XXXI. THE SEPARATION OF CYSTINE AND TYROSINE.

By ROBERT HENRY ADERS PLIMMER.

From the Ludwig Mond Research Laboratory for Biological Chemistry, Institute of Physiology, University College, London.

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Cystine and tyrosine resemble each other so closely in their solubility in water, in alkali and in acid, that their separation is not easily effected. Both Mörner [1899, 1901] and Embden [1900] who first isolated cystine from the products of the acid hydrolysis of scleroproteins obtained it mixed with tyrosine. Mörner effected the separation of the two substances by fractional crystallisation from ammonia; Embden by dissolving out the tyrosine with very dilute nitric acid. Friedmann [1902] separated tyrosine from cystine by solution in ammonia and neutralisation with acetic acid; tyrosine crystallised out in the neutral solution and the cystine was precipitated by making the filtrate strongly acid with acetic acid. Cystine is usually prepared from the products of acid hydrolysis of proteins by nearly neutralising with soda and allowing to crystallise. Folin [1910] described the isolation of cystine and tyrosine by neutralising the hydrochloric acid hydrolysis solution to Congo-red with sodium acetate; cystine crystallises out and on diluting the filtrate and allowing it to stand the tyrosine separates out slowly. Cystine thus seems to be the more insoluble in dilute mineral acids and in strong acetic acid.

Neither Mörner's, Embden's, nor Friedmann's method gives a quantitative separation of the two substances; they only permit of the isolation of a portion of the mixture. Folin's method of preparation was devised for obtaining a quantity of cystine. A method for the separation of these two constituents of a protein is therefore required. On account of their similarity the tyrosine isolated from the products of hydrolysis may contain cystine unless this unit has been completely decomposed during the process of

hydrolysis[1]. For this reason the data of the actual amount of cystine in most proteins are scanty or lacking and except in the scleroproteins and in the protein described by Kotake and Knoop [1911] the relative amount of cystine to tyrosine is very small. The estimations of tyrosine which have been made by Plimmer and Miss Eaves [1913] by the method of bromination are not greatly higher than the amounts of tyrosine obtained by direct isolation and weighing. The presence of a small amount of cystine in the tyrosine may explain the slight differences.

Winterstein [1901] described the precipitation of cystine by phosphotungstic acid and Hopkins and Cole [1902] its precipitation by a solution of mercuric sulphate in five per cent. sulphuric acid. Tyrosine is not precipitated by phosphotungstic acid and its mercury compound is soluble in the five per cent. sulphuric acid. Cystine and tyrosine have been found to differ very greatly in their behaviour to absolute alcohol saturated with hydrochloric acid gas. Tyrosine is readily esterified and goes into solution in the acid alcohol; cystine is not readily esterified and is only very slowly dissolved and the portion which goes into solution can be precipitated by adding an equal volume of absolute alcohol. Neither of these differences has hitherto been used for the specific purpose of separating the two compounds and their applicability has therefore been tested.

Experimental.

Tyrosine was prepared by the hydrolysis of caseinogen by trypsin. The products which crystallised out during the digestion and on the concentration of the filtered solution were mixed and purified by repeatedly dissolving in dilute sulphuric acid and exactly neutralising with caustic soda, 14 g. of pure tyrosine being thus obtained from 600 g. of caseinogen. The substance gave no precipitate with phosphotungstic acid showing the absence of diamino-acid (diaminotrihydroxydodecanic acid) and of cystine.

Cystine was prepared from wool and hair by Folin's method; the final product was not perfectly white but it consisted of the typical hexagonal plates.

[1] Cystine is rapidly decomposed by boiling with alkali with the formation of hydrogen sulphide (loosely bound sulphur) and it is also decomposed by prolonged boiling with acid. In preparing cystine from wool or hair the best yield was obtained when the material was boiled with concentrated hydrochloric acid for 3–4 hours; the yield was very poor when the boiling lasted from 5–8 hours. In purifying the cystine by boiling with charcoal in acid solution loss also occurs. If a solution of cystine in dilute hydrochloric acid be boiled for a long time it becomes yellow or yellow-brown in colour.

(1) *Separation by means of Phosphotungstic Acid.*

Cystine and tyrosine were mixed together in different proportions and dissolved in 50 cc. of 5 per cent. sulphuric acid. A 30 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid was added so long as a precipitate was formed. (20 cc. sufficed for the precipitation of 0·5 g. of cystine.) After standing for 12 hours the precipitate was filtered off and washed repeatedly with a 2·5 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid.

The cystine was recovered from the precipitate either by suspending it in water or in water containing acetone as recommended by Wechsler [1911] and adding baryta water until the solution remained permanently alkaline to phenolphthalein, the decomposition being carried out on the water bath. Excess of baryta was carefully avoided so as to prevent decomposition of the cystine. The filtrate from the barium phosphotungstate was acidified with hydrochloric acid, evaporated to a small volume on the water bath, and neutralised with ammonia. The cystine crystallised out and after being left to stand for 1–2 days was filtered off, washed, dried and weighed.

The recovery of the cystine from its phosphotungstate by decomposition with hydrochloric acid and extraction of the reagent with ether (Winterstein) was also attempted; the whole of the reagent was not dissolved by the ether and the recovered cystine was contaminated with phosphotungstic acid. Experiments were not made with amyl alcohol which Jacobs [1912] recommended as a solvent for extracting phosphotungstic acid.

The tyrosine was recovered from the filtrate by adding ammonia to remove the excess of phosphotungstic acid, filtering off the ammonium phosphotungstate, neutralising and evaporating to a small volume. The crystals so obtained were washed with water to remove ammonium sulphate and the tyrosine residue was dried and weighed. This procedure was preferred to the usual method of removing excess of acid with baryta which entails repeated extraction of a bulky precipitate of barium phosphotungstate and sulphate with hot water and the evaporation of a large volume of liquid.

The amounts of cystine and tyrosine taken and recovered were as follows:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	0·5 g.	0·35 g.	0·35 g.
0·5	1·0	0·47	0·6
0·5	1·0	0·45	0·55
1·0	0·5	0·80	0·25
1·0	0·5	0·82	0·4
0·5	—	0·27	—
0·5	—	0·3	—

Neither the cystine nor the tyrosine was completely recovered. The cystine is precipitated practically completely by the phosphotungstic acid; the loss seems to take place in its recovery from the phosphotungstic acid precipitate; the solution must be made slightly alkaline to ensure complete decomposition of the phosphotungstate and some of the cystine is most probably also decomposed by the alkali. Some decomposition may also occur during the evaporation of the acid solution. Except in the last experiment the cystine always contained tyrosine and this accounts for the loss of tyrosine. The cystine was only obtained free from tyrosine in the last experiment in which the precipitate was washed some twenty times by removing it from the filter, stirring up with the washing reagent and again filtering until the washings showed no reaction with Millon's reagent: over 80 per cent. of the tyrosine was then recovered.

2 g. of the cystine recovered from the earlier experiments were found to contain 0·6 g. of tyrosine which was isolated by means of alcohol saturated with hydrochloric acid (as described below).

(2) *Separation by means of Mercuric Sulphate.*

Mixtures of cystine and tyrosine in various proportions were made and dissolved in 5 per cent. sulphuric acid and treated with mercuric sulphate dissolved in 5 per cent. sulphuric acid (Hopkins and Cole's tryptophane reagent) until no further precipitate occurred. The precipitate was filtered off after standing for 12 hours and washed repeatedly with 5 per cent. sulphuric acid by removing from the filter, stirring up with the acid and again filtering until the washings gave no reaction with Millon's reagent.

The cystine was recovered from the precipitate by suspending in water and decomposing with hydrogen sulphide. The filtrate from the mercuric sulphide was evaporated on the water bath to a small volume and then neutralised with ammonia. The cystine crystallised out and was filtered off, washed, dried and weighed. The acid filtrate containing the tyrosine was evaporated on the water bath to about 400 cc. and filtered from the mercuric sulphate which had separated out. A slight excess of ammonia was added and after again filtering the solution was evaporated almost to dryness. The crystals so obtained were filtered off and washed with water until free from ammonium sulphate and the residue of tyrosine was dried and weighed. This procedure was preferred to the removal of the sulphuric acid with baryta which would have necessitated the repeated extraction of the insoluble barium sulphate with boiling water.

As with the previous method the amounts of cystine and tyrosine recovered were far from quantitative as is shown by the following figures:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	—	0·30 g.	—
0·5	—	0·31	—
0·5	0·5 g.	0·26	0·45 g.
0·5	0·5	0·28	0·47
0·5	1·0	0·31	0·75
0·5	1·0	0·15	0·96

The loss of tyrosine was apparently less than that of cystine but the tyrosine was very impure and contained a brown pigment arising from the decomposition of cystine. On further investigation the precipitation of cystine by mercuric sulphate in 5 per cent. sulphuric acid was found to be incomplete, as was shown by an estimation of the nitrogen in an experiment with cystine alone:

1 g. of cystine was dissolved in 100 cc. of 5 per cent. sulphuric acid; 20 cc. were found to contain 0·0224 g. N by Kjeldahl's method. The remaining 80 cc. (= 0·0896 g. N) were precipitated with 21 cc. mercuric sulphate solution. 70 cc. of the filtrate contained 0·0172 g. N.

Hence the amount precipitated was 0·0724 g. or 81 per cent. of the cystine. The amount of cystine recovered from the precipitate by the procedure described above was 0·3 g. instead of 0·6 g. Loss occurs not only in the precipitation but also in evaporating the solution before neutralising with ammonia. Cystine is much more unstable to acid than one is led to expect from the description of its isolation.

(3) *Absolute alcohol saturated with hydrogen chloride.*

Whilst preparing tyrosine ethyl ester from some tyrosine it was observed that complete solution of the material could not be effected and an examination of the insoluble residue showed it to be cystine; 5 g. of the material yielded 0·05 g. cystine and 4 g. yielded 0·2 g. cystine

This difference in the behaviour of cystine and tyrosine suggested a simple method for effecting their separation.

Preliminary experiments were made with pure cystine and pure tyrosine; 0·5 g. cystine was covered with 20 cc. absolute alcohol saturated with hydrogen chloride, warmed on the water bath and allowed to stand for 12 hours. The undissolved substance was filtered off, washed with absolute alcohol, dried and weighed. Yield = 0·39 g. A white precipitate was produced when the wash alcohol came into contact with the filtrate. This

precipitate was filtered off, washed, dried and weighed. Yield = 0·11 g. On dissolving a test portion of each of these quantities in ammonia and allowing to crystallise the typical hexagonal plates characteristic of cystine were formed.

0·5 g. tyrosine was covered with absolute alcohol saturated with hydrogen chloride and warmed on the water bath. Complete solution readily occurred and the tyrosine was converted into its ethyl ester. On adding water and neutralising with sodium carbonate no tyrosine was precipitated, but on acidifying and boiling for 4–5 hours the ester was hydrolysed and on again neutralising with sodium carbonate tyrosine was precipitated. It was filtered off, washed, dried and weighed. Yield = 0·45 g. A further 0·05 g. was obtained on acidifying the filtrate, boiling, and neutralising once more.

A mixture of 0·5 g. of each was then treated in the same way with 30 cc. absolute alcohol saturated with hydrogen chloride. 0·35 g. was insoluble and the alcohol used for washing the residue precipitated an additional 0·1 g. The filtrate after hydrolysis of the tyrosine ester yielded 0·47 g. tyrosine.

In these experiments the cystine was recovered in two fractions, but if an equal volume of absolute alcohol be added to the absolute alcohol saturated with hydrogen chloride before filtering the whole of the cystine can be obtained in one operation. The cystine which goes into solution seems to be cystine hydrochloride, for it dissolves in water. The insoluble portion seems to consist mainly of cystine but a portion of it dissolves if it be washed with water, and is apparently cystine hydrochloride as the cystine is precipitated in hexagonal plates on neutralising with ammonia. There is no evidence that the cystine is converted into its ester. According to Friedmann cystine is not easily converted into its ethyl ester but its methyl ester is more easily obtained (Fischer and Suzuki).

The separation of cystine and tyrosine by this method has been tested by the following experiments:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	0·0 g.	0·47 g.	0·0 g.
0·5	0·0	0·50	0·0
0·0	0·5	0·0	0·5
0·0	0·5	0·0	0·49
0·5	0·5	0·45	0·47
0·5	1·0	0·49	0·96
1·0	0·5	1·00	0·50

The mixtures were treated with absolute alcohol saturated with hydrogen chloride and warmed on the water bath. An equal volume of alcohol was added and the insoluble cystine filtered off, washed, dried and weighed. The

filtrate was diluted with 2 volumes of water and boiled for 8 hours, water being added when necessary. Tyrosine was precipitated on neutralising; it was filtered off, washed, dried and weighed.

The usefulness of this method is illustrated by the first experiment in which presumably pure tyrosine prepared from wool by Folin's method had been used. The presence of cystine was not observed by microscopic examination and the cystine present was found to be unevenly distributed.

The cystine recovered from the phosphotungstate precipitate above mentioned contained tyrosine; 2 g. contained 1·4 g. cystine and 0·6 g. tyrosine.

A mixture weighing 3 g. was found to contain 1·9 g. cystine; the tyrosine was unfortunately lost. This mixture actually contained 2 g. cystine and 1 g. tyrosine.

SUMMARY.

1. Cystine and tyrosine can be separated from one another by precipitation with phosphotungstic acid. The precipitation of cystine is almost complete, but loss occurs in its recovery from the precipitate. Almost the whole of the tyrosine can be recovered from the filtrate and washings.

2. Cystine and tyrosine can be separated from one another by precipitation with mercuric sulphate in five per cent. sulphuric acid. The cystine is not completely precipitated and the tyrosine which is recovered is impure.

3. Cystine and tyrosine can be completely and quantitatively separated by means of absolute alcohol saturated with hydrogen chloride. The tyrosine is rapidly converted into tyrosine ester and goes into solution. It can be recovered by boiling the solution when diluted with water for eight hours and then neutralising with ammonia. Almost the whole of the cystine is insoluble; the portion which goes into solution (perhaps cystine hydrochloride) is precipitated by adding an equal volume of absolute alcohol. The cystine is not converted into its ethyl ester since on dissolving the insoluble portion in dilute hydrochloric acid and neutralising with ammonia the cystine is precipitated in the typical hexagonal plates.

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