Amino Acids of Bence-Jones Protein. (17865)

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The urinary excretion of Bence-Jones protein, in association with multiple myelomatosis, has been observed repeatedly(1,2), but few chemical investigations, in which present-day methods for the determination of its constituent amino acids were employed, have been reported. Recently Dent and Rose(3) have shown that the sample of Bence-Jones protein isolated by them contained no methionine. The absence of methionine (paper chromatography) was also reported by Ågren (4) and Papastamatis and coworkers(5). Harvier and Rangier(6) found 0.414% of methionine sulfur (equivalent to 1.80% methionine) but made no statement as to the method by which methionine was determined. In the sample of Bence-Jones protein isolated by Devine(7), 0.58% of methionine was present.

Since it is known that the proteins isolated from the urines in multiple myelomatosis differ in physico-chemical properties(2,7,8) and in immunological behavior(9,10), it seemed important to determine whether the absence (or very low content) of methionine is characteristic of all samples of Bence-Jones protein. Calvery and Freyberg(11), in the laboratory of one of us (L), studied the distribution of a limited number of amino acids in a sample of Bence-Jones protein. It was of interest to reexamine this protein by the newer methods of analysis (microbiological assay and two-dimensional paper chromatography). We have been able to account for about 92% of the amino acid residues of the protein. So far as it is known to us, this is the most complete analysis of the Bence-Jones protein which has been reported.

Experimental. Source of protein. The Bence-Jones protein was isolated from the urine of a patient with demonstrated multiple myelomatosis as previously described(11).

Hydrolysis of protein. Samples of the protein were hydrolyzed for 16 hr at 105° with 6N HCl in a sealed tube. The HCl was removed *in vacuo* and the residues were redissolved in water and evaporated repeatedly *in vacuo* to remove all free acid. Samples prepared in this manner were used for chromatography and for the microbiological determination of all of the amino acids except tryptophan.

For the determination of tryptophan, 22.8 mg of protein were placed in a pyrex test tube and dissolved in 0.5 ml of 5N NaOH by heating in a water bath. The mixture was autoclaved at 15 lb pressure for 16 hr, during which time complete racemization of tryptophan was assumed to have taken place. The digest was diluted, adjusted to pH 6.8, made up to 25 ml, and filtered through a dry filter

* Aided by grants from the Charles F. Kettering Foundation and the U. S. Public Health Service.

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TABLE I.
Description of Microbiological Assays.

Amino acid	Test organisms*	Standard amino acid†	Active conc. of reference standard,‡ γ/ml
Alanine	1	DL, Merck, recrystallized	30
Arginine	4	L, HCl	20
Aspartic acid	2	L	40
Cystine	1	L	5
	2	L	5
Glutamic acid	3	L	50
Glycine	2	Merck	10
Histidine	2	L, HCl, H_2O	5
Isoleucine	2	DL	15
Leucine	3	L	15
Lysine	2	L, HCl	50
Methionine	1	DL	8
	5	DL	8
Phenylalanine	2	DL	8
Proline	2	L	10
Serine	2	DL, Merck	20
Threonine	5	DL	15
Tryptophan	3	DL	3
Tyrosine	2	L	10
Valine	3	DL	15

* The test organisms in column 2 are listed as follows: 1, *Leuconostoc citrovorum* 8081; 2, *Leuconostoc mesenteroides* P-60; 3, *Lactobacillus arabinosus* 17-5; 4, *Lactobacillus delbrueckii* 3; 5, *Streptococcus faecalis* R.

† Unless otherwise stated, the amino acids were present in a specially tested composite standard obtained from Dr. L. M. Henderson.

‡ The highest concentration of any amino acid per assay tube was one-half that of the reference standard. It was assumed that the D- and L-isomers of alanine had equal activity for the organism used. For all other DL-isomers, the D-form was considered inactive(16,17).

to remove the silica. The filtrate was diluted suitably for microbiological assay. The value obtained was doubled to give the original L-tryptophan content of the protein.

Chromatography. Volumes of the acid hydrolysate corresponding to 1 mg of the original protein (148 γ of N) were placed on paper for 2-dimensional paper chromatography by the method of Consden, Gordon, and Martin(12). Phenol saturated with water was used as the first solvent and a mixture of collidine and lutidine saturated with water as the second. The spots were treated with hydrogen peroxide(13) prior to chromatography to convert methionine to the sulfone and cystine to cysteic acid. Several samples were treated with hydrogen peroxide and molybdate to insure complete conversion of all of the methionine to the sulfone(14).

Microbiological procedures. The data pertinent to the determination of the individual amino acids are summarized in Table I. The micromethods of Henderson, Brickson, and Snell(15) and Steele, Sauberlich, Reynolds, and Baumann(16) were employed throughout with the basal medium recommended by Henderson and Snell(17). When *L. citrovorum* was used as the test organism(16), the medium was supplemented with a commercial liver preparation (Reticulogen). Alanine was assayed by the method of Sauberlich and Baumann(18) with the use of the

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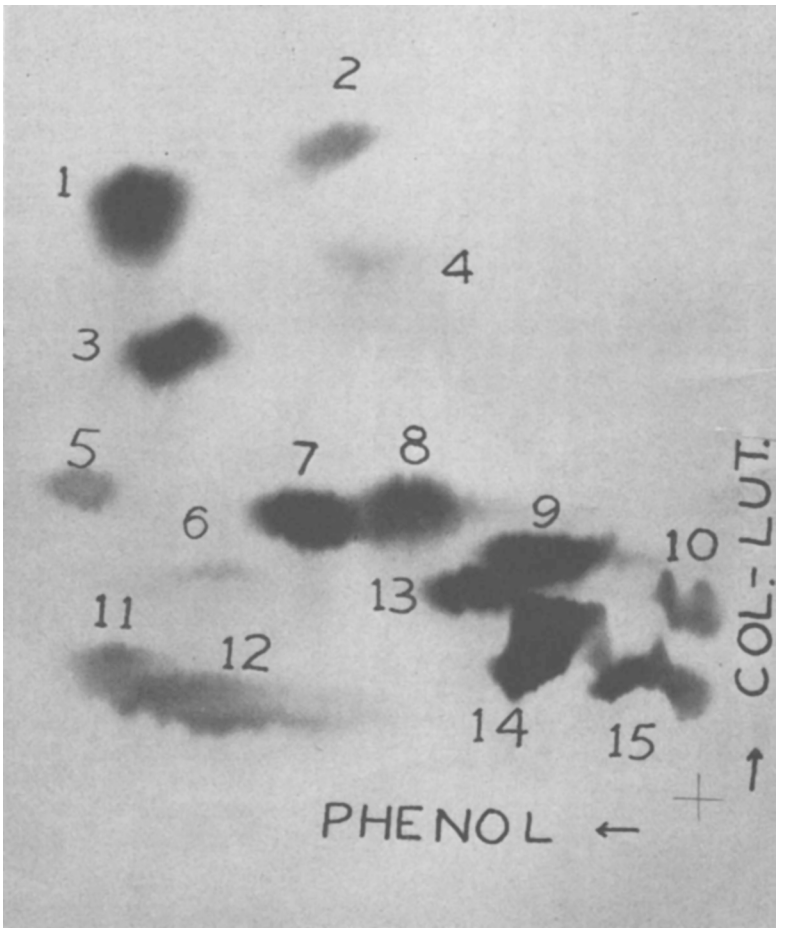

FIG. 1.
Chromatogram of peroxide-treated hydrolysate of Bence-Jones protein. Leucine and isoleucine, 1; tyrosine, 2; valine, 3; glucosamine, 4; proline, 5; histidine, 6; alanine, 7; threonine, 8; serine, 9; cystine (cysteic acid), 10; arginine, 11; lysine, 12; glycine, 13; glutamic acid, 14; aspartic acid, 15.

same organism. Although the blanks were somewhat high, the assays were entirely satisfactory. In the calculation of the results it was assumed that the D- and L-alanine have equal activity for *L. citrovorum* and that all other D- amino acids are completely inactive. Each value reported represents the mean of determinations made at 2 dilutions. The values for each dilution are based on the titration of 18 tubes, *i.e.*, a minimum of 36 determinations for each amino acid was actually carried out. In numerous instances the assay was repeated 2 or 3 times. In view of the importance of the methionine determination, the assays for this amino acid were performed with 2 different microorganisms. The relatively good checks obtained with *L. citrovorum* and *S. faecalis* R make it seem improbable that the active material was an artifact. Good checks were also obtained when cystine was determined with *L. citrovorum* and *L. mesenteroides*.

Other analyses. The water content of the protein was measured by drying to constant weight at 60° *in vacuo*. The ash was estimated by ignition to constant weight at 450° in a muffle furnace. The nitrogen content was determined by a micro-Kjeldahl pro-

TABLE II.
Amino Acid Composition of Bence-Jones Protein.
The ash content was 3.34%; moisture, 7.89%; nitrogen corrected for ash and moisture. 16.70%. All values in Table II are expressed on an ash-free and moisture-free basis.

Amino acid	G per 100 g protein		G residue per 100 g protein	G N per 100 g protein
Glycine	5.41		4.11	0.77
Alanine	8.23		6.56	1.29
Valine	6.87		5.81	0.70
Leucine	6.82		5.88	0.63
Isoleucine	3.61		3.12	0.39
Phenylalanine	2.93		2.61	0.25
Proline	7.10		5.98	0.73
Tryptophan	2.25		2.04	0.31
Cystine	2.71	2.93*	2.51	0.30
		2.48†		
Methionine	0.12	0.11*	0.11	0.01
		0.13‡		
Glutamic acid	12.28		10.79	1.17
Aspartic acid	7.10		6.14	0.75
Histidine	1.91		1.69	0.52
Lysine	6.25		5.47	1.20
Arginine	5.07		4.54	1.63
Serine	12.61		10.48	1.69
Threonine	8.35		7.09	0.98
Tyrosine	5.41		4.88	0.42
Amide-NH_3	2.13		2.13	1.75
Total	107.16		91.94	15.49

* *L. citrovorum* 8081.
† *L. mesenteroides* P-60.
‡ *S. faecalis* R.

cedure. Amide nitrogen determinations were made on the acid hydrolysates by the Conway micro-diffusion method(19). Spectrophotometric examination of solutions of the acid hydrolysates failed to reveal any significant absorption in the ultraviolet attributable to purines and pyrimidines.

Chromatographic findings. Numerous chromatograms were made of acid hydrolysates of the Bence-Jones protein. A typical chromatogram is shown in Fig. 1. In confirmation of previous reports(3-5), methionine and hydroxyproline were not detected in any of the samples. Although phenylalanine is not visible on the photograph, it was present on the original paper as a light halo above the leucine spot. Phenylalanine appeared clearly as a separate spot in other chromatograms which were allowed to run in collidine for longer periods of time. Spot 4 has been tentatively identified as glucosamine. It was found that no new spots appeared when chromatograms were made of samples to which glucosamine had been added and that spot 4 was intensified. Dent also reported the presence of carbohydrate in his sample (3). The large quantities of serine, threonine and proline were of special interest. It is not possible to compare the intensity of the proline spot (spot 5) on the photograph with that of the other amino acids, since proline gives a yellow color while the other amino acids give colors ranging from blue to purple. Tryptophan was not detected since this acid was destroyed during acid hydrolysis.

A semi-quantitative estimation of the amounts of the various amino acids from the chromatograms was of considerable help in establishing the quantities of hydrolysate to be used in the microbiological assays.

Microbiological determinations. It was possible to account for 92% of the weight of the protein and 93% of the nitrogen from the analyses listed in Table II. The methionine assays using two different organisms were in

19. Conway, E. J., Microdiffusion Analysis and Volumetric Error, Crosby Lockwood, & Son, Ltd., London, 1947.

excellent agreement. The amount of methionine found was below the limit of detection of the chromatographic method for the quantities of hydrolysate examined. There was complete qualitative agreement between the chromatographic results and the microbiological assay in the estimation of the relative amounts of the various amino acids.

Discussion. Some analyses have been published previously for the sample of Bence-Jones protein employed in the present study (11). The previous finding of unusually high total nitrogen (18%) and amide nitrogen (5.7%) values has not been confirmed. Significantly lower values of 16.7% and 2.13%, respectively, have been obtained in replicate analyses. The microbiologically determined values for tyrosine, tryptophan, cystine, arginine, histidine, and lysine are in fairly good agreement with those previously reported, while the aspartic and glutamic acid values are considerably higher. Tyrosine and tryptophan have been determined spectrophotometrically, by a procedure in which no hydrolysis of the protein is required, by Mr. D. M. Teague of the Physical Chemical Research Laboratory of the Chrysler Corporation and the laboratory of one of us (L.). Corrected values for the Bence-Jones protein are tyrosine, 6.34%, and tryptophan, 4.34% (unpublished data). Although, in the present study, no methionine was detected by paper chromatography, closely agreeing microbiological assays with two different organisms gave a mean value of 0.12%, an amount below the limit of detection by the chromatographic method. Our small, but definite, values may reflect a difference in the proteins from the two sources or may be the result of the presence of an impurity in our proteins. All of our findings are in essential agreement with those of Dent and Rose(3). No hydroxyproline was detected. Large quantities of serine and threonine were found. The microbiologically determined value for cystine was 2.71% as compared to 2.47%, obtained by chemical analysis in their studies. A comparison of the amino acid composition of our sample of Bence-Jones protein with that of other proteins shows more points of similarity with human γ-globulin than with any of the other proteins cited in Tristram's compilation(20). The high content of serine and threonine in both of these proteins is of special interest, since 3 enzymatically active proteins, pepsin, chymotrypsinogen, and ribonuclease are also reported to have unusually high contents of these amino acids(20).

The examination of the analytical values for the sample of Bence-Jones protein studied does not allow speculation about the etiology of multiple myelomatosis based solely on the similarities in amino acid composition to those of γ-globulin(11) or viruses(3). It may be pointed out, however, that the similarity between virus proteins and Bence-Jones protein(3) with relation to their methionine content may be extended by the present work to include a second common factor, a relatively high content of the aliphatic hydroxy amino acids(21). It may be noted that, with the exception of one strain, the tobacco mosaic virus proteins analyzed by Knight(21) contained a high content (over 16%) of aliphatic hydroxy amino acids. The high content of these acids in Bence-Jones protein is another point of similarity to the virus proteins, in addition to the absence or very low content of methionine(3).

Summary. 1. Hydrolysates of a sample of Bence-Jones protein isolated from the urine of a patient with multiple myelomatosis were examined by paper chromatography. Quantitative estimations of 18 amino acids were also made by microbiological methods.

2. The protein was characterized by a very low content of methionine (0.12%) and by relatively large amounts of serine, threonine, and proline. No evidence of the presence of hydroxyproline was obtained.

3. The analyses accounted for 93% of the nitrogen present in the protein.

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Received March 7, 1950. P.S.E.B.M., 1950, v74.