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The Occurrence of Bis-(2-Amino-2-Carboxyethyl) Trisulphide in Hydrolysates of Wool and other Proteins

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Acid hydrolysates of wool have been found to contain sulphur compounds other than cystine, cysteine and methionine, and experiments in which wool was hydrolysed in the presence of [³⁵S]cystine showed that most of these unidentified sulphur compounds were formed by the decomposition of cystine during hydrolysis (Lewis, Robson & Tiler, 1960). The fact that these decomposition products had the same specific activity as the cystine in the hydrolysate has been made the basis of a method for determining cystine plus cysteine in proteins (Fletcher & Robson, 1962*a*). Most of these compounds reduced phosphotungstic acid, either alone or in the presence of sodium bisulphite, and their presence in wool hydrolysates provided an explanation for the discrepancy between the chromatographic and colorimetric analyses for cystine (Corfield & Robson, 1955). The most abundant of these compounds (given the trivial designation 'S1' by Lewis *et al.* 1960) accounted for 6% of the sulphur in a 40 hr. hydrolysate of wool. The present paper describes its isolation and characterization as bis-(2-amino-2-carboxyethyl) trisulphide. A short account of this work has already appeared (Fletcher & Robson, 1962*b*).

Lewis *et al.* (1960) showed that small amounts of S1 were formed when cystine was boiled in 5*N*-hydrochloric acid in the presence of all the other protein amino acids, and that its formation appeared to depend on the presence of tryptophan. However, the finding of a trace of ³⁵S-labelled S1 in a solution of [³⁵S]cystine in 0.1*N*-hydrochloric acid which had stood for some time suggested that the influence of tryptophan was indirect, and possibly connected with the interaction of cystine and tryptophan to form cysteine (Olcott &

Fraenkel-Conrat, 1947). The chromatographic behaviour of S1 showed that it was very labile under neutral and alkaline conditions, and that cystine and *meso*-cystine were among the products of decomposition, indicating a close structural relationship between S1 and cystine.

Thus far investigations had been conducted on a few milligrams of impure S1, but much larger amounts were required for a more detailed study. However, before the isolation of S1 on a large scale from wool hydrolysates was attempted, other possible sources were investigated. The appearance of traces of S1 in an aged acid solution of [³⁵S]-cystine might have been due to self-irradiation. To test this possibility, an acid solution of inactive cystine was given a massive dose of γ -radiation from a ⁶⁰Co source. Although S1 was present in the irradiated solution, the yield was not sufficient to make this a practicable method of preparation. Subsequently, hydrolysates of insulin, ribonuclease, bovine plasma albumin, lysozyme and β -lactoglobulin were examined for the presence of S1. The amounts of S1 present in hydrolysates of the first three proteins increased with increasing time of hydrolysis: after 40 hr. the percentages of protein sulphur present as S1 were 1.9, 2.0 and 2.7 for insulin, bovine plasma albumin and ribonuclease, respectively; S1 was not found in hydrolysates of lysozyme or β -lactoglobulin. The yields of S1 in these experiments were much less than those obtained from wool hydrolysates, and the latter, therefore, were used exclusively for the preparation of S1 on a large scale.

For the preparative isolation of S1, wool hydrolysates were chromatographed initially on columns of Zeo-Karb 225 (H⁺ form; X4) resin by elution with 2.5*N*-hydrochloric acid. This pro-

cedure, first used for the separation of amino acids by Stein & Moore (1950), was particularly convenient since S1 is stable under acid conditions and the eluent used is completely volatile. The S1 emerged from these columns together with some of the tyrosine and most of the phenylalanine of the wool hydrolysates. The contaminating amino acids were subsequently removed by chromatography on starch, and the S1 was freed from traces of soluble starch by rechromatography on a short column of Zeo-Karb 225 (X4) eluted with 2.5N-hydrochloric acid. After removal of the eluent, S1 was isolated as a glassy deliquescent solid containing Cl^- ion. The free base was obtained by neutralizing a solution of the hydrochloride with pyridine. In this way amounts of S1 varying from 50 mg. to 1 g. were isolated from wool hydrolysates: one of these preparations, obtained from wool hydrolysed in the presence of ^{35}S -cystine, was labelled with ^{35}S .

The S1 so isolated gave a positive reaction with ninhydrin. It did not reduce phosphotungstic acid alone, but did so in the presence of sodium bisulphite. Its electrophoretic mobility at pH 4.5 was similar to that of cystine, indicating that it had a neutral molecule. It appeared as a double peak when chromatographed in analytical amounts under the conditions given in Fig. 1 of Fletcher & Robson (1962a). Peaks 6 and 7 on this chromatogram are both S1, and are believed to be stereoisomers. Elementary analysis gave the formula $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_3$. From the evidence summarized below, it was concluded that the structure of S1 is that of bis-(2-amino-2-carboxyethyl) trisulphide (Cy_2S_3):

When S1 was titrated with potassium bromate-bromide, it was oxidized in accordance with eqn. (1), where, and subsequently, Cy represents $\text{HO}_2\text{C}\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2$.

Confirmation of the course of oxidation was obtained by oxidizing ^{35}S -labelled S1 with free bromine, precipitating and counting the ^{35}S -sulphate as benzidine sulphate, oxidizing the ^{35}S -cysteic acid in the filtrate to sulphate, and again precipitating and counting as benzidine sulphate. The ratio of the two counts was approximately 1:2. The identity of the ^{35}S -labelled products in reaction (1) was confirmed by chromatography on Dowex 1 with cysteic acid and sulphate carriers.

When S1 was reduced with tin (or zinc) and hydrochloric acid, hydrogen sulphide was formed, and chromatography of the resulting solution on paper, or on a column of Zeo-Karb 225 (X4) in

2.5N-hydrochloric acid, failed to reveal the presence of any thiol other than cysteine. Attempts to determine the hydrogen sulphide quantitatively, by using the bismuth reagent of Goa (1961), gave erratic results, but approximately 25% more hydrogen sulphide appears to be formed than is required by eqn. (2).

When S1 was allowed to react with potassium cyanide under the conditions of Sörbo (1957), with Cu^{2+} ions omitted from the reaction mixture, the amount of thiocyanate formed increased with time up to 45 min. and thereafter remained constant. This constant value was equivalent to one-third of the sulphur of S1, in accordance with eqn. (3) (Foss, 1950; Fehér, Krause & Vogelbruch, 1957).

After cyanolysis of S1, only one ninhydrin-positive spot, with an R_f corresponding to that of cystine, appeared on paper chromatograms.

The reduction of phosphotungstic acid by S1 in the presence of sodium bisulphite provided a convenient method for detecting S1 in fractions of effluent from chromatographic columns. Colour development was complete and reproducible after 30 min., but the extinction was less than that given by an equivalent amount of cystine. The reaction between S1 and bisulphite might be expected to take the course indicated by eqns. (4) and (5) (Foss, 1960).

If cysteine were one of the primary products of the reaction between S1 and bisulphite, S1 should give with phosphotungstic acid the same molar extinction as cystine. However, examination of the products of the reaction between S1 and excess of bisulphite at pH 2.2 or 5.2 by paper chromatography indicated that cysteine was absent, although it was clearly detectable when cystine was treated with bisulphite. Cysteinesulphonate was the only major ninhydrin-positive product. A spot, with R_f corresponding to that of thiosulphate, was revealed on staining with silver nitrate and, provided that the concentration of bisulphite was not too high, thiosulphate could be determined in the solution by the method of Sörbo (1957). The species that reduces the phosphotungstic acid, therefore, remains unidentified. When the amount of bisulphite in the reaction is decreased to that equivalent to the S1, hydrogen sulphide can be detected. The reaction given in eqn. (6) may occur under these conditions.

The identity of S1 with Cy_2S_3 was confirmed by synthesis.

The classical method of synthesis of trisulphides is the reaction of 2 mol.prop. of thiol with sulphur dichloride in an inert solvent in which both the reactants are soluble. The difficulty of applying this reaction to water-soluble thiols has been noted by Milligan, Saville & Swan (1961). Balenović & Gašpert (1960) prepared the diphthaloyl derivative of Cy_2S_3 by the interaction of *N*-phthaloyl-L-cysteine with sulphur dichloride in chloroform. Attempts to prepare the free amino acid by the removal of the phthaloyl groups by hydrazinolysis led to loss of sulphur from the compound (Gašpert, Štefanac, Marušić & Balenović, 1960). This suggested that Cy_2S_3 might be prepared by this method if the amino groups of cysteine were first protected by a substituent that could easily be removed under acidic conditions. A suspension of *N*-acetylcysteine was refluxed in a chloroform solution of sulphur dichloride and, after removal of the chloroform, the products were hydrolysed with acid for a short time. A compound was formed, the chromatographic behaviour of which, both on paper and on ion-exchange resin columns, was similar to that of S1. This compound was isolated in a pure state and was shown to have an infrared-absorption spectrum identical with that of S1. A by-product of the reaction, isolated in small yield, possessed a sulphur content close to that calculated for bis-(2-amino-2-carboxyethyl) tetrasulphide (Cy_2S_4), which can be formed from the disulphur dichloride present in the equilibrium:

This compound liberated 2 equiv. of thiocyanate when treated with cyanide (Fig. 1).

Paper-chromatographic evidence suggests that, when Cy_2S_3 is allowed to stand in dilute acid solution, cystine and the tetrasulphide are formed (eqn. 7). There is also evidence that the tetrasulphide

occurs to a small extent in wool hydrolysates. When peak no. 9 of Fig. 1 of Fletcher & Robson (1962*a*) is chromatographed on paper, three spots are obtained, two of which were identified with Cy_2S_3 and Cy_2S_4 by chromatographing these singly and in admixture. This gives an upper limit of 0.022% of sulphur in the form of the tetrasulphide in a 48 hr. hydrolysate of wool.

Two groups of workers studying the chemistry of sulphur-transferring enzyme systems (Hylin & Wood, 1959; Cavallini, De Marco, Mondovì & Mori, 1960*b*) have found that sulphur reacts with cysteine in alkaline solution to form a substance which gives thiocyanate when treated with cyanide at room temperature, and which they

suggested might be involved in the enzyme reactions. Cavallini *et al.* (1960*b*) considered that this substance might be $CySSH$ ('thiocysteine'), or some stable cyanolysable decomposition product thereof. They showed that the compound was stable to hot acid and was retained on columns of Dowex 50.

We have found that, when solutions of sulphur and cysteine are mixed as described by Cavallini *et al.* (1960*b*), hydrogen sulphide can immediately be detected and that, after the solution has been acidified, Cy_2S_3 can be isolated from it. The reaction may be as shown in eqn. (8).

The material prepared in this way was indistinguishable from Cy_2S_3 synthesized by the sulphur dichloride method when examined by chromatography on paper and on starch or resin columns, and gave satisfactory values for N and S contents and cyanide-labile S. However, the infrared-absorption spectra of several preparations, identical among themselves, showed significant differences, as yet unexplained, from that of Cy_2S_3 isolated from wool hydrolysates or synthesized by the sulphur dichloride method. Other reactions in which circumstantial evidence for the participation of thiocysteine has been adduced include the reaction of cysteinesulphonate with sulphide (Sörbo, 1958), the cleavage of cystine by a pyridoxal model (Cavallini, De Marco & Mondovì, 1960*a*) and the spontaneous trans-sulphuration of sulphinates by organic persulphides (De Marco, Coletta & Cavallini, 1961). Although exhibition of the properties attributed to thiocysteine cannot be taken as conclusive evidence of the presence of Cy_2S_3 , it does appear possible that the latter could play a fundamental role as a sulphur carrier in biological systems.

The present evidence suggests that some Cy_2S_3 is formed during the hydrolysis of most cystine-containing proteins with hydrochloric acid. The only exceptions are those proteins containing a high proportion of tryptophan, which interacts with cystine to form cysteine. Concentrations of thiol above a certain limit must therefore inhibit the formation of Cy_2S_3 , although it can certainly be formed in the presence of thiol, and may in fact be formed from cysteine and traces of free sulphur produced by the breakdown of cystine by other routes. Although this reaction is probably very slow in acid media, the stability of Cy_2S_3 under these conditions causes its accumulation in the system.

Because of the occurrence of peptide linkages resistant to acid hydrolysis, it is now customary, when the amino acid composition of a protein is determined, to carry out analyses after different times of hydrolysis. Some of the longer times (up to

72 or 96 hr.) must produce appreciable amounts of Cy_2S_3 . This will not, however, appear as such in ion-exchange resin chromatography, as it is largely decomposed to cystine and other products at the pH of the buffer used (Lewis *et al.* 1960).

Forbes & Savige (1962) observed the presence of a substance ('unidentified product B') on paper chromatograms and electrophoretograms of ultra-violet-irradiated cystine, to which they tentatively ascribed the structure of Cy_2S_3 . The chromatographic behaviour of this substance is consistent with that of Cy_2S_3 .

Lanthionine, first isolated as an artifact from alkali-treated wool (Horn, Jones & Ringel, 1941), was later found to occur naturally (Alderton & Fevold, 1951). If, as the work of Hylin & Wood (1959) and Cavallini *et al.* (1960*b*) suggests, Cy_2S_3 occurs in biological systems, this will present a curious parallel.

EXPERIMENTAL

Proteins. Insulin, bovine plasma albumin, lysozyme and Merino 64s quality wool were the samples used by Fletcher & Robson (1962*a*). Ribonuclease was obtained from British Drug Houses Ltd., and β -lactoglobulin from L. Light and Co. Ltd. These proteins were dried *in vacuo* over P_2O_5 . For the isolation of Cy_2S_3 from wool hydrolysates, commercial dry-combed wool top (Australian Merino 64s A quality) was extracted in a Soxhlet apparatus with azeotropic benzene-methanol (20 siphonings) and washed thoroughly with water.

DL- $[^{35}S]$ cystine. This was obtained from The Radiochemical Centre, Amersham (specific activity 40 mc/m-mole).

Determinations. Thiol and disulphide were determined by the phosphotungstic acid reduction method (Fletcher & Robson, 1962*a*). Total S was determined by the method of Jones & Letham (1956). Total N was determined by the method of Chibnall, Rees & Williams (1943). The ^{35}S activity was determined quantitatively by the method of Henriques, Kistiakowski, Margnetti & Schneider (1946), and for approximate analyses by the counting of dry samples on small polythene trays.

Paper chromatograms. These were run by the descending technique on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:5, by vol.). The ninhydrin spray reagent contained 0.1% ninhydrin in collidine-ethanol (1:19, v/v) (Levy & Chung, 1953). Staining with silver nitrate was done according to the method of Skarzynski & Szczepkowski (1959). The R_F values of the compounds of interest in this system, with the colours that they give with the ninhydrin reagent, are: cystine (0.06, blue-grey); Cy_2S_3 (0.09, brown); cysteinesulphonate (0.15, red-brown); Cy_2S_4 (0.17, brown); cysteine (0.34, blue with yellow centre); thiosulphate (0.10).

Presence of bis-(2-amino-2-carboxyethyl) trisulphide in hydrolysates of wool and other proteins. Protein (20–100 mg.) was hydrolysed in 5.7N-HCl containing $[^{35}S]$ cystine (10–20 μ C) for various times. The HCl was removed *in vacuo* and the residues were chromatographed on a column (either 0.9 cm. $\times$ 40 cm. or 1.8 cm. $\times$ 40 cm.) of Zeo-Karb 225 (X4) in 2.5N-HCl.

The activities in the cysteine, cystine and Cy_2S_3 peaks, which emerged at about fractions 18, 30 and 75–85 respectively, were determined by evaporating and counting a portion of each fraction (3 ml. or 10 ml.) on a polythene dish. Cystine S in the cystine peak was determined by the phosphotungstic acid reduction method, and cysteine S and Cy_2S_3 S were calculated from the ratio of the total counts in the peaks, by assuming the compounds to have the same specific activity. The results are shown in Table 1.

Table 1. *Cystine, cysteine and bis-(2-amino-2-carboxyethyl) trisulphide in hydrolysates of wool and other proteins*

Proteins were hydrolysed in the presence of DL- $[^{35}S]$ cystine, and the three ^{35}S -labelled compounds separated by chromatography on Zeo-Karb 225 (X4). Cystine S and Cy_2S_3 S are calculated from the activities in the three peaks and the cystine S in the cystine peak. Experimental details are given in the text.

Protein	Time of hydrolysis (hr.)	Cystine S (%)	Cysteine S (%)	Cy_2S_3 S (%)
Wool	16	2.90	0.05	0.06
	40	2.85	0.06	0.15
	48	2.70	0.05	0.19
	72	2.44	0.01	0.20
	100	—	—	0.47*
	333	2.14	0.00	0.08
Insulin	16†	2.96	0.01	0.00
	40	3.19	0.03	0.06
	72	3.02	0.06	0.16
Bovine plasma albumin	40	1.52	0.02	0.03
Ribonuclease	40	1.43	0.00	0.04
Lysozyme	16	0.92	0.38	0.00
	40	1.21	0.40	0.00
	72	0.95	0.60	0.00
β -Lactoglobulin	40	0.61	0.30	0.00

* Isolated.

† 0.09% of peptide S.

For wool, in addition to the results obtained by the method described above, results based on the direct determination of Cy_2S_3 by the phosphotungstic acid reduction method in preparative experiments are given.

Isolation of bis-(2-amino-2-carboxyethyl) trisulphide from wool hydrolysates. Wool top (100 g. dry wt.) was refluxed with 900 ml. of 6N-HCl until dissolution was complete (4 hr.). Approx. 1 mc of DL-[^{35}S]cystine (specific activity 40 mc/m-mole) in 0.1N-HCl (5 ml.) was added and the hydrolysis continued for a further 96 hr. After removal of the HCl *in vacuo*, the tarry residue was dissolved in 2.5N-HCl (300 ml.) and run on to a column (9 cm. $\times$ 40 cm.) of Zeo-Karb 225 (X4). The column was eluted with 2.5N-HCl; after a fore-run of 14 l., 200 ml. fractions were collected. The Cy_2S_3 peak was found by evaporating a 0.2 ml. portion of each fraction to dryness on a polythene tray for counting. (For non-radioactive preparations, the peak was found by neutralizing a portion of each fraction and testing with sodium bisulphite and phosphotungstic acid.) Fractions 13-39 were combined and evaporated to dryness *in vacuo*. To remove phenylalanine and tyrosine, and traces of soluble brown impurity derived from the resin, the residue was dissolved in 25 ml. of propanol-0.5N-HCl (2:1, v/v) and run in this solvent on a column of starch (6.4 cm. $\times$ 15 cm.) (Stein & Moore, 1948): 700 ml. of solvent was run off, and then 16 ml. fractions were collected. Fractions 5-63 were combined and the solvent was removed *in vacuo*. Traces of hydrolysed starch were removed by dissolving the residue in 2.5N-HCl (10 ml.) and rechromatographing on a column of Zeo-Karb 225 (X4) (4 cm. $\times$ 4 cm.) in this solvent; 10 ml. fractions were collected and 0.05 ml. of each fraction was plated for counting. Fractions 3-9 gave a positive response to Mölisch's test for sugars (Smith & Jones, 1948). Fractions 12-36, which contained the Cy_2S_3 , were combined and evaporated *in vacuo*, leaving a white, friable, non-crystalline residue. This was dissolved in water, the solution and washings (final vol. 40 ml.) were filtered through sintered glass, and pyridine was added cautiously with stirring until the pH was 4.2. After the mixture had stood at room temperature for 6 days, the ^{35}S -labelled Cy_2S_3 was filtered off, washed with water and ethanol, and dried *in vacuo* over P_2O_5 to give a yield of 1.33 g. (13% of the wool S).

In two non-radioactive preparations, 300 mg. of Cy_2S_3 was isolated from a 48 hr. hydrolysate of 100 g. of wool, and 150 mg. of Cy_2S_3 from a 72 hr. hydrolysate of 50 g. of wool.

The Cy_2S_3 so isolated was a white crystalline slightly-hygroscopic powder, insoluble in water but readily soluble in dilute acid. When chromatographed on paper in butan-1-ol-acetic acid-water (4:1:5, by vol.) and sprayed with ninhydrin, it gave a distinctive brown spot with R_F 0.09. Its electrophoretic mobility on paper (pH 4.5; 82v/cm.) was almost the same as that of cystine [Found: C, 27.1; H, 4.4; N, 10.0; S, 36.4%; acid equiv. (formol titration), 138. $C_6H_{12}N_2O_4S_3$ requires C, 26.5; H, 4.4; N, 10.3; S, 35.3%; acid equiv. 136].

Oxidation of bis-(2-amino-2-carboxyethyl) trisulphide. (a) Titration with bromate-bromide. A sample (9.94 mg.) of Cy_2S_3 was dissolved in 10.0 ml. of 2N-HCl. To samples (2.99 ml.) of this solution, in stoppered flasks, were added 0.100N-KBrO₃ (2.99 ml.), KBr (0.2 g.) and KI (0.2 g.), in that order. The flasks were stoppered and shaken, and the iodine liberated was titrated with 0.0998N-Na₂S₂O₃. The mean of three titrations was 1.270 ml., making 2.97 mg. of

Cy_2S_3 equivalent to 1.720 ml. of 0.100N-KBrO₃. Hence 276 g. of Cy_2S_3 is equivalent to 16 moles of KBrO₃ which is equivalent to 8 g.atoms of oxygen [eqn. (1) above requires 272 g.].

(b) Determination of the ratio of cysteic acid to sulphate formed. A sample (16.07 mg.) of ^{35}S -labelled Cy_2S_3 was dissolved in 8N-HCl (1 ml.), the solution cooled in ice and bromine water added dropwise until a faint yellow colour persisted (Thompson, 1955). Excess of bromine was removed by aspirating N₂ through the ice-cool solution, which was then evaporated to dryness *in vacuo* over KOH. The residue was dissolved in de-ionized water (1.00 ml.) and three samples (0.100 ml. each) run into 10 ml. glass vials. To each vial were added, in the following order, de-ionized water (0.9 ml.), Na₂SO₄ solution (2.69 mg./ml.; 1.0 ml.), cysteic acid solution (2.44 mg./ml.; 1.0 ml.), ethanol (2 ml.) and 2% (w/v) benzidine hydrochloride solution (1.0 ml.). The amounts of 'carrier' sulphate and cysteic acid added were calculated to give a final weight of 7 mg. of benzidine sulphate in the subsequent procedures (Henriques *et al.* 1946). The benzidine [^{35}S]sulphate precipitates were collected on planchets for ^{35}S assay, and the filtrates and washings, containing [^{35}S]cysteic acid, were evaporated to dryness in heavy-walled tubes (1 cm. $\times$ 20 cm.). To each residue were added NaCl (5 mg.) and HNO₃ (0.3 ml.), the tubes sealed, and the contents subjected to combustion for 1.5 hr. at 270° to oxidize cysteic acid to sulphate. The contents of the tubes were evaporated to dryness *in vacuo* over KOH, and assayed for ^{35}S as benzidine sulphate. Three portions of the original ^{35}S -labelled Cy_2S_3 were also subjected to combustion and assayed similarly. Activities found (means of three samples) were: in ^{35}S -labelled Cy_2S_3 samples oxidized, 7.39×10^4 counts/min.; as sulphate, 2.45×10^4 counts/min. (33.2%); as cysteic acid, 4.66×10^4 counts/min. (63.1%); thus ratio of cysteic acid to sulphate was 1.90.

Action of cyanide on bis-(2-amino-2-carboxyethyl) trisulphide. A sample (2 mg.) of Cy_2S_3 was dissolved in 5 ml. of 0.2N-HCl. To 0.5 ml. portions of this solution were added, in turn, water (3.6 ml.), N-NH₃ (0.4 ml.) and 0.1M-KCN (0.5 ml.). The solutions were mixed and left for various times, after which 0.5 ml. of ferric iron reagent (Sörbo, 1957) was added and the extinction at 460 m μ measured. Extinction was plotted against time (Fig. 1): the cyanolysis of Cy_2S_3 was complete after 40 min. The thiocyanate produced in the reaction was determined from a standard curve prepared with KSCN by using the equation:

$$\text{Labile S } (\mu\text{g.}) = 54.0 (E_{460} - 0.025)$$

Three independent determinations on approx. 2 mg. of Cy_2S_3 gave 0.99, 0.98 and 0.95 g.atom of labile S/272 g. of Cy_2S_3 . Cystine gave no thiocyanate under these conditions.

Reduction of bis-(2-amino-2-carboxyethyl) trisulphide. A sample (2 mg.) of Cy_2S_3 was dissolved in 1 ml. of 2N-HCl, and AnalaR zinc dust (50 mg.) was added: H₂S could immediately be detected. The solution was heated to 60° and allowed to cool, filtered, evaporated to dryness, and the residue dissolved in 0.5 ml. of 0.2N-HCl. Portions of this solution were chromatographed on paper with cystine and cysteine controls. When the paper was sprayed with ninhydrin, the reduced Cy_2S_3 gave spots due to cystine (R_F 0.07), cysteine (R_F 0.28) and a faint spot with R_F 0.13. When the paper was sprayed with buffered phosphotungstic acid [20 ml. of phosphotungstic acid solution

Fig. 1. Reaction of cystine, of bis-(2-amino-2-carboxyethyl) trisulphide and of bis-(2-amino-2-carboxyethyl) tetrasulphide with cyanide. Samples (approx. $1 \mu\text{mole}$) of the compounds were cyanolysed by the procedure of Sörbo (1957), with Cu^{2+} ions omitted from the solution. Thiocyanate was determined as described in the text. ▲, Cy_2S_2 ; ○, Cy_2S_3 ; ●, Cy_2S_4 .

(Folin & Marenzi, 1929), 40 ml. of acetate buffer, pH 5.2 (25 ml. of acetic acid and 300 g. of sodium acetate trihydrate/l.), and 40 ml. of water] only one spot, corresponding to that of cystine, appeared.

The remainder of the reduced Cy_2S_3 was chromatographed on a column of Zeo-Karb 225 (X 4) in 2.5N-HCl; 3 ml. fractions were collected and tested for the presence of thiol with phosphotungstic acid. Cysteine emerged in fractions 15–17, but no other thiol peak could be detected up to fraction 100.

Attempts were made to determine the H_2S formed by the reduction of Cy_2S_3 with zinc and HCl (Goa, 1961): AnalR sodium sulphide standardized iodometrically was used as a standard. The reproducibility of the results was poor, but approx. 1.25 g.atoms of S/mole of Cy_2S_3 were liberated as H_2S .

Reduction of phosphotungstic acid and the reaction between bis-(2-amino-2-carboxyethyl) trisulphide and sodium bisulphite. (a) Under the standard conditions described by Fletcher & Robson (1962a) for the reduction of phosphotungstic acid in the presence of sodium bisulphite, $1 \mu\text{mole}$ of Cy_2S_3 gave an extinction of 0.75, compared with the value 0.92 for cystine.

(b) Samples of Cy_2S_3 (1 mg.) were treated with: (1) 0.1M- NaHSO_3 (0.5 ml.) and acetate buffer, pH 5.2 (0.1 ml.); (2) 0.1M- NaHSO_3 (0.5 ml.; pH 2.2); (3) 0.01M- NaHSO_3 (0.5 ml.; pH 2.5). Reaction mixture (1) was kept for 30 min., and reaction mixtures (2) and (3) for 1 hr., before portions were removed for paper chromatography. Chromatograms of the reaction mixtures (1) and (2) showed one strong ninhydrin-positive spot corresponding to cysteine-sulphonate and one very weak spot (R_F 0.19). This latter spot was not present when the reaction mixtures had stood for some days. Staining the chromatograms with silver nitrate revealed a single spot with R_F corresponding to thiosulphate. Mixture (3) smelled strongly of H_2S and the solution was faintly opalescent. Chromatograms showed spots corresponding to cysteine-sulphonate (strong), the

compound with R_F 0.19 (weak), cystine (very weak) and thiosulphate. No unchanged Cy_2S_3 was present. Cysteine was absent from all the samples.

(c) A sample (2.80 mg.) of Cy_2S_3 was dissolved in 0.01M-sodium bisulphite (4 ml.) containing acetate buffer, pH 5.2 (0.1 ml.). After standing for 60 min., with occasional shaking, the clear solution smelled faintly of H_2S . Portions (0.5 ml.) were shaken briefly *in vacuo* to remove H_2S , and thiosulphate was determined by the method of Sörbo (1957) (Found: 0.87 g.ion of thiosulphate/mole of Cy_2S_3). After the solution had stood for 24 hr., this value had increased to 0.91.

Synthesis of bis-(2-amino-2-carboxyethyl) trisulphide from N-acetylcysteine and sulphur dichloride. NN'-Diacylcysteine was prepared according to the method of Pirie (1931) and converted into N-acetylcysteine as described by Pirie & Hele (1933). The latter compound did not crystallize. The oil (2.9 g.) was suspended in chloroform (50 ml.), and a solution of sulphur dichloride (0.9 ml.) in chloroform (10 ml.) was added. The mixture was stirred for 40 hr. at 45° , the chloroform was then distilled off and the dry residue was hydrolysed for 4 hr. with 6N-HCl (50 ml.). The acid solution was filtered and evaporated *in vacuo* to a light-brown tarry residue. A portion of this residue, when chromatographed on paper, showed spots corresponding to cystine, Cy_2S_3 , cysteine and an unidentified substance with R_F 0.17. The bulk of the residue was dissolved in 5 ml. of 2.5N-HCl and chromatographed on a column (4 cm. $\times$ 19 cm.) of Zeo-Karb 225 (X 4) in 2.5N-HCl, changed at fraction 137 (20 ml. fractions) to 4N-HCl. The fractions contributing to the different thiol- or disulphide-positive peaks were combined and analysed by the phosphotungstic acid method. In this way the total residue was estimated to contain cystine (fractions 22–32; 0.62 g.), cystine (fractions 35–46; 0.60 g.), Cy_2S_3 (fractions 87–114; 0.32 g.) and an unidentified substance (fractions 166–188; approx. 0.05 g.), eluted with 4N-HCl, that gave a positive 'disulphide' reaction.

The Cy_2S_3 was rechromatographed in 2.5N-HCl on a column (2.5 cm. $\times$ 6 cm.) of Zeo-Karb 225 (X 4). It emerged in fractions 10–21 (10 ml. fractions), which were combined and evaporated to dryness. The white residue was dissolved in water (10 ml.), and pyridine-water (1:3, v/v) was added cautiously until the pH was 3.5. (Another preparation, in which pyridine was added until the pH was 4.6, gave a product contaminated with cystine.) After standing at 4° for 2 days, the Cy_2S_3 was filtered off, washed with ice-water and ethanol and dried over P_2O_5 . The yield was 283 mg. (12% calc. on N-acetylcysteine) (Found: N, 10.0; S, 34.4%). The infrared-absorption spectrum of this product was identical with that of Cy_2S_3 isolated from wool hydrolysates.

The material that was eluted in 4N-HCl was dissolved in 2.5N-HCl and rechromatographed on a column of Zeo-Karb 225 (X 4); 10 ml. fractions were collected. At fraction 10 the solvent was changed to 4N-HCl. Portions (0.1 ml.) from each fraction were tested with phosphotungstic acid; fractions 17–24 were combined and the acid was removed *in vacuo*. The residue was dissolved in 2 ml. of water and the solution filtered from a small quantity of brown residue. Aqueous pyridine was added to the filtrate until precipitation was complete (the pH was still less than 2.5). The pale-cream solid (32 mg.) was washed with ice-water and ethanol and dried (Found: S, 40.2%. $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_4$ requires S, 42.1%).

When the compound was treated with cyanide, 2.03 g.-atoms of labile S were liberated/mole (304 g.) (Fig. 1). The compound gave a single ninhydrin-positive spot (R_F 0.17; reddish-brown) on paper chromatography. Under the standard conditions for the reduction of phosphotungstic acid in the presence of bisulphite, 1 μ mole of the compound gave an extinction of 0.44 unit.

Synthesis of bis-(2-amino-2-carboxyethyl) trisulphide from cysteine and sulphur. The reaction conditions of Cavallini *et al.* (1960b) were followed, except that the preparation was scaled up 100-fold. To a solution of crystalline sulphur (400 mg.) in chloroform (100 ml.) and ethanol (1 l.) were added aq. ammonia (sp.gr. 0.88) (10 ml.) and, with vigorous shaking, a solution of L-cysteine hydrochloride (1.57 g.) in water (100 ml.). The yellow solution, which smelled of H_2S , was left for 5 min. and then acidified with 2N-HCl (500 ml.). After standing overnight, the solution was filtered from a small quantity of sulphur and evaporated to dryness *in vacuo*, the temperature being kept below 40°. The residue, after being kept *in vacuo* over KOH for several days, was extracted with 25 ml. of 2.5N-HCl and the undissolved ammonium chloride filtered off. The products were chromatographed in this solvent on a column of Zeo-Karb 225 (X 4) (4 cm. $\times$ 19 cm.). The Cy_2S_3 peak, estimated to contain 103 mg., was rechromatographed on a column (2.5 cm. $\times$ 6 cm.) of the same resin. Removal of HCl, dissolution of the residue in water and neutralization to pH 3.5 with pyridine gave 95 mg. of product (Found: N, 9.9; S, 35.7%). Cyanolysis gave 1.04 g.atoms of labile S/272 g.

SUMMARY

1. The sulphur-containing compound formed in highest yield by the decomposition of cystine during the acid hydrolysis of wool and other proteins has been identified as bis-(2-amino-2-carboxyethyl) trisulphide.

2. Confirmation of identity was obtained by synthesis of the trisulphide by two different methods.

3. Bis-(2-amino-2-carboxyethyl) trisulphide may be concerned in certain enzyme reactions involving sulphur.

4. Another of the sulphur-containing compounds present in small amount in wool hydrolysates has been tentatively identified as bis-(2-amino-2-carboxyethyl) tetrasulphide.

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