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A BENCE JONES CRYOGLOBULIN: CHEMICAL, PHYSICAL AND IMMUNOLOGICAL PROPERTIES*

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SUMMARY

A Bence Jones protein with cryoglobulin properties has been isolated from the urine of a myeloma patient. The Bence Jones cryoglobulin was of type K and had a sedimentation coefficient of 3·5S at a concentration of 5 mg/ml. The concentration dependence of cryoglobulin formation suggested that it was due to reversible association. This was confirmed by ultracentrifugal analyses which demonstrated the formation of aggregates with a sedimentation coefficient of approximately 50–100S and higher. Quantitative amino acid analyses revealed that the Bence Jones cryoglobulin was completely devoid of methionine. On starch gel electrophoresis, three to four protein bands could be detected. They were all devoid of methionine and there was no significant difference in their content of other amino acids. On the basis of amino acid and ultracentrifugal analyses the presence of several components on starch gel electrophoresis is consistent with the formation of aggregates by a single molecular species.

INTRODUCTION

Until recently, all cryoglobulins were thought to be intact IgG or IgM immunoglobulins. Several reports of urinary proteins of low molecular weight with cryoglobulin properties, however, indicate that intact immunoglobulin molecules are not essential for cryoglobulin formation (Alper, 1966; Varriale, Ginsberg & Sass, 1962). In studying the urinary protein from a patient with myeloma, a protein fraction was isolated which was found to have the characteristic gelling properties of cryoglobulins with precipitation on cooling and the typical solubility properties of free light chains on heating.

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In this paper evidence is presented based on immunological, chemical and ultracentrifugal analysis which indicates that both the apparent heterogeneity of the protein and its cryoglobulin property are due to the reversible formation of high molecular weight aggregates.

METHODS

Preparation of Bence Jones cryoglobulin

Forty-eight-hour urine samples were collected in bottles containing thymol as preservative. The entire urine sample was adjusted to 90% ammonium sulphate saturation by the addition of 662 g of ammonium sulphate per litre (Franklin, 1959). The sample was stirred at room temperature for 3 hr and then allowed to stand overnight at 4°C in order to obtain maximum sedimentation. The samples were then filtered with suction on a Buchner funnel using a double thickness of Whatman No. 1 filter paper. The protein sediment was recovered from the filter paper and mixed with 200 ml of 0·05 M-$NaHCO_3$. The suspension was stirred for 1 hr and then dialysed overnight at room temperature against 6 litres of 0·05 M-$NaHCO_3$. The dialysate was changed twice. The dialysed sample was recovered and the small amount of undissolved material was removed by centrifugation.

To obtain an enriched cryoglobulin, an 80-ml aliquot of the dialysed solution containing 3 g of protein was applied to a 2 × 30 cm DEAE-cellulose column which had been equilibrated with a 0·05 M-$NaHCO_3$ solution. The column was eluted with a gradient of increasing ionic strength. The mixing vessel contained a 1-litre solution of 0·05 M-$NaHCO_3$ and 0·1 M-NaCl, and the reservoir vessel a 1-litre solution of 0·05 M-$NaHCO_3$ and 0·2 M-NaCl. All column operations were performed at room temperature and 15 ml of eluate was collected in each tube. No proteins were eluted or washed through with 0·05 M-$NaHCO_3$, and the cryoglobulins were eluted only when the ionic strength was increased during the NaCl gradient elution, thus indicating the absence of overloading of the column. The tubes containing the eluant were allowed to stand in the cold room overnight at which time the contents of tubes 19–24 became extremely viscous. Tubes 19–24 were combined after standing at room temperature for 1 hr, at which time the solutions were no longer viscous. The pooled contents of tubes 19–24 contained some non-cryoglobulin material, but the ratio of cryoglobulin to contaminant was much higher than in the dialysed solution applied to the column. The combined fraction was brought to 60% ammonium sulphate saturation and stirred at room temperature for 4 hr. The sediment was recovered by centrifugation at 20,000 rev/min at 5°C for 30 min. The sediment was mixed with 25 ml of 0·05 M-$NaHCO_3$ and dialysed against 2 litres of 0·05 M-$NaHCO_3$ at room temperature. The dialysate was changed once. The sample was resubjected to column chromatography on a 2 × 30 cm DEAE-cellulose column and eluted with the gradient as described.

Amino acid analyses

Samples of protein (2·9–4·6 mg) were hydrolysed in 1 ml of 6 N-HCl for 24 hr at 110°C in vacuum-sealed tubes. The HCl was removed under vacuum and the samples taken up in 3 ml of citrate diluent buffer, pH 2·2. The amino acid analyses were performed on a Beckman/Spinco Model 120B amino acid analyser (Moore, Spackman & Stein, 1958).

Starch gel electrophoresis

A discontinuous borate buffer system was used (Barrett, Friesen & Astwood, 1962).

Ultracentrifugation

Ultracentrifugal analyses were carried out at 27°C in a Spinco model E centrifuge. Photographs of schlieren patterns were taken at the time maximal speed was reached, at 4 min thereafter, and then every 8 min during a total period of 92 min.

Immunology

Antigenic characterization of the Bence Jones cryoglobulin was performed with specific antisera described elsewhere (Vaerman *et al.*, 1965). Gm and Inv typings were done by standard inhibition of agglutination methods (Fudenberg, 1963).

RESULTS

On re-running the enriched cryoglobulin fraction on the second DEAE-cellulose column a single protein peak was obtained (Fig. 1). The contents of tube 32 on standing at 4°C

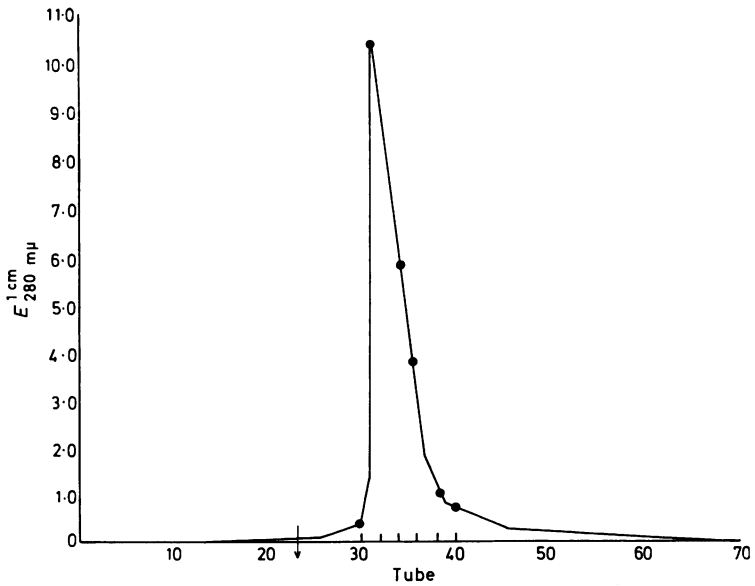

FIG. 1. Chromatography of enriched cryoglobulin fraction on a 2 × 30 cm DEAE-cellulose column which had been equilibrated with 0·05 M-$NaHCO_3$. The column was first eluted with 0·05 M-$NaHCO_3$ and beginning with tube 23, it was eluted with a gradient in which there was a 1-litre solution of 0·05 M-$NaHCO_3$ and 0·1 M-NaCl in the mixing vessel and a 1-litre solution of 0·05 M-$NaHCO_3$ and 0·2 M-NaCl in the reservoir vessel. A volume of 10 ml was collected per tube.

formed a gel and the contents of tubes 33–36 became extremely viscous. At a concentration of approximately 10 mg/ml of a solution containing 0·05 M-$NaHCO_3$ and 0·1 M-NaCl, the proteins in the peak from the DEAE column formed a gel at 4°C, a feature characteristic of cryoglobulins (Fig. 2). The cryoglobulin gelled equally well in Tris buffer. The contents of tubes 32–36 did not exhibit the thermal properties of Bence Jones proteins

when each was suspensed in the eluting buffer. After dilution with 0·1 M-acetate buffer, however, the protein precipitated at 65°C, went into solution again at 100°C, and re-precipitated when cooled to 70°C.

Starch gel electrophoresis of the contents of tube 32 (9 mg/ml), 34 (5 mg/ml) and combined tubes 32–36 (5 mg/ml) as well as the original ammonium sulphate sedimented proteins revealed the presence of three protein bands in the contents of tube 32 and four bands in the contents of tube 34, despite the fact that the latter contains approximately 50% less protein (Fig. 3). Quantitative amino acid analysis of the various protein fractions strongly

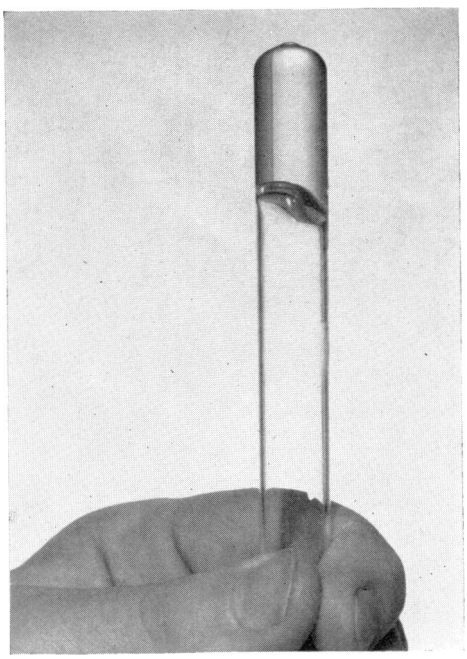

FIG. 2. Cryoglobulin property of Bence Jones protein (concentration, 1%; temperature 4°C).

suggested that the multiple bands represented aggregates of the Bence Jones protein since all of the protein bands in tubes 32 and 34 were found to be completely devoid of methionine (Table 1). Also, there was no significant difference in their content of other amino acids. Since methionine was present in the original ammonium sulphate precipitated proteins, it can be concluded that it was constituent of the two contaminating protein components, which migrated to the anode at the fastest rate (Fig. 3).

Ultracentrifugal analysis of the cryoglobulin at a concentration of 5 mg/ml and a temperature of 27°C revealed the presence of one symmetrical peak which migrated as a single component; the sedimentation coefficient was calculated to be 3·5S (Fig. 4). The protein was homogeneous throughout the run.

On immunoelectrophoresis and Ouchterlony double diffusion analysis with antisera specific for the heavy chains of IgG, IgA and IgM globulins, no bands were visible. The

protein reacted with antisera specific for type K but not type L-light chains and failed to react with antiserum specific for the Fc fragment of IgG globulin. Thus, the primary protein fulfils the antigenic criteria of Bence Jones proteins, type K (Fudenberg, 1967). In addition, it gave bands of identity on Ouchterlony analysis with four other Bence Jones proteins of type K with a specific anti-type K antiserum. Despite previous publications indicating that the H-chain of IgG is necessary for cryoprecipitation (Curtain, Baumgartan & Pye, 1965), no contamination of the Bence Jones protein with IgG could be detected in that the cryo-

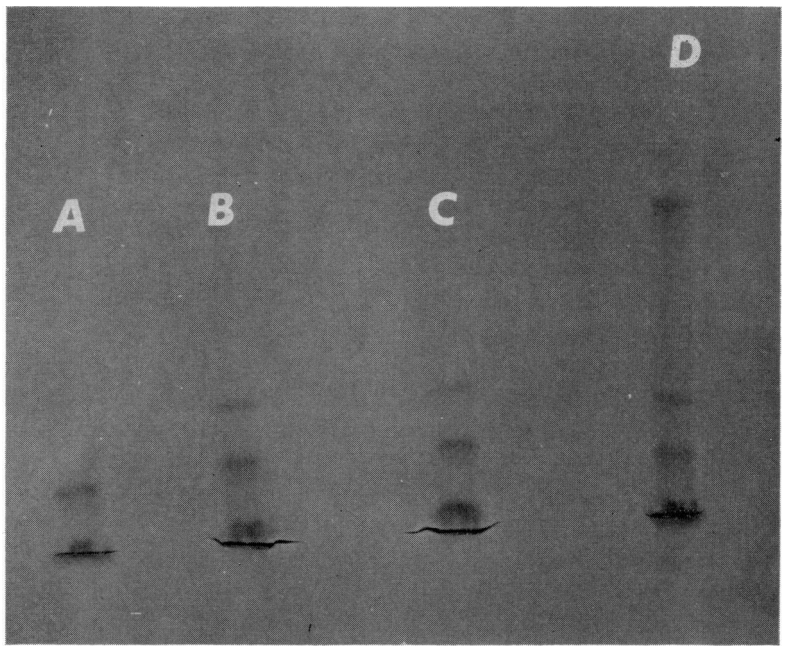

FIG 3. Starch gel electrophoresis of (A) contents of tube 32 from DEAE column; (B) contents of tube 34; (C) contents of pooled tubes 32–36; (D) aliquot of original 0–90% ammonium sulphate protein sediment after dialysis against 0·05 M-$NaHCO_3$ solution but before fractionation by column chromatography. The anode is at the top.

protein was negative for the three Gm factors, Gm(a), (b) and (f), at least one of which is present in normal Caucasian IgG. The Bence Jones cryoprotein was also Inv (1-a-).

The gel electrophoresis, amino acid analysis and concentration dependence of cryoglobulin formation all suggested the reversible formation of aggregates through protein–protein interactions. This was borne out by further ultracentrifugal analysis. At a concentration of 15 mg/ml and at a temperature of 20°C, the formation of aggregates could be detected (Fig. 5). Protein aggregates of 50–100S and higher were seen to be sedimenting while the 3·5S material was just starting to pull away from the meniscus. No low molecular weight aggregates could be detected. Analogous results were obtained at lower cryoglobulin concentrations when the samples were pre-cooled to 4°C for 2 hr and the sedimentation analyses

TABLE 1. Amino acid analysis of Bence Jones cryoglobulin

Amino acid	Tube 32 (moles/100 moles)	Tube 34 (moles/100 moles)	Proteins before column fractionation (moles/100 moles)
Lysine	5·69	5·89	5·78
Histidine	1·49	1·56	1·58
Arginine	3·40	3·49	3·49
Aspartic acid	8·63	8·74	8·90
Threonine	8·70	8·69	8·62
Serine	12·30	12·30	12·20
Glutamic acid	12·20	12·00	12·50
Proline	5·53	5·71	5·29
Glycine	6·26	6·32	6·38
Alanine	6·31	6·33	6·42
1/2 Cystine	2·48	2·26	2·45
Valine	6·86	6·89	6·77
Methionine	0	0	0·17
Isoleucine	3·25	3·27	3·28
Leucine	8·48	8·63	8·50
Tyrosine	4·00	4·06	4·05
Phenylalanine	3·95	3·92	3·76

Results are expressed as moles of amino acid per 100 moles of amino acids recovered from the column after 24 hr acid hydrolysis.

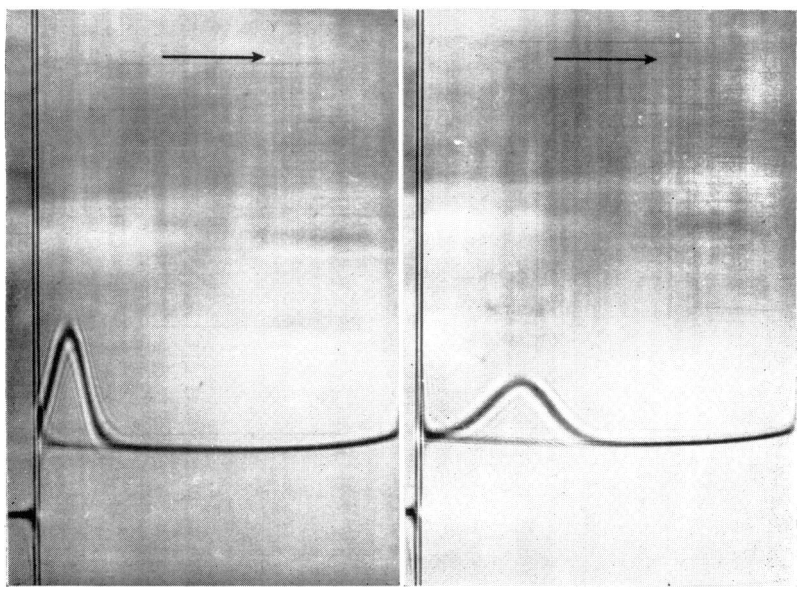

FIG. 4. Sedimentation characteristics of Bence Jones cryoprotein present in tube 32 from DEAE-cellulose column (Fig. 1). The protein concentration was 5 mg/ml in 0·05 M-$NaHCO_3$ and 0·1 M-NaCl. Temperature, 27°C; speed, 59,780 rev/min. Photographs were taken 12 min and 44 min after reaching maximum speed.

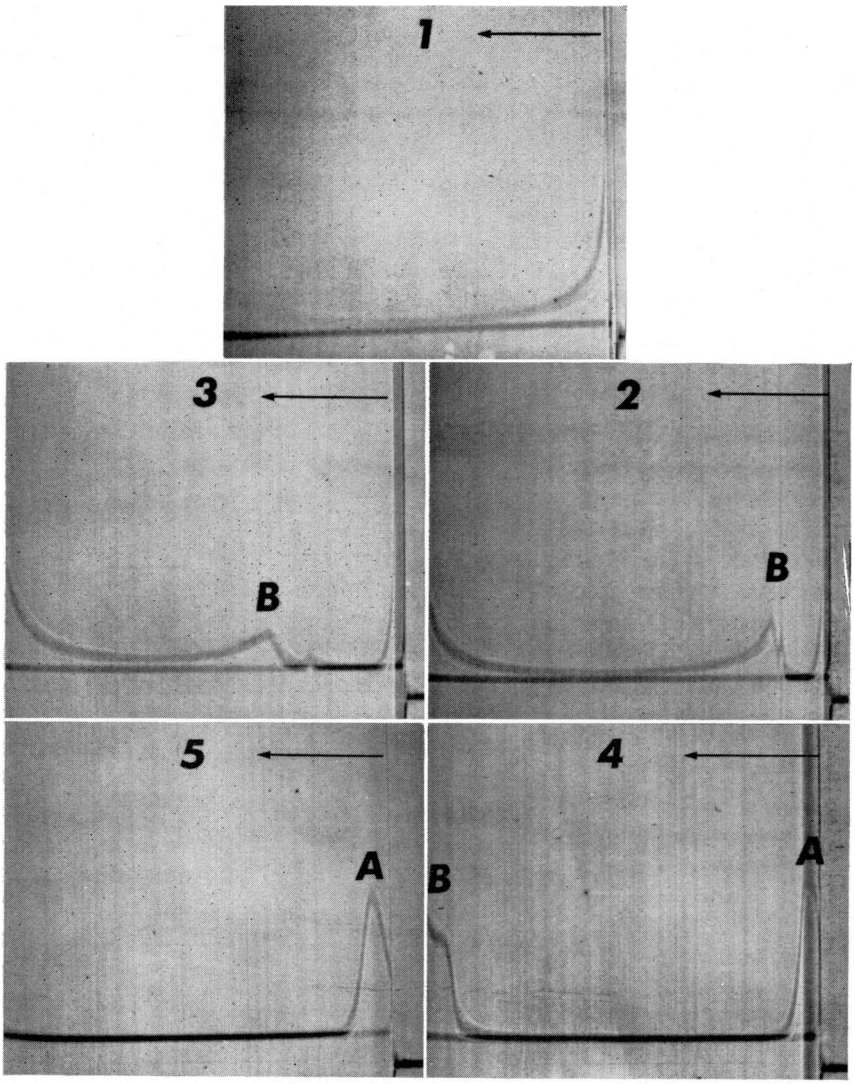

FIG. 5. Demonstration of formation of Bence Jones aggregates 50–100S and higher. Ultracentrifugal analysis was performed at a concentration of 15 mg/ml at 20°C. Arrows indicate the direction of sedimentation. Pictures 1 and 2 were taken at 27,000 and 50,000 rev/min, respectively, during period of acceleration. Picture 3 was taken at 0 time after reaching maximum speed of 59,780 rev/min. Pictures 4 and 5 were taken 4 and 8 min after reaching maximum speed of 59,780 rev/min. Pictures 4 and 5 show emergence of 3·5S peak (A) after sedimentation of high molecular weight aggregates (B).

then performed at 4°C. Under optimal conditions of temperature and concentration, ultracentrifugal analyses showed that as the concentration of aggregates increased, the concentration of 3·5S material decreased. Hence, it can be concluded that the formation of aggregates was produced at the expense of the low molecular weight protein and did not represent a different molecular entity.

The cryoglobulin formation was fully reversible, and the process could be reversed an indefinite number of times without any evidence of alterations in the chemical, physical or immunological properties of the molecule. An un-gelled solution of the Bence Jones protein at concentration of 10 mg/ml was centrifuged at 25,000 *g* for 2 hr at 4°C and sedimented cryoglobulin was recovered, washed with cold buffer and then dissolved in buffer at 25° C. The solution was then recentrifuged at 4°C to permit sedimentation of the cryoglobulin as it formed. This procedure was repeated three times and it was found the washed and re-sedimented cryoglobulin which was finally collected still retained the typical thermal solubility properties of Bence Jones proteins on heating. This demonstrates that the cryoglobulin was not a contaminant, but rather a characteristic property of the Bence Jones protein.

DISCUSSION

The thermal properties of this protein are those characteristic of free L-chains devoid of H-chains (Edelman & Gally, 1962), and this was confirmed by the immunological finding of the absence of H-chain antigenic determinants. These observations and the presence of only one of the two types of L-chain antigenic determinants (K in this case) clearly demonstrate that the protein is a Bence Jones protein. Nevertheless, these purified L-chains had the typical thermal property of cryoprotein, namely gelling at 4°C.

Although it is known that Bence Jones proteins may exist in different polymeric and polymorphic forms (Bernier & Putnam, 1963), it had been generally believed that cryoglobulins were intact immunoglobulins. Work in this laboratory (Kritzman & Liss, 1966) and in other laboratories (Varriale *et al.*, 1962; Alper, 1966) indicates that in general, neither intact immunoglobulins nor their H-chain components are necessary for cryoglobulin formation. This conclusion is based upon the ability of Bence Jones proteins, which are immunoglobulin L-chains, to exhibit cryoglobulin formation. Indeed, it can be inferred that the physical and chemical requirements for cryoglobulin formation in intact immunoglobulin molecules reside in the L-chain segments. However, there may be specific instances where H-chains are necessary for cryoprecipitation (Curtain *et al.*, 1965). Bence Jones cryoglobulins of both type K (Kritzman & Liss, 1966) and type L (Alper, 1966) have been reported. There is some indication of a physical difference in the two types since the latter Bence Jones protein was reported to exhibit cryoglobulin formation at higher protein concentrations. The Bence Jones type K cryoglobulin described in this paper was completely devoid of methionine. Although it has been shown that the absence of methionine is more characteristic of type L it can be seen in either type (Putnam & Easley, 1965). Indeed, the absence of this amino acid in the purified cryoprotein documents the absence of contamination by IgG, since the heavy chains of IgG are relatively high in methionine. To what extent cryoglobulin formation is a general property of Bence Jones proteins is presently unknown. Nevertheless, it can be concluded that at least some Bence Jones proteins of types K and L can be classified as cryoglobulins.

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