THE IODOMETRIC DETERMINATION OF CYSTINE IN THE URINE

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The iodometric titration of thiol compounds, first introduced by Rosenheim and Davidsohn (1) and modified by Klason and Carlsohn (2), was applied to the determination of cysteine and related compounds by Okuda, who studied the reaction of cysteine with iodine in acid solution (3). The reaction would be a valuable one if the conditions for the conversion of \(-\text{SH}\) to \(-\text{SS}\) (cysteine to cystine) could be controlled. In connection with certain metabolic studies concerned with the oxidation of cystine and methionine in the animal organism (4), an attempt was made to use the Okuda method but difficulty was experienced in securing satisfactory values with pure cystine solutions. A detailed investigation of the factors concerned in the more accurate determination of cystine by iodometric titration was accordingly undertaken. As a result of preliminary experiments, the following conditions were found essential: (1) low temperature (0°), (2) fairly high concentration of acid (2 per cent hydrochloric acid), (3) presence of amounts of iodine only slightly in excess of those required for the reaction. After satisfactory conditions for the method to be reported in this paper had been worked out, a similar study by Lucas and King (5) appeared in which a study of optimal conditions for accurate iodometric titrations of thiol derivatives was made. Since these workers present an adequate and detailed discussion of the literature, we have omitted further general discussion of iodometry as applied to thiol compounds. Inasmuch as Lucas and King reported the application of their titration to pure solutions only and did not make a study of the conditions as applied to urine, we are presenting our method for the determination of cystine in urine.
Determination of Cystine (Disulfides) in Urine

Reagents

1. Iodine in potassium iodide (approximately 0.01 N). 1.27 gm. of pure iodine and 2 gm. of potassium iodide are dissolved in approximately 400 cc. of distilled water. The solution is filtered into a liter volumetric flask and made up to volume. It is not necessary to make this solution exactly 0.01 N because a blank titration with the thiosulfate is made in each determination.

2. Sodium thiosulfate (0.02 N). This solution is prepared and preserved with the usual precautions. Standardization is best accomplished by the use of potassium iodate in the usual way.

3. Starch indicator. This is a 2 per cent solution of soluble starch in a saturated solution of sodium chloride.

4. Hydrochloric acid, approximately 2 per cent.

5. Hydrochloric acid, approximately 10 per cent.


Decolorization and Removal of Interfering Substances from Urine

To 25 cc. of urine in a 250 cc. beaker are added 15 cc. of 10 per cent hydrochloric acid, 10 cc. of water, and 0.6 gm. of norit. The mixture is boiled for 1 minute, allowed to cool for 10 minutes (decolorization was unsatisfactory if the cooling was omitted), and filtered through a small filter paper in a Buchner funnel. The norit is washed with 10 cc. of 2 per cent hydrochloric acid, and the filtrate and washings are transferred quantitatively with the aid of distilled water to a volumetric flask of 100 cc. capacity, which contains 3 cc. of 10 per cent hydrochloric acid. The contents of the flask are cooled in an ice bath, made up to volume with ice-cold water, and mixed thoroughly. The final concentration of the hydrochloric acid in the solution is approximately 2 per cent.

Reduction of Cystine—The contents of the flask are now transferred to a dry cylinder (250 cc. capacity),1 about 0.5 gm. of zinc dust is added, and the reduction is allowed to proceed for 30 minutes at room temperature. After reduction, the solution is filtered through a dry filter paper with suction into a dry flask, transferred to a dry cylinder, and returned to the ice bath.

While the reduction is proceeding, a series of Erlenmeyer flasks

1 We have found that the reduction proceeds more satisfactorily in a graduated cylinder than in a volumetric flask.
containing 5 cc. of the standard solution of iodine in potassium iodide is prepared and the contents are frozen in an ice-salt mixture. A blank determination is obtained by adding 15 cc. of 2 per cent hydrochloric acid to one of the flasks containing the standard iodine-iodide solution and titrating with the standard thiosulfate, starch being used as an indicator.

Preliminary Titration—As already stated, the titration with iodine is most satisfactorily made when the amount of iodine present in excess of that required for reaction with the sulfhydryl groups is not great. For this reason, a preliminary titration is carried out to determine the approximate amount of iodine required for the reaction. A definite amount of the urine after reduction (we have usually found 15 cc. a convenient volume) is pipetted from the cylinder in the ice bath into a flask containing 5 cc. of the frozen iodine-potassium iodide solution, 0.5 cc. of the starch indicator is added, and the solution is titrated with the standard thiosulfate. The thiosulfate should be added from the burette at such a rate that the blue color developed as the iodine-iodide solution melts is destroyed. The final addition of thiosulfate should be made just as the last of the iodine-iodide solution melts. This titration gives an approximation of the amount of thiosulfate required to react with the iodine not used for the reaction with —SH groups, but will be slightly less than the amount needed in the final more exact titration, since in the presence of an excess of iodine, the reaction with the sulfhydryl tends to proceed beyond the formation of the disulfide and slightly more iodine will be required.

Final (Accurate) Titration—From the burette an amount of thiosulfate slightly less (e.g., 0.05 cc. less) than that used in the preliminary titration is run into another flask containing the frozen iodine-iodide solution, starch is added, and an aliquot of the ice-cold solution (usually 15 cc.) to be analyzed is pipetted into the flask which is shaken until the ice is melted. As fast as the blue color appears, thiosulfate is added to react with the free iodine. The titration should be completed as the last of the frozen iodine-iodide solution melts. With an accurate microburette,² duplicate determinations agreeing within 0.02 cc. can usually be obtained.

² We have found the Koch microburette (Arthur H. Thomas Company) graduated in 0.01 cc. satisfactory.
Calculation—1 cc. of 0.02 N iodine solution is equivalent to 2.40 mg. of cystine or 2.42 mg. of cysteine. If 15 cc. aliquots of the solution of the reduced urine after charcoal treatment are used, the calculation is as follows: \((a - b) \times \frac{100}{15} \times 2.40\), where \(a\) is the blank titration of the iodine-iodide solution in terms of 0.02 N thiosulfate and \(b\) is the amount of thiosulfate required to titrate the excess of iodine in the unknown. The value obtained represents the mg. of cystine in 25 cc. of the urine.

DISCUSSION

The accuracy of the method was checked with solutions of pure cystine in dilute hydrochloric acid, treated as described except that the preliminary treatment with norit was omitted. The theoretical amount of 0.02 N iodine required to titrate the thiol groups resulting from the reduction of 2 mg. of cystine was 0.833 cc. The values actually observed over a range of concentration of from 0.8 to 4.0 mg. of cystine were from 0.805 to 0.850 cc. per 2 mg. of cystine, with an average of 0.835 cc. The use of the theoretical equation for the calculation of results seemed justifiable and the use of a titration curve as recommended by Okuda was considered unnecessary.

We were concerned primarily with the analysis of urine from rabbits of about 2 to 3 kilos in weight, fed a diet of oats and cabbage. After the dilution of these urines to 150 cc., the 25 cc. aliquots mentioned were used for the analysis. If the volume of the urine was greater than 150 cc., the amount of norit used for the decolorization of the 25 cc. aliquot was diminished proportionally.

The optimal quantities of norit and acid to be used in the decolorization of the urine were determined by experiments with a considerable number of urines, both human and rabbit. With human urines diluted to 1500 cc., it was found that decolorization of a 25 cc. sample could be effected satisfactorily by the procedure outlined. No satisfactory recoveries of cystine could be obtained with dog urine. Other decolorizing carbons could undoubtedly be used, but optimal conditions must be determined for them.

In a series of experiments with 20 mg. of pure cystine, the per cents of cystine recovered after treatment with 0.5, 1.0, 1.5, and 2.0 gm. of norit were 87, 66, 57, and 48, respectively. In urine
acidified as described, norit in the amount recommended appears to remove pigments and other interfering substances without loss of cystine. With larger amounts of norit, losses of cystine added to urine were observed.

In urine, reducing substances are normally present which react with considerable amounts of iodine. Treatment of the acidified urine with norit in the amount recommended removed these substances, so that no iodine was used in the titration of the filtrates obtained if reduction by zinc was omitted. These results were so consistent that titrations for cysteine (i.e., without reduction by zinc and hydrochloric acid) have been omitted except in certain feeding experiments where the presence of cysteine or a derivative was anticipated. The nature of these reducing substances is not known. The failure of normal urine to give a positive ammonium hydroxide-nitroprusside test indicates that mercapto derivatives are not concerned to any important degree.

In Table I are presented the results of a series of experiments in which the recovery of cystine added to urine was studied. The results show satisfactory recovery of cystine and require little comment. When the method was applied to normal human urine, values indicating a normal content of cystine of from 1.1 to 7.5 mg. of cystine per 100 cc. of urine were obtained. In two urines of supposedly normal individuals, higher values, 11.8 and 12.5 mg., were found. We have also determined the cystine content of cystinuric urines by this method. The cystinuric urines examined ranged from those which gave weakly positive cyanide-nitroprusside tests to definitely cystinuric urines, in which the diagnosis of cystinuria was made by microscopic identification of cystine crystals in the urine. Values ranging from 10.2 to 96.5 mg. of cystine per 100 cc. of urine were obtained.

It is realized that satisfactory recovery alone does not constitute an accurate criterion of a method. However, in view of the known relatively low concentration of cystine in normal urines, we believe that the results obtained by this method afford a more satisfactory indication of the cystine (and disulfide) content of urine than the results obtained by the application to the urine of the other common methods. It is true that the method is not entirely specific for cystine. The same objection, however, applies to the other common methods for cystine determination except that of Sullivan,
Cystine Determination in Urine

the practical application of which to urine offers difficulties. The method described has the advantages over the Okuda procedure for urine in a more clear cut end-point, more adequate control of temperature and other conditions which makes possible a stoichiometric reaction and renders unnecessary the use of a correction

**TABLE I**

Recovery of Cystine Added to Normal Rabbit Urine and to Normal or Cystinuric Human Urine

All results are averages of duplicate titrations. Aliquots of 20 cc. were titrated. Although 0.01 N iodine solution was used, since the back titrations were made with 0.02 N thiosulfate, the iodine used is calculated as 0.02 N. The first group of urines includes normal rabbit urines; the second group includes human urines, both normal and cystinuric.

<table>
<thead>
<tr>
<th>Added cystine</th>
<th>Standard thiosulfate (0.02 N) required for back titration of urine</th>
<th>Iodine (0.02 N) used by added cystine</th>
<th>Cystine recovered</th>
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<td>mg.</td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
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* Cystinuric urine.

curve, and in satisfactory removal of interfering substances without loss of cystine.

Since we wished to apply this method to the determination of cystine or related compounds containing the disulfide linkage in urine after feeding cystine and methionine to rabbits (4), it was
necessary to determine the reaction of methionine and its demethyl-
ated oxidation product, homocystine (6), in the method outlined. Methionine gave no titration value either before or after reduction. Homocystine contains a disulfide linkage; it should therefore be quantitatively determined by the proposed method. We prepared homocystine from methionine according to the procedure of Butz and du Vigneaud (6) and obtained a white crystalline material containing 23.4 per cent of sulfur (theory, 23.8 per cent), which gave a positive reaction with cyanide and nitroprusside and with the Folin-Marenzi reagent (7), but a negative Sullivan test. 18.7 mg. of this product were used for the titration, which indicated the presence of 19.0 mg., a recovery of 101 per cent. We have been particularly interested in the values obtained with methionine and homocystine, since, as reported elsewhere (4), after the ad-
ministration of methionine to rabbits, the urines contained a sub-
stance which reacted qualitatively as did homocystine, and in these urines increased iodometric values by the method outlined were also observed.

SUMMARY

A modification of the Okuda iodometric titration method for the determination of cystine (and disulfides) in urine is presented. The optimal conditions for the reaction between —SH groups and iodine have been found to be (1) low temperature (0°), (2) acid reaction (2 per cent hydrochloric acid), and (3) avoidance of amounts of iodine in any considerable excess of those required for the reaction. This method has been used successfully with rabbit and human urine (normal and cystinuric).

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