XXXIV. ANALYSIS OF PROTEINS. VIII.
ESTIMATION OF CYSTINE IN THE MODIFIED VAN SLYKE METHOD.

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In the shortened form of the Van Slyke method of analysis of proteins adopted by Plimmer and Rosedale [1925], in which the determination of the various amino-acids was carried out without removal of phosphotungstic acid from the solutions, the estimation of cystine was not attempted. The direct determination of cystine has now been investigated with satisfactory results.

The estimation of cystine in proteins by this method depends also upon its behaviour in the various stages of the process. Van Slyke [1911] found that only 50 % of the cystine, after hydrolysis with 20 % hydrochloric acid for 24 hours, was precipitated by phosphotungstic acid. It was shown by Hoffman and Gortner [1922] that cystine on boiling with hydrochloric acid was slowly changed into an "isomeric" form which was more soluble, was optically inactive, did not crystallise in the characteristic form of ordinary cystine, and formed a much more soluble phosphotungstate. These observations have been confirmed and extended. The sulphur content of the diamino-fraction really represents from 40 to 50 % of the cystine in the protein. Either a correction could be applied assuming that half the cystine was precipitated by phosphotungstic acid, or a determination of the sulphur content of the monoamino-fraction could be made for the amount of the non-precipitated "isomeric" cystine. This latter determination involves the assumption that no other sulphur-containing compound is present. In view of the isolation of another sulphur-containing amino-acid by Mueller [1923] the sulphur content of the monoamino-fraction cannot be regarded as being due only to "isomeric" cystine. It has been possible for us to estimate the sulphur content of the solutions of the several egg proteins analysed by Plimmer and Rosedale [1925]. The amounts of sulphur in the monoamino-fractions seem to bear no relation to the amounts in the diamino-fractions, but tend to indicate the presence of another sulphur compound which is not precipitated by phosphotungstic acid.

Though the sulphur estimation of the diamino-fraction does not represent the actual amount of cystine in a protein, its estimation is necessary in order to arrive at the amount of lysine. The presence of cystine in this fraction gives

erroneous values for arginine, as it gives off, according to Van Slyke [1911], 18 % of its nitrogen on boiling with 40 % caustic potash. The decomposition has been found to be rather less on boiling with 20 % caustic soda, under the conditions laid down for arginine by Plimmer [1916]. It is greater in the presence of phosphotungstic acid.

Plimmer and Rosedale [1925] found that the monoamino-fraction gave off ammonia on boiling with caustic soda and attributed its origin to arginine which had not been precipitated by phosphotungstic acid. The amount of sulphur in these fractions is too small to account for the quantity of ammonia. On standing for over 15 months these solutions have deposited small quantities of crystals. Their examination has shown them to consist of a mixture of diamino-acids and the solution has still given off ammonia on boiling with caustic soda. The monoamino-acids, glycine, alanine, tyrosine and proline have been boiled with caustic soda and do not evolve any ammonia. It has not yet been possible to test a hydroxyamino-acid, so that the origin of the ammonia still appears to be from non-precipitated arginine. It is most likely that part of the arginine of the protein is racemised on hydrolysis and that racemic arginine forms a more soluble phosphotungstate than the active arginine.

Experimental.

The cystine for these experiments was prepared from feathers. The feathers were hydrolysed by boiling with twice their weight of conc. hydrochloric acid for 6 hours. The acid solution was evaporated *in vacuo* to remove hydrochloric acid as far as possible. The residue was dissolved in water, filtered from black material, boiled with charcoal, again filtered, nearly neutralised with sodium hydroxide (Hoffman and Gortner) and treated with sodium acetate until neutral to Congo red (Folin). The brown product was purified by boiling in 5 % (vol.) hydrochloric acid with charcoal, filtering and precipitating with sodium acetate. The colourless product was then treated with alcohol saturated with hydrogen chloride to remove tyrosine [Plimmer, 1913] and crystallised several times from dilute hydrochloric acid by addition of sodium acetate. After drying in a desiccator, the cystine gave the following figures on analysis:

Found (mean of 5 analyses): N = 11·5 %, S = 26·3 %.
Calculated for $C_6H_{12}O_4N_2S_2$: N = 11·67 %, S = 26·67 %.

Both analytical figures thus indicated a purity of 98·6 %. The sample was found to leave no ash on incineration, and microscopical examination showed only the typical hexagonal crystals of cystine. It is not possible at present to explain the low analytical data for the purified cystine. It may be noted that Hoffman and Gortner obtained similar analytical figures for pure cystine indicating a purity of 97·5 %[1].

[1] [Note added February 7, 1927.] Dr W. H. Hurtley has suggested to us that cystine pre-

I. *Estimation of cystine sulphur in presence of phosphotungstic acid.*

In the Van Slyke method of protein analysis 15 g. of phosphotungstic acid are used in precipitating the diamino-fraction. Known weights of cystine have been oxidised in presence of this large quantity of phosphotungstic acid by the Benedict-Denis method.

On treating the oxidised residue with dilute hydrochloric acid, it was found that the tungstic acid, produced during the incineration, gave a very fine white precipitate which settled out only slowly and was very difficult to separate by filtration. A further quantity of tungstic acid also came down on adding barium chloride for precipitation of the barium sulphate. This treatment was thus not a practical one for the purpose.

As tungstic acid forms a very insoluble yellow oxide on precipitation from strongly acid solutions, whereas it gives the white hydrated oxide in dilute solution, the next experiments were made using conc. hydrochloric acid for solution of the oxidised residue. The greater part of the tungstic acid was then obtained as yellow oxide which could be easily filtered off on a hardened paper and washed with conc. hydrochloric acid. On diluting the solution with water, a small quantity of the white oxide was precipitated; on warming it settled out and could be filtered off and washed with dilute hydrochloric acid. A clear solution was thus obtained for precipitation with barium chloride. The last traces of tungstic acid are not however always removed by this treatment and come down with the barium sulphate. These small amounts are easily soluble in dilute alkali and can be removed by washing the precipitate of barium sulphate with dilute ammonia.

As a routine, the procedure may be described as follows. The solution of cystine phosphotungstate (20 cc. diamino-fraction, or 50 cc. monoamino-fraction) is evaporated to dryness with 5 to 10 cc. of the Benedict-Denis reagent, preferably on a hot plate, until distinct charring is seen. The residue is then heated over a flame for 15 minutes. The oxidised material is boiled with 25 cc. of conc. HCl. After standing (most conveniently overnight) the solution is filtered from insoluble yellow tungstic oxide through a 9 cm.

cipitated from acid solution with sodium acetate may retain traces of acetic acid and thus give low analytical figures for nitrogen and sulphur.

A sample of cystine prepared by Dr Hurtley by precipitation with sodium hydroxide and kindly supplied to us gave the following data:

0·1 g. gave 11·55
0·1 g. gave 11·75 } 11·65 cc. $N/14$ NH_3 N = 11·65 %

0·1 g. gave 0·1941 g. $BaSO_4$ S = 26.65 %.

On recrystallising our own specimen by precipitation from acid with sodium hydroxide, it gave the correct figures:

0·1 g. gave 11·65
0·1 g. gave 11·65 } 11·65 cc. $N/14$ NH_3 N = 11·65 %

0·1 g. gave 0·1930
0·1 g. gave 0·1943 } 0·1936 g. $BaSO_4$ S = 26·60 %.

It thus appears that the low values for cystine nitrogen and sulphur are due to precipitation with sodium acetate and retention of acetic acid.

hardened paper and the precipitate washed with from 25 to 50 cc. of hot conc. HCl and then with hot 50 % HCl. The solution is diluted with 200 to 500 cc. of water. The white precipitate which forms settles out as yellow oxide on warming and is filtered off and washed with dilute HCl (1:1). The clear solution is precipitated with barium chloride. The barium sulphate is washed thoroughly with water and then with dilute ammonia to remove the last traces of tungstic acid. The final treatment of the barium sulphate is carried out by the Folin method of washing with alcohol, burning the paper in a crucible and heating to redness. The following results have been obtained:

0·25 g. cystine + 15 g. phosphotungstic acid gave 0·4806 g. $BaSO_4$
0·25 g. „ „ „ „ 0·4794 g. „ } S = 26·36 %.

These figures correspond with those for the cystine used above, S = 26·31 %.

II. *Estimation of cystine sulphur in cystine phosphotungstate.*

As the main object of these experiments was the estimation of cystine in the phosphotungstic acid precipitate obtained in the modified Van Slyke procedure, known quantities of cystine (0·25 g.) in hydrochloric acid solution were precipitated by an excess of phosphotungstic acid in a total volume of 200 cc. Under these conditions the precipitate came down slowly. After standing for 5 days, the precipitate was filtered off on a special glass filter and washed with 5 portions of 10 cc. dilute hydrochloric acid. The precipitate was dissolved in sufficient caustic soda solution and the volume made up to 100 cc. 20 cc. portions of the solution were taken for total nitrogen and for sulphur determinations.

		Mean			Mean
N	5·5 5·65 / 5·65 5·6	5·6 cc. $N/14$ NH_3,	S	0·0930 0·0934 / 0·0928 0·0928	0·0930 g. $BaSO_4$.

For the corresponding quantity of 0·05 g. cystine the quantities should be 5·75 cc. $N/14$ NH_3 and 0·0958 g. $BaSO_4$.

Both the nitrogen and the sulphur estimations showed that 97·4 and 97·1 % of the cystine were precipitated. The figures correspond with those of Hoffman and Gortner, who determined the extent of the precipitation by nitrogen determinations.

The filtrate from the precipitate of the cystine phosphotungstate was also examined for nitrogen and sulphur: 50 cc. of the filtrate made up to 250 cc. gave:

0·1 and 0·2 / 0·15 „ 0·1 cc. $N/14$ NH_3 0·0022 and 0·0008 g. $BaSO_4$.

The nitrogen was thus equal to 2·5 % of the total nitrogen. The amounts of barium sulphate were insufficient for accurate weighing.

III. *Behaviour of cystine during hydrolysis.*

The observations of Van Slyke [1911] and of Hoffman and Gortner [1922] upon the action of hydrochloric acid upon cystine have been repeated and extended. One portion of 1 g. cystine and one portion of 2·5 g. cystine were boiled for 36 hours under a reflux with 100 and 250 cc. of 25 % HCl. The

solutions became brown in colour, deposited a dirty brown precipitate and gave a distinct odour of hydrogen sulphide. The excess of hydrochloric acid was distilled off *in vacuo* and the volumes made up to 100 cc. and 250 cc. A complete series of analyses was then carried out by the Plimmer-Rosedale procedure, using quantities of the solutions containing 0·25 g. cystine. The following are the data:

		I		II	In % of	
					Total N	Total S
Hydrolysed solution	Total N	5·6		5·6 cc. *N*/14	*Mean*	
		5·7		5·6 cc. „		
	Total S	0·4700		0·4711 g. $BaSO_4$	97·4	98·0
		0·4692		0·4622 g. „		
Amide N		1·3	1·2	1·25 cc. *N*/14	4·3	—
Humin N		0·9	0·65	0·85 cc. „	2·7	—
Phosphotungstic precipitate:						
20 cc. out of 100 cc.		2·1	2·2	2·0 cc. „	—	—
		0·040	0·0412	0·0386 g. $BaSO_4$	36·5	41·8
Phosphotungstic filtrate:						
50 cc. out of 250 cc.		3·2	3·25	2·85 cc. *N*/14	—	—
		0·0538	0·0542	0·0528 g. $BaSO_4$	53·9	56·2

Cystine is thus decomposed by boiling with acid. About 7 % of its nitrogen was lost as amide and humin nitrogen. The sulphur content of the diamino-fraction represents about 40 % of the cystine sulphur; 56 % of the sulphur is found in the monoamino-fraction.

IV. *Estimations of the sulphur content of egg proteins.*

The remainders of the diamino- and monoamino-fractions of the egg proteins analysed by Plimmer and Rosedale [1925] have been analysed for their sulphur content by the method described above. The figures for barium sulphate have been changed to figures for cystine nitrogen by using the equivalent of 1 mg. $BaSO_4$ = 0·06 mg. cystine N. The data are:

	Diamino-fraction 100 cc. g.	Monoamino-fraction 250 cc. g.
Egg-yolk	0·0004	0·0170
Egg-white	0·0038	0·0124
Ovomucoid	0·0026	0·0183
Egg-membrane	0·0065	0·0133
Casein	0·0000	0·0010
Gelatin	0·0002	0·0075

It is of interest to notice the low amount of cystine nitrogen in the diamino-fraction of egg-yolk. The two phosphoproteins, caseinogen and vitellin, thus resemble each other also in their cystine sulphur content.

From the results of the analyses on boiling cystine with hydrochloric acid it might have been expected that the sulphur content of the monoamino-fraction would have been $\frac{3}{2}$ times that of the diamino-fraction, but except in the case of the egg-membrane the amount is considerably greater. It may therefore be inferred that another sulphur compound is present in all the proteins. Egg-membrane appears to contain mostly cystine sulphur.

V. *The presence of arginine in the monoamino-fraction of the egg proteins.*

The monoamino-fractions of the egg proteins and caseinogen analysed by Plimmer and Rosedale [1925] have, on standing for over 15 months, deposited small quantities of crystals. These crystals have been filtered off, dissolved in dilute soda, and analysed for total nitrogen and arginine nitrogen and in one case cystine sulphur. The filtrates have also been analysed for total and arginine nitrogen. The figures are:

	Volume of remaining solution cc.	Original		Filtrate		Precipitate		
		T.N.	Arg. N.	T.N.	Arg. N.	T.N.	Arg. N.	Cyst.
Caseinogen	1875	0·2086	0·0052	0·2114	0·0056	0·0287	0·0098	—
Egg-yolk	600	0·1666	0·0126	0·0166	0·0098	0·0042	0·0036	—
Egg-white	610	0·2016	0·0126	0·1974	0·0098	0·0042	0·0036	—
Ovomucoid	740	0·1946	0·0238	0·1862	0·0238	0·0140	0·0028	—
Egg-membrane	710	0·1960	0·0168	0·1890	0·0154	0·0182	0·0049	0·0020

There has been a small diminution, as expected, in the total nitrogen of the solutions and little change in the amount of arginine nitrogen. The precipitate consists only in part of arginine phosphotungstate. The amounts of "isomeric" cystine in these solutions (section IV) are not sufficient to account for the whole of this arginine nitrogen.

The possibility that the ammonia arose from monoamino-acids, though they were shown to be stable to caustic potash by Van Slyke, has again been tested by boiling solutions of glycine, alanine, proline, and tyrosine with 20 % caustic soda. No ammonia was evolved in any of these experiments. Specimens of hydroxyamino-acids have not been available for testing with caustic soda. The original conclusion that the ammonia arose from non-precipitated arginine is thus strengthened. It is possible that the arginine in the solution comes from racemic arginine which may form a more soluble phosphotungstate.

VI. *Action of sodium hydroxide on cystine.*

The action of caustic potash upon cystine was tested by Van Slyke [1911], who found that 18 % of its nitrogen was given off. To complete the series of observations upon the behaviour of cystine in the Van Slyke method, the action of 20 % caustic soda for 6 hours, the conditions found by Plimmer [1916] for the decomposition of arginine, has been tried upon cystine, "hydrolysed" cystine, cystine precipitated by phosphotungstic acid and unprecipitated "isomeric" cystine. The results were as follows:

Solution boiled with 20 % caustic soda	Total N of solution cc. $N/14$	Ammonia evolved cc. $N/14$		Mean	% of total N
10 cc. 0·1 % pure cystine	11·5	1·0	1·2	1·1	9·6
10 cc. 0·1 % hydrolysed cystine	11·2	1·5	1·3	1·3	11·8
20 cc. cystine phosphotungstate in soda	2·1	0·5	0·4	0·45	21·4
50 cc. filtrate from phosphotungstate	3·1	0·4	0·45	0·4	13·7

The decomposition of pure cystine is thus less with 20 % soda than found by Van Slyke with 40 % potash. The amount is a little greater after hydrolysis. In presence of phosphotungstic acid, the amount of decomposition is greater. This decomposition of cystine by boiling with soda will give results for arginine which are too high. A deduction of one-fifth of the cystine nitrogen should be made before calculating the arginine nitrogen.

Summary.

1. The sulphur of cystine can be estimated in the presence of phosphotungstic acid by a suitable alteration of the Benedict method of determining sulphur.

2. 97 % of pure cystine is precipitated by phosphotungstic acid.

3. Cystine is changed by boiling with acids, losing 7 % of its nitrogen; only 40 % is then precipitated by phosphotungstic acid.

4. Cystine, on boiling with caustic soda, loses 10 % of its nitrogen as ammonia. The decomposition reaches 20 % after boiling with acid and in presence of phosphotungstic acid.

5. The amounts of cystine sulphur in the egg proteins and caseinogen have been determined. The figures indicate the presence of another sulphur compound in these proteins.

6. The probable presence of arginine in the monoamino-fractions obtained in the Van Slyke method of analysis is emphasised by its precipitation as phosphotungstate when the solutions are kept. It is possibly racemic arginine.

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REFERENCES.

Hoffman and Gortner (1922). *J. Amer. Chem. Soc.* **44**, 341.
Mueller (1923). *J. Biol. Chem.* **56**, 157.
Plimmer (1913). *Biochem. J.* **7**, 311.
—— (1916). *Biochem. J.* **10**, 115.
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1015.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.