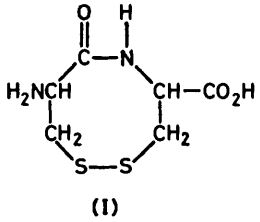
A Study of the Aerial Oxidation of L-Cysteinyl-L-cysteine: Purification of the Product and Equilibrium Relationship involving the Monomeric and Dimeric Cyclic Derivatives

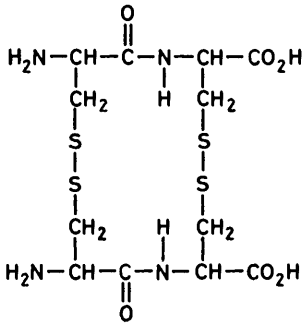
By **Sante Capasso, Carlo Andrea Mattia**, and **Lelio Mazzarella**, Istituto Chimico, Università di Napoli, Via Mezzocannone 4, Napoli, Italy
Raffaella Puliti, Laboratorio per la Chimica di Molecole di Interesse Biologico del C.N.R., Via Toiano 2, Arco Felice-Napoli, Italy

The aerial oxidation of L-cysteinyl-L-cysteine in water at pH 8.5 has been investigated. The reaction products, monomeric cyclic disulphide and parallel and antiparallel cyclic dimers, were fractionated by gel and thin layer chromatography. The relative yields of the products are independent of the type of the catalyst used and strictly obey equilibrium relationships. The equilibrium constant for the reversible isomerization of the two cyclic dimers is of the order of magnitude expected for a random distribution of –S–S– systems at equilibrium (*K ca.* 1), whereas the high values of the equilibrium constants for the dimerization equilibria are in line with the free energy differences expected for a *cis–trans* transformation of two peptide groups.

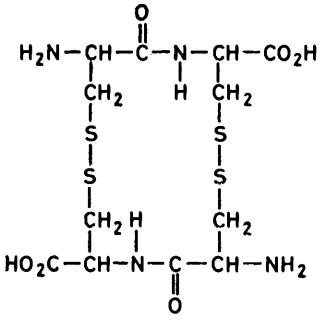
A NUMBER of interesting cyclic disulphides can be obtained by the aerial oxidation of L-cysteinyl-L-cysteine in water. Possible oxidation products are the monomeric cyclic disulphide (I) and the parallel and antiparallel cyclic dimers (II) and (III), as well as higher molecular weight material. The pioneering work of Greenstein *et al.*[1,2] and Heaton *et al.*[3] has demonstrated the presence of (I) in the reaction mixture, but has given contrasting and inconclusive evidence on the presence of the two cyclic dimers (II) and (III).



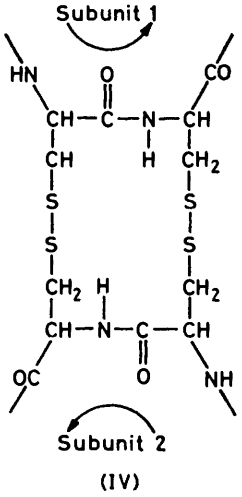
(I)



(II)



(III)



(IV)

The antiparallel dimer (III) is closely related to the sixteen-membered ring compound (IV), identified at the interface of the dimeric protein bovine seminal ribonuclease.[4,5] This enzyme has two identical polypeptide chains covalently linked by two consecutive disulphide bridges through an approximate two-fold axis. Our current interest [6] in the structure of this protein, coupled with the widespread interest on cyclic disulphides, has prompted us to re-examine the preparation of these compounds, with the main purpose of studying the isolation of the cyclic dimers from the reaction mixture and the factors governing their relative yields. Conformational studies in solution and in the solid state of the eight-membered ring compound (I) have already been reported.[7,8] In this paper we show that in basic medium (pH 8.5) the relative yields of the monomeric and dimeric compounds strictly obey equilibrium relationships, and the equilibrium state is reached *via* a fast thiol–disulphide exchange mechanism.[9]

The equilibrium yields of products are in contrast with the results of Sisido *et al.*[10] on the aerial oxidation of terminal thiol groups on polysarcosine chains. In this case no thiol–disulphides exchange was observed, while the relative yields of the products was highly dependent on the catalyst used.

RESULTS AND DISCUSSION

L-Cysteinyl-L-cysteine was prepared from (*N*-benzoyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*S*-benzyl-L-cysteine by removing the protecting groups with sodium in liquid ammonia. After purification the free dipeptide was oxidized by bubbling air through an aqueous solution at pH 8.5 in a thermostat kept at 23 °C. The reaction was allowed to proceed up to the complete disappearance of the thiol groups. Several preparations were performed by varying the peptide concentration (0.3—3.0%) in the presence of Fe^{3+}, Cu^{2+}, and EDTA. In all cases analysis by t.l.c. of the crude product shows at

least five ninhydrin-positive components (Table 1). The isolation of various compounds was carried out by gel-permeation chromatography on Sephadex G-15 as in the typical chromatogram shown in Figure 1.

TABLE 1

R_F of components of air-oxidized mixture from L-cysteinyl-L-cysteine on silica gel in phenol–water mixture

Component	R_F
A	0.35
B	0.25
C	0.23
D	0.18
E	0—0.1

The well resolved component A was identified as the eight-membered ring compound (I) and fully characterized by *X*-ray [7] and n.m.r. studies.[8] Components B and C were eluted together even on Sephadex G-10 and G-25 (superfine), indicating a similar molecular weight for the two compounds. Their separation was finally carried out by t.l.c. of the fraction B + C isolated by gel-permeation chromatography. Chemical assay by sodium cyanide–sodium nitroprusside and the presence of a shoulder at 250 nm in the u.v. spectrum clearly indicate the presence of an –S–S– bridge in both compounds. Hydrolysis in 6N-HCl at 120 °C for 24 h yielded predominantly L-cystine, and t.l.c. fingerprints of the hydrolysis products were indistinguishable from that of pure L-cystine treated under the same conditions. These results, together with the molecular weight obtained by the isopiestic method [11] (431 ± 40 for B and 408 ± 40 for C) clearly identify B and C as the two sixteen-membered ring compounds (II) and (III). The

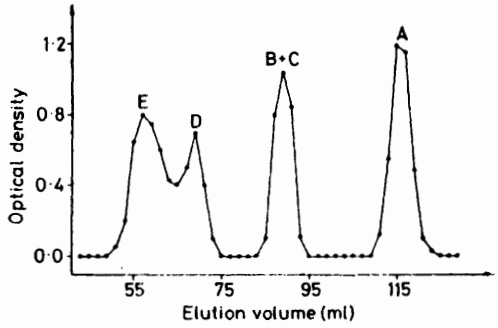


FIGURE 1 Elution diagram from Sephadex G–15 column chromatography of air-oxidized products. Column size 190 × 1.4 cm; samples eluted with 0.2M ammonium hydrogencarbonate. The optical density was determined at 250 nm. Components A—E were defined in Table 1

equilibrium analysis of the reaction mixture (see below) is also in agreement with this result. It is not yet established, however, which of the two components is the parallel and which is the antiparallel peptide.

Component D, which was not completely separated from the higher molecular weight product E, is most probably cyclic trimer as shown by the linear relationship between the elution volume for components A, B + C, and D on Sephadex G-15 and G-25 and the logarithms of the expected molecular weight.

It is well known that the presence of metal ions strongly influences the air-oxidation rate of thiols by forming cysteine–metal ion complexes.[12] The addition of Cu^{2+} or Fe^{3+} to a solution at pH 8.5 of cysteinylcysteine produces the immediate appearance of a yellow colour for Cu^{2+} and purple for Fe^{3+}, which remain unchanged during the oxidation, turning to blue and yellow, respectively, at the end of the reaction. It is thus possible to use the ions Cu^{2+} and Fe^{3+} as internal indicators.

In order to test the dependence of the relative yields of products on the type and amount of the metal ions, several experiments were carried out by adding copper(II) and iron(III) ions and EDTA. As expected the time required for the complete disappearance of thiol groups is strongly dependent on the presence of the catalyst used, ranging from a few hours to several days in the presence

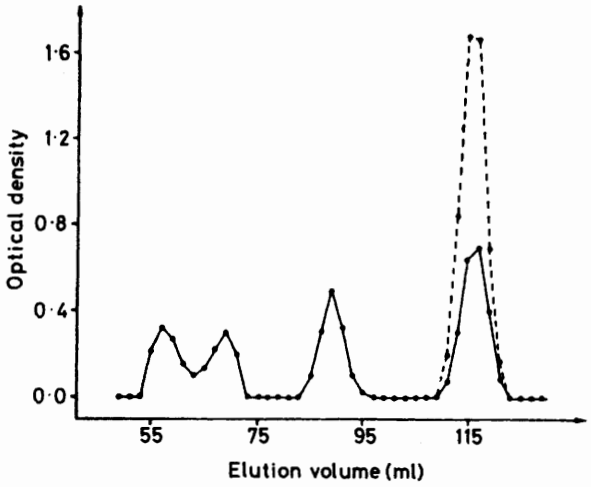


FIGURE 2 Elution diagram of the purified cyclic monomer (dotted line) and after the addition of 1% cysteinylcysteine (solid line)

of EDTA. The relative yields of the products, however, depend only on the concentration of the reactants and not on the type and the amount of the catalyst used.

The above results can be explained in terms of thiol–disulphide exchange reaction.[9] Such a mechanism was tested using the purified cyclic monomer and dimers, which remain stable in solution at pH 8.5 for several days. The addition of a catalytic amount of cysteinylcysteine, in the absence of oxygen, produces in a few minutes all the compounds found in the air-oxidation of the dithiol. Figure 2 shows the chromatogram of the purified cyclic monomer and that obtained two hours after the addition of cysteinylcysteine.

In all cases examined the mole fractions of the three compounds A—C (Table 2) closely follow the equilibrium relationships (1)—(3). Figure 3 shows a correlation plot

$$K_{C\rightarrow B} = x_B/x_C \quad (1)$$

$$K_{A\rightarrow B} = x_B/(x_A)^2 \quad (2)$$

$$K_{A\rightarrow C} = x_C/(x_A)^2 \quad (3)$$

between the mole fractions of B and C. The experimental data clearly indicate that the ratio $K_{C\rightarrow B}$ between the mole fractions of the two compounds is

constant. The slope of the straight line $x_B = K_{C\rightarrow B}x_C$, computed by least-squares, is 1.76 ± 0.04. The average deviation of the experimental points is <8%. This value is reasonably small and ensures the reliability of our experimental procedure.

Simple equilibrium relationships are also satisfied by

TABLE 2

Experimental mole fractions (× 10^5) of A—C in reaction mixtures. Symbols as described in Figure 3

x_A	x_B	x_C	Symbol
8.91	1.64	0.91	□
11.01	2.42	1.27	○
11.83	2.74	1.72	○
14.69	3.58	2.39	○
16.96	5.60	3.73	△
17.20	5.27	2.77	□
17.21	6.30	3.71	○
20.91	8.63	4.79	□
23.36	10.48	6.16	○
23.78	10.09	6.30	○
24.02	11.35	6.30	□
24.31	11.39	5.99	○
26.60	13.44	7.91	□
28.62	17.01	8.96	○

the mole fractions of A and B or A and C. The constancy of the ratio x_B/x_C allows one to plot log x_A as a function of the logarithm of the sum $x_B + x_C$ which can be determined more accurately than the individual

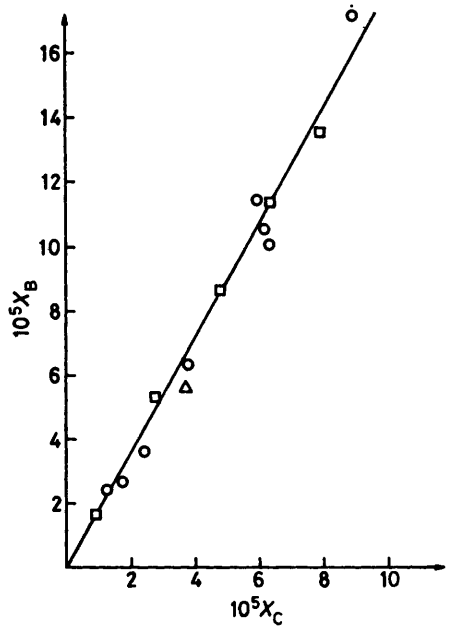


FIGURE 3 Relationship between mole fractions of B and C of mixtures obtained either by direct oxidation of cysteinylcysteine (○), or by adding a catalytic amount of cysteinylcysteine to purified samples of the cyclic monomer (□) and dimer (△)

values of x_B and x_C. The logarithmic plot shown in Figure 4 is a straight line with slope 1.97 ± 0.05. This agrees very well with the theoretical value of 2 expected for the dimerization processes $2A \rightleftarrows B$ and $2A \rightleftarrows C$. Thus the equilibrium data indirectly confirm the identification of B and C as the two dimers (II) and (III).

A least-squares fit of the data, using the theoretical value of 2 for the slope, gives the relationship (4) which allows the values $K_{A\rightarrow B}$ $10^3(1.95 \pm 0.04)$ and $K_{A\rightarrow C}$ $10^3(1.11 \pm 0.02)$ to be obtained where the relation $K_{C\rightarrow B} = K_{A\rightarrow B}/K_{A\rightarrow C} = 1.76 \pm 0.04$ is used.

$$\log(x_B + x_C) - 2\log x_A = \log(K_{A\rightarrow B} + K_{A\rightarrow C}) = 3.485 \pm 0.007 \quad (4)$$

The numerical value of the equilibrium constant $K_{C\rightarrow B}$ is in agreement with a nearly random distri-

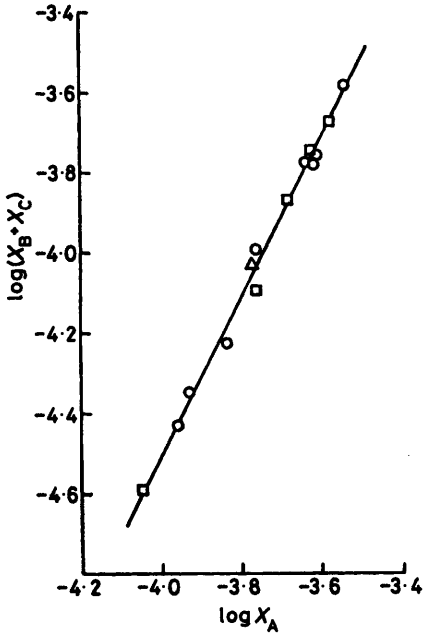


FIGURE 4 Relationship between mole fractions of A and B + C in reaction mixtures. Symbols as described in Figure 3

bution of the S–S group at equilibrium (*K ca.* 1), as found in several cases for disulphide–disulphide exchange reactions.[13] On the other hand the rather high free-energy difference involved in the dimerization equilibria, $\Delta G_{A\rightarrow B}$ −18.6 and $\Delta G_{A\rightarrow C}$ −17.2 kJ mol^{-1}, may be explained by a dominant enthalpy contribution associated with the *cis–trans* transformation of the two peptide groups, which according to the literature is of the order of *ca.* 8.4 kJ/peptide unit.[14] Indeed, ring closure requires a *cis*-peptide to be present in the cyclic monomer (I), as confirmed by the solid-state structure,[7] whereas the larger cyclic dimer can have both peptide units in the *trans*-conformation. This possibility is strongly supported by the low resolution *X*-ray structure [6] of bovine seminal ribonuclease which include the antiparallel ring (IV) at the interface between the two sub-units as part of two α-helices.

In this connection it is of interest to study the solid-state structure and the conformational flexibility in solution of (III), in order to gain useful information on the role of the double interchain disulphide in this interesting dimeric protein.

EXPERIMENTAL

Synthesis and Oxidation of L-*Cysteinyl*-L-*cysteine*.—(*N*-Benzoyloxycarbonyl-*S*-benzyl-L-cysteinyl)-L-cysteine [1] (1.5 g) in liquid ammonia (50 cm^3) was treated with sodium until the blue colour was permanent. Ammonium chloride (1.5 g) was then added and ammonia allowed to evaporate under nitrogen. The solid residue was extracted with cold 0.7N-sulphuric acid (45 cm^3) and treated with an acidic solution of mercury(II) sulphate [15] (10 cm^3). A copious precipitate was collected by centrifugation, and washed with oxygen-free water. A brisk stream of hydrogen sulphide was passed through a suspension in water of the mercury derivative, and, after removal of mercury(II) sulphide by filtration, the L-cysteinyl-L-cysteine solution was freed from excess of hydrogen sulphide by evaporation *in vacuo*. The clear solution was diluted with water or concentrated by evaporation to the desired volume, after which the pH was brought to 8.5 with saturated barium hydroxide solution, and the precipitated barium sulphate removed. Different concentrations (0.3—3%) of L-cysteinyl-L-cysteine at pH 8.5 were prepared. When it was desired a small amount (10^{-4}M) of iron(III) or copper(II) chloride or EDTA was added to the solution and the pH again checked. Oxidative cyclization was accomplished, in a thermostatted bath at 23.0 °C, by a stream of air, previously passed through a water bubbler, until a positive reaction with sodium nitroprusside was no longer evident.

During the air-oxidation experiments the pH did not change appreciably. At the end of the reaction portions of the solution were chromatographed in phenol–water (77%) on silica gel thin-layer plates, thickness 0.25 mm (Merck). The spots were revealed with ninhydrin or cyanide plus nitroprusside. The R_F values are given in Table 1.

Separation of the Oxidation Products by Chromatography.—Gel-permeation chromatography of the oxidation products was carried out on a column of G-15 Sephadex (190 × 1.4 cm) in 0.2M-ammonium hydrogencarbonate solution at a flow rate of 7 ml h^{-1}. The optical density of each fraction (2 ml) was determined at 220 and 250 nm. A typical chromatogram is shown in Figure 1. The amounts of A and B + C in each sample were determined using $E^{1\%}_{250}$ 29.41 for A and $E^{1\%}_{250}$ 24.15 for B + C in ammonium hydrogencarbonate. These values were determined on solutions prepared by weight from pure samples of A and B + C, previously dried on P_2O_5 at 56 °C *in vacuo*. The ratio between B and C was measured by visual comparison of the intensity of the t.l.c. spots with a series of standard spots of known concentration.

The two sixteen-membered ring compounds B and C were obtained pure by preparative t.l.c. on silica gel (20 × 20 cm × 0.25 mm; Merck) in phenol–water. The molecular weights were determined by the isopiestic method [11] in trifluoroacetic acid using L-cystine as the reference at a concentration of 0.7—1.2%. Repeated experiments indicated that the errors involved are not larger than 10%.

Disulphide Interchange.—The pH of an aqueous solution of isolated peptide was adjusted to 8.5 by barium hydroxide and nitrogen was bubbled through the solution for 1 h. After two days no by-products were detected by t.l.c. and gel permeation chromatography. Cysteinylcysteine (1% of oxidized product) was added under nitrogen and the pH again checked. The mixtures were kept at 23 °C until a constant composition was obtained (*ca.* 2 h). Afterward the free dithiol was eliminated by air oxidation and the mixtures were analysed as described.

We thank Drs. Sonia Gatta and Nella Giusto for assistance with the experimental work.

[9/1107 *Received*, 16*th July*, 1979]

REFERENCES

[1] N. Izumiya and J. P. Greenstein, *Arch. Biochem. Biophys.*, 1954, **52**, 203.
[2] R. Wade, M. Winitz, and J. P. Greenstein, *J. Amer. Chem. Soc.*, 1956, **78**, 373.
[3] G. S. Heaton, H. N. Rydon, and J. A. Schofield, *J. Chem. Soc.*, 1956, 3157.
[4] S. Capasso, F. Giordano, L. Mazzarella, and A. Ripamonti, *J. Mol. Biol.*, 1972, **64**, 311.
[5] G. D'Alessio, A. Floridi, R. De Prisco, A. Pignaro, and E. Leone, *Europ. J. Biochem.*, 1972, **26**, 153.
[6] S. Capasso, F. Giordano, C. A. Mattia, L. Mazzarella, and A. Zagari, *Gazzetta*, 1979, **109**, 55.
[7] S. Capasso, C. Mattia, L. Mazzarella, and R. Puliti, *Acta Cryst.*, 1977, **B33**, 2088.
[8] S. Capasso, L. Mazzarella, and T. Tancredi, *Biopolymers*, 1979, **18**, 1555.
[9] P. C. Jocelyn, ' Biochemistry of the SH Group,' Academic Press, London–New York, 1972, p. 121.
[10] M. Sisido, F. Tamura, Y. Imanishi, and T. Higashimura, *Biopolymers*, 1977, **16**, 2723.
[11] Von R. Schwyzer, B. Iselin, W. Rittel, and B. Sieber, *Helv. Chim. Acta*, 1956, **39**, 872.
[12] P. C. Jocelyn, ' Biochemistry of the SH Group,' Academic Press, London–New York, 1972, p. 95.
[13] H. A. Smith, G. Doughty, and G. Gorin, *J. Org. Chem.*, 1964, **29**, 1484; G. Gorin and G. Doughty, *Arch. Biochem. Biophys.*, 1968, **126**, 547.
[14] G. N. Ramachandran and V. Sasikharan, *Adv. Protein Chem.*, 1968, **23**, 283.
[15] E. C. Kendall, B. F. McKenzie, and H. L. Mason, *J. Biol. Chem.*, 1929, **84**, 657.