STUDIES ON OXIDIZED WOOL

II. EXTRACTION OF SOLUBLE PROTEINS FROM WOOL OXIDIZED WITH PERFORMIC ACID

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

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Summary

The variables involved in the alkaline extraction of wool oxidized with performic acid have been studied and reproducible methods of extraction have been developed. The presence of potassium sulphate reduces the amount of protein extracted but, for maximal precipitation of protein at pH 4, potassium sulphate is necessary. The extraction has been followed by estimating the cysteic acid and nitrogen contents of the soluble material. It is shown that the fractions of oxidized wool—α- + γ-, α-, and γ-keratose—contain approximately 9, 5, and 16 g cysteic acid nitrogen per 100 g total nitrogen respectively, whether extraction is carried out at pH 8 or 11.

Comparison is made with wool oxidized with aqueous peracetic acid. The α- and γ-keratoses from the ammonia extraction of peracetic acid-oxidized wool contain less cysteic acid than those from the performic acid-oxidized wool, signifying less complete oxidation of disulphide bonds by peracetic acid. The dialysable nitrogen in the ammonia extract is higher when aqueous peracetic acid replaces performic acid indicating greater splitting of peptide bonds with peracetic acid. No evidence is obtained for the preferential extraction of a sulphur-rich fraction following performic acid oxidation of wool.

I. INTRODUCTION

The heterogeneity of wool fibres as revealed by histological techniques makes it obvious that some fractionation procedure is necessary before chemical characterization of the various components can be achieved. Some progress has been made in separating special histological components, such as the scale cells (Bradbury 1959), but for a knowledge of the major constituent proteins it is necessary to extract them from the fibre and attempt purification by the methods of protein chemistry. To dissolve much of the wool protein material, without hydrolysis of peptide bonds, it is first necessary to break the disulphide linkages of cystine residues.

By fractional extraction with thiols in alkaline solutions evidence for the presence of several components has been obtained (Gillespie and Lennox 1955) and one of these components has been extensively studied (Gillespie 1957). Reduction techniques such as this have an advantage in that they usually modify only the cystine residues. A disadvantage, however, is the fact that the reaction is reversible and special conditions are necessary to ensure complete reduction. Moreover, stabilization of the thiol groups with alkylating agents, such as iodoacetic acid,

*Division of Protein Chemistry (formerly Biochemistry Unit), C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

is essential if oxidation and polymerization reactions are to be avoided. This alkylation reaction may cause side reactions if not carefully controlled (Moore *et al.* 1959).

Examination of tryptic digests of a fractionated sample of *S*-carboxymethylkerateine 2 (Gillespie 1957) by the methods of Hirs, Moore, and Stein (1956) revealed a complex mixture of ninhydrin-positive peaks which did not analyse for amino acids in stoichiometric proportions (Thompson, unpublished data). To explain the complexity of these results it is necessary to assume either that the extracted protein was a complex mixture of closely related proteins with similar physical properties, or that racemization of some amino acid residues had occurred under the conditions, pH 10·5–12·3 at 50°C, used to selectively extract the protein. A study of the proteins isolated from oxidized wool has been undertaken to gather evidence on these two alternative explanations.

The extraction of soluble proteins from wool oxidized with peracetic acid has been studied by Alexander and co-workers (Alexander and Hudson 1954; Alexander and Smith 1956) who treated the oxidized wool with ammonia solution. Some 10 per cent. (β-) of the wool remained undissolved and the soluble protein was fractionated into acid-precipitable (α-, 60 per cent.) and non-acid-precipitable (γ-, 30 per cent.) components.* The amino acid compositions of these fractions have been determined (Corfield, Robson, and Skinner 1958) as well as their *N*-terminal amino groups (Alexander and Smith 1956).

In Part I of the series (Thompson and O'Donnell 1959) it has been shown that performic acid completely oxidizes the cystine residues of wool to cysteic acid residues whereas dilute aqueous peracetic acid does not. With this reagent there is no evidence of racemization (Hill and Smith 1957). Furthermore, the action of performic acid on proteins has been extensively investigated (e.g. Sanger 1949; Hirs 1956) and its action is not complicated by the presence of much free peroxide. In the case of peracetic acid, hydrogen peroxide is formed on dilution with water and it is known that hydrogen peroxide can break peptide bonds (O'Donnell and Woods 1956). For these reasons we have chosen for study wool oxidized with performic acid in formic acid (performic acid reagent). Oxidized wool prepared in this way is not as easy to handle as the peracetic acid product due to its partial solution in the performic acid reagent, but techniques for handling large quantities of wool have been developed.

This paper describes the extraction of soluble proteins from wool oxidized with performic acid and the variables involved. Comparison was made with proteins extracted from wool oxidized with dilute aqueous peracetic acid. The extraction and fractionation procedure was followed by analyses for nitrogen and cysteic acid contents.

II. Materials and Methods

The wool and oxidizing agents used were those as described in Part I (Thompson and O'Donnell 1959).

* The terms α-, β-, and γ-keratoses were introduced by Alexander (Alexander and Hudson 1954). In this paper, while retaining this nomenclature, we consider they are operational definitions rather than definite compounds. "Acid-precipitable" refers to material precipitated at pH 4 in the presence of 0·1M potassium sulphate.

Nitrogen contents of solutions of the proteins and peptides were determined by the Kjeldahl nitrogen procedure (Chibnall, Rees, and Williams 1943). After overnight digestion the nitrogen content of dry wool was 16·9 g/100 g.

When ammonia was present in the protein solution this was first removed by evaporation overnight in the presence of potassium carbonate in a vacuum over concentrated sulphuric acid (Moore and Stein 1951).

For cysteic acid analyses the proteins were hydrolysed overnight under reflux with 6N hydrochloric acid and analysed as described by Thompson and O'Donnell (1959).

III. Experimental

(*a*) *Oxidation of Wool with Performic and Peracetic Acids*

Ten 1-g samples of wool previously cooled to 0°C were each left at 0°C with 30 ml of performic acid reagent for 6 and 16 hr. The wool swelled and partially dissolved. After the completion of oxidation it was poured into 50 ml of distilled water in a dialysis bag and dialysed for 48 hr against running tap water to remove the formic acid and peroxide. This dialysed mixture was then stored in the frozen state until required.

Peracetic acid oxidation of wool was carried out as described by Alexander and Smith (1956). The wool was stood (liquor–wool ratio 50 : 1) with 1·6 per cent. (w/v) aqueous peracetic acid (diluted from the Laporte 40 per cent. (w/v) product) for 24 hr at room temperature with occasional shaking. After this it was thoroughly washed and air-dried.

The percentage of dialysable γ-keratose was always measured after dialysis in 20/32 Visking "Cellophane" tubing (Kjeldahl nitrogen determinations).

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

Oxidized wools (1-g samples) were stirred with approximately 150 ml water at various pH values, the pH being maintained by using either ammonia or potassium hydroxide by means of a pH-stat. Subsequently, before standing for extraction, the volume was increased to 200 ml. The amount of material extracted under various conditions was studied; these conditions included time of standing, pH, treatment with Waring Blendor or glass beads, successive extractions, time of standing before homogenizing, presence and absence of potassium sulphate during extraction, and nature of alkali used.

The supernatant (α-+γ-keratoses) was separated from the undissolved material by centrifugation at 24,000 *g* and subsequent filtration. The extracted (α-+γ-) keratoses were then made 0·1M with respect to potassium sulphate, and glacial acetic acid added to bring the pH to 4; the insoluble α-keratose was separated by centrifugation and was dissolved again at its pH of extraction. It was concentrated, after dialysis to free it from potassium sulphate by pervaporation and stored as a frozen 1 per cent. aqueous solution.

When it was desired to prepare γ-keratose free from salt the oxidized wool was extracted with ammonia, and ammonium acetate was used in place of potassium sulphate before addition of acetic acid. α-, β-, and γ-keratoses were then obtained

in the dried, salt-free form by freeze-drying followed by standing in a vacuum over concentrated sulphuric acid and sodium hydroxide pellets to remove the ammonium acetate and acetic acid.

When 0·3M ammonium acetate or potassium chloride was used in place of 0·1M potassium sulphate the supernatant containing γ-keratose was more cloudy than when potassium sulphate was used and needed longer high-speed centrifugation for its clarification.

TABLE 1

EXTRACTION OF OXIDIZED WOOL AT VARIOUS pH VALUES

Values are per cent. nitrogen

Nitrogen Expressed as:	Wool Oxidized with Performic Acid at pH:					Wool Oxidized with Peracetic Acid at pH:
	6·5	8	9	10	11	11
Extracted nitrogen as per cent. initial wool nitrogen (α-+γ-keratose)	34	48, 53, 53, 49, 52, 47, 58, 51	54	60	79, 79, 82, 82, 81, 82, 79, 75, 76, 78	74
Non-acid-precipitable nitrogen (γ-keratose) as per cent. nitrogen extracted	48	36	38	39	35	33
Dialysable nitrogen as per cent. nitrogen extracted	—	6	5	8	6	12
Dialysable nitrogen as per cent. non-acid-precipitable nitrogen	—	17	14	19	17 (indirect) 11 (direct)	36 (indirect) 22 (direct)

IV. RESULTS

(a) *Variables in the Extraction Process*

(i) *Effect of Alkali Used.*—It was found that ammonia gave no better extraction at pH 11 than did potassium hydroxide in the absence of potassium sulphate, the amount extracted in 24 hr at 2°C being 75–82 per cent. (Table 1) of the total nitrogen. Our work was mainly carried out using potassium hydroxide, since this was more convenient for Kjeldahl nitrogen determinations; however, in the absence of a pH-stat ammonia solution has the advantage that it buffers at pH 11 and is consequently more convenient.

(ii) *Effect of Potassium Sulphate.*—The presence of potassium sulphate reduced the rate of extraction of oxidized wool with alkali and also lowered the yield of soluble material (Table 2).

The effect of the presence of this salt during extraction is certainly dramatic. This retardation in rate of extraction and lowering of its final value appears to be connected with the reduced swelling of the oxidized fibres at pH 11 in the presence of the salt in contrast with the marked swelling in its absence. This reduced swelling may result from the protective effect of the salt on the repulsion between the charged acidic groups in the oxidized wool. In the absence of the salt the oxidized fibre swells (and readily fractures in a blendor) and empties its contents into solution (Plate 1, Fig. 1). In the presence of the salt the less swollen fibre is not so readily dispersed (Plate 1, Fig. 2).

TABLE 2

EFFECT OF POTASSIUM SULPHATE ON THE EXTRACTION OF PERFORMIC ACID-OXIDIZED WOOL AT pH 11

pH adjusted with potassium hydroxide

Extract No.	Time of Extraction (hr)	Values are for Nitrogen Expressed as Percentage Total Initial Wool Nitrogen			
		Without Blendor		With Blendor	
		No K_2SO_4	0·1M K_2SO_4	No K_2SO_4	0·1M K_2SO_4
1	2	61	37	79 (16 hr)	60
2	16	9	8		
3, 4, 5	48	5	5		
Total extracted:		75	50	79	60

If protein is extracted in the absence of the salt and the salt added before separation of the undissolved β-keratose by centrifugation the percentage of soluble protein extracted is only some 2–3 per cent. lower than when the salt is entirely absent.

When oxidized wool, extracted in the absence of the salt, is centrifuged in a "Servall" centrifuge at approximately 24,000 *g* the undissolved material does not pack down well, but if 0·1M potassium sulphate is added before centrifugation it packs down very tightly. Here again the shielding of repulsive charges by the salt is possibly the explanation.

(iii) *Effect of Homogenizing in a Waring Blendor.*—Treatment of the swollen fibres for 2 min in a Waring Blendor increased the rate of extraction of the protein, but here also the presence of potassium sulphate caused the extraction to be slower and the ultimate limit lower in the presence of potassium sulphate than in its absence (Table 2). Despite homogenization the extraction was still slow, 69 per cent. of the nitrogen being extracted in 2 hr and 80 per cent. in 24 hr. The ultimate extractability of the wool was not affected by homogenizing.

With one extraction for 16–24 hr at pH 11, with or without homogenizing, some 75–82 per cent. of the nitrogen was extracted. With successive extractions this could be raised to 86 per cent. but beyond this level a higher pH or prolonged extraction is required.

(iv) *Effect of Shaking with Glass Beads.*—Because homogenizing was effective in breaking up the swollen fibres (more than one homogenizing had no enhanced effect) it was thought that if the fibres could be broken up more finely there might be a higher percentage extraction. Shaking 40 ml of protein suspension at pH 11 for 6 hr with 20 g of either 4-mm, 1-mm, or smaller beads showed that the 4-mm and 1-mm beads broke up the fibres much more effectively than the smaller ones. Nevertheless, glass beads did not improve the extractability in 24 hr over that obtained by either standing alone or by homogenizing. Since solutions being extracted are stood overnight in any case, the use of a Waring Blendor is probably superfluous as far as extractability is concerned. However, it has the advantage of making the residual keratin easier to separate by centrifugation from the solution of $\alpha+\gamma$-keratose.

(v) *Effect of Time of Swelling.*—The time of swelling before homogenizing can cause some variation in the final amount of nitrogen extracted. If the time of swelling at pH 8 (followed by 2 min treatment in the Waring Blendor and 24 hr standing at 2°C) was $1\frac{3}{4}$, 4, and 17 hr then the percentage of nitrogen extracted was 48, 51, and 52 per cent. respectively, while the corresponding figures at pH 11 for 25, 33, and 63 min swelling were 76, 75, and 78 per cent. respectively.

(*b*) *Comparisons of Performic and Peracetic Acid-oxidized Wools*

With the performic acid-oxidized wool it was found that 0·8 per cent. of the total nitrogen was lost on dialysis during its preparation, while for 1·6 per cent. aqueous peracetic acid-oxidized wool 1 per cent. of the nitrogen was lost in the supernatant and washings, this latter value agreeing with that obtained by Corfield, Robson, and Skinner (1958). In the performic acid-oxidation of wool some 40 per cent. of the nitrogen passes into solution in the formic acid. However, after dilution with water followed by dialysis (final pH approx. 4·5) only 4·5 per cent. of the nitrogen remained in the supernatant.

In a single extraction of peracetic acid-oxidized wool, 74 per cent. of the total nitrogen was extracted in 16 hr at pH 11 (KOH) with 2 min homogenizing; this value is very close to that of 75–82 per cent. obtained for the performic acid product. These values can both be increased somewhat by successive extractions.

(*c*) *Separation of Extracted Wool Protein into α- and γ-Fractions*

When potassium hydroxide was used for the extraction of oxidized wool and acetic acid was added to bring the pH to 4 it was found there was only a small, variable amount of precipitate. The supernatant was opalescent and could not be clarified by low-speed centrifugation. The addition of 0·1M potassium sulphate before the acetic acid caused the percentage of α-keratose precipitated to increase and to be reproducible, and the supernatant could then be readily clarified by

centrifugation. Hence all extracts were made 0·1M in potassium sulphate before acidification.

This problem did not arise with ammoniacal extracts of Alexander and Smith (1956) and Corfield, Robson, and Skinner (1958) since they used 0·1N or 3N ammonia solution which would have given rise to adequate salt for complete precipitation of α-keratose on taking the pH to 4 with acetic acid.

(*d*) *Extraction of Oxidized Wools at Various pH Values and their Fractionation into α- and γ-Keratoses*

These extractions were routinely carried out by standing the oxidized wool in potassium hydroxide for 1–4 hr with the required pH being maintained with a pH-stat, the time of standing varying with the pH. At pH 11 the wool was swollen and wet in a few minutes but at pH 6–8, 3–4 hr were required. The suspension was then homogenized for 2 min and allowed to stand 16–24 hr at 2°C before centrifugation.

The combined α-+γ-keratoses were made 0·1M with respect to potassium sulphate and brought to pH 4 with a few drops of glacial acetic acid. The precipitate was centrifuged off and the pellet of α-keratose redissolved, with stirring, at the pH at which it had been extracted from the fibre. This redissolution may take several hours. The solution of γ-keratose was concentrated by freeze-drying.

The values for percentage extraction at various pH values are given in Table 1. With increasing pH the percentage of nitrogen extractable increases. At pH 8 the amount extracted was 52 per cent. and this did not increase above 58 per cent. on two further extractions of the remaining material. Moreover, taking the pellet of remaining protein to pH 11 at this stage only brought the total extraction to 69 per cent. as compared with the 75–82 per cent. normally obtained if the wool was extracted only at pH 11. The wool extracted at pH 8 does not have the exploded appearance of Plate 1, Figure 1, and, even on then taking to pH 11, it does not attain this state. It seems that if oxidized wool is extracted at pH 11 the large immediate osmotic effect causes the fibres to swell rapidly and release their protein. At pH 8 the effect is much smaller and by the time 50 per cent. of the protein has dissolved at this lower pH the cause of the large osmotic effect at pH 11 is lost.

The percentage of extracted material which is γ-keratose is sensibly constant in the range pH 8–11 and slightly higher at pH 6·5. About 16 per cent. of the γ-keratose is dialysable at all the pH values studied. This value is much lower than the value of 36 per cent. obtained by us and the 82 per cent. (Alexander and Smith 1956) obtained for γ-keratose prepared from peracetic acid-oxidized wool. The indirect values for dialysable γ-keratose in the table were obtained from the difference in nitrogen contents on undialysed and dialysed (α-+γ-) solutions as per cent. non-acid-precipitable nitrogen. We cannot account for the difference between the direct and indirect values.

Reprecipitation (in the presence of potassium sulphate) of α-keratose after solution at pH 11 showed that only 2 per cent. of this redissolved material was non-acid-precipitable.

(e) *Cysteic Acid Contents of α- and γ-Keratoses*

Table 3 shows the cysteic acid contents of the hydrolysates of α- and γ-keratoses extracted at various pH values. The values for α- and γ-keratose are higher than those obtained by Corfield, Robson, and Skinner (1958) and by us using aqueous peracetic acid. This is not surprising in view of the incomplete oxidation with peracetic acid (Thompson and O'Donnell 1959).

There is very little difference between the values for α-keratose at pH 6·5, 8, and 11 and only small differences in the γ-keratose values.

TABLE 3

CYSTEIC ACID CONTENTS OF α- AND γ-KERATOSES

Values* are for nitrogen expressed as percentage total nitrogen

Oxidation with:	pH of Extraction	α-+γ-Keratoses	α-Keratose	γ-Keratose
Performic acid	6·5	10·2	5·6	16·5
	8	8·9	5·3	15·3, 15·4 } 15·3
	11	9·0	5·4, 5·2, 5·0 } 5·2	16·1
Peracetic acid	11†		3·72	14·5
	11‡		4·7, 4·7 } 4·7	

*Each value is the mean of duplicate estimations for a separate preparation. The original wool had an equivalent cysteic acid content of 979 μ moles/g. This is 8·1 g nitrogen per 100 g of total nitrogen.

†Corfield, Robson, and Skinner (1958).

‡Two batches of α-keratose extracted at pH 11 were kindly supplied by Mr. E. F. Woods. Higher values were obtained with these samples before reprecipitation at pH 4, probably due to contamination with γ-keratose.

V. DISCUSSION

The cysteic acid sulphur of oxidized wool accounts for 95 per cent. of the total sulphur (Thompson and O'Donnell 1959) and analyses for cysteic acid afford a convenient method for following the sulphur distribution in the isolated fractions.

It appears that, if cysteic acid content (Table 3) and non-acid-precipitability (Table 1) be taken as characteristics for γ-keratose, the proportion of γ-keratose extracted (as a percentage of the total amount extracted) is the same in the pH range 8 to 11 and only slightly higher at pH 6·5. Certainly there is no indication of a large preferential extraction or a considerable amount of "sulphur-rich" (i.e. γ-) fraction emerging first. It is difficult to reconcile these results with those of Corfield, Robson, and Skinner (1958), who, by gradient extraction of peracetic acid-oxidized

wool with 0·2M phosphate in the pH ranges 4·5–7 and 9–11·5, obtained two extracts differing in solubility, and in the approximate quantitative amounts expected for γ- and α-keratoses.

We have explored the extraction of oxidized wool at pH 8 because of a preference for working at pH values near neutrality where dangers of racemization and peptide bond hydrolysis of extracted protein are minimized. At pH's above 10 the extracted protein solution takes on a yellow colour which is possibly due to the action of alkali on the oxidized tryptophan residues. The work reported here indicates that the 50 per cent. of protein extracted at pH 8 is not different from the 80 per cent. extracted at pH 11 (Tables 1 and 3).

The percentage of γ-keratose which is dialysable is much less for γ-keratose prepared from performic acid-oxidized wool than for material prepared from aqueous peracetic-acid oxidized wool. This may point to more severe degradation of wool in the form of peptide-bond breakage when using aqueous peracetic acid (cf. Thompson and O'Donnell 1959). On the other hand, if this were the case it might be expected that the proportion of γ-keratose in the extracts from peracetic acid-oxidized wool would be higher than for performic acid-oxidized wool. The equality of the proportion of γ-keratoses in both extracts in view of the more severe action of peracetic acid may arise from incomplete oxidation of the cystine sulphur–sulphur linkages in the wool by peracetic acid (Thompson and O'Donnell 1959; Maclaren, Leach and O'Donnell, unpublished data).

VI. Acknowledgment

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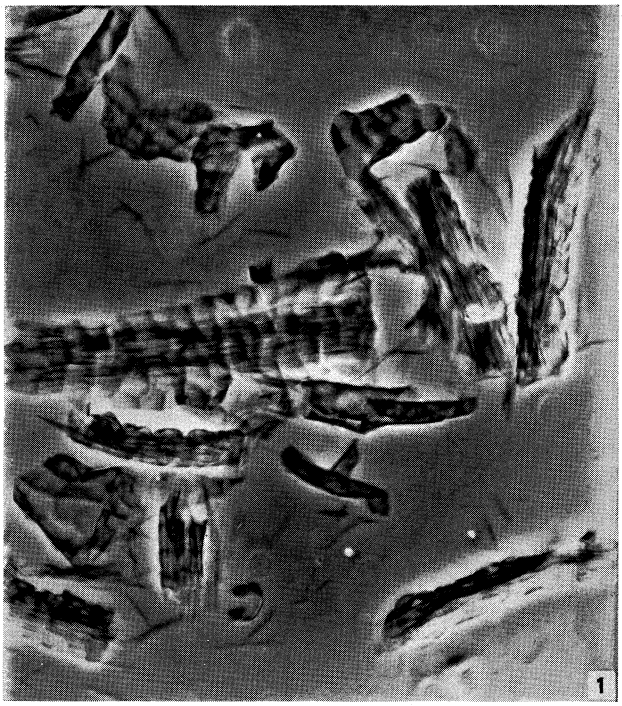

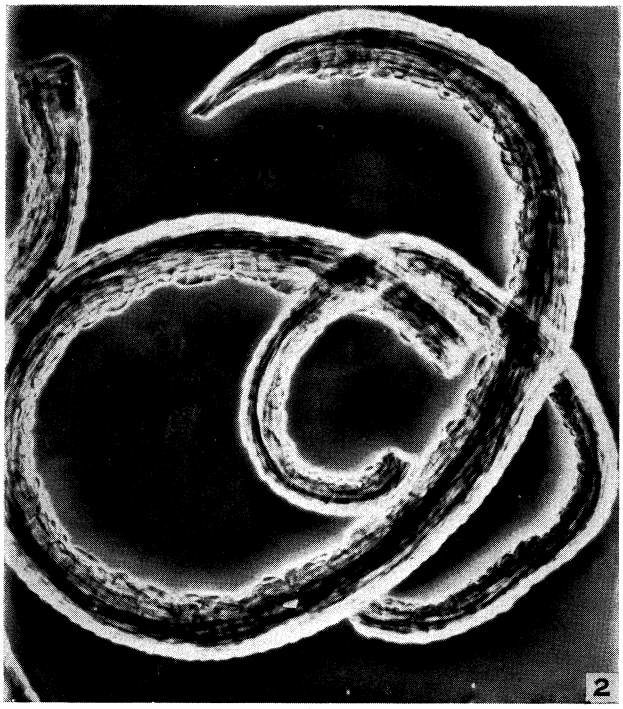

Fibres of performic acid-oxidized wool after extraction at pH 11 and homogenization in a Waring Blendor in the absence of potassium sulphate (Fig. 1) and in the presence of 0·1M potassium sulphate (Fig. 2).