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Evidence of Homology in a High-Sulphur Protein Fraction (SCMK-B2) of Wool and Hair α-Keratins

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Fractions corresponding to the *S*-carboxymethylated high-sulphur protein component SCMK–B2 isolated by Gillespie (1963) from Merino wool were prepared from five different wool samples and also from bovine hair. The six fractions showed great similarities in amino acid composition, and also gave very similar peptide 'maps' after tryptic and chymotryptic digestion. Some of the peptides were isolated from the different samples, and evidence is given that suggests that a sequence of at least 21 amino acids is common to all the fraction SCMK–B2 preparations. Further, all the fractions derived from the wool samples have the same acetylated heptapeptide for the *N*-terminal sequence, but one extra residue may be present in this *N*-terminal sequence in the protein from bovine hair. The general significance of these findings is discussed.

We are attempting to characterize the *S*-carboxymethylated high-sulphur protein fraction of Merino wool, fraction SCMK–B2 (Gillespie, 1963; Lindley, Gillespie & Haylett, 1968; Haylett & Lindley, 1968), and sufficient data have already been accumulated to make it feasible to survey similar protein fractions from other keratins with the hope of uncovering structural regularities. This paper reports the results of a preliminary survey of SCMK–B2-type fractions isolated from a range of wools from various breeds of sheep, and also a similar fraction from bovine hair.

Fraction SCMK–B2 is the *S*-carboxymethylated derivative of a sulphur-rich protein isolated from Merino wool. It has a molecular weight of 23000, and it contains no methionine, lysine or histidine but is rich in CMCys,† proline and serine. Although it gives only a single moving-boundary peak on electrophoresis at pH values in the range 4–11, it has been difficult to obtain conclusive evidence for its homogeneity. This difficulty arises from a number of causes, among which are the absence of a free *N*-terminus, the very high net negative charge at pH values at which the protein is soluble and the extremely poor staining with the dyes commonly used in gel-electrophoretic procedures. It may well be that a sequence study is the only way in which a definite conclusion can be reached on this problem.

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† Abbreviation: CMCys, *S*-carboxymethylcysteine.

MATERIALS AND METHODS

Origin and preparation of the keratin samples. The samples of wool and hair used in this work were obtained from the following sources.

(*a*) Merino wool was produced by a commercial flock (Wintoc) and was similar to that used in earlier studies on fraction SCMK–B2 (Gillespie, 1963).

(*b*) Mutant Merino wool was from a mutant Merino sheep showing the lustre characteristics described by Short (1958).

(*c*) Two samples of English Leicester × Merino wool were obtained from the same animal at the Division of Animal Physiology, Commonwealth Scientific and Industrial Research Organisation. One sample (3% S) was wool produced on a normal diet, and the other sample (4% S) was wool produced after the animal's diet had been supplemented by an abomasal infusion of methionine by the techniques of Reis & Schinckel (1963).

(*d*) Dorset Horn wool came from a stud ewe in a commercial flock (K. G. Luke).

(*e*) Bovine hair came from a young Jersey calf from a commercial herd.

All the keratin samples were thoroughly washed with light petroleum (b.p. 55–70°), ethanol and water, and finally dried in air.

Isolation and purification of fraction SCMK–B2. (*a*) From Merino wool. The standard procedure of Gillespie (1963) was used for the preparation of fraction SCMK–B2 from Merino wool. This used a low-temperature extraction with alkaline thioglycollate followed by alkylation at pH 8–9 with iodoacetate, and a fractionation that involved both precipitation and chromatographic procedures. Details of this method are also given by Crewther, Fraser, Lennox & Lindley (1965).

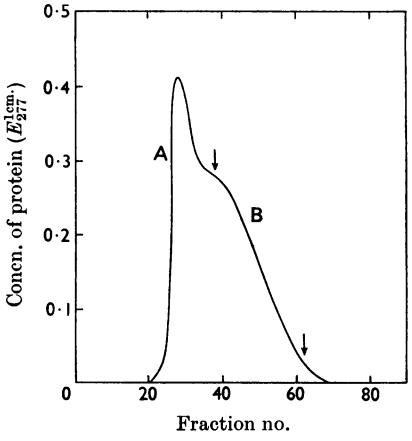


Fig. 1. Column chromatography of the unfractionated high-sulphur proteins from Lustre Mutant Merino wool on DEAE-cellulose at pH 4·5. The buffer was 0·02 *I* acetate with a superimposed linear gradient of NaCl (0·25–0·8 M). Fractions (15 ml.) were collected, and the total volume of eluate was 1400 ml.

(*b*) From other wool and hair samples. A urea–thioglycollate extraction was used in the isolation of fraction SCMK–B2 from hair and from wool samples other than Merino because it was more quantitative than that normally used (Gillespie, 1963), and therefore better suited for use with keratin samples of often limited availability. This change necessitated some modifications to the early stages of the fractionation.

The keratins were solubilized by treatment with a urea–thioglycollate solution by the procedure of Harrap & Gillespie (1963). The extracted proteins were alkylated with iodoacetate at pH 8–9 to convert the cysteine residues into CMCys residues, and the solution was then dialysed and fractionated into low- and high-sulphur protein components by precipitation of the former at pH 4·4 (Gillespie, O'Donnell & Thompson, 1962). The solution was centrifuged and the supernatant, containing the high-sulphur proteins, was dialysed against water and freeze-dried. The yield depended on the keratin sample but was usually within the range 20–30% of the initial dry weight of keratin taken.

The high-sulphur protein fraction (1 g.) was chromatographed on a DEAE-cellulose column (5 cm. × 10 cm.) at pH 4·5 with a NaCl gradient. The initial buffer was 0·02 *I* acetate in 0·25 M-NaCl, and the gradient was linear to 0·8 M-NaCl. Fig. 1 shows a characteristic elution profile; the arrows indicate the fractions that were collected. Tubes containing fraction B were pooled, dialysed and freeze-dried, and the procedure was repeated until about 2 g. had been accumulated.

Fraction B (2 g.) was dissolved in 400 ml. of 0·1 *I* acetate buffer, pH 6·1, and brought to 1·6 M-$(NH_4)_2SO_4$ by the addition of 97·2 g. of $(NH_4)_2SO_4$. The precipitate was centrifuged down, redissolved in 50 ml. of 1% $NaHCO_3$ solution and dialysed for several days against many changes of deionized water. The fractionation procedure was then continued to the phosphoric acid precipitation stage as for Merino-wool proteins (Gillespie, 1963). Scheme 1 shows the steps in a typical fractionation with the yields obtained at each stage.

Amino acid analysis. Amino acid analyses were carried out on either a Technicon AutoAnalyzer or a Beckman model 120C amino acid analyser. The hydrolyses were carried out *in vacuo* with constant-boiling HCl at 110° for 24 hr. in sealed tubes.

High-voltage paper electrophoresis. High-voltage paper electrophoresis was carried out in a Michl-type apparatus. Location of peptides on paper electrophoretograms was carried out with either the Cl_2–tolidine–KI technique or the ninhydrin reagent. When it was necessary to isolate peptides from electrophoretograms they were eluted with 1 N-NH_3 solution.

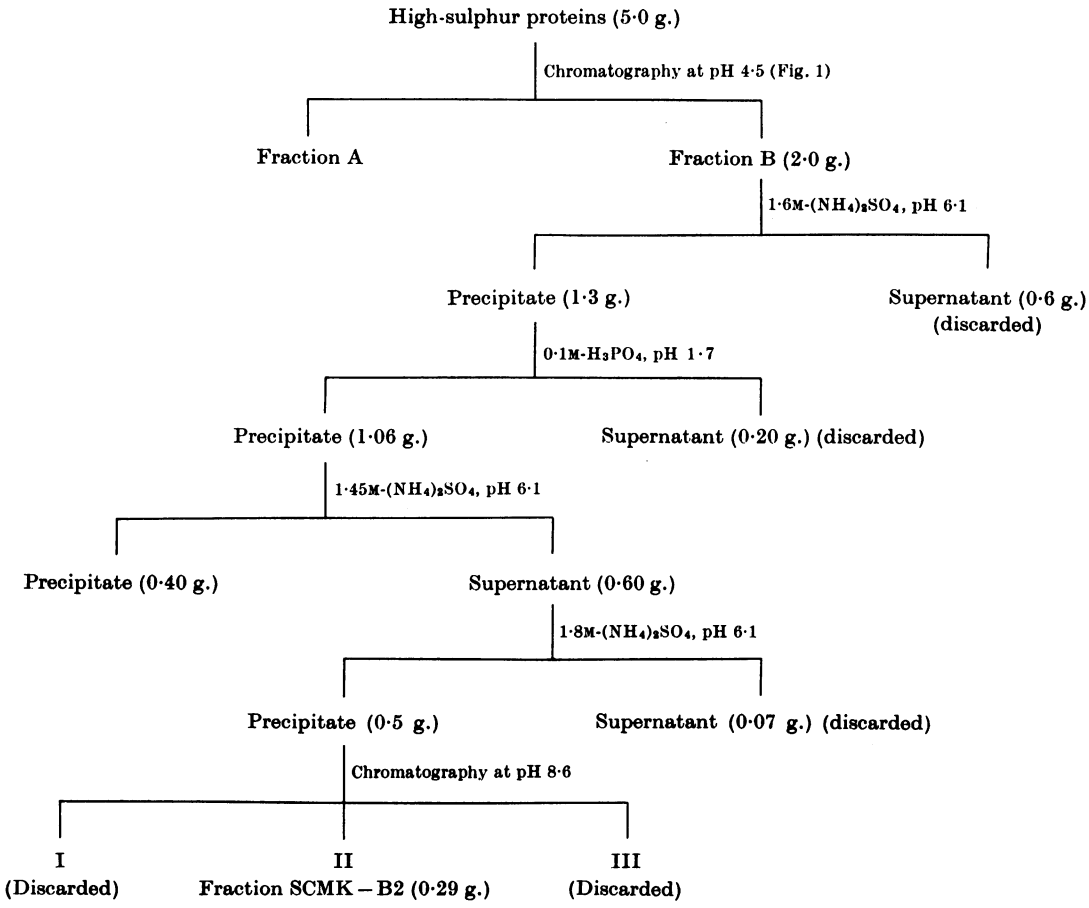
Enzymic digestion. Tryptic and chymotryptic digestions were both carried out at 38° and pH 8·3 in 2% (w/v) $(NH_4)_2CO_3$ solution, with a ratio of protein treated/enzyme used of 100:1 (w/w). The reaction was stopped by the addition of di-isopropyl phosphorofluoridate (10% of the weight of enzyme) and incubation for a further ½ hr. before the solution was freeze-dried overnight. Tryptic digestions were carried out for 3 hr. with trypsin that had been pretreated with L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one to minimize chymotryptic activity. Chymotryptic digestions were allowed to proceed for 4 hr.; the chymotrypsin was preincubated with 50% (w/w) of lima-bean trypsin inhibitor to ensure minimal tryptic activity.

RESULTS

Table 1 shows the amino acid analyses of the fraction SCMK–B2 proteins. For ease of comparison all the results are calculated as residues/mol. of mol.wt. 23000, this value being the molecular weight of fraction SCMK–B2 isolated from Merino wool (Gillespie & Harrap, 1963). There is a close correspondence between the compositions of the proteins from the different wools and this resemblance extends even to bovine hair, though there are significant differences, particularly in the contents of aspartic acid, serine, valine and leucine.

A much more sensitive test for resemblances between proteins is furnished by the peptide pattern given after enzyme digestion. Fig. 2 shows a paper electrophoretogram of tryptic digests of the different preparations run side by side, so that a peptide common to all the samples appears as a continuous band. From the electrophoretic pattern it would seem probable that the two basic peptides are common to all the protein samples. For fraction SCMK–B2 from Merino wool it is known that the faster band (band T1) is Thr-Arg (Haylett & Lindley, 1968). Amino acid analyses after elution showed this to be the case for the fast band from all the keratins in Fig. 2.

The slower of the two basic peptides (band T2) in the tryptic digest of fraction SCMK–B2 from Merino wool was shown to be Trp-CMCys-Arg-Pro-Asp-CMCys-Arg (Haylett & Lindley, 1968). Only qualitative evidence of composition was sought in the corresponding peptides from the other keratins.



Scheme 1. Flow-sheet showing stages in the preparation of fraction SCMK–B2 from Lustre Mutant Merino wool and the yields of fractions at each stage.

These were isolated by elution from individual paper electrophoretograms and all were shown (*a*) to absorb at 280 mμ, (*b*) to give a positive Ehrlich test for tryptophan and (*c*) to yield CMCys, aspartic acid, proline and arginine on hydrolysis as shown by paper electrophoresis at pH 1·9. There is some evidence of contamination with a second peptide in the material eluted from the electrophoretogram of bovine hair fraction SCMK–B2. There is a unique band in the electrophoretogram of the tryptic digest of mutant Merino fraction SCMK–B2 (Fig. 2, band TX). This is evidence for a change in sequence of this protein and is the first inkling of a molecular basis for changes of this kind in the fleece of the sheep.

Fig. 3 shows an electrophoretogram of chymotryptic digests of the fraction SCMK–B2 preparations; again there are similarities in the peptide patterns. We concentrated our attention on the bands labelled C1, C2 and C3. Band C1 is ninhydrin-negative in all the samples, and for the fraction SCMK–B2 from Merino wool is known to be acetyl-Ala-CMCys-CMCys-Ser-Thr-Ser-Phe (Haylett & Lindley, 1968). This band was eluted from individual electrophoretograms of all the samples, hydrolysed and analysed. For this purpose the electrophoresis was run for 3 hr. to resolve the band from other peptides. Table 2 gives the amino acid analyses of the peptides isolated. The peptide from bovine hair was again somewhat anomalous in that the yield was low, and it appears that it may have an extra alanine residue per molecule.

Band C2 was also isolated from the same electrophoretograms as band C1, and Table 3 gives the analyses of these peptides. Band C3 was isolated from another electrophoretogram run for 1 hr. only. For fraction SCMK–B2 from Merino wool its structure is known (Haylett & Lindley, 1968) to be Ser-Arg-Thr-Arg-Trp. Because of the simultaneous occurrence of tryptophan with other sensitive

Table 1. *Amino acid composition of fraction SCMK–B2 from various sources*

	Amino acid composition (residues/mol. of mol.wt. 23000)					
		English Leicester × Merino wool				
Source of fraction SCMK–B2 ...	Merino wool	Normal diet (3% S)	S-enriched diet (4% S)	Lustre Mutant Merino wool	Dorset Horn wool	Bovine hair
CMCys	46·5	47·3	47·4	44·8	47·1	49·8
Asp	1·9	2·1	1·5	1·8	1·7	2·7
Thr	20·6	20·3	20·5	19·7	19·0	16·0
Ser	27·7	29·2	29·4	29·5	29·4	20·7
Glu	23·1	22·0	22·1	20·2	20·5	17·4
Pro	18·8	20·9	20·4	22·4	18·3	23·8
Gly	16·7	15·4	15·8	14·7	15·7	15·4
Ala	6·5	6·4	6·1	5·8	5·9	6·8
Val	7·8	7·5	8·0	8·0	8·1	9·8
Met	0·0	0·0	0·0	0·0	0·0	0·0
Ile	8·1	6·8	7·1	7·0	8·0	7·0
Leu	4·0	4·0	3·7	3·6	3·8	5·0
Tyr	4·6	4·4	4·4	4·7	5·0	4·4
Phe	2·4	1·8	2·1	2·1	2·2	1·9
Arg	8·7	9·0	8·8	10·0	11·2	11·2
Lys	Trace	0·2	0·3	0·2	Trace	0·5
His	Trace	Trace	0·2	0·4	Trace	0·7

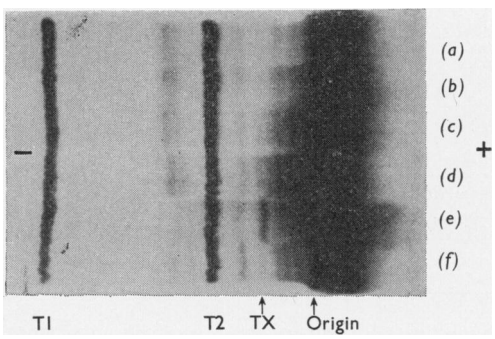


Fig. 2. Electrophoretogram showing comparative peptide 'maps' of tryptic digests of fraction SCMK–B2 of different keratins, run at pH 3·5 and 50 v/cm. for 1 hr. and stained with Cl_2–toluidine. (*a*) Merino wool; (*b*) English Leicester × Merino wool, normal diet; (*c*) English Leicester × Merino wool, sulphur-enriched diet; (*d*) Dorset Horn wool; (*e*) Lustre Mutant Merino wool; (*f*) bovine hair.

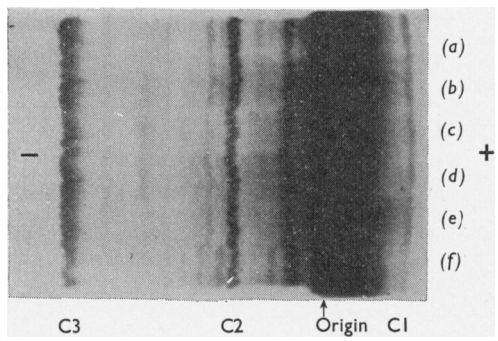


Fig. 3. Electrophoretogram showing comparative peptide 'maps' of chymotryptic digests of fraction SCMK–B2 of different keratins, run at pH 3·5 and 50 v/cm. for 1 hr. and stained with Cl_2–toluidine. The key is as in Fig. 2.

amino acids, it is difficult to get a good amino acid analysis of this peptide. However, all the different samples were shown by paper electrophoresis at pH 1·9 to give serine, threonine and arginine on acid hydrolysis, and they also all gave a positive Ehrlich reaction before hydrolysis.

DISCUSSION

This paper presents evidence that it is possible to prepare a similar *S*-carboxymethylated high-sulphur protein fraction from a number of different types of wool and a related fraction from bovine hair. Significant features of the overall composition are the very high contents of CMCys, serine, threonine, glutamic acid, proline and glycine, which together account for 75% of the residues, the complete absence of methionine and the very low contents of aspartic acid and phenylalanine. The fact that lysine and histidine always occur in amounts less than 1 residue/mol. indicates that they arise from a contaminating protein, and in this connexion it should be stressed that all the SCMK–B2 fractions used in the present work were isolated by a pro-

Table 2. *Amino acid composition of* N*-terminal peptide (band C1, Fig. 3) of fraction SCMK–B2 from various sources*

	Amino acid composition (residues/mol. of peptide)					
		English Leicester × Merino wool				
Source of fraction SCMK–B2 ...	Merino wool	Normal diet (3% S)	S-enriched diet (4% S)	Lustre Mutant Merino woo	Dorset Horn wool	Bovine hair
CMCys	1·7	2·0	1·9	2·1	1·9	2·0
Thr	1·2	1·1	1·1	1·1	1·1	1·0
Ser	2·0	2·0	2·0	2·0	2·1	1·9
Ala	1·1	1·0	1·0	1·0	1·0	1·8
Phe	1·1	0·8	0·9	0·9	0·9	1·3

Table 3. *Amino acid composition of a chymotryptic peptide (band C2, Fig. 3) of fraction SCMK–B2 from various sources*

	Amino acid composition (residues/mol. of peptide)					
		English Leicester × Merino wool				
Source of fraction SCMK–B2 ...	Merino wool	Normal diet (3% S)	S-enriched diet (4% S)	Lustre Mutant Merino wool	Dorset Horn wool	Bovine hair
Ser	2·7	3·0	3·0	3·0	3·1	2·7
Glu	1·0	1·1	1·1	1·1	1·1	1·1
Gly	2·9	2·8	2·8	2·8	2·8	3·1
Ala	1·0	1·1	1·1	1·0	1·0	1·1
Val	1·9	2·0	1·9	1·9	2·0	2·0

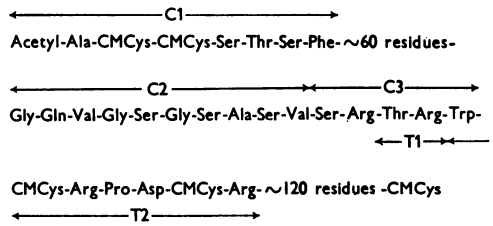


Fig. 4. Amino acid sequences and positions in the molecule of peptides of Merino wool fraction SCMK–B2 discussed in the text.

cedure developed for use with Merino wool, and no attempt was made either to adapt it for any particular keratin or to introduce any subsidiary purification procedures. As a result some of the preparations, particularly that from bovine hair, are obviously contaminated with proteins containing both lysine and histidine. It is quite possible that the overall differences observed between these preparations are a reflection of variable amounts of impurities and not inherent differences in the composition of fraction SCMK–B2, although some differences in a homologous series would not be unexpected.

The reinforcement of this overall similarity of composition by the finding of common amino acid sequences for the fraction SCMK–B2 preparations we have examined is quite a striking finding in a field so far distinguished mainly by its apparent complexity and intractability. In the case of fraction SCMK–B2 from Merino wool it is known (Lindley *et al.* 1968; Haylett & Lindley, 1968) that the sequences and position in the molecule of the peptides discussed in this paper are as shown in Fig. 4.

The evidence we have presented suggests that this sequence may be a common feature of a protein present in all the wool types we have examined, and that they are also present in the protein of bovine hair with perhaps minor variation in the *N*-terminal sequence.

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APPENDIX

The Characterization of some Peptides from the High-Sulphur Protein SCMK-B2 from Merino Wool

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This note records information on the isolation and characterization of the peptides referred to in the main paper (Gillespie, Haylett & Lindley, 1968) as they were originally isolated from fraction SCMK-B2 of Merino wool, together with results on some related bridging peptides.

*Peptide T*1. In addition to the electrophoretic technique referred to by Gillespie *et al.* (1968), this peptide was isolated in 80–90% yield from tryptic digests of fraction SCMK-B2 by (*a*) chromatography on Dowex 50 under the conditions given by Schroeder, Jones, Cormick & McCalla (1962) and (*b*) chromatography on DEAE-cellulose at pH 7 in 0·1 *I* tris–hydrochloric acid buffer with a linear gradient of 0–0·6 M-sodium chloride (Fig. 1). This peptide was shown to be Thr-Arg by quantitative amino acid analysis and the determination of its *N*-terminus by the 'dansyl' technique (Gray & Hartley, 1963*a*,*b*,).

*Peptide T*2. This was isolated by the same techniques as used for peptide T1. It has the formula Trp-CMCys†-Arg-Pro-Asp-CMCys-Arg by complete

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† Abbreviation: CMCyS, *S*-carboxymethylcysteine.

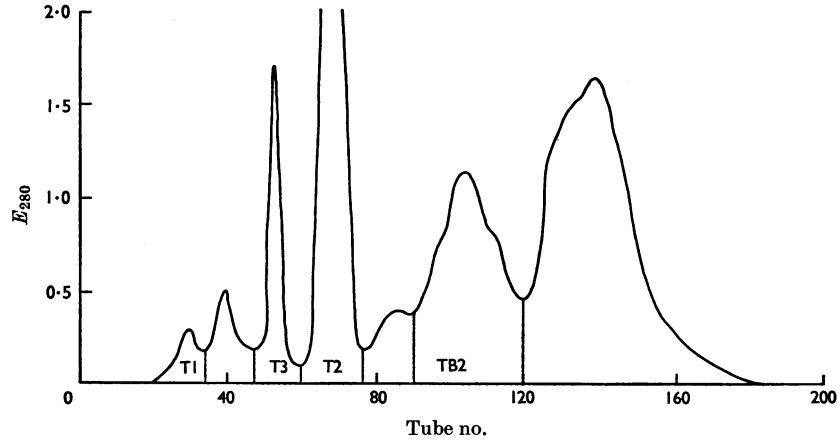

Fig. 1. Fractionation of a tryptic digest of fraction SCMK–B2 (1 g.) on a DEAE-cellulose column (2·5 cm. × 25 cm.). A 1l. gradient of 0–0·6 M-NaCl in tris–HCl buffer, pH 7·0, was used. Fractions (5 ml.) were collected and pooled as indicated to give the tryptic peptides.

sequence determination by the Edman degradative procedure as described by Blombäck, Blombäck, Edman & Hessel (1966) in conjunction with the 'dansyl' technique.

Peptide T3. This was isolated in small yield from tryptic digests by all three procedures; it is a compound of peptides T1 plus T2 with the sequence: Thr-Arg-Trp-CMCys-Arg-Pro-Asp-CMCys-Arg. It obviously arises from an incomplete tryptic split and is accordingly found in highest yield in digests in which tryptic action has only been allowed to proceed for short periods.

Other products of tryptic digestion of fraction SCMK–B2. These are high-molecular-weight peptides that can be fractionated on DEAE-cellulose (Fig. 1). One of these, peptide TB2, has a molecular weight of about 8600, determined by ultracentrifugation and from the empirical formula. Like fraction SCMK–B2, it has no free amino group and we therefore assume that it is the *N*-terminal fragment. [We have been unable to confirm the earlier finding (Gillespie & Harrap, 1963) of an *N*-terminal arginine residue for fraction SCMK–B2]. Carboxypeptidase B liberated arginine from peptide TB2, and subsequent reaction with carboxypeptidase A gave serine as the penultimate residue.

Chymotryptic digestion of peptide TB2. This gave, among other products, three peptides of the following compositions that are readily separated by paper electrophoresis at pH 6·5 for 1 hr. at 50 v/cm.: (1) (Ser,Arg); (2) (Ser_4,Glu,Gly_3,Ala,Val_2,-Arg); (3) (Ser_3,Glu,Gly_3,Ala,Val_2).

Obviously peptide (2) is a composite of peptides (1) and (3), peptide (1) being *C*-terminal. This was confirmed by Edman degradations of peptides (2) and (3), which showed that both peptides have the *N*-terminal sequence Gly-Gln-Val-. However, the degradation could not be carried beyond this point. The action of carboxypeptidase A on peptide (3) gave the *C*-terminal sequence -Ala-Ser-Val, and hence the partial sequence is Gly-Gln-Val-(Gly_2,-Ser_2)-Ala-Ser-Val-Ser-Arg. The complete sequence was established by the isolation of peptides from a papain digest by paper electrophoresis at pH 1·9 and 50 v/cm. Three major peptides were obtained. One of these was (Ser_2,Gly) and gave serine as both *N*- and *C*-terminus and hence must be (Ser-Gly-Ser). The second was (Ser,Ala,Val) and gave *N*-terminal alanine and *C*-terminal valine. Hence the sequence must be Ala-Ser-Val. The third was (Gly_2,Glu,Val) and both *N*- and *C*-terminus were glycine. This is therefore the *N*-terminal fragment of the whole chymotryptic peptide and the complete sequence of peptide (2) is: Gly-Gln-Val-Gly-Ser-Gly-Ser-Ala-Ser-Val-Ser-Arg.

The isolation reported in the main paper (Gillespie *et al.* 1968) of the peptide C3 from a chymotryptic digest of fraction SCMK–B2 [which was (Thr,Ser,Arg_2) and gave a positive Ehrlich reaction for tryptophan] provides a linking peptide between this and the tryptic sequence T3 deduced above, since there is a unique tryptophan residue in the fraction SCMK–B2 molecule. Taken together, these peptides thus establish the following 21-residue sequence in fraction SCMK–B2: -Gly-Gln-Val-Gly-Ser-Gly-Ser-Ala-Ser-Val-Ser-Arg-Thr-Arg-Trp-CMCys-Arg-Pro-Asp-CMCys-Arg-.

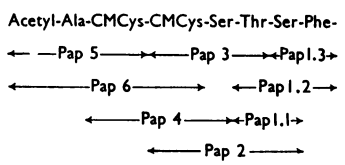

Fig. 2. Complete amino acid sequence of peptide Cl from Merino wool fraction SCMK–B2, and the papain peptides used in determining it.

Peptide C1. Chymotryptic digestion of peptide TB2 or fraction SCMK–B2 itself also produces the peptide C1 referred to in the main paper (Gillespie *et al.* 1968). This has the composition (CMCys$_2$,Thr,Ser$_2$,Ala,Phe) and is ninhydrin-negative, and hence constitutes the *N*-terminal sequence of the protein. The sequence of the peptide was established by the use of carboxypeptidase A and papain digestion. Carboxypeptidase A gave very clear-cut evidence that the *C*-terminal sequence was -Thr-Ser-Phe. Traces of CMCys were observed after very prolonged digestion by the enzyme but alanine was not detected. Papain digestion of the peptide at pH 5·5 and 40° overnight gave a complex mixture of peptides. These were resolved by paper electrophoresis at pH 6·5 and 50 v/cm. for 2 hr. into six fractions. Five of these were single components, but one could be split into three components by paper chromatography on Whatman 3MM paper in pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.). Amino acid analysis was carried out on each fraction, and from the amino acid composition alone the following partial sequence could be deduced: X-(CMCys,Ala)-CMCys-Ser-Thr-Ser-Phe, where X is most probably acetyl. The fact that carboxypeptidase A failed to liberate any alanine suggests that the most probable sequence is acetyl-Ala-CMCys-. Confirmation of this was obtained by showing that synthetic acetyl-Ala-CMCys was indistinguishable in its electrophoretic behaviour at pH 3·5 and 6·5 from the peptide (Pap 5) isolated from the papain digest. The complete sequence and the papain peptides used in deriving it are shown in Fig. 2.

The partial sequence of the *N*-terminal fragment of fraction SCMK–B2 from Merino wool is thus as given in the main paper (Gillespie *et al.* 1968): acetyl-Ala-CMCys-CMCys-Ser-Thr-

Ser-Phe-~60 residues-Gly-Gln-Val-Gly-Ser-Gly-Ser-Ala-Ser-Val-Ser-Arg-Thr-Arg-Trp-CMCys-Arg-Pro-Asp-CMCys-Arg-.

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