# CHEMICAL CHARACTERIZATION OF GLYCOPEPTIDES FROM HUMAN γM-GLOBULINS*

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The three major classes of immunoglobulins γG, γA, and γM contain different amounts of carbohydrate. Considerable data are now available on the number and location of the carbohydrate units on the polypeptide chains of γG-globulins, but as yet little information exists for γM. The γM-globulins have approximately 10% of their mass made up of carbohydrate. In spite of this large mass, neither distribution, linkage, location, nor functional importance of this moiety is known. Among the several reasons for this paucity of information is the difficulty in securing large amounts of pure γM-globulin, plus the problems in structural analysis of complex oligosaccharides.

The present study is devoted to two aspects of the chemistry of the carbohydrate moiety of γM. First is a survey of the carbohydrate composition of several pathological macroglobulins to investigate its constancy of composition. This study demonstrates that macroglobulins can be divided into at least two major groups on the basis of their carbohydrate composition. Second is a study in detail of the carbohydrate portions from highly purified macroglobulins of each group with a view to defining carbohydrate units, including their size and number, as they appear on the parent molecule.

## *Materials and Methods*

*Preparation of γM-Globulin.*—Serum was obtained in the course of plasmapheresis therapy of patients with Waldenström's macroglobulinemia. Sera, selected for euglobulin properties, were either diluted in ten-fold their volumes of distilled water or dialyzed against 1000-fold their volumes of 0.0015 M phosphate, pH 6.0. The centrifuged euglobulin precipitate was dissolved in neutral phosphate-buffered saline, and the solution was cleared by high speed centrifugation or filtration through Millipore filters. Small molecular weight contaminants were removed by filtration through a calibrated column of Sephadex G-200 having a total

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bed volume of 800 ml. Fractions corresponding to the elution volume for γM were pooled and concentrated by pressure dialysis.

Purity of preparations was ascertained by analytical ultracentrifugation using a Beckman model E ultracentrifuge, zone electrophoresis on cellulose acetate membranes using a Beckman microzone electrophoresis apparatus, and immunodiffusion studies in 1% Agarose.

Several grams of individual macroglobulins were prepared prior to carrying out steps to isolate the glycopeptides.

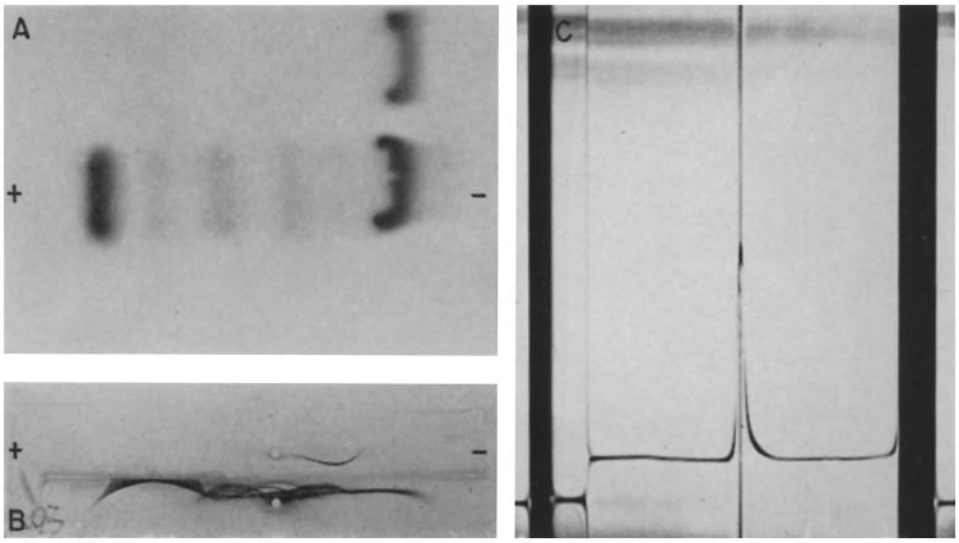


FIG. 1. Analyses of purified γM. A, zone electrophoresis of purified γM compared to whole serum. B, immunoelectrophoresis of purified γM compared to whole serum. C, ultracentrifugation of purified γM.

*Pronase Digestion of Macroglobulin.*—A major part of the protein portion of macroglobulin was removed by pronase digestion. γM at a concentration of 10 ± 2 mg/ml was incubated with the protease from *Streptomyces griseus* (Sigma Chemical Co., St. Louis, Mo.) at a ratio of 100:1 (w/w) in $1 \times 10^{-3}$ M $Ca^{++}$. The protease had been further purified by passage through Sephadex G-100. The solution was incubated at 60°C until ultracentrifugation showed that no macromolecules remained (18–36 hr).

*Chemical Analyses.*—Protein of macroglobulin was quantitated by Folin-Ciocalteu method (1) using a macroglobulin standard. The standard was prepared by drying purified macroglobulin (from serum Elsenrath) to a constant weight at 80°C and redissolving the protein in 1.0 N NaOH. Absorbance at 278 mμ was used as an indicator for protein in most column fractionations.

Carbohydrate was determined in several ways. The phenol-sulfuric acid method of Dubois et al. (2) was used for measuring carbohydrate in column fractions. Glucosamine was determined by the Boas modification of the Elson-Morgan reaction (3) following hydrolysis in 2 N HCl for 3 hr at 100°C, and by ion exchange chromatography on the Beckman amino acid analyzer. Sialic acid was determined by the thiobarbituric acid method of Warren (4)

following hydrolysis in 0.1 N $H_2SO_4$ at 80°C for 1 hr. The neutral sugars mannose, fucose, and galactose were separated from hydrolysates by ion exchange chromatography of sugar-borate complexes using the Technicon carbohydrate analyzer, and were detected by the aniline-acetic-orthophosphoric acid method (5). Samples were hydrolyzed in 2 N $H_2SO_4$ for 4 hr at 100°C, desalted by passage through Amberlite IR-120 ($H^+$) and Amberlite IRA-400 ($formate^-$). The desalted sample was lyophilized and resuspended in 2 ml 0.1 M boric acid, pH 8.00, and subsequently applied to the Technicon ion exchange column for carbohydrate separation.

TABLE I

*Carbohydrate Composition of Macroglobulins*

| γM | Mannose | Fucose | Galactose | NAG | Sialic acid | CHO |
|---|---|---|---|---|---|---|
| | *moles/mole γM (890,000 mol wt)* | | | | | % |
| Group I | | | | | | |
| Loos | 249 | 50 | 72 | 184 | 45 | 11.80 |
| Elsenrath | 218 | 43 | 68 | 136 | 30 | 8.98 |
| Anderson | 242 | 46 | 62 | 208 | 36 | 12.89 |
| Dauphine | 195 | 38 | 67 | 120 | 0 | 9.21 |
| Cousins 1 | 197 | 57 | 96 | 111 | 35 | 10.72 |
| Cousins 2 | 190 | 55 | 93 | 111 | 35 | 10.55 |
| Average | 220 ± 25 | 47 ± 7 | 73 ± 13 | 152 ± 42 | 29 ± 17 | 10.69 ± 1.49 |
| Group II | | | | | | |
| Busby | 143 | 29 | 58 | 179 | 9 | 7.43 |
| Boyer | 122 | 31 | 43 | 144 | 3 | 7.30 |
| Baldwin | 140 | 28 | 47 | 192 | 44 | 8.48 |
| Average | 135 ± 11 | 29 ± 1 | 49 ± 8 | 172 ± 25 | 19 ± 22 | 7.71 ± 0.65 |

Amino acid analyses were performed on a Beckman amino acid analyzer following hydrolysis in 6 N HCl for 22 hr *in vacuo*.

*Electrophoresis of Carbohydrates.*—The glycopeptides were electrophoresed on cellulose acetate membranes in barbital buffer, pH 8.6. The membranes were stained for carbohydrate with a 0.1% solution of Alcian blue in ethanol-acetic acid (9:1) for 15 min and decolorized with 100% ethanol.

## RESULTS

*Isolation of γM-Globulins.*—The macroglobulins studied had euglobulin properties. Steps of euglobulin formation, ion exchange chromatography, and gel filtration generally effected purification of the macroglobulin. In some cases, zone electrophoresis on 0.5% Agarose gel was also used in the purification procedure.

The homogeneity of the purified γM was established using several criteria: Zone electrophoresis on cellulose acetate strips, immunoelectrophoresis, im-

mune diffusion, and ultracentrifugation. Examples of these tests on purified samples are shown in Fig. 1 *a*, *b*, *c*.

*Survey of Macroglobulin for Carbohydrate.*—A survey of several purified macroglobulins was made for carbohydrate composition. Purified whole macroglobulin was hydrolyzed under conditions appropriate for each sugar being analyzed. Sugars were measured in these hydrolysates using standard carbohydrate analyses. The neutral sugars were separated chromatographically prior to analysis. Variation was found in the carbohydrate portions of these proteins, as is shown in Table I. It is seen that not only did the total quantity of carbohydrate vary from 7.30% to 12.89% but also that variation was found for the molar ratios of the individual sugars. However, it is also evident that, based on carbohydrate composition, two major groups of macroglobulin can be described. Five of eight macroglobulins investigated belong to group I which is characterized by total carbohydrate content of 10.69% with a relatively high concentration of mannose. Group II macroglobulin contains only 7.71% carbohydrate with the deficiency evident in the hexoses, especially mannose. *N*-acetylglucosamine (NAG) is apparently constant between the two groups. There was no obvious relationship of L-chain subclass or electrophoretic mobility to these two groups. Evidence that sugar composition did not vary with

TABLE II

*Mole Ratios of Groups I and II Compared to Published Values*

| γM | Mole ratios of sugars | | Total carbohydrate |
|---|---|---|---|
| | NAG | Mannose + galactose | |
| | | | % |
| Group I | 100 | 203 ± 46 | 10.69 ± 1.49 |
| Group II | 100 | 108 ± 9 | 7.71 ± 0.65 |
| Chaplin et al. (9) | 100 | 196 | 8.86 |
| Miller & Metzger (6) | 100 | 182 | 10.22 |
| Müller-Eberhard et al. (11) | 100 | 232 | 12.22 |
| | 100 | 244 | 11.71 |
| | 100 | 218 | 11.39 |
| | 100 | 234 | 11.48 |
| | 100 | 234 | 10.66 |
| | 100 | 218 | 11.08 |
| Müller-Eberhard and Kunkel (10) | 100 | 192 | 9.80 |
| Norberg (13) | 100 | 222 | 9.30 |
| Schultze et al. (12) | 100 | 159 | 11.20 |
| Average of published values..... | 100 | 212 ± 26 | 10.70 ± 1.06 |

the length of storage of frozen whole serum is shown by the similarities of the carbohydrate of the proteins marked Cousins 1 and 2, samples from the same patient taken over 1 yr apart. That these differences in sugar composition are real and not due to analytical error will be shown in the following sections where the differences will be found also in purified carbohydrate preparations. In no case where hexosamine determination was confirmed by chromatography on an amino acid analyzer was galactosamine found.

The relationship of these two groups to published values for the carbohydrate

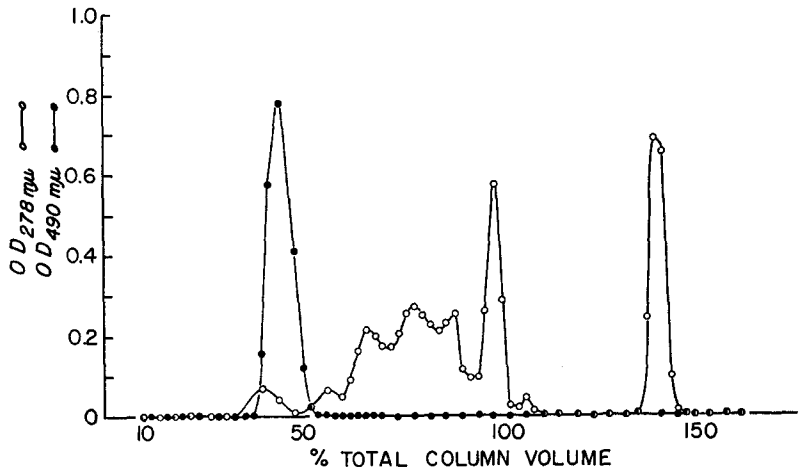


FIG. 2. G-25 Sephadex filtration of γM-glycopeptide. Macroglobulin (Loos) was degraded 22 hr at 60°C with pronase (100:1, w/w). The digest was applied to a G-25 Sephadex column (fine grade, 3.5 × 85 cm) and eluted with water: •, carbohydrate; ∘, $OD_{278}$.

composition of macroglobulin is presented in Table II. Since only one published report has separate values for the neutral sugars (6), we have recalculated our molar ratios so that the sum of mannose and galactose is compared to NAG, to allow comparison between these groups and published values. The total carbohydrate content is also compared. It is apparent that all published values for the carbohydrate of macroglobulin describe group I macroglobulin. The averages of the published values are the same as group I, both with respect to mole ratios of hexoses to NAG and the total carbohydrate content. Therefore, group II represents a previously undescribed macroglobulin subgroup, at least with respect to its carbohydrate. Much greater numbers of macroglobulins will have to be examined before generalizations can be made, of course, but the possibility of subgroups of macroglobulins on the basis of carbohydrate composition is intriguing.

*Isolation of Glycopeptides.*—In an attempt to elucidate the differences in carbohydrate composition between the two groups of macroglobulin, the glyco-

peptide subunits were isolated from one macroglobulin from each group (Loos from group I, Busby from group II). It should be stated at the onset that no significant differences were found between the two macroglobulins during purification of the carbohydrate subunits. The glycopeptide portions of a pronase digest of the two macroglobulins were isolated by passage through Sephadex columns. Initial separation of the glycopeptides from amino acids and small peptides was achieved by filtration through G-25 Sephadex. Fig. 2 shows that glycopeptides were found in only one peak which appeared near the void

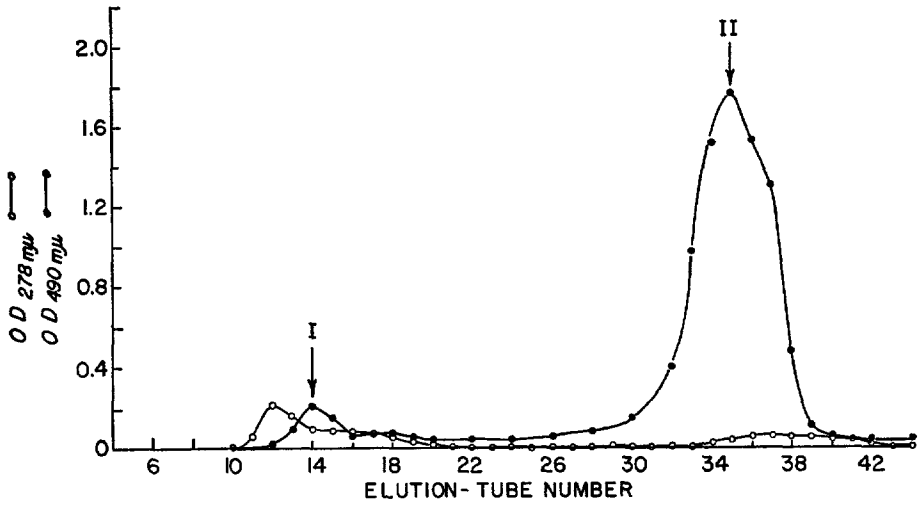


FIG. 3. G-100 Sephadex filtration of γM-glycopeptide. The fractions shown in Fig. 2 which contained carbohydrate were lyophilized and redissolved in water. After Millipore filtration, the filtrate was applied to a G-100 Sephadex column (3.7 × 66 cm) and eluted with water: •, carbohydrate; ○, $OD_{278}$.

volume of this Sephadex column. The fractions containing glycopeptide were concentrated by lyophilization and redissolved in water. After Millipore filtration, the filtrate was applied to a G-100 Sephadex column (Fig. 3) and eluted with water. A small peak which appears to be associated with protein elutes near the void volume. The second peak, which is large and asymmetric, contained the majority of carbohydrate and was submitted to chemical analysis. The absorption spectrum of the crude glycopeptide of γM indicated that the materials contained no tryptophan or tyrosine.

The high concentration of sialic acid in macroglobulin makes the electrophoretic properties of the carbohydrate of interest. A 10 mg/ml water solution of the crude glycopeptide of each γM was electrophoresed in barbital buffer at pH 8.6 for 20 min at 250 v. As is shown in Fig. 4, two distinct bands were formed. It is known that sialic acid may be easily cleaved from glycoproteins

during hydrolysis and preparative procedures. It was therefore thought possible that the two bands could represent the same carbohydrate moiety which had lost varying amounts of sialic acid. However, glycopeptides from four subsequent macroglobulins showed exactly the same two bands on electrophoresis. In addition, analysis showed the bands to be chemically distinct.

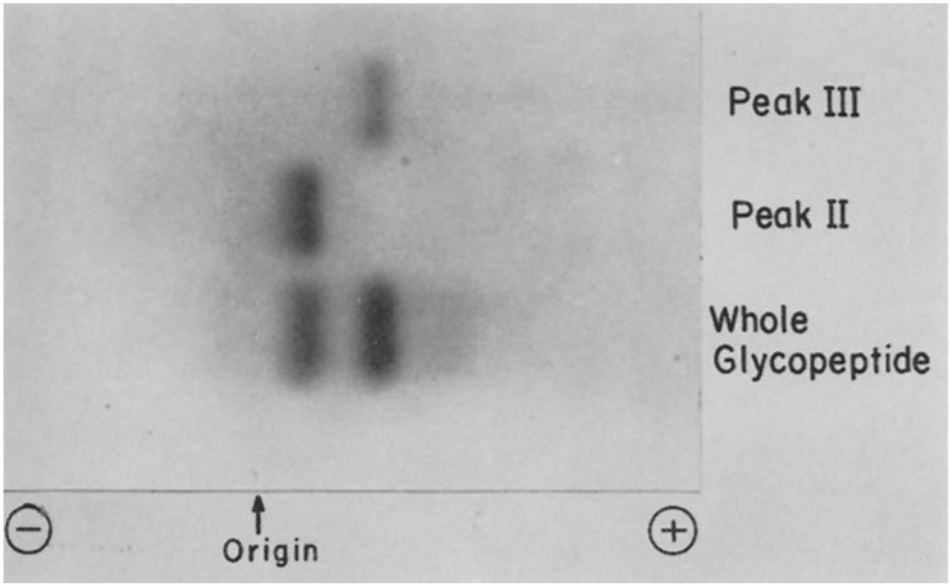


FIG. 4. Electrophoretic pattern of γM glycopeptide. The crude γM-glycopeptide of Loos or DEAE-fractionated glycopeptide (see Fig. 5) was dissolved in water. 3 μl of each solution was applied to a cellulose acetate membrane and electrophoresed in barbital buffer, pH 8.6, in a Beckman Microzone electrophoresis apparatus using the same conditions as for electrophoresis of serum. The membranes were stained for carbohydrate with 0.1% Alcian blue in ethanol-acetic acid (9:1) for 15 min and decolorized in 100% ethanol. Top line, third peak from DEAE chromatography, approximately 4 mg/ml (see Fig. 5). Middle line, second peak from DEAE chromatography, approximately 4 mg/ml (see Fig. 5). Bottom line, crude glycopeptide, 10 mg/ml.

Separation of the two bands was achieved using diethylaminoethyl cellulose. A sample of the glycopeptide was added to a DEAE column which had been charged with phosphate and washed extensively with distilled water. Glycopeptides were eluted using an ionic gradient prepared by a Phoenix four-chamber gradient apparatus; the first three chambers contained 50 ml distilled water, and the final chamber contained 50 ml 0.1 M $(NH_4)_2CO_3$. The fractions were assayed for carbohydrate with the resultant elution pattern as shown in Fig. 5. The three peaks each were lyophilized and redissolved in 1 ml of water.

The isolated peaks were electrophoresed as before (Fig. 4). The first peak, glycopeptide I, was diffuse electrophoretically at this pH and could not be seen. The second peak, glycopeptide II, was shown to correspond to the slow-moving band on electrophoresis. The third peak, glycopeptide III, corresponded to the

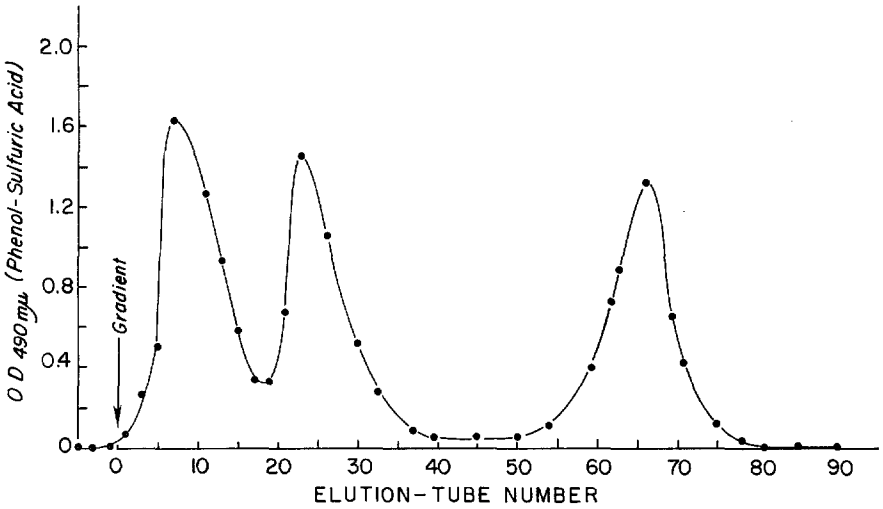


FIG. 5. DEAE chromatography of γM-glycopeptide. 10 mg of crude glycopeptide (Loos) was added to DEAE-cellulose column (1 × 8 cm). Fractions were eluted by ionic gradient (water vs. 0.1 M $(NH_4)_2CO_3$) and assayed for carbohydrate.

TABLE III

*Recovery of Carbohydrate*

| γM | Dry weight | Carbohydrate | | Recovery of carbohydrate |
|---|---|---|---|---|
| | *mg* | %* | *mg*‡ | % |
| Loos γM | 1396 | 11.80 | 164 | — |
| Glycopeptides I + II + III | 459 | 33.60 | 154 | 94 |
| Busby γM | 940.8 | 7.43 | 69.9 | — |
| Glycopeptides I + II + III | 126.0 | 49.00 | 61.8 | 88 |

* Summation of percentage composition of individual sugars.

‡ Per cent × mg dry weight.

fast-moving band on electrophoresis. Again, both macroglobulins showed identical characteristics during purification of the glycopeptides.

*Distribution of Sugars in Glycopeptide Fractions.*—Table III is a summary of yield and purification of the carbohydrate portions from these macroglobulins. It is seen that essentially all of the carbohydrate can be accounted for, with a considerable reduction in protein.

The three glycopeptides from each γM which were separated on DEAE cellulose were analyzed chemically with results shown in Tables IV and V. Considering Loos glycopeptides (Table IV), it is seen that considerable amounts of protein still remain attached to the carbohydrates. Glycopeptides I and II are completely deficient in fucose and sialic acid and therefore consist of the

TABLE IV
*Chemical Composition of Glycopeptides from Group I γM (Loos)*

| | Glycopeptide | | |
|---|---|---|---|
| | I | II | III |
| | *μmoles/mg* | | |
| Sugars | | | |
| Mannose | 0.304 | 1.210 | 0.990 |
| Fucose | <0.01 | <0.01 | 0.408 |
| Galactose | 0.217 | 0.143 | 0.407 |
| NAG | 0.336 | 0.274 | 0.860 |
| Sialic acid | <0.01 | <0.01 | 0.322 |
| Amino acids | | | |
| Aspartic acid | 0.247 | 0.406 | 0.378 |
| Threonine | 0.427 | 0.333 | 0.054 |
| Serine | 0.502 | 0.550 | 0.158 |
| Glutamic acid | 0.345 | 0.200 | 0.421 |
| Proline | 0.753 | 1.653 | 0.129 |
| Glycine | 0.635 | 0.473 | 0.036 |
| Alanine | 0.430 | 0.490 | 0.076 |
| Valine | 0.397 | 0.483 | 0 |
| Isoleucine | 0.162 | 0.103 | 0.058 |
| Leucine | 0.163 | 0.467 | 0 |
| Lysine | 1.530 | 0 | 0.032 |
| Arginine | 0.503 | 0 | 0 |
| Carbohydrate, % | 12.69 | 30.45 | 48.98 |
| Amino acids, % | 85.00 | 66.52 | 46.70 |
| Recovery, % | 97.69 | 96.97 | 95.68 |

three sugars, mannose, galactose, and NAG, but in differing proportions. Glycopeptide III contains all five sugars as well as all of the fucose and sialic acid. Glycopeptide III has a much smaller amount of amino acids remaining which consists primarily of aspartic and glutamic acids. A remarkable point concerning the amino acid composition of the glycopeptides is the high concentration of proline in glycopeptide II.

Busby glycopeptides (Table V), on the other hand, have much smaller peptide fragments. In addition, fucose is present in glycopeptide II in distinction to that found for Loos glycopeptide II. However, the first two glycopeptides are

also deficient in sialic acid. The very high concentration of galactose in glycopeptide II and the lower proline content is also in contrast to the findings in Loos.

Table VI presents the carbohydrate values in terms of residues per molecule

TABLE V

*Chemical Composition of Glycopeptides from Group II γM (Busby)*

| | Glycopeptide | | |
|---|---|---|---|
| | I | II | III |
| | *μmoles/mg* | | |
| Sugars | | | |
| Mannose | 0.750 | 0.744 | 0.544 |
| Fucose | <0.01 | 0.246 | 0.104 |
| Galactose | 0.323 | 1.100 | 0.114 |
| NAG | 0.884 | 0.474 | 0.646 |
| Sialic acid | <0.01 | <0.01 | 0.042 |
| Amino acids | | | |
| Aspartic acid | 0.369 | 0.398 | 0.328 |
| Threonine | 0.255 | 0.120 | 0.127 |
| Serine | 0.261 | 0.302 | 0.214 |
| Glutamic acid | 0.126 | 0.202 | 0.175 |
| Proline | 0.099 | 0.155 | 0.269 |
| Glycine | 0.078 | 0.071 | 0.042 |
| Alanine | 0.102 | 0.131 | 0.114 |
| Valine | 0.105 | 0.172 | 0.039 |
| Isoleucine | 0.144 | 0.095 | 0.071 |
| Leucine | 0.048 | 0.074 | 0.039 |
| Lysine | 0.156 | 0.106 | 0.065 |
| Histidine | 0.093 | 0.052 | 0.084 |
| Carbohydrate, % | 38.93 | 53.30 | 49.28 |
| Amino acids, % | 49.37 | 27.40 | 47.83 |
| Recovery, % | 88.30 | 80.90 | 97·11 |

of γM. In this way differences in yield of glycopeptides are taken into account. The greatest amount of sugars is seen to be present in the glycopeptide III. It was possible that the carbohydrate differences of groups I and II could be determined by the presence or absence of a single glycopeptide. However, analysis of the glycopeptides as shown in Table VI demonstrated this not to be the case. Rather, each glycopeptide differs between the two macroglobulins. However, most of the excess mannose in group I (Loos) macroglobulin is found in glycopeptide II. The relative deficiency of mannose in glycopeptide II of the

group II protein is partly corrected by an increase in galactose content and by the presence of fucose.

*Molecular Weight Determination of Glycopeptide Fractions.*—The absolute number of sugar residues per unit, of course, requires a determination of molecular weight. Estimation of the molecular weight of the glycopeptides was achieved by comparing elution volumes from G-50 Sephadex of proteins of various molecular weights with the glycopeptides. Andrews (7) has demonstrated that analytical Sephadex gel filtration often gives high molecular weight

TABLE VI

*Distribution of Sugars in Glycopeptides*

| Sugars | γM | Glycopeptide | | |
|---|---|---|---|---|
| | | I | II | III |
| | *residues/molecule γM* | | | |
| Group I (Loos) | | | | |
| Mannose | 249 | 27 | 92 | 120 |
| Fucose | 50 | 0 | 0 | 53 |
| Galactose | 72 | 17 | 11 | 52 |
| NAG | 184 | 13 | 21 | 111 |
| Sialic acid | 45 | 0 | 0 | 41 |
| Group II (Busby) | | | | |
| Mannose | 143 | 20 | 22 | 95 |
| Fucose | 29 | 0 | 8 | 18 |
| Galactose | 58 | 9 | 33 | 20 |
| NAG | 179 | 24 | 24 | 113 |
| Sialic acid | 9 | 0 | 0 | 7 |

values for glycoproteins. For this reason the values given here must be considered as approximations until confirmation is obtained by other means. Crude glycopeptides of both Loos and Busby demonstrated heterogeneity of size on G-50 (80 × 1.5 cm) equilibrated with neutral phosphate-buffered saline. Glycopeptide III of Loos eluted at a volume consistent with a molecular weight of 6000 to 7000 whereas glycopeptides I and II corresponded to molecular weights of 3000 to 4000. Similar elution values were obtained from Busby glycopeptides.

Since complete chemical composition is known for the glycopeptides, it was possible to use the estimated molecular weight to assign most probable values for each residue. With these values one can estimate a more precise molecular weight and calculate the number of units present on the molecule. Table VII summarizes these calculations.

It should be emphasized that these values are not the only ones consistent

with the data, but were considered the most probable since they did not require the added assumption of heterogenous distribution among the protein chains.

## DISCUSSION

The data indicate that macroglobulins can be divided, on the basis of carbohydrate composition, into at least two groups. These groups differ in the con-

TABLE VII

*Composition of Carbohydrate Units*

| γM | Glycopeptide | | |
|---|---|---|---|
| | I | II | III |
| | *residues/unit (to nearest ½ integer)* | | |
| Group I (Loos) | | | |
| Mannose | 3.0 | 9.0 | 6.0 |
| Fucose | 0 | 0 | 2.5 |
| Galactose | 2.0 | 1.0 | 2.5 |
| NAG | 1.0 | 2.0 | 5.5 |
| Sialic acid | 0 | 0 | 2.0 |
| Mol wt (calculated) | 1122 | 2244 | 3812 |
| Units/molecule γM | 10 | 10 | 20 |
| Group II (Busby) | | | |
| Mannose | 2.0 | 2.0 | 5.0 |
| Fucose | 0 | 1.0 | 1.0 |
| Galactose | 1.0 | 3.5 | 1.0 |
| NAG | 2.5 | 2.5 | 5.5 |
| Sialic acid | 0 | 0 | 1.0 |
| Mol wt (calculated) | 1095 | 1709 | 2776 |
| Units/molecule γM | 10 | 10 | 20 |

centrations of mannose, fucose, and galactose, and also in their total carbohydrate content. Studies are in progress to determine whether these carbohydrate groups might correspond to any of the antigenic differences known to exist in human macroglobulins. As pointed out previously, there is no correlation with the light chain antigens K and L. During the preparation of this manuscript, Abel et al. (8) published data for γG-glycoprotein showing subgroups separable on the basis of hexose content. These were found to bear no relationship to γ-chain subgroups.

A relatively constant chemical composition has been described for γM-globulin carbohydrate (6, 9–13). All descriptions present in the literature would fall into our group I class of carbohydrate. The reasons why almost 40% of

macroglobulins of the present series represent the undescribed group II, and why none of the previous macroglobulins analyzed for carbohydrate were of this group, is not clear.

Since the present study is the first to separate chromatographically the neutral sugars, it is perhaps appropriate to correct two minor misconceptions concerning macroglobulin carbohydrate. The first is that macroglobulins of carbohydrate group I contain three moles, instead of two (6), of mannose per mole of galactose. Secondly, fucose is present to a higher concentration than is indicated by previous publications. The colorimetric analysis of whole macroglobulins for fucose gives results that are less than that found when fucose is isolated chromatographically (J. M. Davie, personal observation).

One macroglobulin was chosen from each carbohydrate group for detailed analysis of its carbohydrate units. The number and size of the carbohydrate units on a single $\gamma$M-molecule can be estimated. Miller and Metzger (14) have described two glycopeptides from a trypsin-digested macroglobulin which were separable according to size. In addition, Clamp et al. (15) have recently reported in abstract form glycopeptides from $\gamma$M which have similarity to those described here. Two carbohydrate units were described: one containing mannose, galactose, and NAG (3:2:3) with variable amounts of fucose and sialic acid, and the other containing mannose and NAG in ratios from 2:1 to 4:1. In our own studies, it has been possible to isolate three populations of carbohydrate which were separable according to charge and size. Chemical analyses on the separated glycopeptides from both groups show that the carbohydrates are found mainly in a large oligosaccharide consisting of all five sugars, as well as two small units made up of galactose, mannose, and NAG, with the addition of fucose in glycopeptide II of group II. Estimation of the molecular weights of these units with the assumption of homogeneous distribution of glycopeptides among the chains would indicate that the carbohydrates consist of the following. For group I macroglobulins, 10 units per molecule $\gamma$M of a six-sugar oligosaccharide containing mannose, galactose, NAG (3:2:1) with a molecular weight of 1122; 10 units of a twelve-sugar oligosaccharide containing mannose, galactose, NAG (9:1:2) with a molecular weight of 2244; and 20 units of a third type containing mannose, galactose, fucose, NAG, and sialic acid (6:2.5:2.5:5.5:2) with a molecular weight of 3812. The third type is possibly a mixture of two similar oligosaccharides with different mole ratios of fucose, galactose, and NAG. For group II $\gamma$M, carbohydrate subunits consist of 10 units per molecule of a five-sugar moiety of mannose, galactose, and NAG (2:1:2); 10 units of an octasaccharide consisting of mannose, fucose, galactose, and NAG (2:1:3:2); and 20 units of a fourteen-sugar oligosaccharide containing mannose, fucose, galactose, NAG, and sialic acid (5:1:1:6:1).

Multiple carbohydrate units have been shown to exist in four other globulins. Dawson and Clamp (16) in a recent study showed that the carbohydrate of

human γA was distributed among six oligosaccharide units. Two of the units consisted of galactose, and *N*-acetylgalactosamine (3:3). Three of the units contained fucose, mannose, galactose, NAG, and sialic acid (0–1:3:2:3–4:0–3). The last unit contained mannose and NAG (4:3). The distribution of these units between the two heavy chains of γA must therefore be asymmetrical. Dunn and Spiro (17) described 31 carbohydrate units of α2-macroglobulin. In addition, Spiro (18) showed that thyroglobulin contained two distinct carbohydrate units. Abel et al. (8) showed in addition to multiple carbohydrate units, variability in the site of these units on the γG-molecule. The exact location of the carbohydrate units along the polypeptide chain in γM will have to await further structural characterization of the proteins.

Immunoglobulins have been classified in the past according to differences in charge, size, antigenicity, and chemical composition (carbohydrate + amino acids). The present studies suggest that further subclassification may be possible due to qualitative differences in the carbohydrate portion of the γM-globulins. To date it has not been possible to demonstrate any antigenicity of the carbohydrate[1] so that it is doubtful that it plays a role in the antigenic subclassifications.

The precise role of the carbohydrate portion of glycoproteins has not yet been elucidated. Most studies to date have suggested that it plays an important part in the structural and physicochemical properties of the proteins while not being associated with the actual biological activity. The appearance of carbohydrate subgroups on human γM-globulins is of particular interest. Antibody molecules are characterized by a heterogeneity of molecular form and biological activity. It is interesting to speculate on the significance of the carbohydrate on some of the diverse functions of antibody proteins.

## SUMMARY

A survey of human pathological macroglobulins revealed that γM can be divided into at least two groups on the basis of carbohydrate composition. Differences between the two groups exist in the total percentage of carbohydrate (10.69 ± 1.49% for group I, 7.71 ± 0.65% for group II) which is attributable to variation in hexose content. Glycopeptides from macroglobulins of each group were purified from pronase digests and characterized chemically. Macroglobulins from each group contain three types of oligosaccharides. Glycopeptide I for each group consisted of mannose, galactose, and NAG with a ratio of 3:2:1 for group I and a ratio of 2:1:2 for group II. Glycopeptide II consisted of mannose, galactose, and NAG (9:1:2) for group I, and mannose, fucose, galactose, and NAG (2:1:3:2) for group II. Glycopeptide III in both groups consisted of mannose, fucose, galactose, NAG, and sialic acid with a ratio of 6:2.5:2.5:5.5:2 for group I and a ratio of 5:1:1:6:1 for group II. Molecular

[1] Osterland, C. K., and J. M. Davie. Data to be published.

weight estimations by gel filtration indicates that there are 10 glycopeptides I and II and 20 units of glycopeptide III per molecule of γM.

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