CLXXXV. METABOLISM OF SULPHUR.

II. THE QUANTITATIVE DETERMINATION OF CYSTINE IN NORMAL URINE.

By GRACE MEDES.

From the Lankenau Hospital Research Institute.

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In the first paper of this series [Medes & Padis, 1936] a method was described for the quantitative determination of cystine in solutions, the basis of which was the precipitation of the cystine by cuprous chloride, its recovery as cysteine from the copper mercaptide by the use of hydrogen sulphide and its quantitative determination with phospho-18-tungstic acid. The present paper concerns itself with the application of this method to the estimation of cystine in normal urine.

That the compound so determined consists at least in part of cystine has been demonstrated by the isolation of cystine from normal urine by cuprous chloride precipitation.

Isolation of cystine from normal urine. All reagents were prepared as in the method for quantitative determination of cystine reported in the previous paper. Freshly voided urine was brought to pH about 4.6 with acetic acid and preserved in a refrigerator at about 5° until used (not longer than 6 hours). The urine was filtered to remove any uric acid and inorganic salts that had precipitated. To 3 litres of urine was added an equal volume of the sodium acetate-acetic acid buffer at pH 4.6, followed by 25 g. of cuprous chloride dissolved in the KCl-HCl mixture, added in a slow stream with vigorous stirring. The precipitate was allowed to settle for several hours or overnight and the supernatant liquid siphoned off. At this stage the remaining solution including the precipitate was transferred to a small cylinder to permit further settling of the precipitate. After 2-3 hours as much as possible of the supernatant liquid was decanted, the syrupy precipitate was transferred to 100-ml. centrifuge-tubes (usually 3) and centrifuged at first at slow speed, then more rapidly for about $\frac{1}{2}$ hour. The preliminary slow centrifuging causes a layering of the precipitate, the lower whitish layer consisting chiefly of the excess cuprous chloride, and the upper greyish layer containing the copper mercaptide. The latter was scraped away, transferred to a 1-l. beaker and stirred into about 700 ml. of the buffer solution, and 5 g. of cuprous chloride dissolved in the KCl-HCl solution were again added. The precipitate was allowed to settle, the supernatant liquid decanted and the precipitate centrifuged down as before.

The precipitate was stirred into 200 ml. of 0.1 N H_2SO_4 and decomposed with H_2S . The copper sulphide was removed by filtration with suction, the filtrate was decolorized with norite and the H_2SO_4 removed with BaCl_2 , the process being interrupted at pH about 6, thus assuring a slight excess of H_2SO_4 . The clear almost colourless filtrate was concentrated under reduced pressure to a volume of about 10 ml., cooled and filtered. The precipitate was discarded and the filtrate, after being brought to pH about 6.5 with NaHCO_3 , further concentrated by standing in a warm room or in a desiccator. Crystals of cystine sometimes appeared after several days, sometimes only after a number of redilutions and reconcentrations. The crystals assumed various forms, usually needles or minute prisms. These were filtered off, dissolved in hot dilute HCl, the solution neutralized with NaHCO_3 and again concentrated. This procedure was repeated several times before the hexagonal crystals characteristic of pure cystine could be observed. These hexagonal crystals did not appear suddenly but were preceded by a transitional stage in which the spherules of minute prisms underwent a leaf-like lobulation, each leaf gradually approaching the hexagonal form until the mass was transformed into a spherule of plates showing striations corresponding to the original crystal-line structure. After several recrystallizations the clear, hexagonal crystals appeared.

This procedure was repeated twelve times before sufficient crystals were obtained for identification. After further purification they gave, following reduction, the typical reactions of cysteine—the Sullivan reaction, the nitroprusside test and a positive test with phospho-18-tungstic acid. Optical rotation, 1 g. in 100 ml. solution (1 *M* HCl), $[\alpha]_{\text{H}_2\text{O}}^{25^\circ} = -248^\circ$.

Quantitative determination of cystine. For the quantitative determination of cystine, the method as described in the previous paper was employed, using 8 ml. of urine. To the same urine was added standard cystine solution in varying amounts, within the range found in normal urines. The results are given in Table I expressed in mols per hourly excretion, together with the percentages of

Table I. *The cystine content of normal urine by the cuprous chloride precipitation method (a) and by Shinohara's method (b), together with percentages of added cystine recovered.*

<i>cystine recovered.</i>			Cystine per hr. <i>M</i> × 10 ⁶	No. of de- termi- nations	Cystine added		% re- covered	No. of de- termi- nations
Sample	Vol. ml.	Hrs.			0.005 <i>M</i> ml.	0.001 <i>M</i> ml.		
1	450	12	<i>a</i> 7.13	3	—	—	—	—
			<i>b</i> 9.36	1	—	—	—	—
2	680	12	<i>a</i> 6.91	2	1	—	89	4
			<i>b</i> 5.95	1	—	—	—	—
3	450	12	<i>a</i> 6.79	2	1	—	88	2
			<i>b</i> 5.37	1	—	—	—	—
4	570	12	<i>a</i> 6.82	2	—	0.5	88	2
			<i>a</i> 6.82	2	—	1.0	89	2
			<i>a</i> 6.82	2	—	2.0	88	2
			<i>b</i> 6.80	1	—	1.0	41	2
5	820	12	<i>a</i> 5.50	2	—	0.5	86	2
			<i>a</i> 5.50	2	—	1.0	93	2
			<i>a</i> 5.50	2	—	2.0	92	2
			<i>b</i> 5.37	1	—	1.0	66	2
6	735	12	<i>a</i> 6.21	2	—	0.5	92	1
			<i>a</i> 6.21	—	—	1.0	92	1
			<i>a</i> 6.21	—	—	2.0	91	1
			<i>b</i> 6.10	2	—	0.5	62	1
			<i>b</i> 6.10	2	—	1.0	65	1
			<i>b</i> 6.10	2	—	2.0	64	1
Av.			<i>a</i> 6.36				89.4	15
Av.			<i>b</i> 6.49				56.5	7

added cystine recovered. For comparison determinations were made directly upon the urine by Shinohara's modification [1935] of the Folin-Marenzi method and the results are also given in the table. Six samples from the same individual on a constant diet are represented, the various samples being collected over the period from 7 p.m. to 7 a.m. on 6 consecutive days. The output of cystine determined by the cuprous chloride method averaged 6.36×10^{-6} *M* (1.53 mg.) per hour, and by Shinohara's method 6.49×10^{-6} *M* (1.56 mg.) per hour. The individual values by the former ranged from 5.50 to 7.13, and by the latter from 5.37 to 9.36 $M \times 10^{-6}$. By the cuprous chloride method, the recovery of added cystine averaged 89.4 % and by Shinohara's method, 56.5 %.

In Table II are recorded the results of a series of tests on 12 samples of urine with results expressed in mg. per 100 ml. and in mg. per hour.

(a) *Cuprous chloride precipitation* [Medes & Padis, 1936]. Ascorbic acid is not precipitated by cuprous chloride under these conditions. Glutathione (Hoffman La Roche) was subjected to the same process and failed to precipitate. Ergothioneine and uric acid, although precipitated by cuprous chloride, do not

Table II. *Determination of S-S compounds in urine by various methods.*

Subject	Medes		Shinohara		Virtue & Lewis		Medes-Sullivan		Sullivan*		Rossouw & Wilken-Jorden	
	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.
<i>a</i>	4.20	0.87	7.82	1.62	10.10	2.10	—	—	—	—	—	—
<i>a</i>	0.84	0.92	2.88	3.05	1.54	1.63	—	—	—	—	—	—
<i>a</i>	1.56	0.92	4.08	1.67	3.84	2.26	—	—	—	—	—	—
<i>a</i>	2.96	0.92	3.80	1.17	6.59	2.03	—	—	—	—	—	—
<i>a</i>	1.07	1.28	3.24	4.12	1.54	1.84	—	—	—	—	—	—
<i>a</i>	1.90	0.95	0.96	0.48	2.56	1.28	—	—	5.44	2.72	1.50	0.75
<i>a</i>	1.44	1.03	2.76	1.93	2.56	1.79	1.34	0.94	4.00	2.80	1.29	0.90
<i>a</i>	4.68	1.08	4.82	1.09	7.04	1.62	3.65	0.84	9.91	2.28	3.13	0.72
<i>a</i>	2.06	1.08	2.57	1.34	3.52	1.84	—	—	6.03	3.15	1.62	0.85
<i>b</i>	1.80	1.37	1.97	1.50	2.11	1.60	1.67	1.17	4.45	3.38	1.70	1.29
<i>b</i>	1.10	1.21	2.57	2.83	1.47	1.62	—	—	4.35	4.79	1.00	1.10
<i>b</i>	1.66	1.27	1.97	1.50	2.50	1.91	—	—	4.65	3.55	1.35	1.03
Av.	1.08 ±0.14		1.86 ±0.75		1.71 ±0.22		0.98 ±0.12		3.24 ±0.57		0.95 ±0.16	

* Procedure reported at the Kansas City Meeting of the American Chemical Society and given to the author by private communication.

produce colour with uric acid reagent in acid medium. The range of values determined for the two individuals represented in the table extend from 0.87 to 1.28 mg. per hour in one case and from 1.21 to 1.37 mg. per hour in the other.

(b) *Method of Shinohara* [Shinohara, 1935]. All SH and SS compounds of urine except ergothioneine probably respond to this test. Glutathione, if present, would be incompletely determined [Shinohara & Padis, 1936, 1]. The final value is obtained by difference between total intensity of colour produced with phospho-18-tungstic acid in urine and intensity of colour produced with phosphotungstic acid by extraneous reducers alone. In cystinuric urines, the colour developed by extraneous reducers constitutes only a small portion of the total colour, whereas in normal urines that produced by SH *plus* SS becomes the minor factor, and hence a method based on this difference is open to wide possibilities of error. In their own studies on the cystine and cysteine contents of urine, Shinohara & Padis [1936, 2] added amounts of these compounds several times greater than those occurring in normal urines. According to Table II, the hourly output of individual *a* ranged from 0.48 to 4.12 mg. and of the second subject, *b*, from 1.50 to 2.83 mg. In the case of the low value, 0.48, the addition of HgCl_2 to the blank produced a heavy cloud which could not be centrifuged out, and the colorimetric reading could only be approximate. In two instances the values are greater than determined for total S-S by the method of Virtue & Lewis. This fact, together with the general irregularity of the results indicates that the Shinohara method is of questionable applicability to normal urines.

(c) *Method of Virtue & Lewis* [1934]. According to the authors, this method applies to total S-S, and therefore should give the highest values in the table. The only exceptions are the two instances cited under Shinohara's procedure, and the series by Sullivan's method.

(d) *Sullivan's method*. The high values obtained by this method indicate that it is not sensitive enough for direct use in normal urines. The yellow colour of the blank interferes with the pale red of the test.

(e) *Method of Rossouw & Wilken-Jorden* [1935]. The values obtained by this method vary from 0.72 to 1.29 mg. per hour in the two individuals and average

81 % of the values obtained by cuprous chloride precipitation with subsequent estimation by the uric acid reagent. 82 % of the cystine added to normal urines was recovered on the average, so that it is uncertain whether the low values represent error of determination or the presence of some other compound, such as homocystine, in normal urine. That pure cystine, as indicated by its optical rotation, was isolated by the cuprous chloride technique, speaks against this possibility.

(f) *The Sullivan procedure on the filtrate as recovered by H_2S* [Medes & Padis, 1936]. The values obtained by this method are in fair agreement with those by the procedure of Rossouw & Wilken-Jorden and are open to the same considerations. In both these instances 50–100 ml. of urine must be employed and the mercaptides decomposed in 5 ml. of solution. The technical difficulties of such a procedure add to the uncertainty of interpretation of the results.

Production of colour with phospho-18-tungstic acid by oxidation products of cystine. Since in the process of recovering cysteine from its mercaptide by H_2S small amounts of decomposition products may be formed, it is of importance to ascertain the possible effect of these on phospho-18-tungstic acid. Sulphinic acid and cystine disulphoxide were tested through the courtesy of Dr Toennies and Dr Lavine. Sulphinic acid produces no colour with the uric acid reagent under the conditions of these tests and cystine disulphoxide develops colour slowly, approaching about 25 % of the colour developed by a molar equivalent of cysteine. After the first 20 min. when the colorimetric readings in our experiments are taken, only about half of the final colour is developed. The colour produced by traces of disulphoxide which might be formed would therefore be insufficient to affect significantly the final values.

Cysteine in normal urine. Since the cuprous chloride method does not distinguish between cystine and cysteine, attempts were made by Shinohara's method to ascertain if the latter was present. By Shinohara's method, cysteine is determined by difference between colours developed by freshly voided urine with phospho-18-tungstic acid, and by a sample similarly treated, but containing formaldehyde. All tests were performed within 10 min. after the urine was voided. Of 40 samples, 23 gave positive values for cysteine averaging $3 \times 10^{-7} M$ (0.036 mg.) per 100 ml.; 5 samples showed no difference between the two readings and in 12 instances the difference was negative. It seems doubtful, therefore, if cysteine is present in significant amounts in normal urines.

SUMMARY.

The cystine content of specimens of normal urine has been determined by the cuprous chloride precipitation method followed by recovery of cystine as cysteine by decomposition of the mercaptides with hydrogen sulphide and subsequent estimation of the cysteine with phospho-18-tungstic acid.

In this method cystine is differentiated from ergothioneine and glutathione. Ascorbic acid and uric acid are also excluded.

Cysteine probably is not present except possibly occasionally in traces.

The excretion of cystine, as determined by this method, averaged $5.66 \times 10^{-6} M$ per hour, which was approximately 70 % of the total S-S as determined by the iodimetric titration method of Virtue & Lewis.

Determination of cystine by the procedure of Rossouw & Wilken-Jorden gave values approximately 82 % of those by the test as developed by the authors.

Determination of cystine in a filtrate obtained by cuprous chloride precipitation, recovery with H_2S and estimation with the Sullivan reagent gave values approximately in agreement with those obtained by the Rossouw & Wilken-Jorden procedure.

The slightly lower values obtained by these two modifications of the Sullivan method leave open the possibility that some other S-S compound, such as homocystine may be present in urine in small amounts.

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