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ELECTROPHORETIC AND CHROMATOGRAPHIC CHARACTERIZATION OF SULPHUR-RICH PROTEINS FROM WOOL

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SUMMARY

The heterogeneity of the high-sulphur fraction of reduced and S-carboxymethylated proteins from wool was studied by starch-gel electrophoresis in aqueous urea–acetic acid at pH 2.4. This system gives better resolution than previously used alkaline buffers. A typical preparation was found to give twelve major protein zones. A similar degree of resolution was obtained by chromatography on cellulose phosphate in aqueous urea–citrate buffer at pH 2.6. By chromatography of high-sulphur proteins on DEAE-cellulose at pH 4.5 and electrophoresis of the fractions at pH 2.4, a minimum of about thirty-six components could be identified.

INTRODUCTION

The high-sulphur proteins of α-keratins are a group of cystine-rich structural proteins believed to occur in the non-filamentous regions of the keratins. A striking feature of these proteins is their pronounced heterogeneity (for a review see GILLESPIE[1]). Fractionation studies on soluble derivatives of high-sulphur proteins from wool, for example, have revealed the presence of many components that differ in both composition and molecular weight[2,3]; the full extent of the heterogeneity is not known. Presumably these derivatives exist in solution as single polypeptide chains.

Although relatively few of these proteins have so far been isolated in the pure state and their complete amino acid sequence determined[4,5], several studies indicated the existence in the high-sulphur group of several families of proteins within which there may be many common structural features[6,7].

It is not yet known whether the presence of a large number of high-sulphur proteins in what appears to be a single structural role is due to an unusual multiplicity of genes or to some peculiar biosynthetic mechanism. Suggestions that this heterogeneity is introduced during the isolation of the proteins have been largely discounted[8].

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A major obstacle in the study of high-sulphur proteins from wool as well as from other keratins has been the lack of suitable analytical procedures for the characterization of extracts and fractions. Despite the well known high resolving power of electrophoresis in starch or acrylamide gels for protein mixtures, these methods have previously not given good resolution for unfractionated high-sulphur proteins[9–11]. The purpose of this paper is to describe an improved electrophoretic method for the analysis of high-sulphur proteins from wool (in the S-carboxymethyl form), together with its application to a further study of their heterogeneity.

Comparative studies of high-sulphur proteins from various breeds of sheep and from other animals have been greatly facilitated by the improved electrophoretic method[12,13].

EXPERIMENTAL

Preparation of S-carboxymethyl high-sulphur proteins[14]

Sample A was extracted from mixed Merino wool from a commercial flock (batch No. MW 148) with urea-thioglycollate at pH 10.9 (initial) for 2 h at 40°.

Sample B was extracted from a single Merino fleece (batch No. MW 143) at pH 10.5 (initial) for 17 h at 2°.

As the electrophoretic patterns were found to be independent of the method of preparation (DARSKUS AND GILLESPIE[12]), further details are not described.

Starch-gel electrophoresis

Electrophoresis was carried out in horizontal starch gels containing 2.75 *M* urea and 4.8 *M* acetic acid (pH about 2.4). The gels were prepared from 9 g of hydrolysed starch (Connaught Laboratories, Toronto, Canada) and 40 ml of 5.5 *M* acetic acid according to the method of SMITHIES[15], except that 7.5 g of urea were added before vacuum degassing. The gels were allowed to set overnight in trays of dimensions 144 × 43 × 3 mm.

Samples were dissolved in 2.75 *M* urea–4.8 *M* acetic acid and applied on Whatman 3MM paper. Application in free solution in slots cut in the gel gave only a marginal improvement in the sharpness of the bands. Protein concentrations were generally 4% for unfractionated samples, and 0.5–2% for fractions. No change of mobility was found over at least a three-fold concentration range.

The electrode vessels contained 0.05 *M* iminodiacetic acid (B.D.H., Poole, Great Britain; pH about 2.3). With this electrolyte, the current remained almost constant throughout the experiment. During electrophoresis (15 min at 5 V/cm, followed by 6 h at 20 V/cm) the gel, insulated with polyethylene film, was held between two aluminium plates through which water at 20° was circulated. After electrophoresis, the gel was cut into three slices, each 1 mm thick, and the middle slice was stained overnight with 0.01% nigrosine in acetic acid–ethanol–water (10:45:45). This resulted in a light blue–grey background, which could not be removed by destaining. Quantitation was not attempted.

The patterns obtained were reproducible except for the absolute values of the mobilities, which had a range of variation of about ± 10%. Therefore, a standard sample of high-sulphur protein was included in each experiment.

Chromatography on DEAE-cellulose

Chromatography was performed at 20° on a 0.9 × 120 cm column of DEAE-cellulose (Eastman-Kodak) by a modified version of the method described by SWART *et al.*[3]. The protein (200 mg in 3 ml) was dialysed against the starting buffer. The sample was applied to the equilibrated column in a total volume of 7.5 ml. The proteins were eluted at 24 ml/h with a linear gradient formed between 500 ml each of the starting buffer (0.2 *M* sodium acetate, 0.28 *M* acetic acid; pH 4.5) and a solution of 0.2 *M* sodium acetate, 0.9 *M* acetic acid, and 0.4 *M* sodium chloride (pH 3.85). The absorbance at 280 nm of the effluent was recorded continuously with an ISCO Model 222 ultraviolet analyser. Reproducible elution patterns were obtained with this method for a given sample of high-sulphur proteins. Protein fractions were recovered by exhaustive dialysis against deionized water, followed by lyophilization.

Chromatography on cellulose phosphate

A modification of methods used by GILLESPIE AND STEVENSON (unpublished work) and GILLESPIE AND BROAD[16] was used for chromatography at low pH. Cellulose phosphate (Whatman P11, 7.4 mequiv./g) was washed for 45 min with 0.5 *M* potassium hydroxide, with several changes of water, then for 45 min with 0.5 *M* hydrochloric acid. After removal of the finest particles from the ion exchanger and equilibration with buffer, the column was packed and the bed compacted to final dimensions of 2.5 × 40 cm by pumping buffer through it. A tight-fitting end-piece was used to prevent expansion of the bed. Columns prepared in this way shrank very little during elution, so that good reproducibility of elution patterns could be obtained.

The sample (1.5 g in 10 ml) after dialysis for several hours at 20° against the starting buffer (0.2 *M* citric acid in 5 *M* urea, pH about 2.6) was applied to the column, followed by a linear gradient formed between 750 ml each of starting buffer and 5 *M* urea containing 0.4 *M* sodium chloride, 0.05 *M* sodium dihydrogen citrate, and 0.15 *M* citric acid, pH about 2.8. Elution was carried out at 20° and 50 ml/h. Fractions were collected in tubes that contained sufficient saturated sodium acetate to raise the pH to 3.5–4 in order to minimize the danger of protein degradation. Selected fractions were pooled and dialysed free of salts and urea at 2°, and the proteins were recovered by lyophilization.

Amino acid analysis

Samples were hydrolysed for 24 h at 105° in evacuated tubes with constant-boiling hydrochloric acid containing 10^{-3} *M* thioglycollic acid. The freeze-dried hydrolysates were analysed with a Beckman-Spinco automatic amino acid analyser.

RESULTS

Electrophoresis of unfractionated high-sulphur proteins

Two samples of S-carboxymethyl high-sulphur proteins were studied, one from mixed wool from a commercial Merino flock (sample A), the other from an individual Merino (sample B). Starch-gel electrophoresis of these samples was performed at pH 2.4 in urea–acetic acid as solubilizing agent. The results are shown in Fig. 1. Sample A gives twelve major bands, numbered 1–12 in order of increasing mobility, all moving towards the cathode (Fig. 1a). Many of the zones can be seen on the

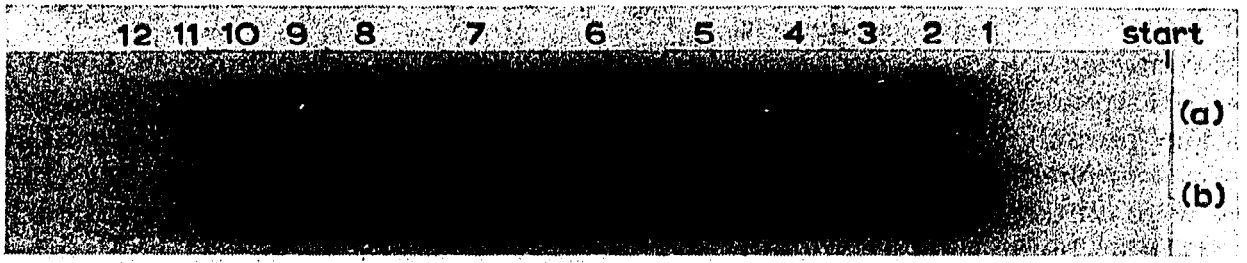

Fig. 1. Starch-gel electropherogram of two preparations of high-sulphur proteins. (a) Sample A; (b) sample B. Migration from right to left.

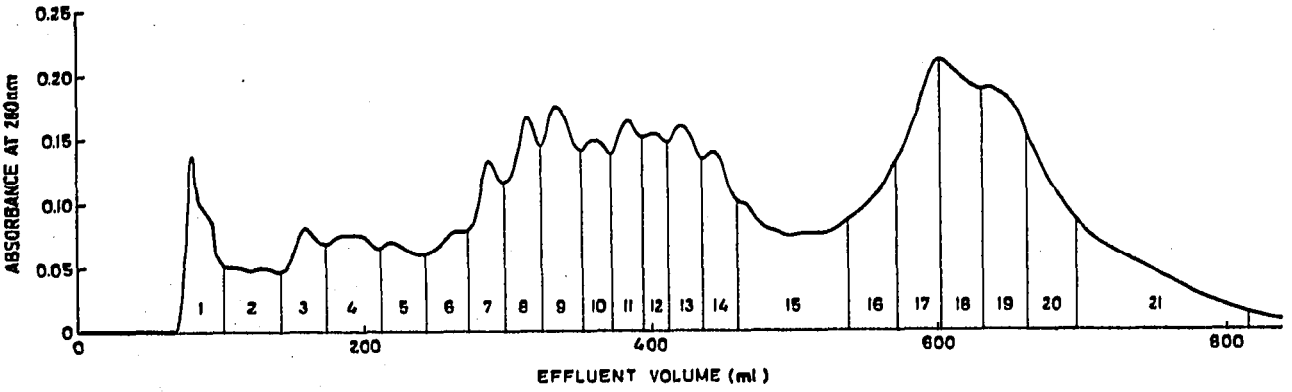

Fig. 2. Elution pattern of high-sulphur proteins (sample A) from DEAE-cellulose. For conditions see text. Optical path length 10 mm.

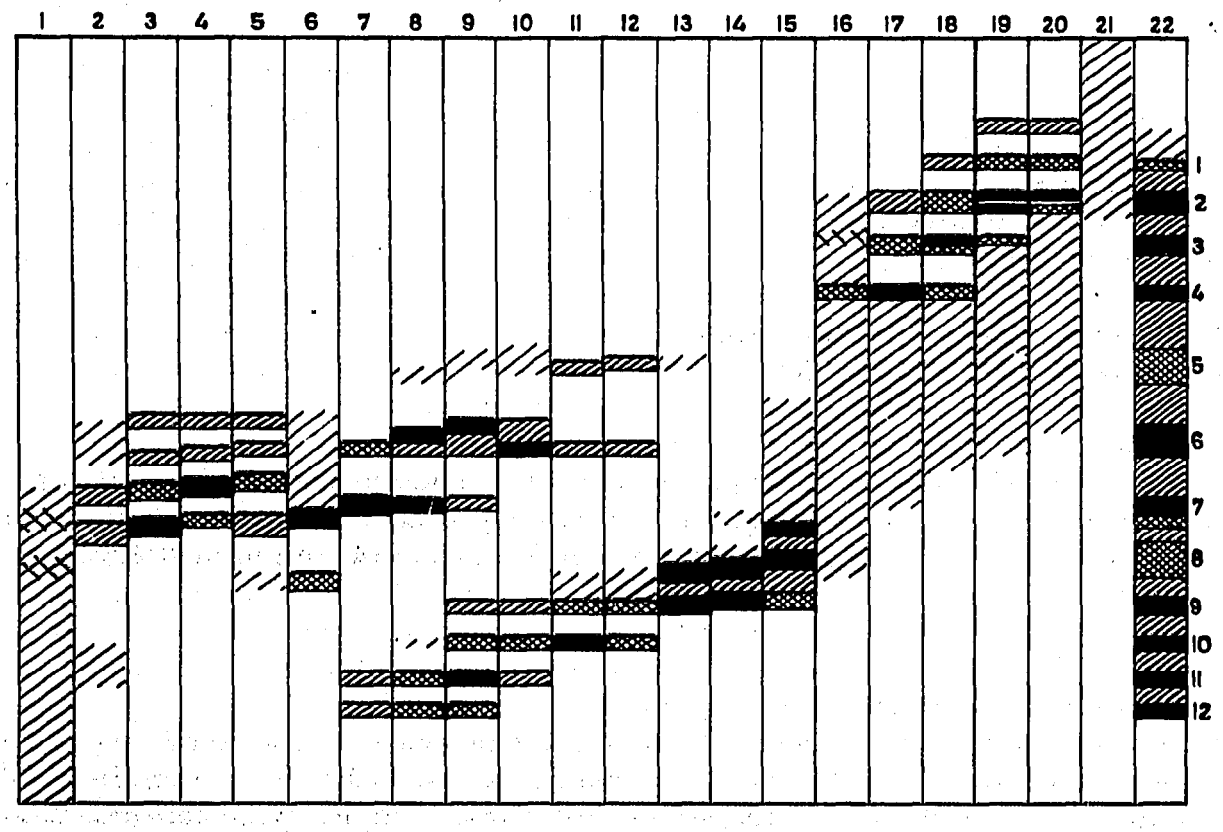

Fig. 3. Schematic drawing of starch-gel electropherograms of fractions obtained from DEAE-cellulose. Numbers 1–21 at the top correspond to fractions 1–21, respectively, in Fig. 2; No. 22 represents the starting material (*cf.* Fig. 1a). Migration from top to bottom. The stained region in fraction 1 extends beyond the lower limit of the diagram.

stained gels to consist of at least two bands, *e.g.*, zones 1–3. Others, particularly zones 5–8, are diffuse, and there is considerable background stain between the major zones. Sample B gives a pattern (Fig. 1b) similar to that of sample A, the most conspicuous difference being the apparent absence of band 12 in Fig. 1b.

Chromatography on DEAE-cellulose

In order to gain further insight into the extent of the heterogeneity observed in Fig. 1, high-sulphur proteins (sample A) were separated by chromatography on DEAE-cellulose.

Twenty-one fractions were isolated as shown in Fig. 2, and examined by electrophoresis at pH 2.4. The results are presented diagrammatically in Fig. 3. From this two-dimensional "map", it can be seen that this sample of high-sulphur proteins from Merino wool contains at least thirty-six components, not including the areas of continuous, faint protein stain present in many fractions. A further conclusion is that it is not possible to define a major constituent of these high-sulphur proteins.

Chromatography on cellulose phosphate

In an attempt to characterize some of the components responsible for particular electrophoretic bands, high-sulphur proteins (sample B; *cf.* Fig. 1b) were fractionated by chromatography on cellulose phosphate in 5 *M* urea at pH 2.6. Arbitrary, narrow fractions were isolated as indicated in Fig. 4.

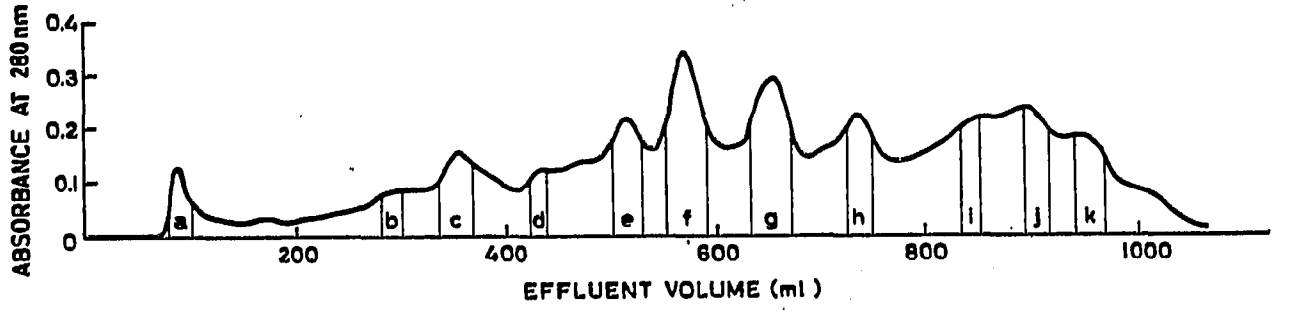

Fig. 4. Elution pattern of high-sulphur proteins (sample B) from cellulose phosphate. For conditions see text. Optical path length 2 mm.

As can be seen in Fig. 5, most of the fractions selected give one major, although sometimes wide, zone on starch-gel electrophoresis at pH 2.4, together with various amounts of diffusely staining material. Fraction a, which is unretarded by cellulose phosphate, yields a diffuse pattern similar to that of fraction 21 in Fig. 3. Although these fractions are impure, it appeared worthwhile to determine their compositions in order to obtain some idea of the differences between some of the electrophoretic components. The amino acid compositions of acid hydrolysates of nine of these fractions are given in Table I. Qualitatively, they show a correlation between the content of basic amino acids, particularly arginine, and the relative electrophoretic mobility and elution sequence of the corresponding fraction.

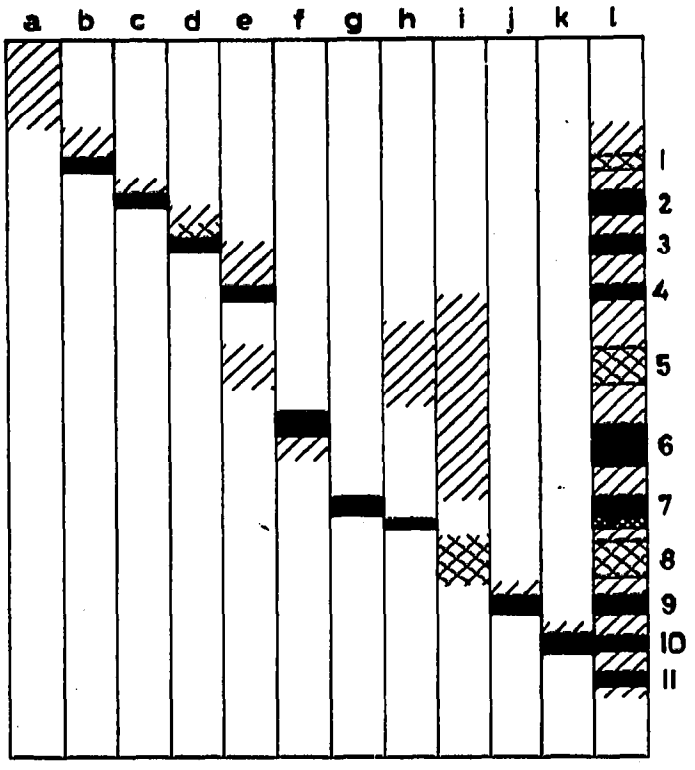

Fig. 5. Schematic drawing of starch-gel electropherograms of fractions obtained from cellulose phosphate. a–k refer to the corresponding fractions defined in Fig. 4; l represents the starting material (*cf.* Fig. 1b). Migration from top to bottom.

TABLE I

AMINO ACID COMPOSITIONS (RESIDUES PER 100 RESIDUES) OF ACID HYDROLYSATES OF FRACTIONS OBTAINED BY CHROMATOGRAPHY ON CELLULOSE PHOSPHATE

Amino acid	*Fraction*								
	b	*c*	*d*	*e*	*f*	*g*	*h*	*j*	*k*
Lys	0.2	0.2	0.2	0.5	0.9	1.1	0.7	0.3	0.3
His	0.1	0.1	0.3	0.6	1.4	1.9	0.9	0.3	0.3
Arg	3.5	3.5	3.9	4.4	3.9	5.3	6.3	10.0	10.7
SCMC[a]	20.7	21.9	20.6	19.8	17.7	16.7	17.2	22.5	22.4
Asp	1.3	1.1	1.4	1.9	4.2	6.4	2.7	1.6	1.6
Thr	10.7	11.1	10.0	10.3	10.3	9.7	10.7	10.3	10.4
Ser	14.2	14.7	15.1	13.8	12.6	10.7	14.3	11.4	11.6
Glu	12.2	11.9	10.4	9.0	7.0	5.2	8.0	7.0	6.7
Pro	10.6	10.2	11.5	11.8	14.9	13.3	12.0	15.1	15.0
Gly	8.8	8.6	8.3	7.6	4.4	5.3	6.1	5.4	5.3
Ala	3.2	3.1	3.6	4.0	3.4	3.2	2.2	2.1	2.2
Val	3.8	3.5	4.3	4.7	5.3	6.3	7.5	6.1	5.9
Ile	4.2	4.1	3.7	2.9	3.3	3.6	3.3	2.1	1.8
Leu	2.4	2.3	2.9	3.9	6.4	6.6	3.8	2.3	2.2
Tyr	2.5	2.2	2.6	2.7	2.2	2.2	2.7	1.7	1.7
Phe	1.6	1.4	1.3	1.7	2.3	2.5	1.7	1.9	2.0

[a] S-Carboxymethylcysteine.

DISCUSSION

The starch-gel electropherograms shown in Fig. 1 represent a significant improvement in the electrophoretic separation of high-sulphur proteins from wool. Although the separation achieved is evidently not complete, many more components can be demonstrated than in previous zone-electrophoretic studies[9–11].

Whereas fractions similar to those of Figs. 2 and 3 have previously been examined by various methods, including disc electrophoresis at pH 8, the improved resolving power of the electrophoretic method described above allows a comprehensive survey to be made for the first time of the heterogeneity of the high-sulphur proteins from wool (Fig. 3). It also provides a method for following the fate of various components during fractionation procedures.

Fig. 3 shows that a given electrophoretic component may occur in several adjacent chromatographic fractions. Such a result is unavoidable because of zone spreading during chromatography. However, the spreading often extends over an unexpectedly large number of successive fractions. Preliminary experiments suggest that at least in several instances this phenomenon is due to the presence in successive fractions of components with slightly different chromatographic properties at pH 4.5, but virtually identical electrophoretic mobilities at pH 2.4. Clearly, then, the estimate of thirty-six components must be regarded as a lower limit.

The results in Figs. 2 and 3 were obtained on mixed wool from a large number of sheep. Similar results were found with an early version of the method for wool from two individual sheep, so that there is no reason to believe that pooling of the wool introduces much additional complexity. In one of these samples, one of the electrophoretic components (band 12) appeared to be absent, as in sample B (*cf.* Fig. 1b). This may be due to polymorphism of the high-sulphur proteins, and has been considered elsewhere[12].

Some evidence was obtained suggesting that the patterns observed are not artefacts of the electrophoretic method. When electrophoresis was carried out at 3° instead of 20°, the only change was an approximately 50% decrease in the mobility of each band. Even when samples were kept at 20° and pH 2.4 for 24 h before electrophoresis, no new components were detectable. Protein degradation during electrophoresis therefore does not contribute to the observed heterogeneity. In gels that contained 0.05 *M* 2-mercaptoethanol, the same patterns were obtained as in standard gels; this result excludes oxidation of S-carboxymethylcysteine residues as a contributor of heterogeneity. As many fractions can be isolated that run as essentially single zones on electrophoresis (Fig. 5), reversible aggregation of proteins and protein–buffer interactions are not major factors affecting the separation.

Several of the fractions listed in Table I show similarities in amino acid composition to fractions obtained by other methods. Thus, fraction g resembles the fraction IIIB obtained by SWART, HAYLETT, AND JOUBERT[4a], and fractions j and k their fraction IIIA and also fraction β1 of GILLESPIE *et al.*[17]. Fractions b–d resemble the fraction SCMKB-2 containing no lysine or histidine, and only 0.9 residue-percent of aspartic acid, which was first prepared by GILLESPIE[18] and which is being studied intensively in these laboratories.

Deviations from identity are probably due to minor contaminants in fractions b–d (Fig. 5). The latter comparison is confirmed by the observation that SCMKB-2

gives mainly bands 1–4 on electrophoresis, whereas fractions b, c, and d give bands 1, 2 and 3, respectively. Of course, not all the material running as bands 1–4 in the unfractionated high-sulphur proteins necessarily corresponds to SCMKB-2. Thus fraction e, running as band 4, contains much more lysine, histidine, aspartic acid, and leucine than SCMKB-2, although again a contribution from impurities is possible. In any event, chromatography on cellulose phosphate appears promising as a first step in the isolation of SCMKB-2 components or other high-sulphur protein components.

The results in Table I agree with earlier findings that the high-sulphur proteins comprise several protein families. The similarities in composition of fractions b–e, and of fractions j and k, suggest that these belong to two such families.

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