STUDIES ON REDUCED WOOL

VI. COMPARISON OF PEPTIDES CONTAINING *S*-CARBOXYMETHYLCYSTEINYL RESIDUES IN DIFFERENT PROTEIN FRACTIONS

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Summary

Wool has been reduced and extracted by urea–mercaptoethanol solution and the cysteine residues labelled by carboxymethylation with 2-[^{14}C]iodoacetate. The extracted protein has been fractionated into the three main classes of protein present in wool, namely the high-sulphur, low-sulphur, and high-glycine–high-aromatic amino acid fractions. After partial acid hydrolysis of each fraction, peptide maps were prepared by paper ionophoretic and chromatographic methods and the *S*-carboxymethyl-containing peptides located by radioautography. The peptide maps given by the three fractions were almost identical in the peptides obtained, although marked differences in intensities were apparent. However, radioautographs of peptide maps of tryptic digests of the three fractions showed marked differences in the peptide patterns obtained. The findings are discussed in relation to the structure and synthesis of wool.

I. Introduction

Wool which has been solubilized after splitting the disulphide bonds and masking the sulphur atoms with stable chemical groups has been shown to consist of three main classes of protein (cf. Crewther *et al.* 1965). Fractions which are higher and lower in sulphur content than the wool (Goddard and Michaelis 1935; Alexander and Earland 1950) are known as high-sulphur and low-sulphur fractions respectively, while a third group is characterized by a high content of glycine and the aromatic amino acid residues (Harrap and Gillespie 1963; O'Donnell and Thompson 1964). These fractions have been assigned to particular morphological components of the wool; the low-sulphur proteins are structural proteins located in the filaments, the high-sulphur proteins originate in the matrix (cf. Mercer 1961); and the high-glycine proteins have been detected in the membrane complex between the cuticular cells (DeDeurwaerder, Dobb, and Sweetman 1964).

In previous papers (O'Donnell and Thompson 1964; Thompson and O'Donnell 1965) a method of separation of these protein fractions from reduced and carboxymethylated wool (SCMK) has been described involving DEAE-cellulose chromatography and gel-filtration on Sephadex G-200 with buffers containing 8M urea. Starch-gel electrophoresis has revealed a marked heterogeneity in each of these groups of proteins. The high-glycine fraction contains many components in small proportions and comprises about 15% by weight of the solubilized wool. The high-sulphur fraction (SCMKB), comprising about 25%, is also markedly heterogeneous as shown also by chromatography and moving boundary electrophoresis (Gillespie

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1963). The low-sulphur fraction (SCMKA) has been shown to consist of two main components, called components 7 and 8, together with aggregates formed predominantly from component 7. In the present work the thiol groups of reduced and extracted wool have been radioactively labelled with 2-[^{14}C]iodoacetate and the three labelled fractions of the soluble wool proteins have been isolated. These have been used to study the sequences of amino acid residues around the half-cystine residues to see if there are similarities between the various fractions.

The amino acid sequences of the components of these three different protein fractions are obviously different judging by the large differences in amino acid composition. It would not be expected that large peptides liberated by specific methods of hydrolysis, e.g. tryptic digestion, would show obvious similarities. However, a random method of hydrolysis, such as that with concentrated HCl which gives a mixture of amino acids and di- and tripeptides (Gordon, Martin, and Synge 1941; Sanger 1952), could reveal similar sequences and throw light on the possibility that these proteins are derived from a common precursor. The half-cystine peptide sequences of partial acid hydrolysates of the various fractions are of interest in view of the study that has been made of them in unfractionated wool in the pioneer work of Gordon, Martin, and Synge (1941) and Consden and Gordon (1950). They are also of interest because of the differential rate of incorporation of radioactive cystine into the high- and low-sulphur protein fractions during the growth of wool (cf. Mercer 1961; Downes, Sharry, and Rogers 1963), and the equal distribution of the readily reducible cysteine residues of wool between the high- and low-sulphur proteins (Blackburn 1961; Gillespie and Springell 1961; Springell *et al.* 1964).

II. Materials and Methods

Labelled 2-[^{14}C]iodoacetic acid (5 mc/m-mole) was supplied by Radiochemical Centre, Amersham, England.

Merino 64's fleece wool MW 138 was used and the method of reduction and labelling was similar to that described in the previous paper (Thompson and O'Donnell 1965) except that the [^{14}C]iodoacetate was not prepared from [^{14}C]bromoacetate. After alkylation with the theoretical amount of [^{14}C]iodoacetate the protein was dialysed against "chromatography buffer" [8M urea – 0·01M Tris – 0·001M Versene (ethylenediaminetetraacetic acid, disodium salt), pH 7·4], re-reduced at pH 10·5 and alkylated with unlabelled iodoacetate to ensure complete absence of disulphide bonds. The extract from 150 mg of wool was dialysed against the urea buffer and fractionated by gel-filtration on a Sephadex G-200 column (2·3 cm int. diam. by 120 cm). The slowest-moving peak (S4, Fig. 1) had little radioactivity and it represents ultraviolet-absorbing non-protein impurity. The slow-moving fraction S3 which is a mixture of high-sulphur and high-glycine components was further fractionated by absorbing the high-sulphur components on a column (0·9 cm int. diam. by 15 cm) of DEAE-cellulose (Serva Entwicklungslabor, Heidelberg) equilibrated with chromatography buffer containing 0·05M KCl at pH 7·4. The bulk of the high-glycine components pass unretarded through the column (Thompson and O'Donnell 1965). After washing the column with 25 ml of equilibrating buffer the high-sulphur fraction was eluted by increasing the concentration of potassium chloride to 1M.

The four fractions [S1, representing low-sulphur aggregates (formed predominantly from component 7), S2, representing low-sulphur components 7 plus 8 (see Fig. 1 and Plate 1, Fig. 1), and the high-sulphur and the high-glycine–high-aromatic amino acid fractions (both prepared from S3)] were dialysed successively against 0·001M borate, 0·3M KCl – 0·001M borate, and water before freeze-drying. A sample (5 mg) of each fraction was partially hydrolysed in 1 ml 10·5N HCl at 39°C for 3 days. The acid was removed by a freeze-drying technique after dilution with water. The residue was dissolved in 0·2 ml of water and 10-μl samples (equivalent to 0·25 mg) were fractionated by ionophoresis at pH 6·5 or pH 3·5 (cf. Thompson and O'Donnell 1962). For tryptic digestions 1 mg of each fraction was mixed with 0·1 ml of 1%

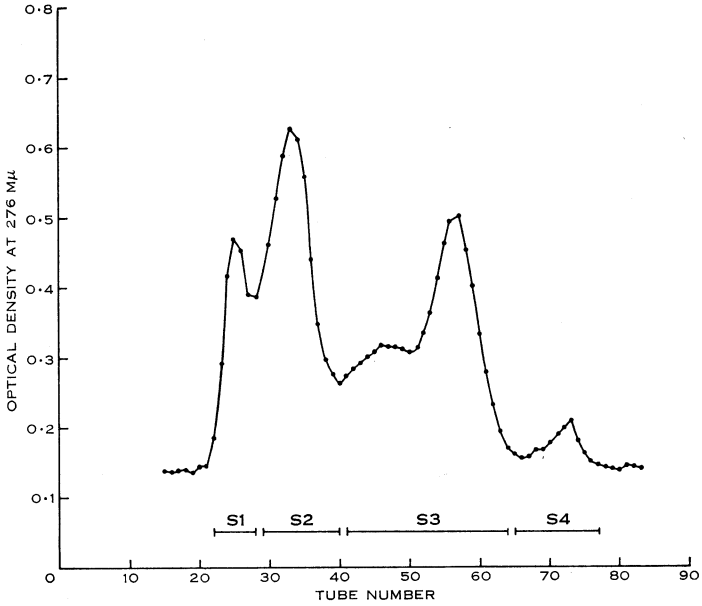

Fig. 1.—Gel-filtration on Sephadex G-200 of a dialysed extract (SCMK) from wool reduced and carboxymethylated with 2-[^{14}C]iodoacetate, in 8M urea–Tris buffer at pH 7·4. Fraction size 8 ml. Column dimensions 2·3 cm internal diameter by 120 cm. Fractions bulked for starch-gel electrophoresis are indicated.

ammonium carbonate solution (pH 8·7) containing 0·01% of crystalline trypsin (Worthington Biochemical Corporation) and 0·01% of merthiolate. After 18 hr at 37°C 25-μl samples were applied directly to a paper strip for ionophoresis at pH 6·5.

For two-dimensional separations the acidic and neutral bands were sewn on a second sheet of Whatman 3 MM paper. For ionophoresis in the second dimension a buffer of pH 3·5 was used, while, for chromatography, a butan-1-ol–acetic acid–water mixture (4 : 1 : 1 v/v) was used. Radioautographs were prepared with Ilford X-ray film (Industrial G) after exposure for 48 hr (Naughton *et al.* 1960).

The dipeptide *S*-carboxymethylcysteinyl.*S*-carboxymethylcysteine was prepared from the dibenzyl derivative of cysteinylcysteine by removing the benzyl

groups with sodium in liquid ammonia (Wood and du Vigneaud 1939) and, after destroying excess sodium, carboxymethylating with the theoretical amount of iodoacetic acid. After evaporating the ammonia the material was adsorbed on Dowex-50 resin (in the H^+ form) and, after washing with water, eluted with ammonia.

III. Results

The separation of the ^{14}C-labelled components by gel-filtration on Sephadex G-200 is shown in Figure 1 and Plate 1, Figure 1. After separation of the high-sulphur and high-glycine fractions on DEAE-cellulose the weights of protein fractions recovered from 150 mg of wool were as follows—aggregates (15 mg), SCMKA components 7 plus 8 (48 mg), SCMKB (18 mg), and high-glycine fraction (9 mg). The degree of contamination of each fraction by other fractions is very small and not sufficient to complicate the interpretation of peptide maps revealed by radioautography.

The radioautographs of the partial acid hydrolysates of the fractions after separation by one-dimensional ionophoresis at pH values of 6·5 and 3·5 are illustrated in Plate 1, Figure 2. The resolution obtained does not reveal obvious differences between the fractions. There are only traces of labelled *S*-carboxymethylcysteinyl residues in peptides that are positively charged at pH 6·5. This result does not agree with the analytical data of Gordon, Martin, and Synge (1941) which suggested the presence of cystinyl residues in considerable amounts in the basic fraction of a partial hydrolysate of wool separated in a three-compartment cell. It is probably due to the increased negative charge introduced by carboxymethylation of thiol groups in reduced wool.

When the acidic and neutral peptides from a pH 6·5 ionophoretic separation were fractionated further by two-dimensional ionophoresis or by chromatography it was possible to examine the radioautographs more critically for differences between the fractions. The resolution of the bulk of peptides was better using chromatography in the second dimension although the spots were more diffuse and some minor components were more readily distinguishable after ionophoresis.

Plate 2, Figures 1–3, shows the patterns obtained by two-dimensional ionophoresis and Plate 2, Figures 4–6, the patterns obtained by ionophoresis–chromatography. In all the partial acid hydrolysates the free amino acid *S*-carboxymethylcysteine was the strongest radioactive fragment since it is formed partially from every *S*-carboxymethylcysteinyl residue.

The patterns given by the three fractions differ in relative intensity of various peptides and it appears as though some peptides present in the other two fractions are absent from the high-glycine fraction. However, the methods have not proved sufficiently definitive to demonstrate that any given strong peptide spot in the pattern from the low-sulphur fraction is entirely absent from the patterns of the high-sulphur fractions although it may be present with a very low intensity. As there is a higher sulphur content in the high-sulphur fraction, more radioactivity will be present in a given weight of its hydrolysate compared with the hydrolysate of an equal weight of the other fractions. However, the general similarity in the peptides obtained, particularly from the high-sulphur and low-sulphur fractions, is very

striking. Only a few minor spots can be seen in the high-sulphur pattern that are absent in the low-sulphur pattern (Plate 2, Figs. 1–6).

The major spot of *S*-carboxymethylcysteine is readily distinguished in Plate 1, Figure 2, and Plate 2, Figures 1–3, from its slight mobility toward the positive electrode at pH 3·5 while at pH 6·5 it is considerably less mobile than some peptides. The peptide of fastest mobility at pH 6·5 (Plate 1, Fig. 2; Plate 2, Figs. 4–6) which remains near the origin at pH 3·5 and which has a similar chromatographic rate to *S*-carboxymethylcysteine has been identified by comparison with the synthetic material as *S*-carboxymethylcysteinyl.*S*-carboxymethylcysteine. It is present in all partial hydrolysates as a major component but its relative intensity will be higher than on a molar basis due to the presence in it of two radioactive residues.

No attempt has been made to compare the other peptides with peptides containing half-cystine residues identified by Consden and Gordon (1950) in partial acid hydrolysates of wool since synthetic peptides of the known sequences were not available. However, the ionophoretic rates and semi-quantitative estimates of these peptides when compared with those found by Consden and Gordon (1950) would assist in their identification. Methods similar to those of Naughton *et al.* (1960) and Milstein and Sanger (1961) could be used for identifying these labelled peptides.

Plate 2, Figures 7–9, shows a comparison of radioautographs of the peptide maps of the acidic and neutral peptides containing *S*-carboxymethyl residues in tryptic digests of the low-sulphur, high-sulphur, and high-glycine fractions. The low-sulphur and high-sulphur digests were run in parallel on the same sheet of paper and their peptide maps are therefore directly comparable. Their patterns are obviously different from that given by the high-glycine fraction (which was not run simultaneously at pH 3·5) and they show many differences from each other in relative intensities of particular spots. There could be some tryptic peptides common to the high- and low-sulphur fractions but there are certainly many more differences than were shown in the corresponding peptide maps of partial acid hydrolysates.

There is an intense area in the high-sulphur pattern, which must represent larger acidic fragments obtained by tryptic digestion, which do not fractionate into discrete spots during paper ionophoresis. Because of this smearing and the similar distribution of peptides on the paper it is not possible to say that some particular peptides from the low-sulphur fraction are entirely absent in the high-sulphur fraction. However, some peptides in the low-sulphur pattern are absent from the pattern of the high-sulphur fraction (noticeably in those peptides neutral at pH 6·5) and there is an overall difference in the distribution of radioactivity.

IV. Discussion

Partial acid hydrolysis of a protein results in partial breakdown of every peptide bond but because of steric and electrostatic factors some bonds are stabilized in the di- and tripeptide derivatives. Certain peptide bonds will be hydrolysed completely after 3 days at 37°C, particularly those involving the amino groups of serine and threonine residues (Gordon, Martin, and Synge 1941; Sanger 1952). Although Gordon, Martin, and Synge (1941) found that bonds involving amino groups of threonine residues in wool were more labile than those involving serine during partial acid hydrolysis, Consden and Gordon (1950) reported that a cystinylthreonine

bond (identified after oxidation as $CySO_3H$.Thr) was present in relatively large quantities after 10 days partial acid hydrolysis under conditions similar to those used in the present study. The same bond, however, may not be as stable once the cystine residues have been converted to *S*-carboxymethylcysteine residues.

Consden and Gordon (1950) identified about 19 peptides containing half-cystine residues, including 13 simple dipeptides, in a partial acid hydrolysate of wool, and Sanger *et al.* (1956) showed that the sequence cystinylcystine was also present. The number of *S*-carboxymethyl-containing peptides revealed in the present experiments is greater than that identified by these authors, and relatively few of them contribute to the major part of the radioactivity. Considering that some isolated protein components of the low-sulphur and high-sulphur fractions contain approximately 21 and 50 moles of *S*-carboxymethylcysteinyl residues per mole of protein respectively and that there are so many different components in the high-sulphur and high-glycine fractions, the limited number of peptides revealed in this work is quite surprising. It suggests the presence of a limited number of amino acid arrangements around the cystine residues in the many proteins present. It is also probable that there are hydroxyamino acid residues situated near the half-cystine residues and the bonds involving amino groups of these residues hydrolyse readily. There is a relatively high content of hydroxyamino acid residues in these proteins, particularly in the high-sulphur components (Gillespie *et al.* 1960; Crewther *et al.* 1965).

It might be expected that if the three fractions of extracted wool proteins were partially hydrolysed with acid there would be noticeable differences between them. However, the patterns of *S*-carboxymethyl peptides obtained are remarkably similar for the low-sulphur and high-sulphur fractions, which are the major proportion of the soluble proteins (i.e. 60% and 25% respectively).

The presence of adjoining half-cystine residues in wool was shown by Sanger *et al.* (1956) and studies by Crewther and Dowling (1960) suggested that this sequence was more prevalent in the low-sulphur fraction. The detection of the dipeptide *S*-carboxymethylcysteinyl.*S*-carboxymethylcysteine in the present studies supports these findings but the peptide was present in all fractions and there was no evidence to support the suggestion that this sequence was concentrated in the low-sulphur proteins.

The similarity of the environment of half-cystine residues in the high-sulphur and low-sulphur fractions may account for the surprising findings of Blackburn (1961), Gillespie and Springell (1961), and Springell *et al.* (1964) that the more reactive disulphide bonds of wool are distributed equally between the high- and low-sulphur fractions even at widely differing levels of reduction or oxidation.

That similarity in sequences in high- and low-sulphur fractions may exist over more extensive regions than the di- and tripeptides containing half-cystinyl residues studied here has been suggested in communications by Blackburn and Lee (1964, 1965). These authors suggest that if the high- and low-sulphur protein fractions represent separate entities (cf. Corfield 1962) then they are probably derived in part from a common precursor. The radioautographs of tryptic digests of the labelled low- and high-sulphur fractions reveal some *S*-carboxymethylcysteine peptides which may be common but there are many distinct differences.

The synthesis of some proteins in wool is thought to take place or be completed in the keratogenous zone (Harkness and Bern 1957; Fleischer, Vidaver, and Haurowitz 1959; De Bersaques and Rothman 1962) since there is a rapid uptake of cysteine (Ryder 1958; Downes, Lyne, and Clarke 1962) which has been shown to be preferentially incorporated into high-sulphur proteins (Downes, Sharry, and Rogers 1963). From studies of soluble high-sulphur wool proteins it has been found (Gillespie 1963; Haylett *et al.* 1963) that there is a correlation between increase in molecular weight and sulphur content of high-sulphur components. From this and other evidence it has been suggested (Gillespie 1965) that high-sulphur proteins could possibly arise from low-sulphur precursors to which are added sulphur-rich peptides. The present evidence of similar environments about the cystinyl residues in both groups of proteins suggests limitations in the sequences around the cysteine residues of such sulphur-rich peptides if this is the mechanism of synthesis. It would be of interest to extend the experiments reported here to the fractions of increasing sulphur content in the high-sulphur group of proteins, and in particular to purified components from these fractions and the other fractions present in wool.

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VI. References

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

Fig. 1.—Starch-gel patterns of the fractions (concentrations *c.* 0·5%) obtained from the chromatography of radioactively labelled SCMK.

Fig. 2.—Radioautograph of partial acid hydrolysates of extracted wool protein fractions labelled with [^{14}C]*S*-carboxymethylcysteinyl (SCMCys) residues. Separations by high-voltage ionophoresis on paper at pH 3·5 and 6·5 are shown. The fractions examined were: (*a*) aggregates S1 (Fig. 1), (*b*) low-sulphur S2 (Fig. 1), (*c*) high-sulphur and (*d*) high-glycine fractions from S3 (Fig. 1).

PLATE 2

Figs. 1–3.—Radioautographs of partial acid hydrolysates of extracted wool protein fractions labelled with [^{14}C]*S*-carboxymethylcysteinyl residues. Separation by high-voltage ionophoresis on paper at pH 6·5 in the first dimension and pH 3·5 in the second dimension of high-glycine (Fig. 1), low-sulphur (Fig. 2), and high-sulphur (Fig. 3) fractions.

Figs. 4–6.—Radioautographs as in Plate 2, Figures 1–3, but separation by high-voltage ionophoresis on paper at pH 6·5 in the first dimension followed by chromatography in butanol–acetic acid–water (4 : 1 : 1 v/v) in the second dimension of high-glycine (Fig. 4), low-sulphur (Fig. 5), and high-sulphur (Fig. 6) fractions.

Figs. 7–9.—Radioautographs of trypsin digests of extracted wool protein fractions labelled with [^{14}C]*S*-carboxymethylcysteinyl residues. Separation by high voltage ionophoresis on paper at pH 6·5 in the first dimension and pH 3·5 in the second dimension of high-glycine (Fig. 7), low-sulphur (Fig. 8), and high-sulphur (Fig. 9) fractions.

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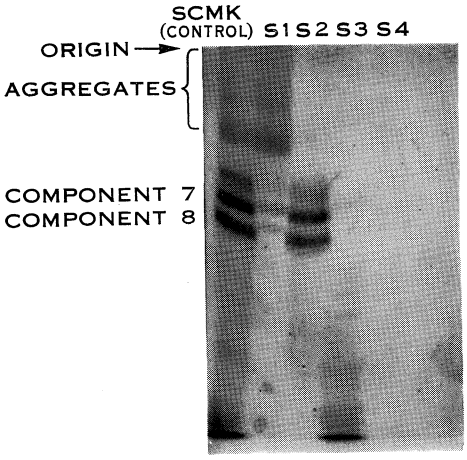

Fig. 1

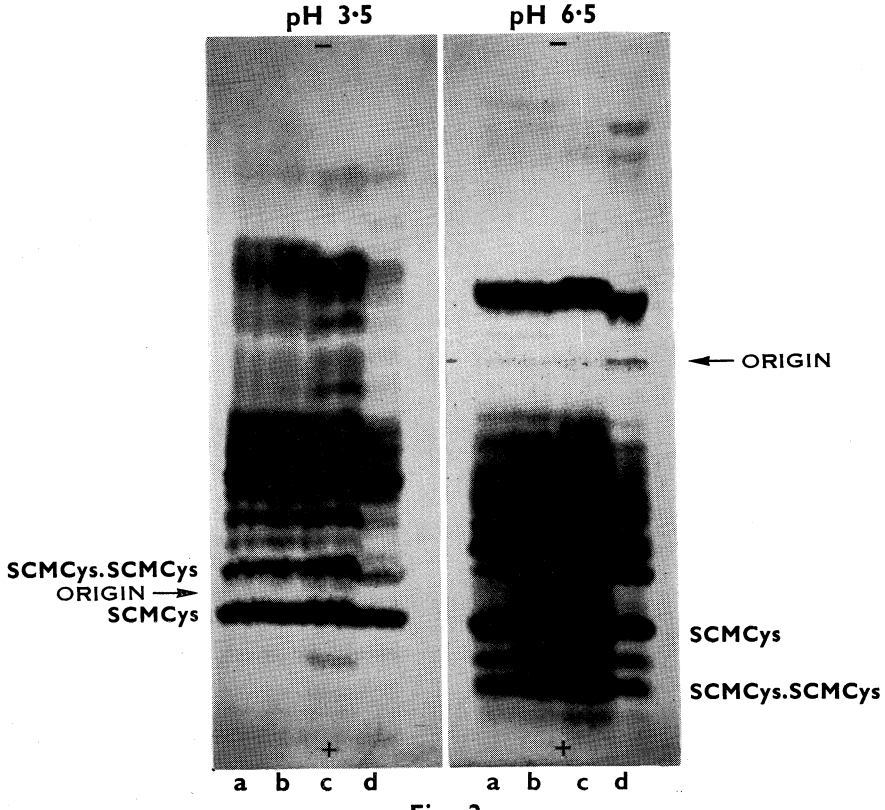

Fig. 2

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