# 340. *Polypeptides. Part VI.*[1] *A Further Study of the Oxidation of* L-*Cysteinyl-tetraglycyl-*L-*cysteine.*

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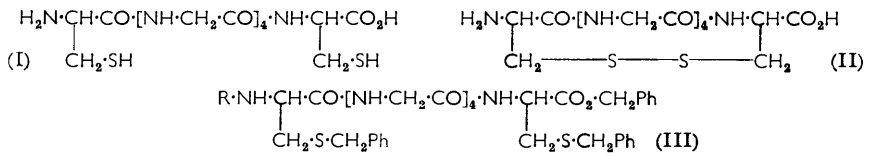
The oxidation of L-cysteinyl-tetraglycyl-L-cysteine (I) in dilute aqueous solution at pH 8·6 has been re-examined and the earlier conclusion [2] that this yields mainly the cyclic monomer, *SS'*-dehydro-L-cysteinyl-tetraglycyl-L-cysteine (II), has been confirmed.

In an earlier paper [2] it was concluded that the principal oxidation product produced by aeration of a dilute aqueous solution of L-cysteinyl-tetraglycyl-L-cysteine (I) at pH 8·6 was the cyclic, monomeric disulphide, *SS'*-dehydro-L-cysteinyl-tetraglycyl-L-cysteine (II). The evidence for this conclusion was somewhat indirect and the main object of the present work was to obtain support for it.



The fully protected hexapeptide (III; R = Ph·$CH_2$·O·CO·) was prepared in considerable quantity. Although the synthetic route was the same as that followed by Hooper *et al.*,[3] the process was considerably improved, giving a much higher overall yield than was obtained by these, and other,[4] workers. The main improvements were the preparation of the intermediates, *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine ethyl ester and *N*-benzyloxycarbonyldiglycyl-*S*-benzyl-L-cysteine benzyl ester, by coupling the appropriate cysteine and glycyl-glycine derivatives by means of bis-*o*-phenylene pyrophosphite [5] and a greatly improved yield in the final coupling of *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine azide with diglycyl-*S*-benzyl-L-cysteine benzyl ester.

In our first experiments, the fully protected hexapeptide (III; R = Ph·$CH_2$·O·CO·), or the hydrobromide of the partially protected peptide (III; R = H) obtained from it by the action of hydrogen bromide in acetic acid,[6] was treated with sodium in liquid ammonia, and the resulting free hexapeptide (I) was oxidised, without isolation, by passing air or oxygen through its 1% aqueous solution at pH 8·6 until the nitroprusside reaction was negative. Paper electrophoresis in N-acetic acid, in the simple apparatus of Durrum,[7] showed the presence of one major, ninhydrin-positive, postively charged component, which was usually accompanied by up to four other similar substances, three moving more slowly (relative migration rates 0·45, 0·6, and 0·85) and one more quickly (relative rate 1·1) than the main product. Dinitrophenylation [8] of the crude oxidation mixture gave, as the main product, a 2,4-dinitrophenyl derivative chromatographically indistinguishable from that prepared earlier.[2] Attempts to isolate the main oxidation product in a state of purity by continuous paper electrophoresis [9] were frustrated, despite the good separation achieved, by poor recoveries of material. More success was achieved by counter-current distribution and a small amount of purified material was obtained by 150 transfers between s-butyl alcohol and 0·5% aqueous trichloroacetic acid. Material so purified still appeared,

[1] Part V, Benoiton and Rydon, *J.*, 1960, 3328.
[2] Heaton, Rydon, and Schofield, *J.*, 1956, 3157.
[3] Hooper, Rydon, Schofield, and Heaton, *J.*, 1956, 3148.
[4] Lautsch and Kraege, *Chem. Ber.*, 1956, **89**, 737.
[5] Crofts, Markes, and Rydon, *J.*, 1959, 3610.
[6] Ben-Ishai and Berger, *J. Org. Chem.*, 1952, **17**, 1564.
[7] Durrum, *J. Amer. Chem. Soc.*, 1950, **72**, 2943.
[8] Sanger, *Biochem. J.*, 1945, **39**, 507; Levy and Chung, *J. Amer. Chem. Soc.*, 1955, **77**, 2899.
[9] Durrum, *J. Amer. Chem. Soc.*, 1951, **73**, 4875.

on paper electrophoresis, to contain traces of two other components (relative migration rates 1·10 and 1·27); these were not removed by a further 100 counter-current transfers and probably arise by a little disulphide interchange during the counter-current distribution or the electrophoresis.

Although the amount of purified material obtained in this way was very small, it was sufficient to enable the correctness of the monomeric cyclic structure (II) to be established in two independent ways. In the first method, the purified oxidation product was treated with a deficiency of 1-fluoro-2,4-dinitrobenzene in aqueous-ethanolic sodium hydrogen carbonate,[8] and the reaction mixture was examined by paper chromatography; apart from unchanged starting material and 2,4-dinitrophenol, only one, yellow, product was detected and it was chromatographically identical with the already mentioned 2,4-dinitrophenyl derivative; no ninhydrin-positive, yellow product, such as would undoubtedly have been produced had the oxidation product contained more than one amino-group,[10] could be detected, and it may safely be concluded that the major oxidation product is the monomeric disulphide (II). This conclusion was supported by cryoscopic determinations of the molecular weight of the oxidation product (Found: $M$, 381 $\pm$ 29. Calc.: $M$, 450) isopiestically in trifluoroacetic acid [11] and of the *N*-2,4-dinitrophenyl derivative (Found: $M$, 534 $\pm$ 7. Calc.: $M$, 617) cryoscopically in phenol. Our work thus confirms the earlier conclusion [2] that the major oxidation product of L-cysteinyl-tetraglycyl-L-cysteine (I) is the monomeric cyclic disulphide (II).

We made numerous attempts to devise a procedure for the preparation of the cyclic disulphide (II) pure and in quantity; although these failed, a number of incidental observations were made which seem worth recording. As with cysteine [12] and cysteinyl-cysteine,[13] the rate of oxidation of L-cysteinyl-tetraglycyl-L-cysteine (I) is markedly affected by pH, being complete at pH 8·6 in 30 minutes but incomplete even after 12 days at pH 4; oxidations at the latter, and lower, pH had, perforce, to be carried out with hydrogen peroxide. The effect of pH on the nature of the oxidation products of (I) is less marked than with cysteinyl-cysteine,[13] although in very strongly acid solution (pH 2) the cyclic monomer (II) was accompanied by another product, present in approximately equal amount, which moved much more slowly on electrophoresis but was not identified. As with cysteine,[14] oxidation of the dithiol (I) is strongly catalysed by metal ions: addition of ferric ions to the oxidation solution considerably reduced the amount of monomeric oxidation product (II) and increased the amount of by-products; conversely, addition of ethylenediaminetetra-acetic acid almost completely repressed the formation of by-products, the monomeric disulphide (II) becoming practically the sole oxidation product, not only at pH 8·6, but also at pH 10 and at pH 4 (oxidation with hydrogen peroxide). It seems that there may be two oxidation processes in operation side-by-side, one metal-catalysed and the other not, the former giving rise to a more complex range of oxidation products than the latter.

## Experimental

*Synthesis of* S-*Benzyl*-N-*benzyloxycarbonyl*-L-*cysteinyl-tetraglycyl*-S-*benzyl*-L-*cysteine Benzyl Ester.*—*S*-Benzyl-L-cysteine [15] had m. p. 214—215°, $[\alpha]_D^{22\cdot5}$ + 29·5° (*c* 1·0 in N-NaOH); care is necessary, when recrystallising the derived benzyl ester toluene-*p*-sulphonate from water, to avoid hydrolysis and consequential lowering of the optical rotation. *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine ethyl ester, prepared in 59—65% yield by the method of

[10] Jarvis, Rydon, and Schofield, following paper.
[11] Schwyzer, Iselin, Rittel, and Sieber, *Helv. Chim. Acta*, 1956, **39**, 872.
[12] Mathews and Walker, *J. Biol. Chem.*, 1909, **6**, 21; Dixon and Tunnicliffe, *Proc. Roy. Soc.*, 1923, *B*, **94**, 266; Benesch and Benesch, *J. Amer. Chem. Soc.*, 1955, **77**, 5877.
[13] Izumiya and Greenstein, *Arch. Biochem. Biophys.*, 1954, **52**, 203; Wade, Winitz, and Greenstein, *J. Amer. Chem. Soc.*, 1956, **78**, 373.
[14] Baumann, *Z. physiol. Chem.*, 1883, **8**, 299; Andreasch, *Monatsh.*, 1885, **6**, 821; Mathews and Walker, *J. Biol. Chem.*, 1909, **6**, 299; Schubert, *J. Amer. Chem. Soc.*, 1932, **54**, 4077.
[15] Wood and du Vigneaud, *J. Biol. Chem.*, 1939, **130**, 109.

Crofts *et al.*,[5] had m. p. 111—112°, $[\alpha]_D^{20}$ −12·2° (*c* 2·2 in EtOH), and was converted into the hydrazide, m. p. 164·5—166°, in 75—87% yield by the method of Hooper *et al.*[3] *N*-Benzyloxycarbonyldiglycyl-*S*-benzyl-L-cysteine benzyl ester, prepared in 75—79% yield by the method of Crofts *et al.*,[5] had m. p. 114—115° or 122—124° (dimorphous), $[\alpha]_D^{22\cdot5}$ −34·6° (*c* 0·5 in EtOH), and was converted into diglycyl-*S*-benzyl-L-cysteine benzyl ester hydrobromide, m. p. 147—149°, in 57—66% yield by treatment with hydrogen bromide in acetic acid.[3,10]

An ethyl acetate solution of the free peptide, obtained by dissolving the last mentioned hydrobromide (17·0 g.) in water (50 ml.) containing triethylamine (5 ml.) and extraction with ethyl acetate (200 ml. and 3 × 100 ml.), was dried over sodium sulphate and cooled to 0°. *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine hydrazide (16·4 g.), in water (10 ml.), acetic acid (140 ml.), and concentrated hydrochloric acid (27·5 ml.), was cooled to 0° and treated with 20% aqueous sodium nitrite solution (17 ml.); the precipitated azide was extracted into cold ethyl acetate (4 × 200 ml.) and the extract washed, at 0°, with saturated sodium hydrogen carbonate solution (2·5 l.) and then dried ($Na_2SO_4$). The two filtered ethyl acetate solutions were mixed at 0° and the mixture was kept overnight at 0°. Next day, the precipitated peptide (27·7 g., 94%), m. p. 190—193°, was collected and washed with a little ethyl acetate. Recrystallisation from aqueous dimethylformamide and then from 90% ethanol gave the protected hexapeptide (20·0 g., 68%), m. p. 206—207°, $[\alpha]_D^{23}$ −34·5° (*c* 1·1 in pyridine) (lit.,[3] m. p. 205—206°, $[\alpha]_D^{20}$ −29·7°). In other experiments the yields varied between 51% and 75%.

This protected peptide (5·0 g.) was dissolved in acetic acid (50 ml.) containing hydrogen bromide (8·1 g.), and the mixture kept at 25° for 35 min., then poured into anhydrous ether (300 ml.) at 0°. The precipitated *S*-benzyl-L-cysteinyl-tetraglycyl-*S*-benzyl-L-cysteine benzyl ester hydrobromide (4·5 g., 95%) was collected by centrifugation and washed repeatedly with dry ether (2 l. in all).

*Oxidation of* L-*Cysteinyl-tetraglycyl-*L-*cysteine.*—Two procedures were used. In method A the sodium was removed, after the reduction, by means of an ion-exchange resin, the final product being substantially free from sodium salts; in method B, resin was not used and the final product was a mixture of the oxidation product and sodium bromide. The details of the two procedures were as follows (all operations involving the free dicysteine peptide were carried out in an inert atmosphere):

*Method* A. *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-tetraglycyl-*S*-benzyl-L-cysteine benzyl ester, or *S*-benzyl-L-cysteinyl-tetraglycyl-L-cysteine benzyl ester hydrobromide, was dissolved in redistilled liquid ammonia (*ca.* 50 ml./g.), and sodium added (either in small pieces or as a solution in liquid ammonia), with stirring, until a permanent blue colour was obtained. Amberlite I.R.-120 resin ($NH_4^+$) was added in excess and the ammonia removed from the product by evaporation, finally under reduced pressure. The residue was treated with boiled-out distilled water, and the resin was filtered off and washed with a little water. After adjustment of the pH to the desired value (with aqueous hydrobromic acid, acetic acid, or carbon dioxide) the solution was diluted with water to bring the concentration of L-cysteinyl-tetraglycyl-L-cysteine to 1%. Oxygen or air was then passed through the solution until the nitroprusside reaction was negative. The pH of the solution was then adjusted to 5·5 and the solution concentrated to small volume under reduced pressure (bath-temp. 30—40°). The residue was treated with an excess of ethanol, and the precipitated solid collected by centrifugation, washed thrice with ethanol and twice with ether, and dried in a vacuum-desiccator.

*Method* B. The partially protected hexapeptide hydrobromide was reduced, by a weighed amount of sodium, as in Method A. When a permanent blue colour was obtained, ammonium bromide equivalent to the sodium used, less two g.-atoms per mole of peptide, was added. The solution was then evaporated and the residue dissolved in water, and oxidised and worked up as in Method A, two equivalents of hydrogen bromide per mole of peptide being added in the last stage.

*Purification of the Oxidation Product by Counter-current Distribution.*—The apparatus was a semi-automatic 50 tube, 25 ml. phase, apparatus (Quickfit and Quartz Ltd.). The solvent system was s-butyl alcohol–0·5% aqueous trichloroacetic acid, equilibrated by mechanical shaking overnight.

The oxidation was carried out, at pH 8·6, by method A. The oxidation product (1·76 g.; containing about 50% of ammonium carbonate) was dissolved in sufficient of the lower phase to bring the volume to 25 ml. and the solution placed in the first tube of the apparatus, lower phase (25 ml.) being placed in the other 49 tubes. Upper phase (25 ml.) was placed in the

first four upper tubes and the distribution process carried out, with a shaking time of 2 min. and a settling time of 2½ min. Additional upper (moving) phase (25 ml.) was added to the first tube after each transfer.

Disulphide-containing material (positive cyanide–nitroprusside reaction) reached tube 50 after 152 transfers. Examination by paper-electrophoresis in N-acetic acid showed that the main product was present in tubes 42—50 (accompanied by very small amounts of material with relative electrophoretic migration rates of 1·30 and 1·10) and in tubes 36—41 (as tubes 42—50, with faint traces of materials with relative electrophoretic migration rates of 0·60 and 0·22).

The contents of the two sets of tubes were evaporated to dryness under reduced pressure; treatment of the residues with ethanol and ether gave the purified oxidation product (142 mg. from tubes 42—50; 308 mg. from tubes 36—41). A further distribution of the material from tubes 42—50 between the same pair of solvents (100 transfers) gave a product (35 mg.) still containing traces of the two faster-moving impurities (relative electrophoretic migration rates, 1·25 and 1·10).

Four determinations of the molecular weight of the twice purified product were carried out by the isopiestic method of Schwyzer *et al.*,[11] with 0·5—1·0% solutions in trifluoroacetic acid and L-cystine as reference substance. The values obtained in successive runs were 457, 349, 390, and 326; mean ± S.E., 381 ± 29.

*Dinitrophenylation of the Oxidation Product.*—The purified oxidation product (90 mg.), in water (7 ml.), containing sodium hydrogen carbonate (100 mg.), was stirred vigorously at room temperature for 3 hr. with 1-fluoro-2,4-dinitrobenzene (0·025 ml.). After acidification, the crude *N*-2,4-dinitrophenyl derivative (61·7 mg., 50%) was collected, washed with a little water, and dried. This derivative was applied, as a band, to Whatman 3 MM filter paper and the chromatogram developed with n-butyl alcohol–pyridine–water (39 : 21 : 39). Apart from some dinitrophenol, the only yellow material had $R_F$ 0·57 and was ninhydrin-negative; this was eluted (from several papers) with 80% acetone and the combined eluates were evaporated under reduced pressure, affording the purified derivative (40 mg.).

Two determinations of the molecular weight of this derivative were carried out cryoscopically in phenol, with *N*-2,4-dinitrophenylglycine as reference substance; the values obtained were 542 and 527.

We thank the Department of Scientific and Industrial Research for a Research Studentship (D. G. L.) and Messrs. Imperial Chemical Industries Ltd. for the loan of apparatus.

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[*Received, November 1st,* 1960.]