PROTEINS IN MULTIPLE MYELOMA

II. BENCE-JONES PROTEINS*

BY FRANK W. PUTNAM AND PETER STELOS

(From the Department of Biochemistry, University of Chicago, Chicago, Illinois)

(Received for publication, January 29, 1953)

The term "Bence-Jones protein" in fact designates a group of proteins often found in the urine of individuals with multiple myeloma and identified by the property of precipitating at 45-55° and redissolving upon boiling (1). Though first reported a century ago by Bence-Jones, these unusual proteins have not yet been fully characterized by physical methods or by chemical analysis. The divergence in sedimentation constants (2-6), the range in electrophoretic mobilities (1, 6, 7), the classification into different serological groups (8, 9), and the varying solubilities (1) of Bence-Jones proteins excreted by different individuals have made it clear that some, at least, of these substances differ in size or structure. In the course of a physicochemical and isotopic study of the proteins in multiple myeloma (10, 11), opportunity arose for the investigation of urinary proteins from eighteen cases. About half of these exhibited the characteristic behavior on heating and were suitable for physicochemical study; the others were grossly heterogeneous. All the homogeneous proteins were found to differ in one or more physical characteristics.

EXPERIMENTAL

Materials and Methods

All the proteins studied were obtained from the urine of patients with verified cases of multiple myeloma. Seven of the subjects were in the group, the serum proteins of which have previously been described (10). Whenever protein was detected by the heating test, it was analyzed electrophoretically and in the ultracentrifuge. For this purpose the urine was

* Aided by research grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

1 The customary heating test was found unsatisfactory for the differentiation of Bence-Jones and other urinary proteins. A study of the effect of pH showed that precipitation occurred only over a narrow range near the isoelectric point. For example, protein A precipitated only from pH 3.7 to 6.2 with maximal coagulation at pH 5.35, compared to an isoelectric point of pH 4.75. The concentration also affected resolution on boiling. Thus protein G, excreted at the rate of 30 gm. daily, was reported to us as "albumin;" physicochemical analysis suggested that it was a Bence-Jones protein, and this was verified by the positive test given after heating.
first dialyzed against water, and, if necessary, was concentrated prior to dialysis against Veronal buffer at pH 8.6, 0.1 ionic strength. In general, the homogeneous proteins were prepared by lyophilization of the dialyzed urine; in some cases (proteins E, F, G) the protein was precipitated by addition of solid (NH₄)₂SO₄ to three-quarters saturation at a pH near the isoelectric point (pI).

Electrophoretic analysis, ultracentrifugation, and the measurement of diffusion constants were carried out as previously described but without refrigeration of the ultracentrifuge (10). Except where stated, the sedimentation constant (s₂₀) and the diffusion constant (D₂₀) are corrected to the water basis at 20°, the former being expressed in Svedberg units (S). Mobilities are corrected to 0° and are given in units of u = 10⁻⁵ cm² sec⁻¹ volt⁻¹. The pH-mobility and pH-stability curves were determined by using buffers of 0.1 ionic strength described by Alberty (13).

Physicochemical Identification of Urinary Proteins

The eighteen urinary protein specimens were analyzed in the Veronal buffer at pH 8.6 to enable comparison of their sedimentation constants and mobilities with those of normal and pathological serum proteins. Table I records the physical constants of all the urinary proteins, and electrophoretic diagrams of proteins A to H are given in Fig. 1.

From a study of the electrical mobilities and the sedimentation constants in Table I, it may be seen that none of the first ten urinary proteins listed (proteins A to J) contained appreciable amounts of serum albumin, a protein which has a mobility of about 6 u in this buffer and an s₂₀ of 4.2 S, or of the serum globulins for which s₂₀ = 6.6 S or greater. Nevertheless, several of these specimens had been reported as "albumin" by the clinical laboratory. To be sure, a number of the specimens contained up to 5 per cent of a component identifiable as serum albumin by its electrical mobility, and the other more heterogeneous urinary proteins (K to R) may have contained more albumin. However, with the exception of specimen N, no major urinary protein fraction was identified as a serum component.

On the basis of their relative homogeneity and their sedimentation con-
### TABLE I

**Physical Constants of Urinary Proteins in Buffer at pH 8.6**

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Group I Mobility</th>
<th>( \lambda_i )</th>
<th>Group II Mobility</th>
<th>( \lambda_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.7</td>
<td>3.41</td>
<td>K</td>
<td>4.0( ^\dagger )</td>
</tr>
<tr>
<td>B</td>
<td>4.2</td>
<td>3.14</td>
<td>L</td>
<td>1.8( ^\ddagger )</td>
</tr>
<tr>
<td>C</td>
<td>3.3</td>
<td>3.03( ^\ddagger )</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2.4</td>
<td>3.44</td>
<td>N</td>
<td>6.9, 1.9( ^\ddagger )</td>
</tr>
<tr>
<td>E</td>
<td>1.4</td>
<td>3.36</td>
<td>O</td>
<td>Very heterogeneous</td>
</tr>
<tr>
<td>F</td>
<td>3.4</td>
<td>3.08</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2.6</td>
<td>3.28</td>
<td>Q</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>3.2</td>
<td>3.30</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.0</td>
<td>3.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td></td>
<td>3.41</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* The proteins may be identified with the Case numbers of the preceding paper (10) as follows: protein A, Case 17; protein B, Case 24; protein C, Case 21; protein D, Case 18; protein P, Case 19; protein Q, Case 9; protein R, Case 8.

\( ^\dagger \) Upon prolonged electrophoresis, split into two components (major 4.0 \( \mu \), minor 3.8 \( \mu \)).

\( ^\ddagger \) Contained three other minor components.

\( ^\ddagger \) In acetate buffer at pH 5.5.

---

**Fig. 1.** Electrophoretic diagrams of Bence-Jones proteins in Veronal buffer, pH 8.6. Ascending boundaries on the left, descending boundaries on the right. A schlieren photograph of the starting boundary is superimposed on the patterns.
stants, the proteins of Table I have been divided into two categories. Group I comprises those specimens which exhibited a single boundary upon electrophoresis or ultracentrifugation, and which have sedimentation constants in the range of 3.1 to 3.6 S. In our hands, all these substances gave a positive test for the Bence-Jones protein and are so classified, although further investigation revealed that some of the specimens contained two components. Group II contains specimens which were more difficult to classify. Specimen K and L appeared to be Bence-Jones proteins of lower molecular weight than those of Group I, but specimen L contained three minor electrophoretic components. Specimen M was an unknown substance, possibly a serum globulin, but specimen N seemed to be a mixture of albumin and \( \gamma \)-globulin. The remaining samples, specimens O to R, were grossly heterogeneous in electrophoresis; the patterns were diffuse and indicated up to five components. The sedimentation diagrams of specimens N to Q were also very diffuse, owing to the presence of unsedimentable material; the mean \( s_{20} \) was about 2 S, indicating that the substances may have been proteoses or degraded proteins. Only the proteins of Group I were studied further.

Electrophoresis of Bence-Jones Proteins

Homogeneity and Stability—Because the proteins of Group I differed greatly in mobility at pH 8.6 and some had skewed patterns (Fig. 1), electrophoretic analysis of the first seven was carried out over a wide pH range. A striking difference in homogeneity, stability, and isoelectric point was found. The patterns of Fig. 1 are fairly representative of the electrical homogeneity of the proteins from about pH 5 to pH 10. Proteins A, C, D, and F were free of contaminants except for 2 to 3 per cent of serum albumin; proteins B, E, and G migrated with skewed patterns, but did not separate into two components in this pH range. However, in the acid region all the proteins except E separated into two distinct components or gave a skewed pattern. In contrast, protein E, though a crystalline preparation, migrated as a single symmetrical boundary only at pH 5 and below. The patterns altered at varying acidity, ranging from pH 3.8 for protein A to pH 5.0 for proteins B and E. Study with the ultracentrifuge suggested that this behavior resulted from lability in acid rather than from a natural heterogeneity. Consequently, the proteins which migrated with a single symmetrical peak from pH 5 to 10 were considered to be at least 95 per cent homogeneous; the other specimens probably were mixtures of two Bence-Jones proteins.

Isoelectric Point—Just as the seven proteins differed significantly in mobility at pH 8.6, their pH-mobility curves and isoelectric points likewise differed, as is illustrated in Figs. 2 and 3 and in Table II. The one excep-
Fig. 2. Mobility curves of Bence-Jones proteins A to E in buffers of 0.1 ionic strength. The dash lines indicate the breakdown into two components. The multiple signs refer to the mobilities obtained in a mixture of the five proteins.

Fig. 3. Mobility curves of Bence-Jones proteins F and G in buffers of 0.1 ionic strength. The dash line indicates the breakdown into two components.

The surprising variation in the electrochemical properties of these five proteins is that the mobility curves for proteins C and F are identical above their common isoelectric point, pH 4.9; however, protein F split into three components just below the pI, whereas protein C was more stable. Fig. 2, containing the pH-mobility curves for proteins A to E, illustrates the surprising variation in the electrochemical properties of these five pro-
BENCE-JONES PROTEINS

teins. The pI ranges from pH 4.6 to 6.7, and at pH 6.5 a mixture will separate into five components. A characteristic feature of the mobility curves is that they merge between pH 4 and 5 where the proteins are unstable. It is noteworthy that the mobilities of the products formed at pH 3 are almost identical in the instances in which these were determined (proteins A, F, and G).

**TABLE II**

*Molecular Constants of Bence-Jones Proteins*

| Protein | pI  | $z_{40}$ | $D_{v0}$, $10^{-7}$ cm$^2$ sec$^{-1}$ (Udin and Putnam) | Mol. wt. | $f/f_4$
|---------|-----|---------|--------------------------------------------------|---------|-----
| A       | 4.75| 3.66*   | 7.70*                                            | 42,200  | 1.16|
| B       | 4.6 | 3.44*   |                                                  |         |     |
| C       | 4.85| 3.63†   |                                                  |         |     |
| D       | 5.5 | 3.44‡   | 7.65*                                            | 43,500  | 1.19|
| E       | 6.7 | 3.53§   |                                                  |         |     |
| F       | 4.9 | 3.37‖   |                                                  |         |     |
| G       | 5.6 | 3.36‖   |                                                  |         |     |

*pH 4.5, ionic strength 0.2, acetate buffer.
† pH 5.5, ionic strength 0.1, acetate buffer.
‡ pH 8.6, ionic strength 0.1, Veronal buffer.
§ pH 4.0, ionic strength 0.1, acetate buffer. This protein appeared homogeneous in electrophoresis only from pH 3.0 to 5.0.
‖ pH 5.0, ionic strength 0.1, acetate buffer.

**Ultracentrifugation of Bence-Jones Proteins**

*Homogeneity* The sedimentation velocity diagrams of four representative Bence-Jones proteins (A to D) given in Fig. 4 reveal a significant difference in molecular homogeneity despite the similarity in $s_{20}$. Proteins B and F sedimented with diffuse boundaries indicative of heterogeneity, whereas the other proteins appeared homogeneous. In the former cases the area under the refractive index gradient accounts for only a fraction of the protein, but in others such as protein C the protein sedimented uniformly and the boundary spreading is almost entirely attributable to the high diffusion constant (see below). In contrast to this rather homogeneous group of proteins with $s_{20} = 3.1$ S to 3.6 S stands the group of electrophoretically heterogeneous proteins (O, P, Q) which consisted largely of unsedimentable material or else material sedimenting with a continuous refractive index gradient with only a slight peak at about $s_{20} = 2$ S.

*pH-Stability*—After early observations suggested that the Bence-Jones proteins are unstable at acid pH, a comparative study of proteins F and G
was made over the range, pH 3 to 10, by means of electrophoretic and sedimentation velocity analyses. Aliquots made up to 1 per cent concentration in buffers of 0.1 ionic strength were equilibrated by dialysis overnight at 4° and then were analyzed simultaneously with the two instruments. The pH-mobility curves of proteins F and G have already been shown in Fig. 3; the corresponding molecular pH-stability curves are given in Fig.

Fig. 4. Sedimentation diagrams of Bence-Jones proteins A to D in buffers of 0.1 ionic strength. Photographs taken at 32 minute intervals at 59,780 r.p.m. with a bar angle of 45°, except for the first photographs of D, in which the bar angle is 60°. Proteins A, B, and D were in Veronal buffer, pH 8.6, protein C in acetate buffer, pH 5.5.

5. In both instances a single symmetrical boundary was obtained and the sedimentation diagrams appeared identical from pH 5 to pH 10. Furthermore, as seen in Fig. 5, there was no significant change in $s_{20}$ in this pH region, except for a drop in the $s_{20}$ of protein F at pH 10. However, at pH 4, in both cases the peak became skewed and diffuse, and at pH 3 there were a large decrease in $s_{20}$ and a pronounced distortion in the boundary. These changes in sedimentation behavior were accompanied by the appearance of three components in the electrophoretic pattern of protein F and

4 The slight scatter in $s_{20}$ from pH 5 to pH 9 may result from the fact that these values were not corrected for buffer density and viscosity.
of two components for protein G. At pH 3 the mobility of the latter substances coincided with the mobility of the two major peaks of protein F. Thus, both ultracentrifugal and electrophoretic data indicate that these proteins are unstable at acid pH just below the isoelectric point; i.e., in the region where the heat test is usually made. Although several other Bence-Jones proteins yielded a similar electrophoretic pattern at pH 4, decomposition at acid pH is not a uniform characteristic. The crystalline specimen is an apparent exception, and Svedberg and Sjögren (2) found that

![Sedimentation constant of Bence-Jones proteins F and G as a function of pH in buffers of 0.1 ionic strength. The values are not corrected for the effect of buffer density and viscosity.](image)

the $s_{20}$ of their protein $\alpha$ was steady from pH 1.2 to 10.5, above which the sedimentation constant dropped progressively.

**Heat Stability**—A few observations on heated Bence-Jones protein were made under the conditions of the clinical laboratory test to ascertain whether the latter depended upon gross changes provoked by heating and detectable by change in physical constants. A 1 per cent solution of protein A was gradually heated for 10 minutes between 40–60° in a phosphate buffer, pH 6.8, in which the protein does not coagulate. Upon ultracentrifugation it was found that the solution contained only unsedimentable material. Two other samples in acetate buffer, pH 5, were heated at

5 Analyses performed by Dr. B. Udin and Dr. F. W. Putnam (7).
6 This protein does, however, precipitate on heating at pH 6.8 in cacodylate buffer and redissolves on boiling.
50–57°, the one briefly to produce clouding and the other for 5 minutes to yield a heavy precipitate. Both samples were dialyzed against Veronal buffer, pH 8.6, whereupon the protein redissolved. The sedimentation diagram and \( s_{20} \) were only slightly altered. Protein A was boiled for 2 minutes in the buffer at pH 8.6, dialyzed, and then analyzed by electrophoresis, the boundary skewed, and the mobility decreased from \(-4.64\) to \(-3.8\) u. Thus, this protein is disrupted by moderate heating in a buffer in which precipitation does not ensue, but paradoxically the protein retains its molecular integrity at a pH where thermal coagulation does occur, and close to the pH where it is unstable. Possibly labile linkages within the molecule are protected by coagulation at 50°, only to be disrupted on boiling with attendant redispersion of the precipitate.

**Molecular Constants of Bence-Jones Proteins**

The variation in \( s_{20} \) for different Bence-Jones proteins described here and elsewhere (4–6) casts doubt on the validity of the molecular weight of 35,000 to 37,000 usually assigned to these substances (4). Accordingly, \( D_{20} \) was measured for two of the better specimens, *i.e.* proteins A and D. These measurements6 were made at pH 4.5 near the isoelectric point. Though this is close to the region of acid instability, the sedimentation diagrams of protein A were unchanged after 21 days at this pH.

Table II contains \( D_{20} \) for proteins A and D, together with the molecular weight calculated on the assumption that the partial specific volume is 0.749, as reported for Bence-Jones protein \( \alpha \) (4). Table II also lists the isoelectric points of the seven proteins, A to G, and gives \( s_{20} \) at a pH in the isoelectric region wherever that is available. The calculation of the molecular weight of proteins A and D depends upon the assumption that \( s_{20} \) and \( D_{20} \) are essentially independent of concentration. The values of the frictional ratio (\( f/\bar{f}_0 \)) are so low that the deviation from unity may largely be attributed to hydration, and the molecules are thus apparently almost symmetrical in shape. Investigation of the effect of protein concentration on \( s_{20} \) was made for protein A at pH 4.5 in the same buffer used for diffusion studies. As would be expected for a symmetrical molecule, \( s_{20} \) was independent of concentration within experimental error (range of 3.56 to 3.61 S for a protein concentration range of 0.4 to 2.8 mg. of N per ml.). Since Bence-Jones proteins \( \alpha \) and \( \beta \) of Svedberg and Pedersen (4) likewise have low frictional ratios, the deviations in \( s_{20} \) reported for different Bence-Jones proteins do not arise from a concentration effect.7

7 The frictional ratio below unity recorded for Bence-Jones protein \( \alpha \) by Svedberg and Pedersen (4) is less than theory and allows doubt about their molecular weight value of 35,000.
This comparison of urinary proteins derived from eighteen cases out of a large group of individuals with multiple myeloma has shown that in no instance are the proteins identical in physicochemical properties. Contrary to the experience of Rundles et al. (6), in almost half our subjects the urinary proteins were grossly heterogeneous, although only one specimen contained much serum protein. Of a group of seven rather homogeneous proteins, all of which gave a positive Bence-Jones test, only two were identical in isoelectric point and pH-mobility curve, and even these differed in s_20 at pH 8.6 and in lability towards acid. Although the s_20 of the seven proteins fell into the narrow interval of 3.1 S to 3.6 S, this range is believed to be outside of experimental error and is not due to a concentration effect. Thus from physicochemical analysis it is concluded that different Bence-Jones proteins are elaborated by different patients. This idea was previously suggested by Gutman (1), who pointed out that various authors (each studying only several specimens) had found differences in mobility at pH 7.4 and a range in s_20 from 2.8 S to 4.0 S. However, the variation in ionic structure of the Bence-Jones proteins was made apparent only by the pH-mobility curves given herein.

Supporting the physicochemical evidence for individuality of the Bence-Jones proteins, immunological study has repeatedly demonstrated that there are at least two antigenically specific groups of these proteins, and that both may appear even in the same urine. It is interesting that large differences in ionic structure are not reflected by the serological reaction, for, when antiserum was prepared to protein A (pI = pH 4.8), the serum reacted at a dilution of 1:100,000 with proteins D and E (pI = pH 5.5 and pH 6.7 respectively), and at a dilution of 1:80,000 with protein B (pI = pH 4.6). However, the grossly heterogeneous protein R did not form a precipitate with the antiserum (7).

The discovery of the thermal and acid lability of the Bence-Jones proteins may aid in explaining some of the characteristics of these substances. Since Bence-Jones proteins of different immunological specificity and different electrical mobility may be detected in serum, it is unlikely that the variety of proteins arises by renal action. However, cleavage in the kidney may produce the heterogeneous low molecular weight specimens encountered in this study. In like manner, fragmentation of the protein on heat-

8 When the mobility at pH 8.6 of the twenty "homogeneous" Bence-Jones proteins in this series and that of Rundles et al. (6) are plotted as a histogram, only 20 per cent of the values clusters at one point and the remainder is randomly distributed from 1.2 to 4.7 u. On the other hand, a histogram of the sedimentation constants of twenty-five specimens reported on here and in the literature reveals that 80 per cent is in the range of 2.9 to 3.7 S and 64 per cent is within the range, 3.0 to 3.4 S.
ing may account for the solubility of the precipitate on boiling. This last hypothesis is attractive because most other proteins are aggregated by heating.

The molecular constants of the Bence-Jones proteins indicate that all we have encountered are smaller than serum albumin. This, together with the greater symmetry of the molecules, affords a ready explanation of their excretion in the urine. Indeed, it has long since been demonstrated that Bence-Jones proteins easily pass through the kidney of the cat or rabbit but normal serum proteins are withheld (14). The small size of the Bence-Jones proteins has also given rise to the idea that they originate by renal cleavage of serum proteins (6). This hypothesis, which is contrary to the evidence for Bence-Jones proteins in the circulation, cannot be judged on the basis of molecular kinetic data. Indeed, isotopic studies in our laboratory indicate that the turnover rates of plasma proteins and Bence-Jones proteins are independent, suggesting a lack of precursor relationship (15).

The profuse synthesis and diverse nature of the proteins elaborated in multiple myeloma constitute the most profound alteration in protein metabolism in any disease. It may be hoped that the biochemical study of this striking phenomenon may have import in the analysis of the mechanism of protein synthesis.

SUMMARY

Physicochemical analysis of the urinary protein excreted by eighteen individuals with multiple myeloma has revealed a striking difference in homogeneity and in electrophoretic mobility. In ten cases (Group I) a single major boundary was obtained on electrophoresis and ultracentrifugation at pH 8.6, and the sedimentation constant was in the range of 3.1 to 3.6 Svedberg units. The other proteins (Group II) were mainly of lower molecular weight and were usually very heterogeneous. Although the substances of Group I satisfied criteria for Bence-Jones proteins, all differed in one or more physical properties such as sedimentation constant, isoelectric point, electrical or molecular homogeneity, and pH-stability. Although the pH-mobility curves of seven of the Bence-Jones proteins varied greatly with a range of isoelectric points from pH 4.6 to pH 6.7, four were immunologically related and all appeared to have molecular weights of about 43,000. These proteins were disrupted by acidification to pH 3 or 4 without heating, and also appeared to be fragmented at neutral pH by heating. The physicochemical analysis, though establishing the diversity of the Bence-Jones proteins, fails to indicate their origin.

Addendum—As the result of study of samples of Bence-Jones proteins prepared by different methods from the urine of a single patient, Jirgensons, Landua, and
Awapara (16) recently concluded: "The discrepancies in properties of Bence-Jones protein as reported by different investigators may be ascribed to the multiplicity of procedures used for isolation." Since these authors also stated that the homogeneity of the protein they studied was questionable, and because they used drastic methods of isolation and drying, it is not surprising that they were able to obtain products differing in chemical and physical properties. Their conclusion cannot apply to our work, for clear differences in the physical properties of Bence-Jones proteins from different patients were demonstrated by electrophoresis and ultracentrifugation of the dialyzed, but otherwise untreated, urines as well as after preparation by lyophilization or precipitation with ammonium sulfate. Nor can their conclusion hold for the previous work of Rundles et al. (6), who observed variation in the physical properties of Bence-Jones proteins obtained by precipitation with ammonium sulfate from the urines of twenty patients.

BIBLIOGRAPHY

PROTEINS IN MULTIPLE MYELOMA:
II. BENCE-JONES PROTEINS
Frank W. Putnam and Peter Stelos


Access the most updated version of this article at
http://www.jbc.org/content/203/1/347.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/203/1/347.citation.full.html#ref-list-1