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High-sulphur Proteins from α-Keratins II.* Isolation and Partial Characterization of Purified Components from Mouse Hair

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Abstract

The present paper continues the study of the reduced and *S*-carboxymethylated high-sulphur proteins from mouse hair. Fractions have been obtained in a substantially purified form by fractional precipitation with ammonium sulphate at pH 6, followed by ion exchange chromatography on cellulose phosphate at pH 2·6.

Approximately 80% by weight of the high-sulphur proteins fall into the ultra-high-sulphur category (carboxymethylcysteine content greater than 26 residues per 100 residues), and they cover a molecular weight range of 17 000–28 000. The components show a remarkable diversity in amino acid composition; for example the contents of arginine and glycine each vary by about 3 : 1. The remainder of the proteins contain 17–20 residues per 100 residues of carboxymethylcysteine, are smaller in size (molecular weight 11 500), and also show great diversity in overall amino acid composition.

Molecular weights were determined by chromatography on controlled-pore glass and confirmed by gel filtration on Sephadex G-100 and Sepharose 6B. A comparison of the results suggests that the values obtained should be reliable to within 10%.

Introduction

Our studies on the high-sulphur proteins from mouse hair are primarily intended to develop methods for handling and studying the apparently similar proteins (termed ultra-high-sulphur proteins) from wool which a sheep produces when its diet is supplemented with sulphur-containing amino acids. The previous paper (Marshall and Gillespie 1976) describes the preparation of the proteins from mouse hair, and procedures for their fractionation and assessment of extent of heterogeneity.

In the present study, components have been isolated in a substantially purified form. An examination of the composition and molecular sizes of these components has thrown light on the nature of the heterogeneity of the proteins.

Materials and Methods

Ammonium Sulphate Fractionation of High-sulphur Proteins from Mouse Hair

Reduced and alkylated high-sulphur proteins from mouse hair were prepared as described previously (Marshall and Gillespie 1976). To a 1% solution of *S*-carboxymethylated high-sulphur proteins in 0·1M sodium acetate adjusted to pH 6 with acetic acid, was added ammonium sulphate to a final concentration of 1·6M (Gillespie 1962). After stirring for 2 h at room temperature, the precipitated proteins were recovered by centrifugation. The supernatant and precipitated proteins (dissolved in 8M urea) were then dialysed against deionized water, and freeze-dried.

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Measurement of Molecular Weight

Estimates of the molecular weights of the isolated components were made by controlled-pore glass (25·5 nm) chromatography in a column of size 175 by 0·9 cm as described by Frenkel and Blagrove (1975). The buffer contained 6M urea, 0·5% sodium dodecyl sulphate (SDS), and 0·05M phosphate, pH 7. The molecular weight protein standards (reduced and *S*-carboxymethylated) used for the determination of the calibration curve were cytochrome C, apomyoglobin, α-chymotrypsinogen A, rabbit tropomyosin, and ovalbumin.

Because of the unusual composition of the mouse hair high-sulphur proteins, molecular weights of some of the components were checked by other techniques *viz.* chromatography on Sepharose 6B and thin-layer gel filtration (Pharmacia TLG-apparatus). In the latter, the experimental procedure was essentially as described by Pharmacia (1972). A 20 by 20 cm glass plate was spread with a 0·6-mm layer of Sephadex G-100 Superfine (Pharmacia) swollen in the development buffer (0·1M ammonium carbonate, 0·1M NaCl, 6M urea, 0·1% mercaptoethanol). After eluting at an angle of 15° for 6·5 h, a paper print was taken, stained and destained using the procedure for polyacrylamide gels described previously (Marshall and Gillespie 1976). Lysozyme, apomyoglobin, and α-chymotrypsinogen A were used as molecular weight standards.

Chromatography on Sepharose 6B (Pharmacia) was carried out in a column of size 165 by 0·9 cm (Fish *et al.* 1969). The phosphate buffer (0·05M, pH 7) contained 6M guanidine hydrochloride (Schwarz–Mann ultra-Pure grade). The calibration curve was determined using the following reduced and *S*-carboxymethylated proteins: apomyoglobin, α-chymotrypsinogen A, and ovalbumin.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out by the method described by Weber and Osborn (1969). The gels contained 12·5% acrylamide, 0·1% SDS, 0·05M phosphate pH 7.

Other Methods

Chromatography on cellulose phosphate, polyacrylamide gel electrophoresis, and amino acid analysis were carried out as described previously (Marshall and Gillespie 1976).

Table 1. Amino acid compositions (as residues %) of mouse hair unfractionated high-sulphur proteins and the ammonium sulphate supernatant and precipitate fractions

Percentage differences between supernatant (S) and precipitate (P) fractions of magnitude greater than 10% are indicated and are calculated as percentages of precipitate fractions

Amino acid	Unfract-ionated	S fraction	P fraction	Difference (%)	Amino acid	Unfract-ionated	S fraction	P fraction	Difference (%)
Lys	0·53	0·45	0·63		Gly	6·53	6·09	8·20	−26
His	0·68	0·43	1·15	−63	Ala	2·19	2·02	2·86	−29
Arg	6·43	6·88	5·53	24	Val	4·33	3·94	5·07	−22
Cys(Cm)	30·50	33·10	24·70	34	Met	0·10	0·14	0·14	
Asp	1·87	1·23	2·79	−56	Ile	2·01	1·61	2·27	−29
Thr	7·03	7·09	7·87	−10	Leu	2·29	1·34	3·17	−58
Ser	12·40	12·80	10·30	24	Tyr	1·55	0·90	2·72	−67
Glu	8·10	8·21	7·66		Phe	1·15	0·82	1·55	−47
Pro	12·40	13·00	13·30						

Results

Characterization of Ammonium Sulphate Fractions

Following fractional precipitation with ammonium sulphate of the mouse hair high-sulphur proteins, approximately equal quantities of proteins were distributed between the supernatant and the precipitate. Amino acid compositions of acid hydrolysates of these two fractions are given in Table 1, and for comparison the composition of the unfractionated material is also reported. It can be seen that the two ammonium sulphate fractions differ greatly in their content of 13 of the amino

acid residues. These differences are significant for the amino acids in high proportion; for *S*-carboxymethylcysteine [cysteine(Cm)] percentage difference is 34%, serine 24%, and glycine 26%. The precipitate fraction with 24 residues per 100 residues (residues %) of cysteine(Cm) is similar to the high-sulphur proteins from guinea pig hair (group-4 protein of Steinert and Rogers 1973) and the SCMK-B1 fraction from wool (Gillespie 1962), while the supernatant proteins from mouse hair with 33 residues % of cysteine (Cm) are apparently similar to the ultra-high-sulphur proteins found in the ammonium sulphate supernatant fraction of high-sulphur proteins from the hairs of Artiodactyla (Gillespie and Broad 1972).

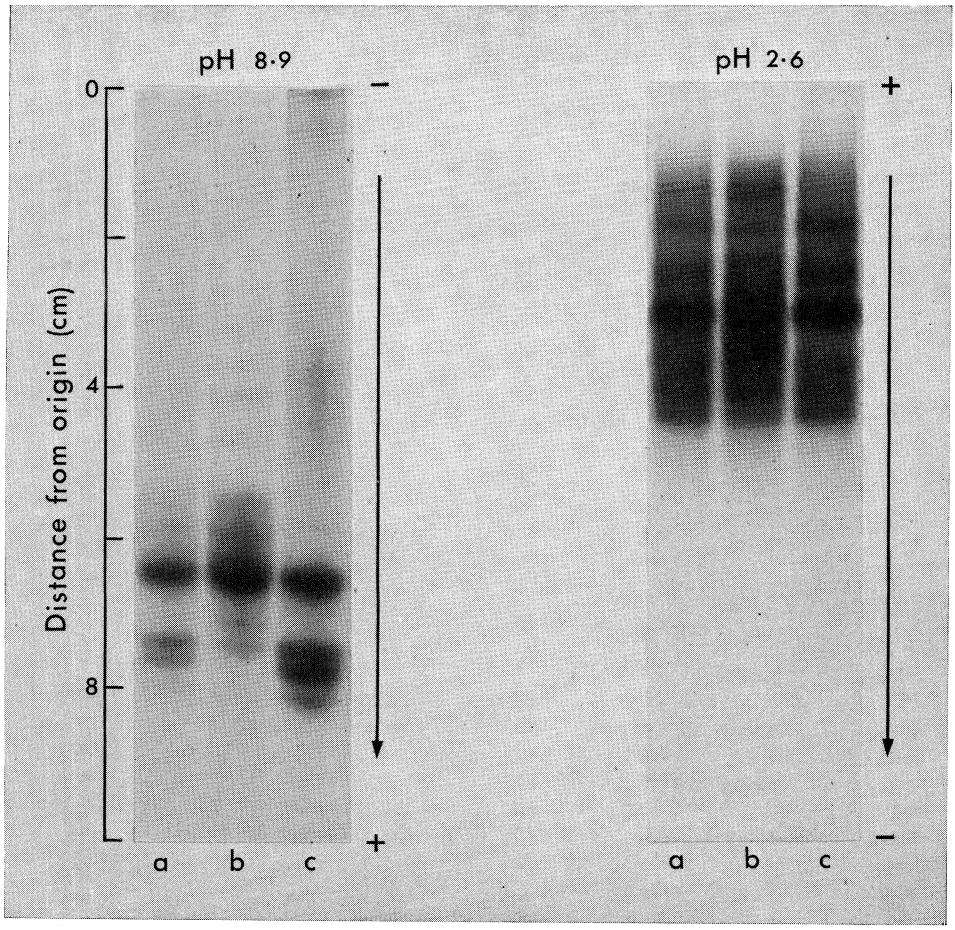

Fig. 1. Polyacrylamide gel electrophoretic patterns at pH 2·6 and pH 8·9 of (*a*) high-sulphur proteins, (*b*) ammonium sulphate supernatant proteins, and (*c*) ammonium sulphate precipitate proteins.

The two ammonium sulphate fractions were examined electrophoretically at pH 2·6 and 8·9. It can be seen (Fig. 1) that at pH 8·9 the two fractions differ markedly in their patterns of components as might be expected from their very different amino acid compositions. The two fast-moving major components are almost exclusively in the precipitate fraction, whilst the supernatant contains a number of slow- and inter-

mediate-mobility bands. The slow-moving major band is either split between the two fractions or represents two materials of similar mobility but different solubility in ammonium sulphate. The results obtained at pH 2·6 are more difficult to interpret because of the complexity and diffuseness of the patterns. Of the distinct bands, the only differences between the two fractions are in the slower components. The slowest component in the supernatant has no corresponding one in the precipitate, while the slowest component in the precipitate has a counterpart in the supernatant but their mobilities are slightly different.

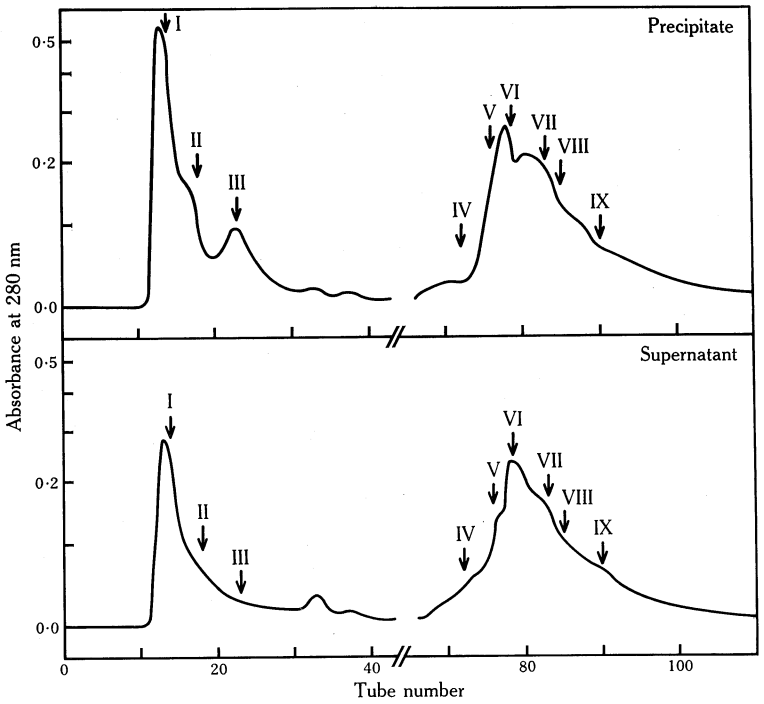

Fig. 2. Elution patterns from cellulose phosphate of ammonium sulphate supernatant (300 mg) and precipitate fractions (300 mg). Column: 67 by 2·0 cm; 10-ml fractions; flow rate, *c.* 10 ml/h; optical path length 5 mm. Starting buffer: 0·2M citric acid, 5M urea (pH 2·6); limiting buffer: 0·2M citric acid, 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. 3. Amino acid compositions of fractions I–VIII are reported in Tables 2 and 3.

Chromatography on Cellulose Phosphate

Ammonium sulphate supernatant and precipitate fractions were chromatographed separately on cellulose phosphate, and the elution profiles are shown in Fig. 2. There is an apparent similarity in elution profile; they both show unbound and bound material although the supernatant fraction contains a lower proportion of unbound material. Superposition of the profiles shows that there are small differences in the exact positions of the peaks. The proteins in the tubes corresponding to fractions I–IX on the elution profile in Fig. 2 were examined by electrophoresis in polyacrylamide

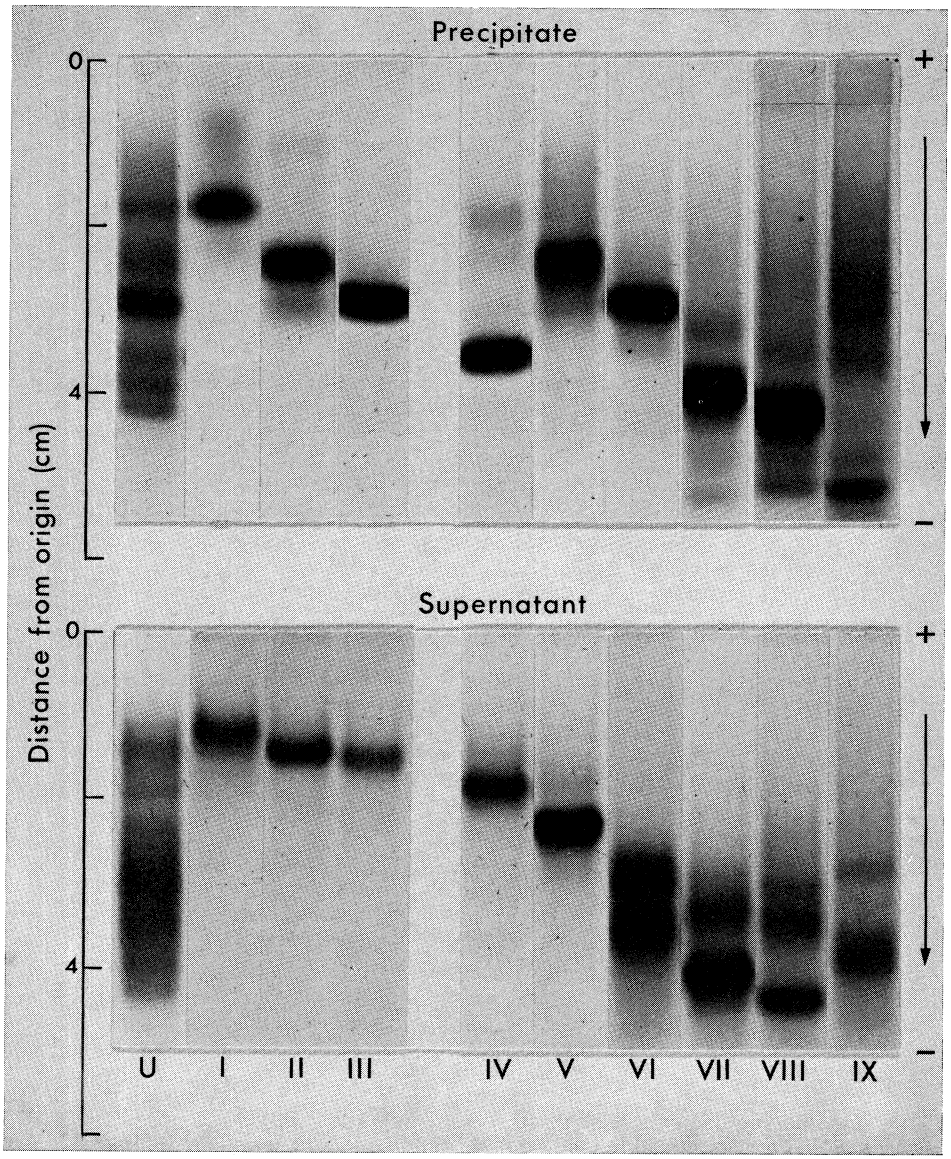

Fig. 3. Polyacrylamide gel electrophoretic patterns at pH 2·6 of representative poolings obtained by cellulose phosphate chromatography of ammonium sulphate supernatant and precipitate fractions (Fig. 2). Unfractionated starting material of the respective ammonium sulphate fraction is designated as U.

gels at pH 2·6 (Fig. 3). It can be seen that the similarity in elution profile is fortuitous as electrophoretic patterns of corresponding individual tubes from the two chromatographic fractionations are markedly different. This would be expected from the different amino acid compositions and electrophoretic patterns at pH 8·9 (Table 1, Fig. 1). The results indicate that although there may be components with similar charge at pH 2·6, they differ in size. To give an example, precipitate components I, II, and III,

although eluting from a cellulose phosphate column at the same position as supernatant components I, II, and III and thus having a similar net charge, migrate much faster in polyacrylamide gels at pH 2·6, suggesting that the precipitate components are smaller in size. If all of the individual tube patterns are examined, an apparent continuum of mobilities, as observed for the mouse hair high-sulphur proteins (Marshall and Gillespie 1976), is found within the regions I–III and VI–VII for the supernatant. Extreme heterogeneity is thus indicated, and this accounts for the diffuseness of the gel pattern of the unfractionated supernatant material. In contrast, only discrete changes in electrophoretic mobilities are observed for the individual tubes resulting from the chromatography of the precipitate.

Table 2. Amino acid compositions (as residues %) of fractions obtained by cellulose phosphate chromatography of ammonium sulphate supernatant (Fig. 2)

Amino acid	Fraction I	II	III	IV	V	VI	VII	VIII
Lys	1·02	0·75	0·75	0·26	0·20	0·14	0·08	0·18
His	0·31	0·34	0·36	0·72	1·07	0·61	0·64	0·53
Arg	3·55	4·87	4·84	5·63	5·81	8·86	9·01	9·54
Cys(Cm)	30·00	32·50	33·20	33·50	29·10	32·40	34·20	33·70
Asp	1·98	2·20	2·03	1·24	1·13	1·33	1·55	1·33
Thr	6·66	7·89	8·18	7·30	6·72	6·94	7·36	6·87
Ser	13·10	13·00	12·70	12·80	12·90	12·30	10·20	12·00
Glu	9·37	10·10	9·94	10·10	10·30	7·88	6·98	6·87
Pro	11·70	12·20	11·60	12·10	11·70	14·00	14·70	14·10
Gly	10·50	5·59	5·49	6·42	8·86	4·75	4·30	4·33
Ala	2·09	2·03	2·17	2·36	3·25	1·98	2·20	1·88
Val	3·99	3·59	3·69	3·37	3·65	4·56	4·51	4·29
Met	0·12	0·12	0·13	0·08	0·09	0·21	0·12	0·20
Ile	1·08	1·04	1·08	0·82	1·06	2·20	2·50	2·40
Leu	2·31	1·78	1·63	1·39	1·45	0·90	0·82	0·87
Tyr	1·27	0·95	0·97	0·90	1·18	0·51	0·32	0·40
Phe	1·00	1·05	1·19	1·06	1·46	0·49	0·50	0·42
Molecular weight	22 500	27 500	27 500	25 500	17 000	17 000	17 000	17 000

In the preceding paper, it was observed that the cellulose phosphate fractions I–IV of mouse hair high-sulphur proteins each contained two major electrophoretically distinct components, each pair being different (Marshall and Gillespie 1976). It now appears from the present study that these components have been cleanly separated, the faster set being found in the ammonium sulphate precipitate fraction and the slower set in the supernatant.

Amino acid compositions of fractions I–VIII from both the supernatant and precipitate are given in Tables 2 and 3. As observed previously (Marshall and Gillespie 1976), the chromatographic separation is according to the content of basic residues which, in most cases, is dominated by arginine. Four fractions from the precipitate have less than 20 residues % of cysteine(Cm) (normal high-sulphur proteins), and all the other fractions contain between 26 and 34 residues % of cysteine(Cm) (ultra-high-sulphur proteins). Threonine, serine, glutamic acid, and proline are other major

amino acid constituents. Aspartic acid content of the ultra-high-sulphur proteins is higher than that of the normal high-sulphur group.

From the weight of recovered protein from individual tubes, it can be estimated that about 80% of the mouse hair high-sulphur proteins fall into the ultra-high-sulphur group. This compares with a maximum value of 10% for wool.

Table 3. Amino acid compositions (as residues %) of fractions obtained by cellulose phosphate chromatography of ammonium sulphate precipitate (Fig. 2)

Amino acid	Fraction I	II	III	IV	V	VI	VII	VIII
Lys	1·12	1·04	1·16	0·90	0·50	0·17	0·13	0·12
His	1·15	1·77	2·67	2·07	1·09	1·29	0·76	0·78
Arg	1·88	2·82	3·32	3·67	4·58	5·66	8·64	8·74
Cys(Cm)	18·40	17·30	20·10	19·00	26·30	26·20	27·60	29·10
Asp	4·06	4·45	5·84	4·64	1·71	1·41	2·20	2·16
Thr	7·18	8·52	9·54	9·59	7·56	6·86	8·72	8·74
Ser	12·50	10·70	7·19	10·10	13·10	10·20	10·10	9·31
Glu	7·21	7·03	6·50	8·20	9·29	7·67	7·57	7·41
Pro	13·50	15·10	15·60	14·30	10·90	11·10	15·00	14·10
Gly	10·30	8·90	5·47	7·07	9·57	12·30	5·80	6·00
Ala	2·42	2·71	3·11	3·44	3·50	3·50	2·72	2·79
Val	5·54	5·74	6·68	5·95	4·10	4·15	4·75	4·85
Met	0·08	0·19	0·00	0·07	0·08	0·07	0·10	0·09
Ile	2·16	2·61	2·78	2·42	1·56	2·39	2·73	2·76
Leu	5·45	5·82	6·67	5·18	2·19	2·00	1·21	1·14
Tyr	5·26	3·82	2·19	1·81	2·04	2·48	0·97	0·91
Phe	1·82	1·47	1·26	1·58	1·96	2·58	0·97	0·96
Molecular weight	11 500	11 500	11 500	11 500	16 500	16 500	16 500	16 500

Heterogeneity of Size

Using controlled-pore glass chromatography (6M urea, 0·5% SDS, pH 7), the molecular weights of fractions I–VIII from the supernatant and precipitate were determined, and are given in the last row of Tables 2 and 3. It can be seen that components cover the molecular weight range from 11 500 to 27 500, a similar range found for the high-sulphur proteins from wool (Gillespie 1965). In the precipitate there appear to be two families of proteins; one with a molecular weight of *c.* 11 500 and the other *c.* 16 500. Apart from having different molecular weights, these families are characterized by different contents of cysteine(Cm); the smaller-molecular-weight family having a cysteine(Cm) content between 18 and 20 residues % and the other family a range of 26–29 residues %. The supernatant fraction, which contains proteins exhibiting only a minor heterogeneity of cysteine(Cm) content (range 29–34 residues %) but major heterogeneity in some other amino acid residues, shows quite a large variation in size within the molecular weight range 17 000–28 000. Fractions V–VIII of both supernatant and precipitate are remarkably similar in size and electrophoretic mobilities at pH 2·6. However, it appears that these fractions from the supernatant and precipitate contain different proteins which have been cleanly separated in the ammonium sulphate fractionation since, as noted above, within the region of fractions

VI–VIII an apparent continuum of electrophoretic mobilities is observed for the supernatant compared with discrete mobility changes for the precipitate. The two fractions V are sufficiently homogeneous for a meaningful comparison to be made of their amino acid composition. Clearly there are substantial differences between them; 10 amino acids differ by more than 10%, including some of the amino acids in highest proportion (cysteine(Cm), threonine, glutamic acid, glycine).

Because the high-sulphur proteins have such an unusual amino acid composition, especially in the high cysteine(Cm) content, compared to the molecular weight protein standards, it was decided to check the calibration curve for the controlled-pore glass column with a high-sulphur protein of known molecular weight. A protein from wool, SCMK-B2A, with 22·8 residues % of cysteine(Cm) and a molecular weight of 20000 from amino acid sequence data (Elleman 1972), was used and the molecular weight estimated from the column was 22000. This discrepancy could partly result from the experimental error in determining peak positions, but also suggests the possibility of an effect on conformation of the protein in SDS because of the high cysteine(Cm) content. In order to assess further the reliability of the molecular weights determined by chromatography on controlled-pore glass, two other gel permeation methods were used. Fractions II and V of the supernatant were chromatographed on Sepharose 6B in guanidine hydrochloride. It has been shown that high concentrations of guanidine hydrochloride denature proteins in the reduced state to linear random coil structures, and all proteins possess the same gross conformation (Tanford 1968). Thus it is the recommended empirical method for determining molecular weights (Fish *et al.* 1969), its major drawback being the time (3–5 days) which is required. Molecular weights of supernatant fractions II and V were estimated to be 24000 and 15500 respectively, compared with 27500 and 17000 from controlled-pore glass chromatography (Table 2). Thin-layer Sephadex G-100 gel filtration (6M urea, pH 9) was used to estimate the molecular weights of the fractions from the precipitate. Comparable, but less precise, results to those given in Table 3 were obtained. It appears that the molecular weights reported in Tables 2 and 3 may be in error by a maximum of 10%, but the relative differences are significant.

SDS polyacrylamide gel electrophoresis was conducted in order to substantiate that there is only one major molecular weight component in each fraction. About half of the fractions were examined, and they confirmed one major component. However, fractions VI–VIII of the supernatant gave broader bands suggesting that the separations observed in the electrophoretic patterns in Fig. 3 derive, at least in part, from size differences. It is also of interest to note that fractions I and II of the precipitate contained a minor fast-moving band at about the same position as high-tyrosine wool proteins (M. J. Frenkel, personal communication). Comparison of the amino acid compositions of these two fractions with the other fractions in Table 3 suggests minor contamination by high-tyrosine proteins because of the higher tyrosine content. The high-tyrosine proteins would not be expected to be observed in the acrylamide gels at pH 2·6 (Fig. 3) as they are insoluble under the experimental conditions.

Discussion

In the preceding paper we showed the complexity and heterogeneity of the high-sulphur proteins from mouse hair, with the best chromatographic separation being obtained at pH 2·6. The present study shows that better separation results if the high-

sulphur proteins are initially fractionated with ammonium sulphate, and the supernatant and precipitate are each chromatographed separately on cellulose phosphate at pH 2·6. Eight fractions from the precipitate have one major component in polyacrylamide gel electrophoresis; four of these contain *c.* 18–20 residues % of cysteine (Cm) and the others have a cysteine(Cm) content of more than 25 residues % (ultra-high-sulphur proteins). The supernatant fraction appears to contain only ultra-high-sulphur proteins.

The supernatant and precipitate derived from the ammonium sulphate fractionation differ markedly in the content of cysteine(Cm); this observation is similar to that reported by Gillespie (1963) for fractionation of wool proteins. Upon chromatography of the precipitate, some components with a cysteine(Cm) content of 26–29 residues % were isolated, and this is only slightly below that in the components of the supernatant. It is probable that in previous studies (Lindley *et al.* 1971; Gillespie and Broad 1972) where fractionation with ammonium sulphate was used to concentrate the ultra-high-sulphur proteins into the supernatant, some ultra-high-sulphur proteins may have been lost in the precipitate.

It was previously realized that there is an almost continuous distribution of cysteine (Cm) content in normal wool high-sulphur protein. If the present results are applicable to wool then this distribution of cysteine(Cm) content extends into the ultra-high-sulphur group, that is from 12 to 33 residues %, and suggests that the ultra-high-sulphur group is not sharply differentiated from the normal high-sulphur group.

Gillespie (1963) has shown that for the high-sulphur proteins from normal wool, the higher the cysteine(Cm) content the larger the protein. Subsequently Lindley *et al.* (1970) confirmed that a highly significant relationship existed between these two parameters. A similar relationship may exist among the components of the ammonium sulphate precipitate fraction of mouse high-sulphur proteins (Table 3), but it certainly does not exist for the proteins of the supernatant fraction for, although their molecular weights vary from 17 000 to 27 500, their cysteine(Cm) contents are almost constant (Table 2).

Many mammalian hairs contain a large proportion of ultra-high-sulphur proteins (Gillespie and Broad 1972). Wool at its lower limit of sulphur content contains little or no protein like this, but these proteins are found in wool of higher sulphur content, their amount increasing linearly with the sulphur content of the wool. The ultra-high-sulphur proteins from wool show a pattern of amino acids similar to many of the isolated components from mouse hair, suggesting that the biosynthetic mechanism in the mouse hair follicle is also present in the sheep and becomes activated under special dietary conditions of the sheep. It is of interest to note that mammals which normally produce a high proportion of ultra-high-sulphur proteins have not lost the power to make high-sulphur proteins of lower cysteine content, less than 25 residues %, which are more characteristic of the sheep (Darskus 1972). Approximately 20% by weight of the mouse hair high-sulphur proteins are of this type. Similar proteins are produced by the guinea pig (Steinert and Rogers 1973) and also by children with the protein deficiency disease 'Kwashiorkor' (Gillespie 1968).

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