THE ISOLATION AND PROPERTIES OF SOME SOLUBLE PROTEINS FROM WOOL

II. THE PREFERENTIAL EXTRACTION OF HIGH-SULPHUR PROTEINS

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Summary

A study has been made of the preferential extraction of high-sulphur protein components from wool by alkaline solutions of potassium thioglycollate. It was found that in general the high-sulphur proteins were extracted in quantity at lower values of pH, temperature, and time than the low-sulphur proteins. The extraction of low-sulphur proteins was repressed by small increases in salt concentration and by the addition of divalent cations such as zinc and calcium, whereas the solubility of the high-sulphur proteins was much less affected by these additions.

When wool was extracted with 0·8M potassium thioglycollate at pH values between 10 and 10·5, the extraction of low-sulphur proteins was almost completely suppressed and a high yield of high-sulphur protein was obtained.

I. INTRODUCTION

During previous studies (Gillespie 1958, 1960*a*), proteins extracted from wool with alkaline solutions of potassium thioglycollate and then alkylated and fractionated into low- and high-sulphur components (containing 2–2·5 and 5·8% S respectively) were observed to yield less of the high-sulphur protein than would have been expected from the amount of γ-keratose (i.e. oxidized high-sulphur protein) obtained from oxidized wool (Alexander and Hudson 1954; Corfield, Robson, and Skinner 1958). As little was known about the best conditions for isolating these high-sulphur proteins from reduced wool, a systematic study has now been made of variables such as pH, temperature, and concentration of extractant likely to influence this procedure.

Methods were also sought for preferential extraction of these proteins similar to those available for the preferential extraction of γ-keratose from oxidized wool (Corfield, Robson, and Skinner 1958; Burley and Horden 1959; Rogers 1959). The availability of high-sulphur protein in the –SH form, substantially free from low-sulphur protein, could open the way for the production of alkylated derivatives with positive charges on the sulphur side-chains (by alkylating with β-bromoethylamine (Lindley 1956)) or with no charge by alkylating, for example, with iodoethanol, methyl iodide, or iodoacetamide and for the re-formation of disulphide-containing proteins.

There was some previous evidence that high-sulphur proteins could be preferentially extracted from reduced wool in Simmonds and Stell's (1956) analysis of "extract A" of Gillespie and Lennox (1955). Protein, prepared by extracting wool

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at 50°C with 0·1M thioglycollate at pH 9·9 for 20 min, was shown to contain more sulphur than wool and also to be richer in serine and threonine residues.

As previous work has shown that these high-sulphur proteins are electrophoretically very heterogeneous (Gillespie 1959, 1960*b*) some attention has also been paid to the preferential isolation of individual components.

Conditions of extraction were chosen such that less than 50% of wool substance was extracted, for, although it was possible to extract larger amounts of protein by using more alkaline solutions of thioglycollate, it was thought that this might cause side reactions such as racemization and amide hydrolysis. When required, the more difficultly extractable protein components of wool were obtained by incorporating urea in the alkaline thioglycollate extractant.

II. Materials and Methods

(*a*) *Wool*

The slubbing used was from dry-combed top prepared from degreased Merino 64's wool (MW 127). This was washed in three changes of petroleum ether, in ethanol, water, again in ethanol, and was finally allowed to dry and equilibrate at 68°F and 70% R.H. Aliquots of wool were weighed in a constant-temperature humidity room and moisture determinations were made on the wool used in each experiment.

(*b*) *Reagents*

A.R. grade thioglycollic acid was distilled under vacuum (20 mm) and the fraction boiling at 107°C collected and stored at −20°C. Solutions were made in freshly boiled and cooled distilled water and adjusted to the required pH by adding potassium hydroxide. Ammonia (0·175M) was added to the solutions to react with any residual thioesters.

Lithium bromide solutions were freed of a purple impurity by adding a few crystals of sodium thiosulphate, the slight excess of the thiosulphate also serving to remove bromine produced during heating.

Colourless iodoacetic acid (Light & Co. Ltd.), was stored at −20°C and dissolved immediately before use.

(*c*) *Optical Density Measurements*

These were made in a Beckman DU spectrophotometer. The extinction coefficients of representative high- and low-sulphur fractions were measured, using solutions of concentration known from dry weight determination (Armstrong *et al.* 1947).

The principal value of the optical density method of protein determination has been its convenience and rapidity in making comparative studies of various methods of extracting the proteins.

(*d*) *Extraction of Wool Proteins*

Unless otherwise stated 1-g samples of wool were immersed in 30 ml of extractant in glass-stoppered test tubes. To wet the wool thoroughly the tubes were evacuated

with a water-pump, then thoroughly shaken and allowed to stand at the required temperature for the appropriate time with occasional swirling. The final pH was measured and undissolved wool removed by filtration on a Buchner funnel under vacuum. It is this final pH value which is referred to in subsequent sections. If the weight of residual wool was needed, the residue was washed with five 20-ml aliquots of distilled water, and dried to constant weight at 105°C.

(*e*) *Conversion to* S-*Carboxymethyl Kerateines* (*SCMK*)

To 20 ml of the extract was added 10 ml of a solution containing 0·95 g iodoacetic acid and 2·0 g trishydroxymethylaminomethane, the final pH being between 8 and 9·0. When the nitroprusside test (Feigl 1947) became negative the excess iodoacetate was allowed to react with a slight excess of potassium thioglycollate. The alkylated proteins were dialysed in "Cellophane" tubing (Visking 18/32) for 18 hr against running tap water, the volume was measured, and, after dilution of an aliquot with an equal volume of 50% v/v acetic acid to give optically clear solutions, the optical density at 277 mμ was determined against an appropriate blank.

(*f*) *Separation into Fractions of High- and Low-sulphur Content*

The low-sulphur protein fraction was precipitated by dialysing the protein solution for 18 hr against acetate buffer (Gillespie 1960*a*). In the initial studies buffer at pH 4·1 and of ionic strength 0·1 was employed but subsequently the pH was increased to 4·4 and the ionic strength to 0·5. The precipitate of low-sulphur protein was recovered from the residual solution of high-sulphur protein by filtration or centrifugation. The protein content of the supernatant fraction was determined from an optical density measurement at 277 mμ.

(*g*) *Calculations*

The difference between the optical densities of the unfractionated proteins in Section II(*e*) and the supernatant derived from it gave the optical density equivalent to the low-sulphur component. This was in excellent agreement with the value determined directly on a solution of the precipitated protein. By using extinction coefficients ($E_{1\,cm}^{1\%}$, 277 mμ) of 8·6 and 5·4 for the low- and high-sulphur proteins, respectively, and making suitable corrections for dilution during dialysis, the concentration of each in the extraction fluid was obtained. Their sum gave the total amount of protein extracted, in good agreement with the value calculated from the weight of residual wool. In some experiments the yields of extracted protein were also measured by a semi-microKjeldahl procedure. Throughout this paper amounts of protein extracted are expressed as a percentage of the weight of dry wool.

(*h*) *Preparation of High-sulphur Protein*

For use in experiments on the separation of low- and high-sulphur proteins from mixtures, a preparation of high-sulphur protein was made by extracting wool for 2 hr at 40°C with 0·8M potassium thioglycollate at pH 10·2. The extracted protein, consisting mostly of high-sulphur protein, was dialysed at 1°C against a large volume of 0·1M potassium thioglycollate at pH 9, then alkylated and fractionated as described in Sections II(*e*) and II(*f*), then dialysed and freeze-dried.

(*i*) *Electrophoresis*

Moving-boundary electrophoresis was carried out as described previously (Gillespie 1960*a*) using an acetic acid–sodium acetate buffer of pH 4·5 and ionic strength 0·1, or a glycine or β-alanine–NaOH buffer of pH 11·0 and ionic strength 0·1.

III. Results

(*a*) *Relation between pH and Solubility at 40°C*

Figure 1 shows the relationship between the pH of the 0·1M potassium thioglycollate extractant and the amounts of high- and low-sulphur protein extracted from wool. Whereas the solubility of the low-sulphur proteins continued to increase with increasing pH, that of the high-sulphur proteins apparently reached a maximum at

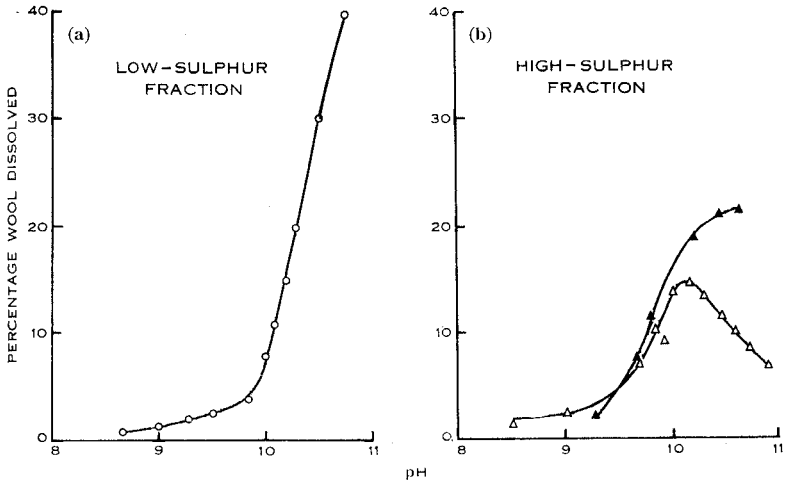

Fig. 1.—Curves relating extraction of proteins from wool by 0·1M potassium thioglycollate at 40°C for 160 min and pH: (*a*) low-sulphur fraction; (*b*) high-sulphur fraction prepared by precipitation at pH 4·1 and ionic strength 0·1 (△), and at pH 4·4 and ionic strength 0·5 (▲).

pH 10·1 when these were measured by precipitation by dialysis against pH 4·1 buffer, and further increases in pH caused a decrease in the amount of these proteins extracted.

Similar results were observed at lower temperatures but the curves were displaced so that, for extraction of equal amounts, a higher pH value or a longer time of extraction was needed. At both high and low temperatures the position of the maximum in the pH extraction curve for high-sulphur protein closely corresponded with the beginning of the steep portion of the corresponding curve for low-sulphur protein, suggesting that the presence of increasing amounts of low-sulphur protein decreased the recovery of high-sulphur protein. This is due to binding of the high-sulphur protein by the low-sulphur protein and their subsequent precipitation together, when the precipitation takes place at a pH between their isoelectric points (Gillespie, O'Donnell, and Thompson 1962). It can be avoided

by precipitation at pH 4·4 in the presence of a high concentration of salt (Fig. 1(*b*)), or glycine or by precipitation outside the inter-isoelectric region with zinc acetate. In the experiments which follow the two types of proteins have been separated by precipitation at pH 4·4 with sodium acetate–acetic acid buffer of ionic strength 0·5.

(*b*) *Relation between pH and Extraction at Various Times and Temperatures*

The effect of temperature and time on the extractability of high-sulphur protein by 0·1M potassium thioglycollate is shown in Figure 2(*a*). It can be seen that at 40°C, as the pH increased above 9, the yield of protein increased, the increase being very marked at pH values near 10. At lower temperatures the corresponding curves were displaced toward higher pH values. Curves showing the relation between pH and the simultaneous extraction of low-sulphur proteins, not recorded here, were similar in shape to the one prepared at 40°C (Fig. 1) but were displaced to higher pH values.

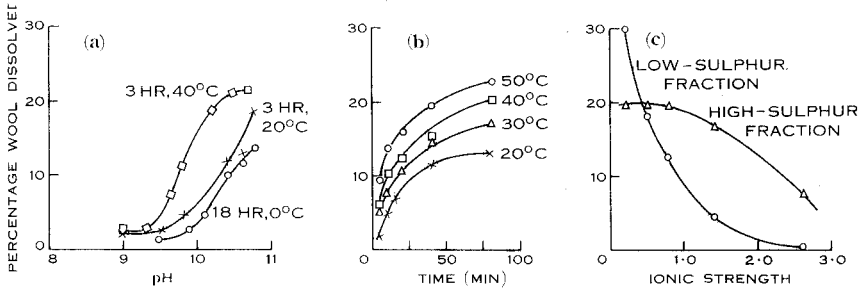

Fig. 2.—Curves showing the extent of extraction of proteins from wool by 0·1M potassium thioglycollate: (*a*) effect of pH on the extraction of high-sulphur protein; (*b*) rate of extraction of high-sulphur protein at pH 10·5; (*c*) effect of ionic strength—extraction period 60 min at pH 10·5 and 40°C.

The rate of extraction of high-sulphur protein by 0·1M potassium thioglycollate was measured at four temperatures. The results (Fig. 2(*b*)) indicate that up to 80 min equilibrium was not reached and that temperature, pH, and time are mutually linked variables. From these curves, the initial rate of reaction and the activation energy of the process were calculated, a value of about 3800 kcal/mole for the latter parameter being obtained.

(*c*) *Effect of Ionic Strength*

High- and low-sulphur proteins were extracted from wool at pH 10·5 by 0·1M potassium thioglycollate at 40°C in the presence of varying amounts of potassium carbonate. The curves in Figure 2(*c*) show that each increase in ionic strength caused a corresponding decrease in the extractability of the low-sulphur proteins, but not of the high-sulphur proteins until the ionic strength exceeded 1·0. Furthermore the curves show that by selecting a suitable ionic strength, preferential extraction of high-sulphur protein could be obtained, although at reduced yield. As might be expected salts other than sodium carbonate showed varying effectiveness in this regard.

(d) Influence of Variation in Liquor : Wool Ratio on the Yield of High-sulphur Protein

Wool was extracted at 40°C with 0·1M potassium thioglycollate containing 0·2M sodium metaborate to buffer the solution at pH 10·0. The increased ionic strength due to sodium metaborate partially suppressed the extraction of low-sulphur proteins. Increasing the liquor : wool ratio up to 100 : 1 (v/w) increased the yield of high-sulphur protein (Fig. 3(*a*)). With this ratio the yield obtained in 100 min at pH

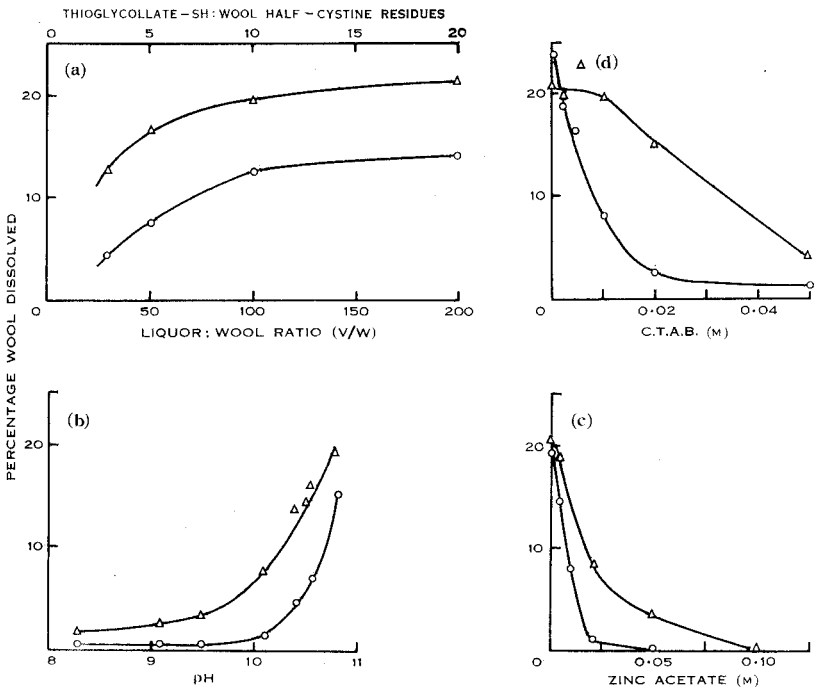

Fig. 3.—(*a*) Effects of variation of liquor : wool ratio in extraction of proteins from wool by 0·1M potassium thioglycollate buffered by 0·2M sodium metaborate at pH 10. Incubation period 3 hr at 40°C. (*b*) Effect of pH on the extraction of proteins from wool by 0·1M potassium thioglycollate in the presence of 0·01M zinc acetate. Incubation period 160 min at 40°C. (*c*) Effect of variation in added zinc acetate concentration on the extraction of proteins from wool by 0·1M potassium thioglycollate at a pH of 10·5. Incubation period 160 min at 40°C. (*d*) Effect of variation in added cetyl trimethyl ammonium bromide (C.T.A.B.) concentration on the extraction of proteins from wool by 0·1M potassium thioglycollate at pH 10·3. Incubation period 160 min at 40°C. ○ Low-sulphur fraction. △ High-sulphur fraction.

10 was comparable with that obtained by extraction for 160 min at pH 10·5 with a 30 : 1 ratio. The graph also indicates the relative ratios of thioglycollate –SH to protein half-cystine residues at each liquor : wool ratio.

(e) Addition of Divalent Metals

The addition of small amounts of calcium, zinc, barium, or magnesium acetate to the 0·1M potassium thioglycollate at pH values above 10 enriched the extracts in high-sulphur protein. Some typical solubility curves with solutions containing

added zinc acetate are shown in Figures 3(*b*) and 3(*c*). So far it has not been possible to obtain a quantitative yield of high-sulphur protein with complete suppression of low-sulphur protein. However, by the addition of 0·01M calcium or zinc acetate to 0·1M potassium thioglycollate (pH 10·1–10·2) it is possible to obtain yields of 10% high-sulphur protein containing only small quantities of low-sulphur protein.

(*f*) *Addition of Cetyl Trimethyl Ammonium Bromide* (*C.T.A.B.*)

The addition of small amounts of C.T.A.B. had a very marked influence on the extraction of protein by potassium thioglycollate at pH 10·3. Figure 3(*d*) shows that as the C.T.A.B. concentration increased there was a steady fall in the yield of low-sulphur protein to about 2% at 0·02M, whereas a yield of about 15% high-sulphur protein was obtained at this C.T.A.B. concentration. At higher concentrations of C.T.A.B. the recovery of this protein was also greatly decreased.

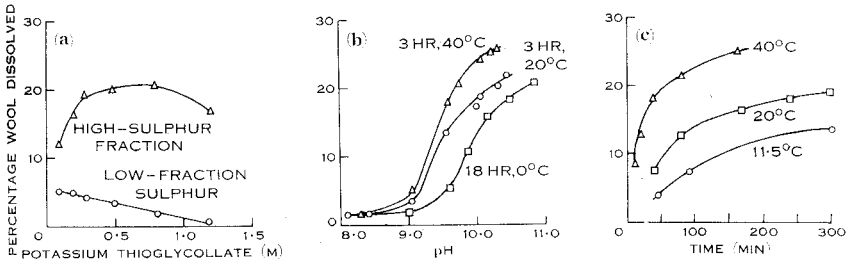

Fig. 4.—Extraction of wool by potassium thioglycollate at 40°C: (*a*) effect of variations in thioglycollate concentration at pH 9·8; (*b*) effect of pH of 0·8M potassium thioglycollate extractant; (*c*) rate of extraction by 0·8M potassium thioglycollate at pH 10 for a series of temperatures.

(*g*) *Effects of Variation in Thioglycollate Concentration at pH* 9·8

Figure 4(*a*) shows that, at pH 9·8, as the concentration of potassium thioglycollate was increased, the yield of high-sulphur protein also increased, reaching a rather flat maximum at about 0·8M. At higher concentrations the yield declined. The low-sulphur proteins were most soluble at the lowest thioglycollate concentrations, being almost completely repressed at thioglycollate concentrations above 0·8M.

(*h*) *Effect of pH and Time on the Extraction of High-sulphur Protein with* 0·8M *Thioglycollate*

The curves relating pH and amount of high-sulphur protein extracted in 0·8M thioglycollate at various temperatures (Fig. 4(*b*)) show that higher yields were obtained and the pH values which could be used were lower than was possible with 0·1M thioglycollate solutions. It can also be seen that high yields could be obtained at 0°C. In most experiments the amount of protein precipitating at pH 4·1 did not exceed 2% even with prolonged extraction. At 40°C it was usually between 0·5 and 1%. It is considered that this protein is not a typical low-sulphur protein of

the SCMKA2* or α-keratose type. Further data on this aspect will be presented in a later section. Variation in the ratio of 0·8M thioglycollate at pH 10·0 to wool over the range 30 : 1 to 100 : 1 had little effect on the amount of protein extracted in 120 min at 40°C, the yield varying between 19 and 21%. The rate of extraction by 0·8M thioglycollate was measured at three temperatures, the results being shown in Figure 4(*c*). Based on the initial extraction rates, the activation energy of the process appeared to be of the order of 3000 kcal/mole.

(*i*) *Extraction of Supercontracted Wool at 40°C*

Wool (20 g) was heated in 600 ml of lithium bromide (approx. 6M at pH 6·0) at 100°C for 10 min and then thoroughly washed and dried. The proteins were more readily extracted from this wool than from normal wool (Fig. 5(*a*)); for example,

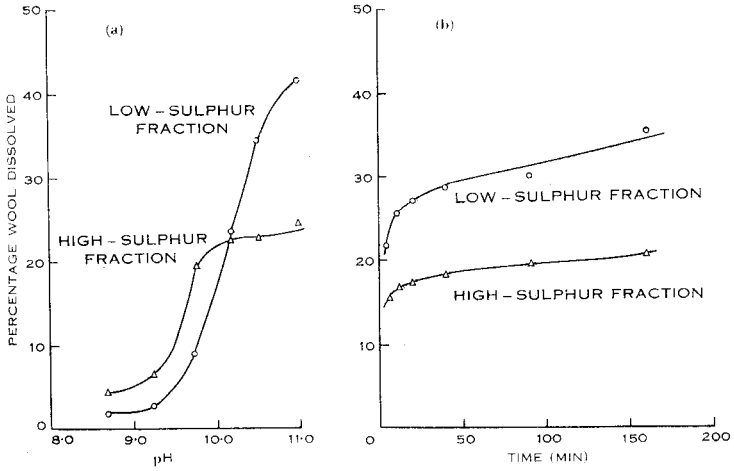

Fig. 5.—Extraction of supercontracted wool by 0·1M potassium thioglycollate at 40°C: (*a*) effect of pH—incubation period 160 min; (*b*) rate of extraction at pH 10·6.

a 6-min extraction at pH 10·6 gave yields of 30% low-sulphur protein and 15% high-sulphur protein. The comparable values for normal wool are about 5–7% respectively. The curves relating pH and amount extracted (Fig. 5(*b*)) show that this ease of extraction was also manifested in a higher extractability at lower pH values than with normal wool. The largest effects in this respect can be seen in pH extraction curves for the high-sulphur proteins; the protein was liberated at a few tenths of a pH unit lower than with normal wool (cf. Fig. 3(*a*)).

(*j*) *Extraction of Alkali-treated Wool*

Wool (10 g) was immersed in 1 l. of potassium carbonate solution (0·1M, pH 11) at 50°C for 30 min then thoroughly washed and dried. It was much more difficult

* See Gillespie (1960*a*, Appendix I).

to extract proteins from this wool than from normal wool (Figs. 6(*a*) and 6(*b*)). For example, at pH 10·5 the yield of low-sulphur protein declined from about 30 to 7% and that of the high-sulphur protein from about 21 to 10%. It is possible that part of the high-sulphur protein dissolved in the sodium carbonate solution, for after heating, the solution had an absorption at 277 mμ equivalent to about 5% of wool, assuming an extinction coefficient of 5·4.

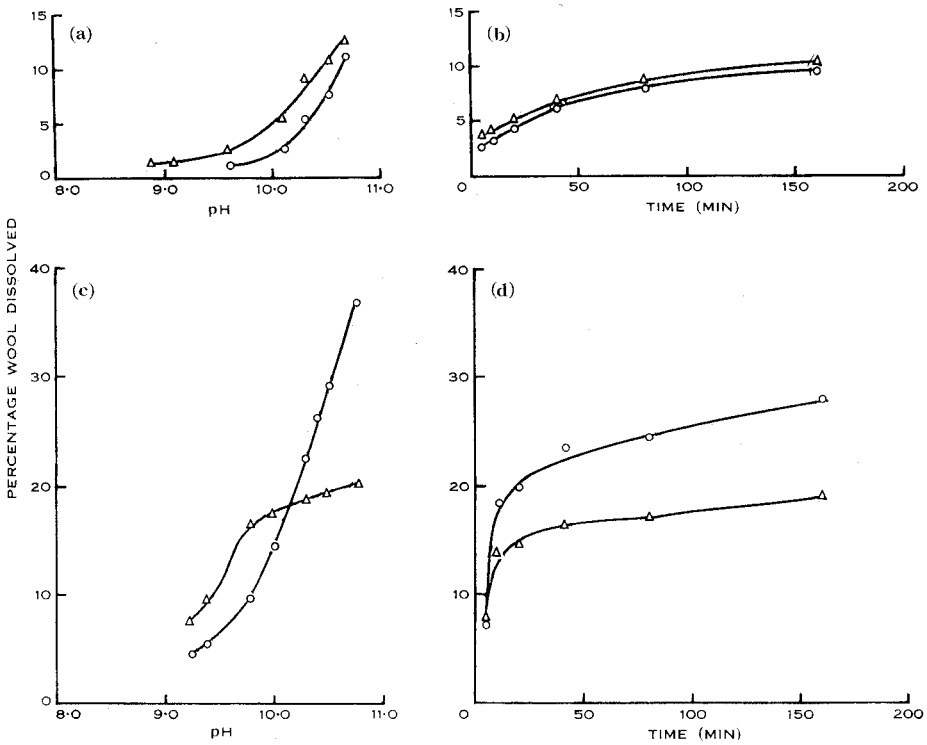

Fig. 6.—(*a*) and (*b*) Extraction of alkali-treated wool by 0·1M potassium thioglycollate at 40°C: (*a*) effect of pH—incubation period 160 min; (*b*) rate of extraction at pH 10·5. (*c*) and (*d*) Extraction of partially reduced and alkylated wool by 0·1M potassium thioglycollate at 40°C: (*c*) effect of pH—incubation period 160 min; (*d*) rate of extraction at pH 10·5. ○ Low-sulphur protein. △ High-sulphur protein.

(*k*) *Extraction of Partially Reduced and Alkylated Wool*

Wool (15 g) was soaked in 750 ml of 1·0M sodium thioglycollate at pH 5·0 for 18 hr at 20°C. The reacted wool was washed with five changes of distilled water (total 10 l.) and then immersed in 750 ml of sodium iodoacetate (0·4%) buffered at pH 9, until the nitroprusside test on single fibres became negative. The wool was thoroughly washed and dried. Polarographic analysis gave a residual disulphide content of 220 μmoles/g which indicated a 57% reduction. This corresponds to reduction of the A+B fraction of wool cystine (Middlebrook and Philips 1942). When this wool was extracted it was found (Figs. 6(*c*), 6(*d*)) that, compared with

normal wool, although the total amount of protein obtained was not increased, the initial rate of extraction was increased and for similar yields of protein the pH of extraction could be decreased.

(l) Comparison of the Properties of High-sulphur Proteins Isolated by Various Methods

The proteins were prepared as previously described (Gillespie 1960 and Section II(*h*) above) and run electrophoretically in sodium acetate buffer of pH 4·5 and ionic strength 0·1. Generally the patterns obtained (Fig. 7) were qualitatively alike,

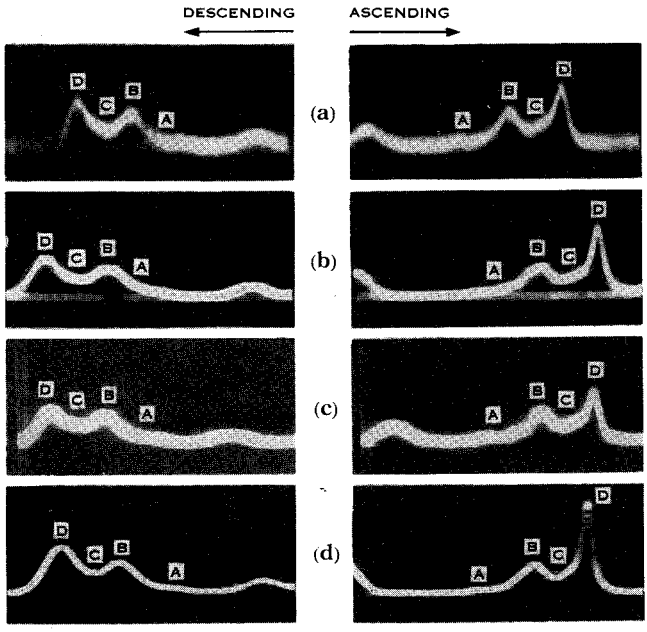

Fig. 7.—Electrophoretic pattern of high-sulphur proteins run in acetate buffer at pH 4·5, ionic strength 0·1. Preparation conditions: (*a*) 0·1M thioglycollate 40°C, 3 hr, fractionated at pH 4·1; (*b*) 0·1M thioglycollate, 40°C, 3 hr, fractionated at pH 4·4; (*c*) 0·8M thioglycollate, 40°C, 3 hr, fractionated at pH 4·1; (*d*) 0·8M thioglycollate, 0°C, 18 hr, fractionated at pH 4·1.

as also were the mobilities, sulphur contents, and areas of the electrophoretic peaks (Table 1), suggesting that the proteins obtained by all methods were the same. There are some small quantitative differences in yield of the different peaks, particularly in the yield of the fastest moving peak, i.e. the material with highest sulphur content (Gillespie, unpublished data). The 0·8M thioglycollate extracts were richer in this protein than the 0·1M thioglycollate extracts, possibly because this protein, having a higher initial content of –SS–, was more difficult to reduce and extract. This is supported by the electrophoretic patterns of protein from successive 1-hr extractions with 0·8M thioglycollate (Fig. 8; Table 1). The second extract appeared to be richer in the faster moving protein than the first. There was also evidence that this protein was somewhat enriched in 0·1M thioglycollate extracts fractionated at pH 4·4, as compared to those fractionated at pH 4·1 in which some high-sulphur

protein was lost, bound to the low-sulphur proteins. This suggests that the low-sulphur protein can to some extent preferentially bind this fast moving protein.

TABLE 1

MOVING-BOUNDARY ELECTROPHORETIC ANALYSIS OF SCMK HIGH-SULPHUR PROTEINS PREPARED BY VARIOUS METHODS

Percentage distribution of protein calculated from areas under peaks of electrophoretic patterns reproduced in Figures 7 and 8. Means of ascending and decending patterns given. All preparations had sulphur contents in the range 5·75–5·9%

Preparation and Electrophoretic Pattern	Percentage Protein with Electrophoretic Mobility (expressed as $cm^2volt^{-1}sec^{-1}$) in Range:			
	$-2\cdot6$ to $-3\cdot2\times10^{-5}$ (peak A*)	$-4\cdot6$ to $-4\cdot8\times10^{-5}$ (peak B)	$-5\cdot8$ to $-6\cdot2\times10^{-5}$ (peak C)	$-6\cdot8$ to $-7\cdot0\times10^{-5}$ (peak D)
Figure 7(*a*)	8·2	36·1	18·1	37·7
Figure 7(*b*)	9·5	33·3	14·5	42·6
Figure 7(*c*)	3·8	42·6	9·6	44·1
Figure 7(*d*)†	7·7	30·9	11·0	50·4
Figure 8(*a*)	12·8	32·9	13·3	41·0
Figure 8(*b*)	11·7	30·6	13·8	43·9

* All protein of mobility lower than that of peak B was included in peak A even though in some preparations (e.g. Fig. 7 (*c*)) more than one peak was observed in this region.

† Means of analysis of three preparations.

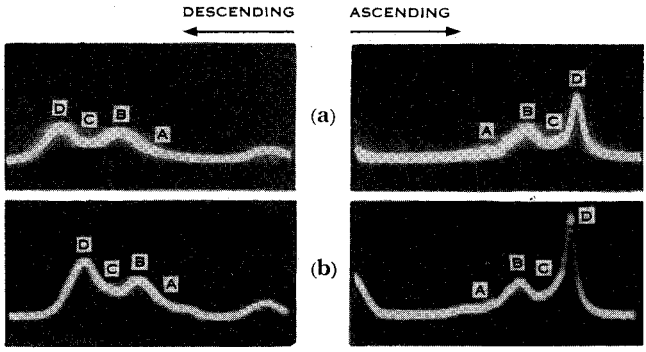

Fig. 8.—Electrophoretic patterns of high-sulphur proteins run in acetate buffer at pH 4·5, ionic strength 0·1: (*a*) protein extracted by 0·8M thioglycollate at 40°C for 1 hr, fractionated at pH 4·1; (*b*) a second extraction on the residue from (*a*) under the same conditions.

(*m*) *Nature of the Low-sulphur Protein Extracted by* 0·8M *Thioglycollate*

Part of this protein in either the –SH or SCMK form was insoluble at pH values below 10. This is probably the same material which was responsible for the turbidity which appeared on cooling the first thioglycollate extract (extract A) of Gillespie and Lennox (1955). It also appears to be the residual non high-sulphur protein in systems where the low-sulphur proteins have been suppressed by high

ionic strength or the presence of a divalent metal or C.T.A.B. This protein was prepared as follows: Wool was extracted with 0·8M potassium thioglycollate at 0°C, dialysed against 0·1M thioglycollate, alkylated, and thoroughly dialysed against running tap water. The alkylated proteins, at this stage quite turbid, were titrated

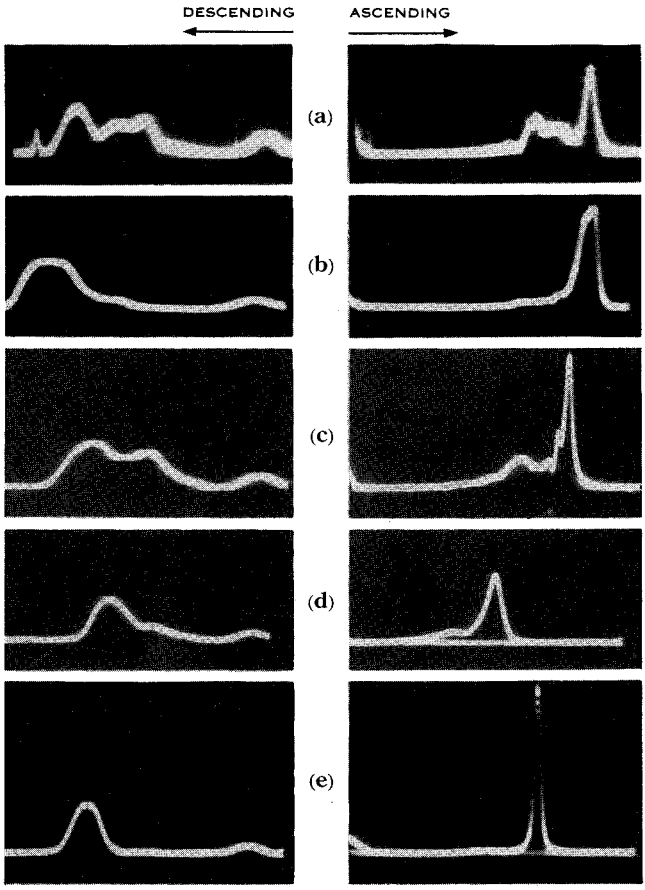

Fig. 9.—(*a*) Electrophoretic pattern of acid-precipitable (pH 5·0) protein from 0·8M thioglycollate extracts, run in glycine–NaOH buffer at pH 11·0, ionic strength 0·1. (*b*) and (*c*) Electrophoretic patterns of high-sulphur proteins run in acetate buffer at pH 4·5, ionic strength 0·1, and showing modification of fast moving peak: (*b*) protein extracted at 0°C with 0·8M thioglycollate for 4 days. Only the fast moving peak is present in this preparation; (*c*) 24-hr extracted protein stored for 24 days at 0°C; (*d*) and (*e*) Electrophoretic patterns of proteins remaining in fibre after repeated extraction with 0·8M thioglycollate: (*d*) residual high-sulphur protein extracted by urea, and run in acetate buffer at pH 4·5, ionic strength 0·1; (*e*) low-sulphur protein extracted by urea, and run in β-alanine–NaOH buffer at pH 11·0, ionic strength 0·1.

to pH 4·4 at an ionic strength of 0·2 and the precipitate which did not sediment readily removed by centrifuging for 1 hr at 40,000 r.p.m. in a Spinco Model L ultracentrifuge. The precipitated protein was dissolved in 0·1M potassium carbonate and reprecipitated by adjusting the pH to 5·5 with acetic acid. An electrophoretic pattern of this material run at pH 11 in glycine–NaOH buffer of ionic strength 0·1 is shown in Figure 9(*a*), which shows it to be very heterogeneous. The mobilities of

components on the descending boundary are shown in Table 2, and are compared with those of high- and low-sulphur proteins run in the same buffer system at similar protein concentrations. The material had a sulphur content of 3·6%.

(*n*) *Effect of Extraction Time on the Nature of the Extracted Protein*

A study of the electrophoretic patterns of *S*-carboxymethyl derivatives of the high-sulphur proteins prepared by extracting wool with 0·8M thioglycollate near pH 10·3 at 0°C has revealed that the fastest peak gives rise to an additional peak as the extraction time increases (Figs. 9(*b*), 9(*c*), compare with Fig. 7(*d*)). On the descending side the peak merely broadens but on the ascending side it is replaced by

TABLE 2

DESCENDING MOBILITIES OF PROTEINS IN THE FRACTION OF INTERMEDIATE SULPHUR CONTENT COMPARED WITH THE MOBILITIES OF HIGH- AND LOW-SULPHUR PROTEIN

Electrophoresis carried out at pH 11 in glycine–NaOH buffer of ionic strength 0·1

Preparation	$10^5 \times$ Descending Mobilities ($cm^2 volt^{-1} sec^{-1}$)
Fraction of intermediate sulphur content from 0·8M potassium thioglycollate extraction (pH 5·0 insoluble material)*	−5·8, −6·9, −9·2,† −10·9
High-sulphur protein from 0·8M potassium thioglycollate extraction at 0°C	−5·0, −6·5, −7·9†
Low-sulphur protein from 0·1M potassium thioglycollate extraction (pH 4·4 insoluble material)	−4·2,‡ −6·8,† −8·2,‡ −10·2‡

* See Figure 9(*a*). † Major peak. ‡ Trace only.

two peaks, the proportion of the faster component increasing with increasing extraction time. In preparations extracted for only 18–24 hr, there was no evidence of splitting but it was quite obvious in preparations extracted for 3 days or more. A 24-hr extract was filtered from the residual wool, stored under nitrogen for 24 days at 0°C, and then converted to the SCMK derivative. Electrophoresis of this protein at pH 4·5 showed that the new faster peak had largely replaced the old peak (Figs. 9(*b*), 9(*c*)).

(*o*) *Extent of Reduction of the Isolated High-sulphur Protein*

High-sulphur protein prepared by extracting wool at 0°C with 0·8M thioglycollate for 18 hr normally contains less than 5μ moles/g of residual disulphide.

(*p*) *Subsequent Extraction of Low-sulphur Protein*

After wool had been extracted several times with 0·8M thioglycollate either at 0 or 40°C, it still retained its fibrous shape and some of its strength. The low-sulphur proteins within this fibre, together with any residual high-sulphur protein, could be extracted by treating the residue with 10M urea. They were conveniently extracted from the residue of 2 g of wool by blending at low speed for a few minutes in a stainless steel Waring microBlendor containing 19 ml of water and 30 g of urea at 40°C.

The suspension was allowed to stand under nitrogen for 30 min, then it was alkylated with iodoacetate and dialysed. After dialysis the undissolved wool and any insoluble protein were removed by centrifugation at 40,000 *g*, washed, dried, and weighed. These steps achieved a total extraction of about 80%. Most of the extracted material was low-sulphur protein (Fig. 9(*e*)) and this was found to be virtually free of disulphide, i.e. it contained less than 5 μmoles/g. The small amount of high-sulphur protein (separated by precipitating the low-sulphur protein at pH 4·4) had an electrophoretic pattern (Fig. 9(*d*)) showing the presence of only two components of mobilities at pH 4·5 of $-3{\cdot}5$ and $-5{\cdot}2\times10^{-5}$ cm^2 volt^{-1} sec^{-1} and a sulphur content of 6·6%.

IV. Discussion

The present results agree with those reported previously (Gillespie and Lennox 1955, 1957; Lennox 1956*a*, 1956*b*), and the additional extraction studies on the high- and low-sulphur proteins of wool have helped to clarify some obscurities in the earlier work. It is of interest that in both the earlier work dealing with mixed proteins and in the present studies on the high-sulphur proteins, the kinetics of the extraction suggest that the process is diffusion-controlled.

The most striking anomaly found in the present work was the maximum in the curve relating yield of high-sulphur protein to pH, which proved to be due to loss of high-sulphur protein by binding to the low-sulphur proteins during the precipitation of the latter (Gillespie, O'Donnell, and Thompson 1962). We now know that the conditions previously used (Gillespie 1960*a*) for fractionating the soluble SCMK extracts (pH 4·1 acetate buffer, ionic strength 0·1) do not give a complete separation of the two types of protein. It is now apparent why it was then found necessary to repeat the precipitation three times in order to free the low-sulphur proteins from the high.

It has been shown in this paper that the high-sulphur proteins can be extracted preferentially from reduced wool, thus bringing the reductive and oxidative methods into line. Because the reductive methods employed here simultaneously reduce and extract wool proteins, the conditions for preferential extraction differ from those employed with oxidized wool. The methods used for extracting γ-keratose preferentially from oxidized wool rely on extraction at pH values near to or below neutrality, under conditions where α-keratose has only a limited solubility. With reductive methods the pH values have to be sufficiently high to permit almost complete reduction; the additional requirement is the presence of a suitable salt in sufficient concentration to repress the solubility of the low-sulphur proteins.

It is of interest that concentrated thioglycollate more completely suppresses the extraction of the low-sulphur proteins than dilute thioglycollate adjusted to the same total ionic strength by adding the salt of a divalent anion. The reason for this must lie in some specific effect of the thioglycollate anion. Extraction with concentrated thioglycollate is a convenient way for preparing completely reduced high-sulphur protein at lower pH values and at lower temperatures than is possible with dilute thioglycollate. For many purposes the small amount of non high-sulphur protein can be ignored but after alkylation it can be quantitatively removed by precipitation at pH 4·1.

The high-sulphur proteins are extractable at lower pH values than the low-sulphur proteins and there are a number of possible explanations for this. The high-sulphur proteins being amorphous (Birbeck and Mercer 1957) may be more rapidly reduced and at lower pH values than the orientated low-sulphur proteins, or the high-sulphur proteins being of smaller size may diffuse more readily. The pronounced effect near pH 10 of small increases in pH causing large increases in the extraction of the low-sulphur proteins may be due to their disaggregation or to some modification to the fibre membranes. The necessity for an adequate degree of reduction is illustrated by the increased yield of both proteins which occurs when the ratio of 0·1M thioglycollate to wool is increased from 30 : 1 to 100 : 1 (v/w) corresponding to an increase in molar ratio of thioglycollate to half-cystine residues in the wool of 3 : 1 to 10 : 1. The importance of repulsion between the charged proteins in facilitating their extraction is illustrated by the experiments with C.T.A.B., where the adsorption of the positively charged cations on the proteins completely suppressed their solubility. As both proteins at pH 10·3 have a large and not greatly dissimilar net negative charge (assuming complete ionization of –SH and tyrosyl residues) the difference in the amount of C.T.A.B. required to suppress them, must lie in differences in solubility of the protein-C.T.A.B. complexes.

It seems improbable that the isolated high-sulphur proteins retain or regain in full their native structure. However, Fraser, MacRae, and Rogers (1962) have shown that high-sulphur proteins when extracted from the fibre, oxidized by contact with air and stained with silver nitrate, give a 20Å X-ray diffraction halo similar to that observed in whole wool fibres stained in the same way. This suggests that these proteins, unlike the low-sulphur proteins, retain much the same molecular shape and size after extraction. The protein used in this study was extracted at 0°C with 0·8M thioglycollate (18 hr, pH 10·3), then oxidized during dialysis against running tap water. The protein, although turbid, remained in solution. The X-ray data was obtained from films cast from this solution.

With at least one of the protein components, prolonged contact with alkaline solutions at 0°C can cause changes which result in the appearance of material of faster electrophoretic mobility. One reaction which could cause this effect is alkaline hydrolysis of amide groups which this protein contains in considerable amount (Gillespie and Simmonds 1960). Although the new component cannot be seen in electrophoretic patterns of proteins extracted for only 18 hr, the assumption must be made that the reaction leading to its formation commences at the start of the extraction. Hence the extraction procedure may introduce a heterogeneity which does not exist in the native protein.

The exact significance of the data relating to the solubility of the alkali-treated wool is not known but there are some significant points which may have some bearing on the ultimate interpretation. It can be seen (Figs. 2(*a*) and 2(*b*)) that in neither the curves relating solubility to pH nor in those relating it to time was equilibrium reached. This may mean that the pretreatment has made the proteins more difficult to extract without altering the equilibrium value and so decreased that rate of extraction. Although Swan (1959) originally thought that this pretreated wool contained little or no lanthionine, traces have since been found (Swan 1960). A few

non-reducible lanthionine cross-linkages in certain positions could effectively limit extraction rates. There would also be some change in molecular weight of the product.

Although only a minor constituent of wool, the acid-precipitable protein discussed above in Section III(*m*) is of interest because it is readily extractable, it is insoluble except at very alkaline pH values, and its sulphur content is intermediate between that of the high- and low-sulphur proteins. It may be located on or close to the surface of the fibre where its extractability could be less affected than proteins elsewhere in the fibre by conditions which repress the extraction of the low-sulphur proteins.

The thioglycollate solubility test developed by Lennox (1956*a*, 1956*b*) has been used as a measure of damage or chemical modification of wool (Maclaren 1958). In the lastest modification of the test (Lennox 1958), the wool is first extracted in a buffered thioglycollate solution of high ionic strength and the residue is then extracted with water. It is now apparent that these are essentially fractional extractions, with the high-sulphur proteins occurring largely in the first extract and the low-sulphur proteins in the second. For this reason the two-stage test provides an index of the extent of modification of each of the two types of wool protein.

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