STUDIES ON OXIDIZED WOOL

VI. INTERACTIONS BETWEEN HIGH- AND LOW-SULPHUR PROTEINS AND THEIR SIGNIFICANCE IN THE PURIFICATION OF EXTRACTED WOOL PROTEINS

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Summary

The amounts of protein extractable from wool following oxidation with performic acid, and the yields of high-sulphur (γ-keratose) and low-sulphur (α-keratose) fractions have been shown to depend markedly on the conditions of dialysis used to remove excess performic acid reagent. The results are explained by assuming that denaturation occurs in warm dilute formic acid. Following denaturation in this way, or following exposure to solutions of trichloroacetic acid or to alkali at pH 11, the extracted proteins are more readily separated into high- and low-sulphur fractions. It is postulated that contaminant high-sulphur protein is responsible, in part, for the chromatographic heterogeneity and for the variation in amino acid composition of separated low-sulphur proteins. Binding of this high-sulphur contaminant protein fraction to the low-sulphur proteins is shown to be by secondary valence bonds. The percentage of high-sulphur proteins in wool is higher than the estimates of earlier workers.

I. INTRODUCTION

In a previous paper (Thompson and O'Donnell 1962) it was shown by the technique of peptide-mapping that fractions from α-keratose, separated by chromatography on DEAE-cellulose in buffers containing 8M urea, were very similar in amino acid sequence despite some marked differences among them in amino acid composition. It was considered that the marked differences between the fractions may be due to a contaminant protein bound to a single α-keratose protein or to a family of very similar proteins and containing amounts of certain amino acids characteristic of the high-sulphur proteins in wool, e.g. those rich in cysteic acid and proline. This paper reports the release of a contaminant protein from α-keratose by trichloroacetic acid or alkali, its amino acid composition, and evidence relating to the way in which it is bound to the low-sulphur protein.

The α-keratose previously used for chromatography was prepared from wool oxidized with performic acid. During the present work it has been found that the percentage extraction of oxidized wool and the relative percentage of γ-keratose (high-sulphur protein) at different pH values of extraction varied with the method of treatment of the wool after oxidation. In particular the rate and temperature at which the excess performic acid reagent was removed by dialysis was found to be important and a study of these variables is reported here. The results have been interpreted as showing that even after the disulphide bonds of wool are completely

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broken the high- and low-sulphur proteins remain, at least in part, bound together by secondary valence forces of the type that have been observed in other protein systems.

II. Experimental

(*a*) *Preparation of Oxidized Wool*

The wools used were a single de-ashed Merino 64's fleece (MW118) and Merino 64's tops. Both were purified as previously described (O'Donnell and Thompson 1961; Thompson and O'Donnell 1962). The nitrogen contents were $17 \cdot 0_5$ and $16 \cdot 8_1$ g per 100 g of dry wool respectively.

Oxidations were carried out at 0°C as described previously (O'Donnell and Thompson 1959). Performic acid (30 ml per gram dry wool) was used for oxidation. After 24 hr, one of two different procedures was used to remove the excess reagent by dialysis. In the tap water dialysis procedure the mixture was diluted with 50 ml of water at room temperature and dialysed in 36/32 Visking cellulose tubing against running tap water at room temperature. In the cold dialysis procedure the oxidized wool and reagent at 0°C were added to 50 ml of cold distilled water (2°C) and the reagent removed by dialysis on a rotating dialyser in a cold room (2°C) against four changes of 2 l. of cold distilled water per gram of wool. The changes were usually made after 1–2 hr, 3–4 hr, 6–7 hr, and 24 hr; the final dialysis was allowed to proceed for at least 6 hr.

(*b*) *Extraction of Proteins from Oxidized Wool*

(i) *Extraction at Room Temperature.*—The oxidized wool samples were extracted (O'Donnell and Thompson 1959) at pH values varying from 7 to 11; the addition of buffer salts was avoided by using a pH-stat to adjust the alkalinity. The sample was stirred at room temperature in approximately 150 ml water at a particular pH for 1–2 hr and, after making the volume to 200 ml, it was then homogenized for 1 min in a Waring Blendor. Extraction was sometimes complete at this stage but it was convenient to stand the mixture at 2°C overnight. The insoluble residue was then centrifuged off leaving ($\alpha+\gamma$)-keratose in solution. The amount of soluble protein extracted was followed by Kjeldahl nitrogen determinations and measurement of optical densities at 276 mμ. Optical densities of ($\alpha+\gamma$)-keratose and α-keratose solutions were measured after suitable dilution with $0 \cdot 1$M phosphate buffer at pH 7, and for the non-acid precipitable fractions they were measured at the pH of fractionation.

(ii) *Extraction at Low Temperature.*—The oxidized wool was extracted either with suitably adjusted potassium hydroxide solution at 0°C in an ice-bath, or with $0 \cdot 3$N aqueous ammonia in the cold room at 2°C.

(*c*) *Fractionation of Keratoses*

The ($\alpha+\gamma$)-keratose solution was brought to pH $4 \cdot 0$ in the presence of $0 \cdot 1$M potassium sulphate and the soluble γ-keratose removed from the insoluble α-keratose by gentle centrifugation. The α-keratose was redissolved at the pH of extraction and the precipitation repeated to remove a further small amount of γ-keratose.

α-Keratose solutions were further fractionated by adding, with vigorous stirring, 10% by volume of a 4N solution of trichloroacetic acid (Mirsky and Anson 1936) and the soluble and insoluble fractions separated by gentle centrifugation. A range of trichloroacetic acid concentrations was also studied. These acid-soluble and acid-insoluble fractions from α-keratose are referred to as X-keratose and (α—X)-keratose respectively.

(*d*) *Preparation of Fractions for Amino Acid Analysis*

All fractions of protein were placed in Visking 18/32 cellulose tubing and dialysed against successive changes of 0·3M potassium chloride to remove bound sulphate ions (which cause destruction of serine and threonine during acid hydrolysis (Gillespie *et al.* 1960)) or trichloroacetate ions or both. Each was finally dialysed against distilled water before freeze-drying. Samples of freeze-dried protein were hydrolysed for amino acid analysis as described previously (Thompson and O'Donnell 1962). Analyses were made with a Beckman-Spinco analyser according to the method of Spackman, Stein, and Moore (1958).

(*e*) *Preparation of* S-*Carboxymethyl Wool Proteins*

Wool was reduced with 4M mercaptoethanol at pH 5 as described previously (Thompson and O'Donnell 1961). It was filtered under nitrogen and thoroughly washed with deoxygenated water. Approximately 6% of the wool dissolved in the mixture and washings and was lost. Attempts were made to extract reduced and carboxymethylated proteins at as low a pH as possible. Vigorous shaking of 1 g of reduced wool in 200 ml 0·02M phosphate buffer at pH 8 with an aqueous solution containing 0·56 g of iodoacetic acid (adjusted to pH 8) extracted only an additional 32% of the original wool in 48 hr; the nitroprusside test on the fibres was still weakly positive after this time. At pH 9 the results were substantially the same.

In attempts to increase the percentage of reduced and alkylated protein extracted under mild conditions of pH, 8M urea solution was used. Washed reduced wool (1 g) was added to 50 ml 0·08M phosphate buffer (pH 7·7) containing 50 g urea and 0·56 g of neutralized iodoacetic acid. After 8 hr at room temperature the nitroprusside test was negative. The reaction mixture was then dialysed against water containing 0·1M potassium chloride, and finally against water. Under these conditions 69% (additional to the 6% lost in the washings) of the original wool was extracted. The low-sulphur *S*-carboxymethyl kerateine A (SCMKA) was prepared as previously described (O'Donnell, Thompson, and Inglis 1962).

(*f*) *Ninhydrin Colour as a Measure of the Effect of High pH Values on Protein Solutions*

The production of groups which give a colour reaction with ninhydrin was followed in solutions of wool proteins or *S*-carboxymethyl lysozyme (Thompson and O'Donnell 1961) stood at room temperature and at pH values of 8, 11, 12, and 13 by the method of Moore and Stein (1948). The reagents of Yemm and Cocking (1955, see Chibnall, Mangan, and Rees 1958) were used for these determinations. Some

solutions at pH 11 were made 0·1M with respect to potassium carbonate to prevent fall in pH due to absorption of CO_2. The ninhydrin colour was then developed with aliquots (containing 0·5–0·75 mg protein) after various times. To determine whether an increase in colour was due to breakage of peptide bonds rather than hydrolysis of amide groups, duplicate samples were evaporated to dryness *in vacuo* in the presence of 0·1M potassium carbonate to remove any liberated ammonia (Moore and Stein 1951) before development of colour with ninhydrin.

(*g*) *Study of Alkali-labile Bonds in Extracted Low-sulphur Wool Proteins*

An "Agla" micrometer syringe was used for the addition of 0·5M alkali to the unbuffered protein solution which was kept at 37°C in the pH-stat in the pH range 8–11. A vigorous stream of nitrogen was passed over the solution to prevent the uptake of CO_2. If alkali-labile bonds such as ester or peptide bonds were present and were broken under these conditions, alkali would be consumed due to the release of carboxyl groups (cf. Narita 1959). If alkali were consumed by hydrolysis of peptide bonds then subsequent back-titration to pH 6 would require an equivalent amount of acid. If, however, ester bonds were being hydrolysed (say at pH 11) by alkali, the back-titration to pH 6 would require fewer equivalents than the original alkali uptake since the liberated carboxyl groups would not be titrated. Moreover subsequent re-titration to pH 11 would then require less alkali than the first titration whereas this would not be so if peptide bonds were being broken.

(*h*) *End-group Determinations*

These were carried out on the extracted proteins using 1-fluoro-2,4-dinitrobenzene (FDNB) by the method of Levy (see Fraenkel-Conrat, Harris, and Levy 1955) by stirring for 2 hr in aqueous solution at 40°C and pH 9·0. The dinitrophenyl (DNP)-amino acids were separated on "Celite" columns (Perrone 1951; Thompson 1957).

III. Results

(*a*) *Fractionation and Nomenclature of Extracted Wool Proteins*

The proteins [($\alpha+\gamma$)-keratose] extracted from oxidized wool can be separated into two fractions according to their solubility in acid solutions. The soluble fraction has a higher sulphur content than the original wool, the insoluble fraction a lower sulphur content. Two alternative procedures have been used for the separation of the high-sulphur protein fraction: fractional precipitation at pH 4·0 in the presence of (1) 0·1M potassium sulphate or (2) 0·38N trichloroacetic acid. The latter preparative procedure gives the higher yield and hence the two high-sulphur fractions are not identical. They are defined operationally as γ-keratose and (γ+X)-keratose respectively. The extra protein remaining in the trichloroacetic acid is shown by amino acid analysis to be a heterogeneous high-sulphur protein and will be referred to as X-keratose. The acid-insoluble fractions are referred to as α-keratose and (α—X)-keratose respectively. This nomenclature does not conflict with the terms introduced by Alexander and Earland (1950) and now generally adopted.

(*b*) *Extraction of Proteins from Oxidized Wool*

In our earlier work dialysis was considered to be the only practical means of removing excess performic acid reagent from the oxidized wool, and dialysis versus tap water was normally used. More recently we have used cold water dialysis. The extraction of proteins from the oxidized wool is not the same for both methods. The results are summarized in Table 1. It is seen that with tap water dialysis the amount of ($\alpha+\gamma$)-keratose extracted varies with the pH of extraction and increases substantially between pH 8 and pH 11. However, when the reagent is removed by cold water dialysis the amounts extracted in range pH 7–11 are much the same.

TABLE 1

COMPARISON OF THE EXTRACTIONS OF OXIDIZED WOOLS

Wool oxidized with performic acid and subsequently dialysed against cold distilled water or tap water at room temperature

Type of Dialysis	Wool Extracted	pH of Extraction*	Nitrogen Content of ($\alpha+\gamma$)-Keratose Extracted (as % of initial wool nitrogen)	Nitrogen Content of γ-Keratose Fraction† (as % of nitrogen extracted)
Cold distilled water	Fleece‡	7	70, 70, 72	21, 21, 22
		8	70, 71, 73, 74, 71, 74, 74	24, 23, 23, 25, 22, 23, 22
		11	78, 79, 81	29, 28, 27
	Top	8	60, 68, 66, 68	30, 28, 26, 30
		11	80, 82, 83	31, 31, 32
Tap water at room temp.	Fleece	8	51, 56	34, 36
		11	80	35
	Top	8	51§	36§
		11	79§	35§

* All extractions carried out in absence of added salt.

† i.e. nitrogen not precipitated at pH 4 in the presence of 0·1M K_2SO_4.

‡ The differences between values obtained for fleece and top are probably significant and may reflect changes ("denaturation") during processing.

§ Average values taken from O'Donnell and Thompson (1959).

Furthermore, the percentage of γ-keratose varies markedly in the two cases between approximately 35% of the extracted nitrogen for both pH values 8 and 11 with tap water dialysis to 21 and 27–32% respectively for extractions at pH 7 and 11 with cold water dialysis. A notable difference (Table 2) as a result of the two dialysis procedures was also observed in preferential-extraction experiments using 0·2M phosphate buffer at pH 6·2–6·8 according to the method of Corfield, Robson, and Skinner (1958).

The difference in the extraction characteristics between the tap water and cold water dialysis procedures is due to the rate of removal of the excess performic acid reagent and the temperature. If the reaction mixture is poured into ice-cold water

and dialysed versus tap water, results similar to cold water dialysis can be obtained. Even pouring into water at room temperature followed by vigorous (rotating) tap water dialysis can give the same results as cold water dialysis. The nature of the changes induced by acid will be discussed in Section IV. Since the results obtained will be influenced by the exact method of preparation, various relevant experiments have been listed in Tables 1, 3, 4, 7, and 8.

TABLE 2

EXTENT OF EXTRACTION OF OXIDIZED WOOL WITH 0·2M PHOSPHATE BUFFER SOLUTIONS

Oxidized wool subjected to tap water dialysis at room temperature or cold distilled water dialysis prior to extraction with the phosphate buffer solutions

Treatment of Oxidized Wool	Conditions of Extraction	Nitrogen Content of Extract (as % of initial wool N)	Extracted Nitrogen not Precipitated at pH 4 (as % of nitrogen extracted)
Tap water dialysis (64's top)	(i) 8 hr, pH-stat, pH 6·2 (no phosphate)	41	56
	(ii) 8 hr, room temp., pH 6·2	17	96
	(iii) 6 hr, room temp., pH 6·6, then stood 72 hr at 2°C	24	n.d.
	(iv) Residue from (iii) re-extracted at pH 6·5	4	n.d.
Cold distilled water dialysis (64's fleece (MW118))	(v) 4 hr, room temp., pH 6·8	31	52
	(vi) 24 hr, 2°C, pH 6·8	26	57
	(vii) Residue from (vi) re-extracted with water only, 4 hr, room temp., pH 6·8 (no additional phosphate)	42	3
	(viii) Residue from (vii) re-extracted for 16 hr, room temp., pH 11 (no phosphate)	13	—

(c) *Release of X-Keratose from α-Keratose by Treatment with Trichloroacetic Acid*

α-Keratose is normally prepared by precipitation at pH 4·0 in the presence of 0·1M potassium sulphate followed by immediate re-solution of the α-keratose at pH 8 and reprecipitation under the same conditions. Approximately 2% of the redissolved α-keratose remains in solution in the second precipitation (and only a further 1% if a third precipitation is given). However, when this doubly precipitated and redissolved α-keratose is precipitated by trichloroacetic acid, some additional material, X-keratose, is released and remains in solution. This is the case with all preparations of α-keratose. The separation of X-keratose by the use of trichloroacetic acid is an inefficient process and up to five successive precipitations and re-solutions are necessary before the amount of X-keratose remaining in solution falls to near 1% of the original protein present. Some experiments of this type are shown in Table 3.

It is seen that if the α-keratose extracted at pH 8 is allowed to stand at pH 10·4–11, even at 2°C, more X-keratose is obtained in one precipitation, and also *in toto*, than for freshly prepared α-keratose. Even on standing at pH 8 and 2°C, X-keratose is released and is recoverable by trichloroacetic acid or pH 4·0 precipitation (Table 4). In all cases studied the X-keratose released by precipitation of (α—X)-keratose with trichloroacetic acid is greater in amount than that staying in solution with a corresponding precipitation at pH 4·0 in the presence of 0·1M potassium sulphate. From Table 1 it will be seen that, of the extracted protein, the percentage of γ-keratose is higher for oxidized wool extracted at pH 11 than that extracted at pH 8. Therefore

TABLE 3

EFFECT OF TRICHLOROACETIC ACID ON THE RELEASE OF PROTEIN MATERIAL FROM α-KERATOSE EXTRACTED FROM OXIDIZED MERINO 64's WOOL

Nitrogen values given as a percentage of original α-keratose nitrogen remaining in the supernatant after each of six cycles of re-solution and precipitation with 10% by volume of trichloroacetic acid

Source of α-Keratose	pH of Extraction	Normality of Trichloroacetic Acid Added	Non-precipitable (i.e. X-keratose) Nitrogen (%)	Total Non-precipitable Nitrogen (%)
Wool top* (cold distilled water dialysis)	8	2	3·9, 3·1, 2·0, 2·0, 1·6, 1·6	14·2
	8	4	5·3, 3·6, 2·0, 2·6, 1·9, 1·5	16·9
	8‡	4	13·9, 3·0, 1·2, 1·6, 0·9, 1·2	21·8
Fleece (MW 118)† (cold distilled water dialysis)	11 (1 hr)	4	2·5, 1·4, 1·0, 0·7, 0·6, 0·9	7·1
	11 (1 hr)§	4	4·4, 1·8, 1·1, 0·8, 0·7, 0·8	9·6

* (α+γ)-keratose = 68% of wool nitrogen; percentage ratio of nitrogen content of γ-keratose to nitrogen content of (α+γ)-keratose = 29.

† (α+γ)-keratose = 77% of wool nitrogen; percentage ratio of nitrogen content of γ-keratose to nitrogen content of (α+γ)-keratose = 27.

‡ Extract allowed to stand at 2°C for 120 hr at pH 10·4–11.

§ Extract allowed to stand at room temperature for 24 hr at pH 11.

it is not surprising that the percentage of X-keratose is higher for α-keratose extracted and prepared at pH 8 and then stood at pH 11 than for that which had no higher pH treatment. However, α-keratose extracted at pH 11 behaves similarly in that further standing at pH 11 releases additional X-keratose (Tables 3 and 4).

The effect of temperature and concentration on the precipitation of the proteins with trichloroacetic acid has been studied and these are shown in Figure 1. At low temperatures the precipitation process becomes less efficient (cf. Richards and Vithayathil 1959). At concentrations of trichloroacetic acid higher than those shown in Figure 1, the protein solubility increases rapidly and most of the protein dissolves. The somewhat arbitrary nature of the conditions of precipitation chosen by us is therefore apparent.

The separation of X-keratose from α-keratose by each precipitation with trichloroacetic acid was immediate and experiments showed that standing the precipitated material with trichloroacetic acid for periods of at least 5 hr did not increase the amount of X-keratose in the supernatant trichloroacetic acid solution.

TABLE 4

PROTEIN NOT PRECIPITATED BY ACID IN PREPARATIONS OF α-KERATOSE FROM MERINO 64'S WOOL

Nitrogen values given as a percentage of original α-keratose nitrogen which remains in the supernatant after a single precipitation with 10% by volume 4N trichloroacetic acid (TCA), or with 0·1M K_2SO_4 at pH 4

Source of α-Keratose	pH of Extraction	(α + γ)-Keratose (%)	Percentage Ratio of γ-Keratose Nitrogen to (α + γ)-Keratose Nitrogen	Additional Treatment		Non-Precipitable Nitrogen (%)	
				pH, Temperature	Time of Standing (hr)	Pptd. at pH 4·0 (0·1M K_2SO_4)	Pptn. with TCA
Top (tap water dialysis at room temp.)	8	51	34	—	—	—	4·6
	11	80	35	—	—	—	3·5
Top (cold distilled water dialysis)	8	68	26	8,* room temp.	0	—	4·4
					66	—	5·5
				10·5,† 2°C	66	—	7·1
				10·5,† room temp.	0	—	5·5
					24	—	11·4
Fleece (MW118) (cold distilled water dialysis)	7	72	21	—	—	—	6·2
	8	74	23	—	—	—	2·8
	11	79	28	—	—	—	1·0
	7	70	21	11,* room temp.	0	2·4	4·8
					2	7·7	11·0
					22	8·6	13·9
					96	11·8	17·2
	8	71	25	8,* room temp.	0	2·1	2·3
					22	3·5	5·8
					96	7·1	9·7
	8	73	22	8,* 2°C	0	—	2·7
					60	—	3·4
	11‡	—	—	11,* room temp.	0	3·7	5·6
					22	6·4	8·5

* Unbuffered solution.

† Buffered at pH 10·5 with sodium borate–NaOH.

‡ Extraction not quantitative.

Following multiple precipitations with trichloroacetic acid of (α+γ)-keratose extracted (and redissolved each time) at pH 8 it was found that exposure to pH 11 at room temperature did not *immediately* produce any additional significant amount of X-keratose soluble in trichloroacetic acid.

The yield of X-keratose is difficult to determine because of the repeated precipitations necessary for its preparation and because (α—X)-keratose will have some slight solubility in the precipitating medium. However, Table 3 shows values in the range 14–22% of the extracted α-keratose for an α-keratose prepared by the cold water dialysis procedure and extracted at pH 8. With preparation of α-keratose by the tap water dialysis procedure and extraction at pH 11 the value for X-keratose will be lower but even here results (see Table 4) indicate it will be of the order of 10% of the α-keratose. For α-keratose prepared by the cold dialysis procedure and extraction at pH 11, X-keratose is 7–10% of the α-keratose (Table 3).

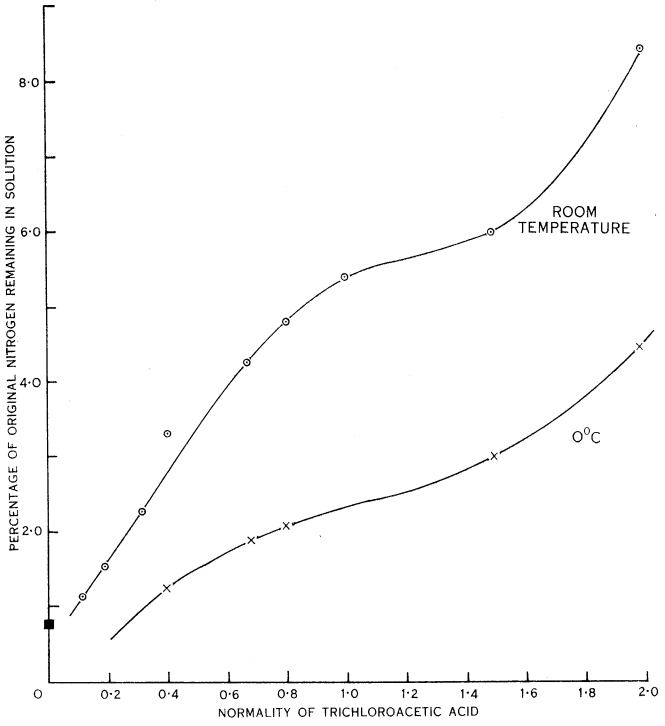

Fig. 1.—Effect of trichloroacetic acid concentration on the non-acid-precipitable nitrogen of a 1·1% α-keratose solution at room temperature and at 0°C. The nitrogen values were determined on the supernatants of mixtures containing 5 ml α-keratose and 5 ml trichloroacetic acid solution. ■ Value obtained when precipitation is carried out at pH 4·0 in the presence of 0·1M K_2SO_4.

(*d*) *Effect of Trichloroacetic Acid on the Breakage of Peptide Bonds*

That trichloroacetic acid (final pH<1) does not cause hydrolysis of peptide bonds under the conditions we have used is suggested by the results of Richards and Vithayathil (1959), Yamamoto *et al.* (1960), and Kotaki (1961). The fluorodinitrobenzene technique applied to (α—X)-keratose from a pH 8 extract precipitated five times with trichloroacetic acid showed that there was no marked increase in the ether-soluble DNP-amino acids compared with the original α-keratose.

(e) *Effect of High pH on the Breakage of Peptide Bonds*

It was found that the proteins α-keratose, *S*-carboxymethyl kerateine A, and *S*-carboxymethyl lysozyme, on standing at pH values around or above 10·5, gave new groups which reacted with ninhydrin. No change occurred in 24 hr at pH 11 at room temperature (see Table 5) but after 48 hr slight increases were detectable and

TABLE 5

EFFECT OF ALKALI ON VARIOUS PROTEIN SOLUTIONS AS MEASURED BY INCREASE IN NINHYDRIN COLOUR

Values given are optical densities at 570 mμ obtained from aliquots of protein solution treated with ninhydrin as described in text (p. 742)

Protein Solution	Treatment of Solution	Optical Density after:				
		0 hr	24 hr	48 hr	94 hr	118 hr
S-Carboxymethyl lysozyme	0·1M K_2CO_3, pH 11·0	0·51	0·48		0·72	
	As above. Aliquot dried to remove any NH_3	0·51	0·49		0·74	
α-Keratose extracted at pH 11	pH 11†	0·32	0·32			0·39
	pH 12†	0·32	0·37			
	pH 13†	0·32	0·68			
α-Keratose extracted at pH 8*	pH 8†	0·27	0·26		0·27	
	pH 11†	0·27	0·26	0·29	0·33	
	pH 12†	0·27	0·34			
	pH 13†	0·27	0·59			
SCMKA	0·1M K_2CO_3, pH 11	0·52	0·53	0·52		0·65
	As above. Aliquot dried to remove any NH_3	0·50	0·56	0·52		0·62
SCMKA+SCMKB	pH 11†	0·47	0·48	0·52‡		

* This α-keratose was extracted after preparation of oxidized wool by the cold dialysis procedure: (α+γ)-keratose = 74% of wool; percentage ratio of γ-keratose nitrogen to (α+γ)-keratose nitrogen = 23. When this α-keratose is taken to pH 11 there will be additional γ-keratose immediately released.

† Unbuffered, initial pH given.

‡ After 68 hr optical density = 0·52, pH then 10·5.

these increased considerably on longer standing. At higher pH values the changes were obvious in 24 hr. The duplicate values obtained after removing any ammonia produced by hydrolysis of amide groups suggests that amide groups are not involved. Horner (1954) has reported that hydrolysis of amide bonds in wool does not occur readily at pH 13·3 at 25°C. The simplest explanation for the increase in ninhydrin colour given by these proteins at pH values above approximately pH 10·5 is that there is breakage of peptide bonds although we cannot definitely exclude the

possibility that there is a release of additional ninhydrin-reacting groups due to unfolding or disaggregation, or hydrolysis of *N*-acetyl residues (O'Donnell, Thompson, and Inglis 1962).

(*f*) *Possibility of Covalent Bonds other than Peptide Bonds in α-Keratose*

When *S*-carboxymethyl kerateine solutions, extracted under the mildest possible conditions (i.e. pH 8, 8M urea, see Section II), were examined in the pH-stat no uptake of alkali occurred at pH 10 (where X-keratose would be released from α-keratose) over a 12-hr period. This data does not favour the existence of ester bonds (Gallup, Seifter, and Meilman 1959; Crewther, unpublished data) in *S*-carboxymethyl kerateine and hence as bonds linking (α—X)-keratose and X-keratose together. Alkali-uptake studies on keratoses are complicated by the formylation of serine and threonine residues during the oxidation in formic acid (Kienhuis, Blaase, and Matze 1959; Narita 1959; Smillie and Neurath 1959); similar studies on *S*-sulphokerateines in the pH range 9–11 were not possible due to decomposition of $-SSO_3^-$ groups.

(*g*) *Reassociation of (α—X)-Keratose and X-Keratose*

Solutions of (α—X)-keratose and X-keratose were prepared from α-keratose extracted at pH 8 (cold dialysis, (α+γ)-keratose = 71% of wool nitrogen, percentage ratio of γ-keratose nitrogen to (α+γ)-keratose nitrogen = 25) were dialysed against potassium chloride and then water. The protein contents were determined and aliquots of the two solutions mixed to give a percentage ratio of X-keratose nitrogen to (α—X)-keratose nitrogen of 27. When this mixture was precipitated with acid at pH 4·0 (0·1M K_2SO_4) and trichloroacetic acid (final concentration 0·38N) the recoveries of X-keratose by nitrogen estimations after a single precipitation were 65 and 85% respectively. Since covalent bonds would not be formed under these conditions it must be assumed that secondary forces are responsible for the apparent reassociation of X-keratose and (α—X)-keratose.

(*h*) *Amino Acid Analysis of Wool and Extracted Proteins*

Table 6 shows the amino acid composition of wool from a single fleece used in many of our experiments. The amino acid compositions of α-keratose and of (α—X)-keratose are given in Table 7 while those of γ-keratose and X-keratose are listed in Table 8. It can be seen that X-keratose shows a general resemblance to γ-keratose and is markedly different from the α-keratose from which it is released. As pointed out previously X-keratose isolated from α-keratose prepared at pH 8 following cold water dialysis should contain a proportion of γ-keratose not released in the initial pH 4·0 fractionation. However, X-keratose differs notably from γ-keratose in its high content of glycine and tyrosine but there are many other significant differences. The amino acid analyses (Tables 7 and 8) suggest that alkali is effective in releasing a bound X-keratose fraction which is similar in amino acid composition to the bulk γ-keratose fraction, e.g. relatively high in proline, threonine, and cysteic acid, while trichloroacetic acid is particularly effective in releasing a fraction very rich in tyrosine

and glycine. Only by treatment with both alkali at pH 11 and trichloroacetic acid are the cysteic acid, proline, tyrosine, and glycine reduced to minimum levels.

Since the percentage of X-keratose is not high, particularly in α-keratose prepared by the tap water dialysis procedure, or by extraction at pH 11, its removal does not have a large effect on the amino acid composition of the residual (α—X)-keratose (Table 7). However, it can be seen that the content of cysteic acid, glycine,

TABLE 6
AMINO ACID COMPOSITION OF HYDROLYSATES OF MERINO 64'S WOOL*

Amino Acid	Amino Acid Nitrogen (as % total N)			
	24-hr Hydrolysate		72-hr Hydrolysate	Mean or Extrapolated Value
Lysine	4·54	4·45	4·66	4·55
Histidine	1·87	1·86	1·89	1·87
Ammonia	8·17	8·04	10·27	(7·02)
Arginine	20·58	20·51	19·38	20·16
Aspartic acid	4·97	4·94	4·91	4·94
Threonine	4·39	4·43	3·96	(4·63)
Serine	6·91	6·80	5·90	(7·33)
Glutamic acid	8·78	8·47	8·53	8·59
Proline	4·66	4·55	4·61	4·61
Glycine	6·78	6·72	6·57	6·69
Alanine	4·22	4·22	4·16	4·20
Cystine†	8·00	8·43	7·32	8·67
Valine	4·51	4·48	4·48	4·49
Methionine	0·38	0·41	0·38	0·39
Isoleucine	2·64	2·55	2·64	2·61
Leucine	5·99	5·95	5·84	5·92
Tyrosine	2·92	2·95	2·57	(3·12)
Phenylalanine	2·27	2·20	2·14	(2·20)
Total				102

* Single fleece MW118. Nitrogen content of wool = $17 \cdot 0_5$%.

† Three overlapping peaks were obtained in the region of cystine and all were included. The initial small peak which was not observed in insulin hydrolysates had a different ratio of absorption at 440 mμ and 570 mμ to meso- and DL-cystine. It has not been identified and remains in both oxidized and reduced wool proteins.

and tyrosine falls on the removal of X-keratose. Hence analysis for cysteic acid, which is rapid and easy to carry out (cf. O'Donnell and Thompson 1961), or tyrosine (e.g. extinction coefficient) can be an aid to the course of purification of (α—X)-keratose. There is more general agreement between the analyses for (α—X)-keratoses exposed to pH 11 at room temperature followed by trichloroacetic acid precipitation (Table 7, columns 6, 9, and 10) than there is for α-keratose fractions extracted at pH 8 and 11 (Table 7, columns 4 and 8).

TABLE 7

AMINO ACID COMPOSITION OF 24-HR HYDROLYSATES OF WOOL FRACTIONS EXTRACTED FROM OXIDIZED MERINO 64's WOOL

Amino acid nitrogen values given are expressed as a percentage of the total nitrogen content of the hydrolysates

	α-Keratose*	α-Keratose	(α−X)-Keratose (TCA, pH8)†	α-Keratose	(α−X)-Keratose		(α−X)-Keratose (TCA, pH11)‡	α-Keratose	(α−X)-Keratose	
					(TCA, pH8)†	(TCA, pH11)†			(TCA, pH8)§	(TCA, pH11)§
Type of wool	Top (MW52)	Fleece (MW118)	Top (MW52)	Fleece (MW118)	Fleece (MW118)		Top (MW129)	Fleece (MW118)	Fleece (MW118)	
Dialysis procedure	Tap water	Tap water	Tap water	Cold water	Cold water		Cold water	Cold water	Cold water	
pH of extraction	8	8	8	8	8		8	11	11	
(α+γ)-Keratose (as % of wool nitrogen)	50	50	51	71	74		67	76	76	
Percentage ratio of γ-keratose nitrogen to (α+γ)-keratose nitrogen	35	35	34	23	23		29	26	26	
Nitrogen content of final preparation (%)	16·5								16·7	16·9
Lysine	3·95	4·51	4·49	4·70	5·44	5·71	5·16	4·76	4·99	5·23
Histidine	1·25	1·44	1·42	1·55	1·39	1·64	1·51	1·50	1·22	1·54
Ammonia	8·40	8·46	8·92	9·21	9·35	8·80	9·26	8·52	8·81	8·68
Arginine	19·96	20·89	20·56	22·05	21·49	21·13	22·16	20·05	19·76	20·32
Aspartic acid	5·57	5·86	6·03	5·94	6·04	6·19	6·36	5·89	6·06	6·21
Threonine	3·32	3·37	3·49	3·53	3·32	3·10	3·21	3·07	3·06	3·03
Serine	6·24	6·57	6·67	6·87	6·04	6·19	5·73	6·27	5·81	5·70
Glutamic acid	9·04	9·82	9·98	10·34	10·08	10·62	10·83	9·96	10·03	10·42
Proline	2·95	2·83	3·01	2·96	2·85	2·73	2·39	2·63	2·49	2·46
Glycine	7·42	6·84	6·44	6·95	5·90	5·16	5·10	6·33	5·50	5·55
Alanine	3·89	4·27	4·37	4·39	4·44	4·37	4·66	4·32	4·43	4·57
Cysteic acid	5·14	4·72	4·49	4·89	4·21	3·80	4·42	4·27	4·31	3·89
Valine	4·01	4·21	4·13	4·00	3·98	3·82	4·16	3·95	4·07	4·11
Methionine sulphone	0·32	0·36	0·35	0·38	0·36	0·36	0·38	0·38	0·36	0·41
Isoleucine	2·18	2·32	2·36	2·38	2·39	2·43	2·46	2·26	2·34	2·41
Leucine	6·45	6·82	6·91	6·93	7·03	7·34	7·30	6·82	6·98	7·24
Tyrosine	2·99	2·81	2·54	2·63	2·39	2·18	2·20	2·31	2·09	2·10
Phenylalanine	2·18	2·33	2·36	2·35	2·32	2·18	2·14	2·26	2·29	2·26
Total	95·3	98·4	98·5	102·0	99·0	97·8	99·4	95·6	94·6	96·1

* A preliminary analysis was reported by Gillespie *et al.* (1960), but the present values are considered the more reliable.

† X-keratose was removed by five precipitations with 10 % by volume 4N trichloroacetic acid (TCA), each precipitation being followed by re-solution at pH 8 or 11. The TCA pH 11 preparation was stood at pH 11·0 for 24 hr at room temperature (final pH = 10·6) before the precipitations with TCA.

‡ X-keratose removed by standing the α-keratose at pH 11 for 120 hr at 3°C and then precipitating five times with TCA.

§ X-keratose removed by six precipitations with TCA, each precipitation being followed by a re-solution at pH 8 or 11. The TCA pH 11 preparation was stood at pH 11·0 for 24 hr at room temperature (final pH = 10·9) before the precipitations with TCA.

It would not be expected that the composition of our α- or (α—X)-keratose fractions would be the same as that of the α-keratose fraction of Corfield, Robson, and Skinner (1958), who used a different wool and a preparative procedure involving peracetic acid under conditions which we have shown to give incomplete oxidation

TABLE 8

AMINO ACID COMPOSITION OF 24-HR HYDROLYSATES OF FRACTIONS EXTRACTED FROM OXIDIZED MERINO 64's WOOL*

Oxidized wool dialysed against cold distilled water prior to extraction at pH 8. Amino acid nitrogen values given are expressed as a percentage of the total nitrogen content of the hydrolysates

Amino Acid	γ-Keratose†	X-Keratose‡	
		TCA, pH 8	TCA, pH 11
Lysine	0·92	0·55	0·86
Histidine	1·62	0·82	1·29
Ammonia	8·63	7·52	7·50
Arginine	17·97	18·34	18·62
Aspartic acid	2·20	1·83	2·16
Threonine	7·88	4·31	5·35
Serine	10·36	9·63	10·52
Glutamic acid	5·79	3·03	3·62
Proline	10·24	5·50	7·84
Glycine	4·94	16·87	11·29
Alanine	2·38	1·37	1·47
Cysteic acid	15·18	13·57	14·66
Valine	4·49	2·38	3·02
Methionine sulphone	(trace)	(trace)	(trace)
Isoleucine	2·33	1·28	1·64
Leucine	3·00	3·76	3·45
Tyrosine	1·24	6·51	3·79
Phenylalanine	1·59	2·11	1·90
Total	100·8	99·4	99·0

* Single fleece MW118.

† Nitrogen content = 14·9%; (α+γ)-keratose = 73% of wool; percentage ratio of γ-keratose nitrogen to (α+γ)-keratose nitrogen = 22.

‡ (α+γ)-keratose = 74% of wool; percentage ratio of γ-keratose nitrogen to (α+γ)-keratose nitrogen = 23. The X-keratose was removed from the α-keratose obtained initially by five precipitations with 10% by volume 4N trichloroacetic acid (TCA), each precipitation being followed by re-solution at pH 8 or pH 11.

of cystine and some evidence of peptide bond breakdown (Thompson and O'Donnell 1959). However, there is closer agreement between the composition of their α-keratose fraction and those of our (α—X)-keratose fractions. In their extraction of α-keratose at pH 10·5–11 they have presumably dissociated off at least some of the X-keratose fraction.

(*i*) *Recommended Procedure for the Preparation of* (α—X)-*Keratose*

It is recommended that the following procedure be used for the preparation of (α—X)-keratose. After oxidation of the wool with performic acid the excess reagent should be removed by the cold dialysis procedure in which a rotating dialyser is used (see Section II). The contents of the bag are then diluted to 200 ml with cold water (2°C) and extraction carried out in the cold room (2°C) by either making the mixture 0·3N in ammonia or by using potassium hydroxide and a pH-stat at pH 11. After 1–2 hr the still cold mixture is blended in a Waring Blendor, filtered or centrifuged, (0·1M K_2SO_4 facilitates these processes), precipitated with 10% (v/v) of a solution of trichloroacetic acid (4N), and allowed to warm to room temperature (1–2 hr). The precipitate is separated by gentle centrifugation in glass cups, redissolved in 0·01M borate at approximately pH 9, and again precipitated with trichloroacetic acid, this time at room temperature. After separation of the precipitate and re-solution at pH 11 the solution (approx. 1% concentration) is allowed to stand in a full stoppered flask for 24 hr at room temperature. It is then precipitated (trichloroacetic acid) and redissolved and this process repeated (up to five times) until the optical density of the supernatant does not change. The (α—X)-keratose is finally redissolved at pH 9 (0·01M borate) and dialysed successively against 0·3M potassium chloride and water before either freeze-drying or storing in frozen solution.

IV. Discussion

The amino acid analyses suggest that exposure to alkali at pH 11 prior to precipitation with trichloroacetic acid gives the (α—X)-keratose of lowest cysteic acid content (equivalent to 1·4% sulphur—see Table 3) and proline content and may represent the purest (α—X)-keratose preparation obtained. However, periods of alkali treatment exceeding 24 hr at room temperature could result in peptide-bond hydrolysis, so that the conditions of treatment at pH 11 where there is no degradation of the protein are limited; hence the preparation of (α—X)-keratose, and its percentage in wool, are determined to some extent by arbitrary experimental parameters. Likewise, it becomes difficult to assess the percentage of the high-sulphur protein fraction in Merino wool since it cannot be assumed at any stage that no high-sulphur protein remains bound non-covalently to (α—X)-keratose. The data in Table 4 suggest that the percentage of high-sulphur protein in wool may be of the order of 40% by weight if it is remembered that about 20% of the wool remains undissolved and could contain some high-sulphur protein. The preferential extraction of high-sulphur proteins from reduced wool has to date only been of the order of 25% by weight of the original wool (Gillespie 1962).

The difference in behaviour of oxidized wool prepared by cold water dialysis and tap water dialysis we believe to be due to a denaturation process which results from prolonged exposure of the oxidized wool proteins to acids at room temperatures. The fact that the characteristics of cold water dialysis can be duplicated by pouring the reaction mixture into cold water prior to tap water dialysis, or by tap water dialyses of the reaction mixture under conditions where the formic acid is removed rapidly, supports this hypothesis. The possibility of peptide-bond breakdown cannot be definitely excluded but the fact that the α-keratose extracted at pH 8 does not

give any immediate increase in ninhydrin colour (Table 5) or *N*-terminal residues when additional non-acid precipitable material is released by exposure to pH 11 (cf. Table 4) or to trichloroacetic acid respectively is evidence against this conclusion.

Following modification by formic acid the low-sulphur proteins have a low solubility in salt in the pH range 4–7 so that preferential extraction of γ-keratose is possible (Table 2) as first reported by Corfield, Robson, and Skinner (1958) for wool oxidized with peracetic acid. If the effect of formic acid is avoided we postulate that the (α—X)-keratose and part of the γ- or X-keratoses remain bound to each other by non-covalent forces, and mixtures of them are more readily extracted at pH values of 8 or less from the oxidized wool (Table 1) even in the presence of salt (Table 2). At pH 11, however, the limit of extraction is the same irrespective of the method of dialysis used in the preparation of the oxidized wool. At this pH the net negative charge on the molecules will be higher due to ionization of tyrosine phenolic groups and repression of ionization of ϵ-amino groups of lysine, thus assisting disaggregation and causing an increase of osmotic forces which leads to membrane rupture.

The fact that reassociation takes place when solutions of (α—X)-keratose and X-keratose are mixed together supports the idea that forces holding them together which are broken by trichloroacetic acid are non-covalent. The work of Richards and Vithayathil (1959) on the binding of a 20-residue peptide to the residual 104-residue protein which results from the splitting of one peptide bond of ribonuclease demonstrates many of the characteristics that we find with extracted wool proteins. Thus Richards (1958) has reported that the peptide–protein complex from subtilisin-digested ribonuclease, where the binding must be due to forces of the secondary valence type (hydrogen bonds and van der Waals' forces), is not broken down by chromatography on "Amberlite IRC-50" or by salt or ethanol fractionation. The only methods reported to break the complex involve prolonged dialysis against 8M urea or treatment with trichloroacetic acid. More recently Brown *et al.* (1961) have reported that 8M urea or exposure to pH 10·5 will disaggregate the protein complex procarboxypeptidase into three discrete protein subunits each approximately one-third in size of the procarboxypeptidase and possessing distinct biological activities. We have previously reported (O'Donnell and Thompson 1961; Thompson and O'Donnell 1962) that chromatography of low-sulphur wool proteins on DEAE-cellulose in buffers containing 8M urea leads to resolution of components which show differences in amino acid compositions consistent with the presence of X-keratose in the more strongly bound fractions. Thus the more acidic fraction is richer in cysteic acid, proline, glycine, serine, and tyrosine and lower in lysine and alanine, characteristics of the composition of X-keratose.

The evidence presented supports the observations of earlier workers (Crewther 1956; Lennox 1956; Farnworth 1957; Gillespie and Lennox 1957; Swan 1959, 1960) that denaturation is possible with the proteins of the wool fibre and has a marked effect on their extraction characteristics. It extends these findings to the extracted proteins, and it appears that the proteins of the fibre should be extracted using delicate techniques but that "fierce" reagents (cf. Synge 1956) may be necessary to separate them. The factors we have discussed here for oxidized wool should be applicable to all extracted wool protein solutions irrespective of the type of modification of the disulphide bonds.

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