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High-sulphur Proteins from α-Keratins
I. Heterogeneity of the Proteins from Mouse Hair

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Abstract

The heterogeneity of the reduced and *S*-carboxymethylated high-sulphur protein fraction from mouse hair has been examined by chromatography and polyacrylamide gel electrophoresis at pH values above and below the isoelectric region. Considerable heterogeneity is observed both in size (molecular weight range 12 000–45 000) and in charge. Amino acid analysis of a number of column chromatographic fractions shows the high-sulphur proteins to be largely composed of proteins with a carboxymethylcysteine content above 25 residues per 100 residues and a pronounced heterogeneity in arginine content. Their chromatographic behaviour is similar to that observed for the ultra-high-sulphur proteins from wool.

Introduction

Three major protein fractions, termed high-sulphur, low-sulphur, and high-tyrosine proteins, are extracted from mammalian keratins after reduction and carboxymethylation of the cystine residues. The high-sulphur protein fraction from wool, a fraction richer in sulphur than the original keratin, is not a material of constant composition as its *S*-carboxymethylcysteine [cysteine(Cm)] content varies directly with the sulphur content of the wool from which it is derived. Thus if the source is wool at the lower end of the range of sulphur content (less than 3%), the cysteine(Cm) content of the high-sulphur fraction is about 18 residues per 100 residues (residues %), but if sulphur-enriched wool produced by abomasal infusion of *S*-amino acids into sheep is examined, then a high-sulphur fraction of much higher cysteine(Cm) content is obtained (Gillespie *et al.* 1964; Gillespie and Reis 1966; Gillespie *et al.* 1969; Broad *et al.* 1970). It has been shown that the increase in cysteine(Cm) content is due to the presence of high-sulphur proteins which contain about 30 residues % of cysteine(Cm) and which can be resolved by moving-boundary electrophoresis at pH 10–11 (Gillespie and Reis 1966; Broad *et al.* 1970). Small amounts of these proteins have been prepared by a lengthy fractionation procedure (Lindley *et al.* 1968). Amino acid analysis shows that, apart from containing a high content of cysteine(Cm), they are rich in proline, threonine, and serine, have less glycine, alanine, and leucine than the other high-sulphur proteins, and are deficient in lysine, histidine, phenylalanine, and aspartic acid (Lindley *et al.* 1968). Little is known about this unusual class of proteins for a number of reasons. Adequate amounts are difficult to obtain because these proteins

account for only about 10% of the weight of wool even in the most highly sulphur-enriched samples, and they are also more difficult to extract than the normal high-sulphur proteins. Because there are only small differences in the properties of the two fractions of high-sulphur proteins, separation is a problem. Study of the ultra-high-sulphur proteins has been made more difficult by the need to use moving-boundary electrophoresis for characterization as no satisfactory zone electrophoretic technique has yet been found.

Proteins with a composition similar to the ultra-high-sulphur proteins of wool are found in many animal hairs (Gillespie and Inglis 1965; Gillespie and Broad 1972; Steinert and Rogers 1973), and they constitute about 30% of mouse hair from which they can be extracted in good yield (Gillespie and Inglis 1965). The present study examines the hetereogeneity of the high-sulphur proteins of mouse hair with the aim that any fractionation and analytical procedures developed for the mouse proteins might be ultimately applicable to the ultra-high-sulphur proteins of wool.

Materials and Methods

Preparation of High-sulphur Protein Fraction from Mouse Hair

Hair from laboratory strain mice was cleaned by immersion in several changes of petroleum ether, ethanol, and water, and was then air-dried. It was solubilized by alkaline reduction in the presence of urea as described previously (Gillespie and Reis 1966). After alkylation with iodoacetate and dialysis, zinc acetate was added to the solution to a concentration of 0·02M. Centrifugation removed the mixture of low-sulphur and high-tyrosine protein fractions from the supernatant high-sulphur proteins. After exhaustive dialysis, the high-sulphur proteins were freeze-dried. In some preparations, it was observed by polyacrylamide gel electrophoresis at pH 8·9 that this protein fraction was slightly contaminated by low-sulphur proteins, and in these cases the latter proteins were precipitated by standing overnight in 0·4M sodium acetate adjusted to pH 4·0 with acetic acid.

Chromatography

Ion-exchange chromatography was performed at room temperature on columns of DEAE-cellulose (Eastman–Kodak) or cellulose phosphate (Serva P23). Gel filtration chromatography was carried out on Sephadex G-100 (Pharmacia). Details of the procedures are indicated in the legend of Fig. 3. Before chromatography, the protein sample was dissolved in and dialysed against the buffer used to equilibrate the column. Poolings were made as described in the legend of Fig. 3, dialysed against deionized water, and lyophilized.

Moving-boundary Electrophoresis

Moving-boundary electrophoresis (Gillespie and Reis 1966) was carried out in the following 0·1 ionic strength buffers: sodium acetate–acetic acid, pH 4·5; diethylbarbituric acid–sodium hydroxide, pH 8·6; β-alanine–sodium hydroxide, pH 11·0. Protein concentrations were about 1·3%.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed with a Beckman model 113 acrylamide gel accessory for the microzone system. With this apparatus the gel slab is horizontal, and the experimental technique required the sample to be set within the polyacrylamide gel. It was observed occasionally that some of the sample remained at the loading position after electrophoresis, apparently trapped within the acrylamide matrix. This probelm was overcome by modifying the apparatus so that the gel slab was in the vertical position. Two electrode buffer reservoirs were attached to the acrylamide gel accessory, and then the electrode buffer solutions, instead of the wicks used in the original apparatus, make direct contact with the acrylamide gel. Each reservoir had a capacity of *c.* 150 ml. Approximate dimensions of the acrylamide gel slab were 9 by 7·5 by 0·4 cm. Eight samples were loaded simultaneously with the Beckman sample applicator. Usually 4·5 μl of a 1% protein

solution was loaded, but occasionally double loadings of more dilute solutions were used with no loss in resolution being observed. Unfractionated starting material was loaded on each gel as a marker.

At pH 8·9, 10% polyacrylamide gels were prepared by the procedure of Davis (1964) except that only the 'small pore' gel was used and the gels contained 8M urea. Proteins were dissolved in the tris–glycine electrode buffer containing 8M urea. Bromophenol blue was added as the tracking dye.

At pH 2·6, 10% polyacrylamide gels containing 4·8M acetic acid and 2·75M urea were prepared by the following procedure. Ten ml of a 0·02% (v/v) hydrogen peroxide solution was added to a degassed solution of 8 ml acetic acid, 8·5 ml acrylamide–bisacrylamide solution (35·3 g acrylamide, 1·29 g *N,N'*-methylenebisacrylamide per 100 ml), 0·06 ml of 0·4% (w/v) ferrous sulphate solution, 4·70 g urea, and 10 mg ascorbic acid. The gel polymerized in 5–10 min at 0°C. Iminodiacetic acid (3·33 g/500 ml) was the electrode buffer solution. Proteins were dissolved in 4·8M acetic acid–8M urea containing a small amount of crystal violet. Resolution in this acrylamide gel electrophoretic system is comparable with that using starch as the support medium at a similar pH (Darskus 1972) but the advantages of the acrylamide system are that it is easier to use, the background staining is minimal, and electrophoretic patterns are reproducible between runs.

For both acid and alkaline gel systems, electrophoresis was carried out at 250 V for 2 h. The gels were stained for 90 min with 0·2% (w/v) Coomassie Brilliant Blue G250 (I.C.I.) in water : methanol : acetic acid (45 : 45 : 10 v/v), and destained overnight in water : methanol : acetic acid (87 : 5 : 8 v/v).

Amino Acid Analysis

Protein samples (2–5 mg) were hydrolysed *in vacuo* at 108°C for 24 h with constant-boiling hydrochloric acid containing 0·5 mM thioglycollic acid. After freeze-drying, the hydrolysate was stored at −20°C until analysed with a Beckman–Spinco 120C amino acid analyser.

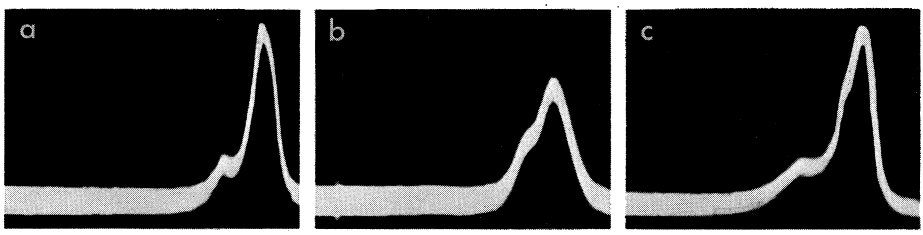

Fig. 1. Moving-boundary electrophoretic patterns (ascending boundary) of high-sulphur proteins from mouse hair at different pH values. (*a*) pH 4·5; (*b*) pH 8·6; (*c*) pH 11·0. Direction of movement from left to right.

Results

Electrophoresis

The ascending moving-boundary electrophoretograms of the mouse hair high-sulphur proteins at pH values of 4·5, 8·6, and 11·0 show limited heterogeneity (Fig. 1). Although only two components are apparent at pH 8·6, electrophoresis at pH 4·5 resolves three components. At pH 11·0 the minor peak plus the asymmetry of the major peak is also indicative that there is a minimum of three proteins.

Greatly increased resolution is obtained by electrophoresis in polyacrylamide gels (Fig. 2). At pH 8·9 three major and four minor bands are separated, while at pH 2·6 further resolution is obtained with one major and seven other bands of about equal intensity being visible. Although the number of bands obtained at these two pH values is similar, the variation in intensities suggests that the components being observed are quite different.

Chromatography on DEAE-cellulose

Fig. 3*a* shows the elution profile obtained with DEAE-cellulose chromatography. There are five distinct peaks compared with the three components observed in moving-boundary electrophoresis at the same pH.

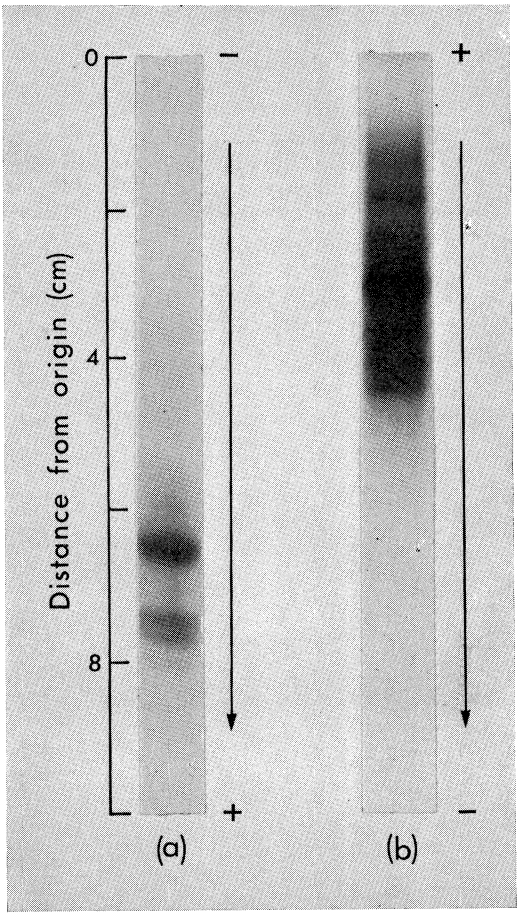

Fig. 2. Polyacrylamide gel electrophoretic patterns of high-sulphur proteins from mouse hair at different pH values. (*a*) pH 8·9; (*b*) pH 2·6.

Polyacrylamide gel electrophoresis at pH 2·6 and 8·9 was carried out on each of the pooled fractions, and the electrophoretic patterns of representative fractions (indicated in Fig. 3*a*) are given in Fig. 4. Amino acid compositions of these fractions are shown

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Fig. 3(*c*). Elution pattern of mouse hair high-sulphur proteins (300 mg) from Sephadex G-100. Column: 110 by 2·5 cm; 5-ml fractions; flow rate, *c.* 15 ml/h under gravity with an effective height of *c.* 60 cm; optical path length 5 mm. Buffer: 0·1M ammonium carbonate, 6M urea, 0·1M NaCl, pH 9. Column was calibrated with the following reduced and *S*-carboxymethylated proteins: ovalbumin, α-chymotrypsinogen A, β-lactoglobulin, lysozyme. Poolings of 25 ml were made across the profile, and examined by polyacrylamide gel electrophoresis at pH 2·6. Electrophoretic patterns and amino acid compositions of fractions I–IV are given in Fig. 6 and Table 3.

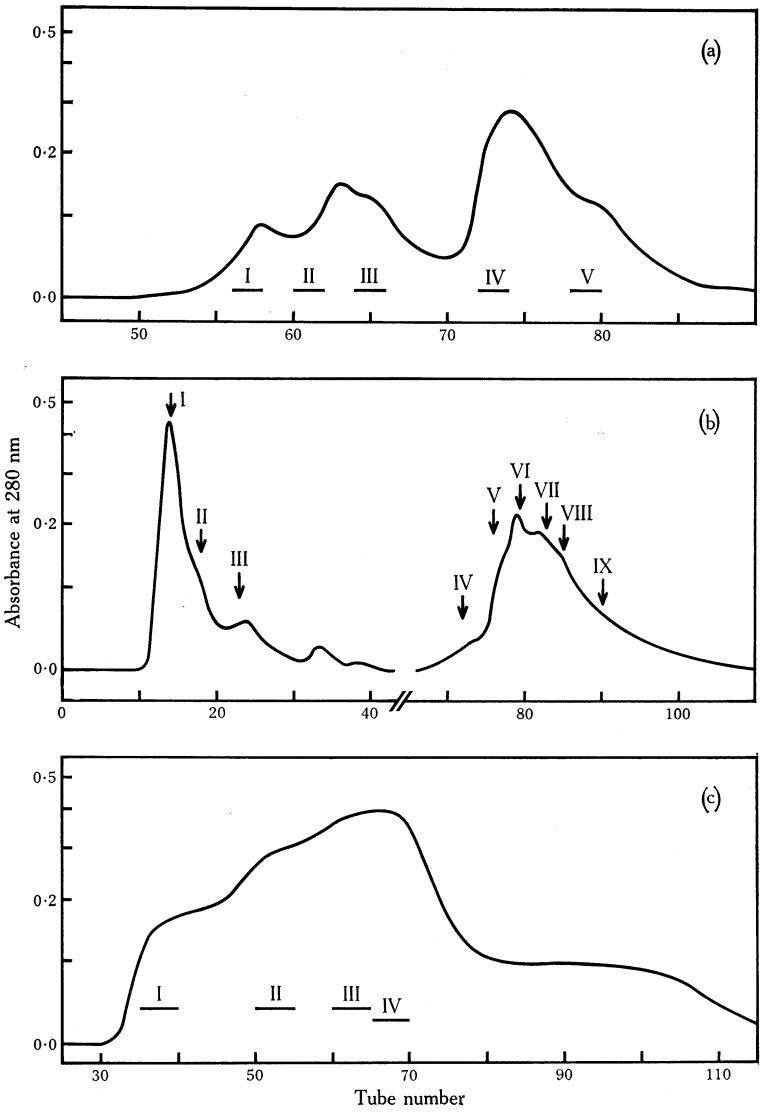

Fig. 3(*a*). Elution pattern of mouse hair high-sulphur proteins (300 mg) from DEAE-cellulose. Column: 70 by 2·0 cm; 10-ml fractions; flow rate, *c.* 10 ml/h; optical path length 5 mm. Starting buffer: 0·05M sodium acetate, adjusted to pH 4·5 with acetic acid; intermediate buffer: 0·05M sodium acetate–acetic acid (pH 4·5), 0·4M NaCl; limiting buffer; 0·05M sodium acetate–acetic acid (pH 4·5), 0·8M NaCl. No protein was eluted by 480 ml of starting buffer. Linear gradient elution with 240 ml each of intermediate and limiting buffer was commenced at tube 48. Poolings of 20 ml were made across the profile, and examined by polyacrylamide gel electrophoresis at pH 2·6 and 8·9. Electrophoretic patterns and amino acid compositions of fractions I–V are given in Fig. 4 and Table 1.

Fig. 3(*b*). Elution pattern of mouse hair high-sulphur proteins (200 mg) from cellulose phosphate. Column: 67 by 2·0 cm; 10-ml fractions; flow rate, *c.* 10 ml/h; optical path length 5 mm. Starting buffer: 0·2M citric acid, 5M urea (pH 2·6); limiting buffer: 0·2M citric acid, 5M urea, 0·2M NaCl. Elution was carried out with *c.* 500 ml of starting buffer, then linear gradient elution with 240 ml each of starting and limiting buffer was commenced at tube 50. Contents of individual tubes were examined by polyacrylamide gel electrophoresis at pH 2·6, and electrophoretic patterns of representative fractions I–IX are given in Fig. 5. Amino acid compositions of fractions IV–VIII are reported in Table 2.

in Table 1. It is of interest to note that there is a general trend towards progressive and significant changes in many amino acid residues in passing from fraction I to fraction V. There are large decreases (greater than 2 : 1) in lysine, histidine, aspartic acid, alanine, and leucine, with smaller decreases in threonine, proline, valine, and isoleucine. These decreases are balanced by increases in cysteine(Cm), serine, and glutamic acid.

Table 1. Amino acid compositions (as residues %) of fractions obtained by chromatography on DEAE-cellulose (Fig. 3*a*)

Amino acid	Fraction I	II	III	IV	V	Amino acid	Fraction I	II	III	IV	V
Lys	0·89	0·64	0·31	0·40	0·39	Gly	4·97	4·67	5·35	4·61	5·48
His	1·97	1·64	0·98	0·46	0·22	Ala	3·20	3·03	3·00	2·19	1·49
Arg	4·37	5·11	6·71	6·95	5·41	Val	6·33	6·47	5·66	4·61	3·75
Cys(Cm)	21·20	23·80	28·00	33·10	34·90	Met	0·38	0·15	0·00	0·20	0·07
Asp	4·84	3·75	2·59	0·86	1·04	Ile	2·90	2·90	2·87	1·95	1·21
Thr	9·78	9·41	9·01	6·65	6·72	Leu	5·25	4·27	2·22	1·65	1·49
Ser	8·36	8·57	8·60	13·80	13·70	Tyr	1·50	1·19	0·70	0·67	0·91
Glu	7·03	7·32	7·66	7·82	9·62	Phe	1·23	0·82	0·76	0·59	1·10
Pro	15·80	16·20	15·80	13·50	12·60						

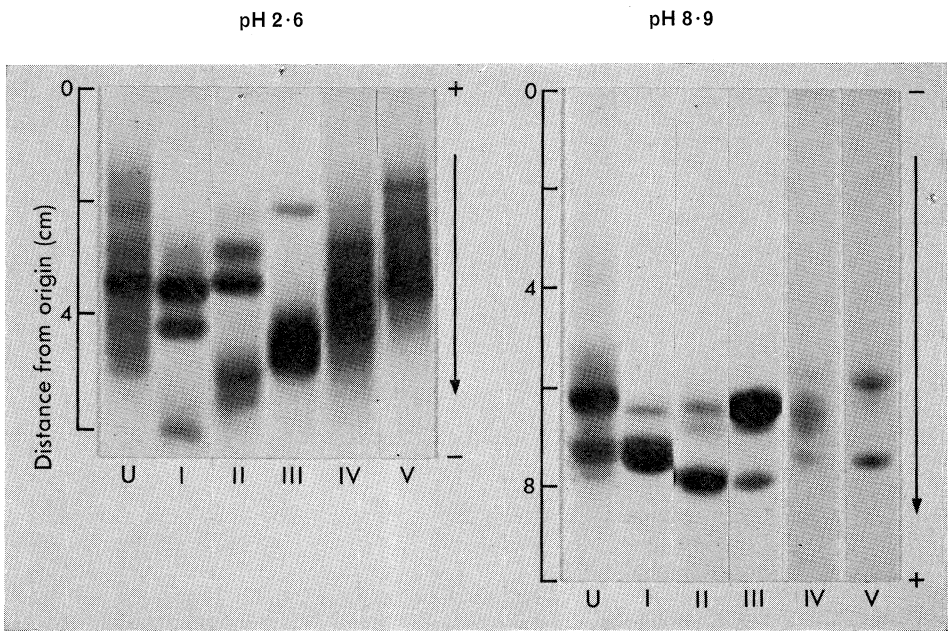

Fig. 4. Polyacrylamide gel electrophoretic patterns (at different pH values) of representative fractions obtained by chromatography on DEAE-cellulose (Fig. 3*a*). Unfractionated starting material is designated as U.

On comparing the patterns for individual poolings at the two pH values, better resolution is obtained at the lower pH; for example, fraction II at pH 8·9 has one major broad band and two minor bands, while at pH 2·6 there are three major resolved bands and two minor ones. The observation that better eletrophoretic resolution for

keratin high-sulphur proteins is obtained at pH 2·6 has been reported by Darskus (1972) for high-sulphur proteins from wool.

Chromatography on Cellulose Phosphate

Chromatography of the high-sulphur proteins on cellulose phosphate at pH 2·6 gave the elution profile shown in Fig. 3*b*. Approximately 36% (by weight) of the protein was not bound to the resin and was collected in tubes 10–40; the remainder of the protein was eluted during the application of a salt gradient.

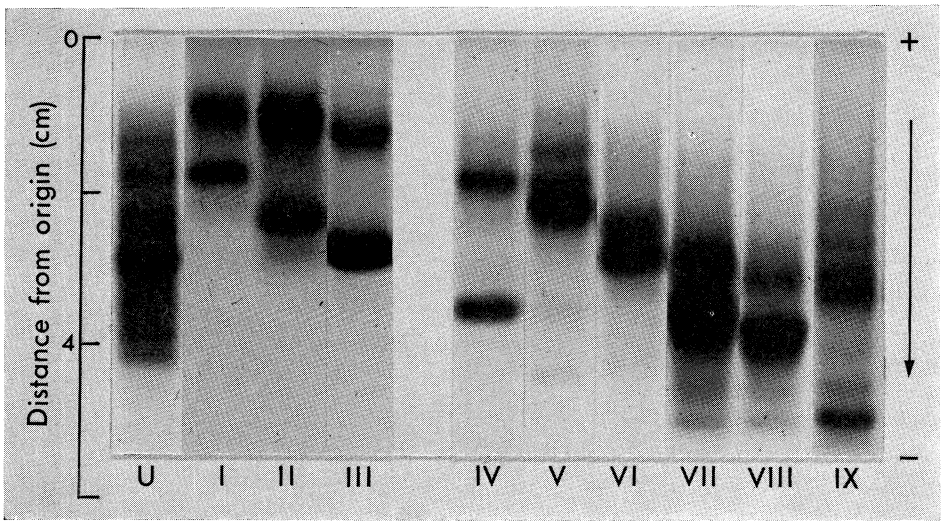

Fig. 5. Polyacrylamide gel electrophoretic patterns at pH 2·6 of representative fractions obtained by chromatography on cellulose phosphate (Fig. 3*b*). Unfractionated starting material is designated as U.

Table 2. Amino acid compositions (as residues %) of fractions obtained by chromatography on cellulose phosphate (Fig. 3*b*)

Amino acid	Fraction IV	V	VI	VII	VIII	Amino acid	Fraction IV	V	VI	VII	VIII
Lys	0·40	0·30	0·21	0·14	0·06	Gly	6·91	9·15	10·70	5·83	4·80
His	0·90	1·05	1·42	0·49	0·71	Ala	2·50	3·31	2·93	1·77	2·60
Arg	4·91	4·95	5·85	8·05	8·88	Val	4·11	3·99	4·38	4·64	5·11
Cys(Cm)	28·80	27·80	28·10	32·90	30·30	Met	0·00	0·04	0·05	0·08	0·03
Asp	2·05	1·14	1·07	1·07	1·93	Ile	1·35	1·24	2·03	1·88	2·85
Thr	8·13	7·27	6·65	6·71	8·79	Leu	2·41	1·81	1·64	1·00	0·91
Ser	12·80	13·00	11·20	12·20	9·35	Tyr	1·19	1·37	1·94	1·03	0·31
Glu	9·93	10·00	8·40	7·84	7·29	Phe	1·33	1·71	2·26	0·81	0·73
Pro	12·30	12·00	11·10	13·50	15·30						

The contents of each tube was examined by acrylamide gel electrophoresis at pH 2·6, and representative patterns, corresponding to fractions designated I–IX in Fig. 3*b*, are given in Fig. 5. At least 20 well-defined components have been resolved. Examination of the electrophoretic patterns for each of the tubes 79–85 (fractions VI–VIII) shows that each component of the doublet forms an apparent continuum of mobilities

indicating extreme heterogeneity and thus making an exact count of the number of discrete components impossible. A similar apparent continuum of mobilities is observed for the slower band of the doublet of the unbound fractions (I–III), and this can be compared with the faster band which shows discrete mobility changes.

Amino acid compositions of acid hydrolysates of fractions IV–VIII are given in Table 2. As expected from the pH of the column chromatographic separation, the content of basic residues (dominated by arginine) increases from fraction IV to fraction VIII. The cysteine(Cm) content of each fraction is near 30 residues %, thus placing the fractions within the ultra-high-sulphur protein group. Proline and serine are the other major amino acid constituents, each having a proportion of about one-half to one-third that of cysteine(Cm). The content of glycine is variable from about 5 to over 10 residues %, while aspartic acid, tyrosine, and phenylalanine are reasonably constant and of low amount. The compositions of the individual fractions are similar to those reported by Lindley *et al.* (1971) for the chromatographically separated ultra-high-sulphur protein components from wool.

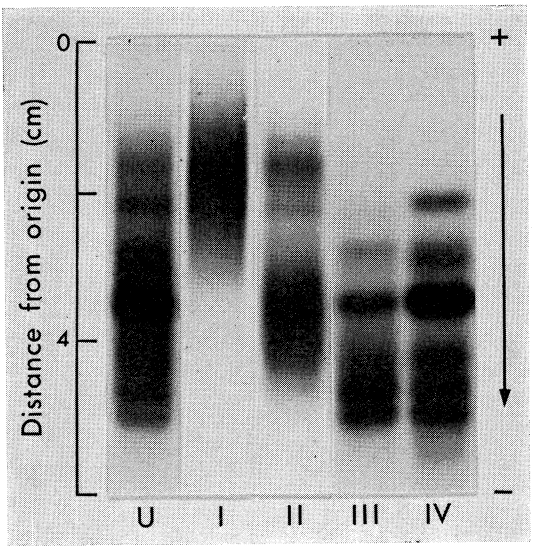

Fig. 6. Polyacrylamide gel electrophoretic patterns at pH 2·6 of representative fractions obtained by chromatography on Sephadex G-100 (Fig. 3*c*). Unfractionated starting material is designated as U.

Heterogeneity of Size

The elution profile from Sephadex G-100 gel filtration chromatography is shown in Fig. 3*c*. It can be seen that the proteins show gross heterogeneity of size with a continuous distribution of molecular weights from 45 000 at tube 38 to 12 000 at tube 68. The major portion of the protein is in the low-molecular-weight end of the spectrum. Thirteen poolings were made and each was examined by polyacrylamide gel electrophoresis at pH 2·6. Four representative patterns (corresponding to fractions I–IV in Fig. 3*c*) are given in Fig. 6. No material stained by Coomassie Blue was observed in the pooled fractions obtained from tubes 83–110. The molecular weights of fractions I and II correspond to approximately 45 000 and 19 500 respectively. Over the tubes from 30 to 55 there is a progressive increase in the electrophoretic mobility of the protein band which is broad and diffuse indicating extreme heterogeneity. Fraction II also shows distinct slower bands, the major one corresponding in mobility to

the slowest component of the unfractionated material. The other major bands of the mouse high-sulphur proteins are observed in fractions III and IV (molecular weights *c.* 14 500 and 12 000 respectively). The diffuseness of the gel patterns for fractions I and II was not present in fractions III and IV.

Amino acid compositions of acid hydrolysates of fractions I–IV are given in Table 3. Only minor variations between fractions are observed. Cysteine(Cm) and serine contents appear to decrease slightly with decreasing molecular size while the amounts of histidine, aspartic acid, threonine, and phenylalanine show the reverse trend.

Table 3. Amino acid compositions (as residues %) of fractions obtained by chromatography on Sephadex G-100 (Fig. 3*c*)

Amino acid	Fraction I	II	III	IV	Amino acid	Fraction I	II	III	IV
Lys	0·77	0·44	0·33	0·50	Gly	4·70	4·52	6·34	6·89
His	0·27	0·29	0·87	1·41	Ala	1·89	1·50	2·81	2·98
Arg	6·40	6·57	6·96	5·65	Val	4·62	3·87	4·97	5·58
Cys(Cm)	33·00	34·50	30·00	26·10	Met	0·13	0·15	0·09	0·08
Asp	0·93	0·97	2·07	2·77	Ile	1·72	1·55	2·29	2·41
Thr	6·07	6·24	7·66	8·46	Leu	1·70	1·48	1·77	3·12
Ser	15·00	14·50	10·20	9·91	Tyr	1·08	0·99	1·06	1·40
Glu	8·25	8·67	8·03	7·64	Phe	0·64	0·80	1·25	1·32
Pro	12·80	13·00	13·70	13·80					

Discussion

Gillespie and Inglis (1965) reported the moving-boundary electrophoretograms of the high-sulphur proteins from a number of α-keratins, and showed that wool had the most components and guinea pig the least. The mouse hair high-sulphur protein fraction has three components in the moving-boundary pattern which is similar in number to that obtained from guinea pig hair. However, by a combination of ion exchange column chromatography and polyacrylamide gel electrophoresis at pH values above and below the isoelectric region (about pH 3), many more components are evident in the mouse hair fraction, the exact number being difficult to estimate because in some regions there appears to be a continuous distribution of components differing in mobility. The complexity of the high-sulphur proteins is seen more readily in the polyacrylamide gel electrophoretic patterns at pH 2·6 than at pH 8·9. The high-sulphur proteins from wool show similar behaviour, giving better resolution below the isoelectric region (Darskus 1972).

The present study shows the value of using multiple criteria in examining the heterogeneity of a complex mixture of proteins. By each of the techniques, the mouse hair high-sulphur protein fraction has been shown to be heterogeneous, and in each case it is almost certain that the set of resolved components is unique to that procedure. For the high-sulphur proteins from guinea pig hair, limited heterogeneity has been observed (Steinert and Rogers 1973). Three relatively homogeneous fractions and a minor fraction containing about three components (as judged by polyacrylamide gel electrophoresis at pH 8·9) were isolated after chromatography on DEAE-cellulose at pH 4·5. It is possible that evidence for further heterogeneity could be obtained if the proteins were characterized by chromatography and electrophoresis below pH 3.

Cellulose phosphate chromatography of mouse hair high-sulphur proteins is qualitatively similar to that observed for the ultra-high-sulphur protein fraction prepared from wool (Lindley *et al.* 1971). There is unbound and bound material, the latter being eluted with an ionic strength gradient. Amino acid compositions of acid hydrolysates of pooled fractions of eluted 'bound-material' from both sources are similar; cysteine(Cm) content is above 25 residues %, arginine content varies from 5 to 10 residues %, whilst lysine, histidine, aspartic acid, and phenylalanine contents are low. The inability to observe by zone electrophoresis the presence of ultra-high-sulphur proteins in wool high-sulphur protein mixtures known to contain them, has always been puzzling. If the ultra-high-sulphur proteins are like the high-sulphur proteins from mouse hair, as the present study suggests, the poor resolution could be due to the presence of a continuous distribution of components differing in charge and size. This would give a smeared pattern resulting merely in an increase in the intensity of the background. A good example of this smearing can be seen in Fig. 6 trace I. If the electrophoretic support medium is starch (Darskus 1972), compared with polyacrylamide gel used in the present study, the background intensity is high and this smearing would be overlooked.

Acknowledgments

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