STUDIES ON OXIDIZED WOOL

IV. FRACTIONATION OF PROTEINS EXTRACTED FROM WOOL ON DEAE-CELLULOSE USING BUFFERS CONTAINING 8M UREA

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Summary

α- and γ-keratoses, which are soluble proteins extracted from oxidized wool, give widely spread zones in their elution curves when examined by gradient elution chromatography on columns of diethylaminoethyl (DEAE)-cellulose in buffers containing 8M urea. Stepwise elution of the proteins indicates that they could consist of many components. An arbitrary chromatographic fractionation of α-keratose into three components followed by amino acid analysis of the fractions revealed distinct differences in amino acid composition among the components. No evidence was obtained for the presence of a major homogeneous protein component in oxidized wool. It is postulated that the original wool itself may consist of families of closely related proteins.

I. Introduction

In the early stages of fractionation of a mixture of proteins, chromatography by stepwise or gradient elution can often be applied successfully. In the case of aggregated or unfolded protein molecules (and often in the case of native protein molecules) it is frequently found that binding to the adsorbent is not truly reversible, In such cases it is probable that differences in states of aggregation or even minor variations in the shape of the protein may result in the elution of the protein in several peaks (cf. Shapira and Parker 1960) which could erroneously be considered a fractionation into proteins of different chemical constitution. One way of limiting such complexity in elution behaviour is to carry out the chromatography with buffers containing disaggregating agents.

In our experiments we have used buffers containing 8M urea in an attempt to minimize complications which may arise in the effluent patterns due to aggregation of the protein molecules. 8M urea has made it possible not only to chromatograph insulin on "Amberlite IRC-50" (Cole 1960) and diethylaminoethyl (DEAE)-cellulose (Thompson and O'Donnell 1960) but has given resolution not possible in the absence of urea. With many proteins (Cole and Mendiola 1960) the presence of 8M urea enables chromatography to be carried out at pH values where the protein normally may be insoluble.

α-keratose and γ-keratose are acidic proteins isolated from oxidized wool (Alexander and Hudson 1954; O'Donnell and Thompson 1959). α-keratose is aggregated in aqueous solution (O'Donnell and Woods 1956*a*, 1956*b*) and it was desirable to study chromatographic separation of this material and γ-keratose under the

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best disaggregating conditions possible. Buffers containing 8M urea are suitable for disaggregating wool proteins (Woods 1959).

This paper reports the fractionation of α- and γ-keratoses on columns of DEAE-cellulose using stepwise elution with buffers containing 8M urea. Amino acid analysis has been used to show that the fractionation of α-keratose involves molecules of different chemical composition.

II. Experimental

(a) *Preparation of Proteins*

The α- and γ-keratoses were extracted at pH 8 from wool (obtained from a single fleece, MW118, 64's quality) oxidized with performic acid. They were freeze-dried from aqueous salt-free solution, or else stored in frozen aqueous solution and freshly freeze-dried before chromatography. The α- and γ-keratoses extracted under these conditions represent only 33 and 16%, respectively, of the wool but it is believed that these are not substantially different from the larger amounts extractable at higher pH values (O'Donnell and Thompson 1959).

Insulin and bovine plasma albumin were oxidized with performic acid reagent at 0°C and isolated by freeze-drying twice from aqueous solutions (Hirs 1956).

(b) *Operation of DEAE-cellulose Columns*

The columns of DEAE-cellulose (0·9 by *c.* 15 cm) were prepared and packed in the 8M urea buffer as described previously (Thompson and O'Donnell 1960). The DEAE-cellulose was used in predominantly the chloride form, being regenerated with 1M potassium chloride in 8M urea buffer at pH 7·4. It was periodically regenerated with 1N sodium hydroxide solution (Sober *et al.* 1956). The protein was eluted either by stepwise or gradient elution and, for the latter, a mixer with a 120-ml mixing chamber of the type described by Moore and Stein (1954) was used, the gradient being calculated according to Bock and Ling (1954). The volume from the outlet of the mixing chamber to the top of the column was 17 ml. Polyethylene tubing was used throughout since the 8M urea buffer dissolved large amounts of ultraviolet-absorbing material from polyvinylchloride tubing. All experiments were carried out at 25°C with water-jacketed columns. The recovery of proteins from the columns was greater than 90% but with the widely spread curves of the extracted wool proteins the value cannot be obtained precisely.

(c) *Buffer and Protein Solutions*

The columns were in all cases washed with the initial buffer consisting of 8M urea (B.D.H. "Analar"), 0·01M Tris (tris(hydroxymethyl)aminomethane), and 0·001M "Versene" adjusted to pH 7·4 until the pH values of the influent and effluent were identical. After regeneration with 1M potassium chloride in 8M urea solution it was necessary to pass at least 140 ml of starting buffer through the column. The buffer was filtered through a separate column of DEAE-cellulose before use. It was used within 2–3 days and stored at 2°C when not in use. The gradient was usually formed with 1·1M potassium chloride in the upper chamber of the mixer.

The freeze-dried proteins were dissolved in the buffer and in some cases dialysed overnight against the initial buffer although this made no difference to the elution pattern of the protein. Usually 10 mg of protein in 0·5 ml buffer were applied to the column.

(*d*) *Analysis of Effluent Fractions*

Approximately 1·1-ml fractions of effluent were collected by means of a drop counter. These were diluted with 3 ml of water before measuring their optical density against a water blank in 1-cm cells at 276 mμ. No correction was made for scattering of light.

(*e*) *Cysteic Acid Analyses*

These were carried out as described in a previous paper (Thompson and O'Donnell 1959) except 1 ml (instead of 3 ml) of the solutions containing cysteic acid was loaded on the column.

(*f*) *Analysis for Basic Amino Acids*

These were carried out on a 15-cm long column of "Dowex 50-X8" (200–400 mesh) according to the method of Moore and Stein (see Moore, Spackman, and Stein 1958). The protein hydrolysate was prepared by hydrolysis of approximately 40 mg of protein in 10 ml redistilled hydrochloric acid (6N) under reflux for 22 hr in an oil-bath at 138°C. This was then made up to 50 ml with water.

Aliquots (5 ml) were freeze-dried and the residue dissolved in 3 ml of citrate buffer (pH 2·2) and 2 ml of this was loaded on the column. 2-ml fractions were collected, the column being developed with pH 5·28 buffer as described by Moore, Spackman, and Stein (1958). The effluent fractions were analysed using a ninhydrin method. To each 2-ml fraction was added 0·2 ml of a 10% solution of recrystallized ninhydrin (Moore and Stein 1948) in purified methyl "Cellosolve" followed by 1 ml of a buffer–cyanide–methyl "Cellosolve" solution (Chibnall, Mangan, and Rees 1958). The tubes were heated in a boiling water-bath for 20 min before dilution with 50% ethanol and estimation in a Coleman Junior spectrophotometer at 570 mμ (Moore and Stein 1948). As standards, commercial samples of purified amino acids were checked and the absorbances per μmole were compared with those obtained using the Beckman–Spinco standard mixture run through our column. The agreement was good (see Table 2) except for the two commercial samples of histidine. The average Spinco absorbances per μmole were used to calculate our results.

(*g*) *Nitrogen Determinations*

These were estimated by the Kjeldahl procedure and amide nitrogen (and extraneous ammonia nitrogen) was driven off prior to each determination by evaporating freeze-dried aliquots (5 ml) of the hydrolysate in the presence of 0·1M potassium carbonate *in vacuo* over concentrated sulphuric acid (Moore and Stein 1951). There is no evidence that this treatment removes other than ammonia nitrogen (Sanger, Thompson, and Kitai 1955). It was deemed necessary to give the hydrolysates this treatment prior to nitrogen determinations because of the

difficulty in ensuring that all the urea nitrogen had been removed by the dialysis procedures.

(*h*) *Preparative Procedures*

For bulk fractionation of α-keratose the stored aqueous solution was precipitated at pH 4 with acetic acid in the presence of 0·3M potassium chloride, centrifuged lightly, and the precipitate dissolved in the 8M urea buffer. This solution was then dialysed overnight versus buffer before loading on a 2-cm dia. column of DEAE-cellulose. Elution was carried out at the rate of 20 ml/sq.cm/hr successively with 40 ml of buffer, and then buffer containing 0·1M, 0·2M, and 0·5M potassium chloride. 5-ml fractions were collected. The fractions comprising the three peaks were dialysed against water, freeze-dried, and the chromatography repeated. Finally they were dialysed successively versus water, 0·1M sodium chloride, and finally water (using a rocking dialyser and 18/32 Visking cellulose tubing) and freeze-dried for analysis.

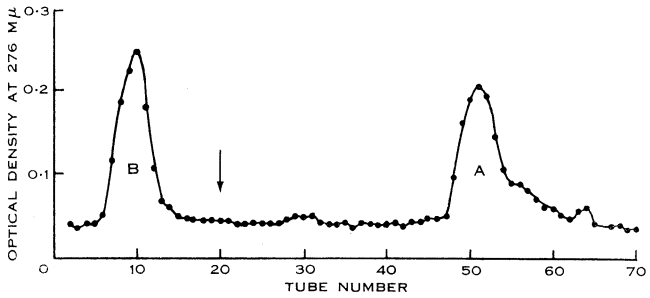

Fig. 1.—Chromatographic behaviour at 25°C of oxidized insulin (1 hr at 0°C) on DEAE-cellulose in 8M urea–Tris buffer at pH 7·4. Fraction size was approximately 1·1 ml. Protein applied in 8M urea–Tris buffer and eluted with a gradient to 0·5M potassium chloride in this buffer. Gradient reached column at tube 20.

III. Results and Discussion

(*a*) *Chromatography of Unfolded Proteins*

Figure 1 shows the gradient elution curve of oxidized insulin in a buffer containing 8M urea. It is obvious that the A and B chains have separated, the more acidic glycyl chain (from absorption spectra) being retarded. It is seen that each of these peaks is itself spread out much more than would be the parent insulin peak under the same gradient (cf. Thompson and O'Donnell 1960). This might be attributable to the incomplete disaggregation of the B chain in 8M urea or to a general variation in shape amongst the molecules comprising each oxidized chain. However, it is apparent that polypeptides in an unfolded state can still be adsorbed and desorbed from a column in spite of the large number of binding sites on these molecules.

The effect of standing oxidized insulin in pH 7·4 or 8 buffer overnight before chromatography was investigated but no difference in pattern due to this standing was noted. Hence it appears that any formylation of the serine and threonine residues

(Kienhuis, Blaase, and Matze 1959; Narita 1959; Smillie and Neurath 1959) caused by formic acid has not affected the chromatographic pattern.

We have also chromatographed bovine plasma albumin after oxidation with performic acid and also after reduction and alkylation of the thiol groups. With a similar gradient to that used for the oxidized insulin a single spread-out peak was obtained in each case (which may contain more than one component). The oxidized bovine plasma albumin was considerably more spread than the reduced and alkylated material (see Fig. 2).

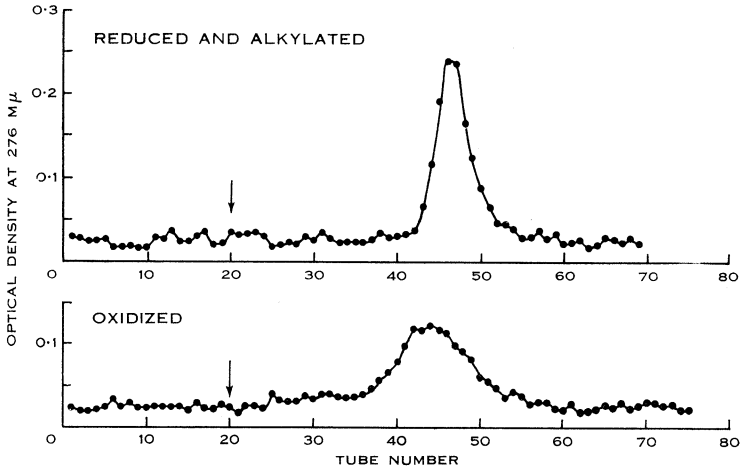

Fig. 2.—Chromatography at 25°C of oxidized, and reduced and alkylated, bovine plasma albumin on DEAE-cellulose. Protein applied in 8M urea–Tris buffer at pH 7·4 and eluted with a gradient to 0·5M potassium chloride in this buffer. Gradient reached column at tube 20. Fraction size was approximately 1·1 ml.

Figure 3(*b*) shows the curve obtained by gradient elution of α-keratose. The pattern recalls the curve obtained by gradient elution of γ-globulin from calcium phosphate columns (Tiselius, Hjerten, and Levin 1956) and by liquid–liquid partition chromatography (Porter 1955). These authors showed that cuts from various positions in the main peak rechromatographed in their original positions and were not unduly spread; hence the wide spreading of the original peak may be indicative of a considerable number of closely related proteins (Porter 1955). We wished to show whether the spreading in the peak of α-keratose was due (at least in part) to chemically distinct proteins and, since Tiselius, Hjerten, and Levin (1956) concluded that stepwise elution gives better resolution than gradient elution, we concentrated on the stepwise procedure; this also gives more concentrated fractions.

One stepwise curve is shown in Figure 3(*a*); the number of peaks obtained is arbitrary and if the increments in ionic strength were made smaller more peaks would almost certainly be obtained.

Figure 4 shows a three-step elution pattern of α-keratose and also the patterns obtained by rechromatography (after dialysis and concentration of each of the three peaks).

(*b*) *Interpretation of Chromatographic Data*

In the stepwise or gradient elution of proteins the interpretation of the effluent curve is not simple (cf. Boardman 1959). Apart from a few relatively small proteins (see Moore and Stein 1956) it is generally difficult to elute a protein from a column of adsorbent without considerable tailing unless the R_F is close to 1. At lower R_F's (for a tailing zone, i.e. a substance which has a strongly curved adsorption isotherm) each change in the eluent concentration (including anomalous changes or double-fronting (Björk 1959)) can give rise to a "false" peak which may not be

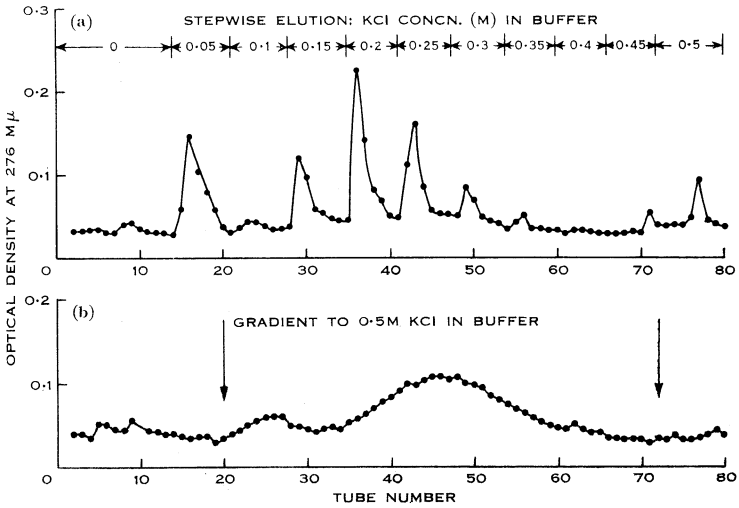

Fig. 3.—Chromatographic behaviour at 25°C of α-keratose on DEAE-cellulose in 8M urea–Tris buffer at pH 7·4. Fraction size was approximately 1·1 ml. (*a*) Stepwise elution with increasing concentrations of potassium chloride in the buffer; (*b*) gradient elution to 0·5M potassium chloride in the buffer.

indicative of chemically differing components. It might be expected that gradient elution would be preferable to stepwise elution in such cases but Tiselius, Hjerten, and Levin (1956) have pointed out that in this case the resolution into separate peaks is never as good as in stepwise elution.

With stepwise elution heterogeneity may be wholly or partly masked since several discrete chemical components may be eluted as a single peak particularly if the increments in ionic strength of the eluent are large.

Despite the difficulties of interpretation gradient or stepwise elution can demonstrate heterogeneity present in a solution of protein. Before claiming definite separation of components, however, each separated fraction should be rechromatographed to show that it runs in its original position, i.e. its behaviour when separated

is the same as when in the mixture. Moreover, the characterization by some specific property (e.g. biological activity or amino acid composition) of the material in the various peaks makes certain whether or not a fractionation has occurred (Moore and Stein 1956; Tiselius, Hjerten, and Levin 1956).

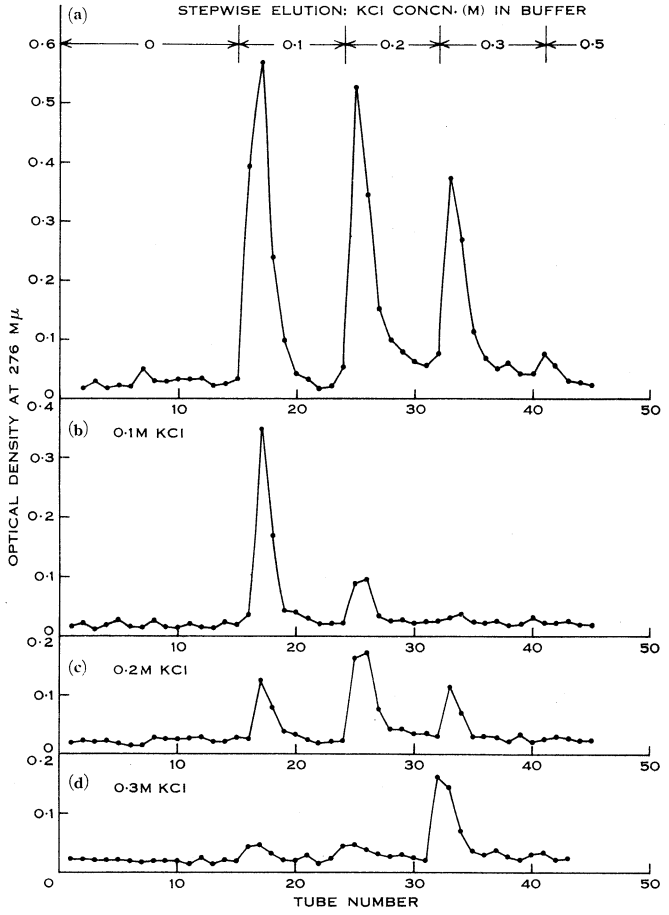

Fig. 4.—Chromatographic behaviour at 25°C of α-keratose on DEAE-cellulose in stepwise elution (*a*) and rechromatography on the same column of fractions obtained (*b*)–(*d*). Protein applied in 8M urea–Tris buffer at pH 7·4 and eluted with 0, 0·1, 0·2, 0·3, and 0·5M potassium chloride in this buffer. Fraction size was approximately 1·1 ml.

Although each cut of our α-keratose patterns (Fig. 4) is not pure, since chromatography by one-step elution is an inefficient process, it is seen there is a considerable enrichment of each component in the rechromatography and this alone points to the fact that a real separation of differing components has been achieved (cf. Boman and Westlund 1956).

(c) *Chemical Characterization of Fractions from α-Keratose*

Ultracentrifugal studies on α-keratose have not yielded any definite evidence on heterogeneity. This is due to the complication introduced by aggregation of the protein (O'Donnell and Woods 1956*a*, 1956*b*). Similarly aggregation makes it impossible to interpret the electrophoretic patterns or to state definitely that electrophoretic heterogeneity is present (O'Donnell and Woods 1956*a*). The electrophoretic pattern is predominantly a single peak. End-group analyses on α-keratose

TABLE 1

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

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

Amino Acid	0·1M KCl Fraction	0·2M KCl Fraction	0·5M KCl Fraction
Cysteic acid	4·70	4·80	6·19
Lysine	6·26	5·53	4·56
Histidine	1·84	1·70	1·94
Arginine	22·8	21·4	20·2
Ammonia	9·45	10·7	10·2
Tryptophan plus oxidized tryptophan*	1·18	1·0	1·55

* These values are not g amino acid nitrogen per 100 g protein nitrogen but are the ratio of absorbances per unit of nitrogen in the hydrolysate, with the 0·2M KCl fraction as standard for the peak eluted between the phenylalanine plus tyrosine and the lysine peak. This is the normal position of tryptophan (Moore, Spackman, and Stein 1958). Our peak had more than one component and no molar absorbance factor could be assumed.

fractions reveal the presence of small amounts of a variety of end-groups, also present in wool, and point to the fact that there are chemically different components in α-keratose (Alexander and Smith 1956) even if it is assumed that masked terminal groups or cyclic molecules are predominant (Thompson 1959).

In order to determine whether the components comprising the elution curve of α-keratose differed radically in their composition or whether only minor differences such as amide content were responsible for the separation an arbitrary fractionation (again by three-step elution) with salt concentrations of 0·1M, 0·2M, and 0·5M potassium chloride was carried out. These fractions were approximately each one-third of the protein put on the column. Each peak was further purified by rechromatography and the isolated fractions were analysed for cysteic acid and the basic amino acids lysine, histidine, and arginine. The hydrolysates of the proteins were prepared simultaneously so that losses during hydrolysis should be identical. The results are shown in Table 1. It is seen that there are large differences in amino

acid composition between the peaks eluted with buffer containing 0·1M and 0·5M potassium chloride, particularly in cysteic acid and lysine. The differences between the peaks eluted with buffer containing 0·1M and 0·2M salt are very much less pronounced. The differences in ammonia values between the 0·1M and 0·2M potassium chloride peaks may be real but no definite conclusions can be drawn due to the possibility that all of the urea has not been removed from one of the fractions.

The ultraviolet absorption curves of the three hydrolysates (of equal protein concentration) in 2N hydrochloric acid are shown in Figure 5; it is seen that the curves for the 0·1M and 0·2M potassium chloride peaks are similar but there is a pronounced difference in the height of the peaks at 270–280 mμ between those for 0·1 or 0·2M and 0·5M potassium chloride.

TABLE 2

NINHYDRIN COLOUR ABSORBANCE VALUES

Ninhydrin colour developed by procedure given in text and measured in matched tubes in a Coleman Junior spectrophotometer at 570 mμ (Moore and Stein 1948). Values given are absorbances per μmole amino acid

Amino Acid	NH_4Cl	Sample 1*	Sample 2†	Sample 3‡
Ammonia	3·62, 3·82 } 3·72			3·68, 4·08 } 3·88
Arginine		3·94	3·86	4·00, 3·90 } 3·95
Histidine		4·16	3·59	3·88, 3·98 } 3·93
Lysine		4·25	4·34	4·32, 4·36 } 4·34

* From California Corporation for Biochemical Research, Los Angeles, California.

† From Nutritional Biochemicals Corporation, Cleveland, Ohio.

‡ Beckman–Spinco standard mixture of amino acids after separation on "Dowex-50".

For the concentrations of amino acids being examined here (except histidine) Moore and Stein (1948, 1954) and Moore, Spackman, and Stein (1958) find that recoveries from standard mixtures of amino acids are 100±3% of theory so that a difference of 6% between duplicate runs is possible. Duplicate runs on a standard Spinco mixture of amino acids showed the variation for lysine, histidine, and arginine to be less than 3% (see Table 2). Hence there are probably real differences in amino acid content between the peaks eluted with buffer containing 0·1M and 0·2M potassium chloride. If the spread pattern obtained by gradient elution is due to a considerable number of closely related proteins it might be expected that the differences between some successive fractions would be quite small and may not involve the amino acids determined here.

It is possible that amide differences alone contribute to the spreading during gradient elution of the original α-keratose. Furthermore it seems probable, in view of the oxidized insulin pattern, that even in 8M urea differences in states of aggregation or molecular shape or both could contribute to the spread.

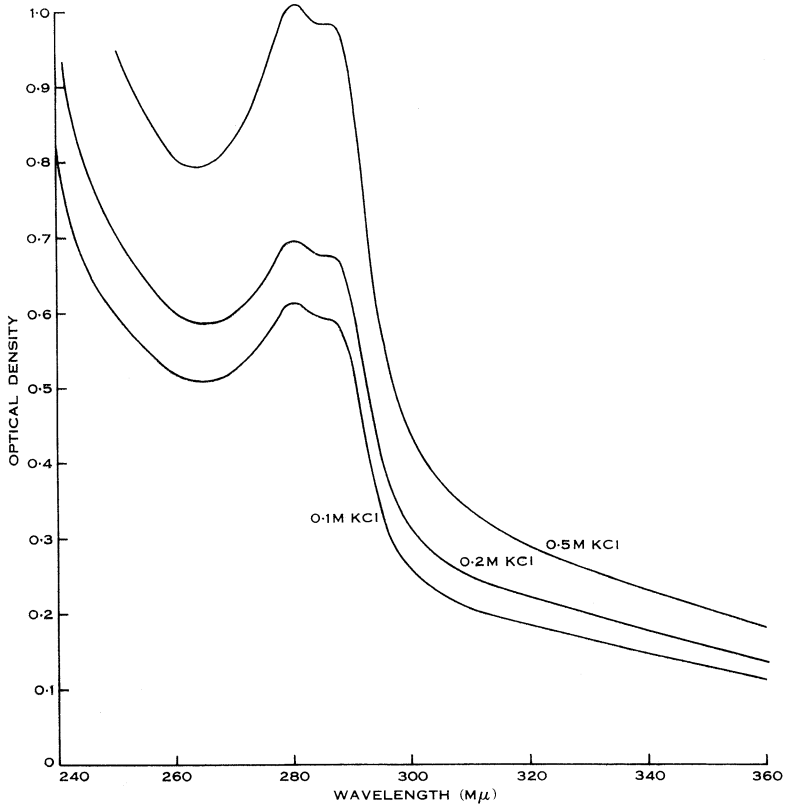

Fig. 5.—Ultraviolet absorption spectra of acid hydrolysate of the three fractions of α-keratose. Concentration was approximately 0·8 mg per ml in 2N HCl.

(*d*) *γ-Keratose*

γ-keratose, the high-sulphur, low molecular weight fraction from oxidized wool, when examined by gradient elution gave a similar type of pattern to α-keratose with maxima at approximately the same elution volumes (see Fig. 6(*b*)). It could also be divided into several peaks by stepwise elution (Fig. 6(*a*)). Since α- and γ-keratose differ markedly in amino acid composition (Gillespie *et al.* 1960) the increased acidity of γ-keratose which would tend to increase the elution volume over that exhibited by α-keratose must be counterbalanced by other factors such as smaller molecular size and shape. It should be noted that preparations of γ-keratose are electrophoretically heterogeneous (Woods, unpublished data) in agree-

ment with the heterogeneity of the high-sulphur protein from reduced and alkylated wool (Gillespie 1959).

(e) *Is there a Major Homogeneous Protein in Wool?*

The question arises as to the whereabouts of the repeat unit in wool which is responsible for the striking regularity in the X-ray diffraction pattern. If it is to come from a major homogeneous protein component then the present work yields no positive evidence that such a component exists; furthermore, end-group analysis on intact wool (Middlebrook 1951) and extracted wool proteins (Alexander and

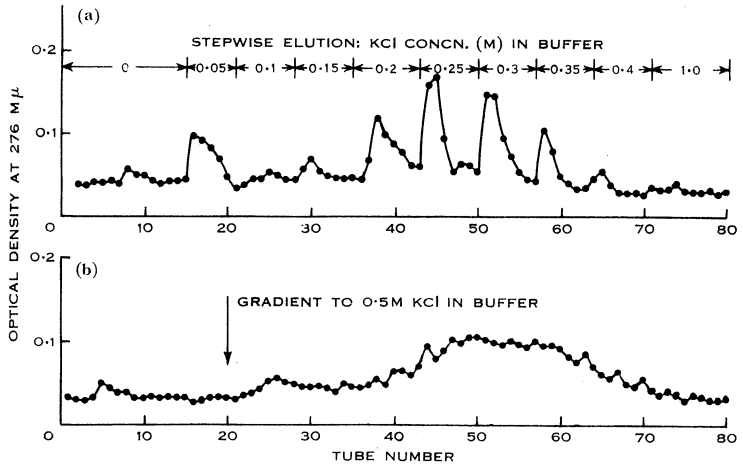

Fig. 6.—Chromatographic behaviour at 25°C of γ-keratose on DEAE-cellulose in 8M urea–Tris buffer at pH 7·4. Fraction size was approximately 1·1 ml. (*a*) Stepwise elution with increasing concentrations of potassium chloride in the buffer; (*b*) gradient elution to 0·5M potassium chloride in the buffer.

Smith 1956; Thompson 1957) reveal a large variety of terminal amino acids each in small amount. One possible interpretation of these facts is that there exists in wool a family (or families) of closely related proteins with different terminal residues. This may be the case even if masked terminal groups or cyclic structures are present and the detected terminal amino acids represent impurities. The possibility of complications arising from the degradative procedures used in the isolation of soluble proteins from wool must always be kept in mind. Oxidation with performic acid is known to modify tryptophan but this does not occur when preparing the *S*-sulphokerateines (Swan 1959); yet the gradient elution diagrams of proteins prepared by these two methods* are similar (the elution of non-acid-precipitable (pH 4) *S*-sulphokerateine required a much steeper gradient than did γ-keratose to elute it in a similar volume).

* The acid-precipitable and non-acid-precipitable *S*-sulphokerateines were prepared in a similar manner to the α- and γ-keratoses from the extract of wool using 8M urea, 0·1M sodium sulphite, and 0·05M sodium tetrathionate (cf. Bailey and Cole 1959). 50% of the wool dissolved in 48–72 hr at room temperature.

An alternative viewpoint could arise from the postulation of multiple-stranded cables of α-helixes (Crick 1952; Pauling and Corey 1953) in the crystalline regions of keratin. The repeat unit could then be visualized as consisting of several distinct protein components interwoven with each other, the percentage of any "pure" component being small.

Because of the difficulties in interpreting the elution patterns during stepwise or gradient elution it is not possible to conclude how many chemically different components there may be in α-keratose, but we feel the stepwise elution pattern (Fig. 3(*a*)) suggests there may well be a considerable number of such components even though some or all of these could be derived from a parent protein.

(*f*) *Heterogeneity of Wool Proteins*

The yields and properties of extracted wool proteins isolated by various methods have recently been summarized (Gillespie *et al.* 1960). It would be expected that α-keratose, which can amount to 53–60% of the wool fibre, would be heterogeneous, in line with the multicomponent nature of wool proteins extracted by reduction and alkylation; here the major electrophoretic component is estimated to be 41% of the wool (Gillespie and Lennox 1955) and possibly consists of two components at least (Gillespie 1960). Thus the large difference in components eluted with 0·1 or 0·2M and 0·5M KCl probably correspond to macroheterogeneity. However, the differences between components eluted with 0·1 and 0·2M KCl according to the properties measured here are very slight and fractionation by smaller increments of salt concentration (e.g. Fig. 3(*a*)) would give fractions which might be difficult to distinguish by even analysis for all of the amino acids. This chromatographic heterogeneity may thus be evidence of microheterogeneity in the extracted wool protein.

When proteins are isolated from tissues and are purified there are many cases where these purified proteins can be further separated chromatographically to give components of identical biological activity (see Colvin, Smith, and Cook 1954). Similar separations can often be achieved by zone electrophoresis and this suggests that the main difference is one of charge and possibly shape. Even with cautious extraction procedures, ribonuclease contains at least two components (Martin and Porter 1951; Hirs, Moore, and Stein 1953; Åquist and Anfinsen 1959), one of which is lacking the *N*-terminal lysine (King, Yphantis, and Craig 1960). Prolactin can be separated into several forms (Cole and Li 1959; Cole and Mendiola 1960). Carsten and Pierce (1960) likewise have demonstrated the presence of several components in beef thyrotropic hormone. More recently Press, Porter, and Cebra (1960) demonstrated the presence of at least 10 molecular forms in cathepsin D by chromatography on DEAE- and carboxymethylcellulose and starch-gel electrophoresis. These authors endeavoured to show that autolysis was not responsible for this complexity. There are many other less well-characterized examples (for summary see Markert and Møller 1959; Miller, Blum, and Hamilton 1960).

Thus the presence of many closely related components in wool would not be surprising particularly as the fibre must undergo many changes in passing from its site of synthesis to the keratinized fibre.

It is currently believed that the material responsible for the regular X-ray diagram of wool is part of the microfibrillar protein (see Fraser, MacRae, and Rogers 1959) and that this protein gives rise to α-keratose on oxidation and subsequent alkaline extraction of wool (see Alexander and Smith 1956; Birbeck and Mercer 1957; Rogers 1959). Proteins derived from this material might be expected to be less heterogeneous than those derived from the matrix, i.e. γ-keratose.

If there are regular repeating lengths of polypeptide chain in a family of closely related proteins such as we have envisaged for the crystalline region, a study of the peptides produced by limited degradation of α-keratose, e.g. by trypsin, and "fingerprinting" should reveal these similarities. Work along these lines is in progress.

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