THE ISOLATION AND PROPERTIES OF SOME SOLUBLE PROTEINS FROM WOOL

I. THE ISOLATION OF A LOW-SULPHUR PROTEIN*

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Summary

Wool was extracted with 0·1M potassium thioglycollate at pH 11·0 for 2 hr at 50°C and the soluble proteins were precipitated by acidifying to pH 5, redissolved in potassium thioglycollate at pH 10, and reacted with sodium iodoacetate at pH 9 to give a mixture of at least five electrophoretically distinct *S*-carboxymethyl kerateines.

Acidification to pH 4·1 precipitated about two-thirds of the protein, including the major protein, and gave two well-defined fractions. Solution of the precipitate fraction in buffer at pH 11·0 showed, on electrophoresis, one main peak and two smaller peaks. Fractional precipitation of this fraction with acetone gave a low-sulphur protein which gave a single boundary on electrophoresis. The composition and some of the properties of this protein are discussed and compared with those of previously isolated wool proteins.

Previous work in this field has been summarized and a suggested nomenclature for wool proteins isolated by reduction and alkylation is given.

I. INTRODUCTION

Interest in the proteins from wool is increasing following improvements in techniques for the preparation and characterization of these materials and with the recent increase in knowledge concerning their location in the fibre (Rogers 1959*a*) and their sites of synthesis in the follicle (Ryder 1956, 1958). It is now generally accepted that the wool fibre can be separated into sulphur-rich and sulphur-deficient protein fractions as summarized in Table 1. It is probable that the low-sulphur proteins, as typified by *S*-carboxymethyl kerateine A2 (SCMKA2)‡ and α-keratose, originate in the microfibrils or α-filaments which are the fibrous units of structure of wool (Birbeck and Mercer 1957) and that these are embedded in a non-fibrous sulphur-rich matrix from which the high-sulphur proteins can be extracted (Rogers 1959*b*). Recognition that these extracted proteins originate in different histological constituents of the fibre heightens interest in their purification and characterization. Reduction followed by carboxymethylation offers one procedure for the separation of these proteins but much still remains to be done in this field, particularly in the study of the purity of the isolated proteins and of the changes undergone by them during the reduction and alkylation reactions.

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‡ Appendix I presents a summary of the separation of reduced proteins from wool performed in this Laboratory, together with a suggested nomenclature.

Gillespie and Lennox (1953, 1955) isolated a protein from wool by successively extracting Merino 64's top five times with potassium thioglycollate at pH 10·5 and once at pH 11·4. The last extract contained kerateine 2, a low-sulphur protein which was converted to the *S*-carboxymethyl derivative (Gillespie 1956) and purified by fractional precipitation with zinc acetate or ammonium sulphate (Gillespie 1957), giving the protein now termed SCMKA2 (see Appendix I).

TABLE 1

SULPHUR-CONTAINING FRACTIONS OF THE WOOL FIBRE

Method of Extraction	Fractions Obtained		References
	Low-sulphur Fraction (% S)	High-sulphur Fraction (% S)	
Alkaline thioglycollate	3·3	4·5	Goddard and Michaelis (1935)
Cetyl sulphonic acid	4·2	7·5	Lindley (1947)
Peracetic–ammonia	2·4	6·1	Alexander and Earland (1950)
Chlorine dioxide	1·3	5·3	Speakman and Das (1950)
Alkaline thioglycollate	1·5*	6·5	Gillespie and Lennox (1953); Gillespie (1958)
Peracetic–ammonia	1·9	5·8	Corfield, Robson, and Skinner (1958)
Urea extract of "wool roots"	1·7	4·1	Rogers (1959*a*)
Performic acid with pH 8 extraction	1·96†	4·72†	O'Donnell and Thompson (1959) and unpublished data

* As *S*-carboxymethyl cysteine.

† As cysteic acid.

The pH 10·5 extracts were shown to be electrophoretically heterogeneous, and to contain at least five peaks, one of which had a similar mobility to kerateine 2. In a preliminary account, Gillespie (1958) reported the isolation of this material as its *S*-carboxymethyl derivative in a form moving with a single boundary on electrophoresis. In this paper the isolation and properties of this easily extractable low-sulphur protein (SCMKA1) will be described in detail. Together with SCMKA2, the low-sulphur proteins in the pH 10·5 extracts account for about 40 per cent. of the weight of wool. SCMKA1 and SCMKA2 may be different forms of the same protein, but until such time as their true identity has been established it is proposed to keep separate names for them.

II. MATERIAL AND METHODS

(*a*) *Preparation of Soluble Wool Proteins*

In the original procedure used for isolating kerateine 2 the pH 10·5 extractable material was spread over five fractions, all of which were in dilute solution (Gillespie and Lennox 1953). In the present study, where the isolation of these proteins was

desired and not their complete removal from the wool prior to the extraction of kerateine 2, a somewhat different procedure was used. 100 g (air dry weight) of solvent-scoured Merino 64's wool top was soaked for 1 hr at 20°C in 3 l. of 0·1M sodium carbonate of initial pH 11·0. The wool was then roughly dried in a cotton towel and transferred to 3 l. of 0·1M potassium thioglycollate, pH 11·0, at 50°C and kept at this temperature for 2 hr. The final pH was 10·4–10·5 and about 40 per cent. of the wool was dissolved. The solution was filtered, rapidly cooled to 20°C, the pH adjusted to 5, and the precipitated protein was separated by filtration. The precipitate was dissolved in about 1 l. of 0·1M potassium thioglycollate at pH 10 using a Waring Blendor* modified to prevent foaming. The pH was then adjusted to 9, 40 g of iodoacetic acid (pH 6) was added, and the pH continuously adjusted to between 8 and 8·5 until the reaction was complete, as evidenced by the disappearance of both the ferrous thioglycollate colour in the protein solution and the nitroprusside colour reaction. The pH was then adjusted to 7·0 and the mixture of *S*-carboxymethyl kerateines thoroughly dialysed for 24 hr in running tap water. This preparation was used in the studies which follow and will be referred to as SCMK.

(*b*) *Spectrophotometry*

Extinction curves were determined and measurements were made of the optical density of protein solutions in a Beckman model DU spectrophotometer. Throughout this paper optical density measurements at 278 mμ will be used to indicate protein concentration even though it is realized that wool proteins have extinction coefficients ($E^{1\%}_{1\,cm}$) varying at least between 4 and 9, but in fractionation experiments, where the analytical data on the proteins were not known, this procedure provided a useful approximation allowing rapid measurements to be made on dilute solutions.

Partially precipitated SCMK solutions were often quite turbid, rendering optical density measurements unreliable. This turbidity could be eliminated by making an initial dilution with an equal volume of glacial acetic acid. At 278 mμ the absorption by acetic acid is small enough to be neglected.

(*c*) *Solubility Curves*

The effect of protein precipitants on the solubility of these proteins was determined by mixing appropriate amounts of protein solution with precipitant in 40-ml glass-stoppered tubes to give final volumes of 20 ml, after which the tubes were gently rocked at 2°C for 24 hr. In experiments with ethanol–water and acetone–water as precipitants, a temperature of −5°C was used and the concentrations were recorded as per cent. v/v of anhydrous solvent. The insoluble protein was centrifuged off at the equilibration temperature and the concentration of protein in the supernatant was measured spectrophotometrically at 278 mμ. When acetone was used as the precipitant, this was not possible and the biuret procedure of Mehl (1945) was used instead.

* The modified Waring Blendor used had an aluminium vessel with screwed lid (Arthur H. Thomas Catalogue No. 4282–D) with a threaded hole in the top of the lid near one edge arranged so that all air could be replaced by fluid and the vessel then completely sealed.

In experiments designed to study the effect of pH on the solubility of SCMK proteins, a somewhat different approach was used, for when acid or buffer was poured into solutions of these proteins a gelatinous lump was formed which came to equilibrium with the system only very slowly. Addition of these reagents by dialysis was less troublesome in this respect. 5-ml aliquots of 0·5 per cent. protein were dialysed in "Cellophane" tubes against 30-ml quantities of buffers of constant ionic strength but varying pH over the range 1–5. These consisted of 0·05M phosphoric acid, 0·1M acetic acid with sufficient sodium hydroxide to give the required pH, and sufficient sodium chloride to give an ionic strength of 0·2. After rocking at 2°C for 24 hr the contents of the dialysis bags were centrifuged and the protein content of the supernatants estimated.

(*d*) *Electrophoresis*

Electrophoresis was carried out in a standard Tiselius apparatus (LKB) at 1°C for about 3 hr at 15 mA using protein solutions dialysed for at least 24 hr with stirring. Unless otherwise stated a "routine" buffer of pH 11·0 and ionic strength 0·1 and containing glycine (0·103M) and sodium hydroxide (0·1M) was employed.

(*e*) *Ultracentrifugation*

This was originally carried out on the Spinco model E ultracentrifuge at the Commonwealth Serum Laboratories, Parkville, through the courtesy of the Director, Dr. P. L. Bazeley, and Dr. J. O'Dea. The experiments were carried out at room temperature and the protein was dissolved in a buffer at pH 11·0 and ionic strength 0·3 (0·2M sodium chloride plus the glycine–sodium hydroxide buffer, ionic strength 0·1).

(*f*) *Analytical Data*

The determination of total sulphur in the various wool protein fractions was made in the C.S.I.R.O. Microanalytical Laboratory. Residual disulphide and mixed disulphide were estimated by the method of Leach (1959).

III. Results

(*a*) *Preliminary Electrophoretic Studies*

Moving-boundary electrophoresis was used to distinguish between the various proteins in the system. The initial experiments were designed to study the effect of *S*-carboxymethylation on the –SH protein and to determine the best conditions for maximum resolution.

Figure 1*A* shows the electrophoretic patterns of an unsubstituted –SH protein run at pH 11·0 in buffer (glycine 0·1M, thioglycollic acid 0·05M, KOH 0·2M, ionic strength 0·2), and Figure 1*B* the same protein after *S*-carboxymethylation and run in the routine buffer with the addition of 0·1M NaCl. It can be seen that both descending boundaries show similar numbers of components; this appears to be true also of the ascending boundaries, but evaluation is difficult in the latter because of the spike on the leading edge of the –SH protein which resembles those observed by Gillespie and Lennox (1955) in other wool protein solutions.

Figure 2 shows the influence of pH on the resolution of the wool proteins in 0·1 ionic strength buffers. It can be seen that resolution improved as the pH was increased to 11, fewer components being observed at lower pH values (cf. Woods 1959). At pH 12, although the ascending boundary showed better resolution, there was poor correspondence between the boundaries and during dialysis a portion of the protein precipitated from solution. In these figures at least three peaks are visible and in some experiments the large slow peak gave evidence of splitting; however, subsequent fractionation experiments have shown that the system was much more complex than would appear from the electrophoretic data.

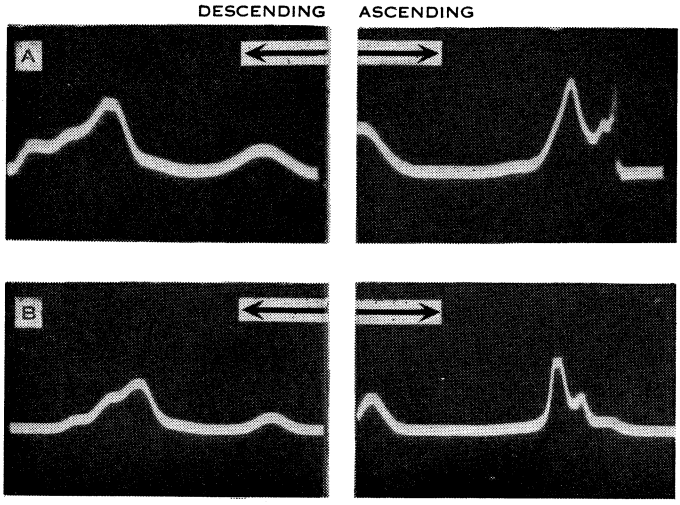

Fig. 1.—Effect of *S*-carboxymethylation on the electrophoretic pattern of the mixed wool proteins. *A*, –SH protein in the pH 11 buffer (glycine 0·1M, thioglycollic acid 0·05M, KOH 0·2M, ionic strength 0·2). Run for 360 min, 2·6 V cm^{-1}. *B*, *S*-carboxymethylated protein in the routine buffer with the addition of 0·1M NaCl. Run for 150 min, 5·2 V cm^{-1}.

Figure 3 shows, for comparative purposes, the pattern obtained by running at pH 11 the *S*-carboxymethyl derivative of the pooled protein from five successive pH 10·5 thioglycollate extracts of wool, made in accordance with the usual procedure employed in preparing kerateine 2 (Gillespie and Lennox 1953). It can be seen that the pattern obtained was qualitatively comparable with that shown in Figure 2*B*, suggesting that the proteins obtained in the single-step extraction are similar electrophoretically to those obtained by the multi-step procedure but are obtained in differing proportions. However, they may differ in their content of –S–S– and bound thioglycollic acid (see Section IV).

(*b*) *Ultracentrifuge Measurements*

SCMK solutions were ultracentrifuged at 59,780 r.p.m. at protein concentrations of 1 per cent. The pattern obtained after 90 min running (Fig. 4) shows the presence of at least two peaks ($S_{20\,w}$ 1·3 and 2·9 respectively). In addition, the

presence of a small amount of rapidly sedimenting aggregated protein was indicated in earlier photographs.

(c) *Solubility Studies*

(i) *Acid Precipitation.*—The effect of pH on the solubility of SCMK was studied over the pH range 1–5 and the results are plotted in Figure 5. It can be seen that about three-quarters of the protein was precipitated sharply as the pH fell from

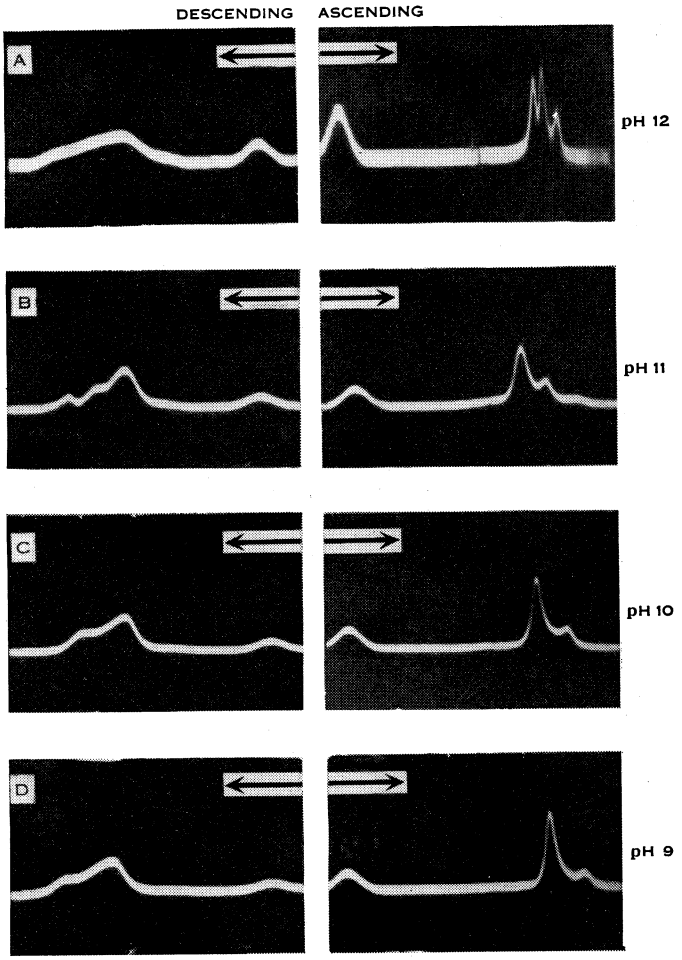

Fig. 2.—Effect of pH on the electrophoresis of SCMK in various buffers of ionic strength 0·1. *A*, pH 12, 135 min, 5·2 V cm^{-1}; *B*, pH 11, 185 min, 4·6 V cm^{-1}; *C*, pH 10, 180 min, 5·4 V cm^{-1}; *D*, pH 9, 190 min, 5·5 V cm^{-1}.

4·7 to 4·5 and then the solubility decreased more gradually as the pH was lowered with the remainder of the protein showing a minimum at about pH 2·9. At lower pH values the solubility increased gradually.

(ii) *Salting Out.*—The solubility of SCMK in ammonium sulphate was measured in the presence of sodium acetate buffer (ionic strength 0·1) at pH 6 (Fig. 6(*a*)). About two-thirds of the protein was precipitated sharply between zero and 0·4M concentration, then followed a zone of little additional precipitation, and finally almost all the remaining protein became insoluble between 1 and 2M ammonium sulphate concentration.

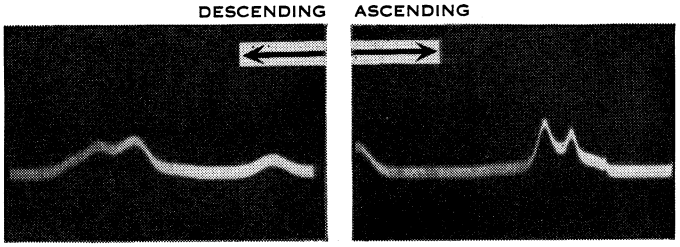

Fig. 3.—Electrophoresis pattern of the *S*-carboxymethyl derivative of the protein obtained by five successive pH 10·5 extracts of wool. Run in the routine buffer at pH 11 for 150 min, 5·3 V cm^{-1}.

(iii) *Precipitation with Ethanol.*—The solubility of SCMK was measured at pH 6 in sodium acetate buffer (ionic strength 0·01) at −5°C with ethanol concentrations ranging from 15 to 70 per cent. The results presented in Figure 6(*b*) show that approximately two-thirds of the protein was precipitated as the ethanol concentration was increased from 30 to 40 per cent. Only small amounts of residual

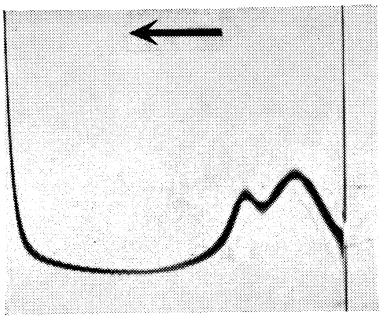

Fig. 4.—Ultracentrifuge pattern of SCMK run at pH 11 in the routine buffer with the addition of 0·2M NaCl.

protein were precipitated even by 70 per cent. ethanol. After removal of ethanol by dialysis, the more easily precipitable protein gave highly turbid solutions at pH 7, whilst the residual proteins showed no turbidity under the same conditions. Sulphur analyses indicated that the precipitate obtained with 40 per cent. ethanol contained low-sulphur protein (2·8 per cent. S), whilst the more soluble proteins were rich in sulphur (6·3 per cent. S).

(iv) *Solubility in the Presence of Zinc.*—At pH 6 in the presence of sodium acetate buffer (ionic strength 0·01), 0·01M zinc acetate precipitated about two-thirds of the protein and the remainder was not precipitated even by increasing the zinc acetate concentration to 0·1M. This gave a very clean separation between the two types of proteins similar to that obtained during the purification of SCMKA2 (Gillespie 1957). But in view of the difficulties associated with the complete removal of bound zinc from the proteins (Gillespie and Springell 1957), this method was not used further.

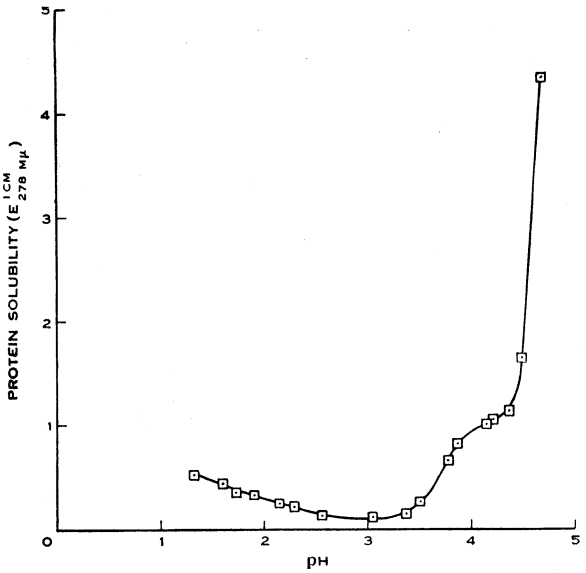

Fig. 5.—Solubility of SCMK as a function of pH in phosphate–acetate buffers, ionic strength 0·2.

(*d*) *Separation of SCMK into Two Main Fractions*

The most successful fractionation of SCMK was obtained by acid precipitation. The procedure followed was to dialyse a 1 per cent. SCMK solution against 10 volumes of 0·1M pH 4·1 acetate buffer (ionic strength 0·1) for 24 hr. The protein which precipitated formed a fibrous rope which had contracted from the walls of the dialysis tubing leaving an annulus of clear fluid. This precipitate was removed and a further quantity of the unprecipitated protein was obtained by washing it in a Waring Blendor with pH 4·1 acetate buffer and then filtering. These two lots of soluble unprecipitated protein, accounting for 30–35 per cent. of original SCMK, were pooled, dialysed, and freeze-dried, and will be referred to as the "pH 4·1 supernatant fraction". The precipitated protein was dissolved at pH 8, precipitated by dialysis, and the precipitate was washed. This procedure was repeated and all traces of the soluble fraction were thereby removed. Finally the "pH 4·1 precipitate fraction" was dissolved at pH 8, dialysed, and stored at −20°C.

The electrophoretic composition of the original protein and of the two fractions was determined using the routine buffer (Fig. 7). The two fractions showed quite

different patterns; the precipitate fraction (Fig. 7*B*) had one peak containing about 60 per cent. of the material and two smaller peaks of faster material, whilst the supernatant fraction (Fig. 7*C*) was more heterogeneous and contained at least four or five components.

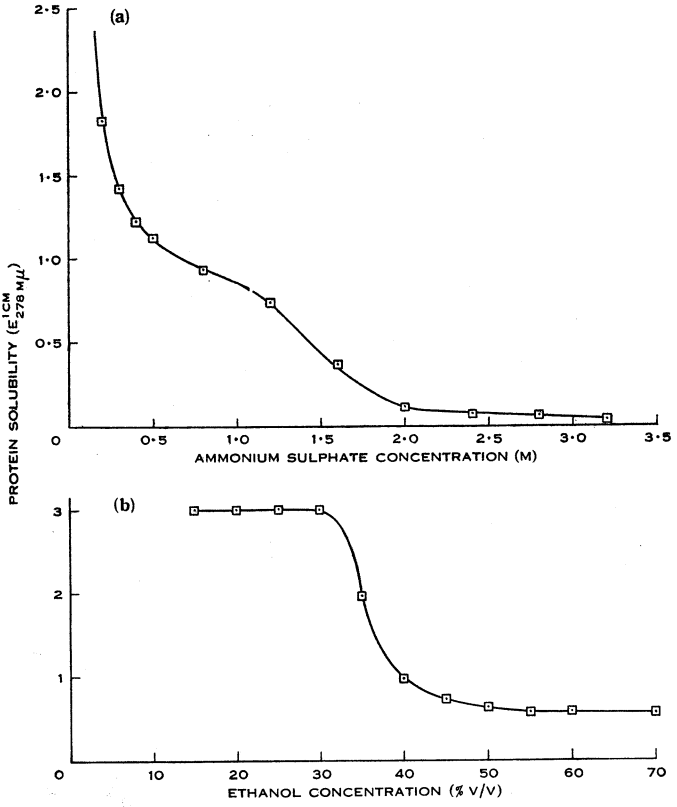

Fig. 6.—Solubility of SCMK at pH 6: (*a*) as a function of ammonium sulphate concentration; (*b*) in acetate buffer of ionic strength 0·01 as a function of ethanol concentration.

Ultracentrifugation of the pH 4·1 precipitate fraction still showed two peaks (Fig. 8) but the amount of material in the slow-moving peak had been much reduced. The approximate sedimentation coefficients $S_{20\,w}$ were now 1·9–2·2 and 2·9 respectively. It is known (Gillespie 1959) that the pH 4·1 supernatant fraction has an $S_{20\,w}$ of 1·0 at a protein concentration of 1 per cent.

These differences were also reflected in the solubilities of the two fractions as measured by salting out and by acid precipitation. Figure 9 shows the pH–solubility relations of the two fractions and it can be seen that the two differ on this basis, the precipitate fraction precipitating sharply as the pH approaches 4·5 and then remaining almost completely insoluble at lower pH values, whilst the supernatant fraction showed a more normal type of solubility curve with a minimum around pH 2·9.

These differences between the fractions were also manifested in salting out experiments as illustrated by the curves in Figure 10. The precipitate fraction was easily salted out whilst the supernatant fraction required higher concentrations of ammonium sulphate. Other differences between these fractions were that the precipitate fraction was comparatively low in sulphur (2·7 per cent.), almost insoluble after freeze-drying, and gave solutions the turbidity of which increased with time.

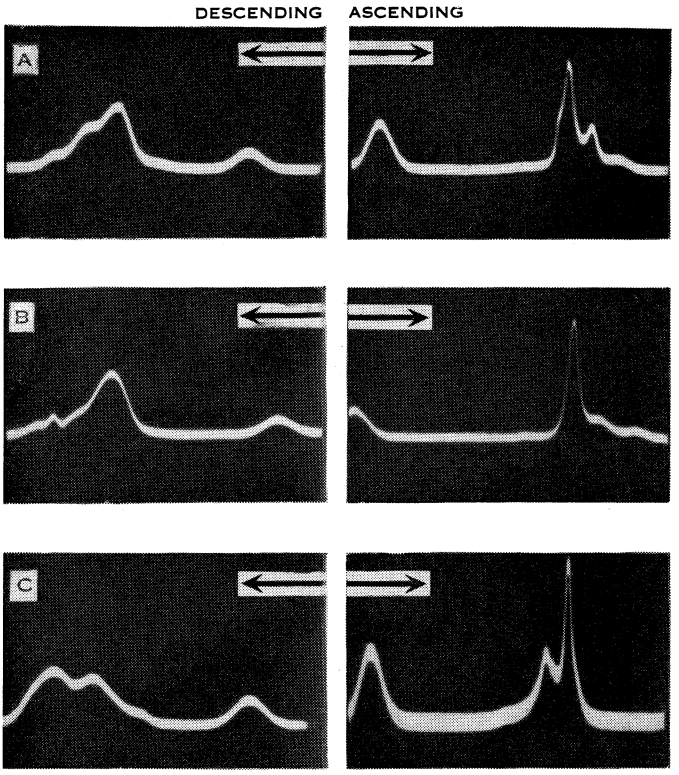

Fig. 7.—Fractionation of SCMK by acid precipitation measured by electrophoresis in the routine buffer at pH 11: *A*, unfractionated SCMK, 180 min, 5·2 V cm^{-1}; *B*, precipitate fraction, 180 min, 5·3 V cm^{-1}; *C*, supernatant fraction, 140 min, 5·3 V cm^{-1}.

On the other hand, the supernatant fraction contained a high concentration of sulphur (6·5 per cent.), gave permanently clear solutions suggesting little aggregation or small molecular weight, and was completely soluble after freeze-drying. A preliminary report on the separation of the proteins in this fraction has already been published (Gillespie 1959).

(*e*) *Purification of the pH* 4·1 *Precipitate Fraction*

The next stage of the fractionation procedures was designed to obtain the main peak protein in this fraction in an electrophoretically single-boundaried form. This

proved to be a difficult problem for the differences between the components were very small and they overlapped in most of their solubility relations.

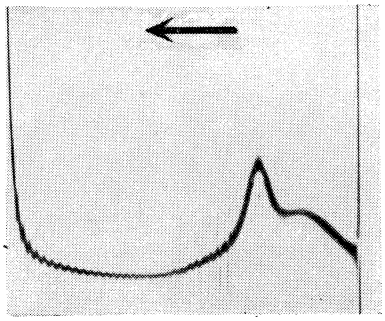

Fig. 8.—Ultracentrifuge pattern of the pH 4·1 precipitate fraction run at pH 11 in the routine buffer with the addition of 0·2M NaCl.

(i) *Salting Out.*—The proteins in this fraction were relatively easily precipitated at pH 6 even from solutions of univalent ions, and a study was undertaken of their

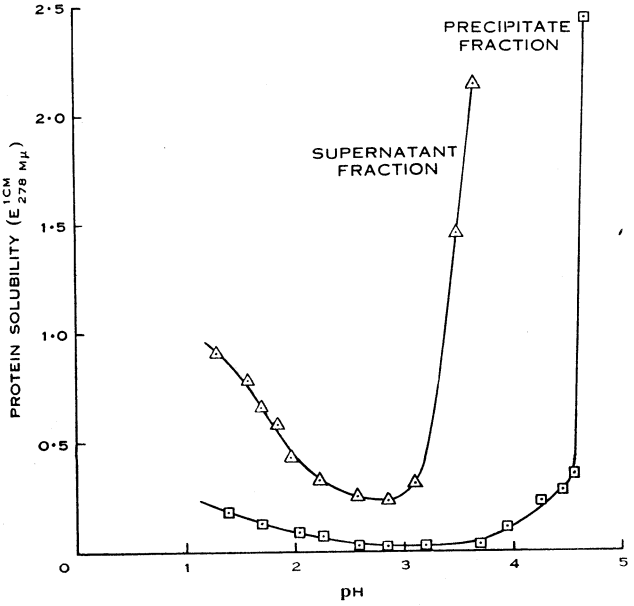

Fig. 9.—Solubility of the pH 4·1 supernatant and precipitate fractions of SCMK as a function of pH in phosphate–acetate buffers of ionic strength 0·2.

solubility in these solutions. Solubility curves at pH 6 (Fig. 11), using sodium chloride and sodium acetate–acetic acid as the precipitants, show that sodium chloride was less effective as a precipitant than ammonium sulphate (Fig. 10) and,

unexpectedly, that sodium acetate–acetic acid was more effective when compared on an ionic strength basis than sodium chloride and almost comparable with ammonium sulphate.

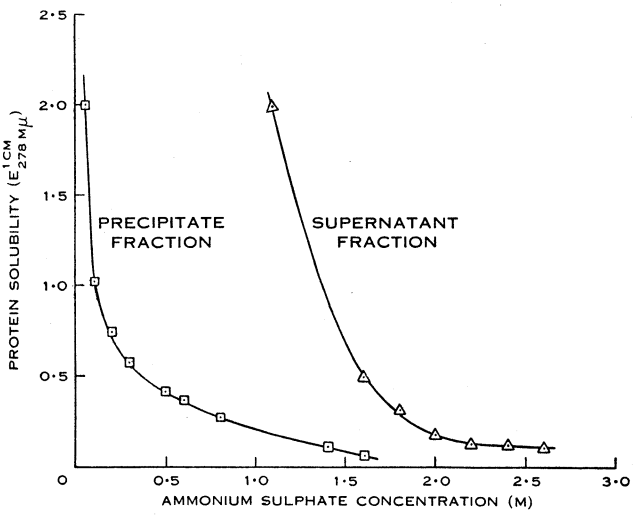

Fig. 10.—Solubility of the pH 4·1 supernatant and precipitate fractions of SCMK as a function of ammonium sulphate concentration.

Both these solubility curves (Fig. 11) in salt solutions showed a sharp initial precipitation and then a levelling off at about 1·5M sodium chloride concentration

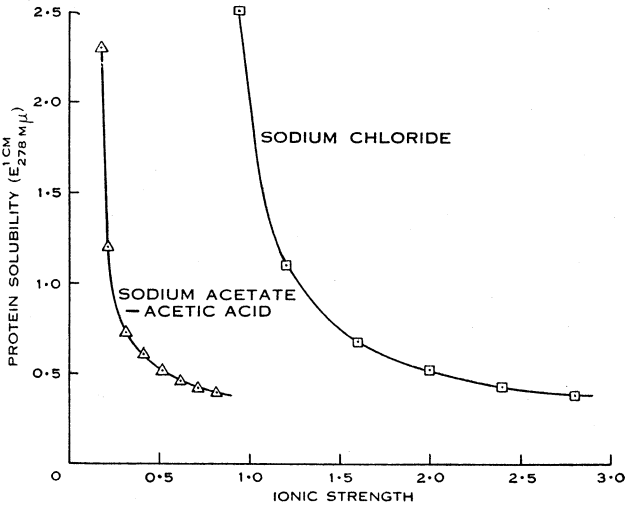

Fig. 11.—Solubility of the pH 4·1 precipitate fraction of SCMK at pH 6 as a function of ionic strength.

and at an ionic strength of 0·3 for sodium acetate–acetic acid. Cuts were therefore made at these concentrations and also at a number of other concentrations on either

side, but electrophoretic analysis showed that only poor separations had been achieved. The best results were obtained at pH 6 with a 0·2 per cent. protein concentration and with a sodium acetate–acetic acid buffer concentration of ionic

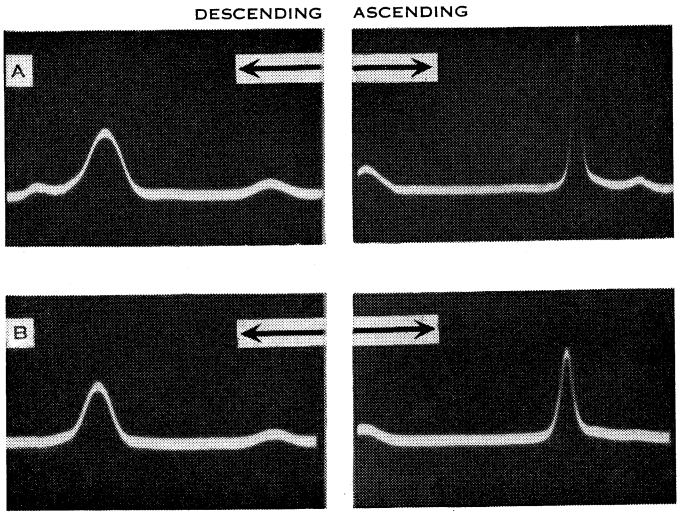

Fig. 12.—Fractionation of the pH 4·1 precipitate fraction measured by electrophoresis in the routine buffer at pH 11: *A*, salting out with sodium acetate–acetic acid buffer at pH 6, 180 min, 5·2 V cm^{-1}; *B*, ethanol precipitation, 180 min, 5·4 V cm^{-1}.

strength 0·195. However, as Figure 12*A* shows, the major protein component was not pure.

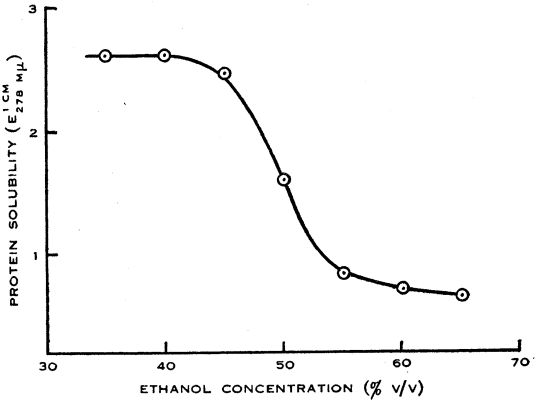

Fig. 13.—Solubility of the pH 4·1 precipitate fraction of SCMK at pH 6 as a function of ethanol concentration.

(ii) *Precipitation with Ethanol.*—The low-temperature, low-ionic strength procedures of Cohn *et al.* (1946, 1950) were employed in the hope that their superior resolving power over salting-out procedures would enable the proteins to be sep-

arated. Precipitation by ethanol was measured at —5°C in sodium acetate–acetic acid buffer at pH 6 and ionic strength 0·01 with a protein concentration of 0·2 per cent. From the results in Figure 13 it can be seen that most of the protein (80 per cent.) was precipitated over a very narrow range of ethanol concentration. Unfortunately the extent of precipitation was not reproducible; for example, in experiments with the same protein preparation under identical conditions of pH, temperature, ionic strength, protein concentration, rate of ethanol addition, and equilibrium time a certain degree of precipitation could be achieved with 50 per cent. ethanol

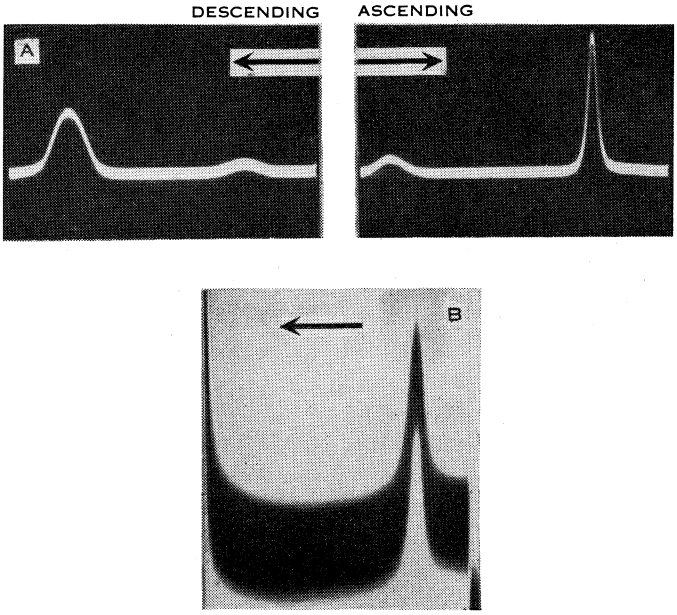

Fig. 14.—*A*, electrophoresis of SCMKA1 in the routine buffer at pH 11. Protein concentration 1·0 per cent., 180 min, 5·4 V cm^{-1}. *B*, ultracentrifugation of SCMKA1 at pH 11 in the routine buffer with added 0·2M NaCl. Protein concentration 0·9 per cent.

on one day, whereas on the next day 40 or 60 per cent. ethanol may be necessary for the same result. There were no obvious reasons for this behaviour, but the appearance of the protein precipitates suggested that they might be coacervates, for they were very bulky and almost transparent. It was found very difficult to obtain partial precipitation of the protein using ethanol. However, when this was accomplished a fairly successful fractionation was obtained as shown by electrophoresis (Fig. 12*B*).

(iii) *Acetone Precipitation.*—The difficulties encountered when using ethanol were not experienced with acetone which gave normal precipitates and completely reproducible results from one experiment to another. The conditions of precipitation were the same as for ethanol. The protein was precipitated twice with an acetone concentration of about 50 per cent. v/v, the first arranged to precipitate about 65 per cent. of the protein and the second 90 per cent. The precipitated protein was dis-

solved and dialysed between precipitations. Because of the steepness of the precipitation curve it was found necessary to add the last 10 per cent. of acetone in several successive portions, estimating the protein concentration in the supernatant at the end of each addition in order to assess the extent of precipitation. For this type of comparative work the biuret test was used with no interval between mixing and reading. Under these conditions a fraction was obtained designated SCMKA1 which, when analysed electrophoretically showed only a single boundary (Fig. 14*A*). The mobility of this protein measured at a protein concentration of 1 per cent. in the routine buffer was $7{\cdot}7\times10^{-5}$ cm^2 volt^{-1} sec^{-1} (cf. SCMKA2 $7{\cdot}2\times10^{-5}$ cm^2 volt^{-1} sec^{-1} (Woods 1959)).

When SCMKA1 was ultracentrifuged at 0·9 per cent. protein concentration in the routine buffer (pH 11) with the addition of 0·2M sodium chloride there was only one peak with a sedimentation coefficient of 2·5 (cf. SCMKA2 = 3·2 (O'Donnell and Woods 1956)) with no evidence of major impurity (Fig. 14*B*). However, the instrument used had a thick schlieren bar which gave a broad baseline and could possibly have obscured small amounts of impurities.

Although quite soluble at neutral pH, the solutions were very turbid and this opacity disappeared sharply as the pH was raised to between 10 and 11. It was at first thought that this might be due to the presence of a minor component of limited solubility because it could be centrifuged down in a Spinco model L centrifuge at 140,000 *g*. However, this is unlikely for, in a preliminary experiment at pH 7 to measure the size of the protein by light-scattering techniques at room temperature, the turbidity of a carefully clarified solution (centrifuged at 140,000 *g*) was observed to increase continuously with time; it had doubled in 2 hr and was visibly turbid after 18 hr. Providing the clarified solution was kept near or below its freezing point it remained clear for long periods.

This propensity for aggregation made physicochemical studies difficult. In electrophoresis experiments, for example, single peaks were obtained in both boundaries only at protein concentrations of about 1 per cent. or less. At higher concentrations, although the ascending limb retained a single peak, the descending limb showed the appearance of a hump, the size of which is increased in relation to the main peak with increasing protein concentration.

(*f*) *Amino Acid Composition of Purified Fractions*

Analysis of the pH 4·1 precipitate fraction and SCMKA1 by the ion-exchange column method (Table 2) reveal that the minor components removed by acetone fractionation must have a quite different amino acid composition from either SCMKA1 or SCMKA2, notably in having a much higher content of *S*-carboxymethyl cysteine (SCMC) and tyrosine. In spite of these and other differences they cannot be differentiated by acid precipitation and fall into the class of proteins precipitating at pH 4·1.

IV. Discussion

It is evident that SCMKA1 and SCMKA2 both fall into the class of proteins of lower sulphur content than whole wool and are possibly derived from the micro-

fibrils. There are many similarities between the two proteins, but there are differences, e.g. solubility, electrophoretic mobility, and sedimentation coefficient. It should be stressed that many of these properties are dependent on the extent of aggregation and possible chemical modification during the extraction procedures used. Physicochemical criteria of purity and homogeneity are difficult to apply to aggregating–disaggregating systems such as the wool proteins. It is not possible at present to establish whether these preparations are in fact distinct proteins or

TABLE 2

AMINO ACID ANALYSIS OF WOOL FRACTIONS

Amino Acid	pH 4·1 Precipitate Fraction* (μmoles/g)	SCMKA1 (μmoles/g)	S. E.	SCMKA2† (μmoles/g)	S. E.
S-Carboxymethyl cysteine	684	455	19·2	452	13·2
Aspartic	583	907	40·9	806	28·9
Threonine	446	471	27·7	415	19·2
Serine	795	801	40·9	721	28·9
Glutamic	888	1467	56·6	1243	39·7
Glycine	1075	702	33·7	600	24·1
Alanine	451	665	21·7	585	15·6
Valine	581	583	33·7	437	24·1
Isoleucine	228	336	13·2	330	9·6
Leucine	717	988	37·3	862	26·5
Tyrosine	538	393	20·5	313	14·4
Phenylalanine	288	249	21·7	248	15·6

* This contains SCMKA1 and other proteins.

† Average results for several preparations.

slightly different forms of the one protein. Possible future lines of approach to this problem include the application of ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose using media favouring disaggregaton. The most interesting differences are in the partial amino acid composition given in Table 2, which shows that in their contents of glutamic acid and tyrosine they differ significantly at the 1 per cent. level.

SCMKA1 contains some disulphide (0·14 per cent. S) probably partly as mixed disulphide and partly as cystine whereas SCMKA2 usually has none as measured by the procedure of Leach (1959) or by a qualitative nitroprusside test in the presence of cyanide. Table 3, taken from Human and Springell (1959), shows the contribution bound thioglycollate makes to this residual disulphide. The value of the re-solution in thioglycollate step in reducing the amount of mixed disulphide is quite obvious and it can also be seen that SCMK is comparable in this respect to the similar mixture of proteins produced by the five-step procedure.

At least 40 per cent. of the wool fibre is thus composed of proteins with a sulphur content less than half that of wool. As the 30–35 per cent. of wool left after exhaustive thioglycollate extraction has an amino acid composition not greatly different from that of wool itself (Gillespie and Simmonds, unpublished data), much of the wool sulphur must be concentrated in other fractions such as the pH 4·1 supernatant. If the thioglycollate extraction procedures remove pre-existing proteins from the fibre, then this observation is consistent with the existence of two histologically distinct classes of proteins in the wool fibre.

TABLE 3

THIOGLYCOLLIC ACID CONTENT OF SCMK PREPARATIONS

Method of SCMK Preparation	Thioglycollic Acid Content (μmoles/g protein)	S.E.
As prepared in this paper but without the steps involving acid precipitation followed by re-solution in alkaline thioglycollate	72·5 (mean of eight determinations)	5·6
As prepared in this paper	41 (mean of six determinations; lowest value 21)	6·5
Pooled five successive pH 10·5 extracts	33·0 (one determination)	—

It is of interest to compare the results presented here with those of Goddard and Michaelis (1934, 1935) who extracted wool with 0·5M sodium thioglycollate at pH 12 (initial pH) for 3 hr, obtaining about 60 per cent. of the wool in solution, and then coupled the dissolved proteins with iodoacetate. Their yield of protein was comparable with that obtained by Gillespie and Lennox (1955) using either one pH 12·6 extraction or a series of five successive pH 10·5 extractions followed by one of pH 12·2 (all initial pH values). Goddard and Michaelis thus had in their extracts, amongst others, the low-sulphur proteins SCMKA1 and SCMKA2 and the sulphur-rich proteins. They fractionated the extracts by salting out with ammonium sulphate from an 0·1M sodium acetate solution, probably at about pH 7·5, thereby obtaining two fractions: fraction A precipitating at 35 per cent. saturation (1·4M) and fraction B at 60 per cent. saturation (2·4M). Figure 15 shows a solubility curve in ammonium sulphate at pH 6 of the *S*-carboxymethyl derivative of a pH 12·6 (initial pH) thioglycollate extract of wool similar to that studied by Goddard and Michaelis. This shows points of inflexion near the ammonium sulphate concentrations used by these workers. Allowing for the greater solubility of the proteins at pH 7·5, their fraction A (3·3 per cent. S) would correspond to a mixture of SCMKA1 and SCMKA2 and other proteins precipitating at pH 4·1. All these proteins are characterized by virtual insolubility below the isolectric point except under certain special conditions. Their fraction B (4·5 per cent. S) would correspond to the high-sulphur protein mentioned in this paper and to the zinc-soluble fraction obtained during the purification of

SCMKA2. The isoelectric point found for fraction B is very similar to the pH of minimum solubility (2·9) reported for the pH 4·1 supernatant fraction protein (Gillespie 1958). Fraction A contained more sulphur than α-keratose (2·4 per cent.) (Alexander and Earland 1950) or SCMKA2 (1·5 per cent. as SCMC); in fact, fraction A had much the same content as the original wool. A possible explanation for these differences is that the "cystine S" recorded in their analysis is in fact mixed disulphide and that some sulphur is incorporated in an unknown form as found by Simmonds (1955), Gillespie (1958), and Human and Springell (1959). However, it should be noted in this connection that not all the pH 4·1 insoluble proteins are low in sulphur for, as Table 1 shows, the minor components removed from the pH 4·1 precipitate fraction during the acetone fractionation step were higher in sulphur than the purified protein,

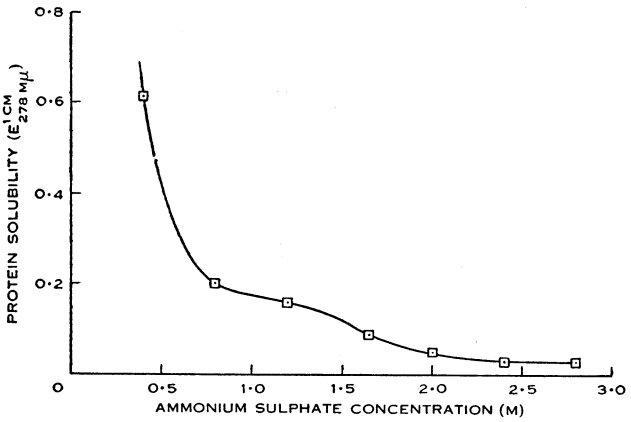

Fig. 15.—Solubility in ammonium sulphate at pH 6 of the *S*-carboxymethyl derivative of a pH 12·6 thioglycollate extract of wool.

the SCMC dropping from 684 to 450 μmoles/g protein during the separation. There appears to be a group of proteins precipitating at pH 4·1 but differing at least in sulphur content. Their fraction B contains a higher concentration of sulphur than does wool, but not as high a concentration as the pH 4·1 supernatant fraction obtained in the present study nor as high as γ-keratose (6·1 per cent.) and it seems likely that the differences found in this Laboratory between the pH 4·1 precipitate fraction and fraction A on the one hand and between the pH 4·1 supernatant fraction and fraction B on the other lie in the imperfect condition obtained by ammonium sulphate salting out as compared with isoelectric precipitation.

In some preparations of SCMKA1 some sulphur is unaccounted for on the basis of the content of sulphur-containing amino acids. This has been previously observed by Fraenkel-Conrat (1942) in experiments on the reduction by thioglycollate of the lactogenic hormone and by Simmonds (1955) in his analyses of wool kerateine. Part of this sulphur is probably mixed disulphide due to incomplete reduction of the protein or to partial oxidation in later handling, as shown by Springell (1958) and Human and Springell (1959) using ^{35}S-thioglycollate. The remainder may come from a contaminant in the thioglycollate, or be produced from it during the extraction,

which combines chemically with the wool. The work of Schoberl (1948) and White (1959) showing that thioesters contaminating thioglycollic acid can condense with the ϵ-amino groups of lysine residues in proteins to introduce new –SH groups is very relevant to this problem and may in fact provide the explanation. All these results serve to emphasize the desirability of carefully purifying thioglycollic acid before using it for the reduction of proteins.

The interpretation of these facts, in the light of knowledge concerning the bilateral structure of wool now available, is difficult. Fraser and Rogers (1953) originally considered that the first fractions of protein extracted from wool by alkaline thioglycollate had their origin in the ortho segment whilst the more difficultly extractable material, e.g. SCMKA2, originated in the para region of the fibre. However, Alexander and Smith (1956) in discussing this problem considered that, in so far as their peracetic–ammonia process was concerned, the difference between the two segments lay in their different proportions of fibrillar and matrix protein rather than in having distinct types of proteins. The recent work of Rogers (1959*b*) would seem to confirm this view.

If the view of these workers is correct it seems difficult to explain the observations we have made on the partial extraction of wool proteins with thioglycollate (Gillespie and Lennox 1955). When wool was extracted with pH 10·5 thioglycollate several extractions were required for the pH to reach 10·5 and there was an increase in the amount of protein extracted in the first two extractions but thereafter the amount in each succeeding extract diminished and after the fifth virtually no more protein came out of the fibre, even though almost all the cystine had been reduced by the end of the third extraction. However, if the pH was raised to about 11·4, further protein could be removed in a series of extractions until a limit was reached again. In order to extract further quantities of protein it was necessary to raise the pH once more or to add a reagent such as urea. Harrap (unpublished data) found that if wool was treated once with 0·5M thioglycollate at pH 10·5, all the thioglycollate-extractable material could be obtained in one operation by washing the fibres with a large volume of distilled water. Microscopic examination showed that the fibres and membranes were disrupted, probably by osmotic action thereby allowing the proteins to escape. This suggests that an important step in the extraction process may be membrane rupture. If SCMKA1 and SCMKA2 are not in different locations in the fibre then they may be contained in membranes of differing degrees of resistance to alkali. As some high-sulphur protein is released with both SCMKA1 and SCMKA2 this implies that both microfibril and matrix are enclosed in these membranes of varying strength. Mercer (1953) suggested that the bulk of the protein occurred as "small packets", each enclosed in a non-keratinous cortical cell membrane which was resistant to reduction but was susceptible to attack by alkaline solutions. Whilst intact or unswollen these membranes would certainly restrict the passage of proteins and if there is a gradation in resistance to alkali, then this could explain our observations.

There is an alternative interpretation of the extraction phenomena: that the matrix–microfibrillar protein complex occurs in different states of molecular organization or different degrees of packing. It is known that only a portion of the wool

fibre is crystalline, estimates varying from 10 per cent. to a maximum of something less than 50 per cent. (Alexander and Hudson 1954; Fraser and MacRae 1957*a*, 1958). Alexander and Hudson suggest that by analogy with the synthetic fibres, the individual micelles of the wool fibre also contain crystalline regions. Presumably this could affect the rate of extraction and the pH and time required to produce a necessary degree of swelling. The well-known differing rates of solubility between the amorphous and crystalline forms of the same protein illustrate the important influence of these two states. The only evidence supporting this view is the observation by Fraser and MacRae (1957*b*) that the residue remaining after wool had been extracted with alkaline thioglycollate under conditions which removed only part of the soluble proteins had a higher density and, presumably, higher crystallinity than the original fibre. These workers point out, however, that the interpretation of their density measurements on partly extracted wools may be open to some question because of possible variations in accessibility to the molecules of the immersion liquid. Yet the results as they stand suggest that the more easily extractable proteins originate in the less crystalline regions of the fibre.

It has been observed in this connection (Gillespie and Simmonds, unpublished data) that the matrix proteins in the native state are basic and this basicity increases with difficulty of extraction, whilst the accompanying microfibrillar proteins are slightly acidic. The matrix–microfibrillar complex isolated at pH 10·5 must be less basic than the material isolated at pH 11·4, and this implies that this latter complex requires a higher net charge by discharge of basic groups before it can be broken into its constituent proteins.

The work of Ryder (1956, 1958), which shows that a considerable proportion of the sulphur in the fully formed wool or hair fibre is introduced at a point in the follicle immediately above the bulb, suggests that the high- and low-sulphur proteins may be produced at separate times and sites and that they may have no covalent bonding between them. This is supported by the work of Lindley (1947) and Blackburn (1959) who were able to separate high- and low-sulphur proteins by methods not involving disulphide bond fission.

With the recognition that the widely used peracetic and performic oxidative methods of disulphide bond rupture also modify proteins in other ways (Corfield, Robson, and Skinner 1958; Moore *et al.* 1958) the study of the alternative method of reduction followed by alkylation has received a new impetus. The role played by thioglycollic acid in introducing new –SH groups into proteins has already been discussed. In addition, however, Michaelis and Schubert (1934) have shown that it is possible for iodoacetate to carboxymethylate the ϵ-amino groups of lysine residues and this reaction has been studied in detail using bromoacetate (Korman and Clarke 1956). The latter workers and Moore *et al.* (1958), and Gundlach, Stein, and Moore (1959) have also shown that iodoacetate can also react with tyrosine and histidine residues. Examination of amino acid analyses made in this Laboratory on soluble proteins from wool such as SCMKA1 and SCMKA2 has failed to reveal the presence of any of the expected modified amino acids. It may well be that our reaction conditions, employing as they do temperatures below 20°C and pH values below 9 for the alkylation reaction followed by dialysis at pH 7, do not favour the

formation of these reaction products. Alternatively, the appropriate residues of wool may be less reactive than those in other proteins.

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APPENDIX I

SUMMARY OF NOMENCLATURE OF PROTEINS EXTRACTED FROM WOOL

It seems desirable at this stage to simplify and clarify the nomenclature of proteins extracted from wool by reduction and subsequent protection of the liberated –SH groups, for example by alkylation. The method used in earlier papers from this Laboratory of numbering on the basis of electrophoretic mobility was sufficient for a simple system but the growing complexity now evident and the isolation of proteins with similar mobilities necessitates some revision of this method. Alexander and Earland (1950) made use of a preliminary subdivision of proteins extracted from oxidized wool on the basis of sulphur content and termed these respectively α- and γ-keratoses. Some years previously Goddard and Michaelis (1935) had named their low-sulphur protein fraction "A" and the high-sulphur fraction "B". In view of the now evident histological localization of these two types of protein, this system would appear to have a good deal of merit. It is proposed therefore to convert to this system, so that all reduced proteins with a sulphur content equal or lower than wool will be termed "kerateine A" and those higher than wool "kerateine B", with the corresponding derivatives, in the case of alkylation with iodoacetic acid, *S*-carboxymethyl kerateine A and B respectively. Then in an arbitrary fashion the individual proteins can be labelled by inserting a number after the letter.

Using this revised nomenclature it is proposed that *S*-carboxymethyl kerateine 2 (SCMK2) be now called *S*-carboxymethyl kerateine A2 (SCMKA2) and the readily extractable low-sulphur protein described in this paper *S*-carboxymethyl kerateine A1 (SCMKA1).

Table 4 summarizes the present knowledge of fractionation of the reduced wool proteins.

TABLE 4

FRACTIONATION OF WOOL (MERINO 64's) BY FRACTIONAL EXTRACTION WITH ALKALINE THIOGLYCOLLATE FOLLOWED BY REACTION WITH IODOACETATE