An Electrophoretic Study of Serum Protein-bound Carbohydrate in Dysproteinemia

David J. Evenson, Warren F. McGuckin, Bernard F. McKenzie, and Albert B. Hagedorn

The glycoprotein hexose in the sera of patients having an electrophoretically homogeneous globulin fraction was studied by paper electrophoresis. The largest diagnostic category was multiple myeloma, but also included were cases of macroglobulinemia, primary systemic amyloidosis, lymphoma, collagen vascular disease, liver disease, and other conditions. The hexose contents of the pathologic globulin fractions were compared; only in the macroglobulins was this value of diagnostic significance, consistently averaging more than 3 times that in normal gamma globulin. In myeloma globulins there was a relationship between the hexose content and the electrophoretic mobility, with only an occasional value approaching the macroglobulin range in the faster electrophoretic mobilities.

It is shown that paper electrophoresis and the PAS staining technic, in conjunction with protein staining on a duplicate sample of serum, constitute a useful screening test for detection of increased concentration of macroglobulin in serum in a large percentage of cases of "hypergammaglobulinemia." Gel electrophoresis, immunologic typing, and ultracentrifugation studies then can be used for further elucidation of the type of abnormality present.

The abnormalities in electrophoretic patterns of serum proteins formerly identified with multiple myeloma are now considered less specific. The electrophoretic demonstration of an increased amount of protein in the beta or gamma mobility regions also has been found associated regularly with primary macroglobulinemia of Waldenström and with primary systemic amyloidosis (1). In addition, globulin

From the Mayo Clinic and Mayo Foundation, Rochester, Minn.

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fractions of sufficiently narrow range of mobility to suggest homogeneity have been found in serum in other diseases in which hypergammaglobulinemia characteristically is present (Fig. 1).

These abnormal globulins, like other serum globulins, contain some glycoprotein. Recent study (2) has indicated that the abnormal globulins in macroglobulinemia generally contain considerably more carbohydrate than those in multiple myeloma, but the reported values have varied over a rather wide range in both conditions. The study described in this paper was undertaken to define further the variations in the carbohydrate content of the abnormal globulins found in the plasmacytic proliferative diseases, and to see if newer laboratory methods could narrow the range in Waldenström’s macroglobulinemia.

Material and Methods

Selection of Patients

Patients were selected for this study on the basis of the electrophoretic pattern of their serum protein. The criterion was the presence of a well-defined, moderately narrow, symmetric band that was sufficiently abnormal in amount, position, or both to suggest the diag-
nosis of multiple myeloma. In multiple myeloma, most abnormal protein bands are characterized by this feature; however, some may be broader and less symmetric—particularly those with electrophoretic mobility faster than gamma myeloma globulin. Occasionally macroglobulin bands also are somewhat less homogeneous. Some lack of homogeneity in molecular size of the macroglobulins and of myeloma globulins has been demonstrated by ultracentrifugal analysis and gel electrophoresis (3-6), and it is possible that these differences in size within the supposed homogeneous protein result in slight differences in migration on paper electrophoresis, producing a wider band. Accordingly, the requirements for electrophoretic homogeneity were less strict in this study than would be necessary for the unequivocal diagnosis of myeloma in order to include some less typical border-line patterns. Figure 1 shows some patterns from patients included in this study that were suggestive but not entirely typical of multiple myeloma.

In almost all cases, stained blood smears and fixed sections of tissue obtained by aspiration of sternal marrow were examined. The diagnosis of macroglobulinemia was confirmed in most instances by demonstration of abnormal amounts of serum protein sedimenting at a velocity in excess of 15 S in the analytical ultracentrifuge. In 3 cases these data were not obtained, and the diagnosis was presumptive, based on failure of the abnormal protein fraction to migrate on gel electrophoresis in 5% acrylamide, on a strongly positive water-dilution euglobulin test, and on a compatible clinical picture. The diagnoses in the miscellaneous group of conditions were based on the usual clinical criteria, including the absence of features of multiple myeloma even though the electrophoretic pattern was suggestive.

Three cases were placed in a separate category designated “mixed” since in each case some features of myeloma, particularly atypical plasmacytosis, were present in addition to a more prominent picture of another disease.

Laboratory Methods

The total serum protein was determined by the Kingsley biuret method.

Paper Electrophoresis

Paper electrophoresis was performed in the Spinco model of the Durrum ridgepole cell* on Whatman 3 MM paper. A constant current

*Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.
of 0.625 ma. per 3-cm.-wide strip was applied for 15 hr. with a diethylbarbiturate buffer at pH 8.6, ionic strength 0.075. The resultant migration averaged 6 cm. from the point of application to the midportion of the albumin band. In an effort to achieve uniform densitometry, the amount of serum protein applied to strips for protein staining was standardized. From the total concentration of protein in each sample, the volume of serum required to supply 0.45 mg. of protein was calculated. As no measure of total carbohydrate was available in advance, 30 μl. of serum was applied to strips for staining for carbohydrate. After electrophoresis, all strips were dried immediately in an oven at 110° for 15 min.

Staining for Protein

The stain used for detecting protein was 0.1% Amidoschwartz 10B in a mixture of methanol and acetic acid (9:1). The dried strips were immersed in this solution for 20 min. and then washed with agitation 3 times for 10 min. each in a mixture of acetic acid, methanol, and water (200:2850:950). After drying at 110°, the strips were made translucent by immersion in light mineral oil. Excess oil was blotted off and the relative protein distribution was determined in the Spinco Model RA Analytrol* using a combination of Wratten filters No. 22 and 58. Quantitative values for each fraction were then calculated from the known total protein.

Staining for Carbohydrate

Carbohydrate distribution on paper strips was determined according to McGuckin and McKenzie’s modification (7) of Köiw and Grönwall’s periodic acid-Schiff (PAS) technic (8). After a preliminary wash of oven-dried strips with 95% ethanol to remove the buffer salts, the strips were redried and subjected to oxidation with periodic acid under carefully controlled conditions of time, pH, and temperature. This treatment was followed by reduction with potassium metabisulfite-thiosulfate, washing, reaction with leukofuchsin sulfite solution, and then prolonged, repeated rinsing in an acid metabisulfite wash. The oxidizing solution was made up fresh in 50% ethanol in order to improve the penetration of the reagents. This procedure yielded durable patterns with sharp definition of the bands and backgrounds of low density.

Densities were measured on dry unoiled strips in the Spinco Model

*Beckman Instruments, Inc., Spinco Division.
RB Analytrol* using 561-m\(\mu\) interference filters. Recent studies (9, 10) with normal and some abnormal sera have demonstrated empirically that the distribution of glycoprotein carbohydrate, as determined by the periodic acid-Schiff technic, closely parallels that obtained by separate quantitative analysis of the protein fractions for their polysaccharide-hexose content. Values for normal distribution of glycoprotein determined by the technic used here are close to those obtained by other workers and in excellent agreement with those obtained by direct analysis of individual fractions for hexose (7).

**Analysis for Total Hexose**

The total hexose was estimated by Shetlar’s anthrone method (10), employing combination with tryptophan and using a mannose standard. The glycoproteins first were precipitated with 10 volumes of absolute ethanol. The precipitate was washed twice with 95% ethanol, and the dried residue was dissolved in 7 ml. of a reagent containing 100 mg. each of anthrone and tryptophan in 100 ml. of 28N H\(_2\)SO\(_4\). This mixture was heated 17 min. at 76° ± 0.5° and examined at 515 m\(\mu\) in the Bausch and Lomb Spectronic 20 colorimeter† against a reagent blank. The normal value for this method is 100 mg. (±7.5, S.D.) per 100 ml. of serum.

**Test for Euglobulin (Water-Dilution Test)**

This screening test for increased amounts of macroglobulin depends on the euglobulin-like behavior of these proteins, which is demonstrated according to a modification of Sia’s original test (11). When 0.5 ml. of serum is diluted with 8 ml. of distilled water, the appearance of a precipitate indicates a positive result which for our study was graded as weak, moderate, or strong, depending on the density and rates of formation and sedimentation of the precipitate. This test was applied to all sera suspected of containing macroglobulins either on a clinical basis or on the basis of abnormal physical behavior of the serum. Waldenström, in a recent review of this test (12, 13) called attention to the ease with which false-negative or false-positive results may be obtained when the dilution ratio is changed slightly. This sensitivity suggests that, even under standardized testing conditions, precipitation might be influenced by the concentration of such factors as beta lipoprotein, total protein or alpha\(_1\) and alpha\(_2\) globulins.

*Beckman Instruments, Inc., Spinco Division.
†Bausch & Lomb, Incorporated, Rochester, N. Y.
Gel Electrophoresis

Serum samples were applied to paper wicks and inserted into a gel of 5% acrylamide polymer (Cyanogum). The gel was prepared by a method similar to that employed by Raymond (14). Electrophoresis was performed using a Tris-EDTA buffer* at pH 9.0. Migration of all normal serum proteins out of the wick and into the gel was obtained by inserting the sample wick close to the cathode vessel. Failure of an abnormal globulin to leave the wick and migrate into the gel was presumptive evidence of its high molecular weight, asymmetry, or both (15). Evaluation of completeness of migration was by inspection of both the sample wick and the gel after staining for protein. Known macroglobulin and the slow gamma globulin of myeloma sera were used as controls. Our earlier gel electrophoresis was done in 13% starch gels, but the synthetic gel was easier to handle and was found to obstruct macroglobulins in a manner similar to that of starch gels.

Ultracentrifuge Studies

When possible, sera suspected of containing increased amounts of macroglobulin were analyzed in the Spinco analytical ultracentrifuge† for rapidly sedimenting components of the macroglobulin class.

Calculations

Hexose Content of Individual Fractions

For quantitation, the amount (milligrams) of hexose per 100 ml. of serum was calculated for each fraction from the percentage color in the stained area (as measured on the Spinco Analytrol) and multiplied by the total hexose in the serum glycoprotein. In order to compare the relative hexose content of the various abnormal globulins under study, the hexose in the abnormal protein fraction was expressed as a percentage of its associated protein. This value was termed hexose content and calculated as percentage carbohydrate

\[
\text{hexose content} = \frac{\text{mg. hexose/100 ml. serum}}{\text{mg. protein/100 ml. serum}} \times 100
\]

Electrophoretic Mobility

In multiple myeloma, the hexose content of the abnormal globulin fraction is related to the relative electrophoretic mobility (on paper) of that fraction. Accordingly, the paper electrophoretic mobility of

*This buffer contained 80.6 gm. tris(hydroxymethyl)aminomethane, 10.6 gm. disodium EDTA, and 6.3 gm. of boric acid per 8 L. of buffer.
†Beckman Instruments, Inc., Spinco Division.
each of the abnormal fractions in this series was measured (in centimeters) from the point of application to the center of its band. To make the mobility values comparable, it was necessary to compensate for the unavoidable slight variation in speed of migration that occurred in different runs in spite of constant conditions of application, duration, and voltage applied during electrophoresis. This was accomplished by correcting the distances traveled on each strip to a mean albumin migration of 6.0 cm. Anodal migration was designated positive (+) and cathodal migration negative (−). No correction for electroendosmosis was made, since only a relative measurement for comparison of cases in this series was desired.

When this calculation was applied to normal sera subjected to the same method of electrophoresis, the gamma globulins covered a range between −2 and +1 cm., with the bulk of this protein lying between 0 and −1 cm. The beta and α₂ fractions moved an average of +1.7 and +3.3 cm., respectively. It was convenient to classify all abnormal globulins according to electrophoretic mobility in the following arbitrary manner: very slow gamma, −2 to −3 cm.; slow gamma, −1 to −2 cm.; intermediate gamma, 0 to −1 cm.; fast gamma, 0 to +1 cm.; beta, +1 to +2 cm.; and fast beta, +2 to +3 cm.

Results

The abnormal globulins studied were from sera from patients with conditions diagnosed as shown in Table 1. The average hexose content

<table>
<thead>
<tr>
<th>Diagnostic category</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple myeloma</td>
<td>44</td>
</tr>
<tr>
<td>Macroglobulinemia</td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>5</td>
</tr>
<tr>
<td>Secondary</td>
<td>2</td>
</tr>
<tr>
<td>Miscellaneous dysproteinemias</td>
<td></td>
</tr>
<tr>
<td>Primary systemic amyloidosis</td>
<td>2</td>
</tr>
<tr>
<td>Malignant lymphoma</td>
<td>2</td>
</tr>
<tr>
<td>Metastatic carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Essential cryoglobulinemia</td>
<td>1</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>2</td>
</tr>
<tr>
<td>Idiopathic hypergamma globulinemia</td>
<td>2</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
</tr>
<tr>
<td>Multiple myeloma and amyloidosis</td>
<td>1</td>
</tr>
<tr>
<td>Multiple myeloma and hepatic cirrhosis</td>
<td>1</td>
</tr>
<tr>
<td>Multiple myeloma and lichen myxedematosis</td>
<td>1</td>
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</tbody>
</table>
Table 2. Hexose Content of Abnormal Protein in Sera of Patients in Various Diagnostic Categories

<table>
<thead>
<tr>
<th>Diagnostic category</th>
<th>No. of patients</th>
<th>Hexose content*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Mode</td>
<td>Range</td>
</tr>
<tr>
<td>Multiple myeloma†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very slow gamma</td>
<td>1</td>
<td>0.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Slow gamma</td>
<td>12</td>
<td>0.71</td>
<td>0.34</td>
<td>0.15-2.42</td>
</tr>
<tr>
<td>Intermediate gamma</td>
<td>14</td>
<td>1.04</td>
<td>0.71</td>
<td>0.34-3.0</td>
</tr>
<tr>
<td>Fast gamma</td>
<td>9</td>
<td>3.40</td>
<td>3.50</td>
<td>0.72-4.8</td>
</tr>
<tr>
<td>Beta</td>
<td>6</td>
<td>3.20</td>
<td>3.15</td>
<td>2.9-3.5</td>
</tr>
<tr>
<td>Fast beta</td>
<td>2</td>
<td>3.20</td>
<td>3.20</td>
<td>2.8-3.6</td>
</tr>
<tr>
<td>All mobilities</td>
<td>44</td>
<td>1.80</td>
<td>—</td>
<td>0.15-4.8</td>
</tr>
<tr>
<td>Macroglobulinemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>5</td>
<td>6.0</td>
<td>5.3</td>
<td>4.6-6.7</td>
</tr>
<tr>
<td>Secondary</td>
<td>2</td>
<td>4.7</td>
<td>4.6</td>
<td>4.6-4.8</td>
</tr>
<tr>
<td>Miscellaneous dysproteinemias</td>
<td>10</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5-2.9</td>
</tr>
<tr>
<td>Mixed</td>
<td>3</td>
<td>1.1</td>
<td>0.9</td>
<td>0.4-1.3</td>
</tr>
<tr>
<td>Normal gamma globulin</td>
<td>—</td>
<td>1.7</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*See text for calculation.
†See text for definition of subcategories.

of the abnormal globulin in each diagnostic category was calculated; these values are given in Table 2. The myeloma category was subdivided according to the electrophoretic mobility of the abnormal component. The hexose content of normal gamma globulin, determined by the same method, averaged 1.7%.

Multiple Myeloma

The slow gamma myeloma proteins contained the least hexose, the values varying for the most part about a mode of 0.34%. A greater mean value, 0.71%, resulted from an exceptionally high hexose content (2.42%) in one case. In this case, the clinical findings were typical of myeloma, and the euglobulin test and gel electrophoresis failed to demonstrate macroglobulin. Nevertheless the presence of some of this protein with high hexose content could not be excluded, since the serum was not analyzed in the ultracentrifuge. The intermediate gamma myeloma globulins had a greater mean hexose content (1.04%), but the mode was only 0.71%. In 3 of the 14 cases, the values for hexose content was 2.4, 2.0, and 3.0%.

Macroglobulinemia

The mean hexose content in all cases of macroglobulinemia, primary and secondary, was 5.8%, and the individual values ranged from 4.6
to 6.7% (Fig. 2). When considered separately, the abnormal fractions of secondary macroglobulinemia contained less hexose (mean, 4.7%) than those of primary macroglobulinemia. The only instances of hexose contents of less than 5%, and consequently in the upper range of the globulins in multiple myeloma, were in the secondary category.

**Fig. 2.** Hexose contents of macroglobulin fractions from individual patients with primary (open circle) and secondary (solid circle) macroglobulinemia plotted against electrophoretic mobility. Broken line indicates maximal hexose contents of myeloma globulins in various mobility ranges.

**Miscellaneous Dysproteinemias**

There were 10 patients with diagnoses in this category. The electrophoretic patterns of sera from these patients, although suggestive of multiple myeloma, were different in a number of ways. The abnormal protein was not present in the amount usually found in multiple myeloma and had a less homogeneous appearance on electrophoresis (Fig. 1). The values for hexose were similar to those found in myeloma globulins of comparable mobility and ranged from 0.5 to 3.9%, with a mean of 1.5%.

**Mixed**

There were 3 patients with dysproteinemia with some features of multiple myeloma. All had atypical plasma cells in the marrow, but the outstanding clinical features were those of primary systemic amyloidosis, hepatic portal cirrhosis, and lichen myxedematosis, respectively. None had bone lesions or pain. All had typical homogeneous, abnormal, fast gamma globulins but the hexose content of these globulins was uniformly low (0.40–1.3%). As shown in Table 2, this is most unusual for myeloma globulins with this mobility.
Discussion

Methods

The method for analysis of the carbohydrate content of the abnormal protein is based on the assumption that the development of color in the PAS reaction quantitatively reflects the hexose content in each of the serum protein fractions (7). Empirical support for this assumption was found by Björnesjö (9), who demonstrated, in a series of normal sera, that the PAS stain yielded results that closely paralleled values derived from the quantitative determination of the hexose units in each fraction. Shetlar (10) also found good agreement in both normal and abnormal sera between the fraction of hexose calculated from the distribution of PAS stain and the quantitative analysis of individual fractions collected by preparative electrophoresis.

That this good agreement may be more than chance is suggested by recent work (16) which indicates that only the hexose and methylpentose sugars react in the PAS staining process. If this is the case, then for all practical purposes the PAS color represents hexose, since serum glycoproteins contain only minor amounts of methylpentose. With this support, we think that the procedures used in this study were capable of yielding results dependable enough for comparison of the carbohydrate contents of the abnormal serum protein components in the dysproteinemias studied.

Results

Multiple myeloma formed the largest diagnostic category in this study (Table 1). It can be seen that the distribution is not bimodal but closely resembles the distribution of gamma globulin in a normal serum.

Figure 3 shows the average hexose content of the abnormal protein fraction in the 44 cases of multiple myeloma grouped according to electrophoretic mobility. The finding of a progressive increase in hexose content of the abnormal protein fraction in multiple myeloma—from a very low value in the slow gamma to a high value in the fast gamma fraction (Table 2)—has been reported by Laurell and associates (2) and by Müller-Eberhard and Kunkel (17). Among normal gamma globulins, those with greater electrophoretic mobility also contain more carbohydrate. Müller-Eberhard and Kunkel (17) fractionated normal gamma globulin by preparative electrophoresis on polyvinylpyrrolidone and found a hexose content of 1.1% in the slow and 1.8% in the fast fractions. Figure 4 shows the superimposed densitometric
tracings of duplicate paper electrophoretic strips of a normal serum stained for carbohydrate and for protein, and also illustrates the increased proportion of carbohydrate in the fast gamma fraction. From these observations it appears that, in any gamma globulin, normal or

Fig. 3. Average hexose content of myeloma protein in 44 cases of multiple myeloma, grouped according to electrophoretic mobility. Vertical bars indicate average hexose content of myeloma proteins in respective mobility ranges. A normal serum glycoprotein pattern is superimposed in broken line.

Fig. 4. Carbohydrate and protein distribution in normal serum. Densitometric tracings of paired electrophoretic strips stained for protein (solid line) and carbohydrate (broken line) are superimposed. Note relative increase in carbohydrate in fast gamma fraction.
abnormal, there is an increase in carbohydrate content with increased electrophoretic mobility. In myeloma proteins, however, the maximal hexose content is attained in the fast gamma fraction with no further increase in hexose content in the beta fractions. This suggests that an increase in content of acidic carbohydrate is not the only factor responsible for the increased electrophoretic mobility of myeloma globulin, as Smith and associates suggested (18).

The singularly high hexose content of the macroglobulin fractions found in this study agrees with the values found by Laurell and associates (2) using similar technics, but the values obtained in our study varied between narrower limits (5.3–6.7 as compared with their range of 3.5–9.4%). Others, measuring the hexose content of isolated macroglobulins quantitatively, have found similar values—6.0 and 6.1% (19, 20). Di Guglielmo and Antonini (21) first suggested the diagnostic value of this feature of macroglobulins. Laurell and associates (2) also mentioned this possibility, since they found no overlapping between myeloma proteins and macroglobulins when the mobility and hexose content were considered together.

Study of the hexose content of the pathologic globulin fraction of those dysproteinemias not classified as myeloma or macroglobulinemia demonstrated little to distinguish them from multiple myeloma. One exception to this was noted in which the occurrence of an increased hexose content accurately predicted the ultracentrifugal demonstration of increased macroglobulin.

Conclusions

Multiple myeloma and other dysproteinemias in which the abnormal entities are of normal molecular weight were not clearly distinguished from each other in all cases by the hexose content of their abnormal fraction. Only macroglobulinemias were distinctive in that their abnormal globulin contained, on the average, 3 times the hexose (on a percentage basis) of myeloma protein or normal gamma globulin. In the cases of primary macroglobulinemia, the range of the amount of hexose was much narrower than previously obtained by similar technics.

The determination of the hexose content from paired paper electrophoretic strips stained for protein and carbohydrate, as used in this study, provides a reliable screening method for indicating the probable presence of macroglobulins (Fig. 5). Exceptionally fast gamma and beta myeloma globulins contain hexose in amounts approaching the
macroglobulin range. The majority of macroglobulins, however, migrate in the slow or intermediate gamma region where myeloma and other abnormal globulins contain even less hexose than does normal gamma globulin. The procedure is more accurate than the water-

Fig. 5. Densitometric tracings of paired electrophoretic strips stained for glycoprotein carbohydrate (broken lines) and protein (solid lines) in cases selected from various disease categories. Note particularly differences in carbohydrate staining of abnormal globulin. A indicates gamma myeloma; B, "M" myeloma; C, beta myeloma; and D, Waldenström's macroglobulinemia.

dilution euglobulin precipitation test which, in this study, yielded a number of false-positive and at least one false-negative result. The electrophoresis method is particularly well suited to laboratories that are equipped with paper electrophoresis apparatus but not with an analytical ultracentrifuge.

References

14. Raymond, S., personal communication to the authors.