Bence-Jones Proteins and Light Chains of Immunoglobulins

I. FORMATION AND CHARACTERIZATION OF AMINO TERMINAL (VARIANT) AND CARBOXYL-TERMINAL (CONSTANT) HALVES*

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SUMMARY

The primary structure of Bence-Jones proteins is characterized by a variable amino acid sequence in the amino-terminal half and a constant amino acid sequence in the carboxyl-terminal half. Proteins corresponding either to the variant half or to the constant half of whole Bence-Jones proteins have been detected in urine of patients with multiple myeloma. Both halves of a Bence-Jones protein were detected in the urine of one patient, and in another patient the constant half of a Bence-Jones protein was found in serum as well as urine. Metabolic studies indicated that the variant half and the constant half can result from catabolism of the whole molecule, and biosynthetic studies suggested that the variant and constant halves may also have synthetic origin.

Bence-Jones proteins and light chains isolated from G- or A-myeloma proteins were cleaved in vitro into the variant half and the constant half. Cleavage was initiated by a proteolytic factor in urine as well as by several types of endopeptidases, and definitive immunochromatographic identification of the cleaved products as variant half and constant half required development of specific antisera. Particular susceptibility to proteolysis of the peptide bond between the variant half and constant half of Bence-Jones proteins and between the variant half and constant half of light chains provides the first direct evidence for the presence of an exposed area in the switch region of these molecules.

The ability to cleave specifically Bence-Jones proteins and light chains makes possible the preparation and isolation of variant halves and constant halves in quantities sufficient for studies of the individual properties of each half. Studies on the isolated variant halves and constant halves of Bence-Jones proteins indicated that the variant half was more resistant to proteolysis than the constant half, was crystallizable, and had thermal solubility behavior identical with that of the intact Bence-Jones protein.

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Over a century passed between the time that Sir Henry Bence-Jones described the urinary protein that bears his name (3) and the time that Edelman and Gally (4) discovered that Bence-Jones proteins were related to the light polypeptide chains of all classes of immunoglobulins. Bence-Jones proteins have assumed particular significance in view of the contributory role of the light chain in antibody specificity (5-7). Detailed analyses of Bence-Jones proteins have provided evidence of a structural basis for this specificity. Putnam, Easley, and Helling (8) and Putnam and Easley (9) compared the tryptic peptide maps of Bence-Jones proteins within each major antigenic group (κ and λ) and found that, for each protein, half of its peptides were group-specific and half were individually specific. From this observation Putnam et al. (8) proposed that the amino acid sequence consisted of a mutable portion and a constant portion. The results of analyses of the primary structure of several human and murine Bence-Jones proteins (summarized in Reference 10) have supported this proposal. The Bence-Jones protein monomer is a single polypeptide chain consisting of approximately 214 amino acids. The NH2-terminal half, the first 107 amino acids, is characterized by variance in amino acid sequence; this half is referred to as the variant or variable half (Vν). The remaining 107 amino acids, the COOH-terminal half, are characterized by constancy in amino acid sequence; this half is referred to as the constant or common half (Cν).

This unique structure of the Bence-Jones protein polypeptide chain has evoked fundamental questions concerning the genetic control of immunoglobulin synthesis and the contributory role of each half in the properties expressed by the intact molecule. Knowledge of the properties of Vν and Cν is of prime importance in providing answers to these basic questions. Such information requires studies on isolated Vν and Cν. Urinary components corresponding to the Vν (11-16) or to the Cν (12, 13) of Bence-Jones proteins provided the necessary source of material for isolation and study. However, the relative infrequency of occurrence and generally low amounts of Vν and Cν in the urine of patients with multiple myeloma (12) limited the usefulness of this source. Our observations that Vν and Cν can have catabolic origin suggested the possibility that the peptide bond between the Vν and Cν may be particularly susceptible to proteolysis. Indeed, subsequent studies showed that Bence-Jones proteins as well as light chains of immunoglobulins were cleaved into Vν and Cν by several types of endopeptidases. The ability to cleave specifically Bence-Jones proteins and light chains into...
Experimental Procedure

Preparative Procedures—Urine samples containing Bence-Jones proteins were obtained from patients with multiple myeloma. Specimens were collected without preservative and each sample was kept at 4° or frozen at –30° prior to processing. Urine specimens were extensively dialyzed at 4° in 23/32 Viasking tubing (Union Carbide) against deionized, double distilled water and lyophilized. For analytical purposes the dried urinary proteins were reconstituted in water or 0.15 M NaCl to a concentration of 10 to 50 mg per ml. Bence-Jones proteins were isolated from the urine by zone (block) electrophoresis (17) or by gel filtration (18) through Bio-Gel P-100 polycrylamide (Bio-Rad) columns, 2.5 × 100 cm. The eluting buffer was composed of 0.15 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, pH 7.6, and contained 0.02% sodium azide.

Immunoochemical Procedures—Antisera against κ and λ type Bence-Jones proteins, isolated V_L of a Bence-Jones protein, and human FII γG-globulin were prepared in albino New Zealand rabbits. The antigen, 5 mg per ml in 0.15 M NaCl, was thoroughly emulsified with 1 ml of complete Freund’s adjuvant. Two rabbits were immunized with the same antigen; each rabbit was injected with 1.25 mg of protein (0.5 ml) in both hind leg muscles weekly for 4 weeks and then at monthly intervals as necessary. Samples of blood were obtained 7 to 10 days after injection. Immunoelectrophoresis and immunodiffusion analyses were carried out as previously described (19, 20).

Analytical Procedures—Protein concentrations were determined by a modification of the Folin-Ciocalteu method (21). Electrophoresis on cellulose acetate membranes was performed with a Microzone apparatus (Beckman). Electrophoresis in alkaline (0.05 M glycine, 0.005 M NaOH, pH 8.8) and acid urea starch gels (0.05 M formic acid, 0.01 M NaOH, pH 3, 8 M urea) was under conditions previously described (22, 23). Proteins (5 mg per ml) were ultrafilter centrifuged in a Spinco model E ultra-centrifuge with an An-D rotor and double sector cells at 59,780 rpm. Schlieren patterns were analyzed with the aid of a Guertner microcomparator and the sedimentation coefficients, s_{20,w}, were corrected to s_{20,w}, according to Schachman (24). The NH_{2}-terminal amino acid was determined by the dansyl technique (25). Proteins were reduced with 0.1 M 2-mercaptoethanol and alkylated with 0.15 M iodoacetamide under conditions described (26).

Tryptic digestion (trypsin twice crystallized, Worthington) of reduced-alkylated Bence-Jones protein and of V_L and C_L prepared from the reduced-alkylated protein was performed according to Condition A described by Easley and Putnam (27). Peptide mapping was performed in one dimension by chromatography on Whatman No. 3MM paper for 16 hours in butanol-acetic acid-water (4:1:5) and then in the second dimension by high voltage electrophoresis in pyridine-acetic acid-water, pH 3.7 (1:10:289). Electrophoresis was carried out on a flat plate apparatus (Savant Instruments, Inc., Hixwick, New York) at 55 volts per cm for 90 min. Papers were treated by chlorination, ninhydrin, and other stains for specific amino acids as described by Easley (28).

Biological Studies—Bone marrow was obtained in a heparinized syringe by aspiration from the sternum or iliac crest of patients with multiple myeloma. The narrow particles were washed three times with sterile Hank’s solution and suspended in an isolucine-lysine-deficient growth medium containing 5 mg per ml of three times crystallized ovalbumin (Nutritional Biochemicals). Radioactive isolucine and lysine were added to yield 1 μCi per ml of ^14C-lysine (198 μCi per mmole, Schwarz BioResearch) and 1 μCi per ml of ^14C-lysine (234 μCi per mmole, New England Nuclear) prior to transferring of 1-ml aliquots to sterile glass screw top tubes, 16 × 123 mm. The tubes were placed in a roller drum at 37° for periods up to 25 hours and culture fluid was harvested by centrifugation at 290 × g for 10 min at 4°. Unincorporated radioactive amino acids were removed by dialysis of the culture fluids against five 400-volume changes of 0.15 M NaCl at 4°, over a 24-hour period. Radioimmunoelectrophoresis was performed as described by Hochwald, Thorbecke, and Asofsky (29).

Turnover Studies—Bence-Jones protein was labeled with carrier-free, reducing agent-free ^14C by the iodo monochloride method of McFarlane (30). Rats were maintained on drinking water containing potassium iodide (0.6 g per liter) for 2 days prior to each study to inhibit the thyroidal uptake of radioactive iodine.

Results

Immunoochemical Identification of Bence-Jones Protein, Variant Half and Constant Half—Urine proteins which are antigenically related to Bence-Jones protein may be identified as having V_L or C_L antigenic determinants by immunoelectrophoretic, immunodiffusion, or immuno-gel filtration (31) analyses. Antisera obtained from several commercial sources have not been satisfactory because of their weak precipitating activity and because their specificity was limited only to the C_L antigenic determinants. To facilitate detection of V_L and C_L antisera to κ and λ type Bence-Jones proteins were prepared in this laboratory. These antisera possessed strong precipitating activity and had marked idiotypic specificity, i.e., recognition of antigenic determinants unique to the protein used for immunization, and thus V_L as well as C_L antigenic determinants could be detected. The specific precipitin reactions of three types of antisera are summarized in Table I.

The use of these antisera permitted the immunoochemical identification, as V_L or C_L, of components antigenically related to Bence-Jones proteins. An immunoelectrophoretic analysis of urine which contained a κ type Bence-Jones protein and both C_L and V_L is shown in Fig. 1. Three antigenically related proteins were detected in this specimen with antiserum H. The anodal and cathodal precipitin arcs were antigenically deficient compared with the middle arc. The cathodal component reacted with antiserum V. The anodal component reacted with antiserum C. Thus, the middle precipitin arc represents whole protein, and the anodal and cathodal antigenically deficient arcs represent C_L and V_L respectively. The finding of a patient excreting both C_L and V_L has not been previously reported. Heretofore, the C_L or V_L of Bence-Jones proteins has been found only in urine; in one patient currently under investigation, the C_L and Bence-Jones protein have been identified in serum as well as urine.

Biosynthetic and Metabolic Studies—Bone marrow cells, obtained from Patient LEN excreting 50 g daily of a κ type Bence-Jones protein and no demonstrable V_L or C_L, were incubated

1 NIH Medium 320, kindly supplied by Dr. Richard Asofsky, National Institutes of Health, Bethesda, Maryland.
Table I

Immunoechemical detection of variant half and constant half antigenic determinants of homologous Bence-Jones protein.

Three types of antisera are used for detection of variant half (VL) and constant half (CL) antigenic determinants of an individual patient's (homologous) Bence-Jones protein. The first type of antiserum (designated as H), prepared against the homologous protein, recognizes both VL and CL antigenic determinants of the homologous protein. The second type of antiserum (designated as V), obtained by absorption of anti-homologous Bence-Jones protein antiserum with a heterologous Bence-Jones protein of the same major antigenic type (κ or λ), recognizes VL antigenic determinants of the homologous protein. Antiserum prepared against isolated homologous VL has similar antibodies directed against shared regions of amino acid sequence in the VL portion (10).

The third type of antiserum (designated as C), prepared against a heterologous Bence-Jones protein, recognizes CL antigenic determinants of the homologous protein.

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<tr>
<th>Antiserum</th>
<th>Detects in homologous Bence-Jones protein</th>
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<tr>
<td>Antihomologous Bence-Jones protein (H)</td>
<td>+</td>
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<tr>
<td>Antihomologous Bence-Jones protein absorbed with heterologous Bence-Jones protein (V)</td>
<td>+</td>
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<tr>
<td>Antiheterologous Bence-Jones protein (C)</td>
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*The VL antigenic determinants of a homologous Bence-Jones protein are detected by certain antiserum to heterologous Bence-Jones proteins (12), presumably because the antiserum possess antibodies directed against shared regions of amino acid sequence in the VL portion (10).*

In growth medium containing 14C-lysine and 14C-isoleucine, cultures were harvested after 4, 8, 18, and 25 hours and the respective supernatant fluids were analyzed for newly synthesized, i.e., labeled, protein. Radioimmunoa electrophoresis was performed with, as carrier proteins, Bence-Jones protein LEN and its isolated VL and CL (vide infra). Labeled Bence-Jones protein, V and CL, were identified in all four culture fluids. The radioautograph of the 25-hour culture fluid, shown in Fig. 2, revealed an intensely labeled line corresponding to the Bence-Jones protein precipitin arc and, in addition, two faintly labeled lines corresponding to the precipitin cathodal arc of V and CL. Labeled VL and CL protein amounted to 1% of the radioactivity of the whole Bence-Jones protein as determined from gel filtration and acid urea starch gel electrophoretic analyses. The Bence-Jones protein in this patient's urine was present as a monomer (mol wt 22,000) and a dimer (92) in a ratio of 4:1. The ratio of labeled monomer to labeled dimer was similar to the ratio of monomer to dimer found in the patient's urine and remained constant in each of the four cultures. Bone marrow cells, obtained from two patients excreting λ type dimeric Bence-Jones proteins and no demonstrable VL or CL, were cultured in similar fashion. In the respective culture fluids the amount of labeled VL and CL represented 1% of the radioactivity of the Bence-Jones protein. The labeled intact protein from each culture was in the dimeric form.

Metabolic studies utilized equimolar amounts of the dimeric and monomeric form of Bence-Jones protein LEN labeled with 14C. Approximately 0.01 mg of the iodinated protein containing 1 μCi of 14C was injected intravenously into a 250-g adult male rat; urine was collected from the bladder 2 hours after injection. The distribution of radioactivity in four peaks obtained by gel filtration of the urine sample together with unlabeled carrier proteins (dimer and monomer Bence-Jones protein, VL and CL) was determined. Catabolism of the injected protein had occurred as indicated by the presence of 60% of the label as non-acid-precipitable 14C activity in the fourth, most slowly eluting, peak. The first three peaks corresponded to the elution pattern of the protein markers and the distribution of radioactivity was as follows: dimer, 3%; monomer, 32%; and

![Fig. 1. Detection of intact Bence-Jones protein (BJP) and both the constant half (CL) and the variant half (VL) in urine by immunoelectrophoresis. Antigen wells contained urine from Patient DR. The upper trough contained an antiserum (C) prepared against a heterologous Bence-Jones protein, the middle trough contained an antiserum (H) prepared against the homologous Bence-Jones protein DR, and the lower trough contained the homologous antiserum absorbed (V) with a heterologous Bence-Jones protein. The use of the three types of antiserum permits the identification, as VL or CL, of urinary components antigenically related to Bence-Jones protein. A component with VL antigenic determinants has the following reactivity: (a) With antiserum H, VL gives a reaction of partial identity with the intact Bence-Jones protein resulting from the reaction of the antiserum with CL as well as VL determinants of the intact molecule. (b) With antiserum V, VL gives a reaction of equal intensity to that obtained with the unabsorbed antiserum; the removal of antibodies to CL determinants results in a reaction of identity between the VL and the intact molecule. With antihomologous VL antiserum, the VL gives a reaction of identity with the intact molecule. (c) With antiserum C, VL gives no reactivity (see Table I, Footnote a). A urinary component with CL antigenic determinants has the following reactivity. (a) With antiserum H, CL gives a reaction of partial identity with the intact Bence-Jones protein resulting from the reaction of the antiserum with VL as well as CL determinants of the intact molecule. (b) With antiserum V, CL gives no reactivity as a result of removal of antibodies to VL determinants. With antihomologous VL antiserum, CL gives no reactivity. (c) With antiserum C, VL gives no reactivity. (d) With antiserum C, CL gives one of three types of reactivity: a reaction of identity with the intact molecule, a reaction of partial identity, the CL deficient to the intact molecule as a result of additional specificity of certain antiserum to VL determinants (see Table I, Footnote a); or a reaction of partial identity, the intact molecule deficient to the CL, as a result of configurational specificity.
In serum collected 2 hours after injection, the ratio of labeled Bence-Jones protein to V_{L}-C_{L} was 4:1 as compared to the 7:1 ratio found in urine. In another study, 0.25 mg of labeled protein containing 25 μCi of ^{125}I was injected and urine was collected directly from the bladder 15, 30, 90, 120, 210, 270, and 360 min after injection. The main peak of radioactivity associated with V_{L}-C_{L} occurred in the 15-min urine sample and again the ratio of labeled intact protein to V_{L}-C_{L} was 7:1. A progressive increase in excretion of labeled dimer and a progressive decrease in excretion of both labeled monomer and V_{L}-C_{L} occurred over the 6-hour period.

**Proteolysis of Bence-Jones Proteins**—Eight lyophilized urine specimens from Patient LEN were examined by immunoelectrophoresis with antiserum to Bence-Jones protein LEN. The precipitin are obtained with seven of the samples was located in the same position as the Bence-Jones protein are obtained with fresh urine. With one sample, only a single more cathodal precipitin arc was noted. Examination of this sample with the three types of antisera H, V, and C identified the cathodally migrating component as V_{L}. No whole protein or protein related to its C_{L} was detected. This specimen, in contrast to the other seven, had been deionized by passage through a mixed bed ion exchange resin^2 prior to lyophilization. The factors responsible for the observed change in the Bence-Jones protein were studied in detail. Results of subsequent experiments provided evidence that both V_{L} and C_{L} can result from proteolytic cleavage of Bence-Jones proteins. The passage of urine through the same position as the Bence-Jones protein.
FIG. 4. Urinary cleavage of Bence-Jones protein into variant half and constant half. Normal urine was added (1:100, v/v) to a 10 mg per ml solution of Bence-Jones protein LEN in 0.05 M glycine-HCl, pH 3.4, and the mixture was incubated at 37°C. Aliquots were withdrawn at 1, 2, and 4 min, neutralized with 1 M Tris base, and compared with untreated Bence-Jones protein (CONTROL) by starch gel electrophoresis, pH 8.8. BJP, Bence-Jones protein.

A cation exchange resin3 produced a marked reduction in the urine pH. The detection of V<sub>L</sub> and C<sub>L</sub> of protein LEN after acidification of urine LEN by passage through the resin column is shown in Fig. 3. In the control specimen only a single precipitin arc representing intact protein was identified. In the specimen maintained at pH 1.5 for 30 min at 25°C, the intensity of the intact protein arc was decreased, and the anodal and cathodal precipitin arcs were identified as C<sub>L</sub> and V<sub>L</sub>, respectively. By starch gel electrophoresis, the intact protein in the control urine appeared as a single band. In the acidified urine, the intensity of the Bence-Jones protein band was considerably diminished; the anodal and cathodal bands were identified as C<sub>L</sub> and V<sub>L</sub>, respectively, by immunoelectrophoresis; all antiserum troughs contained anti-Bence-Jones protein LEN (H). BJP, Bence-Jones protein.

Revealed a progressive decrease in C<sub>L</sub>. Only V<sub>L</sub> was detected immunochemically in an aliquot neutralized after 6 hours at pH 1.5, 25°C. A single cathodal protein band was present on starch gel electrophoresis and no whole protein or C<sub>L</sub> was detected.

Urine specimens from other patients excreting only intact Bence-Jones protein were examined after passage through the resin. Cellulose acetate electrophoresis provided a rapid means for detection of cleavage. In the cases in which homologous antisera were available, the cleaved products were identified immunochemically as V<sub>L</sub> or C<sub>L</sub>.

Cleavage of Bence-Jones proteins into V<sub>L</sub> and C<sub>L</sub> was achieved by acidification of urine directly with 0.05 M glycine-HCl, pH 3.4, or with 1 M acetic acid. Most extensive cleavage occurred between pH 1 and 3; between pH 3 and 5 cleavage occurred more slowly and none was observed at pH greater than 5.8. Further proteolysis could be stopped by raising the pH to neutrality.

FIG. 5. Pepsin cleavage of Bence-Jones protein into variant half and constant half. Bence-Jones protein LEN was treated with pepsin (enzyme to protein ratio, 1:200) in 0.05 M glycine-HCl, pH 3.4, at 37°C. Aliquots were withdrawn at the indicated time periods, neutralized with 1 M Tris base, and analyzed by immunoelectrophoresis; all antiserum troughs contained anti-Bence-Jones protein LEN (H). BJP, Bence-Jones protein.

4 Visualization of the protein bands after Ponceau S staining was enhanced by making the membrane opaque (substitution of 100% ethanol for the acetic acid-ethanol clearing solution) and by viewing the electrophoretogram by transmitted light.

<sup>3</sup>Amberlite IR-120 (H).
FIG. 6. Trypsin cleavage of a λ Bence-Jones protein into vari-
ant half and constant half. Bence-Jones protein COX was treated
with trypsin (enzyme to protein ratio, 1:20) in 0.1 M Tris-HCl,
pH 8.2, for 20 min at 37°. The pH of the reaction mixture was
reduced to 2.8 by the addition of 1 M HCl and the sample was
electrophoresed immediately. The antisera used for detection
of Bence-Jones protein, VL and CL, were anti-Bence-Jones pro-
tein COX (H), the homologous antiserum absorbed (V) with a
heterologous Bence-Jones protein, and antiheterologous Bence-
Jones protein (C). BJP, Bence-Jones protein.

Cleavage by acidification of urine that had been stored at -30°
for as long as 1 year was noted. However, splitting of Bence-
Jones proteins was considerably diminished in reconstituted
specimens of lyophilized urine. Furthermore, there was no
evidence of cleavage into VL and CL of Bence-Jones proteins
isolated from urine even after the isolated protein had been
maintained at pH 3.4 for 8 hours at 37°. The fact that protein
in urine was cleaved into VL and CL by acidification, whereas
the isolated protein was not cleaved, suggested the presence in
urine of a necessary proteolytic factor. Indeed, isolated Bence-
Jones protein was readily cleaved into VL and CL when incubated
in the acid buffer with catalytic amounts of the patient’s urine
or with urine from a normal individual. The extent of proteoly-
sis of isolated protein LEN in the presence of normal urine was
determined by starch gel electrophoresis (Fig. 4). Progressive
splitting of the protein into VL and CL occurred and virtually
no whole protein was evident in the 4-min sample. Similar
division was noted when 10 μl of urine LEN were added to 10
mg of Bence-Jones protein LEN in 1 ml of 0.05 M glycine-HCl,
pH 3.4. The mixture was maintained at 20° for 5 min, neutral-
ized with 1 M Tris base, and analyzed by immunoelectrophoresis.

Two precipitin arcs, both antigenically deficient to the whole
protein arc, were detected with antiserum to the homologous
protein. One precipitin arc was identified as VL and the other
as CL. Boiling or lyophilization of urine markedly diminished,
but did not abolish, the proteolytic effect. Dialysis of urine
containing Bence-Jones protein against 0.05 M glycine-NaOH,
pH 8.6, prior to reduction of pH to 3.4, did not diminish the

proteolytic effect of the urinary factor on the protein. Cleavage
was not inhibited by the presence of a chelating agent (0.001 M
EDTA) or by a sulfhydryl-blocking agent (0.02 M iodoacetamide).

Isolated Bence-Jones protein was cleaved into VL and CL when
incubated under acid conditions with a kidney extract. Extra-
cellular and intracellular extracts were prepared from a rabbit
kidney homogenate (33). An equal volume of extract containing
1 mg of protein per ml in 0.05 M glycine-HCl, pH 3.4, was
added to 20 mg per ml of protein LEN, and the mixture was
maintained at 37°. In the presence of extracellular extract, the
protein was partially cleaved into VL and CL after 30 min and

FIG. 7. Pepsin cleavage of Bence-Jones protein DR and light
chains from A-myeloma protein DR. Bence-Jones protein and
light chains were treated with pepsin (enzyme to protein ratio,
1:200) in 0.05 M glycine-HCl, pH 3.4; the protein-enzyme mixture
was incubated at 37° for 6 min, neutralized with 1 M Tris base,
and compared with untreated Bence-Jones protein and untreated
light chains (CONTROLS). Immunoelectrophoresis was per-
formed with antisera described in Fig. 1. BJP, Bence-Jones
protein; L-CHAIN, light chains.
TABLE II

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<th>Immunological properties</th>
<th>Physicochemical and structural properties</th>
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<tr>
<td></td>
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<td>Antiserum °</td>
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<tr>
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<td>+</td>
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<tr>
<td>V&lt;sub&gt;L&lt;/sub&gt;</td>
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<td>C&lt;sub&gt;L&lt;/sub&gt;</td>
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* Antiserum to homologous protein: anti-Bence-Jones protein LEN.
° Anti-Bence-Jones protein LEN absorbed with a heterologous κ Bence-Jones protein.
+ Antiserum to a heterologous λ Bence-Jones protein.
* P-100 polyacrylamide: effluent volume/total volume (V<sub>e</sub>/V<sub>t</sub>).
* Determined in 0.15 M NaCl.
° Determined in 0.15 M NaCl-0.10 M Tris-HCl, pH 7.6.

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FIG. 5. Immunodiffusion analysis of Bence-Jones protein LEN and its isolated variant half and constant half. All antigen concentrations were 0.25 mg per ml. The center wells contained antisera as described in Fig. 2. BCP, Bence-Jones protein. More completely cleaved after 24-hour incubation. No cleavage occurred in this period with the intracellular extract. Moreover, no change in the protein was observed when incubated with a mixture of the two extracts.

Endopeptidase Treatment of Bence-Jones Proteins—Several types of endopeptidases cleaved κ and λ type Bence-Jones proteins into V<sub>L</sub> and C<sub>L</sub>. The extent of cleavage was related to (a) enzyme to protein ratio, (b) pH of the reaction mixture, (c) time of reaction, and (d) temperature of incubation. The conditions regarding each of these four factors varied somewhat for an individual protein, and the optimum conditions for each protein were determined separately. Pepsin was chosen initially for study because its proteolytic activity, like that of the urinary factor, occurs only at an acid pH. The results of pepsin treatment of κ Bence-Jones protein LEN are shown in Fig. 5. Progressive cleavage of the protein into V<sub>L</sub> and C<sub>L</sub> was evident by immunoelectrophoresis; no intact protein was detected after 10 min of incubation and beyond 30 min the C<sub>L</sub> could no longer be detected. The V<sub>L</sub>, in contrast to C<sub>L</sub>, was resistant to further proteolysis and remained stable even after prolonged incubation.

Cleavage of Bence-Jones proteins occurred with endopeptidases active at neutral or alkaline pH. Pepsin at pH 7.6, subtilisin at pH 8.6, and trypsin at pH 8.2 resulted in formation of V<sub>L</sub> and C<sub>L</sub>. Usually, a higher enzyme to protein ratio was required for these enzymes than for pepsin. Splitting of the proteins was less pronounced at lower ratios, and increasing the period of incubation only resulted in more general proteolysis. When the incubation mixture of certain trypsin-treated proteins was acidified, more pronounced cleavage into V<sub>L</sub> and C<sub>L</sub> occurred. The effect of trypsin on λ Bence-Jones protein COX is shown in Fig. 6. Complete cleavage of the protein into V<sub>L</sub> and C<sub>L</sub> was evident by immunoelectrophoresis.

Those Bence-Jones proteins which were not readily cleaved were rendered more susceptible to proteolysis by prior reduction and alkylation (0.1 M 2-mercaptoethanol, 0.15 M iodoacetamide). However, reduction and alkylation alone of other Bence-Jones proteins yielded a small amount of a component antigenically deficient to intact protein and rendered the protein extremely susceptible to further proteolysis.

Proteolysis of Immunoglobulin Light Chains—Endopeptidase treatment of light chains isolated from G- or A-myeloma proteins resulted in a decrease in the amount of intact protein and formation of two separate components possessing antigenic determinants of V<sub>L</sub> and C<sub>L</sub>, respectively. The light chains were obtained from the serum myeloma protein of patients who were excreting Bence-Jones proteins in their urine. Antisera prepared against the homologous Bence-Jones protein were capable of detecting the V<sub>L</sub> of the homologous light chains. The opti-
Comparative Studies of Bence-Jones Protein, Variant Half and Constant Half—The $V_L$ and $C_L$ products resulting from pepsin cleavage of Bence-Jones protein LEN were isolated by electrophoresis on starch blocks and further purified by gel filtration. The results of certain immunological, physicochemical, and structural analyses of the intact protein and its isolated $V_L$ and $C_L$ are presented in Table II. The precipitin reactions of the isolated $V_L$ and $C_L$ were compared with that of the intact protein LEN (Fig. 8). With homologous antiserum ($H$), the $V_L$ and $C_L$ formed a reaction of partial identity with the whole protein and a reaction of nonidentity with each other. After absorption of this antiserum with a heterologous $\kappa$ type Bence-Jones protein, the $V_L$ formed a line of identity with the Bence-Jones protein; the $C_L$ did not react with this absorbed antiserum ($V$) nor with an antiserum to the $V_L$ of Bence-Jones protein LEN. With heterologous antiserum ($C$), the $C_L$ formed a reaction of identity with the whole protein; the $V_L$ did not react with this antiserum, nor with antisera to six other heterologous $\kappa$ type Bence-Jones proteins.

Endopeptidase cleavage of light chains isolated from human FII $\gamma$-globulin was evident both by immunoelectrophoresis and starch gel electrophoresis. Components sharing $C_L$ determinants with $\kappa$ and $\lambda$ type Bence-Jones proteins were recognized by anti-$\kappa$ and anti-$\lambda$ Bence-Jones protein antisera. Lack of antiserum with specificity for FII light chains limited identification of $V_L$ components.
The monomer Bence-Jones protein and its isolated V\textsubscript{L} and C\textsubscript{L} were relatively homogeneous when analyzed by electrophoresis in an alkaline starch gel and in an acid urea starch gel (Fig. 9). Gel filtration of a mixture of the three proteins yielded two separate peaks. The first peak contained only whole protein and the second contained both V\textsubscript{L} and C\textsubscript{L}. On analytical ultracentrifugation the major portion of C\textsubscript{L} protein sedimented as a homogeneous peak in 0.15 M NaCl, with a $s_{20,w}$ of 1.7. In addition, a small, more rapidly sedimenting broad peak was present. This second peak was not observed when C\textsubscript{L} was centrifuged in 6 M guanidine-HCl, pH 3, or in 0.02 M Tris-NaOH, pH 11. Because of the limited solubility of V\textsubscript{L} in 0.15 M NaCl, sedimentation studies were performed in 0.15 M NaCl-0.1 M Tris-HCl, pH 7.6. The V\textsubscript{L} sedimented as a single peak with a $s_{20,w}$ of 2.6. Centrifugation of V\textsubscript{L} in 6 M guanidine-HCl, pH 3, and in 0.02 M Tris-NaOH, pH 11, resulted in reduction of sedimentation coefficient to 2.0 and 2.3, respectively.

Both Bence-Jones protein LEN and its isolated V\textsubscript{L} had aspartic acid as the NH\textsubscript{2}-terminal amino acid. In contrast, the isolated C\textsubscript{L} had phenylalanine in this position. Twenty-two distinct peptides were identified in the tryptic digest of the reduced-alkylated Bence-Jones protein. Tryptic digests of the V\textsubscript{L} and of the C\textsubscript{L} contained 11 distinct peptides and, with the exception of one peptide, no overlapping peptides were evident (Fig. 10).

The tryptic peptides of V\textsubscript{L} and C\textsubscript{L} were contained in the peptide map of the tryptic digest of the Bence-Jones protein.

The thermal solubility properties of Bence-Jones protein LEN and its isolated V\textsubscript{L} and C\textsubscript{L} were determined in a series of acetate and phosphate buffers, pH 4 to 8, 0.1 ionic strength (34). The initial protein concentration was 2 mg per ml and the amount of protein precipitated by heating to 56$^\circ$C for 30 min was measured. Identical thermal solubility properties were found for protein LEN and its V\textsubscript{L}. Maximum precipitation (98%) of both proteins occurred at pH 4. In contrast, no precipitation of C\textsubscript{L} occurred at 56$^\circ$C and the protein remained soluble even with heating to 100$^\circ$C. Similar results were obtained with other Bence-Jones proteins and their respective V\textsubscript{L} or C\textsubscript{L}. Of special interest was a Bence-Jones protein which partially precipitated (15%) after heating to 56$^\circ$C or to 100$^\circ$C. Its V\textsubscript{L} showed identical thermal solubility properties.

In the course of isolating the V\textsubscript{L} and C\textsubscript{L} of Bence-Jones protein LEN, crystals were noted and immunochemically identified as V\textsubscript{L}. Subsequent studies found V\textsubscript{L} to be readily crystallized in 0.1 M potassium phosphate, pH 6, at 5$^\circ$C from an initial concentration of 10 mg per ml (Fig. 11).

**DISCUSSION**

The NH\textsubscript{2}-terminal (variant) and COOH-terminal (constant) halves of Bence-Jones proteins and light polypeptide chains have an approximately equal number of amino acids (35) and each half contains an intrachain disulfide bond (36). The two halves differ markedly in primary structure as shown by peptide mapping (8, 9, 37) and sequence analyses (10, 35, 38). Proteins, corresponding to one-half of the Bence-Jones protein polypeptide chain, have been detected in urine of patients with multiple myeloma (11-16). These components, found only in the presence of whole Bence-Jones protein, were related to the V\textsubscript{L}, rarely to the C\textsubscript{L}, of the intact molecule. One-third of patients with Bence-Jones proteinuria had such components (12); however, they may occur more commonly. The level of Bence-Jones protein in blood or urine is determined by its rate of synthesis and of catabolism (39, 40). Removal of Bence-Jones protein by catabolism and by urinary excretion is dependent on

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1. In the processing of urine by ammonium sulfate precipitation or by dialysis, loss of V\textsubscript{L} or C\textsubscript{L} may occur if the V\textsubscript{L} or C\textsubscript{L} is more soluble than the Bence-Jones protein or if the dialysis tubing is permeable to V\textsubscript{L} or C\textsubscript{L}. 

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renal function (41, 43). The greater susceptibility of $\kappa$ to proteolysis and differences in catabolism or renal excretion of $\kappa$ and $\lambda$ may explain the predominant occurrence of $\kappa$ in urine. The presence of $V_L$ or $C_L$ has no dependence on either the antigenic type (k or $\lambda$) or the amount of Bence-Jones protein excreted. Some patients excreting 20 to 50 g of Bence-Jones protein daily have no detectable $V_L$ or $C_L$ in their urine, whereas other patients excreting 1 to 5 g have such components. The amount of $V_L$ or $C_L$ in urine usually represents less than 10% of the Bence-Jones protein excreted (12), but in one patient's urine the amount of $V_L$ was equivalent to the whole protein (44). The ratio of $V_L$ or $C_L$ to whole protein has been noted to remain remarkably constant (12, 44).

Biochemical (45, 46) and immunofluorescent studies (47) have shown that Bence-Jones proteins are synthetic products of plasma cells and are formed independent of myeloma proteins (48–50). That $V_L$ and $C_L$ are products of synthesis of de novo has not been conclusively established (44, 51). Biosynthetic studies were performed with bone marrow cells from three patients each excreting only whole Bence-Jones protein. In each case, extracellular culture fluid contained labeled protein, amounting to 1% of the labeled Bence-Jones protein, which corresponded to $V_L$ and $C_L$ by gel filtration. While electrophoretic and gel filtration techniques provided presumptive evidence for the presence of $V_L$ or $C_L$, definitive identification of the low molecular weight proteins as $V_L$ and $C_L$ was made by the application of immunochemical techniques utilizing specific antisera and carrier proteins. In one case both were available, and labeled $V_L$ and $C_L$ were identified by radiouniformed electrophoresis. Since only whole Bence-Jones protein could be detected in this patient's urine, the finding of labeled $V_L$ and $C_L$ suggests a synthetic origin. Whether $V_L$ and $C_L$ are abortive precursors of Bence-Jones protein synthesis or are synthesized independently is not known. Aberrations in immunoglobulin synthesis occur in plasma cell-lymphatic malignancies and have been found in other disease states as well as in normal individuals (53–56). Free light chains, identified in urine and serum of normal individuals, possessed the immunochemical and physicochemical properties of Bence-Jones proteins (57). Fragments of light chains, having filtration properties identical with $V_L$ and $C_L$, have been detected in normal urine (58). The light chain fragments were electrophoretically heterogeneous and contained both $k$ and $\lambda$ antigenic determinants.

While the synthetic origin of $V_L$ and $C_L$ has not been unequivocally shown, the catabolic origin has been established. Cioli and Baglioni (52) have identified $V_L$ as a catabolic product of a Bence-Jones protein. Further, they conclude that the occurrence of Bence-Jones protein fragments in some patients with Bence-Jones proteinuria is due to an inherent structural fragility of the protein, rendering it susceptible to renal catabolic processes. We observed that a Bence-Jones protein, excreted only as intact protein, was partially catabolized into products having gel filtration properties identical with $V_L$ and $C_L$. This protein was cleaved in vitro into its $V_L$ and $C_L$ by a proteolytic factor present in urine and in a kidney extract as well as by several types of endopeptidases.

Some enzymes which cleave Bence-Jones proteins and light chains also cleave the heavy chains of immunoglobulins into well defined components. Papain treatment of immunoglobulin G results in cleavage of the heavy chain into two parts: the Fd fragment which is $\text{NH}_2$-terminal and has a variable amino acid sequence and the Fe fragment which is COOH-terminal and has a relatively constant sequence (59). The proteolytic activity of papain as well as pepsin is directed toward a "hinge" peptide linking the Fd and Fe portion of the heavy chain (60). The susceptibility to proteolytic cleavage of peptide bonds in the "switch" region between $V_L$ and $C_L$ of Bence-Jones proteins and between $V_L$ and $C_L$ of light chains is analogous to the susceptibility to proteolysis of peptide bonds in the hinge region of heavy chains. An intrachain disulfide bond in the $V_L$ and in the $C_L$ of the Bence-Jones protein (36) confers symmetry to the molecule; this symmetry is clearly evident in the molecular model of a Bence-Jones protein presented by Putnam et al. (35). The particular susceptibility to proteolysis of the peptide bonds in the switch region of Bence-Jones proteins and light chains provides direct evidence for an exposed area in this part of the molecule.

Baglioni et al. (44) characterized five urinary components related to Bence-Jones proteins and found three to correspond exactly to the $\text{NH}_2$-terminal, i.e. $V_L$, portion of the whole protein while the others gave correspondences but extended two or three amino acids past the switch region. Pepsin, which has specificity for bonds linking aromatic amino acid residues (61), cleaved $\kappa$ Bence-Jones protein LEN into $V_L$ and $C_L$ and analysis indicated that the $\text{NH}_2$-terminal amino acid of $C_L$ was phenylalanine. This amino acid residue has been located in $\kappa$ type Bence-Jones proteins near the switch region at positions 98 and 116. Tryptsin cleaved isolated Bence-Jones proteins into $V_L$ and $C_L$ and, the radioactivity associated with $V_L$, $C_L$, and whole protein LEN separated by alkaline starch gel electrophoresis.

The numbers indicate the position of the residues along the amino acid sequence of $\kappa$ Bence-Jones protein Ag and $\lambda$ Bence-Jones protein Sh (35).
because of the specificity of trypsin for basic amino acid peptide linkages, it is probable that the Bence-Jones proteins were cleaved at position 107 (lysine) or 108 (arginine) in \( \kappa \) type Bence-Jones proteins and at position 111 (lysine) in \( \lambda \) type proteins. Indeed, the findings of Deutsch (62) and Baglioni et al. (44) indicated the presence of lysine as the COOH-terminal amino acid for two \( \kappa \) type and two \( \lambda \) \( V_L \) proteins.

Comparison of the properties of the isolated \( V_L \) and \( C_L \) with the properties of the Bence-Jones protein or light chain is of prime importance in determining whether a property of the intact molecule is a function of \( V_L \) or \( C_L \) singly or requires the combination of both halves. The \( C_L \) contains the site for covalent linkage between the light and heavy chains (63), while results of recombination experiments (64, 65) indicate that both halves contribute to noncovalent linkage. The Inv genetic factors of \( \kappa \) type Bence-Jones proteins are located in the \( C_L \) (66). It is not known whether the whole molecule is essential for expression of Inv activity. The fact that unfractionated tryptic digests of two Inv(S)-positive Bence-Jones proteins were Inv(S)-negative suggests the importance of configuration for expression of this genetic determinant (27). Genetic determinants localized to the \( V_L \) of Bence-Jones proteins have not been found.

Bence-Jones protein was first recognized because of its unusual thermal solubility properties (3). The unique thermal behavior has been attributed to alterations in the tertiary and quaternary structural organization of the molecule (67). In this study we found that isolated \( V_L \) had thermal behavior identical with that of the homologous Bence-Jones protein, whereas isolated \( C_L \) remained soluble. This observation indicated that the molecular basis for the thermal behavior of Bence-Jones proteins is confined to the \( V_L \) portion of the molecule, and individual differences in the thermal solubility properties result from differences in the primary structure of the \( V_L \) of the molecule.

Specific antisera were required for immunochromatographic detection of protein with \( V_L \) or \( C_L \) antigenic determinants. The development of specific antisera not only permitted the immunochromatographic comparison of \( V_L \), \( C_L \), and the Bence-Jones protein, but also permitted a rapid and sensitive means of ascertaining the purity of isolated \( V_L \) and \( C_L \). With antisera to the homologous Bence-Jones protein, isolated \( V_L \) and \( C_L \) were antigenically distinct, and each was antigenically deficient to whole protein.

The ability to cleave selectively whole Bence-Jones proteins and light chains into \( V_L \) and \( C_L \) permits the preparation and isolation of each half in sufficient quantities needed for study. X-ray diffraction studies of the crystalline \( V_L \), would be invaluable in the elucidation of the molecular conformation of the \( V_L \).

Numerous genetic theories (discussed in Reference 68) have been proposed to explain a variant primary structure in the COOH-terminal half of Bence-Jones proteins, but the exact mechanism has not been established. Studies of properties of \( V_L \) and \( C_L \) made available by selective cleavage of Bence-Jones proteins or light chains should provide further information regarding the genetic control of immunoglobulin synthesis and the relationship between antibody structure and antibody specificity.

Note Added in Proof—The Inv type of intact Bence-Jones protein LEN and its isolated \( V_L \) and \( C_L \) was determined by Dr. Arthur G. Steinberg, Case Western Reserve University. The Bence-Jones protein LEN was Inv(S)-positive whereas the \( V_L \) and the \( C_L \) were Inv(S)-negative. Furthermore, analysis of samples of progressively cleaved Bence-Jones protein LEN (see Fig. 5) showed a progressive decrease in Inv(S) activity with the concomitant decrease in intact Bence-Jones protein.

REFERENCES

65. Mannik, M., Biochemistry, 5, 134 (1967).
Bence-Jones Proteins and Light Chains of Immunoglobulins: I. FORMATION AND CHARACTERIZATION OF AMINO-TERMINAL (VARIANT) AND CARBOXYL-TERMINAL (CONSTANT) HALVES
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