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Studies on the Origin of Sulphur in Wool.

II. A Cuprous Mercaptide Method for the Determination of Cystine or Cysteine.*

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HAVING established the value of the Sullivan method, as modified by the authors (1934), as a specific and quantitative reaction for the determination of cysteine or cystine, it became necessary to study its possible application to the analysis of animal and vegetable materials. The first step in such an analytical procedure would obviously include some process such as acid hydrolysis whereby the cystine or cysteine of the raw material totally liberated and quantitatively brought into solution. In such processes the intensity of acid treatment must be sufficient to liberate all the cystine or cysteine from its protein or protein-like complex, but not so intense as to destroy the cystine thus liberated. It is not the purpose of the present paper to consider this phase of the problem, since investigations in this direction are still under way. On the contrary, it has been considered advisable to assume, for the time being, that the hydrolysis of the raw material can be effected quantitatively. What has been aimed at in the present work is to find a method whereby the cystine or cysteine actually present in such hydrolysates may be determined successfully.

In a few cases of high cystine content, e.g. wool and some pure proteins rich in cystine, the colour of the hydrolysate causes very little interference, and the Sullivan technique as improved by the authors (1934) can be applied directly and with satisfactory results. With low cystine content and a dark-coloured hydrolysate, the problem becomes rather involved. It is obviously quite impossible to apply directly a colour reaction to a hydrolysate obtained from grass or some animal tissue low in cystine, as anyone who has handled

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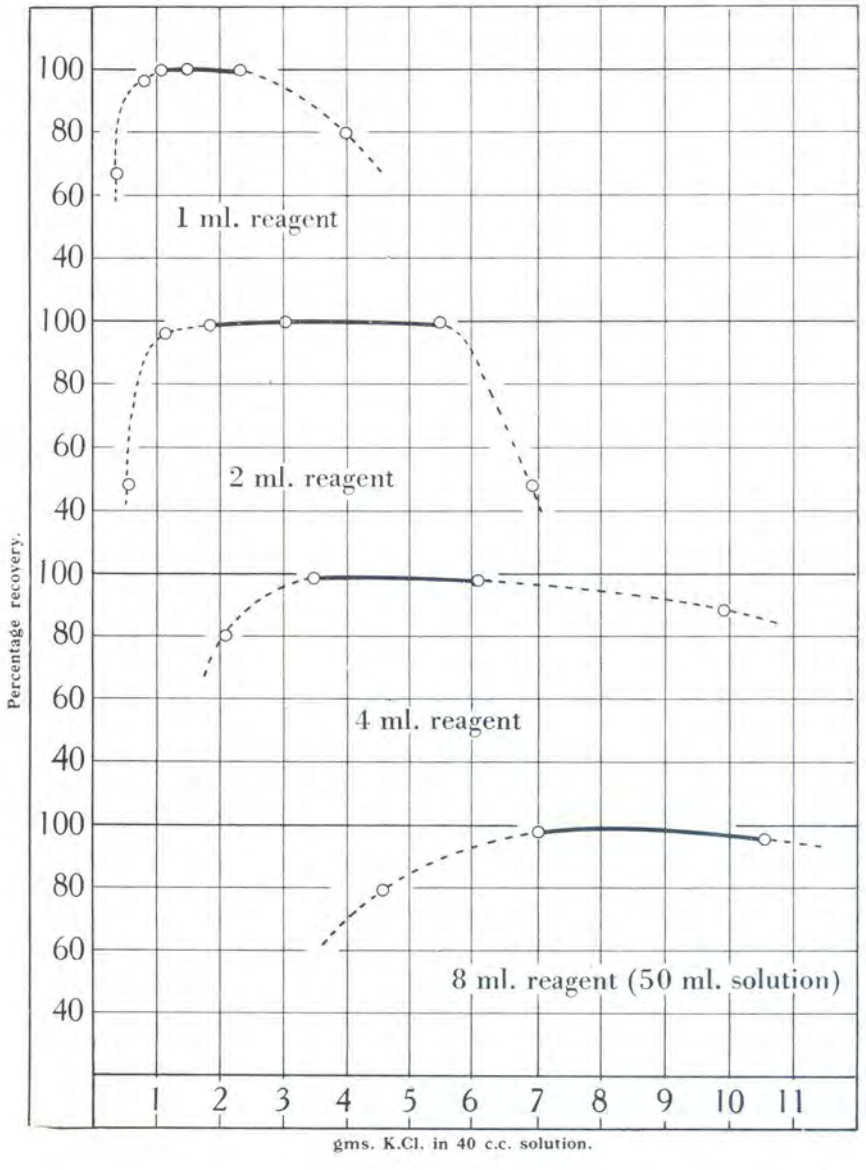
such solutions will readily appreciate. To overcome this difficulty it has been suggested by Henrici (1932) that the cystine should first be precipitated from such a solution. Even after much manipulation it is still difficult however to obtain a solution sufficiently light in colour for colorimetric methods to be applied with any degree of satisfaction. Certain workers (Sullivan, 1929, and Sullivan and Hess, 1930), have suggested the use of active and animal charcoal and other decoloring agents. None of these substances however appears suitable, since the cystine in solution is either adsorbed along with the colouring matter, or it is catalytically decomposed and destroyed (Baur and Wunderly, 1933). Other workers (Lugg, 1933) have tried to inhibit colour development during hydrolysis by adding such reducing agents as stannous or titanous chloride with equally unsuccessful results.

On the other hand it has been shown by Vickery and White (1932), amongst others, that cysteine, in quantities of about 20 mg., can be precipitated quantitatively from solution as the insoluble cysteine cuprous mercaptide. Vickery and White used cuprous oxide, and determined the cystine in the solution by determining the sulphur content of the cuprous mercaptide precipitate. Hopkins (1929) precipitated glutathione in a similar way and found the compound to be $C_{10}H_{16}N_3O_6SCu$. Preliminary experiments on the behaviour of this cuprous mercaptide soon led to the conviction that it embodied a reaction sufficiently sensitive to enable the separation of quantities of cystine or cysteine much smaller than that postulated by Vickery and White.

The addition of cuprous oxide in the solid form appeared somewhat cumbersome, and unsuited for micro-work. Since cuprous chloride is only very slightly soluble in water, initial attempts were made with a solution of cuprous chloride dissolved in potassium acetate and acetic acid mixtures. Although promising results were sometimes obtained with quantities as low as 1 mg. of cystine reduced to cysteine by means of a few mg. of zinc dust, the results were somewhat irregular. At this stage it was resolved to study the precipitation of cystine by making use of the well known solubility of cuprous chloride in potassium chloride solutions. A very interesting fact was soon discovered. Working on 2 mg. quantities of cystine per aliquot volume, the preliminary reduction to cysteine was omitted by mere accident; however, on adding the solution of cuprous chloride in potassium chloride, the cysteine cuprous mercaptide was observed to form as readily as if all the cystine had been present as cysteine. In all subsequent experiments the preliminary reduction of cystine to cysteine was therefore omitted. The cuprous chlorides solution was prepared from Merck's Reagent cuprous chloride. About 0·5 g. of the salt was shaken up with 1 per cent. HCl to remove most of the cupric chloride present, the insoluble cuprous chloride allowed to settle, and the supernatant liquid poured off. This perfectly white residue was then dissolved in a minimum quantity of 25 per cent. KCl containing 0·2 per cent. HCl. The reagent should preferably be prepared freshly before use, although, if left in a well-stoppered bottle it may be kept for a few days.

On studying the precipitation of cystine with this reagent, it was found that the cysteine could be quantitatively precipitated at room temperature at hydrogen ion concentrations between pH 3·0 and 5·5, although the rate of precipitation appeared to be greatest at pH 4·5. It was therefore decided to work at a hydrogen ion concentration of approximately pH 4·5. Aliquot portions of 2 mg. cystine dissolved in very dilute HCl were measured into 50 ml. centrifuge-tubes, and a few drops of glacial acetic acid added to each. These solutions were then brought to pH 4·5 by adding carefully 10 per cent. KOH using bromocresol green (B.D.H.) as an external indicator. To the buffered solution cooled to room temperature 5-10 drops of the curpous chloride solution were added with constant stirring. A fine precipitate was observed to form almost immediately, and coagulated gradually to a flocculent mass. After 40 minutes this precipitate was centrifuged, and the supernatant liquid poured off. The precipitate was washed with alcohol, again centrifuged, and the alcoholic liquid poured off. After draining, the precipitate was dissolved in 5 ml. 1-2 per cent. HCl, and transferred to a 25 ml. measuring cylinder of good accuracy, using about 15 ml. of wash water. 2·5 ml. of a 5 per cent. acetic acid solution were then added, and after mixing 1 ml. of 10 per cent. KCNS was added in order to precipitate the cuprous copper. To remove the varying amounts of cupric copper always present at this stage, use was made of the reaction of Biazzo (1926) by precipitating the cupric copper as the green sulphocyanide-copper-pyridine complex. At this stage pyridine was therefore added until pH 4·5 was reached. Under these conditions no measurable adsorbtion of cystine by the precipitate could be detected. After mixing thoroughly and making up to 25 ml., the precipitates were filtered off. To overcome the buffering action of the acetic acid in solution, 1 ml. of 10 per cent. KOH was added to a suitable aliquot (usually 5 ml.) of the clear filtrate, and the cystine determined by the modified Sullivan method as described by the authors in a previous article (Rossouw and Jorden, 1934).

Using this method, the effect of varying amounts of potassium chloride on the precipitation of the cysteine cuprous mercaptide was studied, since with actual hydrochloric acid hydrolysates the amount of potassium chloride in the aliquot solution must vary with the amount of cystine present in the material. This consideration is most important, since, as the curves reproduced below readily show, the cuprous mercaptide will partly or totally dissolve in an excess of potassium chloride, unless a correspondingly large excess of cuprous chloride reagent is added. However, considering the fact that the presence of potassium acetate in relatively large quantities has been found to depress the solubility of the mercaptide in potassium chloride, the curves show that it is perfectly practicable to determine the cystine in such solutions as a hydrochloric acid hydrolysate of sheep's blood. In dealing with grasses however the position is somewhat different, since in a hydrochloric acid hydrolysate a suitable aliquot containing a determinable amount of cystine would inevitably contain such a large quantity of hydrochloric acid that the amount of cuprous chloride reagent necessary becomes unwieldy and impracticable.



To overcome this difficulty when working with material very low in cystine, the hydrochloric acid was replaced by sulphuric acid. In testing this modification of the method 2 mg. of cystine were added to 5 ml. of 40 per cent. H_2SO_4, 2·5 ml. glacial acetic acid were added and the solution brought to pH 4·5 by neutralising with 10 per cent. KOH. After cooling to room temperature, 5-10 drops of the cuprous chloride solution were stirred in. Precipitation and flocculation were rapid, and 100 per cent. recovery was obtained repeatedly, even after leaving the precipitate to stand for 24 hours.

This procedure of first precipitating the cystine as the cysteine cuprous mercaptide soon proved most successful in the treatment of hydrolysates, especially those highly contaminated with excessive quantities of dark colouring and other matter. On bringing such hydrolysates to pH 4·5 by means of acetic acid and potassium hydroxide, a large amount of objectionable colouring material was precipitated and centrifuged off. That the material thus precipitated could occlude or contain no measurable amount of cystine can be safely accepted, since neither could cystine be found in such precipitates nor could any loss be demonstrated on adding a known amount of cystine to the hydrolysate. On adding the cuprous chloride reagent to the centrifuged and decanted solution, a further quantity of dark-coloured material was precipitated along with the cuprous mercaptide. On dissolving the mercaptide in dilute hydrochloric acid, the accompanying dark material was also brought into solution, but was removed practically completely in the precipitation of the copper with sulphocyanide and pyridine. The final solutions were either perfectly colourless or exhibited only the faintest straw-yellow which has no measurable influence on the cystine determination. As an example of the applicability of the method, the following analytical figures might be given:—

Merino wool: 12·5 per cent.
Dried sheep's blood: 450 mg. per 100 g.
Yellow mealies: 104 mg. per 100 g.
Lucerne hay: 80 mg. per 100 g.
Veld grass: 20-80 mg. per 100 g.

In the successful execution of the method, some experience is undoubtedly necessary. If too large an excess of the cuprous chloride reagent be added to the sulphuric acid hydrolysates, some cuprous chloride may be precipitated along with the mercaptide. Excessive quantities of this cuprous chloride make the dissolution of the mercaptide in the dilute hydrochloric acid more difficult to control, and may further result in some cystine being retained by the cuprous chloride. In such cases the difficulty may be remedied by adding a few mg. of zinc dust to the hydrochloric acid solution and thus converting the cuprous chloride into metallic copper. In such cases however the filtrate, after adding sulphocyanide and pyridine, must be left to stand overnight to allow any cysteine formed to be reoxidised to cystine. Any zinc solution is practically completely removed by pyridine at pH 4·5; the residual traces that may remain in solution have no effect on the determination by the Sullivan method, as also shown by Prunty (1933).

Method.

The method, employing sulphuric acid, may be summarised as follows:—

A suitable quantity of dried material *plus* 8 times its weight of 40 per cent. H_2SO_4 hydrolysed for 24 hours at 125° (oil-bath temperature).

Hydrolysate filtered hot and made up to volume.

A suitable aliquot measured into a 50 ml. centrifuge-tube. 2·5 ml. glacial acetic acid added to this and brought to pH 4·5 with 10 per cent. KOH.

If precipitate is shown here, centrifuge and wash with acetic acid KOH. mixture of pH 4·5 (final volume to be approximately 40 ml.).

Stir in a few drops of cuprous chloride reagent.

Leave until flocculation sets in (a few minutes to 1 hour).

Centrifuge and wash with alcohol.

Dissolve in 5 ml. 1 per cent. HCl.

Transfer, with washings, to a 25 ml. measuring cylinder.

Add 2·5 ml. 5 per cent. acetic acid, and mix.

Add 1 ml. 10 per cent. KCNS and mix.

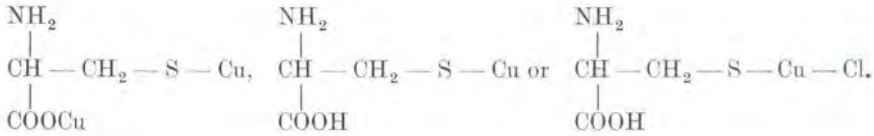
Add pyridine to pH 4·5 and make up to volume.

Filter.

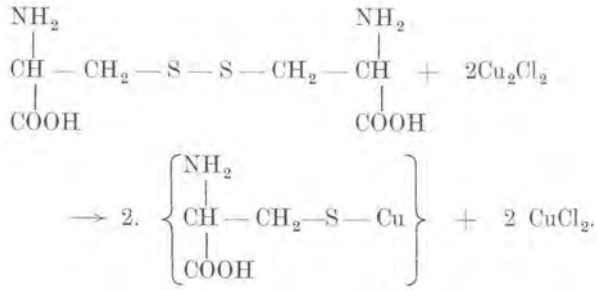
Determine cystine in suitable aliquot (0·4 mg. cystine), by method of Sullivan as modified by the authors, adding 1 ml. of 10 per cent. KOH to counteract buffering effect of acetic acid.

Discussion of Mechanism.

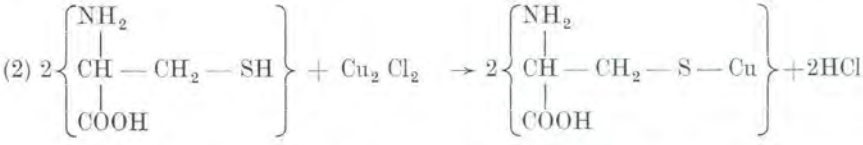
The exact nature of the cuprous mercaptide precipitate has not been determined. Indications (Preisler and Preisler, 1932) have been obtained that the mercaptide which is either—



is apparently associated with either cuprous or cupric chloride, or both. Whatever the composition of the precipitate, it is generally accepted that all the cystine is present in the reduced form as the copper salt of the mercaptan cysteine. It therefore appears that the cystine reacts with the cuprous chloride according to the following reaction:—



In the case of the preformed cysteine, the reaction would naturally be:—



These reactions seem to proceed quantitatively in feebly acid solutions (pH 3·0-5·5) in the presence of an excess of cuprous chloride. In dissolving the precipitates in dilute hydrochloric acid, the initial state of the cystine should be the reduced form. Experimental data can be adduced however to show that its conversion into the oxidised form is extremely rapid in such acid solutions. In the normal course of analysis all the cysteine is quantitatively converted into cystine by the time the copper is precipitated with sulphocyanide and pyridine, even when operations are speeded up to the maximum limit with the object of detecting any unoxidised cysteine. It appears that the transformation to cystine already occurs during the process of dissolution in hydrochloric acid, since, even after adding small quantities of zinc powder no cysteine could be detected. This behaviour of the mercaptide suggests that it is precipitated as a complex containing cupric chloride. In the presence of hydrochloric acid, and in the absence of excess cuprous chloride, the reaction (1) is therefore reversed, the reaction being forced to completeness by the precipitation of the cuprous copper as sulphocyanide. The observation of Andrews (1933) on the catalytic oxidation of cysteine by copper must probably be explained in a similar way.

In the case where a relatively large excess of zinc dust is added to the solution of the mercaptide in dilute hydrochloric acid, it was found possible, by rapid work, to obtain practically all the cystine in the reduced form. Since in the Sullivan method the treatment with cyanide results in the formation of only one molecule of cysteine from one molecule of cystine, and since, for all practical purposes one part by weight of cystine is theoretically equivalent to one part by weight of cysteine, the Sullivan method in reality determines only half of the cystine when present as cystine. When present as cysteine therefore, a colour intensity double that of the standard should be obtained. In actual experiment colour intensities round about 192 were obtained with the standard at 100. On allowing the solution from which the copper had been precipitated to stand for a further four hours, the intensity of the Sullivan colour dropped to 150, while after 20 hours it had dropped further to 100, showing that by that time all the cysteine had been converted into cystine. Since some extractions with chloroform according to Biazzo still revealed minute quantities of copper in the solution, the conversion seems to have been effected catalytically.

The rapid conversion of the cysteine in the mercaptide into cystine was further demonstrated by precipitating the cysteine from a standard cysteine hydrochloride solution with cuprous chloride, and comparing the colour intensity of the thus treated cysteine with that

of the cysteine hydrochloride standard directly treated by the Sullivan method. The cysteine from the mercaptide was found to be only half that of the cysteine treated directly.

The fact however that in the hydrochloric acid solution of the mercaptide the cystine can be quantitatively reduced by means of zinc dust, and the modified Sullivan method applied to this cysteine solution, is considered to be of some practical importance, since double the normal colour intensity is obtained. Along these lines it has been found possible to determine the cystine contents of grasses and other material extremely low in cystine. These and other results will be reported in a following paper.

Summary.

1. A method for the micro-determination of cystine in biological or plant material has been described. The dried material is hydrolysed with 40 per cent. sulphuric acid and the cystine precipitated by cuprous chloride as the cysteine cuprous mercaptide. The cystine content of this is determined by the modified Sullivan method.

2. The interfering colouring matter is automatically eliminated.

3. The method is quick and comparatively easy.

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