LIII. THE NITROGEN-DISTRIBUTION IN BENGE-JONES' PROTEIN, WITH A NOTE UPON A NEW COLORIMETRIC METHOD FOR TRYPTOPHAN-ESTIMATION IN PROTEIN.

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THE physical properties of Bence-Jones' protein have been more or less fully described in all investigated cases. On the other hand, there is but little reference to the exact chemical constitution and especially to the amount of individual amino-acids. Magnus-Levy [1900] determined the N-balance by Haussmann's method [1899]. Abderhalden and Rostoski [1905] estimated the monoamino-acids by esterification and Hopkins and Savory [1911] all the amino-acids by a similar method. This last investigation was carried out in two different cases and it is to one of these that the protein belongs that I have used for the analysis described in this paper. I determined the nitrogen distribution by van Slyke's method and the tryptophan content by the colorimetric estimation of von Fürth and Lieben [1920]. The details of the analysis are described later. For the clinical symptoms of this case and the physical properties of the protein the original paper by Hopkins and Savory [1911] must be consulted in which the case is described as Case C.

Table I gives the results of the different analyses carried out on Bence-Jones' protein.

Table I.

The figures are % of total nitrogen.

	Magnus-Levy	Abderhalden	Hopkins		Present analysis
			Case A	Case C	
Amide-N	8.6	.	8.02	8.00	9.43
Melanin-N	.	.	.	.	.
Total-N of bases	24.7	.	.	.	23.11
Cystine-N	.	.	.	0.41	1.25
Arginine-N	.	+	11.9	12.0	9.27
Histidine-N	.	+	1.44	1.33	4.54
Lysine-N	.	+	.	4.34	8.04
Total-N of filtrate	64.2	.	.	.	66.84
Amino-N of filtrate	.	.	.	.	61.69
Non-amino-N of filtrate	.	.	.	.	5.15
Glycine-N	.	1.95	+	+	.
Alanine-N	.	4.37	+	+	.
Valine-N	.	.	.	4.22	.
Leucine-N	.	6.98	.	4.29	.
Aspartic acid-N	.	2.89	1.34	1.41	.
Glutamic acid-N	.	3.52	4.42	4.73	.
Phenylalanine-N	.	0.78	2.47	2.57	.
Tyrosine-N	.	0.81	1.97	2.02	.
Proline-N	.	1.40	1.93	2.00	.
Oxyproline-N	.	—	—	—	.
Tryptophan-N	.	.	0.69	.	2.44

In the papers of Abderhalden [1905] and Hopkins [1911] the amounts of amino-acids are given in percentage of the protein. Assuming the total nitrogen of the protein to be 16·21 %, a value found by Hopkins, it is possible to calculate the nitrogen of each amino-acid in percentage of the total nitrogen. The above table is expressed according to the latter.

It is a matter of course that only those estimations are directly comparable which have been performed by the same method. This holds good in the two cases examined by Hopkins [1911], which show almost identical values. Moreover Hausmann's estimation of the nitrogen distribution and that by van Slyke are based on the same principle, so that those cases may also be compared. They also show a close agreement. This indicates that in the two cases examined by Hopkins [1911] and in the case of Magnus-Levy [1900] the protein shows the same relative composition of mono- and diamino-acids. The assumption that these three cases have so far the same chemical constitution by mere chance is not probable. It seems more likely that in all cases of Bence-Jones' protein-uria there appears one and the same protein or mixture of proteins in the urine. Abderhalden [1905] found in his case somewhat different values from those of Hopkins in spite of a similar method. But esterification is a very complicated method and does not give quantitative results.

Table II shows the relations between Bence-Jones' protein and those of serum and tissue.

Table II.

	The figures are % of total nitrogen.				
	Bence-Jones' protein	Serum-globulin ¹	Serum-albumin ²	Normal tissues ³	Tumors ⁴
Amide-N	9·43	7·95	6·65	5·82	4·73
Melanin-N	0·90	2·30	0·95	3·09	2·42
Total-N of bases	23·11	25·25	34·75	30·71	30·54
Cystine-N	1·25	1·65	3·10	0·93	1·12
Arginine-N	9·27	8·00	9·90	11·13	10·09
Histidine-N	4·54	4·80	5·85	7·86	7·09
Lysine-N	8·04	10·80	15·90	9·83	12·04
Total-N of filtrate	66·84	64·80	58·15	60·82	62·14
Amino-N of filtrate	61·69	62·65	56·55	55·88	54·24
Non-amino-N of filtrate	5·15	2·15	1·60	4·94	7·90
N-recovered	100·28	100·50	100·30	100·4	99·8
Tryptophan-N	2·44	2·5-3·5 ⁵	—	—	—

¹ and ² carried out by Hartley [1914].

³ and ⁴ carried out by Drummond [1916].

⁵ carried out by von Fürth and Lieben [1920].

Hartley's [1914] analysis and that described in the present paper show the same nitrogen distribution for mono- and diamino-acids in Bence-Jones' protein and serum-globulin. The serum-albumin is characterised by a higher proportion of diamino-acids. Moreover, the tryptophan content is almost identical in serum-globulin and Bence-Jones' protein. On the other hand, the latter as also the serum-albumin yields less melanin. But this fraction depends much on the hydrolysis and is therefore the most uncertain. The only real difference between serum-globulin and Bence-Jones' protein has been found in the non-amino-nitrogen of the filtrate, *i.e.* the proline and oxyproline

fraction. It is much greater in Bence-Jones' protein than in serum-globulin and approaches the amount in tissue proteins. But these, on the other hand, contain the diamino-acids in a greater proportion than Bence-Jones' protein. Analyses of the tumor-proteins in the bone-marrow have not yet been carried out. Those registered in the above table were prepared from breast-cancers. They do not show any close resemblance in their amino-acid balance with Bence-Jones' protein. But it must be understood that Drummond coagulated the proteins in tissues and tumors by heat in acid solution, which also precipitates the nucleo-proteins. It is not known in which fraction the nitrogen of the purine and pyrimidine-ring appears and in what manner the relations are changed. This may make the comparison between his analyses and those in which no nucleo-proteins are present somewhat uncertain. 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.

EXPERIMENTAL DETAILS.

A. Preparation of Bence-Jones' protein: it was precipitated by heat not exceeding 60°, washed with water, alcohol and ether, and dried at 110° till of constant weight. [See Hopkins and Savory, 1911.]

B. Analysis by van Slyke: 3–4 g. of protein were hydrolysed with a volume of 20 % HCl 35 times its weight for 48 hours at 100°. For each of the two analyses the hydrolysis was carried out separately.

The following modifications mentioned by Crowther and Raistrick [1916] were applied:

(a) To the solution of *N*/10 sulphuric acid were added some crystals of KI and 1–2 cc. of saturated solution of KIO₃. The iodine produced was then titrated with sodium thiosulphate using soluble starch as indicator.

(b) The melanin-precipitate was filtered off under slight suction.

(c) The amino-nitrogen was determined by the micro-apparatus. Triplicates done on 2.5 cc. varied within 2 %.

(d) I found it easier to estimate the total nitrogen of the bases in a different portion from arginine as Plimmer [1916] has pointed out. I carried out the arginine estimation itself in the original way described by van Slyke. Bumping was almost entirely avoided by using a rose-burner.

After concentrating the hydrolysed protein the solution was made up to 100 cc. and I proceeded in the following manner:

1. Total-N in 10 cc.: duplicate.

2. Ammonia-N in 75 cc.

3. Melanin-N in 75 cc.

The solution of the bases was made up to 100 cc. In it were estimated:

1. Total-N in 10 cc.

2. Cystine-N in 20 cc.

3. Arginine-N in 50 cc.

4. Amino-N in 2.5 cc.: triplicate.

The filtrate of the bases was made up to 200 cc. and in this were estimated:

1. Total-N in 50 cc.: duplicate.

2. Amino-N: 10 cc. were diluted to 25 cc. and 2.5 cc. taken for estimation of amino-N: triplicate.

Table III gives the results in percentage of total nitrogen of the two analyses carried out, the values corrected for solubility of the bases and according to blank tests.

Table III.

	I	II	Average
Amide-N	9.36	9.50	9.43
Melanin-N	0.92	0.82	0.90
Total-N of bases	23.05	23.18	23.11
Cystine-N	1.26	1.24	1.25
Arginine-N	9.15	9.40	9.27
Histidine-N	4.82	4.27	4.54
Lysine-N	7.82	8.27	8.04
Total-N of filtrate	66.88	66.81	66.84
Amino-N of filtrate	61.98	61.40	61.69
Non-amino-N of filtrate	4.90	5.41	5.15
Total-N recovered	100.26	100.31	100.28

C. Colorimetric estimation of tryptophan by von Fürth and Lieben. For details the original paper must be consulted [1920].

The ordinary cuvettes of the Dubosque colorimeter can be used, putting a rubber ring between the bottom and the upper part and screwing the latter tightly down. Experiments show that the fluid does not come in contact with the metal and the colour remains unchanged.

The colour reaction is better produced in two measuring flasks of 25 cc. than in two test-tubes. Thus the volume of the standard solution and that of the unknown solution can be made equal with greater accuracy.

Each time to 2 cc. of a standard solution of 0.1 % pure tryptophan (prepared in the Biochemical Laboratory of Cambridge) and to 2 cc. of a solution of Bence-Jones' protein of about 5 % were added 1 drop of formaldehyde 2½ % and 20 cc. of saturated hydrochloric acid solution. Ten minutes afterwards sodium nitrite was added, the amount varying from 10–50 drops of a 0.05 solution in order to get the maximum depth of colour.

The following estimations were carried out:

1. 4.80 % solution of Bence-Jones' protein in 0.6 % NaOH. A 20 minutes' heating at 100° dissolved the protein. 20 drops of sodium nitrite produced the maximum colour: triplicate.

2. 6.29 % solution of Bence-Jones' protein in 15 % NaOH. Dissolved in 20 minutes at 100°. 50 drops of sodium nitrite produced the maximum colour.

3. 4.57 % solution of Bence-Jones' protein in 20 % NaOH. Dissolved in 15 minutes at 100°. 50 drops of sodium nitrite produced the maximum colour.

Table IV gives the calculated tryptophan content of Bence-Jones' protein in percentage:

Table IV.

1			2	3	Average
<i>a</i>	<i>b</i>	<i>c</i>			
2.74	2.88	2.95	3.14	2.75	2.89

The difference between the extreme values is 14 % of the highest. The difference in the amount of alkali used in dissolving the protein and therefore the difference in the acidity of the colour solution has no influence on the colour depth. This fact ought to be noticed since von Fürth and Lieben [1920] pointed out that the colour depth in pure tryptophan solutions varies with the acidity.

In the course of the experiments I found two difficulties:

1. The standard solution often changes the character of the colour in a short time and matching becomes uncertain even with a colour-screen. This is probably due to the beginning of clouding. On the other hand, the protein solution does not cloud but reaches its maximum colour more slowly than the pure tryptophan.

2. The colour in pure tryptophan is blue, that in the protein is more or less red, so that matching without a colour-screen is impossible. It is not difficult to find a screen making the two colours identical and a solution of CuSO_4 proved to be best. But the following must be considered. Fearon [1920] has shown that formaldehyde and glyoxylic acid give with tryptophan two coloured compounds, which he calls tryptophan-red and tryptophan-blue respectively. The colour obtained under ordinary conditions is a mixture of both and in pure tryptophan the blue prevails, in protein the red. Since both colours are due to tryptophan it must be considered what influence a colour-screen may have on the estimation. If the screen takes away the red rays, then one part of the tryptophan molecules is eliminated and it is uncertain if the calculation only based on the remaining blue would give right values for the whole amount of tryptophan. As Fearon remarks, a reaction producing two different colours is not suitable for a colorimetric method.

The blue colour of pure tryptophan turns more red by cooling, but at 10° the colour of pure tryptophan and that of protein are not yet matchable.

There is one aldehyde which gives nearly the same blue colour in pure tryptophan and in protein solutions, namely benzaldehyde. Salicylaldehyde gives also red and blue. As to colour, benzaldehyde would therefore be the most suitable reagent for a colorimetric method. The reaction is specific for tryptophan, no other known amino-acid producing it. Pure gelatin does not give any blue colour.

I therefore tried with benzaldehyde and used the following solutions:

1. Solution of pure tryptophan of 0.05 % in distilled water.
2. Solution of pure benzaldehyde of 1.9 % in pure hydrochloric acid of about 38 %.
3. Solution of sodium nitrite of 0.05 % in distilled water.
4. Pure hydrochloric acid of about 38 %. It is most essential to use only

very pure hydrochloric acid without or only with a very slight yellow colour. Otherwise the colour reaction does not reach its maximum. On the other hand, I found it unnecessary to saturate with hydrochloric acid gas if the acid is 38 % or more.

After a good many trials the following technique proved to be best: I mixed 2 cc. of the tryptophan solution, 1 cc. of the benzaldehyde solution and 10 cc. of hydrochloric acid in a measuring flask of 25 cc. After 5 minutes I added 5 drops of the sodium nitrite solution and let it stand together for 15-60 minutes. Then I filled up to the mark with hydrochloric acid.

The experiments revealed several advantages in using benzaldehyde instead of formaldehyde.

1. The colour solution does not cloud and even after several days no precipitate is produced. There is a slight diminution of the colour depth in 24 hours, but it does not change appreciably in the course of 1 or 2 hours.

2. The maximum colour reached with benzaldehyde is about double as deep as with formaldehyde, using in both cases the same concentration of tryptophan. This is, of course, welcome in the case of more or less insoluble proteins. The solution of 0.05 % tryptophan gives a satisfactory colour depth. The reaction goes on as quickly or even more quickly with benzaldehyde.

3. Both pure tryptophan and protein give blue colours. There is a slight difference in shade and sometimes they are not matchable without a colour-screen. On page 560 I discussed the error possibly arising from this source by using formaldehyde and this holds good for benzaldehyde too. But in the latter case the two solutions are nearly the same, whereas in the former they are very different. Therefore one would assume that the error with formaldehyde might be much greater than with benzaldehyde. The estimation is uncertain in both cases with coloured protein solutions or very impure proteins. Traces of iron, for instance, hinder the colour reaction.

In order to decide if the colour depth varies proportionally to the concentration, I estimated solutions of varying concentrations of tryptophan by comparing them with a standard solution and obtained the following results:

Table V.

Number	Calculated tryptophan %	Estimated tryptophan %	Error %
1	0.1092	—	—
2	0.0546	0.0510	-6.6
3	0.0546	0.0574	+5.0
4	0.0546	0.0510	-6.6
5	0.0273	0.0256	-6.0
6	0.0546	0.0549	+0.5
7	0.0546	0.0535	-1.7
8	0.0273	0.0273	±0.0
9	0.0546	0.0549	+0.5
10	0.0546	0.0560	+2.5
11	0.0273	0.0278	+2.0
12	0.0546	0.0546	±0.0
13	0.0546	0.0568	+4.0
14	0.0273	0.0282	+5.0
Average	—	—	-0.2

There is a maximum error of 6.6 %. Von Fürth had one of 20 %, therefore benzaldehyde seems to give more accurate results. I can not give any explanation of the error. The colour reaction is capricious and an unknown influence may sometimes come into play.

I tried the same with caseinogen and got a maximum error of 5 %.

In all cases I used the same amount of benzaldehyde and of sodium nitrite. Within the above limits it is not necessary to vary them proportionally to the amount of tryptophan, since the maximum colour is reached without such a complication of the method. In comparing protein and pure tryptophan the concentration of tryptophan in both solutions ought to be about the same.

In the case of water-insoluble proteins von Fürth dissolved them in more or less alkaline solutions. Since I did the same in my estimations I had to determine the influence of alkali on the colour reaction. Instead of a neutral solution of pure tryptophan different concentrations of NaOH were used as indicated in the following table:

Table VI.

Number	% NaOH	Calculated %	Estimated %	Error %
1	0	0.1092	0.1092	—
2	10	0.1092	0.0729	-50
3	5	0.1092	0.0926	-18
4	2.5	0.1092	0.1051	-4
5	1	0.1092	0.1081	-1
6	0.5	0.1092	0.1050	-4

Up to a concentration of about 2.5 % NaOH there is practically no influence on the maximum colour. Higher concentrations diminish the colour depth.

It is essential to know that things are different in protein solutions. The following table gives the tryptophan content of Witte-Pepton dissolved in different concentrations of NaOH:

Table VII.

Number	% NaOH	Tryptophan %	Error %
1	0.6	3.0	—
2	15	2.85	-5
3	20	3.0	±0
4	30	2.9	-3

After filtering off the precipitate of NaCl the colour depth is the same in all cases. Therefore insoluble proteins may be dissolved in strong alkali of 20–30 % without changing the colour reaction. It is not sure that this holds good for all proteins and it ought to be proved in every special case by using different concentrations of alkali.

The above data show that the colour reaction is not so easily disturbed in proteins as in pure tryptophan. Therefore in making comparisons one is apt to get too high values of tryptophan in proteins.

The following table gives the tryptophan content of several proteins estimated by formaldehyde and benzaldehyde:

Table VIII.

Protein	Tryptophan-content % estimated by		Difference %
	Benzaldehyde	Formaldehyde	
Caseinogen	1.1	1.39	27
Serum-protein	1.65	2.2	33
Ovalbumin	0.97	1.5	53
Witte-Pepton	3.0	4.8	60

In all cases formaldehyde gives values 30–60 % higher than benzaldehyde. Most probably the estimation by benzaldehyde is the more correct, since it avoids several disadvantages of the formaldehyde method. No other method for exact tryptophan estimation is known, so that a direct test is impossible. Therefore the method described in this paper seems to be the best at present available for tryptophan estimation in proteins.

SUMMARY.

1. The nitrogen distribution of Bence-Jones' protein is determined by van Slyke's method.
2. Bence-Jones' protein is a substance, not only characterised by its physical behaviour, but also by the distribution of nitrogen, which differs from that of all the proteins analysed up to the present time.
3. There is some evidence that the same protein appears in the urine in all cases of Bence-Jones' protein uria.
4. The tryptophan content of Bence-Jones' protein is estimated by von Fürth's colorimetric method. Objections to this method are discussed.
5. The writer proposes to use benzaldehyde instead of formaldehyde. There are several advantages in doing so and the method becomes more correct. The values of tryptophan estimated by formaldehyde are probably 30–60 % too high.

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REFERENCES.

- Abderhalden and Rostoski (1905). *Zeitsch. physiol. Chem.* **46**, 125.
 Crowther and Raistrick (1916). *Biochem. J.* **10**, 434.
 Drummond (1916). *Biochem. J.* **10**, 473.
 Fearon (1920). *Biochem. J.* **14**, 548.
 von Fürth and Lieben (1920). *Biochem. Zeitsch.* **109**, 124, 153.
 Hartley (1914). *Biochem. J.* **8**, 541.
 Hausmann (1899). *Zeitsch. physiol. Chem.* **29**, 136.
 Hopkins and Savory (1911). *J. Physiol.* **42**, 189.
 Magnus-Levy (1900). *Zeitsch. physiol. Chem.* **30**, 200.
 Plimmer (1916). *Biochem. J.* **10**, 115.