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The Chemical Composition of Wool XV.* The Cell Membrane Complex

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Abstract

The cell membrane complex of wool has been examined by electron microscopy of stained cross sections after immersion of the wool in formic acid. The cell membrane complex of the cortex is considerably modified by the treatment, but that of the cuticle appears unchanged. Resistant membranes from cuticle cells, cortical cells and wool have been prepared by treatment with performic acid–ammonia. Amino acid analyses show that the resistant membranes from the cuticle contain citrulline but those from cortical cells do not. It is concluded that the cell membrane complex of the cuticle differs from that of the cortex.

Because of the high lysine content of the resistant membranes, their resistance to chemical attack, the hydrophobicity of epicuticle and the observation of a small amount of ε-(γ-glutamyl)lysine, it is postulated that the resistant membranes may contain an appreciable amount of ε-(γ-glutamyl)lysine cross links.

Introduction

The cell membrane complex of keratin fibres has been studied by electron microscopy of cross sections of fibres in conjunction with chemical studies on (1) material extracted from the fibre and (2) resistant membrane† residues obtained after dissolving away the great bulk of the fibre (reviewed by Bradbury 1973). These studies indicate that the cell membrane complex of wool probably consists of about 1% of a readily extractable protein, 0·8% of lipid and 1·5% of a resistant membrane making a total weight of 3·3% of the wool (Bradbury *et al.* 1971). However, it is not known how these components are arranged in the cell membrane complex structure which, as observed by electron microscopy, consists of two lightly stained β segments with a central δ segment of intercellular cement (Rogers 1959*a*, 1959*b*). In this paper we report studies on resistant membranes obtained from cuticle, cortical cells and wool itself and on an examination of the cell membrane complex after extraction with formic acid, which is known to remove lipid and protein from the fibres (Bradbury *et al.* 1965*b*; Bradbury and King 1967).

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† The term 'resistant membranes' describes a system of membranes that occurs in the cell membrane complex of keratin fibres and which remains undissolved when the fibre or separated cuticle or cortical cells are dissolved by various degradative procedures. The epicuticle is that part of this membrane system which is located on the surface of the fibre and thus can be obtained by less severe chemical treatments involving the use of chlorine water (King and Bradbury 1968).

Materials and Methods

Merino 64's virgin wool from a pen-fed sheep was cleaned as described by Bradbury *et al.* (1966). Chemicals were of analytical reagent grade and were used without further purification except for formic acid which was redistilled before use.

Treatment of Wool

Samples of wool were immersed in formic acid (10 : 1 liquor to wool ratio) for various times. They were washed in running, deionized water until the washings were neutral, and then air-dried.

Isolation of Components from Wool

Cuticle and cortical cells were prepared by ultrasonic disintegration of wool in formic acid (Bradbury and Peters 1972*b*) followed by differential screening of an ethanol suspension of the products (Bradbury and Chapman 1964). Resistant membranes were prepared by dissolution of the keratin in performic acid for 2 weeks, followed by gentle agitation in 1M ammonium hydroxide for 1 week (Bradbury *et al.* 1971). The treatment was performed at 100 : 1 liquor to keratin ratio and at 16–18°C. Bradbury *et al.* (1971) showed that a stable residue weight is obtained after this treatment. The membranes, which were examined routinely with the light microscope under phase contrast illumination, were layered onto 1,1,1-trichloroethane and centrifuged to remove heavy contaminants. They were washed with formic acid and freeze-dried. After exposure to laboratory temperature and humidity for 16 h they were dried *in vacuo* at 100°C for 1 h and weighed.

Microscopy and Analysis

Electron microscopy was carried out as described by Peters and Bradbury (1972) using osmium tetroxide with lead citrate poststaining (Reynolds 1963). Amino acid analyses were performed in duplicate on a Technicon amino acid analyser after hydrolysis for 16 h at 105°C in 6M HCl (redistilled) *in vacuo*, as described by Bradbury *et al.* (1965*a*).

Results and Discussion

It is noted in Table 1 that agitation of the ammonia solution is necessary in order to dissolve all material other than the resistant membranes. Since Merino wool consists of approximately 90% cortex and 10% cuticle (Bradbury and King 1967), it is simple to calculate the amount of residue which would be expected from wool fibres using the values given for cortical cells and cuticle in Table 1. This amounts to 1·6% which compares favourably with the results in Table 1.

Amino Acid Analyses

Table 2 shows the results of a collection of amino acid analyses on resistant membranes from wool and from cortical cells and cuticle cells of wool and human hair. The analyses of Elliott *et al.* (1959) are omitted because they are very different from those given here and in fact are much more like those of exocuticle (Bradbury and Ley 1972) than of the other analyses (Bradbury 1973). There is a wide variation between the analyses, even when the method of preparation of the membranes is fairly standardized as is the case for the membranes which give rise to the results given in columns 2, 4 and 5 of Table 2. The wide variability is to be expected because of different wool samples and the very severe methods used to obtain the membranes, which involve the dissolution of all protein material except that of the membranes.

As a result of this variability of analysis between different samples, it is not possible to distinguish clearly between the various membranes on the basis of differences in amino acid analyses. The resistant membranes from whole wool are clearly made up of the membranes of cortical cells and of cuticle and this is, in general, reflected in the analyses. For example citrulline + ornithine occurs in the resistant membrane from

cuticle (and in epicuticle) but not in resistant membranes from cortical cells. This is consistent with its relatively high level in whole cuticle as compared with cortical cells (Bradbury and King 1967), and its occurrence at an intermediate level in resistant membranes from whole wool. The analysis of epicuticle is similar to that of the other resistant membranes from wool except for a lower lysine and higher serine content. The analysis of resistant membranes from the cuticle of human hair is similar to that of membranes from wool except for a lower content of arginine, glutamic acid and proline in the former. In fact the analyses are similar to those of whole wool fibres except for a much larger content of lysine in the resistant membrane samples.

Table 1. Yields of membrane residues obtained by treatment of keratin with performic acid and then ammonia

It was found necessary to agitate the solution during the 1 week of treatment in ammonia, since failure to do this resulted in the observation by microscopy of residual undissolved nuclear remnants, fibrils and endocuticle. Treatments of wool and cortical cells in which no agitation was used in the ammonia stage resulted in increased yields of residues of 3·7 and 2·2% respectively (J. M. O'Shea personal communication). We also obtained 3·5% from wool

Keratin	Initial weight of keratin (g)	Yield of membranes (%)
Wool	2·0	1·6
Wool	5·0	1·4[A]
Wool	—	1·5[B]
Cortical cells	0·245	1·5
Cuticle cells	0·30	2·6

[A] Value obtained by J. M. O'Shea (personal communication).
[B] Value obtained by Bradbury *et al.* (1971).

The significance of the high lysine content of the resistant membranes becomes clear when two other factors are considered. Firstly, the different methods which have been used for the dissolution of protein leaving resistant membranes undissolved involves treatment with (1) performic acid followed by ammonia or urea, (2) chlorine water to produce epicuticle (King and Bradbury 1968) or (3) dithiothreitol and papain (Swift and Bews 1974*a*). These methods involve reagents which dissolve keratin by splitting disulphide bonds and to a lesser extent peptide bonds. However they fail to dissolve the resistant membranes; in fact, epicuticle has been shown to be resistant to a wide range of degradative treatments (Lindberg *et al.* 1948; Leeder and Bradbury 1971). Secondly, amide cross links between the side chains of lysyl and glutamyl residues have been shown to occur in insoluble fibrin (Lorand *et al.* 1968; Matatic and Loewy 1968; Pisano *et al.* 1968), in medulla (Harding and Rogers 1971) and to a very small degree in intact wool (Asquith *et al.* 1970; Cole *et al.* 1971; Milligan *et al.* 1971).

It is therefore postulated that the high lysine content of these membranes coupled with their extreme chemical inertness can be explained in terms of the presence of this cross link. The amount of this cross link found in whole wool by Asquith *et al.* (1970) and by Milligan *et al.* (1971) amounts to 15 and 2·9 μmol/g respectively. A measurement of the amount of the cross link in epicuticle isolated by treatment of wool with chlorine water (King and Bradbury 1968) gave a value of 8 μmol/g (about 0·1 mol %). However this result is considered to be very low because of the inability of the enzyme

Table 2. Amino acid analyses (mol %) of insoluble membranes in resistant membranes isolated from wool and hair cuticle and of whole wool
n.m., Not measured

Amino acid	Membranes from whole wool[A]				Membranes from wool components			Membranes from hair cuticle (Swift and Bews 1974*a*)	Whole wool Merino 64's (Bradbury 1973)
	Bradbury *et al.* (1971) PA/NH_3	Bradbury *et al.* (1971) PA/urea	O'Shea[B]	This work	Cortical cells[C]	Cuticle (this work)	Epicuticle (King and Bradbury 1968)		
Alanine	6·90	6·09	8·16	7·47	7·62	7·82	4·61	5·04	5·3
Arginine	4·26	4·12	5·69	5·49	5·94	4·19	4·27	3·27	6·8
Aspartic acid	5·79	5·02	8·82	7·53	7·62	7·55	5·84	7·17	6·4
Citrulline+ ornithine	n.m.	n.m.	1·29	0·55	0·00	1·43	0·89	n.m.	0·1
Cysteic acid	11·60	14·43	5·61	6·83	6·39	7·42	11·58	—	0·1
½Cystine[D]	0·00	0·00	0·00	0·00	0·00	0·00	0·33	—	10·5
Cysteic acid + ½cystine	11·60	14·43	5·61	6·83	6·39	7·42	11·91	9·90	10·6
Glutamic acid	10·43	10·25	12·13	11·11	11·37	10·91	10·66	7·76	11·9
Glycine	13·90	14·43	8·69	10·18	9·13	9·25	15·35	14·15	8·6
Histidine	1·29	1·37	1·91	1·85	1·33	1·11	1·03	1·67	0·9
Isoleucine	2·65	2·46	3·57	3·69	4·34	3·56	2·52	4·65	3·1
Leucine	5·08	4·67	8·56	7·09	8·37	7·86	5·46	8·20	7·7
Lysine	9·00	7·69	8·39	8·13	7·56	7·12	4·83	7·78	3·1
Methionine[D]	0·00	0·00	0·46	0·00	0·15	0·00	0·03	0·18	0·5
Phenylalanine	1·53	1·52	3·22	2·51	2·96	2·38	1·85	1·52	2·9
Proline	7·01	7·13	6·83	6·40	6·77	7·36	5·80	4·91	5·9
Serine	9·99	10·08	2·78	9·10	8·10	9·54	13·65	8·79	10·2
Threonine	5·89	5·57	3·41	5·52	4·80	5·18	3·59	4·53	6·5
Tyrosine[D]	0·00	0·00	3·18	0·00	0·30	0·36	2·07	3·10	4·0
Valine	4·66	5·16	7·29	6·55	7·25	6·95	5·72	7·38	5·5
Recovery of anhydroamino acids (%)[E]	77	86	61	72	32	58	78	24	96

mixture to digest the membrane (B. Milligan, J. M. O'Shea and J. H. Bradbury, unpublished data).

The low degree of wettability of the surface of wool fibres is well known (Stewart and Whewell 1960; Pittman 1971; Bradbury 1973) and is ascribed to the hydrophobicity of the epicuticle. Since the epicuticle consists of protein with an amino acid analysis similar to that of wool itself, the hydrophobicity may result from the presence of a small amount of lipid (King and Bradbury 1968; Lofts and Truter 1969) and also from the removal of hydrophilic carboxyl and amino groups by the formation of amide cross links.

Thus, although it has not yet been possible to prove directly the presence of large amounts of ε-(γ-glutamyl)lysine cross links in resistant membranes, there is chemical evidence of the presence of at least a small amount. There is also the circumstantial evidence of a high lysine content, together with the extreme chemical resistance and hydrophobicity of the membranes, which would be explained readily in terms of appreciable amounts of ε-(γ-glutamyl)lysine cross links.

Electron Microscopy

Wool fibres were treated with formic acid at room temperature or at 98°C or reduced with tri-n-butylphosphine and alkylated with acrylonitrile (Maclaren and Sweetman 1966; Sweetman and Maclaren 1966; Maclaren *et al.* 1968). The fibres were then examined by electron microscopy and the results are shown in Figs 1–5. Comparison of Figs 1 and 2 demonstrates the rapid attack by formic acid on the cell membrane complex of wool after only 5 min of treatment at room temperature. This is shown by the loss in the cortex of the characteristic β (lightly stained) and δ (darkly stained) structures as described by Rogers (1959*a*, 1959*b*) and shown in Fig. 6. Close examination shows that the β regions are not removed but appear to be concealed by more darkly stained material, presumably originating from the δ region. The β and δ structure remains essentially unaltered in the cuticle, however, and the sharp difference between cortex and cuticle is further exemplified by retention of detail on the cuticle side and removal of detail on the cortical cell side of the cell membrane complex, which occurs at the cuticle–cortex boundary (Fig. 2).

Treatment of wool with formic acid for 2 h and 48 h at room temperature produced slightly larger changes in the cell membrane complex of the cortex than those observed in Fig. 2 after only 5 min immersion. The unusual staining of the cell membrane complex of the cortex is unlikely to result from residual formic acid, since prolonged washing of fibres in water did not appreciably affect the result, as shown in Fig. 3.

Footnotes to Table 2

[A] PA/NH_3 and PA/urea represent the performic acid–ammonia and performic acid–urea treatments.
[B] Sample prepared by PA/NH_3 treatment by Dr J. M. O'Shea.
[C] Mean value of results obtained in this work and by Dr J. M. O'Shea.
[D] Cystine is oxidized to cysteic acid, and methionine and tyrosine are destroyed by treatment with performic acid (Toennies and Homiller 1942; Blackburn and Lowther 1951). These amino acids are also affected by the chlorination treatment used in the preparation of epicuticle by King and Bradbury (1968).
[E] These values are calculated from the amount of amino acids obtained from a given weight of dry material. A low value is indicative of the presence of non-protein material (usually inorganic salts), but in such a case the analysis is still valid for the protein present.

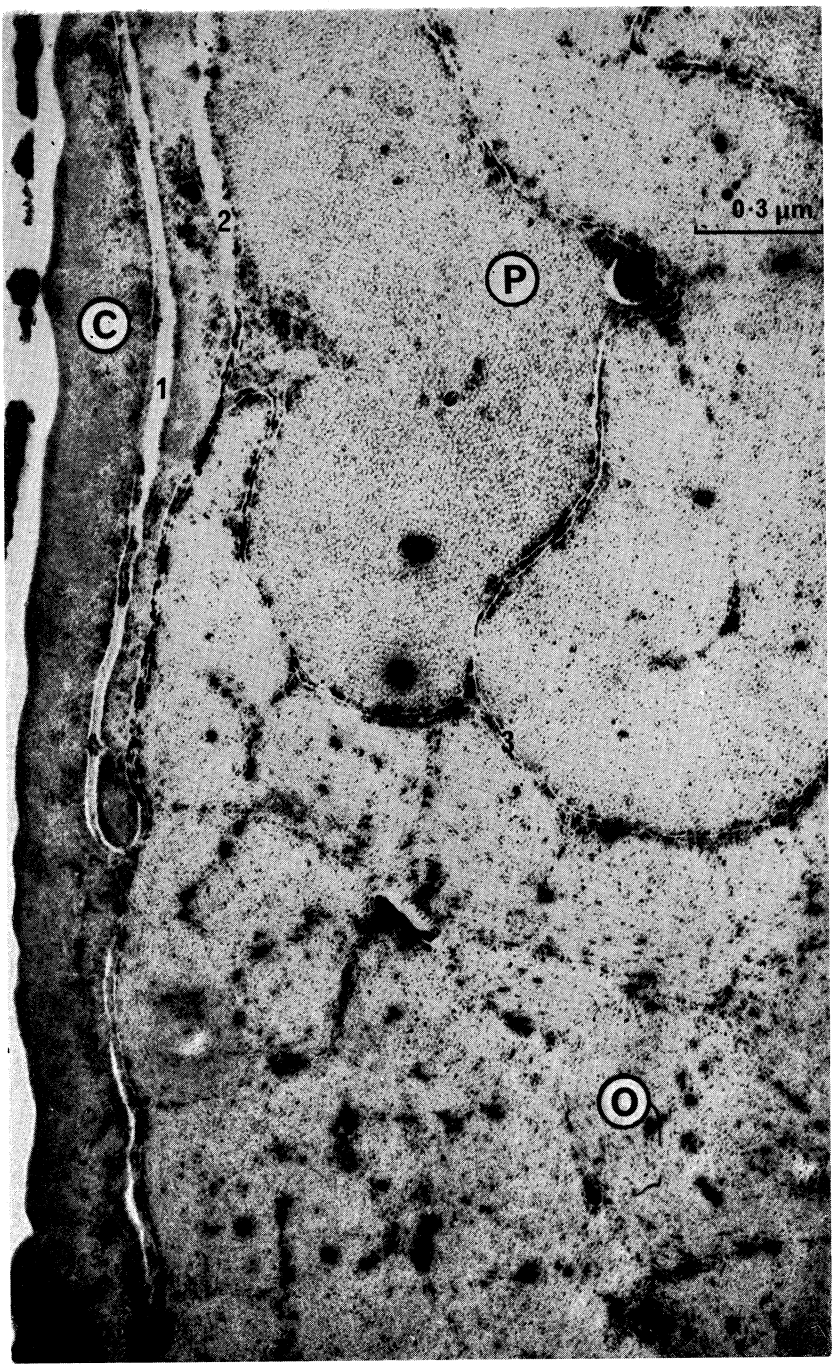

Fig. 1. Electron micrograph of a stained cross section of an untreated Merino fibre showing the cuticle (*C*), orthocortex (*O*), paracortex (*P*) and the cell membrane complex between two cuticle cells at 1, separating cuticle from cortex at 2 and between cortical cells at 3.

Fig. 2. Cross section of a Merino wool fibre after immersion in formic acid for 5 min at room temperature, followed by staining. The cell membrane complex between the three cuticle cells is normal but virtually all detail is removed between cortical cells. At point *B* in the cortex a small amount of detail remains. At point *A* (the junction of cuticle and cortex) the detail is retained on the cuticle side but is absent on the cortical cell side of the membrane.

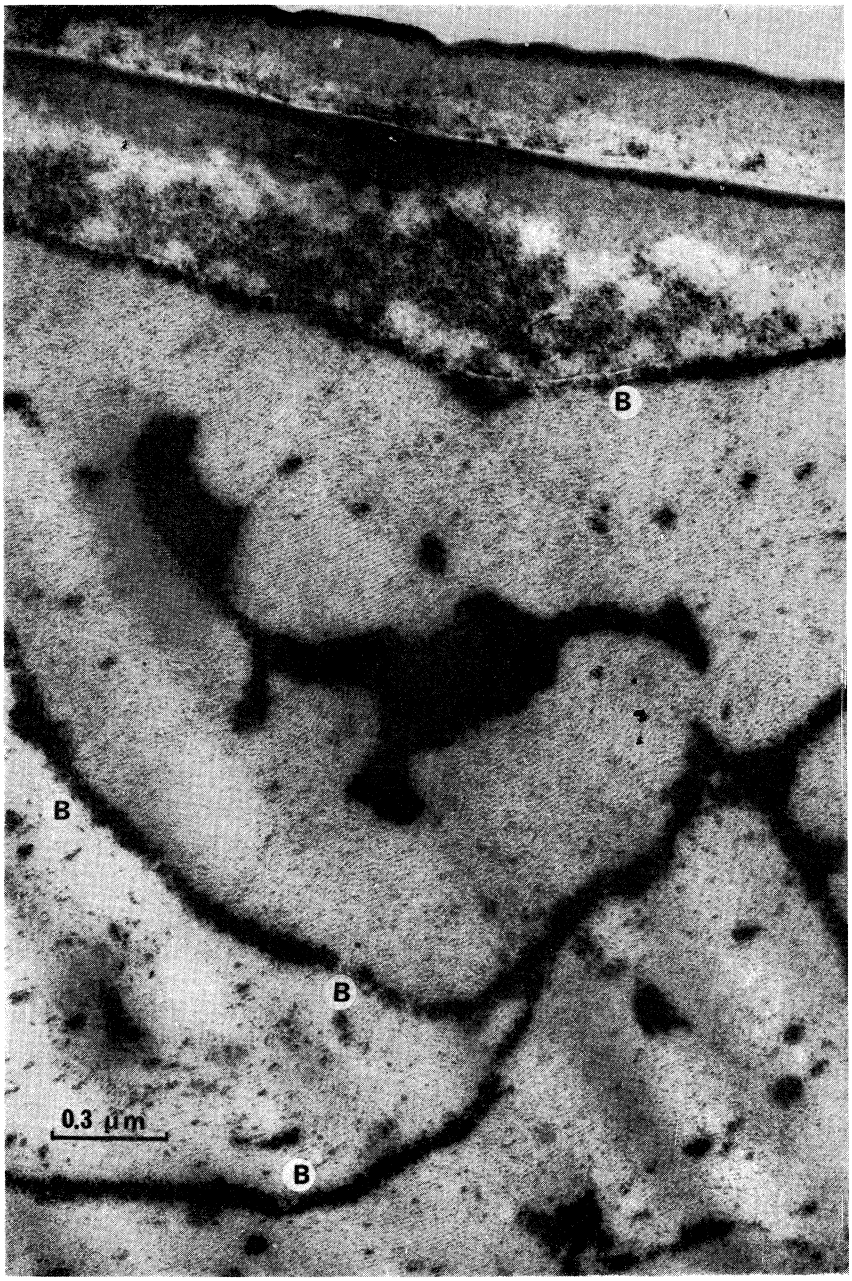

Fig. 3. Cross section of a Merino wool fibre after immersion in formic acid at room temperature for 5 min, washing for 36 h in distilled water and then staining. At the various points labelled *B* there is evidence of residual detail in the cell membrane complex of the cortex. The cell membrane complex of the cuticle is unchanged by the treatment.

The cell membrane complex of the cuticle is still visible after treatment with formic acid at 98°C for 20 min (Bradbury and Peters 1972*a*; Fig. 4). After treatment with formic acid, the normal microfibril–matrix structure of the cortex becomes less distinct. This possibly reflects the disorientation of microfibrils which is known to occur in formic acid as shown by X-ray diffraction studies (Bendit 1966; Chapman and Feughelman 1967).

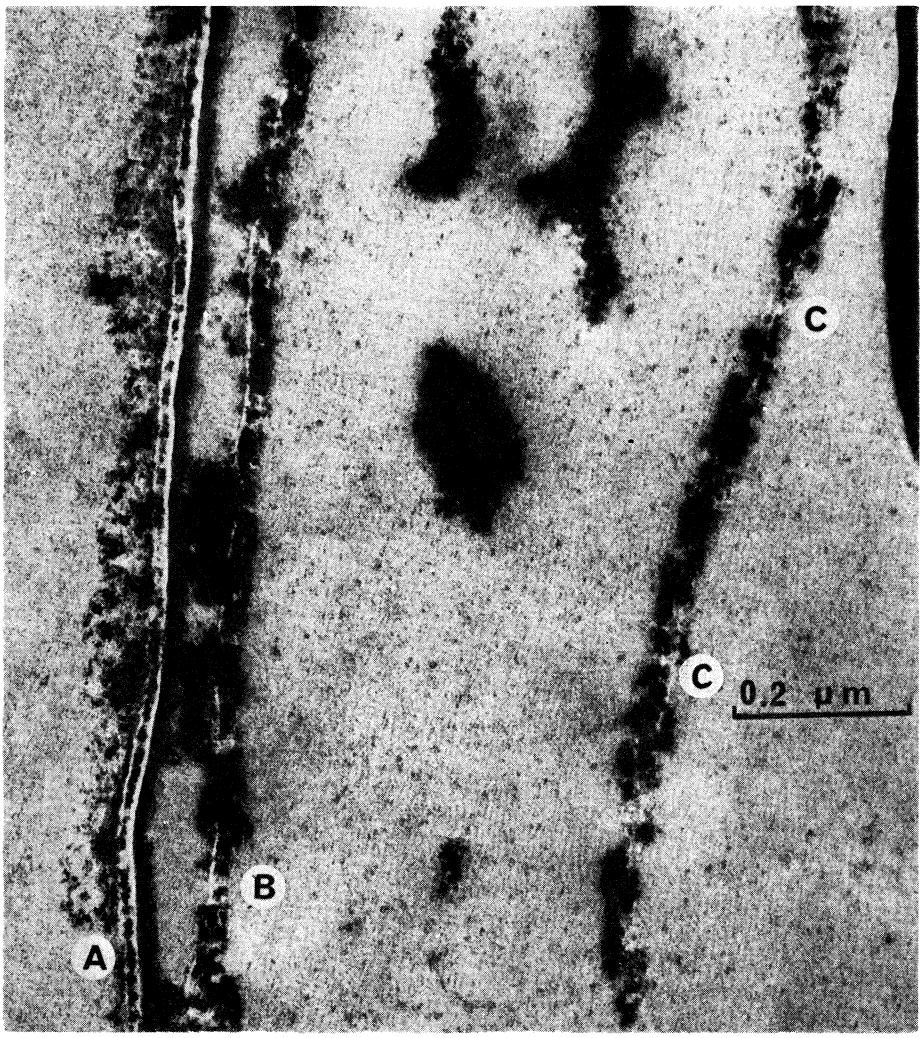

Fig. 4. Cross section of a Merino wool fibre treated with formic acid at 98°C for 20 min and then immersed in formic acid for 16 h at room temperature (Bradbury and Peters 1972*a*). The cell membrane complex at *A* between two cuticle cells is still visible whilst that between cuticle and cortex at *B* is clearly visible on the cuticle side and largely removed on the cortex side of the membrane. The cell membrane complex between cortical cells is still partially visible at *C*.

Wool fibres which have been completely reduced and alkylated with acrylonitrile show no structural detail in the cortex, except for the β region of the cell membrane complex (Fig. 5). In this case, as noted above, the β regions are more obvious and

Fig. 5. Cross section of a Merino wool fibre reduced with tri-n-butylphosphine, alkylated with acrylonitrile and then stained. Note the absence of the microfibril–matrix structure of the cortex and the greatly modified structure of the cell membrane complex, which is still clearly visible in the cuticle and to a lesser degree in the cortex at *A*.

clearly defined in the cuticle than in the cortex. The δ region is not visible as a heavily stained area although the separation between the two β regions is the same as that in an untreated fibre (Fig. 1).

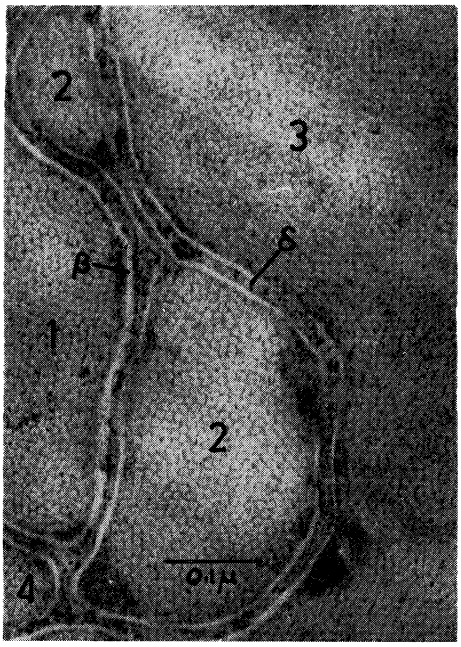

Fig. 6. Electron micrograph of a cross section of a Lincoln wool fibre showing four cortical cells (1, 2, 3 and 4) and the cell membrane complex, which consists of a central dark layer (δ) with a light layer (β) on either side of it (from Rogers 1959*a*).

Differences between Cell Membranes of Cuticle and Cortex

Treatment of wool for 5 min in formic acid at room temperature causes the extraction of about 1% by weight of predominantly lipid material which contains about 10% of a high-glycine–high-tyrosine protein (Bradbury *et al.* 1965*b*; Bradbury and King 1967). This material was considered to come from the cell membrane complex because the examination of cross sections of fibres after treatment with dichloroacetic acid (which with trifluoroacetic acid gave a similar effect to formic acid) showed that the cell membrane complex was modified (Bradbury *et al.* 1965*b*). The present studies show that 5 min of treatment with formic acid does indeed cause modification of the cell membrane complex of the cortex but not of the cuticle. Much more extended treatments in formic acid at room temperature and 98°C do not appreciably change the result obtained after 5 min at room temperature. The exact nature of the modification of the cell membrane complex of the cortex is uncertain, but it appears from Figs 2–4 that the heavy irregular deposit of stain in the cell membrane complex almost completely obscures the normal appearance of the cell membrane complex (Fig. 1) or else replaces it almost completely. It would be worthwhile studying cross sections of formic acid-treated wool using different metal stains.

The second difference between the cell membrane complex of cuticle and cortex is that the resistant membranes from the former contain an appreciable amount of citrulline + ornithine whereas the resistant membranes from cortical cells do not contain any. As mentioned above this is consistent with the presence of citrulline (ornithine is produced by decomposition of citrulline during hydrolysis) in the cuticle

and its virtual absence in the cortex (Bradbury and King 1967). Swift and Bews (1974*b*) have noted that on dissolving human hair with dithiothreitol and papain the residual layers of the cuticle appear to retain their original thickness, whereas the residues at the cortical cell boundaries tend to collapse. This would appear to indicate preferential attack of the membrane complex of the cortex. Finally a difference has been observed in the intensity of staining of the intercellular material in cuticle and corticular cells of the wool follicle which may indicate a difference in composition in the hardened fibre (Orwin and Thomson 1972).

It is therefore concluded that there is a difference between the cell membrane complex of cuticle and cortex in the sense that the former is much more resistant to modification by formic acid. Furthermore, resistant membranes produced from cuticle contain citrulline whereas those from cortical cells do not.

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