# 43. AN ANALYSIS OF BENCE-JONES PROTEIN

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(*Received 21 January 1941*)

BENCE-JONES protein, which is characterized by precipitation from solution at low temperatures (45–50°) and resolution of the coagulum on boiling, is excreted in the urine in about 70 % of the cases of multiple myelomatosis and occasionally also in allied diseases of the bone marrow and blood-forming organs. Of some seven hundred papers on myelomatosis which have appeared since Bence-Jones [1848] first noted the excretion of a peculiar substance in a case of 'mollities ossium', relatively few have dealt in detail with the chemistry of the protein. The purpose of the present communication is to give the results of an analysis of the amino-acid content of a sample of Bence-Jones protein obtained from a case of multiple myelomatosis which recently came under observation in the terminal stages, in the Tropical Ward of the Royal Infirmary, Liverpool; other features of the case will be discussed elsewhere.

The urine, which was collected daily under toluene for about 3 weeks immediately before the death of the patient, showed the typical reactions of Bence-Jones protein and was free from normal plasma proteins. After storage at 0° the different samples were filtered and mixed; part was reserved for isolation of the protein in soluble form, and the rest was coagulated for analysis.

## EXPERIMENTAL

As the amount of material for amino-acid analysis was small, attention was confined to colorimetric and small scale isolation methods. No attempt was made to apply the general solubility method devised by Bergmann & Stein [1939]. All the colorimetric analyses were carried out by means of a Hilger 'Spekker' Absorptiometer. In using this instrument the drum reading obtained by comparing the coloured solution with the appropriate blank is referred to a calibration curve similarly prepared from standard solutions of the pure constituent being estimated. The light filter is so chosen as to give maximum sensitivity. Solutions for the 1 cm. cups should be made up to 10 ml. All the reagents used were of A.R. quality or were purified by standard procedures and were freshly made up in solution. Blank tests were carried out where necessary and all the procedures were submitted to preliminary test against standard protein preparations. Unless otherwise stated, the results obtained with Bence-Jones protein are the mean of two or more determinations and refer to the moisture- and ash-free material.

*Preparation of Bence-Jones protein.* The *p*H of the urine was adjusted with acetic acid to the point of maximum precipitation in the cold and the coagulation completed by heating to 60° for 30 min. Exhaustive washing of the protein by centrifuging was carried out by allowing it to stand 1–2 days in the wash liquor and repeating this several times afresh—first with very dilute acetic acid, then

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water, water at 60°, aqueous alcohol, absolute alcohol, and finally light petroleum twice overnight.[1] The latter was repeated by extracting twice for a day in a Soxhlet apparatus and the protein was then air-dried under cover and finally in a desiccator over $H_2SO_4$.

### *Methods*

*Moisture.* A sample was dried to constant weight at 108° (about 4 hr.) in a silica crucible.

*Ash.* The residue from the moisture determination was ashed over a Bunsen flame and then kept at 600° for 2 hr. in a furnace.

*Total N.* By micro-Kjeldahl analysis. In a repeat experiment, with fresh standard solutions and $CuSO_4$-$K_2SO_4$ mixture as catalyst, the results were identical with those originally obtained by $H_2SO_4$-$H_3PO_4$-$K_2S_2O_8$ digestion.

*Amide-N.* About 0·07 g. was hydrolysed in a 6 × 1 in. test tube by heating for 6 hr. with 5 ml. 2*N* HCl [cf. Lugg, 1938, 3]. Part of the HCl was removed by concentration *in vacuo* and after neutralizing to phenolphthalein and adding 0·15 g. $K_2CO_3$ the solution was rapidly aerated for 2 hr. at 30° [Denis, 1910] and the $NH_3$ determined in *N*/50 acid in the usual manner.

*Humin-N.* The hydrolysate prepared for the determination of the basic amino-acids was filtered through a Whatman No. 1 paper and the residue washed well with distilled water and analysed by the Kjeldahl procedure.

*Total S.* Wet ashing by the Benedict-Denis method is known to yield faulty results as compared with the standard Parr bomb method [Painter & Franke, 1936; Masters, 1939] or the peroxide fusion method [Bailey, 1937] of which use was made in the present case following the directions of Lugg [1938, 2]. The usual procedure for the removal of Group 3 metals was carried out before precipitating $BaSO_4$.

*Total P.* About 0·05 g. protein was digested with 1 ml. conc. $H_2SO_4$ with the dropwise addition of a little perhydrol (Merck). The digest was neutralized and brought to 7 ml. and analysed by the method of Berenblum & Chain [1938]. Filter No. 3 (orange).

### *Tyrosine and tryptophan*

The colorimetric method of Folin & Marenzi [1929] was used as adapted from that of Folin & Ciocalteu [1927]. From the limited data available, this appears to give substantially the same results as the later modification suggested by Lugg [1937]. In three determinations with about 0·08 g. protein, the time of hydrolysis and other details were varied. Separation of the tryptophan as the mercury complex was allowed to proceed overnight at 0° in each case. The tyrosine solutions were read immediately on mixing with the reagents as turbidity developed within a few minutes. The 30 min. development of the tryptophan colour with the Folin reagent was carried out in a bath at 20° or at room temperature and saturated solutions of $Na_2CO_3$ made at 18° and 25° respectively were used; the corresponding calibration curves were identical. Filters: Tyrosine No. 7 (dark blue), tryptophan No. 1 (red).

Dr R. A. Morton and Mr T. W. Goodwin kindly carried out an independent analysis by a slight modification of Holiday's [1936] spectrophotometric method. They conclude from experiments with casein (unpublished) that in order to make the difference between the observed absorption in the ultraviolet region

[1] For determination of the S-distribution according to Baernstein [1936, 1] it is necessary to eliminate traces of alcohol and ether and avoid drying above 100°. Lugg [1938, 2] states, however, that oven-drying does not affect the results appreciably.

and that calculated from the best available chemical data fit in with the extrapolated curve showing general absorption beyond 310m$\mu$, it is necessary to carry out a hydrolysis of the protein with Lugg's [1938, 1] alkaline stannite reagent instead of merely dissolving it in NaOH. Apart from this modification, a preliminary determination on Bence-Jones protein in NaOH showed that the interference due to pigment was so great as to make the corrected (Holiday) result valueless; only a trace of tryptophan was evident. The more reliable results obtained on the colourless Lugg hydrolysate were: tyrosine 8·5 %, tryptophan 2·3 %. The corresponding figures obtained colorimetrically were 8·8 % and 1·4 % respectively, so that while the results for tyrosine compare well, those for tryptophan show a wide and unexplained divergence.

*Sulphur distribution*

The discussions by Lugg [1932; 1933; 1938, 2] and Bailey [1937] of the various procedures for differentiating the total S fraction of proteins and the difficulties attendant on the colorimetric estimation of cystine led to the adoption in the present instance of Baernstein's [1936, 1, 2] elegant and comprehensive method. By hydrolysis with conc. HI a study of the S-distribution can be completed on 0·5 g. protein or less, including the determination of methionine as well as of cystine by titration and the estimation of any volatile S ($H_2S+SO_2$) arising during hydrolysis from $SO_4$ groups, etc., by absorption in $CdCl_2$-$BaCl_2$ mixture. On this basis it is claimed that almost all the S of a number of proteins can be quantitatively accounted for. The scheme does not permit of discrimination between cystine and cysteine. Certain refinements in the method suggested by Kassell & Brand [1938] do not appear to be essential [Macara & Plimmer, 1940].

Two traps instead of one were used for the volatile S, so that the efficiency of absorption could be gauged. These consisted of 10 ml. tapering centrifuge tubes with ground glass connexions, each containing 3·5 ml. wash liquor. A trial of the procedure with casein gave satisfactory results; the volatile S (0·016 mg.) was only enough to produce a faint turbidity with $CaCl_2$-$BaCl_2$. Recoveries of the amino-acids from a known mixture gave 98 % for cystine and 90 % for methionine, in agreement with the values reported by Kassell & Brand [1938] and Lugg [1938, 2].

Application of the method to Bence-Jones protein immediately made it obvious that a relatively large proportion of the S was being volatilized. A heavy precipitate formed in the first trap as soon as boiling commenced and necessitated, at the end of the reaction, careful washing out of the inlet tube with iodate mixture to decompose the deposit of sulphide. One determination on 0·369 g. protein yielded the following results, including the correction for recovery: cystine (cysteine + cystine) 2·91 %, methionine 0·58 %, volatile S 1·56 mg. After correction of the latter value by subtracting 1 % of the methionine-S and 2 % of the cystine-S [Kassell & Brand, 1938] the figures account almost quantitatively for the total S content of the protein: (cystine + methionine)-S 69·7 %; volatile S 28·2 %; total S recovery 97·9 %.

*A. By isolation* *The basic amino-acids*

*Arginine and lysine.* The small scale adaptation by Block [1934] of the classical Kossel & Kutscher [1900] method for the determination of the basic amino-acids has been further modified recently [Block, 1938; Tristram, 1939].

The procedure advocated by Tristram for the isolation of arginine and lysine has been followed in the present investigation, since it avoids the use of excess $AgNO_3$ (which is liable to interfere with the estimation of lysine), gives greater delicacy in the adjustment of *p*H values, and incorporates the finding of Ayre [1938] that ammonium phosphotungstate is not decomposed by dilute $H_2SO_4$ in the presence of amyl alcohol-ether. The removal of $NH_3$ by NaOH before the isolation of lysine as picrate is thereby eliminated.

All the standard operations of concentrations *in vacuo*, etc., were adhered to. In the final stages of both analyses the operations were determined as usual by the total N content (Kjeldahl) of the solutions.

*Histidine.* While the figures recorded by Tristram lend no support to Block's [1938] contention that no more than about 75 % of the histidine present is recoverable as diflavianate, this amino-acid is not easy to deal with on account of the relatively small quantities present in most proteins and the difficulty of isolating the derivative in a state of purity. For this reason Block [1937] has suggested the use of nitranilic acid to precipitate histidine. In a later paper [Block, 1940], the recovery of histidine both from pure solutions and from protein hydrolysates is stated to be quantitative, using the factor 0·403 for the conversion of the nitranilate to histidine. This was checked on histidine solutions as follows: about 0·14 g. histidine hydrochloride monohydrate dissolved in 15 ml. water was precipitated by the addition of 0·24 g. nitranilic acid in 15 ml. methyl alcohol. The derivative crystallized immediately. The solution was left overnight in the ice-chest, filtered on a sintered glass crucible and the residue was washed with methyl alcohol and ether and dried in the air and for a short time at 108°. Recoveries were 100 % from B.D.H. histidine and 98·2 % from a slightly impure sample prepared from ox blood.

In applying this method to Bence-Jones protein, the histidine-Ag fraction was put through the whole procedure in use for the precipitation of histidine as diflavianate, i.e. removal of cystine and other impurities by successive treatment with $HgSO_4$ and $CuCO_3$. This is preferable to the shortened method proposed by Block [1938] in which the solution, after removal of Ag by $H_2S$, is merely treated with charcoal before addition of nitranilic acid. From the final solution (50 ml.) four 1 ml. aliquots were removed for colorimetric estimation as described below. The remainder, which was thereby calculated to contain about 19 mg. histidine, was concentrated *in vacuo* to 3 ml. in a 50 ml. distilling flask and 50 mg. nitranilic acid, dissolved in 7 ml. methyl alcohol, were added at room temperature. Crystallization was completed overnight at 0° and the precipitate collected quantitatively, as described before.

*B. By colorimetric methods.*

Where the available material allows only one gravimetric determination of the basic amino-acids to be made, the relative purity of the final histidine and arginine fractions provides a useful opportunity for checking the results colorimetrically, and safeguarding against the possibility of subsequent loss of material.

*Histidine.* Methods based on diazotization [e.g. Koessler & Hanke, 1919] have found little application. The Knoop [1908] reaction, which has been utilized by Kapeller-Adler [1933] for the quantitative estimation of histidine in pregnancy urines, has been applied recently to protein hydrolysates. An examination of recent papers on this reaction by Armstrong & Walker [1932], Földes [1936], Conrad & Berg [1937], Woolley & Peterson [1937] and Racker [1940] indicates that the procedure described by Block [1938] incorporates the most satisfactory

modifications for use in protein analysis. The solution is treated at room temperature for 10 min. with the bromine reagent, slight excess of which is then destroyed by addition of a drop of arsenite solution. 2 ml. of ammonia-ammonium carbonate solution are added and the mixture is heated in the boiling water bath for 5 min., and then kept at room temperature for a further 10 min. for the complete development of the colour. It soon became evident on analysis of the histidine-Ag fraction from edestin that reasonably consistent results could not be obtained. Unfortunately, there is no record of individual variations apart from that which is implied in Block's directions to develop both unknown and standard solutions in sets of six, and then to mix each set before comparing. Woolley & Peterson [1937] claim a 97 % recovery of histidine from a yeast hydrolysate, whereas Racker [1940], in controlled tests on urine, states that the experimental error is about 10 %.

In experiments on pure histidine solutions no improvement was effected by carrying out the second stage of the reaction, which is probably the source of the trouble, at 90–95° with the object of minimizing the spitting and uneven heating of the liquid consequent on the rapid evolution of $NH_3$. An apparent improvement in this respect was achieved by the use of thin-walled, wider bore tubes ($6 \times \frac{3}{4}$ in.). In studying the effects of temperature and heating period on the intensity and stability of the colour, it was clearly not advisable to aim for conditions of maximum intensity, since this was still not attained after 22 min. heating at 65°, 75° or 100°. The results suggested that the original directions to heat in the boiling water bath for 5 min. are preferable in that the colour is stable or almost so for about 10 min. after taking the first reading, though not for an hour as stated by Kapeller-Adler [1933]. For longer periods at 100° the colour is more intense but fades with increasing rapidity. Colours which were stable for at least 20 min. were also attained by heating at 75° for 12–17 min., but such conditions are less suitable for routine analysis. Under all the other conditions tested the solutions were unstable.

In following Block's directions (5 min. heating in a boiling water bath, etc.) for the construction of a calibration curve with the minor modifications suggested by the above, the mean deviation in the absorptiometer readings (Filter Nos. 5+6) averaged over each set of five determinations was 3·3 %. Over the range investigated (0·4–1·4 mg. histidine) the mean values for each set fell exactly on a straight line. Treatment of the histidine-Ag fraction of Bence-Jones protein with charcoal [Block, 1938] after removal of Ag with $H_2S$ was unsatisfactory. The clear colourless solution gave a white precipitate on adding bromine and finally a turbid brown-violet solution. After further purification of the mother liquor in the usual manner with $HgSO_4$ and $CuCO_3$ the colour developed satisfactorily. The spread of the results was much greater than before, however, the four absorptiometer readings being 0·294, 0·271, 0·278, 0·257.

*Arginine.* Further comments on the procedures of Weber [1930] and of Jorpes & Thorén [1932] for the colorimetric estimation of arginine by means of the Sakaguchi reaction have recently been made by Fisher & Wilhelmi [1937], Davenport *et al.* [1938] and Thomas *et al.* [1939], and the choice of certain empirical factors has been more carefully defined. The time assigned to the first stage of the reaction, between arginine and the alkaline α-naphthol, before the addition of hypobromite, varies from 2–3 min. [Weber, 1930] to 1 hr. [Jorpes & Thorén, 1932]. A constant 0·20 ml. standard hypobromite solution (followed after a certain interval by 1 ml. 40 % urea to destroy the excess) was used by Jorpes & Thorén [1932] in developing the colour, and has been found satisfactory by Fisher & Wilhelmi [1937] and Davenport *et al.* [1938]. Thomas *et al.* [1939],

however, have utilized Weber's [1930] suggestion that the optimum hypobromite concentration should be found by trial; this modification, which is designed for use with the Sheard-Sanford Photelometer, eliminates the preliminary separation of arginine from certain other amino-acids, etc., which interfere with the colour development, and is stated to be directly applicable to protein hydrolysates. For each standard and unknown, the amount of hypobromite is varied until the light absorption remains constant for about 10 min., i.e. the fading of the red colour, which is stable for not more than 2 min., is exactly compensated by the increasing yellow decomposition colour, so that on extrapolating to zero time the reading may be taken to represent 100 % red colour. The safest and most convenient interval allowable between the addition of hypobromite and that of urea has been fixed by Davenport *et al.* [1938], in the light of their experience with the similar estimation of glycocyamine, as 15 sec., in conformity with the earlier recommendations of Jorpes & Thorén [1932]. This allows of more accurate working than the shorter period of 4 sec. used by Weber [1930].

Following the directions of Thomas *et al.* [1939], the amount of hypobromite required to give a satisfactory colour with an aliquot containing 0·12 mg. arginine from the arginine fraction of edestin was found to be 0·20 ml. This amount was optimal also for standard solutions containing 0·08–0·20 mg. arginine. It was difficult to determine with consistency the small range over which the hypobromite could safely be varied so as to give readings which remained constant. On the other hand, excellent agreement was obtained in duplicate determinations, provided that the solutions were read immediately after making up to volume. This point was brought out in the actual determination on the appropriate fraction from Bence-Jones protein. Four groups containing 10 equal aliquots were treated with 0·20, 0·25, 0·30 and 0·35 ml. hypobromite respectively. All the readings were constant within the limits of the experimental error, viz., 1 %.

The method finally adopted, using the solutions recommended by Thomas *et al.* [1939], is as follows. The reagent bottles which are kept in the ice-chest are placed in a bath at 0°. A suitable aliquot of the arginine-Ag fraction (not more than 5 ml.) containing about 0·05–0·20 mg. arginine is placed in a thin-walled $6 \times \frac{3}{4}$ in. test tube graduated at 10 ml., made up to 5 ml. with distilled water and cooled in a separate ice-bath for 30 min. 1 ml. NaOH and 1 ml. α-naphthol are added and the tube replaced in the bath for 5 min. with occasional shaking. 0·20 ml. standard hypobromite solution is added and after exactly 15 sec. vigorous shaking 1 ml. 40 % urea is added with further shaking. The solution is brought to 10 ml. with water and mixed. After warming the tube slightly in the hand to prevent subsequent condensation of moisture, the solution is transferred to the absorptiometer cell as quickly as possible so that the diaphragm can be adjusted within 60–70 sec. after the addition of hypobromite. In adding the reagents the hypobromite is sucked up several times to cool the pipette (straight 1 ml. graduated to the tip) and is then blown directly into the liquid; after this has been shaken the pipette is 'blown off' against the inside of the tube just above the liquid. This allows sufficient time for further mixing before adding the urea solution similarly, blowing this out once from an Ostwald pipette. Holders for the pipettes were not found necessary.

Filter combination Nos. 5+6 is the most sensitive.

*Summary of analysis of the basic amino-acids in 2·490 g. Bence-Jones protein*

With appropriate corrections for aliquots removed for N determination and colorimetric analysis and for the solubility of arginine flavianate and lysine picrate and the incidental losses of procedure [Tristram, 1939], the results are as follows:

| | | Wt. found (mg.) | Mean % of protein |
|---|---|---|---|
| Histidine: | By nitranilate | 21·3 | 0·90 |
| | Colorimetrically | 23·6 | |
| Arginine: | By flavianate | 89·5 | 3·64 |
| | Colorimetrically | 91·5 | |
| Lysine*: | By picrate | 101·0 | 4·04 |

* The lysine picrate exploded at 256° and after recrystallization at 266°.

It may be noted that the minimum mol. wt. of the protein calculated from the histidine content (0·9 %) is 17,200; or assuming 2 mol. histidine per mol. protein, 34,400. This agrees well with the value of 35,000 determined by Svedberg & Sjögren [1929].

*Phenylalanine*

The colorimetric nitration method of Kapeller-Adler [1932] appears to be the only small scale method which has been proposed for the determination of phenylalanine. Jervis *et al.* [1940] have suggested, contrary to the original procedure described by Block [1938], that the removal of histidine and tyrosine may be omitted if the final solution is examined with a suitable filter. The full procedure is outlined here as conducted with slight modifications on one-fifth of the weight of protein suggested by Kapeller-Adler.

0·4 g. protein is refluxed for 20 hr. with 5 ml. 25 % $H_2SO_4$ in a micro-Kjeldahl flask. The humin is removed by centrifuging and washed 3 times with cold water. The solution and washings are brought to 40 ml. (filtered if necessary) and three 10 ml. aliquots are placed in 15 ml. centrifuge tubes and treated with 0·5 ml., 1·0 ml. and 1·5 ml. respectively of 10 % aqueous phosphotungstic acid. Each mixture is well stirred and the precipitate partially dissolved by warming the tube in a water bath. The solution is allowed to cool overnight, the precipitate washed with very dilute $H_2SO_4$ and the filtrate and washings in each case filtered through a small paper into a 50 ml. beaker, the outside of which is marked at the 10 and 20 ml. levels. The filtration ensures the elimination of phosphotungstate scum which is not easily centrifuged down. The solution is treated in the cold with $N/10$ $KMnO_4$ in small portions till the mixture is faintly coloured and then concentrated over a small flame. This operation is too tedious on the water bath. From time to time $KMnO_4$ solution is added to ensure that excess is present. When a volume of about 4 ml. is reached, further concentration to the oily stage (1–2 ml.) is effected very cautiously. 2 ml. of 10 % $KNO_3$ in conc. $H_2SO_4$ are added to the cooled mixture, the whole heated for 20 min. on the water bath and finally brought to about 6 ml. The beaker is next cooled in an ice bath and 5 ml. 15 % hydroxylamine hydrochloride added dropwise with mixing. After cooling to 0° again conc. $NH_4OH$ is added slowly with mixing to a volume of about 20 ml. and the solution is transferred to a 25 ml. flask and brought to volume with conc. $NH_4OH$. The contents are thoroughly mixed and the development of the colour completed by placing the flask in a bath at 40° for 5 min. and then in an ice bath for a further 15 min. The three solutions thus prepared are read with the No. 6 filter (light blue). The determination is repeated

if necessary using only the optimum amount of phosphotungstic acid. Occasionally a solution may develop a brownish shade and should be discarded. A slight deviation in the shade of the unknown from that of the standard solution which would make matching difficult in the visual type of colorimeter can be ignored. It was not found necessary to submit the protein hydrolysate to preliminary fractionation, as recently suggested by Macara & Plimmer [1940] who were unable to develop satisfactory colours with dephosphocaseose.

For the 1 cm. cell a suitable range of values for the calibration curve is 1–8 mg. phenylalanine.

*Hydroxyproline*

No small scale gravimetric method has yet been developed for the determination of hydroxyproline. The colorimetric method of Waldschmidt-Leitz & Akabori [1934] for the micro-estimation of both proline and hydroxyproline in the same sample, which is developed from that of Lang [1933], requires steam distillation in a specially designed apparatus and yields a value of only 8·9–9·4 % for the hydroxyproline content of gelatin as compared with the accepted value of 14·4 % [Bergmann, 1935]. The principle of oxidizing the hydroxyproline and coupling the product with isatin has been modified along simpler lines by McFarlane & Guest [1939] who use as the oxidizing agent alkaline hydrogen peroxide in the presence of a small amount of $CuSO_4$ as catalyst. This method is stated to yield a value of 14·65 %.

Using this procedure on a sample of grade B gelatin (*a*) 0·5 g. refluxed 25 hr. with 8 ml. 20 % HCl, (*b*) 0·5 g. autoclaved with 10 ml. 3*N* HCl for 6 hr. at 145°, lower but identical values in each case of 10·4 % were obtained. As the hydroxyproline contents of most proteins are much less than that of gelatin, the colours developed from more concentrated hydrolysates (with appropriate dilution after development) were compared with those prepared according to the original procedure, but were in each case found to be less intense; no improvement was effected by increasing the concentration of reagents. Hence, where possible the final dilution should be avoided, the colour being developed in a larger volume of solution to enable the 4 cm. cells to be employed in the absorptiometer (Filter No. 6).

Tests made on Bence-Jones protein ((*a*) 0·25 g. refluxed 24 hr. with 5 ml. 20 % HCl and the hydrolysate brought to 25 ml., (*b*) 0·1 g. autoclaved 6 hr. at 145° in 3*N* HCl and the hydrolysate brought to 10 ml.) showed no trace of colour as compared with a control which contained the same reagents but which had not been heated. As an obvious colour was developed from 2 ml. standard solution containing 0·08 mg. hydroxyproline (B.D.H.), which is the smallest amount that could have been present in the test aliquots, assuming only 1 mol. hydroxyproline per mol. protein (mol. wt. 35,000), it can safely be concluded that this amino-acid is completely absent from Bence-Jones protein. This was confirmed later on the filtrate from the proline estimation.

*Proline*

The method of Engeland & Bastian [1937], which involves methylation of the mixed amino-acids and the isolation of dimethylproline by successive precipitation with $HgCl_2$, $H_2PtCl_3$ and $HAuCl_4$, has been used by Bastian [1937] who records a figure of 25–26·5 % for the proline content of gelatin as compared with the accepted figure of 19·7 % [Bergmann, 1935]. Guest [1939] has recently made use of the well-known condensation between oxidized proline and *p*-dimethylaminobenzaldehyde in acid solution in a method in which the oxidation is

effected by means of a suspension of $PbO_2$ in a phosphate buffer *p*H 8·7. As this procedure is stated to yield a satisfactory value for the proline content of casein and depends on there being little or no hydroxyproline present, a considerable number of tests and modifications, which need not be detailed, were made, but the method was finally abandoned. Consistent results could not be obtained with either pure proline or hydrolysates of casein or Bence-Jones protein. Moreover, in preliminary experiments with casein, the colours developed after acid hydrolysis (HCl and $H_2SO_4$) were so weak compared with those of baryta hydrolysates as to make the method suspect. The possibility was therefore examined of precipitating the proline on a small scale by means of ammonium rhodanilate [Bergmann, 1935].

In the large scale precipitation of proline, Bergmann has used various reagents including flavianic acid [Bergmann, 1935], phosphotungstic acid [Bergmann & Niemann, 1936] and alcohol [Bergmann & Niemann, 1937], mainly to remove arginine from the hydrolysate before addition of ammonium rhodanilate. Using aliquots from the same hydrolysate equivalent to 1·6–5·0 g. gelatin (Coignet Gold Label) the addition of flavianic acid was found to be unsatisfactory since it is difficult to remove excess from the precipitate of proline rhodanilate by washing on the filter; on the other hand, phosphotungstic acid results in a much cleaner solution and the amount added can be varied within fairly wide limits without affecting the result. As the final precipitate may easily be contaminated with excess of the ammonium rhodanilate which is not very soluble in water, it is necessary to have a fairly accurate idea of the amount of proline present, which may entail several trials, and to adhere to a fixed ratio between the volume of hydrolysate and that of the reagent. The correction factor for the solubility of proline rhodanilate as determined by Bergmann [1935] can then be applied. The details of the method finally adopted are as follows.

About 0·5 g. Bence-Jones protein was refluxed for 24 hr. with 10 ml. 20 % HCl. The humin was removed by centrifuging and washed twice with cold water. The total solution was concentrated three times *in vacuo* to remove HCl, transferred quantitatively to a 15 ml. centrifuge tube and treated dropwise with 1·0–1·4 ml. 10 % phosphotungstic acid, mixing well with a fine rod. After standing in the ice chest for 2 hr. the mixture was centrifuged and the liquid poured off and concentrated *in vacuo* to a gum. The residue was taken up in 3 ml. water and the solution transferred by means of a small teat pipette to a wide bore graduated test tube and adjusted to 4 ml. A solution of 0·14 g. ammonium rhodanilate in 2·4 ml. methyl alcohol was placed in the flask and the amino-acid solution pipetted in slowly with shaking, the pipette and test tube being washed with 2–3 drops methyl alcohol. After 2 hr. on ice the mixture was filtered on a sintered glass crucible and the residue washed three times with ice-cold water and dried in a desiccator. 0·460 g. protein gave 0·1345 g. proline rhodanilate (M.P. 133° as required). According to Bergmann's [1935] finding, the solubility correction[1] for 6·6 ml. mother liquor (37 % methyl alcohol) is 0·0165 g., giving a total of 0·151 g. proline rhodanilate or $0{\cdot}19 \times 0{\cdot}151$ g. proline. This is equivalent to 6·24 % of the moisture- and ash-free protein.

In one of the determinations the filtrate from the proline precipitation, without washings, was treated with 1 g. ammonium reineckate + 2 drops pyridine and left for 2 hr. on ice as described by Bergmann [1935] for the isolation of hydroxyproline. The mixture was then filtered and the residue washed

[1] Bergmann [1935] precipitated the proline rhodanilate from a solution containing 20 g. in 150 ml. methyl alcohol by adding 250 ml. 0·5 *N* HCl and recovered 19 g., i.e. a solubility of 1 g. in 400 ml. 37 % methyl alcohol which he loosely applies as a + 5 % correction.

well and decomposed on the filter in aqueous suspension by adding 2 ml. pyridine. The resulting filtrate was acidified with acetic acid, the precipitate removed by centrifuging and the solution then tested for hydroxyproline by Guest's [1939] method. No trace of colour was produced, thus confirming the previous results.

## Summary and discussion

The values for the various constituents are recorded in Table 1, along with those found by other investigators.

Table 1. *Comparison of analyses of Bence-Jones protein*

All the values are calculated as percentage of the moisture- and ash-free protein unless otherwise stated.

| Constituent | Hopkins & Savory* | Abderhalden & Rostoski [1905] | Calvery & Freyberg† | Present author | Miscellaneous‡ 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| Ash | Trace | — | 1·2–5·1 | 0·4 | — | — | — |
| Moisture | Nil | — | 4·8–10·6 | 7·5 | — | — | — |
| Total N | 16·2 | — | 18·1 | 14·7 | 14·8 | 16·07 | 16·01 |
| Amino-N | — | — | 14·3 | — | — | — | — |
| Amide-N | 1·3 | — | 1·0 | 1·0 | — | — | — |
| Humin-N | — | — | Nil | 0·14 | — | — | — |
| Sulphur | 1·18 | — | 1·0 | 1·34 | — | — | 1·24 |
| Phosphorus | — | — | Nil | <0·01 | <0·01 | — | — |
| Tyrosine | 4·20 | 1·7 | 6·7 | 8·8 (8·5)§ | 6·1 | — | — |
| Tryptophan | 0·82 | — | 2·5 | 1·4 (2·3)§ | 1·7 | 4·2 | — |
| Cystine | 0·57 | — | 3·0 | 2·9 | — | — | — |
| Methionine | — | — | — | 0·58 | — | — | — |
| Arginine | 6·04 | — | 5·1 | 3·6 | — | — | — |
| Histidine | 0·81 | — | 1·2 | 0·90 | — | — | — |
| Lysine | 3·67 | — | 6·8 | 4·0 | — | — | — |
| Glycine | Present | 1·7 | — | — | — | — | — |
| Alanine | Present? | 4·5 | — | — | — | — | — |
| Leucine | 6·51 | 10·6 | — | — | — | — | — |
| Valine | 5·60 | — | — | — | — | — | — |
| Aspartic acid | 2·12 | 4·5 | 4·6 | — | — | — | — |
| Glutamic acid | 7·8 | 6·0 | 8·6 | — | — | — | — |
| Proline | 2·70 | 1·9 | — | 6·3 | — | — | — |
| Hydroxyproline | — | — | — | Nil | — | — | — |
| Phenylalanine | 4·83 | 1·5 | — | 6·1 | — | — | — |

* The values of Hopkins & Savory [1911] are the mean of two consistent sets obtained from the protein of two separate patients.

† The values of Calvery & Freyberg [1935] are the mean for two specimens of protein excreted at different times by the same patient.

‡ 3, Hewitt [1929]; values uncorrected for ash. 4, Ohlsson & Nordh [1929]. 5, Magnus-Levy [1936].

§ Spectroscopic analysis.

It is of interest to consider these results firstly in relation to other evidence bearing on the nature of Bence-Jones protein. Physicochemical properties of the protein, including the refractive index, optical rotatory power and ultra-violet absorption spectrum have been investigated by Hewitt [1929]. The isoelectric point of different samples has been determined cataphoretically [Svedberg & Sjögren, 1929; Mainzer, 1932, 1, 2, 3; McFarlane, 1935; Magnus-Levy, 1936; Kekwick, 1940] as well as by electrometric titration [Jervell, 1932] and flocculation [cf. Magnus-Levy, 1936]. Svedberg & Sjögren [1929] and Magnus-Levy [1936] have reported measurements of the molecular weight by means of the ultracentrifuge and further work along these lines has been reported by Kekwick [1940] and others. The results of these investigations confirm

the immunological findings of Bayne-Jones & Wilson [1922, 1, 2], Robinson [1927] and others that Bence-Jones protein is of variable composition and is distinct from normal plasma proteins.

The extension of this work to the determination of the chemical structure of the protein has received little attention. Apart from the investigations of Lüscher [1922], Medes *et al.* [1935] and Tsutsui [1935] on the N-distribution, amino-acid analyses have been reported in only three cases [Abderhalden & Rostoski, 1905; Hopkins & Savory, 1911; Calvery & Freyberg, 1935]. While there are not as yet sufficient trustworthy data on the composition of the protein to give any certain indication of its pathological origin, there are however several points of interest in comparing the various values. Medes *et al.* [1935] examined, by the Van Slyke method, six different samples of the protein. Five of these were shown to be chemically identical within the limits of the method, the analyses being in close agreement with that of Lüscher [1922], while the sixth was quite different. Lüscher's [1922] analysis was made on material originally examined by Hopkins & Savory [1911] who considered that despite the admittedly imperfect methods of isolation then available, both the proteins examined by them as obtained from different patients were identical but distinct from the sample analysed by Abderhalden & Rostoski [1905]. Comparison of the figures obtained more recently by Calvery & Freyberg [1935] with those of the present paper and with miscellaneous values shows several divergencies. My N value is low, but it is unlikely that the inconsistency can be explained wholly on the basis of impurity. Hopkins & Savory's [1911] value, which is in agreement with those of Ohlsson & Nordh [1929] and of Magnus-Levy [1936], was obtained on 'practically ash-free' material dried at 110° and hence it is not to be brought into conformity with the higher value of Calvery & Freyberg [1935], as these authors suppose, by 'failure to correct for the ash and moisture content of the protein'. The S value is somewhat higher than has been obtained before but checks well with the determination of the S distribution. The values for the amino-acids also suggest that we are dealing with a protein chemically distinct from that of Calvery & Freyberg [1935]. The results of chemical analysis are therefore in agreement with evidence from other sources that Bence-Jones protein is of variable composition and that it represents a group of distinct but possibly similar proteins. There is little doubt that some of the samples which have been examined were chemically identical.

The origin of the protein remains obscure. The studies of Folin & Denis [1914], Medes *et al.* [1935] and others have shown that it cannot be considered of exogenous origin. The very considerable amount excreted in some cases precludes the possibility of its being a metabolite of the tumour. It seems more reasonable to regard it as the result either of the partial breakdown of a normal protein of higher molecular weight or of some hindrance to the complete synthesis of such a protein. In this way certain allied pathological conditions with which Bence-Jones proteinuria is associated might give rise to different products according to the response to different 'toxins'. On the basis of a chemical similarity first noted by Lüscher [1922], Calvery & Freyberg suggested that Bence-Jones protein may be derived from normal serum globulin by the latter being split into three particles of equal size; this would account for the molecular weight relation and for the excretion of Bence-Jones protein as a 'foreign' protein. This idea appears to entail the assumption that the protein is a single chemical entity of fixed composition. In view of the evidence to the contrary, the alternative suggestion demands consideration, viz. that the protein is the result of a deranged synthetic mechanism which would otherwise produce normal

plasma or other proteins. This would account more easily for the production of a protein which may vary not only in chemical composition and behaviour but also in mol. wt. It is interesting to note in this connexion that according to Bonsdorff *et al.* [1938] the mol. wt. of Bence-Jones protein determined on only a few occasions as 35,000 has once given a value of 70,000 while the myeloma protein discussed by them had a mol. wt. of about 200,000, which is higher than for any of the recognized plasma proteins.

Brief mention should be made of Meyler's [1936] recent experiments on the nature of the protein from bone marrow and leucocytes which suggest that small undetected amounts of Bence-Jones (or similar) protein normally derive from these sources, but that in myelomatosis and certain other conditions the output is so magnified above the rate of destruction in the body that the protein appears in the blood and is excreted as a pathological constituent. Confirmation and further work along these lines is desirable.

The author is much indebted to Prof. H. J. Channon for laboratory facilities and materials, and also to Dr R. A. Morton and Mr T. W. Goodwin for their ready assistance in carrying out spectroscopic analyses. The work was carried out during the tenure of a whole-time grant from the Medical Research Council, to whom the author wishes to express his gratitude.

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