LXXIV. ON GLUTATHIONE. IV. CONSTITUTION.

By JUDA HIRSCH QUASTEL, CORBET PAGE STEWART
AND HUBERT ERLIN TUNNICLIFFE (*Shuttleworth Student, Gonville
and Caius College*).

From the Biochemical Laboratory, Cambridge.

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GLUTATHIONE was isolated from yeast and from various animal tissues by Hopkins [1921]. It is a white amorphous powder, non-hygroscopic when pure. It is very soluble in water, but insoluble in the usual organic solvents. Heated in a capillary tube, it softens at 165–167°, and melts with evolution of carbon dioxide at 182–185°. The yield of glutathione from fresh tissues was about 0.01–0.02 %; from yeast, the usual source, a similar amount was obtained.

On the following evidence, Hopkins [1921] considered glutathione to be a dipeptide of glutaminic acid and cysteine. The elementary analysis agreed with the formula $C_8H_{14}O_5N_2S$, which such a dipeptide would possess. The peptide structure was shown by the fact that the primary-amino nitrogen, as determined by the method of Van Slyke [1911], was half the total nitrogen, and was, moreover, doubled by hydrolysis. Finally, acid hydrolysis of glutathione gave *d*-glutaminic acid and *l*-cystine.

The sulphhydryl group of cysteine is readily oxidised, even by atmospheric oxygen, giving the disulphide cystine.

Similarly, glutathione can be obtained in two forms: one—"reduced glutathione"—containing cysteine; the other—"oxidised glutathione"—containing cystine. The importance of glutathione in tissue oxidation processes depends on the ready inter-convertibility of these two forms.

A dipeptide of glutaminic acid and cysteine must possess a structure represented by one of the following formulae:

Our problem was therefore to find which of these three formulae is that of glutathione.

The first stage was to find through which amino group—that of glutaminic acid, or that of cysteine—the linkage of the two amino-acids was brought about. For this purpose two methods were adopted, both of which had previously been used by Barger and Tutin [1918] in determining the constitution of carnosine.

In the first method, use was made of the power possessed by 2 . 3 . 4- and 2 . 4 . 5-trinitrotoluene, of condensing with primary amino groups.

The condensation product of glutathione and 2 . 3 . 4-trinitrotoluene, on hydrolysis, yielded free cystine. It must be concluded, therefore, that the trinitrotoluene had reacted with the amino group of the glutaminic acid part of glutathione, and that the amino group of cysteine had formed part of the peptide linkage. From the hydrolysis mixture, a yellow substance was also isolated: this was probably the condensation product of glutaminic acid with trinitrotoluene. Its identity was not definitely ascertained however, owing to the failure of all attempts to obtain a satisfactory condensation between trinitrotoluene and free glutaminic acid.

The second method consisted in the replacement of the free primary amino group of the dipeptide by hydroxyl, and identification of the hydroxy-acid obtained after hydrolysis of the resulting compound. In this way, α -hydroxyglutaric acid was isolated, a result confirmatory of that obtained by the first method.

These experiments agree, then, in showing that in glutathione, the free primary amino group is in the glutaminic part of the molecule, and that it is the amino group of the cysteine which is involved in the peptide linkage. Formula I, therefore, is not that of glutathione, but the evidence so far does not differentiate between Formulae II and III.

It remained, therefore, to determine which of the two carboxyl groups of glutaminic acid was, in glutathione, condensed with the amino group of cysteine. For this purpose, use was made of an investigation by Dakin [1905], who found that α -amino-acids, oxidised by means of hydrogen peroxide in presence of a trace of iron salt, gave carboxylic acids containing one carbon atom less than the original amino-acid. The course of the reaction, he has shown, may be represented thus:

Glutaminic acid, oxidised in this way, yielded succinic acid [Dakin, 1908]. Formula II, is that of a γ -amino-acid, Formula III, of an α -amino-acid. A compound of Formula III, oxidised by hydrogen peroxide, would be converted to a substance containing the succinyl radicle in place of the original glutaminyl radicle. This compound would probably be, not succinyl-cysteine,

but succinyl-cysteic acid, the sulphydryl of the cysteine having been oxidised to the sulphonic acid group. After, and only after, hydrolysis of this compound, would free succinic acid be obtained as one of the products.

A compound of Formula II, on the other hand, must behave rather differently. Even if the $>\text{CH.NH}_2$ group be oxidised, the resulting carbonyl must, by continued oxidation, lead to a breakage of the chain, and the immediate formation of succinic acid *before* hydrolysis.

Experiments were carried out on these lines, and it was found that simple oxidation of glutathione by hydrogen peroxide produced no trace of succinic acid, whereas after hydrolysis of the oxidation product, succinic acid was isolated in a yield amounting to 50 % of the theoretical. It is therefore concluded that the structure of glutathione is represented by Formula III.

The ultimate proof of constitution lies in the synthesis, by known reactions, of the substance under consideration. Hence, until glutathione has been synthesised—a task on which the authors are at present engaged—the conclusions set forth in this paper must be regarded as being in no way final. The analytical evidence, however, which is briefly summarised below, appears to be quite strong enough to justify the deductions drawn from it.

1. Glutathione condenses with 2.3.4-trinitrotoluene, its free amino group being thereby bound. Hydrolysis of the resulting compound gives cystine, but no free glutaminic acid.

2. The free amino group of glutathione having been replaced by hydroxyl, a compound is obtained which, by hydrolysis, gives α -hydroxyglutaric acid.

These entirely different methods agree in showing that in glutathione, it is the amino group of the cysteine which is concerned in the peptide linkage.

3. Oxidation of glutathione by hydrogen peroxide yields succinic acid only after hydrolysis of the oxidation product.

Hence glutathione is itself an α -amino-acid, and of Formulae II and III, only the latter satisfies this condition. Moreover, this result affords additional confirmation of the deductions drawn from the previous experiments, since a compound of Formula I could not yield succinic acid under the circumstances.

Owing to the low concentration in which glutathione is found, and the long and complicated method necessary for its separation, the amount available for this work was very small. The whole of the experiments described were carried out on rather less than 2 g. of material.

EXPERIMENTAL.

Condensation of Glutathione with 2.3.4-Trinitrotoluene.

0.3 g. of "oxidised" glutathione and 1.0 g. of 2.3.4-trinitrotoluene, dissolved in 40 cc. of 60 % ethyl alcohol, were heated under a reflux condenser, on the water-bath, for two hours. After ten minutes the liquid began to turn yellow, and the colour gradually deepened. The clear solution was evaporated to dryness on the water-bath: absolute alcohol was added and evaporated off to

remove the last traces of water. The residue was extracted three times with dry benzene, to remove unchanged trinitrotoluene. The yellow crystalline powder remaining behind was dissolved in hot water. The solution was filtered and the filtrate was evaporated to dryness on the water-bath. The yellow residue was washed with absolute alcohol, in which it was only very slightly soluble. Absence of glutathione from this residue was shown by its inability to give any precipitate with mercuric sulphate in acid solutions.

The condensation product thus obtained was a yellow semi-crystalline substance, very soluble in water, only slightly soluble in ethyl alcohol, insoluble in ether and benzene. Heated in a capillary tube, it softened at 115° and melted sharply with decomposition at 202° (uncorr.). Yield 75 % of the theoretical.

Analysis:

0.1113 g. dried at 100° ; 0.1695 g. CO_2 ; 0.0501 g. H_2O .

Found $\text{C} = 41.54\%$; $\text{H} = 4.98\%$.

Calculated for $(\text{C}_{15}\text{H}_{17}\text{O}_9\text{N}_4\text{S})_2$ $\text{C} = 41.94\%$; $\text{H} = 3.97\%$.

Nitrogen was estimated by a modified micro Kjeldahl [Stewart, 1923].

10.0 mg. contained 1.314 mg. Nitrogen $\text{N} = 13.14\%$.

Calculated for $(\text{C}_{15}\text{H}_{17}\text{O}_9\text{N}_4\text{S})_2$ $\text{N} = 13.06\%$.

Hydrolysis of the Condensation Product.

0.4 g. of the condensation product were dissolved in 15 cc. of 30 % sulphuric acid and boiled under a reflux condenser for four hours. At the end of this time, the liquid was cooled, and thoroughly extracted with ether. The deep yellow ethereal extracts, on evaporation left a yellow residue, readily soluble in alcohol and in water, which was probably the condensation product of glutaminic acid and trinitrotoluene. The almost colourless aqueous layer was diluted to 60 cc. with distilled water, and excess of mercuric sulphate solution was added. The voluminous white precipitate was separated by centrifuging, and washed repeatedly with distilled water. It was then suspended in water, and decomposed by hydrogen sulphide. The filtrate from mercuric sulphide was boiled until all the hydrogen sulphide had been expelled. The colourless solution then gave the following reactions:

1. A violet coloration (when neutralised) with triketohydrindene hydrate.
2. A vivid permanganate coloration with sodium nitroprusside in presence of ammonia.

3. A black precipitate of lead sulphide on prolonged boiling with lead acetate and sodium hydroxide.

It was therefore concluded that the solution contained cysteine.

A slight excess of baryta water was added, and after removal of the barium sulphate, the solution was aerated until the nitroprusside reaction was negative—i.e. until the conversion of cysteine to cystine was complete. Barium was then quantitatively removed by addition of sulphuric acid, barium

sulphate was filtered off, and the clear liquid concentrated on the water-bath. On standing, cystine separated in typical hexagon-shaped plates, and on further concentration of the mother liquors, in acicular aggregates.

Nitrogen by micro Kjeldahl:

8.7 mg. contained .999 mg. Nitrogen	N = 11.48 %.
Calculated for $C_6H_{12}O_4N_2S_2$	N = 11.66 %.

De-amination of Glutathione.

0.5 g. of glutathione was dissolved in 10 cc. of water, and 2.0 g. of freshly prepared silver nitrite were suspended in the solution. The liquid was cooled in ice, and 20 cc. *N* HCl were added gradually with constant stirring. The mixture was left in ice for one hour, and was then allowed to stand overnight at room temperature. The precipitated silver chloride was filtered off, and the filtrate was found to be free from silver. The liquid was extracted with ether, and the dried ethereal extracts were evaporated to dryness on the water-bath. There was no residue.

Hydrolysis of De-aminated Glutathione: Isolation of α -Hydroxyglutaric Acid.

An equal volume of 35 % hydrochloric acid was added to the solution containing the de-aminated glutathione, and the mixture was boiled under a reflux condenser for four hours. After cooling, the hydrolysis mixture was thoroughly extracted with ether, and the combined ethereal extracts, dried over anhydrous sodium sulphate, were evaporated on the water-bath. There remained an oily liquid, which, on cooling, solidified to a deliquescent, crystalline mass. Yield 0.10 g. It had a fruity odour: was strongly acid, was readily oxidised by potassium permanganate, and gave no coloration with triketohydrindene. Tests for sulphur and nitrogen were completely negative. The substance melted well below 100°, but owing to its deliquescent nature, no accurate determination of the melting point could be made.

For analysis, the calcium salt was prepared by addition of calcium chloride in slight excess to the neutralised solution of the acid in slightly dilute alcohol. The precipitated salt was separated by centrifuging, washed thoroughly with alcohol and finally with ether. It was dried at 37°. A weighed quantity of the salt was transferred to a centrifuge tube, and dissolved in dilute hydrochloric acid. A slight excess of saturated ammonium oxalate was added, and the liquid was made alkaline with ammonia. It was allowed to stand one hour, and was then centrifuged. Most of the supernatant liquor (all but about 0.3 cc.) was siphoned off, and the precipitate was washed three times with 2 % ammonia by the same process. The calcium oxalate was then dissolved in 2 cc. *N* H_2SO_4 , warmed on the water-bath to 60°, and titrated with 0.01 *N* potassium permanganate from a micro-burette.

6.2 mg. of calcium salt required 6.27 cc. of 0.01 *N* KMnO_4 ;

Ca = 20.22 %.

Calculated for calcium α -hydroxyglutarate, $\text{Ca} \cdot \text{C}_5\text{H}_6\text{O}_5 \cdot \frac{1}{2}\text{H}_2\text{O}$;

Ca = 20.51 %.

Oxidation of Glutathione.

0.5 g. of "oxidised" glutathione was dissolved in a little water, and the solution was made slightly alkaline with ammonia. A trace of ferrous sulphate was added, and then 25 cc. of 5 % hydrogen peroxide. After half an hour at 70°, evolution of gas had ceased, and a further 25 cc. of 5 % hydrogen peroxide were added, the heating being continued to a total of one hour. The solution then gave no coloration with triketohydrindene, whereas glutathione itself gives a strongly positive reaction. Evidently the free amino group had been removed by the oxidation. Further, mercuric sulphate produced no precipitate. After cooling, the solution was acidified with hydrochloric acid, and thoroughly extracted with ether. The ethereal extracts, dried and evaporated, left no residue. No free succinic acid, therefore, had been formed by the oxidation.

Hydrolysis of the Oxidation Product.

The solution of the oxidation product was evaporated to dryness to remove all traces of ether and hydrogen peroxide. The residue was dissolved in 20 cc. of 30 % hydrochloric acid, and boiled under a reflux condenser for three hours. The solution was cooled and extracted with ether. The combined ethereal extracts were dried and evaporated on the water-bath, when there remained a white crystalline solid, in amount equal to 40 % of the theoretical yield of succinic acid. That this substance was succinic acid was shown in the following ways:

1. It formed bunches of thick prismatic needles, identical in form with those of succinic acid.
2. It was not readily soluble in water, and the aqueous solution was strongly acid.
3. It gave no coloration with triketohydrindene.
4. The ammonium salt, distilled with zinc dust gave the pyrrole reaction with a pine splint moistened with hydrochloric acid.
5. Heated with resorcinol and concentrated sulphuric acid, it gave the typical fluorescent solution.
6. Heated alone, it melted and sublimed in feathery needles, and produced a choking odour.
7. Its neutral solution gave a precipitate with ferric chloride solution.
8. From neutral solution, its silver salt was precipitated by the addition of silver nitrate.
9. The acid, after purification through the silver salt, melted at 180° (uncorr.) and the melting point was not altered by the admixture of pure succinic acid.

For analysis, a weighed amount of the acid, purified through the silver salt, was dissolved in water, and titrated with standard alkali from a micro-burette. From this neutral solution, the silver salt was precipitated, and after thorough washing was dissolved in nitric acid and titrated with standard potassium thiocyanate.

1. 15.0 mg. of the acid required 5.050 cc. 0.05*N* NaOH
[= 14.89 mg. succinic acid].
The silver salt required 6.3 cc. 0.04*N* KCNS
[= 14.87 mg. succinic acid].
2. 4.4 mg. of the acid required 1.495 cc. 0.05*N* NaOH
[= 4.41 mg. succinic acid].
The silver salt required 1.850 cc. 0.04*N* KCNS
[= 4.36 mg. succinic acid].

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REFERENCES.

- Barger and Tutin (1918). *Biochem. J.* **12**, 402.
 Dakin (1905). *J. Biol. Chem.* **1**, 171.
 — (1908). *J. Biol. Chem.* **5**, 410.
 Hopkins (1921). *Biochem. J.* **15**, 286.
 Stewart (1923). *Biochem. J.* **17**, 132.
 Van Slyke (1911). *J. Biol. Chem.* **9**, 185.