COMPARISONS OF BENCE-JONES PROTEINS AND L POLYPEPTIDE CHAINS OF MYELOMA GLOBULINS AFTER HYDROLYSIS WITH TRYPSIN

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Previous studies (1–3) have supported the hypothesis that Bence-Jones proteins are composed of free L (light) polypeptide chains similar to those of myeloma globulins. This hypothesis led to the prediction that L chains isolated from normal γ-globulins would behave like Bence-Jones proteins (2) and prompted the identification of the normal low molecular weight plasma and urinary γ-globulins with free L chains (4). The chemical, physical, and immunologic evidence presented in these studies indicated that the Bence-Jones proteins and the L chains of myeloma proteins from the same patient are identical.

In the present paper, we provide further information on the chemical relationship of Bence-Jones proteins and L chains. Peptides resulting from tryptic hydrolyses were compared by two-dimensional, high voltage electrophoresis. Although this comparison revealed marked differences between heterologous proteins, no differences were found between Bence-Jones proteins and L chains of myeloma globulins from the same patients.

Materials and Methods

Bence-Jones Proteins and Myeloma Proteins.—Bence-Jones proteins were isolated from 24 hour collections of urine from patients with multiple myeloma. The urine of the two patients chosen for extensive analysis (Sel and Spi) was concentrated by pervaporation at 4°C. in Visking 23/32 dialysis casing and was then dialyzed against distilled water. The Bence-Jones protein was isolated from the concentrate by zone electrophoresis on starch (5) in sodium barbital buffer at pH 8.6. The other samples of Bence-Jones proteins were isolated by precipitation from urine brought to 50 per cent saturation with $(NH_4)_2SO_4$ at pH 5.0 and 4°C. After they were dialyzed against distilled water, the proteins were lyophilized and stored at 4°C.

The two myeloma proteins (Sel and Spi) were isolated from fresh serum by zone electrophoresis on starch. Previous work by Mannik and Kunkel (6) has shown that these proteins belong to two unrelated antigenic groups.

A genetic analysis of the proteins of patients Sel and Spi has been made by Harboe *et al.* (7).

Starch Gel Electrophoresis in urea.—The method (1) and the conditions of reduction and alkylation of the proteins (2) have been described.

Antigenic Analyses.—These were done by double diffusion in agar with rabbit antisera prepared against whole human 7S γ-globulin. As expected from the work of Mannik and Kunkel

(6), Ouchterlony plate analysis showed that the two Bence-Jones proteins (Sel and Spi) did not cross-react (Fig. 1) when tested with antiserum against whole human 7S γ-globulin. Each Bence-Jones protein showed partial fusion with L chains of normal γ-globulin, consistent with the existence of normal 7S γ-globulins of both antigenic types. Comparison with myeloma proteins of known antigenic grouping revealed that the myeloma protein and the Bence-Jones protein of patient Sel were of antigenic group I; the proteins of patient Spi were of antigenic group II. These results confirmed the original grouping of these proteins by Mannik and Kunkel (6). L chains from myeloma proteins of group I will be called L_I chains; those from myeloma proteins of group II will be designated L_{II}.

Dissociation of Myeloma Proteins.—In order to obtain L chains that were soluble in aqueous buffer, the proteins were reduced in the absence of urea, as first described by Edelman and Poulik (1). 1 per cent solutions of the myeloma proteins were reduced for 1 hour at room temperature in 0.05 M tris buffer, pH 8.0, that was made 0.1 M in 2-mercaptoethanol. Iodo-

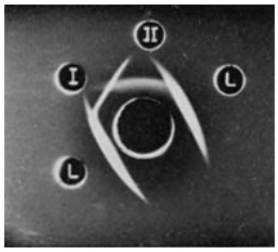

FIG. 1. Comparison of Bence-Jones proteins of antigenic groups I and II with L polypeptide chains of normal human 7S γ-globulin. I, Bence-Jones protein Sel; II, Bence-Jones protein Spi; L, L polypeptide chains. Central well contained rabbit antiserum against human 7S γ-globulin.

acetamide was then added to a final concentration of 0.3 M and after 10 minutes at room temperature the solutions were dialyzed extensively against distilled water at 4°C and lyophilized.

The partially reduced alkylated proteins were dissolved in 0.5 N propionic acid in order to dissociate the chains. The L chains were separated from undissociated material and H (heavy) chains by gel filtration through 100 × 1.5 cm columns of sephadex G-100 in 0.5 N propionic acid. The procedure used was that of Fleischman *et al.* (8). Bence-Jones proteins to be compared were filtered through the same columns. Selected fractions were pooled, dialyzed against distilled water and lyophilized.

Preparation of Proteins for Tryptic Hydrolysis.—All samples to be compared were prepared for tryptic hydrolysis and hydrolyzed at the same time. 10 mg samples were reduced at room temperature in 1.4 ml. of 8 M urea and 0.1 M 2-mercaptoethanol. After 4 hours the solutions were made 0.2 M in iodoacetamide; after 10 minutes at room temperature, they were dialyzed exhaustively against 0.2 M NH_4HCO_3 at 4°C. All of the reduced and alkylated derivatives became insoluble and formed flocculent suspensions.

Tryptic Hydrolysis. Crystalline trypsin (Worthington Biochemical Company, Freehold, New Jersey, batch 5902) amounting to 2 per cent of the weight of the protein to be digested was added to the suspension of reduced alkylated protein in NH_4HCO_3. The hydrolysis proceeded for 24 hours at 37°C. After approximately 6 hours the suspension cleared almost completely, and at 24 hours little or no insoluble material remained. The hydrolysates were frozen and lyophilized.

In an additional experiment, equal amounts of reduced alkylated L chains and Bence-Jones proteins of patient Sel were first mixed and then hydrolyzed with trypsin.

Two-Dimensional High Voltage Electrophoresis.—The hydrolysates were dissolved in 25 μl of the buffer used for the first dimension of electrophoresis (pyridine:acetic acid:water, 25:25:950, pH 4.7) (9). Approximately 10 μl of the sample were streaked in a 0.5 cm band on Whatman 3 MM paper which had previously been dipped in buffer and blotted. Electrophoresis was done for 1 hour in a water-cooled tank (Savant Instrument Company, Hicksville, New York) under varsol (Standard Oil Company, New Jersey) using a potential gradient of 50 volts/cm. The paper sheets were dried at 37°C, and, after analysis of marker strips, a strip (2 inches × 10.5 inches) containing the majority of the ninhydrin-reactive spots (see Fig. 5, Results) was sewn into the origin of another sheet of filter paper (10). The portions of the strips not taken for the second dimension were dipped in ninhydrin, and compared directly. Electrophoresis in the second dimension was done at pH 1.9 (acetic acid:88 per cent formic acid:water, 87:25:888) (9) for 3.5 hours under varsol using a potential gradient of 20 volts/cm.

Staining and Photography.—All electropherograms were dipped in 0.5 per cent ninhydrin in acetone made 0.01 M in pyridine. After full development of the ninhydrin color, the paper sheets were dipped in copper nitrate (saturated aqueous $Cu(NO_3)_2$:10 per cent aqueous HNO_3:acetone, 1:0.2:100) in order to fix the ninhydrin spots. The patterns of the hydrolyzed proteins from patient Sel were also stained for peptides containing arginine (11), tryptophan (12), histidine (13), and proline (14). After staining and fixing, the electropherograms were photographed with a copying camera fitted with an X-2 filter (Eastman Kodak, Rochester, New York) and contrast process panchromatic film (Eastman Kodak, Rochester, New York).

RESULTS

Isolation and Purification of Reduced Alkylated Proteins.—The fractionation by gel filtration of partially reduced alkylated group I myeloma protein (Sel) is given in Fig. 2*a*. The fraction marked III was identified as L chains by immunological criteria (3) and by starch gel electrophoresis in urea. Fractions I and II appeared to be mixtures of undissociated material and of H chains; the latter fraction contained relatively more H chains, as determined by starch gel electrophoresis. The Bence-Jones protein purified by electrophoresis from the urine of the same patient (Sel) was contaminated with an unidentified pigment. In order to purify the protein further and to treat it in the same way as the L chains, it was filtered through a column of sephadex G-100 in 0.5 N propionic acid (Fig. 2*b*). Three peaks were obtained. The first and second peaks contained material which was identified as Bence-Jones protein. The last broad peak contained the pigmented material, which was not further characterized.

A similar separation of chains of a group II myeloma protein and gel filtration of the autologous Bence-Jones protein is shown in Figs. 3*a* and 3*b*. After gel filtration of the reduced alkylated myeloma protein, three peaks were obtained. The first peak (fractions I and II) contained undissociated material and H chains; fractions III and IV consisted of L chains in different states of aggregation. The Bence-Jones protein of this patient, which was pigment-free, also showed a tendency to separate into different polymeric forms in propionic acid (Fig. 3*b*). The fraction of Bence-Jones protein that was most retarded on

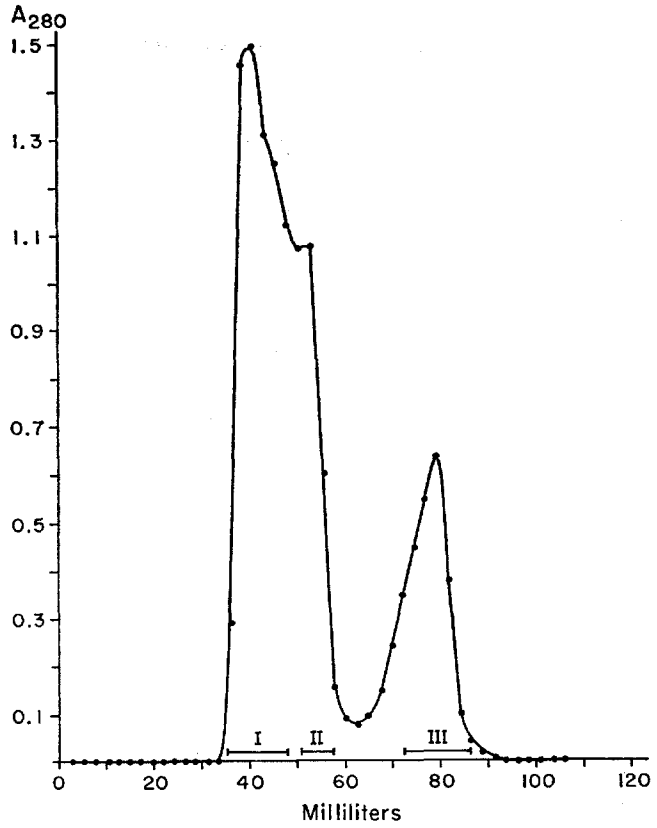

FIG. 2*a*. Separation by gel filtration of L chains of partially reduced alkylated myeloma protein (patient Sel, antigenic group I). I, II, III: fractions pooled for analysis; A_{280}, absorbancy at 280 mμ; sephadex G-100: column dimensions 100 cm × 1.5 cm; solvent: 0.5 N propionic acid.

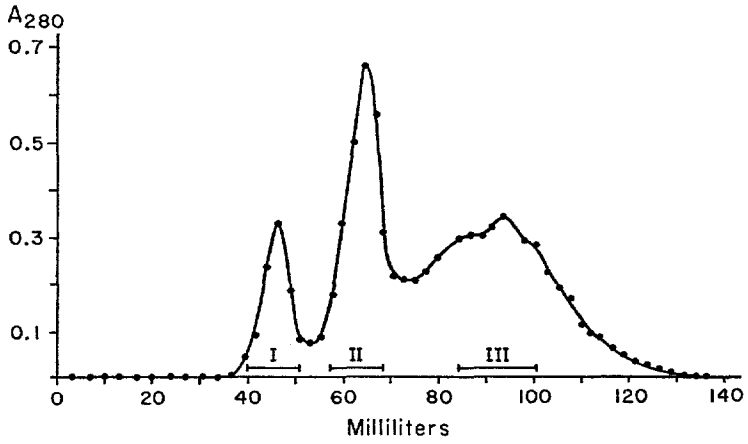

FIG. 2*b*. Gel filtration of Bence-Jones protein (Sel) on sephadex G-100 in 0.5 *N* propionic acid. Column dimensions: 100 cm × 1.5 cm; A_{280}, absorbancy at 280 mμ. I, II, III: pooled fractions.

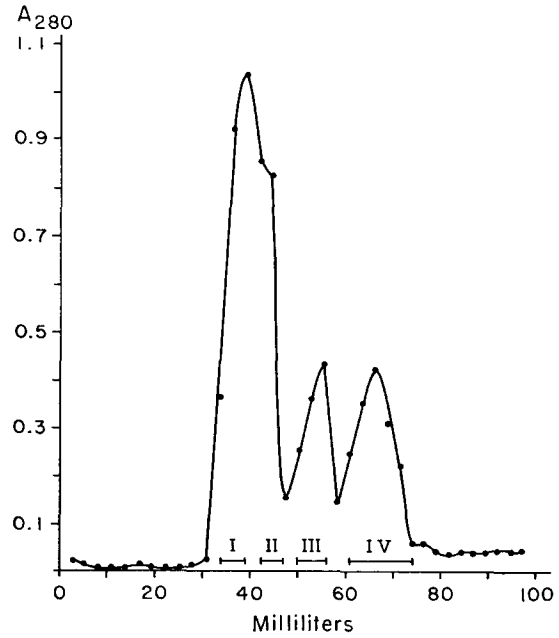

FIG. 3*a*. Separation of L chains of partially reduced alkylated myeloma protein of antigenic Group II (patient Spi). Conditions of gel filtration on sephadex G-100 as in Fig. 2*a*. A_{280}, absorbancy at 280 mμ. I, II, III, IV: pooled fractions.

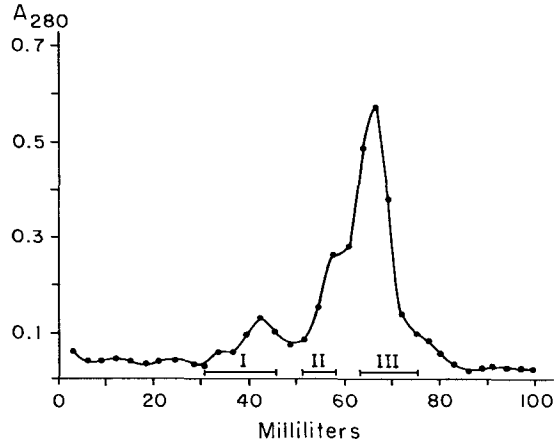

FIG. 3*b*. Gel filtration (sephadex G-100) of Bence-Jones protein (Spi). Conditions as in Fig. 2*b*. A_{280}, absorbancy at 280 mμ. I, II, III: pooled fractions.

the sephadex column was chosen for enzymatic hydrolysis and electrophoretic comparison with L chains.

Electrophoretic Comparisons.—Comparisons by starch gel electrophoresis of the proteins from both patients are shown in Fig. 4. Reduced alkylated Bence-Jones protein migrated with the same mobility as L chains dissociated

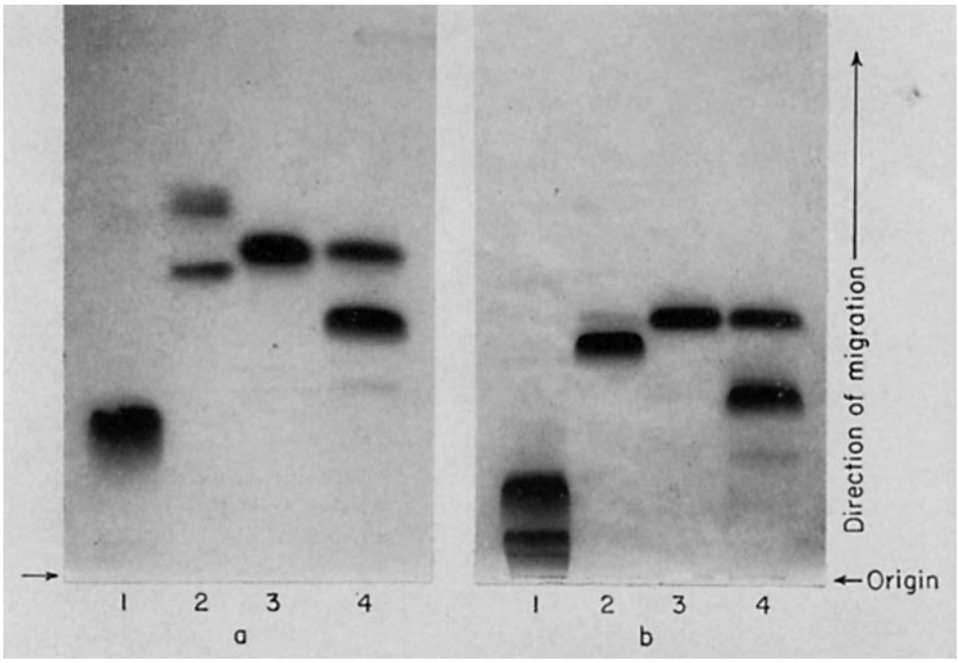

FIG. 4. Starch gel electrophoresis of myeloma proteins and Bence-Jones proteins from the same patient before and after reduction and alkylation.

a. Antigenic group I (patient Sel)

b. Antigenic group II (patient Spi)

1, myeloma protein; 2, Bence-Jones protein; 3, reduced alkylated Bence-Jones protein; 4, reduced alkylated myeloma protein.

from the corresponding myeloma protein, as was expected from earlier findings (2).

The patterns of peptides in the tryptic hydrolysates of L_I chains (Sel) and of Bence-Jones protein (Sel) were indistinguishable when compared by electrophoresis at pH 4.7, as shown in Fig. 5. The presence of free arginine, free lysine, glutamic acid, and aspartic acid was detected by comparison with authentic markers. Note also the presence of peptides more basic than free lysine. A two-dimensional electrophoretic comparison of L_I chains (Sel) and Bence-Jones

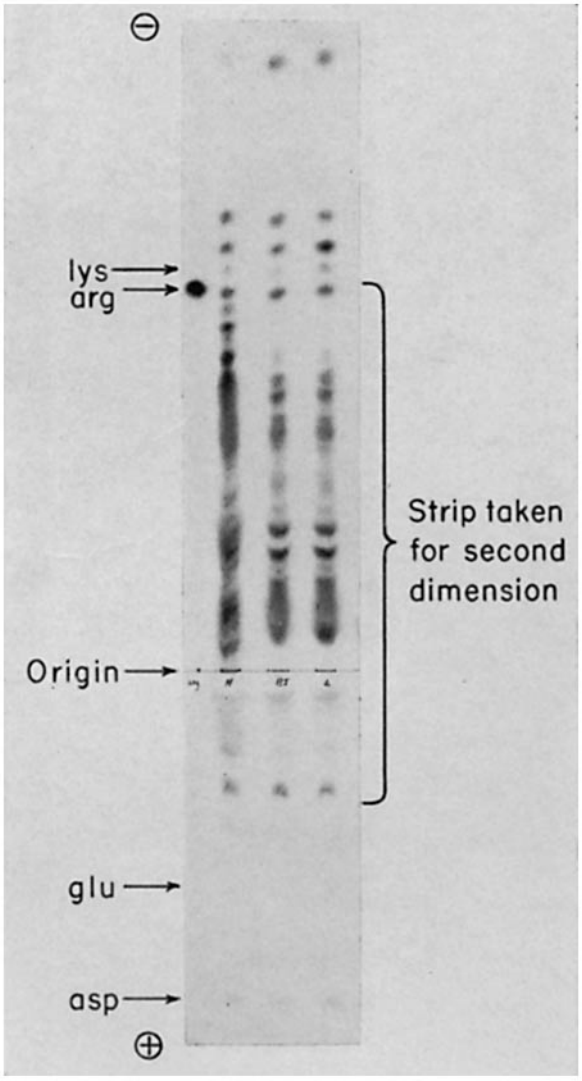

FIG. 5. One-dimensional high voltage electrophoresis of hydrolysates of myeloma protein (M), Bence-Jones proteins (BJ), and L_I chains (L). All of the proteins were from patient Sel.

protein (Sel) is given in Fig. 6. A total of 35 peptides were resolved from each hydrolysate. Similar patterns were obtained in repeated experiments. After they were stained with ninhydrin, the spots corresponded in number, in relative position, in color, and in relative intensity. Moreover, specific staining for arginine, tryptophan, histidine, and proline showed no

difference between the peptides of L_I chains (Sel) and of Bence-Jones protein (Sel). A mixture of these two autologous proteins that was hydrolyzed and electrophorized also showed this same pattern.

Similarly, a comparison of Bence-Jones protein (Spi) and L_{II} chains (Spi)

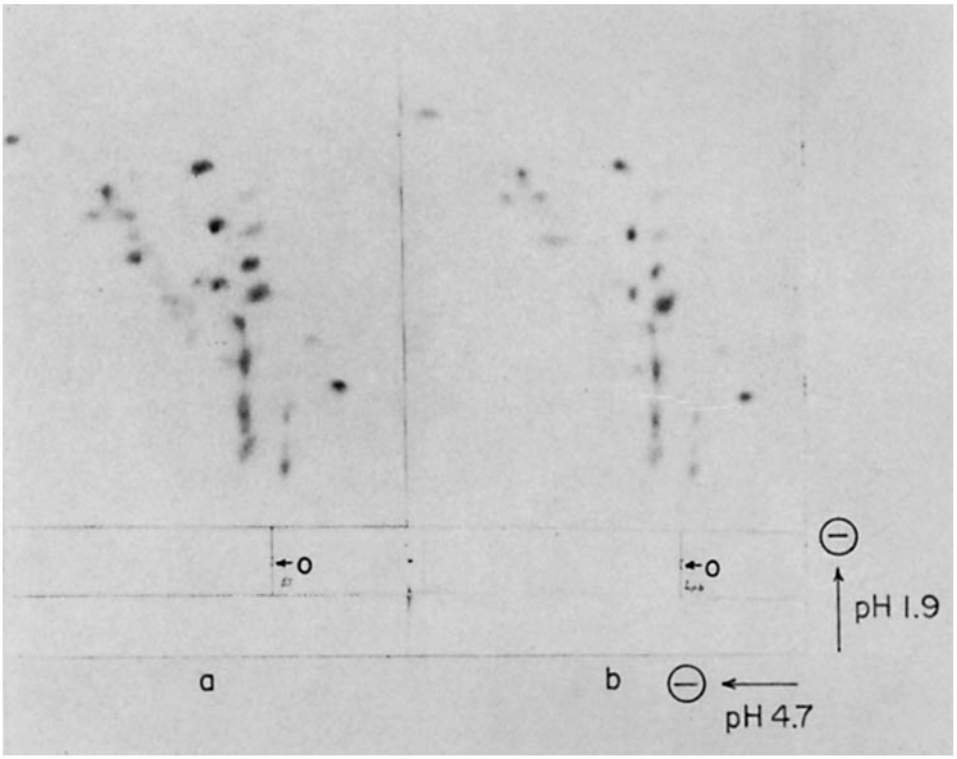

FIG. 6. Two-dimensional high voltage electrophoresis of tryptic hydrolysates of Bence-Jones protein (Sel) and L_I chains (Sel).

a. Hydrolysate of Bence-Jones protein

b. Hydrolysate of L_I chains

O, origin.

(Fig. 7) yielded corresponding patterns. Twenty-four peptides were resolved from the hydrolysates of the Group II proteins.

Marked differences were found between the two antigenic groups. A comparison of the peptides from the Group I Bence-Jones protein with those of the Group II protein is shown in Fig. 8. Comparison of Bence-Jones proteins from different patients within each group revealed differences in a few peptides (15); the differences between groups was much more extensive. These observations

agree with those of Putnam (16) who used different conditions for separation of the peptides.

As noted above, the fractions containing H chains obtained by gel filtration of the reduced alkylated myeloma proteins were contaminated with undisso-

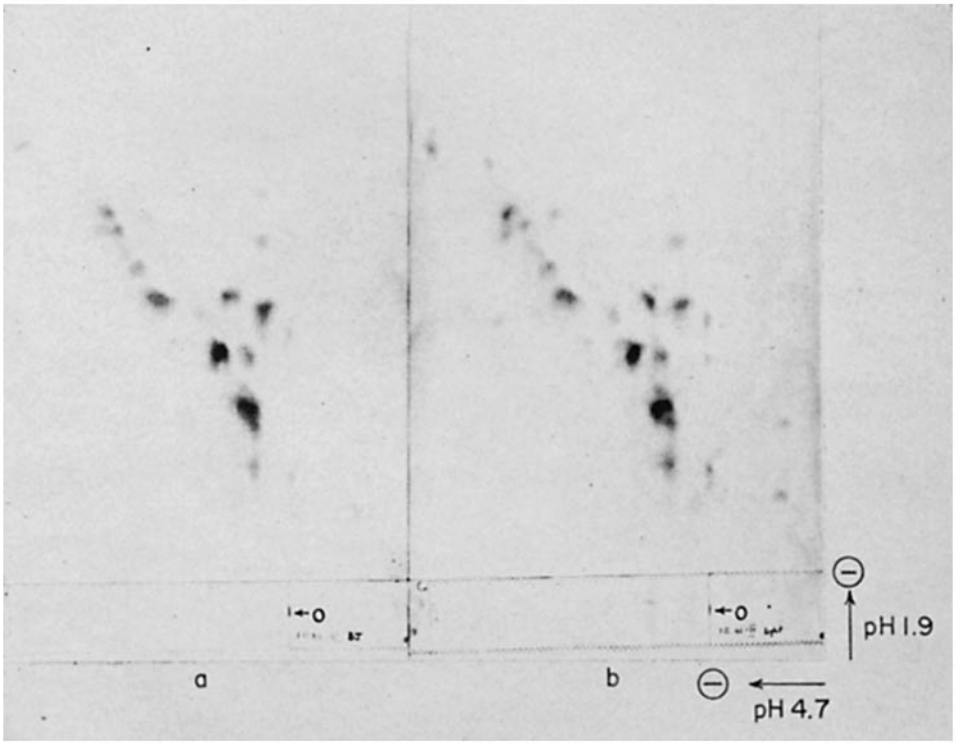

FIG. 7. Two-dimensional high voltage electrophoresis of tryptic hydrolysates of Bence-Jones protein (Spi) and L_{II} chains (Spi).

a. Hydrolysate of Bence-Jones protein.

b. Hydrolysate of L_{II} chains

O, origin.

ciated protein. Nonetheless, a comparison of the tryptic peptides of the L_I and H chain fractions of myeloma protein (Sel) was made. The difference in the peptide patterns is striking (Fig. 9) and adds to the evidence (1–3, 17) indicating that there are chemical differences between L chains and H chains from the same protein.

DISCUSSION

The experiments described here provide additional support for the idea that Bence-Jones proteins and L chains from autologous myeloma proteins are

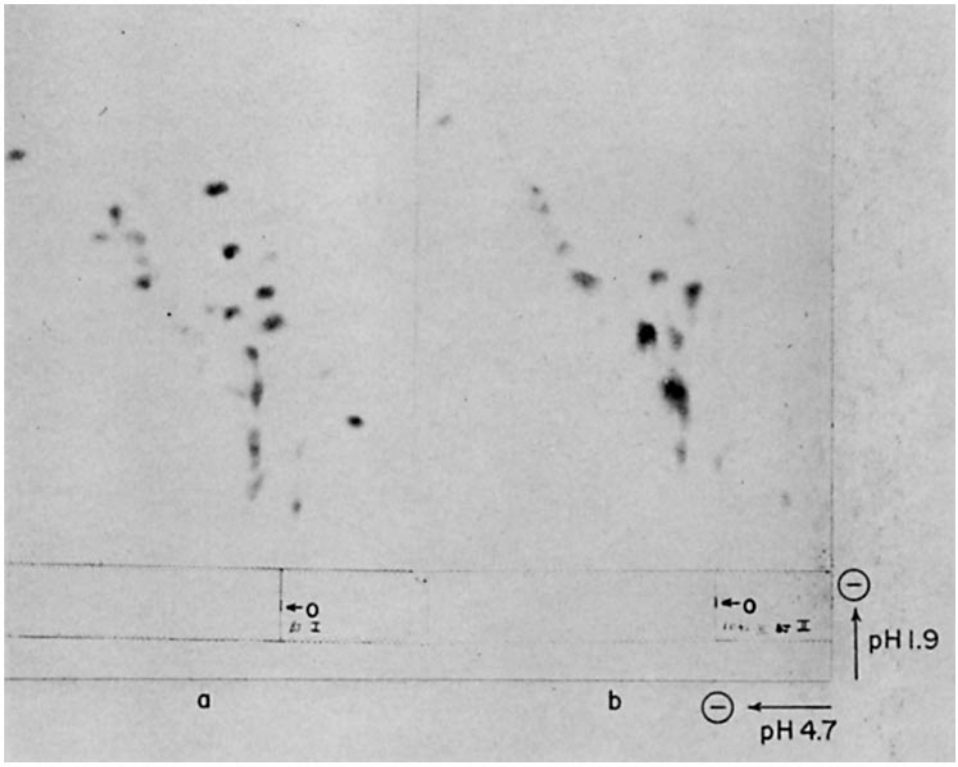

FIG. 8. Comparison of tryptic hydrolysate of Group I Bence-Jones protein (Sel) with that of Group II Bence-Jones protein (Spi).

a. Group I protein

b. Group II protein

O, origin.

chemically identical. Previous studies (2) have shown that the amino acid composition, starch gel electrophoretic behavior, spectrofluorometric properties, and thermosolubility properties of these proteins are the same. The correspondence of the tryptic peptides suggests that Bence-Jones proteins are not aberrant or incomplete polypeptide chains (16) but are in fact entire L chains which have not been incorporated into the autologous myeloma protein. The finding of free L chains in normal serum and urine (4) also makes it unlikely that Bence-Jones proteins are faulty or incomplete chains.

Antigenic analysis has established the existence of two groups of L chains having no antigenic determinants in common (4, 6, 18). The antigenic distinctness of the two groups is correlated with differences in amino acid sequence, since the observations of Putnam (16) as well as the present experiments indi-

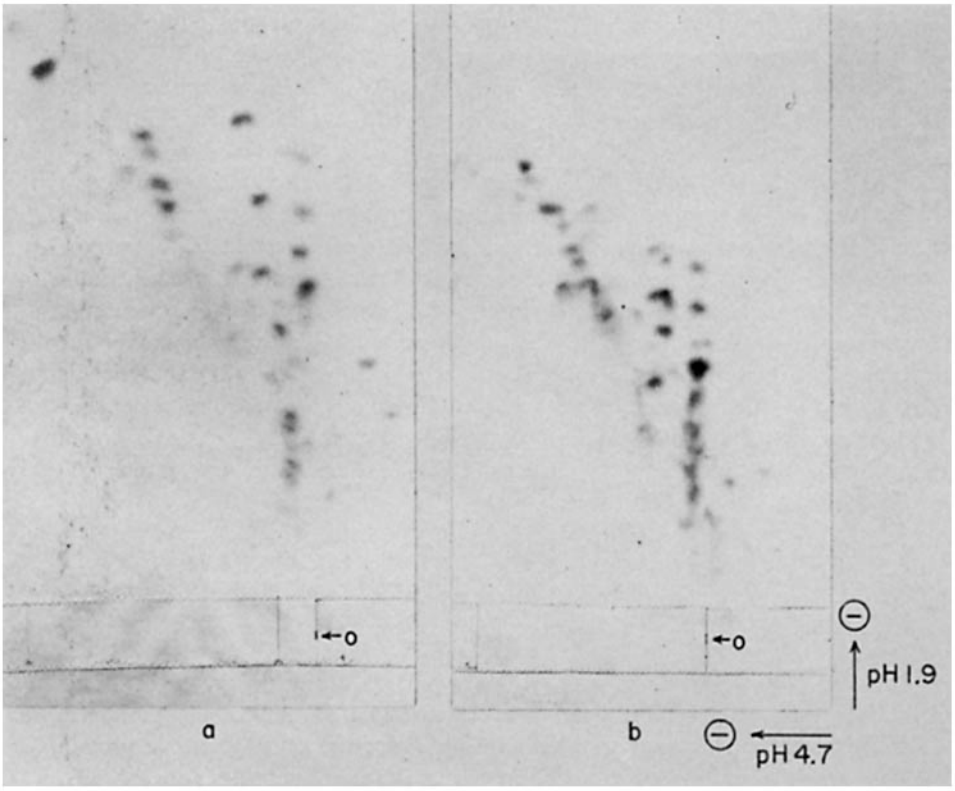

FIG. 9. High voltage electrophoresis of hydrolysates of L and H chains of a γ-myeloma protein (patient Sel).

a. Hydrolysate of L chain fraction

b. Hydrolysate of H chain fraction

O, origin.

cate a marked difference in the tryptic peptide patterns among proteins of the two antigenic classes. The two distinct antigenic groups have been identified in all γ-globulins (γ, γ_{1A}, γ_{1M}) both of normal and pathological origin; L chains are the common structural elements among all classes of γ-globulins (3, 18–20). It is therefore likely that L_I and L_{II} chains of normal γ-globulins will also be found to differ in their amino acid sequences.

Amino acid analyses (15) have shown a total of approximately 12 lysine and arginine residues for each unit of molecular weight 20,000 in Bence-Jones protein (Sel) and 15 lysine and arginine residues for each unit of molecular weight 20,000 in Bence-Jones protein (Spi). The number of peptides found after hydrolysis with trypsin exceeded that expected on the basis of the known

specificity of trypsin. This might be explained by chymotryptic activity in our trypsin preparation or by the presence of more than one kind of L chain in a given Bence-Jones protein or myeloma protein. Whatever the explanation, the conclusion that autologous Bence-Jones proteins and L chains are similar remains valid.

The comparison of H chains isolated by gel filtration on sephadex was hindered by the presence of contaminating, partially dissociated material, and of aggregates of L chains. Nevertheless, the H chain fraction of the group I myeloma protein showed a peptide pattern different from that of the L chains of the same protein. Of particular interest for future studies is the question of whether H chains of all γ-myeloma proteins are alike in their chemical structure. Both immunologic (20) and starch gel electrophoretic (21) analysis suggest that H chains of γ-myeloma proteins (Hγ chains) have structures entirely different from those of the γ_{1A} (β_{2A})-myeloma proteins (Hγ_{1A} chains). A comparison of the peptides of Hγ chains and Hγ_{1A} chains should elucidate further the nature of these differences.

SUMMARY

L polypeptide chains of myeloma globulin and Bence-Jones protein isolated from the same patient were found to be identical after comparison of their tryptic hydrolysates by two-dimensional high voltage electrophoresis. The patterns of peptides from proteins belonging to antigenic group I differed markedly from those of proteins in antigenic group II. A partially purified H chain fraction was compared with L chains from the same myeloma protein. The tryptic hydrolysates yielded dissimilar patterns of peptides.

These data indicate that γ-myeloma proteins contain two kinds of polypeptide chains, Hγ chains and either L_I or L_{II} chains. The L chains appear to be identical with those comprising the Bence-Jones protein from the same patient.

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