STUDIES ON REDUCED WOOL

IV. THE ISOLATION OF A MAJOR COMPONENT

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Summary

Starch-gel electrophoresis in buffers containing 8M urea has been used to follow the fractionation of wool proteins extracted from reduced and carboxymethylated wool. Fractionation on both DEAE-cellulose and Sephadex G-200 in the presence of buffers containing 8M urea is possible but in neither case is a single component obtained. However, a combination of these two methods has enabled one of the major components to be isolated. The amino acid composition of this material is reported.

I. Introduction

In earlier work on the chromatography of extracted wool protein fractions on DEAE-cellulose in buffers containing 8M urea (O'Donnell and Thompson 1961; Gillespie 1962*b*; Thompson and O'Donnell 1962, 1964), it was demonstrated that the fraction which could be precipitated by acid at about pH 4 (low-sulphur proteins) was heterogeneous. However, due to a lack of a revealing diagnostic technique the chromatographic heterogeneity could not be interpreted in terms of the number of discrete components present. Starch-gel electrophoresis in buffers containing 8M urea (Thompson and O'Donnell 1964) revealed the presence of a large number of components in the extracts of reduced and carboxymethylated wool (*S*-carboxymethyl kerateines) and with this technique it was possible to follow the chromatographic fractionation. From these and earlier studies on protein extracts from oxidized wool (O'Donnell and Thompson 1962) it became evident that fractionation at about pH 4 of total extracts of wool proteins was not completely satisfactory since the acid-precipitable fraction was always contaminated with minor components (cf. Gillespie 1960). This was thought to be due to protein–protein interactions and in the present fractionation studies acid precipitation has been avoided where possible. In addition, all operations have been carried out in buffers containing 8M urea to minimize these interactions. The effect of changes in the conditions of chromatography and the behaviour of protein fractions isolated from reduced and carboxymethylated wool by different methods have now been studied by starch-gel electrophoresis and are reported in this paper. Both chromatography on DEAE-cellulose and gel filtration on Sephadex G-200 under these conditions gave a useful separation and by a combination of both techniques one of the major components of reduced and carboxymethylated wool has been isolated. It is a low-sulphur protein or protein fraction.

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II. Experimental

The acid-fractionated (pH 4·4, 0·5M KCl) low-sulphur (SCMKA) and high sulphur (SCMKB) wool protein preparations used (see Gillespie 1960 for nomenclature) were those used in the previous paper (Thompson and O'Donnell 1964). The purified acid-fractionated preparations SCMKA2 Z34 and SCMKA1 were kindly supplied by Dr. J. M. Gillespie.

Total extracts of reduced and carboxymethylated wool were prepared from a single Merino 64's fleece MW138 in the following way (cf. Crestfield, Moore, and Stein 1963; Harrap and Gillespie 1963): the wool was detipped, degreased in the cold with petroleum ether, and isoelectrically washed by the method of Zahn and Blankenburg (1962). Air-dried wool (1·7 g) was wetted with 95 ml 8M urea–0·01M Tris–0·001M Versene (disodium salt of ethylenediaminetetraacetic acid) buffer, pH 7·4, by evacuation at the water-pump and the release of vacuum under nitrogen. Redistilled mercaptoethanol (1 ml) and 5N potassium hydroxide (2 ml) were added to give a pH of 10·5. The reduction was allowed to proceed under nitrogen at room temperature with occasional shaking for 3 hr. Carboxymethylation of the thiol groups was effected by the addition of 2·68 g iodoacetic acid (i.e. the equivalent of 1 ml mercaptoethanol) dissolved in 3M Tris buffer (15 ml), pH 8·5, and 5N potassium hydroxide (0·8 ml). This gave a final pH of 8·5. The extract was filtered after 15 min carboxymethylation and dialysed in 18/32 Visking cellulose tubing against successive changes of chromatography buffer (8M urea–0·01M Tris–0·001M Versene, pH 7·4) until free of surplus salts. Under these conditions approximately 80% of the wool dissolved. Other keratins were extracted in a similar manner except that the reduction was allowed to proceed overnight. The percentages extracted in these cases were not determined.

The urea-soluble proteins of wool roots were freshly prepared from a recently slaughtered sheep by the wax-sheet method (cf. Rogers 1959). The harvested wool roots were extracted with chromatography buffer adjusted to pH 9 and, after centrifugation, the supernatant was reduced with mercaptoethanol and carboxymethylated and dialysed as described above.

The chromatography of the soluble wool proteins on DEAE-cellulose columns in 8M urea buffer was by stepwise elution as described previously. For a column 0·9 cm in diameter and 15 cm in length elution was carried out with 15 ml in each step. The concentrations of potassium chloride in the chromatography buffer were normally 0·025M, 0·05M, 0·1M, 0·15M, 0·2M, and 1M. The temperature was kept constant usually at 25°C and the load could be varied between 1 and 10 ml of a 1% solution with fairly reproducible behaviour, as judged by starch-gel patterns of the fractions. However, fraction B, which was eluted by very low salt concentrations (0·025M KCl), sometimes appeared partially in fraction A. The eluant fractions (1·25 ml) were diluted with 3 ml water before measuring their optical density at 276 mμ. The bulked fractions were prepared for starch-gel electrophoresis as previously described (Thompson and O'Donnell 1964). The DEAE-columns were operated under 2–5 lb/sq in. nitrogen pressure and could be run at approximately 30 ml/hr. To minimize any formation of cyanate from urea (Stark, Stein, and Moore 1960; Stark and Smyth 1963) freshly prepared urea solution containing Versene was acidified to decompose

cyanate before the addition of Tris; also buffer and eluted fractions were kept at 4°C as much as possible. So far there has been no evidence of side reactions due to any traces of cyanate formed.

Horizontal starch-gel electrophoresis (Smithies 1955) was carried out in 8M urea solutions containing 0·01M Versene as previously described (Thompson and O'Donnell 1964).

Sephadex G-200 was sieved to remove fines and the material retained on a 200-mesh screen was equilibrated with 0·5M KCl in the chromatography buffer for 24 hr. After packing, the column (2·3 cm diameter by 125 cm) was washed with 500 ml chromatography buffer at pH 7·4. Amounts of protein up to 20 ml of 1% solutions were successfully fractionated on this column. The fraction collector was operated on a time basis to give fractions of approximately 5–8 ml. The flow rate of the column was approximately 12 ml/hr. Protein concentrations of the fractions and subsequent examination of bulked fractions by starch-gel electrophoresis were carried out as described above.

Amino acid analyses were performed with a Spinco amino acid analyser which was operated under the conditions as described by Spackman, Stein, and Moore (1958). Some hydrolysates were prepared by refluxing for 24 hr with 6N HCl but as there was variable decomposition of the *S*-carboxymethylcysteine in the various fractions from DEAE-columns, these fractions were hydrolysed *in vacuo* as described by Crestfield, Moore, and Stein (1963). Under these conditions there was no destruction of *S*-carboxymethylcysteine.

III. Results

(*a*) *Starch-gel Electrophoresis Patterns of Extracted Wool Proteins*

Plate 1, Figure 1, shows the comparative starch-gel patterns of a total wool extract (SCMK) and the insoluble (SCMKA) and supernatant (SCMKB) fractions prepared by precipitation at pH 4·4 in the presence of 0·5M KCl (Gillespie, O'Donnell, and Thompson 1962). The bands or areas have been arbitrarily numbered and named as shown. Our components are hence defined operationally with reference to a starch-gel pattern under these conditions. No attempt has been made to give individual numbers to bands in the intermediate and fast regions since the minor components obvious in the starch-gel patterns of concentrated fractions from DEAE-cellulose columns (see below) cannot all be seen in the starch-gel pattern of SCMK (Plate 1, Fig. 1). Moreover, the fast-moving components can be resolved further. The staining of minor components is more intense than was the case in the previous paper (Thompson and O'Donnell 1964) where fresh dye solution was not always used. The bulk of the SCMKB proteins run near the fast-moving brown buffer boundary. These faster-moving SCMKB proteins seem to be free of the major SCMKA proteins but the reverse is not so and all preparations of SCMKA prepared in this way are contaminated with intermediate and fast-moving components.

The reduced and carboxymethylated extracts and the products of their acid-fractionation, obtained from wool by widely differing conditions of reduction and extraction, have been examined. For example, the method of reduction of

Maclaren (1962), in which benzyl mercaptan in near-neutral solutions is used, yields protein fractions giving patterns indistinguishable from those of SCMKA and SCMKB shown in Plate 1, Figure 1, which were prepared by reduction at pH 5 with mercaptoethanol and alkylation at pH 9 or pH 11. The SCMK of Plate 1, Figure 1, was the total unfractionated extract prepared in 8M urea as described in Section II.

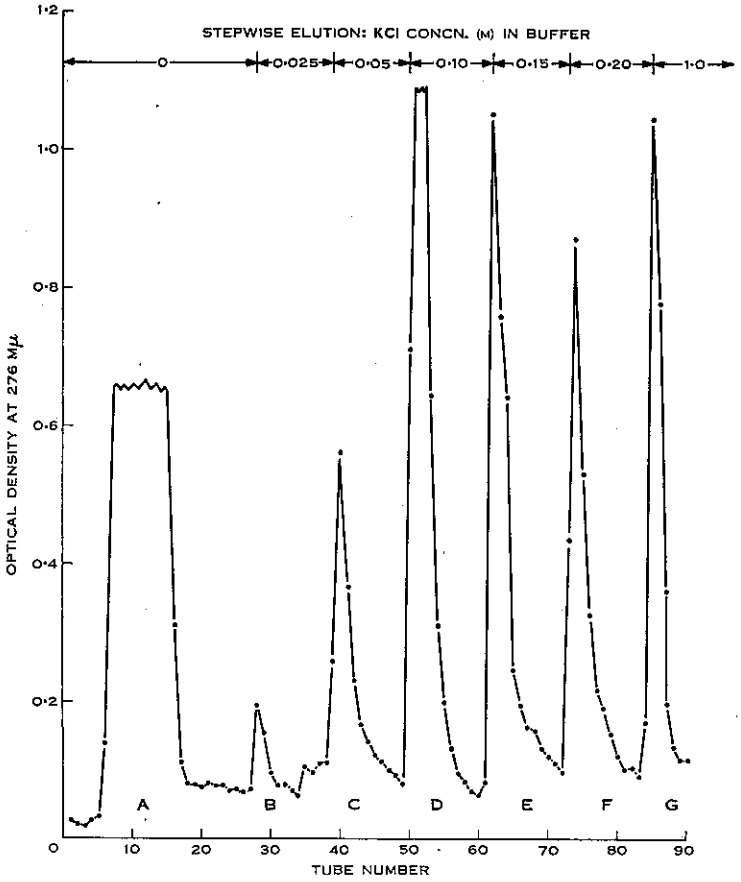

Fig. 1.—Chromatographic fractionation on DEAE-cellulose at 25°C in 8M urea–Tris buffer, pH 7·4, of a total extract (SCMK) from reduced and carboxymethylated wool (100 mg). Fraction size was approximately 1·25 ml. Column size 0·9 cm diameter by 15 cm.

The quantitative aspects of the starch-gel patterns as revealed by the dye-staining technique suffer from some inherent uncertainties since the uptake of dye (nigrosine in this case) is a function of the number and type of basic groups in the various components. However, the chromatographic data (ultraviolet absorption and weights of proteins in fractions A–G) confirm that the majority of the low-sulphur material is contained in the two major bands obvious in the starch-gel pattern of the SCMK or SCMKA fractions. There may be proteins which do not stain well with

the nigrosine due to their low net positive charge under the conditions of staining. The effect of reduction in net positive charge (by the removal of lysine side-chain amino groups) on the uptake of nigrosine dye is illustrated in Plate 2, Figure 3, where a succinylated SCMKA preparation only stained faintly with nigrosine after starch-gel electrophoresis.

(b) Starch-gel Electrophoresis Patterns of Proteins Extracted from Wool Roots

In Plate 1, Figure 2, it is seen that the patterns obtained with protein from freshly prepared wool roots have, as a major feature, bands in the same position as the two predominant ones in the SCMKA fraction (Plate 1, Fig. 1). The slow-moving bands are virtually absent and the faster-moving components seem relatively less intense in these preparations.

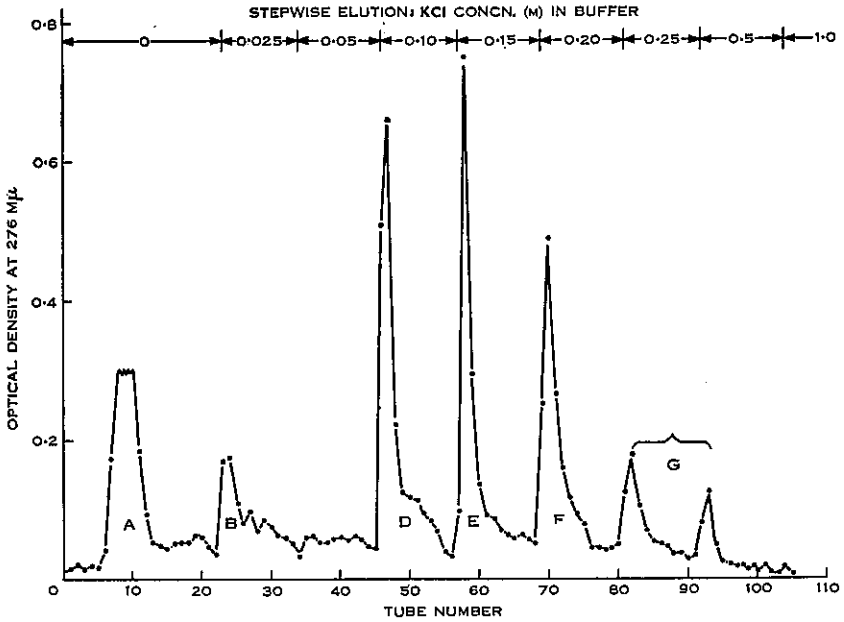

Fig. 2.—Chromatographic fractionation at 25°C on DEAE-cellulose of a total extract (SCMK) from reduced and carboxymethylated wool in 8M urea–Tris buffer at pH 8·6. Fraction size and column dimensions as in Figure 1.

(c) Chromatographic Fractionation of Total Extracts from Reduced and Carboxymethylated Wool

In the previous paper (Thompson and O'Donnell 1964) the chromatographic fractionation and starch-gel patterns of fractions of SCMKA were reported. Figure 1 shows a similar fractionation of a total extract (SCMK) and the corresponding starch-gel patterns of these fractions are shown in Plate 1, Figure 3. The width of the peak corresponding to the unadsorbed fraction is due to the large volume of protein solution (10 ml) applied to the column, and the optical densities of this fraction are

meaningless due to the precipitation of protein in some tubes on dilution with water. The tyrosine and phenylalanine contents of this fraction are also very high, resulting in large extinction coefficients.

In comparison with the elution curve of SCMKA it will be seen that the high-sulphur proteins present in SCMK result in larger peaks (F and G) eluted with 0·2M and 1M KCl in the buffer. The starch-gel patterns showing this increase may be

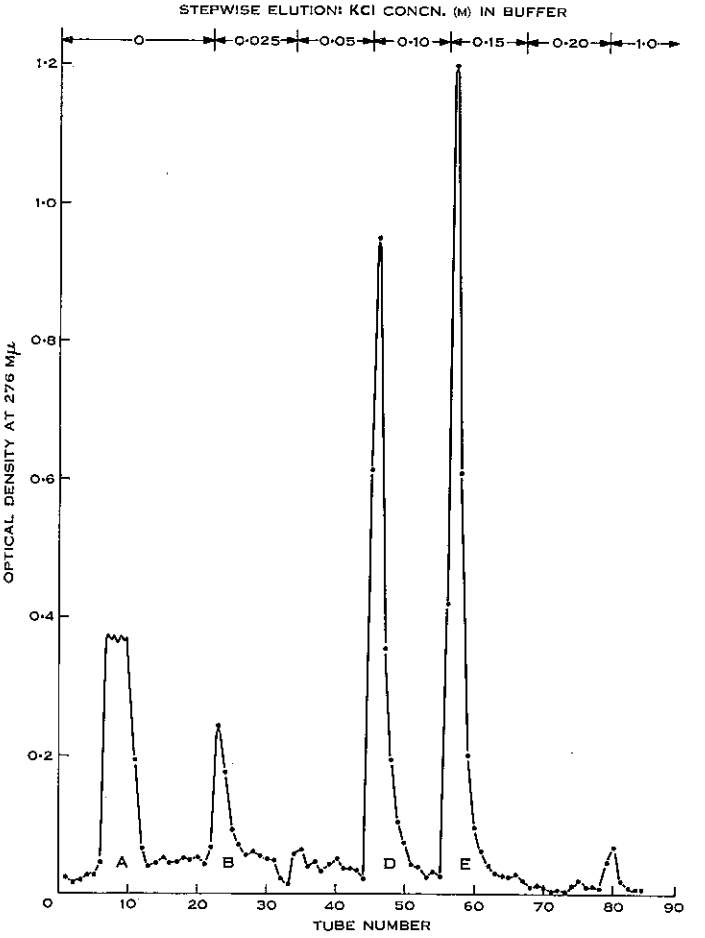

Fig. 3.—Chromatographic fractionation at 10°C on DEAE-cellulose of a total extract (SCMK) from reduced and carboxymethylated wool in 8M urea–Tris buffer at pH 8·6. Fraction size and column dimensions as in Figure 1.

compared with those of similar SCMKA fractions (Thompson and O'Donnell 1964); this behaviour is due to the tighter binding of high-sulphur proteins SCMKB to the DEAE-cellulose.

The effects of changing the arbitrary conditions of chromatography are illustrated in Figures 2 and 3 which show the chromatographic fractionation of a total

extract (SCMK) on DEAE-cellulose at a different pH (8·6) and at two different temperatures (25°C and 10°C), respectively. Corresponding starch-gel patterns of these fractions are shown in Plate 1, Figures 4 and 5. At the lower temperature the proteins are eluted at lower salt concentrations (cf. O'Donnell and Thompson 1960), but the pH change has little effect on the separations. In other experiments not illustrated here the temperature was raised to 50°C, smaller increments of potassium

TABLE 1

AMINO ACID COMPOSITION OF FRACTIONS OF SCMK ELUTED FROM DEAE-CELLULOSE

Amino acid nitrogen values given are expressed as a percentage of the total nitrogen content of the hydrolysates calculated from the number of μmoles of amino acids plus ammonia recovered from the column. Fractions refer to the chromatographic fractions shown in Figure 1

Amino Acid	Fraction No.						
	A*	B†	C†	D†	E†	F†	G*
Lysine	1·0	3·6	5·4	5·4	4·4	2·5	1·0
Histidine	3·0	2·0	1·4	1·2	1·3	2·2	0·8
Arginine	18·3	16·9	18·9	20·8	20·6	15·2	17·6
Aspartic acid	3·1	4·6	5·7	6·5	6·4	4·9	1·6
Threonine‡	3·4	3·2	2·9	3·4	4·5	6·4	7·2
Serine‡	11·1	9·9	7·4	6·3	6·3	7·6	9·7
Glutamic acid	2·6	6·2	9·2	11·3	10·8	7·1	8·1
Proline	4·3	2·9	2·0	2·2	3·4	7·7	8·0
Glycine	15·9	12·2	8·7	5·5	5·5	5·3	5·8
Alanine	1·6	3·8	4·9	5·1	4·5	3·4	2·8
Valine	2·6	3·4	3·9	4·6	4·5	4·5	3·7
Methionine	Trace	0·1	0·3	0·4	0·3	0·2	Trace
Isoleucine	1·0	1·8	2·3	2·7	2·5	2·5	2·4
Leucine	4·7	5·5	6·6	7·4	7·2	5·2	2·5
Tyrosine	7·5	5·6	4·1	2·3	2·2	1·8	2·0
Phenylalanine	7·6	3·4	2·1	1·8	2·1	2·0	1·1
S-Carboxymethylcysteine	4·9	0·8	1·0	3·5	4·8	4·3	15·8 (9·8)§
½Cystine	Nil	Nil	Trace	Trace	Trace	4·1	Nil (4·9)§
Cysteic acid	Nil	0·9	1·2	0·1	0·2	1·0	Nil (0·6)§
Ammonia‖	7·2	13·1	11·9	9·6	8·8	12·3	9·7

* Hydrolysed in a sealed tube under high vacuum.

† Hydrolysed under reflux. Sulphur amino acid values unreliable, see text.

‡ Uncorrected for decomposition.

§ Values in parentheses were obtained when hydrolysed under reflux.

‖ These values are not true amide ammonia values. Ammonia from traces of urea, etc. may be included.

chloride concentration in the eluting buffer were employed, and potassium chloride in the buffer was replaced by Tris to increase the buffering capacity. However, no simplification of the pattern was observed, so far as resolution of individual components was concerned, and no change from pH 7·4 and 25°C as used in most of these experiments seemed warranted.

The starch-gel patterns shown in Plate 1, Figures 3, 4, and 5 give an accurate impression of the complexity of the chromatographic fractions but, because the

eluted fractions are all adjusted to *c.* 1% concentration before re-running on starch gel, the elution curves give a better idea of their relative amounts.

(*d*) *Amino Acid Composition of Chromatographic Fractions*

Table 1 shows the amino acid compositions of the seven fractions eluted during DEAE-chromatography (Fig. 1) of the total extract (SCMK) from reduced and carboxymethylated wool. Each of these fractions contains many components but there is a trend in pattern on the starch gel. Thus fraction A, which is not retarded by DEAE-cellulose, is characterized by very high contents of glycine (15·9% of the total nitrogen), tyrosine (7·5%), and phenylalanine (7·6%), and usually consists mainly of bands running at intermediate rate. Fractions D and E show similarities to one another but there are differences between these fractions. As each is multi-component no conclusions can be drawn from this data as to significant differences between the two main bands. Fractions F and G are characterized by a high content of *S*-carboxymethylcysteine (15·8% for fraction G) and proline (8%).

The stability of *S*-carboxymethylcysteine to hydrolysis under reflux varies throughout these fractions. Fractions A–C decompose readily, giving cysteic acid as the main product; fractions D and E scarcely decompose, as was found previously for the *S*-carboxymethylcysteine of SCMKA and *S*-carboxymethyl plasma albumin (Thompson and O'Donnell 1962); fractions F and G again decompose extensively (*c.* 50%) mainly to cystine, as was found for the high-sulphur fraction SCMKB by Gillespie (1963). However, no decomposition of *S*-carboxymethylcysteine was found in any of the fractions hydrolysed *in vacuo* under the conditions recommended by Crestfield, Moore, and Stein (1963). We have also found that the sum of the sulphur-containing amino acids derived from, and including, *S*-carboxymethyl-cysteine in hydrolysates prepared under reflux for those fractions where decomposition is extensive is not always a good approximation to the true *S*-carboxymethyl-cysteine content of the fraction.

(*e*) *Gel Filtration of Total Extracts from Reduced and Carboxymethylated Wool*

Figure 4(*a*) shows the elution curve of a total extract of reduced and carboxymethylated wool proteins on Sephadex G-200. There are three regions which result from the molecular-sieving effect of the Sephadex and these will be in order of decreasing molecular size as elution proceeds. Plate 2, Figure 1, shows the patterns corresponding to four cuts taken from these three regions and it is seen that, as with DEAE-cellulose, the profile of total SCMK revealed by starch-gel electrophoresis has been broken into distinct areas; the separations produced by the two media are not the same, one being based predominantly on charges on the various proteins present and the other on their sizes. Thus it is concluded that the fast-running material depicted on the starch-gel pattern is of high charge and low molecular weight while the two predominant slower bands of the SCMK fraction have a lower charge and higher molecular weight. The material first eluted from Sephadex appears to consist of aggregated proteins; these tend to be eluted in the later fractions from DEAE-cellulose.

The slowest peak on the Sephadex elution curve [Fig. 4(*a*)] can be seen from the starch-gel patterns (Plate 2, Fig. 1) to contain the high-sulphur components (cf. fractions F and G of Plate 1, Fig. 3, and Table 1) and the high glycine–high aromatic components (cf. fractions A and B of Plate 1, Fig. 3, and Table 1). This agrees with the lower molecular weights of the high-sulphur proteins of 16,000–28,000 (cf. Gillespie and Simmonds 1960; Gillespie 1962*c*; Gillespie and Harrap 1963) and the retardation of aromatic compounds (Porath 1962) by adsorption on Sephadex.

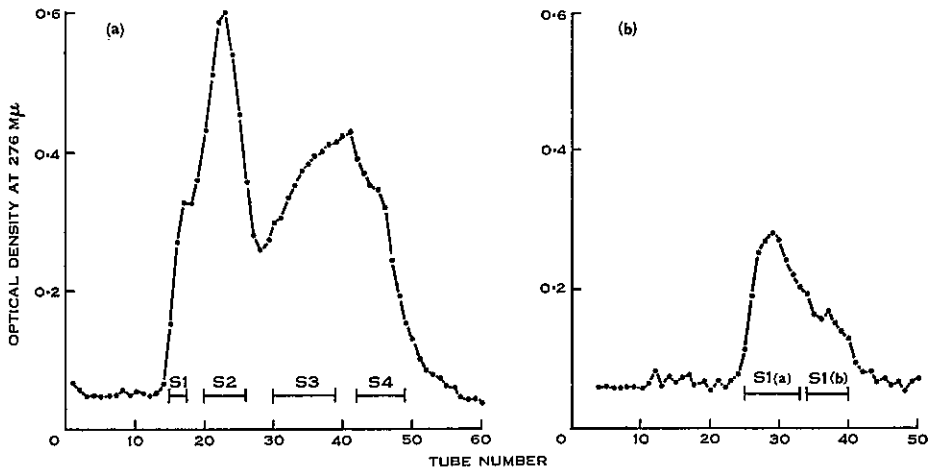

Fig. 4.—(*a*) Gel filtration on Sephadex G-200 of a dialysed total extract (SCMK) from reduced and carboxymethylated wool, in 8M urea–Tris buffer at pH 7·4. Fraction size 5–8 ml. Column dimensions 2·3 cm diameter by 125 cm. Fractions bulked for starch-gel electrophoresis are indicated. (*b*) Repeated gel filtration on Sephadex G-200 of the fastest-moving material of Figure 4(*a*). Fractions bulked for starch-gel electrophoresis are indicated.

The two predominant low-sulphur bands have molecular weights in 8M urea buffers of approximately 50,000 (O'Donnell and Woods 1956; cf. DeDeurwaerder and Harrap 1964). Attempts to fractionate the total extract on Sephadex G-200 after alkylation and without dialysis were less successful, due probably to the increased aggregation in the presence of salt.

The fastest-moving material in the Sephadex separation corresponds to the slow diffuse bands 1–4 of Plate 1, Figure 1, and are presumably of higher molecular weight than the rest of the material present. It was considered that these might be aggregates of the various components present even though overnight centrifugation in 8M urea buffer at 42,000 r.p.m. in a Spinco model L centrifuge did not remove them. This material was purified by a second passage through the Sephadex column [see Fig. 4(*b*)] and cut into two fractions, S1(*a*) and S1(*b*). Starch-gel patterns of these fractions are shown in Plate 2, Figure 2, and their amino acid composition are given in Table 2. The agreement between the amino acid values for the two

fractions is so good that it is concluded that these slow bands (1–4 of Plate 1, Fig. 1) are probably aggregates of component 7 (and perhaps some component 8). It is of interest that these slow bands of aggregated material are absent in fresh preparations of wool root protein (Plate 1, Fig. 2); they are more distinct in starch-gel patterns of some aged SCMKA preparations, particularly if these have been stored in a freeze-dried state.

(*f*) *Isolation of Component 8 from Total Extract of Wool*

A consideration of the different types of fractionation of SCMK produced by DEAE-cellulose and Sephadex G-200 in buffers containing 8M urea suggests that component 8 could be separated in fairly pure form by a combination of these two techniques. Thus when fraction E (Plate 1, Fig. 3) from 1 g of extracted protein

TABLE 2

AMINO ACID COMPOSITION OF TWO FRACTIONS OF SCMK SEPARATED ON SEPHADEX G-200

Starch-gel patterns of the fractions S1(*a*) and S1(*b*) are shown in Plate 2, Figure 2. Amino acid nitrogen values given are expressed as a percentage of the total nitrogen content of the hydrolysates calculated from the number of μmoles of amino acids plus ammonia recovered from the column

Amino Acid	Fraction S1(*a*)*	Fraction S1(*b*)*	Amino Acid	Fraction S1(*a*)*	Fraction S1(*b*)*
Lysine	5·52	5·49	Alanine	5·09	5·16
Histidine	0·88	0·83	Valine	4·55	4·54
Arginine	19·97	20·56	Methionine	0·42	0·41
Aspartic acid	5·95	5·90	Isoleucine	2·83	2·79
Threonine†	3·38	3·34	Leucine	6·77	6·75
Serine†	5·79	5·78	Tyrosine	1·90	1·88
Glutamic acid	10·87	10·87	Phenylalanine	1·71	1·74
Proline	2·48	2·38	*S*-Carboxymethylcysteine	4·60	4·55
Glycine	5·06	5·20	Ammonia‡	12·20	11·64

* Hydrolysed in sealed tube under high vacuum.

† Uncorrected for decomposition.

‡ These values are not true amide ammonia values. Ammonia from traces of urea, etc. may be included.

was isolated on a large column (3 cm diameter) of DEAE-cellulose, a subsequent passage through the Sephadex G-200 column to remove slow and fast components (cf. Plate 2, Fig. 1) and rechromatography on DEAE-cellulose produced material giving a predominantly single band when examined by starch-gel electrophoresis (Plate 2, Fig. 3). On rechromatography of a purified fraction on DEAE-cellulose, where the concentration of a particular protein is much higher than in the original SCMK, it is necessary to examine each peak by starch-gel electrophoresis since displacement effects may result in the purified fraction being eluted at somewhat lower salt concentration than was the case with the original SCMK. With the above procedures the yield of isolated component 8 is approximately 50 mg per gram of

extracted protein (i.e. 4% of wool). The amino acid composition of this purified component 8 is shown in Table 3.

The agreement between the amino acid values for two separate preparations is good. However, the values for arginine and *S*-carboxymethylcysteine would appear to differ significantly. The values for the purified component 8 differ markedly from the values for the original fraction E from DEAE-cellulose in which it is the major component. Comparison between the amino acid composition of component 8 and other mixtures of components, e.g. fraction D (Fig. 1; Plate 1, Fig. 3) therefore seems unjustified at present.

TABLE 3

AMINO ACID COMPOSITION OF COMPONENT 8 ISOLATED FROM REDUCED AND CARBOXYMETHYLATED WOOL

Hydrolyses carried out in a sealed tube under high vacuum for 24 hr at 105°C. Amino acid nitrogen values given are expressed as a percentage of the total nitrogen content of hydrolysates

Amino Acid	Preparation 1*	Preparation 2†	Amino Acid	Preparation 1*	Preparation 2†
Lysine	4·16	4·38	Alanine	4·14	4·21
Histidine	1·17	1·41	Valine	4·39	4·44
Arginine	20·49	23·33	Methionine	0·21	0·23
Aspartic acid	7·45	7·65	Isoleucine	2·67	2·61
Threonine‡	3·64	3·73	Leucine	8·31	8·23
Serine‡	5·23	5·40	Tyrosine	1·79	1·85
Glutamic acid	12·58	12·88	Phenylalanine	1·42	1·50
Proline	2·69	2·48	*S*-Carboxymethylcysteine	3·93	4·45
Glycine	2·84	3·00	Ammonia§	12·88	10·22

* Small sample hydrolysed. Total nitrogen of hydrolysate calculated from the number of μmoles of amino acids plus ammonia recovered from the column.

† Total nitrogen of hydrolysate determined by Kjeldahl method. This agreed within 2% of that calculated from the number of μmoles of amino acids plus ammonia recovered from the column.

‡ Uncorrected for decomposition.

§ The values are not true amide ammonia values. Ammonia from traces of urea, etc. may be included.

Methionine appears to be the least abundant amino acid in component 8 but tryptophan has not yet been estimated. If it is assumed that component 8 consists of a single protein with a nitrogen content of 16·8%, the minimum molecular weight from the methionine content is approximately 38,000.

(*g*) *Examination of Purified Low-sulphur Wool Proteins*

In previous publications from these Laboratories two purified low-sulphur proteins called SCMKA1 (Gillespie 1960) and SCMKA2 (Gillespie 1956, 1957) gave single peaks on moving boundary electrophoresis. These were prepared by different methods, SCMKA1 appearing to be more readily extractable. Although there were many similarities between the proteins, there were differences in solubility, electro-

phoretic mobility, and sedimentation coefficient (Gillespie 1960). The amino acid analyses of SCMKA1 and SCMKA2 were significantly different but a comparison of two different preparations of SCMKA1 (Gillespie 1960; Harrap and Gillespie

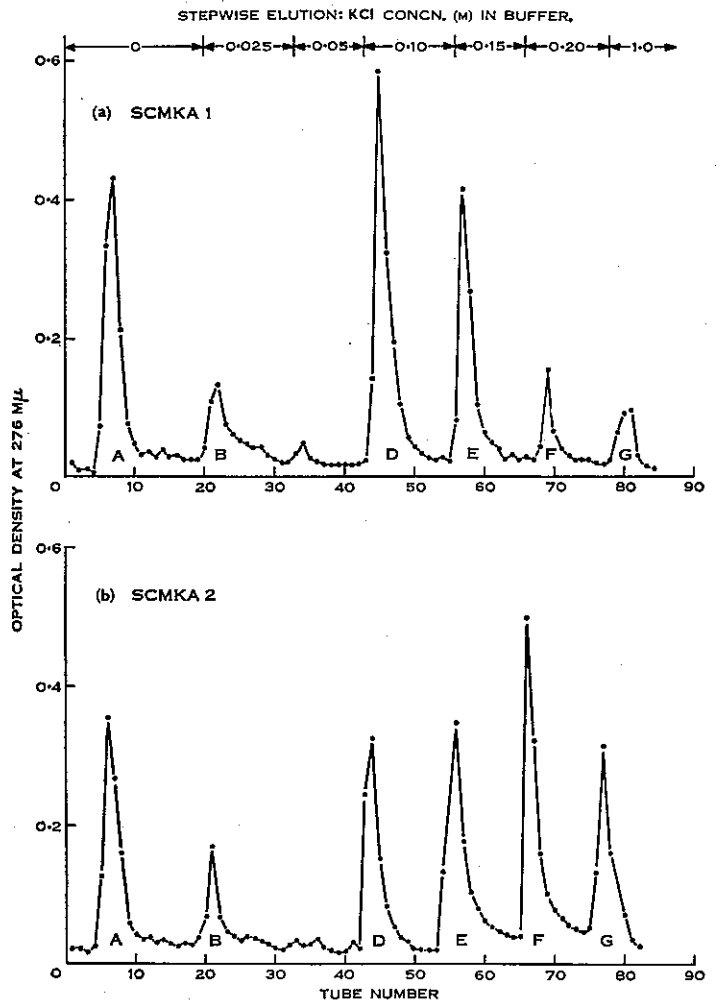

Fig. 5.—(*a*) and (*b*) Chromatographic fractionation (cf. Fig. 1) on DEAE-cellulose in 8M urea–Tris buffer at pH 7·4 and 25°C of low-sulphur protein fractions SCMKA1 (*c.* 50 mg) and SCMKA2 (*c.* 60 mg) separated from reduced and carboxymethylated wool by special procedures.

1963) showed just as large and numerous differences, thus indicating that the preparations are not reproducible and that they are probably mixtures. SCMKA2 was shown to be divisible into fractions of different amino acid composition by chromatography on DEAE-cellulose in 8M urea but both fractions were considered to be significantly different from SCMKA1 (Gillespie 1960).

The present results throw some light on the nature of these differences. As seen in Figure 5 both SCMKA1 and SCMKA2 in 8M urea–Tris buffer at pH 7·4 gave elution profiles from DEAE-cellulose which are quantitatively different from each other and from SCMKA (Thompson and O'Donnell 1964). Furthermore the starch-gel patterns (Plate 2, Fig. 5) of the chromatographic fractions of SCMKA2 are qualitatively similar to those of SCMKA in that they contain the two major components, aggregates, components of intermediate mobility typical of the high glycine–high aromatic fraction (cf. fractions A and B, Plate 1, Fig. 3), and only traces of the fastest moving components. The starch-gel pattern of SCMKA1 fractions in Plate 2, Figure 4, show that while there are two major bands of different mobilities in fractions D and E they have not completely resolved and are faster than normal. This behaviour was also noticed in stored freeze-dried preparations of wool root proteins (Thompson and O'Donnell 1964) which gave a single faster component compared with freshly prepared material (Plate 1, Fig. 2). The nature of the changes which cause these effects are not known. They are not concentration effects. SCMKA2 obviously contains both components 7 and 8. That both components are present also in SCMKA1 is evidenced by the general similarity in the amino acid analyses of SCMKA1 and SCMKA2 (Gillespie 1960; Harrap and Gillespie 1963) and their differences from that of the present component 8. It seems that the correct explanation of these results is that SCMKA and the purified fractions SCMKA1 and SCMKA2 are all mixtures of the two major components 7 and 8 shown in the starch-gel pattern of SCMK (Plate 1, Fig. 1) but each differs in the absolute amounts of each component present and also in the amounts of minor components. In aqueous solution these must be aggregated to move as a single component on moving-boundary electrophoresis (see Harrap and Gillespie 1963 for discussion on mobility differences). In support of this interpretation we have found that in the presence of a disaggregating agent (8M urea) the two main low-sulphur bands (7 and 8 in Plate 1, Fig. 1) can also be separated by ionophoresis in a polyvinylchloride block (Geon No. 427, B. F. Goodrich, C.S.R. Chemicals) (Kunkel and Trautman 1959) where the molecular-sieving effect of starch-gel is not present.

IV. Discussion

It has been common practice since the work of Goddard and Michaelis (1934, 1935) to classify reduced and alkylated soluble wool proteins into two classes on the basis of differing contents of sulphur amino acids. These can be separated by salt, acid, or zinc fractionation (Gillespie 1957, 1960, 1962*a*). These two heterogeneous fractions have been termed the high-sulphur (SCMKB) fraction and the low-sulphur (SCMKA) fraction. In the present studies 8M urea solutions have always been used in an attempt to minimize protein–protein interactions and, under these conditions, both gel filtration and chromatography on DEAE-cellulose readily enables separation of different SCMKA and SCMKB fractions. Harrap (1963) has shown that the proteins are completely unfolded in such a medium but it cannot be assumed that disaggregation is complete. In fact the slow-moving bands evident in the starch-gel patterns of Plate 1, Figure 1, appear to be aggregates of one or both of the major bands. When it is attempted to dissolve in 8M urea any of the low-sulphur wool-protein fractions which have been freeze-dried and stored there is always some gel

left. This happens also with reduced and carboxymethylated bovine plasma albumin and indicates that in both these cases 8M urea will not completely disrupt certain types of aggregation. The aggregates in extracted wool proteins can readily be removed by molecular sieving on Sephadex G-200 and at the same time the material of low molecular weight, which corresponds to the high-sulphur fraction and the high glycine–high aromatic fraction (Table 1), forms a separate peak from the two major low-sulphur components (7 and 8 of Plate 1, Fig. 1). Thus it appears that passage through a column of Sephadex G-200 in buffer containing 8M urea is to date the best single-step method of isolating the major components (7 and 8) of wool substantially free of the other proteins present. In order to separate components 7 and 8 it is necessary to make use of their differences in charge, e.g. by DEAE-chromatography or electrophoresis in 8M urea solutions. Component 8, which has the greatest charge in polyvinylchloride block electrophoresis and the greater mobility in starch gel, is more retarded on Sephadex G-200 than component 7, either due to its size or to adsorption. This difference is being explored as a means of purification of component 7.

The problem of nomenclature of the wool proteins is a difficult one at the present stage. The use of the terms SCMKA1 and SCMKA2 (Gillespie 1960) for what were thought to be different proteins is limited once these protein fractions have been shown to be mixtures. Currently we prefer to call our major components by numbers, i.e. 7 and 8, which is a purely operational definition referring to the starch-gel pattern of Plate 1, Figure 1. They are SCMKA proteins or protein fractions but we have avoided calling them SCMKA7 and SCMKA8 since they may also be heterogeneous and since the bands 1 and 2 of Plate 1, Figure 1, are obviously different from the protein fractions already known as SCMKA1 and SCMKA2.

Component 8 cannot be considered to be homogeneous without further evidence. However, when various pore sizes (3·75, 5·5, and 7·5%) of acrylamide were used, disk electrophoresis (Ornstein and Davis 1962) at pH 9·5 did not result in further fractionation of isolated component 8. Studies on "finger-printing" of tryptic digests will help in deciding this question of homogeneity.

Until each of the bands appearing in starch-gel patterns is isolated and characterized it cannot be said that a particular band, say in the high glycine–high aromatic fraction (A or B of Fig. 1), really is high in its content of glycine and the aromatic amino acids, or that bands of similar mobility on starch gel which occur in different fractions from DEAE-cellulose are identical. Because of such uncertainties and of different dye uptake and extinction coefficients, quantitative estimation of the various components is impossible. However, components 7 and 8 probably represent no more than 20% each of whole wool.

Fractions of wool proteins which are high in glycine, and in tyrosine or phenylalanine or both, have been recognized by various workers (Gillespie 1960; O'Donnell and Thompson 1962; Harrap and Gillespie 1963; Corfield 1963; Dr. M. Fell, personal communication), and a possible location for protein of this type in the membrane complex between the cuticular cells has been found by DeDeurwaerder, Dobb, and Sweetman (1964). The number of components on starch gel of fractions A and B (Plate 1, Fig. 3) isolated in the present work and the various amino acid compositions

reported for these high glycine–high aromatic fractions by the various workers shows that this material is multicomponent in nature (cf. Gillespie 1962*a*).

From amino acid analyses so far, components 7 and 8 seem to show quite large differences but until component 7 is isolated in pure condition and finger-prints obtained the nature of the differences cannot be defined. It is possible that these two components originate in different segments of the fibre or they may reflect genetic changes. It would not be surprising if these two components were heterogeneous. It should be pointed out that the *S*-carboxymethylcysteine content of component 8 is not as low as the cysteic acid or *S*-carboxymethylcysteine content of some other soluble protein fractions isolated from wool. However, correction factors for these latter values may be necessary because of the non-quantitative oxidation of cystine to cysteic acid (in the case of proteins extracted from oxidized wool), or because of destruction of *S*-carboxymethylcysteine during hydrolysis under reflux; alternatively, the heterogeneity of these other preparations may be responsible for their lower values. In considering the possibility that component 8 is heterogeneous, it is of interest that the reduced and carboxymethylated proteins from human hair and finger-nails and from other animal keratins (Plate 2, Figs. 6 and 7) give different starch-gel patterns to the similar total extracts of wool. In some of these there are more than two predominant bands with mobilities similar to those of components 7 and 8 of wool extracts. It does not seem probable from current observations on species variations between similar proteins (for example the insulins or cytochromes) that components from other keratins of different phylogenetic origin with the mobilities of components 7 and 8 from wool will have similar amino acid sequences.

Corfield (1962, 1963) has recently published the results of experiments on fractionation of extracted wool proteins and has interpreted the results to indicate peptide bond breakdown during preparation and chromatography of the protein fractions. The evidence from this Laboratory is against this conclusion (see Harrap and Gillespie 1963), and the results of the present study emphasize the difficulties of interpreting chromatographic experiments with mixtures of aggregating proteins behaving in a non-ideal way. Rechromatography of fractions containing mixtures of components will always lead to partial release of some of them.

It is considered that for more definitive studies, e.g. amino acid sequences on wool proteins, work should be confined to protein material corresponding to some particular component of the starch-gel electrophoretic pattern. Previous work on the fractionation of the low-sulphur proteins from wool which had been either oxidized or reduced and carboxymethylated has helped to illustrate the general problems inherent in fractionation of proteins which are aggregated (cf. O'Donnell and Thompson 1962), but it did not give fractions sufficiently pure to be suitable for chemical degradations. It is apparent that the fractions so far isolated and studied by other workers are also complex mixtures.

V. Acknowledgments

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Explanation of Plates 1 and 2

Plate 1

Fig. 1.—Starch-gel patterns of extracted wool protein fractions showing principal components. (*a*) Reduced and carboxymethylated proteins (SCMK) in a total extract. (*b*) Fraction precipitated at pH 4·4 (SCMKA). (*c*) Fraction not precipitated at pH 4·4 (SCMKB).

Fig. 2.—Starch-gel patterns of reduced and carboxymethylated proteins extracted from wool roots by buffer containing 8M urea. (*a*) As extracted. (*b*) Protein concentrated twofold.

Fig. 3.—Starch-gel patterns of fractions A–G (concentrations *c.* 1%) obtained from the chromatography of SCMK on DEAE-cellulose (cf. Text-fig. 1).

Figs. 4 and 5.—Starch-gel patterns of the fractions (concentrations *c.* 1%) obtained from the chromatography of SCMK at 25°C (cf. Text-fig. 2) and at 10°C (cf. Text-fig. 3) on DEAE-cellulose.

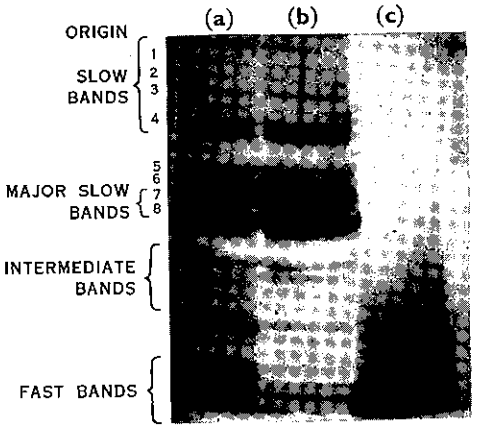

Fig. 1

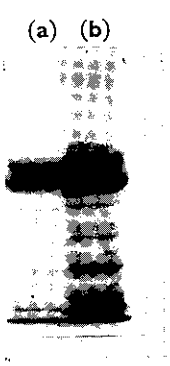

Fig. 2

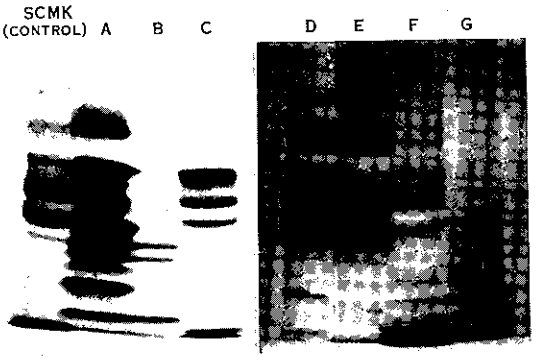

Fig. 3

Fig. 4

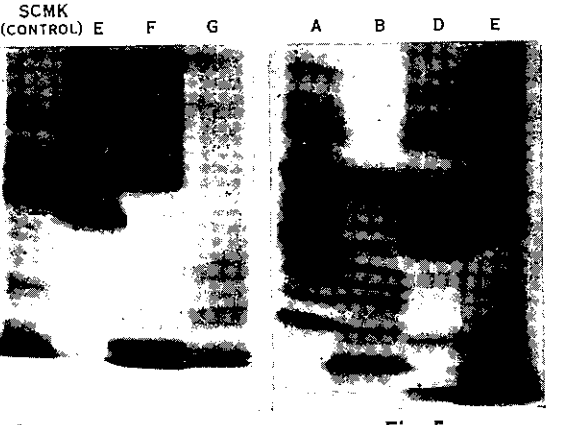

Fig. 5

STUDIES ON REDUCED WOOL. IV

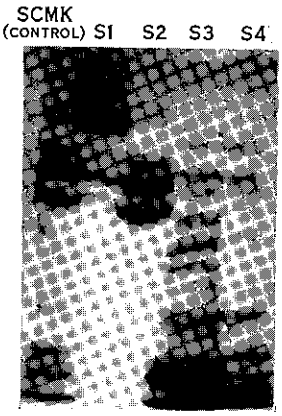

Fig. 1

Fig. 2

Fig. 3

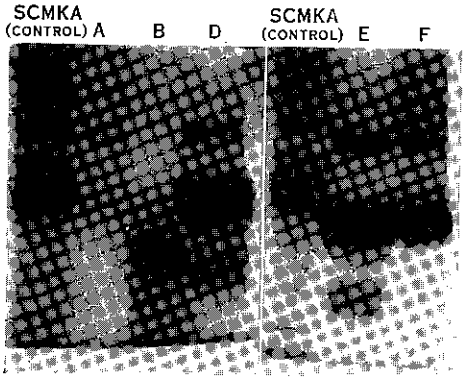

Fig. 4

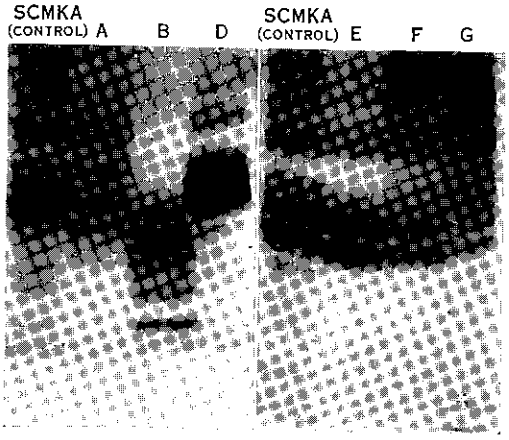

Fig. 5

Fig. 6

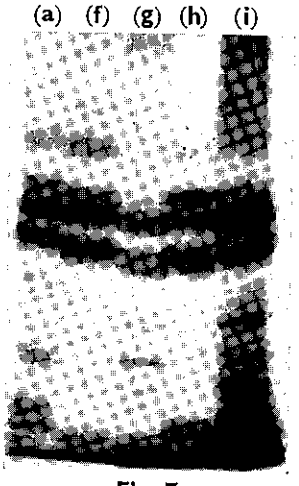

Fig. 7

PLATE 2

Fig. 1.—Starch-gel patterns of protein fractions S1–S4 (concns. *c.* 1%) from the four cuts indicated in Figure 4(*a*) obtained from the gel filtration of SCMK on Sephadex G-200.

Fig. 2.—(*a*) and (*b*) Starch-gel patterns of bulked fractions S1(*a*) and S1(*b*) respectively [cf. Fig. 4(*b*)] from the repeated filtration of the fastest component indicated in Figure 4(*a*) on Sephadex G-200. (*c*) Component 8 (cf. Plate 1, Fig. 1) is included for reference.

Fig. 3.—Starch-gel patterns of (*a*) SCMK, (*b*) succinylated SCMK, and (*c*) component 8 which has been purified by two passages through DEAE-cellulose and one passage through Sephadex G-200, at all times in buffer containing 8M urea.

Figs. 4 and 5.—Starch-gel patterns of fractions of SCMKA1 (Fig. 4) and of SCMKA2 (Fig. 5) separated on DEAE-cellulose as indicated in Text-figures 5(*a*) and 5(*b*), respectively.

Figs. 6 and 7.—Starch-gel patterns of reduced and carboxymethylated proteins from a variety of keratins: extracts from (*a*) wool (i.e. SCMK); (*b*) human hair; (*c*) human finger-nails; (*d*) porcupine quill, exterior; (*e*) porcupine quill, medulla; (*f*) elephant hair; (*g*) seal whisker; (*h*) cow horn; (*i*) echidna quill.