161. THE ANALYSIS OF HAIR KERATIN

1. A METHOD FOR THE QUANTITATIVE REMOVAL OF CYSTINE FROM KERATIN HYDROLYSATES

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THE amount of accurate information concerning the composition of keratin hydrolysates is very limited. Wool has received more study than most keratins, but even in it about 60 % of the protein has still to be accounted for. More knowledge about the amino-acids in keratin hydrolysates should not only prove useful to those interested in the constitution of such proteins but might also have the immediate practical result of revealing a new source for some amino-acids.

It is a curious fact that in spite of the availability of human hair, its chemical composition has never been thoroughly studied. While numerous papers appeared between 1880 and 1920 dealing with this keratin, most of them were concerned with the sulphur and cystine contents and with the effect of age, sex, race etc., on the sulphur content of hair; most of these data are based on unreliable methods. Recent studies have led to the development of more trustworthy procedures [Rimington, 1929] and the results so obtained have invalidated previous conclusions.

Buchtala [1907] attempted to apply Fischer's ester-distillation method to a hair hydrolysate. The mono-amino-acids thus obtained accounted for only about 30 % of the protein and the analytical data for some of the acids isolated did not indicate a very high degree of purity; only limited quantitative significance can be attached to the figures given (glycine 9.1, alanine 6.9, leucine 12.1, phenylalanine 0.6, tyrosine 3.3 %). Buchtala also reported values of 8.0 % for glutamic acid and 14.5 % for cystine, the latter being calculated from the sulphur content of impure fractions obtained by direct crystallization. Block *et al.* [1939] have reported more recent determinations of some of the amino-acids in human hair, but the summation of these latest data accounts for less than 40 % of the hair keratin.

The instability of cystine and its derivatives has been found to lead to difficulties in the analysis of keratins no matter which of the usual methods for the separation of the amino-acids is used; if a procedure could be devised for the complete removal of cystine, without at the same time rendering the solution of the remaining amino-acids unworkable, the problem of keratin analysis would be greatly simplified. Such a method will be described in this paper.

Older procedures for removing cystine from keratin (hair or wool) hydrolysates involved neutralization of the acid used for the hydrolysis by alkali or buffering with sodium acetate, thereby producing large quantities of inorganic salts which remained with the other amino-acids. Not only do such methods introduce much undesirable inorganic matter, but they usually leave one-third

to one-half of the cystine in solution in the mother liquors [Barritt, 1927; Wilson & Lewis, 1927; Toennies & Bennett, 1935-6]. Separation of the basic amino-acids from such a mixture is difficult [Vickery & Leavenworth, 1929] and of the other amino-acids almost impossible. If sulphuric acid be used for the hydrolysis it may be removed with baryta, but there are several disadvantages to this procedure. Addition of powdered baryta or of hot saturated solutions is liable to cause excessive local alkalinity with partial racemization and destruction of cystine; cold saturated baryta solution cannot conveniently be used because of the excessive volume required. In both cases large losses of nitrogen occur by adsorption on the precipitate of BaSO_4 .

A few years ago it occurred to one of us (C. C. L.) that by some modification of the cuprous oxide procedure [Hopkins, 1929; Pirie, 1931] which proved so useful in the hands of Vickery & White [1933] and others, cystine might be removed from keratin hydrolysates without spoiling the mixture for subsequent investigation. It was hoped that cuprous oxide might be used (1) to reduce the cystine quantitatively to cysteine, thus avoiding the introduction of zinc or tin, (2) to precipitate the cysteine as cuprous mercaptide, (3) to neutralize excess (mineral) acid to such a pH as to give quantitative removal of the mercaptide, thus avoiding the addition of alkali or acetate. Preliminary experiments in 1934-5 showed that cuprous oxide would indeed reduce cystine to cysteine and precipitate it under certain conditions, but the observation was not followed up at the time and shortly afterwards Rossouw & Wilken-Jorden [1935] reported a similar finding, viz. that cuprous chloride would reduce cystine and precipitate cysteine cuprous mercaptide. Owing to the pressure of other duties the matter was not touched again until 1937 when the possibility came to mind that, by using a sufficiently concentrated hydrolysate, the cuprous oxide (or chloride formed by the residual HCl) would be dissolved by the amino-acids, allowing the reactions to occur in solution without the necessity of using a salt or acid solvent for the cuprous compound as was done by Rossouw & Wilken-Jorden.

Conditions for the realization of all four of these hopes were soon established. A simple procedure was developed which not only removes cystine from keratin and other hydrolysates in such a way as to leave the mother liquor in a workable condition but which also permits a good recovery of pure, crystalline cystine. This procedure has been used in this laboratory for several years and since it has proved of great value to us for certain types of investigation it may be useful to others.

Two equations have been proposed to explain the appearance of cysteine when heavy metals are used to precipitate cystine:

However, in the presence of Cu_2O the reaction may follow the course indicated by equations (3) and (4) which may be summed to give equation (5):

In order to discover whether equation (1), (2) or (5) represents the mechanism, an attempt was made to recover the cysteine quantitatively. The maximum yield possible, according to equation (1) would be 83.3 %, according to equation (2) 75 % and from (3) 100 %. The recovery in pure crystalline form of 90 % of

the amount of cystine taken, as accomplished in the work described below, eliminates (1) and (2) and strongly supports equation (5).

The use of cuprous oxide for the direct removal of cystine from a protein hydrolysate was studied by adding known amounts of cystine to solutions of pure amino-acids and to hydrolysed gelatin. When the removal and recovery of cystine from these mixtures were shown to be satisfactory, hydrolysates of several types of hair were treated by the procedure adopted; cystine was quantitatively removed (and readily isolated in good yield) leaving the other amino-acids in a form easily amenable to further examination. From many preliminary experiments four only will be described in detail and one separation of cystine from a human hair hydrolysate will be outlined.

EXPERIMENTAL

Exp. 1. Precipitation of cystine added to a gelatin hydrolysate

The first experiment showed that complete removal of cystine from a complex amino-acid mixture could be easily accomplished, without preliminary reduction, by direct addition of cuprous oxide to the hydrolysate.

A known amount of cystine (2.5 g.) was dissolved in *N* HCl and added to 125 ml. of a melanin-free gelatin hydrolysate, 20 % in concentration with respect to amino-acids, from which much of the excess HCl had already been removed. After concentration *in vacuo* to a thick sludge the mixture was diluted to exactly 100 ml. and aliquots of 20 ml. (equivalent to 0.5 g. cystine in about 5.5 g. total amino-acids) were pipetted into a series of mortars. Different quantities of red cuprous oxide (1, 2, 3, 5 and 10 g. respectively) were added to the mixtures. With persistent grinding the oxide dissolved slowly to form a dark greenish-brown solution. In the last two mixtures there was an excess of the red oxide and in them a white turbidity formed. Each of these mixtures was diluted by pouring them separately into 4 vol. of ice-cold water; heavy white precipitates (A) formed at once. These were removed by centrifuging; an equal volume of cold water was added to each of the supernatants and more white precipitate (B) appeared, which was similarly removed. The bluish-green supernatants (volume now 200 ml.) were poured into 200 ml. cold water and left in the refrigerator for several hours. A further small white precipitate (C) appeared.

The use of various volumes of water for the precipitation showed that with less than 10–12 vol. the immediate precipitation was followed by a slower one which required many hours for completion, but which could be accelerated by further dilution. The dilution finally chosen as giving the maximum rate and completeness of precipitation, without excessive dilution, was 15 vol. of water.

The several precipitates (A, B and C) from each mixture were combined, washed twice with cold water, suspended in water and freed from copper with H_2S . The several blue-green supernatants were also freed separately from copper with H_2S . After careful removal of H_2S the supernatants were tested for cystine with sodium nitroprusside in the presence of sodium cyanide. No cystine could be detected in those supernatants which had had 0.4 g. Cu_2O or more per g. of total amino-acids, i.e. the amount of cystine left unprecipitated in a mixture such as the above was less than 4 mg. (0.01 mg. per ml., which is about the limit of sensitivity of this test). The solutions obtained by removing the copper from the white mercaptide precipitates were found (iodimetrically) to contain most of the cystine, the greater part being in the form of cysteine. However, more nitrogen was found in these solutions than could be accounted for as cysteine [cf. Graff *et al.* 1937]. Since some cupric ion is formed during the

reduction of the cystine, part at least of those amino-acids forming insoluble cupric salts (leucine, phenylalanine, tyrosine, aspartic acid) would probably also be precipitated under these conditions. The removal of these by a suitable washing technique was next studied.

Exp. 2. Washing of the cuprous mercaptide precipitate

Some preliminary tests of the washing of the crude cuprous mercaptide precipitate showed that water alone dissolved the "non-cystine-N" very poorly; 0.5 *N* H₂SO₄ was a much better solvent for this purpose but unfortunately it tended to dissolve significant amounts of the mercaptide. This led to the washing procedure being examined in more detail, using both human hair and gelatin hydrolysates (the latter containing known amounts of added cystine), in order to test the general applicability of the precipitation and washing procedures adopted.

A human hair hydrolysate (prepared as described in detail later) was concentrated to give a 40 % solution of amino-acids and the cuprous mercaptide was precipitated from two portions (125 and 25 ml.) in the usual way with a slight excess of cuprous oxide. Both precipitates were washed four times with water, in centrifuge pots, the volume used each time being 5 ml. per g. of hair represented. The first wash-water (intense green in colour) contained much nitrogen, but no cystine could be detected. Subsequent washings became paler in colour, the final one (pale blue) containing very little nitrogen and no cystine. Apparently these washings remove only mother liquor trapped in the rather bulky, amorphous mercaptide precipitate.

The mercaptides were then washed with increasing strengths of dilute H₂SO₄ as shown in Table 1.

Table 1. *Solvent action of dilute H₂SO₄ on crude cuprous mercaptide*

Nature of wash liquid	Vol. used ml.	Total N extracted mg.	Cystine mg.	Cystine-N mg.	Non-cystine-N mg.	Cystine-N as % of total N in extract
A. Mercaptide from 50 g. of hair						
0.1 <i>N</i> H ₂ SO ₄	350	63	2	0.2	62.8	0.4
0.25 <i>N</i> H ₂ SO ₄	300	70	15	1.6	68.4	2.4
0.50 <i>N</i> H ₂ SO ₄	400	49	140	16.3	32.7	49.8
Water	300	27	75	8.7	18.3	47.5
Total		209	232	26.8	182.2	14.7
B. Mercaptide from 10 g. of hair						
0.1 <i>N</i> H ₂ SO ₄	50	12	0.5	0.05	12	0.4
0.1 <i>N</i> H ₂ SO ₄	50	9	0.5	0.05	9	0.6
0.25 <i>N</i> H ₂ SO ₄	50	6	5	0.6	5.4	11.1
0.50 <i>N</i> H ₂ SO ₄	50	12	40	4.6	7.4	62.2
Total		39	46	5.3	33.8	15.7

It is obvious from the above data that the amount of cystine-N lost by washing with 0.5 *N* acid (16.3 mg., i.e. 1.7 % of the accepted cystine-N present, see above) is too large to be neglected. However, 0.25 *N* acid removed only one-tenth this amount of cystine. The data show clearly that acid weaker than 0.25 *N* removes non-cystine-N relatively efficiently without any appreciable loss of cystine. The second experiment (B) confirms the data obtained in the first one and shows that the bulk of the precipitate does not greatly influence the nature of the results.

Exp. 3. Attempted recovery of cystine added to a gelatin hydrolysate

A sample of a cuprous mercaptide from a gelatin hydrolysate (precipitated as described above, using 5 g. Cu_2O for a mixture containing 10 g. hydrolysed gelatin plus 1.0 g. cystine) was washed three times with 75 ml. portions of ice-cold water in a centrifuge pot. As in the case of the mercaptide from the hair hydrolysate, the first wash water was a rich blue-green; the final one was only very pale blue. These washings were added to the main filtrate containing the other amino-acids and the mixture was analysed for total N. No cystine could be detected in this mixture:

	mg.
Total N in original mixture	= 1748
N in filtrate and washings	= 1543
Therefore N in precipitate (by diff.)	= 205
Cystine-N in original mixture	= 116.5
Therefore N precipitated in excess of cystine-N	= 88

The cuprous mercaptide was washed in centrifuge pots three times with 75 ml. portions of very dilute H_2SO_4 (the first two being 0.05 *N*, the third 0.1 *N*), and finally with water. The individual washings were analysed for N, then freed from copper with H_2S and after complete removal of the latter were tested for cystine with cyanide-nitroprusside. Only barely detectable traces of cystine were found although the acid solutions contained much of the non-cystine-N (total of 70 mg.) originally present in the crude mercaptide precipitate. The mercaptide was suspended in water and decomposed with H_2S ; the copper sulphide was washed twice with water saturated with H_2S and once with 0.005 *N* H_2SO_4 . In this experiment the copper sulphide was not analysed for adsorbed N, although subsequent work has shown that 1.5–2 mg. per g. are usually adsorbed. After exact removal of sulphate from the combined solutions (with BaCl_2) the mixture was taken to dryness *in vacuo*. When brought to constant weight over flake NaOH and conc. H_2SO_4 , the cysteine hydrochloride weighed 1.272 g. (corrected for aliquots removed for tests) and contained 8.5 % N; weight expected 1.311 g., theoretical N 8.89 %. On dissolving this hydrochloride in water, making it alkaline with ammonia, aerating to a negative —SH test and bringing to the isoelectric point of cystine with acetic acid, 82 % of the cystine originally taken was recovered. However, if the yield be calculated on the weight of crude cysteine hydrochloride oxidized, the recovery was 85.4 %. The difficulties involved in the quantitative recovery of cystine from cysteine by aeration of an ammoniacal solution are well known, having been discussed by Harris [1923] and others. It was decided in future work to investigate other oxidation procedures.

While the weight of the crude cysteine hydrochloride accounted for 97 % of that expected, its N content was low and the cystine recovered from it was only 82 % of that originally taken. The first figure indicated that equation (5) represents the reaction involved, but the latter figure might be considered to be more in agreement with equation (2). To settle the point the following experiment was conducted.

Exp. 4. Recovery of cystine from a mixture of pure amino-acids

The following mixture of pure amino-acids was dissolved in about 150 ml. of *N* HCl and the mixture was twice taken to dryness *in vacuo* to remove the excess mineral acid.

Amino-acid mixture taken

Amino acid	Wt. g.	N content mg.
Glycine	7.009	1307
Alanine	1.013	159.4
Leucine	1.008	107.8
Glutamic acid	0.404	38.4
Arginine	0.402	129.5
Tyrosine	0.204	15.8
Cystine	2.513	292.5
Total	12.553	2050.4

The hydrochlorides were dissolved in water and made up to 50.0 ml. This solution was ground in a mortar with 4 g. red Cu_2O and then 1 g. portions of Cu_2O were added until a slight excess remained; 12 g. in all were required. The mixture was transferred to a large beaker and 15 vol. of ice-water were added gradually with vigorous mechanical stirring. The resulting white granular precipitate settled quickly. There was a marked contrast between this product and the dark-coloured, bulky, amorphous mercaptides obtained from protein hydrolysates. After 30 min. in the refrigerator the precipitate was filtered on a hardened paper. It was washed thoroughly several times by resuspending in 75 ml. of ice-water and sucking dry on a filter. These water washings were added to the main filtrate. This solution contained 1707 mg. N; therefore the N precipitated was 343.4 or 50.9 mg. in excess of the cystine-N.

The cuprous mercaptide was repeatedly washed as above with very dilute H_2SO_4 . The details are shown in Table 2. Cystine could not be detected in any of the washings.

Table 2

Extract no.	Vol. of wash liquid ml.	Nature of wash liquid	N content of extract mg.
1	75	0.05 N H_2SO_4	12.2
2	75	0.05 N H_2SO_4	10.1
3	75	0.067 N H_2SO_4	8.5
4	75	0.10 N H_2SO_4	10.6
5	75	Water	5.2
			46.6

The cuprous mercaptide was suspended in 250 ml. water and decomposed with H_2S . The mixture was centrifuged and the copper sulphide washed once in a centrifuge pot with 200 ml. H_2S -water, ground in a mortar to a smooth sludge and washed four times more with 0.01 N HCl, saturating each time with H_2S . This exhaustive washing is necessary to remove as much cysteine as possible. The dry weight of the copper sulphide was 13.77 g.; it still retained 16.5 mg. N.

The combined cysteine solutions were evaporated to dryness *in vacuo* under nitrogen. The residue was dissolved in O_2 -free water and made up to 50 ml. An aliquot of 2 ml. was diluted to 100 ml. and portions were analysed for N and cysteine. The cysteine fraction contained 298 mg. N; by Sullivan's highly specific colorimetric procedure [Sullivan & Hess, 1937] 2.40 g. cysteine were found.

The main cysteine solution was made 1.2 N with respect to HCl and oxidized with a strong iodine solution (1.25 N) at a temperature below -5° [Lucas & King, 1932]. The iodine was added dropwise until a yellow colour persisted for over 2 min.; 18.3 milli-equiv. were required, corresponding to 2.195 g. cystine in the 48 ml. or 2.29 g. in the whole solution. The oxidation mixture was concentrated *in vacuo* to remove as much HCl and HI as possible. The concn.

trate, which finally changed in colour from yellow to brown (traces of free iodine?) was diluted to 75 ml. and almost neutralized with NaOH. The pH was then adjusted to 4.8 with sodium acetate. By performing the neutralization very slowly with vigorous mechanical stirring the cystine was obtained in beautifully formed hexagonal plates. Yield (corrected for aliquots) 2.24 g. (89.3 %); N, 11.68, 11.67 % (calc. 11.65 %). Colorimetric comparison by Shinohara's [1935] method with a repeatedly recrystallized sample of cystine also showed the recovered cystine to be pure.

Since our experience with iodimetric oxidation has shown that the recovery of cystine from cysteine rarely exceeds 95 %, the 89.3 % overall recovery which was found indicates that the cysteine solution obtained from the cuprous mercaptide must have contained practically 95 % of the original cystine (confirmed by analytical data above). This effectively excludes reactions of the type shown in equations (1) and (2) and establishes the validity of the assumptions involved in equation (5).

Removal of cystine from keratin hydrolysates

Human hair from a barber's shop, carefully picked over to remove gross impurities, was washed with six changes of distilled water to eliminate dust and other extraneous matter. After being air-dried it was washed in five changes of pure light petroleum, refluxed with several changes of alcohol and finally extracted for some hours with ether in a large Soxhlet apparatus. It was dried in a current of warm dry air (35–40°) and kept in tightly stoppered bottles.

The hair thus prepared had a moisture content of 8.06 % (constant weight over P_2O_5 *in vacuo* at room temperature for 1 month), ash 0.50 %, N 16.2 %, S 5.00 % (the two latter calculated on an ash- and moisture-free basis).

Preliminary experiments showed that boiling with 8–9 *N* HCl on a sand bath caused rapid liberation of amino groups during the first hour, a distinct slowing of the process by the third hour, a gradual increase to a maximum figure at 10.5–12 hr. and then a very gradual decrease over a very long period. The rate of liberation of cystine was also followed, using Lugg's [1933] modification of Sullivan's procedure, and a maximum was noted at about 6–7 hr., in good agreement with the findings of Merrill [1921] and Rimington [1929] who made similar studies on wool. The maximum amino-N by Van Slyke's [1911; 1913] method was 72–79 % of the total N in different hydrolysates, although Kendrick & Hanke's [1940] potassium iodide modification gave figures about 6 % lower. This latter method is claimed to give essentially correct figures for glycine, glyceryl peptides and cystine, all of which give high results by the unmodified procedure. The cystine figure found at the end of the hydrolysis was 16.4 %, in good agreement with Vickery & Block's [1931] figure of 16.5 % for human hair.

CO₂ and H₂S are given off during the hydrolysis, about 350 mg. of the former and 7 mg. of the latter per 100 g. hair, after 11 hr. boiling.

N distribution was determined on one hydrolysate with the results shown in Table 3.

The isolation of cystine was carried out on a hydrolysate from 328 g. hair (equivalent to 300 g. ash- and moisture-free keratin) refluxed with 1600 ml. 9 *N* HCl on a sand bath for 11 hr. After one evaporation *in vacuo* to remove excess HCl the hydrolysate was diluted, melanin was filtered off, washed five times with dilute HCl and the combined filtrate and washings were evaporated to a thick syrup three times before making up to 1200 ml. An aliquot of 1 ml. was removed, diluted to 50 ml. and used for N and cystine determinations.

Table 3. *Nitrogen distribution in hair hydrolysate*

(Total N in hair keratin = 16.04%)

	% of total N	
	Observed	Corrected
Melanin-N	0.56	0.56
Amide-N	7.22	7.22
Basic N	33.4	35.8*
Dicarboxylic N	11.5	11.5
Monoamino-acid-N + non-amino-N	47.32	44.9

* Corrected for solubility of phosphotungstates (Plimmer).

The remaining solution was ground in a mortar with 300 g. red cuprous oxide. Some of the oxide remained unchanged even after 30 min. grinding. The mixture was added slowly with constant stirring to 12 l. of ice-water; after being left in the refrigerator for 2 hr. the greyish white mercaptide was filtered off by suction. It was thoroughly washed by suspending it three times in 500 ml. portions of ice-water, rubbing to a smooth suspension and filtering with suction to a dry hard cake. The total filtrate contained 37.450 g. N, from which figure the N content of the crude mercaptide can be calculated to be 10.69 g., a value considerably in excess of the 5.73 g. N expected if one calculates from the cystine figure obtained by the Sullivan method. The direct cuprous oxide precipitation as used does not possess very great specificity.

The crude mercaptide precipitate was washed as above with three 500 ml. portions of 0.10N H_2SO_4 and given a final wash with 700 ml. cold water. The N thus removed was 1.24 g. The mercaptide was suspended in 3 l. of water and copper removed with H_2S . The copper sulphide was washed four times by suspending in 3 l. of water each time, saturating with H_2S and centrifuging. The dried sulphide weighed 380 g. and contained 697 mg. N.

The filtrate (cysteine hydrochloride solution) was concentrated *in vacuo* and several hundred milli-equiv. of HCl were thus removed. The solution was made up to 500 ml. and 1 ml. was taken for N determinations. The solution, which was found to contain about 3.5 g. non-cystine-N, was ground in a mortar with a slight excess of Cu_2O (100 g.) and the suspension was poured slowly with constant stirring into 15 vol. of ice-water. After 2 hr. in the refrigerator, the white granular mercaptide was filtered with suction, washed by suspending in 1 l. of ice-water and again sucked to a dry hard cake. The filtrate contained 2.71 g. non-cystine-N and traces (125 mg.) of cystine. Other small losses of cystine were noted: 250 mg. in the original filtrate of the other amino-acids and 37 mg. in the acid washings of the mercaptide. The total loss thus accounted for was 412 mg. or less than 1% of the cystine started with.

The reprecipitated mercaptide was decomposed with H_2S and the chocolate-brown Cu_2S was washed as described above. The dried sulphide weighed 128 g. and contained 189 mg. N. The filtrates were concentrated *in vacuo* under N_2 and made to 500 ml. Small aliquots were removed for analysis. The main solution was boiled with 3 g. charcoal to remove a slight brown coloration and the filtrate was concentrated *in vacuo* under N_2 to incipient crystallization. It was warmed slightly, transferred to a beaker and chilled, upon which it formed an almost solid mass of crystals. These were removed on a fritted glass filter, washed with cold conc. HCl and dried *in vacuo* over conc. H_2SO_4 and flake NaOH. A second crop was obtained in the same manner from the filtrate, after concentration. The filtrate was brought to dryness in the same way; the residue was called crop III.

Crystalline cysteine hydrochloride isolated from 300 g. hair

Crop	Wt. g.	N* %	S* %	Yield (calc. as cystine)
I	30.6	8.86	20.14	7.86
II	13.2	8.93	19.45	3.40
III	22.4	9.83	16.38	4.64 (calc. from S)
Total	66.2			15.90

* Calc. for cysteine hydrochloride: N, 8.89; S, 20.3%.

The analytical data on crops I and II were considered satisfactory and the material was accepted as essentially pure cysteine hydrochloride. Crop III, however, was grossly impure and calculations based on the S and N contents indicated contamination with material very high in N. A strongly positive Sakaguchi test was obtained.

By recrystallizing crop III from concentrated HCl another 8.3 g. of fairly pure cysteine hydrochloride were obtained. The separation of arginine from the remaining cysteine was effected as follows. The filtrate was evaporated to dryness to remove excess HCl, taken up in water and treated with hot saturated Ag_2SO_4 solution until an excess of silver ion was present. The mixture was brought to pH 5.5–6 with baryta; the precipitate of silver cysteine, silver chloride and barium sulphate was removed and washed with water. From the filtrate, which gave no test for cystine, 0.97 g. arginine was isolated as flavianate. Although the filtrate still contained about 100 mg. N, no other amino-acid was identified.

The silver precipitate was decomposed by boiling several times with an excess of HCl and the resulting cysteine hydrochloride solution was taken to dryness *in vacuo*. It was dissolved in water, made slightly alkaline with ammonia, treated with a drop of dilute FeCl_3 - CuSO_4 mixture and aerated. After recrystallizing the 6 g. crude amino-acid (N, 11.1; S, 21.4%) several times, only about 4.2 g. of pure cystine (N, 11.64%) were obtained. About 5 g. of crop III were thus left unaccounted for by the products isolated and on the basis of the S content of crop III about 3 g. of the uncrystallizable material were cysteine or some product derived from it.

DISCUSSION

The recovery from the hair hydrolysate of pure, crystalline cystine (14.8%) as such, or as cysteine hydrochloride, accomplished in this experiment exceeds considerably any isolation figure previously reported in the literature. As far as the authors are aware, the highest yield of analytically pure cystine hitherto isolated from a human hair hydrolysate is 10.7% [Vickery & Leavenworth, 1929]. Buchtala [1907] claimed to have isolated 14.5% but this figure was in part arrived at by calculation from the S content of very impure fractions; the same remarks apply to Barritt's figure of 13.0%. Barritt [1927] and others [Hopkins, 1929; Harris, 1923; Rimington, 1929; Toennies & Bennett, 1935–6] have commented on the usual inability to isolate more than 60–70% of the cystine-sulphur of keratins or any other protein as reasonably pure cystine. The cuprous oxide procedure described above affords a considerable improvement in this regard. The cystine figure of 14.8% (material obtained in pure crystalline form) corresponds to 79% of the keratin-S; if the S in impure fractions of cystine be included the fraction accounted for reaches 85%. Further improvements in

the method will doubtless enable still larger proportions of cystine to be isolated in pure crystalline form.

The use of cuprous oxide not only makes it possible to recover the cystine in excellent yield and high purity but separates the cystine quantitatively from the other amino-acids and in such a way that the remainder of the hydrolysate is in a form suitable for the application of other isolation procedures. The value of this reagent is further enhanced by the fact that it removes from the dark hydrolysate practically all of the chloride ion and also colloidal colouring matter which tends to inhibit crystallization [cf. Abderhalden & Fuchs, 1908]. After removal of copper from the main filtrate containing the other amino-acids, an almost water-clear or pale straw-coloured solution is obtained.

In view of the results obtained in this study a note of warning concerning the analytical use of cuprous oxide as a reagent for cysteine seems warranted. Graff *et al.* [1937] claim that the specificity of cuprous oxide for cysteine is so marked that when it is added to a reduced protein hydrolysate the precipitate obtained is pure cysteine cuprous mercaptide. The analytical data presented in their paper certainly appear to support that statement, but none of our experiments have given quite such desirable results. While the direct use of cuprous oxide on an unreduced solution cannot be compared with their procedure, the reprecipitation of the cysteine fraction should be more nearly comparable, yet even here 8.8% of the N in the mercaptide was found to be non-cysteine-N. Experiments with pure amino-acids (as hydrochlorides) either alone or mixed with cysteine, proved that considerable quantities of some, particularly glycine and arginine, are precipitated by cuprous oxide under the conditions used by us. While these findings do not necessarily disprove the results of Graff *et al.* [1937], since our materials and conditions are not identical with theirs, considerable suspicion is cast upon the reliability of cuprous oxide as a highly specific precipitant for cysteine in protein hydrolysates. Certainly little trust should be placed in the accuracy of cysteine determinations based upon the nitrogen content of mercaptide precipitates.

However, in spite of its lack of complete specificity, cuprous oxide should be considered a valuable reagent because of its relative specificity for sulphhydryl compounds. Its specificity has proved sufficient to be of great value in improving the isolation of cysteine, just as it did in the case of glutathione [Hopkins, 1929; Pirie, 1931]. Its value as an analytical reagent depends upon its use in the manner described by Vickery & White [1933], viz. determination of the organic sulphur in the cuprous mercaptide precipitate.

SUMMARY

1. Cuprous oxide can be used to remove cystine quantitatively from protein hydrolysates.
2. Since the cuprous oxide procedure to remove cystine does not require the use of alkalis or inorganic salts, the remainder of the hydrolysate is in a suitable condition for the application of other isolation procedures.
3. Cuprous oxide simultaneously removes most of the chloride ion and all of the colloidal colouring matter from the hydrolysate.
4. Pure crystalline cystine (or cysteine hydrochloride) may be readily obtained in excellent yield from the cuprous mercaptide precipitated.
5. The yield of pure cystine isolated from a human hair hydrolysate has been considerably increased above the usual amount obtained by fractional crystallization of neutralized hydrolysates.

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