STUDIES ON OXIDIZED WOOL

V. COMPARISON OF PROTEIN FRACTIONS EXTRACTED FROM WOOL BY PEPTIDE MAPPING OF ENZYMIC DIGESTS

By E. O. P. THOMPSON* and I. J. O'DONNELL*

[*Manuscript received January* 19, 1962]

Summary

Peptide maps, which were prepared by two-dimensional ionophoresis of various enzymic digests of chromatographically resolvable fractions of α-keratose from Merino wool, have failed to reveal any readily detectable differences despite significant differences in amino acid composition. It is postulated that some contaminant protein which remains bound to the low-sulphur "mother" protein is responsible, in part, for the chromatographic heterogeneity and variation in amino acid composition of separated fractions. Peptide maps of α-keratoses derived from Lincoln and Merino wools are very similar.

Peptide maps of derivatives of bovine plasma albumin or low-sulphur wool proteins are very similar, irrespective of whether the disulphide bonds have been converted to $-SO_3^-$, $-SSO_3^-$, or $-SCH_2COO^-$ side-chains.

The number of peptides found in tryptic digests of α-keratose corresponds to the number that would be expected from a mixture of four or five different proteins of molecular weight around 9000, or one protein of molecular weight around 50,000.

I. INTRODUCTION

In a previous paper (O'Donnell and Thompson 1961) it was shown that α-keratose and γ-keratose, proteins isolated from wool oxidized with performic acid, could be separated into fractions by chromatography on columns of DEAE-cellulose by the use of buffers containing 8M urea. α-Keratose is representative of the class of proteins in wool known as the low-sulphur proteins (Gillespie *et al.* 1960). These proteins are currently thought to be responsible for the regularity of the X-ray diffraction pattern of wool (Fraser, MacRae, and Rogers 1959). Other low-sulphur proteins isolated from wool, in which the disulphide bonds had been broken and modified by different chemical reactions, i.e. sulphitolysis (Bailey and Cole 1959; Swan 1959) or reduction and alkylation (Thompson and O'Donnell 1961; Gillespie 1962), gave similar chromatographic patterns indicative of heterogeneity.

With stepwise elution many components could be obtained from an α-keratose and amino acid analysis of three arbitrary fractions from a three-step elution chromatogram showed that there were significant differences in the contents of cysteic acid, the basic amino acids, and those amino acids which absorb strongly in the region of 276 mμ. More complete amino acid analyses reported in this paper confirm that the three chromatographic components represent a real fractionation. It was further suggested that there probably existed in the wool fibre, and in the

* Division of Protein Chemistry, C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

low-sulphur proteins in particular, proteins of similar structure. Some fine differences in chemical structure could be due to modifications of amino acid residues during growth of the wool or during isolation of protein fractions. Such modifications could result in different chromatographic behaviour.

In order to compare different fractions of low-sulphur wool proteins extracted from the same wool, or similar fractions isolated from different types of wool, we have investigated the possibility of using the peptide-mapping technique which has been used with considerable success for other proteins (e.g. Brown, Sanger, and Kitai 1955; Ingram 1958; Zuckerkandl, Jones, and Pauling 1960; Wilson and Dixon 1961). This technique relies on the fact that proteins of similar amino acid sequence will give many peptides in common when digested by the same proteolytic enzyme, and in some cases the locus of chemical differences between two similar proteins or protein fractions can be detected and identified (Ingram 1957). The results of our application of this technique to the three fractions of α-keratose extracted from Merino 64's wool and to α-keratoses from Lincoln and Merino wools are presented in this paper. An assessment is also given of the relative merits, for peptide mapping, of the three different methods of splitting disulphide bonds that are commonly used. The results focus attention on the initial fractionation into high- and low-sulphur proteins as one possible cause of the complex chromatographic behaviour of extracted wool proteins.

The peptide-mapping technique has also been applied to wool proteins by Burley and Horden (1961) in an attempt to detect differences between wools from normal and copper-deficient animals. Fell, La France, and Ziegler (1960) have identified the basic amino acids and peptides present in tryptic digests of α-keratose extracted from peracetic acid oxidized wool.

II. Experimental

(*a*) *Preparation of Proteins*

The α- and γ-keratoses were extracted at pH 8 from Merino 64's wool top which had been oxidized for 24 hr at 0°C with performic acid (O'Donnell and Thompson 1959). The α-keratose from a single fleece (No. MW118) gave a similar chromatographic pattern (O'Donnell and Thompson 1961). The α-keratose fractions eluted at different salt concentrations (0·1M, 0·2M, and 0·5M KCl) and analysed for amino acid composition were from fleece No. MW118, and it was found that the tyrosine had been completely converted to 3-chloro- and 3,5-dichlorotyrosine (Thompson 1954; Hirs 1956). Such destruction of tyrosine did not occur in the case of α-keratose prepared from the Merino top and was not observed in α-keratose prepared from fleece No. MW118 if this wool was first ion-exchanged with sodium acetate followed by acetic acid at pH 3 (24 hr each) and subsequent washing with water.

The *S*-sulphokerateines were fractionated in a similar manner to the α- and γ-keratoses from the extract of wool obtained with a reagent solution containing 8M urea, 0·4M sodium sulphite, 0·2M sodium tetrathionate, and 0·1M Tris (tris-

(hydroxymethyl)aminomethane) at pH 7–7·5 (cf. Bailey and Cole 1959). Air-dry wool (1·10g) was mixed with 50 ml reagent solution in a Visking cellulose dialysis bag, squeezed to remove air, and dialysed at room temperature with gentle rocking for 24–48 hr against another 50 ml of the reagent. The bag and contents were then dialysed against successive changes of water containing 0·001M sodium borate at 2°C for 48 hr. The contents of the bag were finally diluted to 200 ml with water, homogenized, and allowed to stand overnight at 2°C. About 50–70% of the wool dissolved. The insoluble residue was removed by centrifugation and the combined *S*-sulphokerateines were separated into "acid-precipitable" and "non-acid-precipitable" fractions by the addition of acetic acid to pH 4·0 in the presence of 0·1M potassium sulphate. The acid-precipitation process was repeated on the redissolved low-sulphur (i.e. acid-precipitable) protein. As with the α- and γ-keratoses these low- and high-sulphur proteins were dialysed against water, or against potassium chloride followed by water if it was desired to remove sulphate ions before hydrochloric acid hydrolysis for amino acid analysis (Gillespie *et al.* 1960), and either freeze-dried or stored frozen in aqueous solution.

The *S*-carboxymethyl derivatives of low- and high-sulphur wool proteins (Gillespie 1960) were prepared from fleece No. MW118 as follows (cf. Thompson and O'Donnell 1961): Air-dry wool (1·10 g) was placed in 100 ml of solution comprised of 67 ml deoxygenated water, 28 ml freshly redistilled mercaptoethanol (final concn. 4M), 4ml of 0·5M sodium acetate–hydrochloric acid buffer at pH 5, and 1 ml of 0·1M "Versene" (disodium salt) and stored under nitrogen. The wool was wet by evacuation at the water-pump and release of the vacuum with nitrogen. After 24 hr at room temperature the mixture was diluted to 300 ml with oxygen-free water and precipitated by the addition of 10% (v/v) 4N trichloroacetic acid. After low-speed centrifugation the wool and precipitate were washed by stirring with 100 ml water and centrifuged again after the addition of 10 ml of trichloroacetic acid. The mixture was then transferred in 150 ml water to a reaction vessel coupled to an autotitrator and kept under nitrogen until subsequent alkylation was complete. It was titrated to pH 5 with 4·5M potassium hydroxide and a solution of potassium iodoacetate added (2·5 g iodoacetic acid dissolved in 50 ml oxygen-free water and adjusted to pH 9 with approximately 2·5 ml of 4·5M potassium hydroxide). Titration was continued rapidly to pH 11 and alkylation was complete in less than 3 min (nitroprusside test on the fibres). Mercaptoethanol (1 ml) equivalent to the iodoacetate was then added. The pH fell to below 9 and was kept at pH 9 to destroy the excess iodoacetic acid and thus prevent non-specific alkylation (Gundlach, Stein, and Moore 1959). When the uptake of alkali had ceased (5–10 min) the mixture was dialysed against 0·001M sodium borate and finally centrifuged or filtered free from insoluble residue. Nitrogen determinations on the soluble fraction showed that approximately 70% of the original wool had dissolved.

The soluble *S*-carboxymethyl kerateines were separated into high- and low-sulphur fractions by two precipitations at pH 4·4 in the presence of 0·5M potassium chloride (Gillespie, O'Donnell, and Thompson 1962). Amino acid analysis on the hydrolysed low-sulphur material (SCMKA) showed no trace of cystine. With other

proteins some decomposition of *S*-carboxymethyl cysteine has been reported (Cole, Stein, and Moore 1958).

The corresponding $-SO_3^-$, $-SSO_3^-$, and $-SCH_2COO^-$ (Thompson and O'Donnell 1961) derivatives of bovine plasma albumin were prepared similarly. No detectable thiol or disulphide was present when these samples were analysed by amperometric titration with 0·1M methylmercuric iodide in the presence of sulphite according to the method of Leach (1960). Amino acid analysis of the *S*-carboxymethyl plasma albumin showed no trace of cystine and gave 34 moles of *S*-carboxymethyl cysteine per 69,000 of plasma albumin.

(*b*) *Amino Acid Analyses*

Samples (50 mg) of protein were hydrolysed under reflux in an oil-bath at 138–140°C with 5 ml constant boiling hydrochloric acid for 24 hr. The excess acid was then removed by a freeze-drying technique. The dried hydrolysates were then each diluted to approximately 13 ml with water and filtered. Kjeldahl nitrogen analyses were carried out on 2-ml samples, and 2 ml were quantitatively diluted to 10 ml with pH 2·2 buffer. 2 ml of this solution (i.e. 1·5 mg original protein) were loaded onto the column of a Spinco amino acid analyser operated under the conditions described by Spackman, Stein, and Moore (1958).

(*c*) *Enzymic Digestion*

The enzymes crystalline trypsin, chymotrypsin (Worthington Biochemical Co., New Jersey, U.S.A.), papain (prepared according to Kimmell and Smith 1954), and nagarse (Teikoku Chemical Industry Co. Ltd., Osaka, Japan) were used at a concentration of 1% of the weight of protein taken. Usually 25 ml of 1% solution of protein was taken and made 1 in 10,000 with respect to merthiolate. All digestions were initially carried out at 37°C and pH 8 under a vigorous stream of nitrogen with a Jacobsen–Léonis autotitrator (1951) manufactured by Mr. O. Dich, an instrument maker of Copenhagen, Denmark, in conjunction with a titrator type TTT-1 from the Radiometer Corporation, Copenhagen. Alkali (0·05M) was used in a micrometer syringe in the early stages of digestion and this was changed to 0·01M as digestion approached completion. This step was necessary to ensure that digestion was complete and slow enzymic reaction was not being masked by diffusion of alkali from the tip of the syringe. Likewise the alkali must be free from carbonate since bicarbonate ions buffer around pH 8 and this can give a false picture of the digestion. To make sure that alkali consumption had ceased it was found best to use an expanded pH scale and to remove the alkali delivery tube from the reaction vessel to see that the pH did not fall.

The enzymic reaction was also followed by using the ninhydrin technique of Moore and Stein (1948) and the reagents of Yemm and Cocking (1955) (see Chibnall, Mangan, and Rees 1958) to detect the liberation of amino groups. 100-μl digest samples were used for these tests. This method was less sensitive than following the reaction with a pH-stat.

The rates of digestion of the proteins modified in different ways varied, but none was complete in under 6 hr and 24 hr was adopted as standard procedure. Having found that 24 hr was sufficient, the pH-stat was dispensed with and the digestions were carried out in 1% ammonium carbonate containing merthiolate (1 in 10,000). After 24 hr the solution was brought to about pH 6·5 with hydrochloric acid and heated on a boiling water-bath for 5 min to destroy the enzyme activity. It was then freeze-dried.

(*d*) *Peptide Maps*

(i) *Preparation of Solutions.*—The freeze-dried enzymic digests (50 mg) were dissolved in 0·50 ml buffer and centrifuged to remove the small amount of insoluble material.

(ii) *Ionophoresis of Peptides.*—Ionophoresis was carried out under toluene by the method of Michl (1951) with Michl buffers as used by Sanger and co-workers (cf. Ryle *et al.* 1955). 20 or 40 μl (i.e. 2 or 4 mg) of peptide solution were applied to the filter paper in a 1-in. streak and the paper was then wet with buffer and allowed to wash up to the streak. The paper was then carefully blotted so as not to disturb the applied peptides. These peptides (up to five different samples) were then separated by ionophoresis (2500 V for 1 hr) in one dimension on Whatman No. 3MM paper 7 in. wide by 23 in. long. Buffer at pH 6·5 was used, but no cooling was applied to the system. After drying, strips 1·2 in. wide were then cut and sewn with a sewing machine (Milstein and Sanger 1961) onto, and towards the bottom of, a sheet of Whatman No. 3MM paper of suitable width and 23 in. long. This became the positive end for subsequent electrophoresis. The strip of paper corresponding to the sewn-on strip was carefully removed with a razor-blade. The paper was then wet with pH 3·7 buffer, blotted as before, and the peptides on the paper strip further separated by ionophoresis at 2500 V for $1\frac{1}{4}$–$1\frac{1}{2}$ hr with water cooling.

The buffers used contained pyridine, acetic acid, and water (25 : 1 : 225 v/v, pH 6·5, and 1 : 10 : 289 v/v, pH 3·7) as used by Ryle *et al.* (1955) (see also Katz, Dreyer, and Anfinsen 1959).

The chromatograms, after drying in a current of air at 60°C, were sprayed with 0·5% ninhydrin in 95% ethanol and heated at 60°C to develop the colour. For spraying the paper after ionophoresis at pH 6·5, 1% glacial acetic acid was dissolved in the ninhydrin solution.

III. Results and Discussion

(*a*) *Peptide Maps of Low-sulphur Proteins*

Figure 1 shows a diagram of the peptide map obtained from a tryptic digest of α-keratose after two-dimensional ionophoresis. The spots numbered 1 to 15 were well-defined spots representing basic amino acids or peptides. The shaded areas represent zones which were not sufficiently well resolved to be given distinctive numbers and represent acidic and neutral peptides.

Although the method in which ionophoresis in two dimensions is used was more rapid than those involving chromatography in one of the dimensions, several

solvents were tried with the latter method (in either the first or the second dimension). It was found that solvents such as n-butanol–acetic acid–water (3 : 1 : 1 v/v or 2 : 1 : 1 v/v (Ingram 1958)), n-butanol–acetic acid–pyridine–water, 4 : 1 : 2 : 2 v/v, or pyridine–isoamyl alcohol–water (35 : 35 : 30 v/v (Baglioni 1961)) gave better resolution of some areas but not of others and offered no advantages over the more rapid two-dimensional ionophoresis.

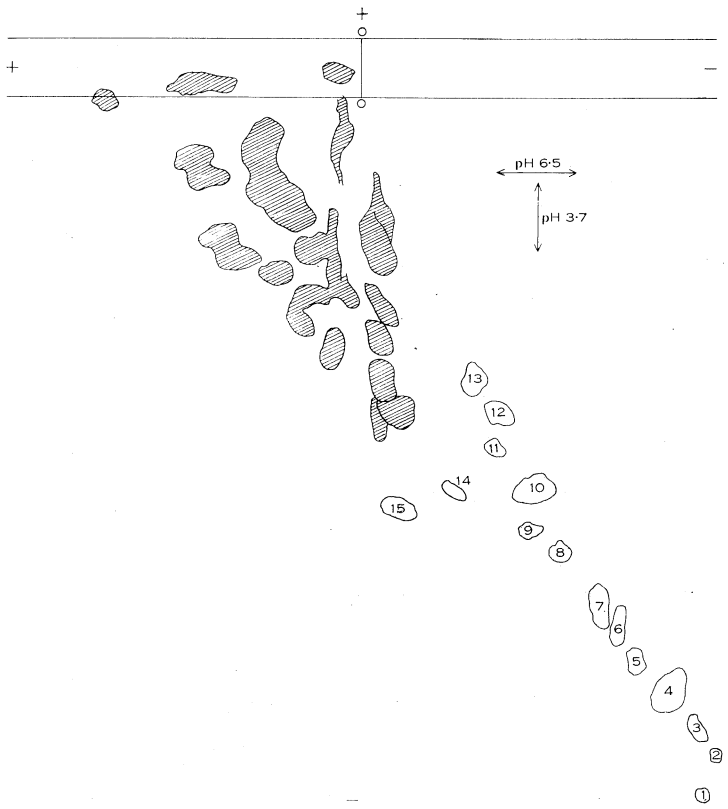


Fig. 1.—Diagram of peptide map obtained by two-dimensional ionophoresis of tryptic digests of α-keratose prepared from Lincoln or Merino wool (see text for explanation of numerals).

The basic amino acids and peptides have the same relative net charge at pH 6·5 and 3·7 (containing no β- or γ-carboxyl groups—see Fell, La France, and Ziegler 1960) and consequently migrate at similar relative rates in both dimensions so that they are spread along a diagonal straight line. The better resolution of the basic amino acids and peptides is due to their smaller size. Fell, La France, and Ziegler (1960) have identified most of these basic peptides, have shown that they are small in size and contain no modified cystine residues, and have concluded that there is a grouping of basic amino acids in certain regions of the peptide chains. Our results as well as those of Burley and Horden (1961) support this interpretation.

Amino acid analyses (Gillespie *et al.* 1960) suggest that if trypsin hydrolyses all arginyl and lysyl bonds completely the average peptides obtained from low-sulphur wool proteins should each contain 10 residues. If the basic amino acids are not evenly distributed in the polypeptide chain so that some small peptides are obtained then the size of the remaining peptides must exceed 10 residues. Moreover, Burley and Horden (1961) have drawn attention to the fact that the tryptic digestion of oxidized wool yields only two-thirds the expected increase in *N*-terminal residues. If this is due to non- or incomplete hydrolysis of some arginyl or lysyl bonds then still larger peptides would be expected.

The poor resolution of the larger acidic and neutral peptides is also apparent in chromatograms obtained with columns of sulphonated polystyrene of a low degree of cross-linkage (Gillespie *et al.* 1960).

One-dimensional ionophoretic separations (pH 6·5) were usually sufficient to give an idea of the degree of resolution to be expected after a second dimension separation. Plate 1, Figure 1, gives a comparison of the one-dimensional patterns obtained with tryptic digests of α-keratose, human haemoglobin, oxidized bovine plasma albumin, and reduced and carboxymethylated bovine plasma albumin. The better resolution with haemoglobin is probably due to a preponderance of basic peptides which suggests a more even distribution of basic amino acids in the peptide chain.

Modified bovine plasma albumin should give about 84 peptides if every arginyl and lysyl bond (Stein and Moore 1949) is split. Although this number of peptides is too large for them to be all clearly resolved, the patterns in Plate 2 show that the resolution is better than that obtained with wool proteins (Fig. 1).

In attempts to obtain smaller well-resolved peptides from wool proteins, enzymes of lower specificity were studied. In general enzymes of exacting specificity, such as trypsin, which give a small number of peptides, should give a more searching comparison between two similar proteins than enzymes of lower specificity (cf. Wilson and Dixon 1961). However, when the peptides obtained are too large to be satisfactorily handled by the techniques available this reasoning is no longer valid and a larger number of spots of more amenable size may be preferable. Digestion of low-sulphur wool proteins with trypsin followed by digestion with chymotrypsin should give about twice as many peptides as with trypsin alone. The pattern obtained with this sequence of enzymes is shown in Plate 1, Figure 2, for the digestion of a low-sulphur protein (*S*-carboxymethyl kerateine) isolated from wool after reduction followed by alkylation. Many more peptides are apparent than with trypsin alone and the resolution in the acidic and neutral area is certainly improved although still not as good as with the basic peptides. Nagarse, an enzyme of low specificity, gave the pattern shown in Plate 1, Figures 3 and 4. This enzyme was found useful in that it gave in one digestion a mixture of peptides showing better resolution than that given by the mixture of peptides obtained after digestion with papain, or with trypsin followed by digestion with chymotrypsin.

(*b*) *Peptide Maps of High-sulphur Proteins*

When the tryptic digest of the high-sulphur protein γ-keratose was examined by peptide mapping in the same way as α-keratose very few peptides could be seen.

This is due in part to its smaller content of basic amino acid residues. Also the high content of cysteic acid residues increases the probability of arginyl or lysyl β-sulphoalanyl bonds which could be resistant to trypsin. Tryptic digestion has been found with other proteins (summarized by Thompson 1960) to be retarded by a build-up of negatively charged acidic groups in the vicinity of the arginyl or lysyl bonds and the larger size of the sulphonic acid group will make it more effective in retarding tryptic action than carboxyl groups. Carboxyl groups are usually inhibitory only if both an α- and β- or γ-carboxyl groups are involved (Thompson 1960). To obtain a reasonable colour from the peptide map on spraying with ninhydrin about four times the load of γ-keratose compared with α-keratose was required. Contamination of α-keratose with γ-keratose would therefore be difficult to detect by peptide mapping of tryptic digests.

With nagarse the increased number of peptides resulted in more ninhydrin-positive spots so that smaller loadings of γ-keratose were required.

(c) *Comparison of Peptide Maps from Proteins in which the Disulphide Bonds had been Modified in Different Ways*

Three methods of modifying the disulphide bonds of bovine plasma albumin and wool were examined—oxidation, which is known to modify tryptophan residues but without breaking peptide bonds (Kimmel, Thompson, and Smith 1955), sulphitolysis, which does not modify any residue other than cystine (Bailey and Cole 1959), and reduction followed by alkylation which, with suitable precautions to minimize non-specific alkylations (Gundlach, Stein, and Moore 1959), can be usually restricted to the disulphide bonds.

Bovine plasma albumin was chosen as a model protein because it contained tryptophan and gave a number of peptides comparable to those obtained from the low-sulphur wool proteins.

Plate 2, Figures 1–3, shows the peptide maps obtained from tryptic digests of the modified plasma albumins. Since the modifying groups are all negatively charged at pH 6·5 the separations based on charge are similar for all three modifications so that almost identical patterns are obtained. Inspection of the peptide maps shows that satisfactory resolution can be obtained with all three methods of disulphide bond splitting. However, it is our impression that sharpness of spots is somewhat greater with the reduced and alkylated material than with the *S*-sulpho-derivatives or the oxidized protein. The added advantage of non-modification of tryptophan and possibly tyrosine suggests that this method would also have advantages if the study progressed to the stage of eluting peptides and determining their amino acid sequence.

Peptide maps of tryptic digests of the low-sulphur proteins gave patterns similar to one another no matter which method of modification of –S–S– bonds was used but, as was the case with plasma albumin, our conclusion was that the one from reduced and alkylated low-sulphur protein gave slightly more clear-cut zones in the acidic and neutral peptide area.

(*d*) *Comparison of Peptide Maps of the α-Keratoses Extracted from Lincoln and Merino Wool*

The α-keratoses from Lincoln and Merino wool were studied by the peptide-mapping technique to see if the method was capable of pinpointing any marked difference between them. Plate 1, Figures 3 and 4, shows the patterns obtained when nagarse digests were examined. They are remarkably similar, a similarity which was also apparent in peptide maps obtained following trypsin digestion or partial acid hydrolysis.

If there is a "mother" protein in Merino wool we can also conclude that a protein of very similar amino acid sequence occurs in Lincoln wool. The fractionation of Merino and Lincoln wool into high- and low-sulphur fractions by the oxidation technique has not revealed any major difference in proportions of these fractions, nor have peptide maps of the low-sulphur proteins shown marked differences. The reason for the differences in the chemical and physical properties between these wools has not so far been made clear in terms of chemical structure but it can be concluded that any differences are not readily located in the low-sulphur fractions.

(*e*) *Comparison of Peptide Maps of the Three Fractions of α-Keratose*

The three fractions of α-keratose obtained by stepwise elution (with 0·1M, 0·2M, and 0·5M KCl) on DEAE-cellulose were each digested with nagarse and peptide maps, prepared by the standard technique, compared. No differences could be observed even though there are differences in amino acid composition (see Table 1). Since the basic peptides were so well defined we assume that no major difference between the three fractions can lie in this part of the peptide chain and the differences must lie in the larger acidic and neutral peptides just as must those differences between Lincoln and Merino wool.

An analysis of Table 1 shows that the main differences are that, as one proceeds from the 0·2M to the 0·5M KCl fraction, there is a decrease in lysine and an increase in cysteic acid, glycine, proline, and tyrosine. This would be consistent with the idea previously postulated (O'Donnell and Thompson 1961) of a family of closely related proteins, possibly derived from a mother protein. As a working hypothesis it is assumed that there is contamination by a protein resembling the high-sulphur protein, γ-keratose. The contaminating high-sulphur protein could be present in different amounts in the three low-sulphur fractions, being most prominent in the 0·5M KCl one, and it may be that the 8M urea buffer used in chromatography is helping to dissociate it. That its presence is not obvious in the peptide maps of the three fractions would be due to the fact that the tryptic or nagarse digests of the high-sulphur protein itself give low colour yields with ninhydrin.

The peptides derived from the contaminant protein or mother protein may be adsorbed on the large neutral and acidic peptides of the tryptic digests of the low-sulphur fractions and be responsible for the smearing and general lack of definition in this area of the peptide maps. Furthermore adsorption of large peptides on the filter paper could contribute to this lack of definition. As a first step towards clarification and improvement of the peptide maps of the low-sulphur proteins from

wool, the original fractionation of extracts from oxidized wool into high- and low-sulphur proteins has been re-examined. Already it seems that trichloroacetic acid can remove an additional amount of protein from α-keratose and this will be discussed in a subsequent paper.

TABLE 1

AMINO ACID CONTENTS OF ACID HYDROLYSATES OF THE THREE FRACTIONS OF α-KERATOSE ISOLATED BY STEPWISE ELUTION FROM A COLUMN OF DEAE-CELLULOSE

Values in grams of amino acid nitrogen per 100 g protein nitrogen (excluding amide nitrogen)

Amino Acid	0·1M KCl Fraction	0·2M KCl Fraction	0·5M KCl Fraction
Lysine	6·26	5·53	4·56
Histidine	1·84	1·70	1·94
Ammonia	9·45	10·7	10·2
Arginine	22·8	21·4	20·2
Cysteic acid	4·70	4·80	6·19
Aspartic acid	7·47	7·43	5·97
Threonine	3·77	3·96	4·03
Serine	6·84	6·66	7·38
Glutamic acid	12·8	13·2	10·1
Proline	2·48	2·89	3·79
Glycine	5·14	4·62	7·11
Alanine	5·51	5·09	4·12
Cystine	Nil	Nil	Nil
Valine	4·91	4·97	4·26
Methionine sulphone	0·48	0·43	0·28
Isoleucine	2·91	2·89	2·42
Leucine	8·33	8·29	6·99
Phenylalanine	2·09	1·88	2·22
Tyrosine*	Nil	Nil	Nil
3-Chlorotyrosine	0·29	0·02	0·30
3,5-Dichlorotyrosine	1·18	1·27	1·94

* Tyrosine was destroyed during performic acid oxidation in the presence of chloride (see text, p. 553). In Part IV of this series (O'Donnell and Thompson 1961, Table 1) the chlorotyrosines were erroneously thought to be tryptophan + oxidized tryptophan.

Furthermore, other enzymes or mixtures of enzymes may break the large acidic and neutral peptides into smaller units and allow for more stringent comparisons between proteins from different wools and different protein fractions from the low-sulphur protein of a particular wool. Trypsin is not a suitable enzyme for these large peptides (cf. Burley and Horden 1961). Further hydrolysis may be best applied only to the larger peptides after separation of the smaller identifiable fragments.

Whether the dissociation of impurities bound to α-keratose will lead to better chromatographic patterns on DEAE-cellulose cannot be predicted with certainty since the dissociation procedure itself may introduce chromatographically distinguishable modifications. In the absence of such modifications an improvement might be expected although it should always be borne in mind that proteins unfolded by modification of the disulphide bonds have not yet been shown to behave satisfactorily on chromatography by elution analysis.

The molecular weight of the low-sulphur wool proteins is still an unresolved problem (see Gillespie *et al.* 1960; Woods 1961) but the number of peptides obtained by tryptic digestion is at least 40. If the molecular weight of 9000 as obtained by Harrap (1956) using the surface balance is correct only nine peptides would be expected from a tryptic digest of a homogeneous low-sulphur wool protein. The fact that many more peptides were detected suggest that, if the minimum molecular weight is around 9000, then there must be a mixture of at least four or five proteins of different amino acid sequence. If, on the other hand, the molecular weight is around 50,000 five times as many peptides would be expected for a homogeneous protein.

IV. Acknowledgments

The authors are indebted to Dr. B. S. Harrap and Mr. A. S. Inglis for the amino acid analyses and to Dr. J. P. E. Human for assistance in the construction of the all-glass equipment used for ionophoretic separations.

V. References

Baglioni, C. (1961).—*Biochim. Biophys. Acta* **48**: 392.

Bailey, J. L., and Cole, R. D. (1959).—*J. Biol. Chem.* **234**: 1733.

Brown, H., Sanger, F., and Kitai, R. (1955).—*Biochem. J.* **60**: 556.

Burley, R. W., and Horden, F. W. A. (1961).—*Text. Res. J.* **31**: 389.

Chibnall, A. C., Mangan, J. L., and Rees, M. W. (1958).—*Biochem. J.* **68**: 111.

Cole, R. D., Stein, W. H., and Moore, S. (1958).—*J. Biol. Chem.* **233**: 1359.

Fell, M., La France, N. H., and Ziegler, K. L. (1960).—*J. Text. Inst.* **51**: T797.

Fraser, R. D. B., MacRae, T. P., and Rogers, G. E. (1959).—*Nature* **183**: 592.

Gillespie, J. M. (1960).—*Aust. J. Biol. Sci.* **13**: 81.

Gillespie, J. M. (1962).—*Aust. J. Biol. Sci.* **15**: 564.

Gillespie, J. M., O'Donnell, I. J., and Thompson, E. O. P. (1962).—*Aust. J. Biol. Sci.* **15**: 409.

Gillespie, J. M., O'Donnell, I. J., Thompson, E. O. P., and Woods, E. F. (1960).—*J. Text. Inst.* **51**: T703.

Gundlach, H. G., Stein, W. H., and Moore, S. (1959).—*J. Biol. Chem.* **234**: 1754.

Harrap, B. S. (1956).—Proc. Int. Wool Text. Res. Conf. Aust., 1955. Vol. B. p. B–86.

Hirs, C. H. W. (1956).—*J. Biol. Chem.* **219**: 611.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1956).—*J. Biol. Chem.* **219**: 623.

Ingram, V. M. (1957).—*Nature* **180**: 326.

Ingram, V. M. (1958).—*Biochim. Biophys. Acta* **28**: 539.

Jacobsen, C. F., and Léonis, J. (1951).—*C. R. Lab. Carlsberg* (Sér. chim.) **27**: 333.

Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959).—*J. Biol. Chem.* **234**: 2897.

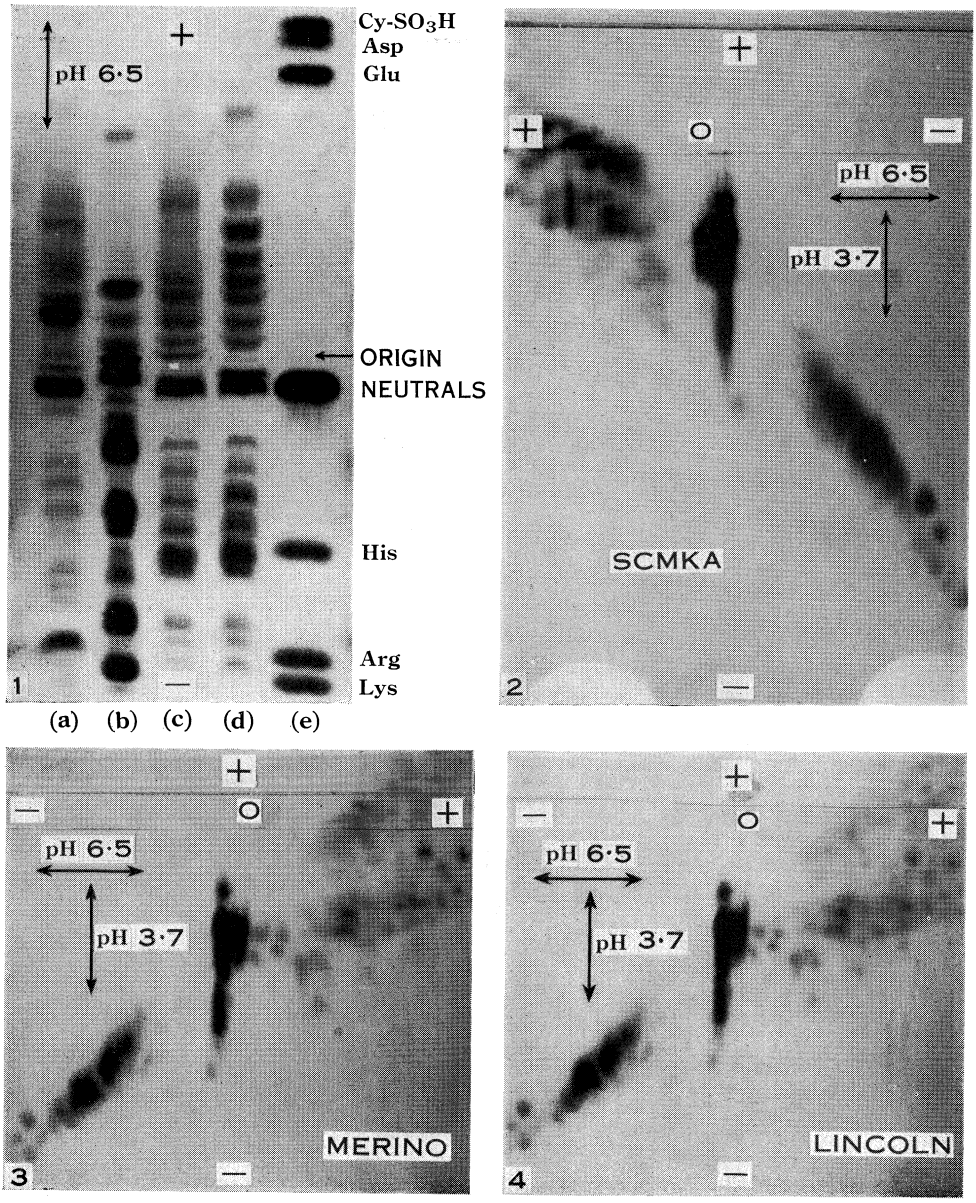
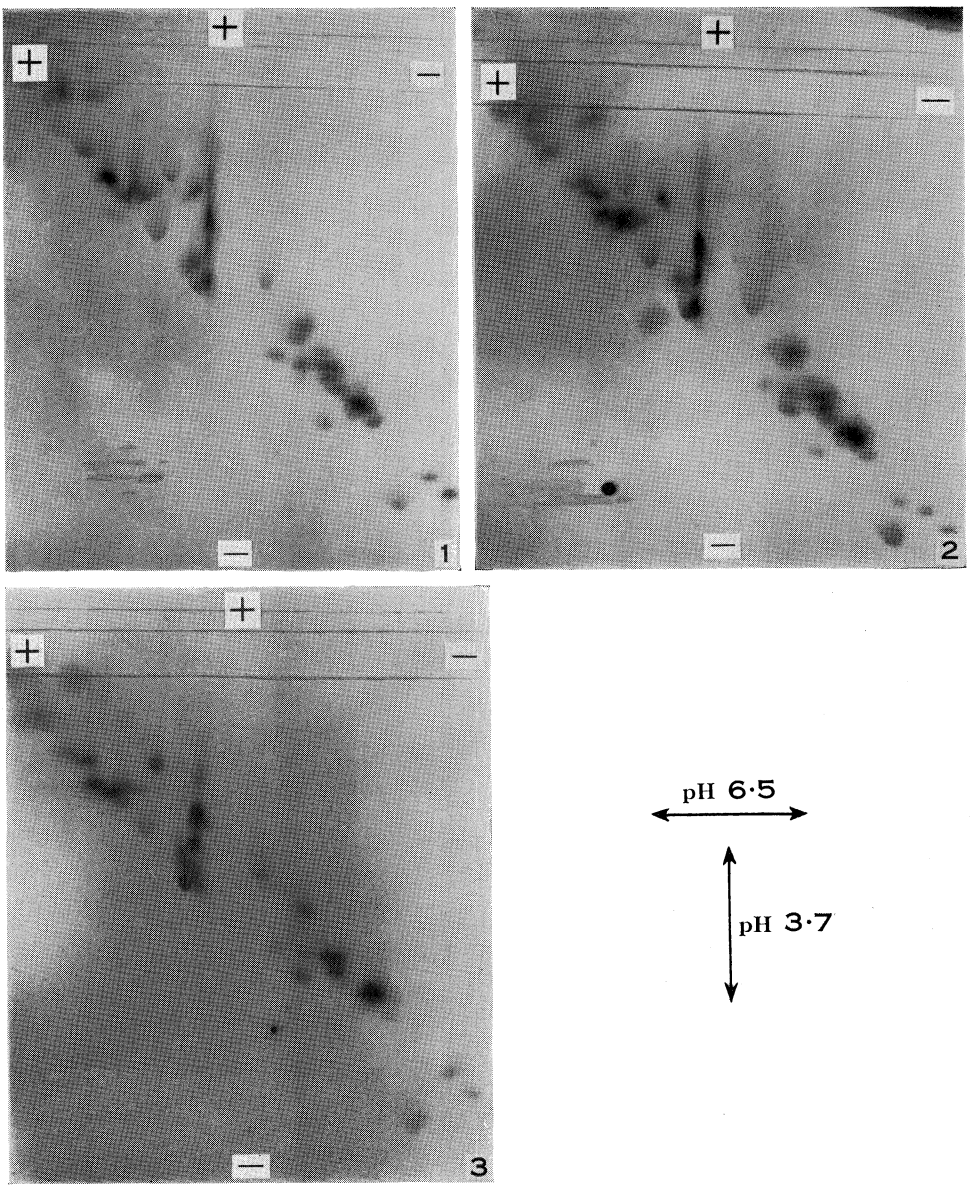


Fig. 1.—One-dimensional ionophoresis at pH 6·5 of tryptic digests of: (*a*) α-keratose; (*b*) human haemoglobin; (*c*) oxidized bovine plasma albumin; (*d*) reduced and carboxymethylated bovine plasma albumin; (*e*)mixture of amino acids showing separation of (from positive end) cysteic acid, aspartic acid, glutamic acid, neutral amino acids, histidine, arginine, lysine.

Fig. 2.—Peptide map obtained by two-dimensional ionophoresis of low-sulphur wool protein (SCMKA) after digestion with trypsin followed by digestion with chymotrypsin.

Figs. 3 and 4.—Peptide maps obtained by two-dimensional ionophoresis of nagarse digests of α-keratose prepared from Merino (Fig. 3) and Lincoln (Fig. 4) wools.



Figs. 1–3.—Comparison of peptide maps obtained from tryptic digests of bovine plasma albumin whose disulphide bonds have been broken and modified, after two-dimensional ionophoresis at the pH values shown: *S*-carboxymethyl bovine plasma albumin (Fig. 1), *S*-sulpho-bovine plasma albumin (Fig. 2), oxidized bovine plasma albumin (Fig. 3).

KIMMEL, J. R., and SMITH, E. L. (1954).—*J. Biol. Chem.* **207**: 515.

KIMMEL, J. R., THOMPSON, E. O. P., and SMITH, E. L. (1955).—*J. Biol. Chem.* **217**: 151.

LEACH, S. J. (1960).—*Aust. J. Chem.* **13**: 547.

MICHL, H. (1951).—*Mh. Chem.* **82**: 489.

MILSTEIN, C., and SANGER, F. (1961).—*Biochem. J.* **79**: 456.

MOORE, S., and STEIN, W. H. (1948).—*J. Biol. Chem.* **176**: 367.

O'DONNELL, I. J., and THOMPSON, E. O. P. (1959).—*Aust. J. Biol. Sci.* **12**: 294.

O'DONNELL, I. J., and THOMPSON, E. O. P. (1961).—*Aust. J. Biol. Sci.* **14**: 461.

RYLE, A. P., SANGER, F., SMITH, L. F., and KITAI, R. (1955).—*Biochem. J.* **60**: 566.

SPACKMAN, D. H., STEIN, W. H., and MOORE, S. (1958).—*Anal. Chem.* **30**: 1190.

STEIN, W. H., and MOORE, S. (1949).—*J. Biol. Chem.* **178**: 79.

SWAN, J. M. (1959).—In "Sulphur in Proteins". Symposium, Falmouth 1958. (Academic Press Inc.: New York.)

THOMPSON, E. O. P. (1954).—*Biochim. Biophys. Acta* **15**: 440.

THOMPSON, E. O. P. (1960).—In "Adavnces in Organic Chemistry: Methods and Results". (Eds. R. A. Raphael, E. C. Taylor, and H. Wynberg.) Vol. 1. p. 149. (Interscience: New York.)

THOMPSON, E. O. P., and O'DONNELL, I. J. (1961).—*Biochim. Biophys. Acta* **53**: 447.

WILSON, S., and DIXON, G. H. (1961).—*Nature* **191**: 876.

WOODS, E. F. (1961).—In "Structure de la Laine". Textes et discussions du colloque, July 1961. p. 143. (Institut Textile de France.)

YEMM, E. W., and COCKING, E. C. (1955).—*Analyst* **80**: 209.

ZUCKERKANDL, E., JONES, R. T., and PAULING, L. (1960).—*Proc. Nat. Acad. Sci.* **46**: 1349.