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A Study of the Peptides of Cystine in Partial Hydrolysates of Wool

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Although much work has been carried out on the disulphide group of wool, no information has been obtained as to which particular amino-acid residues are linked with cystine in the wool polypeptide chain. Such information seems specially desirable because wool and the related keratins are characterized by a high cystine content. Both amino and both carboxyl groups of cystine are probably involved in peptide linkages (Geiger, Harris, Mizell & Patterson, 1941; Lindley & Phillips, 1945), and Phillips (1946), on the basis of differences of reactivity of the combined cystine of wool towards sodium bisulphite and other mild reagents, has suggested that the more reactive fraction is associated with a polar environment. Consden, Gordon & Martin (1949) succeeded in identifying a number of lower peptides of glutamic and aspartic acids in partial hydrolysates of wool, and we were encouraged to apply similar methods to identify the peptides of cystine. Although Consden *et al.* (1949) found that some peptides of cystine with dicarboxylic amino-acids were present in a 3-day partial hydrolysate of wool, much more of the cystine of such hydrolysates occurred in the neutral ionophoretic fraction. The problem was, therefore, to separate the cystine peptides from this complex mixture, before attempting to employ paper chromatography. This has been achieved by oxidation which easily converts the disulphide groups into sulphonic acids. The cysteic acid and cysteic acid compounds thus produced could then be separated

from the non-acidic constituents. The latter would of course include any peptide which contained an equal number of cysteic acid and basic residues. Oxidation has additional advantages: first, acidic substances are the easiest to investigate (Consden *et al.* 1949); secondly, conversion to sulphonic acids avoids working with cystine compounds, which in general do not behave satisfactorily on paper chromatograms.

The object of the present investigation was to demonstrate the presence of as many peptides of cystine as possible (in the derived form of peptides of cysteic acid). This has proved much simpler than the investigation of the peptides of aspartic and glutamic acids, since, among other reasons, only one acidic amino-acid is here involved. The results obtained have already been given in a preliminary report (Consden & Gordon, 1948*a*).

The stages of the analysis are shown in Fig. 1. Acidic peptides and amino-acids in partial hydrolysates of wool were first removed chromatographically on an anion-exchange resin and the non-acidic fraction was then oxidized with bromine water. The cysteic acid and acidic cysteic acid peptides were then separated from non-acidic substances by repeating chromatographic separation on the anion-exchange resin. This was followed by ionophoresis, which separated the acidic peptides into groups with similar electrical mobilities. Each group was then examined by chromatography on paper and the sub-fractions thus obtained were hydrolysed and the constituent amino-acids identified. Further, those amino groups not involved in peptide linkage were destroyed by deamination and the amino-acids re-

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maintaining after hydrolysis were identified. In many cases the sub-fractions from the paper chromatograms were found to consist of single dipeptides of cysteic acid. Identification of some of these was confirmed by comparison of their ionophoretic and chromatographic properties with those of the syn-

however, was precluded, because of the possibility of the production of intermediate oxidation products from the cystine. Evidence for this has been found by Harris & Smith (1937) for the combined cystine of wool, and further direct evidence has been obtained in the present investigation, both for free cystine and combined cystine. These intermediates are undesirable, since of the two probable products, the disulphoxide (Toennies & Lavine, 1936) is a neutral substance and hence would be lost in the preliminary stages, and the sulphinic acid readily dismutates under hydrolysis conditions to cysteic acid and cystine (Lavine, 1936); the latter would introduce complications in the analysis of the peptides.

EXPERIMENTAL

General chromatographic methods. Separations on resin, ionophoresis, working up of ionophoretic fractions and analysis on paper chromatograms were carried out as described by Consden *et al.* (1949). In all cases, two-dimensional chromatography of the ionophoretic fractions was carried out with *s*-collidine as first solvent, and with phenol used in an atmosphere of coal gas and NH_3 as second solvent. (These solvents will henceforth be referred to as 'collidine' and 'phenol', respectively.) Hydrolysis of the chromatogram spots indicated that a large amount of material was present in some of the spots so that when deamination, followed by hydrolysis, was carried out on replicate chromatograms, a small proportion of the extract of these spots was hydrolysed as well to serve as a control for the deamination procedure. Chromatography in phenol was generally sufficient for the identification of the amino-acids produced after hydrolysis or after deamination followed by hydrolysis, but in some cases, where it was necessary to distinguish between fast-running amino-acids, especially valine, leucine, proline and phenylalanine, *o*-cresol was employed (Consden, Gordon & Martin, 1944) in an atmosphere of NH_3 produced from a 0.3% (w/v) solution at the bottom of the chamber. Following Moore & Stein (1948), 8-hydroxyquinoline (0.1%) was added to this solvent. In this system, good chromatograms were obtained in which proline appeared, after ninhydrin treatment, as a yellow spot becoming pink on continued heating, between the spots of leucine and phenylalanine. In some cases extracts of the peptide spots from replicate chromatograms, after hydrolysis, were chromatographed in collidine as well as in *o*-cresol and phenol in order to examine for basic amino-acids. After chromatography of all the treated peptides, acidified ninhydrin was used to spray the chromatograms in order to neutralize the alkaline material formed from the salts introduced during hydrolysis (Consden & Gordon, 1948b).

Hydrolysis of wool and preliminary separation. The wool used was similar to that previously described (Consden *et al.* 1949). Partial hydrolysis was carried out with 10N-HCl at 37°. Hydrolysis for 3 days only was first attempted, but the mixture was found, by analysis on paper chromatograms after separation on resin and oxidation, to be too complex, apparently containing many tripeptides and higher peptides of cysteic acid. Hydrolysis for 10 days proved to give a sufficient amount of lower peptides for further investigation. After removal of most of the HCl by repeated evaporation

Fig. 1. Successive stages of analysis of cystine peptides.

thetic peptides. In other cases, where the synthetic peptide was not available, confirmation was obtained by comparison with closely related synthetic peptides. As in the investigation of peptides of dicarboxylic amino-acids (Consden *et al.* 1949) the amounts of peptides present were assessed roughly by comparison of the intensities of the amino-acid spots produced after hydrolysis with the intensities produced by known amounts of amino-acids (cf. Polson, 1948).

For the oxidation of the cystine fraction, bromine water was used, since the reaction is mild and the conversion is quantitative. The hydrobromic acid formed is easy to remove and there is little likelihood of deep-seated changes in other amino-acid residues except that of tyrosine which undergoes substitution in the benzene ring. Other mild oxidizing agents might also be used, for example, performic acid, which was employed by Sanger (1947) to split insulin into its peptide sub-units. Hydrogen peroxide,

Table 1. Nitrogen distribution after Amberlite separation

	3-day hydrolysate		10-day hydrolysate			
	N		I		II	
	(mg.)	(% of total)	(mg.)	(% of total)	(mg.)	(% of total)
Initial value	232.7	—	212.4	—	212.4	—
Fraction 1-2 (aspartic and glutamic acid fraction)	63.5	27.1	44.8	21.1	40.1	18.9
Fraction 2-1 (neutral and basic fraction)	99.8	42.8	131.7	62.0	134.5	63.4
Fraction 2-2 (cysteic acid fraction)	17.6	7.6	21.7	10.0	19.8	9.3
Recovery (%)	—	77.5	—	93.1	—	91.6

in vacuo, with successive additions of water, the mixture (containing 212.4 mg. N) was treated in the desalting apparatus (Consdén, Gordon & Martin, 1947), brought to a pH of about 4 with N-HCl and transferred quantitatively to a column of Amberlite IR-4 (height 30 cm., diam. 1.5 cm.) which had been adjusted to pH 3-4 (Consdén, Gordon & Martin, 1948). The column was washed with water, the first 100 ml. of eluate were collected (fraction 1-1) and, as judged by chromatography on paper using phenol, contained neutral and basic but no acidic amino-acids or peptides. The acidic fraction (1-2) was then eluted with N-HCl, the movement of the pigment band serving as guide to its complete elution. Fraction 1-1 was then cooled in ice water and Br₂ water added until no further absorption of Br₂ took place. The solution was then repeatedly evaporated *in vacuo* <40° with successive additions of water, to remove excess Br₂ and the bulk of the HBr and finally left overnight *in vacuo* over KOH. The syrup was taken up in 2-3 ml. water, transferred quantitatively to a column of Amberlite precisely similar to that used above, and eluted with water. Portions of the eluate were tested by paper chromatography. The first 50 ml. contained most of the neutral and basic constituents; a further 20 ml. contained only traces of these, whilst no ninhydrin-colouring constituents were present in subsequent eluates. A total of 100 ml. was collected (fraction 2-1), after which the column was washed with N-HCl to elute the acidic fraction, progress being again observed by the movement of the black pigment band. After this had run out, no further ninhydrin-colouring constituents appeared. A total of about 70 ml. was collected (fraction 2-2). Two separate portions (I and II) of a 10-day hydrolysate were fractionated in this way and the N distributions (Kjeldahl) are given in Table 1. For comparison, the data for the 3-day hydrolysate are also given. All figures are corrected for the N of the Amberlite blanks.

Fraction 1-1 from a third portion of the 10-day hydrolysate was treated with excess Br₂ water and the N determined (Kjeldahl). 100% of the N was recovered.

Fractions 2-2 of I and II were found by two-dimensional chromatography in collidine-phenol to contain much cysteic acid and peptides whose spots were relatively weaker in intensity. Many of the latter were yellow at first, slowly changing on standing, or more quickly on continued heating, to purple. Extraction and hydrolysis of the peptide spots from duplicate chromatograms indicated that they were mainly due to lower peptides of cysteic acid. Therefore this material was further investigated as described below.

Ionophoresis and chromatography of fractions. Fraction 2-2 of II was combined with part of fraction 2-2 of I and concentrated to a vol. of 10 ml. The solution contained 23 mg. N.

Fig. 2. Ionophoresis of cysteic acid fraction of wool hydrolysate.

Sodium silicate (4.3 N, 1.1 ml.) was added and the mixture brought to a pH of about 7 with *N*-acetic acid (0.2 ml.), using bromothymol blue as internal indicator. This solution was placed in the gutter of the ionophoresis trough, where it set in a few minutes. Ionophoresis was then carried out at a potential of 300 V. and current of 0.3 amp. After 65 hr. the current was stopped and a paper print taken. Four contiguous bands and a fifth separate band were revealed, all of which had moved towards the anode and, by reference to the print, the jelly was cut into eight sections (A-H) as shown in Fig. 2. After working up of the fractions, each was made up to a volume giving a suitable concentration for chromatography. This was judged by preliminary trials on one-dimensional chromatograms in phenol and in collidine. Two-dimensional chromatography was carried out on all fractions except C, a control mixture of amino-acids being chromatographed in each direction on the same sheet. Peptide spots were examined after extraction from replicate chromatograms coloured with dilute ninhydrin (0.01% in aqueous *n*-butanol). Diagrams of the control chromatograms of fractions D-H, coloured with 0.1% ninhydrin in aqueous *n*-butanol, are shown in Figs. 3-7 and the results of analysis of the peptide spots are given in Table 2. Abbreviations for amino-acids or their residues are as follows (Brand & Edsall, 1947): Ala, alanine; Arg, arginine; Asp, aspartic acid; (CySO₃H), cysteic acid; Glu, glutamic acid; Gly, glycine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine. Where the order of the residues has not been established, the symbols are enclosed in square brackets. Intensities of amino-acid spots after hydrolysis and after deamination followed by hydrolysis are denoted by the symbols 'x' and '?' which have the same significance as in the paper of Consden *et al.* (1949). The spots indicated by shaded areas proved to consist of traces of free amino-acids. The broken lines represent yellow 'salt' spots. In the description which follows, colours and intensities of spots are those developed after treatment with ninhydrin.

Analysis and identification of chromatographic fractions

Fraction A. The results given in Table 2 were obtained from one-dimensional chromatograms developed in phenol, analyses being carried out as described by Consden, Gordon & Martin (1947). Spot 1 was very slow moving (*R_F*, 0.03). Other spots on hydrolysis gave only small amounts of aspartic and glutamic acids, and traces of neutral amino-acids and are not included in Table 2.

Fractions B and C. Fraction B was found to consist largely of free cysteic acid, and also yielded very faint spots similar to those of fraction A. N (Kjeldahl) of this fraction was 0.52 mg. Fraction C consisted of cysteic acid and contained 2.90 mg. N. The N analyses of both these fractions were carried out on the desalted solutions.

Fraction D (Fig. 3). Spots 2 and 3 were cut from a large spot which was more intensely coloured at its right edge. These and spot 4 were yellow at first, finally changing to purple. Spot 1 was faint grey. To confirm the presence of β -sulphoalanylglycine in spots 2 and 3, fraction D (approx. 5%) mixed with the synthetic peptide (approx. 10 μ g.) was chromatographed in two dimensions with the same solvents and for the same time as was done with fraction D alone. Except that the intensity of coloration at the edge of spots 2 and 3 was now somewhat stronger, the mixed

chromatogram was indistinguishable from that of fraction D alone.

Fig. 3. Two-dimensional chromatogram of fraction D. In Figs. 3-7, the arrows show the direction of the developing solvents. The black dot indicates the origin. The spots of the developed chromatogram are outlined by black lines; shaded areas represent traces of free amino-acids; the areas outlined by broken lines represent the yellow 'salt' spots. The small spots at the edges represent the amino-acids of the control mixtures.

Fig. 4. Two-dimensional chromatogram of fraction E. (The position of spot 6 is computed from a chromatogram developed for 41 hr. in each direction.)

Fraction E. Fig. 4 is a composite diagram of two chromatograms of this fraction. One chromatogram was developed for 65 hr. in both directions in order to separate adequately the spots in the upper part of the chromatogram.

Under these conditions, spot 6 was washed off the sheet. The other chromatogram was developed for a shorter time (41 hr.) in both directions for the analysis of spot 6. Spots 2, 5 and 6 were yellow at first, finally becoming strongly purple. Spot 3 was grey. A second deamination and hydro-

served between the mixed chromatograms and that of fraction *E* alone.

Fraction F (Fig. 5). Spots 1-3 were not well separated and weak, and part of this area was yellow at first, turning purple. These spots were not deaminated as they were not

Table 2. *Analysis*

(Amino-acids formed after hydrolysis (*H*) or deamination followed by hydrolysis (*D*) of peptide spots from see Consden *et al.* 1949.)

Frac- tion	Spot no.	$\frac{H}{D}$ or (CySO ₃ H)	Asp	Glu	Ser	Gly	Thr	Ala	Tyr	Val
<i>A</i>	1	<i>H</i>	x x	x	x	?	?	—	—	—
	1	<i>D</i>	x	?	—	—	—	—	—	—
<i>D</i>	1	<i>H</i>	x	—	?	—	—	—	—	—
	2	<i>H</i>	x	—	—	?	—	—	—	—
	2	<i>D</i>	x x	—	—	x	—	—	—	—
	3	<i>H</i>	x x	—	—	x	—	?	—	—
	3	<i>D</i>	—	—	—	x x	—	x	—	—
	4	<i>H</i>	x	—	—	—	—	x	—	—
	4	<i>D</i>	—	—	—	—	—	x x	—	—
<i>E</i>	1	<i>H</i>	x	—	—	x	—	—	—	—
	1	<i>D</i>	x	—	—	—	—	—	—	—
	2	<i>H</i>	x x x	—	—	x x x	—	—	—	—
	2	<i>D</i>	x x	—	—	—	—	—	—	—
	3	<i>H</i>	x	—	—	—	x	—	—	—
	3	<i>D</i>	x	—	?	?	x	—	—	—
	4	<i>H</i>	x x	—	—	—	x x	—	—	—
	4	<i>D</i>	x	—	—	—	—	—	—	—
	5	<i>H</i>	x	—	—	—	—	—	—	x
	5	<i>D</i>	—	—	—	—	—	—	—	x x x
	6	<i>H</i>	x x x	—	—	—	—	—	—	—
	6	<i>D</i>	—	—	—	—	—	—	—	—
<i>F</i>	1	<i>H</i>	x x	—	—	x x	?	?	—	—
	2	<i>H</i>	x x x x x	—	—	x x	x x	x x x x	x	—
	3	<i>H</i>	x x x x	—	—	x	x	x x	x	—
	4	<i>H</i>	x x x	—	—	—	—	—	—	—
	4	<i>D</i>	x x x x	—	—	—	—	—	—	—
	5	<i>H</i>	x	—	—	—	—	—	—	x
	5	<i>D</i>	?	—	—	—	—	—	—	x x
	6	<i>H</i>	x x	—	—	x	—	—	—	x x
	1	<i>H</i>	x x x	—	—	x x	—	—	—	x x x
<i>G</i>	2	<i>H</i>	x x x x x	—	—	—	—	x	—	x x x x
	3	<i>H</i>	x x x x	—	—	—	—	x	—	x x x
	4	<i>H</i>	x x x x	—	—	—	—	—	—	x x x
	4	<i>D</i>	x x x	—	—	—	—	—	—	x x x
	5	<i>H</i>	x x x x x	—	—	—	—	—	—	x x x x x
	1	<i>H</i>	x x	x	x x	—	—	—	—	x
<i>H</i>	2	<i>H</i>	x x	—	—	—	—	—	—	x
	3	<i>H</i>	x	x	—	—	—	—	—	x
	4	<i>H</i>	x	—	—	—	—	—	—	x
	5	<i>H</i>	x x x x	—	—	—	—	—	x x x x	x x
	6	<i>H</i>	x x x x	—	—	—	—	—	x x x x	x x
	7	<i>H</i>	x x	x	?	—	—	—	—	?

lysis of spots 5 and 6 were carried out in order to chromatograph in *o*-cresol. To confirm the presence of glycylcysteic acid and β -sulphoalanylleucine, two separate mixed chromatograms of this fraction (about 2%) with each of the synthetic peptides (about 10 μ g.) were developed, that with the former peptide being developed longer in both solvents than that with the latter peptide. No difference was ob-

servingly well separated and not enough material was available for the separate chromatograms which would have been necessary for their analyses. Spot 6 was faint and did not appear in earlier chromatograms, so that, because of lack of material, deamination could not be carried out. After hydrolysis of all the spots, chromatography was carried out in phenol. After deamination and hydrolysis,

spots 4 and 5 were chromatographed in *o*-cresol. Portions of spots 4 and 5 were also hydrolysed and chromatographed in this solvent.

Fraction G (Fig. 6). The spots of this fraction were faint, 1 and 3 being grey. Chromatography of the hydrolysed spots

graphy of the hydrolysed spots in phenol, *o*-cresol and collidine. Synthetic dibromo-*L*-tyrosine (Zeynek, 1921) had R_F values similar to spot 5 (0.66 and 0.66 in collidine and phenol respectively) and gave a blue-grey colour with ninhydrin.

of fractions

chromatograms of the ionophoretic fractions. Symbols 'x' and '?' refer to the strengths of the spots after *H* or *D*;

Leu	Phe	Pro	Lys	Arg	Percentage of fraction used for chromatography (<i>H</i>)	Peptides	
						Probable	Possible
—	—	—	—	—	25	{ Asp. (CySO ₃ H) Glu. (CySO ₃ H)	[Asp, Glu, (CySO ₃ H)]
—	—	—	—	—	25	Glu. (CySO ₃ H)	—
—	—	—	—	—	5	{ (CySO ₃ H) Gly. (CySO ₃ H) (CySO ₃ H). Gly	—
—	—	—	—	—	5	{ (CySO ₃ H). Gly (CySO ₃ H). Ala	—
—	—	—	—	—	5	(CySO ₃ H). Ala	—
—	—	—	—	—	2	Gly. (CySO ₃ H)	—
—	—	—	—	—	2	Ser. (CySO ₃ H)	—
—	—	—	—	—	2	{ Thr. (CySO ₃ H) (CySO ₃ H). Thr	—
—	—	—	—	—	2	Ala. (CySO ₃ H)	—
—	—	—	—	—	2	(CySO ₃ H). Val	—
x x x	—	—	—	—	17	(CySO ₃ H). Leu	—
x x x	—	—	—	—	17.5	Ser. (CySO ₃ H)	—
—	—	—	—	—	17.5	{ Ser. (CySO ₃ H) Gly. (CySO ₃ H) etc.	—
—	—	—	—	—	17.5	{ Ser. (CySO ₃ H) Gly. (CySO ₃ H) etc.	—
x x x	x	—	—	—	9	{ Leu. (CySO ₃ H) (CySO ₃ H). Leu Phe. (CySO ₃ H)	—
—	—	x	—	—	9	(CySO ₃ H). [Pro, Val]	—
?	—	x	—	—	9	[Val, (CySO ₃ H)]	—
—	—	—	—	—	25	[(CySO ₃ H), Ser, Val]	—
x x x x x	x	x x	—	—	25	—	{ [(CySO ₃ H), Pro, Val] [(CySO ₃ H), Pro, Leu]
x	—	x x	—	—	25	—	[(CySO ₃ H), Pro, Val]
x x	—	—	—	—	25	{ [(CySO ₃ H), Pro, Val] [(CySO ₃ H), Pro, Leu]	—
x x	—	x x x	—	—	25	[(CySO ₃ H), Val]	[(CySO ₃ H), Leu]
x x x	—	x	—	—	17.5	—	—
?	—	x	x x	x x	25	—	—
—	—	x x	x	—	17.5	—	—
x x	—	x	—	—	17.5	—	—
x x x	?	x	—	—	25	—	—
x x x	—	—	x	—	25	—	—
?	—	—	—	—	25	—	—

was first carried out in phenol. From another chromatogram, spots 1-6 were hydrolysed and chromatographed in *o*-cresol; this solvent was used for chromatography of spot 4 after deamination.

Fraction H (Fig. 7). Spot 5 was a strong blue-grey colour. The contiguous spot 6 was a moderately strong purple. Separate chromatograms were prepared for chromato-

R_F values of synthetic peptides of cysteic acid

The R_F values are given in Table 3. Both glycine peptides were rather elongated in collidine. No spots due to contaminants were present in any of the chromatograms.

Ionophoretic rates of synthetic peptides of cysteic acid

These determinations were carried out under almost the same conditions as those used for the ionophoresis of the cysteic acid fraction of the wool hydrolysate. In this case the length of the jelly was 65 cm. and instead of a gutter, holes 1 cm. in diameter were made in the exposed strip of jelly near the cathode. In the first experiment, carried out with L-cysteic acid (control), glycyl-L-cysteic acid and β -sulpho-L-alanylglycine, of each compound approx. 5 mg.

Fig. 5. Two-dimensional chromatogram of fraction F.

were dissolved in a mixture of water (0.6 ml.) and 4.3 N-Na silicate (0.09 ml.), and a very small amount of solid bromothymol blue added. The solutions were brought to pH 6-7 by addition of N-acetic acid (approx. 0.1 ml.) and each solution was transferred to one of the holes. After setting, current was allowed to pass for 42 hr. at a potential of 250 V. At the end of this time, the lid was slid off and the paper print showed three spots which had moved towards the anode. The distance of each from the origin was measured. In the second experiment, carried out under

Fig. 6. Two-dimensional chromatogram of fraction G.

Fig. 7. Two-dimensional chromatogram of fraction H.

Table 3. R_F values of peptides of cysteic acid and of certain amino-acids derived from cystine

(P, purple; B, blue; Y→P, yellow first, turning purple.)

	Solvent systems			Colour with ninhydrin
	Phenol coal-gas NH_3^*	s-Collidine	n-Butanol 8-hydroxy-quinoline†	
Disulphoxide derived from cystine	0.11	0.36	—	P
Cystine sulphinic acid	0.11	0.37	—	P
S-Benzylcysteine	0.91	0.87	0.76	B
Glycyl-L-cysteic acid	0.09	0.34	—	Y→P
β -Sulpho-L-alanylglycine	0.10	0.48	—	Y→P
L-Leucyl-L-cysteic acid	0.40	0.49	—	P
β -Sulpho-L-alanyl-L-leucine	0.40	0.56	—	Y→P

* Aqueous NH_3 (0.3%, w/v) on floor of chamber.

† 0.1% solution.

similar conditions, one solution contained L-leucyl-L-cysteic acid, the second, β -sulpho-L-alanyl-L-leucine and the third, a mixture of all four peptides and L-cysteic acid. The paper print showed single spots from each of the first two. Only four spots were obtained from the third, glycyl-L-cysteic acid and β -sulpho-L-alanyl-L-leucine not having separated. The mean relative rates (cysteic acid=1) are given in Table 4 and plotted on Fig. 2 in such a way as to make the cysteic acid bands coincide.

Table 4. *Relative ionophoretic rates of peptides of cysteic acid*

Peptide	Rate of movement towards anode (cysteic acid = 1)
Glycyl-L-cysteic acid	0.60
β -Sulpho-L-alanylglycine	0.86
L-Leucyl-L-cysteic acid	0.44
β -Sulpho-L-alanyl-L-leucine	0.61

Titration curves of glycyl-L-cysteic acid and β -sulpho-L-alanylglycine

Each peptide, dried to constant weight (approx. 20 mg.), was dissolved in water (5 ml.) and transferred to the chamber of a glass electrode, fitted with a cork through which passed the tips of a burette and of a tube connected with a N_2 cylinder. pH measurements were made after small additions of CO_2 -free 0.1N-NaOH from the burette. A stream of N_2 was passed through the solution during the titration. The results are shown graphically in Fig. 8. The

Fig. 8. Titration curves of glycyl-L-cysteic acid, —○—○—; and β -sulpho-L-alanylglycine, —●—●—.

high value for the titration of glycyl-L-cysteic acid at pH values >10 is probably due to incomplete exclusion of CO_2 and to the effect of salt accumulated from the KCl bridge during the titration. The dotted line represents the theoretical end point.

Interpretation of results of analysis of chromatograms

Fraction A. This fraction contains very little peptide material; most of that present appears to consist of cysteic acid linked with dicarboxylic amino-acid residues. The presence of the tripeptides [Asp, Glu, $(CySO_3H)$] cannot be excluded.

Fraction D (Fig. 3). Spot 1 is considered to be due to a peptide [Glu, $(CySO_3H)$] rather than to a mixture of free glutamic and cysteic acids, each of which would occur in different parts of the chro-

matogram. In fact, a faint glutamic acid spot was present well separated from spot 1 (Fig. 3). The presence of $(CySO_3H)$.Gly in cuts 2 and 3 was confirmed by mixed chromatograms with the synthetic peptide and by the closely similar ionophoretic rate of this fraction and that of the synthetic peptide (Fig. 2). The presence of Gly. $(CySO_3H)$, in smaller proportion than $(CySO_3H)$.Gly in cut 3 is probably due to overlapping with fraction E which contains much more of the former. Free cysteic acid cannot be excluded from cut 2, as this is its expected position and because fraction D is contiguous with Fraction C, which consists mainly of free cysteic acid. $(CySO_3H)$.Ala is clearly present in spot 4 and its presence in cut 3 must arise through the proximity of 3 and 4.

Fraction E (Fig. 4). The interpretation of the results for this fraction is straightforward, and confirmation of the presence of Gly. $(CySO_3H)$ and $(CySO_3H)$.Leu has been obtained both from mixed chromatograms and from ionophoretic rates.

Fraction F (Fig. 5). Spots 1, 2 and 3 probably contain dipeptides arising from overlapping with fraction E. Spot 4 appears to contain much more Leu. $(CySO_3H)$ than $(CySO_3H)$.Leu since a much higher proportion of cysteic acid than leucine remains after deamination. Phe. $(CySO_3H)$ is also considered to be present in spot 4. An alternative interpretation, i.e. that a tripeptide [Phe, $(CySO_3H)$, Leu] is present, is unlikely because such a peptide would be expected to have a higher R_F value in collidine and phenol than spot 4 and to have a lower ionophoretic rate than fraction F. The tripeptide $(CySO_3H)$, [Pro, Val] is considered to be present in spot 5 rather than dipeptides [$(CySO_3H)$, Pro] and [$(CySO_3H)$, Val], both of which would be expected to have much lower R_F values in phenol than spot 5. Moreover, the latter dipeptide is actually present in spot 6, which is well separated from spot 5.

Fraction G (Fig. 6). This fraction must consist mainly of tripeptides and higher peptides of cysteic acid. The presence of dipeptides [$(CySO_3H)$, Ser] and [$(CySO_3H)$, Val] are excluded from spot 1, as they would be expected to occupy different positions on the chromatogram. Too many amino-acids are produced after hydrolysis of spot 2 for the results to be clearly interpretable, but tripeptides [Val, Leu, $(CySO_3H)$] are probably absent since the presence of Val and Leu would be expected to result in a higher R_F value in collidine, but tripeptides, consisting of [$(CySO_3H)$, Pro] with Val or Leu are probably present. These peptides also appear to be present in spot 4, although the proline was at first missed because after hydrolysis chromatography was carried out in phenol. When *o*-cresol was used, however, after deamination, proline was revealed (see Table 2). The very high intensities of the cysteic acid and valine spots after hydrolysis of spot 5 suggests

that [Val, (CySO₃H)] occurs here, which is likely since this spot occupies a position similar to spot 6 of the contiguous fraction *F*.

Fraction H (Fig. 7). Most of the spots in this fraction give too many amino-acids on hydrolysis for the results to be clearly interpretable. Peptides of high molecular weight appear to be present, especially in spots 1 and 3 which give basic amino-acids on hydrolysis. Tripeptides consisting of one residue of cysteic acid with two of the higher monoamino-monocarboxylic acids may be present, e.g. in spots 5 and 6. Such an interpretation would be consistent with their relatively high R_F values and relatively slow ionophoretic rates. Free dibromotyrosine is considered to be present in spot 5 (and probably in the contiguous spot 6) since, from the pK of its OH group (Winneck & Schmidt, 1935), it would be expected to have a small net negative charge at pH about 7 and hence to move slowly towards the anode. Moreover, spot 5 is in the position to be expected for dibromotyrosine and of a similar colour. Di- or tri-peptides containing cysteic acid and dibromotyrosine residues are considered to be absent from spots 5 and 6, as the presence of (CySO₃H) would be expected to have a marked retarding effect in the phenol direction and to confer a higher ionophoretic rate.

Intermediate oxidation products of cystine formed by hydrogen peroxide

The disulphoxide derived from L-cystine was prepared by the method of Toennies & Lavine (1936) and β -sulphinyl-L-alanine was prepared from this by the method of Lavine (1936). Both compounds had similar R_F values in phenol and collidine (Table 3). Mixed chromatograms of either with cysteic acid, developed in phenol, showed two distinct spots. These compounds were each treated with 6*N*-HCl at 105° for (a) 5 hr. and (b) 17 hr. After removal of HCl, chromatograms were developed in phenol. With (a) a faint spot of unchanged material was seen against the background of the grey elongated spot of cystine, which with cysteic acid was present as the strongest component. With (b) only a trace of unchanged material could be seen.

L-Cystine (100 mg.) in 2*N*-HCl (1 ml.) was treated with 26% (w/v) H₂O₂ (0.3 ml.). After shaking for 5 min. (solution still strongly positive to KI), the solution was repeatedly evaporated *in vacuo* below 40°, after successive additions of water, to remove excess HCl and H₂O₂. Treatment with H₂O₂ as above was also carried out in 3% (w/v) NH₃ solution. Phenol chromatograms revealed in the case of treatment in acid, strong spots of cysteic acid and cystine and a moderately strong spot of an intermediate oxidation product, the latter having the same R_F value as the sulphinic acid or the disulphoxide. In the case of treatment in NH₃, both cystine and intermediate oxidation product were relatively much weaker. No intermediate oxidation product was observed in either case when H₂O₂ treatment was prolonged (17 hr.).

To wool (approx. 200 mg.) in 3% (w/v) NH₃ was added 26% H₂O₂ (w/v) (0.5 ml.). The mixture was allowed to stand at room temperature for 2 hr. with occasional shaking. After

rinsing with water, the wool was divided into two equal portions. One portion was hydrolysed with 6*N*-HCl at 105° for 5 hr., the other was hydrolysed for 17 hr. One-dimensional chromatograms in phenol revealed a trace of intermediate oxidation product in the latter and a somewhat larger amount in the former. The intermediate oxidation product was seen on a two-dimensional chromatogram of the 5 hr. hydrolysate (0.5 mg.) developed in collidine (42 hr.) and phenol (17 hr.) as a faint but distinct purple spot occupying the expected position of the disulphoxide or sulphinic acid.

Synthesis of peptides of L-cysteic acid

Glycyl-L-cysteic acid was prepared from diglycyl-L-cystine by oxidation with bromine (White, 1933). β -Sulpho-L-alanylglycine and β -sulpho-L-alanyl-L-leucine were prepared by condensing the acid chloride or azide of *N*-carbobenzyloxy-S-benzyl-L-cysteine (Harington & Pitt-Rivers, 1944) with the esters of glycine and leucine respectively, saponification, removal of the protective groups by sodium in liquid ammonia and oxidation of the cysteinyl peptides with bromine. L-Leucyl-L-cysteic acid was prepared by condensing *N*-carbobenzyloxy-L-leucyl chloride with S-benzyl-L-cysteine ester and then treating as above. Washing of the acid chlorides with water, prior to coupling, was not found to have any advantage (cf. Synge, 1948) and appreciably hydrolysed the acid chloride group of *N*-carbobenzyloxy-S-benzyl-L-cysteinyl chloride. It was found that the latter could be coupled directly with L-leucine and that *N*-carbobenzyloxy-S-benzyl-L-leucyl chloride could be coupled with S-benzyl-L-cysteine, thereby avoiding the stages of preparation of amino-acid ester and of saponification; though the yields were not high, they were comparable with the overall yields of the ester method. Paper chromatography was used to check the progress of the stages of these syntheses.

EXPERIMENTAL

C and H analyses were carried out by Drs Weiler and Strauss, Oxford. N analyses were done by the micro-Kjeldahl method.

Glycyl-L-cysteic acid was prepared by oxidation of diglycyl-L-cystine (Abderhalden & Spinner, 1919) as described by White (1933). It had $[\alpha]_D^{20} - 13.9^\circ$ (l, 2; c, 1.08 in water).

β -Sulpho-L-alanylglycine

S-Benzyl-L-cysteine prepared by reduction of L-cystine by Na in liquid NH₃ and treatment with benzyl chloride as described by Audrieth, du Vigneaud & Loring (1930) had $[\alpha]_D^{20} - 5.15^\circ$ (l, 2; c, 0.97 in *N*-HCl) and $[\alpha]_D^{20} + 26.0^\circ$ (l, 2; c, 0.96 in *N*-NaOH). Hagedüs (1948) gives $[\alpha]_D + 29^\circ$ in *N*-NaOH. (See Table 3 for R_F values.)

N-Carbobenzyloxy-S-benzyl-L-cysteine. To S-benzyl-L-cysteine (5 g.) in *N*-NaOH (30 ml.) was added carbobenzyloxy chloride (4 ml.) and 2*N*-NaOH (16 ml.) in four equal portions with shaking at 0°. After the last addition, the flask was shaken at room temperature for 0.5 hr. The

mixture was alkaline to thymolphthalein throughout. On acidification with HCl (thymol blue), a gummy solid was precipitated which was washed by decantation with *n*-HCl and finally with water. After drying, the compound was crystallized from CHCl_3 -cyclohexane. Yield, 8 g. (needles), m.p. 96–97°. (Found: N, 4.0. $\text{C}_{18}\text{H}_{19}\text{O}_4\text{NS}$ requires N, 4.1%.)

N-Carbobenzoyloxy-*S*-benzyl-L-cysteinylglycine ester. (a) *N*-carbobenzoyloxy-*S*-benzyl-L-cysteine azide was coupled with glycine ester as described by Hagedüs (1948), who did not isolate the product. The compound was crystallized from ethyl acetate-cyclohexane and had m.p. 94–95°. Yield, 2.9 g. from 3.5 g. *N*-carbobenzoyloxy-*S*-benzyl-L-cysteine hydrazide. (Found: C, 61.9; H, 6.0; N, 6.5. $\text{C}_{22}\text{H}_{25}\text{O}_5\text{N}_2\text{S}$ requires C, 61.4; H, 6.1; N, 6.5%.) After hydrolysis with 6*N*-HCl for 17 hr. at 105°, chromatography in phenol gave spots of glycine and of *S*-benzylcysteine of equal intensities.

(b) Carbobenzoyloxy-*S*-benzyl-L-cysteine (1 g.) was shaken with PCl_5 (0.7 g.) in dry ether at 0–10°. The solution was added to solution of glycine ethyl ester in dry ethyl acetate, prepared from 2 g. glycine ethyl ester hydrochloride. The precipitated glycine ester hydrochloride was filtered off and the solution washed with *n*-HCl, saturated KHCO_3 and finally water. After drying over Na_2SO_4 , the organic solvent was removed by distillation *in vacuo*. The gum, after crystallization from ethyl acetate-cyclohexane, had m.p. 94° (0.5 g.) undepressed when mixed with the product from (a). When mixed with carbobenzoyloxy-*S*-benzyl-L-cysteine the m.p. was 74–77°. When the acid chloride from 5 g. *N*-carbobenzoyloxy-*S*-benzylcysteine was washed with water prior to coupling (cf. Synge, 1948), carbobenzoyloxy-*S*-benzyl-L-cysteine (1 g.), m.p. 92°, mixed m.p. 93–95° with an authentic sample, was recovered from the bicarbonate washings of the coupled product.

N-Carbobenzoyloxy-*S*-benzyl-L-cysteinylglycine. This was obtained by saponification of the above ester (3.4 g.) in methanol (20 ml.) with 2*N*-NaOH (4 ml.). The gelatinous precipitate obtained on dilution with water and acidification was filtered and washed with water. The material could not be obtained crystalline. It had m.p. 86° (2.6 g. powder from aqueous ethanol or CHCl_3 -cyclohexane). Hagedüs (1948) gives m.p. 80–82°. The m.p. was depressed 10–20° when mixed with the ester of *N*-carbobenzoyloxy-*S*-benzyl-L-cysteine. (Found: N, 6.8; calc. for $\text{C}_{20}\text{H}_{22}\text{O}_5\text{N}_2\text{S}$ N, 7.0%.) Hydrolysis and chromatography in collidine gave two spots, glycine and *S*-benzylcysteine, of equal intensity.

L-Cysteinylglycine. The above compound (2.5 g.) was dissolved in liquid NH_3 and sufficient Na (0.4 g.) added to give a permanent blue. Powdered $(\text{NH}_4)_2\text{SO}_4$ (3.0 g.) was then added and the mixture left to evaporate. The mixture was taken up in 0.5*N*- H_2SO_4 (25 ml.). The layer of toluene was separated and the aqueous portion filtered. Mercuric sulphate reagent (Kendall, McKenzie & Mason, 1929) was added until no further precipitation took place. The mixture was filtered and the precipitate washed successively with 0.5*N*- H_2SO_4 and water, and then decomposed with H_2S and filtered from HgS . The latter was re-treated with H_2S . The combined filtrates were concentrated *in vacuo* <40° to a volume of about 20 ml. and treated in the desalting apparatus (Consden, Gordon & Martin, 1947) to remove H_2SO_4 and salts. The filtered solution was concentrated *in vacuo* to small bulk. Addition of ethanol precipitated a gum. When this was washed with ethanol several times by decantation, a powder (0.5 g.) was obtained, which was deliquescent. For analysis the peptide was dried *in vacuo* at

room temperature to constant weight. (Found: C, 33.4; H, 5.5; N, 15.7. $\text{C}_5\text{H}_{10}\text{O}_2\text{N}_2\text{S}$ requires C, 33.7; H, 5.6; N, 15.7%.)

β -Sulpho-L-alanyl-L-cysteine. The above peptide (0.3 g.) was dissolved in water, cooled in iced water and treated with a solution of Br_2 in CHCl_3 until no further decoloration took place. The water and CHCl_3 were evaporated *in vacuo* and repeatedly evaporated *in vacuo* with additions of water. After leaving over KOH *in vacuo* for 48 hr., the gum was rubbed with ethanol and washed several times by decantation with ethanol. The peptide formed a deliquescent powder (0.35 g.). For analysis it was dried *in vacuo* at room temperature to constant weight. (Found: N, 12.3. $\text{C}_5\text{H}_{10}\text{O}_6\text{N}_2\text{S}$ requires N, 12.4%.) It had $[\alpha]_D^{20} +22.5^\circ$ (l, 2; c, 0.58 in water). Hydrolysis and chromatography in phenol gave two spots, glycine and cysteic acid, of equal intensity. Deamination followed by hydrolysis gave glycine only.

L-Leucyl-L-cysteic acid

N-Carbobenzoyloxy-*L*-leucyl-*S*-benzyl-L-cysteine ethyl ester. *N*-Carbobenzoyloxy-*L*-leucine (1.3 g.) was shaken in dry ether with PCl_5 (1.5 g.) at 0–10°. The mixture was filtered from the slight excess of PCl_5 through glass wool into an ethyl acetate solution of *S*-benzyl-L-cysteine ethyl ester (4 g.), freed from the hydrochloride as described by Harington & Pitt-Rivers (1944). On shaking, crystals of *S*-benzyl-L-cysteine ethyl ester hydrochloride were formed, which were removed by filtration. The ethyl acetate solution was washed successively with *n*-HCl, saturated KHCO_3 and water, and dried over Na_2SO_4 . The bulk of the ethyl acetate was evaporated *in vacuo*. To the concentrated solution was added cyclohexane. The ester crystallized in clusters of short needles (1.1 g.), m.p. 117°. (Found: C, 64.4; H, 7.1; N, 5.8. $\text{C}_{26}\text{H}_{34}\text{O}_5\text{N}_2\text{S}$ requires C, 64.2; H, 7.0; N, 5.8%.) Yield was not improved when the acid chloride was washed with water prior to coupling with the ester in the presence of saturated KHCO_3 . On hydrolysis and chromatography in collidine, two spots, leucine and *S*-benzylcysteine, of equal intensity, were obtained.

N-Carbobenzoyloxy-*L*-leucyl-*S*-benzyl-L-cysteine. (a) The above ester (4 g.) from several preparations was dissolved in methanol (20 ml.) and treated with 5*N*-NaOH (2.5 ml.). On shaking, a heavy layer was formed which disappeared on continued shaking at room temperature for 1 hr. The solution was diluted with water and acidified with 5*N*-HCl. The white precipitate, after filtration and washing with water, was crystallized from methanol. The compound formed needles (2 g.) m.p. 170°. (Found: C, 62.3; H, 6.4; N, 6.0. $\text{C}_{24}\text{H}_{30}\text{O}_5\text{N}_2\text{S}$ requires C, 62.9; H, 6.6; N, 6.1%.)

(b) To a solution of *S*-benzyl-L-cysteine (0.7 g.) in 2*N*-NaOH (2 ml.) was added, in three equal portions, an ether solution of the acid chloride of *N*-carbobenzoyloxy-*L*-leucine, prepared from 1 g. of the acid and PCl_5 (1 g.). The solution was kept at 0° and shaken vigorously after each addition. Before each addition of acid chloride, sufficient 2*N*-NaOH was added to maintain the mixture alkaline to thymolphthalein. The ether layer was removed and the aqueous solution acidified with HCl. A gum was precipitated which quickly solidified on rubbing. On crystallization from methanol, needles (0.3 g.) were obtained, m.p. 166–167°, undepressed by admixture with (a) above.

The compound from (a) or (b) after hydrolysis and chromatography in collidine gave two spots, leucine and *S*-benzylcysteine, of equal intensity.

L-Leucyl-L-cysteine. The above compound (1 g.) was treated with Na in liquid NH_3 as described for the preparation of L-cysteinylglycine. The crude *peptide* from the desalted solution was obtained from aqueous ethanol as a powder (0.2 g.). (Found: C, 45.8; H, 7.7; N, 11.7. $\text{C}_9\text{H}_{18}\text{O}_5\text{N}_2\text{S}$ requires C, 46.2; H, 7.7; N, 12.0%.)

L-Leucyl-L-cysteic acid was obtained as a white deliquescent powder (aqueous ethanol) from L-leucyl-L-cysteine (0.09 g.) by treatment with Br_2 . Hydrolysis and chromatography in phenol gave two spots, leucine and cysteic acid, of equal intensity. Deamination followed by hydrolysis gave cysteic acid only. For analysis, it was dried *in vacuo* at room temperature to constant weight. The *peptide* had $[\alpha]_D^{20} +22.6^\circ$ (l, 2; c, 0.4 in water). (Found: C, 38.1; H, 6.4; N, 10.0. $\text{C}_9\text{H}_{18}\text{O}_6\text{N}_2\text{S}$ requires C, 38.3; H, 6.4; N, 9.9%.)

β -Sulpho-L-alanyl-L-leucine

N-Carbobenzyloxy-S-benzyl-L-cysteinyl-L-leucine ethyl ester. (a) *N*-carbobenzyloxy-S-benzyl-L-cysteine (1.7 g.) was converted to the acid chloride with PCl_5 (1.1 g.) in ether and the solution was added to an ethyl acetate solution of L-leucine ethyl ester prepared from the ester hydrochloride (4.4 g.); the leucine ethyl ester hydrochloride (1.6 g.) which precipitated was removed by filtration and the solution washed successively with N-HCl , saturated KHCO_3 and water and dried over Na_2SO_4 . The solution was concentrated *in vacuo*. Addition of light petroleum (b.p. 40–60°) precipitated a gum, which became solid after leaving in the refrigerator. On recrystallization from CHCl_3 light petroleum, the *ester* formed clusters of needles (1.2 g.), m.p. 67–69°. (Found: C, 63.6; H, 6.9; N, 5.5. $\text{C}_{28}\text{H}_{34}\text{O}_6\text{N}_2\text{S}$ requires C, 64.2; H, 7.0; N, 5.8%.) Hydrolysis and chromatography in collidine gave two spots, leucine and S-benzylcysteine, of equal intensity. (b) This compound (3.3 g.) m.p. and mixed m.p. with (a), 67–69°, was also prepared by coupling the azide from *N*-carbobenzyloxy-S-benzyl-L-cysteine hydrazide (3 g.) with L-leucine ethyl ester (from 7 g. of the hydrochloride).

N-Carbobenzyloxy-S-benzyl-L-cysteinyl-L-leucine. (a) The ester (3.3 g.) from (b) above, was dissolved in methanol (20 ml.) and treated with 2N-NaOH (4 ml.). After shaking for 1 hr. at room temperature, the solution was diluted with water and acidified with HCl. An oil was precipitated, which on cooling became a sticky solid. It was soluble in ether. Attempts to convert this to crystalline material were unsuccessful. The ester from (a) above (0.7 g.) also gave a gum on saponification which could not be crystallized, but in this case, the substance was not completely soluble in ether. A white powder was left which crystallized in needles (0.15 g.) from aqueous methanol. It melted with decomposition at 182–185° and contained 5.9% N. It was easily soluble in dilute NH_3 , from which it was precipitated unchanged by acid. It gave no colour with ninhydrin, and on hydrolysis and chromatography in collidine gave one spot only which was leucine. The nature of this by-product was not further elucidated. Hydrolysis of the main gummy product and chromatography in collidine gave two spots, leucine and S-benzylcysteine, of approximately equal intensity. (b) *N*-Carbobenzyloxy-L-cysteine (1.2 g.) was converted into the acid chloride with PCl_5 (0.8 g.) in ether and added in several portions to L-leucine (0.4 g.) in 2N-NaOH (2 ml.) at 0° with additions of 2N-NaOH to keep the mixture alkaline to thymolphthalein. After each addition the mixture was shaken vigorously. The ether layer was

removed and acidification of the aqueous solution precipitated a gum which also could not be rendered crystalline. In this case, no by-product was produced. Hydrolysis and chromatography in collidine gave two spots, leucine and S-benzylcysteine, of approximately equal intensity.

L-Cysteinyl-L-leucine. The gum above (2 g.) in liquid NH_3 was treated with Na and worked up as described above for the other peptides of cysteine. The *peptide* was obtained as a fluffy white powder (0.4 g.) from aqueous ethanol. (Found: C, 45.7; H, 7.7; N, 11.7. $\text{C}_9\text{H}_{18}\text{O}_5\text{N}_2\text{S}$ requires C, 46.2; H, 7.7; N, 12.0%.)

β -Sulpho-L-alanyl-L-leucine was obtained by treatment of the above peptide (0.3 g.) with Br_2 in CHCl_3 , as a white powder from aqueous ethanol. It slowly lost weight on heating to 40–50° at <0.1 mm. over P_2O_5 , finally reaching a constant weight. (Found: C, 35.7; H, 6.9; loss in weight, 5.7. $\text{C}_9\text{H}_{18}\text{O}_6\text{N}_2\text{S} \cdot \text{H}_2\text{O}$ requires, C, 36.0; H, 6.7; loss in weight, 6.0%.) N analyses were carried out on the anhydrous substance. (Found: N, 10.2. $\text{C}_9\text{H}_{18}\text{O}_6\text{N}_2\text{S}$ requires N, 9.9%.) Hydrolysis and chromatography in phenol gave two spots, leucine and cysteic acid, of equal intensity. Deamination, followed by hydrolysis, gave only one spot which was leucine. It had $[\alpha]_D^{20} +11.2^\circ$ (l, 2; c, 0.8 in water).

DISCUSSION

Several dipeptides of cysteic acid have been identified. These are given in Table 5; the figures indicate the relative amounts present and have been computed by comparison of the intensities of the spots formed by chromatography of the hydrolysates of each peptide (Table 2) with those of standard amounts of amino-acids as described by Consden *et al.* (1949). Only those peptides for which there is definite evidence have been included in Table 5.

Table 5. *Dipeptides of cysteic acid identified in a partial hydrolysate of wool after oxidation with bromine, with their approximate proportions*

Amino-acid (X) linked with cysteic acid (N not less than 2% total N of wool)	N (mg./100 g. of wool N) present as peptide of X + cysteic acid. NH ₂ group of cysteic acid residue	
	Combined	Free
Aspartic acid	2	—
Glutamic acid	2	—
Serine	60	—
Glycine	24	10
Threonine	20	20
Alanine	30	14
Valine	—	20
Leucine	8	10
Phenylalanine	—	4
Proline	—	—
Tyrosine	—	—
Total	146	78

Valylcysteic acid is probably present (in fraction *F'*), but has not been included because identification was not rigidly established. β -Sulphoalanylserine is probably absent in the hydrolysate, since it has been shown by Casal & Desnuelle (1948) that the peptide bond involving the amino group of serine is particu-

larly labile. But it does not follow that this linkage is absent in the intact protein. Although Casal & Desnuelle (1948) found that the corresponding threonine peptide linkages were also labile, β -sulphoalanylthreonine was found in this investigation, which suggests that a relatively high proportion of threonine may be linked through its amino group with cystine in the original wool. Dipeptides of tyrosine and cysteic acid would have been present as dibromotyrosine peptides and these appear to be absent. The occurrence of peptides consisting of dicarboxylic amino-acids with cysteic acid is difficult to explain, since acidic compounds of aspartic and glutamic acids should have been separated by the first Amberlite treatment. It may, however, be significant that no other dipeptide of glutamic or aspartic acid was found in any of the fractions; if present, these would have appeared on the chromatograms as spots well separated from the cysteic acid peptide spots. Consden *et al.* (1949) found that the peptides of cystine with dicarboxylic amino-acids were present only in small amounts relative to many of the other dipeptides of glutamic and aspartic acids, so that had a portion of the dicarboxylic amino-acid fraction not been retained after the initial Amberlite treatment, peptides of glutamic and aspartic acids with neutral amino-acids would have finally appeared in relatively larger amounts than peptides of cysteic acid with dicarboxylic amino-acids. A possible explanation for the presence of these latter peptides is that they were originally present as peptides of cystine linked to asparagine or glutamine residues; being neutral they would not be retained by the Amberlite. The subsequent treatments prior to ionophoresis might have hydrolysed the amide groups. Rees (1946) has shown that a very small proportion of the original amide nitrogen of proteins is still present after hydrolysis carried out under conditions similar to those employed in this investigation. Nevertheless, whatever the explanation these results confirm that dicarboxylic amino-acids are linked with cystine in wool.

In a control experiment of Consden *et al.* (1949), designed to estimate the losses at each stage, it was found that, with amino-acids, the final spot strengths accounted for only about 30% of the original material. Most of this loss occurred during the final stages. If it is assumed that the peptide losses in the present investigation are similar, then the nitrogen of the peptides identified accounts for about 0.75% of the wool nitrogen or 7.6% of the cysteic acid fraction. Free cysteic acid amounts to 14.9% of this fraction, so that, altogether, 22.5% of this fraction is accounted for. For reasons similar to those given by Consden *et al.* (1949) in the case of glutamic and aspartic acid peptides, the cysteic acid peptides were probably present in the hydrolysate in much larger amounts than indicated by these figures.

Since the disulphide bond of cystine has been broken by oxidation, the present investigation gives no information concerning the residues in combination with the same cystine residue. However, the present results show that cystine is linked with most of the monoamino-monocarboxylic acids and that most of these are linked both through an amino and through a carboxyl group of cystine. This was found to be true also for the dicarboxylic amino-acids (Consden *et al.* 1949), but certain differences may be noted. Apart from those containing phenylalanine and dicarboxylic amino-acids, the amounts of the various peptides of cysteic acid isolated do not differ very widely, suggesting that the monoamino-monocarboxylic acids are more equally distributed between the cystine residues than between dicarboxylic amino-acid residues. Seryl cysteic acid appears to be present in the highest relative concentration, but in the case of the dicarboxylic amino-acids peptides of serine occurred in much smaller relative proportion. This suggests that a larger proportion of serine occurs in combination with cystine than with glutamic and aspartic acids. Conversely, a relatively high proportion of tyrosine was found linked with glutamic acid, in marked contrast to the failure to find any of this amino-acid linked with cysteic acid in the present investigation. These remarks may require modification when data on kinetics of hydrolysis of more of these peptides become available.

No dipeptide of proline with cysteic acid was found, but isolation of proline dipeptides has in our experience proved difficult (Consden, Gordon, Martin & Synge, 1947; Consden *et al.* 1949). However, there would seem to be evidence for the occurrence of tripeptides and higher peptides containing proline residues. It therefore appears likely that proline residues occur near to, if not next to, cystine residues.

An important factor which has contributed to the successful separation of some of the cysteic acid peptides has been the difference between the two dipeptides of cysteic acid and a given amino-acid in respect to chromatographic rates in collidine and in ionophoretic rates. It has been found that the peptide in which the amino group of cysteic acid is free has a higher R_f value in collidine and moves faster towards the anode than the peptide in which the amino group of cysteic acid is protected. The explanation, which is evident from the titration curves of glycyl cysteic acid and β -sulphoalanylglycine (Fig. 8), is that the amino group of the latter is distinctly weaker (pK_a , about 6.9) than the amino group of the former (pK_a , about 8.9), whose pK_a value is similar to, or perhaps slightly higher, than that of other dipeptides (Edsall, 1943). At the pH of the ionophoresis, therefore, the amino group of β -sulphoalanyl peptides would be partially de-

ionized and would carry a higher net negative charge than the peptides in which the cysteic acid-amino group was protected. The latter would also be expected to have smaller R_f values in collidine, due to their more strongly basic character. Similar pairs of peptides of aspartic acid have not been observed to show such marked differences in ionophoretic and chromatographic properties (Consdén *et al.* 1949). It appears, therefore, that the weakening of the amino group is associated with the proximity of the sulphonc acid group.

SUMMARY

1. A method is presented for the identification of lower peptides of cystine in partial hydrolysates of wool.

2. Thirteen dipeptides were identified and a

number of higher peptides of undetermined residue order.

3. It is concluded that, in wool, most of the mono-amino-monocarboxylic acids are combined both through their amino and carboxyl groups to residues of cystine.

4. The results are compared with those of a similar investigation on the peptides of the dicarboxylic amino-acids in partial hydrolysates of wool.

5. Evidence has been obtained for the formation of intermediate oxidation products of combined cystine by the action of hydrogen peroxide on wool.

6. A number of dipeptides of L-cysteine and of L-cysteic acid were synthesized.

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