CCLX. GLUTATHIONE. ITS REACTION WITH ALKALI AND SOME *N* AND *S* DERIVATIVES.

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IN a previous communication [1933] it was observed that reduced glutathione, unlike cysteine, apparently did not react normally with benzyl chloride in the presence of NaOH to give *S*-benzylglutathione. The problem has now been investigated. On shaking GSH with *N* NaOH and benzyl chloride the nitroprusside test gradually became less intense and was only faintly positive after 1.5 hours. On acidification at the end of this time however no precipitate was obtained. The shaking was continued for 10 hours and on careful acidification a compound was obtained which contained an *S*-benzyl group and also a free NH₂ group. In seeking for an explanation of the delayed formation of this compound two lines of argument may be adduced. According to the first, GSH reacts only very slowly¹ with benzyl chloride and the remainder of GSH is oxidised to GSSG in the presence of alkali. GSSG then dismutates into GSH and a higher oxidation product as in equation (2) or (3).

An equation of type (2) was first put forward by Fromm [1908] for dibenzyl disulphide. Equations of type (3) have been put forward by Vickery and Leavenworth [1930] and Shinohara and Kilpatrick [1934] in the case of cystine.

The regenerated GSH then partly reacts with benzyl chloride, the remainder undergoing reoxidation and further dismutation. Ultimately then benzylglutathione and a higher oxidation product are obtained. Support was given to the above suppositions in that the same final product was obtained if GSSG instead of GSH were allowed to react with benzyl chloride and sodium hydroxide.

The reaction between GSSG and alkali is not however as simple as that suggested by equations (2) and (3), for H₂S and sulphur are also found to be products of the reaction. Therefore an alternative possible explanation must not be overlooked. It is possible that prolonged shaking of GSSG with NaOH may give rise to an unsaturated compound analogous with the 3-methylene-6-methyl-2:5-diketopiperazine obtained by Bergmann and Stather [1926] from dialanycystinedianhydride and alkali. The H₂S formed may then react with the NaOH to form NaSH which in turn will react with benzyl chloride to give benzyl mercaptan. Addition of the latter to the unsaturated compound would give the benzyl derivative.

¹ Actually a very small amount of the benzyl derivative is formed at the end of 1.5 hours. This is obtained by evaporating the acid solution to small bulk.

Nicolet [1932] has indeed shown that certain unsaturated substances react readily with mercaptans in alkaline solution, addition taking place across the double bond. Attempts were made to reproduce this mechanism in the present case as follows. Oxidised glutathione was allowed to react with NaOH anaerobically for 4 days. At the end of this time, as was shown by other experiments, GSSG had lost over 90 % of its sulphur. Benzyl chloride was added to the solution and the mixture shaken. No *S*-benzyl derivative could however be detected among the products. The alternative explanation is therefore not acceptable.

The dismutation of GSSG to GSH in alkaline solution is also evident from other considerations. When the nitroprusside reaction is carried out on GSSG by adding ammonia to its aqueous solution and then sodium nitroprusside solution no coloration is produced. If on the other hand NaOH is used instead of ammonia an intense colour is produced which is not due entirely to hydrogen sulphide. This is proved by the fact that nitroprusside gave a pink colour with such solutions in presence of zinc acetate, under which conditions H_2S gives no colour [Shinohara and Kilpatrick, 1934].

GSSG was allowed to react anaerobically with *N* NaOH at room temperature. On acidification after varying intervals it was found that H_2S and sulphur were produced. This is in accordance with the observations of previous workers [Hopkins, 1929]. The acid solution was then boiled to remove H_2S , and a solution was obtained which gave a strong modified nitroprusside test in the early stages. After longer periods the modified nitroprusside test became almost negative, although the solution still readily reduced iodine, bromine water and alkaline permanganate. These facts suggest that the dismutation sets in early with only a small loss of H_2S and sulphur. In the latter stages however GSSG (and GSH) is more or less completely destroyed, giving rise to H_2S , sulphur and a reducing substance.

In the above-mentioned communication by the author it was shown that naphthalene-2-sulphonyl chloride first oxidises cysteine to cystine and then forms dinaphthalene-2-sulphonylcystine. If the SH group in cysteine is blocked as in *S*-benzylcysteine then *S*-benzyl-*N*-naphthalene-2-sulphonylcystine is formed quantitatively. It was found, on the other hand, that GSH reduced naphthalene-2-sulphonyl chloride to naphthalene-2-sulphinic acid without the formation of a sulphonyl derivative. It was with the intention of determining whether naphthalenesulphonyl chloride would react normally with glutathione if the SH group were blocked that the above-mentioned attempt to prepare *S*-benzylglutathione was made.

In seeking a more suitable substance to effect this it seemed reasonable to try 2 : 4-dinitrochlorobenzene, as this compound has a highly reactive chlorine atom and should therefore react rapidly with GSH before the alkali has any marked effect.

Bost *et al.* [1932] have previously used this compound for the identification of simple mercaptans. A modification of their method was used for glutathione. An alcoholic solution of 2 : 4-dinitrochlorobenzene was poured into a cold alkaline solution of GSH. A negative nitroprusside test was given after a few minutes and on acidification a bright yellow crystalline precipitate of *S*-2 : 4-dinitrophenylglutathione separated. This proved to be remarkably stable and could be recrystallised from boiling water in fine canary-yellow needles. Formaldehyde titration showed it to have a free NH_2 group. On account of its facile preparation, high yield and stability it can be recommended for the identification of glutathione.

S-2 : 4-Dinitrophenylglutathione reacted normally with naphthalene-2-sulphonyl chloride according to the general method of Fischer and Bergell [1902] to give *S*-2 : 4-dinitrophenyl-*N*-naphthalene-2-sulphonylglutathione,

together with a small amount of di-(2 : 4-dinitro)-phenyldisulphide due to alkaline hydrolysis. The formation of the latter compound proves that the dinitrophenyl residue is attached to glutathione through the S atom.

Thus the anomaly that the NH_2 of reduced glutathione will not react with naphthalenesulphonyl chloride [Hopkins, 1929] has now been elucidated.

The preparation of *S*-2 : 4-dinitrophenylcysteine is recorded in the experimental part. This compound however crystallises with difficulty and does not possess a sharp melting-point.

EXPERIMENTAL.

Reaction between glutathione and benzyl chloride.

0.5 g. of GSH was dissolved in 5 ml. of *N* NaOH and shaken with about 0.25 g. (excess) of benzyl chloride. No product was obtained on acidification at the end of half an hour. At the end of 1½ hours the nitroprusside test was only faintly positive, but still no product was obtained on acidification. The shaking was continued for a further 10 hours. At the end of this time, the mixture was shaken with ether to remove the excess of benzyl chloride, and the alkaline layer was carefully acidified with very dilute HCl until just green to bromophenol blue. At this p_{H} a white precipitate was produced. The substance was unstable and was recrystallised from water as small white needles: m.p. 181–2°.

(Found: C, 51.31; H, 5.83; N, 10.32; S, 8.27 %. $\text{C}_{17}\text{H}_{23}\text{O}_6\text{N}_3\text{S}$ requires: C, 51.38; H, 5.84; N, 10.58; S, 8.31 %.)

It was insoluble in alcohol, acetone and benzene. It did not give the nitroprusside test when dissolved in water and treated with ammonia and sodium nitroprusside. If however it were dissolved in NaOH with gentle warming a mercaptanic odour became evident, and the solution when treated with nitroprusside gave a marked coloration.

The same product was obtained when GSSG was shaken with NaOH and benzyl chloride in the above manner for 6 hours.

The substance contained a free NH_2 group, and the benzyl group was shown to be attached directly to the S atom as follows:

0.1 g. of the substance was dissolved in a small quantity of *N* NaOH and 0.2 g. of 2 : 4-dinitrochlorobenzene dissolved in a little alcohol was added. The mixture was warmed gently and filtered. Yellow crystals came down which were recrystallised from alcohol: m.p. 130°. (Found S, 11.05 %. 2 : 4-Dinitrophenylbenzylsulphide [Bost *et al.*, 1932] has m.p. 130° and S, 11.07 %.)

Reaction between oxidised glutathione and N NaOH in vacuo.

Several experiments were carried out as follows: 76.5 mg. of GSSG were placed in a Thunberg tube with 5 ml. of *N* NaOH contained in a separate tube. The Thunberg tube was exhausted and the contents mixed. At the end of a specified time the tube was opened and the contents washed into excess of dilute acetic acid and made up to 50 ml. On the addition of the acid the solution be-

came cloudy owing to the separation of sulphur, and the odour of H_2S became evident. To 10 ml. of the solution were added 5 ml. of 0.01 *N* iodine, and the excess was back-titrated with 0.01 *N* thiosulphate. This gave an approximate iodine value for the H_2S *plus* reducing substances. Another 10 ml. were boiled till free from H_2S and titrated as above, the difference giving a rough approximation of the H_2S formed.

Table I.

Time elapsed	Ml. of 0.01 <i>N</i> I for H_2S *	Ml. of 0.01 <i>N</i> I for residue
5 mins.	0.24	3.36
30 mins.	0.54	1.20
2.5 hours	0.70	1.94
1 day	0.97	2.60
3 days	3.58	3.32

* If the GSSG had been completely decomposed into H_2S , 5 ml. of 0.01 *N* I would be required.

The modified nitroprusside test on the residue was most marked in the early stages and disappeared almost entirely during the later stages. It is apparent then that the iodine was titrating reducing substances other than the SH, especially during the later stages.

S-2 : 4-Dinitrophenylglutathione.

1.02 g. of glutathione were dissolved in 10 ml. of *N* NaOH (3 mols.). 0.67 g. of 2 : 4-dinitrochlorobenzene was dissolved in alcohol and added slowly with stirring to the glutathione solution. The mixture was kept for 10 mins., filtered if necessary and made just acid to litmus with very dilute HCl. Bright yellow crystals separated. These were recrystallised from boiling water in radiating clusters of fine canary-yellow needles: m.p. 211° with decomposition. Yield 1.3 g. (90 %). The reaction could be carried out at 0° , the same product being obtained, but in slightly reduced yield.

(Found: C, 39.10; H, 4.48; N, 13.9; S, 6.31; 6.4 %. $\text{C}_{16}\text{H}_{19}\text{O}_{10}\text{N}_5\text{S}$, H_2O requires C, 39.10; H, 4.29; N, 14.28; S, 6.52 %.)

Soluble in concentrated HCl. Very slightly soluble in cold water. Insoluble in alcohol, acetone, chloroform and benzene. Dissolves in *N* NaOH to give a deep yellow solution which obeys de Beer's law on dilution with NaOH of the same strength. 1 mg. of the compound in 20 ml. of *N* NaOH can be estimated colorimetrically in this way.

N-Naphthalene-2-sulphonyl-S-2 : 4-dinitrophenylglutathione.

0.49 g. of *S-2 : 4*-dinitrophenylglutathione was dissolved in 2 ml. of *N* NaOH and shaken with 0.453 g. of naphthalene-2-sulphonyl chloride dissolved in ether. Three further quantities of 1 ml. of *N* NaOH were added at hourly intervals. At the end of 4 hours the alkaline layer was separated and acidified with acetic acid, a gummy mass being deposited. This was dissolved in acetone, water was added until just cloudy and the mixture warmed till clear. On standing, two fractions, A and B, crystallised out.

Fraction A. Yellow-brown crystalline powder: m.p. about 280° . Soluble in hot aniline. (Found: N, 14.01; S, 15.8 %. 2 : 4-Dinitrophenyldisulphide $\text{C}_{12}\text{H}_6\text{N}_4\text{O}_8\text{S}_2$ requires N, 14.07; S, 16.08 %.)

Fraction B. Recrystallised from water as minute pale yellow needles: m.p. 158° . (Found: N, 10.41; S, 9.57 %. $\text{C}_{26}\text{H}_{27}\text{O}_{12}\text{N}_5\text{S}_2$ requires N, 10.56, S, 9.65 %.) Very slightly soluble in water, soluble in alcohol.

S-2 : 4-Dinitrophenylcysteine.

Prepared according to the directions given for the corresponding derivative of glutathione. Yield 75 %. Very slightly soluble in benzene, acetone, ether, acetic acid. Crystallises with difficulty from alcohol or water as a golden yellow powder: m.p. 148–154° with decomposition. (Found: S, 11.32 %. $C_9H_9N_3O_6S$ requires S, 11.2 %.)

SUMMARY.

1. Glutathione and benzyl chloride do not react normally in the presence of alkali to give an *S*-benzyl derivative. A probable explanation of the course of the reaction is given.

2. Alkali has a twofold effect on oxidised glutathione:

(a) Dismutation into GSH and a higher oxidation product is brought about.

(b) Hydrogen sulphide and sulphur are produced freely.

3. Glutathione in alkaline solution reacts rapidly with 2 : 4-dinitrochlorobenzene to give *S*-2 : 4-dinitrophenylglutathione which is a highly crystalline compound stable to boiling water. This substance gives *S*-2 : 4-dinitrophenyl-*N*-naphthalene-2-sulphonylglutathione with naphthalene-2-sulphonyl chloride. Thus the apparent anomaly, originally noticed by Hopkins, that the NH_2 group of GSH fails to react with naphthalene-2-sulphonyl chloride to give a sulphonyl derivative has now been finally elucidated. The matter now stands thus: GSH reduces naphthalene-2-sulphonyl chloride to naphthalene-2-sulphinic acid in alkaline solution. If however the SH group is blocked as in $GSC_6H_3(NO_2)_2$, then the NH_2 group reacts normally with this reagent.

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