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The Reactivity of the Disulphide Bonds of Wool

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1. Fully reduced and *S*-carboxymethylated wool samples were prepared in which either the readily reducible cystine bonds or those that could only be reduced with difficulty were specifically labelled with iodo[2-^{14}C]acetate; these two cystine fractions correspond to the (A+B) and (C+D) cystine fractions respectively, of Middlebrook & Phillips (1942). 2. Radioactively labelled peptides were isolated from partial acid hydrolysates of these wool samples. 3. It appears that the (A+B) cystine residues probably owe their increased reactivity to being in a more polar environment. 4. The implication of these results for the problem of characterizing the disulphide bonds of wool is briefly discussed.

Major advances have been made in the last few years in the isolation, characterization and sequencing of soluble protein fractions from reduced and *S*-carboxymethylated wool. They have led to the recognition of two major protein fractions, the high- and low-sulphur protein fractions, and another fraction of varying importance from wool type to wool type that is characterized by being rich in glycine and tyrosine residues. The complete sequences of seven proteins of the high-sulphur type and one sequence of a high-glycine-tyrosine protein have been published (Haylett & Swart, 1969; Haylett *et al.*, 1971; Elleman, 1972*a*,*b*; Elleman & Dopheide, 1972; Dopheide, 1973; Swart & Haylett, 1971; Swart, 1973). Considerable progress has also been made in sequencing low-sulphur-protein fractions from wool (W. G. Crewther, personal communication) and in a related field the sequence of a protein from emu feather has been reported (O'Donnell, 1973). It seems therefore that the major hurdle of isolating and characterizing individual proteins from the complex mixture that results from the reduction and alkylating procedure necessarily used to solubilize keratins has been successfully negotiated (cf. Lindley & Elleman, 1972) and that in principle there is no reason why all the 100 or so proteins that together make up the wool fibre cannot be isolated and sequenced.

However, even if this were to be achieved, it would still leave unsolved one major structural problem, i.e. the arrangement of the disulphide bonds. Some idea of the complexity of this problem can be gauged from the fact that there are more than 10^{23} possible isomers of one single wool protein (i.e. the parent disulphide protein corresponding to SCMK-B2A) which differ only in disulphide-bond arrangement. Moreover this estimate considers only the case where all the disulphide bonds are intramolecular, and this is certainly an unreal assumption for keratins.

No practical technique exists at present that can solve this problem. The presence of intermolecular disulphide bonds makes impossible the isolation of wool proteins with intact disulphide bonds, since in a sense possibly the whole wool fibre (and certainly some large volume of it considered in terms of molecular dimensions) may be considered as one molecule. However, any attempt to assign specific disulphide bonds on the basis of peptides isolated from whole wool would be impossible because of the inertness of wool to proteolytic enzymes. In any case even if this approach were feasible the known occurrence of repeating sequences would make many assignments ambiguous, and the frequent occurrence of cystinyl-cystine sequences would also pose very difficult technical problems.

The only approach to the problem which would seem worth while at the present time is to look for some underlying pattern to the arrangement of the disulphide bonds in the keratin complex. Although this may seem to be a remote possibility when considered in the light of our present knowledge of disulphide bonds in proteins, two lines of evidence suggest that some regularities may exist in the particular case of keratins. One is that the amino acid sequences of the wool proteins that have so far been determined show evidence of repeating sequences. In turn this periodicity of sequence most probably implies some regularity of structure and in particular some regularity in the arrangement of the disulphide bonds. Secondly quite a strong body of evidence was obtained around 30 years ago to suggest that the disulphide bonds of wool showed quite a definite and reproducible pattern of chemical reactivity, particularly towards sulphite solutions. The initial observations are those of Middlebrook & Phillips (1942), and a review by Lindley (1959) gives references to all the earlier work in this area. The literature is too extensive to review in detail here, but briefly this earlier work showed that: (1) under optimum conditions at room temperature rather more than one-half

of the wool cystine residues would react with bisulphite solution [the (A+B) fraction]; (2) this same (A+B) fraction could be reduced by 0.5M-thioglycollate at pH5.0; (3) the (A+B) fraction represented a genuine difference in reactivity of the wool cystine residues to sulphite solution and not merely an equilibrium value of a reaction involving all the cystine residues (see also Wolfram & Underwood, 1966); (4) although no well-substantiated explanation of this major difference in cystine reactivity was adduced, quite strong evidence was found that subfraction A of the (A+B) fraction owed its special reactivity to charged carboxyl groups of aspartic acid and/or glutamic acid in its vicinity (Lindley & Phillips, 1947; Lindley, 1959); (5) breakage of the less reactive (C+D) cystine bonds causes far-reaching changes in the physical properties of the fibre whether (A + B) bonds are intact or not (Lindley, 1957).

In the circumstances it was considered that there were good grounds for a re-examination of this problem of cystine reactivity, and the present paper is an attempt to see how far the variation in reactivity can be related to neighbouring amino acids.

Materials and Methods

Materials

Wool. Two different wool samples were used, a 64's medium Peppin Merino and wool from a single Lincoln fleece. Initial purification of the wool samples was by washing in cold ethanol followed by rinsing in water. Tips were cut off from each staple and discarded.

Iodo[2-^{14}C]acetic acid was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The particular sample used in this work had a specific radioactivity of 11.8mCi/mmol and was labelled 'CFA 269 Batch 10'.

General methods

Amino acid analyses were carried out on a Spinco 120C analyser equipped with an automatic sample-loading facility (Lindley *et al.*, 1973).

Radioactivity was measured by standard techniques by using a Packard Tri-Carb scintillation counter, model 314 EX, and a dioxan-based scintillant comprising POPOP (1,4-bis-(5-phenyloxazol-2-yl)benzene), 50mg, PPO (2,5-diphenyloxazole), 4g, naphthalene, 120g, and dioxan, 1 litre. POPOP and PPO were purchased from Packard Instrument Company Inc., Downers Grove, Ill., U.S.A. Specific radioactivities were normally determined on the basis of radioactivity of the peptide and its amino acid analysis.

Chromatography on Sephadex G-25 (superfine grade) was through five (120cm×0.9cm) columns in series, with pyridine–1M-NH_3 (7:3, v/v) as eluent as described by Rees *et al.* (1970).

High-voltage paper electrophoresis was performed as described by Ambler (1963).

End-group and sequence determinations were by the dansyl–Edman technique as described by Gray (1967).

Initial reduction of the wool at pH5.0 [the (A+B) fraction]

This was carried out by immersing the wool in a solution of 0.5M-thioglycollic acid previously adjusted to pH5.0 with NaOH, at a liquor/wool ratio of 100:1 (v/w). Any air bubbles entrapped by the wool were removed by evacuating the air space above the solution, re-admitting air and repeating this procedure two or three times. Treatment was at room temperature (approx. 20°C) for 18h and the wool was then filtered off and washed repeatedly with water, left for 1h in 250ml of water and refiltered. This was standard procedure in the original work of Middlebrook & Phillips (1942) and no evidence of reoxidation was ever observed.

Treatment with iodoacetate

The reduced wool (0.5g) was treated with 25ml of ethanol and 25ml of 0.4M-sodium borate buffer, pH8.0 (Maclaren, 1962), containing 200mg of iodoacetate. In the ^{14}C-labelling experiments 10μmol (0.12mCi) of iodo[2-^{14}C]acetate was added to the solution before the addition of the wool. The mixture was gently shaken overnight, the wool filtered off, washed twice with 25ml of 50% (v/v) ethanol and dried.

Second reduction of the wool samples [the (C+D) fraction]

The wool samples (0.5g), which had been reduced and alkylated as described above, were treated with 0.125ml of tributyl phosphine in 50ml of 20% (v/v) propanol for 3 days (Maclaren & Sweetman, 1966). The wool that has previously had its (A+B) cystine residues *S*-carboxymethylated is partially soluble, unlike untreated wool, under these conditions. This leads to practical difficulties in handling, to ensure uniform treatment conditions for the mixture of soluble proteins and insoluble wool residues and also to ensure no mechanical losses. To circumvent this the whole mixture of insoluble wool, soluble protein and excess of reagent was freeze-dried and treated with iodoacetate exactly as described above. For the same reason the whole reaction mixture was again freeze-dried and the whole mixture subjected to partial acid hydrolysis in the presence of the buffer salts and excess of iodoacetate.

Experimental

Two 0.5g samples of both Merino and Lincoln wool were reduced at pH5.0. One each of the Merino and Lincoln samples was alkylated with unlabelled iodoacetate, and the other two samples were alkylated with iodo[2-^{14}C]acetate. All four samples were then reduced with tributyl phosphine and alkylated with iodoacetate, the samples that had been alkylated with unlabelled iodoacetate at the first stage being alkylated with iodo[2-^{14}C]acetate at the second stage and vice versa. Thus for each wool type we have a sample that has been completely reduced and *S*-carboxymethylated and in which the (A+B) fraction of the cystine has been labelled by alkylation with iodo-[2-^{14}C]acetate and another similar sample in which the (C+D) fraction has been radioactively labelled.

On the basis of the known sequences around cystine in wool proteins it was judged that enzyme hydrolysis of the wool samples would not be a profitable approach and accordingly partial acid hydrolysis was used. The standard conditions of 10M-HCl at 37°C for 3 days, which have been shown to be optimum for dipeptide production (Lindley & Haylett, 1967), were adopted and the partial hydrolysates were subsequently evaporated on a rotary evaporator, redissolved in water, re-evaporated and the whole procedure was repeated once more. Finally the material was dissolved in water, made up to 100ml in a graduated flask and stored in the refrigerator.

The same general plan for the separation and characterization of the peptides was followed throughout. An initial separation was made by using high-voltage electrophoresis on paper at pH3.5 and the radioactive peptides were located by radioautography. In all cases this gave six radioactive bands, which were washed off and subjected to further study (see Plate 1). The bands were numbered 1–6 in order,

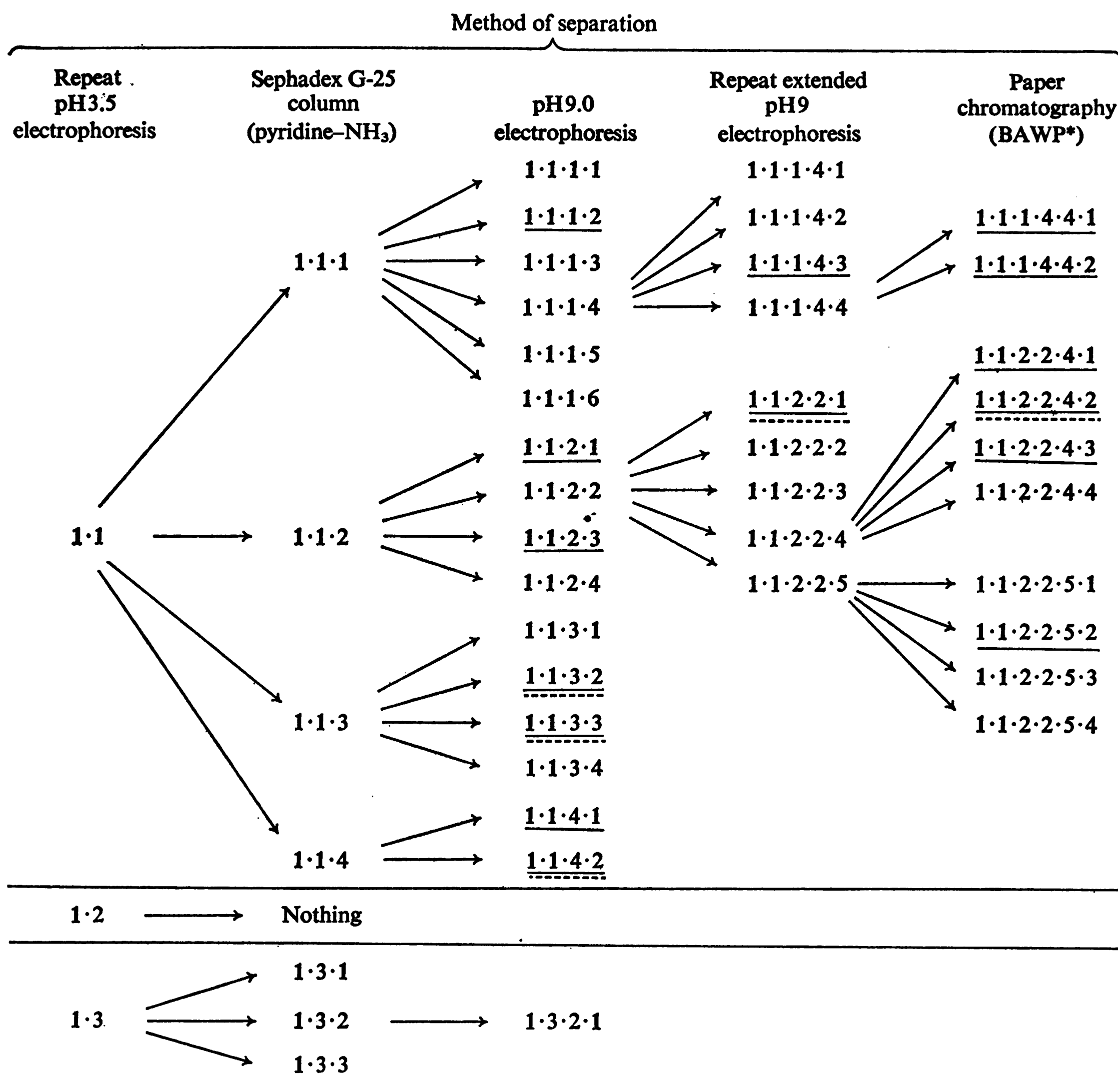
Method of separation				
High-voltage paper electrophoresis at			Paper chromatography (BAWP)*	Identity of peptide
pH3.5 (Plate 1)	pH6.5	pH1.9		
1	See Schemes 2 and 3			
	2·1	2·1·1	2·1·1·1	Ser-Cys(Cm)
2	2·2		2·1·1·2	Thr-Cys(Cm)
	3·1	3·1·1		Cys(Cm)-Gly
3	3·2	3·2·1		Cys(Cm)-Glu
4	4·1	4·1·1	4·1·1·1	Cys(Cm)-Cys(Cm)-Ser†
			4·1·1·2	Cys(Cm)-Cys(Cm)-Thr†
5	5·1			
	5·2	5·2·1		Cys(Cm)-Cys(Cm)
6	6·1	6·1·1		Cys(Cm)

* Abbreviation: BAWP, *n*-butanol–acetic acid–water–pyridine (15:3:12:10, by vol.).

† These two peptides were only characterized in the (A+B) labelled Merino wool. All other samples failed to give identifiable peptides.

Scheme 1. *Fractionation technique for peptides common to all samples*

Fractions that gave identifiable peptides are underlined: other fractions were either complex mixtures or present in too small amounts for satisfactory characterization. ⇀ indicates that the sequence was established by the dansyl–Edman technique. The six fractions referred to in column 1 were obtained from the pH3.5 electrophoresis as shown in Plate 1. Further electrophoresis of these six fractions at the pH values indicated gave further separation as shown. Electrophoresis was by the method of Ambler (1963).



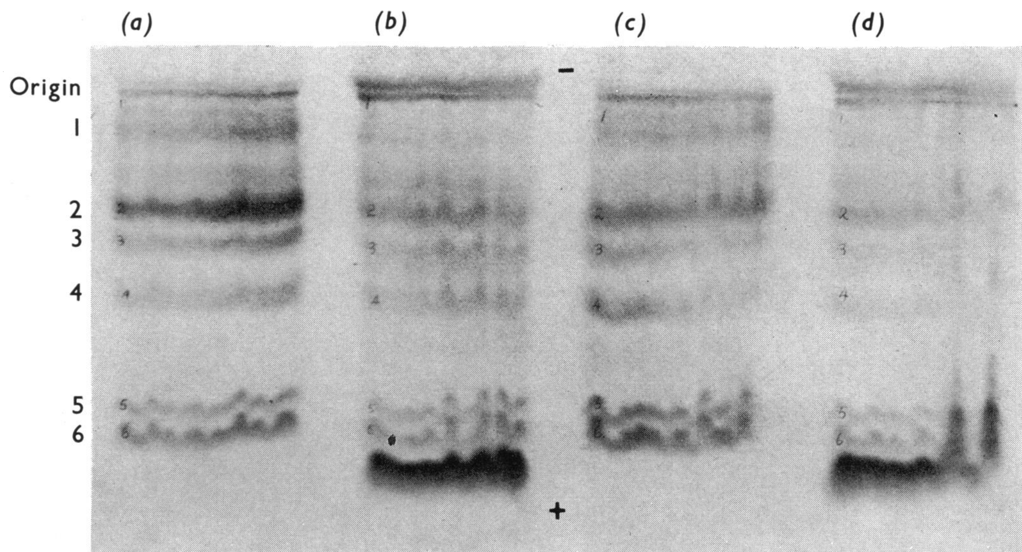
* Abbreviation: BAWP, *n*-butanol–acetic acid–water–pyridine (15:3:12:10, by vol.).

Scheme 2. *Fractionation technique for peptide band* 1 (*Plate* 1) *from* (*A*+*B*) *labelled Merino wool*

Symbols are as in Scheme 1 except an additional symbol ---- indicates that a peptide was isolated and characterized but was identical with previously isolated peptides.

band 6 moving furthest towards the anode. Further separation and characterization of fractions 2–6 was fairly straightforward and involved electrophoresis at pH6.5, followed by electrophoresis at pH1.9 and finally in some cases chromatography on paper with *n*-butanol–acetic acid–water–pyridine (15:3:12:10, by vol.) as developing solvent. The general fractionation procedure used to characterize the peptide fractions is shown in Scheme 1. The peptide was regarded as pure if (1) there was coincidence of ninhydrin colour and radioactivity, (2) it gave an amino acid analysis with nearly integral values and (3) gave only one end group by the dansyl technique. These fractions were studied for both Lincoln and Merino wool and in each case for both the (A+B) and the (C+D) labelled fractions.

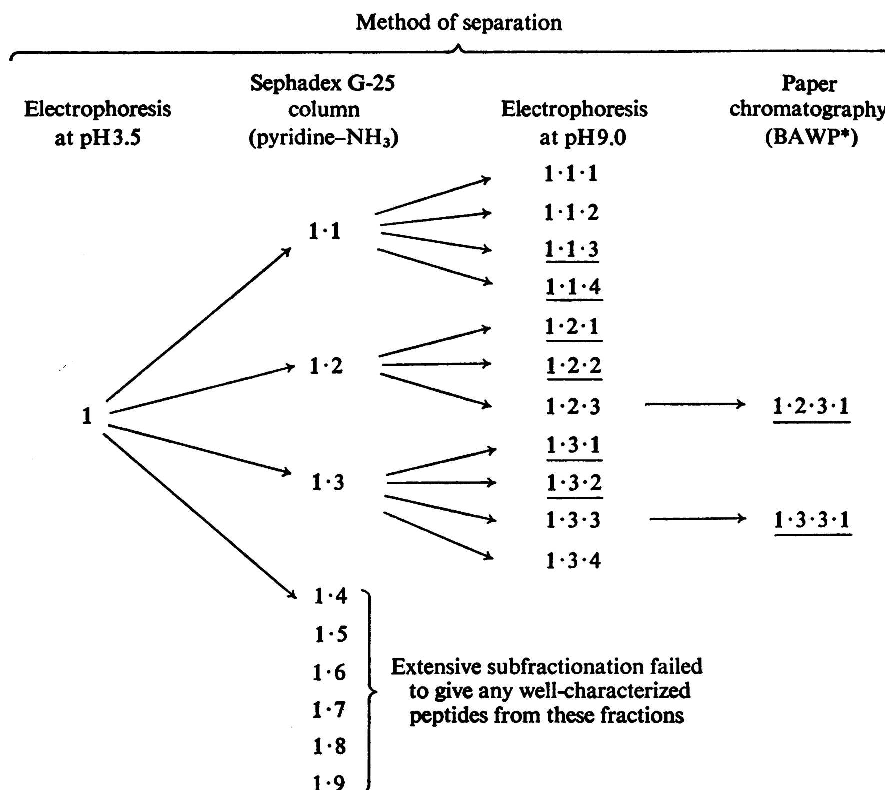
Fraction 1, i.e. the fraction that is almost neutral at pH3.5, proved much more complex and tedious to



EXPLANATION OF PLATE I

Radioautograph of high-voltage-electrophoretic pattern of partial acid hydrolysate of labelled wool samples

Electrophoresis conditions were pyridine–acetate buffer, pH 3.5 (Ambler, 1963), 40V/cm for 2h. (*a*) Merino wool, (A+B) cystine labelled with iodo[2-^{14}C]acetate; (*b*) Merino wool, (C+D) cystine labelled with iodo[2-^{14}C]acetate; (*c*) Lincoln wool, (A+B) cystine labelled with iodo[2-^{14}C]acetate; (*d*) Lincoln wool, (C+D) cystine labelled with iodo[2-^{14}C]acetate. The heavily labelled fast-moving band from the wools with (C+D) labelled cystine is excess of iodo[2-^{14}C]acetic acid that had not reacted and band 6 is free *S*-carboxymethylcysteine.



* Abbreviation: BAWP, *n*-butanol–acetic acid–water–pyridine (15:3:12:10, by vol.).

Scheme 3. *Fractionation technique for peptide band* 1 (*Plate* 1) *from* (*C*+*D*) *labelled Merino wool*

separate and was only studied for the Merino samples. For fractionation of the (A+B) labelled peptides it was necessary to (1) fractionate by longer electrophoresis at pH3.5, followed by (2) chromatography on Sephadex G-25 with pyridine–1M-NH_3 (7:3, v/v) and then (3) one and sometimes two electrophoretic separations at pH9 and finally (4) paper chromatography with *n*-butanol–acetic acid–water–pyridine as developing solvent. The fractionation procedure is shown in Scheme 2.

The (C+D) labelled Merino fraction was treated similarly except that the initial high-voltage electrophoresis at pH3.5 was not repeated. Scheme 3 shows the procedure adopted. In this case complete purification of the peptides proved more difficult, and as noted in the footnotes to Table 4 impurities were sometimes still present in the final fraction. The results of the dansyl–Edman reaction, however, left little doubt as to the identity of the major peptide in the recorded fraction. The complexity of the peptide mixture is in part due to the mixture of proteins present in wool and in part to the relatively non-specific method of hydrolysis used, and is reflected in the comparatively small number of fractions which gave identifiable peptides out of the large number of fractions isolated. Those fractions that were not characterized were either mixtures that were still too complex despite the tedious fractionation techniques used or alternatively they split into a number of minor components each of which was only very weakly radioactive.

Results

Table 1 gives the cystine and carboxymethylcysteine contents of untreated, partially reduced and blocked, and completely reduced and blocked Lincoln and Merino wool samples. This shows that 53% of the total cystine in the Merino wool and 58% in the Lincoln wool can be regarded as (A+B) cystine. Because alkylation conditions with the radioactive label were identical in all cases, it is straightforward to calculate the percentage reduction from the values

Table 1. *Cystine and S-carboxymethylcysteine contents*

(A+B) reduced and alkylated wool refers to wool in which the readily reducible cystine fraction has been reduced with thioglycollate and alkylated with iodoacetate. Fully reduced and alkylated wool refers to wool that has been fully reduced with tributyl phosphine and alkylated with iodoacetate. Cystine and carboxymethylcysteine contents were determined by amino acid analysis.

	Amino acid content (μmol/g of wool)					
	Merino wool			Lincoln wool		
	Whole wool	(A+B) reduced and alkylated wool	Fully reduced and alkylated wool	Whole wool	(A+B) reduced and alkylated wool	Fully reduced and alkylated wool
Cystine	950	425	0	763	289	0
Carboxymethylcysteine	0	500	945	0	444	737

Table 2. *Characterization and specific radioactivity of peptides isolated from both (A+B) and (C+D) fractions of Merino and Lincoln wools*

⟶ indicates that the sequence was established by the dansyl–Edman technique.

Fraction no. (Scheme 1)	Composition and sequence	Specific radioactivity [c.p.m./μmol of Cys(Cm)]			
		Merino (A+B)	Merino (C+D)	Lincoln (A+B)	Lincoln (C+D)
2·1·1·1	$\overrightarrow{\text{Ser}}$-Cys(Cm)	69000	26000	67000	31000
2·1·1·2	$\overrightarrow{\text{Thr}}$-Cys(Cm)	72000	26000	63000	38000
3·1·1	$\overrightarrow{\text{Cys(Cm)}}$-Gly	69000	19000	75000	22000
3·2·1	$\overrightarrow{\text{Cys(Cm)}}$-Glu	54000	28000	45000	32000
5·2·1	$\overrightarrow{\text{Cys(Cm)}}$-Cys(Cm)	51000	25000	45000	32000
5·2·1 after Edman degradation		53000	25000	43000	30000
Average Cys(Cm) from complete hydrolysates		56000	40000	56000	40000

for the specific radioactivity of *S*-carboxymethylcysteine samples isolated from the total hydrolysates (cf. Table 2): this gives a result of 58% for both wool samples.

Table 2 gives the identity and specific radioactivity of some labelled peptides isolated from all four different samples, and Tables 3 and 4 concern peptides isolated respectively from (A+B) and (C+D) labelled Merino wool. The results of Table 2 show that the sequences Ser-Cys(Cm), Thr-Cys(Cm) and Cys(Cm)-Gly derive predominantly from the readily reducible (A+B) fraction of the cystine, whereas Cys(Cm)-Glu and Cys(Cm)-Cys(Cm) show no very obvious trend. The Cys(Cm)-Glu which was isolated is certainly derived from both Cys(Cm)-Glu and Cys(Cm)-Gln sequences in the fibre since the partial acid hydrolysis procedure used converts the glutamine residue into glutamic acid. An interesting point relating to Cys(Cm)-Cys(Cm) which is shown by the results of Table 2 is that the labelling shows no preference for either of the carboxymethylcysteine residues.

Table 3 gives the radioactively labelled peptides that were isolated from the (A+B) labelled Merino wool. The fact that these peptides, with the sole exception of Val-Cys(Cm), were not isolated from the (C+D) labelled wool suggests that they occur mainly in association with the (A+B) cystine. In general this is confirmed by the high specific radioactivity of the

Table 3. *Identity and specific radioactivity of peptides isolated from the (A+B) fraction of Merino wool*

⟶ indicates that the sequence was established by the dansyl–Edman technique.

Fraction no. (Schemes 1 and 2)	Peptide sequence	Specific radioactivity [c.p.m./μmol of Cys(Cm)]
4·1·1·1	Cys(Cm)-Cys(Cm)-Ser	78000
4·1·1·2	Cys(Cm)-Cys(Cm)-Thr	68000
1·1·2·1*	Asp-Cys(Cm)	110000
1·1·2·3	Gly-Cys(Cm)-Cys(Cm)	80000
1·1·4·1	Glu-Glu-Cys(Cm)	73000
1·3·3	Cys(Cm)-Arg-Glu	63000
1·1·1·4·3	Tyr-Cys(Cm)	76000
1·1·4·1	Cys(Cm)-Ile	67000
1·1·4·2	Cys(Cm)-Leu	76000
1·1·2·2·4·3	Val-Cys(Cm)	78000
1·1·2·2·5·2	Cys(Cm)-Ala	69000

* The anomalously high specific radioactivity of this peptide suggests the presence of some non-peptide radioactive impurity.

Table 4. *Identity and specific radioactivity of peptides isolated from the (C+C) labelled fraction of Merino wool*

⟶ indicates that the sequence was established by the dansyl–Edman technique.

Fraction no. (Scheme 3)	Peptide sequence	Specific radioactivity [c.p.m./μmol of Cys(Cm)]
1·1·3	Val-Cys(Cm)-Pro	78000
1·1·4	Val-Cys(Cm)	55000
1·2·1* and 1·3·1	Val-Pro-Cys(Cm)	120000
1·2·2† and 1·3·2	Val-Val-Cys(Cm)-Cys(Cm)	80000 and 72000
1·2·3·1‡	Pro-Ile-Cys(Cm)	84000
1·3·3·1	Pro Pro Cys(Cm) Cys(Cm) Val Val	49000

* Neither fraction was completely pure but undoubtedly this is the major sequence present. The anomalously high specific radioactivity suggests that some non-peptide radioactive impurity may also be present.

† Neither of these fractions was completely pure but the dansyl–Edman results show that this is the major sequence present, but minor contamination with Val-Ile-Cys(Cm)-Cys(Cm) also occurs.

‡ Both amino acid and dansyl–Edman analysis suggest some admixture with Pro-Val-Cys(Cm).

peptides, although none reach the theoretical maximum of 95000c.p.m./μmol of Cys(Cm). [A peptide derived from both (A+B) and (C+D) fractions would of course have a lower specific radioactivity as it would in part be produced by alkylation with unlabelled iodoacetate; see the Experimental section.] All these peptides except Cys(Cm)-Arg-Glu have already been found in wool-protein sequences [high-sulphur proteins SCMK-B2B (Elleman & Dopheide, 1972); SCMKB-IIIB2 and IIIB3 (Swart & Haylett, 1973; T. Haylett, personal communication); SCMK-B2 (Lindley *et al.*, 1968); low-sulphur proteins type I and type II, helical (W. G. Crewther, personal communication); high-tyrosine-glycine protein (Dopheide, 1973)].

The corresponding data for the (C+D) labelled peptides in Table 4 are interesting in the fact that, apart from Val-Cys(Cm), they are tripeptides or larger and involve only proline, valine and isoleucine in addition to the carboxymethylcysteine. The above-average specific radioactivities in general confirm the association with the (C+D) fraction (the argument is the same as that given in the preceding paragraph), but the value for the hexapeptide is lower than might be expected from the general trend. Only the Val-Val-Cys(Cm)-Cys(Cm) sequence has not so far been found in wool proteins.

Discussion

Two quite firm conclusions seem to emerge from the results. First it seems that the major difference between the (A+B) and (C+D) fractions is in the polarity of their environment. Thus the isolated (A+B) labelled peptides generally involve polar amino acids, whereas the less reactive (C+D) cystine seems to be associated with hydrophobic amino acid side chains. With hindsight this conclusion makes good sense of the fact that the subdivision of cystine reactivity is most clear-cut when sulphite solutions are used, since the sulphite ion could presumably only react in an aqueous environment, whereas it is known that reagents like aqueous benzyl mercaptan, which may be able to also penetrate more hydrophobic regions, do bring about more extensive reduction. The hypothesis would thus be in agreement with the suggestion that intrachain disulphide bonds involving relatively small loops would be less reactive but would not imply that the least reactive disulphide bonds were necessarily intrachain (Cecil & Loening, 1960).

A second conclusion would be that the varying reactivity is not directly related to the occurrence of Cys-Cys sequences in the wool. Since from known keratin sequences it appears that about 50% of the cystine residues occur as Cys-Cys sequences (cf. also Lindley & Haylett, 1967), and since it has been shown in model compounds that such sequences are extremely reactive (Lindley & Haylett, 1968), it seemed possible that the more reactive (A+B) fraction might correspond to Cys-Cys sequences in the wool proteins. The present results seem definitely to exclude this.

With regard to the general problem of characterizing disulphide bonds in the keratin complex, our results do not suggest any easy solution. However, the finding that the less reactive cystine bonds occur in non-polar environments suggests that these may well be structurally the most important and may be directly responsible for the chemical inertness of the keratins to chemical attack in aqueous environments. It is also in agreement with previously published observations showing that rupture of the (C+D) cystine fraction has profound structural effects on the wool fibre (Carter *et al.*, 1946; Lindley, 1957). If so, this raises the possibility of isolating soluble proteins from partially reduced and alkylated wool with key disulphide linkages intact. Preliminary work has shown that this is in fact possible and we hope to be able to characterize some cystine linkages that are structurally important in the wool fibre. Looked at realistically and considering the mode of biosynthesis of wool, this is probably the limit of information on characterizing disulphide bonds that is attainable by any presently known technique. This conclusion is further reinforced if the possibility is admitted that, so far as the arrangement of their disulphide bonds is concerned, no two wool fibres may be identical.

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