STUDIES ON REDUCED WOOL

V. A COMPARISON OF THE TWO MAJOR COMPONENTS

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Summary

Starch-gel electrophoresis of wool proteins extracted from reduced and carboxymethylated wool gives a complex pattern in which there are two major protein bands. By a combination of chromatography on DEAE-cellulose and gel-filtration in buffers containing 8M urea these two protein components have been isolated. The amino acid composition and some properties of these two fractions are reported. A comparison of the amino acid composition and of peptide maps of tryptic digests of the two fractions shows distinct differences between them, and by labelling with 2-[^{14}C]iodoacetate the distribution of the peptides containing *S*-carboxymethylcysteine residues were also shown to be different.

The molecular weight of the two protein components was estimated to be about 45,000 by comparison of their elution volumes on gel-filtration with the elution volumes of reduced and carboxymethylated proteins of known molecular weight. Gel-filtration was carried out in 8M urea, 14M formamide, and 5M guanidine hydrochloride, and no evidence of further dissociation of these components was obtained.

I. INTRODUCTION

In previous papers the complexity of the patterns obtained by starch-gel electrophoresis in 8M urea solutions of reduced and carboxymethylated wool proteins (Thompson and O'Donnell 1964; O'Donnell and Thompson 1964*b*) was reported. There are two predominant protein bands (called components 7 and 8—see Plate 1, Fig. 3) present. By working in buffer solutions containing 8M urea to minimize aggregation and using a combination of chromatography on DEAE-cellulose and gel-filtration on Sephadex G-200 one of the components (component 8) was isolated. The second component (component 7) has proved more difficult to isolate in a pure condition but a combination of the same techniques has provided material of sufficient purity for an examination of its properties for comparison with those of the other major component, 8, isolated from the same fleece. The amino acid composition of component 7 is reported. Other differences between the two components have been revealed by peptide mapping of their tryptic digests.

Components 7 and 8 have a high helical content in aqueous solution and must be responsible for the regularity observed in the X-ray diagram of wool. A knowledge of their composition and chemical properties is of interest in relation to the various structures that have been proposed for wool keratin.

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II. Materials

The formamide was a commercial sample (B.D.H. laboratory reagent, 98%) and was used as supplied.

The guanidine hydrochloride was purified by charcoal treatment of the commercial product according to the method of Luck *et al.* (1958). Alternatively, it was made from commercial guanidine carbonate which had been purified by the method of Spackman, Stein, and Moore (1960).

The proteins used were commercial crystalline samples and consisted of bovine plasma albumin and pepsin (Armour & Co.) and β-lactoglobulin and ovalbumin (Pentex Incorporated). Human haemoglobin was a gift from Dr. C. C. Curtain.

The DEAE-celluloses used were obtained from Serva Entwicklungslabor, Heidelberg, and Eastman Organic Chemicals. The guanidinoethyl cellulose was a Serva product.

Dialysis bags of Visking cellulose tubing 18/32 were used. They were extracted before use with boiling water (Hughes and Klotz 1956).

III. Experimental

Extracts of reduced and carboxymethylated wool [*S*-carboxymethylkerateines (SCMK)] were prepared from a single Merino 64's fleece, MW138, as described previously (O'Donnell and Thompson 1964*b*). SCMKA was prepared by dialysis of the carboxymethylated urea–mercaptoethanol extracts against water and repeated precipitation of the SCMKA fraction with citrate or acetate buffer at pH 4·4 as previously described (O'Donnell, Thompson, and Inglis 1962).

For radioactive labellings of the *S*-carboxymethyl groups in the extracted wool proteins 2-[^{14}C]bromoacetate was converted to iodoacetate (Gillespie and Springell 1961) and allowed to react with the urea–mercaptoethanol extract of 150 mg of wool after removal of the thiol reagent by dialysis against trichloroacetic acid (Thompson and O'Donnell 1962*b*). The precipitated protein was dissolved at pH 2 in 10 ml of chromatography buffer [8M urea—0·01M Tris—0·001M Versene (disodium salt of ethylenediaminetetraacetic acid, initial pH 7·4)], for reaction with the labelled iodoacetate which was dissolved in 5 ml chromatography buffer plus 1·5 ml 3M Tris (pH 8·5) and 0·08 ml 5N KOH. Since the yield of iodoacetate from bromoacetate is variable, any thiol groups still present after 3 min (due to the use of insufficient labelled iodoacetate) were carboxymethylated with unlabelled iodoacetate at the same pH of 8·5. The excess iodoacetate was removed by reaction with mercaptoethanol and dialysis as before. These radioactive *S*-carboxymethylkerateines were fractionated first on Sephadex G-200 and then on DEAE-cellulose as described below. The samples used for gel-filtration experiments were obtained from the peak tubes of the main fraction present when the leading half of fraction S2 of a gel-filtration separation of ^{14}C-labelled SCMK [see O'Donnell and Thompson 1964*b*, Fig. 4(*a*)] was re-run on a similar column. Aggregates and lower molecular weight material were still evident in the elution pattern of this fraction of S2.

The *S*-carboxymethyl (SCM) derivatives of reduced bovine plasma albumin, β-lactoglobulin, ovalbumin, and pepsin were prepared by reduction with mercaptoethanol at pH 10·5 in 8M urea, as described for the reduction and extraction of wool,

and were carboxymethylated in the same way at pH 8·5. They gave predominantly single bands on starch-gel electrophoresis in 8M urea but the SCM-β-lactoglobulin seemed to be separating into two bands. The pattern of SCM-pepsin was only very faint because its highly acidic nature results in weak staining with nigrosine. The haem groups of haemoglobin were first removed by the method of Teale (1959) and the residual protein was reduced and carboxymethylated in 8M urea as described above.

Chromatography of proteins on DEAE-cellulose at pH 7·4 in buffers containing 8M urea was carried out at constant temperature by the methods previously described (O'Donnell and Thompson 1964*b*). It is recommended that the urea be purified by passing it through a column of mixed-bed, ion-exchange resin to remove ultraviolet-absorbing impurities and cyanate (Cole 1960). Subsequently the buffer was passed through a pad of DEAE-cellulose to remove dirt (cf. Thompson and O'Donnell 1960). The capacity and performance of batches of DEAE-cellulose varied, as reported by Peterson and Sober (1956), and each batch had to be tested to find the optimum load of SCMK for the best resolution of components 7 and 8. The temperature of chromatography had an effect on the fractionation. In some experiments with Eastman DEAE-cellulose it was found that 50°C gave an improved separation of components 7 and 8. However, at this temperature there was formation of homocitrulline by reaction of the ϵ-amino groups of lysine with cyanate formed in the urea. We have therefore worked at 25°C to lessen this possibility. At 25°C Serva DEAE-cellulose gave better fractionation than did that from Eastman. For preparative experiments the extract from 1·7 g of air-dried wool was dialysed against 8M urea buffer containing 0·01M Tris–0·001M Versene, pH 7·4, and approximately 80% of this was fractionated on a column (15 cm long, 3 cm int. diam.) of DEAE-cellulose equilibrated with similar buffer. Stepwise elution with 165–200 ml of buffer per step was carried out at constant temperature.

The unadsorbed fraction was called A and those eluted with 0·025, 0·05, 0·1, 0·15, 0·2, and 1M KCl in the eluting buffer were called B–G respectively. The fractions A–G were recovered for starch-gel electrophoresis or other operations as was described in Part IV (O'Donnell and Thompson 1964*b*). However, in order to facilitate the re-solution of the lyophilized proteins into urea buffer, the desired amount of urea buffer was added before lyophilization and then the correct amount of water added after the drying process. Gradient-elution experiments were conducted as previously described (O'Donnell and Thompson 1961), shaped gradients produced according to the methods of Bock and Ling (1954) being used.

Both horizontal starch-gel electrophoresis (Smithies 1955) in 8M urea solutions containing 0·01M Versene and gel-filtration on columns of Sephadex G-200 (125 cm long by 2·3 cm int. diam.) were performed as described previously (O'Donnell and Thompson 1964*b*). In order to obtain equal-sized effluent fractions, Beckman Accu-Flo pumps were used to control the flow of buffer to the top of the column to a rate of 16 ml/hr. For studies of molecular weights by gel-filtration it was necessary to incorporate salt in the eluting buffer to minimize any non-ideality effects; the buffers used were 8M urea–0·05M Tris–0·1M KCl–0·001M Versene at pH 7·4, 60% formamide–0·05M phosphate at pH 7 [similar to that used by DeDeurwaerder and Harrap (1964, 1965)], and 5M guanidine hydrochloride–0·01M Versene at pH values ranging from

7 to 8. In general, the methods used to measure elution volumes were similar to those of Whitaker (1963) and Andrews (1964). However, in preparative work separation of aggregates from components 7 and 8 is better at low salt concentrations in the buffer previously used (O'Donnell and Thompson 1964*b*).

For study of the elution curve by frontal analysis (Winzor and Scheraga 1963, 1964) a column (29 cm long by 0·9 cm int. diam.) of Sephadex G-200 was used with chromatography buffer which contained, in addition, 0·1M potassium chloride. The column was run at 5 ml per hour, fractions (0·4 ml) were collected by drop counting, and afterwards weighed. The concentration of the radioactively labelled protein (components 7 and 8) was 0·013% and approximately 11 ml was loaded on to the column.

Liquid-scintillation counting of radioactively labelled proteins was done with a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co. Inc., La Grange, Illinois). The liquid-scintillation solution consisted of 100 μl of urea or formamide effluent or 50 μl (maximum) of 5M guanidine hydrochloride effluent, made to 1 ml with water and mixed with 7·5 ml of scintillation mixture (Butler 1961). Counting was for a period of either 1 or 5 min.

Protein concentrations in urea were determined from optical density measurements at 276 mμ assuming an extinction coefficient ($E^{1\%}_{1cm}$) of 7 for mixtures of components 7 and 8. Harrap (personal communication) noted that the extinction coefficients of our components 7 and 8, determined from refractive index measurements of protein concentration, were considerably lower than the value of approximately 9 of some low-sulphur wool proteins (cf. Harrap and Woods 1958). This is due to the removal of contaminant proteins rich in glycine and the aromatic amino acids present in SCMKA and α-keratose fractions. From Kjeldahl nitrogen analyses, after dialysis against water, 0·3M KCl–0·01M borate, and water to remove urea, and taking the nitrogen contents of components 7 and 8 as 16·8%, the extinction coefficients ($E^{1\%}_{1cm}$) at 276 mμ of components 7 and 8 were approximately 6·9 and 7·5 respectively.

Tryptic digestion of the proteins and peptide mapping were carried out as described previously (Thompson and O'Donnell 1962*a*).

Proteins were carbamylated by the method of Stark and Smyth (1963), and dinitrophenylation (Sanger 1945) in 8M urea–2% potassium bicarbonate was carried out at 40°C. After reaction the proteins were dialysed and further purified as described by these authors. The hydrolysate of dinitrophenylated SCMKA protein was examined for unreacted and reacted lysine and tyrosine residues with a Spinco amino acid analyser (cf. Wofsy and Singer 1963).

In studying the effect of alkali at pH 13 on peptide bonds both SCMKA and carbamylated SCMKA were employed. After standing 24 hr at 25°C both the untreated and treated samples were adjusted to pH 8, made 8M in urea, and dinitrophenylated as above. The carbamylated SCMKA as expected gave no detectable dinitrophenyl (DNP)-amino acids before exposure at pH 13 so that any increase was an indication of peptide-bond hydrolysis. This procedure had advantages when analysing for DNP-arginine in the aqueous layer since no ϵ-DNP-lysine was present.

Quantitative estimation of DNP-amino acids was carried out by two-dimensional paper chromatography in which tertiary amyl alcohol–2% ammonia and 1·5M phosphate and the methods described by Fraenkel-Conrat, Harris, and Levy (1955) were used.

Amino acid analyses were performed with a Spinco amino acid analyser which was operated under the conditions described by Spackman, Stein, and Moore (1958). Hydrolysates were prepared by hydrolysis *in vacuo* for 24 hr at 108°C in 6N HCl (Crestfield, Moore, and Stein 1963).

IV. Results

(*a*) *Isolation of Components 7 and 8*

The resolution of the components of SCMK on DEAE-cellulose columns at 25°C was shown in Part IV (O'Donnell and Thompson 1964*b*). The resolution varies with the load of protein on a particular DEAE-cellulose. With a column (15 cm long, 3 cm int. diam.) of Serva DEAE-cellulose (21 g, degree of substitution 0·69 m-equiv/g) it was found that fraction E contained component 8 with least amount of component 7 if 80% of the extract of 1·7 g of wool was used. Re-running of fractions D and E on DEAE-cellulose was only of limited value in further freeing components 7 and 8 from each other.

It appears that the binding of these highly charged unfolded proteins involves multi-point attachment to the basic groups of the absorbent. There is a range of pK values of the basic groups of the DEAE-cellulose and these factors, together with possible displacement effects with a mixture of proteins (such as SCMK), make the behaviour of a subfraction somewhat unpredictable on rechromatography. With DEAE-Sephadex and guanidinoethyl cellulose no improvement in resolution was found.

The number of elution steps can be reduced by equilibrating the column with a starting buffer containing 0·05M KCl and dialysing the SCMK against this buffer before application to the column. Fractions A, B, and C under these conditions pass unadsorbed through the column as one fraction.

The percentages by weight of protein material recovered from the column in fractions (A plus B plus C), D, E, and (F plus G) were approximately 10, 37, 21, and 32% respectively. The corresponding percentages of optical densities for these fractions were approximately 18, 35, 18, and 29% of the total optical density respectively. (The percentages of fractions D and E vary with the load of protein on a particular column.) The high value of optical density for fraction (A plus B plus C) is a consequence of its high aromatic amino acid content; it also has a high glycine content. On dialysis against water a proportion of this protein material precipitates. The precipitate is much richer in glycine and tyrosine than the supernatant fraction.

Fraction E was used to prepare component 8 by passage through Sephadex G-200 in 8M urea buffer as previously described (O'Donnell and Thompson 1964*b*) and shown in Figure 1 and Plate 1, Figure 1. Groups of tubes from around the main peak area were bulked for examination by starch-gel electrophoresis. It was found that the component 7 present was concentrated in the leading edge of the main

peak and the trailing edge was almost pure component 8. The appropriate fractions were bulked and stored in a deep-freeze in 8M urea solution. By dialysis against water and subsequent freeze-drying, component 8 could be prepared when required, e.g. for tryptic digestion. Component 7 has proved more difficult to isolate than component 8. When component 7 was required in its purest form, fraction D from the DEAE-cellulose fractionation was passed through a Sephadex G-200 column. Figure 1 and Plate 1, Figure 2, show the resolution obtained in a single passage through the Sephadex. The aggregates and component 8 in fraction 2 of Plate 1, Figure 2, can be reduced by further passages through columns on Sephadex G-200 but the yield is of course reduced considerably. The amounts of component 7 thus

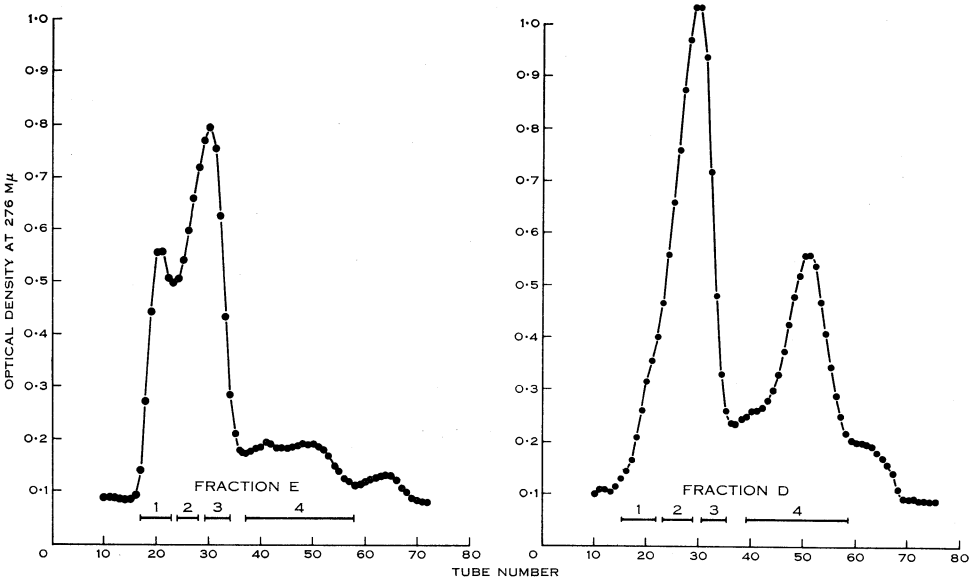

Fig. 1.—Gel-filtration on Sephadex G-200 of fractions from the DEAE-cellulose fractionation of a total extract of wool, in 8M urea – 0·001M Versene – 0·01M Tris buffer, pH 7·4. Fraction size 8 ml. Column dimensions 2·3 cm internal diameter by 125 cm. Fractions bulked for starch-gel electrophoresis are indicated.

obtained which can be used for definitive chemical studies is obviously only a small percentage of that present in the original wool. From 1·7 g of air-dried wool approximately 60 mg of purified component 7 (i.e. 4% of wool) was obtained.

From Figure 1 and from Plate 1, Figures 1 and 2, it can be seen that fraction D contains less aggregates than fraction E. There is a substantial amount of slower-moving material in fraction D which analysed as a high-glycine–high-aromatic amino acid fraction. If this material is included with fraction (A plus B plus C) the total amount of this high-glycine–high-aromatic amino acid fraction accounts for 15% by weight of the extracted wool protein.

Various types of gradients of salt (linear, concave, and convex) have been used for the chromatography on DEAE-cellulose in an effort to improve the resolution of components 7 and 8 but none has proved superior to the simple stepwise elution

procedure outlined. With salt gradients both components are eluted together in a spread curve.

The continuous exposure of extracted wool proteins to buffer solutions containing 8M urea increases the probability of side reactions of protein amino groups with cyanate ions in equilibrium with urea (Stark, Stein, and Moore 1960; Cole 1961; Stark and Smyth 1963). The analytical method of Marier and Rose (1964) shows that even at the lowest concentration of cyanate detectable by their method there would always be ample cyanate ions formed in 8M urea during 24 hr at room temperature for reaction with the amino groups of a protein. However, we have not been able to find homocitrulline in the acid hydrolysates of components 7 and 8 prepared at 25°C or even after exposure for 5 days to 8M urea solutions at 25°C. It must be remembered that homocitrulline is not completely stable to acid hydrolysis and in the case of insulin Cole (1961) has reported that the ϵ-amino groups of lysine do not react as readily with cyanate as do the α-amino groups. One difference between wool proteins and other proteins such as ribonuclease and insulin which have shown reaction with cyanate ions in urea (Stark, Stein, and Moore 1960; Cole 1961) is the much lower content of amino groups; arginyl residues are the predominant basic groups. The amino groups in Tris may also react with cyanate ions or repress their formation (cf. Marier and Rose 1964) and thus have a protective effect on the protein amino groups.

When SCMK was carbamylated at 50°C with potassium cyanate by the method of Stark and Smyth (1963) the subsequent starch-gel pattern did not alter greatly. The bands representing components 7 and 8 were somewhat faster. The intensity of staining of the bands decreased, particularly for the aggregates, but the elution curve from Sephadex G-200 showed no marked change in the percentages of the various sections of the curve. The carbamylated mixture of proteins was held more tightly onto DEAE-cellulose and required higher salt concentrations to release it [carbamylated components 7 and 8 emerged in fraction (F plus G)].

(*b*) *Chemical Characterization of Components 7 and 8*

Table 1 gives an average amino acid composition of the purified component 7 together with the average values of component 8 (taken from O'Donnell and Thompson 1964*b*). Nine different preparations of component 7 were analysed. These preparations did not give a single band on starch-gel electrophoresis since component 7 is usually contaminated with slower-moving aggregates (which are formed, predominantly at least, from component 7) and with small amounts of components 5 and 8. It can be seen that there are marked differences in the amino acid compositions of components 7 and 8, e.g. in the residues glycine, methionine, and lysine; most other residues differ significantly.

The peptide maps of tryptic digests of purified component 7 and component 8 are shown in one dimension (pH 6·5 electrophoresis) in Plate 2, Figure 1, and in two dimensions in Plate 2, Figures 2 and 3. It can be seen from Plate 2, Figure 1, that the peptide bands moving both ways from the origin show major differences in both positions and intensities between components 7 and 8. The two-dimensional patterns (Plate 2, Figs. 2 and 3) show further differences in the peptides produced from

component 7 and component 8 and this is also seen in the radioautographs featuring only the peptides containing *S*-carboxymethylcysteine (Plate 2, Fig. 4). Each component gives a simpler pattern than does a tryptic digest of SCMKA and the peptides, particularly those carrying a positive charge, are well resolved. The negatively charged peptides do not give such compact spots and this is probably an indication of their size or adsorption effects or both.

In previous work (Thompson and O'Donnell 1962*a*) on tryptic digests of SCMKA and α-keratose the number of spots could not be counted with any confidence. From the present patterns, however, at least 40–50 spots can be counted in component 8 and also in component 7. Moreover, there are some negatively charged peptides

TABLE 1

AMINO ACID COMPOSITION OF PROTEINS ISOLATED FROM REDUCED AND CARBOXYMETHYLATED WOOL

Hydrolysis carried out in a sealed tube under high vacuum for 24 hr at 108°C. Amino acid nitrogen values given are expressed as a percentage of the total nitrogen of the hydrolysates calculated from the number of μmoles of amino acids plus ammonia recovered from the column

Amino Acid	Component 8*	Purified Component 7†	Amino Acid	Component 8*	Purified Component 7†
Lysine	4·27	6·14	Valine	4·42	4·54
Histidine	1·29	1·13	Methionine	0·22	0·43
Arginine	21·91	21·15	Isoleucine	2·64	2·73
Aspartic acid	7·55	6·31	Leucine	8·27	6·95
Threonine‡	3·68	3·07	Tyrosine	1·82	2·01
Serine‡	5·32	5·81	Phenylalanine	1·46	1·71
Glutamic acid	12·73	10·97	*S*-Carboxymethyl cysteine	4·19	4·37
Proline	2·58	2·05	Ammonia§	11·55	10·0
Glycine	2·92	5·24			
Alanine	4·18	5·46			

* Average of the two values quoted in Part IV (O'Donnell and Thompson 1964*b*).

† Average of nine analyses of different preparations.

‡ Uncorrected for decomposition.

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

containing *S*-carboxymethylcysteine which are revealed by radioautography but which are not clearly revealed by ninhydrin. At pH 6·5 the radioautographs do not show strongly labelled bands moving with the positively charged peptides, thus confirming the absence (Fell, LaFrance, and Ziegler 1960) of sulphur-containing peptides in this area.

There are probably some peptides in common in the tryptic digests of components 7 and 8. The proof of identity of two similarly placed spots must depend on their isolation and structure determination.

(*c*) *Aggregation of Component 7*

In Part IV, amino acid analyses of mixtures of component 7 and of the slow-moving bands apparent in the starch-gel electrophoretic pattern of SCMK suggested

that these slow-moving proteins were aggregates formed mainly from component 7. In agreement with this conclusion it has now been found (Plate 2, Fig. 1) that the one-dimensional peptide map of a tryptic digest of these aggregates is almost identical to that of component 7 and unlike that of component 8. Furthermore, if these aggregates are obtained by allowing components 7 and 8 to stand for periods of up to 28 days in 0·01M borate buffer at pH 9, subsequent starch-gel electrophoretic patterns (Plate 1, Fig. 3) at pH 8·6 in the buffer containing 8M urea suggest strongly that component 7, rather than component 8, is the one whose intensity is markedly decreased in the formation of this aggregated slow-moving material. It was also noted that this irreversible aggregation occurs more readily in the presence of 0·2M potassium chloride than in its absence. Although the amino acid compositions of components 7 and 8 are significantly different, the differences do not suggest any obvious reason for the greater tendency of component 7 to aggregate.

Sedimentation studies on the material responsible for the aggregate bands visible on the starch-gel electrophoretic patterns showed that its components were of greater molecular weight than component 8. One sample, which was a mixture of the aggregate bands, predominantly component 4 with lesser amounts of components 7 and 8, had a higher sedimentation coefficient in 8M urea buffer containing 0·2M sodium chloride ($S^0_{25,w} = 2·9$) than did component 8 ($S^0_{25,w} = 1·9$). In a limited study of their respective molecular weights by equilibrium ultracentrifugation techniques (short column, cf. Van Holde and Baldwin 1958) the following values were obtained for component 8 by calculation (mid-point method):

Protein concentration (%):	0·6	0·45	0·3	0·22
Molecular weight:	26,200	28,000	33,800	33,800

These values gave a final value of 43,000 when a plot of the reciprocal of molecular weight against concentration was extrapolated to infinite dilution. On the other hand, the aggregated material, which was heterogeneous, gave an average value for the molecular weight across the whole cell of approximately 75,000–80,000 for a solution of protein concentration 0·33% in 8M urea buffer containing 0·15M potassium chloride.

(*d*) *Attempts at Dissociation of Components 7 and 8*

(*i*) *Effect of High pH Values.*—Olofsson and Gralen (1947), O'Donnell and Woods (1956), and Harrap (personal communication) have found that at high pH values there is a decrease in molecular weight of extracted wool proteins. Also it has been reported (Hass and Lewis 1963; Hass 1964) that aldolase dissociates during exposure to pH 12·6 and room temperature [cf., however, Winstead and Wold (1964) and Andrews *et al.* (1964) for evidence of alkaline degradation of aldolase and xanthine oxidase respectively]. We have investigated the effect of keeping SCMKA at pH 13 and 25°C for various times. Starch-gel electrophoretic patterns under normal conditions showed changes after 2 hr. Bands 7 and 8 became diffuse and at the same time material of smaller molecular weight migrated with the buffer boundary. After 8 hr at pH 13 bands 7 and 8 had disappeared almost entirely. End-group estimations by the DNP-technique on SCMKA and carbamylated SCMKA that had been kept at

pH 13 for various lengths of time showed an increase in the number of *N*-terminal residues. The low initial values for the SCMKA (Table 2) compared with those previously reported (Thompson 1957) probably reflect an improvement in extraction procedure of the proteins from wool. It is noteworthy that alkali damage is indicated by a much higher value for serine *N*-terminal residues than for the threonine residues. The SCMKA2 (Gillespie and Lennox 1955) prepared by multiple extractions at 50°C at pH 10·5–12·3 showed similarly a higher value for *N*-terminal serine than for *N*-terminal threonine, thus suggesting that some alkali damage occurred under these conditions. The present values for SCMKA may be compared with those for α-keratose (total 5·1 μmoles per gram after correction for hydrolytic losses) extracted at pH 8 from performic acid-oxidized wool (Thompson, unpublished data, quoted in Crewther *et al.* 1965).

TABLE 2

N-TERMINAL RESIDUES OF SCMKA BEFORE AND AFTER ALKALI TREATMENT

SCMKA exposed to pH 13 at 25°C for 24 hr.* Values in μmoles/g protein

Terminal Amino Acid	Recovery Assumed† (%)	SCMKA	Alkali-treated SCMKA	Alkali-treated Carbamylated SCMKA
Aspartic plus glutamic acids	68	0·6	1·4	1·7
Serine	80	0·9	6·3	8·3
Threonine	86	1·8	2·5	2·0
Glycine	15	1·1	9·9	15·1
Alanine	64	0·3	1·1	1·4
Valine	64	0·2	0·6	0·7
Leucine	64	0	0·7	0·7
Phenylalanine	50	0	1·0	0·9
bis-Tyrosine	50	0	1·3	1·4
bis-Lysine	64	0	0·1	0·1
Total		4·9	24·9	32·3

* After dinitrophenylation the solution was dialysed. Hence the values for alkali-treated wool will be minimum values as no attempt was made to detect dialysable peptides.

† Hydrolysed in a sealed tube at 105°C for 16 hr.

Further evidence of random hydrolysis of the peptide chains of a mixture of components 7 and 8 was obtained by comparing the elution pattern from Sephadex G-200 in 8M urea buffer of the original material with that which had stood in aqueous 0·2N potassium hydroxide (pH 13) for 24 hr. With the treated material very little protein was eluted in the original position and the lower molecular weight material produced was heterogeneous.

(ii) *Effect of Low Protein Concentrations during Gel-filtration in Urea.*— Component 8 or mixtures of components 7 and 8 in the buffer containing 8M urea–

Tris–potassium chloride gave a single peak in the elution diagram from a column of Sephadex G-200 at protein concentrations of 0·02–0·8%. At lower concentrations of components 7 and 8, radioactively labelled material from the peak tube of a larger-scale gel-filtration of a fraction of SCMK was used; this ensured the absence of aggregated material. The trailing edge was steeper than the leading edge at normal working concentrations (0·5–0·8%) and was as steep at very low concentrations (see Fig. 2) and there was no suggestion of a change in the elution volume to larger values as the protein concentration decreased. Behaviour of this type has been

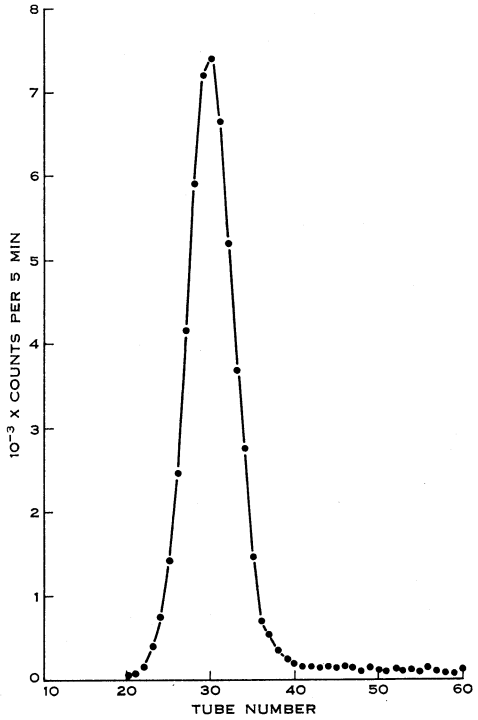

Fig. 2.—Elution curve of a mixture of ^{14}C-labelled components 7 plus 8 (0·02%) on Sephadex G-200 in 8M urea – 0·05M Tris – 0·001M Versene – 0·1M KCl, pH 7·4. Fraction size 8 ml. Column dimensions 2·3 cm internal diameter by 128 cm. Scintillation counting was carried out on 100-μl samples.

observed where there is no reversible association (Winzor and Scheraga 1963, 1964; Andrews 1964; Tracey 1964; Winzor and Nichol 1965). The frontal analysis experiment at a protein concentration of 0·013% (Fig. 3) confirmed that there is no marked difference in the sharpness of the leading and trailing edges, although some evidence of heterogeneity is present. It is known that the sample of protein used in these experiments could be contaminated with smaller amounts of components of lower molecular weight and different amino acid composition evident in the gel-filtration curves of SCMK, or of fractions containing components 7 and 8 (Fig. 1). The irregularity near the plateau in the leading edge of the elution curve is probably

due to the fact that a mixture of component 7 and component 8 was chromatographed and that component 7 emerged ahead of component 8. This is in agreement with the concentration of component 7 in the leading edge during elution analysis (Fig. 1; Plate 1, Fig. 2).

(iii) *Effect of Guanidine Hydrochloride.*—A column of Sephadex G-200 (118 cm long by 1 cm int. diam.) equilibrated with 5M guanidine hydrochloride–0·01M Versene (pH 7·0) gave only a single peak in the elution pattern of a mixture of the SCMK components 7 and 8 at concentrations ranging from 0·02 to 0·8%. There was only a small dependence of elution volume on protein concentration and the elution volume tended to decrease with dilution, as with the urea system above. Again, the trailing edge of the elution pattern was somewhat sharper than the leading edge which does not support the occurrence of a reversible association.

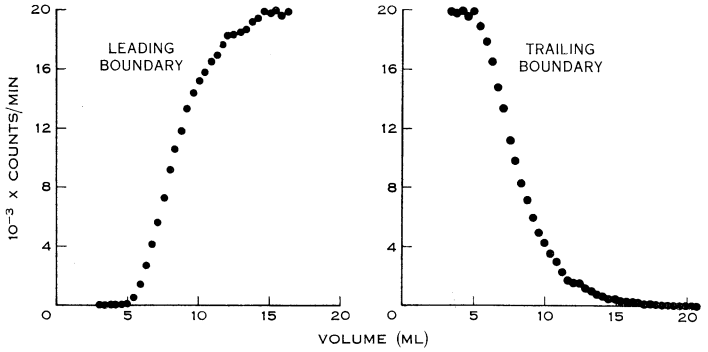

Fig. 3.—Elution profile of the leading and trailing boundaries obtained in frontal analysis chromatography of a mixture of ^{14}C-labelled components 7 plus 8 (0·013%) on Sephadex G-200 in 8M urea – 0·001M Versene – 0·01M Tris – 0·1M KCl, pH 7·4. Fraction size 0·43 ml. Column dimensions 0·9 cm internal diameter by 29 cm. For the trailing boundary zero volume corresponds to the start of elution with buffer. Scintillation counting was carried out on 100-μl samples.

No obvious dissociation of the aggregated material [bands 1–4 of the starch-gel pattern (Plate 1, Fig. 3)], isolated from columns of Sephadex G-200 eluted with 8M urea buffer solutions, occurred when it was dialysed against 5M guanidine hydrochloride for 24 hr and passed through a Sephadex G-200 column equilibrated with 5M guanidine hydrochloride.

(iv) *Effect of Formamide.*—When components 7 plus 8 were passed through Sephadex G-200 columns equilibrated with 14M formamide–phosphate at a protein concentration of 0·8%, the elution curve showed two peaks. At lower concentrations (0·02 and 0·004%) of radioactively labelled material there was a change to an elution pattern showing a single peak. However, even at 0·004% protein concentration the elution volume suggests a molecular weight of the same order (about 45,000) as that found with urea columns. This evidence suggests that at higher protein concentrations reversible aggregation occurs in 14M formamide, which is therefore less effective than 8M urea or 5M guanidine hydrochloride for gel-filtration isolation of components 7 and 8.

(e) Molecular Weight Estimation of Components 7 and 8 on Columns of Sephadex G-200

Andrews (1964) and Whitaker (1963) have shown that for globular proteins in aqueous solution the elution volumes from Sephadex columns of proteins which are not involved in reversible association equilibria are a function of their molecular weight. Over a certain size range, depending on the pore size of the Sephadex, they show that a plot of the elution volume, V_e, relative to the total volume, V_t, of a column (i.e. V_e/V_t) versus the logarithm of the molecular weight is linear. Hence by calibration of a particular column with proteins of known molecular weight an estimate of the molecular weight of another protein can be made to within about 12% (Andrews *et al.* 1964). Other workers, however, find a better correlation between elution volume and Stokes radius (Ackers 1964; Siegel and Monty 1965). In the present work, columns of Sephadex G-200 have been similarly calibrated with *S*-carboxymethyl proteins

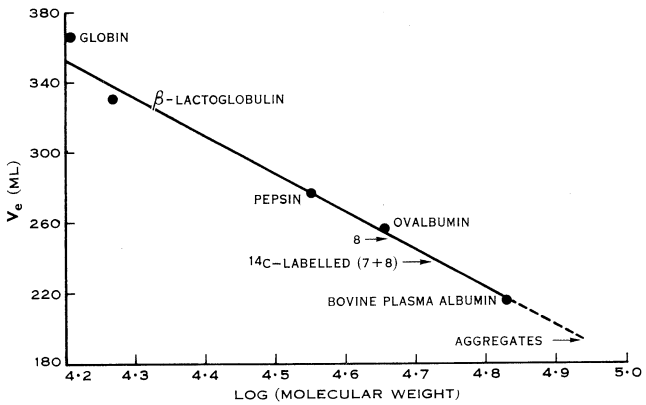

Fig. 4.—Graph of elution volume (V_e) against logarithm of the molecular weight for a series of *S*-carboxymethyl proteins chromatographed on Sephadex G-200 (2·3 cm internal diameter by 128 cm) in 8M urea – 0·001M Versene – 0·1M KCl – 0·05M Tris buffer, pH 7·4. Protein concentrations 0·2–0·8% except for ^{14}C-labelled (7+8) which was 0·02%.

in buffer systems containing the disaggregating agents 8M urea and 5M guandine hydrochloride. Under these conditions the SCM-proteins are all likely to behave as random coils. Figure 4 shows the plots of V_e versus the logarithm of molecular weight for a series of reduced and carboxymethylated proteins of known molecular weight. The molecular weight values taken for the SCM-derivatives of the standard proteins, which are known to be single polypeptide chains or mixtures of chains of the same weight, were: SCM-globin (α plus β chains) 16,000 (Hill *et al.* 1962), SCM-β-lactoglobulin 18,500 (Piez *et al.* 1961), SCM-pepsin 35,500 (Blumenfeld and Perlmann 1959), SCM-ovalbumin 45,000 (Warner 1954), and SCM-bovine plasma albumin 67,000 (Putnam 1965). The behaviour of the SCM-globin and SCM-β-lactoglobulin in urea and guanidine hydrochloride was consistent with the existence of the single dissociated peptide chains. The behaviour of component 8 or mixtures of components 7 plus 8 has also been studied. A range of protein concentrations was

used to see if we could gain any evidence from elution volume values, as distinct from the shape of curve discussed previously, to support the results of DeDeurwaerder and Harrap (1964, 1965). Their results have been interpreted as showing the existence of a reversible dissociation of SCMKA at low protein concentrations in 14M formamide to give a subunit of molecular weight approximately 25,000. Because of the non-ideal behaviour of the SCM-proteins in the solvents used, values of V_e extrapolated to infinite dilution are the best values to use for comparison for estimation of molecular weight. From the V_e values at widely varying concentrations of components 7 plus 8 some non-ideal behaviour was noted but this was not sufficiently different from that of SCM-ovalbumin to suggest that components 7 and 8 differ markedly from other proteins in the degree of non-ideality. Comparison of V_e at 0·5–1% protein concentration (Fig. 4) gives values for the molecular weight of components 7 plus 8 of approximately 45,000 in 8M urea. The corresponding value was 50,000 in 5M guanidine hydrochloride.

Molecular weights for the aggregates of approximately 90,000 in 8M urea and 120,000 in 5M guanidine hydrochloride were calculated from their elution volumes, assuming the curves (e.g. Fig. 4) can be extrapolated for unfolded SCM-proteins of this size. The void volume of the columns was less than the elution volume of the aggregates, suggesting that this extrapolation is justified. These aggregates are heterogeneous and the agreement between these values and the equilibrium ultracentrifugation value (75,000–80,000) is not close, but these values and the chemical evidence provided by almost identical amino acid composition (O'Donnell and Thompson 1964*b*) and peptide maps (Plate 2, Fig. 1) suggest that dimers are the predominant aggregate of component 7.

V. Discussion

From the evidence presented in this paper there is no doubt that components 7 and 8 are distinctly different chemically. The aggregated components 1–4 are formed from component 7 and give a very similar peptide map and amino acid analysis. From the content of basic amino acid residues in component 8 and assuming a single polypeptide chain of molecular weight 45,000, 43 peptides would be expected in a tryptic digest of it. It is difficult to count the number of peptide spots accurately because of weak staining of some of them by ninhydrin and a smearing in the acidic peptide region of the peptide maps. However, the number of peptide spots is of this order. The complexity of the peptide map of component 7 would not be expected to be much greater than that of component 8 since only three extra tryptic peptides would be expected from a single polypeptide chain with its amino acid composition. The map appears to show more spots than this and it is concluded that component 7 is heterogeneous.

In calculating the number of peptides from a tryptic digest of a polypeptide chain of given lysine plus arginine content it should be remembered that six chemically different chains of molecular weight 7,500 would only be expected to give five more peptide spots than a single chain of molecular weight 45,000. For this reason such peptide maps cannot help in fixing the minimum molecular weight of an assembly of chemically different polypeptide chains unless the number of peptides is very small.

It is seen from Plate 2, Figures 2–4, that there appears to be similar peptides in components 7 and 8 but the intensities, i.e. concentrations, show marked differences. This could be due to the fact that components 7 and 8 are not completely disaggregated in 8M urea and each consists of an assembly of polypeptide chains of smaller molecular weight, some of which are either identical or give identical peptides on tryptic digestion. There is some evidence which suggests the existence of subunits in components 7 and 8. Harrap (1956), using a surface balance, has found minimum values of about 8000 for the molecular weights of fractions of low-sulphur proteins from wool. Furthermore DeDeurwaerder and Harrap (1964, 1965) have presented evidence from sedimentation and osmotic pressure data that there is a dissociation of SCMKA to subunits of approximately 25,000. It should be pointed out that SCMKA is less pure than components 7 and 8, as it contains, in addition, aggregated material and some high-glycine–high-aromatic amino acid protein fraction with smaller amounts of high-sulphur proteins [O'Donnell and Thompson (1964*b*) and unpublished data]. The component fractions can be separated by gel-filtration and each isolated fraction runs unchanged and in its original position, showing that it is not aggregating on the column. Nevertheless, in line with this evidence of dissociation, components 7 and 8 might also be expected to dissociate and, because of the implication from the number of peptides in a tryptic digest that the subunits must be different in amino acid sequence, we have attempted to dissociate component 8 and components 7 plus 8 into smaller components by various means. The effect of pH 13 on SCMKA was investigated since this has been shown to produce smaller material. It was found that smaller fragments were produced under such conditions but that peptide bonds were also broken and therefore the effects of alkali in possibly promoting a dissociation cannot be interpreted.

Attempts to get evidence for the existence of subunits of molecular weight 25,000 or less by gel-filtration in the presence of the disaggregating agents 8M urea, 5M guanidine hydrochloride, and 14M formamide were unsuccessful. In the urea and guanidine hydrochloride experiments there was no evidence, even at low protein concentrations, either from the shape of the elution curve or change in V_e to support the idea of a dissociation into smaller molecules. By comparison with reduced and carboxymethylated proteins of known molecular weight the value for the molecular weight of component 8 or components 7 plus 8 is approximately 45,000. Component 7 behaves as if its molecular weight is slightly higher than that of component 8.

When 14M formamide was used there was evidence of a reversible association of components 7 plus 8 but the molecular weight of the smallest unit involved was estimated to be *c.* 50,000. Squire (1964) has developed the exclusion theory of Sephadex gel-chromatography (Porath 1962; Pedersen 1962) and discussed the possible effect of shape factors on the behaviour of a series of protein components. An assumption to which Squire (1964) has drawn attention is that the ratio between the molecular weight and the elution volume is "typical". This can only be assessed if the molecular weight estimated by gel-filtration behaviour is confirmed by classical physicochemical techniques which are not subject to these uncertainties. The molecular weight of component 8 determined from ultracentrifuge data at normal concentrations accords well with the estimates made from its behaviour on Sephadex columns at similar

concentrations and this estimate by gel-filtration did not change at much lower concentrations, even below the concentrations used in the experiments of DeDeurwaerder and Harrap (1965). However, these authors used a different starting material (SCMKA) and Harrap (personal communication) has obtained, by osmotic pressure measurements, values for the molecular weight of our isolated components separated on Sephadex columns consistent with those reported in this paper. A chemical method, e.g. end-group characterization, may throw light on the molecular weight of the minimum units in components 7 and 8. The highest number of end-groups so far reported for low-sulphur wool protein fractions are *N*-acetyl groups, which were present in amounts of 1 mole per 26,000 g of SCMKA and per 33,000 g of α-keratose (O'Donnell, Thompson, and Inglis 1962). Both these fractions contain, in addition to components 7, 8, and aggregates, approximately 10% of contaminant proteins, comprising some rich in glycine and aromatic amino acids and smaller amounts of some rich in sulphur, which can be separated by gel-filtration. These contaminants may have higher *N*-acetyl contents and contribute to the total measured. The analysis of acetyl groups in the purified fractions is being investigated.

Starch-gel electrophoresis of radioactively labelled mixtures of components 7 plus 8 was followed by radioautography of the gel slices but no change in the mobilities of the components was observed at concentrations down to 0·01%. If indeed there are subunits of components 7 and 8 present in SCMKA they must be held together, even in urea, only by non-covalent-type bonds. Dinitrophenylation of SCMKA in 8M urea has shown that all of the side-chain groups of lysine, histidine, and tyrosine are fully substituted since no significant amount of the free amino acids could be detected in the acid hydrolysate of the DNP-protein.

In discussing the origin of components 7 and 8 one question which must be considered is whether they are associated preferentially with either the ortho- or para-segments of the wool fibre. After multiple extractions of wool with alkaline thioglycollate (Gillespie and Lennox 1953) it was shown by Fraser and Rogers (1953) that the more readily extracted material was probably located in the ortho-segment while the later extracts contained material from the para-segments. This extraction procedure of Gillespie and Lennox (1953) has been repeated and the serial extracts re-examined by starch-gel electrophoresis of the carboxymethylated proteins. The results suggest that there is no preferential location of either component 7 or 8 in these ortho- and para-segments. It is also of interest that when SCMK was prepared from cortical cells isolated by the trypsin digestion method of Burgess (1934), the starch-gel electrophoretic pattern did not appear different from that of whole wool. No enzymic degradation of the proteins of the cell could be observed.

A knowledge of the relative amounts of component 7 and component 8 would be of interest in relation to the structural organization of these proteins in the filaments. The methods we have described do not enable precise estimates to be obtained. However, there appears to be considerably more component 7 than component 8, particularly if the aggregates are included in the weight of component 7; the ratio could be of the order of two.

Harrap (1963) has shown that the b_0 value of extracted SCMK wool proteins in buffer containing 8M urea is zero, thus indicating a complete lack of helical regions. On removal of the urea the low-sulphur protein derivatives (SCMKA) return to a conformation with an α-helical content of approximately 50%. Harrap (unpublished data) has also found that components 7 and 8 described in the present work regain a similar α-helical content in aqueous solution. Since measurements of b_0 values on the SCMKB (Harrap 1963) and high-glycine–high-aromatic amino acid fractions (Harrap, personal communication) indicate no helical content present, indications are that components 7 and 8 (and aggregates of them) are the main protein fractions extracted from wool with a helical-forming sequence of amino acid residues.

From X-ray diffraction studies of α-keratin it has been established that a coiled-coil α-helical structure (Crick 1952, 1953; Pauling and Corey 1953) is present. The studies of Fraser and McRae (1961) have refined the earlier models and it would seem that there are either two or three different α-helical chains in the coiled-coil structure (Fraser, McRae, and Miller 1964). The chemical evidence in this paper has not succeeded in revealing the existence, in approximately equal amounts, of two or three different polypeptide chains each of unique chemical sequence which could be assigned to such a two- or three-strand model.

VI. Acknowledgments

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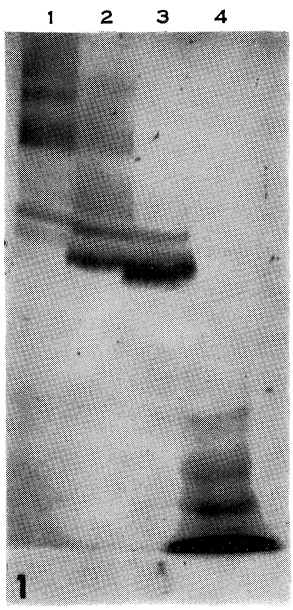
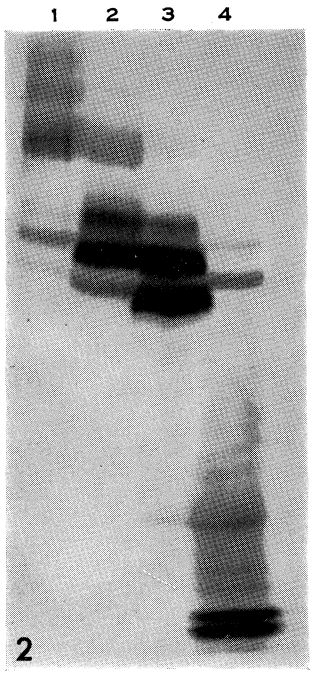
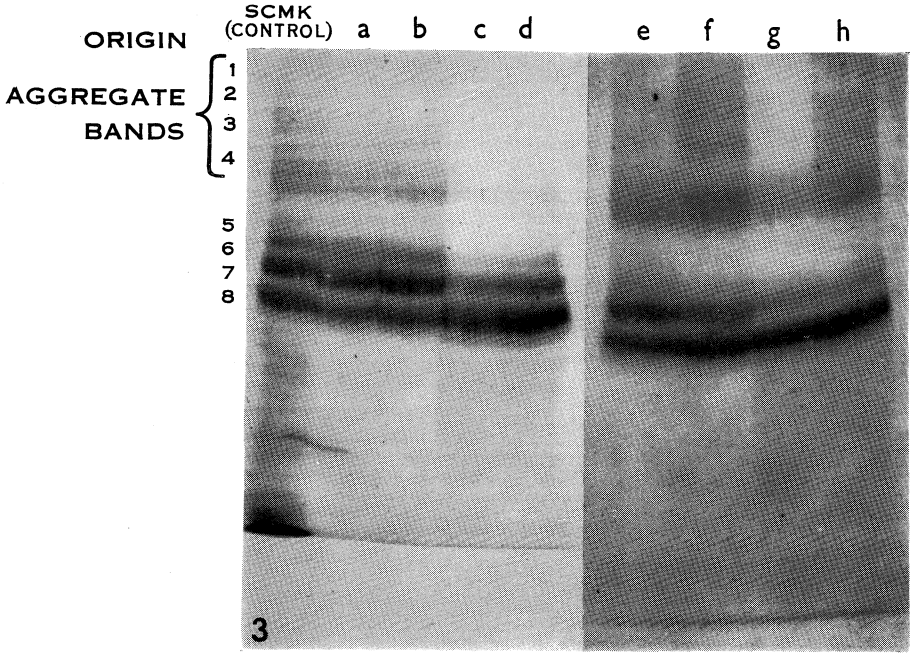
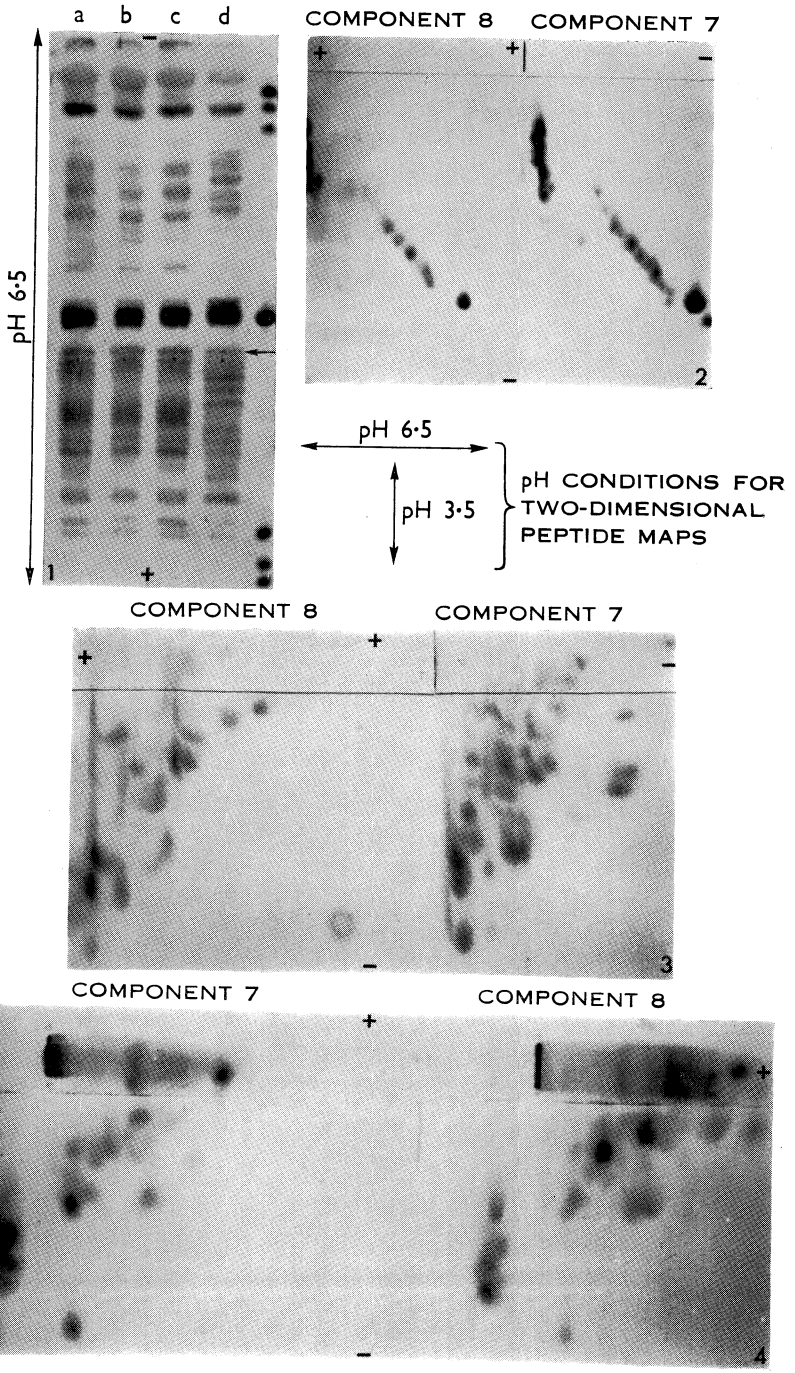
STUDIES ON REDUCED WOOL. V







STUDIES ON REDUCED WOOL. V



Explanation of Plates 1 and 2

Plate 1

Fig. 1.—Starch-gel patterns of protein fractions (concn. *c.* 1%) from the four cuts indicated in Figure 1 obtained from the gel-filtration of fraction E on Sephadex G-200.

Fig. 2.—Starch-gel patterns of protein fractions (concn. *c.* 1%) from the four cuts indicated in Figure 1 obtained from the gel-filtration of fraction D on Sephadex G-200.

Fig. 3.—Starch-gel electrophoresis patterns of mixtures of components 7 and 8 to show formation of aggregates from component 7 on standing in 0·01M borate, pH 9, at room temperature: *a–h*, enriched component 7 at zero time (*a*) and after 28 days (*e*); enriched component 7 plus 0·2M KCl at zero time (*b*) and after 28 days (*f*); enriched component 8 at zero time (*c*) and after 28 days (*g*); enriched component 8 plus 0·2M KCl at zero time (*d*) and after 28 days (*h*).

Plate 2

Fig. 1.—One-dimensional ionophoresis at pH 6·5 of tryptic digests of: *a*, aggregated components 1–4 of SCMK; *b*, mixture of aggregated components 1–4 and component 7; *c*, component 7; *d*, component 8. Arrow indicates origin.

Figs. 2 and 3.—Peptide maps obtained by two-dimensional ionophoresis of the neutral and basic peptides (Fig. 2) and of the acidic peptides (Fig. 3) obtained by tryptic digestion of components 7 and 8.

Fig. 4.—Radioautographs of peptide maps obtained by two-dimensional ionophoresis of the acidic and neutral peptides containing [^{14}C]*S*-carboxymethyl residues obtained by tryptic digestion of components 7 and 8.