CXXXIII. SOME SOURCES OF ERROR IN THE ESTIMATION OF CYSTEINE AND CYSTINE IN COMPLEX MATERIALS WHEN ACID HYDROLYSIS IS EMPLOYED.

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All methods of estimating cysteine and cystine in complex solid materials, that have any claim to specificity, depend in the first instance upon getting these amino-acids into solution, and acid hydrolysis is the means usually employed.

Apart from the errors associated with the final assay of such solutions, some of which have been overcome [Lugg, 1932; 1933], there is the possibility of others that belong strictly to the process of hydrolysis. These are usually overlooked entirely or considered unimportant. It is the purpose of the present article to describe an investigation of two such possible sources of error, decomposition of the cysteine and cystine by the hydrolysing acid once they have been liberated, and reactions of the liberated cysteine and cystine with other substances, either present as such in the material or formed during hydrolysis. Hydrochloric acid was used as hydrolysing agent.

The stability of cysteine and cystine when heated in acid solution was first examined, and the recovery of cysteine and cystine was then tested after they had been "hydrolysed" with much extraneous material including carbohydrates.

On account of the losses that occurred when carbohydrates were present stannous chloride was added [Hlaziwetz and Habermann, 1873] to minimise "humin" formation, but this did not diminish the loss, and humin formation was not much reduced. A necessary preliminary to these experiments was to test the action of stannous chloride on cystine in pure acid solution; this was found to be essentially reduction to cysteine.

In comparison with the loss of cysteine, the loss of cystine when hydrolysed with carbohydrates is quite small, and consequently the possibilities were explored of oxidising the liberated cysteine rapidly to cystine before it could react with the carbohydrate decomposition products. The search for an oxidising agent that would rapidly oxidise cysteine in acid solution to cystine and no further was unsuccessful. The alternative possibilities of protecting the reactive —SH group in cysteine or of rendering the carbohydrate decomposition products unreactive hold little promise of realisation.

At the temperature employed in most of this work (100°) the time allowed for hydrolysis would probably be insufficient for the attainment of a negative biuret reaction in a protein hydrolysate.

Experimental.

The solutions were analysed for their sulphydryl and disulphide contents by the method described by Lugg [1932][1]. These are recorded as such but are calculated in mg. of cysteine and cystine respectively. Such estimations may be made with fair accuracy even in the presence of large amounts of extraneous material. On the other hand the procedure for estimating cysteine and cystine specifically, described by Lugg [1933], fails when large quantities of the decomposition products of carbohydrates are present. Its use in this investigation was therefore limited, and in fact most of the work was done before it became available. The method has been applied to the specific estimation of cystine in experiment D (1) as a check on the possible decomposition of cystine other than that associated with desulphuration.

A. *Cystine heated in HCl solution.* The stability of cystine when boiled with 6*N* HCl for varying periods has been thoroughly investigated, notably by Hoffman and Gortner [1922], who found that the chief change during 20 hours was racemisation of *l*-cystine, but that, with increasing time, chemical decomposition by deamination, decarboxylation and desulphuration became more than slight.

25 mg. cystine were heated with 25 cc. 5*N* HCl at 100° for periods up to 24 hours without detectable loss in the RSSR content (less than 1 %). Such a solution could be evaporated in an open beaker to 2 cc. either on the water-bath or at the boiling-point without loss. There was no loss when the "hydrolyses" were conducted in sealed tubes at 100° with urea present.

B. *Cysteine heated in HCl solution.* (1) 23·6 mg. cysteine in 25 cc. *N* HCl were heated in an open beaker at the boiling-point with renewal of evaporated water for 45 minutes. The solution was diluted to 25 cc. for analysis. RSH content 23·5 mg.; RSSR content nil. (No measurable oxidation to cystine and no loss.)

(2) 23·6 mg. cysteine in 10 cc. 5*N* HCl were heated with 0·5 g. urea in a sealed tube at 100° for 18 hours. There was a little air in the tube when sealed off. A pressure of CO_2 was developed and on opening the tube the odour of H_2S was just perceptible. The contents were evaporated to 5 cc. on the water-bath and diluted to 25 cc. for analysis. RSH content 20·0 mg.; RSSR content 3·3 mg. (84·6 % of the original cysteine was unchanged, 14·1 % had been oxidised to cystine, and just over 1 % of the original S was lost, partly if not wholly as H_2S.)

C. *Cystine heated in HCl solution with $SnCl_2$.* (1) 50 mg. cystine in 10 cc. 5*N* HCl with 2·25 g. $SnCl_2$, $2H_2O$ were heated in a sealed tube for 18 hours at 100°. There were one or two small dark deposits (SnS) at the top of the tube and these dissolved on coming into contact with the liquid contents. On opening the tube the odour of H_2S was faintly perceptible. The solution was diluted to 25 cc. It was necessary to remove any stannous salt present before analysing for RSH and RSSR, as the usual procedure involved the use of mercuric chloride.

Precipitation of the Sn^{++} as SnS. An aliquot of the solution was diluted with three volumes of water, and sufficient NaOH solution was added to reduce the acid concentration to about 0·2 *N* HCl. H_2S was passed in for 20 minutes at room temperature and the SnS was filtered off and washed. Excess H_2S was boiled out of the filtrate, which was then diluted to volume for analysis. RSH content 34·3 mg.; RSSR content nil. (A loss of 32 % of the cystine originally present, the rest occurring as cysteine.)

[1] Unfortunately, on p. 2164 of the article [Lugg, 1932], there are three printer's errors. The corrections are: line 13, *B* instead of *b*; line 33, *A* instead of *a*; line 34, *B* instead of *b*.

Another aliquot was treated in the same way except that CO_2 was blown through the filtrate to remove the excess H_2S. The loss was the same as in the previous experiment.

The loss was not due to the introduction locally of concentrated NaOH solution because zinc and calcium carbonates could be used instead without improving the recovery.

(2) *Substitution of the Sn by Zn.* 50 mg. cystine in 10 cc. 5*N* HCl were heated with 2·25 g. $SnCl_2, 2H_2O$ in a sealed tube for 4 hours at 100°. The solution was then diluted to 25 cc. and treated with 2·5 g. $ZnCO_3$. 4 g. of coarsely powdered zinc were then added in small portions with agitation and the mixture was agitated for a further 7 minutes. It was then filtered through a paper lined with 6 g. of zinc powder. Filtrate and washings were diluted to 50 cc. for analysis. RSH content 49·5 mg.; RSSR content nil. (Conversion into cysteine with a loss of 1·8 %.)

An aliquot of the solution was extracted three times with double its volume of anhydrous ether. It was then warmed to expel ether, cooled and diluted to its original volume for analysis. RSH content 43·8 mg.; RSSR content 5·2 mg. (Partial oxidation of the cysteine present to cystine with a doubtful 1% loss.)

(3) 50 mg. cystine in 10 cc. *N* HCl were heated with 1·125 g. $SnCl_2, 2H_2O$ in a sealed tube for 14 hours at 100°. The solution was diluted to 30 cc., 3·05 g. $ZnCO_3$ were added, and H_2S was passed in for 15 minutes. A further 0·5 g. $ZnCO_3$ was added and H_2S was passed in for a further 20 minutes. The SnS was filtered off and washed and the filtrate boiled to expel H_2S and finally diluted to 50 cc. for analysis. RSH content 39·2 mg.; RSSR content, doubtful trace. (A loss of 22 % of the cystine originally present.)

An aliquot of the solution was treated with powdered zinc as described in C (2). Some tin was removed. This tin, not precipitated by the H_2S, must have been in the stannic condition. RSH content 39·7 mg.; RSSR content nil. (A loss of 21 % of the cystine originally present.)

These experiments show that cystine is reduced to cysteine when heated with stannous chloride in acid solution with very little loss; but that if stannous sulphide is precipitated from a cysteine solution some of the cysteine is carried down by the SnS, the loss increasing with the amount of precipitate. The cysteine is apparently adsorbed on the sulphide particles. Independent experiments show that bismuth sulphide acts in the same way.

D. *Cystine heated in HCl solution with a mixture of amino-acids.* (1) 1 g. of acid-washed gelatin and 50 mg. cystine were hydrolysed with 50 cc. 5*N* HCl at the boiling-point for 20 hours. The hydrolysed mixture was evaporated in a beaker on the water-bath to a small volume. The solution was diluted, filtered, and made up to 50 cc. for analysis. RSH content nil; RSSR content 49·2 mg.; cystine content 47·2 mg. (RSSR loss 2 %; cystine loss 6 %.)

(2) It is known that if any sulphur-containing compound other than cystine occurs in wool keratin, it must be present in excessively minute amounts. A wool keratin hydrolysate in 2*N* acid that had been kept for some years was filtered and analysed: RSSR content 1·18 mg. per cc. 25 cc. of the hydrolysate were heated with an additional 5 cc. 10*N* HCl at 100° in an open beaker until the total volume had been reduced to 5 cc. A very small amount of "humin" material separated. The solution was diluted somewhat, filtered, and made up to 25 cc. for analysis. RSSR content 29·2 mg. (A loss of about 1 %.)

The results of D (1) show that the cystine destruction (deamination *plus* decarboxylation *plus* desulphuration) is more serious than the disulphide destruction (desulphuration), which is only to be expected if the three modes of destruction can occur independently.

E. *Cystine heated in HCl solution with amino-acids, carbohydrates,* etc. (1) 25 cc. of the keratin hydrolysate were heated at 100° in an open beaker with 5 cc. 10*N* HCl, 0·2 g. sucrose, 0·1 g. arabinose, 0·1 g. tartaric acid, and 0·1 g. uric acid until the volume had been reduced to about 7 cc. 20 cc. 5*N* HCl were added and the volume was again reduced, this time to 5 cc. 15 cc. of water were added and were followed by sodium acetate until the p_H was about 2·5 (thymol blue) to precipitate the "humin" materials in the region of their minimum solubility. After standing for several hours the mixture was filtered, the residue was extracted with hot 0·01 *N* HCl, and filtrate and washings were diluted to 50 cc. for analysis. RSH content nil; RSSR content 28·0 mg. (A loss of 5 %.)

An aliquot was extracted with anhydrous ether as in C (2). The colours due to extraneous reducers were diminished, but the RSSR content was unaltered.

(2) 25 mg. cystine were heated on the water-bath in a flask under reflux for 16 hours with 50 cc. 2*N* HCl, 0·5 g. sodium glutamate, 0·25 g. sucrose, 0·05 g. arabinose, 0·125 g. tartaric acid, 0·05 g. tyrosine and 0·025 g. uric acid. The mixture was filtered at the pump and the residue extracted with hot 0·01 *N* HCl. Filtrate and washings were diluted to 50 cc. for analysis. RSH content nil; RSSR content 24·2 mg. (A loss of 3·2 %.)

An aliquot was brought to p_H 2·5 as in E (1). After 24 hours the humins were filtered off and washed, and the filtrate was diluted to volume for analysis. (The loss was increased from 3·2 to 4 %.)

(3) 50 mg. cystine in 20 cc. 5*N* HCl were heated at 100° in a sealed tube for 18 hours with 1·0 g. cellulose, 0·5 g. sucrose, 0·1 g. arabinose, 0·1 g. tartaric acid, 0·5 g. glycine, 0·2 g. aspartic acid, 0·05 g. uric acid, 0·02 g. creatinine and 0·25 g. urea. The mixture was filtered at the pump, the residue was extracted twice with hot *N* HCl solution and washed, and filtrate and washings were evaporated on the water-bath to a syrup (4 cc.). 5 cc. 10*N* HCl and 30 cc. of water were added and the mixture was again evaporated to a syrup. The process was repeated. The syrup then received 5 cc. 5*N* HCl and 10 cc. of water and after 10 minutes' heating on the water-bath sodium citrate buffer (p_H 6) was added until p_H 2·5 was reached. The mixture (about 35 cc.) was allowed to stand 30 hours and was then filtered. The residue, which was very small in bulk, was extracted with 0·01 *N* HCl. Filtrate and washings were diluted to 50 cc. for analysis. RSH content nil; RSSR content 47·2 mg. (A loss of 6 %.)

An aliquot was submitted to ether extractions. The loss was increased by 1 %.

The acid-insoluble residue present at the end of hydrolysis was thoroughly dried after extraction. It weighed 1·05 g. and contained 23·0 mg. nitrogen. There was obviously no evidence of selective adsorption of the cystine. Most of the uric acid would be present in the residue because of its slight solubility. From E (2) it was believed that the bulk of the lost cystine would occur in this acid-insoluble residue and very little in the humins precipitated at p_H 2·5.

(4) 50 mg. cystine in 200 cc. 5*N* HCl were heated at 100° under reflux for 18 hours with 20 g. cellulose, 10 g. sucrose, and 3 g. glycine. The mixture was filtered at the pump and the residue thoroughly extracted with hot *N* HCl. Filtrate and washings were evaporated almost to dryness on the water-bath and then heated with 100 cc. 2*N* HCl for 30 minutes. The mixture was again filtered and the residue thoroughly extracted with hot *N* HCl. Filtrate and washings were evaporated to about 10 cc., partly neutralised with NaOH solution, and brought to p_H 2·5 with sodium citrate. After 24 hours the mixture was filtered, the residue washed and the filtrate diluted to 50 cc. for analysis. RSH content nil; RSSR content 10 mg. (A loss of 80 %.)

An aliquot of the solution was about half decolorised with norite (absorbent carbon). It has been found that very little cystine is removed in this way provided an appreciable brown humin colour remains. RSSR content 9·5 mg. (A loss of 81 %.)

The first residue from hydrolysis when dry weighed 14·5 g., the second 3·5 g., and that precipitated at p_H 2·5, a few decigrams. Less than 2 % of the nitrogen originally present had been lost. Relatively speaking, the cystine was certainly carried down in preference to the glycine.

F. *Cysteine heated in HCl solution with amino-acids, carbohydrates*, etc. 24·7 mg. cysteine in 10 cc. 5*N* HCl were heated in a sealed tube at 100° for 18 hours with 0·5 g. cellulose, 0·25 g. sucrose, 0·05 g. tartaric acid, 0·25 g. glycine, 0·1 g. aspartic acid, 0·025 g. uric acid, 0·01 g. creatinine and 0·5 g. urea. A fair pressure of CO_2 developed but there was no odour of H_2S when the tube was opened. The mixture was filtered at the pump, the residue extracted, and filtrate and washings were evaporated to about 3 cc. About 10 cc. of water were added, and the solution was brought to p_H 2·5 as in E (3). After standing for 4 hours the mixture was filtered and the residue washed, and the filtrate was diluted to 25 cc. for analysis. RSH content 3·0 mg. (doubtful); RSSR content 1·0 mg. (doubtful). (A loss of 85 % or more.)

G. *Cystine heated in HCl solution with $SnCl_2$, amino-acids, carbohydrates*, etc. (1) 50 mg. cystine in 10 cc. 5*N* HCl were heated at 100° in a sealed tube for 16 hours with 2·25 g. $SnCl_2, 2H_2O$, 0·3 g. sucrose, 0·1 g. arabinose, 0·1 g. tartaric acid, 0·5 g. glycine, 0·5 g. aspartic acid, 0·1 g. uric acid and 0·1 g. creatinine. Sedimentation of the insoluble materials in the tube was much better than in E or F, but the solution was still dark brown in colour. The entire contents of the tube were treated as under C (2). There was considerable frothing caused by hydrogen bubbles and the escaping gas carried a peculiar odour reminiscent of carbylamines. The resulting solution diluted for analysis was pale orange-yellow in colour with a tendency to fluoresce. It darkened in colour upon exposure to air. RSH content 5 mg. (doubtful); RSSR content nil. (A loss of at least 90 %.)

(2) 50 mg. cystine in 20 cc. 5*N* HCl were heated at 100° in a sealed tube for 20 hours with 20 g. $SnCl_2, 2H_2O$, 1·0 g. cellulose, 0·5 g. sucrose, 0·1 g. arabinose, 0·1 g. tartaric acid, 0·5 g. glycine, 0·2 g. aspartic acid, 0·05 g. uric acid and 0·02 g. creatinine. Even in the presence of so much $SnCl_2$ the heated solution was dark brown in colour and there was much sediment. The Sn was removed as in C (2), 20 g. zinc being used. Frothing was very pronounced and the hydrogen smelt of carbylamine. The colour of the final solution was the same as in G (1). RSH content 3·0 mg. (doubtful); RSSR content 1·5 mg. (doubtful). (A loss of at least 90 %.)

The effects of oxidising substances upon cysteine and cystine.

Aerial oxidation. Under the conditions described in A, cystine in hot HCl solution appears to be unaffected by atmospheric oxygen, and it is commonly supposed that in the cold such solutions will keep indefinitely. Andrews [1932] has recently shown that cystine is slowly oxidised in HCl solution, mostly, if not entirely to cysteic acid. In H_2SO_4 solution on the other hand there was no loss, even after seven years. Andrews concludes that the oxidation in HCl solution is due either to traces of free halogen produced in the acid or to the presence of catalysts. In strongly alkaline solution any oxidation is probably dependent upon preliminary breakdown of the cystine. Cysteine is readily oxidised to cystine by air at p_H 7–8, the rate being increased enormously by traces of iron,

copper, and other salts [Mathews and Walker, 1909; Harrison, 1924; and others]. The rate of oxidation decreases as the p_H decreases.

(1) A solution containing 1·26 mg. cysteine per cc. in 0·25 *N* HCl was prepared. 20 cc. were placed in a conical flask of 50 cc. capacity. Air was bubbled through the solution through a layer of 2 cm. depth at 10 cc. (N.T.P.) per minute in bubbles of about 0·1 cc. volume at 20° for 1 hour. The solution was then diluted to 25 cc. for analysis. RSH content 25·1 mg.; RSSR content nil. (No measurable oxidation and no loss.)

(2) The experiment was repeated at 100°. An efficient reflux condenser prevented appreciable loss of water vapour. RSH content 25·0 mg.; RSSR content, trace. (Practically no oxidation and no loss.)

(3) (1) was repeated in the presence of 2×10^{-6} mol. of ferrous ammonium sulphate. RSH content 24·8 mg.; RSSR content 0·3 mg. (About 1·5 % of the cysteine had been oxidised to cystine.)

(4) (2) was repeated in the presence of 2×10^{-6} mol. of ferrous ammonium sulphate. RSH content 22·5 mg.; RSSR content 2·4 mg. (10 % of the cysteine had been oxidised and at least 90 % of this could be accounted for as cystine.)

(5) Solution (1) was allowed to stand at room temperature with occasional replacement of the air above it. After 1 day 14 % of the cysteine had been oxidised and was recoverable as cystine. After 4 days 30 % of the cysteine had been oxidised and was recoverable as cystine. After 30 days 50 % of the cysteine had been oxidised and of this at least 96 % was recoverable as cystine. After 180 days all the cysteine had been oxidised. At least 97 % of it was recoverable as cystine, leaving 2 or 3 % unaccounted for.

Oxidation with ferric chloride. (1) Cystine was allowed to stand for 2 hours at 20° with 12 times its molecular quantity of $FeCl_3$ (*a*) in 0·1 *N* HCl, and (*b*) in a citrate buffer at p_H 5·7. In (*b*) there was no oxidation as revealed by the RSSR estimation, but in (*a*) a little ferrous salt was produced and about 2 % of the cystine was lost.

(2) 10 mg. cystine in 12·5 cc. 0·5 *N* HCl with 1 millimol. $FeCl_3$ were heated for 18 hours in a sealed tube containing a little air. The mixture was cooled for analysis. RSH content nil; RSSR content 7·5 mg.; Fe^{++} content 0·018 millimol. (25 % of the cystine was oxidised apparently to cysteic acid. The amount of ferrous salt corresponded with 76 % of the supposed oxidation. Presumably the air in the tube oxidised part of the ferrous salt back to the ferric condition.)

(3) The experiment was repeated with cysteine in place of cystine. The analysis for RSH was of course negative, but only 33 % of the cysteine was recoverable as cystine. The remaining 67 % was apparently oxidised to cysteic acid as the amount of ferrous salt produced corresponded with about 95 % of that required. Re-oxidation of the ferrous salt to ferric by air in the tube would be relatively less pronounced in this case.

(4) In a similar experiment conducted with only 0·5 millimol. $FeCl_3$ and with a heating period of only 4 hours, 40 % of the cysteine was recoverable as cystine, and the ferrous salt present accounted for 90 % of the supposed oxidation.

(5) As a variant to (4), the $FeCl_3$ was placed in the tube with part of the acid and maintained at 100° whilst the cysteine in the remainder of the acid was added slowly in small portions with agitation. The tube was then sealed and heated for 4 hours. The analysis agreed almost perfectly with (4).

It was thought that the rapid breakdown of the cysteine-ferric salt complex in (5) would have resulted in a different distribution between the oxidation products of the cysteine.

Oxidations with various other substances. Over the range p_H 3 to 6 cysteine may be oxidised more or less completely to cystine by ferricyanide, H_2O_2, chromate, persulphate, iodine and iodate. If large excess of the oxidiser is employed, considerable amounts of the cysteine are oxidised beyond cystine, the tendency to the more extensive oxidation increasing with the acidity and the temperature. Cystine itself is destroyed by these same oxidisers, but less easily.

Discussion.

From an analytical point of view, cystine is reasonably stable when heated for 20 hours in 5*N* HCl at 100°, either alone or with such substances as urea and stable amino-acids. Under similar conditions cysteine is decomposed to the extent of about 1 %, one of the decomposition products being H_2S. When heated at the boiling-point, the loss of cystine is about 6 %, but if estimated as disulphide, only 2 %. When heated with stannous chloride in acid solution cystine is reduced to the extent of about 98 % to cysteine, and a little H_2S is produced.

When cystine and cysteine in acid solution are heated with carbohydrates, variable amounts are lost. In actual experiments, when the humins formed were about 20 times the weight of the cystine or cysteine originally present, 6 to 7 % of the cystine and at least 85 % of the cysteine were lost. The cystine loss increased to about 80 % when the weight of humin was about 360 times that of the cystine originally present. When heated with carbohydrates in the presence of stannous chloride, cystine is lost just as extensively as is cysteine in the absence of stannous chloride.

Whereas the loss of cystine appears to be due to ordinary adsorption on the particles of humin, the far more extensive loss of cysteine is presumably due to condensation. Carbohydrate humins are aldehydic in character, and in this connection it is known that the simple aliphatic mercaptans readily condense with aldehydes and ketones forming mercaptals and mercaptoles respectively.

It would appear from these experimental findings that pure proteins on hydrolysis would yield practically all their cysteine and cystine provided that these amino-acids were not decomposed during the rupture of the protein and peptide linkages, that no humins were formed, and that there were nothing present in the protein that would react with either cysteine or cystine. Part of the cysteine would be converted into cystine unless air were excluded.

With relatively small amounts of carbohydrate present, such as might occur in the protein molecule, the loss of cystine originally present would be very small but the loss of cysteine might be very serious. Humins of strictly amino-acid origin, such as those formed from tryptophan and tyrosine, presumably carry cystine down just as do carbohydrate humins, but unless they specifically adsorb cystine the loss in this direction would be very slight.

With relatively large amounts of carbohydrate present, the cystine loss can be very serious and the cysteine loss virtually complete.

The error of estimating the cystine *plus* cysteine content of a material will therefore be the more serious the greater the proportion of the latter split off by hydrolysis. No safe method of oxidising the cysteine rapidly to cystine before it can react with carbohydrate decomposition products has been found.

The practice of adding stannous chloride before hydrolysis to minimise humin formation not only fails to prevent a loss of cysteine but causes the loss of any cystine present by reducing it to cysteine.

If sufficient ferric salts or their precursors were present in a material they could destroy some of the cystine and most of the cysteine during hydrolysis. The salts of certain other heavy metals would also interfere badly.

Summary.

The fates of cysteine and cystine heated in hydrochloric acid solution, with and without the addition of various other materials, have been investigated. In the presence of carbohydrates variable amounts of cysteine and cystine are lost, the loss of cysteine being far more serious than that of cystine. Even such mild oxidising agents as ferric chloride destroy cystine in acid solution.

The dangers of acid hydrolysis as a means of getting the cysteine and cystine of some materials into solution for estimation are emphasised. The practice of adding stannous chloride to minimise humin formation is condemned.

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