

Cra-2 showed a more complicated pattern of glycopeptides than Cra-1. All the glycopeptides in Cra-2 Sephadex fraction 1 had a D-galactose content that was equal to or greater than that of D-mannose, in contrast with those in Cra-1 Sephadex fraction 2, in which the D-mannose content was always greater than that of D-galactose. A glycopeptide containing more D-galactose than D-mannose is difficult to explain on the basis of the structure shown in (I) and (II) that has been postulated (Dawson & Clamp, 1968) for this type of oligosaccharide unit. The higher D-galactose content could arise, however, if there were at least two galactosyltransferases in the cell, one that recognized 2-acetamido-2-deoxy-D-glucose as the acceptor residue, as in the structures shown above, and another that transferred to D-galactose or 2-acetamido-2-deoxy-D-galactose as in glycopeptide no. 3 from Cra-2 Sephadex fraction 1, glycopeptides nos. 1 and 3 from Cra-2 Sephadex fraction 2 and type I glycopeptides in IgA (Dawson & Clamp, 1968). Glycopeptides of this type in which the D-galactose content is greater than that of D-mannose have not so far been isolated from biosynthetically complete myeloma globulins. These results would therefore support the idea that the addition of peripheral monosaccharide residues such as galactose is spatially separated from the synthesis of the core of the oligosaccharide unit (Droz, 1966*a*,*b*; Li, Li & Shetlar, 1968).

The 6-deoxy-L-galactose content differs between the various glycopeptides. Thus the Cra-1 Sephadex fraction 2 glycopeptides all contained 0·7 or more residue of this monosaccharide, whereas the Cra-2 Sephadex fraction 1 glycopeptides contained 0·4 residue or less. The content of 6-deoxy-L-galactose therefore appears to be related to that of D-mannose, and the addition of one or other of these monosaccharides may depend to some extent on the presence of the other.

In a number of glycopeptides from Cra-2 the

Table 7. *Oligosaccharide units of Cra-1 and Cra-2*

The results are given to the nearest whole residue. The content of monosaccharide residues in each protein is given as the totals calculated from those present in the postulated oligosaccharide units (Calc.) and the number found on analysis (Act.).

	No. of monosaccharide residues								
	Cra-1				Cra-2				
	Oligosaccharide units		Total residues		Oligosaccharide units			Total residues	
			Calc.	Act.				Calc.	Act.
No. of units ...	2	2			2	1	1		
Monosaccharide									
6-Deoxy-L-galactose	1	1	4	3–4	0–1	0–1	1	2	2
D-Mannose	3	3	12	10	2	2–3	3	9–10	10
D-Galactose	1	2	6	5–6	2	2–3	2	8	7
2-Acetamido-2-deoxy-D-glucose	3	3	12	12	2–3	3	3	11–12	12
N-Acetylneuraminic acid	0	2	4	4–5	1–3	2–3	2	7	7

content of *N*-acetylneuraminic acid was equal to or greater than that of D-galactose. Thus glycopeptide no. 9 in Sephadex fraction 2 had a D-galactose/sialic acid ratio 2:3. In this glycopeptide the sialic acid was oxidized by periodate whereas the D-galactose was unaffected. These D-galactose residues were therefore either substituted at C-3 as in sialolactose (Kuhn, 1958) or di-*O*-substituted probably with sialic acid at C-2, C-3 or C-4. The D-mannose residues were resistant to oxidation, whereas half the 2-acetamido-2-deoxy-D-glucose was destroyed. These results differ from those obtained with the major glycopeptides and suggest that this oligosaccharide unit has a fundamentally different structure.

A further type of oligosaccharide unit was shown by glycopeptide no. 3 from Cra-2 Sephadex fraction 1 and glycopeptides nos. 1 and 3 from Cra-2 Sephadex fraction 2. These glycopeptides all contained 2-acetamido-2-deoxy-D-galactose, with D-mannose, D-galactose and 2-acetamido-2-deoxy-D-galactose proportions 1·0:2·0:1·0, to which were attached differing amounts of *N*-acetylneuraminic acid. These glycopeptides were minor components constituting less than 5% of the total carbohydrate. Insufficient material was available to determine whether the oligosaccharide was linked *O*-glycosidically to the protein, as might be expected for a unit containing 2-acetamido-2-deoxy-D-galactose as the sole acetamidohexose (A. Neuberger, personal communication).

Although both Cra protein fractions contained a large number of glycopeptides with differing proportions of monosaccharide residues, an attempt has been made in Table 7 to interpret the oligosaccharide units of the proteins in terms of the major glycopeptides that were isolated from each. Thus both proteins appear to contain four oligosaccharide units associated with a dimeric structure of molecular weight approx. 51 000. The calculated and actual values in Table 7 for Cra-1 agree very well in terms of the two major glycopeptides that were isolated from this protein. The range of glycopeptides that were isolated from Cra-2, however, made it difficult to assign precise oligosaccharide units to the protein. The minor glycopeptides containing 2-acetamido-2-deoxy-D-glucose possibly replace the major units in a small number of protein molecules, and may represent incomplete or aberrant units as suggested above. The glycopeptides containing 2-acetamido-2-deoxy-D-galactose, however, are probably attached *O*-glycosidically to hydroxyamino acids and would therefore be additional units in the few molecules that contain them.

J. R. C. and G. D. thank the Medical Research Council and Science Research Council for generous financial support. We also thank Miss Shirley Presland and Mrs Kathlene Greenwood for excellent technical assistance.

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