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# Countercurrent-Distribution Studies with Bence-Jones Protein in a Dissociating System*

Norbert Hilschmann and Lyman C. Craig

ABSTRACT: The Bence-Jones protein isolated from the urine of a selected patient has been subjected to fractionation experiments with the objective of preparing starting material of sufficient purity for reliable sequence studies. Of the fractionation methods tried, countercurrent distribution in a dissociating system revealed the most heterogeneity. A major fraction could be isolated by countercurrent distribution from the crude protein, which appeared to have adequate purity by the criteria of countercurrent distribution, gel filtration, starch-gel electrophoresis, and quantitative amino acid analysis.

Bence-Jones proteins are excreted in the urine by patients with multiple myeloma (Jones, 1847, 1848). While these proteins obtained from different patients differ in their physical, chemical, and antigenic properties (Putnam, 1957, 1960, 1962; Putnam *et al.*, 1962; Mannik and Kunkel, 1963), a Bence-Jones protein from one patient has been widely assumed to be homogeneous (Putnam, 1960).

Bence-Jones proteins have recently been shown (Edelman and Gally, 1962) to be very similar to if not identical with the L-polypeptide chain of the myeloma globulin from the same patient. The problem of their nature is therefore part of the much larger and more important problem of the nature of the $\gamma$-globulins. In the latter case the problem of preparing a sufficient amount of a sample of pure protein for structural study, to say nothing of the proof of purity, is a formidable one. Perhaps it is even beyond the capabilities of existing methods.

On the other hand, gram quantities of Bence-Jones protein can be recovered rather easily from a single patient. On the basis of these considerations the study of Bence-Jones proteins has been taken up in a number of laboratories. There remains, however, a fundamental question of their purity, which has been considered (Van Eijk *et al.*, 1963; Bernier and Putnam, 1964) but not completely solved.

Even those preparations considered most homogeneous show a considerable heterogeneity when investigated by starch-gel electrophoresis. However, earlier studies with the ultracentrifuge indicated heterogeneity caused by aggregation. Fractions with an $s_{20,w}$ of 3.5 S appeared to dissociate into smaller units at an acidic *p*H (Putnam and Stelos, 1953). Such behavior seemed to explain the values for the molecular weights of Bence-Jones proteins from different patients which range from 20,000 to 45,000 and even higher (Putnam, 1960). In addition to the ultracentrifuge investigations purity studies by gel filtration and ion-exchange chromatography have been made (Van Eijk *et al.*, 1963; Bernier and Putnam, 1964).

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In spite of this and particularly because the Bence-Jones proteins have been shown to aggregate strongly, it seemed especially important to undertake the study of the purity question by countercurrent distribution under strongly dissociating conditions, such as those which proved effective in the case of insulin (Craig *et al.*, 1960), ACTH (L. C. Craig and T. P. King, data to be published), and hemoglobin (Hill and Craig, 1959). This paper will report the results obtained thus far in such a study.

## Materials and Methods

*Isolation of Bence-Jones Protein.* The urine of a patient (Roy) was selected for the larger-scale isolation of the protein after a preliminary survey of a number of proteins isolated from different patients. The survey was based on their behavior in gel filtration, thin-film dialysis with a calibrated membrane of suitable porosity, and antigenic data. This patient had multiple myeloma of the 7-S-$\gamma$ type and both the myeloma protein and the Bence-Jones protein were of antigenic type I.

The urine was collected in 24-hour specimens, filtered, and, after addition of thymol, stored at $-20°$. The protein was precipitated from pooled samples of urine by 65% saturated ammonium sulfate at *p*H 5.5. The precipitate was either centrifuged or filtered (Schleicher and Schuell filter paper No. 576). The supernatant or filtrate was discarded. The precipitate was suspended in water, and the suspension was placed in 23/32 Visking seamless cellulose tubing and dialyzed at 4° until all the precipitate had dissolved. The solution was lyophilized and the product was stored at $-20°$.

*Thin-Film Dialysis.* A dialysis cell was prepared as described by Craig and Konigsberg (1961). The membrane was from 20/32 Visking dialysis casing stretched to make it more porous. This cell gave a 50% escape time of 2 hours with chymotrypsinogen, 24,500 in 0.01 N acetic acid at 40°.

With the crude Bence-Jones protein preparation in 0.01 N acetic acid at 40° a straight-line escape plot was observed until 72% of the protein had diffused through the membrane. The 50% escape time was 4.3 hours, a value to be expected from a globular protein approximating a molecular weight of 40,000.

A faster rate of dialysis, 3.5 hours, however, was noted when the solvent was 0.1 N acetic acid. Propionic acid proved even more effective. The half-escape times noted with 0.03, 0.1, and 0.5 N propionic acid were 3.0, 2.3, and 3.0 hours, respectively.

*Gel Filtration.* Sephadex G-100 proved to be the optimum size for this work. Columns 0.9 × 150 cm were packed in the usual way, and chromatography was carried out at room temperature. The fractionation behavior was definitely improved by the presence of propionic acid in the solvent, as found by Fleischman *et al.* (1962) for reduced carboxymethylated $\gamma$-globulin. Of four concentrations tried (0.1, 0.25, 0.5, and 1.0 N) the most favorable was 0.5 N propionic acid.

A 15-mg sample of the crude Bence-Jones protein gave the pattern in Figure 1. The first small peak on the left emerged at the interstitial volume. The flow rate approximated 2 ml/hour. Effluent fractions of 1 ml were collected. Appropriate cuts were lyophilized and stored at $-20°$.

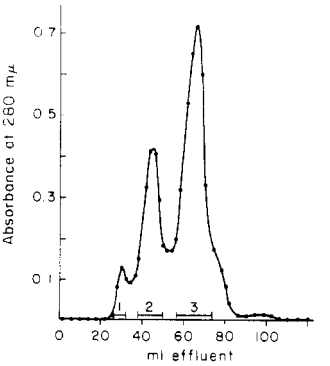


FIGURE 1: Gel filtration of crude Bence-Jones protein on Sephadex G-100 (0.9 × 150 cm) in 0.5 N propionic acid. Flow rate about 8 ml/hour.

*Countercurrent Distribution.* The system used consisted of water, a solution of 0.086% trichloroacetic acid in 2-butanol, and propionic acid in the volume proportions of 11.0:8.7:1.5. The 2-butanol was purified immediately before use by fractionation in a 200-cm distilling column packed with glass helices. Only solvent having an absorbance below an $E^{1cm}_{280m\mu}$ of 0.200 was used.

The amount of protein initially placed in the system appeared to be important. The protein (0.5 g) was dissolved in the propionic acid–water solution so that the protein concentration did not exceed 0.5%. After complete solution the 2-butanol solution containing the trichloroacetic acid was added. After gentle equilibration and settling of the two phases a very small amount of solid material at the interphase was removed and discarded. The phases were separated and scattered in the first 33 tubes of the distribution train which contained 1020 tubes of 5/3 capacity. It was automatically operated and was of the type previously described (Craig and King, 1958). The volume ratio was 4.7 ml/3 ml. The temperature was 25°. The settling time was at first 15 minutes, 8 minutes after 25 transfers, and 4 minutes after 100 transfers. No phase shift was observed when a cocurrent of about 0.2 ml was maintained throughout the run. Analysis was made by absorbance at 280 m$\mu$ in a Beckman DU spectrophotometer after 395 and 865 transfers. The result is shown in Figure 2.

For recovery the selected cuts were pooled and dialyzed in 23/32 Visking cellophane casing first against running tap water, then against distilled water. The retentate was concentrated about 10:1 in a rotary

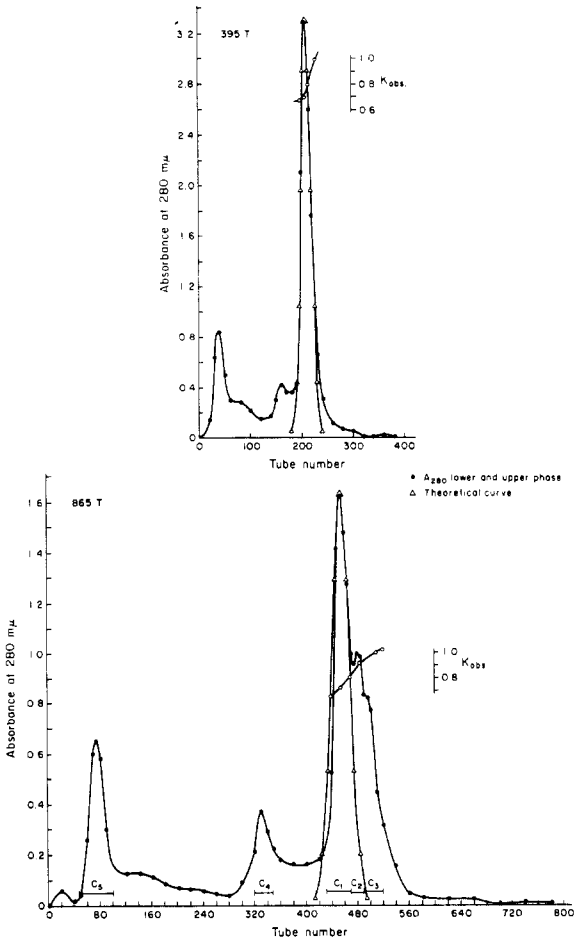


FIGURE 2: Countercurrent distribution of Bence-Jones protein. System: water, 0.086% trichloroacetic acid in 2-butanol, propionic acid (11.0:8.7:1.5), lower phase 3 ml, upper phase 4.7 ml. A (upper), after 495 transfers; B (lower), after 865 transfers.

evaporator with continuous feed-in and lyophilized. The residues were stored at −20°.

*Ion-Exchange Chromatography.* For chromatography, Cellex-CM, a carboxymethylated cellulose from Bio-Rad Laboratories which had an exchange capacity of 0.6 meq/g (control No. B-2340), was used. The resin was prepared by first washing it with 0.1 N NaOH plus 1 M NaCl, and then with 1 N HCl. The fines were removed by repeated sedimentation and decantation. The column was equilibrated with a 0.01 N sodium acetate buffer, *p*H 5.0, overnight. Chromatography was carried out in a 2 × 22-cm column at 4°, using a concentration gradient. Protein (100 mg) was dissolved in 10 cc of water and adjusted with acetic acid to *p*H 4.5. The protein solution, which was slightly turbid, was put on top of the column and at first eluted with primary buffer. After 30 fractions a linear gradient was started which was provided by an initial 400 cc of 0.01 N sodium acetate buffer, *p*H 5.0, to which was continuously added 400 cc 0.2 N sodium acetate buffer, *p*H 5.0. The flow rate was 60 cc/hour; the fractions collected contained 14.5 cc. The run was analyzed by optical density at 280 mμ, and the increase of ionic strength was followed by a Model RC 16B2 conductivity bridge (Industrial Instruments, Cedar Grove, N.J.). The result is shown in Figure 3.

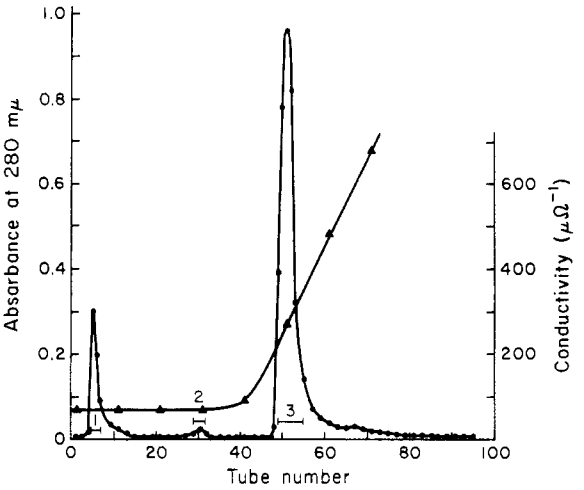


FIGURE 3: Chromatography of 100 mg Bence-Jones protein on CM-cellulose (2 × 22 cm). Equilibrated with 0.01 N sodium acetate, *p*H 5.0. Linear gradient of 0.2 N sodium acetate starting with fraction 30. Flow rate, 60 ml/hour, fractions 14.5 ml.

The fractions under the individual cuts were pooled and dialyzed in 23/32 Visking seamless cellulose tubings. The protein was recovered by lyophilization and stored at −20°.

*Starch-Gel Electrophoresis.* The starch (Connaught Laboratories, Toronto, Canada) was prepared as described by Smithies for vertical gels (Smithies, 1959) using a Tris-borate buffer, *p*H 8.9 (Poulik, 1957). A 1% protein solution (25 μl) was applied to the gel. The electrophoresis was carried out for 16 hours at 5 v/cm. The gels were sliced and stained with Amido-Schwarz.

*Amino Acid Analysis.* About 5 mg of protein was hydrolyzed in constant-boiling (5.7 M) HCl in evacuated sealed glass tubes for 20 hours. Afterward the hydrolysates were taken to dryness with a rotary evaporator and then dissolved in 0.2 N sodium acetate buffer, *p*H 2.2. The amino acid analyses of the hydrolysates were performed on the Spinco automatic amino acid analyzer by the method of Spackman *et al.* (1958). Half-cystine was determined as cysteic acid after performic acid oxidation according to the method of Schram *et al.* (1954). The number of tryptophan residues was determined spectrophotometrically after Goodwin and Morton (1946) with a Cary Model 14 spectrophotometer. The values of the labile amino acids were corrected for decomposition during hydrolysis. The factors used for this were Ser 1.11, Thr 1.05, Tyr 1.05 (Crestfield *et al.*, 1963).

TABLE I: Amino Acid Composition of Different Preparations of Bence-Jones Protein.[a]

| Amino Acid | 1 Cut 3 Fig. 4A | 2 Cut 1 Fig. 4B | 3 Cut 2 Fig. 4B | 4 Cut 3 Fig. 4B | 5 Cut 1 Fig. 4C | 6 Cut 2 Fig. 4C | 7 Cut 3 Fig. 4C | 8 Cut 1 Fig. 6 | 9 Cut 2 Fig. 1 | 10 Cut 3 Fig. 1 | 11 Cut 3 Fig. 1 | 12 Cut 3, Fig. 3 after Sephadex Chromatography |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lysine | 13.8 | 13.7 | 14.2 | 13.9 | 13.9 | 13.6 | 13.8 | 13.6 | 12.9 | 13.0 | 13.7 | 14.4 |
| Histidine | 2.0 | 2.3 | 1.9 | 2.0 | 2.0 | 2.0 | 1.9 | 2.2 | 2.0 | 2.1 | 2.0 | 2.0 |
| Arginine | 5.2 | 5.0 | 4.8 | 4.8 | 4.9 | 5.3 | 4.9 | 4.9 | 5.1 | 5.3 | 4.6 | 4.8 |
| Aspartic acid | 19.6 | 20.4 | 20.2 | 19.8 | 19.3 | 20.4 | 18.9 | 20.0 | 19.4 | 19.9 | 19.0 | 19.7 |
| Threonine[b] | 17.2 | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 | 16.7 | 17.3 | 17.1 | 17.0 | 16.6 | 17.5 |
| Serine[b] | 26.0 | 27.6 | 28.6 | 27.7 | 27.9 | 28.2 | 28.1 | 28.6 | 28.1 | 26.2 | 27.8 | 28.6 |
| Glutamic acid | 24.6 | 24.5 | 24.4 | 24.4 | 24.6 | 25.1 | 24.0 | 24.8 | 23.8 | 24.5 | 24.7 | 24.0 |
| Proline | 11.7 | 10.0 | 10.6 | 10.7 | 10.1 | 10.0 | 11.2 | 11.0 | 12.6 | 12.7 | 11.2 | 10.6 |
| Glycine | 13.4 | 12.9 | 13.0 | 13.3 | 13.6 | 12.5 | 13.2 | 12.5 | 13.3 | 14.5 | 14.8 | 12.7 |
| Alanine | 13.2 | 12.3 | 13.1 | 12.9 | 13.6 | 12.5 | 13.1 | 12.5 | 13.3 | 13.2 | 14.1 | 12.6 |
| Half-cystine | 3.6<br>4.7[c] | 3.4 | 3.8 | 4.9 | 4.0 | 4.2 | 4.4 | 4.0<br>4.8[c] | 3.5 | 4.0 | 4.2 | 4.3<br>4.9[c] |
| Valine | 12.6 | 12.5 | 12.5 | 12.9 | 12.8 | 12.0 | 13.3 | 12.2 | 12.5 | 12.2 | 12.2 | 11.9 |
| Methionine | 0.92 | 1.00 | 0.98 | 1.00 | 0.84 | 1.00 | 1.10 | 0.82 | 1.00 | 0.87 | 0.80 | 0.88 |
| Isoleucine | 8.2 | 8.1 | 7.9 | 8.0 | 8.1 | 8.4 | 8.1 | 7.9 | 7.6 | 8.0 | 7.8 | 8.0 |
| Leucine | 17.4 | 17.7 | 17.3 | 17.2 | 17.1 | 18.0 | 17.0 | 17.3 | 16.6 | 16.3 | 16.7 | 17.5 |
| Tyrosine[b] | 7.6 | 7.1 | 7.6 | 7.9 | 7.6 | 7.9 | 7.9 | 7.9 | 7.3 | 7.9 | 7.0 | 8.0 |
| Phenyl-alanine | 10.8 | 10.0 | 9.9 | 9.6 | 9.9 | 9.8 | 9.2 | 9.5 | 10.2 | 10.5 | 10.3 | 9.8 |
| Tryptophan[d] | 2.5 | | | 2.2 | | | 2.5 | 2.3 | | | | |

[a] Expressed in numbers of residues per monomer (mw 23,160). [b] Values corrected for decomposition after 20 hours of acid hydrolysis. [c] Determined as cysteic acid after performic acid oxidation. [d] Determined spectrophotometrically.

## Results and Discussion

As a preliminary step before engaging in a long and costly study of the sequence of a protein containing over 200 residues, every reasonable effort should be made to ensure that the work is performed on, or at least related to, a preparation homogeneous with respect to its amino acid content. For such a purpose conformational or association heterogeneity is not important but a difficulty arises in deciding whether or not the several components separated by a fractionation procedure, if more than one are found, in fact differ only by conformation or association (stable dimers, trimers, and so on).

Salt precipitation, centrifugation (Putnam, 1957, 1960; Edelman and Gally, 1962), electrophoresis (Putnam, 1957, 1960), gel filtration (Van Eijk *et al.*, 1963; Bernier and Putnam, 1964), and ion-exchange chromatography (Van Eijk *et al.*, 1963; Bernier and Putnam, 1964) have been applied to Bence-Jones proteins. The best crude preparations from a single patient of type I have been thought to contain 75–95% of a single component by these criteria. The higher figure is certainly an overly optimistic one.

Experience in many laboratories has shown that gel filtration is a useful way of detecting and separating the stable association products of proteins, in amounts sufficient for further study. It, however, often brings about separation on the basis of parameters other than size. In any case it is an ideal tool for the first step in the examination of homogeneity. Figure 1 gives the result when 0.5 N propionic acid is the solvent for the Bence-Jones proteins chosen for the present study.

The first small band to emerge occurs at the interstitial volume. It consists of association products larger than dimers. In addition amino acid analysis indicated a composition widely different from the main component. The second component gave an amino acid analysis agreeing very closely with that found for the main component (Table I, columns 9 and 10) except possibly for a small difference in the glycine figure. It would therefore seem that the second band represents a stable dimer of the third band which contains the monomer.

Thin-film dialysis (Craig and Konigsberg, 1961) also is an easy way of recognizing stable polymers and with a selective membrane often permits the diffusion of the monomer while practically rejecting the dimer. Apparently this occurs with the present preparation since an experiment with a membrane permitting a 50% escape time of 2 hours for chymotrypsinogen, mw 24,500, gave a relatively straight line for the crude Bence-Jones protein until 72% of the protein had escaped. The rate of dialysis was markedly influenced by the solvent. In 0.01 N acetic acid it was that expected from a molecule larger than chymotrypsinogen, but 0.1 N propionic acid gave the most rapid dialysis with a half-escape time of 2.3 hours. The behavior gave an indication of reversible association or conformational change with different solvents. With other Bence-Jones proteins concentration dependence in sedimentation had previously been reported (Bernier and Putnam, 1963). Propionic acid was more effective in affording the smallest diffusional size than was acetic acid. This behavior corresponds to the effect of propionic acid in gel filtration found by Fleischman *et al.* (1962) with reduced γ-globulin. Reversible association occurs under these conditions in Bence-Jones proteins in addition to the formation of stable dimers separable by gel filtration.

In searching for a suitable system for countercurrent distribution all the systems previously found suitable for distributing insulin, ribonuclease, lysozyme, parathyroid hormone, serum albumin, and globin were unsatisfactory from the standpoint of solubility or of $K$ value. Since propionic acid had shown a dissociating effect in Sephadex chromatography and also in thin-film-dialysis experiments it seemed of interest to try it in the countercurrent-distribution system. A favorable effect was noted and led to the system used in Figure 2. Isobutyric acid was even better but was not used because of its unpleasant properties.

Through repeated runs it was found that the relative height of the first band was a function of the load and the way the protein mixture was introduced to the system. This band appeared to be mostly denaturation products of higher molecular weight. The mixture was therefore first dissolved in aqueous propionic acid before exposing it to the trichloroacetic acid. After a rather slow settling time at first a normal distribution was effected.

Comparison of Figure 2B at 865 transfers with 2A at 395 transfers indicates that a satisfactory distribution has taken place. The main band agrees well with the theoretical at 395 transfers but a determination of $K$ across the band indicates that a small amount of closely related components is being slowly moved to the right. The agreement with the theoretical is therefore coincidental. Systems with trichloroacetic acid are not entirely ideal and may give a band with a single solute which is either too narrow or too broad, depending on the solute load. Two minor components (apparently aggregates) on the right side of the main band are clearly revealed at 865 transfers.

At this point the distribution approach seemed to offer promise as a way of fractionating crude Bence-Jones proteins but there was the question of the recovery of the material without causing a change in its properties. This was accomplished by dialysis, careful concentration by rotary evaporation, and finally passage through a Sephadex column. Cut $C_1$ from the main band gave the pattern shown in Figure 4A, cut 2 next to it gave the pattern shown in Figure 4B, while cut 3 gave 4C. The three bands common to all in Figure 4A,B,C are ascribable to the monomer, dimer, and trimer of the main component in the crude preparation. Amino acid analysis of each showed they were very close in composition (Table I, columns 1 to 7).

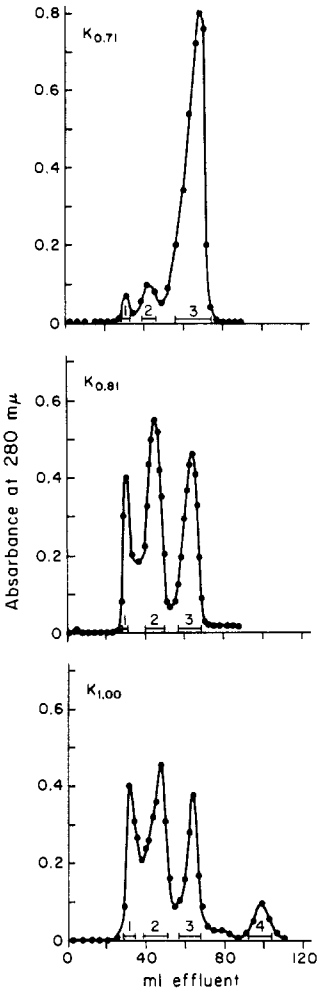


FIGURE 4: Gel filtration of cuts of the countercurrent distribution of Figure 2B on Sephadex G-100 (0.9 × 150 cm) in 0.5 N propionic acid. Flow rate about 8 ml/hour. A (top), cut $C_1$; B (center), cut $C_2$; C (bottom), cut $C_3$.

It would thus appear that countercurrent distribution shows rather poor selectivity for resolving the stable polymers of the main component. In fact it is interesting that such a strong dissociating system fails to dissociate them completely to monomers. This could suggest that a covalent linkage such as an S—S bond is involved.

If the gel-filtration pattern of Figure 1 truly reveals the polymeric composition, it follows that the con-

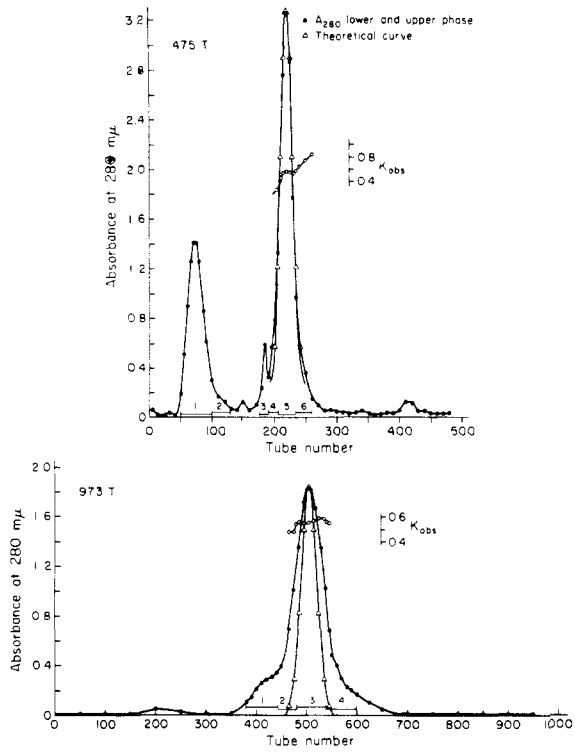


FIGURE 5: Countercurrent distribution of 1 g Bence-Jones protein. System: water, 0.086% trichloroacetic acid in 2-butanol, propionic acid (11.0:8.7:1.5), lower phase 5 ml, upper phase 7 ml. A (upper), after 475 transfers; B (lower) redistribution of cut 5 of (A) up to 973 transfers with the same system; lower phase 3 ml, upper phase 4.2 ml.

siderable material between the main component of Figure 2B and the first band represents inhomogeneity other than that caused by association. Amino acid analysis of cut 4 indicated it to be entirely different. It is not known how much of the first band, $C_5$, is ascribable to denaturation.

As a further step in confirming the indication that the inhomogeneity shown was not owing to denaturation or transformation during the run, another more extensive distribution was carried out. In this case the load was double that of Figure 2B, although the initial concentration was the same. It was begun in a 500-tube train with larger tubes, 10/5 ml volume. At 475 transfers the pattern of Figure 5A was obtained.

At this point a central cut of the main band was transferred to the 1020-tube train previously loaded with the system. The sequential order of the tubes in the cut was maintained. An additional 973 transfers were then applied, making a total of 1448 transfers. This gave the pattern of Figure 5B. Although the band was considerably broader than the theoretical, partition ratios across the main band did not show much drift. The shoulders and partition ratios in the region of the shoulders indicated a certain amount of impurity still being removed. The fact that the band is too broad is not necessarily significant with a trichloroacetic acid system.

Cuts were taken for recovery as indicated. The gel-filtration pattern for cut 3 is given in Figure 6. Compared with Figure 4A there is less polymeric material on the left side of the main band but a small amount of smaller-sized material now has appeared on the right. The source or reason for this is not obvious.

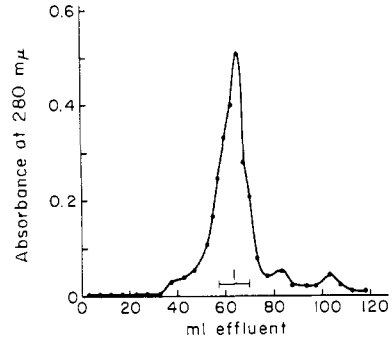


FIGURE 6: Gel filtration of cut 3 of Figure 5B on Sephadex G-100 (0.9 × 150 cm) in 0.5 N propionic acid. Flow rate about 8 ml/hour.

When material from cut 1 in Figure 6 as well as from cut 3 in Figure 4A was further checked for purity by starch-gel electrophoresis, only a single band could be obtained.

When all factors are considered it seems quite certain that this Bence-Jones protein maintains its identity through a prolonged countercurrent distribution in the trichloroacetic acid system at 25°. Countercurrent distribution reveals considerably more inhomogeneity than the other separation techniques tried. This is apparent when the countercurrent distribution results are compared with the CM-cellulose pattern of Figure 3. Both were made on the same crude preparation of the Bence-Jones protein.

Since very little of the dimer and higher polymer (cuts 2 and 1 of Figures 1 and 4A) was revealed in Figure 6, it follows that they do not arise as a result of the prolonged countercurrent distribution. On the other hand, the fact that they are slowly separable by countercurrent distribution and readily separable by gel filtration indicates that once formed they do not revert to the monomer under the gentle conditions presented by these methods. Whether or not more severe conditions will cause reversion remains to be investigated.

It appears that the central cut of an extensive distribution such as in Figure 5B will prove to be a reasonably reliable preparation for sequence study or as a standard to control degradations made on less pure preparations.

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# The Chemistry of Tyrocidine. VI. The Amino Acid Sequence of Tyrocidine C*

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ABSTRACT: A third major component of the antibiotic cyclic decapeptide series of tyrocidine, designated tyrocidine C, has been isolated by countercurrent distribution and its amino acid composition has been determined. A novel peptide cleavage reaction employing $LiAlH_4$ resulted in a single split at the acyl-proline linkage and thereby converted the peptide from a cyclic to a linear structure. This linear structure, in contrast to its cyclic precursor, could be digested by pepsin, and it was subsequently degraded in a manner which permitted the following unique sequence to be assigned:

┌L-prolyl-L-tryptophanyl-D-tryptophanyl-L-
| asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-
| L-ornithyl-L-leucyl-D-phenylalanyl-┐
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The discovery more than 25 years ago of the antibiotic polypeptides produced by *Bacillus brevis* (Dubos, 1939; Hotchkiss and Dubos, 1940) was an event of considerable interest not only because of the therapeutic potential of such substances but because at the time it was thought they would serve as readily available "pure" models to develop methods for the study of proteins. However, with the introduction of countercurrent distribution it was found that the two polypeptide fractions, gramicidin (Craig *et al.*, 1950) and tyrocidine (Battersby and Craig, 1952a), were each mixtures of three or more closely related polypeptides differing in their amino acid composition.

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