PROTEINS IN MULTIPLE MYELOMA

VII. THE FREE AMINO GROUPS OF BENCE-JONES PROTEINS*

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A century of intermittent investigation has failed to produce a systematic physical and chemical characterization of Bence-Jones proteins that would serve for the classification of these unique urinary constituents in multiple myeloma or for the clarification of their possible metabolic relationship to normal proteins or to pathological serum globulins. Early observations on occasional specimens have suggested that the Bence-Jones proteins excreted by different individuals were not identical (1-4). More recently it has been shown by extensive physical (5, 6), end group (7), and immunological analyses (8, 9) that different patients with multiple myeloma do, in fact, excrete biologically and molecularly dissimilar urinary proteins. However, quantitative amino acid analyses are limited to single specimens without adequate evidence of molecular or electrophoretic homogeneity. Indeed, several hypotheses as to the metabolic origin of Bence-Jones proteins are based upon incomplete analyses, suggesting a methionine deficiency (9, 10). Hence, it seemed imperative to undertake a chemical characterization of a number of specimens that had been well investigated by both electrophoretic and ultracentrifugal analyses. For this purpose, analysis for N-terminal amino acids rather than for total composition has been chosen, for the end group determination serves both as a means of structural characterization and as a criterion of chemical homogeneity. This communication summarizes the molecular properties, the N-terminal groups, and the antigenic types of fourteen Bence-Jones proteins, four of which were crystallized. Although no two of these proteins proved to be identical with each other in all the properties studied, significant correlations have appeared, particularly between the antigenic type and the nature of the N-terminal group. The importance of these observations is considered in reference to the metabolic origin of the proteins.

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EXPERIMENTAL

Isolation of Bence-Jones Proteins—For this study, subjects were selected in whose urine Bence-Jones protein comprised 95 per cent or more of the electrophoretically demonstrable protein.\(^1\) This greatly facilitated the isolation procedure. The Bence-Jones proteins (designated A, B, etc.) include eight specimens previously described (7), five isolated more recently (T, Lu, Bo, He, and Be), and a crystalline protein (Ho) received from another laboratory.\(^2\) The proteins were isolated by ammonium sulfate precipitation; crystallization in some instances was effected by exhaustive dialysis against distilled water (11).

Electrophoretic and Ultracentrifugal Analyses—Electrophoretic and ultracentrifugal analyses were carried out as previously described (5). Mobilities at 0° in Veronal buffer, pH 8.6, are given for the descending boundary and are expressed in units of \(10^{-5}\) cm.\(^2\) sec.\(^{-1}\) volt\(^{-1}\) designated “u.” Sedimentation constants were corrected to water as solvent at 20° (\(s_{20,\text{w}}\)) and are expressed in Svedberg units (S). Because no appreciable effect of concentration upon \(s_{20,\text{w}}\) was found for several specimens, the values given are uncorrected for the protein concentration.

With the exception of preparation He, each of the proteins migrated with a single electrophoretic boundary and sedimented with a single ultracentrifugal boundary under the usual conditions of study. However, as previously illustrated (5), the boundaries were sometimes skewed or hyper-sharp and were not always enantiomorphic. Upon protracted electrophoresis at pH 8.6, several proteins gave some evidence of electrophoretic anomalies, for example a splitting of either the ascending or descending boundary (see Putnam and Stelos (5), Fig. 1, F-G)). Although this phenomenon is often interpreted as arising from the electrophoretic dissociation of complexes, no evidence was found for distinct separation into several components over a wide pH range above pH 5, at which level the proteins retain molecular stability. The sedimentation diagrams of all the proteins were similar to those already published for specimens A, B, C, and D (5).

Analysis for N-Terminal Amino Acids—N-Terminal amino acid residues were identified and estimated by use of the fluorodinitrobenzene (FDNB) method of Sanger (12), with modifications introduced by other authors and by ourselves. To assure uniformity of corrections for loss, the conditions of hydrolysis were kept constant (24 hours in a sealed tube in 6 N HCl in an oven at 110°), exposure to light was minimized, and the column separation and spectrophotometric estimation were completed rapidly.

\(^1\) Our criteria for Bence-Jones proteins have been defined (5).
\(^2\) We are indebted to Dr. J. E. Hotchin of the University of British Columbia, Vancouver, B. C., for specimen Ho.
The ether-soluble dinitrophenol (DNP) amino acids were separated by two chromatographic steps, the first on Celite columns with ethyl acetate as an eluent, the second on silica gel with solvent systems containing various mixtures of methyl ethyl ketone and chloroform (5:95, 15:85, 30:70, v/v). Both columns were buffered at pH 6.5 with phosphate. The separated DNP amino acids (except lysine) were estimated quantitatively from the light absorption at 350 nm in the Beckman spectrophotometer (12). The identification was completed by chromatography on paper with at least two different solvent systems, e.g. tert-amyl alcohol-phthalate and decalin-glacial acetic acid (14, 15). In some cases, the amino acid residues were verified by barium hydroxide cleavage of the DNP derivative and paper chromatography of the free amino acid (16). It should be noted that none of these procedures distinguishes between a dicarboxylic acid and its amide in the N-terminal position; moreover, proline cannot be rigorously excluded as an end group because its DNP derivative decomposes readily. Most determinations were repeated several times on samples of approximately 100 mg. of protein. Corrections for the recovery of individual amino acids and for the protein content of the DNP derivatives were made in accord with published procedures (18). To permit comparison for proteins of different SzO, all the results are expressed as moles of N-terminal residues per 44,000 gm. (the molecular weight of proteins A and D and others of similar SzO).

Analysis for ε-Lysine Residues—Because of the apparent absence of N-terminal amino acids in some Bence-Jones proteins, as judged by the failure to detect appreciable quantities of ether-soluble DNP amino acids, the acid hydrolysates were rigorously examined for water-soluble DNP derivatives of basic amino acids. The hydrolysates were freed from HCl by use of a flash evaporator and aliquots were chromatographed on a column of Celite previously triturated with 1N HCl. With a mobile phase of 66 per cent (v/v) methyl ethyl ketone-ether, the Rf of DNP-ε-lysine is about 0.35. The presence of DNP-arginine and DNP-histidine was excluded by the use of the same mobile phase on a Celite column prepared by triturating with formaldehyde-water (1:9, v/v) neutralized to pH 7.

* Celite, Johns Manville No. 545. Silica gel was prepared according to Tristram (13). Aspartic acid, glutamic acid, serine, threonine, and glycine (listed in the order of increasing Rf) are adsorbed by the Celite. Silica gel separates the other DNP amino acids and the colored artifacts.

*N-Terminal aspartic acid is sometimes cleaved in the reaction with FDNB, and this may account for the fractional quantities of other apparent end groups (17). Some evidence for adsorbed amino acids or peptides was found by the more sensitive carboxypeptidase method for ascertaining C-terminal groups. However, the same results were obtained with proteins devoid of detectable amino end groups as for those having N-terminal aspartic acid (J. L. Blatt and F. W. Putnam, unpublished data). Further work on the stoichiometry of the end groups is in progress.
The ε-lysine residues were estimated quantitatively from the absorption at 390 μm (12).

Antigenic Typing—Dr. Leonhard Korngold of the Sloan-Kettering Institute for Cancer Research kindly determined the antigenic types of twelve of our specimens by use of the agar diffusion technique. Because of the immunological relationship between Bence-Jones proteins and γ-globulin, anti-γ-globulin sera as well as anti-Bence-Jones sera could be used for the typing (8). Although only two major antigenic types are reported (A and B), subtler differences among the specimens in each group could be detected by use of a variety of sera. It is not known whether types A and B accord with the two major antigenic types earlier reported (2, 3).

Results

The results of the determination of the physical constants (Table I) and of the free amino groups (Table II) reveal the wide spectrum of types occurring among the fourteen individual Bence-Jones proteins. However, all proteins of Group A have an \( s_{20, w} \) of about 3.4 S and lack N-terminal aspartic acid, whereas all proteins with N-terminal aspartic acid (or asparagine) are in Group B, though, to be sure, Group B falls into the two major sedimentation classes \( (s_{20, w} = 3.4 \text{ S or } 2.2 \text{ S}) \) first reported by Svedberg and Sjögren (1). This is the first evidence for an accord of physical and chemical properties with immunological classification.

In a preliminary report covering eight of the above specimens, we concluded that no two were identical in all physical or chemical properties studied (7). Hence, it appeared almost as if each patient excreted his individual Bence-Jones protein. This impression persists despite the new evidence for a correlation of antigenic types with physicochemical factors. For example, of the four crystalline proteins, all were in antigenic Group A, had an \( s_{20, w} = 3.4 \text{ S} \), were homogeneous in electrophoresis and ultracentrifugation, and lacked N-terminal aspartic acid. Indeed, preparations Lu and Bo were alike in all properties observed except crystal habit. Three of the four proteins had no detectable amino end groups (E, Lu, and Bo), whereas the fourth (Ho) had N-terminal isoleucine plus an unidentified

5 Qualitative end group analyses by other workers indicate that still other types exist. Biserte (19) found aspartic acid as the N-terminal group in five specimens, valine in a sixth, and valine plus lysine in a seventh. Glutamic acid has been identified as the only amino end group in another Bence-Jones protein (20).

6 In a private communication, K. Woods and R. L. Engle of the Cornell University Medical College, New York, have confirmed the relationship of antigenic type and amino end groups reported in this paper. Of four Bence-Jones proteins which they analyzed, two with aspartic acid end groups were in the immunologic Group B of Korngold, while two proteins without detectable amino end groups were in Group A.
end group. Yet, at pH 8.6, the mobility of the four ranged from $-1.4$ u (E) to $-4.3$ u (Ho). Although no two proteins of either type A or type B are entirely identical in all properties listed, there is a close similarity among the five proteins without a detectable end group. It would seem,

**Table I**

*Physical Constants of Bence-Jones Proteins*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Serum type</th>
<th>Isoelectric point</th>
<th>Mobility (pH 8.6)</th>
<th>$s$</th>
<th>Crystals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A, antigenic type A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>?</td>
<td>6.7</td>
<td>$-1.4$</td>
<td>3.36</td>
<td>*</td>
</tr>
<tr>
<td>T</td>
<td>N†</td>
<td>6.5</td>
<td>$-1.8$</td>
<td>3.36</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>$\beta$</td>
<td>5.5</td>
<td>$-2.4$</td>
<td>3.44</td>
<td></td>
</tr>
<tr>
<td>Lu</td>
<td>?</td>
<td>5.6</td>
<td>$-2.6$</td>
<td>3.44</td>
<td>†</td>
</tr>
<tr>
<td>Bo</td>
<td>N</td>
<td>4.6</td>
<td>$-4.2$</td>
<td>3.14</td>
<td>‡</td>
</tr>
<tr>
<td>G</td>
<td>&quot;</td>
<td>4.6</td>
<td>$-4.3$</td>
<td>3.46</td>
<td>*</td>
</tr>
<tr>
<td><strong>Group B, antigenic type B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag</td>
<td>$\gamma$</td>
<td>4.9</td>
<td>$-2.2$</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>$\beta$</td>
<td>4.9</td>
<td>$-3.4$</td>
<td>3.08</td>
<td></td>
</tr>
<tr>
<td>Ma</td>
<td>N</td>
<td>4.9</td>
<td>$-4.0$</td>
<td>2.25</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>$\beta$</td>
<td>4.75</td>
<td>$-4.7$</td>
<td>3.41</td>
<td></td>
</tr>
<tr>
<td><strong>Group C, antigenic type undetermined</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs</td>
<td>$\gamma$</td>
<td>4.9</td>
<td>$-3.4$</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>Be</td>
<td>N</td>
<td>4.9</td>
<td>$-2.5$</td>
<td>2.50</td>
<td></td>
</tr>
</tbody>
</table>

* Proteins crystallized by salt precipitation.
† N signifies "normal" type of serum, i.e. no increase in total protein and no predominant myeloma globulin peak.
‡ Proteins crystallized by dialysis against distilled water.

then, that the Bence-Jones proteins conform in most properties to a restricted number of classes despite the clear variation observed among individual specimens.

The most sensitive criterion for differentiating the individual specimens was electrophoretic mobility. Since this property is greatly affected by a difference in a number of even a few basic groups as well as by carbohydrate content, investigation was made of each factor. However, the four specimens with about the same mobility at pH 8.6 showed almost as great a
range in the number of ε-lysine residues as did the whole group (see Table II). Carbohydrate determinations on five of the specimens showed a content below 1 per cent and no significant difference in distribution. It is

<table>
<thead>
<tr>
<th>Protein</th>
<th>Aspartic acid</th>
<th>Glutamic acid</th>
<th>Serine</th>
<th>Threonine</th>
<th>Tyrosine</th>
<th>Valine</th>
<th>Isoleucine</th>
<th>ε-Lysine</th>
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<tbody>
<tr>
<td>Group A, antigenic type A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>E†</td>
<td>0.01</td>
<td>0.05</td>
<td></td>
<td>0.23</td>
<td>25.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.06</td>
<td>0.04</td>
<td>0.04</td>
<td>27.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
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<td></td>
<td>(20)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lu†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bo†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26.8</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G</td>
<td>Trace</td>
<td>Trace</td>
<td>0.57</td>
<td>22.4</td>
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<td></td>
<td></td>
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<tr>
<td>Ho†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.37‡</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group B, antigenic type B</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Ag</td>
<td>1.84</td>
<td></td>
<td></td>
<td></td>
<td>29.0</td>
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</tr>
<tr>
<td>F</td>
<td>1.69</td>
<td>0.13</td>
<td>0.06</td>
<td>24.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ma</td>
<td>1.17</td>
<td>0.07</td>
<td>0.03</td>
<td>22.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A</td>
<td>1.50</td>
<td>0.07</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group C, antigenic type undetermined</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>He</td>
<td>Trace</td>
<td>0.12</td>
<td>0.08</td>
<td>1.37§</td>
<td>23.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Be</td>
<td></td>
<td>0.27</td>
<td>0.04</td>
<td>0.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The major end groups are italicized. The parentheses indicate a single determination on an inadequate sample. The procedure does not distinguish between a dicarboxylic acid and its amide in the N-terminal position.

* Blank spaces indicate undetected amino end groups and undetermined ε-lysine groups.
† Crystalline proteins.
‡ Plus 0.64 mole of an unidentified residue (value uncorrected for loss).
§ Plus 0.19 mole of alanine.

of interest that a similar variation in mobility and isoelectric point of four myeloma globulins was attributable both to amino acid composition and to carbohydrate content (21).

To ascertain whether the differences in properties among the Bence-Jones proteins

7 Carbohydrate determinations were kindly reported to us by R. J. Winzler of the University of Illinois, College of Medicine, Chicago.
proteins are secondary owing to degradative changes during the excretory process, studies are being made of the properties of proteins reinjected into the donor patient or into experimental animals. In one such experiment, an apparently electrophoretically homogeneous sample of an isotopically labeled preparation, He, was reinjected into the patient. Labeled protein recovered from the urine had suffered very little change in mobility and electrophoretic pattern at pH 8.6. We are now studying the physicochemical properties of protein recovered from the urine after an isotopically labeled preparation, Bo, was injected into rabbits.

DISCUSSION

Study of Table I fails to reveal any relationship of the nature of the serum electrophoretic pattern with antigenic type or with physical or chemical properties of the Bence-Jones proteins. Likewise, no relationship of the Bence-Jones proteins to the abnormal serum globulins can be deduced either from the nature or the abundance of the free amino groups. All myeloma globulins thus far investigated contain from one to four N-terminal groups per 160,000 gm., usually aspartic or glutamic acid, and normal γ-globulin has approximately 1 mole of each dicarboxylic acid as a free amino group (21–24). On the other hand, five of the Bence-Jones proteins had no detectable free amino groups in stoichiometric proportions, whereas the specimens with measurable N-terminal groups such as aspartic acid had from 5 to 7 moles per 160,000 gm. In other words, the peptide chain of Bence-Jones proteins in some cases is much shorter than that of the serum globulins, and in other cases the peptide chain is possibly cyclic, but again differs from that of the serum globulins. In only two cases (preparations Ag and F) have we determined the amino end groups of the Bence-Jones protein and of the myeloma globulin of the same patient. The Ag globulin had 2.0 moles of N-terminal aspartic acid per 160,000 gm. or a chain length about 4 times that of the Ag Bence-Jones protein. The F globulin had both aspartic and glutamic acids in the N-terminal position and also had a longer average chain length than that of the F Bence-Jones protein.

A similar lack of evidence for relationship between Bence-Jones proteins and myeloma globulins prevails in regard to physical properties. The proteins of Table I have isoelectric points from 1 to 3 pH units lower than those of most myeloma globulins. Correspondingly, the Bence-Jones proteins usually have greater mobilities at pH 8.6. Both the low frictional ratio

8 Unpublished experiments of Franz Meyer and Frank W. Putnam.
9 The e-lysine content per 10⁴ gm. of all the Bence-Jones proteins examined except D, G, and A exceeds that of certain myeloma globulins and of normal human γ-globulin fractions II-1, 2 and II-3, but approximates that of other myeloma globulins (20).
and the lack of dependence of $s_{20}$ on protein concentration for Bence-Jones proteins suggest that the latter are less asymmetric than the myeloma globulins.

None of the data are in accord with the hypothesis that Bence-Jones proteins are derived by degradative cleavage of the abnormal globulins in the kidney (6). In such a case one might expect to find large open chain polypeptides that were (1) asymmetric, (2) heterogeneous with regard to physical properties and amino end-groups, (3) and thus non-crystalizable. The listed attributes, in fact, apply to normal $\gamma$-globulin rather than to Bence-Jones proteins, and the lack of amino end groups in five specimens in Table II is incompatible with the cleavage hypothesis. Indeed, the remarkable feature of the Bence-Jones proteins that must be explained in any hypothesis of their origin is the physical and chemical homogeneity of the immunologically distinct specimens from different patients.

The diversity in properties of the homogeneous Bence-Jones proteins obtained from different individuals is also difficult to reconcile with the hypothesis of a common metabolic block at methionine for all patients.\textsuperscript{10} However, the data are not incompatible with the occurrence of a number of individually characteristic metabolic blocks resulting in a failure to complete protein synthesis within the plasma cells, which are considered to be sites of antibody globulin production.

**SUMMARY**

Purified Bence-Jones proteins from fourteen patients with multiple myeloma have been studied by ultracentrifugal, electrophoretic, amino end group, and immunological analyses. Although apparently homogeneous, the proteins differed markedly in physical constants and in ease of crystalizability. They could be classified into two antigenic types, Groups A and B; these types accorded, respectively, with the absence and presence of $N$-terminal aspartic acid. However, within Group A, five proteins lacked detectable $N$-terminal groups whereas other specimens had $N$-terminal tyrosine, isoleucine, etc. Similarly, proteins of Group B differed in size or electrophoretic mobility. These results suggest that Bence-Jones proteins are individually specific, thus raising a question as to their origin and possible metabolic function.

**BIBLIOGRAPHY**


\textsuperscript{10} Although some Bence-Jones proteins lack methionine, others contain about 1 mole per 40,000 gm. (9, 10).
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