341. *Polypeptides. Part VII.*[1] *The Synthesis and Oxidation of* L-*Cysteinyl-pentaglycyl-*L-*cysteine and* L-*Cysteinyl-hexaglycyl-*L-*cysteine.*

By D. Jarvis, H. N. Rydon, and (in part) J. A. Schofield.

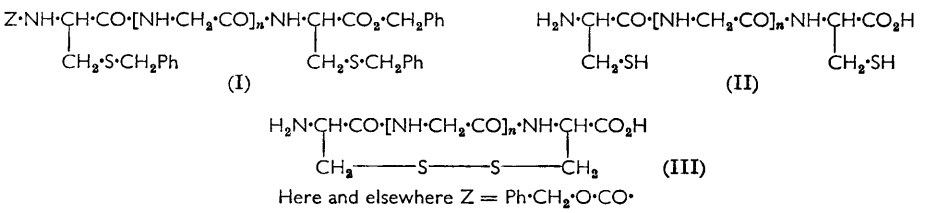
N-Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-pentaglycyl-*S*-benzyl-L-cysteine benzyl ester (I; $n = 5$) and the corresponding hexaglycyl compound (I; $n = 6$) have been synthesised. Oxidation of L-cysteinyl-pentaglycyl- and -hexaglycyl-L-cysteine (II; $n = 5$ and 6), obtained by removal of the protecting groups, in dilute aqueous solution at pH 8·6, has been shown to produce almost exclusively the cyclic disulphides, *SS'*-dehydro-L-cysteinyl-pentaglycyl- and -hexaglycyl-L-cysteine (III; $n = 5$ and 6).

Earlier papers in this series described the preparation [2] of a number of protected L-cysteinyl-polyglycyl-L-cysteines (I; $n = 0$—4) and the oxidation,[1,3] in dilute aqueous solution, of the derived free peptides (II; $n = 0$—4). It was found that the proportion

[1] Part VI, Large, Rydon, and Schofield, preceding paper.
[2] Hooper, Rydon, Schofield, and Heaton, *J.*, 1956, 3148.
[3] Heaton, Rydon, and Schofield, *J.*, 1956, 3157.

of monomeric cyclic disulphide (III) present in the oxidation products increased with increasing separation of the cysteine residues, the cyclic monomer becoming the major product in the case of the tetraglycyl compound (II; $n = 4$). At about the same time, Lautsch and Kraege [4] reported the oxidation of the hexapeptide (II; $n = 4$) to the monomeric cyclic disulphide (III; $n = 4$), but did not adduce any evidence for the structure assigned to their oxidation product.

Since the twenty-membered disulphide ring system present in (III; $n = 4$) is also found in Nature, in the hormones insulin,[5] oxytocin,[6] and the vasopressins,[7] it seemed important to study higher members of the series (II) in order to ascertain whether there was some critical factor tending to make the formation of such twenty-membered ring compounds particularly easy. While our work was in progress, Lautsch and his colleagues [8,9,10] reported, without experimental detail, the oxidation of L-cysteinyl-penta-, -hexa-, and -hepta-glycyl-L-cysteine (II; $n = 5$, 6, and 7) to the monomeric cyclic disulphides (III; $n = 5$, 6, and 7); only in the case of the octapeptide (III; $n = 6$) was the structure claimed for the oxidation product supported by experimental evidence (molecular weight). The same school has also announced [9,10,11] similar findings with other peptides in which two cysteine residues are separated by 4—6 other amino-acid residues.

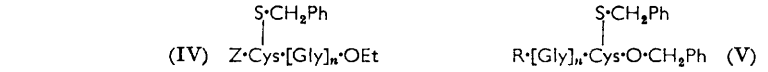


Here and elsewhere Z = Ph·CH_2·O·CO·

The key intermediates for the synthesis of the protected heptapeptide (I; $n = 5$) were *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-diglycyl-glycine ethyl ester (IV; $n = 3$) and *N*-benzyloxycarbonyldiglycyl-*S*-benzyl-L-cysteine benzyl ester (V; $n = 2$, R = Z). Both intermediates had previously been prepared by Hooper *et al.*,[2] but in both cases improved results were obtained by using di-*o*-phenylene pyrophosphite as coupling reagent.[12] The use of this reagent in the coupling of *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteine with diglycyl-glycine ethyl ester gave the first-named intermediate (IV; $n = 3$) with a much higher rotation than was obtained by the earlier workers [2] who had used the mixed anhydride method of coupling; [13] although racemisation does not generally occur in coupling reactions involving mixed anhydrides derived from *N*-benzyloxycarbonyl amino-acids,[14,15] the lability of derivatives of *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteine has been encountered by several workers [16] and it is not altogether surprising that couplings using

[4] Lautsch and Kraege, *Chem. Ber.*, 1956, **89**, 737.
[5] Ryle, Sanger, Smith, and Kitai, *Biochem. J.*, 1955, **60**, 541.
[6] du Vigneaud, Ressler, and Trippett, *J. Biol. Chem.*, 1953, **205**, 949; du Vigneaud, Ressler, Swan, Roberts, and Katsoyannis, *J. Amer. Chem. Soc.*, 1954, **76**, 3115; Tuppy, *Biochim. Biophys. Acta*, 1953, **11**, 449; Tuppy and Michl, *Monatsh.*, 1953, **84**, 1011.
[7] du Vigneaud, Lawler, and Popenoe, *J. Amer. Chem. Soc.*, 1953, **75**, 4880; du Vigneaud, Gish, and Katsoyannis, *ibid.*, 1954, **76**, 4751; Acher and Chauvet, *Biochim. Biophys. Acta*, 1953, **12**, 487.
[8] Lautsch, Wiechert, Gnichtel, Schuchardt, Kraege, Singewald, Broser, Becker, Rauhut, and Grimm, *Österr. Chem.-Ztg.*, 1957, **58**, 33.
[9] Lautsch, Wiemer, Zschenderlein, Kraege, Bandel, Günther, Schulz, and Gnichtel, *Kolloid Z.*, 1958, **161**, 36.
[10] Lautsch, Boroschewski, Gante, Shingte, Rauhut, Grimm, Rimpler, Schulz, and Paetzke, *Chimia (Switz.)*, 1959, **13**, 142.
[11] Lautsch and Günther, *Naturwiss.*, 1957, **44**, 492; Lautsch and Schulz, *ibid.*, 1958, **45**, 58.
[12] Crofts, Markes, and Rydon, *J.*, 1959, 3610.
[13] Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874.
[14] Goodman and Kenner, *Adv. Protein Chem.*, 1957, **12**, 465.
[15] Vaughan, *J. Amer. Chem. Soc.*, 1952, **74**, 6137.
[16] For refs. see Young, *Coll. Czech. Chem. Comm.*, 1959, **24**, Special Issue, 114.

the derived mixed anhydride may be accompanied by racemisation. The second intermediate (V; $n = 2$, R = Z) was prepared in good yield by coupling *N*-benzyloxycarbonylglycyl-glycine and *S*-benzyl-L-cysteine benzyl ester with the aid of di-*o*-phenylene pyrophosphite;[12] this method gave a much better yield than the mixed anhydride procedure.[13] The *N*-benzyloxycarbonyl group was selectively removed from the protected peptide (V; $n = 2$; R = Z) with hydrogen bromide in acetic acid,[17] and the product (V; $n = 2$; R = H) treated with the azide obtained, through the hydrazide, from the protected tetrapeptide ester (IV; $n = 3$); the required protected heptapeptide (I; $n = 5$), so obtained, had properties in satisfactory agreement with those recorded by Lautsch and Kraege [4] for material synthesised by a different route.

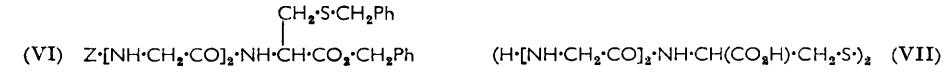


(IV) Z·Cys(S·CH$_2$Ph)·[Gly]$_n$·OEt R·[Gly]$_n$·Cys(S·CH$_2$Ph)·O·CH$_2$Ph (V)

Two methods were used for the synthesis of the protected octapeptide (I; $n = 6$). In the first, the azide derived from *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine ethyl ester (IV; $n = 2$) [1,12] was coupled with glycyl-glycine ethyl ester to give *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-triglycyl-glycine ethyl ester (IV; $n = 4$). This, in its turn, was converted, through the hydrazide, into the azide which was then coupled with diglycyl-*S*-benzyl-L-cysteine benzyl ester (V; $n = 2$; R = H) to give the required product (I; $n = 6$). In the second method, *N*-benzyloxycarbonylglycyl-glycine was coupled, by means of di-*o*-phenylene pyrophosphite, with diglycyl-*S*-benzyl-L-cysteine benzyl ester (V; $n = 2$; R = H), and the benzyloxycarbonyl group was selectively removed from the resulting protected pentapeptide (V; $n = 4$; R = Z) with hydrogen bromide in acetic acid.[17] Tetraglycyl-*S*-benzyl-L-cysteine benzyl ester (V; $n = 4$; R = H), so obtained, was treated with the azide from *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine ethyl ester (IV; $n = 2$), yielding the required protected octapeptide (I; $n = 6$). The products obtained by the two methods were identical, but the second method was preferred as being more economical.

As in the earlier work,[3] the protected peptides (I; $n = 5$ and 6) were reduced with an alkali metal in liquid ammonia, and the resulting cysteinyl-polyglycyl-cysteines (II; $n = 5$ and 6) were oxidised in aqueous solution, at a concentration of 0·5% and a pH of 8·6; since, however, we wished to isolate the oxidation products the earlier technique required modification. Further work [1,18] having shown the course of the oxidation of cysteinyl-tetraglycyl-cysteine to be markedly affected by traces of metal ions, the reductions were carried out in redistilled ammonia in an apparatus (Fig. 1) designed to allow the oxidation to be carried out in the vessel used for the prior removal of the protecting groups.

In view of the relative inaccessibility of the dicysteine peptides, exploratory experiments were carried out with the more easily available *N*-benzyloxycarbonyldiglycyl-*S*-benzyl-L-cysteine benzyl ester (VI). This was reduced, in liquid ammonia, with either lithium or potassium, to diglycyl-L-cysteine which was then oxidised as usual. Attempts to purify



(VI) Z·[NH·CH$_2$·CO]$_2$·NH·CH(CH$_2$·S·CH$_2$Ph)·CO$_2$·CH$_2$Ph (H·[NH·CH$_2$·CO]$_2$·NH·CH(CO$_2$H)·CH$_2$·S·)$_2$ (VII)

the product from the lithium reduction by extraction of the lithium salts with ethanol were unsuccessful, but it proved possible to isolate the oxidation product, bisdiglycyl-L-cystine (VII) by use of an ion-exchange resin; the bis-2,4-dinitrophenyl derivative was also isolated in satisfactory yield. A satisfactory experimental procedure having been thus established, attention was turned to the dicysteine peptides.

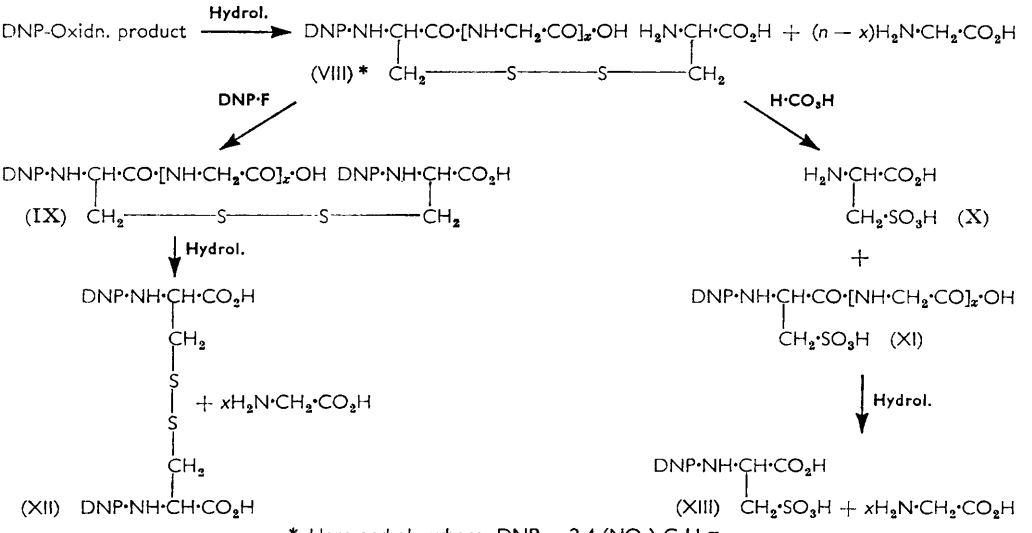
The protected hepta- and octa-peptides (I; $n = 5$ and 6) were reduced with sodium in liquid ammonia, and the resulting L-cysteinyl-penta- and -hexa-glycyl-L-cysteine (II;

[17] Ben-Ishai and Berger, *J. Org. Chem.*, 1952, **17**, 1564.
[18] Large, Ph.D. Thesis, Manchester, 1959.

$n = 5$ and 6) were then oxidised, as usual, by passing oxygen through 0·5% aqueous solutions at pH 8·6. Paper chromatography revealed the presence in the oxidation solutions of only one product in each case, but paper electrophoresis showed this to be contaminated by a small amount of a second product; in each case, both the major and the minor products contained free amino-groups and disulphide linkages. It is noteworthy that a similar minor product accompanied the major product (bisdiglycyl-L-cystine) of the oxidation of diglycyl-L-cysteine and it seems likely that all the minor products are diastereoisomerides of the major products, arising from the occurrence of a little racemisation during the synthesis of the protected peptides. The simplicity of the oxidation products of the hepta- and the octa-peptide (II; $n = 5$ and 6) is in marked contrast to the complexity of the mixtures resulting from the oxidation of the analogous lower peptides (II; $n = 0$—3).[3]

Although the use of an ion-exchange resin had proved successful in the isolation of the oxidation product of diglycyl-cysteine, it was thought inadvisable, owing to the possibility of disulphide interchange, to use such a procedure for isolating the oxidation products of the dicysteine peptides. Accordingly, salt-free aqueous solutions of the free peptides (II; $n = 5$ and 6) were prepared from the protected peptides by way of the mercury derivatives, du Vigneaud and Miller's general procedure[19] being used. The oxidation products were precipitated, by addition of ethanol to the concentrated oxidised solutions, as white, hygroscopic powders, contaminated by small amounts (3—4%) of barium sulphate; in both cases the chromatographic and the electrophoretic behaviour of the products (isolated in about 50% yield) were the same as those of the major component of the corresponding crude oxidation mixture. Treatment of the oxidation products with 1-fluoro-2,4-dinitrobenzene in aqueous-ethanolic sodium carbonate gave the chromatographically homogeneous *N*-2,4-dinitrophenyl derivatives; as in the work of Heaton, Rydon, and Schofield,[3] the structures of the oxidation products were determined by studying the partial acid-hydrolyses of these derivatives.

The *N*-2,4-dinitrophenyl derivative of the oxidation product of cysteinyl-pentaglycyl-cysteine was heated with a mixture of acetic and hydrochloric acid, samples being removed



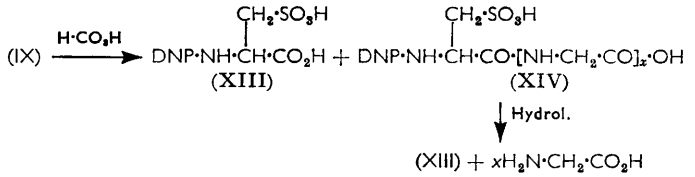
* Here and elsewhere, DNP = 2,4-$(NO_2)_2C_6H_3$–.

for chromatography from time to time; the progress of the hydrolysis is represented in Fig. 2. An initial hydrolysis product, *A*, yellow and ninhydrin-positive, appeared after

[19] du Vigneaud and Miller, *Biochem. Preps.*, 1952, **2**, 87.

10 min.; as the hydrolysis proceeded, the spot corresponding to this product developed into a streak and spots corresponding to glycine, cystine, and the mono- and bis-*N*-2,4-dinitrophenyl derivatives of the latter appeared. A further quantity of the *N*-2,4-dinitrophenyl derivative of the oxidation product was hydrolysed for 15 min. and the initial hydrolysis product, *A*, was isolated by elution from paper chromatograms. Oxidation of *A* with performic acid [20] gave cysteic acid (X) and a yellow, ninhydrin-negative product, *B*, which, on complete acid hydrolysis gave only glycine and *N*-2,4-dinitrophenylcysteic acid (XIII). It follows that the initial hydrolysis product, *A*, has the structure (VIII), the oxidation product, *B*, being the derived cysteic acid peptide (XI); the course of the degradation is shown in the chart. The presence of a free amino-group in the initial hydrolysis product (VIII) was confirmed by the observation that treatment with 1-fluoro-2,4-dinitrobenzene gave a new product (IX), yellow and ninhydrin-negative, which on complete hydrolysis yielded glycine and bis-*N*-2,4-dinitrophenylcystine (XII).

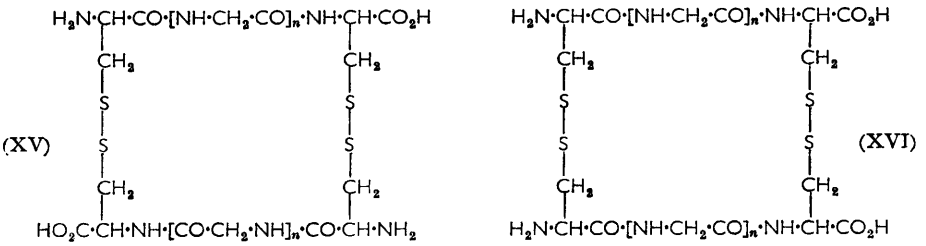
The *N*-2,4-dinitrophenyl derivative of the oxidation product of cysteinyl-hexaglycyl-cysteine was subjected to progressive hydrolysis in similar fashion to that used with the pentaglycyl compound; the relevant chromatogram is shown in Fig. 3. The pattern was similar to that observed with the heptapeptide, except that the initial hydrolysis product, *C*, gave no obvious reaction with ninhydrin and neither cystine nor its dinitrophenyl derivatives appeared to be formed. Nevertheless, the chemical behaviour of the initial hydrolysis product, *C*, was very similar to that of the product similarly obtained from cysteinyl-pentaglycyl-cysteine; oxidation of *C* gave cysteic acid (X) and a yellow, ninhydrin-negative product (XI), which yielded *N*-2,4-dinitrophenylcysteic acid (XIII) and glycine on complete hydrolysis. The presence of a free amino-group in *C*, despite its failure to react visibly with ninhydrin, was established by treating it with 1-fluoro-2,4-dinitrobenzene, which converted it into another yellow compound, *D*. Oxidation of this compound *D* with performic acid gave 2,4-dinitrophenylcysteic acid and a yellow, ninhydrin-negative product, *E*, which gave 2,4-dinitrophenylcysteic acid and glycine on complete hydrolysis. These observations show that *C*, like *A*, must be represented by (VIII), *D* and *E* being (IX) and (XIV), respectively, thus:



The difference in behaviour of *A* and *C* towards ninhydrin, although both are represented by the same general structure (VIII), is most easily explained as being due to a difference in the number of glycine residues they contain; it seems probable that *A* is the monoglycine compound (VIII; $x = 1$), encountered in the earlier work,[3] which it closely resembles in chromatographic behaviour, whereas *C* contains more (up to six) glycine residues, a circumstance which would explain its failure to give a pronounced colour with ninhydrin.[21] The implied difference in the mode of hydrolysis of the parent oxidation products (III; $n = 5$ and 6) indicates a difference in conformation of the peptide chains in these two compounds; abrupt discontinuities in physicochemical properties, ascribed to a conformational change, have been encountered at about the same length of peptide chain in ascending other series of peptides.[22]

[20] Sanger, *Nature*, 1947, **160**, 295; *Biochem. J.*, 1949, **44**, 126.
[21] Rydon and Smith, *Nature*, 1952, **169**, 922.
[22] Cf., *inter alia*, Idelson and Blout, *J. Amer. Chem. Soc.*, 1957, **79**, 3948; Mitchell, Woodward, and Doty, *ibid.*, p. 3955; Goodman and Schmitt, *ibid.*, 1959, **81**, 5507.

The structure (VIII) assigned to the initial hydrolysis products of the *N*-2,4-dinitrophenyl derivatives of the oxidation products of cysteinyl-pentaglycyl- and -hexaglycyl-cysteine shows that the oxidation products themselves must be the cyclic monomers (III),



the anti-parallel dimers (XV), or higher, anti-parallel, polymers; parallel structures, such as (XVI), are definitely excluded since these would necessarily give rise to a mixture of a bisdinitrophenyl derivative and a compound containing no dinitrophenyl groups as the initial products of hydrolysis.

The chromatographic behaviour of the *N*-2,4-dinitrophenyl derivatives of the two oxidation products suggests strongly that these are, indeed, monomeric; the derivatives have R_F values of 0·37 and 0·35, respectively, in butanol–pyridine–water, whereas the corresponding derivatives of dimers, such as (XV), would be expected to have much lower R_F values.[3] Conclusive evidence in favour of the monomeric structure (III) for both oxidation products was obtained by partial dinitrophenylation.[23] On treatment with a deficiency of 1-fluoro-2,4-dinitrobenzene both oxidation products gave, in addition to unchanged starting material, only one yellow, dinitrophenylated product. This is to be expected on the basis of the monomeric structure (III), which contains only one amino-group, whereas a dimer such as (XV), containing two amino-groups, would be expected to give two yellow products (the mono- and the bis-dinitrophenyl derivative) in addition to unchanged starting material, as bisdiglycyl-cystine (VII) was shown to do in a control experiment.

We conclude, therefore, that, under the conditions of our experiments, oxidation of L-cysteinyl-pentaglycyl-L-cysteine (II; $n = 5$) and L-cysteinyl-hexaglycyl-L-cysteine (II; $n = 6$) gives, as almost the only products, the cyclic disulphides (III; $n = 5$ and 6).

This conclusion is similar to that previously reached [3] in the case of L-cysteinyl-tetraglycyl-L-cysteine (II; $n = 4$), which was likewise shown to yield mainly the cyclic monomer (III; $n = 4$) on oxidation; it is clear that the circumstance that the 20-membered ring present in the oxidation product (III; $n = 4$) from L-cysteinyl-tetraglycyl-L-cysteine is also found in a number of peptide hormones [5–7] is not attributable to any tendency for such rings to be formed more easily than the 23- and 26-membered disulphide rings encountered in the present work. It was very noticeable that the amount of by-product accompanying the cyclic monomer in the oxidation products of the hepta- and the octapeptide (II; $n = 5$ and 6) was much less than that encountered [1,18] in the case of the hexapeptide (II; $n = 4$); others [24] have noted the instability of 20-membered disulphide rings in aqueous solution and it is tempting to speculate that the fact that disulphide rings of this size, rather than those containing fewer or more amino-acid residues, occur in insulin,[5] oxytocin,[6] and the vasopressins [7] is not without significance. Rings of this size may be optimal in being the smallest which are sufficiently stable to have a useful life in body fluids while being the largest which are sufficiently reactive to be capable of reaction with suitable receptor groups in proteins.[25]

[23] Cf. Battersby and Craig, *J. Amer. Chem. Soc.*, 1951, **73**, 1887.
[24] Ressler, *Science*, 1958, **128**, 1281; Bodanszky and du Vigneaud, *J. Amer. Chem. Soc.*, 1959, **81**, 2504.
[25] Cf. Fong, Schwartz, Popenoe, Silver, and Schoessler, *J. Amer. Chem. Soc.*, 1959, **81**, 2592.

Neither of the two cyclic disulphides (III; $n = 5$ and 6) exhibited avian depressor activity in the chicken or pressor activity in the rat, and neither inhibited the actions of oxytocin or vasopressin.

Approximate statistical calculations [26] have shown that the yield (90% in 1% solution) of cyclic monomer (III; $n = 4$) obtained from L-cysteinyl-tetraglycyl-L-cysteine (II; $n = 4$) is higher than that to be expected (58%) on a purely statistical basis. The same is true of the present cases, the calculated yields of cyclic monomer to be expected from the heptapeptide (II; $n = 5$) and the octapeptide (II; $n = 6$) being, for 0·5% solutions, 67% and 61%, respectively, as compared with the observed all but theoretical yields. Such discrepancies are not beyond the uncertainty of the calculations, and only direct experiment can show whether, as the number of glycine residues separating the two cysteines in (II) is increased, the yield of monomeric disulphide will fall as expected or whether some other factor, such as hydrogen-bonding within the peptide chain, is involved; such experiments are now in progress in these laboratories.

The optical rotatory powers of the known *SS'*-dehydro-L-cysteinyl-polyglycyl-L-cysteines (III) are collected in the following Table:

n in (III)	0	4	5	6	7
$[\alpha]_D$	−29° (N-HCl)	−25° (H_2O)	−4·5° (H_2O)	−4·3° (H_2O)	−1·4° (H_2O)
Reference	27	9	Present work	Present work	10

The fall in rotatory power in passing from the tetraglycyl to the pentaglycyl compound is very marked and must be connected with a conformational change. Inspection of models shows that, as the number of glycine residues in (III) increases, the number of possible conformations with the peptide linkages having the preferred *trans*-configuration [28] increases; the small rotatory powers of the compounds containing five or more glycine residues may be due either to their being able to take up a preferred conformation in which the individual contributions of the two cysteine residues to the optical rotatory power very nearly cancel one another out, or to the increased flexibility of the molecules and the consequent multiplication of optically cancelling conformations.[29]

Experimental

Optical rotations were measured in 2 dm. tubes (estimated error ±0·01°).

Paper chromatograms were run on Whatman No. 2 paper using the following solvent systems and conditions: *A*, Butan-1-ol–pyridine–water (2 : 1 : 2), equilibrated for 7 days; descending development. *B*, Butanol–acetic acid–water (1500 : 250 : 564), equilibrated for 4 weeks; descending development. *C*, Acetone–urea–water (60 : 0·5 : 40); [30] ascending development.

Paper electrophoresis was carried out on Whatman No. 2 paper in a horizontal and a vertical apparatus; distances moved from the origin towards the cathode are designated as + and towards the anode −. The following experimental conditions were used: *K*, Vertical; 0·5N-acetic acid (pH 2·8); 650 v; 2 hr. *L*, Vertical; borate buffer (pH 9·3); [31] 650 v; 1 hr. *M*, Horizontal; 0·5N-acetic acid; 600 v; 2 hr. *N*, Horizontal; borate buffer (pH 9·4); [31] 250 v; 2 hr.

Spots were revealed with ninhydrin, chlorine–starch–iodine [21] or nitroprusside, with or without added cyanide.[32]

S-Benzyl-L-cysteine, m. p. 212—214°, $[\alpha]_D^{23·5}$ +26·8° (*c* 1·0 in N-NaOH), was prepared by the method of Wood and du Vigneaud [33] in an average yield of 73% and converted (64—83%

[26] Rydon, Ciba Foundation Symposium on Amino-acids and Peptides with Antimetabolic Activity, 1958, p. 200; cf. Boissonnas and Schumann, *Helv. Chim. Acta*, 1952, **35**, 2229.
[27] Wade, Winitz, and Greenstein, *J. Amer. Chem. Soc.*, 1956, **78**, 373.
[28] Pauling, Corey, and Branson, *Proc. Nat. Acad. Sci.*, 1951, **37**, 205.
[29] Cf. Brewster, *J. Amer. Chem. Soc.*, 1959, **81**, 5483.
[30] Bentley and Whitehead, *Biochem. J.*, 1950, **46**, 341.
[31] Britton, "Hydrogen Ions," Chapman and Hall Ltd., London, 4th edn., 1955, Vol. I, p. 363.
[32] Toennies and Kolb, *Analyt. Chem.*, 1951, **23**, 823.
[33] Wood and du Vigneaud, *J. Biol. Chem.*, 1939, **130**, 109.

yield) into the benzyl ester toluene-*p*-sulphonate, m. p. 158—159°, $[\alpha]_D^{18}$ −22·0° (*c* 1·0 in 90% EtOH), as described by Hooper *et al.*[2] and into the *N*-benzyloxycarbonyl derivative (70—95% yield), m. p. 97—98°, $[\alpha]_D^{19}$ −44·5° (*c* 1·0 in EtOAc), as described by Harington and Mead.[34]

Synthesis of Protected L-*Cysteinyl-pentaglycyl-*L-*cysteine* (with J. A. Schofield).

S-*Benzyl*-N-*benzyloxycarbonyl-*L-*cysteinyl-diglycyl-glycine Ether Ester* (IV; $n = 3$).—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteine (3·45 g.) and diglycyl-glycine ethyl ester hydrochloride[35] (2·54 g.) in anhydrous pyridine (8 ml.) were treated with di-*o*-phenylene pyrophosphite[36] (3·5 g.) in a flask fitted with a calcium chloride guard-tube. After the contents had been swirled for 5 min., the flask was heated on a boiling-water bath for 45 min. After cooling, ice and water (200 g.) were added and the mixture was kept overnight at 0°. The precipitate was collected by filtration and washed (slurrying) successively with half-saturated sodium hydrogen carbonate solution (200 ml.), N-hydrochloric acid (100 ml.), and water (100 ml.). The dried product (3·4 g., 63%), m. p. 118·5—120·5°, was recrystallised from chloroform–ether, giving the required ester (2·58 g.; 47%), m. p. 124·5—126°, $[\alpha]_D^{21\cdot5}$ −23·8° (*c* 1·0 in dioxan) (Found: N, 10·3. Calc. for $C_{26}H_{32}N_4O_7S$: N, 10·3%); other preparations gave yields of 39% and 45%.

The method of Hooper *et al.*[2] gave higher yields (62—65%) but the product, m. p. 125—127°, was partially racemised, $[\alpha]_D^{26}$ −17·7° (*c* 4·7 in dioxan); Hooper *et al.*[2] record m. p. 120°, $[\alpha]_D^{20}$ −2·2° (*c* 4·6 in dioxan), and their material was obviously extensively racemised.

*Diglycyl-*S-*benzyl-*L-*cysteine Benzyl Ester* (V; $n = 2$; R = H).—*N*-Benzyloxycarbonyl-diglycyl-*S*-benzyl-L-cysteine benzyl ester, prepared as described by Crofts *et al.*[12] in 75—92% yield, had m. p. 113·5—114·5°, $[\alpha]_D^{22}$ −34·1° (*c* 1·0 in 50% aqueous pyridine); the yield obtained by a mixed anhydride coupling of *N*-benzyloxycarbonyl-glycyl-glycine[37] and *S*-benzyl-L-cysteine benzyl ester, by means of ethyl chloroformate and triethylamine in chloroform, was only 49%.

This ester (2·25 g.) was kept at room temperature with a solution of dry hydrogen bromide (4·1 g.) in acetic acid (25 ml.). After 1 hr., anhydrous ether (150—200 ml.) was added and the precipitate collected after 40 min. Recrystallisation from ethanol–ether afforded *diglycyl-*S-*benzyl-*L-*cysteine benzyl ester hydrobromide* (1·0 g., 49%), m. p. 147—148°, $[\alpha]_D^{14}$ −38·0° (*c* 1·0 in 50% aqueous pyridine), $[\alpha]_D^{16\cdot5}$ −39·3° (*c* 1·0 in H_2O), chromatographically homogeneous (R_F 0·58 in system *A*) (Found: C, 50·6, 50·4; H, 5·4, 5·3; N, 8·6. $C_{21}H_{26}BrN_3O_4S$ requires C, 50·8; H, 5·3; N, 8·5%); other preparations gave yields varying from 51 to 78%.

S-*Benzyl*-N-*benzyloxycarbonyl-*L-*cysteinyl-pentaglycyl-*S-*benzyl-*L-*cysteine Benzyl Ester* (I; $n = 5$).—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-diglycyl-glycine ethyl ester (2·93 g.) was added to hot ethanol (70 ml.) containing hydrazine hydrate (3·8 ml.); the mixture was shaken vigorously and then left to cool overnight. The precipitated hydrazide (2·57 g., 90%), m. p. 180—185°, was recrystallised from aqueous ethanol, affording the pure compound (2·2 g., 76%), m. p. 184·5—186°, $[\alpha]_D^{19}$ −27·8° (*c* 1·0 in 50% aqueous pyridine) (Found: C, 53·6; H, 5·7; N, 15·2. Calc. for $C_{24}H_{30}N_6O_6S$: C, 54·3; H, 5·7; N, 15·8%) (Hooper *et al.*[2] record m. p. 196°).

This hydrazide (2·65 g.) was dissolved by gentle warming in 80% acetic acid (80 ml.); the solution was cooled in ice and treated with ice-cold concentrated hydrochloric acid (5 ml.), followed by ice-cold 10% sodium nitrite solution (4 ml.). The azide formed was extracted into chloroform and the extract washed thrice with saturated sodium hydrogen carbonate solution and then dried (Na_2SO_4), the temperature being kept below 5° throughout. At the same time, a chloroform solution of diglycyl-*S*-benzyl-L-cysteine benzyl ester was prepared by dissolving the hydrobromide (2·48 g.) in a little water, adding chloroform (140 ml.) and triethylamine (1·6 ml.), and then, finally, shaking with sufficient anhydrous potassium carbonate to absorb all the water; the solution was then dried (Na_2SO_4). The two filtered chloroform solutions were mixed at 0° and the mixture was allowed to warm to room temperature overnight. Most of the chloroform was removed from the resulting gel under reduced pressure and the residue was then treated with ether. The resulting off-white solid (4·35 g., 95%) was collected and recrystallised, once from aqueous pyridine and once from aqueous ethanol, affording the fully protected heptapeptide (I; $n = 5$) (2·27 g., 50%), m. p. 225—226° (decomp.),

[34] Harington and Mead, *Biochem. J.*, 1936, **30**, 1598.
[35] Cook and Levy, *J.*, 1950, 646.
[36] Crofts, Markes, and Rydon, *J.*, 1958, 4250.
[37] Bergmann and Zervas, *Ber.*, 1932, **65**, 1192.

$[\alpha]_D^{17}$ −32·5° (*c* 0·5 in 50% aqueous pyridine), $[\alpha]_D^{22}$ −25·3° (*c* 0·6 in acetic acid) (Found: C, 58·7; H, 5·8; N, 10·9. Calc. for $C_{45}H_{51}N_7O_{10}S_2$: C, 59·1; H, 5·6; N, 10·7%); Lautsch and Kraege [4] give m. p. 224—225°, $[\alpha]_D^{23}$ −27·2° (*c* 1·0 in acetic acid), for material synthesised by a different route. In eight other preparations the yield varied from 33 to 61%.

Synthesis of Protected L-*Cysteinyl-hexaglycyl-*L-*cysteine.*

Derivatives of S-*Benzyl-*N-*benzyloxycarbonyl-*L-*cysteinyl-triglycyl-glycine.*—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine ether ester (IV; $n = 2$), prepared in 85% yield by the method of Crofts *et al.*,[12] had m. p. 112—114°, $[\alpha]_D^{20}$ −13·5° (*c* 2·0 in EtOH); a much poorer yield (40%) was obtained by coupling *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteine and glycyl-glycine ethyl ester with the aid of dicyclohexylcarbodi-imide in chloroform or methylene chloride. This ester was converted into the hydrazide, m. p. 163—164°, $[\alpha]_D^{18}$ −29·4° (*c* 1·0 in 50% aqueous pyridine), after recrystallisation from aqueous ethanol, in 55% yield as described by Hooper *et al.*,[2] who record m. p. 164° but give no optical rotation.

This hydrazide (2·07 g.) was dissolved in warm 50% acetic acid (60 ml.), and treated at 0° with concentrated hydrochloric acid (7 ml.), followed by 10% aqueous sodium nitrite (5 ml.). The azide formed was extracted with chloroform, washed thrice with saturated sodium hydrogen carbonate solution, and dried (Na_2SO_4), at <5° throughout. At the same time, a chloroform solution of glycyl-glycine ethyl ester was prepared as usual from the hydrochloride (0·86 g.) and triethylamine. The filtered chloroform solutions were mixed at 0° and allowed to attain room temperature overnight. Working up as usual and recrystallisation from aqueous ethanol gave the *ethyl ester* (IV; $n = 4$) (2·0 g., 76%), m. p. 159—161°, $[\alpha]_D^{18}$ −28·9° (*c* 0·5 in 50% aqueous pyridine) (Found: C, 56·7; H, 6·5; N, 11·6. $C_{28}H_{35}N_5O_8S$ requires C, 55·9; H, 5·9; N, 11·6%). This ester (1·1 g.) was treated in hot ethanol (50 ml.) with hydrazine hydrate (2 ml.); after the mixture had cooled overnight, the precipitate (0·9 g., 84%), m. p. 187—190°, was recrystallised from methanol and from ethylene glycol, affording the *hydrazide* (0·61 g., 57%), m. p. 209—211° (Found: C, 52·5; H, 5·6. $C_{26}H_{33}N_7O_7S$ requires C, 53·1; H, 5·7%).

*Derivatives of Tetraglycyl-*S-*benzyl-*L-*cysteine.*—*N*-Benzyloxycarbonyl-glycyl-glycine [37] (2·66 g.) and diglycyl-*S*-benzyl-L-cysteine benzyl ester hydrobromide (4·96 g.) in anhydrous pyridine (20 ml.) were treated with di-*o*-phenylene pyrophosphite [36] (3·5 g.). The mixture was swirled for 5 min. and then heated on a boiling-water bath for 40 min. with intermittent shaking. Working up as usual, followed by recrystallisation from, successively, aqueous ethanol, aqueous acetone, and aqueous dimethylformamide, gave the *N*-benzyloxycarbonyl benzyl ester (3·95 g., 60%), m. p. 216—217°, $[\alpha]_D^{29}$ −24·6° (*c* 1·0 in acetic acid) (Found: N, 10·9. Calc. for $C_{33}H_{37}N_5O_8S$: N, 10·6%); Lautsch and Kraege [4] give m. p. 217—218·5°, $[\alpha]_D^{23}$ −24·5°, for material synthesised by a different route. In subsequent preparations the yields ranged from 62 to 77%.

This compound (1·0 g.) was kept for 1 hr. at room temperature, with occasional shaking, with a solution of dry hydrogen bromide (2·8 g.) in acetic acid (17 ml.); anhydrous ether (200 ml.) was then added and the mixture set aside overnight. Collection of the precipitate and recrystallisation from ethanol–ether gave the *benzyl ester hydrobromide* (0·57 g., 53%), m. p. 152—154° (Found: N, 11·9. $C_{25}H_{32}BrN_5O_6S$ requires N, 11·5%), chromatographically homogeneous (R_F 0·41 in system *A*).

S-*Benzyl-*N-*benzyloxycarbonyl-*L-*cysteinyl-hexaglycyl-*S-*benzyl-*L-*cysteine Benzyl Ester* (I; $n = 6$).—(*a*)Tetraglycyl-*S*-benzyl-L-cysteine benzyl ester hydrobromide (1·0 g.) was dissolved in warm water (15 ml.), and the solution mixed with ethyl acetate (60 ml.). Triethylamine (2·5 ml.) was added, followed by sufficient anhydrous potassium carbonate to absorb the water; the ethyl acetate layer was then separated and dried (Na_2SO_4). At the same time, *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-glycyl-glycine hydrazide (0·8 g.) was dissolved in warm 50% acetic acid (14 ml.) and treated at 0° with ice-cold concentrated hydrochloric acid (4 ml.), followed by ice-cold 10% aqueous sodium nitrite (3 ml.); the azide was extracted with ethyl acetate, washed thrice with saturated sodium hydrogen carbonate solution, and dried (Na_2SO_4), at <5° throughout. The two filtered ethyl acetate solutions were mixed at 0° and allowed to attain room temperature overnight. The solvent was then removed under reduced pressure and the residue twice recrystallised from acetic acid (charcoal), giving the protected *octapeptide* (0·68 g., 45%), m. p. 244—246° (decomp.), $[\alpha]_D^{22}$ −30·3° (*c* 0·4 in 50% aqueous pyridine), $[\alpha]_D^{21}$ −20·7° (*c* 0·6 in acetic acid) (Found: C, 57·9; H, 5·9; N, 10·6. $C_{47}H_{54}N_8O_{11}S_2$ requires C, 58·1; H, 5·6; N, 11·5%); Lautsch *et al.*[8] give m. p. 258—260°, $[\alpha]_D$ −25·7° (in acetic acid)

for material synthesised by a different route but record no analysis. In further preparations, chloroform was found to be as good as ethyl acetate as solvent for this coupling, and ether to be much inferior; the yields obtained varied from 35 to 61%.

(*b*) *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-triglycyl-glycine azide (from the hydrazide, 0·85 g.) and diglycyl-*S*-benzyl-L-cysteine benzyl ester (from the hydrobromide, 0·72 g.) were coupled, as usual, in chloroform. Recrystallisation of the crude product (0·94 g., 67%) from aqueous pyridine gave the same protected octapeptide, m. p. 246—247° (decomp.) (Found: N, 11·2%).

Oxidations.

Removal of the protecting groups from the protected peptides and oxidation of the resulting cysteine peptides were carried out in the apparatus shown in Fig. 1, consisting of a flat-bottomed cylindrical vessel fitted with a gas inlet tube *A* and a side-arm *B* and provided with two externally-fitting ground glass caps (B40 joints), one (*C*) provided with a stirrer guide, the other (*D*) being a simple cap.

The protected peptide was dissolved in liquid ammonia in the reaction vessel, fitted with *C* and a stirrer, tap *A* being closed and *B* fitted with a soda-lime guard tube. Small pieces of alkali metal were added, with stirring, until a blue colour was stable for 15 min. Ammonium chloride equivalent to the amount of alkali metal used in excess of the amount required to form the alkali-metal salt of the cysteine peptide was added, with stirring, and the cap *C* replaced by cap *D*. The ammonia was then allowed to evaporate, initially at atmospheric pressure and then for 2—3 hr. at 0·1 mm. The vessel was then filled, through *A*, with nitrogen, and a current of nitrogen passed through the apparatus while boiled-out distilled water was added to dissolve the alkali-metal salt of the cysteine peptide. The pH was then brought to 8·6 with 2N-hydrochloric acid, and the volume finally made up with boiled-out distilled water to give the desired (usually 0·5%) concentration of the peptide. Oxygen was then passed through the solution at about 500 ml./min. until oxidation was complete (negative nitroprusside reaction). The resulting solution was extracted with ether, evaporated almost to dryness under reduced pressure, brought to pH 5 with hydrochloric acid, and then evaporated to dryness under reduced pressure at room temperature and finally over calcium chloride or phosphorus pentoxide in a vacuum-desiccator.

Oxidation of Diglycyl-L-cysteine.—(*a*) *N*-Benzyloxycarbonyldiglycyl-*S*-benzyl-L-cysteine benzyl ester (1·2 g.) was reduced with potassium in liquid ammonia (50 ml.), and the resulting diglycyl-L-cysteine oxidised in water (100 ml.) at pH 8·6 (6·5 hr.).

Paper chromatography showed only one, ninhydrin-positive, product, having R_F 0·04 in system *A* and R_F 0·00 in system *B*. Paper electrophoresis showed the main oxidation product to be accompanied by a small amount of another product, thus:

Conditions (see p. 1758)	*K*	*L*	*M*
Movement of major component (cm.)	+9·5	−3·5	+14·0
Movement of minor component (cm.)	+12·0	−2·5	+18·5

(*b*) The product of another reduction was dissolved in water (100 ml.) and run through a column (3 × 19 cm.) of Zeo-Karb 225 ion-exchange resin; water was then run through the column until the effluent was free from chloride ions. The peptide was then eluted with 0·2N-ammonia, and the eluate evaporated to dryness under reduced pressure. The residue was dissolved in water (30 ml.) and again evaporated to dryness. Six such treatments gave a product (0·25 g., 24%) which was free from ammonium and potassium ions; a specimen, recrystallised from acetic acid–ether and dried at 80°, had m. p. 190—200° (decomp.) and was shown by analysis to be *bisdiglycyl-L-cystine dihydrate* (Found: C, 33·3; H, 6·3; N, 15·9. $C_{14}H_{24}N_6O_8S_2,2H_2O$ requires C, 33·3; H, 5·6; N, 16·7%).

(*c*) The product from another experiment was dissolved in water (15 ml.), containing sodium hydrogen carbonate (1·0 g.), and shaken for 5 hr. with 1-fluoro-2,4-dinitrobenzene (0·9 ml.) in ethanol (20 ml.); a further 0·3 ml. of fluorodinitrobenzene was then added and the mixture shaken for a further 3 hr. The mixture was then extracted repeatedly with ether, and the residual solution acidified. Recrystallisation of the precipitate, first from aqueous methanol and then from acetic acid, gave *bis*-(N-2,4-*dinitrophenyldiglycyl*)-L-*cystine*, m. p. 174—176° (decomp.) (Found: C, 38·3; H, 3·8; N, 16·8. $C_{26}H_{28}N_{10}O_{16}S_2$ requires C, 39·0; H, 3·5; N, 17·5%), chromatographically homogeneous (R_F 0·38 in system *A*).

Oxidation of L-*Cysteinyl-pentaglycyl-*L-*cysteine.*—(*a*) *S*-Benzyl-N-benzyloxycarbonyl-L-cysteinyl-pentaglycyl-*S*-benzyl-L-cysteine benzyl ester (0·9 g.) was reduced with sodium (0·51 g.) in liquid ammonia (50 ml.), and the resulting L-cysteinyl-pentaglycyl-L-cysteine oxidised in water (100 ml.) at pH 8·6 (25 min.).

Paper chromatography revealed only one ninhydrin-positive product, appearing as a single, precisely defined, spot (R_F 0·78) in system *C* and as a single, tailed spot (R_F 0·13 and 0·02, respectively) in systems *A* and *B*. Paper electrophoresis revealed the major component accompanied by a small amount of a slower-moving product, thus:

Conditions	*M*	*N*
Movement of major component (cm.)	+13·0	−4·0
Movement of minor component (cm.)	+9·0	−1·5

(*b*) The protected heptapeptide (2·26 g.) was reduced with sodium in liquid ammonia (110 ml.). Excess of sodium was destroyed with ammonium sulphate, and the ammonia evaporated as usual. The residue was dissolved, under nitrogen, in 0·5N-sulphuric acid (120 ml.)

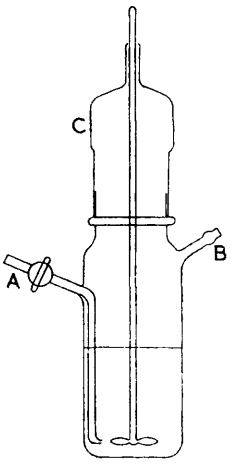


FIG. 1. *Apparatus for removal of protecting groups from protected dicysteine peptides and oxidation of the resulting free peptides.*

and treated with 20 ml. of an acid solution of mercuric sulphate, prepared by mixing mercuric sulphate (200 g.), 10N-sulphuric acid (500 ml.), concentrated sulphuric acid (40 ml.), and water (600 ml.) in the order stated. After 18 hr., the copious white precipitate was collected by centrifugation, washed thrice with 0·5N-sulphuric acid, and dried overnight in a vacuum (P_2O_5).

A brisk stream of hydrogen sulphide was passed through a suspension of the product (*ca.* 3 g.) in water (25 ml.) for 2·5 hr. with occasional shaking; mercuric sulphide was then removed by centrifugation and washed thrice with water. To the supernatant liquid and washings sulphuric acid was added to give a concentration of 0·5N. Nitrogen was passed through the solution for 3 hr. to remove hydrogen sulphide, after which the pH was brought to 8·6 with saturated barium hydroxide solution, and the precipitated barium sulphate removed. The filtrate and washings therefrom were made up to 280 ml. with boiled-out distilled water and oxygen passed through at 500 ml./min. until oxidation was complete (15 min.). The solution was concentrated under reduced pressure to 80 ml. and sulphuric acid added to remove all barium ions. The filtered solution was evaporated almost to dryness under reduced pressure and the residue treated with an excess of absolute ethanol. The precipitated SS′-*dehydro*-L-*cysteinyl-pentaglycyl*-L-*cysteine* (0·71 g., 57%), collected by centrifugation, washed with ethanol and ether, and dried to constant weight in a vacuum ($CaCl_2$), had m. p. 197—200° (decomp.), $[\alpha]_D^{21}$ −4·5° (*c* 0·4 in H_2O) (Found: C, 37·3; H, 5·1; N, 18·1; residue, 3·6. Corrected for residue: C, 38·7; H, 5·3; N, 18·8. $C_{16}H_{25}N_7O_8S_2$ requires C, 37·9; H, 5·0; N, 19·3%); paper electrophoresis (conditions *M* and *N*) gave patterns similar to those obtained with the oxidation product from experiment (*a*).

(*c*) In another experiment the oxidation product, from the protected heptapeptide (0·9 g.), was dissolved in 0·4N-sodium carbonate (25 ml.) and converted into the 2,4-dinitrophenyl

derivative as described for the oxidation product of diglycyl-cysteine. The product was freed from 2,4-dinitrophenol by extraction (Soxhlet) with ether; the resulting N-2,4-*dinitrophenyl*-SS′-*dehydro*-L-*cysteinyl-pentaglycyl*-L-*cysteine*, m. p. 190—196° (decomp.), was chromatographically homogeneous (R_F 0·37 in system *A*) and analysed as the *dihydrate* (Found: C, 37·3; H, 4·1; N, 17·7. $C_{22}H_{27}N_9O_{12}S_2,2H_2O$ requires C, 37·2; H, 4·4; N, 17·7%).

Oxidation of L-*Cysteinyl-hexaglycyl*-L-*cysteine*.—(*a*) *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-hexaglycyl-*S*-benzyl-L-cysteine benzyl ester (0·86 g.) was reduced and the product oxidised as described for the pentaglycyl compound; the final oxidation required 35 min. Paper chromatography showed the presence of only one product (R_F 0·08 in system *A* and 0·02 in system *B*), but paper electrophoresis (conditions *M*) showed the main product (migration + 12·0 cm.) to be accompanied by a small amount of a second product (migration + 8·0 cm.).

(*b*) The protected octapeptide (2·0 g.) was reduced and the product oxidised by the mercaptide procedure described above for the heptapeptide. The resulting SS′-*dehydro*-L-*cysteinyl-hexaglycyl*-L-*cysteine* (0·54 g., 47%) had m. p. 189—191° (decomp.), $[\alpha]_D^{20}$ −4·3° (*c* 0·5 in water), and analysed as the *monohydrate* (Found: C, 36·3; H, 5·5; N, 18·2; S, 10·6; residue, 3·0. Corrected for residue: C, 37·7; H, 5·7; N, 18·7; S, 11·7. $C_{18}H_{28}N_8O_9S_2,H_2O$ requires C, 37·1; H, 5·2; N, 19·2; S, 11·0%).

(*c*) In another experiment the oxidation product, without isolation, was converted as usual into the 2,4-dinitrophenyl derivative. The resulting N-2,4-*dinitrophenyl*-SS′-*dehydro*-L-*cysteinyl-hexaglycyl*-L-*cysteine*, m. p. 159—163° (decomp.), was chromatographically homogeneous (R_F 0·35 in system *A*) and analysed as the *monohydrate* (Found: C, 38·1; H, 3·7; N, 16·4. $C_{24}H_{30}N_{10}O_{13}S_2,H_2O$ requires C, 38·1; H, 3·7; N, 17·1%).

Partial Hydrolysis of Oxidation Products.

(a) *Dehydro-cysteinyl-pentaglycyl-cysteine*.—(i) The 2,4-dinitrophenyl derivative (10 mg.) was heated at 98° with a hydrolysis mixture (0·5 ml.) containing equal volumes of acetic acid and 20% hydrochloric acid; from time to time a little of the solution was spotted on paper. After addition of marker spots (glycine and the product of heating a mixture of L-cystine and its bis-*N*-2,4-dinitrophenyl derivative in the same hydrolysis mixture) the chromatogram was developed with solvent system *A*. After having been sprayed with ninhydrin and heated, the chromatogram had the appearance shown in Fig. 2; the markers are cystine (R_F 0·04), glycine (R_F 0·10), mono-*N*-2,4-dinitrophenylcystine (R_F 0·41), and bis-*N*-2,4-dinitrophenylcystine (R_F 0·60).

(ii) In another experiment, the 2,4-dinitrophenyl derivative (3 mg.) was heated for 15 min. at 98° with the same hydrolysis mixture (0·15 ml.). The solution was rapidly cooled and evaporated to dryness at room temperature; the residue was dissolved in a little dimethylformamide and applied as a band to a strip of filter paper. After development (system *A*), the band corresponding to the main product (R_F 0·38) was cut out and eluted with dimethylformamide. The eluate was evaporated to dryness under reduced pressure and the residue dissolved in 0·05 ml. of a solution of performic acid (from formic acid, 6 ml., and 20-vol. hydrogen peroxide, 1 ml.). After an hour the solution was evaporated to dryness under reduced pressure and the residue applied in dimethylformamide as a band to a strip of filter paper. Development with system *A* revealed the presence of cysteic acid (R_F 0·08; colourless and ninhydrin-positive) and a yellow, ninhydrin-negative substance (R_F 0·34). The latter was eluted with dimethylformamide and heated at 98° for 3·5 hr. with a few drops of the usual hydrolysis mixture. Chromatography (system *A*) showed the hydrolysate to contain only glycine (R_F 0·10; colourless and ninhydrin-positive) and *N*-2,4-dinitrophenylcysteic acid (R_F 0·38; yellow and ninhydrin-negative).

(iii) In a third experiment, the initial hydrolysis product, prepared as in (ii) from the *N*-2,4-dinitrophenyl derivative (5 mg.), was dissolved in 2% aqueous sodium hydrogen carbonate (0·5 ml.) and shaken mechanically for 4 hr. with 1-fluoro-2,4-dinitrobenzene (0·05 ml.) in a little ethanol. The solution was concentrated, extracted with ether, and acidified; the yellow precipitate, collected by centrifugation, was applied to paper in dimethylformamide, and the chromatogram was developed with system *A*. The main product (R_F 0·46; yellow and ninhydrin-negative), which was accompanied by a trace of 2,4-dinitrophenylglycine (R_F 0·66), was eluted with dimethylformamide and heated at 98° for 3 hr. with a few drops of the usual hydrolysis mixture. The only products detected in the hydrolysate by paper chromatography (system *A*) were glycine (R_F 0·10) and bis-*N*-2,4-dinitrophenylcystine (R_F 0·59).

(b) *Dehydro-cysteinyl-hexaglycyl-cysteine.*—The procedures were the same as those used with the pentaglycyl compound; in the following, R_F values are all for system *A*.

(i) The paper chromatogram of the progressive hydrolysis of the 2,4-dinitrophenyl derivative had the appearance shown in Fig. 3.

(ii) Oxidation of the initial hydrolysis product (R_F 0·39) gave cysteic acid (R_F 0·08) and a yellow, ninhydrin-negative substance (R_F 0·34); hydrolysis of the latter gave only glycine (R_F 0·10) and *N*-2,4-dinitrophenylcysteic acid (R_F 0·38).

FIG. 2. *Chromatogram showing the effect of various periods of hydrolysis on the dinitrophenyl derivative of the oxidation product of* L-*cysteinyl-pentaglycyl-*L-*cysteine.*

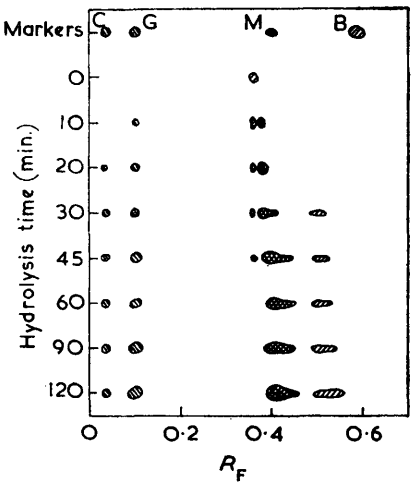
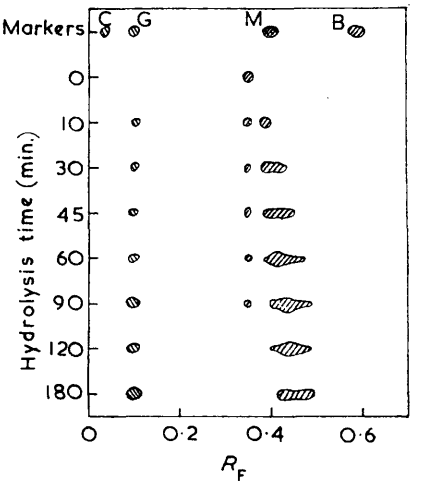


FIG. 3. *Chromatogram showing the effect of various periods of hydrolysis on the dinitrophenyl derivative of the oxidation product of* L-*cysteinyl-hexaglycyl-*L-*cysteine.*



//// *Yellow.* \\\\ *Ninhydrin positive.*

C = L-*Cystine.* G = *Glycine.* M = *Mono*-N-2,4-*dinitrophenyl-*L-*cystine.* B = *Bis*-N-2,4-*dinitrophenyl-*L-*cystine.*

(iii) Dinitrophenylation of the initial hydrolysis product (R_F 0·39) gave a yellow, ninhydrin-negative product (R_F 0·47) which, on oxidation with performic acid, gave *N*-2,4-dinitrophenylcysteic acid (R_F 0·38) and another yellow, ninhydrin-negative product (R_F 0·34). Hydrolysis of the latter gave only glycine (R_F 0·10) and *N*-2,4-dinitrophenylcysteic acid (R_F 0·38).

Partial Dinitrophenylation of Oxidation Products.

(a) *Bisdiglycyl-*L-*cystine.*—The crude oxidation product, from *N*-benzyloxycarbonyl-diglycyl-*S*-benzyl-L-cysteine benzyl ester (1·2 g.), was dissolved in water (15 ml.) containing sodium hydrogen carbonate (1 g.), and the solution shaken mechanically for 2 hr. with 1-fluoro-2,4-dinitrobenzene (0·23 ml., 0·85 equiv.) in ethanol (10 ml.). Ethanol was removed under reduced pressure and the residue diluted with water and extracted with ether. Paper chromatography of the residual solution (system *A*) showed three spots: (i) R_F 0·04; colourless, ninhydrin and cyanide–nitroprusside positive; bisdiglycyl-L-cystine. (ii) R_F 0·30; yellow, ninhydrin and cyanide–nitroprusside positive; mono-*N*-2,4-dinitrophenyl-diglycyl-L-cystine. (iii) R_F 0·39; yellow, ninhydrin negative, cyanide–nitroprusside positive; bis-*N*-2,4-dinitrophenyl-diglycyl-L-cystine.

(b) *Dehydro-cysteinyl-pentaglycyl-cysteine.*—The crude oxidation product, from the protected heptapeptide, (I; $n = 5$) (0·9 g.), was treated similarly with 1-fluoro-2,4-dinitrobenzene (0·08 g., 0·43 equiv.). Paper chromatography of the product (system *A*) showed the presence of only the dehydroheptapeptide (R_F 0·10; colourless; ninhydrin and cyanide–nitroprusside positive) and its *N*-2,4-dinitrophenyl derivative (R_F 0·37; yellow; ninhydrin negative and cyanide–nitroprusside positive).

(c) *Dehydro-cysteinyl-hexaglycyl-cysteine.*—A similar experiment with the protected octapeptide (I; $n = 6$) gave only the dehydro-octapeptide (R_F 0·06; colourless; ninhydrin and

cyanide–nitroprusside positive) and its *N*-2,4-dinitrophenyl derivative (R_F 0·35; yellow; ninhydrin-negative and cyanide–nitroprusside positive).

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