A SPONTANEOUS CRYSTALLIZATION OF A BENCE-JONES PROTEIN.

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Many observations have been made on the protein material designated as Bence-Jones protein which is found occasionally in urine from patients suffering from certain disorders. Attempts to crystallize some of these preparations have rarely been successful and then usually only by accident. Below is reported an observation of spontaneous crystallization which is unique in two respects. It is apparently the first time that a Bence-Jones protein has been observed to crystallize spontaneously from the urine as passed and, with one exception, is the only observation of any protein material crystallizing spontaneously from urine.

Henry Bence-Jones reported in 1847 (1) the peculiar reactions of the protein material which now is designated by his name. He stated that 2 years previously there was found in urine from a patient with osteomalacia a protein which gave no precipitate with nitric acid in the cold except on long standing, but when the solution was heated and allowed to cool it became solid. The precipitate redissolved on heating and formed again on cooling.

Heller (2) described somewhat similar observations in a text-book bearing the date of 1846. He had found several cases where the urine contained protein material which he recognized to be different from albumin and casein. It coagulated at about 50°, but the addition of small amounts of nitric acid completely hindered the coagulation by heat. The most unique property of Bence-Jones protein, solubility at the boiling point and precipitation on cooling, was not mentioned.

Similar observations were not made for many years. Kühne (3) in reporting a case which was observed in 1869 states that Bence-Jones had written in a personal communication that he had not seen another case of Bence-Jones proteinuria between 1845 and 1869. Within recent years, however, a number of cases have been reported.
Spontaneous precipitation of an amorphous precipitate of Bence-Jones protein seems to be not uncommon. Bradshaw (4) states that a patient under his observation passed, two or three times a week, milky urine which deposited a copious amorphous sediment giving protein reactions. Rosenbloom (5) reported a case where an amorphous precipitate of Bence-Jones protein occupied one-third of the volume of each specimen of urine. Several authors report milky urine while others record that the protein did not precipitate on standing. Several of the urines which I have examined deposited an amorphous precipitate of protein after standing several weeks.

Magnus-Levy (6) attempted to crystallize a Bence-Jones protein and accidentally obtained crystals from one preparation after standing for months. He was unable to repeat the experiment. Guttercrink and de Graaff (7) studied a case of Bence-Jones proteinuria and succeeded in causing the protein to crystallize. They precipitated the protein with 2 volumes of saturated ammonium sulfate and dialyzed a solution of the precipitate for 4 days. To the resulting solution was added one-ninth its volume of saturated ammonium sulfate, an equal volume of water, and a few drops of sulfuric acid. Crystals formed within 24 hours. They were said to be too insoluble to recrystallize.

Schumm and Kimmerle (8) report that they accidentally obtained crystals of a Bence-Jones protein on one occasion under very peculiar conditions. Some sodium chloride and acetic acid were added to urine containing a Bence-Jones protein, the mixture was boiled and filtered hot. The protein in the filtrate was precipitated with alcohol, washed once, and the damp precipitate dissolved with the aid of heat in water containing a little acetic acid. After filtering, the turbid solution was placed in an ice box. The next morning, a small quantity of crystals was found which contained but little ash and responded to all of the simple tests for Bence-Jones protein.

Since the preliminary report (9) was made of the preparation described below, Krauss (10) has succeeded in crystallizing a Bence-Jones protein. Many liters of urine were half saturated with ammonium sulfate. The precipitate was dissolved in water, the solution dialyzed until free from ammonia and then allowed to evaporate at room temperature. After several weeks crystals appeared.

Loehlein (11) reported finding numerous crystals, having the forms of needles, prisms, and plates, in the tubules of a kidney at autopsy from a case of myeloma which had excreted Bence-Jones protein. The crystals were not soluble in liquid solvents; they swelled in dilute alkali, were anisotropic, and stained with dyes which indicated that they were protein. This is the first report of what may have been spontaneously crystallized Bence-Jones protein.

Glaus (12) observed myeloma cells in which there were crystal-like needles and Hedinger (13) states that he had observed a similar phenomenon.

A protein which crystallized spontaneously from the urine was reported by Bramwell and Paton (14). It crystallized from the urine often within 2
or 3 days and some times not for several weeks. They concluded that it was a globulin. Huppert (15) thought from the description that it might be a Bence-Jones protein, but after studying some of the material (16) agreed that it was a true globulin and quite dissimilar from the Bence-Jones protein.

We may state then that up until the present time a spontaneously crystallized Bence-Jones protein has not been observed with certainty. Several cases are recorded where crystallization was accomplished by artificial means. The spontaneously crystallized Bence-Jones protein described below seems, therefore, to be unique.

A patient at the Mayo Clinic thought to be excreting Bence-Jones protein was transferred to Dr. Rowntree's service for special study. My attention was called by Dr. Rowntree to the peculiar milkiness which developed in the urine from this patient soon after it was passed. Under the high power of the microscope there was a slight suggestion that the precipitate might be crystalline. The precipitate showed on the next day a more definite crystalline structure, in appearance similar to freshly crystallized egg albumin. The turbid fluid also showed a marked sheen upon being shaken.

Some of the crystalline material was separated by centrifugation, washed once with cold water, and dissolved in water by adding a trace of sodium hydroxide. The solution was acidified slightly with acetic acid, a small quantity of sodium chloride added, and the whole heated slowly. The protein began to coagulate at 57°. Coagulation seemed to be complete at about 70°. The precipitate dissolved almost completely between 80-85°, leaving a slightly opalescent solution which did not clear up on boiling. The precipitate reappeared as the solution cooled to 80°. Heating and cooling again caused the same phenomena.

The acid urine gave the same reactions. In the more concentrated solutions of the crystalline material, as well as the urine itself, the precipitate dissolved on heating, leaving a small residue which was gummy and stuck to the sides of the test-tube. Magnus-Levy (6) reported the same phenomenon. From these

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1 The case was referred by Dr. Eusterman to the service of Dr. Rowntree and was reported by Dr. Walters (17). My investigations were begun in Rochester and completed in Baltimore.
preliminary observations it seemed justifiable to conclude that the patient was excreting a Bence-Jones protein which crystallized spontaneously on standing a few hours.

In order to obtain a quantity of material, the urine was collected, preserved with toluene, and allowed to stand at room temperature. The volume of urine in 12 hours varied from 300 to 1,000 cc. It was usually acid and was always clear when voided and contained no casts. About 15 gm. of protein were excreted per day. Some time later metabolic experiments were carried out by Walters (17) who found the excretion varying from 10 to 28 gm. daily. Spontaneous crystallization occurred after varying intervals, depending apparently on the concentration and acidity of the urine. In one instance crystallization occurred within 8 hours after the 12 hour specimen had been completed.

On standing the crystals increased in size until they could be easily seen by the low power of the microscope. The largest crystals appeared as long rectangular plates which were very thin. They were so thin that, while they seemed to be doubly refractive, the observation was somewhat doubtful.

After the urine had stood several days and crystallization seemed to be complete, a considerable quantity of protein still remained in solution. The dissolved protein coagulated at the same temperature as a solution of crystals. Attempts were made to bring about a more complete crystallization from the urine artificially. By experimenting on portions of a concentrated urine it was found that no crystals formed in neutral or alkaline solutions. Crystallization occurred readily when the urine was made acid to litmus with a small quantity of acetic acid, while no crystals formed when the urine was made as acid as pH 4. No crystals were obtained when ammonium sulfate, ammonium chloride, or sodium chloride were added in amounts to form varying quantities of precipitate, even when the tubes were seeded with crystals previously obtained. These reagents seemed to prevent crystallization completely. Long standing in the ice box and at room temperature failed. But the crystalline material could easily be recrystallized by dissolving in water made slightly alkaline with sodium hydroxide, acidifying with acetic or sulfuric acid, and allowing to stand over night. Crystals formed more rapidly when the solutions were seeded.

In order to obtain a dry preparation of this protein, Taylor and Miller's method (18), slightly modified, was used.

To 100 cc. of filtered urine were added 15 cc. of supersaturated sodium sulfate solution (200 gm. of crystalline sodium sulfate dissolved in 100 cc. of warm water) and 20 cc. of glacial acetic acid. After standing 15 to 20 minutes, the solution was centrifuged and the clear supernatant fluid decanted. The precipitate was washed by centrifugation once with acidulated water, once with 50 per cent alcohol, twice with 85 per cent
alcohol, once with absolute alcohol, once with absolute alcohol and ether, and finally with ether. It was then suspended in ether, transferred to a Buchner funnel, and pumped dry. A fine, colorless powder was obtained. A fresh 24 hour specimen of urine, 1,370 cc. in volume, yielded 15 gm. of air-dry protein.

Attempts were made to crystallize some of this dry preparation. The procedure which was successful in recrystallizing the moist crystals was entirely unsuccessful when applied to the dried preparation.

A quantity of crystals from urine was centrifuged, then washed with alcohol and ether as described above. This dry preparation yielded small quantities of crystals when recrystallization was attempted. The yields were so small, however, that it was concluded that the drying with alcohol and ether damaged the preparation.

After some experimentation the following procedure was adopted:

The supernatant liquid was decanted from the crystals which formed in the urine on standing. The remaining fluid was centrifuged, the liquid decanted, and the colorless crystals were suspended in four times their volume of acidulated water and centrifuged again. After decanting the supernatant liquid, the crystals were dissolved in a small amount of water with the aid of a few drops of sodium hydroxide, and the solution was filtered. The filtration was slow. The slightly opalescent filtrate was acidified with acetic acid and a drop of crystal suspension was added. The fluid became turbid in about 2 minutes and within 5 minutes it was milky white. Under the microscope small wart-like masses of needle plates could be seen. After standing over night, the suspension was centrifuged. The supernatant liquid contained considerable protein, but no further crystallization occurred on standing in the ice box. The crystals were again dissolved and recrystallized from a nearly colorless solution. On standing over night, large burrs were observed which broke up easily. See Fig. 1. After centrifuging, the cream of crystals was transferred to a porous plate, spread into a thin layer, and allowed to dry partially. When nearly dry, the now transparent film (crystals had disappeared) was scraped off. It was allowed to dry and then ground to a powder. 9 gm. of material were prepared in this way. This preparation could be crystallized easily by dissolving in water with a trace of alkali, then acidifying, and allowing to stand over night.

The recrystallized preparation gave all the typical reactions of Bence-Jones protein. A comparison was made between the
coagulation temperatures of the original urine, the sodium sulfate preparation, and the recrystallized preparation. They all reacted similarly. Coagulation began between 47 and 52°, depending on the concentration of protein and concentration of salts and acid. The coagulum dissolved at about 80°, leaving a slight residue which did not dissolve even when a dilute solution was boiled. If the solution were cooled quickly, a considerable precipitate formed.

Some studies were made to analyze the conditions influencing the crystallization of the precipitated Bence-Jones protein. The preparations isolated by the use of sodium sulfate and glacial acetic acid, as described above, could not be crystallized. When smaller quantities of acetic acid were used in the precipitation, some crystallization occurred. 25 cc. of urine were precipitated with 12.5 cc. of sodium sulfate after adding 3 cc. of glacial acetic acid. The pH was roughly 5. The precipitate was removed by centrifugation and dissolved in a small quantity of water with the aid of a little sodium hydroxide. The solution was slightly acidified, seeded, and allowed to stand at room temperature. A small quantity of crystals was obtained after several days.

Experiments were made acidifying the urine with sulfuric acid instead of acetic and precipitating with sodium sulfate. Good
yields of crystals were obtained quickly when from 5 to 10 drops of 10 per cent sulfuric acid were added to 25 cc. of urine, the most acid solution being neutral to methyl orange. When 15 drops were added (pH ± 3) the yield was much poorer and the crystallization slower. Too much acid seemed to damage the protein and decrease the ease of crystallization. Ammonium chloride and sulfate could be used just as well as sodium sulfate. When ammonium sulfate or sodium sulfate with sulfuric acid was used, the precipitates were highly colored. When ammonium chloride or sodium sulfate with acetic acid was used, the precipitates were nearly colorless.

Dialysis of the solutions of the precipitates seemed to aid crystallization. But dialysis of the mother liquor from which crystals had been obtained caused no further crystallization even when the solution contained a large quantity of protein which gave the typical reactions for Bence-Jones protein.

After these experiments were carried out, many attempts were made to crystallize the Bence-Jones proteins from various sources. The method of Grutterink and de Graaff was also used. The experiments were uniformly unsuccessful.

The blood of the patient who excreted the crystallizable material was examined for Bence-Jones protein.

15 cc. of serum were diluted with 75 cc. of water and 1 gm. of sodium chloride was added. 40 cc. of this solution were taken, heated to the boiling point after adding a few drops of acetic acid, and filtered through a heated funnel into a hot flask, the filtrate being kept near the boiling point. The filtrate was slightly acid and water-clear. It was allowed to cool and at about 75° a marked turbidity developed which increased in density on further cooling, but did not form a flocculent precipitate. The solution was again heated and the precipitate dissolved almost entirely, leaving the solution only faintly opalescent. A precipitate started to form at about 85°. The solution was placed in a water bath at 60° and kept between 55 and 65° for 4 hours. It was then centrifuged and the supernatant liquid which was slightly opalescent was decanted. The precipitate was washed once with 50 per cent alcohol and dried at 100°. 3.8 mg. of dry protein were obtained which was equivalent to 0.057 gm. per 100 cc. of blood. A second determination yielded 0.039 gm. per 100 cc. These values are certainly minimum values because the total quantity of Bence-Jones protein does not precipitate after boiling.

Normal serum was treated in the same way and no trace of opalescence appeared on cooling the hot filtrate.
Many isolated studies of the properties of Bence-Jones protein have been carried out, but preparations from different sources have not often been compared, under identical conditions. Different investigators have reported coagulation temperatures varying from 40 to 60°. The solutions some times became clear on boiling and some times not. Hopkins and Savory (19) pointed out the enormous effect of the presence of neutral salts on the precipitation and solution with heat. The temperature at which the protein coagulates varies also with the concentration of protein and the acidity.

In order to minimize the effects of these variables, the urines from several cases were diluted with normal urine until only a slight cloud formed on heating. Portions of 5 cc. were acidified with 5 drops of 10 per cent acetic acid and heated slowly in the same water bath. The temperatures at which precipitation and solution occurred were noted.

<table>
<thead>
<tr>
<th>Precipitate forming</th>
<th>R (X5) °C.</th>
<th>CND (X10) °C.</th>
<th>JED (X4) °C.</th>
<th>RL (X30) °C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>forming</td>
<td>51</td>
<td>51</td>
<td>76</td>
<td>59</td>
</tr>
<tr>
<td>&quot; maximum</td>
<td>56</td>
<td>56</td>
<td>82</td>
<td>60</td>
</tr>
<tr>
<td>&quot; dissolving</td>
<td>89</td>
<td>89</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>&quot; dissolved</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>99</td>
</tr>
</tbody>
</table>

The figures in parenthesis show the volumes of normal urine used in diluting the pathological urines.

It is at once apparent that considerable variations in the temperature of coagulation and solution may occur among different Bence-Jones proteins. It is interesting to note that while the proteins from R and from CND reacted identically with heat, the two proteins have been shown to be different in other respects. The protein from R crystallized spontaneously and the other could not be crystallized. Immunological studies by Bayne-Jones and the author (20) have also brought out differences between them.

On account of the peculiar physical properties of Bence-Jones protein, many investigators in the past have considered the material to be an albumose. Hopkins and Savory (19) first showed that the material is more nearly similar to the native proteins than to the albumoses because they were able to isolate practically all of the amino-acids usually found in native proteins.
In an attempt to gain added information regarding the nature of the molecule, the free amino nitrogen of the purified preparations of Bence-Jones protein was compared with the total nitrogen. Van Slyke (21) and Van Slyke and Birchard (22) have found that the free amino nitrogen of native proteins varies between 0 and 6 per cent of the total nitrogen, while derived proteins such as albumoses yield from 8 to 10 per cent. An unexpected difficulty which is described in the previous paper was encountered in the use of Van Slyke’s method for the determination of free amino nitrogen in Bence-Jones protein. Sörensen’s method of formol titration (23) was therefore employed in obtaining the data reported in this paper. Various protein preparations were examined in order to make possible a comparison of results obtained under similar conditions. Besides the solutions described in the previous paper, the Bence-Jones protein (No. R 4) was examined. It had been crystallized three times and dried on a porous plate. It was quickly dissolved in water to which had been added 2 or 3 drops of sodium hydroxide and the solution was neutralized as soon as possible.

The solutions were made up, with the exception of the stronger peptone and the edestin, so that similar concentrations of free amino nitrogen would be present in solution. The results obtained with the formol titration are recorded in Table I together with the total nitrogen and the calculated ratio of free amino nitrogen to total nitrogen.


<table>
<thead>
<tr>
<th>Material</th>
<th>Free amino N.</th>
<th>Total N.</th>
<th>Free NH₃-N Total N.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per cc.</td>
<td>mg. per cc.</td>
<td>per cent</td>
</tr>
<tr>
<td>Peptone, 2 per cent</td>
<td>0.730</td>
<td>2.73</td>
<td>26.8</td>
</tr>
<tr>
<td>&quot; 0.5 per cent</td>
<td>0.180</td>
<td>0.68</td>
<td>26.4</td>
</tr>
<tr>
<td>Proteose from beef</td>
<td>0.170</td>
<td>2.12</td>
<td>8.02</td>
</tr>
<tr>
<td>Bence-Jones protein, No. R 4, crystalline</td>
<td>0.139</td>
<td>2.86</td>
<td>4.86</td>
</tr>
<tr>
<td>&quot; No. R 5</td>
<td>0.138</td>
<td>2.50</td>
<td>5.52</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>0.127</td>
<td>2.49</td>
<td>5.10</td>
</tr>
<tr>
<td>Serum globulin</td>
<td>0.189</td>
<td>4.50</td>
<td>4.20</td>
</tr>
<tr>
<td>Edestin</td>
<td>0.052</td>
<td>2.70</td>
<td>1.93</td>
</tr>
</tbody>
</table>
The crystallized Bence-Jones protein contained 4.86 per cent of the total nitrogen in the form of free amino nitrogen which is slightly less than that in crystallized egg albumin. The proteose preparation contained about 8 per cent free amino nitrogen while the peptone yielded much higher values. The evidence would indicate that the Bence-Jones protein is more nearly similar to the proteins than to the proteoses or albumoses.

Further consideration must be given this evidence before drawing a definite conclusion. The ratio of the free amino nitrogen to total nitrogen was found to be much lower in edestin than in any of the other proteins examined. Similar variations have been reported by several investigators (22, 24, 25). Van Slyke and Birchard (22) were the first to point out that the amount of free amino nitrogen in proteins is very close to half the nitrogen of the lysine present and concluded that the ε-amino group of lysine is practically the only free amino group in the protein molecule, while other amino groups are free in proteoses.

The lysine content of a Bence-Jones protein had not, until recently, been determined by Van Slyke's method. Hopkins and Savory (19) isolated 3.67 per cent of lysine from a Bence-Jones protein, obtaining 4.32 per cent of the total nitrogen in the form of lysine nitrogen. Grutterink and de Graaff (26), determining the partition of nitrogen by the procedure of Kossel and Kutscher found 8.05 per cent of the total nitrogen in the lysine fraction.

Lüscher (27) in Hopkins' laboratory, using Van Slyke's method for studying the nitrogen distribution, has recently found 8.04 per cent of the total nitrogen in the lysine fraction.

There seems to be enough similarity in the chemical composition between the various specimens of Bence-Jones protein which have been analyzed in this way to permit a rough comparison of them with the crystalline Bence-Jones protein reported in this paper. It was found to contain 4.86 per cent of nitrogen in the form of free amino nitrogen which is not far from one-half of the lysine nitrogen (4.02 per cent) as determined by the analyses reported above. It is not evident why the precipitated preparation (No. R 5) should contain a higher percentage of free amino to total nitrogen unless there was slight hydrolysis on standing for several months in preserved urine before it was isolated.
The free amino nitrogen is somewhat higher than one-half the lysine nitrogen, but the comparison is obviously rough. As the ratio of free amino nitrogen to half the lysine nitrogen is not as great as that found by Van Slyke for albumoses and as the percentage of free amino nitrogen to total nitrogen is similar to a number of other characteristic proteins it seems justifiable to conclude that the Bence-Jones protein is a true protein and not an albumose.

Lüscher states in his recent article: "On the whole, Bence-Jones' protein seems to be a substance, not only characterised by its physical behaviour, but also by its distribution of amino-acids, which differs from all the proteins analysed up to the present time." An examination of his data, however, shows that there is a great similarity between his analyses of Bence-Jones protein and those of the serum globulin studied by Hartley (24). The agreement in the analyses is as good as is met with in comparisons of proteins placed in the same class such as the vegetable globulins studied by Johns and his coworkers (28, 29).

The statement is also made that: "There is some evidence that the same protein appears in the urine in all cases of Bence-Jones' protein uria." The only evidence presented is that, in two cases, the isolation of amino-acids; and in two cases, a study of the distribution of some forms of nitrogen yielded results which were similar and probably within the errors of the methods used. It is well recognized that these methods can be used only in making relatively rough comparisons of the chemical constitution of similar proteins.

By studying the immunological reactions of Bence-Jones proteins from various sources, Bayne-Jones and the author have obtained data which argue against the conclusion of Lüscher. These methods are far more sensitive than any ordinary chemical methods and seem to depend on the chemical constitution of the protein molecule. The experiments demonstrated that different Bence-Jones proteins do exist and may be obtained from different patients.

**SUMMARY.**

A Bence-Jones protein was found which crystallized spontaneously from the urine in which it was excreted. Dried preparations were made which could be reecrystallized.

\[Lüscher (27), p. 563.\]
Crystallization of Bence-Jones Protein

Attempts were made to crystallize other Bence-Jones proteins, but without success.

The crystalline Bence-Jones protein contains a ratio of amino nitrogen to total nitrogen of 4.86 per cent. This is added evidence that the compound should be classed as a protein and not as an albumose.

BIBLIOGRAPHY.

13. Hedinger, see Loehlein (11).
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