

KERATIN FIBRES

VII.* PROTEINS OF THE HISTOLOGICAL COMPONENTS OF KANGAROO FIBRES

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Abstract

Cortical cells, cuticle, and an impure sample of medulla were prepared from kangaroo fibres by vigorous agitation in formic acid at room temperature, which control experiments show causes no degradation of soluble high- or low-sulphur proteins. Many reductive extraction procedures were examined and the best method appears to be treatment with 0.2M thioglycollate at pH 11.0 in 8M urea for 3 hr at 40°C, followed by coupling with iodoacetate. The high- and low-sulphur proteins from kangaroo fibres and its histological components were prepared and examined by gel filtration using Agarose in 8M urea at pH 8 with a column calibrated for measurement of molecular weight.

The amount of protein extracted by thioglycollate from kangaroo fibres (57%) is less than that from wool because of the presence of non-extractable medulla in the former case. The 42% of soluble protein extracted from cuticle by thioglycollate does not contain low-sulphur proteins and most of the high-sulphur proteins have molecular weights < 12,500. The high-sulphur proteins of molecular weight range 15,000–30,000 and the low-sulphur proteins of molecular weight range 40,000–70,000 from kangaroo fibres arise almost entirely from the cortical cells. The proteins which are rich in glycine and aromatic amino acids originate from the cortical cells of kangaroo fibres. In wool, the bulk of these proteins are from the microfibril-matrix structure of the cortical cells and a small fraction from the cell membrane complex.

I. INTRODUCTION

A very large amount of work has been done over many years on the development of methods, which are relatively mild and (hopefully) specific, for the scission of a particular type of bond in the three-dimensional protein network of keratin fibres (reviewed by Crewther *et al.* 1965*a*). By this means solubilization of the bulk of the proteins in the keratin are achieved. Thus, it becomes possible to apply the normal techniques of protein chemistry for the separation of the complex mixture of proteins (O'Donnell and Thompson 1964; Cole *et al.* 1965; Crewther *et al.* 1965*b*; Swart, Haylett, and Joubert 1969; Swart, Joubert, and Strydom 1969) and the determination of the sequences of particular proteins (Haylett and Swart 1969; Haylett *et al.* 1971; Corfield and Fletcher 1969; Lindley *et al.* 1971).

The question of the sites in the fibre from which the various proteins originate has been inferred by various indirect methods (reviewed by Crewther *et al.* 1965*a*), but can only be finally resolved by a direct approach which involves separation of the histological components by *mild* methods, followed by dissolution of the insoluble

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keratin. This has been attempted for cortical cells (Ward and Bartulovich 1956; Thompson and O'Donnell 1965; Asquith and Parkinson 1966), orthocortical and paracortical cells (Bradbury, Chapman, and King 1968; Kulkarni, Robson, and Robson 1971), and cuticle (Asquith and Parkinson 1966). Unfortunately, almost all of the methods for separation of histological components are degradative to some extent and we have spent many years developing methods which are as mild as possible (Bradbury, Chapman, and King 1968; Bradbury and O'Shea 1969; Bradbury and Ley 1972; Peters and Bradbury 1972).

In this paper we are concerned with the dissolution and preliminary separation and analysis of the proteins of the kangaroo fibre and of the cuticle, cortical cells, and medulla therefrom. Kangaroo fibres were chosen for study because, unlike fine wool, they contain medulla, and yet are similar in appearance (Bradbury and Leeder 1970) and in the amino acid composition of their cuticle and cortical cells to wool (Bradbury *et al.* 1970).

II. EXPERIMENTAL

Kangaroo fibres were obtained from the belly of a red kangaroo (*Megaleia rufa*) and were supplied by the Division of Wildlife Research, CSIRO, Canberra. They were cleaned by successive extractions, with agitation, in methanol, chloroform, and light petroleum at room temperature. The fibres were air-dried, washed in four changes of warm water (temp. < 35°C) for 10 min and dried in air. Merino 64's fibres were cleaned as described by Bradbury *et al.* (1966). Insulin (bovine pancreas) and ribonuclease-A (five times crystallized) were obtained from Sigma Chemical Co., pepsin (twice crystallized) from Worthington Biochemicals, and bovine serum albumin and sperm whale myoglobin from Mann Laboratories. The first four proteins were reduced and coupled with iodoacetic acid using the method described by Hirs (1967). The formic acid used was reagent grade which was distilled before use to remove solid residues. Other chemicals were of reagent grade purity and were used without further purification.

(a) Preparation of Histological Components

Samples (1 g) of red kangaroo fibres were agitated for 10 min in formic acid using a Vibromix agitator, in order to remove skin flakes (Bradbury *et al.* 1966). The fibres were separated from dispersed material by pouring through a coarse stainless steel sieve and the dispersed material separated from the formic acid by sedimentation at 4000 *g* for 30 min. The dispersed material was found by light microscopy to consist of skin flakes and cuticle and was discarded. The formic acid solution was lyophilized.

The fibres were suspended in 50 ml of formic acid and agitated in the Vibromix stirrer for four successive 1-hr periods. At the end of each period the dispersed material was collected as previously described, as was the material soluble in each of the 50-ml aliquots of formic acid. The combined samples of dispersed material consisted entirely of pieces of cuticle cells and some cortical cells with no medullary cells. The formation of medullary cells was avoided by not cutting the fibres into short lengths prior to treatment. The formic acid was removed by successive washings with ethanol and centrifugations and a pure sample of cuticle was prepared from this material by the sieving procedure of Bradbury and Chapman (1964).

The residual fibres were again suspended in 50 ml of formic acid at 4°C and chopped in a Polytron stirrer (Kinematica, G.M.B.H., Luzern, model PT20) operating at minimum speed for 5 min. This treatment produced a mixture of cuticle pieces, cortical cells, disrupted cortical cells (Bradbury and Chapman 1964; Peters 1971), and medullary cells. The treatment was repeated for a total of 12 periods each of 5 min duration after which no appreciable amount of residual fibres remained. The dispersed material from the latter treatments was free of cuticle and thus it was possible, by sieving through the 18- μ m screen (Bradbury and Chapman 1964), to obtain a sample of medullary cells free of intact cortical cells (which were held on the screen) and cuticle. However, the sample of medulla contained a considerable quantity of small, disrupted cortical cells which were produced by the Polytron treatment and could not be removed by sieving.

Cortical cells were readily obtained as the material which was held back by a 40- μ m screen during all stages of the treatment, yield 39.5%. A sample of cuticle, the purity of which was checked by light microscopy, was obtained by the Vibromix treatment followed by sieving, yield 3.9%. A mixed sample of medulla and disrupted cortical cells was obtained from the latter stages of the Polytron treatment, yield 3.8%. The material soluble in formic acid was obtained by combination of all the formic acid solutions and sedimentation at 4°C and 4000 *g* for 18 hr after which the solution was still slightly opalescent. The formic acid supernatant was then lyophilized and the soluble protein obtained with a yield of 10%.

(b) *Extraction of Soluble Proteins from Kangaroo Fibres and Histological Components*

Protein has been extracted from insoluble keratins by many different methods (Crewther *et al.* 1965a) and we have compared various methods which involve fission of the disulphide bonds of kangaroo fibres, in an attempt to achieve the best method.

(i) *Reduction and Coupling of Keratin followed by Extraction*

The reduction of the kangaroo fibres was carried out essentially using the "sequential one-bath process" of Maclaren and Sweetman (1966) with tri-*n*-butylphosphine and the reduced proteins coupled with iodoacetic acid (24 hr alkylation, Hirs 1967). Various solution conditions were used including 100% formamide (Sweetman and Maclaren 1966), aqueous borate buffer at pH 8 containing 30 or 50% *n*-propanol or 8M urea. The extent of reduction was determined by the amount of cystine left in the reduced and alkylated fibres, which was determined by amino acid analysis using the Technicon system essentially as described by Bradbury, Chapman, and King (1965a). The lowest residual cystine content was 0.12 mole per 100 moles (compared with 9.3 moles per 100 moles for untreated kangaroo fibres) which was obtained when the reaction in 30% *n*-propanol was carried out in a sealed tube over nitrogen. Other values ranged up to 0.33 with one value as high as 0.82 mole per 100 moles. Similar, low residual values of cystine (1.2–8.9% of that in untreated kangaroo fibres) have been observed by Maclaren and Sweetman (1966) and Peters (1971) in the reduction and coupling of wool. They may perhaps result from incomplete reduction of disulphide bonds, even in the presence of denaturants such as 8M urea. However, this is unlikely since Maclaren (1971) has achieved quantitative reduction and coupling using 4-vinylpyridine as coupling reagent (Friedman and Noma 1970). It is probable that there is some reoxidation of the reduced keratin during the coupling with iodoacetic acid, which would also explain the results of other workers (Maclaren and Sweetman 1966; Maclaren 1971; Peters 1971). The formation of cystine from *S*-carboxymethyl proteins during acid hydrolysis occurs under conditions of reflux (Gillespie 1963), but apparently not *in vacuo*, as used in this work.

The extraction procedure consisted of taking a known weight of keratin (dried *in vacuo* for 8 hr at room temperature), and shaking it in formic acid (liquor to keratin ratio of $\leq 50 : 1$) at room temperature for the requisite period. Residual kangaroo fibres were separated from the formic acid by passage through a coarse stainless steel mesh and the fine material separated by centrifugation at 4°C at 4000 *g* for 18 hr. The total solid material was washed, dried *in vacuo* for 8 hr at room temperature, and weighed. The amounts of material extracted by an 8-hr formic acid treatment of (1) untreated kangaroo fibres, (2) reduced and alkylated kangaroo fibres, and (3) reduced and alkylated cortical cells were 4, 38, and 74% respectively. Extraction of reduced and alkylated kangaroo fibres for 12 hr in formic acid removed 52%. Increase of the time of alkylation with iodoacetic acid from 4 hr as in (2) and (3) above to 16 hr caused an appreciable reduction in the amount of material subsequently extracted by the formic acid treatment as observed by Maclaren, Kilpatrick, and Kirkpatrick (1968). Whilst there is a considerable increase in the amount of material extracted by formic acid from kangaroo fibres as a result of the reduction and coupling procedure, nevertheless the 52%* extraction after 12 hr is less than that obtained by direct extraction of fibres with thioglycollate (see below). Since the latter procedure is also much simpler it was used in preference to the former.

* This result is in reasonable agreement with the 59% extracted by formic acid in a much longer treatment (2 days) from reduced and alkylated wool by Maclaren, Kilpatrick, and Kirkpatrick (1968).

(ii) *Direct Extraction Methods on Kangaroo Fibres*

Preliminary experiments on the extraction of soluble protein using tri-*n*-butylphosphine in 100% molar excess over the disulphide content at pH 10.4 for 24 hr at room temperature in 8M urea or 6M guanidine hydrochloride produced disappointingly low yields of 30 and 23% respectively. These procedures were discontinued (O'Shea 1970), although it is worth noting that Maclaren and Kilpatrick (1969) at about the same time achieved 70% solubilization of wool by similar reductions with tri-*n*-butylphosphine in aqueous media containing 19% *n*-propanol and 5M sodium iodide.

Fibres were extracted by gentle shaking in a solution (liquor to wool ratio 30 : 1) containing 0.2M thioglycollate at pH 11.0 in 8M urea either for 18 hr at 4°C or for 3 hr at 40°C (Gillespie 1964). The insoluble residue was separated by filtration through a No. 1 glass filter and the extract was alkylated with iodoacetic acid (Hirs 1967). The extractions at 4 and 40°C yielded 44 and 57% of soluble proteins respectively. The latter method was considered to be the most suitable for further studies on histological components.

(iii) *Extraction of Histological Components with Thioglycollate*

A known dry weight of the histological components was wet out by repeated evacuation at the water vacuum pump in an aqueous solution (liquor to wool ratio 30 : 1) containing 8M urea and 0.2M thioglycollate and adjusted to pH 11.0. The mixture was gently agitated at 40°C for 3 hr and then centrifuged at 40,000 *g* for 30 min. The supernatant solution was removed and the protein coupled with iodoacetic acid (Hirs 1967). The solution was dialysed for 3 days against running tap water, for 1 day against about six changes of distilled water, and the solution lyophilized to obtain the yield of *S*-carboxymethyl protein. The yields are shown in Table 1.

TABLE 1
S-CARBOXYMETHYL PROTEINS FROM KANGAROO FIBRES AND HISTOLOGICAL COMPONENTS

Compound	Extraction of SCM-protein (%)	Percentage SCM-protein in:	
		High-sulphur fraction	Low-sulphur fraction
Kangaroo fibre	57	53	47
Cortical cells	75	46	54
Cuticle cells	42	100	—
Medulla + disrupted cortical cells	26	42	58
Formic acid-soluble fraction	100	22	78

(c) *Separation of High- and Low-sulphur Proteins*

The SCM-proteins were dissolved in formic acid at room temperature, dialysed against tap water for 3 days, and then against acetic acid-sodium acetate buffer (0.4M) at pH 4.4. The low-sulphur protein precipitated was centrifuged and washed with buffer solution (stage 1 of Gillespie 1964). It was precipitated twice more according to stages 2 and 3 of Gillespie (1964), in order to remove contaminating high-sulphur protein. The supernatant solutions from the three centrifugations were dialysed against distilled water for 3 days, and the solution lyophilized to obtain the high-sulphur protein.

(d) *Gel Filtration in Urea*

A jacketed chromatographic column 3.8 cm in diameter was filled to a height of 76 cm with 6% cross-linked Agarose (Biorad Laboratories, Biogel A-5M) dispersed in a buffer at pH 8 containing 0.1M tris(hydroxymethyl)aminomethane (Tris), 8M urea, 0.01M ethylenediamine-tetraacetic acid (EDTA), and 0.02% sodium azide. Protein (20 mg) dissolved in the buffer and

sucrose was applied at the top of the column and the column was eluted at 4°C with the buffered solution using a flow rate of 25 ml/hr. The effluent was monitored at 280 nm by an L.K.B. Uvicord II recording spectrophotometer and then 5-ml fractions were collected in an L.K.B. fraction collector. The elution volume of a particular peak was obtained with considerable precision by use of the recorder, although there was some change of elution volume over a period of several months. The column was calibrated with regard to molecular weight measurements of random-coil proteins devoid of disulphide bonds in 8M urea, by measurement of the elution volume of SCM-insulin, SCM-ribonuclease, myoglobin, SCM-pepsin, and SCM-bovine serum albumin and construction of a graph of elution volume against log (molecular weight) as shown in Figure 1 (Ackers 1970). For preparative purposes the appropriate fractions obtained by the fraction collector were combined, dialysed against running water for 3 days, and then concentrated to small volume in a Diaflo ultrafiltration apparatus using UM-05 membranes. The solution was then lyophilized and the residue subjected to amino acid analysis essentially as described by Bradbury, Chapman, and King (1965a).

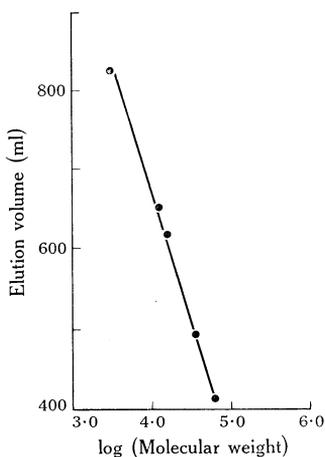


Fig. 1.—Calibration of elution volume against log (molecular weight) for SCM-insulin, SCM-ribonuclease, myoglobin, SCM-pepsin, and SCM-bovine serum albumin, respectively, in ascending order of molecular weight.

(e) Preparation of High- and Low-sulphur Proteins from Wool

Wool was reduced for 22 hr in a solution of 0.2M thioglycollate in 8M urea at pH 10.6 and was then alkylated with excess iodoacetate at pH 8–9 until the nitroprusside test for thiol was negative. The solution was dialysed for 3 days and the low-sulphur proteins separated from high-sulphur proteins by the three-stage precipitation procedure of Gillespie (1964).

III. RESULTS AND DISCUSSION

(a) Possibility of Degradation of Histological Components during Preparation

The possible degradation of proteins because of immersion and ultrasonic disintegration in formic acid has been reviewed and studied by Bradbury, Chapman, and King (1965a) and is the subject of a more searching study by O'Shea and Bradbury (1972). As part of this latter study we have compared the gel-filtration patterns of the low-sulphur protein from wool (SCMKA) before and after a 4-hr Vibromix treatment in formic acid followed by 30 min treatment in the Polytron [Figs. 2(a) and 2(b)]. The gel-filtration patterns of the high-sulphur proteins from wool (SCMKB) have also been compared before and after a 4-hr Vibromix treatment in formic acid as shown in Figures 2(c) and 2(d). It is noted that the chromatograms of both SCMKA and SCMKB are unchanged, within experimental error, as a result of

the treatments which are essentially the same as those used in the preparation of the histological components. A reduction in molecular weight of the soluble proteins resultant on, say, peptide-bond hydrolysis would have been observed as an increase in the area of peaks at higher elution volume. The absence of any appreciable change in the elution profile of these *soluble* proteins, which together make up the bulk of the wool fibre [which is very closely related to the kangaroo fibre (Bradbury *et al.* 1970)] is good evidence that there is little likelihood of chemical attack of the *insoluble* and hence less reactive proteins of kangaroo keratin.

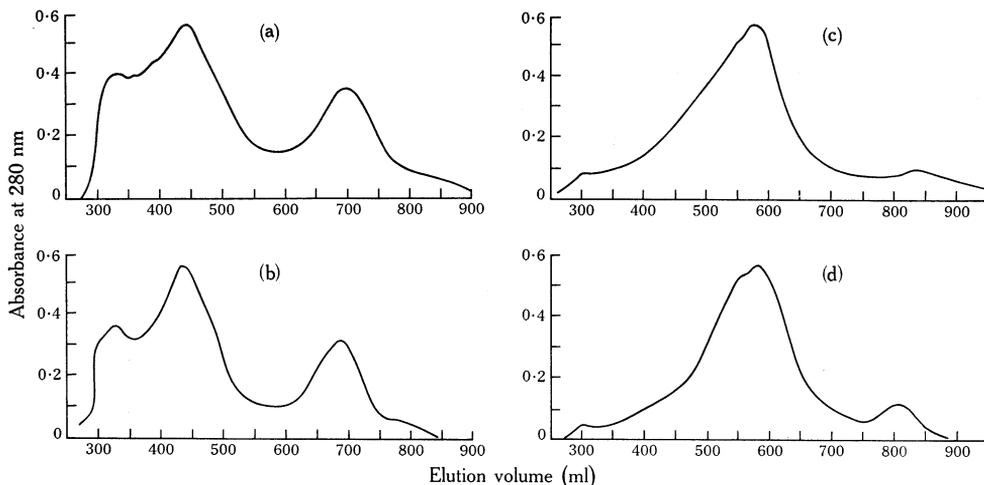


Fig. 2.—Gel filtration chromatogram of (a) low-sulphur protein from wool (SCMKA); (b) SCMKA after Vibromix stirring for 4 hr in formic acid at room temperature, and Polytron mixing for 30 min; (c) high-sulphur protein from wool (SCMKB); (d) SCMKB after 4 hr Vibromix stirring in formic acid at room temperature.

(b) *Extraction of Proteins from Kangaroo Keratin*

The maximum amount of material extractable from wool by oxidation with peracetic acid or performic acid or by reduction with thioglycollate is about 75–90% (Alexander and Hudson 1954; Harrap and Gillespie 1963; Gillespie 1964; O'Donnell and Thompson 1964). By comparison, we have obtained 57% extraction with kangaroo fibres (see Table 1) and Gillespie 67% (J. M. Gillespie, personal communication). The major reason for the lower value with kangaroo as compared with wool is the presence of about 10% medulla in kangaroo fibres (Bradbury *et al.* 1970), which is virtually completely resistant to extraction by alkaline thioglycollate. This resistance is shown by the fact that only 26% of the sample of medulla and disrupted cortical cells is extracted by thioglycollate and the extract has the same fraction of high- and low-sulphur proteins as the cortical cells (Table 1). In view of the very large difference in amino acid analysis of medulla and cortical cells, it is clear that the 26% of extracted material must originate from the disrupted cortical cells and not the medulla. Harding and Rogers (1971) have recently established the presence of γ -glutamyl- ϵ -lysyl cross-links in medulla and this seems to be the origin of its well-known resistance to alkaline solutions (Matoltsy 1953; Bradbury and O'Shea 1969).

A small concentration of such cross-links (Asquith and Otterburn 1971; Cole *et al.* 1971; Milligan, Holt, and Caldwell 1971) or the possibility of a low concentration of disulphide bonds, which are resistant to thioglycollate attack even in 8M urea, or both, is the probable explanation for the extraction of only 42% from cuticle cells as compared with 75% from cortical cells (Table 1). On the other hand the cortical cells produced by dispersion of wool in 6M HCl at room temperature for 28-90 hr by Ward and Bartulovich (1956) were dissolved nearly completely by mercaptoethanol at pH 8 and the cortical cells and cuticle produced by treatment with papain-sodium sulphite by Asquith and Parkinson (1966) were virtually completely soluble in performic acid. The apparent discrepancy between these results and our own (Table 1) results from the severity of their methods of preparation of histological components, which most likely produced an appreciable amount of peptide-bond hydrolysis.

(c) *High-sulphur Proteins*

The gel-filtration patterns for the high-sulphur proteins from the kangaroo whole fibres and of cortical cells, cuticle, and of the material soluble in formic acid are given in Figure 3, and the amino acid analyses of representative fractions (cut as

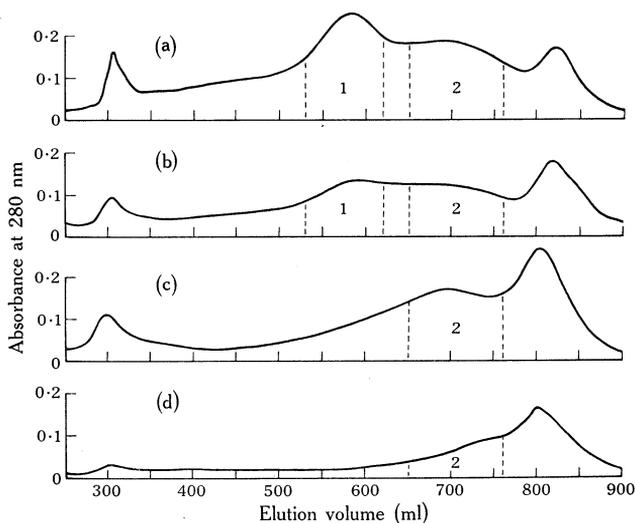


Fig. 3.—Gel-filtration chromatogram of high-sulphur proteins from (a) kangaroo fibres, (b) kangaroo cortical cells, (c) kangaroo cuticle, and (d) material soluble in formic acid.

shown in Fig. 3) are given in Table 2. One notes the presence of a small amount of highly aggregated material which appears at an elution volume of about 310 ml, and corresponds to the void volume of the column.

Fraction 1, which corresponds to material with a molecular weight in the range of 15,000–30,000, is most prominent in the whole fibre and cortical cells, occurs to a small extent in the cuticle, and is almost non-existent in the material from the fraction soluble in formic acid. It obviously constitutes a major fraction of the high-sulphur proteins from kangaroo fibre, which have molecular weights of the same order as those

from wool (Haylett *et al.* 1971). Furthermore the two amino acid analyses of fraction 1 proteins from kangaroo whole fibre and cortical cells are the same within experimental error, except for tyrosine which is disregarded because it has been very low and extremely variable in all the fractions obtained from the gel-filtration column. It is therefore reasonable to conclude that the high-sulphur proteins of molecular weight > 15,000 originate from the cortical cells.

TABLE 2

AMINO ACID ANALYSES OF HIGH-SULPHUR PROTEIN FRACTIONS FROM GEL-FILTRATION STUDIES*

Values expressed as moles per 100 moles

Amino acid	Unfractionated high-sulphur protein from kangaroo fibre	Fraction 1 (Fig. 3) from		Fraction 2 (Fig. 3) from			
		Fibre	Cortical cells	Fibre	Cortical cells	Cuticle	Material soluble in formic acid
Alanine	3.10	3.48	3.66	5.15	4.98	6.42	5.49
Arginine	6.10	6.60	6.45	6.06	5.96	1.90	2.96
Aspartic acid	4.28	4.79	4.71	4.78	5.47	3.87	6.66
Cysteic acid†	0.07	0.72	1.05	1.16	1.44	1.55	2.10
SCM-cysteine	21.82	18.95	16.23	13.25	15.21	2.42	0.00
$\frac{1}{2}$ Cystine†	0.26	0.98	2.45	1.17	1.51	6.77	2.59
Cysteic acid + SCM-cysteine + $\frac{1}{2}$ cystine	22.15	20.65	19.73	15.58	18.16	10.74	4.69
Glutamic acid	8.27	9.07	10.07	8.03	9.09	6.97	9.62
Glycine	7.25	7.01	6.91	10.02	8.31	16.94	10.80
Histidine	1.25	1.65	1.51	2.04	1.12	1.19	4.94
Isoleucine	2.18	2.66	2.75	2.78	2.67	1.94	3.02
Leucine	3.29	3.69	4.00	4.70	4.02	4.65	5.92
Lysine	0.83	1.07	1.05	1.69	1.64	2.68	5.12
Methionine	0.24	0.25	0.22	0.49	0.37	0.97	1.23
Phenylalanine	1.72	1.90	1.74	2.53	2.11	1.77	3.02
Proline	11.59	12.63	13.13	11.25	12.84	11.29	10.12
Serine	11.12	10.10	10.03	10.19	9.97	10.48	13.58
Threonine	10.00	8.49	8.41	7.62	7.82	6.00	5.98
Tyrosine‡	2.11	1.21	0.68	1.77	0.37	0.48	1.48
Valine	4.52	4.75	4.95	5.32	5.10	11.71	5.37

* Recovery of anhydroamino acids from the unfractionated protein was high (> 88%) but from all the fractions was low (about 30%) due to the solubility of Agarose in 8M urea (Determann 1968).

† Occurrence of cystine and cysteic acid in acid hydrolysates of SCM-proteins may occur due to the instability of SCM-cysteine under acid oxidizing conditions (Gillespie 1963; Harrap and Gillespie 1963). However, the large value of cystine (column 7) is probably due to incomplete reduction of cystine.

‡ The tyrosine values for the unfractionated proteins (column 2) are correct, but for all fractions from the gel-filtration columns the tyrosine results are low and variable, probably due to the presence of carbohydrate (Agarose) during the acid hydrolyses.

Fraction 2 occurs to a considerable extent in whole fibre, cortical cells, and cuticle and slightly in the material which is soluble in formic acid. It corresponds to the molecular weight range 7000–12,500, and contains within it a group of high-sulphur proteins, some of which from wool (molecular weight 11,000) have been sequenced

(Haylett *et al.* 1971). The amino acid analyses of the fraction 2 proteins from whole fibre and cortical cells in Table 2 are similar but show significant differences for aspartic acid, cysteic acid + SCM-cysteine + $\frac{1}{2}$ cystine, glycine, histidine, methionine, phenylalanine, and proline. In all cases except for aspartic acid and phenylalanine which are doubtful the amino acid analysis of the whole fibre can be rationalized in terms of the analyses of the three fractions from cortical cells, cuticle, and material soluble in formic acid respectively. Of course the relative contributions of the last two will be small relative to that from cortical cells because of the relatively small amount of soluble, high-sulphur* cuticle (4% of original fibre) and high-sulphur* formic acid-soluble material (2% of original fibre). One notes also the large differences in composition between the analyses of fraction 2 proteins from (1) cuticle, (2) the material soluble in formic acid, and (3) whole fibre or cortical cells. The amino acid analyses in Table 2 show that the high-sulphur proteins of molecular weight between 7000 and 12,500 originate not only from cortical cells but also from the cuticle and probably the cell membrane complex too, which is one source of the material that is soluble in formic acid (Bradbury, Leeder, and Watt 1971).

The low molecular weight fraction in Figure 3 is material of molecular weight <7000 which is shown to occur in all cases, but appears to be the major constituent from cuticle and the material soluble in formic acid. Although it is possible that some of this material may be degraded products resulting from peptide-bond fission during preparation of fractions, nevertheless there is evidence of the extraction of material of low molecular weight from wool by mild methods (Zahn and Meienhofer 1956; Zahn and Biela 1968*a*, 1968*b*). It is interesting that this material was not lost during the dialysis procedure used to prepare the high-sulphur protein; perhaps it aggregates or is loosely bound to higher molecular weight protein in the dialysis bag (Peters 1971), but the aggregation is largely removed during chromatography in 8M urea.

In Table 2 it is observed that all fractions contain a small but appreciable amount of methionine, whereas the high-sulphur fractions obtained by Gillespie and Inglis (1965) from a wide range of keratin fibres including kangaroo are all free of this amino acid. We have used their preparative procedures, hence do not consider it likely that our high-sulphur fractions are contaminated with low-sulphur protein. However, the integrity of the separation is dependent on the protein concentrations used (J. M. Gillespie, personal communication) and it is possible that the protein concentrations used in our work were lower (due to dilution during dialysis) than those of Gillespie (1964), which were also indeterminate because of dialysis. The presence of a small amount of highly aggregated material in the gel-filtration patterns in Figure 3, could also be due to some low-sulphur protein contaminant.

(d) *Low-sulphur Proteins*

The gel-filtration chromatograms in Figure 4 show that there is a large amount of material of molecular weight >70,000 from the whole fibre, an even greater concentration from cortical cells, no low-sulphur protein at all extracted from cuticle (see Table 1),

* The term "high-sulphur protein" is used throughout the paper for the fraction which remains in solution during the fractionation procedure of Gillespie (1964). In these particular cases the actual protein has less sulphur than the whole fibre (Bradbury *et al.* 1970) and thus the term is somewhat of a misnomer.

and some from the material soluble in formic acid. By analogy with work on the low-sulphur proteins from wool (Thompson and O'Donnell 1965) it is believed that this material is an aggregate of the main fraction [fraction 2, Fig. 4(a)]. This is confirmed by the similarity between the amino acid analyses of fractions 1 and 2 of Figure 4(a) as shown in Table 3. However, a simple, slow aggregation of material in fraction 2 to form fraction 1 is obviously an oversimplification because of the differences in the ratio of the relevant peak areas in Figures 4(a), 4(b), and 4(c).

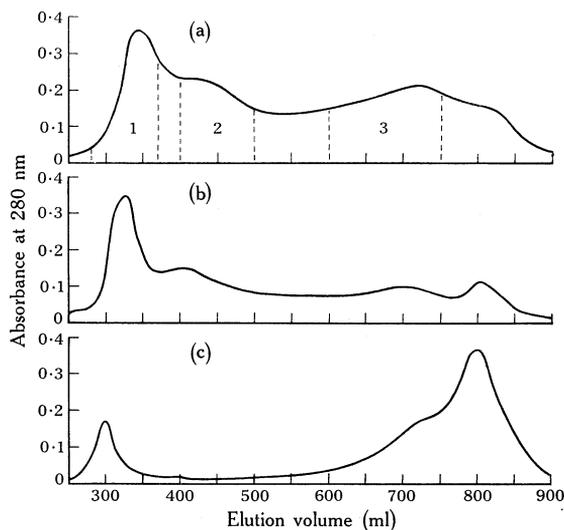


Fig. 4.—Gel-filtration chromatogram of low sulphur proteins from (a) kangaroo fibres, (b) kangaroo cortical cells, and (c) material soluble in formic acid.

Fraction 2 in Figure 4(a) covers the range of molecular weight of 40,000–70,000 and as already stated is probably the main fraction of the low-sulphur protein from kangaroo. By comparison two of the major components of the low-sulphur protein of wool have molecular weights of 45,000 and 51,000 (Thompson and O'Donnell 1965; Jeffrey 1969, 1970). This material occurs to a greater degree in whole fibre than in cortical cells and to a very small extent in the material soluble in formic acid.

Fraction 3 covers the molecular weight range 7000–18,000 and occurs to a larger extent in the whole fibre than in cortical cells and is also evident in the material soluble in formic acid. The amino acid analysis of this fraction (Table 3) shows a very high content of glycine and phenylalanine as does the corresponding high glycine fraction from wool (Harrap and Gillespie 1963; Crewther *et al.* 1965a; Gillespie and Darskus 1971), but unfortunately we were unable to compare the tyrosine content because of the very low values for all fractions from the column. Confirmation that this is the glycine–aromatic-rich fraction comes from its position on the chromatogram, which agrees with that obtained for the same protein fraction from wool run on Sephadex G200 in 8M urea at pH 7.4 (O'Donnell and Thompson 1965). Furthermore, its occurrence in the low-sulphur fraction in the molecular weight range 7000–18,000 agrees with the molecular weight of 12,000 obtained by Gillespie and Darskus (1971).

(e) Site in Fibre of Glycine-Aromatic-rich Proteins

These proteins occur in fraction 3 of the low-sulphur proteins from kangaroo. Amino acid analyses of fractions 3 and 4 of the material soluble in formic acid [Fig. 4(c)] shows no evidence of a high glycine or phenylalanine content (O'Shea 1970), hence the material does not originate from this source, although previous work on wool has shown that formic acid removes a small amount of a glycine-aromatic-rich fraction, probably from the cell membrane complex (Bradbury, Chapman, and King 1968). It cannot be produced from the cuticle, since there are no low-sulphur proteins produced from this source. We therefore conclude that this material must come from the cortical cells.

TABLE 3

AMINO ACID ANALYSES OF LOW-SULPHUR PROTEIN FRACTIONS FROM GEL-FILTRATION STUDIES*
Values expressed as moles per 100 moles

Amino acid	Unfractionated sample	Fraction 1 (Fig. 4a)	Fraction 2 (Fig. 4a)	Fraction 3 (Fig. 4a)
Alanine	6.26	6.86	7.33	6.49
Arginine	6.40	6.40	6.25	5.58
Aspartic acid	8.64	9.08	9.46	7.57
Cysteic acid†	0.22	0.52	0.36	0.69
SCM-cysteine	7.25	7.09	5.98	2.91
$\frac{1}{2}$ Cystine†	0.00	0.22	0.00	0.00
Cysteic acid + SCM-cysteine + $\frac{1}{2}$ cystine	7.47	7.83	6.34	3.60
Glutamic acid	14.35	14.35	16.88	9.46
Glycine	8.69	8.45	6.84	22.54
Histidine	1.35	1.55	1.77	2.55
Isoleucine	3.83	3.77	4.48	3.21
Leucine	9.62	9.54	10.81	8.45
Lysine	4.43	4.69	5.43	3.67
Methionine	1.15	1.09	1.45	0.83
Phenylalanine	2.77	2.57	2.44	5.33
Proline	4.85	5.37	4.53	4.42
Serine	5.90	6.68	5.21	7.18
Threonine	4.98	5.83	4.21	3.91
Tyrosine‡	4.14	0.22	0.23	0.49
Valine	5.17	5.72	6.34	4.72

*, †, ‡ See corresponding footnotes in Table 2.

In the case of wool there is evidence which points to the extraction of small amounts (about 1%, Bradbury, Leeder, and Watt 1971) of a similar protein fraction from the cell membrane complex by formic acid (Bradbury, Chapman, and King 1965*b*), 50% formic acid (Zahn and Biela 1968*b*) or by treatment with a reducing agent in formamide (Dedeurwaerder, Dobb, and Sweetman 1964). However, the small total amount of this extract in comparison with the amount of the glycine-aromatic-rich fraction in wool (approx. 12%, Gillespie and Darskus 1971) indicates that it can only form a small part of the total. The remainder of this fraction must come from another source and by analogy with the results for kangaroo fibres it is reasonable to suggest that this is from the cortical cells. It is known that the protein extracted from the nuclear

remnants of the cortical cells by treatment with enzymes is not rich in glycine or aromatic amino acids (Peters and Bradbury 1972). Thus the site of the glycine–aromatic-rich fraction in wool is mainly within the microfibrils–matrix structure of the cortical cells, with a small amount in the cell membrane complex. Gillespie and Darskus (1971) have independently concluded, by somewhat similar arguments, that this protein originates from some internal structure, perhaps the matrix.

(f) *Cuticle Proteins*

The difficulty of solution of the protein of the cuticle is shown by the 42% extraction in Table 1 and is probably due to disulphide bonds which are difficult to reduce in the heavily cross-linked structure (Bradbury, Chapman, and King, 1965*a*; Bradbury and Ley 1972) and the possibility of resistant cross-links of a different type (Harding and Rogers 1971). The SCM-protein produced is a high-sulphur protein, in agreement with the results of Asquith and Parkinson (1966), obtained by using performic acid. Gel-filtration studies [Fig. 3(c)] show that the high-sulphur protein is largely in the lower molecular weight range (7000–12,500). This means that the high-sulphur proteins from kangaroo fibre (and probably also those of wool) of molecular weight > 12,500 do not originate from the cuticle, but those of molecular weight about 11,000 (Haylett *et al.* 1971) probably contain components from the cuticle of the fibre.

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