LXXI. THE CUPROUS DERIVATIVES OF SOME SULPHYDRYL COMPOUNDS.

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METALLIC derivatives of mercaptans have been long known and in the cases of mercury and lead extensively studied. The copper derivatives on the other hand, though they have been prepared on numerous occasions, have received comparatively little attention and have rarely even been analysed. It is one of the purposes of this paper to point out that the formation of a cuprous compound is a general reaction of mercaptans, and that such compounds are frequently very well suited for their isolation or purification.

Precipitates have frequently been obtained on adding a solution of a cupric salt to a mercaptan in neutral or slightly acid solution, but Klason [1877], using thioglycollic acid, first pointed out that it was a cuprous compound that was formed while some of the mercaptan was oxidised to disulphide. Before this Carius [1862] had prepared copper derivatives of mono-, di- and tri-thioglycerol; his analytical figures show that these contain univalent copper.

Suter [1895] and Gabriel [1905] as well as several more recent workers, have prepared mixtures of cuprous cysteine and cuprous chloride by adding copper sulphate solution to a solution of cysteine hydrochloride, while Mörner [1904] and Friedmann [1903] have investigated the cuprous derivatives of α - and β -thiolactic acids. It is probable also that the greyish-black precipitate which Lewis and McGinty [1922] obtained on adding copper sulphate to an extract of the urine of a rabbit which had been fed on phenylcarbamidocystine was the cuprous compound of phenylcarbamidocystine.

So far the methods for the preparation of cuprous mercaptides have all involved the conversion of half the mercaptan to the corresponding disulphide, and this renders them unsuitable for preparing pure mercaptans. Hopkins's [1929] striking observation, that cuprous oxide would react with a mercaptan in acid solution to form the cuprous mercaptide, has changed the whole situation, since by this method, with a little care, a mercaptan may be quantitatively precipitated as its cuprous derivative and so obtained pure.

EXPERIMENTAL.

Part I. *The preparation of some cysteine and cystine derivatives.*

Acetylcystine. Diacetylcystine was first made by Inouye [1929] by the hydrolysis of various diacetylcystine esters. Working at 37°, and adding the

theoretical amount of sodium hydroxide gradually so that the p_H never exceeds 9.5 (0.2 g. of glycine may conveniently be added to buffer the fluid at this p_H) I find that the hydrolysis of 5 g. of the methyl ester of diacetylcystine dissolved in 200 cc. of water takes 6 hours. The propyl ester, the use of which Inouye recommends, is much less soluble and the saponification of 5 g. suspended in 600 cc. of water requires 9 hours at 60°.

These methods are laborious and there is great risk of racemisation during the hydrolysis. A simpler method was therefore sought. Neither acetyl chloride nor acetic anhydride will react with cystine in pyridine suspension, but the latter, as Nicolet [1930] and Du Vigneaud and Hollander [1930] have also found, reacts smoothly in alkaline solution.

10 g. of cystine are dissolved in 50 cc. of 10 % sodium hydroxide, 16 cc. of acetic anhydride are added and the mixture is kept cool and stirred until homogeneous. The excess of acetic acid and acetic anhydride is removed by distillation at 40° *in vacuo* till a viscous gum remains. This is dissolved in 300 cc. of 4 % (by volume) sulphuric acid and reduced with 5 g. of zinc dust; the mixture is stirred occasionally and filtered after about 30 minutes. An aqueous suspension of cuprous oxide is cautiously added to the filtrate, care being taken that, after each addition, the precipitate has lost its initial pink colour before more cuprous oxide is added. The end-point is easily found by centrifuging a sample and adding cuprous oxide to the centrifugate, but, should it be overshoot and the cuprous compound of acetylcysteine redissolve, the latter may be quantitatively re-precipitated by aeration. This phenomenon has already been described in the case of glutathione [Pirie, 1930].

The colourless cuprous compound is washed by repeatedly stirring up the precipitate in distilled water and centrifuging. After 3–4 washings the centrifugate remains cloudy even after 15 minutes at 3500 R.P.M. It is then sulphate-free and contains some dissolved cuprous acetylcysteine. On suspending the thoroughly washed solid in water and decomposing with H_2S (by the closed vessel method) a suspension of cuprous sulphide resembling strong coffee is obtained. This is perfectly transparent when diluted and the sulphide cannot be removed by centrifuging or filtering through kieselguhr. The solution is evaporated to complete dryness *in vacuo* at 45° and the residue is boiled up with glacial acetic acid. The cuprous sulphide may now be removed by filtration and the filtrate, after concentration by vacuum distillation, is evaporated to crystallisation in a desiccator over soda-lime. Whetstone-shaped crystals of acetylcysteine form readily on scratching the concentrated acetic acid solution with a glass rod.

The yield is variable, depending on the completeness of the precipitation with cuprous oxide but is generally about 6.5 g. M.P. (uncorr.) 107°–109°. (Found: N, 8.52 %; S, 19.9 %; Calc.: N, 8.59 %; S, 19.63 %.)

Chloroacetylcysteine. Fischer and Suzuki [1904] isolated dichloroacetylcystine by extracting with ether the solution obtained when chloroacetyl chloride is added to an alkaline solution of cystine. This method gives a pure

unhydrated product, but it is troublesome when used on a large scale. The hydrate, slightly contaminated with coloured products, will however crystallise from the acidified reaction mixture on standing for a day in the ice-chest.

To 22.3 g. of cystine, dissolved in 188 cc. of *N* NaOH, 16.7 cc. *i.e.* 25 g. of chloroacetyl chloride and 275 cc. of *N* NaOH are added concurrently in the course of an hour, the temperature being kept below 5° throughout. After standing at room temperature for 2 hours 34 cc. of concentrated hydrochloric acid are added and the mixture is filtered after being well shaken. It is put in the ice-chest and next day the hydrated dichloroacetylcystine is filtered off and washed with cold water. The yield at this stage is 27 g. and a further 3–5 g. of a less pure material may be obtained by distilling the filtrate and washings *in vacuo* until an oil separates, and cooling.

4 g. of hydrated dichloroacetylcystine and 1 g. of zinc dust are shaken at room temperature for an hour with 120 cc. of 2.5 % H_2SO_4 . The dichloroacetylcystine and most of the zinc dissolve. Some H_2S is formed. If the reaction mixture is heated, or if a large excess of zinc is used, there is more of this destruction and some free hydrochloric acid, which interferes with the subsequent precipitation by cuprous oxide, is also formed. Dewar and Gamgee [1871] observed the production of H_2S during the reduction of cystine by tin, and some H_2S is almost always formed during the reduction of a disulphide by zinc even in the cold, but it is more marked in the case of dichloroacetylcystine than in that of any other compound that I have used.

The solution is filtered and, if necessary, air is blown through it to remove the H_2S ; after warming to about 40° cuprous oxide suspension is added very gradually. When about half of the cuprous oxide has been added the action proceeds more rapidly and the mixture frequently sets to a perfectly rigid jelly which breaks up into a curd on stirring. Cuprous chloroacetylcysteine is soluble in presence of excess of cuprous oxide but is re-precipitated on aeration. Owing to the relative insolubility of chloroacetylcysteine in water it is unnecessary to wash the cuprous compound very thoroughly and a few washings with water on a Büchner funnel are adequate.

As in the case of acetylcysteine a colloidal solution of cuprous sulphide is formed if cuprous chloroacetylcysteine is decomposed with H_2S in aqueous suspension. When suspended in glacial acetic acid, however, especially in the presence of the electrolytes which were not removed by washing, the Cu_2S separates after a few hours as a precipitate. It is filtered and the filtrate, after concentration by distillation *in vacuo*, is evaporated in a vacuum desiccator over soda-lime. The crystals are recrystallised from water. Chloroacetylcysteine generally forms irregular angular masses but occasionally small regular octohedra are obtained. Yield 2–2.5 g. M.P. 126°. (Found: N, 6.95 %; S, 16.67 %; Cl, 17.34 %; Calc.: N, 7.09 %; S, 16.2 %; Cl, 17.97 %.)

Glycylcysteine. 4 g. of hydrated dichloroacetylcystine are put in a strong 100 cc. flask and 20 cc. of ammonia solution (sp. gr. 0.880) are added. Since the neutralisation of the acid by ammonia evolves considerable heat it is best

to add 5 cc. of ammonia first with cooling and then, when the solid has again reached room temperature, the remainder. The flask is tightly stoppered and kept in an incubator at 37° for 4 days. So far the method is simply a modification of that of Abderhalden and Spinner [1919] for the preparation of diglycylcystine.

As much of the excess ammonia as possible is removed by distilling the brownish-coloured solution *in vacuo* till it forms a gum adhering to the sides of the flask, this is dissolved in 20 cc. of 2 % sulphuric acid. To the acid solution 2.9 g. of finely powdered silver sulphate are added and the mixture is left, with occasional stirring, for some hours. It is important to precipitate all the chlorine at this stage. After filtering, 40 cc. of 2 % sulphuric acid are added to the filtrate, followed, after removing any excess of silver with H_2S , by 1–2 g. of zinc dust. Reduction is complete in about 20 minutes. The excess of zinc is filtered off and the filtrate heated to about 80° before any cuprous oxide is added. Precipitation will occur at a lower temperature if all the hydrochloric acid has not been removed or if a considerable amount of cuprous glycylcysteine has already been formed. If however cuprous oxide is added to a cold acid solution of glycylcysteine most of it is converted to cupric oxide and copper. As in the case of the two compounds already described an excess of cuprous oxide will cause re-solution of the already precipitated cuprous compound. On aeration it is re-precipitated as a bright yellow powder which, apart from its colour, is identical with the flocculent white mass which is formed at first.

The removal of the last traces of sulphuric acid from this substance is difficult, and as the properties of the resulting glycylcysteine show, I have probably never accomplished it quite completely. It is advisable to use a filtration method of washing, similar to that which has been described already [Pirie, 1930], rather than to wash by centrifuging. The cuprous glycylcysteine is somewhat unstable in the absence of acid and gradually becomes greenish on exposed surfaces when the washing has been carried nearly to completion. It is suspended in water and decomposed with H_2S . Decomposition is very rapid and after a few hours the solution may be filtered from cuprous sulphide. It is advisable to keep the volume of water used to suspend the cuprous compound as small as possible and to evaporate the filtrate (still containing some H_2S) rapidly in a vacuum desiccator over sulphuric acid and caustic soda. Residual sulphuric acid keeps the p_{H} of the resulting gum down to about 4 and makes crystallisation difficult. It will always crystallise however when seeded.¹ Owing to the excessive solubility of glycylcysteine in water the mother-liquor in contact with it is very viscous and filtration is slow. A

¹ After several early failures crystals were obtained by adding pyridine to the hydrochloric acid-containing gum which resulted when the treatment with silver sulphate was omitted. On washing with alcohol fairly pure glycylcysteine was obtained, but it is difficult to make the removal of pyridine hydrochloride by alcohol under these conditions complete. Since then I have experienced no difficulty with crystallisation.

further quantity of less pure material may be obtained by adding alcohol to the filtrate. As usual the yield is variable but 5–6 g. may be expected.

Glycylcysteine has no definite melting-point. It sinters at 130° and then gradually swells to a semi-solid foam which melts again at 177° and goes brown. (Found: N, 15.28 %; S, 18.08 %; Calc.: N, 15.73 %; S, 17.97 %.)

Methyl ester of acetylcysteine. The disulphide is prepared by a method based on that of Inouye [1929]. A suspension of 6.8 g. of cystine dimethyl ester hydrochloride in 50 cc. of dry pyridine is cooled to 0° and 4 cc. (excess) of acetyl chloride added gradually. Considerable heat is developed and the ester hydrochloride dissolves when about half the acetyl chloride has been added; crystals separate on adding the remainder. After standing at room temperature for 3 hours it is filtered through a small Hirsch funnel. Great care must be exercised here since the white crystals which remain on the funnel are apt to turn brown and melt, and occasionally ignite, on exposure to air. As soon as the fluid has been sucked through the funnel is removed from the flask and washed out. I have attempted to avoid this by adding the theoretical amount of acetyl chloride, but this results in a very much diminished yield. The filtrate is evaporated in a vacuum desiccator over sulphuric acid till crystallisation commences. On mixing with its own volume of water a mass of crystals is obtained and filtered off. The filtrate is brought to about p_H 6 with 10 % sodium hydroxide; on removing most of the liberated pyridine in a vacuum desiccator further crystallisation takes place. The crystals are washed with cold water.

5.9 g. of colourless crystals, M.P. 128–9°, are obtained. Unlike Inouye, I find that the dimethyl ester of diacetylcysteine is perfectly stable in air and not at all hygroscopic. (Found: N, 7.95 %; S, 18.19 %; Calc.: N, 7.95 %; S, 18.18 %.)

0.380 g. was made up to 10 cc. with water and the rotation measured in a 19.95 cm. polarimeter tube.

Using the two mercury lines the rotations were -7.35° and -6.53° these values gave $[\alpha]_{577}^{17} = -96.94^\circ$ and $[\alpha]_{546}^{17} = -86.13^\circ$. The latter figure is in good agreement with the value (-85.59°) given by Voss, Guttmann and Klemm [1930].

Zinc dust is added to 2 g. of diacetylcysteine dimethyl ester dissolved in 150 cc. of 2 % sulphuric acid in the cold. The solution is filtered and treated with cuprous oxide at about 30° in the usual way. After washing, the cuprous acetylcysteine ester is suspended in glacial acetic acid and decomposed with H_2S . The cuprous sulphide remains colloidal even on boiling so the suspension is evaporated to dryness *in vacuo* and boiled with benzene. The benzene solution is filtered and evaporated in a desiccator over paraffin after most of the benzene has been removed on a water-bath. The residue may be recrystallised from water containing a little acetic acid and melts at 80°. (Found: N, 7.94 %; S, 18.35 %; Calc.: N, 7.91 %; S, 18.08 %.)

Diacetylcysteine dipropyl ester. Propyl alcohol is dried by distilling from lime and 6 g. of cystine are suspended in a litre. Hydrogen chloride is passed

into the mixture, boiling under a reflux condenser, for 2 hours. It is filtered hot and evaporated to small bulk by vacuum distillation. 6 g. (M.P. 178–9°) of cystine dipropyl ester hydrochloride separate out on cooling with ice. A further crop of crystals separates on adding ether.

7 g. of ester hydrochloride are acetylated in 100 cc. of pyridine with 4.2 cc. of acetyl chloride. After lying overnight in the ice-chest the solution is filtered and the filtrate distilled *in vacuo* to remove most of the pyridine. On adding ether 5.9 g. of diacetylcystine dipropyl ester separate. On recrystallising from water masses of fine needles are formed which are not hygroscopic and melt at 124–5°. (Inouye gave 175° and 117–8° as the melting-points of cystine dipropyl ester hydrochloride and diacetylcystine dipropyl ester respectively.) (Found: N, 6.51 %; S, 15.55 %; Calc.: N, 6.86 %; S, 15.69 %.)

Dicinnamylcystine dimethyl ester. 12 g. of cinnamyl chloride are added in the course of 5 minutes to a cooled suspension of 10.5 g. of cystine dimethyl ester hydrochloride in 200 cc. of dry pyridine. The mixture is then left for 8 hours at room temperature. The pyridine is distilled off *in vacuo* till crystals separate. These are filtered off, after standing overnight in the ice-chest, and the filtrate is poured into water. Both the crystals and the solid which separates when the pyridine filtrate is poured into water are recrystallised from alcohol. The product crystallises in very pale yellow leaflets which melt at 161–2°. (Found: N, 5.06 %; S, 12.21 %; Calc.: N, 5.30 %; S, 12.12 %.)

0.3024 g. of dicinnamylcystine dimethyl ester are dissolved in acetic acid and made up to 10 cc. At 17° the rotations for the mercury lines are -1.21° and -0.82° in a 2 dm. tube, *i.e.* $[\alpha]_{577}^{17} = -20^\circ$ and $[\alpha]_{546}^{17} = -13.6^\circ$.

Oxidised glutathione. Hopkins [1929] observed that when glutathione was oxidised by aeration at p_H 7.6 in the presence of a trace of iron, the product isolated, after adding sulphuric acid to remove the baryta used to adjust the p_H , by evaporation followed by treatment with alcohol, had a low nitrogen and sulphur content. Very slow oxidation in the absence of iron was found to give similar results so other oxidising agents were tried. Hydrogen peroxide proved to be very much the most suitable.

A 10–20 % solution of glutathione is used and exactly the theoretical amount of 30 % hydrogen peroxide is added. Copper catalyses the oxidation of the —SH group at this p_H (2.8) and in the presence of 1 mg. of copper the oxidation of 10 g. of reduced glutathione is complete in 3 hours. So long as an excess of hydrogen peroxide is not added no side reactions occur and 1 mol. of H_2O_2 will cause the complete disappearance of the nitroprusside reaction from a solution containing 2 mols. of glutathione. Glutathione prepared from its cuprous compound may contain less than 1 part of copper per million. It is generally necessary therefore to add a trace of copper sulphate or oxide. When oxidation is complete the solution is cooled to 0°, put in a vacuum desiccator, and exhausted rapidly so that it freezes. The vacuum is maintained and in the course of a day all the ice evaporates leaving the oxidised glutathione as a white feathery non-hygroscopic mass easily soluble in water. It has not

yet been crystallised. Oxidised glutathione prepared in this way gives analytical figures for N and S identical with those of the reduced glutathione used in its preparation.

Mason [1931, 2] in a very recent paper has pointed out that oxidised glutathione holds alcohol tenaciously. It is obvious therefore that, apart from its very great convenience, the method of preparation of oxidised glutathione just described has no merit except the avoidance of alcohol. The presence of alcohol explains Pirie and Pinhey's [1929] observation that the oxidised glutathione used contained 17 % of an impurity which had no titratable groups.

Part II. *Preparation and properties of the cuprous derivatives of some thiol compounds.*

Cysteine. Although mixtures of cuprous cysteine and cuprous chloride have been prepared by several different workers, no one appears to have prepared pure cuprous cysteine.

l-Cystine (*dl*-cystine behaves in a substantially similar way though the cuprous compound of *dl*-cystine is rather more soluble in weak acid than that of *d*-cystine) is dissolved in 2 % sulphuric acid and some zinc dust is added. The solution is boiled and filtered. Both titration with iodine and polarimetric observation indicate that 97–100 % of the cystine has been reduced.

Cuprous cysteine is not readily precipitated from this solution in the cold. A reddish or grey precipitate is obtained and a considerable amount of copper sulphate is formed. Precipitation occurs smoothly in the cold if a trace of hydrochloric acid or a chloride is present, but in the absence of chloride it will take place at the boiling point. Being fairly soluble in boiling dilute sulphuric acid, cuprous cysteine may remain dissolved, but it precipitates on cooling. It is soluble in 5 % sulphuric acid even in the cold, but shows no sign of being soluble in presence of excess of cuprous oxide.

For analysis it is washed a little on the centrifuge and then transferred to a small Jena glass filter of the type used in micro-analysis. Washing is continued, with suction, until the wash-water is sulphate-free or until the cuprous compound begins to turn green. The latter happens first in the case of cysteine. The filter is put in a desiccator over P_2O_5 at room temperature and exhausted to 1 mm. A hard cake is obtained in this way which is much less readily oxidised by air than the loose mass which results if the precipitate in the centrifuge tube is dried.

A sample, containing about 6 mg. of copper, is transferred to a weighed 100 cc. Kjeldahl flask, with all but 8 cm. of its neck cut off, and incinerated with 0.5 cc. of sulphuric acid and some potassium sulphate. When cold, saturated sodium carbonate solution is added till the solution is alkaline. It is then acidified with acetic acid and, after the addition of some solid potassium iodide, titrated with *N*/100 thiosulphate using starch as indicator. If the incineration has been thorough the end-point is perfectly sharp. The

ordinary Pregl method of analysis for copper has been found not to work satisfactorily with many of these compounds.

21.1 mg. of the dried material required 11.1 cc. of *N*/98 thiosulphate whence, Cu, 34.2 %; Calc. Cu, 34.6 %.

Like other compounds of this type, cuprous cysteine is soluble in dilute HCl and in alkalis, it is also soluble in unusually dilute sulphuric acid, for most cuprous compounds are not appreciably soluble in sulphuric acid weaker than 20 %.

Acetylcysteine and chloroacetylcysteine. The cuprous derivatives of these two substances are similar in their general properties. Both are soluble in presence of excess of cuprous oxide and in moderately concentrated (10–20 %) sulphuric acid. They are also soluble in water in the absence of acid; thus on washing a sample by centrifuging, the p_H of each successive centrifugate is higher till a p_H of about 3.6 is reached. At this point the compound goes into solution and the wash-water is found to be sulphate-free. A saturated solution of cuprous acetylcysteine at p_H 3.6 contains 44 mg. of copper per 100 cc. On adding a little sulphuric acid, mixing rapidly and allowing the mixture to stand, a rigid jelly is obtained in about 15 minutes. The concentration of cuprous acetylcysteine in this jelly is 0.15 % so that it shows a gel-forming capacity of the same order as that of dibenzoylcystine [Wolf and Rideal, 1922]. Cuprous chloroacetylcysteine is less soluble in water (it forms a solution of about 0.07 %) and, although it also will form remarkably rigid jellies, gel-formation is not so reliable as with cuprous acetylcysteine.

When re-precipitated from solution cuprous acetylcysteine does not dry readily and tends to blacken. As a result the analytical figures are generally low: *e.g.* directly precipitated; different preparations contained 27.9, 27.5 and 28.1 % Cu; re-precipitated; different samples contained 25.1, 22.1, 25.0, 24.8 and 26 % Cu; Calc. for $C_5H_9O_3NSCu$ 28.2 % Cu.

Cuprous chloroacetylcysteine also is not very stable on drying and always gives rather low analytical figures. (Found: 23.1 % Cu; Calc.: 24.5 % Cu.)

The p_H of an aqueous solution of cuprous acetylcysteine gradually rises on exposure to air and may reach 7. At this stage nothing is precipitated by acid and the fluid will dissolve further quantities of solid with consequent fall in p_H . It is probable that this rise in p_H is due to the oxidation of cuprous copper to the cupric form. This then combines with the carboxyl instead of with the sulphhydryl group. A titration curve indicated that, while there was a group with a p_K in the neighbourhood of 4, there was no other titratable group with a p_K less than 12. This suggests that, as would be expected, the copper in cuprous acetylcysteine is linked to the sulphur atom.

If either compound is dissolved in 2 % sulphuric acid and put in a narrow-mouthed vessel, with a stream of hydrogen passing through to prevent oxidation, the initially formed cuprous compound is completely redissolved on the gradual addition of two atomic proportions of copper, in the form of cuprous oxide suspension of known strength. If too little cuprous oxide is

added, traces of the normal cuprous compound remain undissolved, if too much is added metallic copper deposits. The solution formed is yellow or brown. For every 10 cc. of solution 100 cc. of 97 % alcohol are put into a Büchner flask and the solution is filtered directly into it through a previously moistened paper. A yellowish gelatinous precipitate is formed but it is too unstable to give consistent analytical figures; probably, by analogy with glutathione, it is a complex of two molecules of cuprous acetylcysteine and one of cuprous sulphate.

Glycylcysteine. Like cysteine this substance forms a cuprous compound in the cold in the presence of a chloride, but in the absence of chloride it is necessary to heat to 80° before adding the cuprous oxide. It resembles cysteine also in forming a cuprous compound from which the sulphuric acid is not readily removed by washing. It is perfectly stable when dried. (Found: 27.36 %, 26.77 % and 26.6 % Cu; Calc.: 26.44 % Cu.)

Cuprous glycylcysteine reacts readily with more cuprous oxide when it is suspended in 6 % sulphuric acid in the absence of air. The product obtained by filtering the solution into alcohol and then washing the precipitate with alcohol till it starts to turn green is stable when dry. (Found: Cu, 30.7 %; SO₄, 23.06 %)

Calc. for (C ₅ H ₉ O ₃ N ₂ SCu) ₂ Cu ₂ SO ₄	Cu 36.1 %, SO ₄ , 13.63 %.
Calc. for C ₅ H ₉ O ₃ N ₂ SCu.CuSO ₄	Cu 31.7 %, SO ₄ , 24.0 %.

It is thus apparent that the analytical figures are in much better agreement with a formula in which there is one atom of cupric copper than with one in which both are cuprous. Nevertheless, the fact that re-solution of cuprous glycylcysteine occurs on the addition of one atomic proportion of cuprous copper in absence of air and without any free copper being formed, taken in conjunction with the fact that cuprous glycylcysteine is re-formed on aeration (analysis of the yellow material shows that it contains 26.7 % Cu), seems to me to indicate that both atoms of copper are in the cuprous state. Sulphuric acid is a probable contaminant in view of the necessarily incomplete washing, although 9.43 % is undoubtedly more than would have been anticipated.

Methyl and propyl esters of acetylcysteine. Both react normally in acid solution and give cuprous derivatives which dissolve on the addition of another atomic proportion of cuprous copper. So far I have been unable to isolate these double copper compounds since they are not only soluble in alcohol but are also very readily autoxidisable.

Propyl acetylcysteine was prepared from the disulphide by suspending it in 2 % sulphuric acid at about 50° and adding zinc dust. The disulphide is rather difficult to wet but it is reduced in about half an hour. The cuprous derivatives of both these compounds are insoluble in water but tend to remain in suspension in water when they have been washed free from acid. The cuprous sulphide suspensions formed on decomposing either of these with

H_2S are remarkably stable and, if free from electrolytes, will withstand boiling with alcohol or glacial acetic acid. Acetylcysteine propyl ester has not been obtained crystalline.

Both cuprous compounds are stable on drying:

$\text{CH}_3\text{CONHCH}(\text{COOCH}_3)\text{CH}_2\text{SCu}$ Found; 26.00 % Cu; Calc. 26.55 % Cu.

$\text{CH}_3\text{CONHCH}(\text{COOC}_3\text{H}_7)\text{CH}_2\text{SCu}$ Found; 23.2 % Cu; Calc. 23.77 % Cu.

Glutathione. Cuprous glutathione has been more extensively studied than the other cuprous compounds. Hopkins [1929] observed that it redissolved in presence of an excess of cuprous oxide. For some reason it will not re-precipitate on aeration when an excess of cuprous oxide has been added to a solution recovered from a mercury precipitate from a boiled yeast extract, whereas, as I have already pointed out [Pirie, 1930], it may be re-precipitated from a yeast filtrate prepared directly. This is due, in part at any rate, to the formation of interfering substances (cysteinylglycine?) on boiling and to their concentration in the filtrate from mercuric sulphate. A certain amount of re-precipitation is obtained if such a solution is largely diluted with water and aerated.

On dissolving glutathione in dilute sulphuric acid and adding cuprous oxide in a current of hydrogen to exclude air, maximum precipitation is obtained after the addition of one atomic proportion of copper. The addition of a second atom of copper results in complete re-solution, but a third may be added before any turbidity due to metallic copper appears. A white precipitate is obtained if the filtrate from this mixture is allowed to run into alcohol. On washing this precipitate it turns slightly green but seems to be stable.

The analytical figures obtained depend on the thoroughness with which it is washed with alcohol. On very thorough washing: Cu, 25.5 % and 25.45 %; SO_4 , 14.2 % and 14.9 %; Calc. for $[(\text{CuSG})_2\text{Cu}_2\text{SO}_4]_2\text{SO}_4$:¹ Cu, 25.1 % and SO_4 , 14.25 %. With less thorough washing the figures are 26.44 % and 16.13 % respectively.

In this case it is obvious that all the extra copper cannot be in the cupric state since there is not enough sulphate present. The figures show further that there are only two atoms of copper present for each atom of sulphur whereas three may be added without any deposition of copper occurring. It is noteworthy that products having the same analytical figures are obtained irrespective of whether two or three atomic proportions of copper have been used to dissolve the glutathione. In the latter case there is some copper in solution in the alcohol. Probably therefore a compound of the type $\text{CuSG} \cdot \text{Cu}_2\text{SO}_4$ is present in aqueous solution, but on pouring the solution into alcohol it is broken down. In view of the extreme difficulty experienced in washing all the sulphuric acid out of certain cuprous compounds, *e.g.* that of glycylcysteine,

¹ GSH is reduced glutathione.

it seems probable that the substance actually precipitated by alcohol is $(\text{CuSG})_2\text{Cu}_2\text{SO}_4$ and that the extra sulphate is adventitious.

The behaviour of the soluble copper compound towards carbon monoxide is interesting. Joannis [1903] showed that sulphuric acid saturated with carbon monoxide would dissolve finely divided copper and isolated Cu_2SO_4 , 2CO , H_2O . If, without admitting any air, the hydrogen which was used to exclude air during the solution of cuprous glutathione in an excess of copper oxide, is replaced by a stream of carbon monoxide, precipitation of cuprous glutathione occurs. Precipitation is nearly complete in 3 hours. At this stage, if the CO is displaced by a current of hydrogen, there is complete solution of the precipitate. On passing in more CO precipitation occurs again, and so on. The behaviour of CO is thus entirely different from that of oxygen and seems to involve the reaction:

If the suspension obtained by saturating with carbon monoxide is centrifuged, with a stopper in the centrifuge tube to prevent too much loss of CO, and the centrifugate is exhausted until it boils and then left for a day with occasional re-exhaustion, a precipitate of copper is formed. The fluid, which initially liberated very little iodine from potassium iodide, now liberates a large amount. This behaviour is what would be expected if the centrifugate contained the carbon monoxide complex of cuprous sulphate.

Glycylcysteine anhydride. Oxidised glutathione, prepared by the method already described, is boiled in water to form the diketopiperazine by the method of Hopkins [1929]. The resulting disulphide is reduced with zinc. Towards cuprous oxide the reduced product behaves exactly like glycylcysteine except that it is unnecessary to heat the solution to more than 30° before adding the cuprous oxide. The cuprous compound turns greenish when washed and is not very stable. (Found: Cu, 28.48 %; Calc.: Cu, 28.57 %.)

In acid solution the cuprous compound is soluble in presence of an excess of cuprous oxide but it does not appear to stabilise a whole molecule of cuprous sulphate as does glutathione although it might have been expected to do so since it also has two peptide linkages. The gelatinous product obtained on pouring this solution into alcohol is not sufficiently stable to give consistent analyses.

Methyl ester of cinnamylcysteine. Since the dimethyl ester of dicinnamylcystine is insoluble in water it is reduced with zinc in glacial acetic acid containing a few drops of concentrated sulphuric acid per 100 cc. The solution is boiled, filtered and then, when again boiling, treated with cuprous oxide in aqueous suspension. A yellowish curd separates which is washed with acetic acid and then with water till sulphate-free. (Found: Cu, 18.9 %; Calc. for $\text{C}_{13}\text{H}_{14}\text{O}_3\text{NSCu}$: Cu, 19.4 %.)

A suspension of cuprous sulphide formed on decomposing this, suspended in glacial acetic acid, with H_2S but it filtered after being boiled. A white curd

separated on adding water to the concentrated acetic acid solution. (Found: N, 4.81 %; S, 11.50 %; Calc. for $C_{13}H_{15}O_3NS$: N, 5.3 %; S, 12.12 %.)

It gives a nitroprusside reaction and, when oxidised, decolorises an acetic acid solution of bromine. On pouring this solution into water a curd containing 13 % of bromine is obtained. It is therefore improbable that the reduction has saturated the double bond.

Benzoylcysteine. This compound has not been obtained pure. Dibenzoylcystine is shaken with zinc in a mixture of equal volumes of 2 % sulphuric acid and alcohol. It is advisable to add a trace of copper sulphate to catalyse the action. If necessary the mixture is warmed but as a rule all the dibenzoylcystine dissolves easily. After filtering from undissolved zinc and removing the alcohol by distillation *in vacuo*, an oil separates which does not solidify on cooling. This is washed by boiling up again with water and dried in a vacuum. It remains viscous.

Equal volumes of 3 % sulphuric acid and alcohol are used to dissolve this gum and a trace of sodium chloride is added. On adding cuprous oxide to the hot fluid a fawn-coloured precipitate is formed. There is no sign of this being soluble in presence of excess of cuprous oxide. For analysis it is suspended in water in an atmosphere of hydrogen, and dilute sodium hydroxide solution is added. The suspension is centrifuged and the centrifugate poured into dilute sulphuric acid. There is no sign of gel formation and the white precipitate is washed and dried as usual. (Found: Cu, 21.4 %; Calc. for $C_{10}H_{10}O_3NSCu$: Cu, 22.1 %.)

When suspended in alcohol and decomposed with H_2S the cuprous sulphide is not colloidal though it is when suspended in acetic acid. Benzene precipitates benzoylcysteine from its concentrated alcoholic solution as a stringy mass and I have not yet obtained it in satisfactory form.

Cysteinylglycine. Mason [1931, 1] showed that cysteinylglycine did not form a cuprous compound insoluble in sulphuric acid. The compound, prepared by the method of Kendall, Mason and McKenzie [1930], dissolves cuprous oxide in acid solution. There is no precipitation on adding sodium hydroxide till the p_H is about 4, but on evaporating this brown fluid *in vacuo*, a grey precipitate is formed. On exposing this to air it immediately turns chocolate in colour. This abnormal behaviour of cysteinylglycine is difficult to understand, but further investigation of it is in progress. Preliminary work shows that the benzoyl derivative of cysteinylglycine forms a cuprous compound of the usual character.

Cysteine methyl ester. The amount of saturated silver sulphate solution necessary to combine with all the hydrochloric acid is added to some cystine dimethyl ester hydrochloride together with the same volume of 2 % sulphuric acid. The mixture is centrifuged and some zinc is added, after about 10 minutes it is filtered. To the filtrate, cuprous oxide suspension is added, air being excluded by a current of hydrogen. No insoluble cuprous compound is formed, but the copper oxide dissolves with the formation of a perfectly colourless

solution. Two atomic proportions of copper may be added in this way but a third results in a pale green colour and a faint turbidity.

When poured into alcohol a white precipitate forms and the alcohol takes on a bluish tinge. The precipitate rapidly turns grey and does not give consistent analyses. It appears however to have the composition

The alcohol contains more copper than the precipitate. This indicates that, as in the case of glutathione, the more complex copper compound is unstable in alcohol.

DISCUSSION.

Several reasons for the formation of soluble cuprous compounds suggested themselves at the beginning of this work but all have been excluded except one.

A carboxyl group cannot be concerned since the methyl ester of acetylcysteine and glycylcysteine anhydride both dissolve when an excess of cuprous oxide is present.

An amino-group cannot be concerned since acetylcysteine, chloroacetylcysteine, acetylcysteine methyl ester and glycylcysteine anhydride all form soluble compounds.

It is improbable that subsidiary valencies of the sulphur atom are concerned since cysteine and thioglycollic acid do not form soluble compounds. (The cuprous compounds of thiourea and thioglycollic acid have been prepared by several different workers; I find that neither will dissolve on adding an excess of cuprous oxide.)

The peptide linkage remains as a possibility and it seems to be a highly plausible one. Cuprous benzoylcysteine it is true does not dissolve in presence of an excess of cuprous oxide; but benzoylcysteine itself is insoluble in dilute acids unlike the other compounds used. A more serious objection is that none of the sulphur-free peptides which I have tried (*i.e.* glycylglycine, diglycylglycine and leucylglycylglycine) will stabilise cuprous oxide in dilute sulphuric acid. It seems therefore that, if the theory is correct, the copper mercaptide linkage in the molecule is a necessary precursor of any further action.

The examples given in this paper should serve to show the value of the cuprous compound as a means of separating mercaptans from the other products of a reaction and of getting a pure product without having to recrystallise. Different mercaptans cannot of course be easily separated from each other, though in some cases, *e.g.* a mixture of cuprous acetylcysteine and cuprous glycylcysteine, it is possible to concentrate the two compounds in different fractions.

With the exception of some purines, mercaptans seem to be the only compounds which form cuprous derivatives insoluble in acid solution. A purine-like body, containing 30–36 % of nitrogen, has in fact been isolated from a yeast extract, prepared in the way already described [Pirie, 1930], by adding a large amount of cuprous oxide to the fluid after as much glutathione as possible

has been removed. A yellowish precipitate forms which on washing and decomposing with H_2S gives a white solid soluble in acids, alkalis and H_2S solution but insoluble in water. It has not been further investigated.

In view of the solubility of the cuprous derivatives of cysteine methyl ester and cysteinylglycine and of the difficulty experienced in washing cuprous glycylcysteine or cuprous cysteine free from sulphuric acid, it is advisable to work with acidic rather than with neutral or basic compounds. When dealing with an unknown sulphydryl compound it would therefore be wise to acetylate or benzoylate an amino-group before proceeding to cuprous precipitation.

Attempts to utilise the specificity of this cuprous compound precipitation in a method for the estimation of glutathione have so far been unsuccessful. The most hopeful results were obtained when a tissue extract, warmed to $50\text{--}60^\circ$, was titrated with a mixture of copper sulphate and sodium hypophosphite. A precipitate was obtained and, on adding an excess of the reagent, it redissolved giving a fairly sharp end point. The figures obtained were however always very high and were much less reliable than those obtained by iodimetric methods.

The specific rotations of the sulphydryl compounds prepared have not been given in the text since they are unreliable, partly because of the possibility that some of the disulphide is present and partly because the observed angle is so small. The following values for $[\alpha]_{5461}^{17}$ were observed in acid solution:

Acetylcysteine + 0.42° ; chloroacetylcysteine + 8.7° ; glycylcysteine + 6.9° ; methyl ester of acetylcysteine - 31.0° .

In describing the effect of carbon monoxide on the cuprous sulphate complex of a copper mercaptide only glutathione has been mentioned. All the other sulphydryl compounds which form soluble cuprous derivatives with an excess of cuprous oxide, but insoluble derivatives with one atom of copper, behave in precisely the same way.

SUMMARY.

Acetylcysteine, chloroacetylcysteine, glycylcysteine, the methyl ester of acetylcysteine, and the dimethyl ester of cinnamylcysteine have been prepared and the method of preparation is given in each case.

Improvements are suggested in the preparation of oxidised glutathione, dichloroacetylcystine and the dimethyl and dipropyl esters of diacetylcystine.

Cinnamylcysteine methyl ester, acetylcysteine propyl ester and benzoylcysteine have been prepared in an impure state.

The cuprous derivatives of these compounds have been prepared and complexes of these derivatives with cuprous sulphate have in many cases been isolated and analysed. These complexes are decomposed reversibly by carbon monoxide and irreversibly by oxygen.

The suitability of cuprous derivatives for the isolation and purification of sulphydryl compounds is emphasised.

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