607. *Polypeptides. Part III.* The Oxidation of Some Peptides of Cysteine and Glycine.†*

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The oxidation, in aqueous solution at pH 8·5, of a series of L-cysteinyl-polyglycyl-L-cysteines, in which the number of glycine residues varies from 0 to 4, has been studied. The proportion of cyclic monomer (III) has been found to be maximal when four glycine residues separate the two cysteine residues. All the cyclic polymers isolated have been found to have anti-parallel structures, as (V), while appreciable amounts of linear high polymers have not been encountered. The significance of these results is briefly discussed, with special reference to the chemistry of insulin and oxytocin.

It is generally agreed that disulphide bridges between pairs of cysteine residues are the most important covalent cross-linkages found in protein molecules, serving both to bind together pairs of peptide chains and to form loops in single peptide chains; thus, in insulin [1] the two peptide chains are held together by two disulphide linkages while a third such linkage, between two cysteine residues separated by four other amino-acid residues, forms a loop in one of the peptide chains. Such linkages are, furthermore, generally held [2] to play an important part in the phenomena of denaturation and gelation which are so typical of native globular proteins. Holding, with Edsall,[3] that the next phase in the development of our understanding of protein chemistry requires an intensive study of the chemistry of model synthetic polypeptides, we have instituted research on the disulphide cross-linking of selected peptides containing cysteine residues. The controlled establishment of disulphide cross-linkages is one of the major unsolved problems which must be overcome if proteins are ever to be synthesised; we hope that our work may also be of assistance in this connexion.

The present paper, which presents the first results of this programme, describes a preliminary study of the oxidation of five peptides (I; $n = 0$—4), in which two cysteine residues are separated from each other by various numbers of glycine residues. When our work was begun, in 1950, the only studies of this kind reported in the literature were those of Greenstein [4,5] on the oxidation of cysteinylcysteine (I; $n = 0$); recently Greenstein and his colleagues [6,7] have reported further studies of this problem.

The fully protected peptides (II; R = Ph·CH_2·O·CO, R′ = Ph·CH_2), the preparation of which is described in the preceding paper,[8] were converted into the free peptides (I) either directly, by treatment with sodium in liquid ammonia,[9] or, better, in two stages by first removing the *N*-benzyloxycarbonyl and *O*-benzyl groups by treatment with hydrogen bromide in acetic acid [10] and then treating the resulting *S*-benzylcysteine peptides (II; R = R′ = H) with sodium in liquid ammonia. The free peptides (I) were not, in general,

* Part II, preceding paper.

† An account of this work was given at the Symposium on Peptide Chemistry held in London on March 30th, 1955 (see *Chem. Soc. Special Publ.* No. 2 (1955), p. 66).

[1] Ryle, Sanger, Smith, and Kitai, *Biochem. J.*, 1955, **60**, 541.

[2] Neurath, Greenstein, Putnam, and Erickson, *Chem. Rev.*, 1944, **34**, 157; Ferry and Morrison, *J. Amer. Chem. Soc.*, 1947, **69**, 388; Huggins, Tapley, and Jensen, *Nature*, 1951, **167**, 592; Frensdorff, Watson, and Kauzmann, *J. Amer. Chem. Soc.*, 1953, **75**, 5157, 5167; Halwer, *ibid.*, 1954, **76**, 183; Hospelhorn, Cross, and Jensen, *ibid.*, p. 2827; Hospelhorn and Jensen, *ibid.*, p. 2830.

[3] Edsall, *Proc. Roy. Soc.*, 1953, *B*, **141**, 97.

[4] Greenstein, *J. Biol. Chem.*, 1937, **118**, 321.

[5] *Idem, ibid.*, 1937, **121**, 9.

[6] Izumiya and Greenstein, *Arch. Biochem. Biophys.*, 1954, **52**, 203.

[7] Otey and Greenstein, *ibid.*, 1954, **53**, 501.

[8] Hooper, Rydon, Schofield, and Heaton, preceding paper.

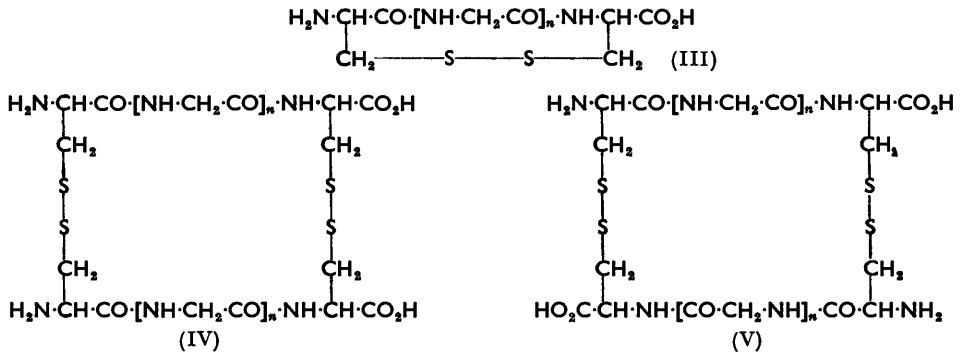
[9] Sifferd and Du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753.

[10] Ben-Ishai and Berger, *J. Org. Chem.*, 1952, **17**, 1564; Ben-Ishai, *ibid.*, 1954, **19**, 62.

isolated but were directly oxidised by bubbling air through 1% or 0·1% solutions at pH 8·5; we have not detected any marked effect of variation of the peptide concentration within these limits.

Possible oxidation products are the monomeric cyclic disulphides (III), the parallel (IV) and the anti-parallel (V) cyclic dimers, analogous higher cyclic polymers, and acyclic polymers. In theory, these possibilities are best distinguished from one another by introduction of an *N*-substituent into the free amino-group or groups, followed by hydrolysis; on such treatment the parallel cyclic polymers, *e.g.*, (IV), should yield one mol. of cystine and one of the *NN'*-disubstituted cystine, whereas the monomers (III) and the antiparallel cyclic polymers, *e.g.*, (V), should yield only the *N*-monosubstituted cystine. In practice, the issue is complicated by the occurrence of disulphide interchange reactions [11] which result in the conversion of *N*-monosubstituted cystines into equimolecular mixtures of cystine and *NN'*-disubstituted cystines.

This interchange was forcibly brought to our notice when we found, by paper electrophoresis, that the action of hydrochloric acid on the chromatographically pure bis-*N*-phenylcarbamoyl derivative of the oxidation product of L-cysteinylglycyl-L-cysteine gave, in addition to glycine, both cystine, corresponding to the dimer (IV; $n = 1$), and cystine



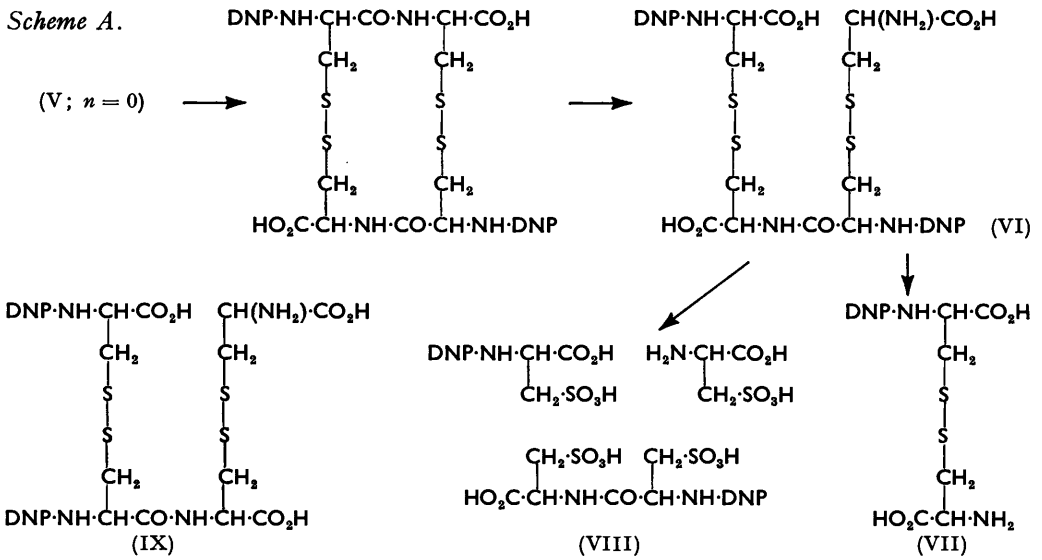
monophenylhydantoin, corresponding to the isomer (V; $n = 1$). Attempts to devise conditions under which *N*-phenylcarbamoyl derivatives could be cyclised without concomitant interchange having failed, we turned our attention to *N*-2 : 4-dinitrophenyl derivatives, since Ryle and Sanger [12] had reported success with these. Although the conditions employed by Sanger and his colleagues [1, 11] for the partial hydrolysis of insulin were unsuitable for our purpose, we found conditions under which the disulphide interchange was slow relatively to the hydrolysis of the peptide linkages in our peptides (I). Under these conditions unsymmetrical *N*-2 : 4-dinitrophenyl derivatives of cystine and cystine–glycine peptides could be recognised on paper chromatograms with confidence, appearing in the early stages of hydrolysis as single, yellow, ninhydrin-positive spots which were replaced, as the hydrolysis proceeded, by two spots, one yellow and ninhydrin-negative (the *NN'*-bis-2 : 4-dinitrophenyl derivative) and the other colourless and ninhydrin-positive (the *N*-unsubstituted compound).

Examination of the oxidation product of L-cysteinyl-L-cysteine (I; $n = 0$) by paper chromatography and paper electrophoresis showed the presence of at least four components. Direct conversion of the oxidation product into its *N*-2 : 4-dinitrophenyl derivative, by Levy and Chung's method,[13] gave, in 36% yield, a product shown by paper chromatography to be a mixture of five components. The major component (52% of the total) was subjected to progressive hydrolysis (see Fig. 4, p. 3165); the first hydrolysis product was a yellow, ninhydrin-positive material (VI), which passed, on further hydrolysis, first into monodinitrophenylcystine (VII) and then, by disulphide interchange, into bisdinitrophenylcystine and cystine. The formation of an intermediate hydrolysis product as

[11] Sanger, *Nature*, 1953, **171**, 1025; Ryle and Sanger, *Biochem. J.*, 1955, **60**, 535.
[12] Ryle and Sanger, *Biochem. J.*, 1954, **58**, v.
[13] Levy and Chung, *J. Amer. Chem. Soc.*, 1955, **77**, 2899.

a precursor of monodinitrophenylcystine rules out the monomeric structure (III; $n = 0$) for the oxidation product, since this would necessarily yield monodinitrophenylcystine directly. Oxidation of the intermediate (VI) by performic acid gave cysteic acid, *N*-2 : 4-dinitrophenylcysteic acid, and a third, yellow, ninhydrin-negative product which was shown to be the acid (VIII) by hydrolysis to dinitrophenylcysteic acid and cysteic acid. These findings are consistent only with the formulation of the oxidation product as the antiparallel dimer (V; $n = 0$), the various reactions being those set out in scheme A. Had the oxidation product had the alternative parallel structure (IV; $n = 0$), the initial hydrolysis product would have been (IX) rather than (VI) and would have given, on further hydrolysis, bisdinitrophenylcystine and cystine directly and not by way of monodinitrophenylcystine. It may therefore be concluded that this oxidation product, which was however isolated in only 20% yield, is the antiparallel dimer (V; $n = 0$). The nature of the other oxidation products was not further investigated in view of the renewed interest of Greenstein and his colleagues [6, 7, 14] in this problem; the chromatographically slower-moving dinitrophenyl derivatives are clearly those of higher cyclic polymers.

Scheme A.



Here and elsewhere DNP = 2 : 4-$(NO_2)_2C_6H_3$.

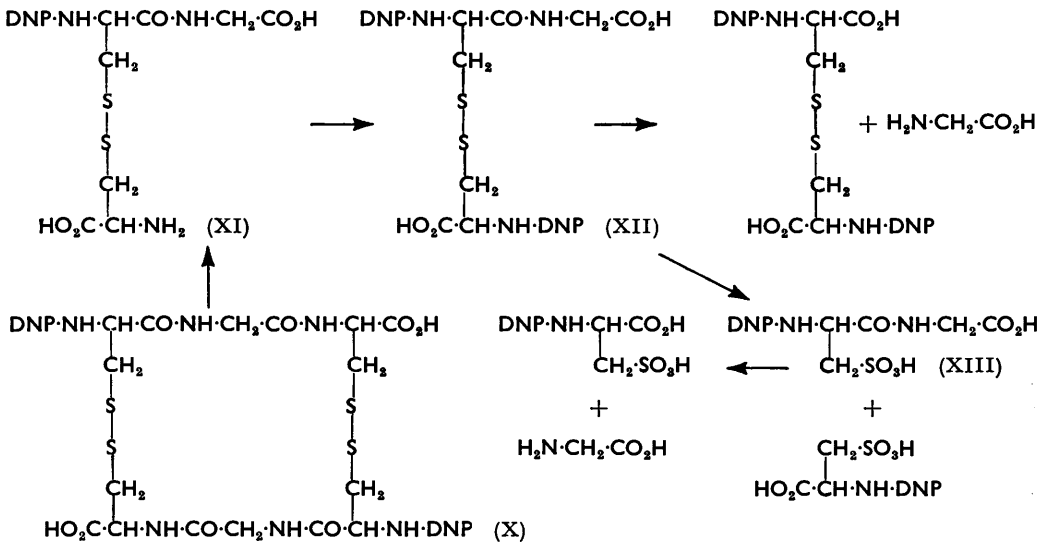
The antiparallel structure (V; $n = 0$) was originally assigned by Greenstein [5] to the oxidation product of L-cysteine-L-cysteine but without supporting evidence. Izumiya and Greenstein [6] assigned the parallel structure (IV; $n = 0$) to crystalline oxidation products obtained, in 25—30% yield, from D-cysteinyl-L-cysteine and L-cysteinyl-D-cysteine, on the basis of their hydrolysis to DL- rather than to *meso*-cystine. The same authors obtained two crystalline oxidation products (25—30% yield) from L-cysteinyl-L-cysteine and suggested that these were the parallel and antiparallel isomerides (IV and V; $n = 0$); Dr. Greenstein had kindly sent us an advance summary of a recent paper [14] in which one of these products is shown to be the cyclic monomer (III; $n = 0$) and the other a cyclic dimer, the precise nature of which is not conclusively established.*

* [*Added, March 14th*, 1956.] This paper has now been published in full.[14] The dimeric material is considered to be largely the parallel compound (IV; $n = 0$). We do not, however, find the evidence for this completely convincing; in particular, the apparent absence of disulphide interchange during the hydrolysis of the dinitrophenyl derivative is very surprising. It is possible that the two groups were dealing with different products, or with different components of the same mixture, and it is perhaps significant that colour changes, ascribed to the presence of traces of heavy metals, were observed by Dr. Greenstein and his colleagues during the oxidation of cysteinylcysteine; no such changes were observed in any of our oxidations, the solutions being initially colourless and remaining so throughout; heavy-metal catalysis might well have a profound effect on the course of the oxidation.

[14] Wade, Winitz, and Greenstein, *J. Amer. Chem. Soc.*, 1956, **78**, 373.

Paper electrophoresis of the oxidation product of L-cysteinylglycyl-L-cysteine (I; $n = 1$) showed the presence of three products, one of which preponderated and was isolated, electrophoretically pure, in 80% yield. The crude 2 : 4-dinitrophenyl derivative, prepared directly from the oxidation product, was a mixture of two components in the molar ratio 3 : 1. The more abundant, chromatographically faster-moving component was subjected to progressive hydrolysis (see Fig. 5, p. 3165); the initial hydrolysis product was a yellow, ninhydrin-positive material which passed, on further hydrolysis, first into monodinitrophenylcystine and glycine and then into bisdinitrophenylcystine, cystine, and glycine. The initial hydrolysis product, isolated in 80% yield after 15 minutes' hydrolysis, was shown to be *N*-mono-2 : 4-dinitrophenyl-L-cystinyl-glycine * (XI) by the transformations in Scheme B. The initial hydrolysis product (XI) was first converted into the bisdinitrophenyl derivative (XII) which, on hydrolysis, gave bisdinitrophenylcystine and glycine, and, on performic acid oxidation, dinitrophenylcysteic acid and a yellow, ninhydrin-negative product shown to be the cysteic acid derivative (XIII) by hydrolysis to dinitrophenylcysteic acid and glycine. The dinitrophenyl derivative (XI) can only arise by partial hydrolysis of the dinitrophenyl derivative of the cyclic monomer (III; $n = 1$), the antiparallel cyclic dimer (V; $n = 1$), or higher antiparallel cyclic polymers. The dimeric nature of the dinitrophenyl derivative (X) was established by partial esterification with a deficiency of diazomethane which afforded, in addition to unchanged material, two esters, *viz.*, the mono- and the di-methyl ester of (X); a monomer could only give one ester while a higher polymer would give more than two. It is therefore concluded that the major oxidation product of L-cysteinylglycyl-L-cysteine (I; $n = 1$) is the antiparallel cyclic dimer (V; $n = 1$). Owing to lack of material the nature of the chromatographically slower-moving dinitrophenyl derivative was not investigated but it is believed, on the basis of its R_F value, to be derived from a cyclic trimeric oxidation product.

Scheme B.



Paper chromatography showed the 2 : 4-dinitrophenyl derivative of the oxidation product of L-cysteinyldiglycyl-L-cysteine (I; $n = 2$) to be a mixture of four components. The three chromatographically faster-moving of these were purified and subjected to brief hydrolysis; each gave as the main product the same dinitrophenylcystinyl glycine (XI) as was obtained similarly from the oxidation product of L-cysteinylglycyl-L-cysteine. These products must, therefore, all have cyclic monomeric or antiparallel cyclic polymeric

* Here and elsewhere hyphens are inserted into names where it is believed that it will help to elucidate the positions of substituents.

structures; unfortunately, they could not be esterified with diazomethane and it has been necessary to deduce their molecular complexity from their R_F values.

The R_F values, in butanol-1-ol–pyridine–water (2 : 1 : 2), of the *N*-2 : 4-dinitrophenyl derivatives of the oxidation products of the peptides (I) are plotted against the number of glycine residues (n) in Fig. 1. It has been shown above that the fastest-moving products when $n = 0$ and 1 are dimers and the finding that the dinitrophenyl derivatives of the other oxidation products of L-cysteinyl-L-cysteine move more slowly on chromatograms than that of the dimer indicates clearly that the R_F value decreases with increasing molecular complexity, as would be expected from the work of Pardee.[15] The line in Fig. 1 indicates the most probable relationship between the R_F value and the value of n for antiparallel dimers; points falling above this line are taken to correspond to monomers and those falling below it to trimers and higher polymers. On this basis we assign to the three faster-moving dinitrophenyl derivatives from L-cysteinyldiglycyl-L-cysteine structures

FIG. 1. *R_F values for dinitrophenyl derivatives of the oxidation products of L-cysteinylpolyglycyl-L-cysteines* (I).

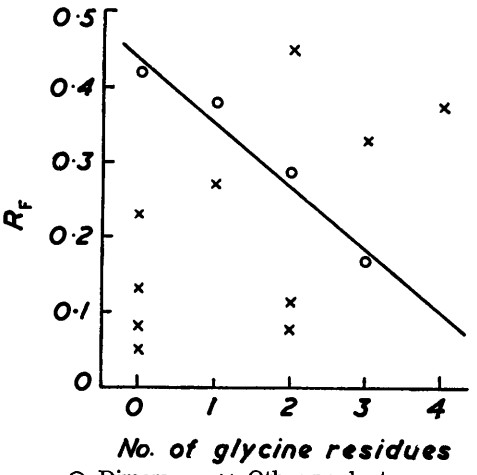
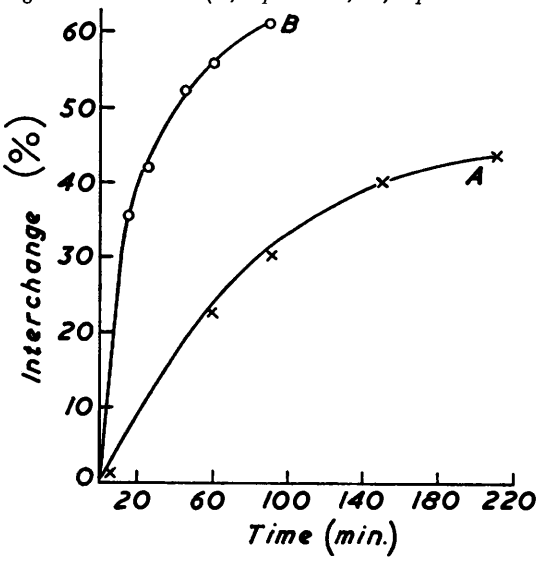


FIG. 2. *Curves showing the rate of formation of mono-N-2 : 4-dinitrophenyl-L-cystine from L-cystine and bis-N-2 : 4-dinitrophenyl-L-cystine under the conditions given in Table 2.* (*A*, *expt. no.* 9; *B*, *expt. no.* 10.



corresponding to the cyclic monomer (III; $n = 2$), the antiparallel dimer (V; $n = 2$), and the corresponding antiparallel trimer; the very slow-moving derivative no doubt arises from a higher polymer.

Paper chromatography showed the *N*-2 : 4-dinitrophenyl derivative of the oxidation product of L-cysteinyltriglycyl-L-cysteine (I; $n = 3$), isolated in 90% yield, to be a mixture of only two components. These were isolated chromatographically and, on brief hydrolysis, gave the dinitrophenylcystinyl-glycine (XI), the isolation of which excludes all parallel cyclic polymers. Attempted partial esterification was again unsuccessful, but, on the basis of the R_F values of the dinitrophenyl derivatives (Fig. 1), the two oxidation products of L-cysteinyltriglycyl-L-cysteine are regarded as the cyclic monomer (III; $n = 3$) and the antiparallel dimer (V; $n = 3$).

The *N*-2 : 4-dinitrophenyl derivative obtained in 90% yield from the oxidation product of L-cysteinyltetraglycyl-L-cysteine (III; $n = 4$) was chromatographically homogeneous, although paper electrophoresis of the isolated oxidation product itself showed the presence of three other components in addition to the major oxidation product. Brief hydrolysis of the dinitrophenyl derivative again gave as the major product the dinitrophenylcystinyl-glycine (XI). It was, unfortunately, not possible, owing to solubility difficulties, to bring

[15] Pardee, *J. Biol. Chem.*, 1951, **190**, 757; cf. Waley, *J.*, 1955, 517.

about partial esterification of the dinitrophenyl derivative; for the same reason, coupled with lack of material, we were not able to obtain a molecular weight by physicochemical methods. The high R_F value (see Fig. 1), however, leaves little doubt that the derivative is monomeric and thus that the major oxidation product of L-cysteinyltetraglycyl-L-cysteine is the cyclic monomer (III; $n = 4$). Work is at present in progress with the object of preparing this compound in sufficient quantity for a detailed study of its properties.

The compounds which have been isolated from the oxidation products of the five

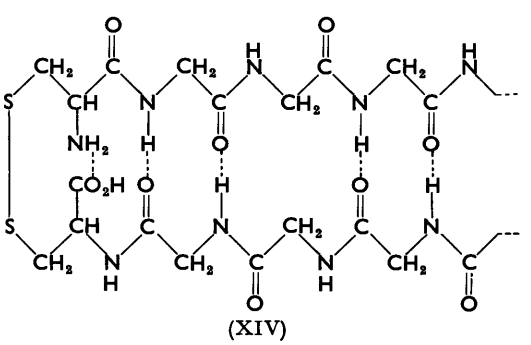
TABLE 1. *Yields (%) of oxidation products of* L-*cysteinylpolyglycyl-*L-*cysteines* (I).

	n	0	1	2	3	4
Monomer (III)		*a*	—	15	40	90
Dimer	parallel (IV)	*b*	—	—	—	—
	antiparallel (V)	20	80	20	55	—
Trimer	parallel	×	×	—	—	—
	antiparallel			25	—	—
Higher polymers		×	—	×	—	—

× denotes that the compound was detected but not isolated.
a, Greenstein *et al.*[6, 14] obtained yields of up to 70% at pH 6·5 but only about 35% at pH 8·5.
b, Greenstein *et al.*[6, 14] report a yield of about 35% at pH 8·5 (but see footnote, p. 3159).

peptides (I; $n = 0$—4) are listed in Table 1, together with the yields in which they were obtained; these, although only approximate, give some indication of the proportions of the various products. The ease with which these large rings, up to 42-membered in the case of the cyclic trimer from L-cysteinyldiglycyl-L-cysteine, are formed is very remarkable, as also is the absence of substantial amounts of linear high polymers.

The tendency for formation of the cyclic monomer at pH 8·5 clearly increases in passing from the dipeptide (I; $n = 0$) to the hexapeptide (I; $n = 4$). Inspection of models shows that the molecules of the cyclic monomers (III) become much less congested as the number of glycine residues increases, there being, in consequence, more and more conformations in which the peptide linkage can adopt the preferred *trans*-configuration;[16] it seems likely that this decreasing congestion as the peptides become larger is responsible for the observed results. The formation of the cyclic monomer with a 20-membered ring (III; $n = 4$) as the main oxidation product of L-cysteinyltetraglycyl-L-cysteine is completely analogous to the behaviour of oxytocin, which contains a similar 20-membered disulphide ring formed,

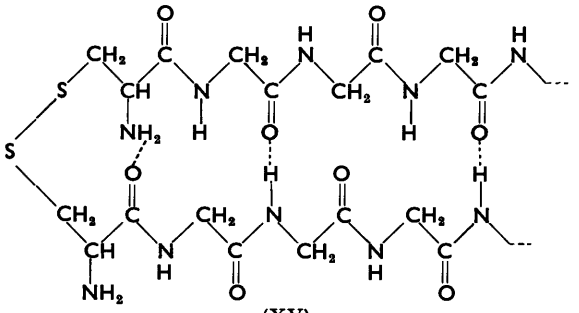


(XIV)

on oxidation and reduction,[17] from two L-cysteine residues separated by four other amino-acid residues[18]—behaviour which was of fundamental importance in its synthesis.[19] It is also noteworthy that the same type of 20-membered ring (or "loop") is also found in

[16] Pauling, Corey, and Branson, *Proc. Nat. Acad. Sci. U.S.A.*, 1951, **37**, 205.
[17] Sealock and Du Vigneaud, *J. Pharmacol.*, 1935, **54**, 433; Gordon and Du Vigneaud, *Proc. Soc. Exp. Biol. Med.*, 1953, **84**, 723.
[18] Du Vigneaud, Ressler, and Trippett, *J. Biol. Chem.*, 1953, **205**, 949; Tuppy and Michl, *Monatsh.*, 1953, **84**, 1011.
[19] Du Vigneaud, Ressler, Swan, Roberts, and Katsoyannis, *J. Amer. Chem. Soc.*, 1954, **76**, 3115.

insulin [1] and in vasopressin.[20] It is clearly of interest to determine whether cyclic monomer formation is maximal when $n = 4$, whether it is affected by side-chains present in the amino-acid residues separating the two L-cysteine residues, and whether the formation of the larger disulphide rings is subject to the remarkable pH effect observed by Greenstein and his colleagues [6, 14] in the case of L-cysteinyl-L-cysteine; work on these problems is in hand.



(XV)

It is noteworthy that we have not found any parallel cyclic polymers, such as (IV). In the first stage of polymerisation it seems probable that, owing to interaction (salt-formation or hydrogen bonding) between amino- and carboxyl groups, pairs of peptide molecules will come together at unlike, rather than like, ends. Establishment of the first disulphide linkage will then result in a structure in which the two peptide chains, in their fully extended forms, can readily be brought, by inter-chain hydrogen-bonding, to lie side by side in a position favourable to closure of the second disulphide linkage, as in (XIV). Completion of the process would lead to the formation of the antiparallel cyclic dimer (V). Formation of the parallel cyclic dimer (IV) seems likely to be more difficult. In the first place, the necessary establishment of a disulphide linkage between like ends of two peptide molecules is less probable than the formation of such a linkage between unlike ends, and, in the second place, the resulting structure is less prone to inter-chain hydrogen-bonding [cf. (XV)], and hence less likely to assume a conformation favourable to closure of the second disulphide linkage. In this connexion it is perhaps significant that insulin, which has a parallel structure, is not effectively regenerated by re-oxidation of its reduction product.[21] It may well be possible to establish conditions under which parallel cyclic polymers are formed; such conditions would clearly be of importance for any projected synthesis of insulin and we are, for this reason, actively seeking them.

Experimental

Unless otherwise stated, paper chromatography was carried out with butan-1-ol–pyridine–water (2 : 1 : 2) on Whatman No. 1 filter paper. Apparatus of the Durrum type (Shandon Scientific Co. Ltd.) was used for paper electrophoresis. Spots were detected by treatment with ninhydrin and sometimes also with cyanide–nitroprusside.[22] *N*-2 : 4-Dinitrophenyl derivatives were estimated colorimetrically in aqueous pyridine with a Spekker photoelectric absorptiometer and Ilford Spectrum Violet filters (No. 601).

Disulphide Interchange Reaction of Bis-N-2 : 4-*dinitrophenyl*-L-*cystine and* L-*Cystine.*—Bis-*N*-2 : 4-dinitrophenyl-L-cystine [23] and L-cystine were heated together under the conditions indicated in Table 2. In the qualitative experiments (Nos. 1—8) the resulting solutions were examined by paper chromatography for bis-*N*-2 : 4-dinitrophenyl-L-cystine (R_F 0·51; yellow,

[20] Du Vigneaud, Lawler, and Popenoe, *J. Amer. Chem. Soc.*, 1953, **75**, 4880; Du Vigneaud, Gish, and Katsoyannis, *ibid.*, 1954, **76**, 4751; Archer and Chauvet, *Biochim. Biophys. Acta*, 1953, **12**, 487.

[21] Du Vigneaud, Fitch, Pekarek, and Lockwood, *J. Biol. Chem.*, 1931—1932, **94**, 233; Freudenberg and Wegmann, *Z. physiol. Chem.*, 1935, **233**, 159.

[22] Toennies and Kolb, *Analyt. Chem.*, 1951, **23**, 823.

[23] Porter and Sanger, *Biochem. J.*, 1948, **42**, 287.

ninhydrin-negative), mono-*N*-2 : 4-dinitrophenyl-L-cystine (R_F 0·35; yellow, ninhydrin-positive), and L-cystine (R_F 0·06; colourless, ninhydrin-positive). In the quantitative experiments (Nos. 9 and 10) aliquot parts were removed from time to time, evaporated to dryness under reduced pressure, applied to paper in acetic acid, and chromatographed as usual; the spots corresponding to the mono- and the bis-dinitrophenyl derivative were eluted with aqueous

TABLE 2. *Disulphide interchange between bis*-N-2 : 4-*dinitrophenyl*-L-*cystine and* L-*cystine.*

Expt. no.	Reactants (moles/l.) Bis-DNP-cystine	Cystine	Thioglycollic acid	H^+	Solvent, % of AcOH (% by vol.)	Temp.	Time reqd. for interchange
1	0·002	0·002	0	1·7	44	100°	No interchange within 2 hr.
2	0·002	0·002	0·001	2·0	30	100	2 hr.
3	0·002	0·002	0·001	3·0	30	100	30 min.
4	0·001	0·001	0	5·0	0	37	<12 hr.
5	0·007	0·008	0	6·0	40	100	30 min.
6	0·004	0·004	0·001	6·0	40	100	<1 hr.
7	0·003	0·003	0·1	6·0	33	100	5 min.*
8	0·002	0·002	0·001	10·0	30	100	2 hr.
9	0·029	0·029	0	2·5	60	100	See Fig. 2, curve A
10	0·012	0·012	0	4·0	50	100	See Fig. 2, curve B

* Two additional spots (R_F *ca.* 0·15, colourless, ninhydrin-positive, and R_F *ca.* 0·8, yellow, ninhydrin-negative), probably the mixed disulphides of thioglycollic acid with cysteine and dinitrophenylcysteine, respectively, appeared on the chromatograms from this experiment.

pyridine and the solutions submitted to colorimetry. The results are summarised in Table 2 and Fig. 2; hydrochloric acid was used in all experiments except No. 8, in which sulphuric acid was used.

Oxidation of L-*Cysteinyl*-L-*cysteine.*—(*a*) *S*-Benzyl-L-cysteinyl-*S*-benzyl-L-cysteine [8] (300 mg.), in liquid ammonia (50 ml.), was treated with sodium (120 mg.) until the blue colour was permanent. Acetic acid (0·3 ml.) was then added and the ammonia allowed to evaporate. The residue was dissolved in water (10 ml.), the pH brought to 8·5 with ammonia, and a current of air passed through the solution until the nitroprusside test was negative. The solution was then neutralised with acetic acid and evaporated to dryness under reduced pressure. The residue was triturated with ethanol, and the solid oxidation product (100 mg., 61%) filtered off and washed with ethanol.

Paper chromatography, with phenol saturated with water, showed the presence of three components (R_F 0·0, 0·45, and 0·64), the fastest-moving being the major one. Paper electrophoresis, with 5N-acetic acid on Whatman No. 5 paper, showed the presence of at least three components; better separation was achieved with N-acetic acid, four components being visible.

Similar results were obtained when the peptide concentration during the oxidation was varied between 0·1% and 1·0%.

(*b*) *S*-Benzyl-L-cysteinyl-*S*-benzyl-L-cysteine (500 mg.) was similarly debenzylated and the product oxidised in 1% solution at pH 8·5. The crude evaporated product, in 2N-sodium carbonate (5 ml.), was shaken at room temperature with 2 : 4-dinitrofluorobenzene (0·4 ml.) for 2 hr. The mixture was then filtered and extracted with ether. Acidification of the residual aqueous solution precipitated the 2 : 4-dinitrophenyl derivative (170 mg., 36%), which was collected and washed with a little cold water.

Difficulty was experienced in the paper chromatography of this material and its components. When it was applied as a spot in the usual manner little movement occurred and the chromatogram was a mere streak extending from a position close to the origin (see Fig. 4, points for 0, 1, and 5 min.). Satisfactory separation was, however, achieved when the material was applied as a rather heavy band extending across the whole width of a narrow strip of paper (see Fig. 3); this technique was used in all paper chromatographic experiments with this material. This unexplained behaviour was not shown by the dinitrophenyl derivatives derived from the oxidation products of other cysteinylpolyglycylcysteines (see, for example, Fig. 5).

A paper chromatogram of the crude dinitrophenyl derivative had the appearance shown in Fig. 3; band 4 was by far the most intense. Elution and rechromatography of bands 1, 2, and 3 gave only bands 1, 2, and 4. Bands 1 and 2 were shown by direct comparison to be

2 : 4-dinitroaniline and 2 : 4-dinitrophenol, respectively. Elution of bands 4—8 with aqueous pyridine and colorimetric estimation showed the following distribution of material :

Band	4	5	6	7	8
Mols. (%)	52	19	11	12	6

Column chromatography, on both cellulose and alumina, having proved fruitless, a larger amount (*ca.* 20 mg.) of the crude dinitrophenyl derivative was chromatographed on thick paper (Whatman No. 3 MM), and the various components (bands 4—8) were eluted with aqueous

FIG. 3. *Chromatogram of the dinitrophenyl derivatives from the oxidation product of* L-*cysteinyl*-L-*cysteine* (I; $n = 0$).

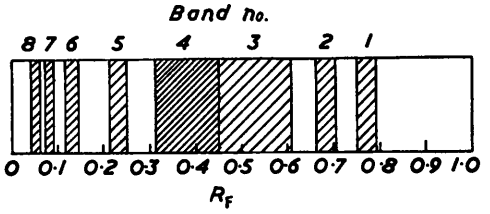
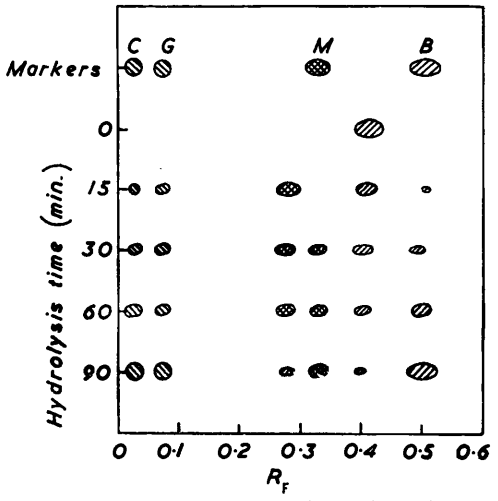
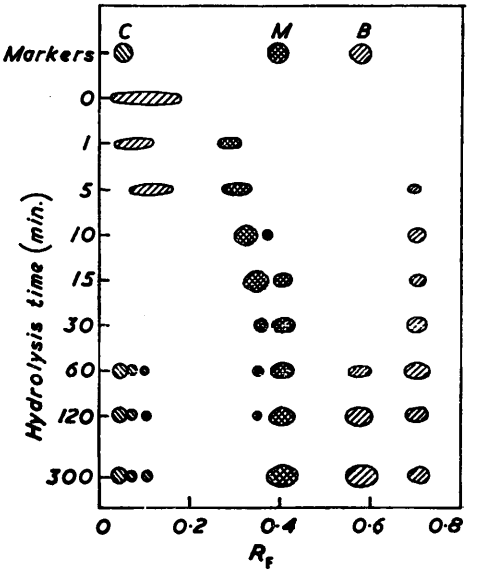


FIG. 5. *Chromatogram showing the effect of various periods of hydrolysis on the dinitrophenyl derivative of the antiparallel cyclic dimer from* L-*cysteinyl-glycyl*-L-*cysteine.*



//// = *Yellow.* \\\\ = *Ninhydrin positive.*
C = L-*Cystine.*
G = *Glycine.*
M = *Mono*-N-2 : 4-*dinitrophenyl*-L-*cystine.*
B = *Bis*-N-2 : 4-*dinitrophenyl*-L-*cystine.*

FIG. 4. *Chromatogram showing the effect of various periods of hydrolysis on the dinitrophenyl derivative of the antiparallel cyclic dimer from* L-*cysteinyl*-L-*cysteine.*



//// = *Yellow.* \\\\ = *Ninhydrin positive.*
C = L-*Cystine.*
M = *Mono*-N-2 : 4-*dinitrophenyl*-L-*cystine.*
B = *Bis*-N-2 : 4-*dinitrophenyl*-L-*cystine.*

pyridine and isolated by evaporation of the eluates in a vacuum-desiccator over sulphuric acid.

The material from band 4 was purified by further paper chromatography (R_F 0·42) and the product (*ca.* 3 mg.) heated at 100° with 1 : 1 acetic acid–20% hydrochloric acid. From time to time a little of the solution was spotted on to paper. After addition of a marker spot, from a solution prepared by heating L-cystine (1 mg.) and *N*-bis-2 : 4-dinitrophenyl-L-cystine (1 mg.) in 1 : 1 acetic acid–20% hydrochloric acid at 100° for 1 hr., the chromatogram was run as usual. After spraying with ninhydrin and heating as usual the chromatogram had the appearance shown in Fig. 4. The yellow spots, R_F 0·7, are due to 2 : 4-dinitrophenol, produced by some hydrolysis, and no doubt this hydrolysis is responsible also for the additional colourless,

ninhydrin-positive spots with R_F 0·06—0·07 and 0·1. The three marker spots are cystine (R_F 0·06), monodinitrophenylcystine (R_F 0·39), and bisdinitrophenylcystine (R_F 0·58).

More material from band 4 (*ca.* 1 mg.) was hydrolysed, as just described, for 10 min. and the product subjected to paper chromatography. The yellow, ninhydrin-positive band, R_F 0·3—0·35, was oxidised on the paper with performic acid (from formic acid and 20-vol. hydrogen peroxide). After an hour at room temperature, the product was eluted and again subjected to paper chromatography. Three spots appeared having R_F 0·08 (colourless and ninhydrin-positive), R_F 0·10—0·22 (yellow and ninhydrin-negative), and R_F 0·3 (yellow and ninhydrin-negative). The first and the third were shown, by direct comparison, to be *L*-cysteic acid and *N*-2 : 4-dinitrophenyl-L-cysteic acid, respectively. The second spot was eluted, hydrolysed overnight at 100° with 20% hydrochloric acid, and rechromatographed; spots appeared with R_F 0·08 (colourless and ninhydrin-positive) and R_F 0·30 (yellow and ninhydrin-negative) and were shown by direct comparison to be L-cysteic acid and *N*-2 : 4-dinitrophenyl-L-cysteic acid, respectively.

Oxidation of L-*Cysteinylglycyl*-L-*cysteine*.—(*a*) *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinylglycyl-*S*-benzyl-L-cysteine benzyl ester [8] (II; R = $CH_2Ph{\cdot}O{\cdot}CO$, R′ = $Ph{\cdot}CH_2$, n = 1) (1·0 g.), in liquid ammonia (40 ml.), was treated with sodium (0·3 g.) in small portions with stirring. After addition of ammonium chloride (0·7 g.), the solution was evaporated and the residue dissolved in water (400 ml.). The pH was adjusted to 8·5 with barium hydroxide and a current of air passed through until the nitroprusside test was negative (90 min.). The pH was then brought to 7·0 with sulphuric acid and the solution filtered and evaporated to dryness under reduced pressure; recrystallisation of the residue from aqueous ethanol gave the oxidation product (350 mg., 87%) as a greyish solid, not entirely free from inorganic matter. Paper electrophoresis in 5N-acetic acid showed two slow-moving ninhydrin-positive components and a third faster-moving one.

(*b*) The same fully protected peptide (500 mg.) was similarly treated with sodium (230 mg.) in liquid ammonia (50 ml.). Dry Zeocarb 225 (10 ml.) was added and the ammonia allowed to evaporate. The residue was extracted with water (2 × 20 ml.); after adjustment of the pH of the extract to 8·6 with hydriodic acid, air was passed through the solution until the nitroprusside reaction was negative (3 hr.). The pH was then adjusted to 7·0 and ethanol (50 ml.) was added; the precipitated oxidation product (160 mg., 79%), collected by filtration, showed only one ninhydrin-positive component on paper electrophoresis in 5N-acetic acid.

(*c*) The fully protected peptide (1·0 g.) was treated with sodium and liquid ammonia and the product oxidised as before, in 1% solution at pH 8·5. The resulting solution was brought to pH 7·0, treated with sodium hydrogen carbonate (300 mg.), and shaken mechanically for 2 hr. with 2 : 4-dinitrofluorobenzene (300 mg.) in ethanol (30 ml.). Removal of the ethanol under reduced pressure, extraction with ether, and acidification of the residual aqueous solution precipitated the crude 2 : 4-dinitrophenyl derivative (200 mg., 31%). Paper chromatography showed the presence of two components, R_F 0·38 and 0·27. A similar product was obtained when the oxidation was carried out in 0·1% solution.

The crude product was chromatographed on cellulose with *tert.*-amyl alcohol–butan-1-ol–water (1 : 5 : 1); the yield of the faster-moving component (A) was 70% and that of the slower-moving one (B), 25%.

The purified dinitrophenyl derivative (A) (10 mg.) was heated at 100° with acetic acid (0·25 ml.) and 20% hydrochloric acid (0·2 ml.). Portions were removed from time to time and spotted on to filter paper; after chromatography, spraying with ninhydrin, and heating, the chromatogram had the appearance shown in Fig. 5. In another experiment the dinitrophenyl derivative (A) (0·2 mg.) was heated at 100° for 15 min. with acetic acid (0·25 ml.) and 20% hydrochloric acid (0·2 ml.). The solution was evaporated to dryness under reduced pressure and the residue subjected to paper chromatography; elution of the band R_F 0·25—0·30 and colorimetric estimation showed the yield of this initial hydrolysis product to be 80%.

The purified dinitrophenyl derivative (A) (20 mg.) was heated at 100° for 15 min. with acetic acid (0·5 ml.) and 20% hydrochloric acid (0·4 ml.). The solution was then evaporated to dryness under reduced pressure and the residue taken up in 1% sodium hydrogen carbonate solution (2 ml.) and shaken for 2 hr. with 2 : 4-dinitrofluorobenzene (10 mg.) in ethanol (1 ml.). Removal of ethanol under reduced pressure and extraction with ether, followed by acidification, precipitated the bis-2 : 4-dinitrophenyl derivative (XII) (20 mg., 73%), shown to be homogeneous (R_F 0·48) by paper chromatography. This derivative (5 mg.) was left for 1 hr. at room temperature in performic acid (0·1 ml.) [from formic acid (6 ml.) and 20-vol. hydrogen peroxide (1 ml.)]. Paper chromatography afforded two bands; one (R_F 0·3; yellow, ninhydrin-negative)

was shown by direct comparison to be 2 : 4-dinitrophenyl-L-cysteic acid; the other (R_F 0·13; yellow, ninhydrin-negative) was eluted with aqueous pyridine. The eluate was evaporated and the residue heated at 100° for 2 hr. with acetic acid (0·01 ml.) and 20% hydrochloric acid (0·02 ml.); paper chromatography then showed the presence of 2 : 4-dinitrophenylcysteic acid (R_F 0·3) and glycine (R_F 0·08). The bisdinitrophenyl derivative (XII) (1 mg.) was heated at 100° for 2 hr. with acetic acid (0·01 ml.) and 20% hydrochloric acid (0·02 ml.); paper chromatography showed the hydrolysate to contain only bis-2 : 4-dinitrophenyl-L-cystine (R_F 0·50) and glycine (R_F 0·08).

The purified dinitrophenyl derivative (A) (10 mg.), in anhydrous dioxan (5 ml.), was treated with ethereal diazomethane (1 ml.; from methylnitrosourea, 2·7 mg.). After 15 min. at room temperature and 5 min. at 50°, the mixture was evaporated to dryness. Paper chromatography showed the presence of the original derivative (R_F 0·42) and its mono- and di-methyl esters (R_F 0·72 and 0·87). In a control experiment bis-2 : 4-dinitrophenyl-L-cystine afforded unchanged material (R_F 0·49) and mono- and di-methyl esters (R_F 0·68 and 0·82).

Oxidation of L-*Cysteinyldiglycyl*-L-*cysteine.*—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-diglycyl-*S*-benzyl-L-cysteine benzyl ester [8] (II; R = Ph·CH_2·O·CO, R′ = Ph·CH_2, n = 2) (1·0 g.) was treated with sodium (450 mg.) in liquid ammonia (50 ml.) in the usual manner and the product oxidised by aeration at pH 8·5 in 1% solution. The solution was brought to pH 7·0 and then shaken for 2 hr. at room temperature with sodium hydrogen carbonate (230 mg.) and a solution of 2 : 4-dinitrofluorobenzene (250 mg.) in ethanol (30 ml.). Working up as usual gave the crude 2 : 4-dinitrophenyl derivative.

Paper chromatography showed the presence of the following components, in addition to 5 mols. % of 2 : 4-dinitrophenol :

Band no.	1	2	3	4
R_F	0·45	0·285	0·115	0·02—0·08
Mols. %	22	30	38	10

Components 1, 2, and 3 were separated on a larger scale by preliminary column chromatography on cellulose with *tert.*-amyl alcohol–butan-1-ol–water (1 : 5 : 1) followed by paper chromatography with butan-1-ol–pyridine–water (2 : 1 : 2). Each purified component (0·5 mg.) was heated at 100° for 15 min. with acetic acid (0·01 ml.) and 20% hydrochloric acid (0·02 ml.); paper chromatography showed that in every case the main product (45, 33, and 41% yield, respectively, from components 1, 2, and 3) was the same dinitrophenyl-L-cystinylglycine (XI) (R_F 0·29) as was obtained similarly from the oxidation product of L-cysteinylglycyl-L-cysteine.

Oxidation of L-*Cysteinyltriglycyl*-L-*cysteine.*—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-triglycyl-*S*-benzyl-L-cysteine benzyl ester [8] (II; R = Ph·CH_2·O·CO, R′ = Ph·CH_2, n = 3) (100 mg.), in acetic acid (4 ml.), was heated to 50° while dry hydrogen bromide was passed through the solution for 2 hr. Ether precipitated *S*-benzyl-L-cysteinyl-triglycyl-*S*-benzyl-L-cysteine hydrobromide (80 mg., 98%), shown to be homogeneous by paper chromatography (R_F 0·60). This pentapeptide (50 mg.) was reduced, and oxidised in 1% solution, and the product converted into the 2 : 4-dinitrophenyl derivative (43 mg., 93%) in the usual manner.

Paper chromatography showed the presence of two components, R_F 0·33 (41 mols. %) and R_F 0·17 (59 mols. %). These were separated by larger-scale paper chromatography and each (0·5 mg.) was heated at 100° for 15 min. with acetic acid (0·01 ml.) and 20% hydrochloric acid (0·02 ml.). In both cases paper chromatography showed the main product to be the dinitrophenyl-L-cystinylglycine (XI) (R_F 0·30).

Oxidation of L-*Cysteinyltetraglycyl*-L-*cysteine.*—(*a*) *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-tetraglycyl-*S*-benzyl-L-cysteine benzyl ester [8] (II; R = Ph·CH_2·O·CO, R′ = Ph·CH_2, n = 4) (100 mg.), in acetic acid (4 ml.), was heated at 50° while dry hydrogen bromide was passed through the solution for 2 hr. Ether (40 ml.) precipitated *S*-benzyl-L-cysteinyl-tetraglycyl-*S*-benzyl-L-cysteine hydrobromide (50 mg., 60%), which was chromatographically homogeneous (R_F 0·59). This, reduced, oxidised, and treated with 2 : 4-dinitrofluorobenzene in the usual manner, afforded a 2 : 4-dinitrophenyl derivative (40 mg., 92%), shown by paper chromatography to be homogeneous (R_F 0·375). This derivative (1 mg.), was heated at 100° for 15 min. with acetic acid (0·1 ml.) and 20% hydrochloric acid (0·2 ml.); paper chromatography again showed the main hydrolysis product to be the dinitrophenylcystinyl-glycine (XI) (R_F 0·30).

(*b*) In another experiment the hexapeptide hydrobromide (70 mg.) was treated with sodium (30 mg.) in liquid ammonia. Dry Zeocarb 225 (2 ml.) was added and the mixture evaporated to dryness. The residue was taken up in water (5 ml.) and filtered. The pH was brought to 8·5

with hydriodic acid, and air bubbled through the solution for 3 hr., after which the pH was brought to 7·0 with aqueous ammonia. Addition of ethanol precipitated the oxidation product (40 mg., 90%); paper electrophoresis in 5N-acetic acid showed the presence of 3 minor in addition to the major component. Conversion of this mixture into the 2 : 4-dinitrophenyl derivative in the usual way afforded a chromatographically homogeneous product (R_F 0·35), identical with that obtained directly by procedure (*a*).

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