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The Keratin Proteins of Wool, Horn and Hoof from Sheep

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Abstract

This paper compares the constituent low-sulphur and high-sulphur proteins isolated from the three hard keratins—wool, horn and hoof—produced by the sheep. In general the low-sulphur protein fractions from the three keratins are similar in composition and range of molecular sizes. The same can be said for the high-sulphur protein fractions. However the relative proportions of the component proteins within each fraction vary from keratin to keratin. Horn and hoof are more similar to each other than they are to wool and, compared to wool, they contain less total high-sulphur protein and a smaller proportion of those high-sulphur protein fractions of highest sulphur content. There is a changed distribution of protein components in the low-sulphur protein fractions, and one of the wool components is apparently missing from horn and hoof.

Since these keratins contain similar proteins in different proportions they should make ideal materials for studying the relation between protein composition and the mechanico-chemical properties of keratins.

Introduction

Most land mammals produce at least two forms of hard keratin—hair and either claw, nail or hoof. Sometimes a third form, either horn or quill, may be produced. Although it has been known for many years that these keratins differ markedly in amino acid composition (Block and Bolling 1951), it has been shown only recently that this variability in composition is due to variations in their content of three constituent protein groups which have vastly different compositions (Gillespie and Frenkel 1974*a*). The keratins appear to be built to the same plan with filaments (microfibrils), often aligned, of about 7·0 nm diameter embedded in a non-filamentous matrix (Fraser *et al.* 1972). The filaments appear to be composed of proteins (low-sulphur) which are lower in sulphur content than the parent keratin, whilst the matrix contains two groups of proteins—one group is rich in cystine (high-sulphur proteins) and the other is rich in glycine and tyrosine (high-tyrosine proteins) (Fraser *et al.* 1972; Gillespie and Frenkel 1974*a*). Within each group the proteins are not identical in composition and they thus provide an additional cause for the diversity in composition of keratins.

Keratins differ not only in composition but also in function, and it is assumed that these attributes are interconnected although virtually nothing is known of the connection between them. A study of the relation between function and protein composition of keratins is of particular relevance to the long-term aim of gaining a better understanding of the mechanico-chemical properties of wool as a textile fibre. One

method of studying this relation is to take keratins of various types from different species—keratins selected for large differences in protein composition (see Gillespie and Frenkel 1974*a*)—and attempt to relate composition to chemical and physical properties. Preliminary results have already shown that a positive correlation exists between transverse compressional moduli of different keratins and their relative content of the matrix proteins (Bendit and Gillespie, unpublished data).

Evolutionary changes in amino acid sequence of the proteins from different species can complicate the interpretation of mechanico-chemical properties. If, however, a set of keratins were available which contained similar proteins, but in differing proportions, then structure–function data from these keratins might be more easily interpreted. Gillespie (1968) suggested that such a set was produced by the sheep, this suggestion being made on the basis of results obtained with the relatively low-resolution electrophoretic methods then available. The present paper re-examines the relationships between the keratin proteins produced by an individual animal, and modern methods better able to assess the identity of proteins have been used. The study comprises two sections. In the first we have examined in some detail the high-sulphur and low-sulphur proteins of the ovine keratins. The methods employed were amino acid analysis and various high-resolution electrophoretic techniques. In the second, described in the accompanying paper (Gillespie and Marshall 1977), we have looked to see whether these findings can be extended to keratins from other genera, examining in less detail the keratin proteins of echidna, hedgehog, rabbit, ox and man. We have not included the comparison of the high-tyrosine proteins because the similarity of those of wool and hoof has already been established (Gillespie 1972) and because they are absent from some keratins, e.g. human hair, used in this study.

Materials and Methods

Origin and Preparation of Keratins

Samples of horn and hoof (referred to as horny keratins) and wool were obtained from two Dorset Horn sheep. Only the outer portion of horn about 2·5 cm beyond the skull was used and the shell of hoof keratin was prepared by carefully dissecting away the adhering subunguis. These horn and hoof samples were cleaned by scrubbing with a non-ionic detergent solution, and cut into 20-μm sections with a milling machine for horn and a hand microtome for hoof. The sections of ground horny keratins were washed with repeated changes of 0·15 M NaCl over a period of 8 h to remove soluble constituents, and then washed with water and dried with ethanol. All three keratins were defatted with petroleum ether and ethanol, washed with water and dried. The high-sulphur protein fraction SCMK-B2 (Gillespie 1963*a*) was prepared from the horns of four sheep and the hooves of eight sheep of unknown breeds.

Preparation of Soluble Proteins

The keratins were solubilized to the extent of 70–80% by alkaline reduction in 6 M urea, alkylated with iodoacetate and the solution dialysed. Zinc acetate was added to a concentration of 0·02 M (pH 6·0) and the solution centrifuged for 30 min at 4000 g. The high-sulphur proteins were recovered from the zinc acetate supernatant by first adding sodium citrate to 0·02 M, dialysing against deionized water, and finally freeze-drying. The zinc acetate precipitate, which contained the high-tyrosine (types I and II) and low-sulphur proteins, was stirred with 0·02 M sodium citrate and the usually turbid solution dialysed against deionized water. The high-tyrosine type I proteins were removed as a precipitate by centrifugation for 1 h at 40000 *g*. An acetone–ammonium sulphate fractionation of the supernatant separated the high-tyrosine type II proteins from the low-sulphur proteins. The latter proteins were dissolved in 0·05 M borate, dialysed against deionized water and stored frozen. The full details of the procedures are given by Gillespie and Reis (1966) and Gillespie (1972).

Preparation of a High-sulphur Protein Fraction

A high-sulphur protein fraction, SCMK-B2, was prepared from wool, horn, and hoof using the procedures of Gillespie (1963*a*) and Gillespie *et al.* (1968). The fraction from wool contains three sequenced components—SCMK-B2A, B2B and B2C (Elleman 1972; Lindley and Elleman 1972).

Electrophoretic Methods

Electrophoresis at pH 2·6 was carried out in 10% polyacrylamide gels (acrylamide–bisacrylamide, 27:1) in a buffer containing 4·8 M acetic acid and 2·75 M urea for 2 h at 250 V. At pH 8·9, 7·5% polyacrylamide gels containing 8 M urea and prepared by the method of Davis (1964) were used. Full details of the procedures employed are described by Marshall and Gillespie (1976).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was carried out in 4–27% continuous gradient polyacrylamide gels (Gradipore) (Margolis and Kenrick 1968). The electrode buffer system contained 0·1% SDS and 0·05 M phosphate, pH 7. Electrophoresis of the samples was carried out for 3·5 h at 50 mA after a pre-run for 3 h at the same current. Before loading, the samples were heated at 100°C for 10 min (pH 7) with an SDS:protein ratio of about 3:1. As the experiments were designed to compare the molecular sizes of proteins rather than to determine their exact molecular sizes, the gels were not calibrated with proteins of known sizes.

In order to obtain the relative proportions of components, polyacrylamide gels were first stained with Coomassie Brilliant Blue G250 (Marshall and Gillespie 1976) and then scanned in the transmission mode with a Shimadzu CS-900 TLC scanner. Sample and reference wavelengths were 590 and 400 nm respectively. Because the amount of protein in the gels varied, comparisons were made only between the relative proportions of components found in each protein preparation, and not between the absolute intensities of bands in different electrophoretic patterns. In these comparisons it is assumed that intensity of staining is proportional to protein concentration.

Moving boundary electrophoresis (Gillespie and Reis 1966) was carried out in two 0·1 ionic strength buffers: sodium acetate–acetic acid, pH 4·5, and β-alanine–sodium hydroxide, pH 11·0. Protein concentrations were about 1·3%. The relative proportions of components in the proteins were estimated from the relative areas under peaks in the ascending patterns of runs at pH 4·5. On tracings, the peaks were arbitrarily defined by vertical lines, the horizontal distance covered under corresponding peaks being kept the same for wool, horn, and hoof to make the results strictly comparable.

Analytical Methods

For amino acid analysis the samples were hydrolysed for 22 h *in vacuo* with constant-boiling hydrochloric acid. Each hydrolysate was freeze-dried and the amino acid composition determined with a Beckman–Spinco 120C amino acid analyser.

The sulphur content of proteins was determined by an oxygen flask combustion method (MacDonald 1965).

Results

Composition of Wool, Horn, and Hoof

The sulphur content and the amino acid composition of wool, horn and hoof obtained from two Dorset Horn sheep from the same flock were determined. As comparable keratins from the two sheep had virtually identical compositions and as no differences could be detected between their corresponding proteins the results for keratins from only one sheep are presented in this paper. These keratin samples were used for all the studies which follow except for the preparation of SCMK-B2 which required larger amounts of hoof and horn than could be provided by one sheep.

It can be seen (Table 1) that the most striking difference between the compositions of the horny keratins and of wool lies in their very different sulphur contents. In this sheep, horn and hoof have only 57% of the sulphur content of wool; this is a reflection of the different cystine contents. The general amino acid compositions of horn and hoof are more similar to each other than they are to wool but the horny keratin compositions are not identical and differ by more than 10% in some amino

Table 1. Amino acid composition (as residues per 100 residues) of wool, horn and hoof produced by one sheep, and the high-sulphur, SCMK-B2 and low-sulphur protein fractions isolated from these keratins

Amino acid	Keratin			High-sulphur proteins			SCMK-B2 proteins			Low-sulphur proteins		
	Wool	Horn	Hoof	Wool	Horn	Hoof	Wool	Horn[B]	Hoof[B]	Wool	Horn	Hoof
Lys	2·66	3·76	3·96	0·61	1·01	0·98	0·00	0·00	0·00	4·14	4·14	5·01
His	0·79	1·33	0·94	0·73	0·99	0·89	0·00	0·00	0·00	0·61	0·84	0·85
Arg	6·24	6·68	7·16	6·24	5·43	5·97	3·63	3·91	3·97	7·85	7·80	7·19
Cys(Cm)	0·00	0·00	0·00	22·10	16·30	16·90	22·30	20·70	23·10	6·03	4·74	3·77
Asp	5·93	7·80	8·39	2·28	4·67	4·26	0·71	0·97	0·87	9·64	8·88	10·10
Thr[A]	6·53	4·78	4·95	10·20	9·60	10·20	11·80	10·90	10·60	4·84	4·94	4·49
Ser[A]	10·80	9·56	9·54	13·20	11·20	11·80	14·60	15·00	14·80	8·14	8·44	7·87
Glu	11·10	12·90	13·70	7·90	6·06	6·86	10·60	10·90	10·50	16·90	15·80	17·70
Pro	6·60	3·83	3·99	12·60	12·40	13·00	10·70	10·90	10·00	3·34	3·58	2·43
Gly	8·56	11·10	9·10	6·18	9·04	7·22	8·89	9·40	9·20	5·23	6·93	6·31
Ala	5·20	5·90	6·37	2·91	3·20	3·21	3·32	3·42	3·25	7·66	7·40	7·26
½Cys	13·10	6·24	5·66	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
Val	5·68	5·21	5·66	5·30	5·61	5·79	3·55	3·55	3·45	6·35	6·17	5·91
Met	0·54	0·81	0·80	0·00	0·00	0·00	0·00	0·00	0·00	0·56	0·72	0·73
Ile	2·98	3·31	3·56	2·62	3·25	3·15	4·01	3·88	4·16	3·79	3·78	3·95
Leu	7·20	9·13	9·51	3·35	5·34	4·87	1·94	2·16	2·03	10·20	10·10	11·10
Tyr	3·78	5·00	4·03	2·14	3·33	2·28	2·40	2·54	2·59	2·67	3·34	3·29
Phe	2·48	2·64	2·65	1·57	2·55	2·44	1·33	1·23	1·33	1·97	2·47	2·09
Sulphur content (%)	3·75	2·13	2·15									

[A] Uncorrected for decomposition. [B] Isolated from a number of sheep.

acids. The horny keratins not only contain less cystine than wool but also significantly less threonine and proline, and contain more lysine, aspartic acid, glutamic acid, and leucine. Horn contains more histidine, glycine and tyrosine than the other keratins.

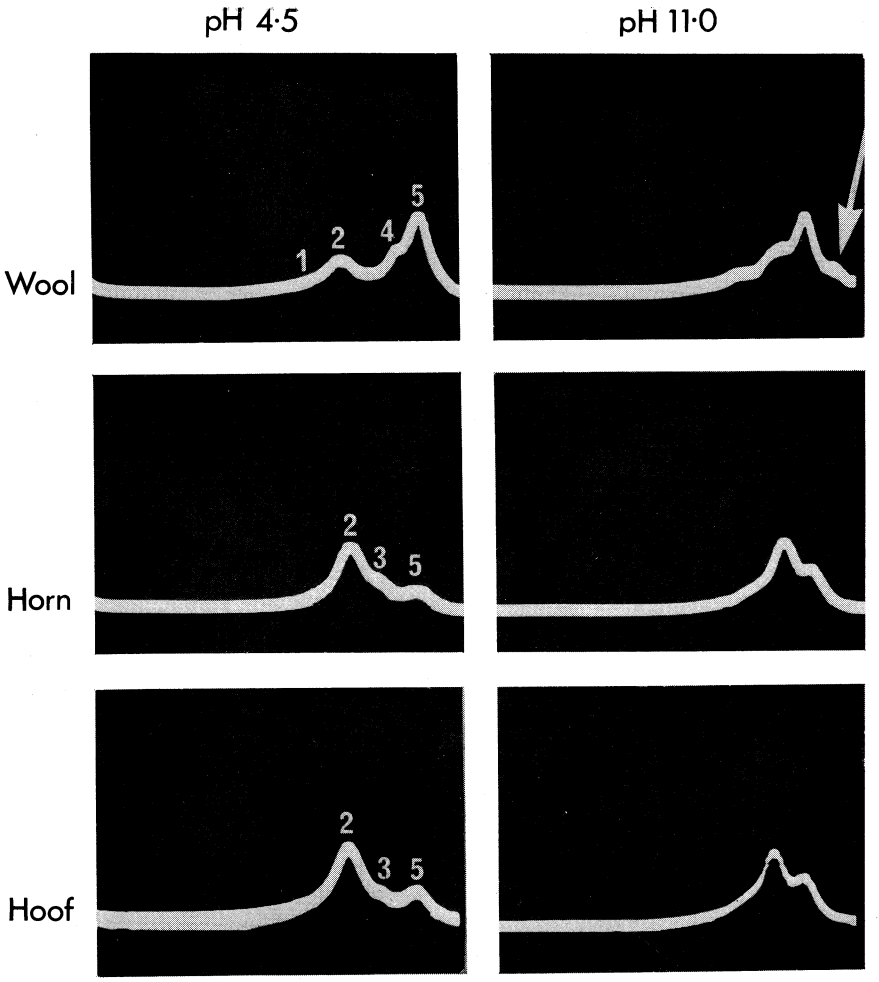

Fig. 1. Moving boundary electrophoretic patterns at pH 4·5 and 11·0 of high-sulphur proteins isolated from three keratins produced by a Dorset Horn sheep. Direction of movement from left to right. Corresponding resolved peaks in the runs at pH 4·5 are labelled 1–5. The peak corresponding to the ultra-high-sulphur proteins resolved in the runs at pH 11·0 is indicated by an arrow.

High-sulphur Proteins

(i) *Composition*

An examination of the amino acid analyses of the high-sulphur protein fractions from wool, horn, and hoof (Table 1) shows the characteristic high content of *S*-carboxymethylcysteine [cysteine(Cm)], proline, serine, and threonine, the small amounts of lysine and histidine, and no more than traces of methionine. In general, horn and hoof are more similar to each other than they are to wool although the horn fraction contains significantly more glycine than the hoof fraction. Compared with

wool, the horny proteins contain much less cysteine(Cm) but significantly more aspartic acid, leucine, isoleucine and phenylalanine.

(ii) *Moving boundary electrophoresis*

The high sulphur proteins of wool, horn and hoof were examined electrophoretically by the moving boundary method. The patterns obtained at pH 4·5 (Fig. 1) show that the high-sulphur proteins of horn and hoof are similar to but somewhat different from that of wool. For each keratin the electrophoretic components in Fig. 1 corresponding to peak 2 have about the same mobilities. The same can be said for peak 5. Horn and hoof have another peak at position 3 and wool has a peak at position 4. Because the Schlieren curves are above the baseline in the appropriate places, it has been assumed that fraction 3 is present in wool and fraction 4 in horn and hoof. The most notable difference between the electrophoretic patterns is that peak 2 dominates in the horny keratins whilst peak 5 is the major component in wool.

Hoof and horn have similar proportions of electrophoretic components (Table 2). As the mobility of a high-sulphur component is approximately proportional to its cysteine(Cm) content (Gillespie 1963*b*), the ratios of the components give some indication of the difference in distribution of sulphur content amongst the high-sulphur proteins. The ratio of peaks 3+4+5 to peaks 1+2 (from Table 2), i.e. the ratio of sulphur-rich to sulphur-poor components, is about 0·5:1 for horn and hoof, and about 2:1 for wool. These differences in the proportions of components are responsible for the differences already referred to in the amino acid composition of the high-sulphur proteins (Table 1).

The reasonably high sulphur content of the wool (3·75%) indicates the presence of ultra-high-sulphur proteins (Gillespie and Reis 1966), and the presence of these proteins was confirmed by an electrophoretic run at pH 11 (Fig. 1, indicated by arrow). This protein component does not appear to be present in significant amounts in either horn or hoof (see Fig. 1 and Table 2).

(iii) *Polyacrylamide gel electrophoresis*

The high-sulphur proteins from the three keratins were examined electrophoretically in polyacrylamide gels at pH 2·6 (Fig. 2*a*). Fig. 3 shows scans of the gel patterns. The patterns for the three proteins are strikingly similar both in the number and in the mobility of components. The horn and hoof patterns show only minor differences in the relative proportions of their components, but they differ from the wool pattern. On a proportional basis wool contains more of the slow-moving components and these include the SCMK-B2 proteins.

Polyacrylamide gel electrophoresis at pH 8·9 of each high-sulphur protein preparation gave patterns showing at least nine resolved bands, corresponding bands having apparently identical mobilities (Fig. 2*b*). Scans of these gels (not shown here) indicate that horn and hoof contain similar proportions of components but that they differ from wool in the relative proportions of many components, particularly the fastest major one.

Polyacrylamide gel electrophoresis in the presence of SDS of the high-sulphur proteins from the three keratins gave the patterns shown in Fig. 2*c*. Apart from the major low-molecular-weight component in horn and hoof occurring near the front, the three patterns are very similar, corresponding bands having similar mobilities and hence molecular weights. The three major components in wool are present in about

equal proportions whereas in the corresponding bands of horn and hoof the lowest-molecular-weight component is present in the greatest proportion.

(iv) *SCMK-B2 proteins*

The amino acid compositions of the three preparations of SCMK-B2 are shown in Table 1. In overall composition the preparations are similar. There is no lysine, histidine or methionine, and for 11 of the other amino acids, including the most plentiful ones, the differences are not significant (less than 10%).

Table 2. Proportion (as %) of electrophoretic components in wool, horn and hoof high-sulphur protein estimated from electrophoresis at pH 4·5 (peaks 1–5) and pH 11 (ultra-high-sulphur proteins; see Fig. 1)

	Peak No.					
	1	2	3	4	5	UHS proteins
Wool	7	27	11	12	44	15
Horn	9	57	16	5	13	<1
Hoof	14	52	13	5	17	<1

From the electrophoretic patterns at pH 2·6 (not shown) of the three keratins it is clear that for the three major components in each preparation, corresponding bands have the same mobilities. Horn and hoof contain a greater proportion of the fastest-moving component (SCMK-B2C) than does wool.

The alkaline gel (not shown) also resolved three components (one major, two minor) in each preparation, corresponding bands having identical mobilities. The major component probably contains the SCMK-B2 subfractions A, B, and C which were resolved at acid pH, whilst the two minor bands reflect the microheterogeneity of the A, B, and C subfractions (Elleman 1972; Lindley and Elleman 1972).

Low-sulphur Proteins

(i) *Composition*

In a comparison of low-sulphur proteins it is difficult to be sure that contaminating proteins, mainly high-tyrosine proteins (Gillespie 1972; Gillespie and Frenkel 1974*b*), are completely removed. Even with wool one cannot be sure that this can be reliably achieved. The fractionation procedure developed specifically for the purification of low-sulphur proteins of wool was applied to the other keratin types. Although there is an overall similarity in amino acid composition of the three low-sulphur proteins (Table 1) there are significant differences between them in the content of lysine, cysteine(Cm), proline and aspartic acid. The proteins of horn and hoof also contain more glycine and tyrosine than do the wool proteins, possibly because of residual high-tyrosine proteins (Gillespie and Frenkel 1974*a*, 1974*b*).

(ii) *Polyacrylamide gel electrophoresis*

In polyacrylamide gel electrophoresis at pH 8·9 (patterns not shown), the major low-sulphur protein components of wool—labelled 5, 7 and 8 (O'Donnell and Thompson 1964; Sparrow and Crewther 1972; Crewther *et al.* 1976)—are also present in horn and hoof, corresponding components having identical mobilities. Horn and hoof contain about the same proportions of components 7 and 8 whereas wool is richer in component 8.

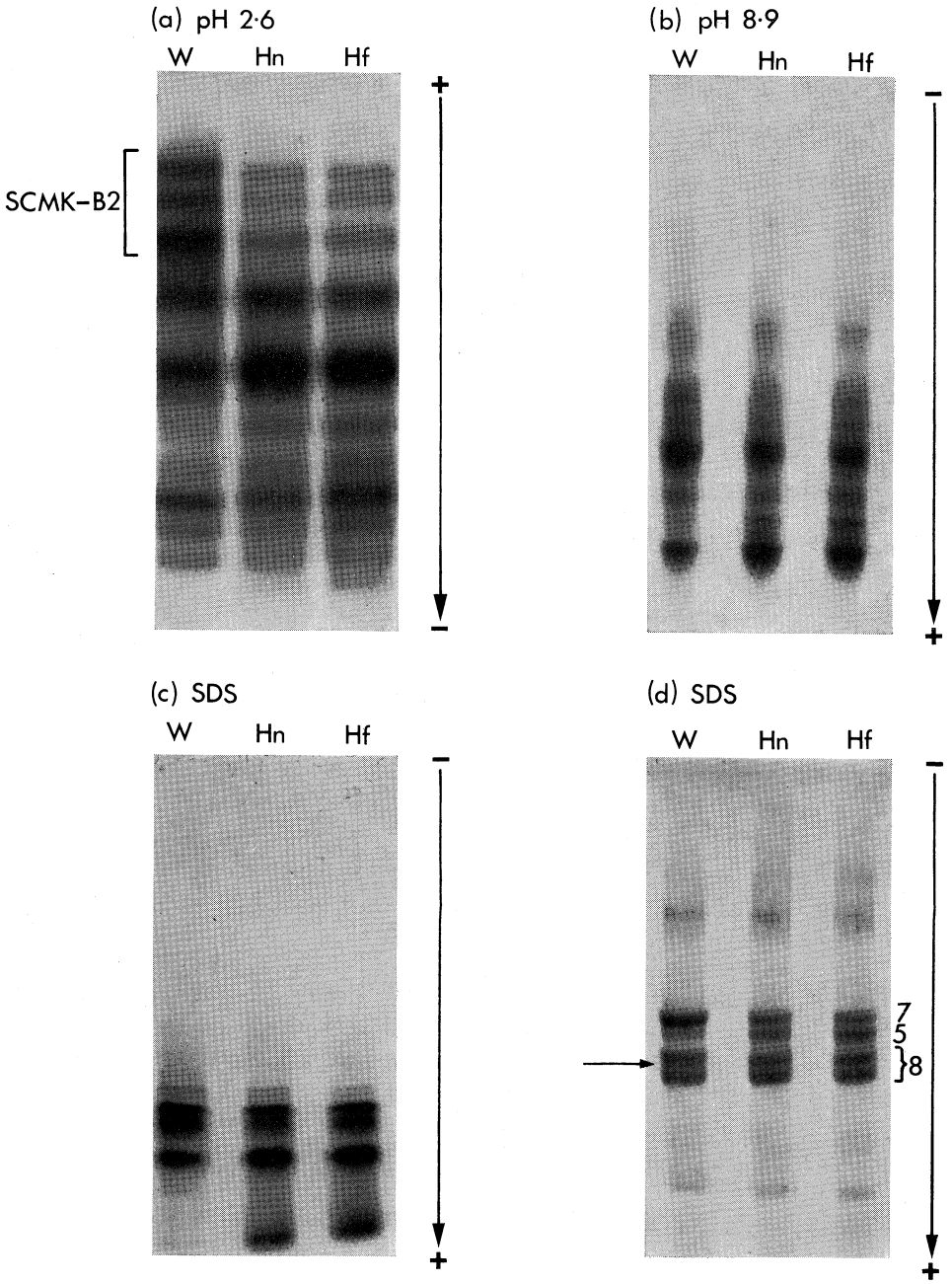

Fig. 2. Polyacrylamide gel electrophoretic patterns of protein fractions isolated from the sheep keratins—wool (W), horn (Hn) and hoof (Hf). (*a*) High-sulphur proteins, pH 2·6, about 70 μg loaded. (*b*) High-sulphur proteins, pH 8·9, about 50 μg loaded. (*c*) High-sulphur proteins, SDS, about 30 μg loaded. (*d*) Low-sulphur proteins, SDS, about 10 μg loaded. Bands on Fig. 2*d* are labelled using the nomenclature of Crewther *et al.* (1976). The arrow indicates the split into two components in the component 8 region of the wool proteins.

The electrophoretic patterns in polyacrylamide gel in the presence of SDS (Fig. 2*d*) show that, qualitatively, the low-sulphur proteins of the three keratins are alike in that there are two groups of components falling into the same corresponding molecular weight ranges. For horn and hoof each group contains two resolved components, the corresponding components having identical mobilities and being present in about the same relative proportions. Three of the components of horn and hoof are present in wool (components 5, 7 and 8). In the component 8 region (Crewther *et al.* 1976) of the wool pattern, two bands are just resolved (indicated by an arrow in Fig. 2*d*), but there is no corresponding resolution in the patterns of horn and hoof. The wool components are not present in equal proportions and this is especially true of component 5.

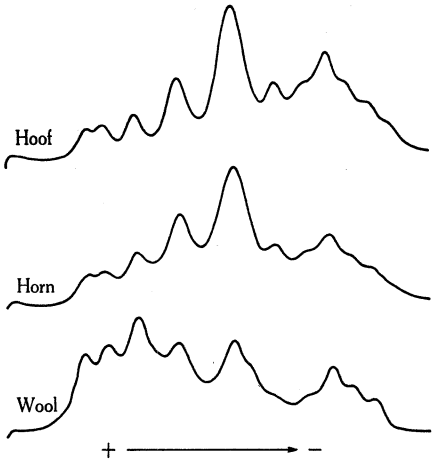

Fig. 3. Densitometric tracings of the polyacrylamide gel electrophoretic patterns obtained at pH 2·6 of the high-sulphur proteins isolated from the sheep keratins—wool, horn and hoof.

Discussion

It has been found that the Dorset Horn wool high-sulphur and low-sulphur proteins have electrophoretic patterns indistinguishable from those previously obtained for Merino wool (O'Donnell and Thompson 1964; Gillespie and Reis 1966; Fraser *et al.* 1972; Crewther *et al.* 1976). It was also found, in agreement with earlier observations (Gillespie 1968, 1972), that the constituent protein fractions are present in different relative proportions in these keratins—for example, the high-sulphur protein content of wool, horn, and hoof is 29, 11 and 11% respectively; the high-tyrosine protein content of wool, horn and hoof is 9, 13 and 10% respectively.

There is a major low-sulphur protein band in horn and hoof with an apparent molecular weight similar to the minor component 5 of wool. Without isolation and characterization of this fraction from each keratin we cannot be sure that these components are identical, for it is possible that there exists a protein, unique to the horny keratins, with the same mobility in the SDS system as component 5. However, the lower sulphur content of the low-sulphur proteins of hoof and horn compared with those of wool is consistent with increased levels of component 5 and decreased levels of component 7 since these components have quite different sulphur contents (Crewther *et al.* 1976).

It is well established that wool, horn and hoof contain microfibrils of about the same dimensions in which the low-sulphur proteins are precisely arranged giving rise to the characteristic X-ray diffraction pattern of α-keratin (Fraser *et al.* 1972). It is

of interest to note that the quite variable proportions of components 5, 7 and 8 in wool, horn and hoof appear to be readily accommodated within the structure of the microfibrils without any apparent effect on structure. Because there is an approximate inverse relationship between the proportions of components 5 and 7 in the three keratins and because these components are of similar molecular size, it is tempting to speculate that they are interchangeable in the microfibril structure. Perhaps these components contain regions with the same amino acid sequence. Proof of this, however, must await the completion of sequence studies now in progress (Crewther *et al.* 1976).

The high-sulphur proteins from the three keratins contained components of identical mobility in moving boundary electrophoresis (i.e. the same charge distribution at pH 4·5), in SDS–polyacrylamide gel electrophoresis (i.e. the same size distribution), and in polyacrylamide gel electrophoresis at pH 2·6 and 8·9 (i.e. the same distribution of size–charge ratios). However, there were differences in the relative proportions of components. From the moving boundary electrophoretic results, where a linear relation exists between sulphur content and mobility, we can say unequivocally that hoof and horn are more enriched in components of lower sulphur content than is wool. This is consistent with the amino acid analytical data. Electrophoresis in polyacrylamide gels at pH 2·6 showed a decrease in the relative proportions of the lower-mobility components in the horny keratins compared with wool, and this corresponds to the SCMK-B2 protein region. Thus at least part of the depletion in the higher sulphur high-sulphur components of the horny keratins may be due to a lower proportion of the SCMK-B2 fraction. An examination of this particular fraction isolated from each keratin revealed that each fraction had the same amino acid composition and contained the same components as shown by electrophoresis at two pH values.

No trace of the sulphur-enriched high-sulphur proteins (Gillespie and Reis 1966) was found in the horny keratins even though there was a substantial amount in the wool. The absence of this protein fraction from horn has been confirmed in another experiment using samples of wool and horn grown at the same time by a sheep on a sulphur-enriched diet.

It therefore appears that the growing regions for the horny keratins normally do not produce ultra-high-sulphur proteins. It would be of interest to know whether this synthetic activity is permanently repressed or whether it becomes operative only under nutritional or other conditions not experienced by the sheep used in this study.

The results reported here show that the basic framework of wool, horn, and hoof is formed from essentially the same low-sulphur and high-sulphur protein components, but the relative proportions of protein components are different. Horn and hoof resemble each other more than they do wool. The horny keratins contain more microfibrillar and less matrix material than wool, and the proteins in the matrix of horn and hoof have fewer disulphide bonds. Such differences in composition would be expected to cause changes in the mechanico-chemical properties of the keratins, but unfortunately the necessary measurements (e.g. torsional modulus and compressibility) have not been made. Potentially these measurements could provide help in assessing the relation between composition, structure and function. With essentially the same proteins being involved, the interpretation of the results would be expected to be simpler than for comparative studies made on keratins from different species.

It is incidental to the main purpose of this study that the amino acid compositions of sheep horn and hoof included here appear to be the first modern and complete analyses. Partial analyses of horn have been given by other workers (Block 1951; Graham *et al.* 1949; Marecek 1959) but these analyses referred to bovine horn which is known to contain more sulphur than sheep horn (Lindley 1947).

Acknowledgments

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