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PHYSICOCHEMICAL AND IMMUNOLOGICAL ANALYSIS OF A VARIABLE HALF MOLECULE OF A K-TYPE BENCE JONES PROTEIN

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Introduction

Low molecular components related to Bence Jones proteins were detected in the urine of patients with multiple myeloma by several investigators (1-11). Recently, some of these components have been characterized and found to correspond to the variable (6-10) or constant (2, 3) halves of Bence Jones proteins. It still remains obscure as to whether these components arise from abortive synthesis de novo or enzymic degradation of intact Bence Jones protein.

In the present paper, we have described a case in which a patient with myeloma excreted a variable half in the urine together with intact Bence Jones protein. The origin of the fragment protein was also discussed.

Clinical Summary

The patient was a 61-year-old male admitted on Aug. 5th, 1968, with complaints of low back pain, general arthralgia, weakness and fatigue. Physical findings included swelling with tenderness over the vertebral column and both knee joints. X-ray examination revealed widespread osteolytic lesions in the femur, pelvis and vertebral bodies and several punched out areas in the skull. Laboratory studies showed hemoglobin of 8.4 Gm/dl, total serum protein of 7.5 Gm/dl with relative hypogammaglobulinemia and marrow smears exhibiting poorly differentiated myeloma cells of 32.5%. Bence Jones protein in the urine was strongly positive by Jacobson's method (12). Following predonisolone and cytoxan therapy, the clinical and laboratory status of the patient remarkably improved. The patient was discharged after 10 months hospitalization and a follow up was carried out for 2 years at the out-patient clinic.

Experimental Procedure

Preparative procedure

Urine samples of the patient treated with activated carbon was filtrated with ditomaceous earth in order to remove the pigmented impurities. The filtrate was salted out with 60% saturated ammonium sulfate and kept at $4^{o}C$ overnight. This precipitation procedure was repeated three times. The precipitated protein was dialyzed against distilled water and continuous 0.15 M NaCl. Then 25 ml of protein solution was applied for gel-filtration to Sephadex G-100 column 150 x 3.0

cm equilibrated in 0.15 M NaCl and eluted at a flow rate of 25 ml per hour. Three fractions were obtained. Fraction 2 and 3 were collected and concentrated with a collodion bag (Sartorius membrane filter No. 13200), and dialyzed against 0.02 M potassium phosphate buffer, pH 8.0. Fractions were subsequently chromatographed on DEAE Sephadex A-50 column 90 x 2 cm with a starting buffer of 0.02 M potassium phosphate, pH 8.0 and a linear gradient to the limiting buffer of 0.02 M potassium phosphate, pH 8.0, 1 M NaCl.

The Fraction A and B obtained from DEAE Sephadex column were rechromatographed on Sephadex G-100 column 120 x 2 cm, equilibrated in 0.15 M NaCl with a flow rate of 10 ml per hour. Through the whole procedure, absorbancy of each fraction was read at 280 mμ with a Hitachi spectrophotometer 124. All chromatography procedures were carried out at 4°C.

The materials were finally dialyzed against distilled water, lyophilized and kept at 4°C for further analysis.

Cellulose acetate membrane electrophoresis

Electrophoresis was performed on a Separax membrane, Fuji Film Co., in 0.06 M barbital barbital-Na buffer, pH 8.6, with a constant current of 0.8 mA/cm for 45 min. As the staining dye, Nigrosin was sellected.

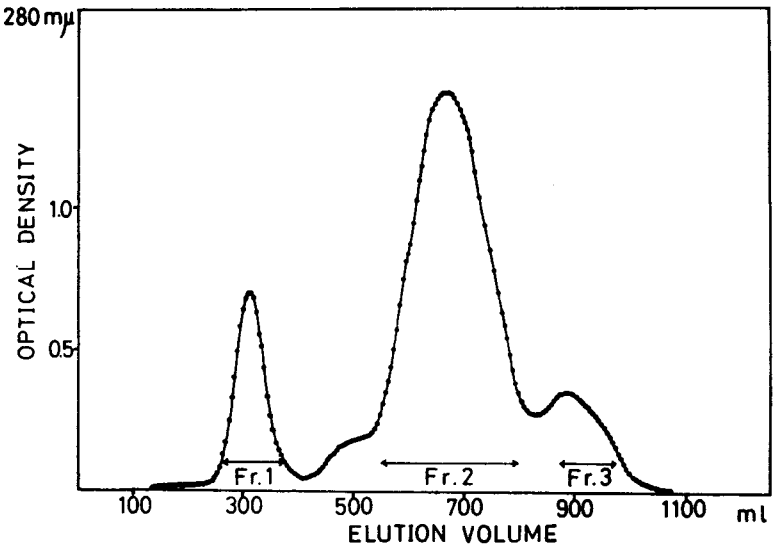


Fig. 1. Sephadex G-100 column chromatography of urinary protein precipitated with 60% ammonium sulfate.

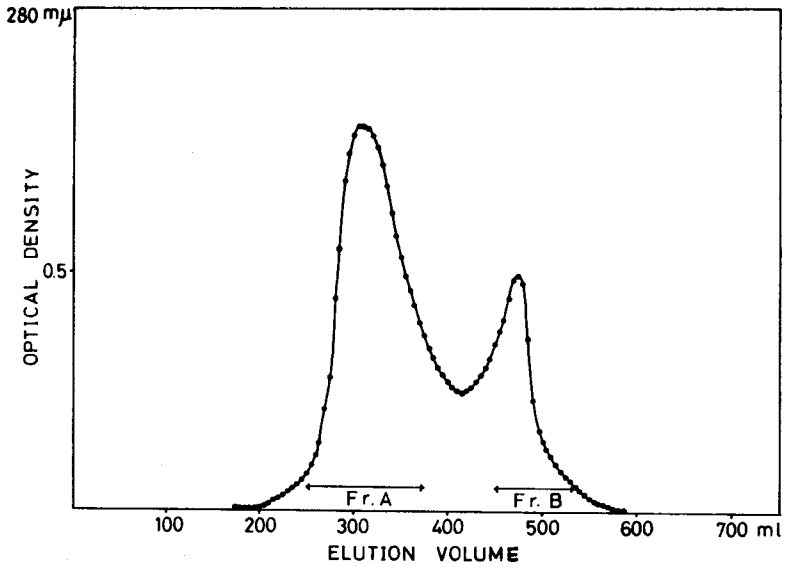


Fig. 2. DEAE Sephadex A-50 column chromatography of Fr. 2 of the urine of the patient.

Column gle filtration

Sephadex G-100 column 100 x 2.5 cm, equilibrated with 0.1 M tris HCl buffer, pH 8.0, 1 M NaCl was used for the estimation of molecular weight of the proteins (13) using the following proteins as standards of molecular weight; human albumin, human r-globulin and equine cytochrome C. The column was developed upwards at a flow rate of 25 ml per hour. Transmission tracing was obtained with a LKB Uvicord Absorptionmeter and LKB recorder Type 6520H. Void volume was determined by blue dextran 2000 from Pharmacia Fine Chemicals, A. B., Uppsala, Sweden.

Ultracentifugation

Samples were centrifuged at 20°C and 54000 R. P. M. by a Spinco Model-E analytical centrifuge in two different solvents: 1) 0.15 M KCl 2) 1 M propionic acid. The S values were not corrected.

Antisera

Anti K-type Bence Jones protein antisera was purchased form Behring Werke, A. G. Marburg / Lahn, Germany. In addition to the commercial antisera, specific antisera against the patient Bence Jones protein was obtained by immunizing rabbits subcutaneously with 10 mg of purified protein with Freund's adjuvant. Stimulation was done by weekly subcutaneous injections of 5 mg of the protein with the adjuvant.

Double diffusion

Double diffusion was performed by the method of Campbell (14).

Amino acid analysis

Samples were hydrolyzed in 6 N HCl for 18, 24, 48, 72 and 96 hrs. at 110°C and analyzed in a Hitachi automatic Amino Acid Analyzer model KLA-3B. The procedure of analysis was essentially the same as that of Spackman et al. (15).

Results

Preparation and purification

Urinary protein precipitated with 60% ammonium sulfate showed three peaks on Sephadex G-100 column chromatography as shown in Fig. 1. These distinct peaks were designated as Fraction 1, 2 and 3. Fraction 1 was discarded as aggregated materials. Fr. 2 and 3 were collected separately and dialyzed against 0.02 M phosphate buffer, pH 8.0 for subsequent ion-exchange chromatography. The chromatographic pattern on DEAE Sephadex A-50 column showed that Fr. 2 contained two peaks, namely Fr. A and B as shown in Fig. 2, whereas Fr. 3 gave a homogenous profile. The rechromatographic pattern of Fr. A was shown in Fig. 3a. The Fr. A furthermore was separated into three peaks appearing as Fr. A-1, A-2 and A-3 out of which Fr. A-2 and A-3 were shown to be identical with Fr. B and Fr. 3 by cellulose acetate membrane electrophoresis. Fr. A-1 was considered to be aggregated material of dimers with low molecular weight protein because these two components were demonstrated separately in considerable amounts together with the original aggregated material when gel filtration was performed in 1 M propionic acid.

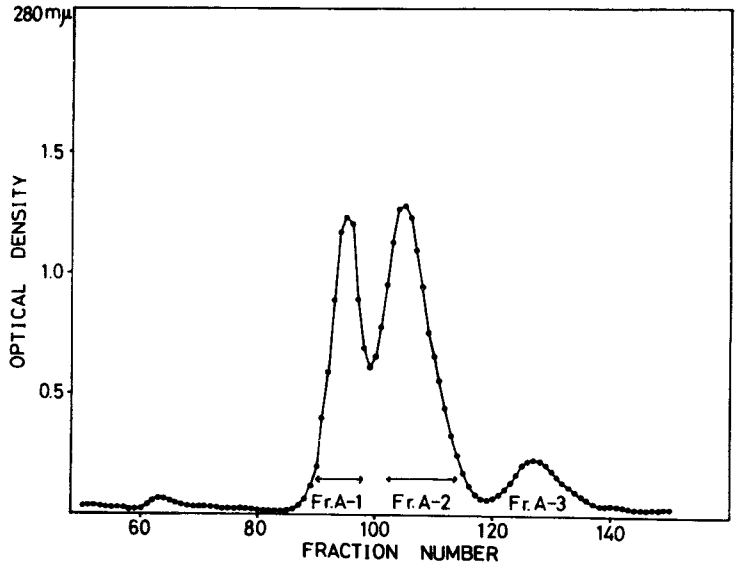


Fig. 3a. Sephadex G-100 column rechromatography of Fr. A.

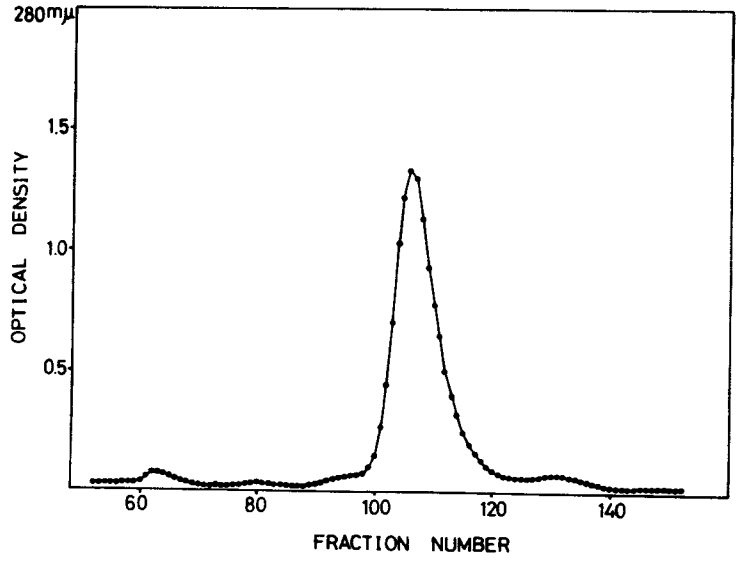


Fig. 3b. Sephadex G-100 column rechromatography of Fr. B.

On the other hand, the rechromatographic profile of Fr. B was homogenous as shown in Fig. 3b.

Electrophoresis

The purity of the fractions was examined by starch gel-, paper- and cellulose acetate membrane electrophoresis. Among them, the most satisfactory patterns were obtained by cellulose acetate membrane electrophoresis when Nigrosin was used for staining of the protein. As shown in Fig. 4, Fr. B and 3 were demonstrated to be electrophoretically homogenous and differed in mobility with each other. Fr. A-1 migrated to an intermediate position between the bands of Fr. B and 3 with a small amount of contamination of both fractions. As shown in Fig. 5, a weakly stained band which gave identical electrophoretic mobility with that of Fr. 3 was exhibited in the patient serum.

Molecular weight estimation

Molecular weights of Fr. B and 3 were estimated by gel filtration on a calibrated column of Sephadex G-100 with 0.1 M tris-HCl buffer, pH8.0. The values were calculated from the equation:

$$\log M = \log M_o - (6.062 - 5.00\ d) \times (V_e / V_o)$$

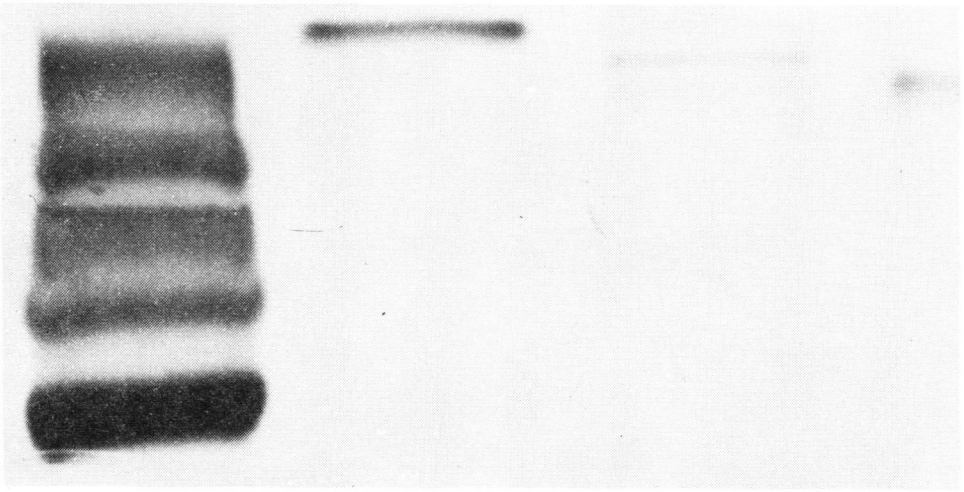
V_o : void volume

V_e : elution volume

d : density of the gel

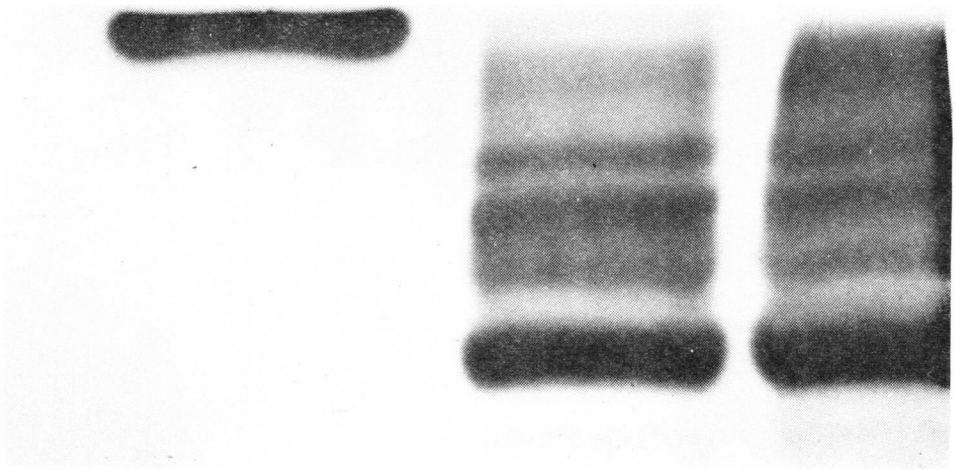
M_o : point of intersection at the M-axis

Fr. B and 3 showed values of 44000 and 21000, respectively.



Normal serum | Fr. 3 | Fr. A-1 | Fr. B

Fig. 4. Cellulose acetate membrane electrophoresis of each fraction.



Fr. 3 | Patient serum | Normal serum

Fig. 5. Cellulose acetate membrane electrophoresis of patient serum.

Ultracentrifugal analysis

The ultracentrifugal analysis of Fr. 3 was carried out in two different solvents as shown in Fig. 6. The sedimentation coefficient of this protein was 2.3 in 0.15 M KCl and 1.4 in 1 M propionic acid. These results indicated that Fr. 3 was not a stable monomer but was composed of two basic subunits which existed in dissociated form in the organic solvent.

Immunological analysis

Antigenic properties of Fr. B and 3 were studied by double diffusion technique as shown in Fig. 7. Fr. 3 formed a feebler precipitin as compared with the apparent precipitation line of Fr. B, using anti K-type Bonce Jones protein antisera. Using anti Fr. B serum, Fr. B formed a spur over Fr. 3. These findings suggested that Fr. 3 lacked in most of type K common determinants and possessed unique determinants of the patient Bence Jones protein.

Amino acid analysis

Amino acid analysis clearly demonstrated the difference in amino acid composition between Fr. B and 3, as shown in Table 1. When the amino acid composition of Fr. 3 were subtracted from that of Fr. B, the values were almost identical with that of a common part of k-chain (16).

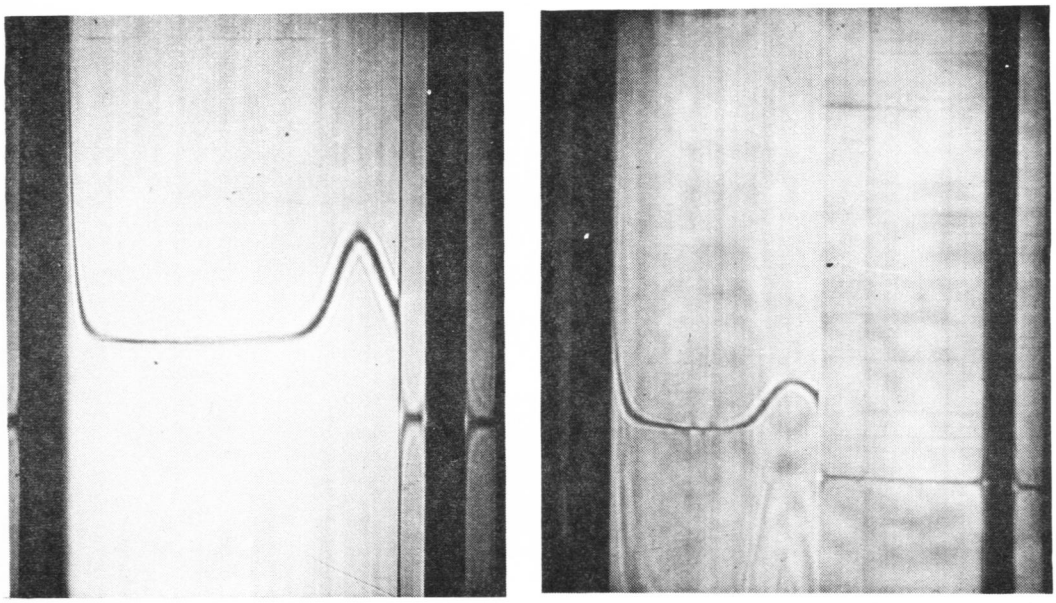


Fig. 6. Ultracentrifugal analysis of Fr. 3 in 0.15 MKCl (left side) and in 1 M propionic acid (right side).

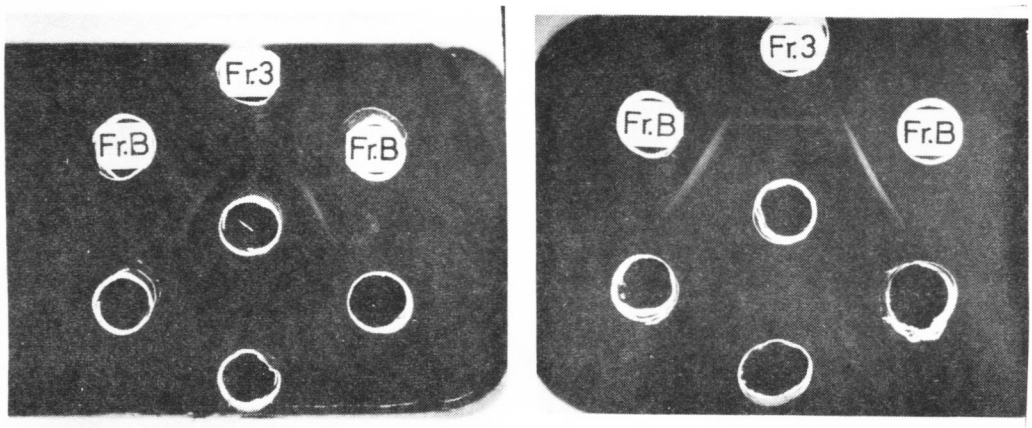


Fig. 7. Immunological analysis of Fr. B and 3, using anti type k Bence Jones protein antiserum (left side) and anti Fr. B serum (right side).

Table 1. Amino Acid Analysis of Fr. B and Fr. 3.

	Fr.B	Fr. 3	Fr.B-3	Common part of B-J protein(K)
Lys.	9.4	3.8	5.6	8
His.	1.7	0	1.7	2
Arg.	9.6	7.7	1.9	2
$CySO_3H$.	4.8	2.0	2.8	3
Asp.	14.5	4.4	10.1	10
Thr.	21.3	13.2	8.1	8
Ser.	32.2	16.3	15.9	16
Glu.	25.9	12.2	13.7	13
Pro.	13.2	7.9	5.2	5
Gly.	14.8	10.4	4.4	4
Ala.	11.0	4.5	6.5	7
Val.	12.1	2.3	9.8	10
Ileu.	8.0	6.9	1.1	1
Leu.	17.6	8.8	8.7	9
Phen.	8.3	4.8	3.5	4
Tyr.	7.6	3.7	3.9	4

Discussion

Two kinds of protein, Fr. 3 and B were isolated from the urine of patient with myeloma by repeated chromatography on Sephadex G-100 and DEAE Sephadex A-50 column. The Fr. B and 3 were shown to be electrophoretically homogeneous and differed in electrophoretic mobility. The molecular weights of Fr. B and 3 estimated by gel filtration on a calibrated Sephadex G-100 column were 44000 and 21000 respectively. During the purification of Fr. B and 3, aggregated materials composed of both

fractions were also demonstrated by column gel filtration. On electrophoresis, the aggregated material migrated to a intermediate position between Fr. B and Fr. 3, indicating that electrophoretic heterogeneity was attributed not only to polymorphism but also to polymerism as hitherto accepted (17). By ultracentrifugation, Fr. 3 was shown to be dissociated into two basic units with S value of 1.4 in 1 M propionic acid whereas in 0.15 M KCl, the value was 2.3. These findings suggested that Fr. 3 existed in dimer form of subunits which interacted each other noncovalently. The observations in gel diffusion clearly elucidated the relationship between Fr. B and 3. Both fractions shared unique determinants of the patient Bence Jones protein and Fr. 3 was lacking in most of the type k common determinants, suggesting that Fr. 3 was derived from a variable half of the Bence Jones protein. The above considerations received additional support by the amino acid analysis, i.e. the amino acids of Fr. 3 were almost identical with that of the variable half of Fr. B.

Studies of the fragment proteins related to Bence Jones proteins have been reported by several workers (1-11). Most of these proteins were characterized as the variable halves (6-10) or constant halves (2,3) of the L chain by physicochemical and immunological analysis. The presence of the fragments has no dependance on either the antigenic type or the amount of Bence Jones protein excreted. Solomon et al. (2) noted that these components were present in one-third of the patients with Bence Jones proteinuria. In addition, Karlson et al. (9) found that even normal

urine contained such components which had quite similar molecular sizes as that from patients with myeloma. These facts raise the question as to whether the fragment proteins have a catabolic origin or a synthetic origin. Recently, Cioli and Baglioni identified the fragment as a catabolic product of Bence Jones protein (4). Further, they concluded that the occurrence of Bence Jones protein fragments is due to an inherent structural fragility of the protein, rendering it susceptible to the renal catabolic process. To support this conclusion, studies have been attempted to cleave the light chains into two halves using a method of limited proteolysis. Solomon and McLaughlin (11) succeeded in the demonstration of the cleaved products in vitro and characterized them as a variable half and a constant half by immunochemical analysis. The relative infrequency of the occurrence of a constant half in the urine of the patients with myeloma may be explained by the fact that the variable half is more resistant to the proteolysis than the constant half. Although the other possibility, the synthetic origin, has not been unequivocally established, contradictions of the above interpretation arose (20). Recent evidence by which these two halves are encoded by separate genes (18, 19) seems to provide indirect backing of the contradictions. In our case, the findings in which the patient serum showed an electrophoretical corresponding band to Fr. 3 justifies the speculation that the fragment protein is derived from serum origin.

Summary

A fragment protein of type k Bence Jones protein was isolated from the urine of a patient with multiple myeloma. Sedimentation coefficient of the fragment protein was 2.3 S in 0.15 M KCl and its molecular weight estimated by Sephadex G-100 column gel filtration was 21000 at neutral pH. In 1 M propionic acid, the protein dissociated into two basic units with an S value of 1.4. The antigenic analysis of the protein showed that the fragment lacked the type k common determinants and had unique determinants of patient Bence Jones protein. The amino acid composition of this protein was almost identical with that of the variable half of intact Bence Jones protein from the same patient. In cellulose acetate electrophoresis, a corresponding band to the fragment protein was exhibited by the patient serum.

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