167. *Syntheses from Phthalimido-acids. Part VIII.* Synthesis of Glutathione by a New Route to Cysteinyl-peptides.*

By F. E. King, J. W. Clark-Lewis, and Roy Wade.

L-4-*N*-Methoxycarbonylmethylcarbamoyl-2 : 2-dimethylthiazolidine, which has been prepared from L-cysteine hydrochloride by way of L-2 : 2-dimethylthiazolidine-4-carboxylic acid, reacts with phthalyl-L-glutamic anhydride to give the γ-derivative (phthalyl*iso*propylideneglutathione monomethyl ester) in 40% yield from cysteine. Glutathione (12% yield as copper derivative) was obtained from the protected tripeptide by removal of the phthalyl, methyl, and *iso*propylidene groups.

Glutathione (I) was shown by Harington and Mead [1] to be γ-L-glutamyl-L-cysteinylglycine by synthesis of the tripeptide after it had been isolated and investigated by others, notably by Hopkins [2] and by Kendall, MacKenzie, and Mason.[3] The biological function and chemistry of glutathione, which is an important constituent of all living cells, was the subject of a recent symposium.[4] Synthesis [1, 5-8] of glutathione is accomplished only with difficulty and in low over-all yield so that the peptide is usually prepared from natural sources, *e.g.*, yeast.[2, 3] On the other hand, the synthetical methods are of potential value to the study of analogues of glutathione, among them the penicillamine derivative, γ-L-glutamyl-D-penicillaminylglycine, which is of considerable interest in regard to a proposed mechanism [9] for the antibiotic action of the penicillins.

In the syntheses so far described, benzyloxycarbonylglutamic acid is generally used, and special procedures are necessary in order to ensure coupling of the γ-carbonyl group with the cysteinylglycine fragment, which in all but the method of Harington and Mead [1] contains the grouping $-S\cdot CH_2Ph$ and must therefore be debenzylated with sodium and liquid ammonia in the final stage.† We have now investigated the application to this problem of phthalyl-L-glutamic anhydride which in its reactions with amines directly affords γ-derivatives.[10] When seeking to modify the existing methods in this respect it seemed desirable also to avoid the use of *S*-benzyl ethers, and an entirely new synthesis of cysteinyl-peptides has thus been devised which is exemplified as follows by glutathione.

First, L-2 : 2-dimethylthiazolidine-4-carboxylic acid hydrochloride [11] (II) was prepared

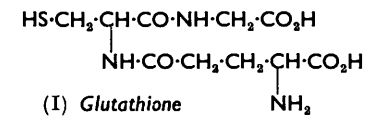
* Part VII, preceding paper.

† After this paper had been written, a synthesis of glutathione (6% yield on L-cysteine) using tritylated intermediates was reported by Amiard, Heymès, and Velluz (*Bull. Soc. chim. France*, 1956, 698).

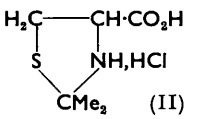
[1] Harington and Mead, *Biochem. J.*, 1935, **29**, 1602.
[2] Hopkins, *ibid.*, 1921, **15**, 286; *J. Biol. Chem.*, 1929, **84**, 269.
[3] Kendall, MacKenzie, and Mason, *ibid.*, p. 657.
[4] "Glutathione. Proceedings of the Symposium held at Ridgefield, Connecticut," Academic Press, New York, 1954.
[5] du Vigneaud and Miller, (*a*) *J. Biol. Chem.*, 1936, **116**, 469; (*b*) *Biochemical Preparations*, 1952, **2**, 74.
[6] Hegedüs, *Helv. Chim. Acta*, 1948, **31**, 737.
[7] Rudinger and Šorm, *Coll. Czech. Chem. Comm.*, 1951, **16**, 214.
[8] Goldschmidt and Jutz, *Chem. Ber.*, 1953, **86**, 1116.
[9] Fischer, *Science*, 1947, **106**, 146.
[10] King and Kidd, *J.*, 1949, 3315, and later papers in this series.
[11] Micheel and Emde, *Ber.*, 1939, **72**, 1724; cf. Woodward and Schroeder, *J. Amer. Chem. Soc.*, 1937, **59**, 1690.

from L-cysteine hydrochloride and acetone, and the free base formylated with formic–acetic anhydride to give 3-formyl-L-2 : 2-dimethylthiazolidine-4-carboxylic acid. This product (III) was converted into mixed anhydrides with *n*- and *iso*-butyl chloroformate, and although they failed to react in the desired manner with an aqueous solution of glycine sodium salt, both gave satisfactory yields of 3-formyl-L-4-*N*-methoxycarbonylmethylcarbamoyl-2 : 2-dimethylthiazolidine (IV) with glycine methyl ester. When ethyl chloroformate was used instead of the butyl chloroformates a much lower yield of the ester (IV) was obtained. A similar preparation of the ester (IV) was described in an abstract by Sheehan and Armstrong [12] while this work was in progress.

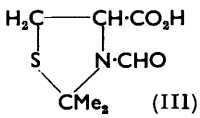
Deformylation of the thiazolidine ester (IV) with methanolic hydrogen chloride (1—5%) gave an almost quantitative yield of L-4-*N*-methoxycarbonylmethylcarbamoyl-2 : 2-dimethylthiazolidine hydrochloride (V), which with triethylamine and phthalyl-L-glutamic anhydride gave the fully protected tripeptide (VI) in 40% yield from L-cysteine hydrochloride. The first four intermediates crystallised readily but the fully protected glutathione (VI) separated as a viscous oil from various solvents, although the amorphous *S*-benzylthiuronium salt gave satisfactory analytical results for nitrogen and sulphur. The procedure outlined above for the preparation of the glutathione derivative (VI) was found to give reproducible and satisfactory results, and other coupling procedures were unsuccessful. Attempts [13] to prepare an acid chloride from the formylthiazolidinecarboxylic acid (III) with phosphorus chlorides or with thionyl chloride under various conditions invariably led to extensive decomposition, and similar results with 5 : 5-dimethyl-3-phenylacetylthiazolidine-4-carboxylic acid are recorded by Süs and Rosenberger.[14] Coupling of the L-formyl acid (III) with glycine ester by the phosphorus trichloride procedure developed by Süs and Hoffmann [15] gave an optically inactive product, and the anilide of the acid (III) prepared in a similar manner had an optical purity of 64%.[13]



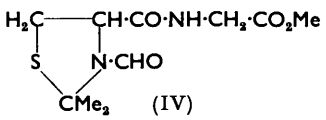
(I) *Glutathione*



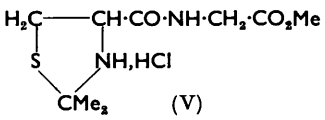
(II)



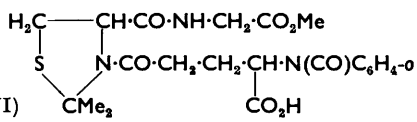
(III)



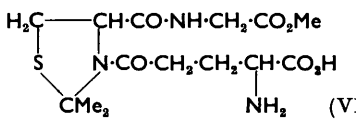
(IV)



(V)



(VI)



(VII)

Removal of the protecting groups from the glutathione derivative (VI) was accomplished by hydrazine, followed by alkaline hydrolysis of the resulting ester (VII). Preliminary experiments had shown that acid hydrolysis of the ester (VI) by the method of Sheehan, Chapman, and Roth [16] caused preferential hydrolysis of the γ-glutamyl amide link and that simultaneous treatment with hydrazine and alkali effected only partial dephthalylation while causing hydrolysis to five ninhydrin-positive substances. Removal of the phthalyl group was quantitative when the compound (VI) was heated for 2 hr. with ethanolic hydrazine, and led to the *iso*propylideneglutathione monomethyl ester (VII)

[12] Sheehan and Armstrong, 122nd Meeting Amer. Chem. Soc., 1952, Abs. no. 23, p. 15M.
[13] See succeeding paper.
[14] Süs and Rosenberger, *Annalen*, 1949, **564**, 54.
[15] Süs and Hoffmann, *ibid.*, 1951, **572**, 96.
[16] Sheehan, Chapman, and Roth, *J. Amer. Chem. Soc.*, 1952, **74**, 3822.

which was proved homogeneous by paper chromatography. It did not respond to tests for the thiol group, and a mercury derivative was obtained only by destruction of the thiazolidine ring. The ester (VII) was readily soluble in water and in ethanol and, as it did not crystallise, it was not isolated in subsequent work. The mixture of phthalhydrazide and *iso*propylidene methyl ester (VII) resulting from dephthalylation and evaporation of the ethanol was therefore treated with saturated barium hydroxide solution, and when hydrolysis of the ester group was complete (2 hr.) the solution was acidified with sulphuric acid and thiol compounds were isolated from the filtrate as mercury derivatives (70%). Glutathione was then isolated as described by du Vigneaud and Miller,[5] and its copper derivative obtained in 5% yield from L-cysteine hydrochloride. Glutathione was regenerated [5] from the copper derivative and obtained, by freeze-drying the aqueous solution, as an amorphous solid with $[\alpha]_D^{21}$ −20·3° (2% in H_2O). The product was chromatographically homogeneous and behaved on paper chromatograms in the same way as authentic glutathione. Crystallisation of the amorphous material from aqueous ethanol gave a limited quantity of crystalline glutathione, m. p. 191—192° (decomp.), and the remainder suffered oxidation in solution. The material recovered by precipitation with alcohol had $[\alpha]_D^{25}$ −38°, which corresponds to a mixture of glutathione with its disulphide (20%), and was shown to consist of the γ-glutamyl derivatives by determination of carboxyl nitrogen.[17]

Yields and specific rotations of glutathione obtained by synthesis are shown in the Table. Du Vigneaud and Miller [5] improved Harington and Mead's synthesis [1] by

	Specific rotation	Yield of glutathione (over-all %)	Yield in debenzylation stage (%)
Harington and Mead [1]	$[\alpha]_D^{17}$ −21°	1·7	—
du Vigneaud and Miller [5a]	$[\alpha]_D^{27}$ −21·3°	8—10	27
du Vigneaud and Miller [5b]	$[\alpha]_D^{27}$ −21·3°	10—13	40
Hegedüs [6]	$[\alpha]_D$ −16 ± 1·5°	below 1·5	23
Rudinger and Šorm [7]	Not recorded	3·3	14
Goldschmidt and Jutz [8]	$[\alpha]_D^{17}$ −17·4°	12—13	27
Amiard, Heymès, and Velluz (see footnote, p. 880)	$[\alpha]_D^{27}$ −21·3°	6	—
Present synthesis	$[\alpha]_D^{21}$ −20·3°	4	—

protection of the thiol group as the *S*-benzyl derivative, and syntheses described by later investigators differ only in the methods for preparing the final intermediate, benzyloxycarbonyl-*S*-benzylglutathione (cf., however, footnote, p. 880). Debenzylation of this intermediate by sodium–liquid ammonia reduction was achieved in various yields (Table) by the different authors, and it is not clear whether this is due to variations in technique or in the quality of the benzyloxycarbonyl-*S*-benzylglutathione obtained by the four methods, as the recorded properties [5-8] of this amorphous intermediate differ considerably. The lower rotation recorded by Goldschmidt and Jutz may originate from the phosphorus trichloride coupling procedure which was used, as we have found [13] that in a different solvent (benzene or dioxan instead of pyridine) the method causes racemisation of our intermediates. The new synthesis with thiazolidine intermediates gave glutathione with a satisfactorily high specific rotation and in a yield comparing favourably with four of the alternative methods, and it is probable that the yield could be increased, *e.g.*, by experiments to determine the optimum conditions for the preferential hydrolysis of the ester group in (VII). Hydrolysis of cysteine peptides with alkali is undesirable (cf. Hopkins,[2] 1929) but necessary with the synthesis in its present form, and is mainly responsible for the disappointingly low yield of glutathione from the ester (VII), which can be quickly and easily prepared in *ca.* 40% over-all yield from L-cysteine hydrochloride. It was not found possible to obviate the alkaline hydrolysis by keeping the glycine-carboxyl group free. Acetone was initially chosen to protect the thiol group of L-cysteine because of the ease of

[17] Van Slyke, MacFadyen, and Hamilton, *J. Biol. Chem.*, 1941, **141**, 671.

fission of 2 : 2-dimethylthiazolidines [18] to amino-thiols, but it is clear that other aldehydes and ketones could be used in the same way, and we now consider that the greater stability of 2-phenylthiazolidines may prove an advantage for the preparation of glutathione and other cysteinyl-peptides by this method.

Experimental

L-2 : 2-*Dimethylthiazolidine-4-carboxylic Acid Hydrochloride* (II).—A suspension of powdered L-cysteine hydrochloride (5 g.) in acetone (350 c.c.) was boiled for 30 min., whereupon the amino-acid dissolved and large plates of the thiazolidine hydrochloride separated from the hot acetone. The cold suspension was filtered from the product (6 g., 95%), m. p. 164—165° raised to 168—170° (decomp.) by recrystallisation from acetone (lit.,[11] m. p. 165—168°). On a larger scale (20 g. of L-cysteine hydrochloride) the yield of thiazolidine was 80%.

L-3-*Formyl*-2 : 2-*dimethylthiazolidine-4-carboxylic Acid* (III).—Acetic anhydride (84 c.c.) was added to a suspension of anhydrous sodium formate (13 g.) and L-2 : 2-dimethylthiazolidine-4-carboxylic acid hydrochloride (37·4 g.) in formic acid (90%; 175 c.c.) during 1 hr. while the mixture was kept below 20°. Next day the crystalline product (27 g., 76%), prisms, m. p. 225° (decomp.), was collected and the filtrate, after evaporation under reduced pressure (bath-temp. 40°) and dilution with water, gave further L-3-*formyl*-2 : 2-*dimethylthiazolidine-4-carboxylic acid* (total 32·5 g., 90%), m. p. 225° (decomp.), $[\alpha]_D^{21}$ −181° (2·4% in 0·333N-Na_2CO_3) (Found : C, 44·2; H, 5·7; N, 7·0. $C_7H_{11}O_3NS$ requires C, 44·4; H, 5·8; N, 7·4%). The formyl compound crystallised in plates from water, and gave an anilide,[13] m. p. 189—190°

L-3-*Formyl*-4-N-*methoxycarbonylmethylcarbamoyl*-2 : 2-*dimethylthiazolidine* (IV).—The above formylthiazolidine (31·6 g.) and triethylamine (23·3 c.c., 1 equiv.) were dissolved in dried ($CaCl_2$) chloroform (800 c.c.) and cooled to 5°. *iso*Butyl chloroformate (21·8 c.c., 1 equiv.) was added dropwise during *ca.* 10 min. to the solution which was kept between 0° and 5°. A mixture of glycine methyl ester hydrochloride (21 g., 1 equiv.), triethylamine (23·3 c.c., 1 equiv.), and dry chloroform (*ca.* 200 c.c.) was cooled to 0° and added in portions during *ca.* 5 min. to the stirred and cooled mixed anhydride solution; carbon dioxide was evolved and the temperature rose to 9°. Next day the chloroform solution was washed with water, aqueous sodium hydrogen carbonate, and water, and the chloroform was removed by evaporation under reduced pressure (bath-temp. 30°). The residue was dissolved in ethyl acetate and after the solution had been diluted with light petroleum (b. p. 60—80°) L-3-*formyl*-4-N-*methoxycarbonylmethylcarbamoyl*-2 : 2-*dimethylthiazolidine* (29 g., 67%) crystallised in prisms, m. p. 108—109°, $[\alpha]_D^{20}$ −161·2° (1·5% in $CHCl_3$) (Found : C, 46·1; H, 5·8; N, 10·5. $C_{10}H_{16}O_4N_2S$ requires C, 46·1; H, 6·2; N, 10·8%). In a similar experiment the formylthiazolidine (32 g.) was converted with *n*-butyl chloroformate into the mixed anhydride which gave the above methyl ester (29·8 g., 66%). On a smaller scale (1·9 g. of formylthiazolidine) the yields were 70% with *iso*butyl chloroformate and 30% with ethyl chloroformate. L-3-Formyl-2 : 2-dimethylthiazolidine-4-carboxylic acid was recovered after the addition of an aqueous solution of the sodium salt of glycine to the above mixed anhydride solution.

4-N-*Methoxycarbonylmethylcarbamoyl*-L-2 : 2-*dimethylthiazolidine Hydrochloride* (V).—A solution of the foregoing formyl compound (IV) (29·2 g.) in dried (Mg) methanol (750 c.c.) containing hydrogen chloride (1·5%) was heated to boiling during 5 min., boiled for 5 min., then cooled and evaporated to small volume under reduced pressure (bath-temp. 30°). The residue was dissolved in a small volume of dried (Mg) methanol, and the solution was diluted with dry ether to 500 c.c. 4-N-*Methoxycarbonylmethylcarbamoyl*-L-2 : 2-*dimethylthiazolidine hydrochloride* (30·2 g., *ca.* 100%) separated in needles, m. p. 128° (decomp.), which were collected, washed with ether, and dried in a vacuum-desiccator over sulphuric acid; it had $[\alpha]_D^{20}$ −45·8° (2·4% in MeOH) (Found : C, 40·8; H, 6·4; N, 10·2. $C_9H_{16}O_3N_2S,HCl$ requires C, 40·3; H, 6·3; N, 10·4%). Repetition of the methanolysis gave the hydrochloride (32 g., 89%) from the formyl compound (35 g.).

L-4-N-*Methoxycarbonylmethylcarbamoyl*-2 : 2-*dimethyl*-3-*phthalyl*-γ-L-*glutamylthiazolidine* (VI).—The foregoing thiazolidine hydrochloride (30·2 g.) was dissolved in dried ($CaCl_2$) chloroform (200 c.c.) and cooled to 0° before the addition of triethylamine (15·6 c.c., 1 equiv.) and phthalyl-L-glutamic anhydride [10, 19] (32 g., 1·1 equiv.), $[\alpha]_D^{18}$ −43° (3% in dioxan). Next day the solution

[18] Cook and Heilbron, " Chemistry of Penicillin," Princeton Univ. Press, 1949, Chapter 25.
[19] Clark-Lewis and Fruton, *J. Biol. Chem.*, 1954, **207**, 477.

was filtered from a small amount of unchanged anhydride and the filtrate was washed with 2N-hydrochloric acid and with water, and then extracted with saturated aqueous sodium hydrogen carbonate. The extract was cooled to 0° and acidified with 12N-hydrochloric acid, the precipitated γ-glutamyl derivative was extracted into ethyl acetate, and the ethyl acetate solution was washed with water and dried (Na_2SO_4). Evaporation of the ethyl acetate under reduced pressure (bath-temp. 30°) left a viscous residue (41 g., 74%) of L-4-*N*-methoxycarbonylmethylcarbamoyl-2 : 2-dimethyl-3-phthalyl-γ-L-glutamylthiazolidine, which did not crystallise and gave an amorphous S-*benzylthiuronium salt* (Found: N, 10·6; S, 9·4. $C_{22}H_{25}O_8N_3S,C_8H_{10}N_2S$ requires N, 10·7; S, 9·7%).

A solution of the tripeptide derivative (2 g.) in acetone (25 c.c.) and 4N-hydrochloric acid (25 c.c.) was boiled for 30 min. Evaporation of the solution under reduced pressure to 10 c.c. caused the separation of an oil which solidified after trituration. Crystallisation of the solid from water gave phthalyl-L-glutamic acid [10] in prisms, m. p. and mixed m. p. 158—159°.

Removal of the Protecting Groups from (VI) *and Isolation of Glutathione* (I).—(a) *Removal of the phthalyl group.* (i) The above tripeptide derivative (41 g.) was heated with ethanol (400 c.c.) and aqueous hydrazine hydrate (90%, 10 c.c., 2·2 equiv.) on a steam-bath for 2 hr. and then evaporated to dryness under reduced pressure. The solid residue (A), which was treated as described below under (b), contained phthalhydrazide (14·3 g., *ca.* 100%) and 3-γ-L-glutamyl-L-4-*N*-methoxycarbonylmethylcarbamoyl-2 : 2-dimethylthiazolidine which did not respond to thiol tests with ferric chloride and sodium nitroprusside, and moved as a single ninhydrin positive component with R_F 0·21 in butanol–acetic acid–water (4 : 1 : 5) and R_F *ca.* 1·0 in phenol–water. The procedure for removal of the phthalyl group gave satisfactory and reproducible results and removal was shown to be complete under the conditions described by a parallel experiment in which the phthalyltripeptide derivative (10·4 g.) yielded phthalhydrazide (3·4 g., 100%) after acidification with 2N-sulphuric acid.

(ii) A solution of the phthalyltripeptide (31·2 g.) was treated with hydrazine as described in (i) above and the ethanol solution (300 c.c.) was kept for 14 hr. before filtration from a solid (12·5 g.), apparently a mixture of phthalhydrazide with the phthalhydrazide salt of 3-γ-L-glutamyl-L-4-*N*-methoxycarbonylmethylcarbamoyl-2 : 2-dimethylthiazolidine. The solid (12·5 g.) was suspended in water (40 c.c.) and acidified with N-sulphuric acid, and the filtrate from phthalhydrazide (6 g.) was treated with mercuric sulphate reagent [2,3,5] which precipitated the *mercury derivative* of glutathione monomethyl ester (7·8 g.) (Found: OMe, 3·1; N, 5·2; Hg, 48·0. $C_{11}H_{19}O_7N_3SHg,HgSO_4$ requires OMe, 3·7; N, 5·0; Hg, 48·1%). Evaporation of the ethanol filtrate from the solid (12·5 g.) and treatment of the residue with mercuric sulphate reagent after removal of phthalhydrazide gave further quantities of the mercury derivative (total 30·9 g., 59%).

(iii) A solution of the phthalyltripeptide (2·4 g.) in dioxan (15 c.c.) was treated with 0·5N-sodium hydroxide (20 c.c., 2 equiv.) as described for the hydrolysis of other glutathione esters,[1,5-8] and after 1 hr. at room temperature 40% aqueous hydrazine hydrate (0·63 c.c.) was added and the mixture was set aside for 3 days. The solution was acidified with 2N-hydrochloric acid and evaporated to 15 c.c. under reduced pressure (bath-temp. below 40°) and filtered from phthalhydrazide (0·4 g., 50%) before continuing the evaporation, to obtain a viscous residue. The residue was treated with silver carbonate before development on a paper chromatogram with butanol–acetic acid–water (4 : 1 : 5). The chromatogram was sprayed with ninhydrin and showed spots corresponding to glycine (R_F 0·12), glutamic acid (R_F 0·18), and three other spots with R_F between 0·5 and 0·9 attributed to peptide fragments.

(b) *Alkaline hydrolysis of the methyl ester and isolation of the mercury and copper derivatives of glutathione.* The material (A) obtained from the phthalyltripeptide derivative (41 g.) as described in (a) (i) was treated with saturated aqueous barium hydroxide (500 c.c.), and the solution (pH 10·5) was kept at room temperature for 2 hr. before acidification to pH 2·0 with 10N-sulphuric acid (with cooling) and filtered from phthalhydrazide and barium sulphate. The filtrate, which gave a positive reaction for thiols, was treated with an equal volume of N-sulphuric acid, mercuric sulphate reagent [2,3,5] was added until no further precipitation occurred, and the mercury derivative of glutathione (48 g., 70% calculated from the HgS obtained later) was collected and washed at the centrifuge with water (Found: N, 5·3; Hg, 49·0. Calc. for $C_{10}H_{17}O_7N_3SHg,HgSO_4$: N, 5·1; Hg, 48·9%). The mercury derivative was suspended in water (200 c.c.), and mercury was removed as the sulphide (27·3 g.) by passing hydrogen sulphide under slight positive pressure for 2 hr. through the mechanically shaken suspension. The

supernatant liquid was separated and the sulphide was washed at the centrifuge three times with water (50 c.c.). Hydrogen was passed through the combined aqueous solution (350 c.c.) until the issuing gas was free from hydrogen sulphide and the solution was then adjusted to contain 0·5N-sulphuric acid by the addition of 2N-sulphuric acid. A suspension of freshly prepared cuprous oxide [5] was then added dropwise to the solution at 40—50° until precipitation appeared to be complete and the copper derivative of glutathione [3·4 g., 11% calculated with reference to the phthalyltripeptide (41 g.)], which had a characteristic sheen,[1,2,5] was collected and washed at the centrifuge with water (10 times) until free from sulphate ions. The supernatant liquid remaining after removal of the copper derivative gave with the mercuric sulphate reagent a precipitate of the mercury derivative (15·2 g.) of an unidentified substance which, after treatment with hydrogen sulphide, hydrogen, and cuprous oxide as already described, gave a further yield of the copper derivative (0·5 g., total 3·9 g., 12·5%). The unidentified thiol is probably L-cysteinylglycine, and it was isolated again as the mercury derivative (13·7 g., 90% recovery) by precipitation with the mercuric sulphate reagent.

(c) *Isolation of glutathione.* A suspension of the copper derivative (3·4 g.), obtained as described in (b), was suspended in water (60 c.c.), and hydrogen sulphide under slight positive pressure was passed through the mechanically shaken suspension for 2 hr. The supernatant liquid was separated and the copper sulphide was washed with three quantities of water (20 c.c.) at the centrifuge. The combined aqueous solutions (120 c.c.) were freeze-dried in a conventional apparatus (3 flasks). Glutathione (2·2 g., 78% from the copper derivative) remained as a bulky, white, amorphous solid, decomp. 145—150° with evolution of gas, $[\alpha]_D^{21}$ −20·3° (2% in H_2O) (Found : N, 13·5. Calc. for $C_{10}H_{17}O_6N_3S$: N, 13·7%). The behaviour of the synthetic glutathione on paper chromatograms developed with phenol–water (4 : 1) was identical with that of a commercial (B.D.H.) sample of glutathione. The chromatogram showed a main ninhydrin-positive spot corresponding to glutathione (R_F 0·45) with a faint spot corresponding to glutathione disulphide (R_F 0·095) in both samples, and amino-acids run for comparison were absent, *viz.*, glycine (R_F 0·38), glutamic acid (R_F 0·27), and L-cysteine (R_F 0·81 and R_F 0·27). Crystallisation of the amorphous glutathione (1 g.) from aqueous ethanol [5] gave a small quantity of small prisms, m. p. 190—191° (decomp., gas) alone and when mixed with authentic glutathione, m. p. 192° (decomp.). Material which failed to crystallise from aqueous ethanol after storage at 0° for several weeks was recovered by evaporating the solution over phosphoric oxide in a desiccator filled with hydrogen. The syrupy residue was treated with ethanol, and the amorphous, white solid was collected; it decomposed at *ca.* 130° with evolution of gas. Partial oxidation had occurred in solution as the material was found to be a mixture of glutathione (80%) and glutathione disulphide (20%), the mixture having $[\alpha]_D^{25}$ −38·2° (1·1% in H_2O) (Found : N, 13·3. Calc. for $C_{10}H_{17}O_6N_3S$ and $C_{20}H_{32}O_{12}N_6S_2$: N, 13·7%). Glutamic acid was shown to be combined through the γ-carboxyl group by determination of carboxyl nitrogen [17] with samples (0·011 g.) dissolved in water (2 c.c.) containing citrate buffer (pH, 2·5; 0·1 g.) and ninhydrin (0·1 g.) (Found : Carboxyl N, 4·54, 4·55. Calc. for $C_{10}H_{17}O_6N_3S$ and $C_{20}H_{32}O_{12}N_6S_2$: carboxyl N, 4·56%).

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