CXXVI. THE COLORIMETRIC DETERMINATION OF CYSTINE BY MEANS OF THE URIC ACID REAGENT.

By CLAUDE RIMINGTON.

*From the Biochemical Department, British Research Association for
the Woollen and Worsted Industries, Torridon, Headingley, Leeds.*

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THE colorimetric method for the determination of cystine, originally proposed by Folin and Looney [1922], is based upon the fact that cystine in the presence of sodium sulphite is reduced to cysteine, which reacts in alkaline solution with the phosphotungstic acid reagent of Folin and Denis [1912], termed by them the "uric acid reagent," giving rise to a blue colour.

The original procedure has been modified from time to time. Folin and Trimble [1924] sought to make the reagent more specific, in particular to eliminate the sensitivity towards tyrosine and other phenols, by employing a reagent from which molybdenum compounds had been substantially removed. In a more recent investigation, Folin and Marenzi [1929, 1] have discovered another defect from which the original method suffered, namely, that in order to obtain complete reaction with the cystine relatively much more of the uric acid reagent has to be added than was employed by Folin and Looney.

In this paper Folin and Marenzi also recommend adding the sulphite (to reduce the cystine to cysteine) before and not after the alkali is added, and they use a reagent from which all traces of molybdate have been removed [1929, 2].

Whilst former procedures have given only a limited degree of proportionality between cystine content and colour intensity, that now described by Folin and Marenzi shows strict proportionality over a range at least from 1 to 4 mg. of cystine.

In attempting to use this latest reagent and procedure, the writer encountered two difficulties.

- (1) Turbidity very frequently formed in both standard and test solutions.
- (2) Small inaccuracies in measuring out the saturated sodium carbonate solution appeared to produce quite appreciable changes in colour intensity.

Since the procedure appears in all other respects to be eminently suitable for quantitative colorimetric work, it was decided to make studies in the following directions.

- (1) The effect upon the colour intensity of variations in the quantity of

sodium carbonate added. In view of the marked influence of the latter, "saturated" sodium carbonate as recommended by Folin and Marenzi does not define the concentration with sufficient precision.

2. The use of sodium hydroxide, as recommended by Hunter and Eagles [1927] in place of sodium carbonate, the use of which they criticise on the grounds that on the addition of the acid reagent, carbon dioxide is liberated, which in turn liberates sulphurous acid from the sulphite thereby causing quite a large blank owing to the reduction of the phosphotungstic acid.

(3) The conditions giving maximum colour intensity with the minimum of interference from turbidity. Having had little success himself in the search for a substance inhibiting turbidity, the author wrote to Prof. Folin who drew attention to his use of urea to achieve this object, when determining uric acid, in a paper just then published [Folin, 1930].

The author is indebted to Prof. Folin for his suggestion, which, as will appear in the experimental part, was adopted with complete success in suppressing turbidity in the present investigation.

EXPERIMENTAL.

The following solutions were employed.

- (1) Uric acid reagent of Folin and Marenzi.
- (2) Standard cystine solution containing 1 mg. of cystine in 2 cc. of 0.1 *N* hydrochloric acid.
- (3) "Saturated" sodium carbonate solution, actually 3.6 *N*, also a 3 *N* solution, both made from A.R. sodium carbonate.
- (4) CO₂-free sodium hydroxide, *N* solution.
- (5) Freshly prepared 20 % solution of Merck's sodium sulphite (for reduction of cystine to cysteine).
- (6) Freshly prepared 3 % solution of Merck's sodium sulphite (for diluting to final volume).
- (7) A 20 % solution of lithium sulphate.
- (8) A 40 % solution of urea.

Tests were made using either 1 or 1.5 mg. of cystine, as stated, together with 1 cc. of 20 % sulphite, and 4 cc. of reagent, the final volume being made up to 50 cc. (unless otherwise stated). Thus, although the quantities are one half of those employed by Folin and Marenzi, the relative proportions are the same.

Influence upon colour intensity of quantity of sodium carbonate solution added.

A series of eight tests was performed, made up as shown in Table I, in which the quantity of sodium carbonate was the only factor varied. The flask to which 5 cc. of sodium carbonate had been added served as the standard against which all the others were matched. 1 cc. of lithium sulphate solution was included in each flask according to the Folin-Marenzi procedure.

Table I.

1.5 mg. cystine; final volume 50 cc.

	<i>a</i>	<i>b</i>	<i>c</i>	<i>S</i>	<i>d</i>	<i>e</i>	<i>f</i> *	<i>g</i> *
Saturated sodium carbonate cc.	1	3	4	5	6	7	8	10
Water cc. 	0	7	6	5	4	3	2	0
Colour value 	90.0	89.5	89.5	100.0	115.5	116.5	118.0	118.0

* Turbidity formed in each of these flasks whilst matching.

Folin and Marenzi recommend the use of 18 cc. of saturated sodium carbonate solution, equivalent to 9 cc. on the half-scale employed here. Although at this alkalinity the colour value tends to become constant, yet in practice it was found that turbidity invariably developed in those solutions containing 8 cc. or more of sodium carbonate. The addition of greater quantities of lithium sulphate failed to counteract this tendency.

A trial in the present instance showed that quite appreciable colour formed in a blank to which 5 cc. of saturated carbonate had been added, but a blank containing 9 cc. even after 10 minutes' standing, although turbid, remained practically without colour.

Influence upon colour intensity of quantity of N sodium hydroxide solution added.

These tests were carried out in the same way as those previously described, the flask selected as standard containing 5 cc. of *N* sodium hydroxide (Table II). Lithium sulphate (1 cc.) was added to each flask.

Table II.

1.5 mg. cystine; final volume 50 cc.

	<i>a</i>	<i>b</i>	<i>S</i>	<i>c</i>	<i>d</i>	<i>e</i> *	<i>f</i> *	<i>g</i> *
<i>N</i> Sodium hydroxide cc.	1	3	5	7	8	8.5	9	10
Water cc. 	0	7	5	3	2	1.5	1	0
Colour value 	99.5	100.5	100.0	100.0	105.0	136.5	149.0	170.0

* Turbidity formed in each of these flasks.

To make 4 cc. of reagent alkaline to phenolphthalein 7.3 cc. *N* sodium hydroxide solution were required.

The general inference to be drawn from the two sets of results is that high colour intensity is not to be obtained, either with sodium carbonate or hydroxide, without accompanying liability to turbidity. In the case of the sodium hydroxide experiments, it was impossible to follow the colour value any further owing to the immediate clouding of the solutions. It is presumed that it would tend to become constant as in the case of sodium carbonate.

It is also clear that colour formation, due to the interaction of cysteine and reagent, takes place in acid solution although, since the colour developed is less than in alkaline solutions and the blank is considerably larger, this

region is not suitable for quantitative work. It will be noted that in the case of the sodium hydroxide experiments the increase in colour value occurs immediately the reaction becomes alkaline (7.3 cc. alkali).

The equivalent of sodium carbonate solution is 2 cc., however the increase did not occur until over 4 cc. had been added. Evidence obtained later on, however, showed that here also as soon as the p_H of the complete reaction mixture exceeded 7.0, greater colour intensity resulted.

Alkaline solutions are desirable in cystine determination provided turbidity can be avoided. It is not permissible to purchase stability by cutting down the alkali since, almost immediately, a range is reached where colour intensity varies directly with alkali added. In this connection it is difficult to see how Hunter and Eagles could have obtained very satisfactory results when using sodium hydroxide as alkali.

Before passing to the question of turbidity, the following experiments may be recorded which demonstrated that dilution and actual volume during colour formation do not untowardly affect final colour values. In addition, it was found that small variations in the quantity of reagent (from 4 cc. to 1.5 mg. cystine) are without effect.

(1) Tests were compared, each containing 1.5 mg. cystine, but brought to final volumes of 25 cc. and 50 cc. respectively. The weaker at 40.5 matched the stronger set at 20.

(2) Colour development was allowed to take place in similar tests, one having had 5 cc. of water added making volumes of 17 cc. and 22 cc. respectively. Diluted to 50 cc. each, the one at 21 matched the other set at 20.

The suppression of turbidity.

As stated above, it was found impossible to work with sodium carbonate in the range giving maximum colour intensity owing to the development of turbidity. Investigation showed that it was the sodium salt of the phospho-17-tungstic acid described by Wu [1920] which was being precipitated in the form of minute, glistening, rectangular platelets.

In the following experiments "saturated" sodium carbonate was replaced by a solution of exactly specified concentration. 3 *N* was selected as being suitable since this is near to saturation but sufficiently far removed to obviate the possibility of crystallisation occurring during winter months.

A mixture containing 2 cc. of cystine solution (1 mg.), 4 cc. of reagent, 5 cc. of 3 *N* sodium carbonate solution and the other reactants in their customary proportions has a p_H , measured by the hydrogen electrode, of 6.9. Inflection of the curve relating quantity of sodium carbonate and colour intensity occurs at this point.

The experiments (see Table III) illustrating the action of urea, referred to above, in suppressing the precipitation of the interfering sodium phospho-17-tungstate, also serve to show that urea, in varying quantity, does not affect

the final colour developed, and it was speedily found that all trouble could be eliminated by using 5 cc. of a 40 % aqueous solution added to the flasks immediately prior to the reagent.

Table III.

1.0 mg. cystine; final volume 25 cc., 8 cc. of 3 <i>N</i> sodium carbonate to each; no lithium sulphate added.						
	<i>S</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	
40 % urea solution (cc.) ...	5	4	3	2	1	
Water (cc.) ...	0	1	2	3	4	
Colour value ...	100.0	101.5	102.5	98.0	*	
Time after mixing before (Not in turbidity develops (mins.) 18 hours)		30	20	15	<5	

* Almost immediate development of turbidity prevented any accurate comparison being made with this solution.

A complete determination should thus be carried out in the following manner.

To 2 cc. of cystine solution, or volume equivalent to about 1 or 1.5 mg. cystine, add 1 cc. of 20 % sodium sulphite (Merck's) and wait 1 minute.

Add 8 cc. of 3 *N* sodium carbonate (A.R.) solution, 5 cc. of 40 % urea solution, mix and run in 4 cc. of Folin and Marenzi's uric acid reagent.

After 5 minutes make up to 25 cc. (or 50 cc. if preferred) with 3 % sodium sulphite (Merck's) solution and proceed with the colorimetric comparison.

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