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A New Fraction from Oxidized Wool

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The isolation of a large number of acid dipeptides from wool by Consden, Gordon & Martin (1949) indicated that a material considerably less complex than whole wool is needed if determinations of amino acid sequence are to have a successful outcome. Since then chemists devoting themselves to this end (Alexander & Earland, 1950; Speakman & Das, 1950; O'Donnell & Thompson, 1959; Gillespie & Lennox, 1953; Gillespie, O'Donnell, Thompson & Woods, 1960; Thompson, 1961; Blackburn, 1962; Corfield, 1962) have produced many subfractions of wool, but none of these has proved to be homogeneous and only from one, α-keratose, have peptides been isolated and characterized (Fell, La France & Ziegler, 1960). The fission of the disulphide bonds of cystine and the subsequent treatment with alkaline solutions usually adopted to render wool soluble as a preliminary to fractionation cause so much peptide-bond hydrolysis that the subfractions ultimately obtained are always very complex. Wool fractions are very susceptible to hydrolysis by alkali (Corfield, 1962) and therefore the adoption of a fractionation procedure that avoids entirely the use of alkaline reagents ought to provide components of wool more suitable for sequence determinations. Such a method is described in the present paper.

EXPERIMENTAL

Fractionation procedure. Peracetic acid-oxidized Merino 64s wool was prepared as described by Corfield, Robson & Skinner (1958). A sample (5 g.) of oxidized wool mixed with 25 g. of Celite 545 was triturated with 250 ml. of 0·1 M-acetic acid and the resultant slurry was poured in several portions to form a column (2·8 cm. diam. × 20 cm. long). Britton–Robinson universal buffer at pH 3·4 (Britton, 1942), containing 5 moles of urea/l., was transferred to the column from a stirred 2 l. flask at 15 ml./hr. through two tubes of a three-tube peristaltic pump: 16 ml. effluent fractions were collected. When 35 fractions had been collected, the volume of buffer in the mixing flask was adjusted to 1700 ml. and addition of a second buffer at 7·5 ml./hr. was begun through the third tube of the peristaltic pump. This buffer was Britton–Robinson universal buffer at pH 11·2 also containing 5 moles of urea/l. In this way oxidized wool was continually treated with 5 M-urea solution of gradually increasing pH. The dependence of the pH on fraction number is shown in Fig. 1 (*a*). The distribution of protein material in the effluent fractions was determined spectrophotometrically by measuring the ultraviolet absorption at 276 mμ in a Unicam SP. 600 spectrophotometer with silica cells of 1 cm. path-length. Strongly absorbing solutions were measured in a 0·2 cm. cell. Fig 1 (*b*) shows the relationship between extinction and fraction number in a typical effluent pattern. Although several different batches of oxidized wool were used, the relative peak heights and positions of the four peaks were completely reproducible.

Fractions contributing to peaks in the effluent pattern as shown in Fig. 1 were combined, filtered (to remove dirt) into a Visking cellophan U-tube and dialysed against water and ethanol alternately to remove urea, buffer salts and the bulk of the water. Visking tubing of 2 in. diam. was often required initially, but the final dialysis, when the volumes were smaller, could be performed with $\frac{3}{4}$ in. tubing. After the final dialysis against ethanol, the solid in the U-tube was centrifuged, washed with ethanol and ether, and finally dried in a vacuum desiccator. The yields by weight of the components isolated in this way, given in Table 1, showed little variation with different samples of oxidized wool, indicating that both the oxidation and fractionation procedures were reproducible and that complete removal of

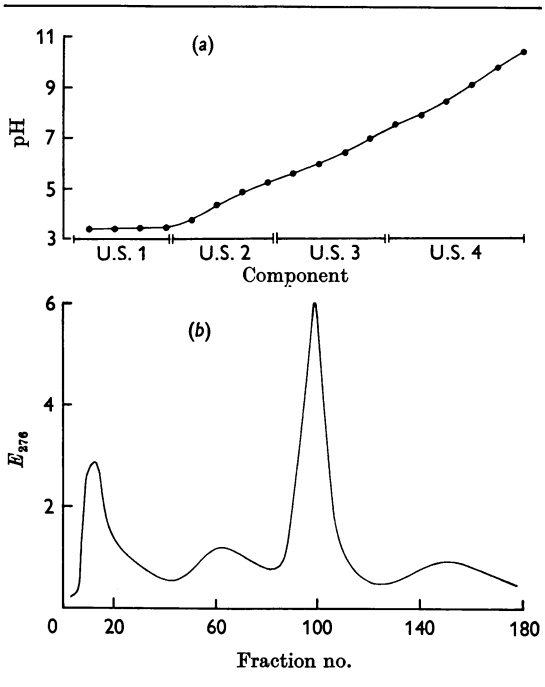

Fig. 1. Fractionation of oxidized wool by treatment with 5 M-urea solutions of continuously increasing pH. (*a*), Extinction at 276 mμ of the effluent fractions; (*b*), pH of the effluent fractions. Components were recovered from the effluent fractions combined as indicated.

urea and buffer salts had been effected in the dialysis. Table 1 also gives the yields of the components produced in an experiment with stepwise, instead of gradient, elution. In this experiment 10 g. of oxidized wool was successively treated for 24 hr. at room temperature with 1700 ml. of 5 M-urea solutions buffered with Britton–Robinson universal buffer at pH 3·4, 4·4, 5·8, 8·4 and 11·2. Between treatments the mother liquors were separated from the residual undissolved wool by filtration and the dissolved component was recovered by the method described above. The combined weights of the recovered components together with the residual wool accounted for 101 % of the starting material, showing that the buffer salts had been effectively removed without loss of protein material.

Table 1. *Yields of components obtained from oxidized wool by treatment with* 5 M-*urea solutions of increasing* pH

The pH of the urea solution was changed continuously during gradient elution and five times to give the yields under the heading 'stepwise elution'. For details of these methods see the text. Components, after prolonged dialysis against water and ethanol, were recovered in the dry form by centrifuging.

Gradient elution		Stepwise elution	
Fraction	Yield (%, w/w)	pH of eluent	Yield (%, w/w)
U.S. 1	10	3·4	12·2
U.S. 2	7	4·4	8·5
U.S. 3	36	5·8	36·0
U.S. 4	23	8·4	28·0
		11·2	6·3
		Residue	10·0

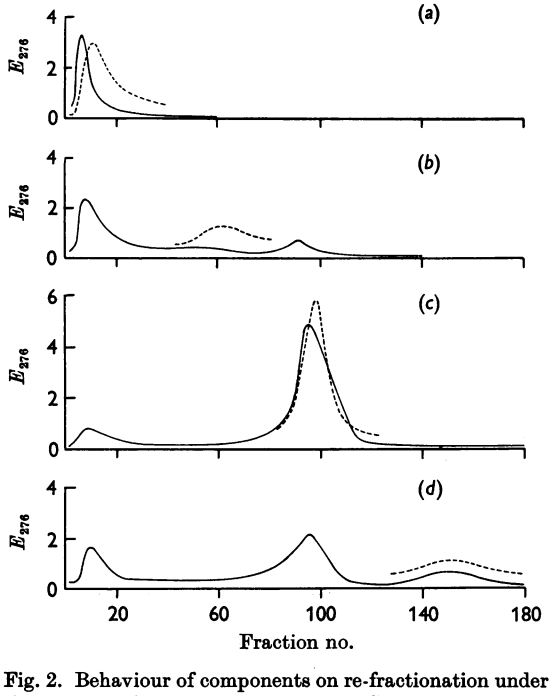

Fig. 2. Behaviour of components on re-fractionation under the same conditions as in Fig. 1. (*a*), Component U.S. 1; (*b*), component U.S. 2; (*c*), component U.S. 3; (*d*), component U.S. 4. The locations of the components in the original fractionation are indicated by broken lines.

Amino acid analysis. The amino acid compositions of the components were determined by the method of Moore, Spackman & Stein (1958). Hydrolysates were prepared by refluxing 200 mg. of sample with 20 ml. of 5·7 N-HCl for 24 hr. and removing the HCl *in vacuo*. Results are expressed as the means of five determinations and are not corrected for hydrolytic decomposition. Total N was determined by the micro-Kjeldahl procedure (Chibnall, Rees & Williams, 1943). Total S was determined by the method of Jones & Letham (1956).

Sedimentation diagrams. The components were examined in a Spinco model E ultracentrifuge. When the components were allowed to form a precipitate, subsequent solutions contained aggregates that prevented an assessment of the homogeneity of the dissociated protein. The presence of a dispersing agent in freshly prepared solutions of neutral pH was also necessary to prevent aggregation. A sample of oxidized wool was fractionated as described above and the effluent solutions of components were examined without any additional treatment. Sedimentation was performed at 56 000 rev./min. and photographs of the sedimentation pattern were taken at 64 min. intervals.

N-*Terminal groups.* The *N*-terminal groups of component U.S. 3 were determined by interaction with *p*[^{131}I]-iodobenzenesulphonyl chloride (Keston, Udenfriend & Cannan, 1949; Keston, Udenfriend & Levy, 1950). After acid hydrolysis, the *p*-iodobenzenesulphonyl derivatives of the *N*-terminal residues, and also *O*-*p*-iodobenzenesulphonyltyrosine and N_ϵ-*p*-iodobenzenesulphonyl-lysine, were separated on buffered Celite columns (Corfield, Fletcher & Robson, 1956) by the automatic procedure of Corfield, Dilworth, Fletcher & Gibson (1959).

RESULTS

Re-treatment of components. The four components isolated by the above methods were subjected to the fractionation procedure for a second time to test their reproducibility. Fig. 2 shows the results obtained. All of component U.S. 1 and more than 85 % of component U.S. 3 were eluted in the pH range over which they had been collected in the original fractionation. Component U.S. 2 was eluted in the position characteristic of component U.S. 1; it was also contaminated with some component U.S. 3, which appeared at the correct position in the effluent pattern. The effluent pattern given by component U.S. 4 on re-fractionation resembled that of oxidized wool (Fig. 1) but was not reproducible; the peak at pH 8·4 varied in height and with one sample did not appear at all.

These facts imply that components U.S. 1 and U.S. 3 are not seriously decomposed under the conditions required for their isolation. Components U.S. 2 and U.S. 4, on the other hand, are modified during fractionation.

Amino acid analysis of components. Table 2 presents the amino acid composition of the four components isolated by the method employing gradient elution. Two analyses of samples of U.S. 3 are included, one of which was prepared without precipitation from solution before hydrolysis, the buffer salts and urea being removed by prolonged dialysis against water. The last column gives the analysis of the component prepared in the pH 5·8 treatment of the stepwise fractionation of 10 g. of oxidized wool described above, which corresponds to the component U.S. 3 of the gradient elution. Despite the differences in the methods of preparation of these three samples, their amino acid compositions are remarkably constant. The variation that does occur is much less than that observed between different preparations of other wool components, such as α-keratose (Corfield *et al.* 1958) and the 'component III' prepared by Blackburn (1962). This reproducibility indicates that this component is not markedly heterogeneous.

The compositions of all four components are different in many respects from those of other reported components derived from wool. Component U.S. 1 has an unusual composition, especially with respect to its content of tyrosine and phenylalanine, although it has some features in common with the soluble protein liberated from wool during oxidation with peracetic acid. Component U.S. 2 shows several similarities to γ-keratose but differences also occur, especially in the content of lysine. The relative proportions of amino acids present in component U.S. 3 are, in general, intermediate between the corresponding values for α-keratose and oxidized wool. Although the sulphur content of component U.S. 3 is lower than that of wool, its cysteic acid content, which is very close to that of oxidized wool, is too high for it to be classed as a low-cystine constituent of wool. The amino acid composition of component U.S. 4 is very similar to that of oxidized wool.

Sedimentation diagrams. The sedimentation diagrams of the four components are presented in Fig. 3. Component U.S. 1 gave a broad slowly sedimenting peak indicative of a mixture of polypeptides with a range of relatively low molecular weights. Component U.S. 2 behaved similarly except that the boundary was much sharper and moved faster; this component probably consisted of polypeptides of an average molecular weight that was higher than that of component U.S. 1. The sedimentation diagram of component U.S. 3 showed a sharp fast-moving boundary that would be expected from polypeptides with a narrow range of relatively high molecular weights. Component U.S. 4, in contrast with the other components, did

Table 2. *Amino acid composition of wool and components isolated from oxidized wool by treatment with* 5 M-*urea solutions of increasing* pH

For experimental details see the text.

	N (% of total N)							
	Gradient elution					Stepwise elution		
	U.S. 1	U.S. 2	U.S. 4	U.S. 3	U.S. 3*	pH 5·8 fraction	Mean of columns 4, 5 and 6	Wool†
Alanine	1·92	2·36	3·91	4·64	5·13	4·38	4·72	4·12
Arginine	16·9	15·7	20·1	22·4	21·5	20·7	21·5	19·1
Aspartic acid	2·97	1·88	4·27	6·23	6·51	5·57	6·10	4·38
Cysteic acid/cystine	8·67	11·7	9·69	5·32	5·10	5·42	5·28	7·30
Glutamic acid	3·78	7·06	9·22	10·4	10·5	10·2	10·4	8·48
Glycine	13·7	7·75	4·96	4·67	4·81	4·76	4·75	6·29
Histidine	1·71	1·75	1·59	1·52	1·34	1·47	1·44	1·91
Isoleucine	1·53	2·19	2·12	2·66	2·71	2·51	2·63	2·44
Leucine	4·71	2·40	5·12	6·87	7·20	6·38	6·82	5·85
Lysine	0·91	8·04	4·23	5·57	5·54	4·66	5·26	3·92
Phenylalanine	5·07	1·76	1·35	1·70	1·95	1·90	1·85	2·07
Proline	7·06	8·68	6·25	2·94	3·37	3·69	3·33	5·05
Serine	12·2	11·3	8·07	6·27	6·46	6·95	6·56	7·83
Threonine	4·91	6·67	5·78	4·06	4·54	4·23	4·28	4·71
Tyrosine	6·76	2·41	2·45	2·25	2·44	2·32	2·34	2·62
Valine	3·52	3·87	4·18	4·15	4·39	4·14	4·23	4·16
NH_3	—	—	—	—	10·1	—	—	—
Total	—	—	—	—	103·6	—	—	—
N (%)	12·1	13·7	13·0	15·8	15·3	15·7	—	—
S (%)	—	—	—	—	2·32	—	—	—

* Prepared without precipitation.

† The results given for wool are compiled from the results of Corfield & Robson (1955).

not provide a single boundary on sedimentation; it clearly contained a mixture of peptides whose molecular weights covered the entire range represented by the other three components.

N-Terminal groups. The *N*-terminal groups of component U.S. 3 are given in Table 3. These results are corrected for decomposition of the *p*-iodobenzenesulphonyl derivatives during hydrolysis of the component after reaction with *p*-iodobenzenesulphonyl chloride. In all, 88 % of the tyrosine was recovered as *O*-*p*-iodobenzenesulphonyltyrosine and 76 % of the lysine as N_ϵ-*p*-iodobenzenesulphonyl-lysine. If a 75 % reaction is assumed, the amount of *N*-terminal groups found corresponds to an average molecular weight of about 220 000.

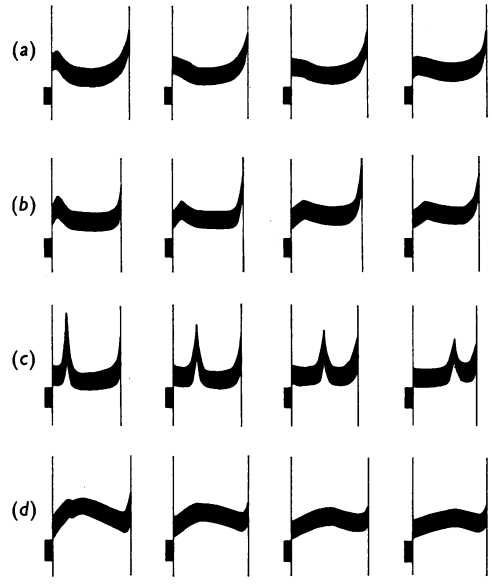

Fig. 3. Sedimentation diagrams of components isolated from oxidized wool by continuous extraction with 5 M-urea solutions of increasing pH. (*a*), Component U.S. 1; (*b*), component U.S. 2; (*c*), component U.S. 3; (*d*), component U.S. 4. Sedimentation was performed at 56 000 rev./min. in a Spinco model E ultracentrifuge. The first photographs, shown on the left, were taken about 30 min. after the attainment of maximum speed and subsequent exposures were made at 64 min. intervals. The meniscus is seen as a fine line at the left of each diagram.

Table 3. *N-Terminal residues of component* U.S. 3

Component U.S. 3 was isolated by gradient elution (see the text and Table 1 for details). *N*-Terminal residues were determined by reaction with *p*-iodobenzenesulphonyl chloride.

Amino acid	Amino acid content (μmoles/g. of protein)
Alanine	0·15
Aspartic acid	0·08
Glutamic acid	0·50
Glycine	1·02
Serine	0·47
Threonine	1·11

DISCUSSION

The results given in this paper support the hypothesis that a large proportion of the wool structure embodies long protein chains, of a definite composition similar to that of whole wool, whose molecules contain certain labile peptide bonds. The nature and complexity of components obtained from wool depend on the degree of hydrolytic fission that occurs during the fractionation procedure; derived components, consisting of mixed polypeptides, will only closely resemble the parent protein chains if they have been isolated by a method involving limited fission of labile peptide bonds. In this respect the fractionation procedure described above is probably superior to methods previously used to obtain components from wool.

Components U.S. 1, U.S. 2 and U.S. 3 described in this paper are believed to be derived from the parent protein of wool as a result of peptide-bond fission concomitant with the splitting of the cystine disulphide groups by oxidation with peracetic acid.

Components U.S. 1 and U.S. 2 are considered to be mixtures of polypeptides of relatively low molecular weight formed by the hydrolysis of single peptide bonds near the ends of the parent protein chains and of pairs of bonds situated near to each other in the same chain. Such polypeptides originate from all parts of the parent protein chain and their amino acid compositions and hence their physical properties vary accordingly. The behaviour of these components on sedimentation and re-fractionation is a reflexion of this variation.

Component U.S. 3, which comprises 36 % of the weight of wool, is thought to be a mixture of the polypeptides of high molecular weight that are left after the removal of components U.S. 1 and U.S. 2 during the fractionation and are soluble in 5 M-urea at pH 5·8. These represent large pieces of the parent protein and therefore their amino acid compositions and physical properties are similar. Component U.S. 3 does in fact behave like a mixture of closely related long-chain polypeptides both on re-fractionation and sedimentation, and the number and quantities of its *N*-terminal amino acids are also consistent with this view.

Component U.S. 4 behaves in a non-reproducible manner on re-fractionation, and sedimentation shows it to be heterogeneous. The conditions necessary for its isolation, i.e. 5 M-urea at pH 8·4, are probably sufficiently alkaline to cause rapid

hydrolysis of peptide bonds in the insoluble wool residue accompanied by dissolution of the liberated polypeptides. The nature of component U.S. 4 depends on the duration of the treatment with pH 8·4 buffer, and the variations in behaviour of different preparations of this component on refractionation are due to this cause.

Previously reported fractionations of wool have obtained soluble components that always fall into two groups of widely different but complementary amino acid compositions. For example, oxidized wool is easily broken down by treatment with alkali into two soluble components, one of which, α-keratose, is precipitated by acid leaving the other, γ-keratose, in solution. α-Keratose contains more aspartic acid, glutamic acid, leucine and lysine but smaller amounts of cysteic acid, proline, serine and threonine than does oxidized wool. γ-Keratose contains less of the former group of amino acids and more of the latter than does oxidized wool. It is now believed that the isolation of these two types of component, the so-called low- and high-sulphur fractions of wool, is symptomatic of the peptide-bond hydrolysis that occurs during the alkaline stage of the fractionation procedure and the subsequent separation of the liberated polypeptides into two groups, one soluble and the other insoluble in acid media. A method of fractionation, such as the one described in the present work, that does not involve treatment of wool by alkaline reagents should therefore give rise to a component with a structure very like that of the original wool. Table 2 shows the average of the three analyses of component U.S. 3 and the amino acid composition of an acid hydrolysate of wool compiled from the results of Corfield & Robson (1955). Component U.S. 3 resembles wool more closely than any other soluble component yet isolated from wool. However, the differences between the two analyses given in Table 2 are still considerable and are probably indicative of the peptide-bond hydrolysis that occurs when wool is oxidized with peracetic acid.

Although differences have been observed in amino acid composition of wools of different quality, wool is endowed with a remarkably constant composition (Simmonds, 1955) despite its complex histological structure. Because of the wide variations that occur in the relative proportions of cortex, cuticle, and other histological regions of the fibre, the unchanging chemical composition of wool can reasonably be accounted for in terms of a single keratin precursor in the developing cells of the follicle. The postulation of two structures in wool with such widely different compositions as the low- and high-sulphur fractions isolated from wool (Crewther & Dowling, 1960) is untenable for the same reason.

SUMMARY

1. Wool that had been oxidized with peracetic acid was fractionated into four components by continuous extraction with 5 M-urea solutions of increasing pH.

2. The main component, which comprises 36 % of the weight of the wool, has an amino acid composition similar to that of wool. The other components have compositions unlike those of wool components reported previously.

3. An explanation of the formation of the components is given in terms of the peptide-bond fission that occurs during the oxidation of wool with peracetic acid.

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