THE ISOLATION AND PROPERTIES OF SOME SOLUBLE PROTEINS FROM WOOL

IV. THE ISOLATION OF THE HIGH-SULPHUR PROTEIN SCMKB1*

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Summary

A protein of high sulphur content (6·7% S) has been separated from the *S*-carboxymethyl derivatives of the high-sulphur proteins of Merino wool by salting-out and chromatography on DEAE-cellulose.

Amino acid analysis shows that this modified protein is extremely rich in *S*-carboxymethyl cysteine, almost one residue in every four being accounted for by this residue, and it is also rich in serine, threonine, and proline; these four residues together account for almost two-thirds of the weight of the protein. It contains no methionine and only small amounts of lysine, histidine, phenylalanine, and aspartic acid. About 0·6% sulphur, based on the dry weight of the protein, is unaccounted for. This protein appears to have a molecular weight of between 25,000 and 28,000.

I. INTRODUCTION

About one-third of the weight of Merino wool is protein material which is very rich in sulphur, and contains about two-thirds of the cystine of wool. This material in the native state in wool is probably basic and thought to be unoriented and possibly in a globular form (Alexander and Hudson 1954; Corfield, Robson, and Skinner 1958; Gillespie and Simmonds 1960; Sikorski and Woods 1960; Fraser, MacRae, and Rogers 1962). Previous studies (Gillespie 1959, 1960*a*, 1960*b*, 1962) showed that this protein fraction contained a number of components which were resolvable by electrophoresis and chromatography. The work to be described in this paper was carried out as part of a programme to isolate as many as possible of these high-sulphur protein components in a form suitable for characterization. Ultimately the properties of a particular wool and differences between wools with different physical characteristics will probably be related to the properties of the proteins within the fibre and particularly to those of the high-sulphur proteins.

This paper describes the isolation and characterization of the high-sulphur protein SCMKB1. This protein occurs in the fastest moving electrophoretic peak and was chosen for isolation because it seemed to be the richest in sulphur.

* A scheme of nomenclature defining SCMKB1 and other wool proteins is given by Gillespie (1960*a*, p. 102).

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II. Materials and Methods

(*a*) *Extraction of Proteins*

(i) *Preparation of Wool.*—The wool used in these experiments was a Merino 64's of the 1959 shearing from the Wintoc property in Victoria. It was solvent-scoured, dry-combed, and converted to top. Before use it was washed three times with petroleum ether, once with alcohol, twice with distilled water, and finally air-dried. Wool from this property from earlier shearings has also been used in previous work on the isolation of low- and high-sulphur proteins.

(ii) *Extraction with* 0·1M *Thioglycollate at 40°C.*—The extraction of protein and conversion to *S*-carboxymethyl kerateines was done as described previously (Gillespie 1960*a*) with the following modifications: (1) the wool was not soaked in sodium carbonate solution as this was found to be unnecessary; (2) ammonia (0·175N) was added to the potassium thioglycollate to react with thiol esters; (3) after the completion of alkylation, unused iodoacetate was removed by reaction with either thioglycollate or sulphite to prevent side reactions. The mixed *S*-carboxymethyl kerateines were thoroughly dialysed and then separated into low- and high-sulphur components by precipitation at pH 4·1 at an ionic strength of 0·1. The supernatant containing the high-sulphur protein was separated by filtration, then dialysed, and freeze-dried, or titrated to pH 2·9 and the precipitate separated by filtration, dissolved in sodium bicarbonate solution, dialysed, and freeze-dried. Wool proteins prepared by this method will be referred to as "high temperature preparations".

(iii) *Preferential Extraction at 0°C.*—50 g of wool was cooled to 0°C and extracted at this temperature for 18 hr with 1500 ml 0·8M potassium thioglycollate of pH 10·3 and containing 0·1M NH_3. The undissolved wool was filtered off at 0°C and the protein precipitated from the filtrate by acidification with 50 ml of glacial acetic acid. The precipitate was allowed to settle at room temperature for several hours under nitrogen and then the supernatant was decanted, the precipitate allowed to drain and then dissolved in 1l. of 0·1M potassium thioglycollate at pH 9 by stirring in a completely filled and sealed Waring Blendor jar. The solution was allowed to warm to 20°C, then treated with between 30 and 40 g of iodoacetic acid, the pH being maintained between 8 and 9 until the nitroprusside test became negative. When the alkylation was complete the unused iodoacetate was removed with thioglycollate or sulphite. At this stage the solution was either thoroughly dialysed and the small amount of protein precipitable at pH 4·1 removed or it was subjected to fractionation with ammonium sulphate as described in Section III(*b*). Finally the fractionated proteins were dialysed and freeze-dried. Fractions prepared in this way will be referred to as "low temperature preparations" and were employed in most of the studies reported in this paper.

(*b*) *Electrophoresis*

The progress of purification of the proteins was followed by electrophoresis in a Tiselius moving boundary apparatus (made by LKB Produktor, Stockholm). When sufficient protein was available 1% solutions which had been dialysed against the appropriate buffer were used. Routinely, an acetate buffer at pH 4·5 and of

ionic strength 0·1 was employed, as this gave the best resolution of the unfractionated protein. The purified protein was also examined in other buffers of similar ionic strength: phosphate and imidazole–HCl at pH 7·0, Tris–HCl at pH 8·0, veronal at pH 8·6, and β-alanine–NaOH at pH 11·0. Most experiments were performed in standard, long, intermediate cells but when only small amounts of protein were available a pair of short cell sections with a 1·5-mm channel width were employed. Although much less protein was needed, the distance over which the protein peak could migrate was halved and some high-sulphur proteins could not be resolved. By back-compensating, the protein could be run for a length of time comparable to that used with the longer cells but occasionally the counter-flowing liquid disturbed the protein concentration boundaries.

(*c*) *Spectrophotometry*

Absorption measurements at 276 mμ, made on a Beckman DU spectrophotometer, were used to estimate the protein content of solutions. The extinction coefficient ($E^{1\%}_{1cm}$) of the unfractionated protein at this wavelength was about 5·5. Spectral curves were determined on a Beckman DK spectrophotometer.

(*d*) *Chromatography*

The proteins were chromatographed at room temperature on DEAE-cellulose (EK 7392) by gradient elution. Before use the resin was repeatedly washed with 1M NaCl to remove material absorbing in the ultraviolet region, the resin was then washed repeatedly with water (during which a quantity of finely divided resin was removed by decantation), and finally equilibrated with the appropriate buffer. The resin was supported on sintered-glass disks in jacketed columns and packed under a pressure of 10 lb/in^2. The protein dissolved in the same buffer was applied to the column and allowed to adsorb under gravity. A linear sodium chloride concentration gradient was applied to the column and the effluent collected in equal-volume samples, the protein concentration being measured spectrophotometrically.

(*e*) *Sedimentation*

Measurement of sedimentation coefficients and molecular weight by the Archibald technique was made in a Spinco model E ultracentrifuge. As a preliminary the proteins were thoroughly dialysed against a buffer of pH 6·8 containing 0·2M NaCl and 0·025M each of monosodium and disodium orthophosphates and then clarified by contrifuging for 1 hr at 40,000 r.p.m. in a Spinco model L centrifuge. This clarification enabled reproducible molecular weights to be obtained with different preparations and presumably removed some aggregated protein.

(*f*) *Viscosity*

The intrinsic viscosity of the protein was measured in the same buffer employed for the determination of sedimentation coefficients. An Ostwald viscometer was employed with a time of flow for buffer at 25°C of about 200 sec. The protein stock solution and dilutions from it were clarified by filtration through a sintered-glass filter (porosity 3). The procedures and calculations employed were as described by Schachman (1957).

(*g*) *Amino Acid Analysis*

The proteins were hydrolysed prior to amino acid analysis by refluxing for either 24 or 72 hr with 6N HCl, and then the HCl was removed by freeze-drying, and the amino acid content of the hydrolysate estimated. A Spinco automatic amino acid analyser was used for these estimations. The nitrogen content of the intact protein was measured by a microKjeldahl procedure, using solutions with the protein concentration known from a dry weight determination.

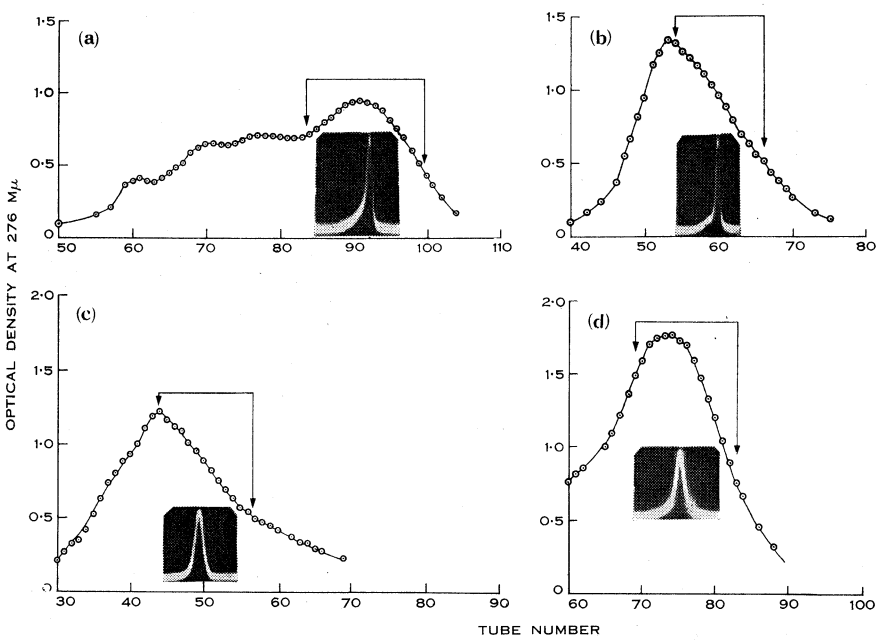


Fig. 1.—(*a*)–(*c*) Chromatography of high-sulphur wool proteins on DEAE-cellulose: stages in the purification of a high temperature preparation. (*a*) First chromatography at pH 4·5: NaCl gradient 0–0·65M, 600 ml eluant, 5-ml fractions collected. (*b*) Rechromatography at pH 4·5: NaCl gradient 0·2–0·65M, 400 ml eluant, 5-ml fractions collected. (*c*) Rechromatography at pH 7·0: NaCl gradient 0·2–0·7M, 400 ml eluant, 4·7-ml fractions collected. (*d*) Final purification of ammonium sulphate-fractionated low temperature preparation. Chromatography at pH 4·5, NaCl gradient 0–0·65M, 600 ml eluant, 6-ml fractions collected. Electrophoresis patterns shown in (*a*)–(*d*) are of ascending boundaries.

(*h*) *Solubility Measurements*

The solubility of the various protein components was measured as described previously (Gillespie 1960*a*).

III. Results

(*a*) *Isolation of SCMKB1 by Repeated Column Chromatography of a High Temperature Preparation*

About 10 g of a high temperature preparation was fractionated by repeated gradient elution chromatography on DEAE-cellulose. A 3-cm column was used with a loading of up to 0·5 g protein. Preliminary experiments showed that just

High temperature preparation of high-sulphur wool proteins

Merino wool top extracted for 3 hr at 40°C with 0·1M potassium thioglycollate at initial pH 10·5 (liquor : wool ratio 100 : 1). Extract alkylated with iodoacetate, dialysed. Precipitated at pH 4·1 with acetate buffer of ionic strength 0·1

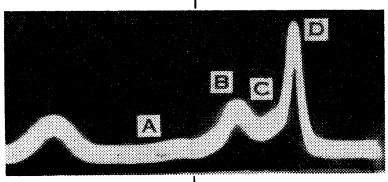
Precipitate (non high-sulphur protein)

Supernatant (high-sulphur protein)

Titrate to pH 2·9 with HCl

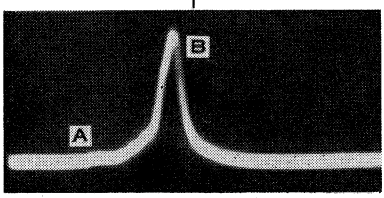
Supernatant (minor components)

Precipitate (high-sulphur protein, 5·7–5·8% S)

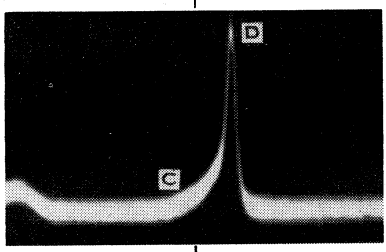


Chromatograph on DEAE-cellulose at pH 4·5

Lower charge components

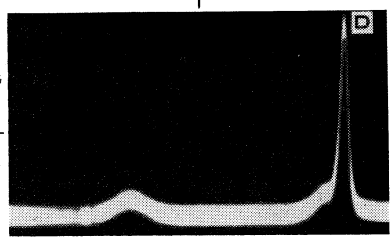


SCMKB1

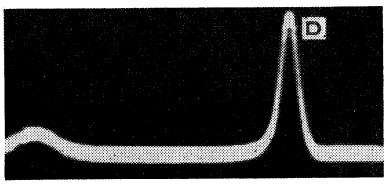


Rechromatograph at pH 4·5

SCMKB1



Chromatograph at pH 7·0



SCMKB1 (6·7%S)

Fig. 2.—Flow sheet of the various stages in the isolation of SCMKB1 from a high temperature preparation by repeated chromatography. Lettering of ascending electrophoretic components as used by Gillespie (1962).

as electrophoresis gave the best resolution around pH 4·5 so the best chromatographic resolution was also obtained at about this pH value. In general the proteins were eluted in the inverse order of their electrophoretic mobilities. As a consequence, a minor component, if of lower mobility than SCMKB1, could be eliminated by discarding the more easily eluted components and if of higher mobility by discarding the less easily eluted fractions.

The chromatographic pattern shown in Figure 1(*a*) was obtained at pH 4·5 in acetate buffer, ionic strength 0·01, with a gradient of 0 to 0·65M NaCl. Protein collected from the fractions between the indicated points gave the electrophoretic pattern shown in the same figure. This fraction was enriched in SCMKB1 but contained considerable amounts of impurities of lower mobility. The protein collected from five replicate separations was pooled and rechromatographed at pH 4·5 with a gradient of 0·2M to 0·65M NaCl when the curve shown in Figure 1(*b*) was obtained. Protein obtained by pooling those fractions between the indicated points gave the electrophoretic pattern shown in the same figure. The impurity of lower mobility had been considerably reduced in amount but further rechromatography at this pH did not significantly increase the purity. However, the lower charge component could be completely removed by rechromatographing at pH 7·0 in imidazole–HCl buffer of ionic strength 0·015, with a gradient of 0·2 to 0·7M NaCl. The protein collected from five replicate runs, similar to the second chromatography at pH 4·5, was rechromatographed at pH 7·0 and gave the pattern shown in Figure 1(*c*). Protein collected from fractions between the indicated points gave the electrophoretic pattern also shown on this figure. It can be seen that there is now no obvious electrophoretic impurity.

This method used to isolate SCMKB1 is summarized in the flow sheet of Figure 2. This protein had a sulphur content of 6·7% and at a concentration of 1% at pH 4·5 it had a mobility of $-6{\cdot}8\times10^{-5}$ cm^2 volt^{-1} sec^{-1}. However, the method gave an extremely low yield of SCMKB1 and was wasteful of protein, largely because of poor chromatographic resolution and closeness in charge of the components.

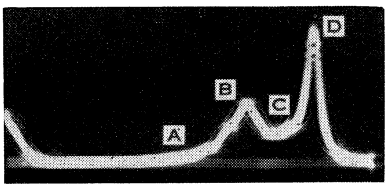
(*b*) *Preparation of SCMKB1 from a Low Temperature Preparation by Ammonium Sulphate Fractionation followed by Chromatography*

A sharp separation of SCMKB1 from the other proteins in the low temperature preparation was obtained by fractional precipitation of the latter with ammonium sulphate. Immediately after the kerateines had been alkylated (Section II(*a*)(iii)) the pH of the solution was adjusted to 6·2 by the addition of dilute acetic acid, and then solid ammonium sulphate was added with stirring to give a final concentration of 1·6M. A curd of brown sticky protein floated to the surface and, after standing for about 1 hr, it was skimmed off. The remaining turbid solution was clarified by centrifugation at 40,000 *g* for 1 hr. The protein responsible for the turbidity centrifuged down as a viscous brown oil.

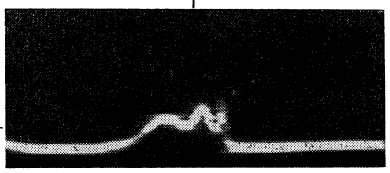
SCMKB1 was precipitated from the clarified supernatant by titrating the solution to pH 4 with HCl and then adding sufficient ammonium sulphate to bring the concentration to 2M. The protein separated as a white granular precipitate

Low temperature preparation of high-sulphur wool proteins

Merino wool top extracted for 18 hr at 0°C with 0·8M potassium thioglycollate at initial pH 10·3 (liquor : wool ratio 30 : 1). Protein precipitated at pH 6, dissolved in 0·1M potassium thioglycollate at pH 9



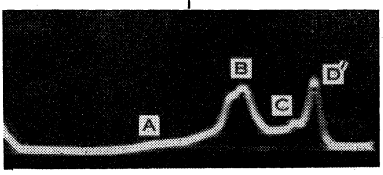
Alkylate with iodoacetate



5·7–5·8%S

Adjust pH to 6·2, add solid ammonium sulphate to give a final concentration of 1·6M. Temperature 20°C

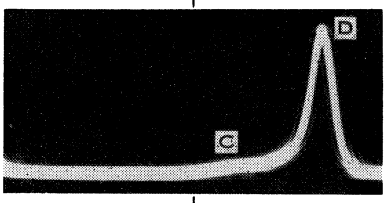
Precipitate (minor components, 5·2%S)



Supernatant (higher charge components)

Titrate solution to pH 4 with HCl, Add ammonium sulphate to bring concentration to 2·0M

Precipitate (SCMKB1, 6·5%S)

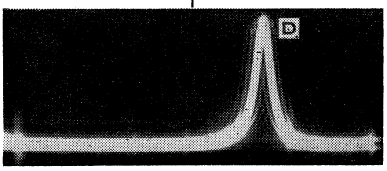


Supernatant (minor components)

Chromatography on DEAE-cellulose

Lower charge components

SCMKB1 (6·7%S)



Higher charge components (traces only)

Fig. 3.—Flow sheet of the various stages in the isolation of SCMKB1 from a low temperature preparation by combined salting-out and chromatography. *D'*, minor protein component. Lettering of other ascending electrophoretic components as used by Gillespie (1962).

which was usually collected by filtration, dissolved in dilute sodium bicarbonate, dialysed, and freeze-dried. The electrophoretic pattern of this fraction is shown in Figure 3. The protein is largely SCMKB1 with the main impurity of lower charge and well separated in electrophoretic mobility from that of SCMKB1. Repeated fractional precipitation with ammonium sulphate at pH 4, discarding the material not precipitating between 20 and 30% saturation, resulted in some further purification but the simplest procedure to achieve electrophoretic purity was to chromatograph on DEAE-cellulose at pH 4·5. The chromatographic pattern obtained is shown in Figure 1(*d*). Protein collected from fractions between the indicated points gave the electrophoretic pattern on the same figure. It can be seen that virtually all the electrophoretically (at pH 4·5) distinct impurities had been removed. The steps in this fractionation are summarized in the flow sheet of Figure 3. The various chemical and physical properites of SCMKB1 were determined on protein preparations prepared and purified by this latter procedure.

Preparations were made by this procedure from several other types of Merino wool. These showed no significant amounts of impurities on electrophoresis at pH 4·5 but small amounts of both slow- and fast-moving impurities were apparent on electrophoresis at pH 8·6. These impurities could be removed by an additional chromatographic separation at pH 8·5 with a sodium chloride gradient rising from 0·2 to 0·7M, and discarding the first and last 20% of protein which was eluted. It has not been established whether these preparations are identical with SCMKB1.

The proteins insoluble in 1·6M ammonium sulphate at pH 6 (i.e. the brown floating protein and the centrifuged oil) were pooled, dissolved in dilute sodium bicarbonate solution, and again precipitated at pH 6·2 with ammonium sulphate (1·6M). The precipitate was dissolved in dilute sodium bicarbonate solution and dialysed. A small quantity of non high-sulphur protein was removed by precipitation at pH 4·1 (ionic strength = 0·5), the supernatant then being dialysed and freeze-dried. The electrophoretic pattern of this protein (Fig. 3) shows that in addition to the slow-moving components observed in the unfractionated protein there is some protein (D′)* present with similar mobility to SCMKB1 but less soluble in 1·6M ammonium sulphate. For the purposes of discussion the proteins in this fraction will be referred to as "minor components". A study is at present being made of their fractionation and properties.

(*c*) *Solubility of SCMKB1 and the Minor Components*

The solubilities of SCMKB1 and a fraction containing the minor components were measured at 20°C in acetate–phosphate buffers of constant ionic strength, over the pH range 1·5–4. The results are shown in Figures 4(*a*) and 4(*b*). With all these fractions the pH of minimum solubility varied with ionic strength, shifting to more alkaline pH values as the ionic strength decreased. At an ionic strength of 0·2 the minimum solubility of SCMKB1 was observed close to pH 2·65, and that

* This material (now termed SCMKB2), when separated from the other proteins has a mobility at a protein concentration of 1% considerably less than that of SCMKB1 and is clearly distinguishable from the latter protein in physicochemical properties (Gillespie, unpublished observations).

of the minor components close to pH 3·0. Variation in ionic strength over the range studied had practically no effect on the solubility of these proteins at pH values alkaline to the pH of minimum solubility, but at pH values acid to these points the proteins became more soluble as the ionic strength decreased. SCMKB1 was much less soluble at pH values below the point of minimum solubility than were the minor components.

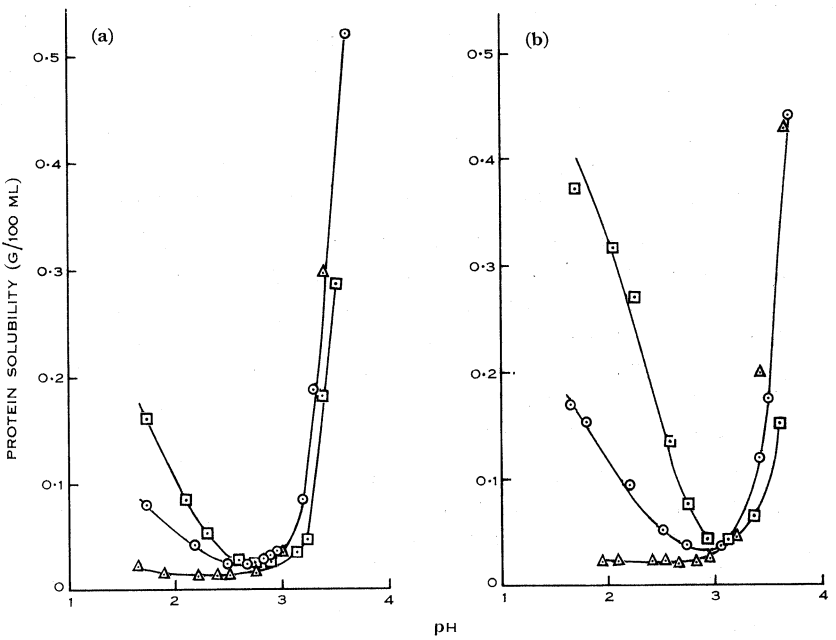


Fig. 4.—Solubility curves of high-sulphur wool proteins in acetate–phosphate buffer of varying ionic strength. (*a*) SCMKB1. (*b*) Minor protein components. □ Ionic strength 0·06. ○ Ionic strength 0·2. △ Ionic strength 0·5.

(*d*) *Amino Acid Analysis of SCMKB1 and the Minor Components*

Analyses were made on both a 24-hr and 72-hr hydrolysate of SCMKB1. Table 1 contains the analytical values together with the mean or extrapolated values for each amino acid. SCMKB1 is extremely rich in *S*-carboxymethyl cysteine, proline, serine, and threonine but contains only small amounts of lysine, aspartic acid, phenylalanine, and histidine. Almost 1 in every 4 residues consists of *S*-carboxymethyl cysteine, about 1 in every 9 is threonine or proline and about 1 in every 6 or 7 is serine, these four residues between them representing almost two-thirds of the weight of the protein.

For comparison the results of one analysis on a 24-hr hydrolysate of minor components are shown in Table 2. This particular fraction was prepared by chromatography and did not contain either SCMKB1 or the other protein of similar mobility. These proteins too are rich in serine, threonine, proline, and *S*-carboxymethyl cysteine, these four residues making up 54% of the protein. The differences

between SCMKB1 and the minor components are that the latter contain less *S*-carboxymethyl cysteine but more proline, although the sum of the two residues is almost the same in both proteins. There are large differences also in many other residues.

TABLE 1

AMINO ACID COMPOSITION OF SCMKB1

Amino Acid	Amino Acid Nitrogen (as % total N)			Amino Acid Content* (μmoles/g)
	24-hr Hydrolysate	72-hr Hydrolysate	Mean, Maximum, or Extrapolated Value	
Alanine	2·30	2·29	2·29	238
Amide	9·33	11·60	8·56‡	888
Arginine	15·84	14·84	15·34	398
Aspartic acid	0·58	0·57	0·57	60
S-Carboxymethyl cysteine	17·67	17·00	17·90‡	1859
Cystine†	0·13	0·77	—	—
Glutamic acid	7·46	7·41	7·43	772
Glycine	4·82	4·77	4·79	497
Histidine	1·35	1·25	1·30	45
Isoleucine	2·08	2·06	2·07	215
Leucine	1·39	1·39	1·39	144
Lysine	0·72	0·73	0·72	38
Methionine	0	0	0	0
Phenylalanine	0·57	0·56	0·56	59
Proline	9·35	9·31	9·33	969
Serine	10·40	8·15	11·2‡	1163
Threonine	8·22	7·15	8·6‡	893
Tyrosine	1·58	1·23	1·58	164
Valine	3·19	3·02	3·19	331
Total	96·96	94·10	96·82	

* Calculated on a protein nitrogen content of 14·5%.

† No disulphide was detectable in the intact protein.

‡ Extrapolated to zero hydrolysis time.

(*e*) *General Solubility Data*

SCMKB1 was precipitated from aqueous solution by trichloroacetic acid (5%), phosphotungstic acid (2%), and sulphosalicylic acid (5%). It was not precipitated by boiling, although a faint haze developed on cooling. At pH 6 it was not precipitated by calcium, magnesium, or zinc ions but was precipitated by lead, mercury, cadmium, and cupric ions at a concentration of 0·02M.

(*f*) *Electrophoresis and Ultracentrifugation of SCMKB1*

Analysis by moving boundary electrophoresis carried out at pH 4·5, 8·6, and 11 showed only one peak (Fig. 5) although some skewness was evident at pH

11·0, particularly at protein concentrations over 1%. The mobilities measured from the descending boundaries at these pH values are given in Table 3. At alkaline pH values and particularly in the range pH 7–8·5 in Tris–HCl, phosphate, and imidazole–HCl buffers, a concentration-dependent interaction of some sort occurred, which resulted in hypersharp ascending and very spread out descending boundaries.

TABLE 2

AMINO ACID COMPOSITION OF THE HIGH-SULPHUR MINOR COMPONENTS

Amino Acid	Amino Acid Nitrogen* (as % total N)	Amino Acid Content† (μmoles/g)
Alanine	1·98	215
Amide	7·22	784
Arginine	18·74	509
Aspartic acid	2·64	287
S-Carboxymethyl cysteine‡	12·85	1395
Cystine	1·70	185
Glutamic acid	4·72	512
Glycine	3·59	390
Histidine	2·15	78
Isoleucine	2·26	245
Leucine	3·59	390
Lysine	0·76	41
Methionine	0	0
Phenylalanine	1·51	164
Proline	11·05	1200
Serine‡	8·50	923
Threonine‡	7·75	841
Tyrosine	0·85	93
Valine	4·34	472
Total	96·20	

* Determined on 24-hr hydrolysates.

† Calculated on an estimated protein nitrogen content of 15·2%.

‡ Not corrected for destruction.

Further experiments concerning the state of purity of SCMKB1 were carried out by ultracentrifugation of the solutions employed previously for electrophoretic experiments, after adding 0·2M NaCl. Within the pH range 4·5–11 a single symmetrical peak was observed (Figs. 5(*d*) and 5(*e*)).

(*g*) *Optical Rotary Dispersion*

The optical rotation of a 1% solution was measured at a number of wavelengths between 334 and 578 mμ. The specific optical rotation extrapolated to the sodium D line (589 mμ) was −120°. When the results were plotted according to

the method of Moffitt (1956) the curve had zero slope indicating a b_0 close to 0, the implication being that the protein was in the form of a random coil.

(h) Determination of Molecular Weight

The sedimentation coefficients of SCMKB1 determined at 20°C at a protein concentration range between 0·125 and 1·6% are shown in Figure 6(*a*), a value for $S^0_{20,w}$ of 1·65 S being obtained. The partial specific volume of this protein,

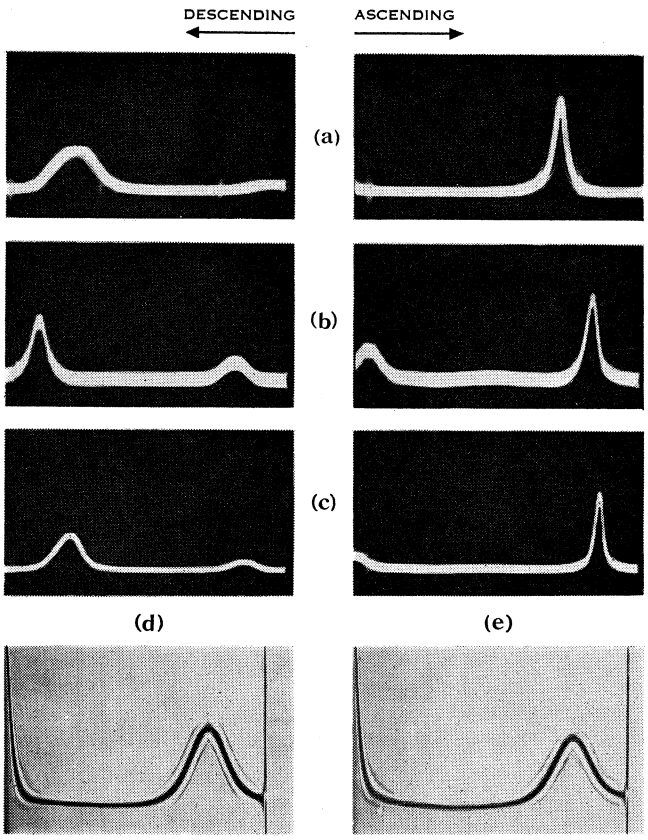


Fig. 5.—(*a*)–(*c*) Moving boundary electrophoresis of SCMKB1 in buffers of ionic strength 0·1: (*a*) acetate buffer, pH 4·5, protein concentration 1%; (*b*) veronal buffer, pH 8·6, protein concentration 0·8%; (*c*) β-alanine–NaOH buffer, pH 11·0, protein concentration 0·7%. (*d*)–(*e*) Ultracentrifugation of SCMKB1 (protein concentration 1%) in buffers of ionic strength 0·1: (*d*) acetate buffer containing 0·2M NaCl, pH 4·5; (*e*) β-alanine–NaOH buffer containing 0·2M NaCl, pH 11·0.

calculated from its amino acid composition (Table 1) by the method of Edsall (1943) was found to be 0·717 (ml/g). No data for the specific volume of the *S*-carboxymethyl cysteine residue being found in the literature, a value of 0·594 was calculated for this residue using the data of Edsall (1943) and McMeekin and Marshall's (1952) value of 0·63 for cystine. The intrinsic viscosity of SCMKB1 was 0·215 $(g/100ml)^{-1}$,

(Fig. 6(*b*)). The molecular weight of SCMKB1 derived from these data by the equation of Scheraga and Mandelkern (1953),

$$M^{\frac{1}{2}} = \{S^0_{20,\,w} \cdot [\eta]^{\frac{1}{2}} . N . \eta_0\} / \beta(1 - \overline{V}\rho),$$

was found to be 24,800. A value of $2 \cdot 55 \times 10^6$ was assumed for β because the rotatary dispersion measurements were consistent with SCMKB1 being in the form of a random coil.

TABLE 3

PHYSICOCHEMICAL AND ANALYTICAL CHARACTERISTICS OF SCMKB1

Physicochemical Data	
Specific refractive index increment (pH 7·0, 5460 Å)	$1 \cdot 88 \times 10^{-3}$
Electrophoretic mobility ($cm^2\ volt^{-1}\ sec^{-1}$):	
pH 4·5	$-6 \cdot 8 \times 10^{-5}$
pH 8·6	$-7 \cdot 2 \times 10^{-5}$
pH 11·0	$-7 \cdot 9 \times 10^{-5}$
Sedimentation coefficient ($S^0_{20,\,w}$)	1·75 S
Specific optical rotation (a_D^{25})	−120°
Calculated partial specific volume, $\overline{V}$ (ml/g)	0·717
Molecular weight:	
Light-scattering technique (Harrap 1962)	27,400
Sedimentation coefficient, intrinsic viscosity, and partial specific volume measurements	24,800
Archibald technique, extrapolated to zero protein concentration	28,100
pH of minimum solubility (in buffer of ionic strength 0·2)	2·65
Extinction coefficient ($E^{1\%}_{1cm}$, 276 mμ), pH = 7·0	5·7
Intrinsic viscosity $(g/100\ ml)^{-1}$	0·215
Analytical Data	
Percentage sulphur, direct analysis	6·7
Percentage sulphur, amino acid analysis	6·1
Percentage nitrogen (microKjeldahl method)	14·54
Amide nitrogen (μmoles/g)	824

Molecular weight determinations by the Archibald (1947) method were conducted as outlined by Schachman (1958). An ultracentrifuge speed of 14,290 r.p.m. was employed and measurements were made only at the air–water meniscus, the corresponding equation

$$M_m = \frac{RT}{(1 - \overline{V}\rho)\omega^2} \cdot \frac{(dc/dx)_m}{x_m C_m},$$

being used in the calculations. The equation of Klainer and Kegeles (1955) was used for correction of change in concentration at the meniscus,

$$C_m = C_0 - \frac{1}{x_m^2} \int_{x_m}^{X} x^2 \cdot \frac{dc}{dx} . dx,$$

where M, R, T, $\overline{V}$, and ω have their usual designations, C_m and $(dc/dx)_m$ are the concentration and concentration gradient at the meniscus, x_m is the distance from the centre of rotation to the meniscus, and X refers to a position in the plateau region where $(dc/dx) = 0$; C_0 is the initial concentration in the cell and was determined by measuring the refractive index of the protein solution and taking into consideration the known optical constants of the ultracentrifuge. The necessary measurements of the photographic plates were made with a microcomparator. Archibald measurements were made at three protein concentrations, 0·25, 0·5, and 1%, and when extrapolated to zero concentration gave a molecular weight of 28,100. For the extrapolation the protein concentration at the meniscus, C_m, was used rather than the initial protein concentration.

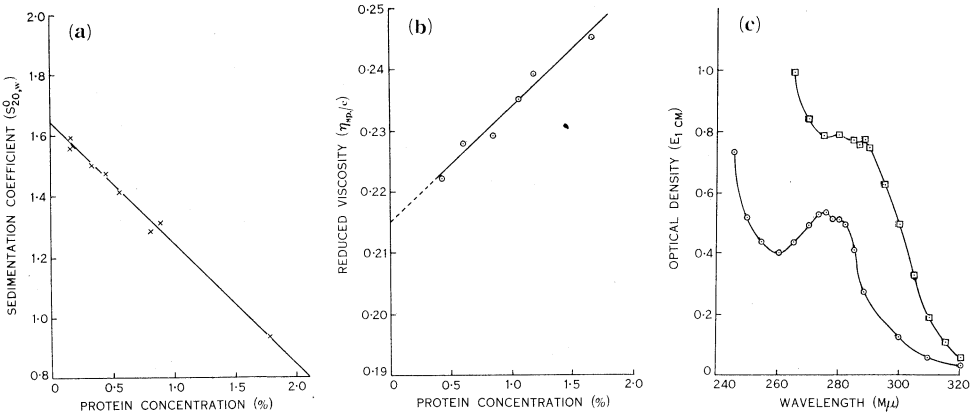


Fig. 6.—(*a*) Sedimentation coefficients of SCMKB1 plotted as a function of protein concentration. Buffer contains 0·2M NaCl and 0·025M each of monosodium and disodium orthophosphates, pH 6·8. (*b*) Intrinsic viscosity of SCMKB1, measured at pH 6·8 in a buffer containing 0·2M NaCl and 0·025M each of monosodium and disodium orthophosphates. (*c*) Ultraviolet absorption curves of SCMKB1 at a protein concentration of 0·092%. ○ pH 7, 0·1N KCl. □ pH 13, 0·1N NaOH.

(*i*) *Spectral Data*

The ultraviolet absorption curve of SCMKB1 was determined at pH 7 and 13, (Fig. 6(*c*)). From the pH 7 curve, which shows a minimum at 259 mμ and a maximum at approximately 276 mμ, an extinction coefficient ($E^{1\%}_{1cm}$, 276 mμ) of 5·7 was calculated. At pH 13 the expected curve with two maxima resulting from the known shift of tyrosine absorption and with a minimum between 250 and 260 mμ (Bencze and Schmid 1957) was not observed but instead there was an enhancement in general absorption. For this reason it was not possible to measure the tryptophan content of the protein by spectrophotometric means.

IV. Discussion

Although SCMKB1 gives single boundaries on electrophoresis and ultracentrifugation over a wide range of pH and concentration it has not yet been established that it is in fact a homogeneous protein.* However, purification to the

* At present SCMKB1 should be regarded simply as a fraction.

single-boundary stage has enabled a study to be made of the composition of this fraction in relation to that of the high-sulphur proteins as a whole. SCMKB1 is obviously related to the group of high-sulphur proteins which are characterized by richness in *S*-carboxymethyl cysteine, proline, serine, and threonine. Nevertheless there are major differences in the amino acid composition of this fraction and of the remaining high-sulphur proteins in practically every amino acid residue. As the extinction coefficients of the two fractions are very similar, the low value of tyrosine in the analytical values of the minor components may be in error or may be compensated for by more tryptophan and phenylalanine. SCMKB1 is estimated to contain 7·5% sulphur when in the native state in wool. As far as is known the only protein containing a higher concentration of sulphur appears to be the recently isolated metallothioneine from equine renal cortex (Kaji and Vallie 1961) which contains 8·5% sulphur.

SCMKB1 also contains a comparatively large amount of amide nitrogen. Analysis by the method of Leach and Parkhill (1956) showed the presence of 824 μmoles/g, suggesting that most, if not all, of the aspartic and glutamic acid residues (835 μmoles/g) are present as their amides. These values for amide content are a little higher than those obtained with high temperature preparations and extraction of the high-sulphur proteins with concentrated thioglycollate at 0°C is therefore the preferable procedure on this account.

Because there are apparently so few free carboxyl groups the isoelectric point of SCMKB1 in the native state should lie between the pK_a values for the tyrosyl and arginyl residues and nearer the latter than the former. But moving boundary electrophoresis of an unfractionated high-sulphur *S*-carbamidomethyl kerateine does not support this for, at pH 11, all components moved to the anode. If this is a true measure of the location of the isoelectric point and not an aberration caused by ion binding, then the results suggest that acidic groups are present which have not been assayed, or that some basic groups are masked. Dr. B. S. Harrap of this Laboratory has pointed out to the author that a similar effect might be realized if some of the amide groups were attached to the *C*-terminal ends of the peptide chains. That there are several *C*-terminal groups per molecule is suggested by the recent work of O'Donnell, Thompson, and Inglis (1962).

The sulphur content of SCMKB1 has been measured in two ways; by direct microanalysis and by determination of the *S*-carboxymethyl cysteine content (no methionine and only traces of cysteine are present). The two values (Table 3) obtained are in poor agreement, the microanalytical value being the greater by 9·8%. If the analysis for *S*-carboxymethyl cysteine is correct, then some unidentified compound must be present in the intact protein, either bound strongly enough to resist removal by exchange with chloride and acetate or in covalent linkage. Lindley (1948) reported the presence of variable amounts of unidentified sulphur compounds in a wide variety of α-keratins and so the presence of some such compound in the high-sulphur proteins would not be unexpected. The unusual spectral curve in alkaline solution may also be due to the presence of this material. However, *S*-carboxymethyl cysteine is partially destroyed during hydrolysis and there is the

possibility that this residue may be underestimated by using a linear extrapolation to zero hydrolysis time. Work is in progress on this problem.

The concentration-dependent anomalous behaviour during electrophoresis at pH values around 7 and 8 may be due to protein–buffer or protein–protein interaction. The slight skewness observable at pH 8·6 and 11 may also stem from these causes. However, Woods (1959) after his study on the electrophoresis of the low-sulphur wool proteins suggested that this sort of behaviour can reflect heterogeneity.

There is satisfactory agreement between the molecular weight estimates for SCMKB1 by the Archibald and light-scattering methods (Harrap 1962). Whether these values represent an aggregated form, in view of the molecular weight of 9000 found by Harrap (1957) with a surface balance technique for an unfractionated high-sulphur protein, can only be decided by further study. However, the high-sulphur proteins of Merino wool appear to be of lower molecular weight than the low-sulphur proteins (Harrap and Woods 1958).

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