CLXXV. THE DETERMINATION OF TRYPTOPHAN AND TYROSINE.

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THE colorimetric estimation of tryptophan and tyrosine, published by Folin and Ciocalteu [1927], has been used lately by several workers. Marston [1928] determined the amounts of these amino-acids in keratin from sheep's wool and considered the determinations to be satisfactory. Hanke [1928] found that the method could not be used for the estimation of tyrosine in the presence of cystine. He noted further, that a crude protein hydrolysate, either acid or alkaline, appeared to contain something other than tyrosine and tryptophan which interferes with the determination of these amino-acids. McFarlane and Fulmer [1930], using the method for the estimation of these amino-acids in some food proteins, experienced similar difficulties. More recently, Carpenter [1931] has used the method in determining the molecular weight of caseinogen. He does not appear to have recognised any difficulty except that he observes that only 81.3 % of added tryptophan was recovered. He estimated tryptophan, not only colorimetrically, but also by bromination.

This latter modification has been subjected to an investigation in this laboratory for the past two years which seems to demonstrate some important difficulties which are likely to be encountered in carrying out the procedure. The work was undertaken as part of an investigation into certain discrepancies in the methods of protein analysis, particularly with reference to the Van Slyke method. It was found that the decomposition products of tryptophan are likely to become involved, so that it was desirable to use a rapid and simple method of estimating tryptophan. As will be shown, this object has not altogether been achieved, but when using this procedure it is necessary to take into account the presence of histidine. The method appears to offer as good an estimation of histidine as it does of tryptophan. The main part of the investigation will form the subject of a further communication, but in view of a somewhat general adoption of the method of estimating tryptophan and tyrosine, it is thought that publication of these experiments, at this time, may be of interest.

EXPERIMENTAL.

Plimmer and his associates [1913, 1924] have shown that bromine may be absorbed by tryptophan, tyrosine, histidine, and cystine. Homer [1915] obtained slightly higher results for tryptophan.

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Two cc. of a 1 % solution of each amino-acid (except tyrosine) were used for each determination, and were brominated according to the procedure of Plimmer and Phillips [1924], using 10 cc. of 0.2 *N* sodium bromate, 10 cc. of a 20 % solution of sodium bromide, and 5 cc. conc. HCl. After standing for 15 minutes, 10 cc. of a 4 % solution of sodium iodide were added and the liberated iodine was titrated with standard sodium thiosulphate. The thio-sulphate solutions were carefully standardised by potassium dichromate, and frequent blank estimations were carried out with the above reagents.

Tryptophan.

cc. thiosulphate used	g. bromine absorbed
7.7	0.05760
7.9	0.05504
7.7	0.05760
7.6	0.05888

Blank experiments required 12.2 cc. thiosulphate.

1 cc. thiosulphate = 0.0128 g. bromine.

This corresponds closely to an absorption of 7 atoms of bromine by 1 molecule of tryptophan.

Tyrosine. 5 cc. of a 1 % solution were used for each determination.

cc. thiosulphate used	g. bromine absorbed
7.1	0.06784
7.2	0.06656
6.7	0.07296
7.1	0.06784

Blank experiments required 12.4 cc. thiosulphate.

1 cc. thiosulphate = 0.0128 g. bromine.

This corresponds to an absorption of 3 atoms of bromine by the molecule of tyrosine—a value, higher than that given by Plimmer and Phillips.

Histidine. 2 cc. of a 1 % solution were used for each determination.

cc. thiosulphate used	g. bromine absorbed
17.9	0.020185
17.9	0.020185
*17.3	0.025029

* This experiment was allowed to stand for 30 minutes.

Blank experiments required 20.4 cc. thiosulphate.

1 cc. thiosulphate = 0.008074 g. bromine.

This corresponds to an absorption of 2 atoms of bromine by 1 molecule of histidine.

Cystine. 1 cc. of a 1 % solution was used for each determination.

cc. thiosulphate used	g. bromine absorbed
9.85	0.03136
9.80	0.03072
9.85	0.03136

Blank experiments required 12.2 cc. thiosulphate.

1 cc. thiosulphate = 0.0128 g. bromine.

This corresponds to an absorption of 10 atoms of bromine by the molecule of cystine.

Hydrolysis and precipitation of the proteins.

Hydrolysis has been carried out for 20–24 hours with 20 % NaOH in such quantity that 4 g. NaOH were present for each g. of protein. Two cc. of butyl alcohol were added. At the end of the period the condenser was removed and the butyl alcohol driven off. Sufficient 14*N* H₂SO₄ to neutralise the NaOH was added in accordance with the directions of Folin and Ciocalteu [1927], and after shaking, the flask was allowed to cool. The solution was then made approximately *N* with respect to H₂SO₄. Filtering was carried out using a fairly large funnel (11 cm. diameter). The precipitate and paper were thoroughly washed in a beaker with 7*N* H₂SO₄. The washings were added to the solution and the final volume was made up to 100 cc. It was found that a Jena flask gave considerably less residue than a Pyrex flask, and accordingly less nitrogen was lost by adsorption.

Before precipitating, aliquot portions of this solution were titrated, and the bulk was made *N* with respect to H₂SO₄. Quantities of 8 cc. were placed in centrifuge tubes, precipitated with 15 % HgSO₄, washed, and decomposed in accordance with the procedure of Folin and Ciocalteu. The precipitate and the filtrate portions were made up to suitable volume and the determinations carried out on aliquot portions. Each experiment was carried out at least in duplicate.

Experiment with pure amino-acids.

A mixture containing 15.198 mg. histidine, 120.2 mg. tyrosine, 113.4 mg. tryptophan, and 167.0 mg. cystine was hydrolysed with NaOH in accordance with the above procedure. The precipitate portion was examined for total nitrogen, amino-nitrogen, using Van Slyke's apparatus, and for bromine absorption.

	mg.
Total nitrogen found	= 19.631
Calculated for 113.4 mg. tryptophan	= 15.588
Difference +	4.043
Amino-nitrogen found	= 9.139
Calculated for 113.4 mg. tryptophan	= 7.794
Difference +	1.345

Since the extra amino-nitrogen is approximately one-third of the extra total nitrogen, and is therefore in the same proportion as in histidine, it appears probable that histidine has been precipitated with the tryptophan under the conditions of experiment. It may be recalled that precipitation with HgSO₄ in 5 % H₂SO₄ is a regular procedure for the isolation of histidine [see also,

Onslow, 1921]. If histidine and tryptophan alone are present, the amount of histidine-nitrogen may be calculated from the formula

$$\text{Histidine-N} = 3 \times \text{total N} - 6 \times \text{amino-N.}$$

Applying this to the above figures

	mg.
Histidine-N	= 4.059
Calculated for 15.198 mg. histidine	= 4.088
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	- 0.029

which would represent a loss of 0.7 %.

	mg.
The amount of bromine absorbed by this solution	= 254.08
Calculated for histidine + tryptophan	= 282.12
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	- 28.04

This represents a loss of about 9.9 % on the combined values for histidine and tryptophan, and 11.6 % if the loss is merely attributed to the tryptophan. The experiment has shown that in the presence of histidine, the tryptophan may not be estimated accurately, but on account of the nitrogen figures it does not appear that either tyrosine or cystine has contributed to the error.

When the filtrate from the mercuric sulphate precipitate was examined, loss was found to have occurred.

	mg.
The amount of bromine absorbed	= 353.33
Calculated for tyrosine + cystine	= 714.72
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	- 361.39
 Total nitrogen found	 = 29.731
Calculated	= 31.355
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	- 1.624

only 49.4 % of the calculated bromine absorption and 94.8 % of the total nitrogen have been secured. If we assume that the loss is entirely due to cystine, and calculate accordingly, we find that the bromination represents a loss of 65 % cystine, although 93 % of the cystine-nitrogen is still present. Consequently, therefore, there is the probability that some cystine has escaped destruction, and may, as pointed out by Hanke [1928], interfere with the estimation of tyrosine. This interference would be experienced with bromination methods just as much as with colorimetric methods as cystine absorbs rather a large quantity of bromine (10 atoms). It will be shown that the extent of the destruction of cystine by alkali is variable.

On acidifying the alkaline hydrolysate, a strong smell of H_2S may be obtained, if the amount of cystine originally present is large. Cystine has therefore been hydrolysed by the above procedure, and directly after acidifying the hydrolysate, attempts have been made to estimate the H_2S by sucking it

through a solution of cadmium chloride and weighing the CdS formed. In one experiment where 0.2132 g. cystine was used, an amount of CdS equivalent to 0.0629 g. cystine was weighed, which represents 29.5 %. In another case using 0.2524 g. cystine, an equivalent of 0.02749 g. was recovered—a loss of 10.8 %. On another occasion the hydrolysis was continued for 100 hours and no CdS was obtained.

Continuing the usual procedure of precipitating with HgSO_4 , bromination could be carried out with the filtrate, but no bromination was found to take place with the precipitate portion. In the above three experiments with cystine 2.8 %, 10.5 % and 5.2 % cystine have been accounted for by bromination. The results with cystine are thus very variable.

Experiments with proteins.

In view of the above results with pure amino-acids, it was of interest to carry out the procedure with a protein from which tryptophan was absent. Gelatin was selected and 1.0492 g. was hydrolysed as above, and the procedure of precipitating with HgSO_4 carried out.

Total N of the precipitated portion = 1.3737 mg., which if calculated as histidine = 0.5 %. Amino-N = 0.4691 mg., which equals 0.49 % histidine in gelatin, and the bromine absorption of 1 cc. = 0.0612 mg., which represents 0.46 % histidine present. Rosedale (unpublished data) has carried out a series of determinations by Van Slyke's method on this sample of gelatin and finds the value for histidine to be 0.55 %.

In another experiment tryptophan (0.0956 g.) was added to gelatin (0.8632 g.). The procedure was identical with the experiment above, and the results from the precipitate portion were:

	mg.
Total N	= 14.237
Amino-N	= 6.934

From the previous experiment with gelatin alone, 4.92 mg. histidine were calculated, = 1.114 mg. histidine-N. Applying the formula used in the first experiment, we get 1.107 mg. histidine-N giving a loss of 0.007 mg., which represents 0.7 %.

Bromination showed an absorption of 2.823 mg. bromine. Allowing for 5.072 mg. the amount calculated for 4.92 mg. histidine, this represents 78.588 mg. tryptophan present, which is 82.2 % of the amount originally added, and is in agreement with the experiments of Carpenter [1931].

A quantity of 0.9886 g. caseinogen was treated similarly, and the following results were obtained from the precipitate portion:

	mg.
Total N	= 9.7342
Amino-N	= 3.4626
Bromine absorbed	= 56.600

If the histidine is calculated by the formula from the nitrogen figures, we find 31.3 mg., which represents 3.1 % histidine in caseinogen. In the remainder, 1.3072 mg. tryptophan-nitrogen are present, representing 0.9 % of tryptophan in caseinogen, a figure which is low even when compared with results obtained by isolation. According to the bromination, if we allow for the calculated amount of histidine, we find only 0.86 % tryptophan. If on the other hand the presence of histidine were ignored, the bromination would give 2 % tryptophan.

It seems doubtful, however, whether tryptophan may be estimated from the nitrogen figures without taking the histidine into account, and even then it appears that low results are likely to be obtained.

Folin and Ciocalteu did not attempt to estimate tryptophan by bromine absorption and the question arises as to whether the interference of histidine might not be avoided by the use of the colorimetric determination. No series of such determinations has been made in the present work, partly because it seems evident that some of the tryptophan has been destroyed in the process of hydrolysis, and partly on account of the fact that it was found that histidine dihydrochloride imparted to the phenol reagent a greenish tint which it was considered would cause too high a reading to be made for tryptophan. The addition of sodium cyanide did not prevent this, so that any further attempt to use this method was abandoned.

CONCLUSIONS.

If tryptophan is to be estimated after alkaline hydrolysis and precipitation with HgSO_4 , histidine, which is precipitated at the same time, must be taken into consideration. It appears that this may best be done by carrying out determinations of total nitrogen and amino-nitrogen at the same time that the colorimetric or bromination estimations are made. If the histidine is thus allowed for, it appears that the amount of tryptophan present in the solution at the time may be determined. From the results with caseinogen, it seems evident that tryptophan may be lost owing to decomposition during hydrolysis. Onslow [1921] has used baryta as hydrolytic agent, but the difficulty in applying this to such small quantities of material is that, on acidifying before precipitation with HgSO_4 , a relatively large precipitate of BaSO_4 would be formed and would be difficult to wash. Onslow points out that after hydrolysis with baryta peptides are found in the mercury precipitate. This seems to have been avoided in this instance owing to the use of strong alkali. Accurate estimations of tryptophan in a protein appear to require application of Onslow's method upon larger quantities of material.

SUMMARY.

1. An attempt has been made to estimate tryptophan and tyrosine by bromination after alkaline hydrolysis and separation by HgSO_4 .
2. Any cystine which may escape decomposition by hydrolysis interferes with the estimation of tyrosine.

3. Histidine appears to be precipitated with tryptophan and must be taken into account. A method of doing this by means of nitrogen determinations is suggested.

In conclusion, I desire to thank Prof. Rosedale, for his interest in the work, and for much help during its progress.

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