The Fractionation of α -Keratose

By M. C. CORFIELD

Wool Industries Research Association, Torridon, Leeds 6

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Goddard & Michaelis (1935) first isolated fractions from wool which differed in their sulphur content. They considered that 'the high sulphur content of Fraction B indicates that the native keratin probably consists of two fractions'. Since that time protein fractions have been isolated from wool by many investigators using oxidation (Alexander & Earland, 1950; Speakman & Das, 1950; Corfield, Robson & Skinner, 1958; O'Donnell & Thompson, 1959), reduction (Gillespie & Lennox, 1953; Gillespie, O'Donnell, Thompson & Woods, 1960), or partial acid hydrolysis (Lindley, 1947; Blackburn, 1962) for the initial solubilization. Analyses have shown that, despite the differences in the methods of extraction, protein fractions from wool fall into two closely defined groups of strikingly different amino acid composition (Gillespie *et al.* 1960; Corfield *et al.* 1958; Lindley, 1947; Earland & Wiseman, 1959; Blackburn, 1959, 1962). This surprising fact, perhaps taken together with the analogous behaviour of insulin, has led to the belief that these two fractions, although not yet isolated in a pure state, represent two chemical entities in wool. The fractions have been given distinctive names and have been characterized by determinations of *N*- and *C*-terminal amino acids (Alexander & Smith, 1955; Thompson, 1957; Bradbury, 1958), electrophoretic mobility (Gillespie & Lennox, 1955), sedimentation coefficient (O'Donnell & Woods, 1955; Peacock & O'Callaghan, 1959), molecular weight (Harrap, 1955), and intrinsic viscosity (Gillespie & Lennox, 1955; O'Donnell & Woods, 1955). Speculations have been made concerning the mode of incorporation of the precursors of the fractions in wool (Blackburn, 1962), the arrangement of their cystine residues (Earland & Wiseman, 1959) and even their actual positions in the histological structure of the wool fibre (Crewther & Dowling, 1960). Although doubts concerning the homogeneity of these protein fractions had been expressed (Gillespie, 1957), it was not until amino acid-sequence studies had begun on SCMK2 (a fraction with a low sulphur content), believed to represent the purest wool protein available (Gillespie, 1957), that these doubts were confirmed. This protein fraction gave more peptides when digested with trypsin than were compatible with both its amino acid composition and molecular weight, and it was suggested that the fraction did

not represent a chemical entity but was a complex mixture of closely related proteins (Gillespie *et al.* 1960). This point of view is supported by the observations that both α - and γ -keratose (Alexander & Hudson, 1954; Corfield *et al.* 1958) can be fractionated into several components by chromatography on columns of diethylaminoethylcellulose (Thompson, 1961).

If the fractions with low and high sulphur contents isolated from wool do not represent chemical entities corresponding to two components, then some other explanation of their existence must be postulated. The present paper describes some experiments with α -keratose that shed some light on this matter.

METHODS AND RESULTS

The α -keratose used in all the experiments was part of the sample prepared and analysed by Corfield *et al.* (1958).

Chromatography of α -keratose and related components on columns of hydroxyapatite

Hydroxyapatite was prepared as described by Tiselius, Hjerten & Levin (1956). Small columns (1 cm. diam. $\times$ 10 cm.) were used for the fractionation of up to 20 mg. of material, but larger columns (up to 4.2 cm. diam.) were required for preparative chromatography.

α -Keratose was prepared for chromatography by dissolving 500 mg. in 50 ml. of 8 *M*-NaOH with stirring for 3 hr. The solution was titrated to pH 6.8 with H_3PO_4 and dialysed overnight against 400 ml. of water with 24/32 Visking cellophan. A second overnight dialysis against 400 ml. of 1 *mM*-sodium phosphate buffer, pH 6.8, prepared by mixing equal volumes of 1 *mM*- NaH_2PO_4 and 1 *mM*- Na_2HPO_4 , was followed by filtration and storage at 2°. During the dialysis about 2% of the dissolved α -keratose passed through the membrane.

The chromatogram was developed by stepwise elution by using in turn 0.001, 0.01, 0.1 and 1.0 *M*-phosphate buffer, pH 6.8, and finally 0.1 *M*- Na_3PO_4 . Five hold-up volumes of each buffer, about 20 ml. for the 1 cm. diam. columns, were run through under gravity. Equal volumes of effluent were collected and the extinctions at 276 *m* μ measured in a Unicam SP. 600 spectrophotometer. Protein concentrations were calculated from the extinction coefficient, $E_{1\%}^{1\text{cm}}$, of α -keratose, namely 8.52. This is an approximation, since the extinction coefficients of the derived proteins depend on their compositions. A more reliable estimate of the protein contents of the fractions was obtained by alkaline hydrolysis and subsequent interaction with ninhydrin. During hydrolysis more than 80% of the peptide bonds were

broken, but the destruction of certain amino acids by alkali caused loss of ammonia and only 50% of the expected colour intensity was obtained with ninhydrin. Although the method is not strictly quantitative, experience with many large and small peptides from wool shows that it gives a better assessment of peptide concentration than the measurement of the extinction at 276 $m\mu$. A sample of every fraction was treated for 1 hr. at 100° with 1 ml. of 2.5N-NaOH in an open test tube. After the solution had been cooled, 1 ml. of aq. 30% (v/v) acetic acid was added and the resultant mixture treated with ninhydrin as described by Moore & Stein (1954*b*). The extinction at 570 $m\mu$, after deduction of the contribution due to the reagent blank, is approximately proportional to the weight of the protein hydrolysed.

Fig. 1 shows the fractionation of α -keratose into six components. The sequence of solvent changes is not ideal since component HA 4 is only partially separated from

component HA 3. Evidence that these two components are distinct was provided by a column with linear gradient elution, when two peaks in this region were still obtained. The chromatogram obtained with 0.1M- Na_3PO_4 as the sole eluting buffer has two peaks which together represent 97% of the applied load, showing that α -keratose is completely eluted by sodium phosphate buffers.

Recovery of components from solution proved to be unexpectedly difficult. Removal of buffer salts by dialysis was impracticable because the proteins themselves also dialysed at appreciable rates. Desalting on ion-exchange resins also resulted in large losses of material. On titration to pH 4 with H_3PO_4 components HA 1 and HA 2 remained in solution and the other components were only incompletely precipitated. For subsequent amino acid analysis components HA 1 and HA 2 were evaporated to dryness *in vacuo* and hydrolysed in 5.7N-HCl without removal of buffer salts. The approximate weights of protein in the

Fig. 1. Fractionation by chromatography on hydroxyapatite. (a) α -Keratose; (b) component HA 1; (c) component HA 3.

Table 1. Amino acid composition of components isolated from α -keratose by chromatography on hydroxyapatite columns with phosphate buffers

The yields of components HA1 to HA5 were determined from their colour yields with ninhydrin after alkaline hydrolysis. The composition of α -keratose was calculated as a mixture of components HA1 to HA5, inclusive, in the proportions of 14:3.55:19:6, by wt. The results for α -keratose, γ -keratose and oxidized wool were taken from the results of Corfield *et al.* (1958).

Amino acid	N (% of total N)							Oxidized wool
	HA1	HA2	HA3	HA4	HA5	α -Keratose (calc.)	γ -Keratose	
Alanine	2.3	3.4	5.5	5.7	5.2	4.8	2.6	4.0
Aspartic acid	2.4	2.8	7.4	7.8	6.9	6.4	1.8	4.6
Cysteic acid	9.5	11.8	3.1	2.4	2.5	4.1	3.7	6.5
Glutamic acid	3.6	6.5	14.1	13.2	11.8	11.7	14.5	8.5
Glycine	10.2	6.1	3.7	3.5	1.9	4.4	5.9	6.1
Isoleucine	1.9	2.1	2.8	3.5	3.2	2.8	5.0	2.3
Leucine	3.9	3.7	9.2	10.2	9.0	8.1	2.1	2.3
Phenylalanine	2.9	1.7	3.4	2.2	3.4	2.2	2.6	5.8
Proline	6.4	6.6	1.8	2.1	2.1	2.4	1.2	1.9
Serine	11.4	9.7	6.2	4.8	6.3	6.6	9.9	4.9
Threonine	4.4	5.8	2.9	2.7	3.1	3.1	9.7	7.5
Tyrosine	3.8	2.6	2.5	3.0	3.9	2.8	7.5	4.7
Valine	3.2	3.8	4.0	3.7	5.9	2.4	1.4	2.4
Yield of component (%)	14	3	55	19	6	3.8	4.2	4.0

hydrolysates were determined by reaction with ninhydrin. Components HA3, HA4, HA5 and HA6 were recovered from the effluent fractions, grouped as shown in Fig. 1, by concentration and precipitation with H_3PO_4 . After being centrifuged, the components were washed with a small quantity of water and dried with ethanol and diethyl ether. Known weights were hydrolysed under reflux with 20 ml. of 5.7N-HCl for 24 hr. and HCl was removed by distillation *in vacuo*. Amino acid analyses were performed by the method of Moore & Stein (1951, 1954a) except that Zeo-Karb 225 (X8) resin, in the form of spheres passing a 200-mesh sieve, was used for chromatography. The basic amino acids were not determined. The results, together with those for α -keratose, γ -keratose and oxidized wool (Corfield *et al.* 1958), are given in Table 1. Contamination of the components by inorganic salts precluded direct determination of their yields, but sufficiently accurate estimates were made from the reactivity of the hydrolysed fractions with ninhydrin. The yield of a component was taken as the sum of the extinctions at 570 $m\mu$ of the fractions contributing to a peak expressed as a percentage of the total extinction of all components eluted from the column.

Table 2 shows the recoveries of the acid-insoluble components obtained in a typical experiment starting with 210 mg. of α -keratose. Components HA4, HA5 and HA6 thus had appreciable solubility in acid solutions, the losses in the mother and wash liquors being more significant for the components present in small amounts. If, as seems likely, the components obtained by this procedure were heterogeneous mixtures of similar proteins, then there is no guarantee that the material recovered was identical with that eluted from the column. For this reason amino acid analyses of the components recovered in low yield can only be regarded as approximate. Against this, 178.7 mg. of material, equivalent to 85% of the applied load, was actually recovered, together with 11.0 mg. of component HA1 and 6.9 mg. of component HA2, determined by the reactivity of their acid hydrolysates with ninhydrin. Also, the calculated amino acid composition of α -keratose, estimated from the compositions of the five components and the proportions in which they occur, agrees well with that found by direct determination. The values for components HA1, HA2 and HA3, which were completely recovered, are not subject to this error.

The amino acid compositions of the components show striking differences. Components HA3, HA4 and HA5 resemble α -keratose but their amino acid compositions differ even more from that of oxidized wool. Components HA1 and HA2 resemble γ -keratose in amino acid composition although the resemblance does not seem close at first sight. The fractionation of α -keratose into soluble (HA1 and HA2) and insoluble (HA3, HA4 and HA5) components is, however, exactly paralleled in the formation of α - and γ -keratose from oxidized wool. The most striking differences in the composition of α - and γ -keratose and oxidized wool are in the proportions of cysteic acid, threonine, serine, proline, aspartic acid, glutamic acid, alanine and leucine. The amounts of these amino acids in the components given in Table 1 have been recast in Table 3 as percentages of the amounts of the same amino acids in their parent proteins. The relationship between the amino acid composition of γ -keratose and that of oxidized wool is the same as the relationship between the amino acid compositions of HA1 and HA2 and that of γ -keratose. In addition,

Table 2. *Components recovered after chromatography of 210 mg. of α -keratose on hydroxyapatite*

The yields of components were determined from their colour yields with ninhydrin after alkaline hydrolysis.

Component	Yield (percentage E_{270})	Wt. eluted (mg.)	Wt. recovered (mg.)	Recovery (%)
HA3	55	116	116.5	100
HA4	19	40	31.7	79
HA5	6	12.6	9.5	75
HA6	3	6.3	3.1	49

Table 3. *Distribution of amino acid residues of components isolated from α -keratose and oxidized wool*

The weight of amino acids in components HA1 to HA5, inclusive, are expressed as percentages of the weights of the corresponding amino acids in α -keratose. Weights of the amino acids in α - and γ -keratose are expressed as percentages of the weights of the corresponding amino acids in oxidized wool.

Amino acid	HA1	HA2	γ -Keratose	HA3	HA4	HA5	α -Keratose
Alanine	48	71	65	115	119	108	120
Aspartic acid	38	44	39	117	124	110	137
Cysteic acid	257	314	223	84	65	68	57
Glutamic acid	33	60	69	130	121	109	128
Leucine	53	51	45	126	140	123	126
Proline	237	244	202	67	78	78	55
Serine	170	145	129	93	72	94	89
Threonine	126	166	160	83	77	89	75

Table 4. *Behaviour of components HA1 and HA3 on re-chromatography under the same conditions used for their isolation*

The yields of protein materials emerging in the chromatographic positions characteristic of the components HA1 to HA6, inclusive, were determined from their extinctions at 276 $m\mu$.

Component eluted	Yield (percentage E_{270})		
	From α -keratose	From HA1	From HA3
HA1	13	34	10
HA2	4	1	5
HA3	64	47	66
HA4	8	3	12
HA5	8	11	4
HA6	3	2	2

α -keratose and oxidized wool are related to each other in the same way as components HA3, HA4 and HA5 are to α -keratose.

The components are modified to some extent as a result of peptide-bond fission during the separation. This is borne out by the behaviour of components HA1 and HA3 on re-fractionation. Component HA3 was treated in the same way as α -keratose before rechromatography: only 65% of the dissolved component was retained during the dialysis. The solution of HA1 in 1 mm-sodium phosphate buffer, pH 6.8, was concentrated to 5 ml. *in vacuo* and dialysed for 3 hr. against 500 ml. of water before application to the column. About 30% of the dissolved fraction was lost during the dialysis. Fig. 1 shows that the two components, which had been chromatographically purified, behaved as mixtures of degradation products. The yields of the new fractions, calculated in terms of the extinction at 276 $m\mu$, are compared with those obtained from α -keratose in Table 4. The pattern of products obtained from HA3 is

similar to that from α -keratose; however, the products themselves differ in that they all rapidly dialyse through Visking cellophan and are soluble in acid solution.

Fractionation of α -keratose by fractional solubility in ethanol-aqueous 0.01N-ammonia

A column (1 cm. $\times$ 30 cm.) was poured in ten sections from a slurry of 5 g. of α -keratose and 10 g. of Celite 545 in 50 ml. of ethanol. Components were eluted from the column by perfusion with a solvent of continually changing composition in which the α -keratose finally became completely soluble. A peristaltic pump transferred solvent at 14 ml./hr. to the column from a magnetically stirred mixing vessel that initially held 1200 ml. of 98% (v/v) ethanol containing 10 ml. of aq. 1 N-NH₃/l. Simultaneously aq. 0.01 N-NH₃ was pumped at 7 ml./hr. into the mixing vessel. The concentration gradient thus produced has a linear dependence on the volume pumped, and the rate of change of concentration depends on the initial volume of solvent in the mixing vessel. Fractions (20 ml.) of effluent were collected and their protein content was determined spectrophotometrically.

This system of fractionation was chosen because of the relative ease of recovery of fractionation products from solution in a salt-free form. Fig. 2 shows the effluent pattern obtained with α -keratose. This method of fractionation of α -keratose depends on its solubility behaviour, which is known to vary with the age of the sample, and would, therefore, be expected to be non-reproducible. In fact, although the relative heights of the peaks varied, the maxima always corresponded to a characteristic concentration of ethanol. This unexpected result implies that the peaks have a real significance and that α -keratose consists of three major components; these could be chemically distinct or represent different physical configurations. The components were recovered from the combined effluent from several experiments divided into four parts as shown

in Fig. 2. Solutions were treated with 40 ml. of 2*N*-acetic acid/l. to adjust the pH to the value required in the original preparation of α -keratose. Ethanol was then added to bring its concentration to 95% (v/v). If the protein did not precipitate immediately, flocculation was brought about by the addition of a few crystals of ammonium acetate. After standing for 24 hr. the precipitated material was centrifuged off, washed with ethanol and diethyl ether and finally dried *in vacuo* over KOH and H₂SO₄. Table 5 shows the distribution of components isolated from each group in a typical experiment in which 96% of the fractionated α -keratose was recovered. The column headed ΣE_{276} is the sum of the extinctions of the fractions in the group; these

values divided by the corresponding weights are given in the next column. The variability of these values, which are proportional to the extinction coefficients of the components, indicates that there are chemical differences between the components. This is confirmed by the amino acid analyses presented in Table 6, but the differences, except with tyrosine, are small.

Further evidence concerning the nature of the components was provided by again subjecting them to the fractionation procedure. If they were physical modifications of α -keratose then their effluent patterns on re-treatment would be identical with that of α -keratose; if they were chemically distinct then each component would produce a pattern with a peak in the position it occupied in the original fractionation. The actual behaviour of the components on re-treatment is shown in Fig. 2. Attempts to isolate the dissolved components from the three characteristic peak positions revealed that they were much more soluble than the parent materials in 95% (v/v) ethanol solutions and that they diffused rapidly through Visking 24/32 dialysis tubing. It is evident that these components from α -keratose are heterogeneous and their complexity is, at least in part, due to changes brought about during fractionation.

Solubility curves of α -keratose

In ammonium sulphate solutions buffered at pH 6.8. A series of solutions of known, gradually changing, salt concentration was prepared from 502.5 ml. of 64% saturated (NH₄)₂SO₄ solution as follows: a 5.0 ml. sample was transferred to a stoppered Pyrex test tube and 2.5 ml. of water was then stirred into the remainder of the solution. This procedure was repeated until 150 test tubes had been prepared. The concentration of salt in the *n*th tube, *c_n*, is given by

$$c_n = \left(\frac{502.5 - 2.5n}{500} \right) c_1$$

where *c₁* is the initial concentration of (NH₄)₂SO₄.

A sample (3.0 ml.) of α -keratose solution, prepared by dissolving 3.71 g. in 1l. of 0.02*M*-sodium phosphate buffer, pH 6.8 [0.02*M*-NaH₂PO₄-0.02*M*-Na₂HPO₄ (1:1, v/v)], was added to each test tube, and the contents were shaken and allowed to equilibrate at room temperature for 3 days. The light-absorption at 276 m μ of the supernatant solution was measured after filtration through pleated Whatman no. 42 paper.

In potassium phosphate solutions at pH 6.8. The experimental procedure was the same as that described for ammonium sulphate solutions with the following exceptions. The α -keratose solution was made by dissolving

Fig. 2. Fractionation in ethanol-aq. 0.1*N*-NH₃. (a) α -Keratose; (b) component FS1; (c) component FS2; (d) component FS4.

Table 5. Components recovered after fractionation of α -keratose by fractional solubility in ethanol-aqueous 0.01*N*-ammonia

α -Keratose (602 mg.) was supported on a column of Celite 545 and perfused with a solution of ethanol-aq. ammonia of continually changing composition.

Component	Wt. recovered (mg.)	Yield (%)	ΣE_{276}	$\Sigma E_{276}/\text{wt.}$
FS1	151.2	25	9.94	0.066
FS2	202.9	34	6.81	0.034
FS3	52.7	9	1.89	0.036
FS4	169.7	28	6.80	0.040

the same weight of α -keratose in 700 ml. of 0.01 M-potassium phosphate buffer, pH 6.8, and diluting to 1 l. with water. A total of 200 solutions of potassium sulphate was prepared from 627.5 ml. of 3.5 M-potassium phosphate [$3.5\text{M-KH}_2\text{PO}_4$ - $3.5\text{M-K}_2\text{HPO}_4$ (1:1, v/v)] by alternate removal of a 5 ml. sample and addition of 2.5 ml. of water. The molarity of PO_4^{3-} in the n th tube is given by

$$c_n = \left(\frac{627.5 - 1.5n}{625} \right) 2.1875 + 0.0263$$

In ethanol solution at pH 4.7. The α -keratose solution was prepared as described for potassium phosphate solutions except that 20 ml. of N-NaOH was added before making up to 1 l. Altogether 150 aqueous ethanol solutions, 5 ml. in volume, were prepared from 470 ml. of aq. 75% (v/v) ethanol solution in the manner previously described. A sample (3 ml.) of ethanol containing 0.6% (w/v) of trichloroacetic acid was added to each tube, and then 3 ml. of α -keratose solution was also added. The percentage (v/v) of ethanol in the n th tube is given by

$$c_n = \left(\frac{470 - 2.5n}{467.5} \right) 34.09 + 27.27$$

Fig. 3 shows the dependence of the solubility of α -keratose on the concentration of three protein precipitants. The solubility of α -keratose, and to some extent the shape of the solubility curves, depend on the method of preparation and the age of the sample. Freshly prepared samples were usually more soluble than those that had been stored in the dry state. Solubility is also dependent on the pH of the medium. Curves (a) and (b) show a high solubility in regions of low salt concentration because the solutions were at pH 6.8. Curve (c) was obtained at pH 4.7, near to the isoelectric point of α -keratose, and consequently the solubility was low at low ethanol concentrations, but increased on the addition of ethanol. In solutions of ethanol concentration greater than 90% (v/v), α -keratose again became virtually insoluble.

The solubility behaviour of α -keratose in the three systems studied indicates that it is a heterogeneous pre-

paration. The many irregularities in the curves are symptomatic of the presence of substances with varying solubility characteristics. It is evident that fractionation of these substances can be achieved by fractional solution of α -keratose in any of these systems.

Fractionation of α -keratose in urea-water at pH 4.7

The method described above for the fractionation of α -keratose by fractional solubility in ethanolic ammonia was adopted except that the mixing flask initially contained 800 ml. of McIlvaine buffer, pH 4.7 (Na_2HPO_4 , 3.41 g./l.; citric acid monohydrate, 2.73 g./l.). This solution was pumped at 7 ml./hr. to a column (0.9 cm. $\times$ 25 cm.) prepared from 8 g. of Celite 545 and 100 mg. of α -keratose. A similar buffer containing 50% (w/v) of urea was simultaneously pumped into the mixing vessel at 3.5 ml./hr. Effluent fractions of 10.5 ml. were collected. With an old sample that had been stored as a dry powder, a reproducible effluent pattern, showing two broad peaks, was obtained (Fig. 4). Freshly precipitated α -keratose exhibited more components although the peaks near 20% (w/v) urea concentration did not always appear. When attempts were made to recover the dissolved components from the

Fig. 3. Solubility curves of α -keratose. (a) In $(\text{NH}_4)_2\text{SO}_4$ at pH 6.8; (b) in potassium phosphate at pH 6.8; (c) in aqueous ethanol at pH 4.7.

Table 6. Amino acid compositions of components isolated from α -keratose by fractional solubility in ethanol-aqueous 0.01 N-ammonia

Amino acid	N (% of total N)		
	FS1	FS2	FS4
Alanine	6.38	4.92	4.58
Ammonia	10.1	14.1	10.1
Arginine	20.8	20.8	22.2
Aspartic acid	5.34	5.46	7.10
Cysteic acid	3.83	2.65	2.81
Glutamic acid	10.0	13.4	12.0
Glycine	6.57	5.20	5.12
Histidine	1.36	1.03	1.09
Isoleucine	2.55	3.30	3.54
Leucine	7.20	8.60	8.70
Lysine	4.89	5.00	5.96
Phenylalanine	1.73	1.48	1.67
Proline	2.25	1.67	2.20
Serine	6.70	4.15	4.78
Threonine	3.09	2.53	3.25
Tyrosine	3.58	1.67	0.50
Valine	3.41	4.28	4.32

Fig. 4. Fractionation of α -keratose. (a) In aqueous urea solutions at pH 4.7 with an old sample of α -keratose; (b) as for (a) but with a freshly precipitated sample; (c) by dialysis in aqueous urea at pH 6.8.

Fig. 5. Apparatus for fractionation by dialysis. A, Solution inlet tube; B, stirrer; C, tube supporting dialysis sac; D, outer vessel; E, to fraction collector.

effluent fractions by dialysis, it was evident that in the presence of urea the components passed through Visking 24/32 cellophan.

Fractionation by dialysis in urea solutions

A small celloidin sac (0.8 cm. diam. $\times$ 2 cm. long) of about 1 ml. capacity was prepared from a solution of 2% (w/v) celloidin in ethanol-diethyl ether-ethane-1,2-diol (20:30:1, by vol.) by the method of Smithies (1953). α -Keratose (30 mg. dissolved in 1 ml. of 0.01M-sodium phosphate buffer, pH 6.8) was placed inside the sac, which was set up in the assembly shown in Fig. 5. The volume of solution in the outer vessel was kept as small as possible and was about 2 ml. Solvent of gradually changing composition was pumped to the outer vessel at 7 ml./hr. from a mixing vessel containing initially 200 ml. of 0.01M-sodium phosphate buffer, pH 6.8. A similar buffer solution containing 60% (w/v) of urea was simultaneously pumped at 3.5 ml./hr. into the mixing vessel. After collection of 115 fractions, each of volume 3 ml., the contents of the dialysis sac were washed out and examined spectrophotometrically for residual protein.

The effluent pattern obtained was dependent on the membrane used. Fig. 4 shows one such effluent pattern. The values in parenthesis are the yields in each peak determined from the extinctions of the fractions. Of the original material 12% remained in the sac at the end of the experiment.

DISCUSSION

The evidence presented supports the hypothesis that α -keratose is a heterogeneous mixture of related polypeptides whose molecules contain certain easily hydrolysed peptide bonds. The existence of similar labile peptide bonds in oxidized wool accounts for both the formation of α -keratose and the ease of its degradation during subsequent fractionation.

The complex nature of the solubility curves, and the many components produced by fractionation in urea solutions and by dialysis, all demonstrate that α -keratose is heterogeneous. Appreciable chemical degradation is unlikely to have occurred during these experiments because the methods involve only dissolution in neutral solutions in the presence of salts, urea or ethanol. Heterogeneity is also apparent from the number of components revealed by fractional solubility in ethanolic ammonia solutions and by chromatography on hydroxyapatite columns. However, re-treatment of some of the components, derived by the latter methods, by subjecting them to the fractionation procedure for a second time has indicated that their existence may be due to decomposition during fractionation. Such decomposition most probably occurs by hydrolysis of labile peptide bonds when α -keratose is subjected to alkaline conditions. The fission of relatively few peptide bonds would result in a large amount of residual material, similar in composition to α -keratose but of slightly lower mole-

cular weight, and smaller amounts of relatively low-molecular-weight polypeptides of random composition. In the preparation of a solution for chromatographic examination on columns of hydroxyapatite, α -keratose is treated with dilute alkali: the six components found represent resolved groups of similar polypeptides that were derived from α -keratose as a result of this treatment. Component HA 3, obtained in about 60% yield, is more easily dialysed than α -keratose but resembles it in amino acid composition and the behaviour on rechromatography. The extreme differences in amino acid composition of the other components are due mainly to the resolving power of the hydroxyapatite column but also, in part, to the fact that all the α -type components were isolated by incomplete precipitation from acid solution and would thus be richer in amino acids with side chains that confer insolubility in acids.

With the fractionation of α -keratose by treatment with ethanolic ammonia solutions, the alkaline conditions prevail throughout the fractionation procedure and decomposition products should emerge continuously as wide tailing peaks in the effluent pattern. This kind of behaviour does, in fact, occur and is especially evident in the samples subjected to a second fractionation. Component DS 4 is here considered to be the residual α -keratose-like material; in fact, its effluent pattern on re-treatment is similar in several respects to that of α -keratose itself. The other components do not, however, show the large differences in amino acid composition that are observed in the components from hydroxyapatite columns. This may stem from the fact that they were isolated by complete precipitation from acid solution.

An explanation of the heterogeneity of α -keratose is afforded when the methods of its isolation from wool are examined. The first stage of preparation consists in oxidizing wool with peracetic acid with the object of converting all cystine residues into cysteic acid, although there is some evidence that intermediate oxidation products of cystine are also formed (Gillespie *et al.* 1960). The procedure necessarily involves the concomitant oxidation of methionine to methionine sulphone, and the conversion of tryptophan into some unidentified compound, but appears to have little or no effect on the other amino acids (Corfield *et al.* 1958). It is generally believed that no degradation of protein chains takes place, but 1.6% of the total wool nitrogen goes into solution, and this contains (by weight) 20% of glycine, 10% of serine, and 10% of cysteic acid, as well as smaller amounts of other amino acids. Hence some fission of peptide bonds can be assumed to have occurred. During the second stage of preparation of α -keratose, the

oxidized wool is stirred for 24 hr. in 0.1 N-ammonia solution, when 80–90% dissolves. Wool is very susceptible to attack by alkali, and under these conditions further peptide-bond fission is inevitable. Indeed, if the dissolution period is prolonged, the amount of α -keratose eventually obtained decreases and the solution becomes less viscous (Peacock & O'Callaghan, 1959; Booth, 1952; O'Donnell & Woods, 1955). Addition of acetic acid to the alkaline solution until the pH is about 4 gives a clean separation of α -keratose as a white precipitate, leaving γ -keratose in solution. A crude fractionation of the polypeptides in solution into α - and γ -keratose in this way is not surprising, since the former is rich in those amino acids with side chains that could be expected to confer insolubility in acid media, namely, aspartic acid, glutamic acid and leucine, whereas the latter is rich in the amino acids cysteic acid, threonine, serine and proline, whose side chains could be expected to confer solubility. The amino acid compositions of different samples of α - and γ -keratose vary according to their methods of preparation (Corfield *et al.* 1958), and a large but variable proportion of γ -keratose will easily dialyse through cellophan. These facts imply that α - and γ -keratose do not represent distinct stages in the hydrolytic degradation of wool but that their composition is a function of the extent of bond fission involved in their preparation.

The isolation of α -type and γ -type components from α -keratose by chromatography on columns of hydroxyapatite is an extension of the process of formation of α - and γ -keratose from wool. α -Keratose is subjected to further peptide-bond hydrolysis and the subfractions produced fall into two groups according to their solubility in acid media.

The direct measurement of *N*-terminal residues in components should give a positive indication of the extent of peptide-bond rupture. In practice increased amounts of end group are offset by losses of low-molecular-weight components during the isolation of a component or during reaction with the *N*-terminal reagent. This is exemplified by the work of Thompson (1957), who could find no change in the *N*-terminal residues of wool after prolonged washing with hot water or after dinitrophenylation in highly alkaline media. These treatments are known to release low-molecular-weight peptides from wool (Zahn & Meienhofer, 1955).

The reported fractionations of wool depend on separation procedures similar to those required for the isolation of α -keratose. The disulphide bonds of cystine residues are broken and fractions then obtained on the basis of their solubility in solutions of various pH values. Treatment with alkali is required at some stage of the procedure. Black-

burn (1962) has recently subjected wool to deliberate partial acid hydrolysis, and obtained two components by dissolving the degraded wool in sodium hydroxide solution and precipitating one component by the addition of acid. As pointed out above, the isolated components, whatever their method of preparation, invariably show a marked similarity to α - and γ -keratose. It is probable that the fractions are merely different chemical forms of the same family of polypeptides; none of them can be considered as a chemical entity even in an impure form. All the evidence presented here shows that treatment of wool with alkaline solutions causes peptide-bond fission; this is more pronounced when the wool has previously been treated with acids. Components obtained after such treatments by methods depending on solubility in aqueous media are merely manifestations of the procedure and do not necessarily represent definite intrinsic structures in wool. Speculations concerning the role of such nebulous components in the histological structure of wool seem pointless.

SUMMARY

1. α -Keratose, a protein isolated from oxidized wool, has been shown to be heterogeneous by measurement of its solubility curves in three solvent systems.

2. α -Keratose has been fractionated into several components by chromatography on columns of hydroxyapatite, fractional solubility in aqueous ethanol solutions and dialysis in buffered urea solutions. Some of the derived components exhibit properties similar to γ -keratose and other proteins with a high sulphur content derived from wool.

3. An explanation of the heterogeneity of α -keratose is afforded in terms of peptide-bond fission during its preparation. This concept also accounts for the formation of the same proteins with high and low sulphur contents prepared from wool by different degradative procedures.

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