CXXXVII. SULPHUR METABOLISM.

I. THE PRECIPITATION OF CYSTINE FROM SOLUTIONS BY MERCURIC AND CUPROUS CHLORIDES AND ITS RECOVERY, AS PRELIMINARY TO ITS QUANTITATIVE DETERMINATION IN URINE.¹

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It has been shown by a number of investigators that cystine may be precipitated almost quantitatively from solution by the heavy metals, silver, copper and mercury. The insoluble character of the resulting metal mercaptides has been utilised by Rossouw and Wilken-Jorden [1935] and Vickery and White [1933] in the quantitative determination of cystine in protein hydrolysates. The problem of the estimation of the cystine content of normal urine offers many points in common with its determination in hydrolysates, such as the relatively low content of cystine and the relatively high concentration of colouring matter (humin or urinary pigments) and of other compounds reacting with the reagents employed in the various quantitative methods. A study is here presented of conditions for precipitation of cystine from solutions containing amounts comparable with those of normal urine and its recovery in media suitable for colorimetric determinations.

EXPERIMENTAL.

For the cuprous chloride precipitation the general procedure of Rossouw and Wilken-Jorden [1935] was followed. The mercury mercaptide was decomposed with hydrogen sulphide and the recovered cysteine determined with Folin's [1934] uric acid reagent.

Solutions.

(a) *Standard cystine solution*, 0.001 *M* in 0.1 *M* H₂SO₄. This solution is relatively stable [Shinohara and Kilpatrick, 1934].

(b) *Standard cysteine solution*, 0.002 *M* in 0.1 *M* H₂SO₄. This solution must be kept cold and renewed every 4 or 5 days. The colour standard is relatively stable and may be used throughout a day's work.

(c) *Sodium acetate-acetic acid buffer* at *p*_H 4.6, made by combining equal vols. of 2 *M* sodium acetate and 2 *M* acetic acid.

(d) *Sodium acetate-acetic acid buffer* at *p*_H 5.2, made by combining 10 vols. of 2 *M* sodium acetate with 3 vols. of 2 *M* acetic acid.

(e) *Hydrochloric acid*, 1%.

(f) *Potassium chloride*, 25% in 0.2% HCl.

(g) *Cuprous chloride*. Immediately before using 0.3 g. of well-powdered cuprous chloride is washed in 25 ml. of 1% HCl, allowed to settle and after decantation of the supernatant liquid dissolved in 2 ml. of the KCl solution.

(h) *Uric acid reagent* [Folin, 1934].

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Technique.

To 8 ml. of the standard cystine or cysteine solution in a graduated 15 ml. centrifuge-tube were added 5 ml. of buffer at p_H 4.6 and 10 to 12 drops of the freshly prepared cuprous chloride solution. The mixture was kept for 30–40 min., centrifuged for 5 min. at moderate speed and the supernatant liquid decanted. The precipitate was stirred with a fine glass rod in buffer at p_H 5.2 added to the 8 ml. mark, after which H_2S was bubbled through for 3–4 min. with constant stirring to break up any particles of the mercaptide. The solution was filtered through a small fine filter-paper into a test-tube and nitrogen or carbon dioxide gas bubbled through vigorously for about 20 min. to remove H_2S . A 5 ml. aliquot part was pipetted into a 25 ml. volumetric flask containing 1.5 ml. of buffer at p_H 5.2, followed by 2 ml. of the uric acid reagent. At the same time 6.5 ml. of the same buffer were added to 2 ml. of the standard cysteine solution in a second 25 ml. volumetric flask and 2 ml. of the uric acid reagent added. Both solutions were made up to volume with water and read in a colorimeter after 20 min.

$$\frac{\text{Cysteine standard in mol.}}{2} \times \frac{20}{R} = \text{mol. cystine recovered.}$$

Mercuric chloride precipitation.

Solutions. In addition to solutions *a*, *b*, *d* and *h* listed above, 2 *M* sodium acetate solution and a saturated solution of mercuric chloride were employed.

Procedure. The same procedure as in the cuprous chloride precipitation was followed, except that 5 ml. of 2 *M* sodium acetate were added to the standard solution, followed by 2 ml. of saturated $HgCl_2$. Since precipitation is slow, the mixture was kept several hours, usually overnight, after which the tubes were centrifuged and the precipitate treated as described above.

Vickery and Leavenworth [1930], upon decomposing their silver mercaptide with H_2S , found 97% of the nitrogen in the form of cysteine and 3% in the form of cystine. In the experiments reported in this paper, repeated attempts were made to demonstrate the presence of disulphide in the solution after treatment of the copper or mercury precipitate with H_2S and aeration of the filtrate with CO_2 . In no case did the addition of sodium bisulphite to the test solution before

Table I. *Recovery of cystine and cysteine as cysteine after precipitation with Cu_2Cl_2 under varying conditions.*

Cystine 0.001 <i>M</i> ml.	Cysteine 0.002 <i>M</i> ml.	Cu_2Cl_2 drops	Time ppt. stood min.	Recovery in % of 5 ml. aliquot			No. tests
				Max.	Min.	Av.	
8	—	10–12	40	97.7	88.0	93.5	30
—	8	10–12	40	98.3	87.3	94.4	25
Effect of varying the quantity of cystine or cysteine.							
2	—	10	40	98.3	87.2	93.0	6
4	—	10	40	97.0	89.0	92.3	6
—	2	10	40	97.0	90.1	92.5	6
—	4	10	40	98.1	87.4	91.3	6
Effect of varying the amount of precipitant.							
8	—	5	40	91.6	85.0	87.2	7
8	—	8	40	94.2	84.6	93.1	10
8	—	15	40	77.3	74.2	75.1	4
—	8	15	40	—	—	—	—
Effect of standing for varying periods after precipitation.							
8	—	10–12	0	90.5	82.5	86.9	5
—	8	10–12	0	93.8	72.2	83.6	8
8	—	10–12	20	95.8	84.9	93.2	8
—	8	10–12	20	96.8	87.3	95.0	8
8	—	10–12	120	97.0	87.2	93.0	6
—	8	10–12	120	97.7	86.4	95.7	6

introduction of the uric acid reagent bring about development of more than twice the colour produced with the uric acid reagent alone, as should theoretically have occurred if mixtures of cystine and cystine instead of cystine alone had been present.

The results upon varying the conditions of the precipitation of cystine and cysteine and their recoveries as cysteine are reported in Tables I and II.

Table II. *Recovery of cystine and cysteine as cysteine after precipitation with HgCl_2 under varying conditions.*

Cystine 0.001 M ml.	Cysteine 0.002 M ml.	Medium			HgCl_2 ml.	Recovery % theoretical range	No. tests
		2 M NaAc ml.	2 M HAc ml.	M NaOH ml.			
5	—	5	—	—	1	32.9–47.3	3
5	—	5	—	—	2	65.9–71.1	30
5	—	5	—	—	3	63.7–70.6	5
5	—	5	—	—	4	62.6–71.2	5
5	—	5	—	—	6	60.3–71.4	5
5	—	5	—	0.2	1	40.0	1
5	—	5	—	0.2	2	42.9–48.9	7
5	—	5	—	0.2	3	43.0–62.7	5
5	—	5	—	0.2	4	49.6	1
5	—	5	—	0.2	6	49.0–56.3	4
5	—	5	1.5	—	—	60.2–65.8	5
—	5	5	—	—	1	80.1–82.0	3
—	5	5	—	—	2	88.0–95.0	10
—	5	5	—	—	4	86.8–90.0	3
—	5	5	0.2	—	2	Reading inconstant	3
—	5	5	0.2	—	4	Reading inconstant	3

Results.

(a) Cu_2Cl_2 .

Recovery under standard conditions. The average yield of cystine from cystine was 93.5% in thirty tests (88–97.7%). The recovery of cysteine from cystine was not significantly greater, averaging 94.4% (87.3–98.3%).

Effect of varying the quantity of the precipitant. When 5 drops of the cuprous chloride solution were added, the recovery was slightly lower. The failure of the precipitate to settle out promptly indicated that an insufficient amount of the precipitant had been used. When 15 drops of the cuprous chloride were added, the recovery again was low, possibly because of the solubility of the mercaptide in the large amount of potassium chloride added.

Effect of varying the quantity of cystine and cysteine. Since the presence of excess precipitant affects the solubility of the precipitate, the recovery of smaller amounts of the test solutions under otherwise standard conditions was studied. Aliquot parts of 2 and 4 ml. of cystine or cysteine were recovered equally well as 8 ml.

Optimum period for allowing the precipitate to stand before centrifuging. When the precipitate was centrifuged immediately after the addition of cuprous chloride the average recovery fell to 87%. With a delay of 20 min. the average recovery was about the same as with the 40-min. interval, but as the individual values were somewhat erratic, the shorter period was deemed hardly sufficient. Keeping the precipitate overnight did not further increase the yield.

(b) $HgCl_2$.

The yield of cysteine from cystine precipitated with mercuric chloride was lower than when cuprous chloride was used as the precipitating agent (Table II), 69.0% as average under optimum conditions and 71.1 and 65.9% as maximum and minimum respectively. 1 ml. of the mercuric chloride proved insufficient for the precipitation of the amount of cystine employed, and 6 ml. did not further increase the yield. In more acid or alkaline media the yields were lower and greater amounts were determined in the filtrates. The recovery of cysteine was comparable with that of cysteine under the cuprous chloride precipitation.

Effect of varying the temperature. Since it was shown by Vickery and Leavenworth [1930] that, in the silver precipitation, maintaining the solution at 10° or lower tends to prevent decomposition of the mercaptide, the cuprous and mercuric precipitations were carried out at 2°, the reagents being embedded in ice or allowed to stand in a refrigerator at that temperature. Under these conditions the copper mercaptide separated in colloidal form and could not be successfully centrifuged even after 12 hours. The yield averaged about 12% lower than at room temperature. The yield from cysteine was only slightly higher. In the case of the mercury precipitation, the same colloidal effect was not produced, but the yield was not increased above that reported in Table II. Slight improvement in the yield occurred when cysteine was precipitated, the average value being about 95% as against 94% at room temperature. The range of values however was essentially the same, so it was concluded that this difference was not significant.

DISCUSSION.

The average recovery of about 95% of the cystine precipitated with cuprous chloride is in close agreement with the figures recorded by Vickery and White [1933], whose recovery averaged 94.6% and who employed cuprous oxide as the precipitant, after preliminary reduction of the cystine to cysteine by tin and acid. They believed this preliminary reduction to be essential for a nearly quantitative yield, as it had been previously demonstrated that the reduction of cystine to cysteine by tin is quantitative, whereas in the reduction which takes place during the precipitation by mercury and silver salts, one-sixth of the cystine had been reported to be oxidised to cysteic acid [Vickery and Leavenworth, 1930; Preisler and Preisler, 1930; Simonsen, 1931-32]. The findings of the present authors that the percentage of cystine recovered without previous reduction is as great as that of cysteine, corroborating the conclusions of Rossouw and Wilken-Jorden, indicates that this simultaneous oxidation and reduction does not take place when cuprous chloride is used as the precipitant under the conditions of these experiments.

Rossouw and Wilken-Jorden, on the basis of 100% recovery of cystine after cuprous chloride precipitation, postulate that each mol. of cystine gives rise to 2 mols. of cysteine as mercaptide instead of 5 from 3 of cystine. According to them the excess cuprous chloride used in the precipitation drives the reaction to the SH side; while in the process of dissolving the mercaptide in dilute acid, the initial state of the cystine is recovered owing to the presence of excess of the cupric ion in the complex molecule. It may be expressed:

and is forced in either direction depending upon whether cupric or cuprous copper is in excess. A second reduction of S—S to SH must be postulated during the subsequent treatment with H_2S .

Andrews and Wyman [1930] rejected the possibility of the reduction by H_2S since they believed that in this event free sulphur would appear in the solution according to the equation:

The authors are able to confirm their observation that no free sulphur appears in these solutions. It is known, however, that the rapid reduction of cystine to cysteine by H_2S does occur in the presence of ions of the heavy metals [Mauthner, 1901], which were not present in the solution tested by Andrews and Wyman. It is reasonable therefore to assume that in the transfer of electrons the copper ions are involved and that the reaction is more complex than these authors suggest. The markedly different results obtained under the mercuric chloride treatment lead to the conclusion that a different set of reactions must be occurring when cystine is being precipitated by the mercuric ion. Here, a yield of 40–75 % of the original cystine is obtained from the precipitate according to the conditions of the experiment, whereas cysteine may be recovered to about the same extent as when cuprous chloride is used. Since the fundamental difference between these two procedures consists in the substitution of the mercuric ion for the strongly reducing cuprous ion, the conclusion seems justifiable that in this case simultaneous oxidation and reduction occur in some such manner as that suggested under the first equation above, and that the reaction goes to completion because of the insoluble character of the mercury mercaptide which is one of the reaction products. At no time, however, could a value as great as 83 % of the original cystine be obtained from the combined precipitate and filtrate, the actual figures varying between 68 and 74%. Hence some other reaction is possibly also involved.

Since cystine, precipitated by cuprous chloride, could be recovered only to about 95 % on the average following decomposition of the mercaptide with hydrogen sulphide, an attempt was made to ascertain at what step the loss occurred, by substituting decomposition by potassium thiocyanate, as employed by Rossouw and Wilken-Jorden. The following modification of their method was made.

The cuprous precipitate in the centrifuge-tube is stirred into 1 ml. of 0.5 *N* HCl and 1 ml. of 0.1 *N* acetic acid until all particles have disappeared. Water is added to about 5 ml. followed by 0.4 ml. of 10 % KSCN to precipitate cuprous ions and after 2 min. of vigorous stirring, 2 drops of pyridine are added from a finely pointed pipette to precipitate cupric ions. The volume is adjusted to the 8 ml. mark with water and the solution filtered through a fine filter-paper. No SH can be demonstrated at this stage, as stated by Rossouw and Wilken-Jorden. A 5 ml. aliquot part of the filtrate is pipetted into a 25 ml. volumetric flask followed by 6.5 ml. of sodium acetate-acetic acid buffer at p_{H} 5.2, 1.5 ml. of 0.1 *M* NaHSO_3 and 2 ml. of the uric acid reagent. A white precipitate is formed by the excess KSCN and the uric acid reagent. If this precipitate is large in amount, additional reagent must be added. The solution is centrifuged after 15 min. and compared with a cysteine standard prepared 20 min. previously.

The error of a single determination by the phospho-18-tungstic acid method with the filtrate as prepared above is somewhat greater than with the filtrate from the H_2S method, *i.e.* the proportionality between colour developed and amount of cystine present is less strict, probably owing to the varying amount of the uric acid reagent remaining in the solution after partial precipitation. In all cases, however, the final readings indicated a recovery of 100 % (100 ± 1.5 %).

The filtrate obtained after either method of precipitation may also be used with the Sullivan method as modified by Rossouw and Wilken-Jorden [1934].

In this case however the proportionality between initial amount of cystine and amount recovered was even less strict in the hands of the present authors than with either procedure described above, although recovery of approximately 100 % is indicated. Table III gives a comparison of results by the three methods.

Table III. *Comparison of quantitative determinations of cystine following its precipitation with Cu_2Cl_2 and its subsequent recovery. (a) Decomposition of mercaptide with H_2S and determination with the uric acid reagent. (b) Decomposition of the mercaptide with KSCN and determination with the uric acid reagent. (c) Decomposition of the mercaptide with KSCN and determination by the Sullivan method. In each case results of 5 tests were averaged and the recovery expressed in percentage.*

Cystine pptd.		Standard		Method	Recovered %
0.001 M ml.	0.005 M ml.	0.001 M ml.	0.005 M ml.		
5	—	5	—	(a)	96.3 ± 0.4
4	—	5	—	(a)	94.9 ± 0.5
3	—	5	—	(a)	96.2 ± 0.4
2	—	5	—	(a)	93.9 ± 0.5
1	—	5	—	(a)	94.2 ± 0.5
5	—	5	—	(b)	100.1 ± 0.6
4	—	5	—	(b)	100.2 ± 0.5
3	—	5	—	(b)	98.3 ± 3.4
2	—	5	—	(b)	101.3 ± 4.1
1	—	5	—	(b)	102.0 ± 6.8
—	5	—	5	(c)	100.3 ± 2.3
—	4	—	5	(c)	105.8 ± 3.8
—	3	—	5	(c)	109.6 ± 4.9

These results indicate that the loss of 5 % of the initial cystine in the cuprous chloride and hydrogen sulphide method occurs during the decomposition of the mercaptide by H_2S and the subsequent reduction of the cystine to cysteine. The following experiments were performed: 8 ml. of 0.001 M cystine were introduced into a test-tube, 2 drops of 5 % cupric chloride added and H_2S bubbled through for 0.5 min., then removed with CO_2 . Recovery of cystine from a 5 ml. aliquot part was 100 %, of which about 98 % was cysteine and 2 % cystine. When H_2S was bubbled for 1 min., 100 % was recovered as cysteine. The same experiment was performed with mercuric instead of cupric chloride and similar results were obtained. Since the loss of 5 % of the initial cystine cannot be accounted for in any of these steps, it seems most probable that it occurs by occlusion of cysteine on the copper sulphide. Attempts were made to recover a greater amount by washing the copper sulphide in hot buffer solution, but the yield was increased only by about 1 %.

SUMMARY.

A study has been made of the precipitation of cystine and cysteine from solutions by mercuric chloride and cuprous chloride and its recovery as cysteine in sodium acetate-acetic acid buffer solutions suitable for subsequent use in quantitative determination by phospho-18-tungstic acid.

Recovery following decomposition of the mercury mercaptide by hydrogen sulphide averaged 69 % of the original cystine. Recovery from cuprous mercaptide by the same method averaged 95 % of the original cystine.

Recovery of cystine from cuprous mercaptide by precipitation of the copper with potassium thiocyanate and pyridine yields 100 % of the cystine, but the

filtrate does not lend itself to accurate quantitative determinations by phospho-18-tungstic acid.

The filtrates obtained by either procedure may be used also with the Sullivan method of cystine determination. The proportionality is still less strict than with the phospho-18-tungstic acid method.

The procedures described above, except for the less sensitive Sullivan test, may be carried out with 8 ml. or less of the solution to be tested, containing as little as 1×10^{-5} g. mol. of cystine (0.24 mg.) in the sample. This value, as will be shown in a later paper, is comparable with those found in normal urine.

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