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Studies on the Origin of the Sulphur in Wool.

I. A Study of the Sullivan Technique for Cystine.

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THE solution of biological and nutritional problems centering around sulphur metabolism has been appreciably advanced, since Sullivan's (1926) discovery that the reaction with 1,2-Naphthoquinone-4-Sodium sulphonate under certain conditions constitutes a highly specific reaction for cysteine or cystine. Admittedly, the other methods such as the iodometric methods of Okuda (1925) and of Baernstein (1930), and the colorimetric method of Folin and Marenzi (1929) may sometimes be applied with advantage when dealing with relatively pure solutions of amino-acids or protein hydrolysates; their non-specificity, however, limits their general application, especially when dealing with more complex biological material.

The accuracy of the Sullivan technique, as a quantitative method, has however been criticised by various workers, and it is to be admitted that such criticism has for the greater part shown itself to be justified. The Sullivan technique as described by Sullivan himself contains several weaknesses and inherent errors. To circumvent these difficulties various modifications of Sullivan's original procedure have been suggested. Apparently none of these suggested modifications can be claimed to be based on a systematic study of the Sullivan technique. In the present paper an endeavour will be made to give experimental data to indicate along what lines the Sullivan procedure may be modified with advantage. On the other hand, certain objections to the method, such as the interference of other amino acids, radicles, ions and adventitious colouring matter, cannot be avoided by modification of the method as such. This aspect of the matter will be dealt with in a later publication, when a new micro method for the determination of cystine or cysteine in biological matter, depending on the quantitative isolation of cystine as cysteine cuprous mercaptide with subsequent determination of the cystine by the Sullivan method, will be described.

The chief difficulty with the original Sullivan method would seem to lie in the application of the cysteine reaction to the determination of cystine. Sullivan (1926) seems to have come to the conclusion that the degree of correspondence of colour intensity between cystine and cysteine varied with cystine concentration, and that the reduction of cystine indicated only 50-75 per cent. of the theoretical. This statement would appear to have caused considerable confusion. It must not be taken to signify that up to 75 per cent. of a given amount of cystine can be transformed to cysteine by the action of cyanide or sulphite. As Pulewka and Winzer (1928) have shown, one molecule of cystine reacts with cyanide to form only one molecule of cysteine, according to the equation:—

$$R-S-S-R + NaCN \rightarrow R-SNa + R-SCN$$

Similarly Clarke (1932) has shown that from one molecule of cystine only one molecule of cysteine is obtained by the action of sulphite:—

$$R-S-S-R + Na_2SO_3 \rightarrow R-SNa + R-SSO_3Na$$

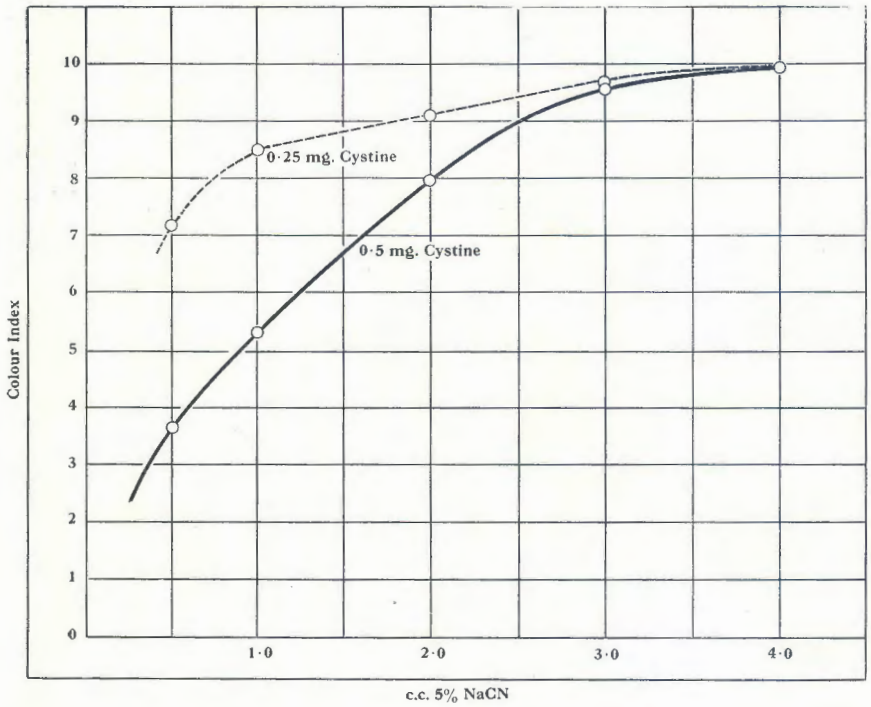
It is clear, therefore, that reduction by this means cannot transform more than 50 per cent. of the cystine to cysteine. Sullivan's findings can therefore be interpreted only as signifying that the above reactions proceeded to the extent of 50 to 75 per cent., i.e. that one molecule of cystine produced only 0·5 to 0·75 of a molecule of cysteine.

In this connection it is interesting to note that Lugg (1933) states that, even if the conditions be varied considerably, the above reaction with cyanide proceeds so nearly to completion that any deviation from it cannot be detected colorimetrically. Lugg gives no details as to which conditions may be varied, but states further on in his paper that if the amount of cyanide is halved, a 25 per cent. loss in colour intensity occurs, while if the cyanide be doubled or trebled the final colour gives a transient brown on agitation in air.

It is therefore necessary that we should consider the influence of cyanide concentration on the reduction and resultant colour development more carefully. To 0·25 mg. and 0·50 mg. respectively of cystine contained in suitable aliquot volumes, increasing amounts of cyanide were added, the volumes of the mixtures being kept constant at 9·0 c.c. After 10 minutes 1·0 c.c. of 0·5 per cent. of the fresh naththoquinone reagent was added, the solution well mixed, and 25 seconds later 5 c.c. of 10 per cent. anhydrous Na_2SO_3 in 0·5 N NaOH solution added. The solution was mixed and left to stand for 30 minutes. Finally 1·0 c.c. of a 1-2 per cent. $Na_2S_2O_4$ in 0·5 N NaOH solution was added, and the solutions compared colorimetrically, using the solution containing 2·0 c.c. 5 per cent. NaCN as standard. The results have been represented graphically in Fig. 1.

Fig. 1.

EFFECT OF NaCN ON COLOUR DEVELOPMENT.



As will be seen from the figure, the curves for both the 0·25 and 0·50 mg. cystine show a strong tendency to run horizontally when a cyanide concentration of 4·0 c.c. 5 per cent. NaCN per 9·0 c.c. solution is reached.

In order to show that at this cyanide concentration the reaction

$$R-S-S-R + NaCN \rightarrow R-SNa + R-SCN$$

had, for all practical purposes, attained completeness, a cystine standard containing 0·40 mg. cystine was compared colorimetrically with two cysteine standards each containing 0·205 mg. of cysteine, the one treated precisely in the same way as the cystine standard, and the other according to the Sullivan procedure for cysteine (with the exception that the time factor of 25 seconds was observed). No measurable difference in colour intensity of the three solutions could be obtained. In addition to this it was shown that for this higher cyanide concentration a variation in the time interval from 5 to 15 minutes produced no measurable effect on the ultimate colour intensity.

If, then, the addition of 4·0 c.c. 5 per cent. NaCN liberates 1 molecule of cysteine from one molecule of cystine, Sullivan's findings with 1·0 or 2·0 c.c. 5 per cent. NaCN may easily be explained. As the 0·5 mg. cystine curve shows, the use of 1·0 c.c. cyanide would

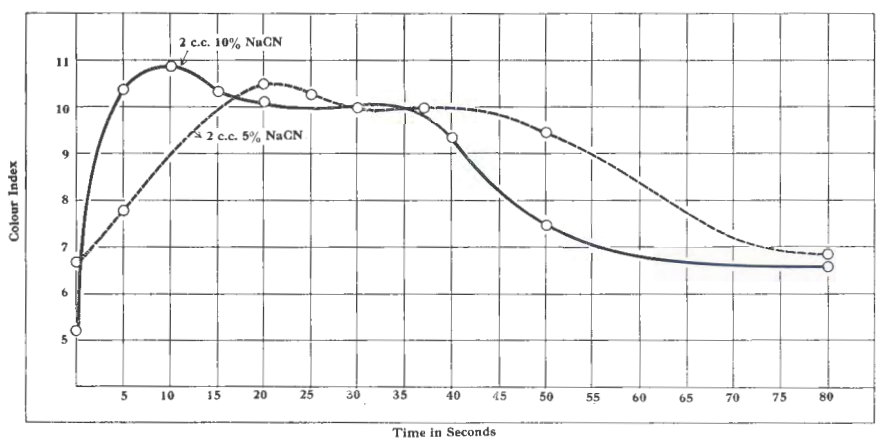
result in the liberation of just over 0·5 molecule cysteine, whereas the use of 2·0 c.c. cyanide yields about 0·8 molecule cysteine. In the 0·25 mg. cystine curve the cysteine yields for 1·0 and 2·0 c.c. cyanide are respectively appreciably higher. Thus, when working under conditions of cyanide deficiency, Sullivan is essentially correct in stating that the correlation in colour intensity between cystine and cysteine depends on the amount of cystine present. Theoretically, of course, any of the cyanide concentrations mentioned should lead to correct assays when all the material is present as cystine, provided both assay and standard are treated in exactly the same way. In practice, however, the difficulty of duplicating any set of conditions increases with the steepness of the curve, and for this reason alone it would be desirable to work at a cyanide concentration represented by 4·0 c.c. 5 per cent. NaCN, or what is more practical, 2·0 c.c. 10 per cent. NaCN.

Apart from the influence of cyanide concentration on the completeness of the cyanide-cystine reaction, there is another step in the Sullivan procedure which may lead to serious errors. Sullivan gives no indication that the time interval between the adding of the naphthoquinone reagent and the alkaline sulphite must be carefully controlled. In Fig. 2 the colour intensity has been plotted against this time interval in seconds, using 0·40 mg. of cystine. Both the 2·0 c.c. 10 per cent. NaCN curve and the 2·0 c.c.

Fig. II.

Effect of Napthoquinone Sulphite.

Time Interval on Colour Development.



5 per cent. NaCN curve show the same peculiar form; the only difference would appear to lie in the acceleratory influence of the higher cyanide concentration, thereby shifting the higher cyanide curve to the left. It should be observed, however, that when the napthoquinone-sulphite time interval is below a certain minimum, depending on cyanide concentration, the curves are steep. Thus, if we were to adopt Sullivan's original procedure of adding the sulphite

immediately after adding the naphthoquinone and mixing, we would invariably be working on this steep part of the curve. It is due mainly to the failure of Sullivan to stipulate the control of this time factor, that his method, as applied to relatively pure solutions has been found to give irregular results, as stressed by Rimington (1929), Prunty (1933), and Lugg (1933).

Apparently Csonka (1932) was the first to suspect the existence of a time factor at this stage of the process, since in his modification of the Sullivan technique he stipulates that after adding the naphthoquinone reagent the solution be shaken for 10 seconds before adding the alkaline sulphite. Lugg (1933) on the other hand employed a time interval of no less than 5 minutes. Although it would appear that Lugg was working on a fairly horizontal part of the curve, the employment of a 5-minute time interval is not to be recommended, chiefly for two reasons. In the first place Lugg was working under conditions where the final colour intensity fell far below the optimum maximum intensity; and in the second place his 5-minute time interval would seem to make the whole process too susceptible to variations in cyanide concentrations. In this connection it should be observed that Lugg adds 2·0 c.c. of molar or 4·9 per cent. NaCN solution to a mixture of about 16 c.c. of cystine and buffer mixture. Under such conditions it is inconceivable that one molecule of cysteine can be freed from one molecule of cystine, unless the buffer solution exercises a strongly positive catalytic acceleration of the reaction. In spite of this Lugg reports that doubling or trebling the amount of cyanide adversely affects the final colour tint obtained.

In contrast to Lugg's observations it was found that the colour tint is in no way impaired by adding 2·0 c.c. 10 per cent. NaCN to 5·0 c.c. cystine solution, provided the time interval between naphthoquinone and sulphite is regulated to 20-30 seconds. As a matter of fact the choice of this time interval is strongly suggested by the curve in Fig. 2, as representing the most suitable conditions for obtaining the optimum maximum colour development.

The method as ultimately adopted in this laboratory is as follows:—

5 c.c. of standard cystine solution, slightly acid with HCl and containing 0·4 mg. of cystine.

2 c.c. 10 per cent. aqueous solution of NaCN. Mix and wait 10 minutes.

1 c.c. of a 0·5 per cent. naphthoquinone solution. Mix and allow 20 seconds.

5 c.c. 10 per cent. sodium sulphite in 0·5 N NaOH. Mix and allow 30 minutes.

1 c.c. 1-2 per cent. sodium hydrosulphite solution in 0·5 N NaOH.

Mix and compare colours after being left in the colorimeter cups for about three minutes. All the reagents must be freshly prepared, especially the naphthoquinone and the sodium hydrosulphite.

Apart from the factors already discussed various other objections have been raised against the Sullivan method. For the greater part all these objections centre around the influence of substances and ions other than cystine in solution on the ultimate intensity of colour development.

Thus Lugg (1933) states that the presence of other amino acids in relatively large quantities diminish the colour obtained, while the yellow colour given by these amino acids after addition of hydrosulphite ($Na_2S_2O_4$) tends to interfere with the colorimetric comparison. This interference on the part of other amino acids has been verified here, as the results in the table below clearly show.

Influence of Amino Acids on Cystine Colour Development.

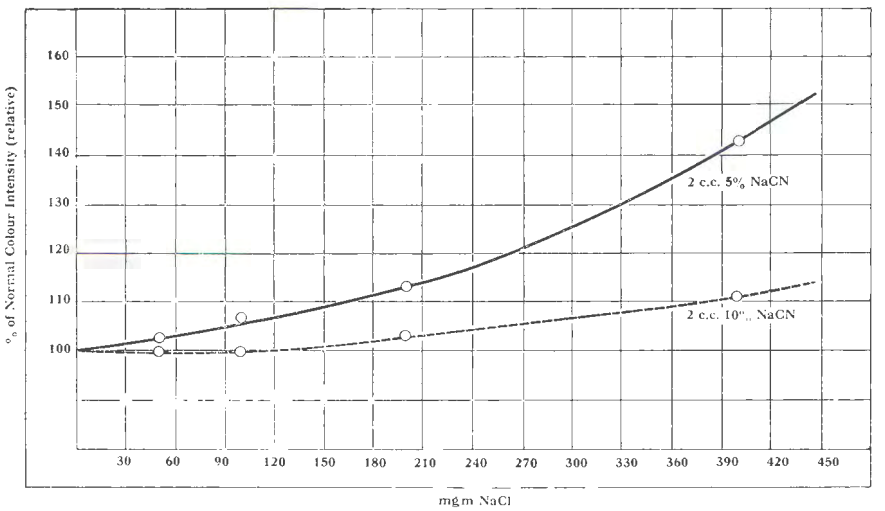
Amino Acids + 0·4 mg. Cystine.	Percentage Normal Colour.	
	50 × wt. of Cystine.	100 × wt. of Cystine.
Glutamic acid hydrochloride	97	—
Histidiue hydrochloride	—	95
Alanine	—	94
Tyrosine	87	83
Tryptophane	90	81
Aspartic acid	85	71

Thus Lugg in his method " swamps " both assay and standard with glycine. The soundness of the principle of such a procedure would seem rather doubtful, since it involves the adding of an excess amount of interfering material in order to counterbalance the influence of interfering material already present.

Apart from the presence of other amino acids, the solutions in which the cystine is to be determined usually contain smaller or larger amounts of sodium chloride or sulphate depending on the cystine content of the original material and the amount of acid used for hydrolysis. In one of his later publications Sullivan (1929) notes that the larger amounts of sodium chloride may lead to appreciable errors. In this connection it would appear that sodium chloride has both an acceleratory and intensifying action. According to the curves in Fig. 3 the action of sodium chloride is not very marked when employing 2·0 c.c. 10 per cent. NaCN for the reduction of cystine to cysteine.

Fig. 3.

INTENSIFYING EFFECT OF NaCl.



Since with such cyanide concentrations the cystine-cyanide double reaction is practically complete, the observed effect of the sodium chloride must be ascribed to its intensifying action. On the other hand, the 2·0 c.c. 5 per cent. NaCN curve indicates that under such conditions the sodium chloride effect is fairly marked at higher concentrations. Since the cystine-cyanide reaction under conditions of cyanide deficiency has been shown not to be complete, the sodium chloride in this case would seem to have an acceleratory as well as intensifying action. Incidentally this appreciable difference in the influence of sodium chloride as exhibited by the two curves in Fig. 3, illustrates another disadvantage in the original Sullivan technique.

The interference of inorganic salts is, however, not limited to sodium chloride; it has been found that sodium sulphate, potassium sulphate and potassium chloride produce a similar effect, although to a somewhat lesser extent. Lugg has shown that the presence of ferric iron in appreciable concentrations may lead to serious errors. The same was found to apply to zinc. The suggestion made by Prunty (1933) first to reduce the cystine to cysteine by means of zinc dust must, therefore, be considered with the greatest caution. According to our own experiments, minute amounts of zinc seem to exercise no measurable influence; on the other hand, larger amounts of zinc cause turbidity and produce unreliable colours. As an example of a strong inhibitor sodium borate may be mentioned, 60 mg. with 0·4 mg. cystine producing only 64 per cent. of the normal colour development. More remarkable still is the effect of ammonium salts. On adding 400 mg. of ammonium sulphate to 0·4 mg. cystine a perfect blank was obtained on the subsequent addition of the hydro-sulphite; 100 mg. showed a recovery of 56 per cent. and even 10 mg. still showed a loss of 10 per cent. Ammonium chloride produced the same effect.

As most protein hydrolysates will contain ammonium salts in small and even considerable quantities, this shows a further disadvantage of the Sullivan method when applied directly.

In view of the interference so far established, the direct applicability of the Sullivan method to all types of hydrolysates and other solutions would seem to be open to serious doubt. The great specificity of the reaction as shown by Sullivan merely indicates that no other substance is known to give the reaction by itself, yet such a finding in no way proves that the same substance may not appreciably influence the colour reaction when present together with cystine. As far as is known, this latter type of interference is to a greater or lesser degree common to most colorimetric methods.

SUMMARY.

It has been shown that the Sullivan reaction as a quantitative colorimetric method for the determination of cystine can be considerably improved by better regulation of the conditions necessary for optimum maximum colour development. This improvement has been brought about, chiefly by increasing the cyanide concentration and regulating the time interval between the adding of the napthoquinone reagent and the alkaline sulphite.

It has been found that various substances interfere with the intensity of the final colour obtained. Thus other amino-acids, ammonium salts and sodium borate have been shown to retard or inhibit the reaction, while sodium and potassium chlorides and sulphates have been found to exercise an acceleratory and intensifying action.

REFERENCES.

BAERNSTEIN, H. D. (1930). The Gasometric Determination of Cysteine and Cystine. *J.B.C.*, Vol. 89, p. 128.

CLARKE, H. T. (1932). The Action of Sulphite upon Cystine. *J.B.C.*, Vol. 97, p. 235.

CSONKA, F. A. (1932). Studies on Glutelins, VII. Cystine, Tryptophane. and Tyrosine Content of Glutelins. *J.B.C.*, Vol. 97, p. 281.

FOLIN, O., AND MARENZI, A. D. (1929). An Improved Colorimetric Method for the Determination of Cystine in Proteins. *J.B.C.*, Vol. 83, p. 103.

LUGG, J. W. H. (1933). Sullivan's Reaction for the Quantitative Determination of Cysteine and Cystine. *Bioch. Jnl.*, Vol. 27, p. 668.

OKUDA (1925). *J. Biochem.* (Tokio), Vol. 5, p. 217. See Prunty (1933).

PRUNTY, F. T. G. (1933). The Determination of Cystine in Biological Material. *Biochem. Jnl.*, Vol. 27, p. 387.

PULEWKA AND WINZER (1928). Untersuchungen über die Reaktionen des Cystins mit Schwefel-und Cyanalkali. *Arch. Exp. Path. u. Pharmakol*, Bd. 138, s. 154.

RIMINGTON, C. (1929). The Relation between Cystine Yield and Total Sulphur in Wool. *Biochem. Jnl.*, Vol. 23, p. 41.

SULLIVAN, M. X. (1926). A Distinctive Test for Cysteine. *U.S. Publ. Health Reports*, 28th May, 1926, p. 1030.

SULLIVAN, M. X. (1929). Studies on the Biochemistry of Sulphur, IV. The Colorimetric Estimation of Cystine in Casein by Means of the Beta-Naphthoquinone Reaction. *Supplement No. 78 to U.S. Publ. Health Reports* (1929).