THE ISOLATION AND PROPERTIES OF SOME SOLUBLE PROTEINS FROM WOOL

VIII. THE PROTEINS OF COPPER-DEFICIENT WOOL

By J. M. GILLESPIE*

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Summary

The proteins in wools obtained from copper-deficient sheep before and after supplementing the diet with copper have been solubilized with urea–potassium thioglycollate at pH 11, alkylated with iodoacetate, and then separated into protein fractions of high- and low-sulphur content. The isolated proteins from these wools have been examined by moving boundary electrophoresis and the mobilities and relative proportions of the various components measured.

It has been found that there are marked changes in the type of high-sulphur proteins synthesized during copper deficiency, there being a shift in synthesis to protein components of lower sulphur content but with a significantly higher content of certain amino acids including aspartic acid, leucine, and phenylalanine.

It is considered that these changes reflect an upset in cystine availability or utilization but may not be responsible for the marked changes in the physical properties of these wools. The changes in fibre properties could be due to changes in protein conformation and the type of cross-linking between high- and low-sulphur proteins consequent upon the slow keratinization observed by Marston (1946).

I. INTRODUCTION

It has long been considered that the composition of wool, at least within a breed, was fixed. This idea probably originated with the studies of Marston (1928) who showed that in carefully purified wool the sulphur content was constant from sample to sample. The apparent constancy of wool in this regard is remarkable, for Fletcher, Robson, and Todd (1963) have recently shown that the sulphur content of Merino wools of 60's–70's count has not varied significantly over the past 36 years. The amino acid analyses of Simmonds (1955, 1956) have been used to support this idea and it was again reiterated when Corfield (1963) stated that "wool is endowed with a remarkably *constant composition*" and that "the *unchanging chemical composition* of wool can be reasonably accounted for in terms of a single keratin precursor in the developing cells of the follicle."

There is, however, much recorded information which suggests that wool does not always have a fixed composition. This is most evident in analyses of cystine and other amino acids (Simmonds 1955, 1956, 1958; Human 1958) and in the study of straight steely wool from copper-deficient sheep (Burley 1960; Burley and Hordern 1959, 1960, 1961). The latter workers compared normal wool and steely wool from the same animal and showed that the latter contained less sulphur, less γ-keratose, and a γ-keratose which was lower in sulphur than the control. Ross

* Division of Protein Chemistry, CSIRO Wool Research Laboratories, Parkville, Vic.

(1961) has shown that in normal Romney Marsh sheep there is a seasonal variation in the sulphur content of the wool, the sulphur content being lowest when the wool production was greatest and vice versa. Recently Reis and Schinckel (1963) were able to increase the sulphur content of wool by as much as 30% thus providing further evidence that the composition of wool is not fixed. Mercer (1961) goes so far as to say that "wool keratin has no precisely fixed composition". One aim of the present series of investigations is to locate the protein fraction which is responsible for this variable sulphur content of wool.

The work of Burley and Hordern (1959, 1960, 1961) left unanswered the question of whether the changes in amount and sulphur content of the high-sulphur proteins were due to overall changes or to alterations in specific protein components. It is the purpose of the present study to answer these questions. In Part IX of this series (Gillespie, Reis, and Schinckel 1964) similar studies will be reported on the proteins from wools of increased sulphur content.

In the present paper, wool showing the specific copper-deficient lesion will be termed steely wool according to South Australian usage (Lee 1956). This author summarizes the names by which this syndrome is known in other parts of Australia and elsewhere.

II. Materials and Methods

(*a*) *Origin and Preparation of Wools*

The wools used in these studies came from two sources. For comparative purposes, Merino wool from the Wintoc flock (MW148) was used (Harrap and Gillespie 1963). The steely wools came in the form of staples or skin pieces which contained in the one staple approximately equal lengths of steely wool and a control wool grown after the animal had been dosed with copper. The transition point was clearly defined as a change in crimp. About 1 cm of tip and 1 cm in the junction region were rejected. These wools were obtained through the courtesy of Mr. H. J. Lee, Division of Biochemistry and General Nutrition, CSIRO, and came from experiments performed at the Robe Field Station from 1943 to 1952 (Marston and Lee 1948*a*, 1948*b*; Marston 1952; Lee 1956).

All wool samples were repeatedly washed in petroleum ether, then in several changes of ethanol and water, and finally equilibrated in a conditioned room (68°F, 60% R.H.).

(*b*) *Estimation of Proportion of High-sulphur Protein in Wool*

The total extractable protein and the proportion of high-sulphur protein in the extract were measured by the urea–thioglycollate extraction procedure described by Harrap and Gillespie (1963). The reasons for choosing an alkaline-reduction procedure for these estimations in preference to the oxidative procedure of Burley and Hordern (1959) are presented in Section III(*a*).

Conditioned wool (1 g, 0·86 g dry wt.) was extracted at 40°C in a glass-stoppered test tube with 30 ml of a solution containing 6M or 10M urea and 0·2M potassium thioglycollate. In preparing this solution 0·4M potassium thioglycollate was adjusted to pH 11, and urea and water added to give the desired final concentration of reagents.

The extraction tubes were frequently inverted over a 2-hr period. The extracted proteins were separated from the residue by centrifugation at 40,000 *g* for 30 min. For analytical purposes a 10-ml aliquot of this extract was alkylated with a mixture of 0·8 g iodoacetic acid and 1·6 g Tris, quantitatively transferred to a Cellophane bag (18/32), and dialysed against running tap water for 2 days. The remainder of the extract was alkylated and treated in a similar but non-quantitative manner.

(*c*) *Separation of High- and Low-sulphur Proteins*

Three methods were examined for precipitating the low-sulphur proteins from dialysed alkylated extracts:

(1) Alkylated extract (10 ml) was dialysed against 2 l. of acetic acid–sodium acetate buffer at pH 4·4 and ionic strength 0·4 (Gillespie, O'Donnell, and Thompson 1962).

(2) To 10 ml of alkylated extract was added 10 ml of acetic acid–sodium acetate buffer of pH 4·4 and ionic strength 0·8. The final pH of the mixture was carefully adjusted to 4·4.

(3) To 10 ml of alkylated extract was added 2 ml of a solution containing 0·1M zinc acetate and acetic acid–sodium acetate buffer of ionic strength 0·1 at pH 6 (Gillespie 1957).

In each procedure the precipitate of low-sulphur protein was centrifuged down, leaving the high-sulphur proteins in solution. The concentration of protein in these supernatant solutions was then estimated as described in Section II(*d*). For preparative purposes the supernatants were dialysed and freeze-dried. Sufficient sodium citrate was added before dialysis to complex the Zn^{2+} in zinc supernatants.

The precipitated low-sulphur proteins were dissolved in saturated sodium borate (in the case of zinc precipitates in 0·2M sodium citrate) and dialysed. In order to effect a maximum separation from contaminant high-sulphur proteins the low-sulphur proteins were precipitated twice with zinc acetate by method (3) and once with acetate by method (2). After each precipitation the precipitates were dissolved and dialysed against tap water. Finally the precipitates were dissolved in saturated sodium borate, the solution dialysed first against tap water, then against distilled water, and finally freeze-dried.

Each time the low-sulphur fractions are reprecipitated small additional amounts of high-sulphur protein appear in the supernatants which in the aggregate probably do not represent more than 2% of wool. The estimates of high-sulphur protein contents of extracts described in this paper are therefore low by this amount but since this error affects extracts of control and steely wools equally a comparison between them is not affected.

(*d*) *Determination of Protein Concentration*

(i) *Total Amount of Protein Extracted (based on the weight of undissolved wool).*— The undissolved wool residue, together with occluded soluble protein and extraction fluid, was placed in 100 ml 0·1M potassium thioglycollate at pH 10·0 for 30 min and then alkylated with a mixture of 5 g iodoacetic acid and 10 g Tris. The residue

was washed on to a tared filter paper, washed with 1 l. of hot distilled water, and finally dried to constant weight at 105°C.

(ii) *Total Amount of Protein Extracted (based on measurements of refractive index increment).*—Five ml of extract was dialysed in Cellophane tubing (18/32) against two changes of 1 l. 0·02M potassium thioglycollate–0·2M KCl at pH 10·5. After 2 days the protein concentration in the dialysed extract was determined from the difference in refractive index between the solution and buffer using a Brice–Phoenix differential refractometer, assuming $dn/dc = 1{\cdot}85 \times 10^{-3}$ (Harrap and Gillespie 1963).

(iii) *Protein Concentrations by the Biuret Procedure.*—The biuret procedure of Mehl (1945), employing a standard of previously isolated high-sulphur protein, was used to estimate the protein concentration of dialysed alkylated extracts and of supernatants from zinc acetate precipitations of these extracts.

(iv) *High-sulphur Protein Concentrations by Refractive Index Increment Measurements.*—The protein concentrations of supernatants from pH 4·4 precipitations by the dialysis procedure [Section II(*c*)(2)] were measured from the difference in refractive index n between the buffer and the supernatant, assuming a specific refractive index increment (dn/dc) of $1{\cdot}85 \times 10^{-3}$.

(v) *High-sulphur Protein Concentrations by Optical Density Measurements.*—The protein concentrations of supernatants from the precipitation of the low-sulphur proteins by any of the three methods of Section II(*c*) were also estimated from the measurement of optical densities at 276 mμ using a value of $E^{1\%}_{1\,cm}$ of 5·5. Suitable reagent blanks were used in every case.

In all the procedures employing dialysis the changes in volume were measured by a weighing technique and suitable corrections were employed in the calculations of protein concentrations.

(*e*) *Electrophoresis*

The freeze-dried high-sulphur proteins were dissolved in acetic acid–sodium acetate buffer (pH 4·5, ionic strength 0·1) to give a 1·1–1·2% solution and dialysed against 2 l. of this buffer. Electrophoresis in this buffer system appears to give the best resolution of these proteins. The runs were carried out in a Tiselius moving boundary apparatus (LKB-Produkter, Stockholm) for 4 hr at a voltage gradient of about 5·5 V/cm. Mobilities were calculated from the rate of peak movement in the descending boundary. The proportions of components in the proteins were estimated from the mean of the relative areas under peaks in the ascending patterns. On tracings, the peaks were arbitrarily defined by vertical lines, the horizontal distance covered under each peak being kept the same in control and steely wool patterns in order to make the results strictly comparable.

(*f*) *Analysis of Amino Acid Content*

Samples (30 mg) of freeze-dried protein were hydrolysed under reflux with 5 ml constant-boiling HCl for 24 hr, then freeze-dried. The analyses were made with a Beckman Spinco automatic amino acid analyser. As found previously partial destruction of *S*-carboxymethylcysteine (SCMC) occurs during hydrolysis, resulting

in the formation of some cystine. The sum of these two residues approximates to the amount of SCMC in the unhydrolysed protein (Gillespie 1963*a*).

(*g*) *Starch-gel Electrophoresis*

Both low- and high-sulphur proteins from normal and copper-deficient wools were compared by starch-gel electrophoresis. The apparatus and techniques used are described by Bodman (1960) and Thompson and O'Donnell (1964). The Tris–citrate buffer system of Poulik (1957) was employed, in the presence of 8M urea (Wake and Baldwin 1959).

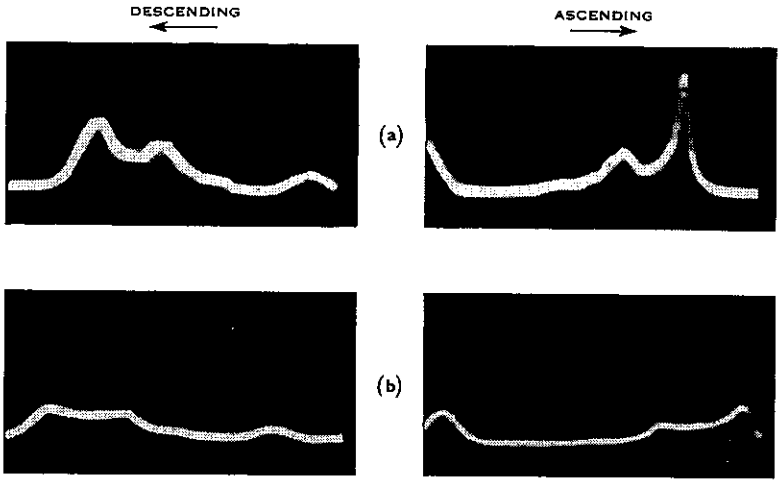

Fig. 1.—Moving boundary electrophoresis of high-sulphur proteins in acetic acid-sodium acetate buffer, 0·1 ionic strength, at pH 4·5 and a protein concentration of 1%. (*a*) *S*-carboxymethyl kerateines; (*b*) γ-keratose (performic acid method of O'Donnell and Thompson 1959).

III. Results

(*a*) *Quantitative Methods for Estimating Wool Proteins*

(i) *Consideration of Methods for Quantitatively Solubilizing Wool*

Because of the preferential extraction of one or other protein component from wool (Corfield, Robson, and Skinner 1958; Gillespie 1962) any method designed to measure the relative proportions of protein components in wool must aim at extracting essentially all the protein from the fibre, otherwise the results can be misleading. The upper limit for extractability of wool is generally regarded as being about 90% (Alexander and Hudson 1954). The residue called β-keratose was suggested to be mostly membranous (Alexander and Earland 1950; Mercer 1961) but as its composition is not unlike that of wool (Corfield, Robson, and Skinner 1958), it may still contain α- and γ-keratoses. In selecting a method for dissolving wool for this study, two alternatives were considered: performic acid oxidation (Blackburn and Lowther 1951) as modified by Burley and Hordern (1959, 1960), followed by extraction with ammonia or buffers; and the urea–thioglycollate extraction method used by Jones and Mecham (1943) and by Harrap and Gillespie (1963).

Although each method has its own merits, the reductive procedure followed by alkylation was selected largely because the *S*-carboxymethyl high-sulphur proteins are better resolved into components than is γ-keratose by both electrophoresis (Fig. 1) and by chromatography (Thompson, personal communication). Because the former proteins have been more thoroughly studied than γ-keratose, changes in the synthetic pattern of the components can also be more easily recognized.

In general, the experimental wools were more difficult to dissolve than MW148 Merino wool, and the steely wools were more resistant than their controls, as was also found by Burley and Hordern (1959). In order to extract comparable amounts of protein from sets of control and steely wool from each sheep, it was necessary to

TABLE 1

COMPARISON OF METHODS FOR THE ESTIMATION OF TOTAL PROTEIN AND PERCENTAGE OF HIGH-SULPHUR PROTEIN

Merino wool (MW148) extracted with urea–thioglycollate

Method of Estimation	Total Protein Extracted (as % of dry wool)	High-sulphur Protein in Supernatant (as % of dry wool) after Precipitation* with:		
		Zinc Acetate, pH 6·0	Acetate Buffer, pH 4·4	
			Reagent Added in Solution	Reagent Added by Dialysis
Dry weight of undissolved wool	91	†	†	†
Refractive index ($dn/dc = 1 \cdot 85 \times 10^{-3}$)	89‡	†	†	27·5
Biuret (using high-sulphur protein as standard)	105§	28·6	†	†
Optical density at 277 mμ [$E^{1\%}_{1\ cm}$ (277 mμ) = 5·5 with reagent blank]	†	35·9	31·3	27·9

* To remove low-sulphur protein.

† Method either unsuitable or not used for this analysis.

‡ Measured on –SH protein.

§ After alkylation and dialysis.

extract the latter wools more vigorously—either by increasing the urea concentration to 10M or the liquor–wool ratio to 100 : 1. Although at first sight the extracts obtained in this way from normal and steely wools are not strictly comparable, control experiments showed that, regardless of which of these three extraction conditions were employed, the high-sulphur proteins obtained from normal wool had the same amino acid and electrophoretic compositions. For this reason a valid comparison is possible.

(ii) *Estimation of Total Protein Extracted*

Employing Merino wool MW148, the three methods for estimating the total protein [Section II(*d*)] in urea–thioglycollate extracts were compared. The results are given in Table 1 and show a good agreement between the refractive index and

dry weight methods, but poor agreement with the biuret method. When a protein standard of high-sulphur protein is used in the latter method, results are obtained which are too high, and when a low-sulphur protein is used as a standard results are too low. Thus when dealing with a wool extract, a mixture of indefinite composition, it appears impossible to obtain a suitable standard protein for estimation by the biuret method.

(iii) *Separation and Estimation of the High-sulphur Proteins*

Three methods were used for precipitating the low-sulphur proteins [Section II(*c*)] and the three methods for the estimation of protein concentration in the supernatants from these precipitations were compared.

The results in Table 1 show that there was good agreement between the values obtained by the biuret method on the zinc supernatant and specific refractive index increment and optical density measurements on the supernatant from a dialysis precipitation.

The disagreement with the other estimations is due to contaminating material in the system which strongly absorbs in the ultraviolet and is characterized by a decrease in absorbency when the pH is lowered from 6 to 4·5. This material can be removed from the proteins by dialysis against 0·5M acetate buffer at pH 4·5 but not by dialysis against a similar buffer at pH 7. Of the three likely protein-bound impurities—dithioglycollic acid, biscarboxymethylsulphide, and iodoacetic acid—only the latter material shows the required drop in absorption at 276 mμ as a neutral solution is acidified.

Because of possible complications with this material the biuret method has been used with zinc supernatants and the specific refractive index method with supernatants obtained by precipitation at pH 4·4 by the acetate dialysis method.

(iv) *Dialysable Protein*

Little is known about the extent of dialysability of the proteins prepared by thioglycollate extraction of wool. Certainly at high pH values and temperature some dialysable protein is produced (Gillespie and Lennox 1955). However, the good agreement between the two methods for determining total extraction suggests that little dialysable protein is produced by the urea–thioglycollate procedure. The following tabulation shows the results obtained with the specific refractive index method for measuring total protein concentration when the time of dialysis was extended over several days:

Period of dialysis (days):	1	2	3	6	10
Total protein concentration (% of dry wool):	87·2	87·8	85·8	86·0	86·5

Up to 10 days, within experimental error, only traces of protein have disappeared from the system.

(*b*) *Examination of the Differences Between Normal and Steely Wools*

(i) *Analytical Differences*

In confirmation of the results of other workers (Marston 1946; Burley and Hordern 1959) it has been found that, as compared with normal wool from the same

staple, steely wool contains slightly less sulphur (normal 3·58%; steely 3·43%), but considerably more –SH (normal 25 μmoles/g; steely 105 μmoles/g). This analytical data applies to Merino wool MW150/83.

(ii) *Difference in the Content of High-sulphur Protein*

Because of the difficulty of quantitatively extracting reduced protein from steely wools as compared with controls it has been found hard to make comparisons between their apparent contents of high-sulphur proteins. Steely wool (Table 2) apparently contains less high-sulphur protein than normal wools. However, when steely wools are extracted to give a yield of total protein approaching that of the controls (Table 2) then the two wools do not appear to differ much in their content

TABLE 2

COMPARISON OF THE HIGH-SULPHUR PROTEIN CONTENT OF NORMAL AND STEELY WOOLS FROM THE SAME STAPLE

Liquor–wool ratio 30 : 1 except where indicated

Wool Sample	Normal Wool		Steely Wool	
	Protein Extracted (%)	High-sulphur Protein (as % of dry wool)	Protein Extracted (%)	High-sulphur Protein (as % of dry wool)
Merino (MW38)	91·5	26·9	81·9 89·9*	22·4 25·5
Merino (MW150/83)	81·3	31·8	68·9 79·5*	23·4 28·6
Merino (MW46/47)	85·5	27·5	78·4 86·0*	21·8 27·9
Romney Marsh (MW150/86)	75·6	22·3	67·3	19·2
Border Leicester–Merino cross (MW150/84)	69·4	21·9	66·0	20·7

* Liquor–wool ratio 100 : 1.

of high-sulphur protein. Furthermore, calculations suggest that at exactly equal extractions the percentages of high-sulphur protein would not differ significantly. The position is not clear in the case of the Romney Marsh and Border Leicester–Merino cross wools from which unsatisfactory yields of protein were obtained. Certainly from the three Merino wools, where fairly satisfactory extractions were obtained, there was no indication of any big differences in high-sulphur protein content such as was found by Burley and Hordern (1959, 1960).

(iii) *Composition of the Insoluble Residue*

Although it was possible to extract equal amounts of proteins from normal and steely wool in each pair, the level obtained usually did not reach the desired 90%. If the wool was preferentially extracted for low-sulphur protein or high-

TABLE 3

AMINO ACID ANALYSES OF HIGH-SULPHUR PROTEINS FROM PAIRS OF NORMAL AND STEELY WOOL FROM THE SAME STAPLES

Analytical values are given as amino acid nitrogen expressed as a percentage of total nitrogen

Amino Acid	Merino Wool (MW38)			Merino Wool (MW46/47)			Merino Wool (MW150/83)					Border Leicester–Merino Cross Wool (MW150/84)		
								Steely						
	Normal	Steely	Difference (%)	Normal	Steely	Difference (%)	Normal	Liquor–Wool Ratio 30:1	Difference (%)	Liquor–Wool Ratio 100:1	Difference (%)	Normal	Steely	Difference (%)
Lysine	0·82	0·86	5	0·70	0·74	6	1·32	1·18	−11*	1·26	−5	0·71	0·77	8
Histidine	1·24	1·33	7	1·69	1·78	5	1·32	1·18	−11*	1·26	−5	1·42	1·31	−8
Arginine	13·30	14·11	6	16·66	12·86	−23*	14·56	13·93	−4	14·85	2	15·80	14·66	−7
SCMC	12·84	10·19	—	12·97	10·53	—	13·21	10·80	—	10·44	—	13·26	9·36	—
SCMC plus ½ cystine	16·02	13·98	−13*	16·23	14·16	−13*	15·58	13·40	−14*	12·51	−20*	15·85	12·43	−22*
Aspartic acid	1·85	2·15	16*	1·75	2·14	22*	1·90	2·38	25*	2·45	29*	1·96	2·47	26*
Threonine	7·28	7·06	−3	7·61	7·64	0	7·36	7·25	−2	6·84	−7	7·41	6·97	−6
Serine	8·74	8·36	−4	9·14	9·35	2	9·08	9·86	8	9·01	−1	9·37	9·18	−2
Glutamic acid	5·69	5·75	1	5·75	5·89	2	5·74	5·86	2	5·58	−2	5·60	5·37	−4
Proline	8·87	8·63	−3	9·51	10·03	5	9·17	8·79	4	8·53	7	8·99	8·72	−3
Glycine	4·37	4·44	2	4·62	4·83	5	4·24	4·70	11*	4·71	11*	4·55	4·53	0
Alanine	2·12	2·22	5	2·13	2·51	18*	2·16	2·46	14*	2·41	13*	2·05	2·20	7
½ Cystine†	3·18	3·79	—	3·35	3·63	—	2·37	2·60	—	2·07	—	2·59	3·07	—
Valine	4·10	4·18	2	4·22	4·41	5	4·07	4·17	2	4·16	2	3·99	3·91	−2
Isoleucine	2·12	2·22	5	2·13	2·26	6	2·09	2·14	2	2·09	0	2·17	2·16	0
Leucine	2·52	2·82	12*	2·62	3·04	16*	2·41	2·72	14*	2·76	14*	2·51	2·76	10*
Tyrosine	0·66	0·65	−2	1·23	0·94	−24*	0·96	1·11	16*	0·77	−20*	1·21	0·96	−21*
Phenylalanine	1·19	1·31	10*	1·13	1·25	11*	1·11	1·28	17*	1·32	17*	1·21	1·36	12*

* Differences exceeding 10%. † The unhydrolysed protein contained no detectable –S–S–.

sulphur protein, analyses of the solubilized proteins would give an entirely false picture of the proportions of these proteins in the wools. To check this possibility, residues were analysed for their content of sulphur, since any inequalities in extraction would be reflected in wide differences of this element. In every case there were essentially no differences; as an example, with sample No. MW150/83 the normal wool residue contained 3·58% sulphur and the steely wool residue 3·51% sulphur.

(iv) *Amino Acid Analyses of High-sulphur Proteins*

The results of amino acid analyses made on pairs of high-sulphur proteins prepared for normal and steely wools are shown in Table 3. In each set there are very significant differences between the proteins. In contrast to the controls, high-sulphur proteins of steely wool contain considerably less SCMC but more aspartic acid, leucine, and phenylalanine, and in individual cases more alanine and glycine. Tyrosine is very variable and as there is no consistent change, no conclusions can be made concerning this amino acid.

(v) *Electrophoresis of High-sulphur Proteins*

A comparison of moving boundary electrophoresis patterns of high-sulphur proteins from normal and steely wools show marked differences in the distribution of components (Fig. 2). In every case calculation of the areas under peaks (Table 4) indicates that steely wool contains less of the fastest-moving proteins (peak D) and usually an equal increase in the amount of the slower-moving material in peak C but with no changes in the other components. However, with the sample of Romney Marsh wool (MW150/86) peak D protein may possibly have been replaced with both peak B and peak C material.

It has been shown with both Merino and Southdown wools that there is a linear relation between sulphur content and the electrophoretic mobility at pH 4·5 of high-sulphur protein components (Gillespie 1963*b* and unpublished data). Therefore the electrophoretic findings indicate that in steely wool those components richest in sulphur have been replaced by components of lower sulphur content. This, then, is the origin of the decrease in SCMC content reported in Table 4.

Mobilities have been calculated for each of the major peaks in each of the protein preparations (Table 5). It can be seen that within experimental error the mobilities of the various components in pairs of proteins are identical. As judged by moving boundary electrophoresis then, the same types of proteins are present in normal and steely wool but in differing proportions.

The electrophoretic runs described in this section were usually made on high-sulphur proteins prepared from extracts made by the procedure in which the ratio of liquor to wool is 30 : 1, even though the high-sulphur protein so obtained did not constitute all that could be extracted. To make certain that the changes found in proportions of peaks C and D were not distortions due to the preferential extraction of peak C material, in several cases high-sulphur proteins prepared from extracts in which the liquor–wool ratio was 100 : 1 were also run. Comparable results for MW150/83 show no differences between the two types of extract (Tables 4 and 5) and thus suggests that regardless of the extent of extraction a fair sample of the

TABLE 4

COMPARISON OF THE PERCENTAGES OF RESOLVABLE COMPONENTS IN HIGH-SULPHUR FRACTIONS FROM NORMAL AND STEELY WOOLS FROM THE SAME STAPLES

Results are from the ascending electrophoretic patterns shown in Figure 2

Wool Sample	Type of Wool	Peak A	Peak B	Peak C	Peak D
Merino (MW38)	Normal	10	35	18	38
	Steely	9	36	25	30
Merino (MW150/83)	Normal	14	26	16	45
	Steely				
	Liquor–wool ratio 30 : 1	16	26	21	37
	Liquor–wool ratio 100 : 1	11	28	23	37
Merino (MW46/47)	Normal	10	35	14	41
	Steely	9	34	23	33
Romney Marsh (MW150/86)	Normal	8	36	21	35
	Steely	8	39	23	30
Border Leicester–Merino cross (MW150/84)	Normal	13	29	23	35
	Steely	12	29	27	32

TABLE 5

COMPARISON OF MOBILITIES OF RESOLVABLE COMPONENTS IN HIGH-SULPHUR FRACTIONS FROM NORMAL AND STEELY WOOLS FROM THE SAME STAPLES

Results are from descending electrophoretic patterns (same run as ascending pattern shown in Fig. 2)

Wool Sample	Type of Wool	Mobilities ($\times 10^5$)* ($cm^2\ sec^{-1}\ volt^{-1}$)			
		Peak A	Peak B	Peak C	Peak D
Merino (MW38)	Normal	−2·8	−4·6	−5·5	−6·5
	Steely	†	−4·9	−5·8	−6·5
Merino (MW150/83)	Normal	†	−4·8	−5·7	−6·6
	Steely				
	Liquor–wool ratio 30 : 1	†	−5·0	−5·6	−6·6
	Liquor–wool ratio 100 : 1		−4·8	−5·5	−6·7
Merino (MW46/47)	Normal	†	−4·5	−5·7	−6·5
	Steely	†	−4·2	†	−6·4
Romney Marsh (MW150/86)	Normal	−3·1	−4·9	−5·5	−6·6
	Steely	†	−5·1	−5·6	−6·6
Border Leicester–Merino cross (MW150/84)	Normal	−3·1	−4·8	−5·7	−6·6
	Steely	−3·3	−5·2	†	−6·6

* To nearest 0·1 unit. † Peak not resolved in descending boundary.

high-sulphur proteins is obtained. This observation is in agreement with previous studies on Merino wool which indicated that apparently no preferential extraction

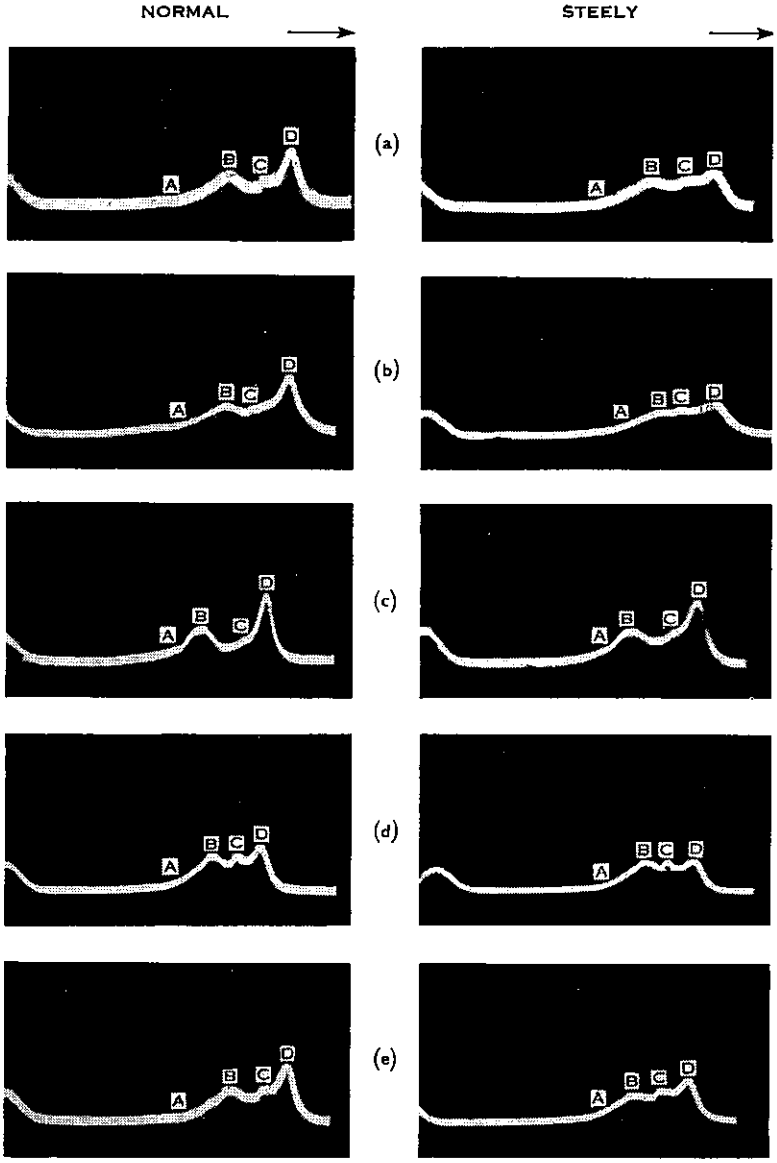

Fig. 2.—Moving boundary electrophoresis of high-sulphur proteins from normal and steely wools run in acetic acid-sodium acetate buffer, 0·1 ionic strength, at pH 4·5 and a protein concentration of 1·0–1·2%. (*a*) Merino (MW38); (*b*) Merino (MW150/83); (*c*) Merino (MW46/47); (*d*) Romney-Marsh (MW150/85); (*e*) Border Leicester–Merino (MW150/84).

of individual high-sulphur components could be achieved by incomplete extraction with alkaline thioglycollate solutions (Gillespie 1962).

(vi) *Amino Acid Analyses of Low-sulphur Proteins*

The content of amino acids in hydrolysates of low-sulphur proteins from samples of normal and steely wools from the same staple is given in Table 6. In general, the proteins do not appear to differ significantly from each other, as the differences in most residues fall within or very close to the commonly accepted $\pm 3\%$. Threonine and serine fall outside this limit but in view of the variable hydrolytic destruction which occurs with these amino acids, the differences may be of little significance.

TABLE 6

AMINO ACID ANALYSES OF LOW-SULPHUR PROTEINS FROM NORMAL AND STEELY WOOLS FROM THE SAME STAPLE

Analytical values for Merino wool sample MW38 are given as amino acid nitrogen expressed as a percentage of total nitrogen

Amino Acid	Normal Wool	Steely Wool	Difference (%)
Lysine	5·42	5·23	−4
Histidine	1·36	1·37	0
Arginine	21·00	19·50	−7
SCMC	4·10	3·74	—
SCMC+½ cystine	4·82	4·45	−7
Aspartic acid	6·35	6·55	3
Threonine	3·41	3·70	8
Serine	6·32	6·85	9
Glutamic acid	11·20	11·52	3
Proline	2·33	2·46	6
Glycine	5·63	5·74	2
Alanine	4·84	4·92	2
½ Cystine*	0·72	0·71	—
Valine	4·40	4·40	0
Methionine	0·33	0·33	0
Isoleucine	2·60	2·61	0
Leucine	7·20	7·33	2
Tyrosine	1·54	1·61	5
Phenylalanine	1·97	2·03	3

* Unhydrolysed protein contained no cystine.

(vii) *Electrophoresis of Low-sulphur Proteins*

Moving boundary electrophoresis at pH 11·0 of low-sulphur proteins from normal and steely wools showed in each case single symmetrical peaks with no evidence of resolvable impurities (Fig. 3). Descending mobilities were not significantly different, being respectively $-7{\cdot}4\times10^{-5}$ and $-7{\cdot}3\times10^{-5}$ cm²/volt/sec at a protein concentration of 1·1–1·2%.

(viii) *Starch-gel Electrophoresis*

The high-sulphur proteins from pairs of normal and steely wools have been compared by starch-gel electrophoresis (Fig. 4). No difference can be seen in the number of bands and therefore judged by this method the proteins are identical. It appears therefore that in the shift in synthesis of the high-sulphur proteins in copper deficiency the regular proteins are produced but in different amounts.

A similar comparison of the low-sulphur proteins, whilst making evident no new bands, shows that in the protein from steely wool there is a slight increase in mobility of the faster major band and a decrease in the amount of fast-moving contaminant proteins.

IV. Discussion

In going from the normal to the copper-deficient state, the amino acid composition of the low-sulphur protein mixture is unaltered, as Burley and Hordern (1960) also found, and there are no detectable differences in electrophoretic patterns or mobilities. As this is also the situation with wool of increased sulphur content (Gillespie, Reis, and Schinckel 1964), it seems that although the rate of production of these proteins can be controlled by variations in diet, no changes in composition are allowable by the cell.

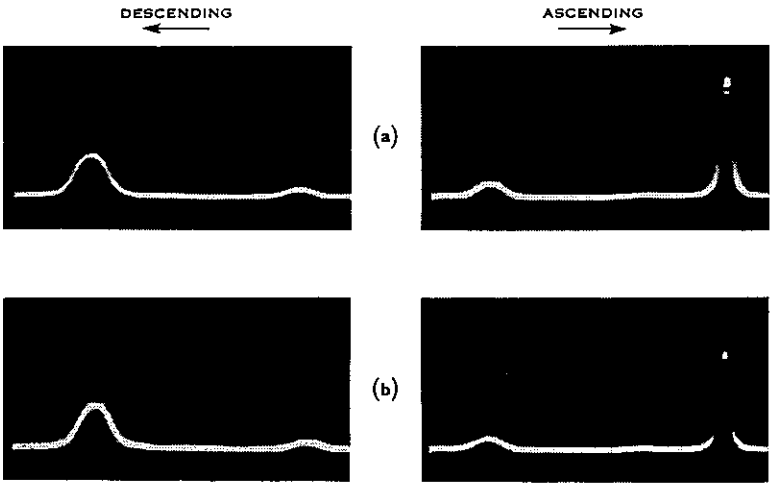

Fig. 3.—Moving boundary electrophoresis of low-sulphur protein from normal and steely Merino wool (MW38) run in β-alanine–NaOH buffer, 0·1 ionic strength, at pH 11·0 and a protein concentration of 1·2%.

Any changes in protein composition appear to be confined to the high-sulphur protein fraction, and in steely wool, a defect in the synthesis of these proteins is evidenced by a partial shift in synthesis from those components richest in sulphur to those having intermediate sulphur contents. Amino acid analyses of the proteins show the expected decrease in SCMC content as compared with the control but also an unexpectedly large increase in aspartic acid, phenylalanine, and leucine, and in some cases in glycine and alanine. It adds to the significance of these results that in parallel experiments on high-sulphur proteins from wools with increased sulphur content, an increase in SCMC content is accompanied by a decrease in these amino acids (Gillespie, Reis, and Schinckel 1964).

In contrast with the present work, Burley and Hordern (1960) found quite large decreases in total high-sulphur protein in their steely wools as compared with the controls. It is possible that this is a spurious result caused by their inability to extract as much total protein from steely wool as from the controls. However, there are

several facts which should be borne in mind in comparing the two sets of work. Firstly, that steely wool is but one (and the earliest) syndrome in a pathological condition of which anaemia is the end result (Underwood 1962) and it it is possible to get varying degrees of changes in wool by adjustment of copper intake. The effects of stepwise increments in copper feeding to deficient animals are well illustrated by the series of photographs showing changes in fleece properties given by Marston and Lee (1948*b*) and Lee (1956) and by the changes in physical properties discussed by Palmer (1949). It is quite possible therefore that the South Australian wools examined

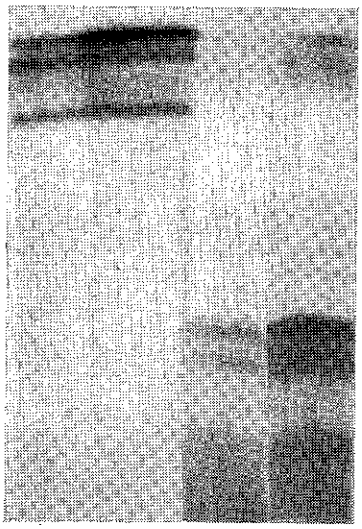

Fig. 4.—Starch-gel electrophoresis of proteins from normal and steely wool. Run in Tris–citrate–8M urea at pH 8·6.

in the present study and the South African wools used by Burley and Hordern are representative respectively of mild and more severe deficiency states. The first change in the proteins may be the shift in the synthetic pattern of the high-sulphur proteins, followed in more severe deficiency states by a decrease in the total amount of high-sulphur protein formed. In the present work the data of Figure 2 and Table 4 would suggest that MW150/84 wool is less altered than for example MW150/83 wool. Secondly, as Dick (1956) has pointed out, the steely wool of coastal South Australia is seldom due to a true deficiency of copper intake but is usually the result of an upset in absorption and retention of copper caused by high molybdenum intake. It could well be that the high-sulphur proteins are altered in different ways depending on the way in which the copper deficiency is induced. It will require a comparative investigation on the proteins of steely wool produced in both ways to settle this point.

It is possible that the altered synthesis of high-sulphur proteins in steely wool is not directly related to a deficiency of copper. In experiments on sulphur-enriched

wools (Gillespie, Reis, and Schinckel 1964) it has been found that the availability of sulphur-containing amino acids in the abomasum of the sheep controls not only the extent of wool growth but also the amount and type of high-sulphur proteins synthesized. The greater the concentration of sulphur-containing amino acids, the greater the growth of wool and also of the proportion of high-sulphur components richest in sulphur. Steely wool may represent the reverse of this situation, in which the concentration of sulphur-containing amino acids in the abomasum is reduced below normal, leading to a decrease in wool growth of as much as 40% (Marston and Lee 1948*a*, 1948*b*; Palmer 1949)* and to a decrease in the synthesis of those high-sulphur components which are richest in sulphur. The evidence thus suggests that this is another example of the control of wool synthesis by sulphur-containing amino acids.

The role of copper in this situation is unknown. Perhaps, as in cobalt deficiency, there is a change in rumen flora (Marston 1952) and with it increased losses of sulphur-containing amino acids, or alternatively some copper enzyme essential to cysteine metabolism may be present in reduced amount. In support of the former proposition is the work of Mills and Quarterman (1963) who found an increase in sulphide concentration in the rumen of sheep with a molybdenum-induced copper deficiency, which suggested to them that a change had occurred in the pattern of sulphur metabolism of the digestive tract. However, Underwood (1962) has suggested that the " . . . reduced food consumption by the copper-deficient animals" is responsible for the decreased wool growth and therefore the change in the pattern of high-sulphur protein metabolism may just reflect a decrease in intake of sulphur-containing amino acids.

The most striking feature of copper-deficient fleeces is the presence of straight lustrous fibres entirely devoid of the crimp which is characteristic of the wool of the normal animal. This change is accompanied by a decrease of strength of from 35–45%, depending on the method of measurement (Palmer 1949), a reduced affinity for dyes, abnormal elastic properties, and a tendency to become permanently set when stretched (Marston 1946, 1952; Marston and Lee 1948*a*, 1948*b*; Haly 1957). Besides the changes in the high-sulphur proteins already mentioned there are general increases in –SH groups (Marston 1946) and in end-groups (Burley and de Kock 1957). Changes of the magnitude described in fibre physical properties are unlikely to be explained on the basis of the observed changes in the proteins. It may be that more subtle changes have occurred in the proteins within the fibre.

A clue to the causes of these fibre changes may lie in the findings and conclusions of Marston (1946, 1950). He found that the keratinization stage in fibre formation involving the conversion of –SH to –SS–, which normally occurred in about 6–8 hr, was very much slower in copper-deficient sheep and was not complete in 3 days. He proposed that in the normal animal, as the unkeratinized fibre is extruded through the constriction at the base of the follicle, the molecules are subjected to an orientating

* Palmer (1949) reported only the comparative lengths and diameters of fibres grown for the same periods of time in the experiments of Marston and Lee (1948*b*). The change in growth rate has been calculated from these measurements assuming no change in the density of the fibre proteins. However, this decrease in wool growth in copper-deficient animals has been recognized for a long time (Bennetts 1932).

force, become aligned, and are retained in this arrangement until the completion of keratinization which permanently fixes the orientation; whereas in the copper-deficient animal, because of the slow-setting of the fibre, there is more opportunity for disorientation. Fraser (1961) has pointed out that dehydration is one force which even in normal fibre formation introduces disorder into the molecular chains.

At the molecular level three types of disorder might occur: a more random arrangement of helical polypeptide chains, an abnormal arrangement of cross-linkages, and a change in conformation of the helical chains. Conformational changes may also occur in the unorientated matrix proteins. It is not possible to decide which of these changes comes first; for example, an abnormal arrangement of disulphide bonds might allow the other changes to occur. Alternatively an initial randomization of polypeptide chains might prevent the normal pattern of cross-linking. There is strong evidence for a change in protein conformation in the marked decrease in solubility of the steely wool. For it has been repeatedly observed that when wool undergoes conformational changes by mild heating (Swan 1959, 1960; Gillespie 1962) or by reduction and alkylation (Maclaren, unpublished data) it changes to a much-less-soluble form. The extremely important idea of Marston (1952) that copper is needed for the conversion of prekeratin to keratin and that normal cross-linking is produced catalytically, by a copper enzyme, by the oxidative closure of the thiol groups present has not yet been proved. It is supported, however, by much recent work. Goldberger, Epstein, and Anfinsen (1963) found a microsomal system from rat liver which accelerated the reoxidation of reduced ribonuclease. These workers do not state whether their active material is a copper protein; however, a large group of oxidases are known to be copper proteins (Singer and Kearney 1954) and at least one of them, cytochrome oxidase, is reduced in amount in the brain tissue of lambs with ataxia due to a "conditioned" copper deficiency (Mills and Quarterman 1963). The current view is that in or near the zone of keratinization in the normal follicle, high-sulphur proteins are produced in their final form and the whole fibre is rendered insoluble (Mercer 1961; Downes, Sharry, and Rogers 1963). As a substantial proportion of the disulphide bonds appear to be between high- and low-sulphur proteins (Springell *et al.* 1964) it is probable that in the final stage of the synthetic mechanism that both types of protein chains are arranged in the correct positions for disulphide bonds to form. In copper-deficiency it seems reasonable to assume that because of the relative absence of a specific enzyme, normal cross-linking does not occur and the disorientation referred to by Fraser (1961) may so alter the relative positions of the protein chains that the correct inter-chain links cannot form, and disulphide formation may be mostly within the one chain or between chains of the same protein type. These changes would be sufficient to account for many of the altered properties of steely wool. The decrease in solubility could result from conformational changes or a change in the type of cross-linking, and the latter change could also explain the decrease in strength. The unusual elastic properties, too, could stem from these changes and also in part from the residual –SH which would facilitate interchange reactions.

A successful solution to the problem of copper deficiency in sheep by Marston and his colleagues has diverted attention from this problem. However, the insight

that this system affords into the mechanism of high-sulphur protein synthesis and of the keratinzation process suggests that it is worthy of renewed study.

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