XLIH. THE ANALYSIS OF PROTEINS.

IV. SOME ANALYSES OF GELATIN.

BY ROBERT HENRY ADERS PLIMMER AND T. SHIMAMURA.

From the Chemical Department, St Thomas's Hospital Medical School, London.

(Received January 16th, 1924.)

IN connection with the experiments of Plimmer and Phillips [1924] upon the estimation of histidine, after its addition to gelatin, by the method of bromination, some analyses of gelatin by Van Slyke's method [1911], carried out by us during the summer of 1919 at University College, are not without interest. The purpose of these analyses was to test the accuracy of the Van Slyke method with the addition of extra quantities of histidine to gelatin. At the same time the histidine content was estimated by the colorimetric method of Weiss and Ssobolew [1913]. In this way the two methods could be compared. By the latter method the presence of tyrosine is liable to lead to errors as it gives a similar colour with diazotised sulphanilic acid. Tyrosine was therefore also introduced to ascertain if it was completely removed from the phosphotungstate precipitate by washing. The colorimetric method was not used by Weiss and Ssobolew for estimating histidine in proteins, but has since been adapted for this purpose by Koessler and Hanke [1919, 1920] whose results did not appear till after our experiments had been completed. The desired accuracy has not been given by either method in our experience; further work in this connection to locate the source of error is in progress.

A valuable adjunct to the Van Slyke nitrogen distribution figures of proteins would be the determination of tyrosine in the mono-amino fraction. Its estimation is possible by bromination or by the colorimetric method of Folin and Denis [1912]. The bromination method was considered in Part III. The colorimetric method gives results which are higher than the gravimetric and are not considered to be accurate. The method has generally been applied to the hydrolysed protein directly and not to the fraction containing the mono-amino acids. If applied in this way very accurate results were obtained for amounts of tyrosine added to gelatin.

EXPERIMENTAL.

Seven analyses in duplicate of gelatin were made by Van Slyke's method; the first three were carried out on gelatin alone, the other four with the addition of histidine and tyrosine. In the first five experiments gold label gelatin was used, in the last two an English variety.

In each case about 8 g. of gelatin were hydrolysed by boiling with 20 parts of 20 % hydrochloric acid for 10 to 20 hours; after the hydrolysis, extra histidine was added in the form of the monohydrochloride, and tyrosine as such in those analyses in which they were introduced. The hydrolysed solution was evaporated *in vacuo* to a syrup, dissolved in water and made up to 250 cc. with water. Portions of 10 cc. were taken for determination of total nitrogen and two portions of 100 cc. each for the nitrogen distribution estimations. Except for the filtration of the hexone base phosphotungstate precipitate, the process was that given by Van Slyke. In Exps. 1 to 3 the filtration was carried out on an 8 cm. Buchner with a hardened filter paper, in Exps. 4 and 5 on one of 5 cm.; moderate suction was used. The quantity of wash liquid in Exps. 1 and 2 was not noted, but in the others it was 200 cc.; the time occupied in the filtration was from two to three hours; in Exps. 4 and 5 from four to five hours.

Arginine was estimated by the modification of Plimmer [1916].

The results of the experiments are given in Table I, corrections having been made for solubilities of the phosphotungstates, and the percentages of the different forms of nitrogen are given in Table II.

Table I. *Nitrogen Distribution Results.*

	I		II		III		IV				V			
	(a) (b)		(a) (b)		(a) (b)		(a) (b)		(c)	(d)	(a) (b)		(c)	(d)
	mg.		mg.		mg.		mg.		mg.		mg.		mg.	
Total N of gelatin	624.4		568.4		553.0		635.6		635.6		609.0		609.0	
N of added histidine	—		—		—		4.1		4.1	4.1	20.5		10.3	
N of added tyrosine	—		—		—		3.8		3.8	7.6	15.2		7.6	
Total N of experiment	624.4		568.4		553.0		643.5		643.5	647.3	644.7		626.9	
Amide N	10.6	10.5	10.1	9.2	8.3	8.8	5.9	10.1	9.9	9.4	17.9	17.6	16.8	16.1
Humin N	3.5	3.1	5.6	6.6	3.2	2.5	2.1	3.5	2.0	2.8	3.4	3.1	2.8	3.5
Diamino:														
Total N	145.6	134.4	134.4	121.8	127.4	124.6	170.8	159.6	190.4	179.4	159.8	173.6	147.0	156.8
Amino N	61.4	56.5	57.3	54.0	56.9	56.9	80.1	73.5	89.8	73.5	67.3	81.2	65.6	71.4
Arginine N	94.7	88.1	86.2	85.5	86.2	84.9	96.1	94.7	97.6	95.2	91.4	88.9	94.6	88.8
Histidine N	29.9	26.5	26.6	12.5	16.7	14.8	38.3	32.9	51.9	60.7	40.1	46.6	24.6	37.0
Lysine N	31.7	28.8	29.8	32.0	33.7	33.1	47.6	43.0	52.6	33.2	36.7	46.4	38.1	40.2
Mono-amino:														
Amino N	352.2	354.6	335.3	324.2	310.9	306.6	349.6	348.4	335.7	355.9	343.7	327.6	337.0	333.2
Non-amino N	91.6	131.4	104.6	115.2	99.5	102.3	118.5	125.4	123.8	124.4	99.1	109.4	108.4	101.9

As may be seen from the table of percentages, the data for amide nitrogen, except IV (a), were within the usual experimental errors; IV (a) was too low. The higher data for the experiments V were evidently connected with the variety of gelatin employed; in this case an English sample was used and it contained more amide nitrogen. The figures for humin nitrogen, except in experiment II, were fairly constant.

Table II. *Nitrogen Distribution Results in Percentages of Total Nitrogen.*

	I		II		III		IV				V			
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
Added histidine N	—	—	—	—	—	—	0.64	—	0.64	0.63	3.18	3.18	1.64	1.64
Added tyrosine N	—	—	—	—	—	—	0.59	—	0.59	1.17	2.36	2.36	1.21	1.21
Amide N	1.70	1.68	1.78	1.62	1.50	1.59	0.92	1.59	1.51	1.45	2.78	2.73	2.68	2.57
Humin N	0.56	0.50	0.99	1.16	0.58	0.45	0.33	0.54	0.27	0.43	0.53	0.48	0.45	0.56
Arginine N	15.17	14.11	15.17	15.05	15.59	15.36	14.94	14.72	15.17	14.71	14.18	13.79	15.09	14.17
Histidine N	4.79	4.24	4.79	2.20	3.02	2.62	5.95	5.11	8.00	9.38	6.24	7.23	3.92	5.90
Lysine N	5.08	4.61	5.24	5.63	6.09	5.99	7.40	6.82	8.18	5.13	5.69	7.20	6.08	6.41
Mono-amino N	56.39	56.82	58.99	57.05	56.22	55.46	54.34	54.16	52.17	54.98	53.31	50.81	53.75	53.15
Non-amino N	14.67	21.04	18.40	20.27	18.01	18.58	18.42	19.49	19.24	19.22	15.37	16.97	17.30	16.25
Total	98.36	103.00	105.36	102.98	101.01	100.05	102.30	102.43	104.54	105.30	98.10	99.21	99.27	99.01

The values for arginine nitrogen were on the whole very constant; with the gelatin alone one figure was lower than the remainder. The figures for histidine nitrogen were very irregular and did not show a proper increase in those experiments in which there was an addition. Considering that the lysine values are found by difference and that the histidine values are so erratic, it is remarkable that these data were so close to one another.

The mono-amino figures also showed great variations and no indication was shown of the addition of tyrosine by an extra amount of amino nitrogen.

On the whole, the data by the Van Slyke method cannot be looked upon as accurate, though they may give a good approximation. The irregularity may be connected with gelatin and may not be so great with other proteins, although great differences have also been found in their analyses. There is an indication that the error is in connection with the phosphotungstate precipitate; further work is confirming this preliminary observation.

The Colorimetric Estimation of Histidine.

Histidine was estimated in the above solutions of hydrolysed gelatin (except I (a), II (a), III (a) and (b)) according to the method of Weiss and Ssobolew [1913]. Special attention was given to those solutions to which histidine and tyrosine had been added, the object being to test the accuracy of the method. Tyrosine was added, as it gives a similar colour to histidine and it was of importance to ascertain if it was completely removed by washing from the precipitate of the phosphotungstates.

It was first necessary to determine the quantity of reagent which gave an optimal red colour with the standard histidine solution¹. Using the reagents A and B of the same strength as described by Weiss and Ssobolew and a solution of histidine monohydrochloride ($C_6H_9O_2N_3 \cdot HCl \cdot H_2O$) containing 1 mg. in 10 cc. (= 0.74 mg. histidine or 0.2005 mg. histidine nitrogen), it was found that the colour intensity was optimal with 0.3 cc. reagent (0.1A + 0.2B) and 1 cc. of sodium carbonate in a total volume of 10 cc. and with 5 cc. of

¹ The method is described in Plimmer's *Chemical Constitution of the Proteins*, Part I, 3rd edition.

the histidine solution. These proportions were then used in the preparation of the standards for the colour comparisons.

Preliminary experiments were made with the various hydrolysed gelatin solutions in test tubes to ascertain the best dilution and quantity of reagent required to produce a colour of similar intensity to the above standard. A suitable quantity (1 or 2 cc.) was then diluted, mixed with the quantity of reagent and compared with the standard in a Duboscq colorimeter.

As gelatin contains a small amount of histidine, a colour comparison was made with the hydrolysed solution before the addition of the histidine hydrochloride in order to obtain the histidine content by this method. It was necessary for this purpose to assume that the tyrosine content of gelatin was negligible. The total histidine content of the solution was then determined in the diamino fraction as prepared by the Van Slyke method.

The data and calculations of the histidine content are shown in Table III. The values are calculated to a volume of 100 cc. hydrolysed gelatin according to the scheme

$$\frac{10}{11.9} \times 10 \times 0.2005.$$

In the case of the diamino solutions which were made up to a volume of 50 cc., from 100 cc. of original solution, the resultant figure is divided by two. A correction of 3.8 mg. has then been added for the solubility of histidine phosphotungstate.

Table III. *Colorimetric Estimations of Histidine.*

Experiment	Dilution	Colorimeter	Histidine N corr. for solubility mg.	Additional histidine N found mg.	Histidine N actually added mg.
I b. Diamino	1 : 10	10 : 11.9	12.1	...	...
II. Original	1 : 4	10 : 8.7	9.2	...	...
IV. Original	1 : 8	5 : 4.8	16.7	...	...
IV a. Diamino	1 : 10	5 : 4.5	14.9	- 1.8	4.1
IV b. "	1 : 10	5 : 4.4	15.2	- 1.5	4.1
IV c. "	1 : 20	5 : 5.2	23.1	+ 6.4	4.1
IV d. "	1 : 20	5 : 5.0	23.8	+ 7.1	4.1
V. Original	1 : 8	10 : 12	10.0	...	...
V a. Diamino	1 : 40	10 : 11	40.2	+ 30.2	20.5
V b. "	1.4 : 50	10 : 9	43.6	+ 33.6	20.5
V c. "	1 : 20	10 : 12.5	19.8	+ 9.8	10.3
V d. "	1 : 20	10 : 12.1	20.0	+ 10.0	10.3

The results were thus very variable. Only in the last experiment were data obtained which corresponded with the amount of histidine nitrogen added. The variations may be due to errors inherent in the complicated procedure of the colorimetric method, especially with the choice of the most suitable dilution to give the optimal red colour, or to some error with the preparation of the diamino fraction. Since this work was completed the adaptation of the colorimetric method to the estimation of histidine in proteins has been published by Hanke and Koessler [1920], who made special provision for the solubility of histidine phosphotungstate. In view of the erratic results

by the Van Slyke method, the main error would appear to be connected with the treatment of the phosphotungstate precipitate; the colorimetric procedure of Hanke and Koessler seems to be accurate.

The Colorimetric Estimation of Tyrosine.

Folin and Denis' method [1912] of estimating tyrosine has been tested by adding definite amounts of tyrosine to solutions of hydrolysed gelatin prepared in the same way as in the Van Slyke method. The several fractions were separated and tyrosine was estimated in each solution by the colorimetric method; from 1 to 3 cc. of the solutions were required to give a colour comparable with the standard. The figures in Table IV show that a loss occurs between the original solution and the solution obtained after removal of the humin nitrogen; it was not possible to recover this loss by prolonged washing of the humin. No loss was found in the phosphotungstate precipitate if this was sufficiently washed.

Table IV. *Colorimetric Estimations of Tyrosine.*

Experiment	Tyrosine found mg.	Tyrosine added mg.	Experiment	Tyrosine found mg.	Tyrosine added mg.
IV. Original solution	29.0	—	V a. Lime filtrate	187.0	200
IV a. Lime filtrate	57.0	50	V b. "	187.0	200
IV b. "	58.0	50	V c. "	105.3	100
IV c. "	57.0	50	V d. "	102.6	100
IV d. "	108.0	100	V a. Mono-fraction	182.8	200
IV a. Mono-fraction	59.5	50	V b. "	186.1	200
IV b. "	61.6	50	V c. "	114.8	100
IV c. "	52.7	50	V d. "	106.1	100
IV d. "	104.8	100	VI. Original solution	38.5	—
V. Original solution	28.6	—	VI a. Lime filtrate	75.2	50
			VI b. "	77.5	50

The loss that occurs between the original solution and the lime filtrate may in part be due to the removal of some substance giving the colour reaction and causing the high results generally obtained by the method. In all experiments except those with 200 mg. of tyrosine, the amount of tyrosine found has been slightly greater than the amount added. The estimation of tyrosine after removal of humin thus appears to give accurate results, if the solution does not contain more than 100 mg.

In order to avoid loss in the stage of removal of humin by lime, some further estimations were made omitting this step, but clearing the solution by means of phosphotungstic acid according to the Van Slyke procedure. In some experiments the solution and precipitate were made up to a definite volume and the estimation made in the mono-amino filtrate; in other experiments the phosphotungstic acid precipitate was filtered off and washed and filtrate and washings made up to a known volume. The results are here shown as percentages of tyrosine in gelatin, including the blank experiment figure in the amount of added tyrosine.

	Gelatin + tyrosine			Gelatin + tyrosine		
	Direct %	Filtrate %	Calculated %	Direct %	Filtrate %	Calculated %
I <i>a</i>	1.5	1.1	—	1.2	0.9	—
I <i>b</i>	1.5	1.1	—	—	0.9	—
II <i>a</i>	2.4	2.3	2.7	2.3	2.0	2.3
II <i>b</i>	2.4	2.3	2.7	—	2.0	2.3
III <i>a</i>	3.1	3.1	3.9	3.2	3.0	3.5
III <i>b</i>	3.4	3.4	3.9	—	3.0	3.5
IV <i>a</i>	5.1	4.7	5.0	—	4.0	4.7
IV <i>b</i>	5.2	4.7	5.0	—	4.0	4.7
V <i>a</i>	6.2	5.8	6.2	—	5.5	5.8
V <i>b</i>	6.2	5.8	6.2	—	5.2	5.8
VI <i>a</i>	7.5	7.4	7.4	—	6.2	6.7
VI <i>b</i>	7.6	—	—	—	6.2	6.7

By avoiding the lime precipitation the figures for tyrosine are thus very close to the calculated quantities; some interfering substance is removed by the phosphotungstic acid.

The same process was applied to the estimation of tyrosine in wool, caseinogen and egg albumin with the following results:

Caseinogen		Wool		Egg albumin	
Direct %	Phosphotungstic filtrate %	Direct %	Phosphotungstic filtrate %	Direct %	Phosphotungstic filtrate %
Using 1 cc. of solution:					
7.3	6.2	6.9	6.1	6.3	5.7
7.3	6.2	7.0	6.1	6.4	5.7
7.4	6.4	6.9	6.2	6.4	5.6
7.4	6.2	6.8	6.0	—	—
Using 2 cc. of solution:					
6.7	5.4	6.4	5.6	5.8	5.1
6.8	5.3	6.4	5.6	5.7	5.0
6.8	5.7	6.4	5.6	5.7	5.0
6.8	6.0	—	—	—	—
Using 2 cc. and filtering and washing phosphotungstates:					
6.5	6.2	6.4	6.0	6.0	5.5
6.5	6.2	6.4	6.0	6.0	5.3

The estimation of tyrosine can thus be carried out in the mono-amino fraction of the products of hydrolysis of proteins; the results are lower than those obtained directly. It is not possible to say whether they are more accurate; if there is an interfering substance it is removed by phosphotungstic acid.

SUMMARY.

1. Duplicate analyses of gelatin by Van Slyke's method have given very erratic results.
2. Known quantities of histidine added to hydrolysed gelatin have not been accurately estimated.
3. The colorimetric method of estimating histidine has not given reliable results; the error seems to be in connection with the phosphotungstic precipitate.

4. Tyrosine can be estimated by the colorimetric method in solutions of hydrolysed proteins either after removing humin, or in the mono-amino fraction. The results are lower than by the direct estimation.

Grateful acknowledgement is made to the Government Grant Committee of the Royal Society for a grant to one of us for defraying the expenses of this research.

REFERENCES.

- Folin and Denis (1912). *J. Biol. Chem.* **12**, 245.
Hanke and Koessler (1920). *J. Biol. Chem.* **43**, 521.
Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 497.
Plimmer (1916). *Biochem. J.* **10**, 115.
Plimmer and Phillips (1924). *Biochem. J.* **18**, 312.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.
Weiss and Ssobolew (1913). *Biochem. Z.* **58**, 119.