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Characterization of the Proteins of Guinea-Pig Hair and Hair-Follicle Tissue

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This work forms a part of a study of the mechanism and control of protein synthesis in the hair follicle and concerns the characterization of the proteins of hair-follicle tissue and for comparative reasons those of the hair itself. 1. Five different groups of reduced carboxymethylated proteins were delineated from both tissues; these were: group 1A proteins, which appeared to be aggregates of the group 2 proteins; group 1B proteins, soluble at pH4.4, which were thought to originate from the medulla and inner-root-sheath layers; group 2 proteins, which were defined as the main low-sulphur keratin proteins insoluble at pH4.4; group 3 proteins, the precise origin of which is not known; and the group 4 proteins, which were defined as the main high-sulphur keratin proteins soluble at pH4.4. 2. With the single exception of the group 1B proteins, the types and properties of all hair and hair-follicle proteins were identical as far as could be determined by use of such criteria as multiplicity of components, molecular charge, molecular weight and amino acid composition. 3. Two significant quantitative differences were noted: in follicle extracts there were more group 2 proteins but less group 3 and group 4 proteins than in hair extracts; and secondly, in the follicle group 4 proteins, there were more proteins of lowest molecular weight and *S*-carboxymethylcysteine content, but fewer proteins of the highest molecular weight and *S*-carboxymethylcysteine content than in the hair group 4 proteins. 4. These quantitative differences are discussed in terms of the mechanism of synthesis of the keratin proteins. 5. Follicle group 1B proteins are postulated to have arisen from the trichohyalin droplets of the developing medulla and inner-root-sheath layers of the follicle and may be precursors of the proteins of the mature medulla and inner root sheath.

The processes of development in the hair follicle involve a complex series of macromolecular events leading to the biosynthesis and structural organization of several different groups of proteins. The major group of proteins is the α-keratins.

An important aspect in the investigation of the mechanism of hair (and wool) growth has been the detailed characterization of the chemistry and properties of the mature keratin proteins (Crewther *et al.*, 1965; Fraser *et al.*, 1972). These studies have shown that most of the wool fibre can be converted into soluble protein by use of reducing reagents that cleave disulphide bonds. These proteins are then changed to more stable derivatives by reaction with iodoacetic acid to form the *S*-carboxymethylcysteine proteins. The reduced and alkylated proteins of wool can be fractionated into two major classes; one of a lower *S*-carboxymethylcysteine content than the total proteins (the low-sulphur keratin, or 'LoS' proteins) and the other of higher *S*-carboxymethylcysteine content (the high-sulphur keratin, or 'HiS' proteins). From a number of detailed physical and physicochemical studies it has been concluded that the low-sulphur proteins are α-helical and are thought to comprise the microfibrils of the mature fibre, whereas the high-sulphur proteins lack an ordered structure and are throught to comprise the matrix of the fibre (Fraser *et al.*, 1972).

Studies were undertaken to determine whether the proteins that are deposited during development within the cells of the follicle (the prekeratin) are in fact identical with or different from the proteins of the mature fibre. It was shown that the prekeratin could be fractionated into low-sulphur and high-sulphur proteins (Rogers, 1959; Downes *et al.*, 1966; Fraser, 1969; Clarke & Rogers, 1970). Although there were several marked similarities in the properties of the proteins, there were also some important differences. Notably, the high-sulphur proteins of the follicle had a markedly lower *S*-carboxymethylcysteine content and a rather different amino acid composition than those of the fibre. Further, a number of additional protein components were identified in the follicle proteins, whose origin and relation to the proteins of the mature fibre was not known. The earlier studies did not extend these observations to more detailed comparisons of the proteins. Several possibilities exist which could account for the differences observed.

For example the lower *S*-carboxymethylcysteine content of the follicle high-sulphur proteins may be due to major differences between the chemical structure of the follicle and the fibre high-sulphur proteins. Clearly, clarification of these possibilities is important in studies on the mechanism of protein synthesis in the hair follicle.

The experiments reported here were undertaken with two particular aims. The first was to define more precisely the properties of the proteins of prekeratin in relation to those of the mature fibre. A concurrent aim was to provide basic procedures for the isolation and characterization of the various types of proteins. Information on the first and the development of the techniques in the second are an important prerequisite to studies on the mechanism and control of synthesis of the proteins of the follicle.

Experimental

Materials

Source of tissue. Albino guinea pigs of age less than 3 weeks of both sexes were used for the source of hair and hair-follicle tissue in this work.

Methods

Extraction of hair-follicle tissue. Hair follicles were isolated from guinea-pig skin by the wax-sheet procedure described by Clarke & Rogers (1970). This tissue was disrupted by homogenization in a loose-fitting Potter–Elvehjem vessel in a buffer of 8M-urea, 0.1M-Tris–HCl (pH7.6), 1mM-EDTA and 25mM-2-mercaptoethanol (25ml/g of follicle tissue). The solution was stirred for 10min to ensure complete reduction of the thiol groups of the proteins. At this pH and 2-mercaptoethanol concentration, no significant amount of polypeptide material was extracted from the mature hair fibres present in the hair-follicle preparations. The pH was raised to 8.5 and the solution was treated with iodoacetic acid (200mg/ml) dissolved in 3M-Tris–HCl (pH8.5), to a final concentration of 50mM. The alkylation reaction was allowed to proceed until free thiol groups could no longer be detected (negative nitroprusside test), whereupon excess of iodoacetic acid was destroyed by addition of 2-mercaptoethanol. The solution was filtered through nylon gauze (pore size about 0.2mm), centrifuged at 38000*g* for 10min to remove debris and then dialysed against 10mM-Tris–HCl (pH7.6)–1mM-EDTA buffer with four changes of 100vol. All procedures, except the alkylation step, were carried out at 0°C.

Extraction of hair. Guinea-pig hair was cleaned by immersion in several changes of light petroleum, ethanol and water and was then air-dried. It was ground in a mill to through-40-mesh size and the proteins were extracted by the procedure of O'Donnell & Thompson (1964) with 0.1M-2-mercaptoethanol at pH9.2 for 3h before being alkylated with iodoacetic acid as described above.

Preparation of the pH4.4-soluble and -insoluble protein fractions. These fractions were prepared by a modification of the procedure of Crewther & Harrap (1967). The dialysed extracted proteins were adjusted to 0.5M-KCl and centrifuged at 38000*g* for 10min to remove precipitated material. The pH was lowered to 4.4 by addition of 0.5vol. of 0.2M-sodium acetate buffer (pH4.4, $I = 0.2$). The solution was stirred for 15min and the precipitate (fraction A) was collected by centrifugation at 1000*g* for 10min. This was redissolved in 50mM-sodium tetraborate (pH9.2) (to about 10mg/ml), adjusted to 0.5M-KCl and reprecipitated with the acetate buffer. This process was repeated a third time. The pH4.4-soluble proteins (fraction B) from the first precipitation were dialysed against a buffer of 10mM-Tris–HCl (pH7.6)–1mM-EDTA with three changes of 100vol., concentrated to about 10mg/ml by rotary film evaporation under reduced pressure at 40°C and then treated with the acetate buffer as described above. This process was repeated a third time. Finally, both pH fractions were concentrated to about 20mg/ml by rotary evaporation. The yield of protein based on the weight of hair was about 45% for the fraction insoluble at pH4.4 and about 20% for the soluble fraction, and the yields from hair follicle tissue were about 35% and 15%, respectively.

Preparation of urea buffers. Wherever possible, 8M-urea solutions were used to prevent aggregation of the keratin proteins. However, constant exposures to urea solutions increased the possibility of the reaction of protein amino groups with cyanate ions, which exist in equilibrium with urea in solution. Therefore, the precautions of Thompson & O'Donnell (1965) were adopted, involving treatment of the 8M-urea solution with mixed ion-exchange resin. All 8M-urea buffers contained Tris base, which acts as a scavenger for cyanate ions. This base and other components were added as solid reagents to the desired concentration and the pH finally was corrected with 1M-HCl.

Chromatography with Sephadex. Dry Sephadex G-200 (medium grade) was washed in sodium-dried ether to remove fine beads (Kawata & Chase, 1968), soaked in water with several changes for 1 week to ensure complete swelling and then in a urea buffer of 0.2M-KCl, 50mM-Tris–HCl (pH7.6) and 1mM-EDTA for 2 days before packing the column by established procedures. The direction of flow in these columns was upwards with a net hydrostatic pressure head of 15–20cm of urea buffer (approximately 2000Pa). The columns equilibrated and operated in this manner maintained a constant flow rate of about 2ml/h per cm^2 for periods in excess of 2 years. Sephadex G-100 was first swollen in water before

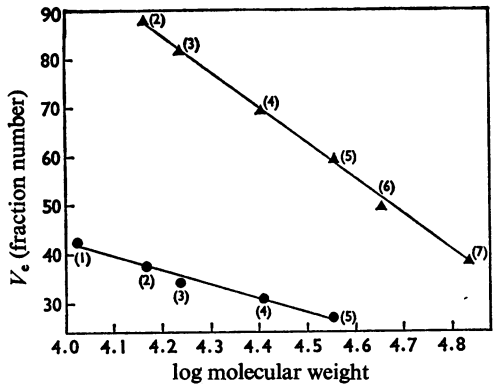


Fig. 1. *Calibration of Sephadex columns with S-carboxymethyl-proteins for molecular-weight estimations*

The relationship between fraction number (V_e) and the logarithms of the molecular weights of certain *S*-carboxymethyl-proteins is shown for Sephadex G-100 and G-200. The Sephadex G-200 column (165cm×1.6cm) was equilibrated with a urea buffer [8M-urea, 0.2M-KCl, 50mM-Tris–HCl (pH7.6) and 1mM-EDTA]. About 40mg of protein was chromatographed at a flow rate of 4ml/h; the fraction size was 3.0ml (see Fig. 2). The Sephadex G-100 column (92cm×1.0cm) was prepared as described under 'Methods' and equilibrated with 50% (v/v) aq. formic acid. About 25mg of each protein was chromatographed at a flow rate of 5ml/h; the fraction size was 1.25ml (see Fig. 7). The molecular-weight values of the proteins have been corrected to allow for the addition of *S*-carboxymethyl groups on to all of the half-cystine residues of each protein at the rate of 60 daltons/group. Sephadex G-100, ●; Sephadex G-200, ▲. (1), Feather keratin; (2), lysozyme; (3), apomyoglobin; (4), chymotrypsinogen A; (5), pepsin; (6), ovalbumin; (7), bovine serum albumin.

transfer to aq. 50% (v/v) formic acid. Columns were prepared by standard procedures but operated at a flow rate of 3ml/h per cm² by means of a Beckman Accu-Flo pump.

Before use all columns were calibrated with proteins of known molecular weights (Fig. 1). These proteins had been converted into their *S*-carboxymethyl derivatives by the same procedure as that used for extraction of guinea-pig hair. Each modified protein was checked by chromatography on a Sephadex column and by polyacrylamide-gel electrophoresis and was used only if homogeneous with respect to size and charge. Calibration with the alkylated proteins was necessary as they were used for the estimation of the molecular weights of *S*-carboxymethylated proteins from keratin tissues. The volume of the sample loaded and the fraction size collected were about 1% of the total column volume (V_t).

Column chromatography with DEAE-cellulose. This was performed by standard procedures (O'Donnell & Thompson, 1964).

Measurement of protein concentrations. Approximate protein concentrations were determined spectrophotometrically at 276nm by using the $E^{1\%}_{1cm}$ values of: SCMK-A (proteins insoluble at pH4.4), 7.2; SCMK-B (proteins soluble at pH4.4), 4.3. These values were calculated from the absorption of a solution containing a known amount of dry salt-free protein.

Polyacrylamide-gel electrophoresis. The 7.5% acrylamide gels (0.6cm×10cm) at pH9.0 containing 5M-urea were made by established procedures (Davis, 1964) by using riboflavin for polymerization. Samples (up to 200μg), usually dissolved in buffer containing 8M-urea from the chromatography column experiments, were applied to the top of the 'stacking' gel and subjected to electrophoresis at 1mA/gel until the tracking dye band (of Methylene Blue) had entered the 'separation' gel and then at 3mA/gel for the desired time. After electrophoresis, the gels were washed with 10% (w/v) trichloroacetic acid to remove urea and then stained with 0.05% (w/v) Coomassie Brilliant Blue (Chrambach *et al.*, 1967) in 10% (w/v) trichloroacetic acid for 2 days. Excess of stain was removed from the gels by rinsing with 50% (v/v) aq. ethanol.

Amino acid analysis. Protein samples were thoroughly desalted by dialysis against water and freeze-dried. Samples were hydrolysed in 6M-HCl *in vacuo* at 110°C for 30h. Hydrolysates contained 0.1% (w/v) phenol to prevent modification (by chlorination) of tyrosine residues (Sanger & Thompson, 1963). *S*-Carboxymethylcysteine was partially destroyed on hydrolysis, but the amount lost was rendered insignificant if 2–5mg of protein were hydrolysed (compare with Cole *et al.*, 1958). Samples were freed of HCl by rotary film evaporation and analysed on a Beckman 120C amino acid analyser.

Results

Nomenclature of fractions

In Plate 1 are shown polyacrylamide-gel electrophoresis patterns of proteins extracted from guinea-pig hair and hair-follicle tissue. The *S*-carboxymethylkeratin (SCMK) proteins from hair (H) or hair-follicle (F) tissue are respectively denoted as H-SCMK and F-SCMK. The proteins from both these sources are referred to as SCMK-A for those that are insoluble at pH4.4 and SCMK-B for those

that are soluble at pH4.4. The gel patterns are divided operationally into four groups of increasing electrophoretic mobility as shown. Plate 1 shows that although there were quantitative differences in the proteins extracted from both sources, there were few significant differences in the types extracted. In the F-SCMK proteins, there were more group 1 but fewer group 3 and group 4 protein bands than in the H-SCMK proteins. The background of stain in the F-SCMK gel was usually denser than that in the H-SCMK gel, presumably owing to the presence in the former of numerous cytoplasmic proteins.

The polyacrylamide-gel patterns of the SCMK-A and SCMK-B proteins from both hair and hair-follicle tissue are also shown in Plate 1. Both the SCMK-A and SCMK-B proteins contained group 1 protein bands. The group 1 bands from SCMK-A proteins were termed the group 1A proteins and those bands from the SCMK-B proteins were termed group 1B proteins. Except for differences in the hair and hair-follicle group 1B proteins, the corresponding A and B fractions from both tissues were very similar. Thus, in both tissues, fractions A contained group 2 and group 3 proteins and the fractions B contained group 4 proteins. The bands which appear in the gels of the B fractions of Plate 1 in the group 2 region were possibly due to nucleic acid material (see below).

The amino acid compositions of the SCMK proteins extracted from both sources and their A and B fractions are given in Table 1. The compositions of the F-SCMK-A and H-SCMK-A proteins were similar but the analyses of the F-SCMK and H-SCMK proteins and their B fractions showed marked differences in their contents of the amino acids *S*-carboxymethylcysteine, serine, threonine, glutamic acid, proline and leucine. These differences were analogous to those observed between wool and wool-follicle proteins (Rogers, 1959; Downes *et al.*, 1966). Additional experiments further characterized these differences.

Fractionation of the different groups of proteins from hair and hair-follicle tissue

Chromatography on Sephadex G-200. The elution profiles of the F-SCMK and H-SCMK proteins on a calibrated Sephadex G-200 column are shown in Fig. 2. The profiles were similar but there was more material in peak 3 of the F-SCMK proteins, which was presumably due to the presence of nucleic acid

Table 1. *Amino acid composition of the unfractionated and pH4.4-fractionated hair and hair-follicle proteins*

For experimental details see the text. The values are expressed as residues/100 residues and represent only one analysis of each sample because of variability between different batches of proteins. Amounts indicated by 'Trace' were present in <0.05 residues/100 residues. During acid hydrolysis, citrulline undergoes partial decomposition to ornithine, therefore the citrulline value given is the sum of the citrulline remaining and the ornithine produced during acid hydrolysis.

Amino acid	Unfractionated SCMK protein		pH4.4-fractionated SCMK protein			
			SCMK-A		SCMK-B	
	Hair follicle	Hair	Hair follicle	Hair	Hair follicle	Hair
S-Carboxymethylcysteine	6.10	10.75	6.15	6.25	12.10	19.10
Aspartic acid	7.10	6.05	7.95	7.85	4.85	3.30
Threonine	4.80	6.95	5.15	5.05	5.35	8.85
Serine	8.25	11.30	8.20	8.15	8.30	11.05
Glutamic acid	17.65	12.55	18.65	18.35	18.25	13.45
Citrulline		0.65				1.55
Proline	3.95	7.15	3.35	3.25	8.05	11.85
Glycine	8.90	9.25	8.45	8.55	5.70	5.45
Alanine	5.65	4.90	6.25	6.45	4.10	3.35
Valine	4.85	4.85	4.90	5.15	4.65	4.45
Half-cystine			Trace		Trace	
Methionine	0.75	0.65	0.75	0.70	0.45	0.20
Isoleucine	2.95	3.15	3.45	3.65	2.65	2.60
Leucine	9.45	6.95	9.45	9.65	6.05	4.15
Tyrosine	3.75	3.85	3.65	3.80	1.60	1.40
Phenylalanine	2.45	2.30	2.40	2.30	1.90	1.25
Lysine	2.95	2.60	3.45	3.75	2.60	1.45
Histidine	0.75	0.65	0.75	0.75	0.80	0.80
Arginine	7.40	7.65	7.40	7.90	6.95	6.60

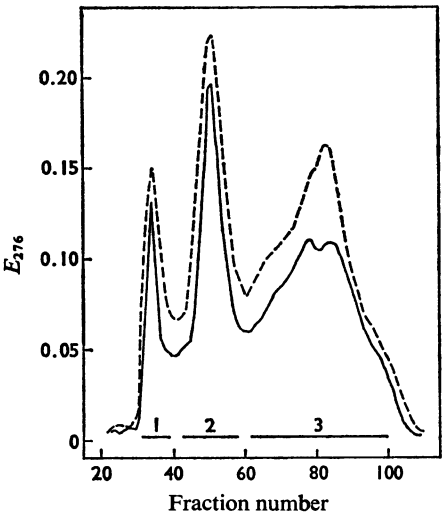


Fig. 2. *Chromatography of the SCMK protein extracts on Sephadex G-200*

A Sephadex G-200 column identical with that used in Fig. 1 was employed. In both cases about 40mg of protein was chromatographed. ——, H-SCMK; ----, F-SCMK. The bars 1–3 refer to the tubes that were pooled for characterization of their contents (see Plate 2).

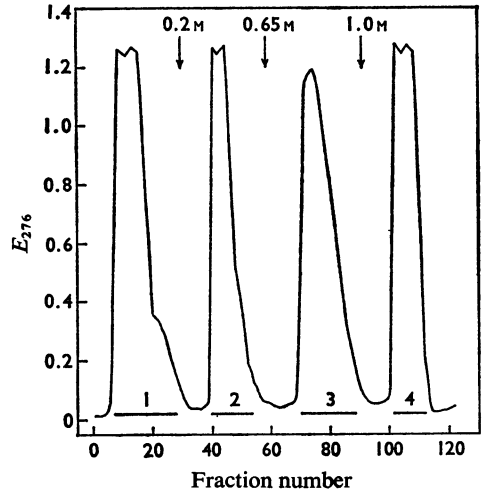


Fig. 3. *Chromatography of the F-SCMK proteins on DEAE-cellulose*

A column (50cm×2.2cm) of DEAE-cellulose was prepared and equilibrated with a starting buffer of 8M-urea, 50mM-KCl, 20mM-Tris–HCl (pH7.6) and 1mM-EDTA. The flow rate was 100ml/h and the fraction size was 20ml. Approximately 1g of F-SCMK proteins was applied. Buffers of the same composition but with the increased KCl concentrations of 0.2, 0.65 and 1M were applied at tubes 31, 62 and 93 respectively. The bars 1–4 refer to the tubes that were pooled for characterization of their contents (see Plate 3). An identical experiment was performed on a sample of H-SCMK proteins.

(see below). The polyacrylamide-gel patterns of peaks 1–3 are shown in Plate 2 and it is evident that although there were differences in the types of proteins of peak 1 from the two samples (compare gel F-1 with gel H-1), the proteins present in peaks 2 and 3 from hair were similar to those from the follicle. The dense stain at the top of the gel H-1 arose because the protein material migrated down the outside of the gel. Peak 1 contained mostly group 1 and some group 2 proteins. Peak 2 contained mostly group 2 proteins and peak 3 contained group 3 and group 4 proteins.

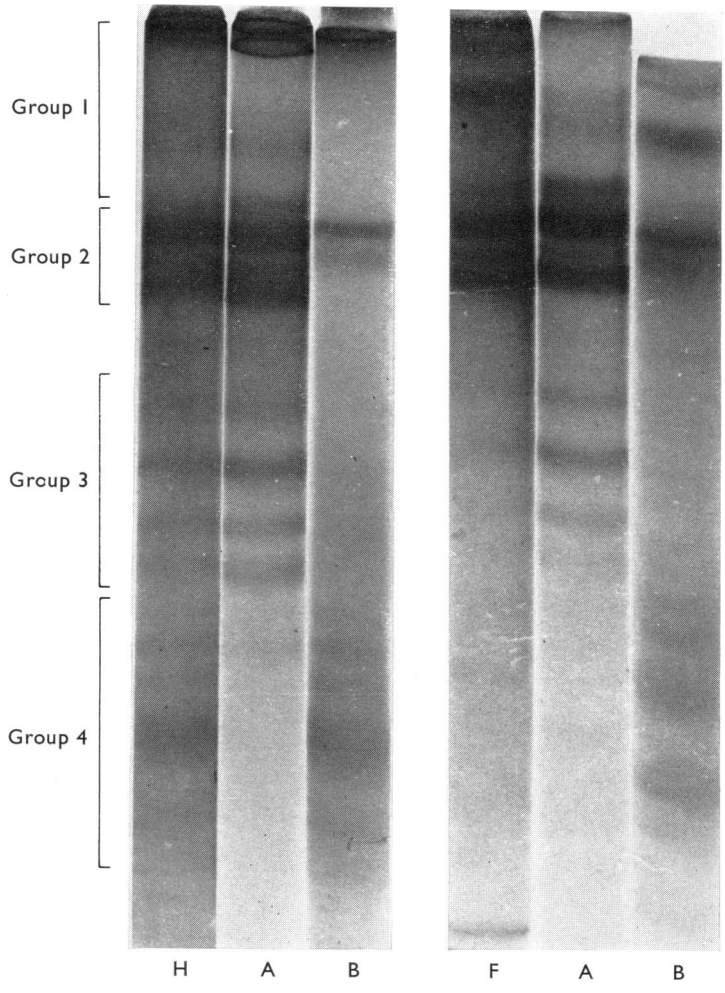
Chromatography on DEAE-cellulose. O'Donnell & Thompson (1964) showed that appreciable fractionation of the wool keratin proteins was possible by stepwise elution from DEAE-cellulose by using urea buffers and increasing KCl concentrations. The present studies showed that this procedure was also effective in resolving the components of the H-SCMK and F-SCMK proteins and the elution profile of such an experiment with the F-SCMK proteins only is shown in Fig. 3. The elution profiles of both were similar but there was markedly more peak 4 material in the F-SCMK proteins. This was most likely nucleic acid, since it absorbed maximally at 260nm. Polyacrylamide-gel patterns of the peaks 1–4 from the F-SCMK sample and from a similar experiment with an H-SCMK protein sample are shown in Plate 3. Peak 1 contained group 1, part of group 2 and group 3 proteins. Peaks 2 and 3 contained mostly the group 2 and group 4 proteins respectively. The only significant differences revealed by the DEAE-cellulose fractionation were in the group 1 and group 3 proteins of peak 1 (compare gel F-1 with gel H-1).

Complete fractionation of the protein extracts. The three procedures, namely acid precipitation at pH4.4, chromatography on Sephadex G-200 and DEAE-cellulose provided several convenient means of fractionation of the F-SCMK and H-SCMK proteins into the five groups. At least two different variations of these techniques for preparing each group were investigated, and the most frequent methods were as follows (and see below for further details): group 1A: protein fraction SCMK-A, Sephadex G-200 (peak 1); group 1B: protein fraction SCMK-B, Sephadex G-200 (peak 1); group 2: protein fraction SCMK-A, DEAE-cellulose (peak 2); group 3: protein fraction SCMK-A, Sephadex G-200 (peak 3); group 4: SCMK extract, DEAE-cellulose (peak 3).

Table 2. *Amino acid composition of the different groups of hair and hair-follicle proteins*

For experimental details see the text. The values are expressed as residues/100 residues and represent the averages of at least two different analyses on different batches of proteins. Amounts indicated by 'Trace' were present <0.05 residues/100 residues. Molecular weights were determined by chromatography on the calibrated Sephadex G-200 column (Fig. 1). The values of tryptic peptides of guinea pig are from Steinert *et al.* (1969).

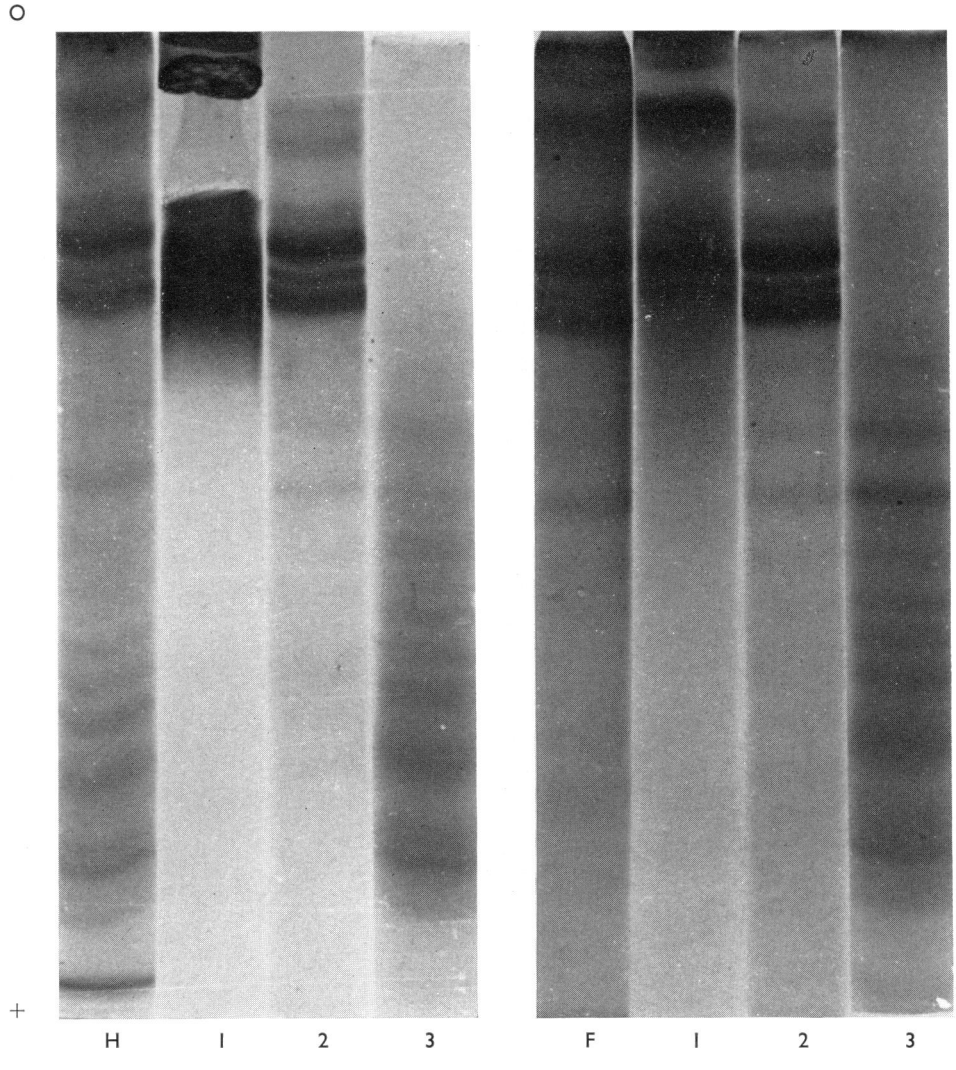
Column ...	1	2	3	4	5	6	7	8	9	10	11	12
Amino acid	Group 1A		Group 1B		Tryptic peptides of guinea pig		Group 2		Group 3		Group 4	
	Hair follicle	Hair	Hair follicle	Hair	Hair medulla	Inner root sheath	Hair follicle	Hair	Hair follicle	Hair	Hair follicle	Hair
S-Carboxymethylcysteine	5.70	5.55	2.45	1.95			6.25	6.15	12.85	12.30	21.65	23.40
Aspartic acid	8.55	8.40	7.35	6.95	5.95	7.25	8.15	7.95	3.70	3.65	2.30	2.35
Threonine	5.05	4.95	2.65	2.85	1.80	3.20	5.00	4.90	4.25	4.40	9.95	10.40
Serine	7.45	7.60	5.00	4.85	3.35	7.20	8.60	8.75	11.25	12.00	12.45	12.55
Glutamic acid	18.65	19.05	26.85	27.85	33.95	21.55	18.05	18.40	3.45	3.15	9.90	10.20
Citrulline			Trace	7.95	9.85	3.35						
Proline	3.10	3.05	2.70	2.20	2.15	3.50	3.40	3.45	6.00	5.85	13.45	13.05
Glycine	7.15	6.95	5.60	5.35	4.00	7.40	8.95	9.15	23.75	24.15	5.85	5.45
Alanine	7.05	6.70	4.85	4.65	4.80	6.25	6.45	6.15	1.95	1.75	3.00	2.95
Valine	5.35	5.50	4.55	4.15	4.25	4.90	5.00	4.95	3.00	2.85	4.90	4.45
Half-cystine	Trace	Trace	Trace	Trace								
Methionine	0.90	0.75	0.85	0.90	0.60	2.30	0.80	0.70	0.05	Trace		
Isoleucine	3.80	3.85	2.65	2.45	1.75	3.55	3.60	3.70	1.15	1.05	2.70	2.55
Leucine	9.50	9.30	9.75	9.95	9.20	9.70	9.15	8.85	5.75	5.85	3.10	2.80
Tyrosine	3.25	3.25	2.15	2.20	2.25	2.40	3.95	4.15	11.05	11.45	1.15	1.20
Phenylalanine	2.20	2.35	3.00	3.05	2.80	3.15	2.50	2.60	5.05	4.85	0.75	0.75
Lysine	3.70	3.85	4.70	4.90	8.75	9.00	3.35	3.50	0.35	0.35	0.50	0.55
Histidine	0.90	0.85	0.95	0.95	1.25	1.40	0.85	0.80	0.40	0.40	0.65	0.65
Arginine	7.75	7.60	11.95	5.65	3.35	3.75	7.10	7.90	4.50	4.75	6.30	6.80
Molecular weight:	⩾90000		⩾90000				45000		10000–14000		10000–30000	



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of SCMK protein extracts and SCMK-A and SCMK-B protein fractions

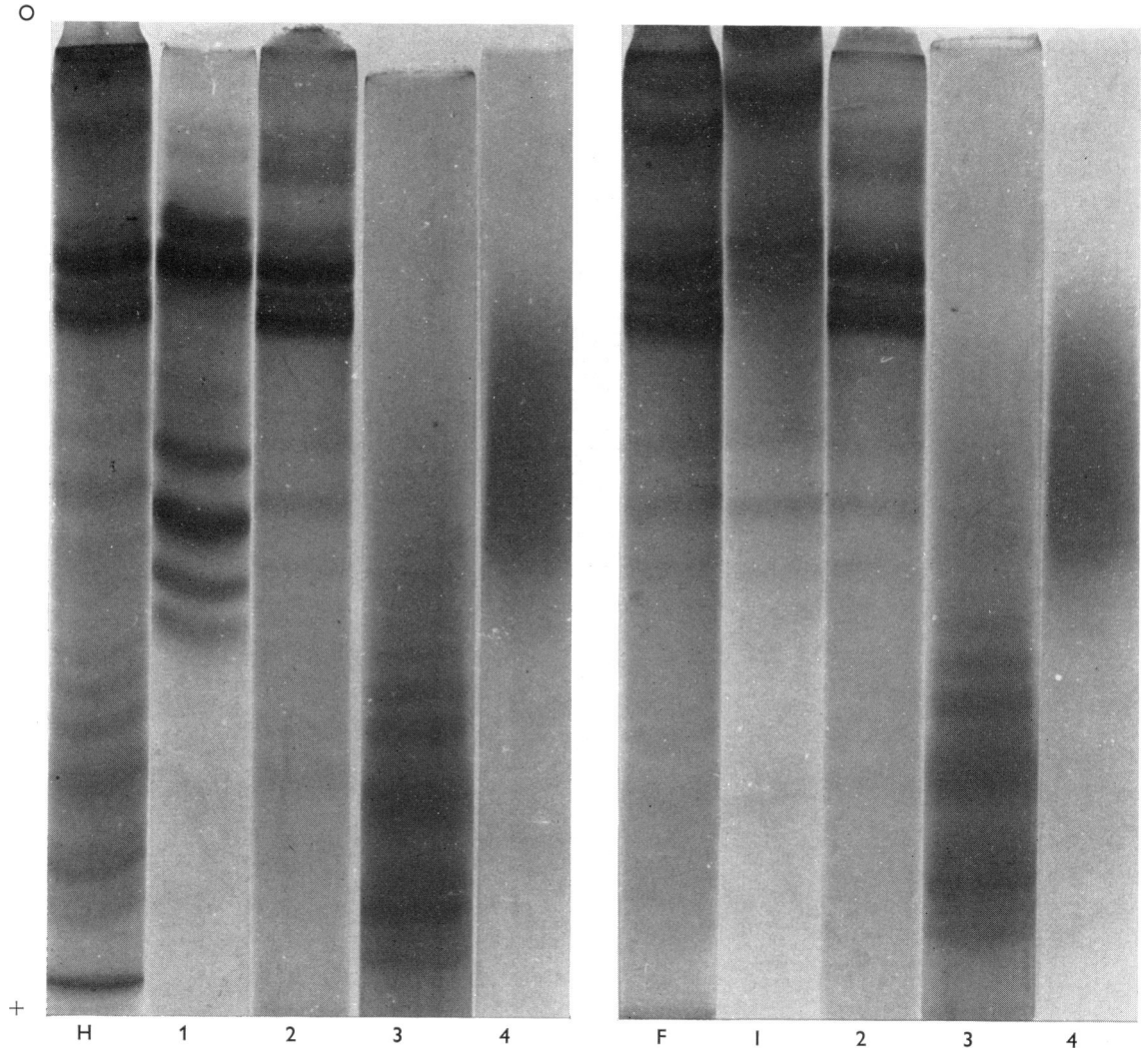
The gels were prepared, used and stained as described under 'Methods' and electrophoresis was done for 3h. Each gel was loaded with 80μg of sample. *O*, origin; +, anode end of gel. The band at the end of the gel F is the band of Bromophenol Blue used as a tracking dye. The bands were operationally divided into the groups as described in the text. H = H-SCMK; F = F-SCMK; A = SCMK-A; B = SCMK-B.



EXPLANATION OF PLATE 2

Polyacrylamide gels of fractions from Sephadex G-200 column of Fig. 2

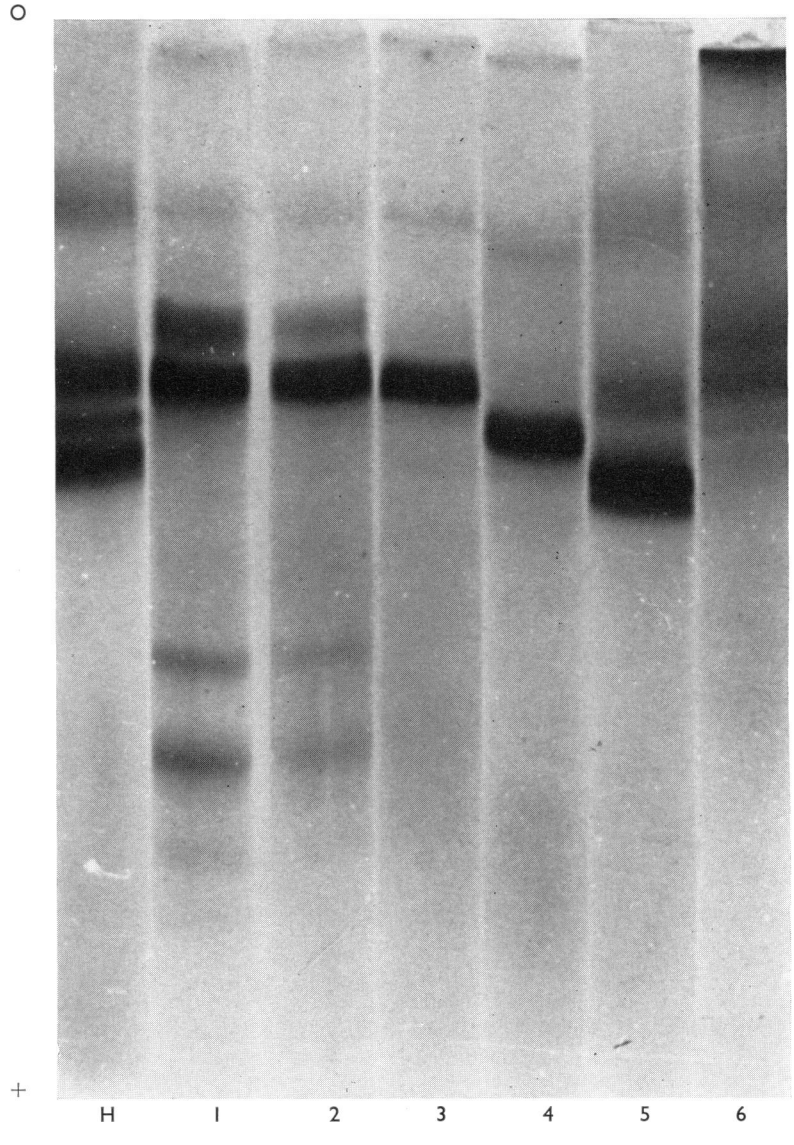
Samples from the pooled tubes of Fig. 2 were applied to the gels without prior desalting. The volume loaded was adjusted so that it contained about 50μg of protein and the gels were electrophoresed for 3h. H = H-SCMK; F = F-SCMK; 1–3 are from the pooled fractions of Fig. 2.



EXPLANATION OF PLATE 3

Polyacrylamide gels of fractions from the DEAE-cellulose column of Fig. 3

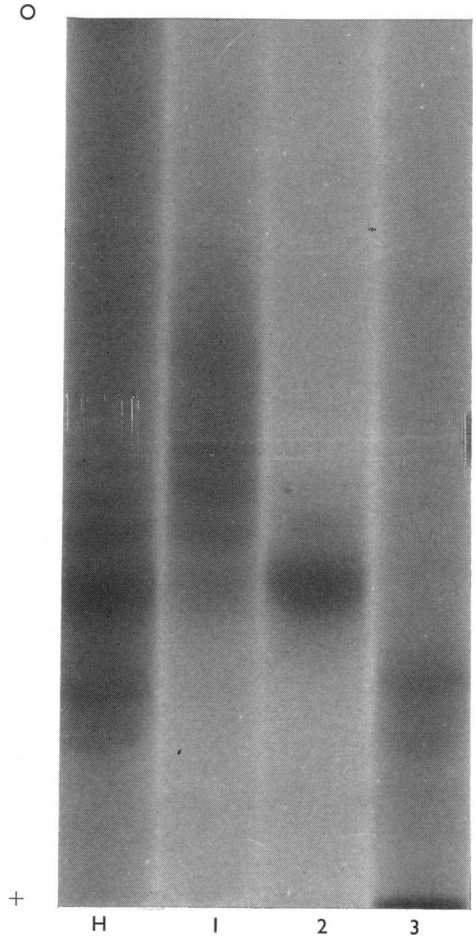
Samples from the pooled tubes of Fig. 3 were applied to the gels without prior desalting. The volume of sample loaded in each case was adjusted so that it contained about 80μg of protein. The samples in high salt electrophoresed more slowly than others and the electrophoresis was stopped when the tracking-dye band reached the end of the gel. Electrophoresis was for 3–4.5 h. H = H-SCMK; F = F-SCMK; 1–4 are from the pooled fractions of Fig. 3.



EXPLANATION OF PLATE 4

Polyacrylamide gels of fractions from the DEAE-cellulose column of Fig. 5

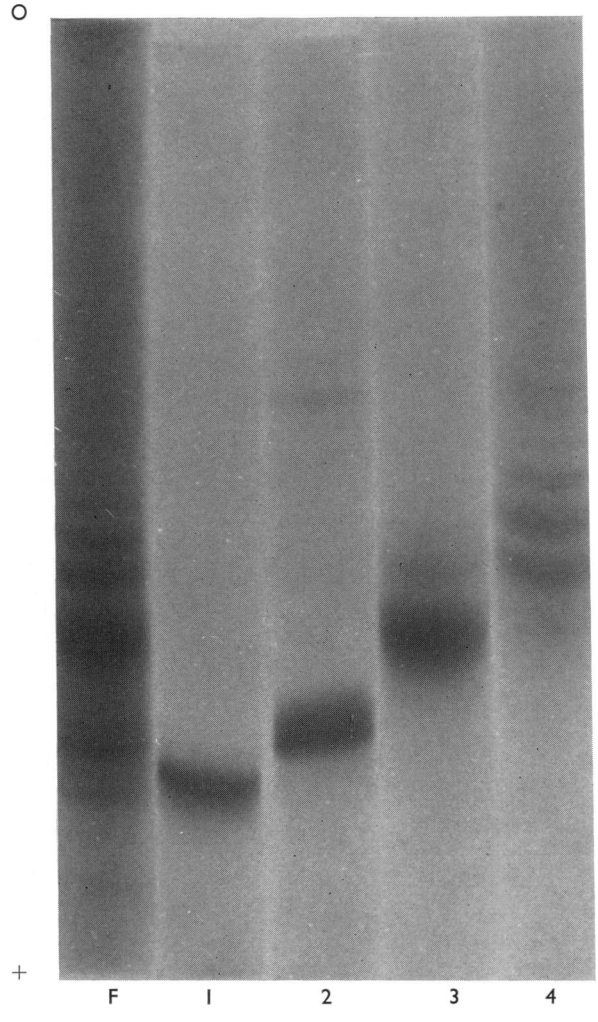
Samples from the pooled tubes of Fig. 5 were applied to the gels without prior desalting. The volume of sample was adjusted so that it contained about 50μg of protein. Electrophoresis was for 3–4h. H = H-SCMK; 1–6 are gels of the pooled fractions of Fig. 5.



EXPLANATION OF PLATE 5

Polyacrylamide gels of fractions from the Sephadex G-100 column of Fig. 7

Samples from the pooled fractions of the experiment with group 4 proteins of hair were evaporated to dryness *in vacuo* and a sample equivalent to about 50μg of protein was electrophoresed for 3h. H = group 4 proteins of hair; 1–3 are from the pooled fractions of Fig. 7. The band at the end of gel 3 is the tracker dye band. Gels of the corresponding fractions from follicle group 4 proteins were identical.



EXPLANATION OF PLATE 6

Polyacrylamide gels of fractions from the DEAE-cellulose column of Fig. 8

Samples from the pooled fractions of the experiment with group 4 proteins of the follicle were applied to the gels without prior desalting. The volume of sample was adjusted so that each gel was loaded with about 50μg of protein. Electrophoresis was stopped when the tracking dye reached the end of the gel. Electrophoresis was done for 3–3.5 h. F = group 4 proteins of the follicle, 1–4 are from the pooled fractions of Fig. 8. Gels of the corresponding fractions from the hair group 4 proteins were identical.

Table 3. *Yields of groups of proteins from the hair and hair-follicle tissues*

The groups of proteins were fractionated as described in the text. Protein samples were dialysed exhaustively against water and the weight of protein was determined after freeze-drying. The values are expressed as a percentage of the total weight of proteins recovered. The percentage yields given refer to one particular batch of proteins; the ranges are derived from the results of four (three) different batches.

Protein group	Percentage content from F-SCMK	Percentage content from H-SCMK
1A	7±2	2±1
1B	9±3	5±1
2	71±5	57±3
3	3±1	8±2
4	10±3	28±2

With the exception of the amounts of arginine and citrulline in the group 1B proteins (see below), the amino acid composition of the groups from both extracts were the same within experimental error (±5%) (Table 2). The amounts of the various groups of proteins present in both hair and hair-follicle preparations were determined during these experiments and are given in Table 3. Some variations in yields were apparent between different batches of tissue extracts, but these may have been due to the experimental conditions used. Nevertheless, the F-SCMK proteins, relative to the H-SCMK proteins, contained comparatively more group 1 and group 2 proteins but less group 3 and group 4 proteins.

Characterization of group 1 proteins

The different electrophoretic mobilities (Plate 1) and amino acid compositions (Table 2) support the conclusion that the group 1A proteins are different from the group 1B proteins. The amino acid analyses of the group 1A proteins from both sources were very similar (Table 2) and were similar to those of the group 2 proteins (Table 2; compare columns 1 and 2 with columns 7 and 8). Because the group 1A proteins also possessed a molecular weight of at least twice that of the group 2 proteins, it is suggested that the group 1A proteins were high-molecular-weight aggregates of the group 2 proteins.

In the group 1B proteins, the contents of arginine and citrulline were different. The presence of citrulline in the group 1B proteins from hair suggested that they might have arisen from contaminating medulla and inner-root-sheath proteins, as there were pronounced similarities in the amino acid contents of these proteins (Table 2; compare columns 3 and 4 with columns 5 and 6). The arginine content of the group 1B proteins from the follicle was approximately the same as the sum of the contents of arginine and citrulline in the same group of proteins from the hair. These observations might mean that the group 1B proteins from the follicle are precursors of the medulla and inner-root-sheath proteins, which are also synthesized in the hair follicle, and which presumably were solubilized by the urea extraction procedure. The group 1B proteins of hair may have arisen from contaminating small pieces of medulla tissue, which was broken up during grinding of the guinea-pig hair before extraction.

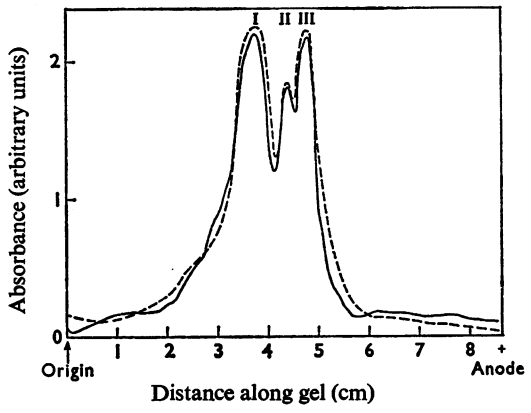


Fig. 4. *Quantitative estimations of the relative amounts of the group 2 components of both tissues by polyacrylamide-gel electrophoresis*

Approximately 50μg samples of the group 2 proteins prepared from both hair and hair follicle as in Fig. 3 were electrophoresed for 4h. The gels were stained and the densitometer traces were determined with a Uvicord apparatus. ——, Group 2 proteins of hair; ----, group 2 proteins of hair follicle. I, II and III are the different components of group 2 proteins of increasing mobility as described in the text.

Characterization of group 2 proteins

Quantitative estimations by polyacrylamide-gel electrophoresis. The group 2 proteins consisted of three main protein bands and these were termed components I, II and III, of increasing electrophoretic mobility as shown in Fig. 4. In Fig. 4 is a polyacrylamide-gel experiment designed to determine the relative amount of each component in the group 2 protein fractions from the follicle and hair. The densitometer traces of the two samples were almost identical, and this implies that each component was present in approximately the same amount in both extracts.

Fractionation and purification. The experiment

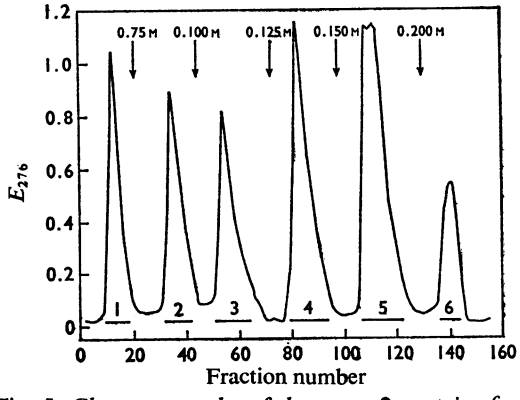


Fig. 5. *Chromatography of the group 2 proteins from hair on DEAE-cellulose*

The group 2 proteins employed were obtained from an H-SCMK-A fraction by the method described in Fig. 3. These group 2 proteins were then chromatographed on DEAE-cellulose with steps of increasing KCl concentrations of 0.075, 0.10, 0.125, 0.15 and 0.20M. A column (20cm×2.5cm) was loaded with about 0.5g of hair group 2 proteins in a starting buffer of 8M-urea, 20mM-Tris–HCl (pH7.6), 50mM-KCl and 1mM-EDTA. The flow rate was 80ml/h and fraction size 15ml. The bars 1–6 refer to the tubes that were pooled for characterization of their contents (see Plate 4).

shown in Fig. 3 demonstrated that the group 2 proteins could be separated largely from other protein groups by chromatography on DEAE-cellulose in the KCl concentration range 0.05–0.2M. Further studies on the group 2 proteins showed that the three components could be separated from each other simply by utilizing smaller steps of increasing KCl concentration within this range. The elution profile of the group 2 proteins of hair is shown in Fig. 5 and the polyacrylamide-gel-patterns corresponding to the peaks 1–6 are shown in Plate 4. Each component was completely separated from the others. Similar fractionation was obtained under identical conditions for the group 2 proteins of the follicle. The group 1 and group 3 protein impurities that were still present in the separated components could be removed by repeated re-chromatography on Sephadex G-200 until the components showed homogeneous molecular weights and gave constant amino acid compositions.

Properties of the purified group 2 proteins. When equal weights of the corresponding purified protein components of follicle and hair origin were examined by polyacrylamide-gel electrophoresis, they migrated together precisely, indicating close similarity. A noteworthy observation was that on extended electrophoresis, component III from both sources appeared as two distinct bands.

The amino acid analyses of the purified group 2

Table 4. *Amino acid composition of the purified group 2 protein components*

For experimental details see the text. The values are expressed as residues/100 residues and are the averages of analyses on three different batches of proteins. Amounts indicated by 'Trace' were present <0.05 residue/100 residues.The molecular-weight values were determined by chromatography on the calibrated Sephadex G-200 column (see Fig. 1).

Amino acid	Follicle components			Hair components		
	I	II	III	I	II	III
S-Carboxymethylcysteine	5.85	5.70	5.45	5.60	6.00	5.70
Aspartic acid	8.25	9.40	10.15	8.50	9.45	9.95
Threonine	4.85	5.85	4.85	4.65	5.65	4.90
Serine	7.00	7.80	7.90	6.90	8.05	8.00
Glutamic acid	19.10	21.90	22.75	18.65	22.05	22.75
Proline	3.00	3.25	3.65	2.90	3.40	3.55
Glycine	8.05	5.35	5.05	8.10	5.30	4.70
Alanine	8.05	5.85	6.10	8.35	6.00	5.80
Valine	5.80	4.80	4.50	5.60	5.00	4.75
Half-cystine	Trace	0.05	Trace	0.05	Trace	0.05
Methionine	1.15	0.25	0.25	1.10	0.25	0.25
Isoleucine	4.00	3.25	3.40	3.85	3.25	3.45
Leucine	9.15	10.45	11.55	9.05	10.15	11.50
Tyrosine	3.15	3.25	3.05	2.85	3.40	3.15
Phenylalanine	2.70	1.75	1.80	2.65	1.95	1.75
Lysine	4.60	2.35	2.90	4.45	2.15	2.75
Histidine	0.90	0.90	0.75	0.90	0.85	0.80
Arginine	7.90	8.15	7.75	7.85	8.55	7.85
Molecular weight	48000	43000	43000	48500	43500	43000

components are given in Table 4, which shows that values for the corresponding follicle and hair components were very similar.

Samples of the purified components were chromatographed on calibrated columns of Sephadex G-200. The molecular-weight values of the proteins obtained by this method are summarized in Table 4. These values can be subject to errors of ±10% (Whitaker, 1963), despite the use of rigorously controlled experimental conditions. Nevertheless, component I from both sources had a higher molecular weight (about 48000) than components II and III (about 43000 each).

Identification of the group 3 proteins

The amino acid analyses of the group 3 proteins prepared from both hair and hair-follicle extracts by the method stated above are given in Table 2, which shows that there were no significant differences. There are high contents of serine, glycine and tyrosine. These proteins may have arisen in part from cuticle or membrane components of the hair fibre (Crewther *et al.*, 1965) as well as from some cortical component (Darskus & Gillespie, 1971).

Characterization of group 4 proteins

Quantitative estimations by polyacrylamide-gel electrophoresis. The group 4 proteins consist of a complex number of components when separated by polyacrylamide-gel electrophoresis. Fig. 6 shows an experiment comparing the densitometer traces of the group 4 proteins from both hair follicle and hair after separation on polyacrylamide gels. Although the traces of both samples are similar, there were marked differences in the amounts of the bands: follicle group 4 proteins contained fewer slower-migrating but more faster-migrating bands than the hair group 4 proteins. The proportions of the amounts of these bands varied between different batches of proteins, but the same relationship was always evident.

Chromatography on Sephadex G-100. The elution profiles of the follicle and hair group 4 proteins from a calibrated column of Sephadex G-100 are shown in Fig. 7. The profiles were similar but quantitative

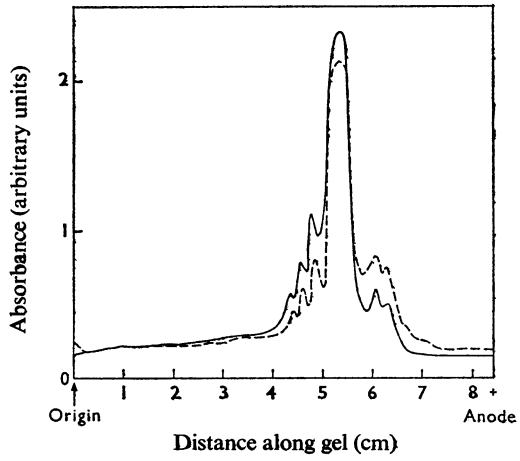


Fig. 6. *Quantitative estimations of the relative amounts of the group 4 proteins of both tissues by polyacrylamide-gel electrophoresis*

Approximately 50 μg samples of the group 4 proteins prepared from both hair and hair-follicle tissue as in Fig. 3 were electrophoresed for 3 h. The gels were stained and the densitometer traces were prepared. ——, Group 4 proteins of hair; ----, group 4 proteins of the follicle.

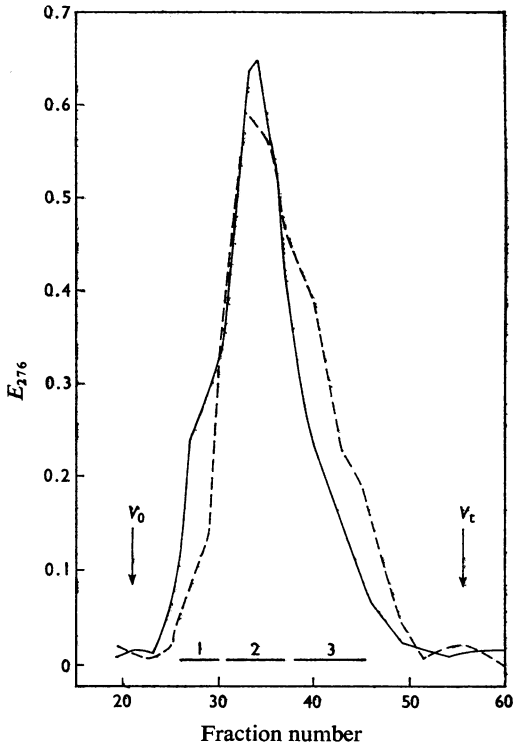


Fig. 7. *Chromatography of group 4 proteins on Sephadex G-100*

The Sephadex G-100 column used in this experiment was identical with that used in Fig. 1. In each case about 25 mg of group 4 proteins prepared as in Fig. 3 was chromatographed. ——, Group 4 proteins of hair; ----, group 4 proteins of the follicle. The bars 1–3 refer to the tubes that were pooled for characterization of their contents (see Plate 5).

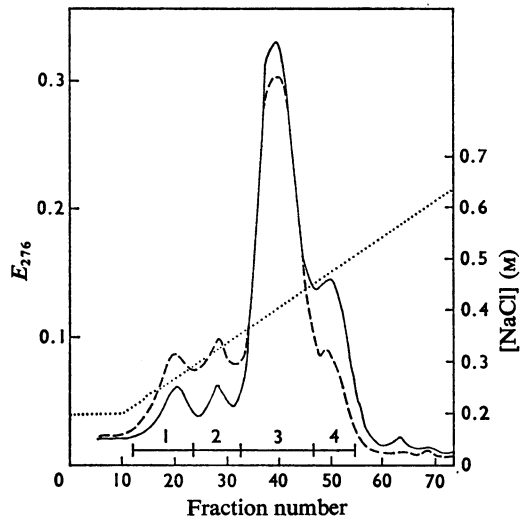


Fig. 8. *Chromatography of group 4 proteins on DEAE-cellulose*

A 15cm×1.6cm column of DEAE-cellulose was prepared and equilibrated in a starting buffer of 20mM-sodium acetate (pH4.5) and 0.2M-NaCl (Gillespie, 1963). The flow rate was 10ml/h and fraction size 3.0ml. Approximately 50mg of each group 4 protein fraction prepared as in Fig. 3 was loaded. At tube 10 a linear gradient of increasing NaCl concentration was applied (from 0.2 to 0.75M; two chambers, 100ml in each). ——, Group 4 proteins of hair; – – – –, group 4 proteins of the follicle; · · · ·, gradient of NaCl. The bars 1–4 refer to the tubes that were pooled for characterization of their contents (see Plate 6). The numbers 1–4 also refer to the subgroups of group 4 proteins as described in the text.

differences were apparent: follicle group 4 proteins contained fewer high-molecular-weight proteins but more low-molecular-weight proteins than the hair proteins. The polyacrylamide-gel patterns of the peaks 1–3 of both samples were similar. The patterns for the hair proteins are shown in Plate 5 and indicate that the high-molecular-weight proteins migrated least rapidly.

Chromatography on DEAE-cellulose. The experiments of Gillespie (1963) showed that wool high-sulphur proteins (proteins soluble at pH4.4) could be fractionated most satisfactorily on DEAE-cellulose at pH4.5. When guinea pig group 4 proteins were chromatographed under similar conditions the elution profiles shown in Fig. 8 were obtained. Again, the profiles were similar, but quantitative differences were observed. The gel patterns of the peaks 1–4 are given in Plate 6 and in conjunction with Fig. 8 it is seen that the group 4 proteins of greatest mobility were more abundant in the follicle whereas the proteins of lowest mobility were less abundant.

Table 5. *Yields of subgroups of proteins from hair and hair-follicle group 4 proteins*

The subgroups of proteins were fractionated on DEAE-cellulose as shown in Fig. 8. The pooled fractions were prepared for quantitation and are expressed in the same way as in Table 3.

Subgroup of group 4	Percentage content of group 4 proteins of	
	Follicle	Hair
1	13±3	5±2
2	18±4	8±1
3	61±5	69±6
4	8±1	18±3

Since the four peaks of Fig. 8 contained different proteins, the peaks 1–4 were classified as subgroups of group 4 proteins. The relative amounts of the subgroups were determined (Table 5). Although variations were evident between different batches of proteins, there were always two or three times as much subgroup 1 proteins in the follicle group 4 proteins but only 0.5 to 0.3 of the subgroup 4 proteins in comparison with the group 4 proteins from hair. The amino acid compositions of the subgroups are given in Table 6 and the molecular weights, which were determined by chromatography on Sephadex G-100, are also given. The values from follicle and hair protein subgroups were the same within the limits of experimental error.

Discussion

In the present paper methods are described for the fractionation and characterization of the different groups of proteins that can be extracted from the hair and hair-follicle tissue of the guinea pig. Five different groups of proteins that were chemically distinct are described from both hair and hair follicle. From the results presented, it is concluded that the types and properties of all proteins from both tissues are identical, with the single exception of the group 1B proteins. There were, however, two significant differences in the amounts of the protein groups. The group 1 and group 2 species were more abundant in the F-SCMK proteins than in the H-SCMK proteins and the group 3 and group 4 proteins were markedly less abundant. Secondly, there were quantitative variations within the group 4 proteins themselves: there were more proteins of lowest molecular weight and *S*-carboxymethylcysteine content in the group 4 proteins from the follicle than in the group 4 proteins

Table 6. *Amino acid composition of the group 4 protein subgroups from extracts of hair and hair follicle*

For experimental details see the text. The values are expressed as residues/100 residues and are the averages of analyses on at least two different batches of proteins. Molecular weights were determined by chromatography on the calibrated Sephadex G-100 column (see Fig. 1). The values for group 4 are from Table 2.

Amino acid	Group 4		Subgroup 1		Subgroup 2		Subgroup 3		Subgroup 4	
	Hair follicle	Hair	Hair follicle	Hair	Hair follicle	Hair	Hair follicle	Hair	Hair follicle	Hair
S-Carboxymethylcysteine	21.65	23.40	18.90	19.10	20.90	20.80	24.60	23.85	26.80	27.45
Aspartic acid	2.30	2.35	4.05	3.90	3.20	3.20	2.25	2.10	1.10	1.20
Threonine	9.95	10.40	9.55	9.90	10.35	10.25	10.75	10.50	9.55	8.95
Serine	12.45	12.55	11.20	11.00	11.95	11.90	11.75	12.00	14.55	14.25
Glutamic acid	9.90	10.20	9.20	9.50	10.35	10.30	10.35	10.25	10.25	10.70
Proline	13.45	13.05	11.00	11.55	12.65	12.45	13.05	13.05	14.20	13.85
Glycine	5.85	5.45	8.05	7.70	7.10	6.75	5.45	5.55	3.95	3.70
Alanine	3.00	2.95	3.85	3.70	3.50	3.25	2.65	2.95	1.95	1.90
Valine	4.90	4.45	6.95	6.60	5.75	5.40	4.55	4.50	3.00	3.35
Methionine			0.05	0.10						
Isoleucine	2.70	2.55	3.05	2.95	2.60	2.65	2.30	2.60	2.35	2.40
Leucine	3.10	2.80	3.55	3.45	2.95	2.95	2.65	2.85	1.75	1.95
Tyrosine	1.15	1.20	1.20	1.15	1.45	1.30	1.10	1.20	0.90	0.85
Phenylalanine	0.75	0.75	1.20	1.35	0.95	1.00	0.70	0.75	0.55	0.65
Lysine	0.50	0.55	0.70	0.75	0.60	0.60	0.45	0.45	0.40	0.35
Histidine	0.65	0.65	1.20	0.95	0.70	0.80	0.55	0.55	0.40	0.45
Arginine	6.30	6.80	4.85	5.05	6.20	6.15	6.40	6.35	7.30	7.35
Molecular weight			10000		14000		22000		28000	

from hair and less proteins of highest molecular weight and *S*-carboxymethylcysteine content.

There were marked differences in the amino acid compositions of the SCMK and SCMK-B proteins from hair and hair follicle (Table 1) which were similar to those observed by Rogers (1959) and Downes *et al.* (1966) in wool. Thus the present results show that these differences can be readily accounted for, because the SCMK and SCMK-B proteins contained different amounts of the various groups of proteins with markedly different amino acid compositions. This consideration had not been appreciated in the previous studies with wool.

In studies on wool keratin proteins (e.g. Thompson & O'Donnell, 1964), the SCMK-A fraction (proteins insoluble at pH4.4) was termed the 'low-sulphur keratin' (LoS) protein fraction and the SCMK-B fraction (proteins soluble at pH4.4) was termed the 'high-sulphur keratin' (HiS) protein fraction. Both the A and B fractions in the present study contained several different protein species of different properties. Since the group 2 proteins were the most abundant group in the SCMK-A fraction and the group 4 proteins were the most prominent in the SCMK-B fraction, it would seem appropriate to term the guinea-pig LoS and HiS proteins the group 2 and group 4 proteins respectively.

From the present findings, certain predictions can reasonably be made on the mechanism of synthesis of the keratin proteins. The first concerns the fact that the corresponding components of the group 2 and group 4 proteins from both hair and hair follicle are chemically indistinguishable. To account for the differences in the content of *S*-carboxymethylcysteine and that of the other amino acids of the wool follicle and wool proteins, and the heterogeneity of the wool HiS proteins (proteins of the SCMK-B fraction), Rogers (1959) and Gillespie (1965) suggested that some of them might be synthesized by addition of cysteine to pre-existing proteins during terminal stages of development in the wool follicle. From the results of the present work, it is difficult to conceive how such a mechanism could account for the identity of the follicle and hair proteins. The only mechanism for their synthesis that can reasonably be considered is one involving the ordered addition of amino acids by ribosomes and mRNA. Other experimental evidence supporting this conclusion has been obtained for both groups of proteins in the guinea-pig and wool follicle tissues: cell-free protein-synthesis systems containing polyribosomes from hair or wool follicles have been shown to direct the synthesis of completed proteins of both groups (Steinert & Rogers, 1971, 1973; Wilkinson, 1971). Accordingly, the quantitative differences in the amounts of the subgroups of group 4 proteins and between the group 2 and group 4 proteins from hair and hair follicle must reflect differences in their rates of synthesis during terminal stages of development. Thus the relative rates of synthesis of the group 4 proteins must increase in comparison with the group 2 proteins. Further, it is evident that the proteins with the highest *S*-carboxymethylcysteine content in the group 4 proteins must be synthesized at a rate greater than the other species in the group during terminal stages of development in the follicle.

One definitive approach to these problems was adopted by Fraser (1969), in which the properties of the proteins isolated from various levels of the developing wool follicle were compared with those extracted from fully keratinized wool. The method of Fraser (1969) used for the separation of the wool follicle cells could be criticized as it involved use of proteolytic digestion. It is possible that significant damage to the cellular contents could have occurred, especially at the highest levels of the follicle, since the cells here were exposed to the highest concentrations of enzyme for the longest times. Notwithstanding this criticism, Fraser (1969) showed that the wool LoS proteins (the SCMK-A fraction in his study) were synthesized at an approximately constant rate throughout the follicle. The rate of HiS protein (SCMK-B fraction in his study) synthesis was not constant but increased 'exponentially' in the higher levels of the follicle, such that about 50% of the HiS proteins were deposited at the terminal stages of development. These observations would therefore appear to explain the differences in the temporal rates of synthesis of the proteins mentioned above if they are applicable to the guinea-pig hair-follicle system.

Fraser (1969) reported the presence in wool-follicle HiS protein fractions (SCMK-B fraction) of a major group of proteins of low *S*-carboxymethylcysteine content which he termed 'low-sulphur non-microfibrillar proteins'. Together with the studies on [^{35}S]-cystine incorporation *in vivo*, he postulated that these were 'precursor proteins requiring hydrolysis before ^{35}S is incorporated into structural fibrous protein or are subunits which in turn polymerize to form microfibrillar protein'. No protein species that could fulfil the role of this 'subunit' protein was found in any protein fraction in the present work. Therefore, in the absence of more detailed information on the molecular weight and chemical structure of this protein of Fraser (1969), it is not possible to speculate on this difference between the wool and guinea-pig hair-follicle systems.

The only significant difference between the proteins extracted from hair and hair-follicle tissue was in the group 1B proteins. The amino acid analyses of the group 1B proteins of hair were very similar to those of the medulla and inner-root-sheath proteins, and it was suggested that the group 1B proteins of the follicle might be precursors of them. The other major group of proteins present in hair-follicle cells is those of the trichohyalin droplets of both the medulla and

inner-root-sheath layers, and it is possible that the group 1B proteins from the follicle originated from the trichohyalin droplets. If this assertion is true, then it implies that the trichohyalin droplets may serve as the precursor protein of the mature proteins of the medulla and inner root sheath. However, this theory must await characterization of isolated trichohyalin droplets before validation. Nevertheless, from the amino acid composition, in which citrulline and arginine are prominent, there exists some support for the hypothesis of Rogers (1959, 1963, 1964) that desimidation takes place for the formation of citrulline.

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References

Chrambach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967) *Anal. Biochem.* **20**, 150–154
Clarke, R. M. & Rogers, G. E. (1970) *J. Invest. Dermatol.* **55**, 419–424
Cole, R. D., Stein, W. H. & Moore, S. (1958) *J. Biol. Chem.* **233**, 1359–1363
Crewther, W. G. & Harrap, B. S. (1967) *J. Biol. Chem.* **242**, 4310–4319
Crewther, W. G., Fraser, R. D. B., Lennox, F. G. & Lindley, H. (1965) *Advan. Protein Chem.* **20**, 191–346
Darskus, R. L. & Gillespie, J. M. (1971) *Aust. J. Biol. Sci.* **24**, 1189–1197
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Downes, A. M., Ferguson, K. A., Gillespie, J. M. & Harrap, B. S. (1966) *Aust. J. Biol. Sci.* **19**, 319–333
Fraser, I. E. B. (1969) *Aust. J. Biol. Sci.* **22**, 231–238
Fraser, R. D. B., MacRae, T. P. & Rogers, G. E. (1972) *Keratins, Their Composition, Structure and Biosynthesis*, Charles C. Thomas, Springfield, Ill.
Gillespie, J. M. (1963) *Aust. J. Biol. Sci.* **16**, 259–280
Gillespie, J. M. (1965) in *The Biology of Skin and Hair Growth* (Lyne, A. G. & Short, B. F., Eds.), pp. 377–398, Angus and Robertson, Sydney
Kawata, H. & Chase, M. W. (1968) *J. Chromatogr.* **35**, 565–566
O'Donnell, I. J. & Thompson, E. O. P. (1964) *Aust. J. Biol. Sci.* **17**, 973–989
Rogers, G. E. (1959) *Ann. N.Y. Acad. Sci.* **83**, 408–428
Rogers, G. E. (1963) *J. Histochem. Cytochem.* **11**, 700–705
Rogers, G. E. (1964) in *The Epidermis* (Montagna, W. & Lobitz, W., eds.), pp. 179–236, Academic Press, New York
Sanger, F. & Thompson, E. O. P. (1963) *Biochim. Biophys. Acta* **71**, 468–471
Steinert, P. M. & Rogers, G. E. (1971) *Biochim. Biophys. Acta* **238**, 150–155
Steinert, P. M. & Rogers, G. E. (1973) *Biochim. Biophys. Acta* **312**, 403–412
Steinert, P. M., Harding, H. W. J. & Rogers, G. E. (1969) *Biochim. Biophys. Acta* **175**, 1–9
Thompson, E. O. P. & O'Donnell, I. J. (1964) *Aust. J. Biol. Sci.* **17**, 277–281
Thompson, E. O. P. & O'Donnell, I. J. (1965) *Aust. J. Biol. Sci.* **18**, 1207–1225
Whitaker, J. R. (1963) *Anal. Chem.* **35**, 1950–1953
Wilkinson, B. R. (1971) *Biochem. J.* **125**, 371–373