

PROTEINS OF KERATIN AND THEIR SYNTHESIS

I. PROTEINS OF PREKERATIN AND KERATIN

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Summary

The presence within prekeratin cells of the wool follicle of both matrix and microfibrillar proteins analogous to proteins within keratinized tissue has been demonstrated.

An increased or enriched sulphur content of both high- and low-sulphur protein extracts of keratinized tissue, as compared with similar extracts from prekeratin tissue, is almost totally the result of a high proportion of non-matrix and non-microfibrillar proteins within the cells. Though little indication of increasing sulphur content within matrix and microfibrillar proteins during cell maturation is recorded, there is a large and progressive increase in the concentration of both groups of proteins in cells up to the level of keratinization.

The increase in cell concentration of microfibrillar proteins has been shown to be approximately linear throughout the length of the prekeratin region. The increase in cell content of matrix proteins appears to be exponential, approximately half of the final matrix protein content of wool being formed within the proximal two-thirds of the prekeratin region and approximately half in the distal one-third.

The results presented dispute the currently held concept of a "two-stage" process of keratin synthesis. Rather, results indicate a "dual" synthesis of both matrix and microfibrillar proteins throughout the prekeratin or protein-synthesizing region of the wool follicle.

I. INTRODUCTION

The process of "hard" keratinization was considered by earlier workers to be associated with an increase in sulphur content during, rather than before, keratinization (Giroud, Bulliard, and Leblond 1934; Giroud and Bulliard 1935). Later results from autoradiographic and chemical studies of hair follicles indicated that sulphur enrichment occurs during keratinization (Van Scott and Flesch 1954; Bern, Harkness, and Blair 1955; Rudall 1956; Harkness and Bern 1957). Because of the simultaneous incorporation into keratinized material of several labelled amino acids it was proposed that keratins are formed *de novo* from amino acids arising from hydrolysis of cellular proteins in the keratogenous zone (Rothman 1954; de Bersaques and Rothman 1962).

It is well-known that reduced and alkylated α -keratin can be fractionated into two major classes of proteins, one high and the other low in sulphur (Goddard and Michaelis 1935; Gillespie and Lennox 1953; O'Donnell and Thompson 1959).

In the case of wool keratin, the high-sulphur proteins are considered to be amorphous proteins constituting the interfibrillar matrix (Birbeck and Mercer 1957; Rogers 1959; Crewther and Dowling 1960). The low-sulphur proteins are composed

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of at least two distinct groups arising from different sources (Crewther *et al.* 1965). One group arises from the cortical cell microfibrillar protein complex (Birkbeck and Mercer 1957; Rogers 1959; Mercer 1961), and another group, rich in glycine and aromatic amino acids (Gillespie 1960; O'Donnell and Thompson 1962; Harrap and Gillespie 1963; de Deurwaerder, Dobb, and Sweetman 1964), from the cuticle and other cell membranes (de Deurwaerder, Dobb, and Sweetman 1964).

On the basis of electron microscopic, X-ray diffraction, and chemical studies of the prekeratin tissue of the hair follicle, the current view is that the fibrous proteins form as filaments at low levels in the follicle root, and matrix proteins form at high levels in the keratogenous zone (Rudall 1956; Mercer 1961); hence, keratin synthesis has been called a "two-stage" process. Mercer (1961) considered the matrix proteins to be new proteins formed at the zone of keratinization.

A more recent study of proteins from the wool follicle was aimed at providing additional support for the two-stage hypothesis (Downes *et al.* 1966). Further acclimation for the two-stage process of keratin synthesis was considered justified on the basis of the results which were reported.

Results reported here are from a study designed to extend knowledge of the proteins formed during keratin synthesis, using not only keratinized (wool) and prekeratin (wool root tissue below the level of keratinization) proteins, but also proteins extracted from different levels within the prekeratin tissue.

The vertical array of keratin-synthesizing cells contained within the prekeratin region of hair and wool follicles (see Fig. 1) provides an excellent system for a study of the sequential processes occurring within differentiating and protein-synthesizing tissue between mitosis and full keratinization.

In order to compare proteins at different stages of development as the cells grow up the follicle shaft within the prekeratin regions, root cells from above the follicle bulb have been isolated into three groups. Cell groups 1, 2, and 3 represent cells from the base of the root up to the level of keratinization respectively (Fig. 1).

II. MATERIALS AND METHODS

(a) Preparation and Collection of Wool Roots and Wool

Six sheep fed cut pasture and lucerne chaff (*ad lib.*) were used in these experiments. Animals 1, 2, 3, and 4 were used for the collection of wool root material. The animals used for the collection of wool roots and wool were slaughtered. The animals were flayed and the wool clipped from the skin surface. Approximately 1 cm of wool from above skin surface of each animal was retained for protein analysis. Each clipped skin was stripped of its wool roots by the wax-sheet method (Ellis 1948), the wool roots and the keratinized fibres from below skin surface being treated as described below. The collection of wool roots was completed 75 min after slaughter.

In all experiments, more than 95% of the wool roots were removed with the bulb region detectable.

(b) Separation of Prekeratin Cells from Wool Roots

Preliminary investigations showed that all of the thiol-positive region of wool roots between the follicle bulb and the level of keratinization, henceforth referred to as the prekeratin region, could be removed in the form of discrete cells by using controlled trypsin treatment or ultrasonic disintegration. The former treatment gave more consistent results and was adopted.

A portion of the harvested wool roots from animals 1, 2, 3, and 4 were treated for the separation of the prekeratin region into three groups of cells in proximodistal progression as

follows: four successive treatments, each at 37°C, of the roots followed by washing for 15 min in calcium-free Lockes solution (9 g NaCl, 0.42 g KCl, 0.1–0.2 g NaHCO₃, made up to 1 litre with distilled water, and made 0.001M with respect to EDTA). The treatment consisted firstly of rapid magnetic stirring of a suspension of roots (1:30, w/v) in a homogenizing flask containing 0.05% trypsin (Difco) in Lockes solution for 4 min. The roots were washed, and pressure-jetted on a 300 μ sieve with Lockes solution until free from separated cells. This treatment removed bulb cells and large quantities of inner root sheath cells, which were discarded. The length of the prekeratin region remaining intact after this treatment was measured on 300 fibres from each treatment.

This preparative step was followed by the same treatment of the remaining root material but using 0.1% trypsin. After washing and jetting the residual intact roots free from separated cells (as above), the length of the remaining prekeratin region was again measured. The separated cells were passed through two sieves, again through a 300 μ sieve, and finally through a 120 μ sieve to remove plates of cells, mainly inner root sheath cells and single fibres. The cell suspension was centrifuged three times in distilled water. The final suspension was freeze-dried in a tared container, and dried over silica gel.

Two more consecutive treatments, identical to above were carried out on the wool roots, but using 0.2% trypsin, again for approximately 4 min, and 0.4% trypsin for approximately 5 min. Slight variations in time were required to ensure that the entire prekeratin region was as evenly divided as possible into three regions.

Figure 1(a) illustrates the effect on the root and shows the separated cells from each of the three levels. Figure 1(b) illustrates the residual keratinized fibres after cell isolation.

(c) *Protein Extraction from Intact Wool Roots, Residual Keratinized Fibres, Wool, and Cell Groups*

Newly stripped, intact wool roots were either extracted with Lockes solution for 30 min at 40°C, followed by extraction with urea-Tris buffer solution (Sigma) (8M urea, 0.1M Tris buffer, 0.001M EDTA, pH 9.4) at 40°C, or for 2 hr directly after stripping with the urea-Tris buffer solution at 40°C with constant shaking. A preliminary comparison was made between two methods of extracting newly stripped roots. The urea-Tris solution at pH 9.4 at 40°C was used in one method, and the second method utilized 8M urea–0.001M EDTA solution at pH 7.1 at room temperature. Both extractions were carried out with constant shaking.

The residual keratinized fibres and wool were washed in light petroleum, ethanol, and distilled water, and dried. A tared weight of clean wool or residual keratinized fibres was extracted by the urea-thioglycollate method of Harrap and Gillespie (1963) (6M urea, 0.24M potassium thioglycollate, adjusted to pH 10.5) at 40°C for 2 hr with constant shaking.

Proteins were extracted from tared individual wool root cell groups by the same potassium thioglycollate reduction method.

After extraction of protein, the solids from wool roots, residual keratinized fibres, and wool were separated by filtering through terylene gauze. The solids were washed three times with the original extracting solution. The solids remaining after extraction of the cell groups were separated by vacuum filtration using a sintered-disk filter, and washed with extracting solution.

The solutions of extracted proteins from intact wool roots were made 0.2M with respect to potassium thioglycollate at pH 10.5. After standing for 30 min the solutions were alkylated, as also were the extracts from residual keratinized fibres, wool, and cell groups, by addition of 0.8 g of iodoacetic acid and 1.6 g of Tris buffer per 10 ml of extract. After completion of the reaction, determined by a negative nitroprusside test, excess 1-mercaptoethanol was added and the solutions dialysed against running tap water for 24–36 hr. All dialyses were carried out using Visking 18/32 cellulose tubing which had been extracted before use with boiling water.

(d) *Protein Fractionation*

Fractional precipitation of the *S*-carboxymethylated low-sulphur proteins from all the sources was made by addition of an equal volume of acetic acid–sodium acetate buffer of ionic strength 1.0 and pH 4.4, in the presence of 0.5M KCl (Gillespie, O'Donnell, and Thompson 1962). The supernatant solution containing the high-sulphur proteins was decanted. The low-sulphur

proteins were reprecipitated twice at pH 4.4 from saturated sodium borate made 0.5M with respect to KCl before solubilizing in sodium borate and dialysing against running tap water for 36 hr. The combined supernatant solutions of high-sulphur proteins after each precipitation were also dialysed against running tap water for 36 hr. After dialysis all solutions were freeze-dried.

When weight of proteins was required, freeze-drying was carried out in tared containers and the protein dried *in vacuo* at 40°C over silica gel.

(e) *Starch-gel Electrophoresis*

Comparison of protein fractions from all sources was carried out by starch-gel electrophoresis, the low-sulphur proteins by the urea-starch-gel (Connaught hydrolysed starch) technique according to Thompson and O'Donnell (1964) and the high-sulphur proteins by essentially the same method, but making the gel buffer 0.004M with respect to sodium tetraborate. 1% solutions of all low-sulphur proteins and 2% solutions of all high-sulphur proteins were used for gel loading, using 3 MM Whatman paper (Thompson and O'Donnell 1964). A voltage gradient of 16 V/cm was used. Gels of high-sulphur proteins were stained with nigrosine-amido black in a solution of acetic acid-25% trichloroacetic acid-distilled water (10:5:85 v/v/v). For low-sulphur protein gels, nigrosine alone was used and methanol replaced acetic acid in the staining and clearing solution.

(f) *Chromatography and Gel Filtration*

The *S*-carboxymethylated high- and low-sulphur proteins from the four sources were fractionated by chromatography on DEAE-cellulose (Gillespie 1963) and by gel filtration using Sephadex G-200 (O'Donnell and Thompson 1964) respectively. The DEAE-cellulose columns (2 by 10 cm) were equilibrated with acetic acid-sodium acetate buffer (ionic strength 0.01, pH 4.5) and a weighed amount of high-sulphur protein was applied to the column following dialysis for 24 hr against the acetate buffer solution. Elution was carried out using a linear gradient of sodium chloride (0-1.0M) in the acetate buffer, and 4-ml fractions were collected in tubes using an LKB fraction collector. Ultraviolet absorption of these fractions was determined at 276 m μ .

Sephadex G-200 gel filtration was carried out strictly according to O'Donnell and Thompson (1964). Fractions of 8 ml were collected using an LKB fraction collector and the ultraviolet absorbance of each fraction read on a Beckman spectrophotometer.

(g) *Chemicals*

Freshly redistilled potassium thioglycollate was used.

(h) *Sulphur Analysis*

Sulphur contents were determined by the Schöniger (1954) oxygen-flash technique.

(i) *Amino Acid Analysis*

The freeze-dried proteins (20 mg) were oven-vacuum-dried and hydrolysed in a vacuum-sealed tube in 6M HCl at 104°C for 24 hr. After removal of HCl by freeze-drying, aliquots of a solution of the hydrolysate were analysed on a Beckman-Spenco automatic amino acid analyser.

III. RESULTS

(a) *Extraction of Intact Wool Roots*

The amino acid analyses of high- and low-sulphur proteins from wool roots of the same population, extracted for 2 hr with 8M urea-0.001M EDTA at pH 7.1 (analyses 1 and 2) and 8M urea-Tris buffer at pH 9.4 (analyses 3 and 4) immediately after stripping are given in Table 1. Differences are observed between both extracts. Lysine values for the high-sulphur proteins are much larger in the urea-EDTA

extract, and are of an order similar to that for lysine in the low-sulphur proteins from both urea-EDTA (analysis 2) and urea-Tris extracts (analysis 4).

Fig. 1

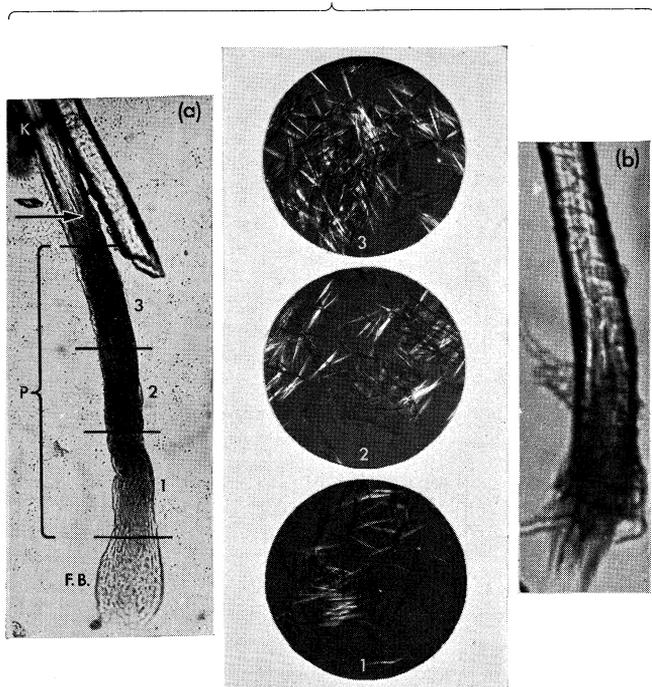


Fig. 2

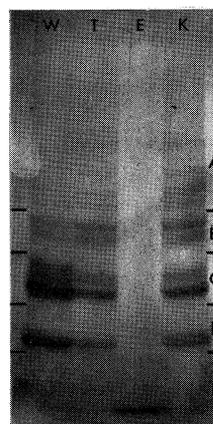


Fig. 1.—(a) Thiol-reactive prekeratin region (*P*) of the wool follicle proximal to the level of keratinization (arrow), and the keratinized fibre distal to this level. The approximate division of the prekeratin region into three cell groups (1, 2, 3) above the follicle bulb (*F.B.*) is shown together with cells separated from each of the three levels (inserts 1, 2, 3 respectively). *K*, keratin. (b) Keratinized wool root residue after separation of root cells up to the level of keratinization.

Fig. 2.—Starch-gel patterns of *S*-carboxymethylated high-sulphur proteins from wool (*W*) and wool roots (*T*, *E*) from the same animal. The origin is at the top of the figure. *T*, *E* denote proteins extracted from wool roots with urea-Tris buffer at pH 9.4 and urea-EDTA buffer at pH 7.1 respectively. *K*, proteins from keratinized wool root residues. Bands occurring in delineated zones *A-E* are discussed in the text.

The *S*-carboxymethylcysteine (SCM-cysteine) values for both high- and low-sulphur proteins are appreciably lower in the urea-EDTA extracts than in the urea-Tris extracts. More methionine is present in both urea-EDTA-extracted proteins. Whilst proline values in both extracts are much larger for the high- than for the low-sulphur proteins, the urea-Tris extract has considerably more proline than the urea-EDTA extract. Higher serine and threonine values occur in the urea-Tris extract.

Whilst lysine, arginine, and methionine values in the urea-EDTA extracts are similar for both high- and low-sulphur proteins, proline and SCM-cysteine values are

markedly different, being much higher in the high-sulphur proteins. On the other hand, isoleucine and leucine values are lower. These differences enable the high- and low-sulphur proteins to be distinguished.

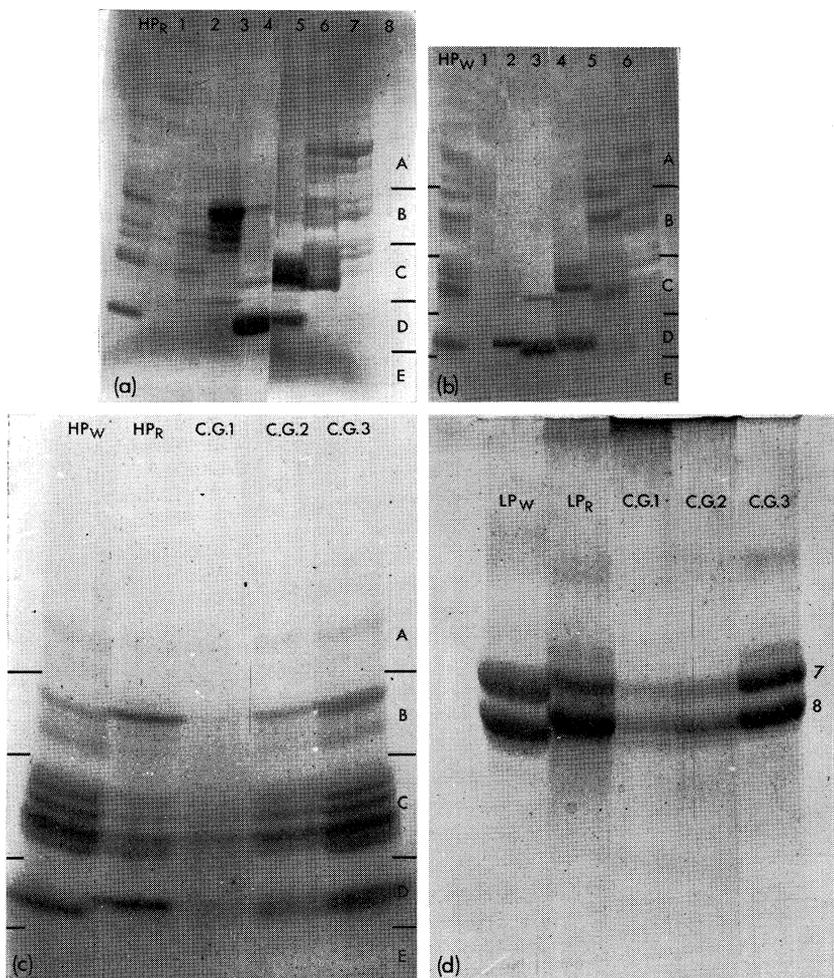


Fig. 3.—(a), (b) Starch-gel electrophoretograms from 2% solutions of *S*-carboxymethylated high-sulphur proteins from intact wool roots (HP_R) and from wool (HP_W). Patterns 1–8 in (a) and 1–6 in (b) refer to pooled fractions obtained after chromatography on DEAE-cellulose of intact wool roots and wool extracts [see Fig. 4(a)]. (c), (d) Starch-gel electrophoretograms from 2% solutions of *S*-carboxymethylated high- and low-sulphur proteins from wool (HP_W , LP_W), intact wool roots (HP_R , LP_R), and cell groups (*C.G.*) 1, 2, and 3 respectively. In all figures the origin for electrophoresis is at the top of each pattern. Zones A–E delineated in (a), (b), and (c), and bands numbered 7 and 8 in (d) are discussed in the text.

Starch-gel electrophoretograms of high-sulphur proteins extracted by both methods show marked differences (Fig. 2). The urea-Tris extracts show a very close similarity to high-sulphur proteins from wool and from keratinized wool root residues

TABLE 1

AMINO ACID ANALYSIS OF HIGH- AND LOW-SULPHUR PROTEINS EXTRACTED FROM INTACT WOOL ROOTS AND WOOL

Amino acid nitrogen expressed as a percentage of total nitrogen of hydrolysates. Numbers in parenthesis refer to analysis number

Amino Acid	Wool Root (urea-EDTA extract, pH 7.1)		Wool Root (urea-Tris extract, pH 9.4)		Wool (urea-TGA* extract, pH 10.5)		Wool Root High-sulphur Proteins†		
	High S (1)	Low S (2)	High S (3)	Low S (4)	High S (5)	Low S (6)	45-53 (7)	54-71 (8)	72-98 (9)
Lysine	5.86	6.51	1.27	6.28	1.25	5.86	1.45	2.59	0.32
Histidine	1.98	2.27	1.76	1.32	2.30	1.30	1.64	3.75	0.42
Arginine	12.74	14.20	12.54	20.33	12.75	20.44	16.66	12.25	10.95
Aspartic acid	6.43	4.98	2.33	6.99	2.36	6.84	1.79	5.16	0.62
Threonine‡	3.62	2.55	7.02	3.08	6.42	3.12	7.60	7.42	6.53
Serine‡	4.86	5.14	9.06	5.11	8.57	5.80	13.00	7.49	8.70
Glutamic acid	9.88	7.88	6.01	11.84	5.84	11.60	5.87	5.00	6.79
Proline	5.81	1.10	7.90	2.20	7.98	2.18	6.43	10.45	6.85
Glycine	3.96	4.00	4.32	3.45	4.63	4.20	3.85	3.82	4.90
Alanine	4.18	4.00	2.25	5.11	2.22	5.10	1.67	2.81	2.10
Valine	3.16	3.40	4.00	4.37	4.41	4.45	6.55	4.48	2.56
Methionine	0.50	0.96	Trace	0.51	Trace	0.50	Trace	Trace	Trace
Isoleucine	2.48	2.68	3.10	2.65	2.95	4.45	2.36	3.18	3.07
Leucine	4.23	6.10	3.36	7.93	3.14	7.89	3.20	4.95	1.78
Tyrosine	2.36	1.73	1.78	1.63	1.86	1.75	2.04	1.40	1.91
Phenylalanine	1.27	2.35	1.50	1.25	1.51	1.65	1.08	2.60	0.83
SCM-cysteine	8.18	1.01	12.40	3.80	12.65	3.75	8.44	10.60	16.55
Citrulline	—	1.64	—	—	—	—	—	—	—
Ammonia	10.50	9.52	11.20	12.00	9.42	9.80	12.60	11.90	11.50

* Thioglycollic acid. † Effluent fraction numbers refer to Figure 4(b). ‡ Not corrected for decomposition.

extracted by the urea-thioglycollate reduction method. The urea-EDTA extract is markedly lacking many of the protein components and most of the positive staining occurs in the gel front. Such a gel pattern for the urea-EDTA extract as shown in Figure 2 is not always consistent. Some extracts contain protein material with lower mobility in zone *C* and zone *D* (Fig. 2), but which show poor resolution into distinct bands. Because of the above differences, only urea-Tris buffer has been used in further work. It is likely that urea-EDTA buffer at pH 7.1 either does not extract all, or does not extract representative proteins from the wool root, whilst urea-Tris buffer at higher pH extracts proteins similar to wool fibre proteins.

TABLE 2

PERCENTAGE BY WEIGHT AND SULPHUR CONTENT OF HIGH- AND LOW-SULPHUR PROTEINS FROM INTACT WOOL ROOTS, FRACTIONATED WOOL ROOT CELLS, AND WOOL FROM THREE SEPARATE ANIMALS

Animal No.	Wool Fraction	Total Material Extracted (%)	High-sulphur Extract		Low-sulphur Extract	
			Protein (%)*	Sulphur (%)	Protein (%)*	Sulphur (%)
1	Intact wool roots			3.84		1.50
	Wool root fractions:					
	Cell group 1	77.4	22.9	3.99	54.5	1.57
	Cell group 2	81.6	26.9	4.71	54.7	1.58
	Cell group 3	81.5	28.0	5.00	53.5	1.79
Wool	83.5	29.2	5.70	54.3	2.12	
2	Intact wool roots		18.4†	3.4	81.6†	1.40
	Wool root fractions:					
	Cell group 1	86.2	16.2	3.68	70.0	1.94
	Cell group 2	84.2	17.8	3.89	66.4	1.94
	Cell group 3	86.7	23.7	4.11	63.0	1.98
Wool	87.0	20.7	5.12	66.3	2.28	
3	Intact wool roots					
	Wool root fractions:					
	Cell group 1	70.1	22.1	3.76	48.0	1.82
	Cell group 2	85.1	29.3	4.01	55.8	1.84
	Cell group 3	84.6	30.6	4.98	54.0	2.00
Wool	80.0	25.6	5.53	54.5	2.09	

* As percentage of total material extracted.

† As percentage of total protein extracted.

(b) *Material Extracted from Wool, Intact Wool Roots, and Cell Groups*

The amounts of high- and low-sulphur proteins extracted from wool and fractionated cell groups expressed as a percentage of the total weight of wool and cell groups are given in Table 2. Because of the difficulty in extracting proteins from wool root tissue after drying, without recourse to reducing conditions, the percentage by weight of high- and low-sulphur proteins from intact wool roots was not determined. The percentage of high- and low-sulphur proteins in the total protein extract is given for animal 2 only.

Whilst there is an increase in the percentage of high-sulphur proteins from cell group 1 to cell group 3, the low-sulphur proteins show no marked trends. The

percentages by weight of high- and low-sulphur proteins in wool are of the same order as in cell group 3.

The sulphur content of the high- and low-sulphur fractions from intact root and cell groups is substantially lower than their counterparts from wool, and the sulphur content of both proteins from intact root tissue is lower than in any of the cell groups. An increasing sulphur content in both the high- and low-sulphur proteins from cell group 1 to cell group 3 and wool is evident (Table 2).

(c) *Column Fractionation and Electrophoresis of High-sulphur Proteins Extracted from Wool, Intact Wool Roots, and Cell Groups*

Figure 4(a), curve *B*, is a typical ultraviolet elution curve for the high-sulphur proteins extracted from intact wool roots with urea-Tris buffer immediately after stripping, and chromatographed on DEAE-cellulose, whilst Figure 4(a), curve *A*, is that for the high-sulphur proteins from wool from the same animal, chromatographed in the same way. The elution pattern for wool root proteins shows better resolution than that for wool. Wool roots contain absorbing material in effluent fractions 97-140 which is not present in wool.

The percentage sulphur in pooled effluent fractions from wool and wool root extracts is given in the legend to Figure 4. As noted by Gillespie (1963), increasing sulphur content is associated with increasing effluent volume. This is apparent for both wool and wool root extracts.

Elution patterns of the high-sulphur proteins extracted from wool roots with urea-Tris buffer and with Lockes solution immediately after stripping are compared in Figure 4(b), curves *C* and *D* respectively. It will be noted that more material was extracted by Lockes solution initially (cf. unshaded areas under both curves, corresponding to effluent fractions 25-45) than by urea-Tris buffer, but the converse was the case for effluent fractions 46-93 (see shaded areas) and also for the final effluent fractions. In these final fractions, however, the Lockes solution extract also contained strongly absorbing material.

The total extract obtained with Lockes solution was fractionally precipitated at pH 4.4 resulting in a precipitate low in sulphur (1.0%) and a supernatant with a higher sulphur content (3.4%). The higher sulphur content of high-sulphur proteins from cell groups as compared with intact root extracts (Table 2) can be explained by the removal of the proteins soluble in Lockes solution from cells during separation.

The elution patterns of 50 mg of high-sulphur-protein-containing extracts of wool root cell groups 1, 2, and 3 chromatographed on DEAE-cellulose are shown in Figure 4(c). Each cell group differs from extracts of intact roots in that the proteins contained in effluent fractions 30-45 [Fig. 4(a)] are not present, having been largely removed by Lockes solution during cell separation. Otherwise the ultraviolet absorption peaks in the elution patterns appear identical for intact roots [Fig. 4(b)] and the cell groups [Fig. 4(c)]. Though the same weight of extract from each of the cell groups was chromatographed, there is a progressive increase in the total absorbance in proteins eluted in fractions 0-95 from cell group 1 to cell group 3, and there is a corresponding decrease in absorbance of material in fractions 96-130 in all cell groups. Approximately 85% by weight of the extract is contained in fractions 0-95 from cell group 3. The total material in effluent fractions 0-95 from cell groups 1 and 2 repre-

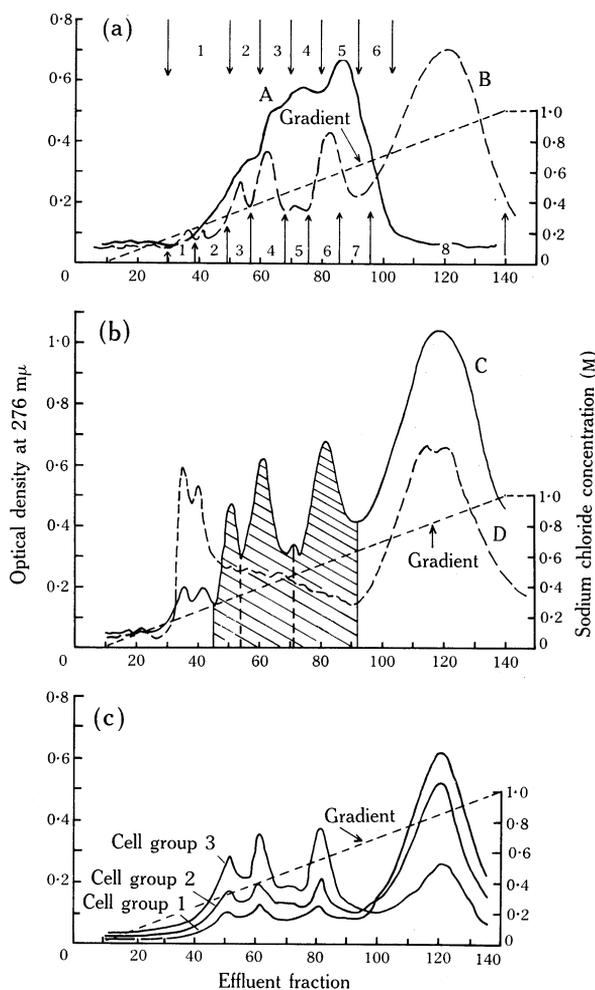


Fig. 4.—(a)–(c) Chromatography on DEAE-cellulose with sodium chloride gradients of 0–1.0M of *S*-carboxymethylated high-sulphur proteins extracted from wool [(a), curve A], intact wool roots [(a), curve B; (b)], and wool root cell groups 1, 2, and 3 (c). Starch-gel electrophoretograms of pooled fractions 1–6 (curve A) and 1–8 (curve B), delineated by arrows in (a), are shown in Figures 3(b) and 3(a) respectively. The sulphur content of these fractions is as follows:

Pooled fraction No. (curve A):	2	3	4	5	6
Effluent fraction Nos.	48–57	58–67	68–75	76–91	92–101
Sulphur content (%):	4.69	5.39	5.85	6.22	6.40
Pooled fraction No. (curve B):	1+2	3	4	6+7	
Effluent fraction Nos.	30–48	49–56	57–68	76–96	
Sulphur content (%):	3.86	4.11	5.70	5.90	

In (b), curves D and C were obtained from extracts of intact wool roots with calcium-free Lockes solution and urea-Tris buffer at pH 9.4 respectively, immediately after stripping. 66 and 80 mg respectively of material were chromatographed. In (c) column loads were 50 mg in each case.

sented 37 and 48% respectively when expressed as a percentage of the ultraviolet absorbance of material extracted in fractions 0-95 from cell group 3.

The sulphur content of proteins in the pooled effluent fractions 0-95 from cell groups 1, 2, and 3 was 5.26, 5.43, and 5.49% respectively. These values are considerably higher than the sulphur content of the total extracts from the cell groups (Table 2) and approach the values of the sulphur content of the wool matrix (e.g. 5.53% for animal 3, Table 2).

Starch-gel electrophoretograms of the high-sulphur proteins from wool, intact wool roots, and each of the three cell groups are shown in Figure 3(c). Very close similarity is observed between all patterns. However, one marked difference is evident between the patterns for the wool root proteins (HP_R) and for wool (HP_W), this being the presence of an intense band (in zone *B*) in the former pattern.

The electrophoretograms of high-sulphur proteins from cell groups 1, 2, and 3 [Fig. 3(c)], using identical concentrations of the total extracts from cells, indicate that the proportion of high-sulphur proteins in cells increases towards the level of keratinization. This is in accord with the results of column chromatography presented in Figure 4(c).

Starch-gel electrophoretograms of high-sulphur proteins from wool roots and pooled fractions 1-8 obtained when these proteins were chromatographed on DEAE-cellulose [see Fig. 4(a), curve *B*] are shown in Figure 3(a). Likewise those of high-sulphur proteins from wool and pooled fractions 1-6 [see Fig. 4(a), curve *A*] are shown in Figure 3(b). The most obvious difference between the two sets of electrophoretograms is the presence of bands in zone *B* of pooled fraction 3 [effluent fractions 49-57 of Fig. 4(a), curve *B*] from wool roots [Fig. 3(a)], and the absence of such bands in any of the patterns in Figure 3(b). However, the electrophoretic patterns of proteins in effluent fractions 60-95 from both sources are similar.

The strongly absorbing material present in effluent fractions 97-140 of wool root extract [Fig. 4(a), curve *B*] is absent in extracts of wool [Fig. 4(a), curve *A*], and does not show any clearly demarcated protein bands on electrophoresis [pattern 8, Fig. 3(a)]. On staining the gel with pyronine-methyl green, an intense pink band develops at the gel front of pattern 8. As RNA also moves in the gel front and stains pink with pyronine-methyl green, this indicates that some of the material in fractions 97-140 from wool roots is RNA or a derivative. Another indication is that this material has absorption maxima in the range 262-282 $m\mu$.

(d) Amino Acid Analysis of High-sulphur Proteins Extracted from Wool and Wool Roots

Amino acid analyses of high-sulphur proteins in pooled effluent fractions 0-96 from wool roots [Fig. 4(a), curve *B*] and from wool [Fig. 4(a), curve *A*] are given in Table 1, analyses 3 and 5 respectively. Analyses of protein in effluent fractions 45-53, 54-71, and 72-93 [shaded areas in Fig. 4(b)] of the urea-Tris extract of wool roots are also given in Table 1, analyses 7, 8, and 9 respectively.

The amino acid concentrations in high-sulphur proteins from wool roots are similar to those from wool, particularly in terms of the high threonine, serine, proline, and SCM-cysteine values and the low lysine, aspartic acid, and glutamic acid values

which typify matrix proteins as compared with the low-sulphur or microfibrillar proteins (Table 1, analyses 4 and 6). However, the amino acid analysis of wool root effluent fractions 45–53 [Fig. 4(b); analysis 7, Table 1] indicates a lower SCM-cysteine content than other component high-sulphur proteins, and a higher serine content. Valine also tends to be higher than in the other proteins. Analyses 8 and 9 (Table 1) of the remaining high-sulphur proteins from wool roots [effluent fractions 54–71 and 72–93 respectively, Fig. 4(b)] are typical of analyses for matrix proteins from wool (Gillespie 1963). Decreased values for lysine and histidine are associated with increased SCM-cysteine values in analysis 9. These proteins are eluted last from DEAE-cellulose [Fig. 4(b)], and have a lower electrophoretic mobility than other high-sulphur proteins from wool roots [Fig. 3(a)].

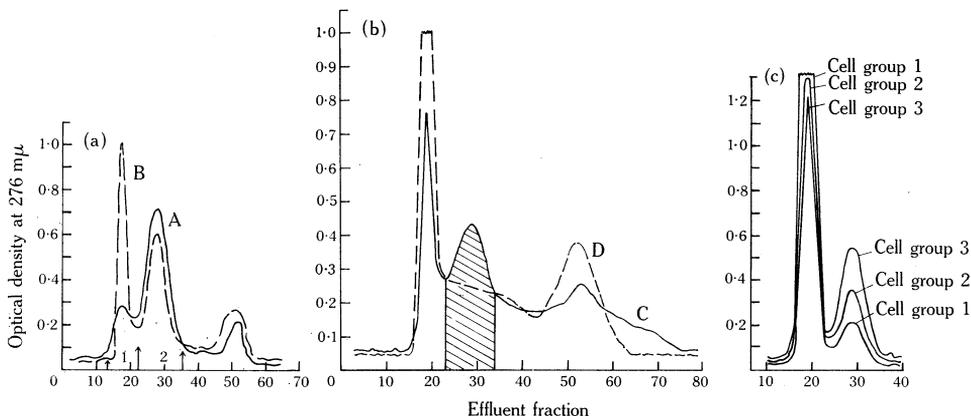


Fig. 5.—(a)–(c) Gel filtration on Sephadex G-200 of *S*-carboxymethylated low-sulphur proteins from wool [(a), curve A], intact wool roots [(a), curve B; (b)], and wool root cell groups 1, 2, and 3 (c), all being from the same animal. Starch-gel electrophoretograms of these low-sulphur proteins are shown in Figure 3(d), and the sulphur content of pooled fractions 1 and 2, delineated by arrows in (a), is as follows:

Pooled fraction No.:	Wool		Wool Roots	
	1	2	1	2
Effluent fraction Nos.	13–22	23–36	13–22	23–36
Sulphur content (%):	2.01	2.20	1.00	1.86

In (b), curves D and C were obtained from extracts of intact wool roots with calcium-free Lockes solution and urea–Tris buffer at pH 9.4 respectively, immediately after stripping. 33.6 and 100 mg respectively of material were chromatographed. In (c), 100 mg of material were chromatographed in each case.

(e) *Column Fractionation and Electrophoresis of Low-sulphur Protein Extracts from Wool, Intact Wool Roots, and Cell Groups*

Figure 5(a), curve B, shows a typical ultraviolet absorption pattern of low-sulphur proteins of intact wool roots eluted from Sephadex G-200, whilst Figure 5(a), curve A, shows a typical pattern of the low-sulphur proteins of wool chromatographed in the same way. The sulphur content of the material contained in pooled fractions 1 and 2 in Figure 5(a) are given in the legend to Figure 5.

Figure 5(b) compares elution patterns of low-sulphur proteins extracted with urea-Tris buffer (curve *C*) with the material extracted by Lockes solution, precipitated at pH 4.4, and prepared as described previously (curve *D*). These patterns are similar, except that the absorption peak in effluent fractions 23-34 of the urea-Tris extract (shaded area under curve *C*) does not occur in the Lockes solution extract, although there is appreciable ultraviolet absorption at 276 m μ in these fractions in the latter.

The elution patterns of effluent fractions 10-40 from Sephadex G-200 columns of 100 mg of low-sulphur protein extract from each of the three cell groups are shown in Figure 5(c). The concentration of microfibrillar proteins in the low-sulphur protein extract increases in effluent fractions 23-34 [Fig. 5(c)]. The proportion of material in effluent fractions 12-22 decreased from cell group 1 to cell group 3. Proteins in effluent fractions 23-34 from cell groups 1, 2, and 3 represent 31, 55, and 91% by weight of the total low-sulphur extracts from these cell groups, and contain 1.87, 1.73, and 2.01% sulphur respectively.

Starch-gel electrophoretograms, using equal quantities of low-sulphur proteins from both intact wool roots and cell groups, are compared with each other and the low-sulphur proteins from wool in Figure 3(d). The numbering of protein bands 7 and 8 in Figure 3(d) is in accord with terminology used by O'Donnell and Thompson (1964).

The principal difference between wool and root tissue proteins is in the protein material remaining at the gel origin. This apparently highly aggregated protein is eluted from Sephadex G-200 in effluent fractions 12-22 [Figs. 5(a) and 5(c)]. The decreasing amounts of this material from cell group 1 to cell group 3 as shown in Figure 5(c) is also shown in the electrophoretograms in Figure 3(d) as bands at the origin.

The low-sulphur protein from wool which is eluted in the first peak has been shown to run electrophoretically between the origin and bands 7 and 8 (O'Donnell and Thompson 1964). Bands 7 and 8 are the major components of wool and correspond to the second peak eluted from Sephadex G-200 [shaded area in Fig. 5(b), curve *C*] (O'Donnell and Thompson 1964; Thompson and O'Donnell 1965). These proteins are considered to be the helical proteins of the microfibrils (Crewther *et al.* 1965; Thompson and O'Donnell 1965). The material in the third peak in Figure 5(a) is reported to have higher electrophoretic mobility than bands 7 and 8 and analyses high in glycine and tyrosine (O'Donnell and Thompson 1964). No further reference will be made to these proteins.

Figure 3(d), in accord with Figure 5(c), indicates that the microfibrillar proteins from cell groups increase in concentration from cell group 1 to cell group 3.

(f) *Amino Acid Analysis of Low-sulphur Protein Extracts from Wool and Wool Roots*

Amino acid analysis of microfibrillar proteins contained in pooled effluent fractions 23-34, Figures 5(a) and 5(c), from wool roots is given in Table 1 (analysis 4). The analysis is in close accord with microfibrillar proteins from wool off the same animal (Table 1, analysis 6) and values reported by other workers for the microfibrillar proteins from wool (O'Donnell and Thompson 1964).

Both wool and wool root microfibrillar protein analyses are distinct from high-sulphur proteins in having higher lysine and glutamic acid values, lower serine, threonine and proline contents, and a lower SCM-cysteine content; also, methionine

is present in the former, and leucine is present in the low-sulphur proteins in much larger amounts than isoleucine.

IV. DISCUSSION AND CONCLUSIONS

Column fractionation of the proteins in extracts of intact wool roots and wool root cells has resulted in the isolation of both high- and low-sulphur proteins [shaded areas under curve *C* in Figs. 4(*b*) and 5(*b*) respectively] which, on the basis of electrophoresis, amino acid analysis, and sulphur content, are respectively analogous to the matrix and microfibrillar proteins of wool keratin.

In the case of microfibrillar proteins, gel filtration, gel electrophoresis, and amino acid analysis serve to indicate the close similarity between wool root and wool proteins. These results are in accord with earlier work (Frater 1966) showing that wool root and wool microfibrillar proteins are very similar. Very small differences reported have been considered as resulting from changes during keratinization.

In the case of matrix proteins, whilst those fractionated from contaminating material within wool root tissue have amino acid analyses typical of matrix proteins from wool, and the majority show similar electrophoretic behaviour, there are distinct differences. These differences are observed on DEAE-cellulose chromatography, electrophoresis, and amino acid analysis. A group of proteins is present in the wool root proteins [pooled fraction 3, Fig. 4(*a*), curve *B*] which have no counterpart that can be isolated from wool [compare zone *B* in electrophoretogram 3 of Fig. 3(*a*) with that in Fig. 3(*b*)]. The extracts of cell groups [Fig. 4(*c*)] show that such proteins increase in cell concentration, along with other matrix proteins, throughout the length of the prekeratin region, i.e. from cell group 1 to cell group 3. These proteins (Table 1, analysis 7), typical of wool matrix proteins in terms of low values for SCM-cysteine, lysine, and histidine and high values for threonine, serine, and proline as compared with microfibrillar proteins (Table 1), tend to characterize all wool root extracts studied. They differ from other matrix proteins (Table 1, analyses 8 and 9) in having lower SCM-cysteine and appreciably higher serine values. As these proteins are present in the prekeratin cells nearest to the level of keratinization and have increased in concentration in the cells up to this level, as do associated proteins [Fig. 4(*c*)], it is proposed that they are incorporated into keratin along with the other root matrix proteins which are typical of wool matrix proteins in terms of electrophoretic mobility [Fig. 3(*a*)] and amino acid analysis (Table 1, analyses 8 and 9).

The inability to isolate the high-serine proteins of wool roots from wool extracts as such is believed to result from the keratinization process. The less-resolved elution pattern of matrix proteins from wool as compared with root matrix proteins [Fig. 4(*a*)], and also from cells near to the site of keratinization [Fig. 4(*c*), cell group 3], gives evidence of changes resulting from keratinization. Comparison of both elution patterns in Figure 4(*a*), and of the electrophoretic patterns of proteins within the pooled fractions numbered in this figure [electrophoretograms in Figs. 3(*a*) and 3(*b*)], may indicate specific changes. Whilst pooled fraction 5 of wool root matrix proteins [electrophoretogram 5, Fig. 3(*a*)] contains proteins with identical electrophoretic behaviour to the wool proteins in pooled fraction 4 [electrophoretogram 4, Fig. 3(*b*)], their concentration relative to the other matrix proteins is markedly less

in prekeratin regions than after keratinization. Whether the disappearance of the prekeratin proteins of high serine content is significant in terms of an increase in other proteins present in lesser amounts in prekeratin, as a result of keratinization, cannot yet be determined. However, such changes in matrix proteins appear to be associated with the process of keratinization.

A further distinction between the high-sulphur proteins extracted from prekeratin and keratin is the presence in the former and absence in the latter of strongly absorbing material eluted from DEAE-cellulose [pooled fraction 8, Fig. 4(a), curve B]. That this material contains some RNA or a derivative is indicated by its presence in the electrophoretogram front as a pink band after staining with pyronine-methyl green. Maximum ultraviolet absorption between 262 and 268 $m\mu$ further indicates its nucleic acid nature. The concentration of this material relative to matrix proteins is seen to decrease towards the site of keratinization [Fig. 4(c), cell groups 1-3], and the material could be lost from cells before keratinization occurs.

The low-sulphur proteins extracted from prekeratin clearly contain microfibrillar proteins analogous to such proteins from wool. This is verified by elution patterns during gel filtration [second peak, Fig. 5(a)], electrophoretic patterns of proteins from this peak [Fig. 3(d), bands 7 and 8], and amino acid analysis of these proteins as compared with those from wool (Table 1, analyses 4 and 6).

The first peak in Figure 5(a) indicates that together with microfibrillar proteins, low-sulphur extracts of wool roots contain proteins which are eluted first during gel filtration and which remain at the gel origin on electrophoresis [Fig. 3(d)]. Low-sulphur protein extracts from wool, though showing a small absorption peak at similar effluent volumes [Fig. 5(a)] do not have the same concentration relative to microfibrillar proteins in the second effluent peak. Such proteins from wool are considered to be aggregates of the microfibrillar proteins (O'Donnell and Thompson 1964) and, unlike the wool root material, have been shown to move away from the origin on gel electrophoresis.

Unlike the fully keratinized proteins, extraction of prekeratin tissue with Lockes solution removes considerable quantities of non-dialysable protein which can be separated by fractional precipitation at pH 4.4, as used for wool proteins, into low- and high-sulphur containing groups (1.0 and 3.40% sulphur respectively). Neither of these groups of proteins contain matrix or microfibrillar proteins as indicated by DEAE-cellulose chromatography [Fig. 4(b)] and gel filtration [Fig. 5(b)].

The study of cell groups 1, 2, and 3, representing the cell sequence from differentiation to near keratinization within the prekeratin region of the developing wool fibre (Fig. 1), clearly indicates from chromatography, gel filtration, and electrophoresis a progressive increase in matrix [Fig. 4(c)] and microfibrillar [Fig. 5(c)] protein concentration within cells as they grow towards the level of keratinization. Correction for the contribution to cell content by non-microfibrillar protein at the three prekeratin levels can be approximated from the results presented.

The weight of total high- and low-sulphur protein extracts as a percentage of the total weight of cells and wool is given in Table 2. Only small differences exist between percentage values of each group of proteins at the different cell levels. Though all cell groups show similar percentage values to those in wool, the sulphur

contents increase from cell group 1 to cell group 3 to wool, particularly in the high-sulphur proteins.

Correction of the percentage values for animal 3 in Table 2 on the basis of the matrix and microfibrillar proteins separated from the total extracts by chromatography and gel filtration give the following percentage values:

	Cell Group 1		Cell Group 2		Cell Group 3		Wool	
	Matrix	Micro-fibrillar	Matrix	Micro-fibrillar	Matrix	Micro-fibrillar	High S	Low S
Material extracted (%)	8.0	15.0	14.0	31.0	26.0	49.0	25.6	54.5
Sulphur content (%)	5.26	1.87	5.43	1.72	5.49	2.01	5.53	2.09

Both matrix and microfibrillar proteins are seen to increase in concentration as prekeratin wool fibre cells grow up the follicle. The addition of matrix protein tends to be exponential, something of the order of 50% of the wool matrix protein content being found in cells in the lower two-thirds, with addition of approximately 50% in the upper third of the prekeratin region. The microfibrillar proteins, however, appear to increase linearly in concentration in cells with maturation. The above tabulation shows little difference in sulphur content between the same proteins at the three prekeratin levels and wool. Whilst such differences do not indicate significant sulphur enrichment of structural proteins as growth proceeds, sulphur values in Table 2 do indicate a sulphur enrichment within the total high-sulphur-containing protein of cells. This apparent enrichment, however, is the result of a decreasing proportion of the material present as non-matrix protein (3.4% sulphur), with increasing matrix protein (5.53% sulphur) as growth proceeds.

The presence of both matrix and microfibrillar proteins throughout the length of the prekeratin region provides the first evidence to refute the hypothesis of a "two-stage" process of keratin synthesis. The fact that microfibrillar proteins are present in the lowest levels of the wool root, and show an approximately linear increase in concentration in the cells proximodistally, is not in accord with complete synthesis occurring only at proximal levels as suggested by Rudall (1956) and Mercer (1961) and supported by Downes, Sharry, and Rogers (1963), and Downes *et al.* (1966). Rather than "two-stage", a "dual" synthesis of matrix and fibrillar proteins in keratin is evident throughout the length of the prekeratin region.

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VI. REFERENCES

- BERN, H. A., HARKNESS, D. R., and BLAIR, S. M. (1955).—*Proc. natn. Acad. Sci. U.S.A.* **41**, 55.
 BERSAQUES, J. DE, and ROTHMAN, S. (1962).—*Nature, Lond.* **193**, 147.
 BIRBECK, M. S. C., and MERCER, E. H. (1957).—*J. biophys. biochem. Cytol.* **3**, 202.

- CREWETHER, W. G., and DOWLING, L. M. (1960).—*Trans. Text. Inst. Manchr* **51**, T775.
- CREWETHER, W. G., GILLESPIE, J. M., HARRAP, B. S., O'DONNELL, I. J., and THOMPSON, E. O. P. (1965).—*Proc. 3rd Int. Wool Text. Res. Conf., Paris. Vol. I*, p. 303.
- DEURWAERDER, R. E. DE, DOBB, M. G., and SWEETMAN, B. J. (1964).—*Nature, Lond.* **203**, 48.
- DOWNES, A. M., FERGUSON, K. A., GILLESPIE, J. M., and HARRAP, B. S. (1966).—*Aust. J. biol. Sci.* **19**, 319.
- DOWNES, A. M., SHARRY, L. F., and ROGERS, G. E. (1963).—*Nature, Lond.* **199**, 1059.
- ELLIS, W. J. (1948).—*Nature, Lond.* **162**, 957.
- FRATER, R. (1966).—*Aust. J. biol. Sci.* **19**, 699.
- GILLESPIE, J. M., and LENNOX, F. G. (1953).—*Biochim. biophys. Acta* **12**, 481.
- GILLESPIE, J. M. (1960).—*Aust. J. biol. Sci.* **13**, 81.
- GILLESPIE, J. M. (1963).—*Aust. J. biol. Sci.* **16**, 259.
- GIROUD, A., BULLIARD, J., and LEBLOND, C. P. (1934).—*Bull. Histol. appl. Physiol. Path.* **11**, 129.
- GIROUD, A., and BULLIARD, J. (1935).—*Archs Anat. microsc.* **31**, 271.
- GODDARD, D. R., and MICHAELIS, L. (1935).—*J. biol. Chem.* **112**, 361.
- HARKNESS, D. R., and BERN, H. A. (1957).—*Acta Anat.* **31**, 35.
- HARRAP, B. S., and GILLESPIE, J. M. (1963).—*Aust. J. biol. Sci.* **16**, 542.
- MERCER, E. H. (1961).—"Keratin and Keratinization." (Pergamon Press: London and New York.)
- O'DONNELL, I. J., and THOMPSON, E. O. P. (1959).—*Aust. J. biol. Sci.* **12**, 294.
- O'DONNELL, I. J., and THOMPSON, E. O. P. (1962).—*Aust. J. biol. Sci.* **15**, 740.
- O'DONNELL, I. J., and THOMPSON, E. O. P. (1964).—*Aust. J. biol. Sci.* **17**, 973.
- ROGERS, G. E. (1959).—*Ann. N.Y. Acad. Sci.* **83**, 408.
- ROTHMAN, S. (1954).—"Physiology and Biochemistry of the Skin." (Chicago Univ. Press.)
- RUDALL, K. M. (1956).—*Proc. Int. Wool Text. Res. Conf., Aust., 1955. Vol. F.* p. F176.
- SCHÖNIGER, W. (1954).—*Mikrochim. Acta* **1954**, 74.
- THOMPSON, E. O. P., and O'DONNELL, I. J. (1964).—*Aust. J. biol. Sci.* **17**, 277.
- THOMPSON, E. O. P., and O'DONNELL, I. J. (1965).—*Aust. J. biol. Sci.* **18**, 1207.
- VAN SCOTT, E. J., and FLESCHE, P. (1954).—*Archs Derm. Syph.* **70**, 141.

