LXXXVII. SULLIVAN'S REACTION FOR THE QUANTITATIVE DETERMINATION OF CYSTEINE AND CYSTINE.

By JOSEPH W. H. LUGG.

*From the Division of Animal Nutrition of the Commonwealth Council for Scientific
and Industrial Research, University of Adelaide, South Australia.*

(Received March 28th, 1933.)

SULLIVAN [1926] has adduced reasons for believing that his colour test for cysteine, depending upon the production of a red-coloured soluble complex of cysteine and sodium 1 : 2-naphthoquinone-4-sulphonate, is specific for cysteine among the sulphydryl compounds. Various substances form coloured complexes with the quinone, but Sullivan found that in the presence of cyanide under conditions referred to later, the complex with cysteine was the only one that would withstand subsequent treatment with $\text{Na}_2\text{S}_2\text{O}_4$.

In later communications Sullivan [1929, 1, 2] develops his methods, explains his preference for the "sulphite modification," and briefly mentions the effects of the buffering capacity of a hydrolysate.

Sullivan's procedure for the estimation of cysteine consists essentially in allowing the cysteine to react with the reagent in alkaline solution. Subsequently sulphite and more alkali are added and are followed after the lapse of from 10 to 30 minutes by an alkaline solution of sodium hydrosulphite. After the addition of the $\text{Na}_2\text{S}_2\text{O}_4$ the red colour due to cysteine is retained whilst colours due to other amino-acids are changed to yellow. Sullivan advocates addition of a small quantity of cyanide before the reagent "to check oxidation" of the cysteine, and states that the addition of a little more cyanide after the hydrosulphite will "stabilise" the colour. The procedure for cystine consists in first treating the solution with plenty of cyanide and then proceeding as for cysteine. Sullivan believed that the cyanide "reduced" the cystine to cysteine to a varying degree depending upon the amount of cystine present, and that a cysteine unknown must therefore be compared with a cysteine standard similarly prepared, and a cystine unknown with a cystine standard.

Sullivan's procedure provides a valuable colour test for cysteine, but experience of his quantitative methods in this laboratory has not been satisfactory. Thus it was found: (1) The depth of red colour is variable and far from proportional to the amount of cysteine or cystine present. Rimington [1929] had already noted this poor proportionality and advised having the unknown within 15 % of the standard in an estimation. (2) Even when compared with a standard of nearly the same colour, the results for the unknown are often irregular. (3) The presence of relatively considerable amounts of other amino-acids diminishes the amount of red colour due to cysteine or cystine, and the yellow colour due to the other amino-acids interferes when a pure cysteine or cystine standard is used for comparison. The diminution in red colour was found to be

attributable in part to the buffering effects of the amino-acids present upon the p_H of the solution¹.

Two directions in which the accuracy of the method might be improved suggested themselves: (1) to maintain the same p_H in standard and assay, and (2) to swamp both standard and assay with a considerable amount of amino-acid.

It was found possible to effect both objects by adding glycine with appropriate amounts of alkali, and by this means, two of the most serious drawbacks to Sullivan's procedure (unreliability and lack of proportionality) were overcome.

It was found that the reaction between the cystine and cyanide was the double decomposition:

discovered by Pulewka and Winzer [1928]. Under conditions that may be varied considerably, this reaction proceeds so nearly to completion that any deviation from it cannot be detected colorimetrically.

Reagents required.

A. N NaOH solution.

B. M Glycine buffer in $0.4 M$ tertiary sodium citrate². 7.50 g. of glycine and 14.3 g. of the salt $\text{C}_6\text{H}_5\text{O}_7\text{Na}_3$, 5.5 H_2O , are dissolved in 50 cc. of water + 25 cc. of solution A and diluted to 100 cc.

C. M NaCN solution in $0.1 N$ NaOH solution.

D. $0.02 M$ Solution of sodium 1 : 2-naphthoquinone-4-sulphonate.

E. $0.5 M$ Na_2SO_3 solution in $0.5 N$ NaOH solution.

F. $2.5 N$ NaOH solution.

G. $0.1 M$ $\text{Na}_2\text{S}_2\text{O}_4$ solution in $0.5 N$ NaOH solution.

Solutions B and C may not be mixed and stored as one, because a slow reaction proceeds, leading to the development of a brown colour not removed by $\text{Na}_2\text{S}_2\text{O}_4$. Solutions A, B, E and F may be stored for months in well-stoppered bottles. Solution C may be kept for several weeks. Solutions D and G must be prepared immediately before they are required.

THE DETERMINATION OF CYSTEINE AND CYSTINE IN PURE SOLUTIONS³.

(a) *When either cysteine or cystine alone is present.*

An aliquot (a cc.) of the unknown solution containing preferably between 1 and 2 mg. of cystine or 0.5 and 1 mg. of cysteine, is titrated with b cc. solution A (or F if necessary) to p_H 9.5 using thymol blue indicator. This mixture is titrated with solution F (c cc.) to about p_H 12.5 using orange G.G. (alizarin yellow R is hardly satisfactory because its range does not extend much above p_H 12.0).

5 cc. of solution B and 2 cc. of solution C are transferred to a 25 cc. stoppered cylinder; b cc. of solution A (or F, if used) are added and followed by $5 - a - b$ cc. of water. The contents are mixed and a cc. of the unknown solution are added. The contents are thoroughly mixed and are then allowed to react for 10 minutes. Solution D is made up during this interval and 1 cc. of the fresh solution D is added to the contents of the cylinder, which are again mixed. After a further interval of 5 minutes, 5 cc. of solution E are added and mixed and are followed

¹ The work recorded in this article was performed at temperatures in the vicinity of 25° .

² The presence of citrate in solution B is desirable when calcium salts occur in the unknown. The amount may be increased or diminished if desired.

³ Various steps in the procedures are explained in the discussion.

by $1 - c$ cc. of water and then by $5 + c$ cc. of solution F. The cylinder's contents are again thoroughly mixed and allowed to react for 20 minutes. 5 minutes before this period is completed, solution G is prepared and at the conclusion of the 20-minute interval, 1 cc. of solution G is added, and the contents of the cylinder are again mixed. After standing for 10 minutes, the solution is ready for colorimetric comparison with a cystine standard similarly prepared at the same time and between 0.7 and 1.5 times as intense in colour. The maximum safe red colour intensity is that obtained with 3 mg. of cystine. Solutions must be allowed to stand in the colorimeter cups with plungers immersed approximately to the right depths for at least 3 minutes before final readings are taken, and these should be completed within 30 or 40 minutes of adding solution G. With the standard containing s mg. of cystine and set at y mm. in the colorimeter and with the unknown matching at x mm., the unknown will contain sy/x mg. of cystine, or $121sy/240x$ mg. of cysteine.

The above procedure has been found satisfactory for estimating cysteine or cystine in pure solution to within 1 or 2 %. The final colour solutions which ordinarily have a volume of 25 cc. cannot safely be diluted to a different volume because of the risk of oxidation. For this reason, the different reagents should be pipetted accurately into the cylinders.

(b) *When both cystine and cysteine are present.*

Except by recourse to the somewhat unsatisfactory procedure of blowing air through a mixed cysteine-cystine solution first rendered slightly alkaline at p_H 7.5-8, to oxidise the cysteine present, it has not been found possible to estimate the two amino-acids when present together. If the red colour from an aliquot of the unoxidised solution corresponds with u mg. of cystine, and the colour from an equivalent aliquot after aerial oxidation corresponds with v mg., then the actual amounts present are $(u - v)$ 121/120 mg. of cysteine and $2v - u$ mg. of cystine.

DETERMINATION OF CYSTEINE AND CYSTINE IN PRESENCE OF OTHER SUBSTANCES.

The methods are in every way the same as those already given for the determinations in pure solutions. Errors may arise from two causes. (1) Substances present in the unknown solution may interfere with the colour reaction. (2) The initial colour of the unknown solution may contribute towards the final colour to be read.

Sullivan has dealt very thoroughly with the question of specificity. He claims that the cysteine colour reaction is most probably confined to compounds containing the group $-\overset{|}{\text{C}}(\text{SH})-\overset{|}{\text{C}}(\text{NH}_2)-\text{COOH}$.

His conclusions concerning the interference of iron salts may be amplified somewhat. Ferric salts may interfere badly in the estimation of cystine. If added before the cystine and allowed to stand with the buffer and cyanide, they may cause only slight loss of colour. If added after the cystine, however, the loss may be very serious. In the table of results given below, this contrast in effects is very striking. Unfortunately, it has not been found possible to eliminate these effects and on this score there will always be some degree of uncertainty in reporting the cystine contents of solutions containing unknown amounts of ferric salts. With ordinary tissue hydrolysates, this error will be unlikely to exceed 1 or 2 %. Ferric salts form a complex with cysteine which is later liberated in the oxidised form whilst the iron appears in the reduced form. The cystine so formed can then react with more cyanide to form cysteine and

RSCN, and these processes will continue with gradual conversion of the cysteine or cystine into RSCN until all the ferric iron is reduced or all the cystine is converted into RSCN. This is probably the explanation of the effects mentioned above. The iron could also act as a carrier between the cysteine and atmospheric oxygen, if present.

In so far as many heavy metal salts are unlikely to be present in solutions to be tested, their effects have not been investigated in detail. Had it been found that zinc salts would not interfere, a simpler procedure depending upon reduction by metallic zinc might have been evolved for the estimations of cysteine and cystine when present together. One-tenth of a millimol of ZnSO_4 added after the cyanide and before the cystine caused a loss of about 20 % in colour and a rapid fading later.

Errors of type (2) may sometimes be eliminated by colorimetric compensation. The colour of a hydrolysate due to humin may sometimes be removed by adsorbents without appreciable loss of cystine, if particular precautions are taken. The brown humin colour, contrary to what might be expected, is not appreciably bleached in any stage of the colour reaction. Blanks for use in compensating colorimeters may be prepared if required. One is made from the standard cystine solution and one from the unknown by introducing 0.1 cc. of M FeCl_3 solution 5 minutes before adding the quinone reagent. Alternatively, the blanks may be made to contain no iron by omitting the quinone reagent and substituting pure water.

Tissue hydrolysates may sometimes give slightly cloudy colour solutions, particularly following the addition of the hydrosulphite. It is essential that the solutions be crystal clear for accurate comparison, and in such cases the colour solutions (standard as well as cloudy unknown) should be filtered through rapid paper with the least possible agitation before comparison.

ANALYTICAL RECOVERY OF CYSTINE FROM ADDED SUBSTANCES.

A gelatin and a gelatin *plus* carbohydrate hydrolysate were prepared by hydrolysing 5 g. of acid-washed gelatin, and 5 g. of acid-washed gelatin *plus* 1 g. of filter-paper cellulose *plus* 1 g. of sucrose, separately each with 50 cc. of $5N$ HCl solution. After remaining for 24 hours at the boiling-point the solutions were filtered, evaporated to syrups upon the water-bath, diluted to 15 cc. with water and brought to p_H 2.5 (spot indicator) with M tertiary sodium citrate solution (about 25 cc.). At this p_H the humins generally are least soluble. After standing for 48 hours, the solutions were filtered and diluted each to 50 cc. These solutions constitute the A 1 (gelatin only) and B 1 (gelatin *plus* carbohydrate) referred to in Table I. From each of these solutions, 25 cc. were taken and treated with 1 g. of acid-washed kieselguhr and then after filtration with two successive portions each of 0.5 g. of acid-washed fine norite charcoal. The solutions were filtered after each treatment with norite and constitute finally the A 2 and B 2 of Table I. Neither cysteine nor cystine could be detected in A 2 or B 2. A 1 and more particularly B 1 did not give pure yellow blanks in the cysteine and cystine tests, but the amount of brown colour was very small and presumably owed its origin entirely to the initial brown colour of the solutions. The initial brown colours of A 1, B 1, A 2 and B 2 have been compared colorimetrically with a fresh ferric ferri cyanide solution made by adding 5 cc. of $M/20$ FeCl_3 solution to 5 cc. of $M/30$ $\text{K}_3\text{Fe}(\text{CN})_6$ solution. Matchings were not perfect by any means. The brown colour intensities in terms of this standard were approximately 0.5, 1.2, 0.01, and 0.08 respectively.

Table I.

Extraneous material added		Cystine present mg.	Cystine estimated mg.	% recovery
1 cc. A 2.	Just before the cystine	2.00	2.03	101.5
1 cc. A 2.	"	1.00	1.01	101.0
2 cc. A 2.	"	1.00	1.025	102.5
1 cc. B 2.	"	2.00	2.00	100.0
1 cc. A 1.	"	2.00	1.98	99.0
1 cc. B 1.	"	2.00	1.93	96.5
1 cc. B 1.	"	1.00	0.955	95.5
100 mg. arginine.	Just before the cystine	1.05	1.07	102.0
10 mg. tyrosine.	"	1.00	1.015	101.5
1/300 millimol FeCl ₃ .	5 min. before the cystine	2.00	1.83	91.5
1/150	" "	2.00	1.68	84.0
1/30	" "	2.00	1.15	57.5
1/150	" 5 min. after the cystine	2.00	0.53	26.5
1/30	" "	2.00	Trace	?
1/12	" 5 min. after the cystine	(1.00)	(0)	0

The effects of varying amounts of these hydrolysates and of a few pure amino-acids upon the estimation of cystine are recorded in Table I. Readings were made with the aid of an ordinary Klett (non-compensating) colorimeter. The results demonstrate the applicability of this method to the estimation of cystine in hydrolysates, when the cystine amounts to approximately 2, 1, or even 0.5 % of the amino-acids present, and when the decomposition products of a considerable amount of carbohydrate are present also. At the same time, they reveal a tendency to underestimate slightly the cystine present when humin substances occur in a solution. Mercaptans generally condense with aldehydes and ketones and the loss might be accounted for on these grounds. Clearly it is not always essential to decolorise a hydrolysate or to employ a compensating instrument with appropriate blanks.

DISCUSSION.

During the reaction between the cystine and cyanide, the p_H of the solution is approximately 9.5 in the procedure given, and colour development in the final period takes place at about p_H 12.5. At this higher p_H cystine undergoes some decomposition, but if the p_H is lowered by only 0.5 unit the amount of red colour is diminished 20–30 %. Further, if the reaction between the cystine and cyanide takes place at p_H 12.5, little colour develops on subsequently adding the quinone and other reagents, and what develops fades rapidly. For these reasons it is necessary to control the p_H in the two regions, such control being achieved with the aid of the glycine buffer.

If the amount of cyanide is halved, the red colour due to 1 mg. of cystine is diminished by about 25 %; if doubled or trebled the final colour solutions become brown transiently upon slight agitation in air. The canary-yellow colour obtained in the absence of cystine and cystine varies more or less directly with the amount of quinone present. If the quinone be reduced to half, the loss of red colour developed by cystine is not appreciable unless more than 3 mg. of cystine are present. On the other hand, the colour developed by cystine is unaccountably reduced if the amount of quinone be much increased.

In consequence of this anomalous behaviour, an attempt to derive the stoichiometric relation between cystine and the quinone colorimetrically could not lead to definite results, the more so because of the recognised instability of the quinone. The indications from colour readings with varying excesses of cystine suggested that two molecules of cystine react with one of quinone.

Employing Sullivan's procedure with 1 mg. (1/240 millimol) of cystine the red colours obtained with (approximately) 1/50 millimol of the quinone are of the same order, whether 1/5 millimol of mixed amino-acids are present or not. As all these amino-acids can react with the quinone, one is led to conclude either that the group or groups involved in the reaction with cysteine are not the same as those involved in the reaction with other amino-acids, or failing that, that the cysteine complex will always be formed at the expense of any other. The first alternative is supported by the known influence of $\text{Na}_2\text{S}_2\text{O}_4$ upon the colours produced. Amino-acids almost certainly react at the sulphonate group in the reagent in the ordinary amino-acid reaction [but see Folin, 1922], leaving the carbonyl groups for reduction by $\text{Na}_2\text{S}_2\text{O}_4$ in alkaline solution or by sulphite at p_{H} 6-7, whereas presumably, the cysteine reacts in some way with the carbonyl groups which cannot then be attacked readily by $\text{Na}_2\text{S}_2\text{O}_4$ or acid sulphite.

The colour solutions bleach slowly on standing in absence of air and turn brown (transiently if excess $\text{Na}_2\text{S}_2\text{O}_4$ is present) when agitated with air. In the early stages, the fading is sensibly proportional to the cysteine or cystine content and, therefore, does not lead to errors in comparison. The amount of hydro-sulphite may be increased to check aerial oxidation with consequent browning, but only at the expense of increased fading of the red colour from cysteine.

Both sulphite and cyanide react with cystine in such manner that for each molecule of cystine, one molecule of cysteine and one of something else are produced¹. It would, therefore, appear that sulphite or cyanide alone would suffice for the colour reaction. With sulphite alone, very little colour develops, whereas with cyanide alone the full colour is obtained with pure cystine solutions, and addition of sulphite, as in the procedure given, may be regarded as a pre-cautionary measure against adventitious oxidation.

SUMMARY.

Sullivan's colour test for cysteine has been examined with the object of improving his procedure for estimating cysteine and cystine in solutions containing these amino-acids. A method has been evolved which permits the colorimetric estimation of cysteine or cystine (to within 1 or 2 %) in pure solution. The same method has been applied successfully to the estimation of cystine in the presence of large quantities of other amino-acids and of the hydrolysis products of carbohydrates. Some of the possible sources of error have been discussed.

I wish to record my indebtedness to Sir C. J. Martin, at whose suggestion this work was done, for his notes on earlier experiments with Sullivan's reaction and for his criticisms and assistance.

¹ In a previous communication [Lugg, 1932], I discussed the reaction with sulphite. At the time it was submitted for publication, I was not aware that Clarke [1932] had arrived at the same conclusions.

REFERENCES.

- Clarke (1932). *J. Biol. Chem.* **97**, 235.
Folin (1922). *J. Biol. Chem.* **51**, 377.
Lugg (1932). *Biochem. J.* **26**, 2160.
Pulewka and Winzer (1928). *Arch. exp. Path. Pharm.* **138**, 154.
Rimington (1929). *Biochem. J.* **23**, 41.
Sullivan (1926). *U.S. Pub. Health Reports*, **41**, No. 22, 1030.
— (1929, 1). *U.S. Pub. Health Reports*, **44**, No. 24, 1421.
— (1929, 2). *U.S. Pub. Health Reports*, **44**, No. 27, 1599.

OLD SY