COMPARISON OF SIMILAR PROTEIN COMPONENTS ISOLATED FROM WOOL AND WOOL ROOTS

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Summary

Soluble derivatives of wool and wool-root proteins have been extracted by reduction with mercaptoethanol in the presence of 8M urea followed by alkylation with acrylonitrile. Using chromatography on DEAE-cellulose, followed by gel-filtration on Sephadex, one of the major low-sulphur proteins present in the extract has been isolated in a pure state as determined by starch-gel electrophoresis. Such pure proteins were isolated from extracts of wool and wool roots taken from the same animal. Proteins from these two sources were then compared on the basis of amino acid composition and peptide maps prepared from tryptic digests of them. The results show that small but significant differences do exist between the wool and wool-root proteins. Comparisons of the protein from different wools show that differences also occur here. It is concluded that small changes must occur in the protein composition during the keratinization process.

I. Introduction

The growing wool fibre may be divided into three distinct zones: the root (or follicle region), the keratinization zone, and the region of fully keratinized fibre. The wool root is the region where growth of the fibre starts and where keratin precursors are formed. The proteins in this region are rich in sulphydryl groups (cf. Rogers 1959). In the zone of keratinization, oxidation of these groups to disulphide bonds occurs (cf. Mercer 1961) and the fully matured fibre begins to form. It has also been implicated as a zone where sulphur enrichment of particular proteins occurs (Downes, Sharry, and Rogers 1963; Downes, Ferguson, Gillespie, and Harrap 1966). The last zone, where the fibre is fully hardened or keratinized represents the fully mature fibre, and most studies of wool have been carried out on this material.

To obtain soluble preparations of wool it is first necessary to break the disulphide bonds in the presence of alkali or a disaggregating agent (cf. Crewther *et al.* 1965) such as 8M urea.

A particular fraction of the extracted wool proteins called the low-sulphur fraction represents the major portion of wool (cf. Alexander and Hudson 1963) and has been further fractionated in this laboratory to yield some electrophoretically pure proteins (Thompson and O'Donnell 1965). At the same time it was shown by starch-gel electrophoresis that a similar group of proteins exist in the wool root (Thompson and O'Donnell 1964) although no pure components were isolated. In

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this study, similar techniques have been applied to the fractionation of wool-root proteins, and one of the principal low-sulphur proteins has been isolated as a component giving one band on starch-gel electrophoresis. Its composition and properties have been compared with the equivalent protein isolated from the wool of the same animal.

Some modifications to the methods as used by Thompson and O'Donnell (1962*a*, 1962*b*) were, however, made in an attempt to obtain better fractionation and higher yields of the low-sulphur protein. The wool (or wool-root) proteins were reduced with mercaptoethanol in the presence of 8M urea, and then alkylated with acrylonitrile to yield the *S*-cyanoethyl derivatives. This alkylating reagent has been used with several proteins in recent years (Bartulovich, Tomimatsu, and Ward 1960; Weil and Seibles 1961; Plummer and Hirs 1964), the reaction being quantitative and yielding stable derivatives. One advantage of the alkylating reagent compared with the widely used iodoacetic acid is that no extra charges are introduced into the proteins, and fractionation methods based on charge difference may be expected to be more efficient than with reduced proteins whose –SH groups have been carboxymethylated.

II. Materials and Methods

Urea (B.D.H. Analar reagent) was purified before use as follows: 8M solutions in deionized water were filtered through a mixed-bed, ion-exchange resin to remove solid impurities and dissolved salts (in particular, cyanate). As a further precaution, the filtered solution was acidified to pH 3·0 with 6N HCl and stirred at room temperature for 30 min. The solution was then made 0·001M with respect to EDTA (Analar reagent, B.D.H.) and 0·01M with Tris buffer (Sigma) and the pH adjusted to 7·4. This was the buffered urea solution used in all of the experiments to be described.

β-Mercaptoethanol was a sample from Fluka A.G. and was distilled before use. Acrylonitrile was from B.D.H. (Analar reagent). All other reagents used were of A.R. quality.

Samples of wool and wool root were obtained from freshly killed sheep.* The wool was removed with electric clippers to within about 1 mm of the skin surface. On the flesh side of the skin, any adhering fat or muscular tissue was removed so that the skin could be laid out as flat as possible. Wool roots were then removed from the skin by the method of Ellis (1948). Generally, 1–2 g of wool roots were obtained from one skin. The roots were dropped immediately into 400 ml of 8M urea buffer, adjusted to pH 9·0, and the suspension stirred for 1 hr at room temperature. Undissolved material was removed by filtration through cheesecloth followed by centrifugation at 14,600 *g* (11,000 r.p.m.) for 30 min. The clear supernatant was used for the preparation of soluble wool-root proteins. The total time which elapsed between killing the sheep and harvesting of the roots was approximately 2 hr. As the sulphur in the wool root occurs predominantly in the reduced state, it is possible that some oxidation to disulphide occurs in this process. The subsequent reduction will, however, reverse this.

* Three different breeds of sheep—crossbred, Lincoln, and Border Leicester—were used.

For the wool sample, a portion was selected from the mid-back region of the fleece and degreased with several washes of light petroleum followed by isoelectric washing in salt detergent (Zahn and Blankenberg 1962). The wool was finally air-dried.

(a) Reduction and Alkylation of Wool and Root Proteins

The supernatant fraction from extracted wool roots was adjusted to pH 10·5 with 5N NaOH and 2 ml of mercaptoethanol per 400 ml solution added. Dissolved air in the solution was replaced by evacuation and release under nitrogen. The reduction was allowed to proceed for 3 hr at 25°C, after which the pH was adjusted to 8·0 and 4 ml acrylonitrile added (twofold excess over mercaptoethanol added). After 30 min the reaction was complete (negative nitroprusside test) and excess acrylonitrile was destroyed by the further addition of 2 ml mercaptoethanol. The solution was dialysed exhaustively against urea buffer, pH 7·4, to remove excess salts. The preparation contains *S*-cyanoethyl kerateines from wool roots (abbreviated SCEK). Extracts containing SCEK derivatives of wool were obtained in the following manner. Degreased and washed wool (1·7 g) was added to a solution containing 95 ml 8M urea buffer and 1 ml of mercaptoethanol. The pH was adjusted to 10·5 and the solution flushed with nitrogen. After 3 hr reduction at 25°C, the pH was adjusted to 8·0 and 2 ml of acrylonitrile added. When reaction was complete (30 min) 1 ml of mercaptoethanol was added and the solution filtered to remove undissolved wool residues. The filtrate was dialysed against 8M urea buffer for 24 hr at room temperature.

(b) Preparation and Purification of a Single Low-sulphur Protein

SCEK prepared from either wool or wool root was fractionated in two stages to yield an electrophoretically pure component.

(i) *DEAE-cellulose Separation.*—A column (2·3 cm int. diam. by 20 cm) of DEAE-cellulose (BioRad. Laboratories, California, 0·9 m-equiv/g) was used in the first stage. The DEAE-cellulose was prepared for use by washing in ethanol, decanting fines, and finally washing with 8M urea buffer, pH 7·4, containing 1·0M KCl. Before use the column was regenerated by washing with urea buffer containing no KCl until the pH had returned to 7·4. All column operations were carried out at room temperature.

A sample of SCEK, prepared from 0·85 g of wool (or wool root), was added to the column and washed through with urea buffer. The elution of this and subsequent fractions was monitored by ultraviolet absorption at 276 mμ. Subsequent fractions were obtained by elution with 0·02, 0·05, and 1·0M KCl in the buffered urea solution (200 ml each) in a stepwise manner. Gravity pressure was used and samples were collected at the rate of 10–15 ml/15 min. Fractions containing ultraviolet-absorbing material were pooled and dialysed against deionized water until free of urea (48 hr with frequent changes). The solutions were then freeze-dried.

(ii) *Sephadex Separation.*—A column (2·3 cm int. diam. by 120 cm) of Sephadex G-200 (bead form) was set up from Sephadex previously washed with urea buffer containing 1M KCl. Before use the column was washed with urea buffer (no KCl). All column operations were carried out at room temperature, with a flow rate of

16 ml per hour (Beckman Accu Flo pump). The elution of proteins was followed by ultraviolet absorption at 276 mμ. Major fractions were pooled, dialysed exhaustively against deionized water, and finally freeze-dried.

(*c*) *Starch-gel Electrophoresis*

The number of components in each fraction at different stages of purification was monitored by electrophoresis on starch gels containing 8M urea (cf. Thompson and O'Donnell 1964). After 6·8 hr electrophoresis at 40 V/cm the gels were stained with nigrosine.

(*d*) *Amino Acid Analysis*

Samples were prepared for analysis by dialysis of the 8M urea buffer solution against deionized water, then against a solution of KCl–borate (0·3M KCl, 0·0001M sodium tetraborate, pH 9·0), and finally exhaustively against water. The solutions were freeze-dried and a sample for analysis hydrolysed at 110°C in high vacuum (20 micron Hg) by the method of Crestfield, Moore, and Stein (1963). After hydrolysis for 24 hr, the contents were freeze-dried and used directly for amino acid analysis on the Beckman amino acid analyser.

(*e*) *Tryptic Digestion*

Samples (5 mg) of the purified proteins (i.e. after Sephadex separation) were treated with 0·5 ml of a solution containing 5 mg ammonium carbonate, 0·05 mg of trypsin (Worthington, twice crystallized), and 0·05 mg of merthiolate as a preservative. The pH of the solution was adjusted to 8·5 and the digestion allowed to proceed for 24 hr at 40°C. The contents were then freeze-dried and two dimensional paper electrophoresis carried out on a sample as described by Thompson and O'Donnell (1962*a*, 1962*b*). The first dimension was carried out at pH 6·5 with pyridine acetate buffer on Whatman 3MM paper for 45 min (125 V/cm). For the second dimension, strips were cut from the paper and sewn onto a larger sheet of paper (45 by 60 cm). Electrophoresis was then carried out at pH 3·5 with the acetic acid buffer for 70 min (70 V/cm). The papers were air dried and stained with ninhydrin–collidine reagent. Tryptophan peptides were detected on the same sheet by subsequent dipping in Ehrlich reagent and tyrosine peptides were detected by spraying the thoroughly dried paper with Pauly reagent.

III. Results

The isolation of two electrophoretically pure low-sulphur proteins from wool has been recently reported by Thompson and O'Donnell (1965). These two proteins, components 7 and 8, were isolated from *S*-carboxymethyl kerateine (SCMK), and since a different derivative is being used in these experiments (SCEK), some experiments were carried out in order to identify and characterize these same two proteins.

Starch-gel electrophoresis patterns of SCMK and SCEK prepared from the same wool (MW 138) are shown in Figure 1. The two principal bands in the SCMK preparation (called components 7 and 8) together represent 45–50% of the total protein extracted (Thompson and O'Donnell 1965, and personal communication),

the remainder being made up of the high-glycine–high-tyrosine fraction and high-sulphur fraction. Two major bands are indicated on the SCEK pattern and evidence will be presented later that these are derived from the same proteins which give components 7 and 8 as found in SCMK. As these SCEK proteins do not have extra negative charges such as are introduced by alkylation with iodoacetic acid, their electrophoretic mobility is lower and their separation is greater; however, component 7 is smeared due to aggregation.

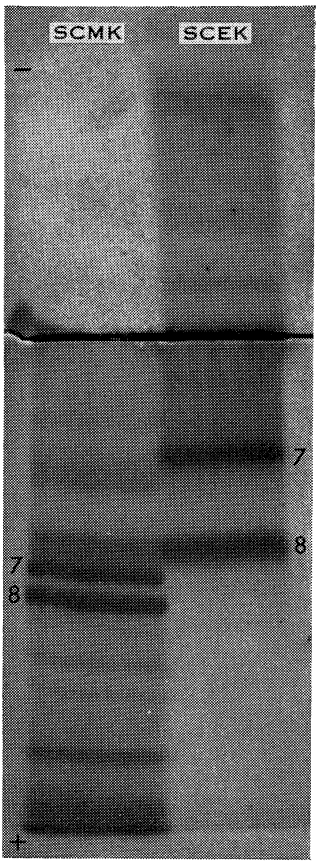

Fig. 1.—Starch-gel electrophoresis patterns of SCEK and SCMK prepared from MW 138 wool. The positions of the two major proteins, components 7 and 8, are indicated.

(*a*) *Separation of SCEK on DEAE-cellulose*

A typical elution curve of SCEK from MW 138 wool on DEAE-cellulose is shown in Figure 2. Fractions *A*, *B*, and *C* were cut as shown and the starch-gel pattern of each fraction is seen in Figure 3. Samples A_1 and B_1 are peak tubes from fractions *A* and *B*. Note the complete separation of components 7 and 8 obtained. Further purification of fractions *B* and *C* was effected by gel filtration on Sephadex G-200 in the 8M urea buffer. The amino acid composition of each DEAE fraction (after

purification on Sephadex G-200) is shown in Table 1 and is compared with the composition of the two major proteins (component 7 and 8) as obtained from SCMK (MW 138 wool). Fraction *A* from DEAE-celluose has been analysed and found to

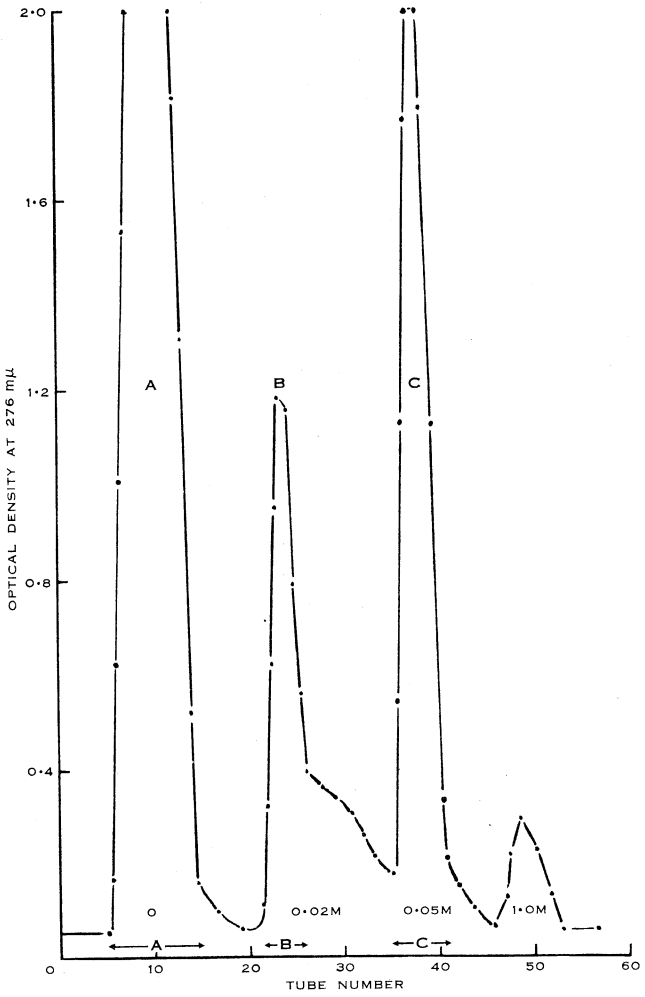

Fig. 2.—Chromatography of SCEK from MW 138 wool on DEAE-cellulose (2·3 cm int. diam. by 20 cm column) at 25°C in 8M urea–Tris buffer, pH 7·4. Stepwise elution (50 ml/hr) with increasing concentrations of KCl. Fraction size was approximately 10 ml.

consist of a mixture of the high-sulphur and high-glycine–high-tyrosine proteins. This fraction also contains a component with similar mobility to component 7, and after purification by gel filtration was shown to be a high-sulphur protein.

The fraction eluted with 1·0M KCl contains mostly component 8, but since it was in such small yield no further work was done with it.

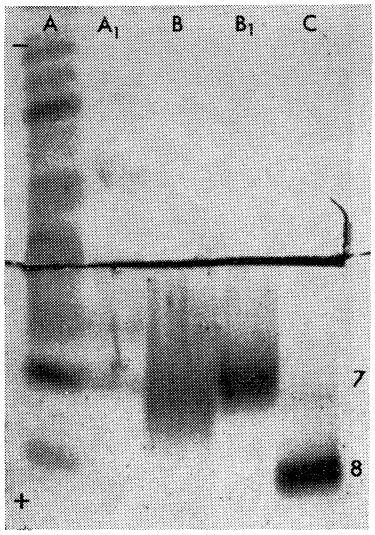

Fig. 3.—Starch-gel electrophoresis patterns of fractions *A*, *B*, and *C* from chromatography on DEAE-cellulose of MW 138 SCEK. Samples A_1 and B_1 are peak tubes from the main fractions. The positions of the two major proteins, components 7 and 8, are indicated. The band with the same mobility as component 7 occurring in fraction A is a high-sulphur protein.

TABLE 1

AMINO ACID COMPOSITION OF COMPONENTS 7 AND 8 PREPARED FROM SCEK AND SCMK DERIVATIVES

Amino acid nitrogen values are expressed as a percentage of the total nitrogen content (Kjeldahl) of the hydrolysate

Amino Acid	Component 7*		Component 8*	
	SCMK† Derivative	SCEK Derivative	SCMK† Derivative	SCEK Derivative
Lysine	6·14	6·15	4·27	4·40
Histidine	1·13	1·20	1·29	1·40
Arginine	21·15	21·40	21·91	21·25
Aspartic acid	6·31	5·82	7·55	7·55
Threonine‡	3·07	3·03	3·68	3·35
Serine‡	5·81	4·95	5·32	5·20
Glutamic acid	10·97	10·12	12·73	12·15
Proline	2·05	2·15	2·58	2·55
Glycine	5·24	5·81	2·92	3·05
Alanine	5·46	5·81	4·18	4·55
Valine	4·54	4·75	4·42	4·50
Methionine	0·43	0·46	0·22	0·24
Isoleucine	2·73	2·52	2·64	2·50
Leucine	6·95	6·30	8·27	8·20
Tyrosine	2·01	2·02	1·82	2·00
Phenylalanine	1·71	1·81	1·46	1·45
S-Carboxyethyl cysteine	—	4·15	—	3·82
S-Carboxymethyl cysteine	4·37	—	4·19	—

* Prepared from MW 138 wool.

† Results from Thompson and O'Donnell (1965).

‡ Uncorrected for decomposition.

By comparison of the amino acid compositions of components 7 and 8 from SCEK and SCMK (Table 1) it is apparent that SCEK may be fractionated on DEAE-cellulose to yield the same major fractions as obtained from SCMK proteins. Fractionation on DEAE-cellulose of SCEK proteins prepared from wool roots gave similar results with the exception that nucleic acid was also eluted at 1·0M KCl. This did not interfere with the fractionation of components 7 and 8 in any way.

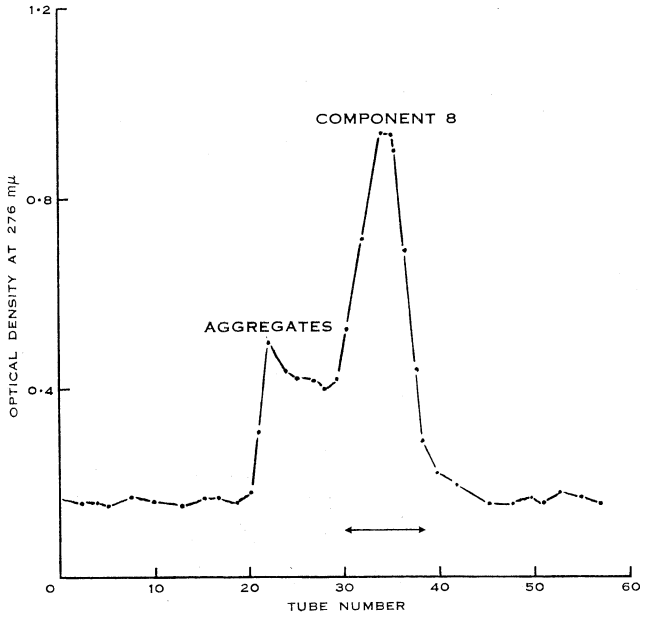

Fig. 4.—Purification of fraction *C* from MW 138 SCEK on Sephadex G-200 (2·3 cm int. diam. by 120 cm column). The fraction was obtained by chromatography on DEAE-cellulose of SCEK and contains some aggregated material and component 8. Tubes pooled as shown contained electrophoretically pure component 8. The elution rate was 16 ml/hr and 8-ml fractions were collected.

(*b*) *Sephadex Purification*

A typical elution curve of component 8 obtained from DEAE fractionation is shown in Figure 4. It can be seen that contaminants of higher molecular weight are present and may be removed by this method. Similar results were found for component 7 (fraction *B* from DEAE-cellulose separation) except that the amount of high-molecular weight material was greater. The elution volumes of high-molecular weight material (aggregates) and components 7 and 8 from SCEK correspond with the elution volumes obtained with the same fractions from SCMK, indicating that they are of the same order of molecular weight.

An estimate of the amounts of components 7 and 8 obtained from either SCMK preparations or SCEK preparations showed that the two proteins (together with aggregates) represent approximately 60% of the extracted material in each case. In agreement with the results of Thompson and O'Donnell (1965) it was found that the aggregated material was predominantly derived from component 7 and that component 7 plus aggregates is present at about twice the amount of component 8.

The actual yields of component 8 obtainable in a pure state are approximately 4% from the SCMK preparation and 16% from the SCEK preparation.

Experiments were then carried out to compare the composition and properties of component 8 isolated from wool and wool root obtained from the same animal. Three different types of fleece were used—crossbred, Lincoln, and Border Leicester. In one case (crossbred) an SCMK preparation was used, and in the other two cases, SCEK preparations were used. Component 8 was isolated from each of the protein preparations by a combination of DEAE-cellulose and Sephadex fractionation, and starch-gel patterns of the purified components are shown in Figure 5. Faint bands with a lower mobility than the major band are aggregates of component 8 (see Section IV).

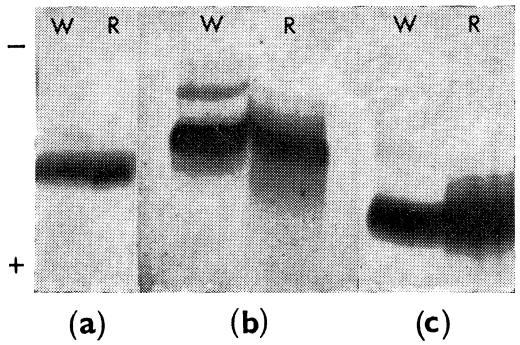

Fig. 5.—Starch-gel electrophoresis patterns of component 8 isolated from the wool (*W*) and wool roots (*R*) of crossbred (*a*), Lincoln (*b*), and Border Leicester (*c*) sheep.

The percentage yields of component 8 from wool and wool root were approximately equal. The amino acid composition of the purified components is shown in Table 2, and it is apparent that the proteins from the wool and wool root from the same animal are similar in each case, although differences do exist. In the crossbred, there are differences in the lysine, histidine, arginine, and alanine contents. Differences between Lincoln wool and wool root are in the lysine, serine, proline, alanine, and methionine contents, and in Border Leicester between the lysine, alanine, methionine, and leucine contents.

A sample of each protein was digested with trypsin and peptide maps of the digests are shown in Plate 1. On these maps, spots encircled and labelled *E* gave a strong positive reaction with the Ehrlich reagent. Note that in some cases, the Ehrlich-positive peptides gave no colour with the ninhydrin reagent. Other spots encircled are peptides containing tyrosine or histidine.

For more critical comparison, the acidic and neutral peptides have been run on one sheet and the basic peptides on another sheet.

IV. Discussion

When comparing proteins from the wool and the wool root of a particular sheep the peptide maps of the tryptic digests show two features. For each of the three types of sheep studied there are differences, either in the presence of completely new peptides, seen particularly in the case of the tryptophan and tyrosine peptides, or in those peptides present in differing amounts between the two digests.

However, for a particular sheep, the majority of peptides are common to both the wool and the wool root, and it must be assumed that there is great similarity in the amino acid sequences in proteins from these two sources. This fact is also supported by the results of the amino acid analysis; there is an overall similarity in each case, although significant differences do exist in several of the amino acid contents. In considering possible explanations for the differences in component 8 from the wool and the wool root of the same animal as found by peptide-mapping and amino acid

TABLE 2

AMINO ACID COMPOSITIONS OF COMPONENT 8 ISOLATED FROM WOOL AND WOOL ROOTS

Amino acid nitrogen values are expressed as a percentage of the total nitrogen content of the hydrolysates

Amino Acid	Crossbred Fleece*		Lincoln Fleece†		Border Leicester† Fleece	
	Wool	Wool Root	Wool	Wool Root	Wool	Wool Root
Lysine	4·05	4·60	4·63	6·17	4·45	6·26
Histidine	1·26	1·60	1·31	1·42	1·15	1·46
Arginine	24·75	21·40	22·87	20·62	22·23	21·82
Aspartic acid	6·80	7·40	7·94	7·48	7·29	7·62
Threonine‡	3·62	3·82	3·15	2·70	3·31	3·16
Serine‡	5·41	5·53	3·96	3·39	5·30	5·08
Glutamic acid	11·30	11·50	12·89	12·54	12·04	13·12
Proline	2·73	2·72	2·59	2·13	2·44	2·09
Glycine	2·91	2·61	2·39	3·33	2·72	3·32
Alanine	4·02	4·60	4·49	5·54	4·19	5·41
Valine	4·20	4·54	4·56	4·92	4·38	4·95
Methionine	0·23	0·25	0·21	0·43	0·23	0·48
Isoleucine	2·33	2·61	2·65	2·99	2·53	2·88
Leucine	8·23	8·62	8·90	8·43	8·00	8·83
Tyrosine	1·81	1·72	1·67	1·46	1·77	1·78
Phenylalanine	1·52	1·44	1·43	1·50	1·38	1·37
S-Carboxyethyl cysteine	—	—	3·40	2·95	3·57	3·42
S-Carboxylmethylcystine	4·52	4·03	—	—	—	—

* Prepared from SCMK derivative.

† Prepared from SCEK derivative.

‡ Uncorrected for decomposition.

analysis, some thought must always be given to the possibility of contamination by other proteins. However, the peptides that are different in each case are present in more than trace amounts so that any contaminant would have to be present in large amounts. That the isolated protein components are reproducible is supported by the good agreement between the amino composition of component 8 prepared by two different methods (SCMK and SCEK) from the same sample of wool. It might be expected that a mixture of independent proteins would not fractionate in the same way if a charged substituent was replaced by a neutral group, yet in fact there is no marked difference in the fractionation of the two derivatives.

It may be noticed that some of the starch-gel patterns of purified component 8 from SCEK show faint bands with a lower mobility than that of the main band. In preparations of electrophoretically pure component from SCEK it was found that the constituents giving rise to these bands slowly accumulate when the freeze-dried protein was stored in the cold. Gel-filtration of such samples showed the presence of a faster-eluting fraction, and it was therefore assumed that these bands were due to aggregates of component 8.

The above evidence indicates therefore that there is a real chemical difference between the component 8 of wool and wool root, even though the similarities outweigh the differences.

Evidence has been presented that a sulphur-enrichment process occurs in the keratinization zone and that the high-sulphur proteins are involved (Downes, Sharry, and Rogers 1963; Downes *et al.* 1966). This process must occur by *de novo* synthesis or by exchange of covalent bonds. There is no reason to suppose that the latter process could not just as easily occur with the low-sulphur proteins without necessarily involving sulphur enrichment.

The use of acrylonitrile instead of iodoacetic acid as an alkylating reagent in these experiments has shown that a more complete separation and higher yields of component 8 may be achieved. Evidently the great excess of negative charge introduced into the protein as the *S*-carboxymethyl group greatly reduces the percentage charge difference between components 7 and 8. This can be seen from the relative mobilities of SCEK and SCMK derivatives on starch-gel (Fig. 1), and the more complete separation of the SCEK derivative by chromatography on DEAE-cellulose. As no extra charges are introduced by alkylation with acrylonitrile the proteins are in their natural charged state, and it can be seen that component 8 is the most acidic on starch-gel. Consequently this protein emerges from the DEAE-cellulose column last.

One disadvantage of the use of cyanoethyl derivatives is that component 7 has been found to aggregate more easily than the corresponding SCMK protein after removal of urea and freeze-drying. To prevent this, the dilute protein solution from DEAE-cellulose or Sephadex fractionation may be concentrated by pressure dialysis (Everall and Wright 1958) or by the use of Sephadex G-25 (Flodin, Gelotte, and Porath 1960) and stored in 8M urea solution.

It has been shown that acrylonitrile may react with lysine groups, particularly above pH 9 (Plummer and Hirs 1964; Kalan, Neistadt, and Weil 1965), but in the present work no such reaction was apparent. This can be seen by a comparison of the lysine contents of component 8 prepared from SCMK and SCEK (Table 1). It is apparent that the short time period and the low pH used in these experiments have prevented any reaction of acrylonitrile other than with the sulphydryl group.

The work presented in this paper was an attempt to compare a purified protein, called component 8, from the wool and wool-root of the same animal, but since several different types of sheep were used, conclusions may also be drawn from the data concerning a comparison of component 8 from different wools. If the peptide maps of component 8 from Lincoln and Border Leicester wools are compared it can be seen that differences also exist here. The maps from the crossbred wool cannot be used in these comparisons as the *S*-carboxymethyl derivative was used to prepare

component 8. Amino acid compositions of component 8 from the three wools may, however, be compared, and differences may be seen here. Again, it can be said that the major feature is the overall similarity of the components from the different wools, but the differences may be of significance in reflecting the characteristic properties of each wool.

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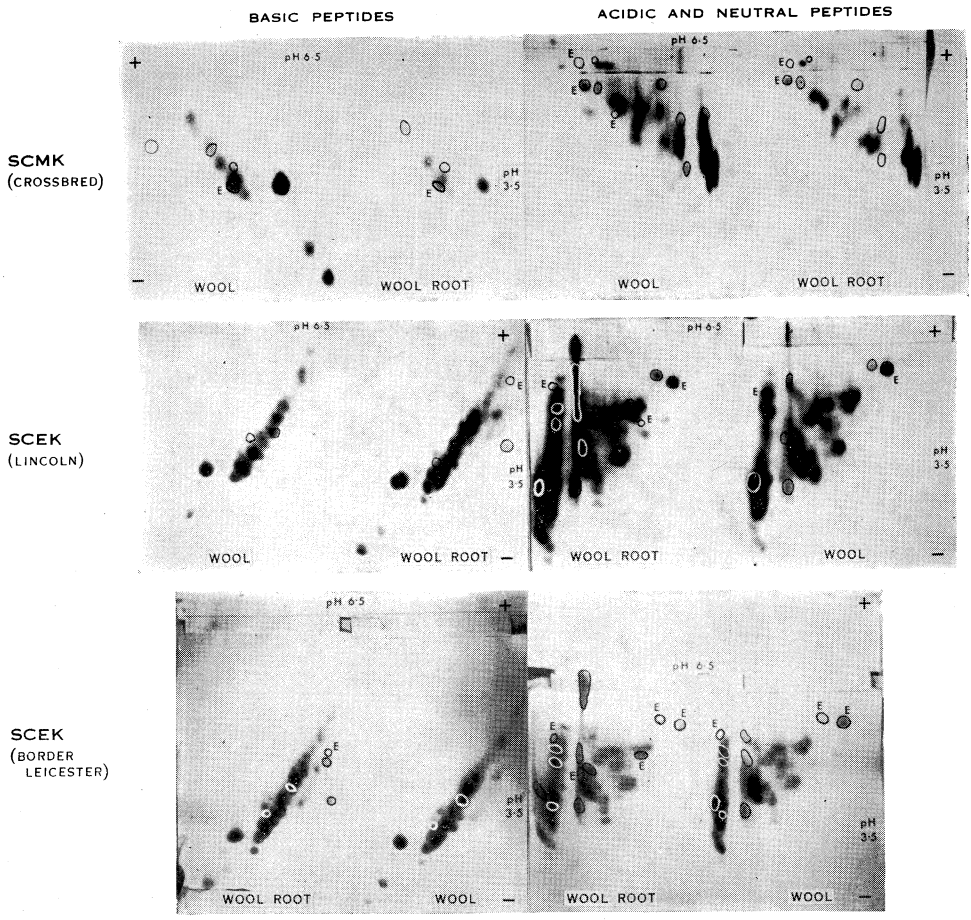
PROTEIN COMPONENTS FROM WOOL AND WOOL ROOTS



Peptide maps of tryptic digests of component 8 from wool and wool roots. Spots encircled and labelled *E* are tryptophan-containing peptides, and other encircled spots are peptides containing tyrosine or histidine or both amino acids.