VIII. CUPROUS GLUTATHIONE.

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WITH A NOTE ON THE CRYSTALLOGRAPHY OF GLUTATHIONE.

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IN a recent paper Kozlowski [1931] has described the preparation of a compound, containing 16.45 % Cu, by addition of excess of cuprous oxide to a solution of oxidised glutathione in $N/2$ sulphuric acid, filtering and aerating. This compound he assumes to be a derivative of oxidised glutathione, presumably of the composition Cu_2GSSG . In view of this claim it has been thought necessary to reinvestigate this reaction.

Since cuprous oxide is a reducing agent it is to be expected that it will first reduce the oxidised glutathione and then combine with the reduced molecule to form a soluble derivative of the type $(\text{CuSG})_2\text{Cu}_2\text{SO}_4$ [Pirie, 1931, p. 623], which, on exposure to air, breaks down with the formation of cuprous glutathione and cupric sulphate. There are five independent lines of evidence for this view.

(a) A solution of oxidised glutathione will, after the addition of a small amount of cuprous oxide, give a nitroprusside reaction; whereas, after the addition of a cupric salt, it will not give a nitroprusside reaction unless a reducing agent such as KCN is added.

This is not conclusive evidence that the reduction can occur in acid solution; it may only take place after the addition of the ammonia.

(b) If a de-aerated solution of oxidised glutathione in $N/2$ sulphuric acid is mixed *in vacuo* with an air-free suspension of cuprous oxide containing not more than two molecules of Cu_2O for each molecule of GSSG a clear blue solution results. This suggests that a cupric salt has been formed. If more cuprous oxide is added the solution turns yellow or green.

(c) The specific rotation of a solution of cuprous glutathione prepared by the ordinary Hopkins [1929] method is identical with that of a solution of cuprous glutathione prepared by Kozlowski's method.

(d) When titrated with iodine solution in the presence of potassium iodide and acetic acid cuprous glutathione prepared by either method takes up one atom of iodine for each atom of copper, as would be expected if its formula were CuSG . If the formula were Cu_2GSSG there is clearly no reason why, under these conditions, it should take up any iodine at all.

(e) A molecule of the type suggested by Kozłowski should, on decomposition with H_2S , give rise either to cupric sulphide or to a mixture of cuprous sulphide and sulphur if at the same time reduced glutathione is formed. I find that reduced glutathione is formed quantitatively while the ratio of copper to sulphur in the precipitate is 2 : 1. The analytical data in support of (c), (d) and (e) are given in the experimental section.

EXPERIMENTAL.

Reduced glutathione was prepared by the method already described [Pirie, 1930], except that for 2 kg. of yeast 120 cc. of ether are now used instead of the 80 cc. which were used in the original method. It is a matter of some importance that there should not be too much peroxide in the ether used since this oxidises the glutathione and renders the precipitation incomplete. In one or two preparations I have replaced the ether by the same amount of phenol with satisfactory results; any substance which will kill yeast cells when present in fairly low concentration and which does not interfere with the subsequent cuprous precipitation would probably do equally well. The killing of the cell appears to be necessary for the liberation of glutathione since I have been unable to find any reagent which will make glutathione diffuse into the fluid part of a suspension of yeast cells without causing a proportionate fall in the viable count of the suspension.

If large crystals of glutathione are not required alcohol may be added to the filtrate from cuprous sulphide as in Hopkins's [1929] method, but crystallisation is improved if the use of alcohol is avoided as in the method which I have already described. By this method crystals 1–2 mm. long are always obtained and they are sometimes as large as $10 \times 2 \times 2$ mm. (See Fig. 1.)

Crystallographic data.

Mr J. D. Bernal has kindly examined these crystals and gives the following description of them, a more complete account will be published shortly in another place.

"The crystals occur in orthorhombic prisms or plates bounded by many narrow faces, chiefly (001) (302), and terminated by (011) (?). The extinctions are straight. No optic picture could be obtained but the γ direction is probably b .

Oscillation and Weissenberg photographs have been taken establishing a cell $a = 28.0$, $b = 8.74$, $c = 5.57$ Å., containing 4 asymmetric molecules with density 1.50 and with the space group $V^4 - 2_12_12_1$. The positions of the molecules in the cell cannot be determined from such a preliminary investigation but from the fact that the (200) planes are fairly normal it is most probable that they form two layers 14.0 Å. thick and are of a straight chain character."

When preparing cuprous glutathione from pure glutathione it is advisable to heat the solution to about 40° to get a perfectly white product; with crude

preparations or yeast extracts or in the presence of a trace of hydrochloric acid this is unnecessary and no copper or cupric oxide is formed even in the cold. The precipitate is washed on the centrifuge in the usual way and, since a light

Fig. 1. Glutathione crystals from water. Magnification $\times 130$.

friable product is required, dried in the centrifuge-tube *in vacuo* over phosphorus pentoxide.

Cuprous glutathione (Kozłowski) is prepared from oxidised glutathione by

adding about 4 mols. of cuprous oxide, as an aqueous suspension, to 1 mol. of oxidised glutathione, in the form of a 1 % solution in $N/2$ H_2SO_4 . After shaking for about a minute the mixture is filtered by suction and air is then bubbled through the filtrate for an hour or two. This white precipitate is dried like the last. When prepared in this way cuprous glutathione is soluble in an acid cuprous oxide suspension and precipitates again on aeration in the manner already described [Pirie, 1930].

Cuprous glutathione is readily soluble in dilute hydrochloric acid and gives a pale brown solution which is not very stable but apparently gradually dissociates into free glutathione (or its decomposition products) and a copper salt. For example:

89 mg. of CuSG prepared from GSH dissolved in 6 cc. of 1.4 *N* hydrochloric acid.

Time ...	7 mins.	20 mins.	37 mins.	6 hrs.	9 hrs. 40 mins.
Rotation in 2 dm. tube	+1.34°	+1.33°	+1.27°	+0.97°	+0.63°
Time ...	12 hrs. 20 mins.	22 hrs.	31 hrs. 27 mins.	36 hrs.	
Rotation in 2 dm. tube	+0.32°	-0.34°	-1.05°	-1.50°	

The initial reading gives $[\alpha]_{5461}^{16.5} = +45.1^\circ$.

If stronger acid is used as solvent the rotation, initially low, rises in the course of an hour and then falls again but much more slowly than in the case of the less acid solution. For example:

90 mg. of the same CuSG dissolved in 6 cc. of 4*N* HCl.

Time ...	5 mins.	9 mins.	28 mins.	50 mins.	85 mins.
Rotation in 2 dm. tube	+0.53°	+0.63°	+0.76°	+0.80°	+0.80°
Time ...	4 hrs. 25 mins.	9 hrs. 28 mins.	12 hrs. 15 mins.	21 hrs. 45 mins.	31 hrs. 20 mins.
Rotation in 2 dm. tube	+0.72°	+0.60°	+0.56°	+0.36°	+0.13°

Reduced glutathione itself when dissolved in 4*N* HCl does not show a comparable change in rotation although the specific rotation in strongly acid solution is -9° for the mercury-green line instead of -18.5° as it is in aqueous solution. The rise in the rotation during the first hour is not accompanied by any change in the formaldehyde titration; it has not been investigated further.

When prepared from oxidised glutathione the cuprous compound behaves in an identical manner on keeping in hydrochloric acid solution and has, under the same conditions as before, $[\alpha]_{5461}^{16.5} + 45.4^\circ$ to $+45.8^\circ$.

It is already well known that cuprous mercaptides, suspended in a weakly acid solution, become soluble on the addition of small amounts of a chloride. As might be expected iodides have a similar effect. The cuprous iodide which results is itself held in solution by the thiol compound and does not separate until some of this has been oxidised to the disulphide. On the addition of more thiol compound it again goes into solution but reprecipitates on adding iodine.

58 mg. of cuprous glutathione prepared from GSSG were suspended in 3 cc. of water and 0.5 cc. of glacial acetic acid; addition of 0.2-0.3 g. of potassium

iodide caused complete solution. Using starch as indicator the mixture was titrated with *N*/100 iodine. 15 cc. were required (theoretical 15·7). Cuprous glutathione prepared directly from reduced glutathione showed identical equivalence to iodine.

108·4 mg. of cuprous glutathione prepared from oxidised glutathione were suspended in about 10 cc. of water in a 50 cc. round-bottomed flask. The mixture was decomposed with H_2S , with rigid exclusion of air for 2–3 hours and was then connected to an efficient pump and exhausted cautiously, the flask was kept warm to prevent the solution from freezing. After an hour the cuprous sulphide and any free sulphur that might have formed were removed by centrifuging and the precipitate washed once on the centrifuge. The centrifugates, which were water-clear, were taken nearly to dryness in a vacuum desiccator over sulphuric acid and sodium hydroxide.

The precipitate remaining in the centrifuge-tube contained 5·23 mg. S and 19·4 mg. Cu, giving a Cu : S ratio of 3·78 (calculated for Cu_2S , 3·97). This agreement is as close as could be expected and certainly excludes the possibility that either cupric sulphide or an equimolecular mixture of cuprous sulphide and copper is formed.

The centrifugate which contained glutathione was titrated with *N*/1000 iodine; 5·4 cc., corresponding to 84 mg. of GSH, were required. This indicates that at least 95 % of the glutathione was now in the reduced form.

SUMMARY.

A modification in the method of preparation of glutathione is described.

Crystallographic data and photographs of the crystals are given.

The specific rotation of cuprous glutathione has been measured and evidence is brought forward to show that there is no reason to assume the existence of a cuprous derivative of oxidised glutathione, insoluble in weak acids.

I am deeply indebted to Sir F. G. Hopkins for his interest in this work.

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