The Carbohydrate Content of Fragments and Polypeptide Chains of Human γG-Myeloma Proteins of Different Heavy-Chain Subclasses*

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ABSTRACT: The carbohydrate composition and its distribution within the polypeptide chains and enzymatic fragments of γG-myeloma proteins of different subclasses have been determined; 29% of 76 proteins studied had a hexose content higher than 19 moles/mole of protein compared to values ranging from 9 to 13 moles of hexose/mole of protein in the remaining 71%. No correlation was observed between hexose content and antigenic subclass of heavy- or light-chain type. The excess carbohydrate on the high carbohydrate-containing proteins was localized to the Fab fragment whereas the Fc fragment contained the same amounts of carbohydrate as that found in normal γG-globulin. Detailed analysis of nine proteins with high carbohydrate content localized the Fab carbohydrate to the Fd fragment in three proteins and to the light chains in four proteins, and in two instances both Fd fragment and light chains contained carbohydrate. Comparable amounts of hexose, glucosamine, and fucose were present in the Fab carbohydrate as was present in the Fc carbohydrate, but the sialic acid content was four times as high in the Fab fragment. No evidence was obtained for the presence of a glycopeptide in the $F(ab')_2$ fragment which was not in the Fab fragment. Starch gel electrophoretic banding of γG-myeloma proteins was not correlated with carbohydrate content.

In one case, however, the banding of the isolated light chains was shown to be due to variations in sialic acid content.

It is well established that both normal human and normal rabbit γG-immunoglobulins contain covalently linked carbohydrate residues (Smith *et al.*, 1946; Nolan and Smith, 1962; Clamp and Putnam, 1964). Conflicting data, however, have been reported with regard to the number of the glycopeptides and their position on the four polypeptide chains of the γG-immunoglobulin molecule.

Myeloma proteins have been very useful in clarifying various structural features of the γ-globulin molecule which were not apparent from analysis of the heterogeneous population of molecules present in normal γ-globulin. Previous studies on the carbohydrate content of γG-myeloma proteins indicated that these proteins varied with respect to the quantity of carbohydrate they contained (Müller-Eberhard and Kunkel, 1956; Froelich, 1963).

The present study was undertaken to investigate the carbohydrate content of human γG-myeloma proteins of the four heavy-chain subclasses. Also, in order to localize the carbohydrate moiety in the γG molecule, the carbohydrate content of fragments and polypeptide chains was studied. Furthermore, it was investigated whether the banding of the intact myeloma proteins and their light polypeptide chains observed in gel electrophoresis was the result of differences in carbohydrate content.

Materials and Methods

Myeloma Proteins. Human γG-myeloma proteins were isolated from the sera of patients with multiple myeloma by Pevikon block electrophoresis (Kunkel, 1954; Müller-Eberhard, 1960). Sections (0.5 in.) of the block, comprising the γ-globulin peak, were eluted and the tubes containing the myeloma peak were identified by immunoelectrophoresis (Scheidegger, 1955) using a rabbit antiserum against whole human serum. In the case of electrophoretically slow proteins DEAE chromatography was also used to isolate the myeloma protein (Levy and Sober, 1960). Normal γG-globulin (HGG) was obtained by DEAE chromatography of fraction II of normal human serum (kindly supplied by the American Red Cross).

Separation of Heavy and Light Chains. The isolated myeloma proteins were partially reduced with 0.2 M 2-mercaptoethanol in 0.55 M Tris for 1 hr at room temperature and alkylated with a 20% excess of iodoacetamide in 0.55 M Tris (pH 8.2) for 1 hr at 4°. The reduced and alkylated proteins were dialyzed against 1 M acetic acid for 16–18 hr and heavy and light chains were separated by gel filtration through Sephadex

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G-100 columns equilibrated in 1 M acetic acid (Fleischman *et al.*, 1962).

Enzymatic Digestion. Fab and Fc fragments were obtained by treating the isolated proteins for 45 min at 37° with 1% papain (w/w) (Worthington, two-times crystallized) in 0.1 M phosphate buffer (pH 7.5) made 0.01 M in cysteine and 0.002 M in EDTA (Porter, 1959). The reaction was stopped by the addition of a fivefold molar excess of iodoacetamide. The alkylation reaction was allowed to proceed for 30 min at 4°, after which the samples were dialyzed against a large volume of cold isotonic saline. Fab fragments were separated from Fc fragments by Pevikon block electrophoresis, after which the former were subjected to gel filtration on G-100 Sephadex equilibrated in 1 M acetic acid. $F(ab')_2$ fragments were obtained by treating the isolated proteins with 1% (w/w) pepsin (Worthington, two-times crystallized) in 0.1 M acetate buffer (pH 4.0) at 37° for 16 hr (Nisonoff *et al.*, 1960). The products of digestion were subsequently dialyzed against 0.1 M phosphate buffer (pH 7.5) and subjected to gel filtration on G-100 Sephadex equilibrated with 1 M acetic acid. Neuraminidase digests were carried out using *Vibrio cholerae* neuraminidase (Mann Research Laboratories); 40 RDE[1] units of enzyme/mg of protein was used. Digestion was carried out for 48 hr at 37° in 0.05 M acetate buffer (pH 5.5) to which calcium chloride was added to a final concentration of 0.01 M. To determine the extent of cleavage of sialic acid from the proteins following neuraminidase treatment, the thiobarbituric acid assay was performed without previous acid hydrolysis.

Starch Gel Electrophoresis. Vertical starch gel electrophoresis (Smithies, 1959) employing a glycine buffer (Fahey, 1963) or an acid urea–formate buffer (Poulik, 1960) was performed as previously described. Glycine gels were electrophoresed for 5 hr at 450 V in the cold, and acid urea gels for 16 hr at 170 v at room temperature.

Protein Determinations. Routine protein quantitation was done with the Folin–Ciocalteau reagent (Lowry *et al.*, 1951) using normal human γ-globulin as standard. Protein nitrogen determinations were done on all samples subjected to quantitative carbohydrate analysis by a modified method of the micro-Kjeldahl technique using a Technicon Auto Analyzer (Ferrari, 1960).

Carbohydrate Analysis. Hexoses were determined by the orcinol reaction (Svennerholm, 1956) reducing the sample and reagent volumes to 1 and 2 ml, respectively, and using an equimolar mixture of galactose and mannose as standard in concentrations of 0.058–0.233 μmole/ml. Spectrophotometric readings were taken at 505 mμ. Blanks eluted from Pevikon blocks were included when Pevikon block electrophoresis isolated material was assayed since it was found that certain batches of Pevikon contained orcinol-reactive material. Hexosamines were determined on an amino acid analyzer. Protein samples (0.25–0.5 mg) were hydrolyzed with 4 N hydrochloric acid at 105° for 6 hr. The hydrolyzed samples were dried *in vacuo* over sodium hydroxide. The dried residues were dissolved in citrate buffer (pH 2.2) and analyzed with the Beckman 120C amino acid analyzer using the short column with an 11.5 cm "Aminex A-5" resin bed and the standard pH 5.28 citrate buffer for elution (Walborg *et al.*, 1963). Under these conditions clear separation of D-glucosamine from D-galactosamine and L-tryptophan was obtained. Standards containing 0.05–0.1 μmole of D-glucosamine were used. Recoveries after acid hydrolysis of D-glucosamine standard were 87–90%. L-Fucose was determined by the cysteine reaction (Dische and Shettles, 1948), using a 3-min boiling period. Differential spectrophotometric readings were taken at 396 and 426 mμ after allowing the samples to stand 90–120 min at room temperature. L-Fucose standards in concentrations of 0.012–0.048 μmole/ml, as well as HGG preparations at concentrations of 0.5–1.0 mg/ml with internal L-fucose standards, were included with each determination. Sialic acid was measured by the thiobarbituric acid assay (Warren, 1959) after its hydrolytic cleavage from the protein with 0.1 N sulfuric acid for 1 hr at 80°. Crystalline *N*-acetylneuraminic acid in concentrations of 0.0485–0.194 μmole/ml was used as a standard.

Results

Determination of the hexose content of 76 γG-myeloma proteins showed marked individual variations; 54 (71%) had hexose contents similar to normal human γG-globulin (9–13 moles of hexose/mole of protein), whereas the remaining 22 (29%) contained 19–37 moles of hexose/mole of protein.[2] These two groups were clearly distinguishable from one another, the average molar values of hexose being 11.7 for the low group and 26.2 for the high group. These 76 myeloma proteins were analyzed with regard to light-chain type and heavy-chain subclass (Table I). Proteins with high and low carbohydrate values were found in each of the antigenic types and subclasses indicating that the presence of additional carbohydrate does not correlate with a particular antigenic structure. Also, within the γG_1 subclass no correlation was observed between carbohydrate content and the Gm allotypes, a or f, of the myeloma proteins.

In order to study the distribution of the carbohydrate within the γ-globulin molecule, the carbohydrate content of Fab, $F(ab')_2$, and Fc fragments as well as heavy and light chains of 17 myeloma proteins were determined. All proteins that had high carbohydrate content had a carbohydrate moiety both on the Fc and on the Fab fragment. In contrast, the carbohydrate moiety was localized on the Fc fragment alone in all myeloma proteins studied with low carbohydrate content. Analysis of the Fc fragments isolated from

[1] RDE = receptor-destroying enzyme; HGG = human γ-globulin.

[2] All but three proteins in the high group had 19–28 moles of hexose/mole of protein.

TABLE I

Antigenic Classification	Proteins Examined	Number with High Hexose[a]	Number with Low Hexose[a]
Light-Chain Type			
K	56	20	36
λ	20	2	18
Heavy-Chain Subclass			
γG_1	49	14	35
γG_2	14	2	12
γG_3	9	4	5
γG_4	4	2	2
Total	76	22	54

[a] High hexose refers to proteins with hexose values greater than 19 moles/mole of protein. Low-hexose proteins had hexose contents between 9 and 13 moles.

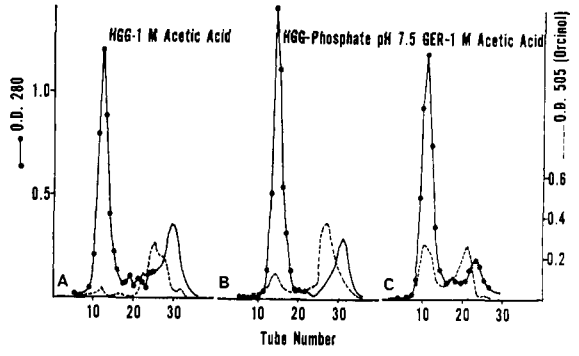

FIGURE 1: Sephadex G-100 gel filtration of pepsin-digested γG-proteins. Dashed lines show carbohydrate by the orcinol reaction and solid lines represent protein by 280-mμ absorption. $F(ab')_2$ was eluted with the void volume; this was followed by the nondialyzable Fc fragment peptides. Protein Ger (C) was a high carbohydrate protein and contained $F(ab')_2$-bound hexose. The $F(ab')_2$ peak from normal HGG showed a negligible orcinol reaction when eluted with acetic acid (A) and a small but measurable peak when eluted with phosphate buffer (B).

myeloma proteins with high and low carbohydrate content revealed no significant differences. For this reason, no further studies on the Fc fragment carbohydrate were done. In order to evaluate if the carbohydrate moiety was covalently linked to the Fab and $F(ab')_2$ fragments, prior to carbohydrate quantitation, the isolated fragments were subjected to G-100 gel filtration in 1 M acetic acid in order to dissociate any noncovalently linked carbohydrate from these fragments. Figure 1 shows the elution patterns obtained when Sephadex G-100 column effluents of the pepsin-digested material from a myeloma protein with a high carbohydrate content and that from normal γG-globulin were tested for hexose by the orcinol reaction. The $F(ab')_2$ was eluted with the void volume of the column. In protein Ger this peak showed a positive orcinol reaction (Figure 1C) whereas the $F(ab')_2$ peak obtained from normal γ-globulin showed no detectable hexose at the concentrations tested (Figure 1A). All proteins with high carbohydrate content gave results similar to those observed in Figure 1C, whereas low carbohydrate proteins gave patterns similar to that of normal HGG. In all proteins studied, a nondialyzable glycopeptide was also found which appeared after the $F(ab')_2$ peak but before the major peak of nondialyzable Fc fragment peptides. When the pepsin digest of normal γ-globulin was placed on a G-100 column equilibrated in 0.1 M phosphate buffer (pH 7.5) rather than one equilibrated in 1 M acetic acid, only a small amount of orcinol reactivity was present in the $F(ab')_2$ peak (Figure 1B). This indicates that the Fc fragment glycopeptide which is the product of pepsin digestion has no appreciable affinity for the $F(ab')_2$ fragment since the bulk of it could be easily separated from the $F(ab')_2$ in the absence of any dissociating reagent.

In order to further localize the Fab fragment carbohydrate to the Fd fragment of the heavy chain or to the light chain, the hexose content of the heavy and light chains of myeloma proteins were determined. G-100 Sephadex column fractions were tested for protein at 280 mμ and for carbohydrate by the orcinol reaction. Figure 2 shows the carbohydrate content of heavy and light chains obtained from normal γG-globulin (Figure 2A) compared with that of two γG-myeloma proteins with high carbohydrate content, one having excessive amounts limited to the heavy chain (Figure 2B) and another one showing the presence of carbohydrate on the light chains (Figure 2C). The myeloma proteins with low carbohydrate content showed a distribution similar to that observed in normal γG. Of 17 proteins studied with high carbohydrate values, all of them had the excess carbohydrate in the Fab fragment. Nine of these were further investigated with regard to heavy- and light-chain distribution. Three of nine proteins had Fd fragment carbohydrate; four of nine had light-chain carbohydrate; and two of nine had carbohydrate on both Fd fragment and light chains. In all instances hexose determinations were repeated on the pooled and concentrated heavy and light chains[3] to corroborate the data obtained from the orcinol reactions done on the individual fractions obtained from the G-100 Sephadex columns. Of the six proteins with light-chain carbohydrate three were K type and one was λ. Of the five proteins analyzed with Fd carbohydrate three were γG1 proteins and two were γG2.

Further analysis for sugars other than hexoses was performed on the following proteins: two proteins in which the extra carbohydrate was localized to the light chain; two proteins in which extra carbohydrate was localized to the Fab portion of the heavy chain (Fd fragment); three proteins which had low carbohydrate values; and normal γG-globulin. In order to analyze the composition of the non-Fc fragment glycopeptide, whether on the light chain or Fd fragment,

[3] Calculations were based on the following molecular weights: light chains = 23,000; heavy chains = 55,000.

TABLE II

Antigenic Classification:	Fd Carbohydrate Proteins		Light-Chain Carbohydrate Proteins		Low Carbohydrate Proteins			Normal
	Cut. K, γG1	Dah λ, γG2	Pet. λ, γG1	Ger K, γG4	Ger K, γG1	Heb λ, γG4	Bro K, γG1	γG
				Whole γG-Protein[a]				
Hexose	24.9	25.1	23.2	24.1	10.9	8.2	9.5	11.4
Fucose	4.6	6.2	4.2	6.6	2.2	2.7	2.2	2.2
Sialic acid	4.5	3.4	2.9	2.9	0.4	0.7	0.6	0.8
Glucosamine	17.5	17.6	18.7	21.0	8.1	11.4	8.5	10.1
				Fab[a]				
Hexose	6.0	6.9	6.2	6.2	0.3	0.0		0.3
Fucose	1.2	1.0	1.4	0.9	0.6	0.1	0.2	0.2
Sialic acid	1.7	1.3	1.0	0.8	0.4	0.0	0.0	0.14
Glucosamine	4.1	5.9	6.5	4.1	0.0	0.0	0.0	0.7
				Fab'[a]				
Glucosamine	4.5	5.4				0.0	0.0	0.7

[a] Molar values of sugars based on the following molecular weights: whole γG-protein, 160,000; Fab fragment, 50,000; and Fab' fragment, 50,000.

analyses were performed on the Fab fragments of the myeloma proteins with high carbohydrate content. Also, to evaluate the possibility of a glycopeptide being present in the region of the heavy chain between which papain and pepsin act, glucosamine determinations were also performed on some $F(ab')_2$ fragments. The data are presented in Table II. The values obtained for the two proteins with Fd fragment carbohydrate were quite similar to those obtained for the two proteins that had light-chain carbohydrate, with the possible exception of the sialic acid values, the two proteins with Fd fragment carbohydrate containing on the average 1 more mole of sialic acid. When compared to the three proteins with low carbohydrate content, the average values for hexose, glucosamine, and fucose were all 2.0–2.5 times as high, whereas the average value for sialic acid was six times as high in the high carbohydrate proteins. Little or no carbohydrate was found in the Fab fragments of the proteins with low total carbohydrate content. No glucosamine at all was detected. The test used to detect glucosamine was the most sensitive[4] and reproducible of the various tests employed. Also, since glucosamine is the sugar which is covalently bound to the peptide chain, the complete absence of this sugar indicates that the occasional low values obtained for other sugars were probably the result of nonspecific color reactions in the other assays. On the other hand, the Fab fragments from the four proteins with elevated carbohydrate all had considerable amounts of the four sugars tested for. If consideration is taken for the presence of two Fab fragments per γ-globulin molecule, 30–70% of the total hexose, glucosamine, and fucose were present in the Fab fragments along with 60–75% of the total sialic acid. The relatively high content of sialic acid on the Fab fragment glycopeptides is demonstrated by the sialic acid/fucose ratio of these preparations. Whereas the low carbohydrate proteins had S/F ratios of 0.18–0.27, the high carbohydrate proteins had ratios of 0.44–0.98 with an average ratio of 0.67 and the Fab fragments, of the high carbohydrate proteins had an average ratio of 1.1. No significant difference between the glucosamine values of the Fab' fragments and Fab fragments was observed. Both of the low carbohydrate myeloma proteins tested had no detectable glucosamine. In one of the two high carbohydrate proteins the figure obtained for glucosamine in the Fab' was 0.4 mole higher than that obtained in the Fab fragment, and in the other protein it was 0.5 mole lower than in the corresponding Fab fragment.

It has been recognized for several years that a large percentage of γG-myeloma proteins show multiple bands when examined by starch gel electrophoresis. It was considered of interest to determine whether the presence or absence of electrophoretic banding could be correlated with the carbohydrate content since variation in the sialic acid content could generate the heterogeneity observed. Figure 3 shows the starch gel patterns of the whole sera of two high carbohydrate (slots 1 and 2) and five low carbohydrate (slots 3–7) containing myeloma proteins. Electrophoretic heterogeneity was observed in both groups of proteins (slots 1, 3,

[4] Less than 0.1 mole of glucosamine/mole of protein could be detected.

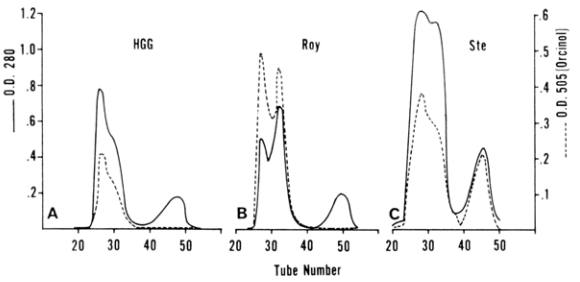

FIGURE 2: Sephadex G-100 gel filtration in 1 M acetic acid of partially reduced and alkylated proteins. Elution diagram shows carbohydrate by the orcinol reaction (dashed line), protein by 280-mμ absorption (solid line). Roy myeloma protein showed extra carbohydrate on the heavy chain; Ste on the light chain.

and 5) as was relative electrophoretic homogeneity (slots 2, 4, 6, and 7). This demonstrated that there was no correlation between carbohydrate content and the electrophoretic banding, and since the low carbohydrate proteins contained 1 mole or less of sialic acid per mole of protein, it was not possible that in proteins such as the one shown in slot 3 of Figure 3, where there were five bands, that variations in sialic acid content could account for the great multiplicity of bands. It was, however, still possible that sialic acid variations, in those proteins in which the sialic content was high, could account for the observed banding. For this reason further studies were performed on protein Ger (slot 1, Figure 3) which, as shown in Table II, had a carbohydrate moiety on the light chains and had an average of 2.9 moles of sialic acid/mole of protein. As can be seen from Figure 3, this protein exhibited six to eight bands in starch gel electrophoresis with the most predominant ones occurring in the middle. It was possible, then, that each successive band represented a molar increase in sialic acid starting from 0 and that the average sialic acid content of the total protein would be about 3. When the isolated light chains from this protein were examined in urea–starch gel, pH 3.0, it was seen that the light chains exhibited three bands. In order to test whether variations in sialic acid content could account for the electrophoretic heterogeneity of the whole myeloma protein as well as that of isolated light chains, these proteins were digested with neuraminidase to cleave sialic acid from the protein. Figure 4 shows the starch gel patterns before and after neuraminidase treatment of Ger myeloma protein and light chains. Following neuraminidase treatment, the three light-chain bands were converted into a single band (Figure 4b). However, when the intact protein was examined after neuraminidase treatment, there was still marked electrophoretic banding present, although the average mobility of the protein was shifted toward the cathode. Quantitative assay for release of sialic acid from the protein indicated that over 95% had been released from the protein. Next, the effect of neuraminidase treatment on several high carbohydrate containing, partially reduced and alkylated, proteins was examined and is shown in Figure 5. This demonstrated the cathodic shift in the mobility of light chains that contained carbohydrate (arrows, Figure 5). However, little or no shift in mobility could be seen after neuraminidase treatment of proteins that contained carbohydrate on the Fd fragment (Cut., Dah, and Bou) despite data that indicated that 93–100% of the sialic acid had been released from these proteins.

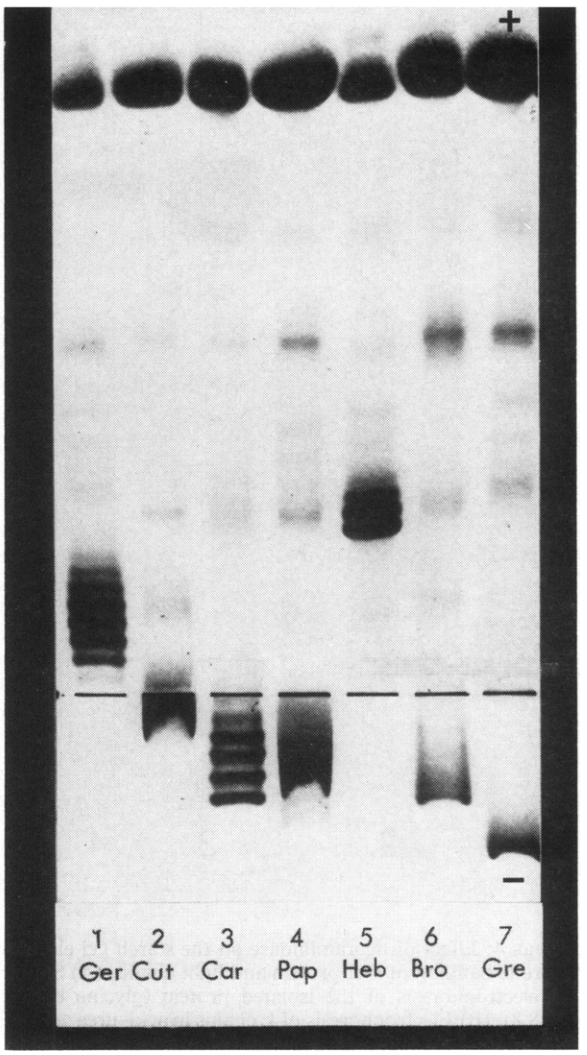

FIGURE 3: Starch gel electrophoresis of γG-myeloma sera. Electrophoresis in glycine buffer (pH 8.8). Samples 1 and 2 had high carbohydrate content, 24 and 25 moles of hexose per mole of protein, respectively; all other proteins had low carbohydrate content (8.5–10.5 moles of hexose per mole of protein).

Discussion

In the present study a large number of human γG-myeloma proteins were analyzed for their carbohydrate content and submolecular distribution. Of 76 proteins examined, 29% had a hexose content greater than 19 moles/mole of protein. These relatively high carbohydrate-containing proteins were distributed among all heavy-chain subclasses and both light-chain types so that no correlation with the known structural variants could be demonstrated. Also, microhetero-

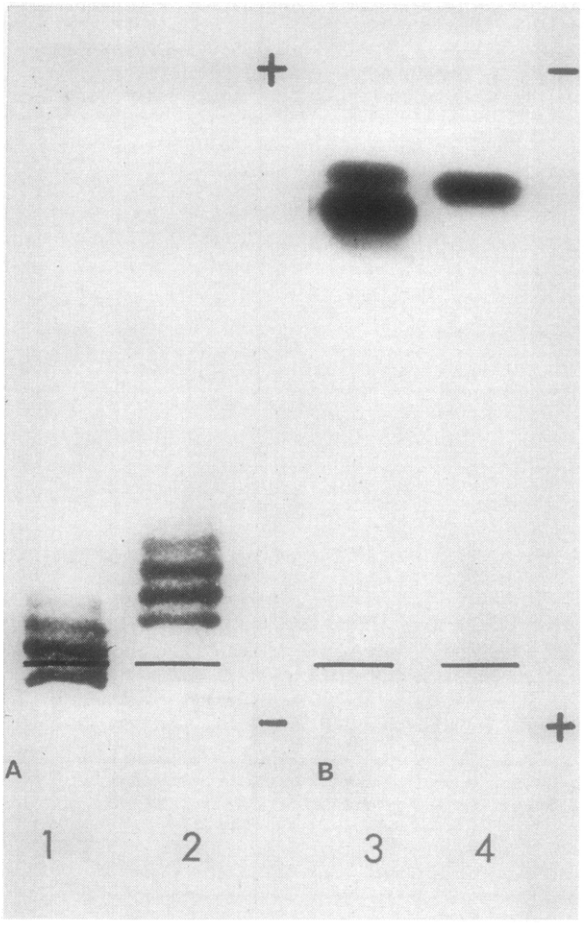

FIGURE 4: Effect of neuraminidase on the starch gel electrophoretic banding of Ger protein and light chains. (A) Starch gel electrophoresis of the isolated protein (glycine buffer, pH 8.8). (B) Electrophoresis of L chains in acid–urea gel (pH 3.0). Slots 2 and 3 before neuraminidase treatment; slots 1 and 4 after treatment.

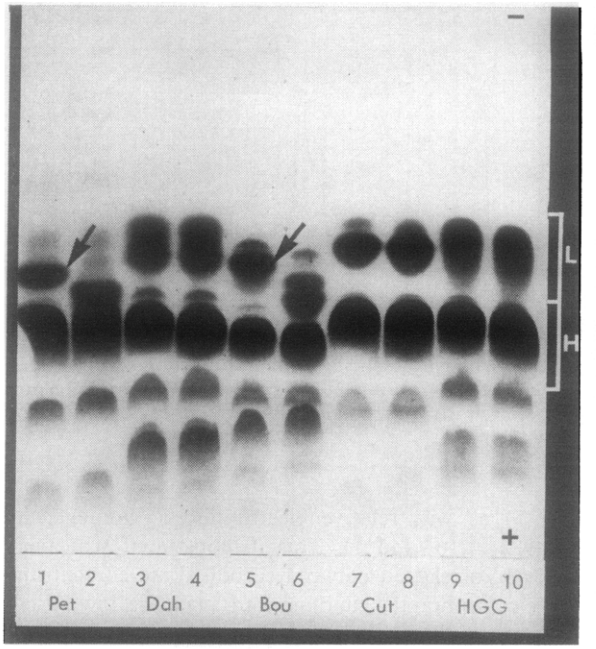

FIGURE 5: Starch gel electrophoresis of partially reduced and alkylated proteins before and after neuraminidase treatment. Electrophoresis in acid–urea gels at pH 3.0. Pet. protein contains carbohydrate on the L chain. Cut. and Dah proteins contain carbohydrate on the Fd fragment. Bou protein contains carbohydrate on both the Fd fragment and L chain. Arrows indicate the changed mobility of Pet. and Bou light chains after neuraminidase treatment. Slots 1, 3, 5, 7, and 9 after neuraminidase treatment; 2, 4, 6, and 8 before treatment.

geneity as judged by starch gel electrophoresis was not related to carbohydrate content since some proteins with high carbohydrate content showed no heterogeneity, while others having normal carbohydrate composition were heterogeneous. All proteins with high carbohydrate content had carbohydrate on the Fab fragment, approximately one-half of the total carbohydrate being present on the Fab fragments. Since the composition of these fragments was determined after gel filtration through columns equilibrated with 1 M acetic acid, it seemed evident that the monosaccharides analyzed were covalently bound. No indication of a glycopeptide in the region between papain and pepsin cleavage of the heavy chain was obtained when Fab and $F(ab')_2$ fragments were analyzed. Of nine proteins in which detailed analysis of the monosaccharide residues were performed, four had carbohydrate associated with the light chain, three with the Fd fragment of the heavy chain, and the remaining two had carbohydrate on both the light chain as well as on the Fd fragment. It is of interest to note that proteins which had a 2–2.5-fold excess in carbohydrate had extra carbohydrate either in the light chain or in the Fd fragment, while those having a three- to fourfold excess contained carbohydrate on both the light chain and Fd fragment. Further localization of the glycopeptide within the Fd fragment or light chain was not done. However, the lack of correlation between the presence of carbohydrate and any known class or subclass of heavy or light chains suggests that the carbohydrate may be linked to residues within the variable portion of a polypeptide chain rather than to the constant region.

The question remains as to how relevant these findings in myeloma proteins are to the carbohydrate composition of normal γG-globulin. In the past when a specific structural feature of a myeloma protein has been looked for in normal γ-globulin, it has been found. Also, the frequency of finding a specific structural feature in myeloma proteins has correlated with the incidence of this characteristic in the normal γ-globulin population. For instance, about two-thirds of myeloma proteins have light chains of the K type and approximately two-thirds of the molecules of normal γG-globulin are also K type (Mannik and Kunkel, 1963). Similar criteria can be used to evaluate the relevancy of the presence of Fab fragment carbohydrate in myeloma proteins to the carbohydrate content of normal γG-globulin. Studies on normal Fab fragment (Dische and Franklin, 1963) suggest that there is a minor population of molecules in normal γ-globulin with a carbohydrate moiety on the Fab fragment which in its sialic acid/fucose ratio resembles the Fab fragment carbohydrate observed in the present study in the

myeloma proteins. A calculation can be made based on the carbohydrate content of normal γG, and that of the high and low carbohydrate myeloma proteins, to determine what the theroetical proportion of high and low proteins would be in order to obtain a carbohydrate content equal to that found in normal γ-globulin. Such a calculation was done based on the values obtained for glucosamine and sialic acid, since in our hands the tests for these sugars were the most accurate. Both calculations gave remarkably similar results and indicated that a mixture of 9% high carbohydrate proteins and 91% low carbohydrate proteins would have a glucosamine and sialic acid content equal to that of normal γ-globulin. This theoretical incidence is less than one-third of the actually observed figure of 29%. The reason for this discrepancy is not clear. It might be that the γG-immunoglobulin used in this study was not representative of the entire γG-globulin population of whole serum. The γG-globulin was obtained by DEAE-cellulose chromatography, a procedure known to yield only 60–70% of the total γ-globulin. Since the myeloma proteins with high carbohydrate values had a high sialic acid content as well, the fraction of normal γ-globulin with high carbohydrate content would be expected to be relatively more acidic than the low carbohydrate proteins. This would result in a major portion of the normal γ-globulin with high carbohydrate being retained by the DEAE-cellulose under the conditions used in this study. The testing of this hypothesis is complicated by the difficulty in getting the less basic 30–40% of the γ-globulin in pure form. Another possible explanation for the large incidence of high carbohydrate-containing myeloma proteins is that with respect to carbohydrate content the myeloma proteins are truly abnormal and do not accurately represent the situation as it exists in normal γ-globulin. At present, it is not possible to choose between these two possibilities.

Association of carbohydrate with the light chain of γG-globulin has not been previously reported in humans. In mice, the presence of covalently bound carbohydrate in a light-chain preparation obtained from BALB/c mice bearing a Bence-Jones protein producing plasma cell tumor has been demonstrated (Melchers *et al.*, 1966). That study indicated, as has been shown for the Ger light chains in the present study, that sialic acid is responsible for the banding of the carbohydrate-containing light chains in acid urea–starch gel electrophoresis. Although light-chain mobility and electrophoretic heterogeneity were greatly influenced by neuraminidase treatment, neuraminidase treatment of reduced and alkylated whole protein indicated that heavy-chain mobility was not appreciably changed although quantitative cleavage of the terminal sialic acid was obtained. Also, the electrophoretic heterogeneity of the whole myeloma protein was only slightly altered after digestion with the enzyme, although the over-all mobility of the protein was markedly changed. It seems plausible to infer from this that the sialic acid content of these proteins is, at best, only partially responsible for their electrophoretic heterogeneity and that other factors such as the loss of amide groups are more important in determining the degree of banding (Reisfeld, 1968).

From the results obtained from study of the distribution of monosaccharide residues, it can be concluded that glycopeptide chains are associated with both the Fd fragment and/or the light chain in all proteins having high carbohydrate content. That these glycopeptides are not a mere duplication of the prosthetic group associated with the Fc fragment is indicated by the fact that the sialic acid content of the Fab fragment carbohydrate is four to five times higher than that seen in whole proteins that have all the carbohydrate on the Fc fragment. This difference is also reflected in the sialic acid/fucose ratio of the Fab carbohydrate being 1.1 and the ratio in the low carbohydrate proteins being 0.24.

In the rabbit, most recent evidence indicates two carbohydrate moieties to be present on normal γG. The findings (Fleischman *et al.*, 1963) indicated the attachment of two-thirds of the carbohydrate to the Fc fragment, while the remaining one-third was present as a small glycopeptide which was apparently cleaved from the parent molecule by papain. These results have been corroborated and extended in that a carbohydrate moiety has been identified on that portion of the heavy chain which is present on the pepsin $F(ab')_2$ fragment but absent on the papain Fab fragment (Utsumi and Karush, 1965, 1967). These findings were also confirmed by analysis of the cyanogen bromide peptides of rabbit heavy chains (Piggot and Press, 1967). Sequence studies of the Fc fragment of rabbit γG indicate that the Fc fragment glycopeptide is positioned approximately 150 residues from the C-terminal end of the heavy chain (Hill *et al.*, 1966). In the human, however, it appears that a different situation exists in that both the papain and pepsin Fab fragments of normal γG-globulin contain the same amount of glucosamine (Table II) and the myeloma protein studies gave no definite evidence for a glycopeptide in the region of papain cleavage.

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