Heterogeneity and Incomplete Disulfide Reduction in the High-sulfur Proteins of Wool

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Abstract
During the course of a study into the causes of heterogeneity of the high-sulfur proteins of wool, it was observed that the powerful reducing agent tributylphosphine although able to completely reduce the high-sulfur proteins in 8 M urea at pH 7.5 was unable to do so in the solvent system 5 M sodium iodide-25% (v/v) propanol. In a preparation prepared in the latter solvent system each electrophoretically resolvable high-sulfur protein was found to contain at least one intrachain disulfide bond and on average there were two of these resistant disulfides to every polypeptide chain, assuming a mean molecular weight of 20000. It is suggested that the high-sulfur proteins take up a particular conformation in 5 M sodium iodide-25% propanol which makes certain disulfides inaccessible to tributylphosphine. The nature of this conformation is unknown but it may be significant that high-sulfur proteins, normally thought to have no ordered structure, were found by optical rotatory dispersion measurements to contain about 10% α-helix in 5 M sodium iodide-25% propanol.

No evidence was forthcoming from these studies that the solvent system 0.2 M potassium thioglycollate-6 M urea, pH 11, often used to prepare high-sulfur proteins, is responsible for inducing heterogeneity in these proteins.

Introduction
In order to obtain high yields of soluble proteins from wool and other keratins it has been customary to use comparatively drastic conditions of pH and temperature. The high-sulfur proteins separated from the total protein extract have been found to be extremely heterogeneous by all workers in the field (for example Joubert et al. 1967; Lindley et al. 1970; Marshall and Gillespie 1976). The question has often been asked, how much of the heterogeneity, which encompasses both charge and size, is caused by the degradative effects of the conditions of extraction. With the evidence then available Lindley et al. (1970) could find no evidence of such degradative effects. Recently, however, Frenkel (1977) has shown that mild alkali causes changes in the high-tyrosine proteins of wool. This has led us to re-examine the effects of high pH on the heterogeneity of high-sulfur proteins by using the high-resolution electrophoretic procedures and extraction methods operating near neutral pH which have become available since the review of Lindley et al. (1970). In this work we have examined the differences in molecular size distribution, and heterogeneity of charge at pH 2.6 and 8.9 for high-sulfur proteins prepared at pH 11 and 40°C and at pH 7.8 and 20°C.

In the course of this work (Marshall 1977) it was observed that high-sulfur proteins prepared by extraction with the system 0.2% (v/v) tributylphosphine-5 M sodium iodide-25% (v/v) propan-1-ol (Maclaren and Kilpatrick 1969) had a lower net
positive charge at pH 2·6 than other preparations. The major part of the work described in this paper was devoted to gaining an understanding of the causes of this observation.

Materials and Methods

Preparation of High-sulfur Proteins as the S-Carboxymethyl Derivatives

The procedure routinely used in this laboratory for the quantitative extraction of keratins involves the treatment of 1 g of wool for 2 h at 40°C with 50 ml of 0·2 M potassium thioglycollate in 6 M urea at an initial pH of 11. After alkylation with iodoacetate at pH 8 (5 min at room temperature) excess iodoacetate was reacted with β-mercaptoethanol and the proteins dialysed (Gillespie and Reis 1966).

Using the procedure of Maclaren and Kilpatrick (1969) 1 g of wool was extracted with 100 ml of 0·2% (v/v) tributylphosphine in 5 M sodium iodide–25% (v/v) propan-1-ol. The pH of the system was between 7 and 8 and the reaction took place over 24 h at room temperature with gentle agitation in an atmosphere of N₂. In one experiment the concentration of tributylphosphine was 0·8% (v/v). After filtration, the soluble proteins were alkylated at pH 8 with a slight excess of iodoacetate and after 15 min excess iodoacetate was reacted with sodium sulfite. The proteins were then dialysed. In some experiments described in the text no sodium sulfite was added.

Wool (1 g) was also solubilized at a pH between 7 and 8 by treatment at room temperature for 24 h with either 0·2% (v/v) tributylphosphine in 8 M urea (100 ml) or 0·5 M β-mercaptoethanol in 6 M guanidine hydrochloride (50 ml). The latter procedure, developed by Frenkel (1977), gives a yield of protein from wool of about 80%. In both preparations the proteins were alkylated with iodoacetate and dialysed.

All preparations were treated with zinc acetate at pH 6·0 to precipitate low-sulfur and high-tyrosine fractions, leaving the high-sulfur proteins in the supernatant from which they were recovered by dialysis and freeze-drying. Full details are given by Gillespie and Reis (1966).

Preparation of High-sulfur Proteins in Sulfhydril Form

Wool (10 g) was treated at 0°C for 18 h with 300 ml of 0·8 M potassium thioglycollate at pH 10·3 containing 0·1 M NH₃; this preferentially extracts the high-sulfur proteins (Gillespie 1963). The residue was filtered off and the high-sulfur proteins precipitated from the filtrate by acidification to pH 5 with about 10 ml of glacial acetic acid. The precipitate was allowed to settle for several hours, the supernatant was decanted, and the precipitate was allowed to drain and consolidate and was then dissolved in 60 ml of 0·2% tributylphosphine–5 M sodium iodide–25% propanol in a small Waring Blendor jar. Aliquots were dialysed against either several changes of the sodium iodide–propanol solvent or 0·1 M potassium thioglycollate (pH 10·2) before optical rotatory dispersion measurements were carried out.

Chromatography

High-sulfur proteins were chromatographed on a column (67 by 2·0 cm) of cellulose phosphate (Serva P23) in the buffer 0·2 M citric acid–5 M urea (pH 2·6). Before chromatography, the protein sample (250 mg in 10 ml) was dialysed against this buffer. Elution was carried out at room temperature first with about 650 ml of buffer, and then with a linear gradient of 0·0–0·2 M sodium chloride in citrate–urea (pH 2·6) using a total volume of 720 ml. Flow rate was about 15 ml/h and 15-ml fractions were collected. The absorbance at 280 nm (Isco Model UA5) and conductivity (Beckman Conductivity Meter) of the effluent were monitored continuously.

Polyacrylamide Gel Electrophoresis

Electrophoresis at pH 2·6 was carried out in 10% polyacrylamide gels in a buffer containing 4·8 M acetic acid and 2·8 M urea for 2 h at 250 V. At pH 8·9, 7·5% polyacrylamide gels, containing 8 M urea, prepared by the method of Davis (1964) were used. Electrophoresis was for 2 h at 200 V. Polyacrylamide gel electrophoresis in the presence of 0·1% sodium dodecyl sulphate (SDS) at pH 7 (0·05 M phosphate) was carried out in 4–27% continuous gradient polyacrylamide gels (Gradipore) for 3·5 h at 50 mA. In general, about 70, 50 and 30 mg of protein was loaded for pH 2·6, pH 8·9, and SDS electrophoresis respectively. At the completion of electrophoresis the proteins were located by staining with Coomassie brilliant blue G250 (Marshall and Gillespie 1976, 1977).
Optical Rotatory Dispersion

Optical rotatory dispersion measurements were made with a Perkin Elmer spectropolarimeter model 141 at wavelengths of 365, 405, 436, 546 and 578 nm. Values of \( b_0 \) from the Moffit equation were determined by a linear least-squares fit to the data (Urnes and Doty 1961). A mean residue weight of 116 was used. Measurements were made on a high-sulfur protein fraction preferentially extracted from wool, in the solvents 0.2 M tributylphosphine-5 M sodium iodide-25% propanol (pH 7-8) and 0.1 M potassium thioglycollate (pH 10). Protein concentrations in 0.1 M potassium thioglycollate were determined by refractive index measurements using a dn/dc value of 1.85\times10^{-3}. Similar measurements were made on proteins in sodium iodide-propanol after replacing this solvent by dialysis against potassium thioglycollate. Corrections for volume changes were made by weighing.

Analytical Procedures

The proteins were hydrolysed with constant-boiling hydrochloric acid containing 2 mm phenol and 0.3 mm sodium tetrathionate for 22 h in vacuo and the content of amino acids was estimated using a Beckman–Spinco 120C amino acid analyser. At the completion of hydrolysis but before analysis, any cystine in the hydrolysate, both that derived from cystinyl residues in the protein and that produced during the hydrolytic decomposition of S-sulfocysteiny1 residues, was reduced to cysteine with dithiothreitol and then oxidized with tetrathionate to S-sulfocysteine (Inglis and Liu 1970). Cystine is not obtained in 100% yield from the destruction of S-sulfocysteiny1 residues, therefore the value given in Table 1 must be regarded as a minimum value (Bailey and Cole 1959).

Amides were estimated by the procedure of Inglis et al. (1974). The protein (2–3 mg) was treated with 1 ml of constant-boiling hydriodic acid at 108°C for 1/2 h. The released ammonia was estimated on the amino acid analyser.

Table 1. Amino acid composition (as residues per 100 residues) of acid hydrolysates of high-sulfur protein fractions prepared by extracting wool with 0.2 M thioglycollate-6 M urea, 0.5 M mercaptoethanol-6 M guanidine hydrochloride, and 0.2 M tributylphosphine-5 M sodium iodide-25 % propanol with addition of sulfite

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Thioglycollate</th>
<th>Mercaptoethanol</th>
<th>Tributylphosphine</th>
<th>Amino acid</th>
<th>Thioglycollate</th>
<th>Mercaptoethanol</th>
<th>Tributylphosphine</th>
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<tr>
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<td>0.7</td>
<td>0.6</td>
<td>Pro</td>
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<td>0.8</td>
<td>0.8</td>
<td>Gly</td>
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<td>5.8</td>
<td>6.6</td>
</tr>
<tr>
<td>Arg</td>
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<td>6.8</td>
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<td>Ala</td>
<td>3.0</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Cys (O=S)</td>
<td>0.6</td>
<td>0.4</td>
<td>0.9</td>
<td>Val</td>
<td>5.3</td>
<td>5.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Cys(Cm)</td>
<td>0.3</td>
<td>0.2</td>
<td>2.2</td>
<td>Ile</td>
<td>2.7</td>
<td>2.9</td>
<td>2.8</td>
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<tr>
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<td>2.3</td>
<td>2.4</td>
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<td>7.9</td>
<td>Amide</td>
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</tbody>
</table>

\( ^{a} \) Determined as S-sulfocysteine, by conversion of cystine in hydrolysate to S-sulfocysteine and estimation as such.

\( ^{b} \) Presumptive origin from S-sulfocysteiny1 residues in protein.

\( ^{c} \) Uncorrected for decomposition.

Results

Effect of Extraction Conditions on the Heterogeneity of High-sulfur Proteins

High-sulfur protein fractions, extracted with thioglycollate-urea at pH 10.5–11 and with mercaptoethanol-guanidine hydrochloride (mercaptoethanol fraction) at pH 7-8, were compared to see if the method of preparation affected the amino acid composition or the electrophoretic heterogeneity. The amino acid compositions of the two fractions given in Table 1 are the same within experimental error.
The two fractions were examined by polyacrylamide gel electrophoresis at pH 2.6 and 8.9 and in the presence of SDS at pH 7. It can be seen (Fig. 1) that within the limits of resolution of the techniques the two types of preparation contain the same distribution of charged species at both pH 2.6 and 8.9, and appear similar in their distribution of molecular sizes. There are differences in the proportions of some components, probably resulting from differences in the level of extraction. As might be expected no change in molecular size distribution occurred if the low pH preparation was subsequently treated at pH 11 with urea-thioglycollate.

At pH 8.9 the charge on the proteins is dominated by the S-carboxymethylcysteinyl residues. Hydrolysis of amide bonds of glutaminyl and asparaginyl residues would increase the net negative charge but the proportional change would be comparatively small and might not cause a detectable change in electrophoretic mobility. Direct estimates were therefore made of amide nitrogen in the two preparations (Table 1) but no significant difference was found.

**Fig. 1.** Polyacrylamide gel electrophoretic patterns at pH 2.6 and 8.9, and in SDS at pH 7 of high-sulfur protein fractions extracted by (a) 0.2 M thioglycollate–6 M urea at pH 11, and (b) 0.5 M mercaptoethanol–6 M guanidine hydrochloride at pH 7.5.

*Heterogeneity of High-sulfur Proteins Extracted With Tributylphosphine–Sodium Iodide–Propanol*

Tributylphosphine high-sulfur protein fractions examined electrophoretically at pH 8.9 and at pH 7 in the presence of SDS (Figs 2f, i) gave similar patterns to those of the mercaptoethanol fraction (Figs 2e, h), indicating that both fractions contained a spectrum of molecules of similar charge and size. However, this was not true at
pH 2.6 since the proteins in the tributylphosphine fraction (Fig. 2b) had lost the bands of highest charge (region B) and gained the slower bands of region A, and thus appeared to have an overall net positive charge lower than those in the mercapto-ethanol fraction (Fig. 2a). This effect persisted if the concentration of tributylphosphine in the extraction medium was increased from 0.2 to 0.8% (v/v), but disappeared if 8 M urea replaced the sodium iodide–propanol mixture.

Fig. 2. Polyacrylamide gel electrophoretic patterns at pH 2.6 and 8.9, and in SDS at pH 7 of high-sulfur protein fractions extracted with (a, e, h) 0.5 M mercaptoethanol–6 M guanidine hydrochloride, or (b, f, i) 0.2% tributylphosphine–5 M sodium iodide–25% propanol with the standard addition of sulfite. (c, g, j) As for (b) but without addition of sulfite. (d) As for (c) but with a post-treatment of sulfite.

Identification of Highly Acidic Residues in the Tributylphosphine Protein Fraction

The decrease in net positive charge mentioned above could arise from an actual decrease in the number of basic amino acids (not supported by the amino acid analyses of Table 1) or from an increase in negative charges. As carboxyls are virtually uncharged at pH 2.6 the additional negative charges must be originating in amino acids such as cysteic acid or S-sulfocysteine which retain their charge at this pH. Cysteic acid residues can probably be eliminated from consideration for although the tributylphosphine fraction contains more cysteic acid than the mercaptoethanol fraction (Table 1), this difference is too small to account for the observed electrophoretic differences between these two fractions. In any case cysteic acid is frequently formed during the acid hydrolysis of proteins containing S-carboxymethylcysteine. It is more likely that the highly acidic residue is S-sulfocysteine formed by reaction of sulfite (added to destroy excess iodoacetate) with unreduced cystine residues. Unfortunately during acid hydrolysis S-sulfocysteine is decomposed to cystine (and to some minor products including cysteic acid) and its presence must be sought by indirect means.

The evidence supporting the presence of S-sulfocysteine is summarized as follows:

1. The hydrolysed tributylphosphine protein fraction contains less S-carboxymethylcysteine and more cystine (a hydrolysis product of S-sulfocysteine) than
a similarly treated mercaptoethanol fraction (Table 1). Alone, this evidence is inconclusive as cystine could also be derived from cystinyl residues in the protein.

(2) The abnormal electrophoretic pattern (Fig. 2b) at pH 2·6 of the tributylphosphine fraction becomes normal and identical with that of the mercaptoethanol fraction (Fig. 2a), i.e. it shows an overall increase in net positive charge, with the appearance of the fast-moving bands of region B, when treated with a reducing agent in a disaggregating medium (e.g. dithiothreitol–6 M guanidine hydrochloride at pH 8) and then alkylated with iodoacetate. The content of S-carboxymethylcysteine then becomes similar to that of the mercaptoethanol fraction. This behaviour is in accord with the known reaction of S-sulfocysteine with a reducing agent which, when followed by alkylation with iodoacetate, results in the conversion of the highly acidic S-sulfocysteinyl residue to the mildly acidic S-carboxymethylcysteinyl residue. This behaviour excludes a reaction involving cystinyl residues.

(3) A tributylphosphine high-sulfur fraction made without adding sulfite during preparation gives a normal electrophoretic pattern at pH 2·6 (Fig. 2c) in showing the presence of bands in region B and the relative absence of bands in region A. However, if the preparation is then treated with sulfite the abnormal pattern appears (Fig. 2d) with the loss of bands in region B, the gain of bands in region A and an overall decreased net positive charge. Although the background staining, characteristic of this type of preparation, makes it difficult to establish the complete identity of the patterns of Figs 2b and 2d, it is clear that they show a greater resemblance to each other than to the patterns of Figs 2a and 2c.

This evidence is consistent with the presence of S-sulfocysteinyl residues in the tributylphosphine fraction and strongly suggests that some cystinyl residues are not reduced by tributylphosphine in the sodium iodide–propanol reaction medium but are subsequently cleaved by sulfite.

![Fig. 3. Elution profile obtained by chromatography on cellulose phosphate at pH 2·6 of high-sulfur proteins prepared by the tributylphosphine–sodium iodide–propanol method with addition of sulfite. A linear salt gradient of 0–0·2 M NaCl was applied, its progress being indicated by the broken line. Fractions were pooled as indicated. Optical path length 1 cm.](image-url)
Characteristics of Tributylphosphine-resistant Disulfides

We have attempted to answer two questions concerning the disulfide bonds resistant to tributylphosphine in sodium iodide–propanol. Are they in an inter- or intrachain arrangement, and how widely are they distributed amongst the spectrum of high-sulfur protein components?

The SDS electrophoretic pattern of a tributylphosphine high-sulfur protein fraction made without the addition of sulfite, and therefore containing some intact disulfides, does not show any increase in mobilities of resolved bands, i.e. no decrease in molecular weights, after the protein is reacted with sulfite (Figs 2i, j). Thus the bulk of tributylphosphine-resistant disulfides must be in an intrachain configuration. However, small amounts of interchain disulfides may be present as sulfite treatment largely eliminates minor high-molecular-weight components evident as a smeared-out background stain in the gel.

In order to provide an answer to the second question tributylphosphine proteins were chromatographed on cellulose phosphate at pH 2.6 in citrate–urea buffer (Fig. 3). Seven pooled fractions were collected as shown in Fig. 3. Each of the seven pooled fractions was electrophoresed at pH 2.6 before and after reduction and alkylation (Fig. 4). In general, each pooled fraction contained components which increased in mobility and hence net positive charge after reduction and alkylation. (In some cases, particularly fractions II and III, this is not easy to see, as the new material covered the lower half of the gel with a large number of trace bands which did not photograph well.) We conclude that most proteins contained S-sulfocysteine derived from tributylphosphine-resistant disulfides. The analytical data of Table 1 suggests that, assuming an average molecular weight of 20,000, each high-sulfur protein molecule could contain two disulfides.

Three pooled fractions (I, V and VII) were examined by SDS electrophoresis for changes in apparent molecular weight before and after reductive treatment (Fig. 5). In most cases there were no changes in molecular weight. However, the molecular weight of a minor component in fraction I decreased on reduction, suggesting that a small amount of interchain disulfide bonding survived both tributylphosphine and sulfite treatment.

Evidence for Structure Induced in High-sulfur Proteins in 5 M Sodium Iodide–25% Propanol

A tributylphosphine fraction prepared without sulfite and therefore containing residual disulfide was shaken with tributylphosphine in aqueous buffer suspension and then alkylated with iodoacetate. This reduced and alkylated fraction was found to be devoid of disulfide. This suggests that 5 M sodium iodide–25% propanol induces some ordered structure which prevents access of tributylphosphine to certain disulfides.

A search for an ordered structure in this solvent is difficult because its intense ultraviolet absorption restricts optical rotatory dispersion studies to the visible region of the spectrum. A high-sulfur protein fraction in the sulfhydryl form, prepared by the preferential extraction method, was found to have a b_o value of +10 in 0.1 M potassium thioglycollate (pH 10.2), indicating the absence of x-helix. However, the same protein dissolved in 0.2% tributylphosphine–5 M sodium iodide–25% propanol had a statistically significant b_o value of −68. This corresponds to about 10% x-helix, indicating that this solvent can induce some order in the high-sulfur protein conformation.
Fig. 4. Polyacrylamide gel electrophoretic patterns at pH 2.6 of fractions I-VII, obtained by chromatography of a tributylphosphine high-sulfur protein preparation on cellulose phosphate (Fig. 3). The unfractionated protein is included for comparison (U). Each sample was run (a) before and (b) after reduction and alkylation. The reduction step was carried out by treating each sample (0.5 mg) with 50 μl of a solution which contained 5 mM dithiothreitol, 6 M guanidine hydrochloride, 2 mM EDTA, 0.5 M Tris (pH 8.1) for 2 h at 40°C. The proteins were then alkylated by adding 10 μl of a solution containing 0.08 M iodoacetic acid, 0.5 M Tris and after 15 min they were precipitated by the addition of ethanol to 75%. Before electrophoresis the precipitate was dissolved in 50 μl of 4.8 M acetic acid-8 M urea and 5 μl was loaded on the gel.
Incomplete Disulfide Reduction of Wool Proteins

Discussion

Gillespie (1964) has shown that high yields of protein can be obtained from keratins by extraction with potassium thioglycollate–urea solution at pH 11 and 40°C, but it has been suggested that this severe extraction procedure may induce some of the heterogeneity in the high-sulfur protein fraction. The present study, however, has shown that within the limits of resolution of the electrophoretic methods employed and within the accuracy of amino acid and amide analyses, there is no detectable contribution made to the heterogeneity of the high-sulfur proteins as the result of using alkaline extraction media. Although this confirms the conclusions of Lindley et al. (1970), the results are in contrast to those obtained with high-tyrosine proteins where some heterogeneity is induced by treatment at pH 11 (Frenkel 1977).

Tributylphosphine is regarded as a powerful reducing agent (Sweetman and Maclaren 1966) and it is therefore surprising that when this reagent in 5 M sodium iodide–25% propanol is employed to solubilize wool the high-sulfur proteins contain residual disulfide. These disulfides (average of two –SS– per 20 000 molecular weight) are largely intrachain and can be reduced by tributylphosphine in dilute

Fig. 5. Polyacrylamide gel electrophoretic patterns in SDS at pH 7 of fractions I, V, and VII obtained by chromatography of a tributylphosphine high-sulfur protein preparation on cellulose phosphate (Fig. 3). Each sample was run (a) before and (b) after reduction. The reduction step was carried out by treating each sample (0·3 mg) with 30 μl of solution which contained 8 mM dithiothreitol, 4% SDS, 0·05 M phosphate (pH 7). The samples were then heated for 5 min at 100°C before electrophoresis.
aqueous buffer at pH 7.5. They are also cleaved when the proteins are extracted from wool at pH 7–8 with tributylphosphine in 8 M urea. Thus it appears that the high-sulfur proteins have a particular configuration in 5 M sodium iodide–25% propanol which makes certain intrachain disulfides inaccessible to tributylphosphine. About 10% α-helix is induced in these proteins by the solvent, but without further work it is not possible to say if this structure is responsible for the inaccessibility. Since most of resolved high-sulfur proteins contain at least one unreduced disulfide, the particular sequence of amino acids involved in the accessibility must be widespread. It would be interesting to know whether the small residual disulfide found by Sweetman and Maclaren (1966) in tributylphosphine-treated wool is confined to the high-sulfur proteins.

The reason for the differences in the accessibility of sulfite and tributylphosphine to these residual disulfides is unknown but may be merely one of size. Sulfite in aqueous buffer at pH 5–7 is able to break only about 50% of the disulfides in wool and these have generally been regarded as being the most accessible (Lindley 1959). Lindley and Cranston (1974) have shown that these groups are generally located in regions rich in polar amino acids. If the residual disulfides in the present study are also found in polar regions then it is understandable why they should be susceptible to fission with sulfite.

The location of the individual residual disulfides is unknown. However, if the results of Cecil and Loening (1960) can be applied to wool it can be postulated that the unreactive disulfides are within a large loop in the polypeptide chain. Also it is not known if the same cystinyl residues are linked in the same protein species. As most of the proteins each contain at least one residual disulfide (Fig. 4), it should be possible from known sequences (e.g. Elleman 1972) to locate and identify the appropriate regions of the sequences. A serious gap in present knowledge concerns the arrangement of disulfide bonds in wool proteins in situ. If we could be sure that the tributylphosphine-resistant disulfides are in fact in their native arrangement then a study of their arrangement could be of considerable value. Unfortunately at present it is not possible to rule out the existence of sulfhydryl–disulfide interchange which will cause rearrangements of the disulfide bonds.

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References


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