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The cell membrane complex: Three related but different cellular cohesion components of mammalian hair fibers

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Synopsis

The structure, chemistry and physical properties of the cell membrane complex (CMC) of keratin fibers are reviewed, highlighting differences in the three types of CMC. Starting with Rogers' initial description of the CMC in animal hairs, several important developments have occurred that will be described, adding new details to this important structure in mammalian hair fibers. These developments show that essentially all of the covalently bound fatty acids of the beta layers are in the cuticle and exist as monolayers. The beta layers of the cortex are bilayers that are not covalently bonded but are attached by ionic and polar linkages on one side to the cortical cell membranes and on the other side to the delta layer. The delta layer between cortical cells consists of five sublayers; its proteins are clearly different from the delta layer that exists between cuticle cells. The cell membranes of cuticle cells are also markedly different from the cell membranes of cortical cells. Models with supporting evidence are presented for the three different types of cell membrane complex: cuticle–cuticle CMC, cuticle–cortex CMC, and cortex–cortex CMC.

INTRODUCTION

GENERAL STRUCTURE OF THE CMC

The cell membrane complex (CMC) consists of cell membranes and adhesive material that binds the cuticle and cortical cells together in keratin fibers. G. E. Rogers from his seminal high-resolution transmission electron microscope (TEM) studies (1,2) provided evidence for the current structure of the CMC, consisting of a central delta layer approximately 15-nm thick sandwiched by two lipid layers called beta layers each about 5-nm thick; see Figure 1, adapted from Fraser *et al.* (3).

Questions still exist about the relative thickness and composition of the beta layers between cuticle cells versus the beta layers of cortical cells (see Figures 2,3) and between the upper beta layer versus the lower beta layer of cuticle cells (see Figure 2). Although most authors quote the thicknesses of the beta layers between 2.5 (6) and 5.0 nm, 6.0 nm has also been cited (11). Swift (7) in his review of the human hair cuticle describes in detail the difficulty of obtaining accurate measurements of the beta layers in the high-resolution TEM, and his explanation clarifies the uncertainty that exists in ascribing monolayers or bilayers to these lipid strata on the basis of TEM measurements alone.

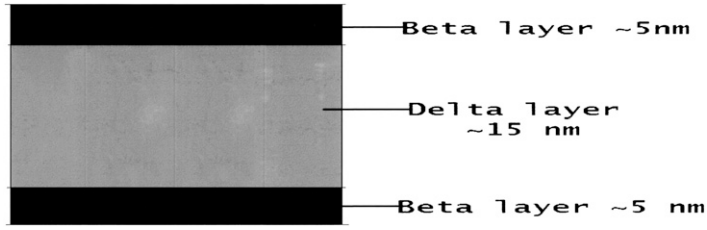

Figure 1. Rogers' view of the CMC in 1959 (not drawn to scale).

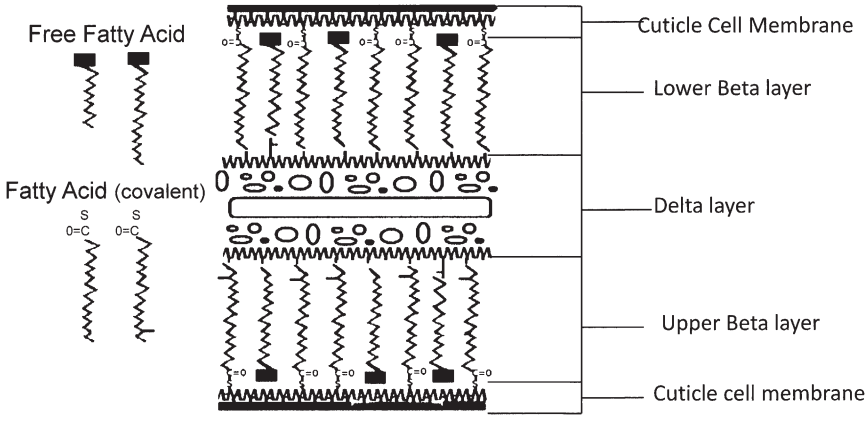

Figure 2. Schematic representing the cuticle–cuticle CMC. This schematic was first modified by Robbins *et al.* (4) from a schematic by Bryson *et al.* (5) of the cortex–cortex CMC and has been subsequently modified into its current form (not drawn to scale).

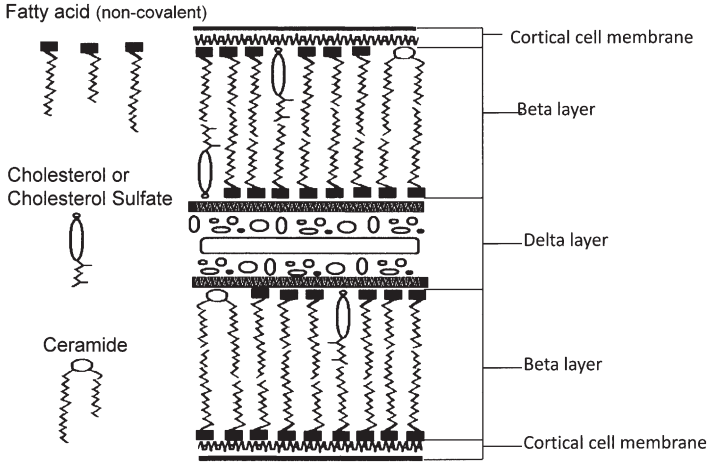

Figure 3. Schematic representing the cortex–cortex CMC.

Three types of CMC have been described in the literature (8): cuticle–cuticle CMC, representing CMC between cuticle cells; cortex–cortex CMC, representing CMC between cortical cells; and cuticle–cortex CMC, representing CMC at the cuticle cortex boundary

(see Figure 4). Since Rogers' (1,2) initial description of the CMC and his additional work demonstrating that the delta layer of the cortex consists of five sub-layers (9), several additional important developments have occurred that will be described in this paper, adding new details to this important structure in animal hairs.

SUPPORT FOR CUTICLE–CUTICLE CMC AND CORTEX–CORTEX CMC STRUCTURES

GENERAL DIFFERENCES FOR CUTICLE–CUTICLE CMC VS CORTEX–CORTEX CMC

Jones and Rivett (10,11) provided evidence that the CMC of the cuticle contains 18-methyl eicosanoic acid (18-MEA) in its upper beta layer while the CMC of the cortex has virtually no 18-MEA. The facts strongly suggest that the CMC of the cuticle has monolayer lipids that are attached by covalent bonds (primarily thioester) (12,13), with some ester or amide linkages (13) to proteins of the cell membranes on one end, and by van der Waals attractive forces to proteins of the delta layer on the hydrophobic end of the fatty acids (Figure 2). On the other hand, the CMC between cortical cells consists of lipid bilayers that are not attached by covalent bonding to protein layers but are bound by salt linkages and polar bonding to the cortical cell membrane proteins on one side and similarly attached to the delta layer on the other side of the bilayer (see Figure 3). References and supporting facts for these conclusions are presented in the next sections of this paper.

CUTICLE–CUTICLE CMC

In 1916, Allworden (14) discovered that chlorine water reacts with the cuticle cells of wool fiber to produce large bulbous sacs on the fiber surface. Chlorine water degrades proteins beneath the cuticle cell membranes (most likely cleaving disulfide linkages between the epicuticle and the A-layer (15)), producing water-soluble species too large to

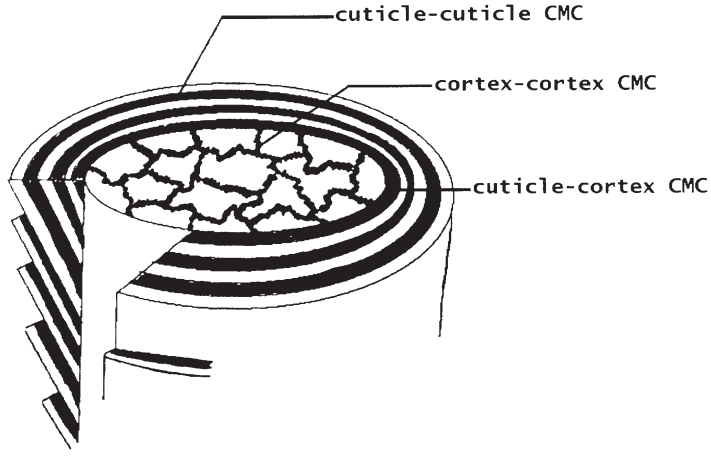

Figure 4. Location of the three different types of CMC (not drawn to scale).

diffuse out of the semipermeable cuticle cell membrane. Swelling results from osmotic forces, and the cuticle membrane stretches, producing the so-called Allworden sacs that separate from the underlying proteinaceous intracellular matter.

The epicuticle membrane was first isolated and named by Lindberg *et al.* in 1949 (16,17). Several years later, in 1968, Leeder and Bradbury (18) defined the epicuticle as the "thin outer membrane which is raised on the surface of fibers as sacs by treatment with chlorine water" in the Allworden reaction. The epicuticle provides the protein supporting structure for 18-MEA in cuticle cells (see Figures 2 and 5). It is also attached to the A-layer of cuticle cells of wool and human hair, and together with 18-MEA is perhaps the most thoroughly studied part of the CMC. Leeder and Rippon (19), in 1985, suggested that the epicuticle was proteinaceous and covered with a strongly bound lipid layer that could not be removed by lipid solvents, but could be removed with alcoholic alkali; they called this lipid layer the F-layer (see Figures 5 and 6).

The F-layer of covalently bound fatty acids, together with the cuticle cell membranes (essentially the epicuticle), is analogous to the cornified envelopes or the cellular envelope of

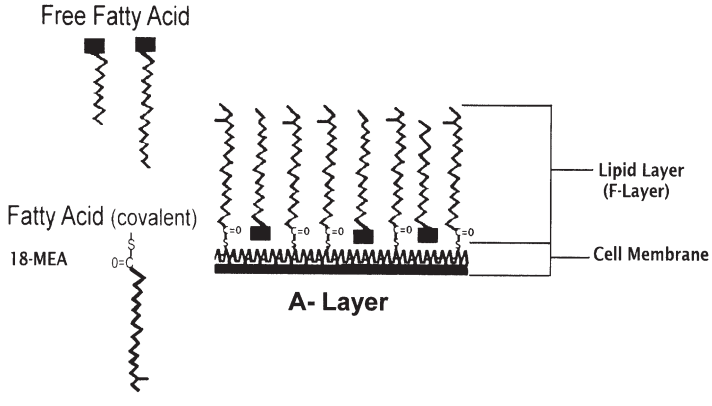

Figure 5. Schematic of the hair surface showing the lipid layer (F-layer) and the cell membranes (not drawn to scale).

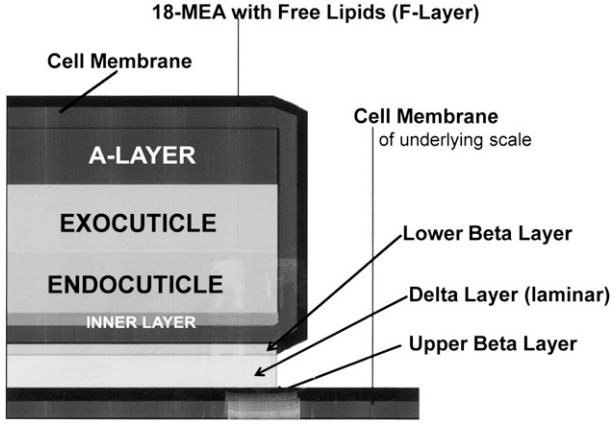

Figure 6. Schematic of a transverse section of a cuticle cell with the CMC (not drawn to scale).

stratum corneum. As early as 1945, Weitkamp (20) reported 18-MEA in wool wax (degras). In 1985, Evans *et al*. (21) demonstrated that 18-MEA is covalently bonded to the keratin fiber surface by reacting wool fiber with anhydrous alkali after solvent-extractable lipids have been removed. The cleavage of 18-MEA with chlorine water by Negri *et al*. (12) and by hydroxyl amine at neutral pH by Evans and Lanczki (13) support the attachment by a thioester linkage rather than an ester or amide linkage, although there is evidence for ester and/or amide attachment of some fatty acids (primarily palmitic, stearic, oleic, and others), mainly in the lower beta layer in cuticle–cuticle CMC by Evans and Lanczki (13) and by Korner and Wortmann (22).

Essentially all of the MEA is in the upper beta layer of the cuticle–cuticle CMC, as demonstrated in a paper by Jones *et al*. (23). Maple syrup urine disease (MSUD) is a genetic defect in humans and Poll Herford cattle (24) involving 18-MEA. MSUD is caused by a deficiency in an enzyme involved in the complicated synthesis of 18-MEA. Isoleucine serves as a precursor in the biosynthesis of 18-MEA via the branched chain 2-oxo acid dehydrogenase, which is the enzyme that is deficient in this genetic defect (10). Jones and Rivett in their TEM studies of MSUD (10,23) found that the structural defect of MSUD in human hair occurs only on the upper surface of cuticle cells (upper beta layer), where 18-MEA is replaced by straight chain C18 and C20 fatty acids, and that the undersides of cuticle cells (lower beta layer) are not affected. These facts confirm that 18-MEA is attached to the top surface of cuticle cells (upper beta layer) and not to the underside.

Only a few years after the discovery by Evans *et al*. (21) that 18-MEA is covalently bound to the keratin fiber surface, Negri *et al*. (12) proposed a model for the keratin fiber surface consisting of a monolayer of 18-MEA covalently bonded to an ultra-high sulfur protein through a thioester linkage at approximately 1-nm spacings, and they suggested that the protein support was in the beta configuration and that it might be attached to the Allworden membrane. Although widely varying estimates of the thickness of the epicuticle have been made from 5 to 14 nm, one of the more recent and perhaps reliable estimates is by Swift and Smith (25), who examined wool fiber, human hair, and several other mammalian hairs using high-resolution TEM and identified that the epicuticle is approximately 13-nm thick and is rich in cystine, and that thioester-bound lipids might be present within its bulk. Swift's estimate of the epicuticle thickness is consistent with the maximum thickness reported by several other workers (26–28).

Leeder and Bradbury (18,29) discovered that the Allworden reaction takes place with isolated cuticle cells from several different animal hairs including wool and human hair fiber, proving that this proteinaceous membrane material completely surrounds each cuticle cell and is not a continuous external membrane on hair fibers. In this important scientific effort, cuticle cells were isolated by shaking fibers in formic acid and then exposing the isolated cells to chlorine water. Formic acid is known to attack and to solubilize some proteins believed to be largely from the delta layer of the cell membrane complex, and its effects will be discussed later in the section entitled "Proteins of the CMC." In the intact fiber Allworden sacs form over the top of cuticle cells (the exposed surface). Leeder and Bradbury suggested that "the sac always occurs on only one side of the cuticle cell," i.e., the top of cuticle cells and not the bottom (15,18,29). They explained that this effect occurs because the connecting bonds on the top of cuticle cells are between the epicuticle and the A-layer and therefore are most likely through disulfide crosslinks that are vulnerable to chlorine water oxidation (15). Furthermore, they suggested that the connecting bonds on the underside of cuticle cells are between the

membrane and the endocuticle (actually the inner layer, a layer about 10- to 40-nm thick (7) between the endocuticle and the cell membrane and similar in composition to the exocuticle (see Figure 6), through bonding that is resistant to chlorine water oxidation (15) (possibly isopeptide linkages).

Negri *et al*. (12) have shown that the Allworden reaction is an effect of the membranous proteins around cuticle cells and that 18-MEA is not required for the formation of Allworden sacs because the sacs can be produced from cuticle in which 18-MEA has been removed by prior treatment with either methanolic KOH or potassium t-butoxide in t-butanol. Because of the bulky nature of the t-butoxide anion, it removes only covalently bound fatty acid at or near the fiber surface. Furthermore, Negri *et al*. (12) have shown that removal of the covalently bound fatty acid facilitates the formation of Allworden sacs because the rate of formation of the sacs increases with prior removal of the covalently bound 18-MEA.

The separation and analysis of the Allworden membrane by Allen *et al.* (30), together with the work of Zahn *et al*. (31), provided indirect evidence for the cuticle cell membrane containing an ultra-high sulfur protein, loricrin, and involucrin. This important contribution will be described in more detail later in this paper in the section entitled "Proteins of the CMC."

Bilayers versus monolayers in the cuticle–cuticle CMC. Whether or not the covalently bound lipids of the cuticle–cuticle CMC are bonded to another lipid layer on their hydrophobic end, forming a bilayer, or they are bonded to a hydrophobic protein in the delta layer is still debated, but this author believes the evidence clearly favors the monolayer model (4,7) for the following reasons:

- If the beta layers are monolayers, then 18-MEA is linked to the delta layer through short hydrophobic bonds, making the upper beta layer susceptible to failure at the delta layer where it has been shown to occur (4,7,32,33).
- Swift (7) has pointed out that a monolayer model fits better from the point of view of CMC measurements.
- If bilayers exist (a schematic of the bilayer model is shown in reference 4), then there are two options for bonding of the second fatty acid layer to the delta layer. One option is for fatty acids to be covalently bonded to the delta layer, but this option is not plausible because in human hair and wool fiber 40% to 50% of the covalently bonded fatty acids are 18-MEA (34–36); therefore, there are insufficient covalently bound fatty acids in human hair and wool fiber to account for this type of bonding. The other option is bonding of the second layer of fatty acids through hydrophobic linkages to the covalently bound fatty acids and bonding to the delta layer through polar attachments and ionic bonding. However, this type of bonding would provide beta–beta failure, not beta–delta failure, and it would allow for solvent removal of the non-covalently bound lipid layer by chloroform/methanol extraction, which has been shown to occur in cortex–cortex CMC but not in cuticle–cuticle CMC, which is more resistant to this type of solvent system (37,38). To provide beta–delta failure from this bilayer model, the new hair surface would form a bilayer consisting primarily of hydrophilic acid groups at the very surface, and so this bilayer model is also not plausible.
- Negri *et al*. (39) noted that formic acid removes proteins more readily from the cortex–cortex CMC and that it modifies CMC junctions of the cortex more than those of the

cuticle, which is consistent with covalent and hydrophobic bonding of the cuticle–cuticle CMC, as shown by the monolayer model in Figure 2, rather than a bilayer model.

Another point of contention concerning the CMC is whether or not the delta layer contains globular proteins or glycoproteins. Allen *et al*. (40) found evidence for glycoproteins in several different animal hairs in formic acid extracts, which they suggested could be from the CMC; however, they also suggested that these materials could be remains of cell membrane glycoproteins from the follicle or that they could be functional adhesive materials in the CMC. I believe the current evidence favors globular proteins in the delta layer as functional adhesive materials for the following reasons:

- The delta layer resists solubilization by aqueous reducing or oxidizing agents and by acids and alkalies (5). If the CMC contains globular proteins similar to those in many other membranes containing large domains of hydrophobic amino acids on their surfaces (41), that would provide the ideal delta layer surface for the hydrophobic ends of the covalently bound fatty acids to adhere to, and this type of globular protein should be resistant to aqueous reagents, as Bryson *et al*. (5) found.
- Bryson *et al*. in 1995 (5) isolated lipid-soluble lipoproteins from the delta layer of cortex–cortex CMC and not glycoprotein.
- The delta layer stains with phosphotungstic acid (PTA). This is either a reaction of hydroxyl groups of a polysaccharide or of a primary amine function. Swift (7) has explained that this reaction is blocked with fluro dinitro benzene (FDNB); therefore, it is more likely a reaction involving primary amine groups, consistent with a globular protein.
- The delta layer reacts with periodic acid/silver methenamine (7), a method for polysaccharides; however, Swift (7) has also pointed out that since cystine interferes with this reaction, it is still consistent with a globular protein in the delta layer.

Thus, the globular protein model is consistent with the currently known reactivity of the cuticle–cuticle CMC and with the proposed structure in Figure 2, and therefore the glycoproteins that Allen *et al*. (40) found were most likely remains of cell membrane material from the follicle.

CORTEX–CORTEX CMC

Wertz and Downing (35) found in five different mammalian hairs, including those of sheep, humans, dogs, pigs, and cattle, that the percentage of 18-MEA relative to the total amount of covalently bound fatty acids varied from 38% to 48%. Table I summarizes a tabulation of analyses of the covalently bound lipids of wool and human hair from several different laboratories. These results were all obtained after the fibers had been exhaustively extracted with chloroform/methanol to remove the non-covalently bound fatty acids and then by saponifying the residue with methanolic alkali, showing that 18-MEA accounts for about 50% of the covalently bound fatty acids in these wool fibers and about 40% in human hair.

Covalently bound internal lipids of animal hairs. Korner and Wortmann (22) (Table I) analyzed covalently bound fatty acids in isolated wool cuticle and found 55% 18-MEA, 25% stearic acid, and 20% palmitic acid, with "only traces of other straight and odd number carbon chain fatty acids." For wool fiber Wertz and Downing (35) found 48% 18-MEA, 17% palmitic acid, 10% stearic acid, and 5% oleic acid, and the remaining covalently

Table I
Covalently Bound Fatty Acids in Wool and Human Hair Fiber

	Data for wool fiber						Data for human hair
Fatty acid	[13]	[43]	[44]	[36]	[22]	Averages	[35]
16:0	8	11	8	17	20	12.8	18
18:0	8	12	6	10	25	12.2	7
18:1	7	8	5	5	0	5	4
MEA	51	43	72	48	55	53.8	41
Others	26	26	9	20	trace	16.4	30

Data are expressed as percentages.

bound fatty acids ranged from C16 through C20, with 6% uncharacterized. For human hair, Wertz and Downing (34) found 41% 18-MEA, 18%, palmitic acid, 7% stearic acid, and 4% oleic acid, and the remaining small percentages of fatty acids ranged from C16 through C20, with 9% uncharacterized. Negri *et al*. (42) found 72% 18-MEA, 8% palmitic acid, 6% stearic acid, and 5% oleic acid in wool fiber.

The variation in these data from different laboratories is quite large. Part of the variance has been suggested to be related to fiber diameter, which determines the number of layers of covalently bound fatty acids in the fibers; however, certainly part of the variance is due to experimental error. The bottom line is that somewhere in the vicinity of 50% ± at least 10% of the covalently bound fatty acids in most keratin fibers is 18-MEA (attached mainly on the top surface of cuticle cells). Furthermore, hair fibers from sheep, humans, dogs, pigs, and cattle, and likely most keratin fibers, contain the remaining nearly one half of the covalently bonded fatty acids, primarily as palmitic, stearic, and oleic acids attached mainly on the bottom of cuticle cells.

In 1990, Kalkbrenner *et al*. (43) demonstrated with isolated cuticle cells that 18-MEA is essentially all in the cuticle. Since 18-MEA represents more than 40% of the total covalently bound fatty acids in human hair and about 50% in wool fiber, and since 18-MEA is confined to the upper beta layer of the cuticle (23,24) while most (essentially an amount equal to the 18-MEA) of the other covalently bound fatty acids are confined to the lower beta layer, then most of the covalently bound fatty acids in wool and hair fiber must be in the cuticle–cuticle CMC, with some in the cuticle–cortex CMC (to be described later) and virtually none in the cortex–cortex CMC. Therefore, if most of the covalently bound fatty acids are in the cuticle–cuticle CMC, then most of the lipids of the cortex–cortex CMC must be bound to the membranes on one side and to the delta layer on the other side by non-covalent bonds. The fact that most of the remaining lipids can be removed by solvent extraction confirms that this is the case, as will be shown below and in the section entitled "Lipids in the CMC."

Leeder *et al*. (44) first found that there are virtually no phospholipids in keratin fibers. This fact was confirmed by Schwan and Zahn (45) and by Rivett (46), casting doubt on whether lipid bilayers could be involved in the cell membranes of keratin fibers (44). However, Wertz *et al*. (47) demonstrated that liposomes (lipid bilayers and a presumed precursor to the formation of lipid bilayers in the CMC of keratin fibers) can form in the absence of phospholipids if an acid species such as cholesterol sulfate is present with other lipids.

Furthermore, evidence has been provided confirming the existence of cholesterol sulfate in human hair by Wertz and Downing (34) and by Korner *et al*. in wool fiber (48).

The work of Korner *et al*. (48) builds upon the findings of Wertz *et al*. on liposome formation and lipids from stratum corneum (47), where Korner *et al*. (48) demonstrated that cell membrane lipids extracted from human hair and wool fiber with chloroform/methanol/aqueous potassium chloride can form liposomes, providing evidence for a bilayer structure of the internal lipids of the beta layers of the cortical CMC in wool fiber and in human hair (see Figure 3). Such extracts must come primarily from the cortex–cortex CMC because covalently bound MEA and the other covalently bound lipids of the cuticle CMC are not removed with this solvent system.

Therefore, if the beta layers of the cuticle cells are primarily covalently bound fatty acids with some free lipids (see Figure 2) and the beta layers of cortical cells consist primarily of lipid bilayers (Figure 3), then it is highly likely that the proteins that these very different lipid layers are attached to, which are the cell membrane proteins, and the delta layer proteins of the cuticle cells and cortical cells are also different (see the section entitled "Proteins of the CMC" and the next part of this section).

Additional differences between cuticle–cuticle, cuticle–cortex, and cortex–cortex CMC. As early as 1975, Nakamura *et al.* (8) provided evidence by staining reactions that the disulfide content in the delta layer in cuticle–cuticle CMC is lower than the disulfide content of the delta layer in either cuticle–cortex or cortex–cortex CMC and that the carboxyl content in the cuticle–cuticle CMC is higher than that of the cortex–cortex CMC. In addition, Nakamura *et al.* added that the delta layer of the cuticle–cuticle CMC stains similar to the endocuticle.

In 1983, Leeder *et al.* (44) in a TEM study involving the effect of solvents on wool fibers found that formic acid treatment of wool fiber modified the CMC of the fibers, but this effect was only observed between adjacent cortical cells and not between cuticle and cortical cells. These scientists suggested that these results are consistent with differences in the CMC between cuticle cells versus the CMC between cuticle and cortical cells.

Peters and Bradbury (49) observed by electron microscopy that formic acid treatment of wool modified the cell membrane complex of the cortex, "but that of the cuticle appears unchanged." They also analyzed "resistant membranes" from cuticle and cortical cells isolated by shaking wool fibers in formic acid and oxidized with performic acid, and found differences, although their results for the Allworden membrane provided considerably lower values for cystine than the analysis of Allworden membranes by Allen *et al.* (30). Peters and Bradbury concluded that the "CMC of the cuticle differs from that of the cortex."

Leeder *et al.* in 1985 (50) described differences in the staining characteristics of the cuticle–cuticle CMC, the cuticle–cortex CMC, and the cortex–cortex CMC. After dyeing the fibers with a uranyl dye, these scientists found a layer of dye around each cuticle cell, which was restricted to the CMC of the cuticle and not in the CMC of the cortex. They found only one dye layer at the cuticle–cortex junction and none in the cortex–cortex CMC, but two layers of dye in the cuticle–cuticle CMC, and they referred to the observations of Nakamura *et al.* (8) on differences in the staining characteristics of these three types of CMC.

Mansour and Jones, in 1989 (37), treated wool by Soxhlet extraction with chloroform/methanol for five hours, and subsequently in boiling water for 15 minutes, and examined

the fibers by electron microscopy after each stage of treatment. After the initial solvent extraction, the cuticle–cortex CMC appeared unmodified, while the staining intensity of the beta layers between cortical cells were changed and appeared "intermittent." After solvent extraction for five hours and hydrolysis for 15 minutes, significant structural changes were observed. The cortex–cortex CMC showed an overall reduction in definition in the delta layer, and the beta layers displayed a lack of clear definition. These scientists suggested that solvent extraction of intercellular lipids makes the hair more vulnerable to hydrolytic damage, with the largest changes in the CMC occurring in the cortex–cortex CMC, which these scientists believe is related to a reduction in tear strength of wool fiber by solvent extraction and hydrolysis. These results show that the cuticle–cortex CMC behaves differently from the cortex–cortex CMC in reaction to solvent extraction. The cuticle–cortex CMC is damaged by solvent extraction and subsequent hydrolysis, but not as severely as the cortex–cortex CMC.

Logan *et al.,* in 1990 (38), examined wool fibers by TEM after extraction with chloroform/methanol and found that the cuticle–cuticle CMC appeared unchanged compared to untreated fibers. On the other hand, they found that the delta layer in the cortex was smaller and displayed variable staining intensity in most regions, which they deduced as "incomplete or preferential extraction." These scientists examined fiber sections after chloroform/methanol extraction, followed by treatment with formic acid, and noted large changes in the beta and delta layers of the cortex–cortex CMC that were "rarely observed" in the cuticle–cuticle CMC. They concluded that these results show that "inherent differences exist between CMC's of cuticle and those of cortical cells."

Negri *et al.* (39) in a 1996 paper referred to the work of Leeder *et al.* (50) and cited the work of Leeder, Bishop, and Jones (44), who showed that the unstained beta layers of the cuticle and cortex react differently to formic acid treatment. Formic acid removes proteins (51) from the cortex–cortex CMC (40), and it modifies the CMC junctions of the cortex but not the cuticle–cuticle CMC junctions; they referenced Nakamura (8), Leeder, Bishop, and Jones (44), and Peters and Bradbury (49) on these effects. They concluded that these observations suggest that only the beta layers of the cuticle–cuticle CMC contain covalently bound lipids, while the beta layers of the cortex contain lipids and a "stain-resistant membrane protein" that is "likely to be of a different structure than the cuticle membrane."

Inoue *et al.* in 2007 (52), by microbeam X-ray diffraction described that extraction of human hair with polar organic solvents (methanol or chloroform/methanol) at 37 degrees C for six hours removed some material from the delta layer of the cuticle–cuticle CMC, but that the beta layers were unaffected. On the other hand, the beta layers of the cuticle–cuticle CMC appeared to be affected by hexane extraction under the same conditions. The observation that changes in the delta layer of the cuticle–cuticle CMC by chloroform/methanol extraction could be detected suggests that this method is more sensitive than TEM (38). The fact that Inoue *et al.* observed changes in the beta layers of the cuticle–cuticle CMC by hexane extraction could result from removal of free lipids between the covalently bound fatty acids of the cuticle–cuticle CMC (Figure 2), resulting in the folding back of the covalently bound fatty acids in the beta layers and accounting for the differences found.

The above discussion shows clearly that both the lipid beta layers and the proteins of the cell membranes and those of the delta layer of the cuticle–cuticle CMC differ from those of the cortex–cortex CMC, with evidence for differences from the cuticle–cortex CMC also.

A PROPOSAL FOR THE STRUCTURE OF THE CUTICLE–CORTEX CMC

The following proposal for the cuticle–cortex CMC (Figure 7) is based on logic and the following supporting evidence. The work of Nakamura *et al*. (8) suggests that the cuticle–cortex CMC differs from both the cuticle–cuticle CMC and the cortex–cortex CMC. The work of Leeder *et al*. (44) and of Mansour and Jones (37) shows that the cuticle–cortex CMC is more resistant to solvents than the cortex–cortex CMC. But the most convincing evidence for this model (Figure 7) is the uranyl dye study by Leeder *et al*. (50) which showed layers of dye around each cuticle cell, i.e., two layers of dye in the cuticle–cuticle CMC, one layer of dye in the cuticle–cortex CMC, and no layers of dye in the cortex–cortex CMC.

Since the cuticle–cortex CMC bridges cuticle and cortical cells, it is logical to assume that it is a hybrid based partly on the cuticle–cuticle CMC and the cortex–cortex CMC. Therefore, the membrane on the cuticle side would be the cuticle cell membrane that supports covalently bound fatty acids that are bonded either through thioester, ester, or amide linkages, and these covalently bound fatty acids are connected on their hydrophobic end to a hydrophobic protein in the delta layer.

The membrane on the cortex side is a cortical cell membrane that supports fatty acids bound through polar and salt linkages, as illustrated in the schematic of Figure 7, and these fatty acids form a lipid bilayer. The delta layer of the cuticle–cortex CMC should then contain a hydrophobic protein on one side (to bond to the beta layer on the cuticle side) and a hydrophilic protein on the opposite side (to bond through polar and salt linkages to the lipid bilayer). Leeder *et al*. (50) in their TEM study on dyeing and diffusion suggested that either the cuticle beta layer or the resistant membrane surrounding cuticle cells has an affinity for the uranyl dye, whereas the cortical cell membrane or the delta layer between cortical cells does not. The models in Figure 7 (for the cuticle–cortex CMC), Figure 2 (for the cuticle–cuticle CMC), and Figure 3 (for the cortex–cortex CMC) are consistent with the results and explanation by Leeder *et al*. (50) of the uranyl dye binding in the three different CMCs.

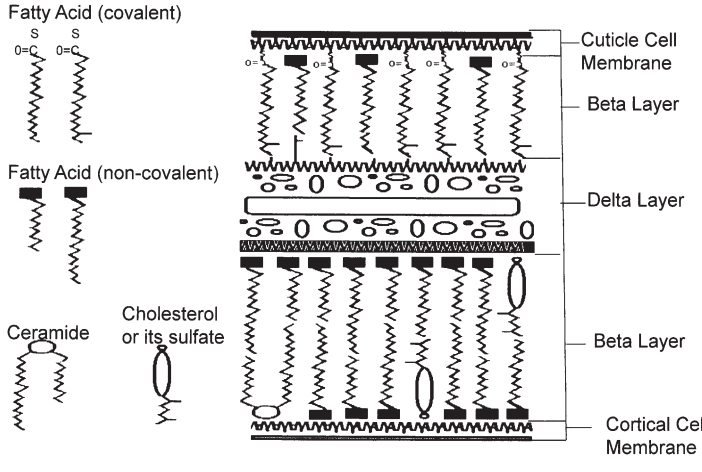

Figure 7. Schematic representing the cuticle–cortex CMC (not drawn to scale).

THE FORMATION OF THE CMC IN DEVELOPING HAIRS

The following description of the formation of the CMC in the developing hair fiber was taken from the work of Rogers (53), and also from the early work by Orwin and coworkers (54), with more recent work by Jones and coworkers (55). For more details of the formation of the CMC in developing hair fibers, I recommend the review by Jones and Rivett (11) and the recent paper by Jones *et al*. (55).

In the latter stages of development of the hair fiber, desmosomes or intercellular bridges, gap junctions (where cells exchange molecules), and tight junctions (intercellular junctions where cell membranes fuse) are established between differentiating keratinocytes of the hair fiber and the inner root sheath to varying extents as they move upward in the hair follicle (see Figure 8). Orwin *et al*. (54) observed that gap junctions and desmosomes cover about 10% of the plasma membrane of cortical cells in the bulb region and then gradually degenerate.

Tight junctions are established between Henle's outermost layer of the inner root sheath and Huxley's layer of the inner root sheath and between Henle cells and the close companion layer of the outer root sheath (see Figure 8). These junctions are replaced with a new cell membrane complex that gradually develops as a continuous complex between the cells. Similar events should occur for cuticle–cuticle CMC, cuticle–cortex CMC, and cortex–cortex CMC, with appropriate distinctions to allow for the differences that arise between these three different cell-connecting units.

LIPIDS OF THE CMC OF KERATIN FIBERS

METHODS TO REMOVE LIPIDS FROM ANIMAL HAIRS FOR ANALYSIS

Removal of external lipids. Wool fibers are normally processed by scouring with a nonionic agent such as Lissapol TN 450 (a nonylphenol ethoxylate with an average of 8.5 ethoxy units; CTFA = nonoxynol-8.5) and then in scientific studies treated with one or more solvents to remove any remaining external lipids. Non-swelling solvents and/or solvents of bulky molecules (like t-butyl alcohol) have been used to remove external lipids from keratin fibers, that is, lipids that are believed to be soil and not part of the internal structure of animal hairs. Solvents such as hexane, t-butyl alcohol, or heptane, and sometimes

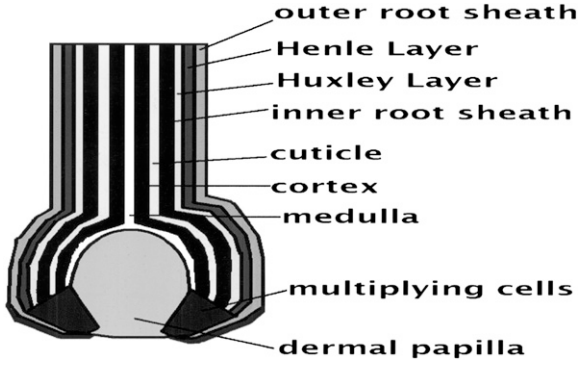

Figure 8. Schematic of the different layers of an active hair follicle (not drawn to scale).

t-butanol and heptane sequentially (36,56) have been used to extract "external lipids" such as wool wax or sebaceous matter from animal hairs. Such lipids are sometimes called external, extrinsic, or even exogenous (57) and are primarily sebaceous in origin in humans. A large percentage of these lipids are not believed to be involved in the intercellular structure of animal hairs, but there is evidence (58) that some of this solvent-extractable lipid may be part of the structure of the surface lipids of hair fibers.

In the case of human hair, external lipids are normally removed by shampoo or sodium lauryl sulfate washing, by a combination of shampoo followed by incubation in hexane for only five minutes (57), or in some cases by other solvents that most likely do not remove substantial amounts of internal hair lipids (e.g, ether or heptane).

Removal of internal lipids not covalently bound to hair. Hair-swelling organic solvents alone or in combination with a second lipid solvent are used to remove internal lipids that are part of the internal structure of hair fibers (of the CMC) but not covalently bonded to the hair protein structures. Solvents such as chloroform/methanol (44,56), methanol (52), ethanol (6), formic acid (50), n-propanol/water (50), or acetone (52) have been used to extract internal matter from animal hairs. The most frequently used solvent for removal of internal lipids has been chloroform/methanol (70/30), although other mixtures have been used. Normally soxhlet extraction is employed; however, multiple room temperature extractions have also been employed (57). Although formic acid and n-propanol/water (generally 1:1) do remove some internal lipids, these two solvents also remove some hair proteins of the CMC (most likely from the delta layers and possibly from other regions of the fibers), as will be described in the section entitled "Proteins of the CMC."

Removal of covalently bound hair lipids plus salts insoluble in lipid solvents. Alkaline hydrolysis or methanolic alkali is used to remove covalently bound hair lipids. This technique can be used to remove total hair lipids, but it is generally used after extraction of external and internal lipids that are not covalently bound to the fibers. Those lipids that are covalently bound at or near the fiber surface in the cuticle are generally removed with potassium t-butoxide in t-butanol (bulky cleaving agent in a bulky solvent) (59), and total covalently bound lipids are generally removed with potassium hydroxide in methanol because alkali in a swelling solvent like methanol penetrates well into hair.

In addition to covalently bound lipids, Wertz and Downing (34) have suggested that salts of cholesterol sulfate bind ionically to cationic groups on the hair proteins and will be insoluble in chloroform/methanol and therefore will remain in the fibrous residue after extraction with organic solvents. Korner *et al*. (48) used a solution of chloroform/methanol/aqueous potassium chloride to extract CMC lipids from wool and human hair and formed liposomes from the extracts. Since cholesterol sulfate is essential for liposome formation of keratin fiber extracts, this solvent system must extract cholesterol sulfate too.

TOTAL LIPIDS IN HAIR FIBERS

The total amount of lipid extractable from hair is generally 1% to 9% of the weight of the hair (57,60). Masukawa *et al*. (57) have provided a relatively thorough analysis of human hair lipids. In their study, the total hair lipid composition from 44 Japanese women, ages 1 to 81, was examined. The lipids were extracted/removed from hair in varying procedures, to allow for analysis of several lipids and covalently bound 18-MEA. In this study, total fatty acids and 18-MEA were determined, but other important fatty acids,

both covalently bound and non-covalently bound, such as palmitic, stearic, oleic and palmitoleic acids, were not quantitated and were found in significant quantities in other studies (13,34,35,42,56). Cholesterol sulfate was also not determined in this effort by Masukawa *et al*.

Logan *et al*. (56) analyzed human hair by extracting it with a chloroform/methanol azeotrope for five hours after surface lipids had been removed with t-butanol and heptane, and they found 23% palmitic, 25% palmitoleic, 4% stearic, and 13% oleic acids, as well as other fatty acids. These are all non-covalently bound fatty acids, with 39% of the total fatty acids being unsaturated (palmitoleic and oleic acids, but it is possible that other unsaturated fatty acids were present). Weitkamp *et al*. (61), by analysis of solvent-extracted lipids from pooled adult Caucasian human hair clippings, found 51% of the total fatty acids to be unsaturated, with palmitoleic and oleic acids as the principal unsaturated fatty acids, but other unsaturated fatty acids were found in these extracts.

Masukawa *et al*. (57) initially shampooed the hair and then washed it with hexane, allowing a five-minute incubation time. The hexane wash was determined by plotting the amount of lipid extracted versus the square root of the time of the hexane wash. The time that diffusion of lipids from the interior of the fiber began was determined graphically, a reasonable approach to removing external lipid soils from the fibers, presumably leaving most of the internal and structural lipids in the hair.

The lipids removed were separated into eight groups by Masukawa *et al*., and their data are summarized in Table II. These data show that approximately 58% of the total lipids in hair under these conditions are fatty acids, some covalently bonded, with others existing as free and non-covalently bound fatty acids. The total fatty acids found were 14.4 mg/gm of hair, but only 0.3 mg/gm hair of 18-MEA were found. Wertz and Downing (34) found 1.31 to 2.1 mg/gm of 18-MEA in four different human hair samples (three from individuals and one pooled hair sample, presumably Caucasian hair) and in a later paper (35) cited 4.0 mg/gm total integral (covalently bound) fatty acids with 40.5% as 18-MEA for human hair or 1.6 mg/gm 18-MEA. Since most 18-MEA estimates in wool fiber are close to 1 mg/gm or higher and human hair contains more cuticle layers than wool fiber, one would expect more covalently bound fatty acids in human hair than in wool

Table II
Lipids in Human Hair, from Masukawa *et al*. (57) and Wertz and Downing (35)

Type of lipid	mg/gm hair	Percentage of total lipid
Hydrocarbons	2.4	9.7
Squalene	0.7	2.8
Wax easters	4.9	19.8
Triglycerides	0.5	2.0
Total fatty acids	14.4	58.1 (58)**
Total covalent fatty acids	— (4.0)*	
Cholesterol	1.3 (0.6)*	5.2
Cholesterol sulfate	— (2.9)*	
Ceramides	0.29 (0.5)*	1.2
18-MEA	0.30 (1.6)*	1.2
Totals	24.79	100%

*Data in parenthesis by Wertz and Downing (35), not in parenthesis by Masukawa *et al*. (57).
**See Logan *et al*. (56) for a breakdown of the actual fatty acids in human hair.

fiber. Masukawa did not list amounts for total covalently bound fatty acids, only for 18-MEA. Therefore, I will use the data for Wertz and Downing (35) for total covalently bound fatty acids.

Since Masukawa *et al*. (57) found 14.3 mg/gm total fatty acid, but did not determine the total covalently bound fatty acids, and Wertz and Downing found 4 mg/gm total covalently bound fatty acids, then the Masukawa *et al*. finding most likely represents or is closer to the total amount of non-covalently bound fatty acids in human hair. Therefore, if we assume human hair has approximately 14 mg/gm of non-covalently bound fatty acids and we assume about ½ the equivalent amount of free lipid in the cuticle relative to covalently bound fatty acid, this provides 2 mg/gm of free fatty acid in cuticle layers and leaves about 12 mg/gm of non-covalently bound fatty acids. If we then assume 2 mg/gm of fatty acid as intracellular lipid, that leaves 10 mg/gm of fatty acids in the cortex–cortex CMC. Therefore, with these approximations, about 10 mg/gm of fatty acids will exist in the "bilayers" of the CMC of the cortex of human hair along with cholesterol, cholesterol sulfate, and ceramide, (see Figure 3).

Wertz and Downing (35) also found cholesterol (0.6 mg/gm), cholesterol sulfate (2.9 mg/gm), and ceramides (0.5 mg/gm) in their alkaline hydrolysates from human hair after removal of all free lipids by chloroform–methanol extraction. These same scientists also found these same lipid components in hair from sheep, dogs, pigs, cattle, and humans: cholesterol (0.3–1.4 mg/gm), ceramides (0.6–1.4 mg/gm), and cholesterol sulfate (0.7–3.3 mg/gm) (36). Examination of these data from different laboratories suggests the following ingredients in these approximate ratios as the principal components of the bilayers of the cortex–cortex CMC for human hair:

Lipid component	Approximate amount	Approximate relative amount
Fatty acids	10 mg/gm hair	10
Cholesterol sulfate	0.7 to 3.3 mg/gm	2
Cholesterol	0.6 to 1.2 mg/gm	1
Ceramides	0.6 to 1.4 mg/gm	1

These ratios are most likely not exact, but they show a large amount of fatty acid followed by cholesterol sulfate and smaller amounts of cholesterol and ceramide.

After shampooing an appreciable amount of free lipid remains on the hair surface. Shaw (62) suggested that washing hair with ether or shampoos in a one-step application leaves virtually the entire hair surface free of lipid (removable by surfactants or solvent) and that differences in cleaning efficiencies of surfactants relate to the amounts of internal lipid removed. Recent XPS data show that shampooing does remove some free lipid from the surface of hair, but even after shampooing a significant amount of free lipid remains in the surface layers, that is in the top 3 to 5 nm (58).

Free lipid in surface layers affects the isoelectric point of wool and hair. Capablanca and Watt (63) examined wool fiber that had been washed with detergent (Lissapol) and extracted with various solvents using a streaming potential method to estimate the effect of free-lipid (non-covalently bound fatty acids) in the surface layers on the isoelectric point of wool fiber. These scientists found an appreciable effect of free lipid on the isoelectric point. The surfactant-washed wool (containing the most free lipid) provided an isoelectric point of

3.3. The isoelectric point of wool increased as the effectiveness of the solvent system increased, with the most effective lipid solvent providing wool with an isoelectric point of 4.5. These data show that the true isoelectric point of the surface proteins of wool fiber is closer to 4.5 than 3.5 and that free fatty acids in the surface layers are an important and essential component of the surface of animal hairs. Thus, the more free lipid that is present in these surface layers, the lower the isoelectric point of keratin fibers.

Therefore, all free lipid is not totally removed and should not be totally removed from the surface layers by the shampooing of hair or surfactant scouring of wool fiber. In addition, free fatty acids are important to the isoelectric point of animal hair fibers. Furthermore, the amount of free lipid on the surface of hair fibers will influence hair friction, surface energy, and a whole range of important properties, including the adsorption of surfactants and other ingredients onto human hair and wool fibers.

PROTEINS OF THE CMC

The schematic in Figure 2 depicts cell membrane proteins and multiple layers of proteins in the delta layer of the cuticle–cuticle CMC analogous to the delta layer of the cortex (5,9). The structures and composition of the proteins of the CMC are still not adequately characterized. The primary reason for this gap is that it is extremely difficult to isolate proteins from only the cell membranes or only the delta layer, and this difficulty has been the primary obstacle to our understanding the composition and structure of the proteins of this important region of the fiber, but nevertheless I will review this area in this section. To date, much more scientific attention has been given to the analysis of cuticle cell membranes than to those of the cortex; therefore, I will begin this discussion on the proteins in the cuticle cell membranes.

PROTEINS IN THE CUTICLE CELL MEMBRANES

The proteins of the cuticle cell membranes are associated with the Allworden reaction (14) as described earlier. The membranous epicuticle supports 18-MEA and is attached to the A-layer on the top of cuticle cells and has been isolated by shaking animal hair fibers during Allworden sac formation with chlorine water and has subsequently been analyzed for amino acids. Perhaps the most quoted and "reliable" amino acid analysis of the Allworden membrane has been provided by Allen and coworkers (30), summarized in Table III.

Since the attachment of 18-MEA to hair proteins is through thioester linkages and the cuticle cell membrane protein is cross-linked by cystine bridges, Negri *et al*. (12) proposed that the lipid layer must be attached to an ultra-high sulfur protein (UHSP) that can provide attachment sites at approximately 1-nm spacings along the top of its folded chains.

Zahn *et al*. (31) have provided indirect evidence by a multiple regression technique for the presence of approximately 51% UHSP, 42% loricrin, and 7% involucrin in the Allworden membrane as analyzed by Allen *et al*. (30), that is, in the cuticle cell membranes of wool fiber or the 13–15 nm of the hair fiber surface underlying the F-layer or 18-MEA. See Table III describing the amino acid analyses of these and other important proteins adapted from the paper by Zahn, *et al*. (31).

From the results of this work and from previous work on the cell envelope of stratum corneum by Steinert and Marekov (68) and Jarnik *et al*. (69), Zahn *et al*. concluded that

Table III
Amino Acids (in mole%) of Allworden Membrane vs Calculated Values for Wool CE by Zahn *et al*. (31) and Proteins Believed to Be Part of This Membrane

Amino acids	Wool CE	Human loricrin	Human involucrin	Human UHSP	Human SPRP	Allworden membrane
Asp	2.7	0.3	2.8	3.4	0	3
Glu	9.8	4.4	45.8	8.2	28	8.6
Thr	2.2	2.2	1.6	10.3	2.4	2.1
Ser	15	22.8	1.6	10.9	0.4	14.3
Tyr	0.2	2.5	0.8	1	0	0
Pro	4	2.9	5.7	9	31.2	4.2
Gly	24.5	46.8	6.7	5	0	23.8
Ala	3.2	1	1.5	1.4	0	3.2
Val	3.5	3.5	3.7	3.8	9.6	5.6
Iso	1.1	1.6	0.4	1.6	0	1.2
Leu	2.4	0	14.6	2.4	1.6	2.9
Trp	0	0.3	0	0	0	
Phe	0.8	2.9	0.6	0.8	0	0.4
His	0.9	0.3	4.7	0.7	0.8	0.2
Lys	5.3	2.2	7.4	3.7	12. 8	4.5
Arg	1.7	0	0.7	5.6	0	2.5
Met	0	0	0.9	0	0	0
Cys	22.7	6	0.3	32.2	11.2	21.1
Totals	100	99.7	99.8	100	98	97.6

Wool CE calculated by Zahn *et al*. (31).
Human loricin from Hohl *et al*. (64).
Human involucrin from Eckert and Green (65).
Human SPRP from Marvin *et al*. (66).
Human UHSP from Tezuka and Takahashi (67).
Allworden membrane by Allen *et al*. (30).

loricrin and involucrin are most likely in the cellular envelopes of wool (CE wool) and thus of human hair (CE hair) and other animal hairs. Loricrin is the major component (60% to 80%) of the cornified cell envelope of stratum corneum in most animals, and involucrin, which is rich in glutamine, has an almost ideal structure to provide cross-linking sites to other proteins via isopeptide bonds. Therefore, involucrin is likely a major component of the cuticle cell membrane.

Isopeptide bonds are formed between glutamine and lysine, reacting in the presence of a transglutaminase enzyme similar to what Steinert and Marekov (68) have shown occurs in the cellular envelope of human skin. Exactly how these proteins might be arranged in the cuticle cell membranes is not known at this time. One reasonable conjecture is that two layers exist in the epicuticle, the outermost (upper) layer being comprised primarily of UHSP to provide anchoring sites for 18-MEA on the top portion of the membrane and also covalent anchoring sites for other fatty acids on the bottom part of epicuticle cells.

Involucrin is likely interspersed in the UHSP to provide isopeptide crosslinks to it and also to loricin and other proteins in the second layer of the cuticle cell membrane. Zahn *et al*. (31) also pointed out that small proline-rich proteins (SPRP) that are rich in glutamine and lysine are also most likely a part of this membrane because SPRPs (70) have been shown to participate in isopeptide cross-linking in the CE of stratum corneum and

because we know that isopeptide bonding is critical to the chemical resistance (attack by oxidizing and reducing agents) displayed by these membranes in human hair and wool fiber in the Allworden reaction and other reactions. Thus, the stability and resistance to chemical attack by this membranous material is provided through two types of crosslinks: cystine crosslinks (vulnerable to oxidizing and reducing agents) and isopeptide crosslinks that are resistant to oxidizing and reducing agents.

PROTEINS IN THE CORTICAL CELL MEMBRANES

Proteinaceous material called "resistant membranes" have been isolated from both the oxidation of wool and/or hair with performic or peracetic acid followed by treatment with either ammonia or alkaline urea (71). The authors of this paper state that this material from the performic acid reaction is similar to that of their own analysis of the Allworden membrane; nevertheless, it is clearly different from the Allworden membrane analysis by Allen *et al*. (30) as summarized in Table IV.

Treatment of keratin fibers with either peracetic or performic acids and separation into three fractions according to solubilities has been called the "keratose" method by Alexander and Earland (72). After oxidation, adjustment of the pH to the alkaline side provides an insoluble fraction called beta-keratose, about 10% of the weight of the hair. Acidification to pH 4 provides a fraction greater than 50%, called alpha-keratose, containing crystalline material, by X-ray diffraction, and the third fraction is called gamma-keratose. The beta-keratose fraction is believed to be proteins derived primarily from cell membrane material; however, other proteins are likely to be present. According to a different workup procedure by Bradbury *et al*. (71), only 1–1.55% residue is provided. Other workup procedures have been applied to the keratose method (73).

Table IV
Allen's (30) Analysis of the Allworden Membrane vs Resistant Membranes from Performic Acid Reaction with Wool Fiber (71)

Amino acids	Allworden membrane (30)	Resistant membranes (71)
Asp	3	5.4
Glu	8.6	10.3
Thr	2.1	5.7
Ser	14.3	10
Tyr	0	0
Pro	4.2	7.1
Gly	23.8	14.2
Ala	3.2	6.5
Val	5.6	4.9
Iso	1.2	2.6
Leu	2.9	4.9
Trp	—	0
Phe	0.4	1.5
His	0.2	1.3
Lys	4.5	8.4
Arg	2.5	4.2
Met	0	0
Cys	21.1	13
Totals	97.6	100

Since the cell membrane lipids of cortical cells are not bound by thioester linkages as in cuticle cells, but are bound by polar bonding and ionic bonds, a high level of cysteine links via an UHSP is not necessary for the cell membrane proteins of cortical cells. The resistant membrane material from the reaction of performic acid on wool fiber by Bradbury *et al*. (71) provides only about 62% of the amount of cystine as the composition of the Allworden membrane by Allen *et al*. (30) and is consistent with the expectation of a lower cystine content in cortical cell membranes versus cuticle cell membranes. In addition, one would expect the proteins of the cortical cell membranes to contain a larger number of basic amino acids (for bonding to cholesterol sulfate and to fatty acids), and more polar sites such as carboxyl groups would be preferred for polar bonding to fatty acid groups of lipids; we find nearly twice the basic amino acid content for salt link formation to cholesterol sulfate and fatty acids and more carboxyl groups of acidic amino acids in the membranes by Bradbury *et al*. (71). Furthermore, one would expect this performic acid-derived membrane material by Bradbury *et al*. to be richer in cortical cell membranes than cuticle cell membranes, since the cortical cell membranes should be a higher percentage of the total membrane matter in total wool fiber, and the data is certainly consistent with this expectation.

A cleaner experimental scheme to isolate pure cortical cell membranes would be to start with pure cortex to exclude cuticle cell membranes and A-layer proteins. Pure cortex from human hair could be provided by the glass fiber method of Wortmann *et al*. (74) and then perhaps by the performic acid reaction or another scheme to provide cortical cell membranes in the absence of cuticle contamination for further workup and analysis.

PROTEINS EXTRACTED FROM HAIR/WOOL BELIEVED TO BE FROM THE DELTA LAYER OF THE CMC

Leeder and Marshall (51) extracted Merino wool with formic acid and also with n-propanol/water (50/50). Proteinaceous matter was removed from the hair fibers with each of these solvent systems. With formic acid, these scientists concluded that the proteins were at least partially derived from the CMC, most likely from the delta layer because the extract contained virtually no cystine. If this proteinaceous material is from the delta layer, it most likely is not from the central proteins sometimes called the contact zone (or it contains small amounts of these proteins) because Naito *et al*. (75) have provided evidence that the contact zone contains hydrophilic protein with disulfide bonding.

Leeder and Marshall (51) concluded that the proteins derived by their own propanol/water extraction of wool is not entirely from the cell membrane complex, but that they also contain high glycine–tyrosine proteins possibly derived from the cortex. The amino acid composition of proteins extracted by formic acid, by n-propanol/water, and by chloroform/methanol are compared with that of the Allworden membrane in Table V.

Logan *et al*. (56) have shown that a chloroform–methanol azeotropic mixture provides a very different mixture of proteins than the high temperature propanol/water extraction (see Table V). Could this chloroform–methanol extraction be partially derived from cortical cell membranes or part of the outer lamella (outermost layer) of the delta layer proteins of the CMC? Since Mansour and Jones (37) have shown that chloroform/methanol extraction provides large changes to the cortex–cortex CMC in wool, it is possible that these proteins removed by chloroform/methanol extraction are at least partially attached to beta layers and are at least in part delta layer proteins of the cortex–cortex CMC.

Table V
Proteins Extracted from Wool with Formic Acid and n-Propanol/Water Compared with the Allworden Membrane

Amino acids	Allworden membrane (30)	Formic acid (50)	n-propanol (51)	$CHCl_3$/MeOH (56)
Asp	3	5.7	3.7	6.2
Glu	8.6	7.2	2.4	7.5
Thr	2.1	3.8	3.2	5.1
Ser	14.3	8.1	11.7	13.3
Tyr	0	12	16.4	3
Pro	4.2	4	5.2	6.4
Gly	23.8	19.2	25	11.9
Ala	3.2	5.2	2.2	8
Val	5.6	4.2	2.8	5.9
Ile	1.2	3.3	0.8	3.5
Leu	2.9	9.2	6.1	8.1
Trp				
Phe	0.4	5.2	7.8	3.6
His	0.2	1.2	0.7	1
Lys	4.5	4	0.8	2.7
Arg	2.5	6.2	5.2	4.1
Met	0	0.9	0.2	0.8
Cys	21.1	0.4	5.5	9
Totals	97.6	99.8	99.7	100.1

The method of Swift and Holmes (6) has been used by several different researchers to obtain proteinaceous matter believed to be partially derived from the CMC. This method involves dissolving matter from hair using papain with a reducing agent such as bisulfite or dithiothreitol (DTT). Bryson (76) conducted a series of experiments from which he concluded that the laminated structure observed under the TEM following a 72-hour digestion of wool fibers with papain and reducing solution (somewhat standard procedure) is not derived entirely from the CMC. Prolonging the digestion beyond 72 hours increased the number of laminated layers beyond what could be accounted for by the number of cortical cells in a fiber cross section. Bryson concluded that the CMC lipids were rearranging with other proteins and peptides to form these laminated layers.

Mass spectrometric analysis of the proteins of the digestion residue indicated that the majority of the protein component was papain, suggesting that the CMC lipids had rearranged with papain to form the laminated structures. Therefore, Bryson concluded that it is not possible to isolate pure proteinaceous CMC by papain digestion. These conclusions by Bryson are consistent with those of Swift and Bews (77), who concluded that although treatments of keratin fibers with enzymes and reducing agents do cause separation of cells, they could find no evidence of dissolution of the cuticle CMC via critical electron microscopic examination of treated hair sections. Therefore, the value of this method for isolation of CMC proteins is limited because of contamination with papain.

To repeat, the difficulty in isolating pure cell membrane proteins and pure delta layer proteins that are free of extraneous proteins and proteins from other regions of the fiber is the primary obstacle to our understanding of the composition and structures of the proteins of this important region of the fiber and the reason that the composition of the CMC proteins is still not adequately characterized.

CHEMICAL AND PHYSICAL ACTIONS ON THE CMC OF HAIR

The covalently bound lipids of the CMC of the cuticle are sensitive to oxidation, reduction, and alcoholic alkalinity, while the lipid beta layers of the cortex are affected more by lipid solvents and free radical chemistry. The beta layers of the cuticle are more sensitive to nucleophilic attack by species such as the hydroperoxide anion and mercaptans, but the beta layers of the cortex, with their multiplicity of double bonds (oleic plus palmitoleic acids, plus cholesterol and cholesterol sulfate) and tertiary hydrogen atoms (cholesterol and cholesterol sulfate) are more sensitive to free-radical chemistry. On the other hand, the membranes of the CMC are resistant to oxidizing and reducing agents (78). Several of these chemical actions make the CMC more vulnerable to fracture, to cuticle fragmentation, and to the propagation of cracks through the cortex, as will be described in this section.

There is evidence that a significant amount of free lipid (not covalently bound to hair proteins) is in the beta layers of the cuticle and likely in all lipid layers of keratin fibers (58). Since about 50% of free lipid in human hair is fatty acid, free lipid provides acidic groups to the hair surface and decreases the isoelectric point, as shown by Capablanca and Watt (63).

As hair is exposed to repeated shampooing, blow drying, and rubbing, and to sunlight, changes occur on and in the surface layers. These changes involve removal of some free lipids by shampoos and photo-degradation of 18-MEA, disulfide, and other functional groups, and consequently, fractures form in or between layers from bending, stretching, and abrasive actions.

These actions expose "new" protein material and sulfur acids, primarily sulfonate groups, with an accompanying decrease in the free lipid content of the hair surface, thereby converting the virgin hair surface from a hydrophobic entity with little surface charge to a hydrophilic, polar, and negatively charged surface. The more the exposure of the hair to chemical and abrasive actions, the further from the root ends the more hydrophilic the hair becomes and the more polar and more negatively charged the surface becomes.

DAMAGE BY SHAMPOOS AND CONDITIONERS

The dissolution or the removal of structural lipids or proteinaceous matter from hair, primarily from the CMC or endocuticle, by shaking keratin fibers either in surfactant solutions, shampoo solutions, or even water has been demonstrated by several different scientists. For example, Marshall and Ley (79) demonstrated the extraction of proteinaceous components from the cuticle of wool fiber by shaking wool fiber in surfactant solutions of sodium dodecyl sulfate, a common surfactant in many shampoos. Gould and Sneath (80) examined root and tip end sections of scalp hair by TEM. This hair had never been chemically treated. Gould and Sneath observed holes or vacancies in the thin cross sections, and these holes were more frequent and larger in tip ends than in root ends. These scientists attributed these holes to damaging effects by shampooing involving the breakdown and removal of the non-keratin portions (CMC and endocuticle) of the hair, leaving the intercellular regions more susceptible to fracturing.

One of the most common types of fractures in hair fibers forms in the dry state, and it occurs in the cuticle–cuticle CMC between the upper beta layer and the adjoining delta layer (see Figure 2) and is called beta-delta failure (39). Gamez-Garcia (32) noted that the

lower the relative humidity or the moisture content of hair, the lower the strain level at which beta-delta failure occurs. Beta-delta failure was observed by Negri *et al*. (39) on wool fiber, and they noted disruption of the cuticle–cuticle CMC along the upper beta layer in TEM sections. With this type of fracture, the delta layer and the lower beta layer are both retained on the underside of the "old" outermost cuticle cell, leaving 18-MEA as the "new" hair surface once the "old" outermost cuticle cell is abraded away. This type of fragmentation has been described in detail by Feughelman and Willis (33), who proposed that the failure of adhesion between overlapping scales involves 18-MEA, and that because of its branching, it provides mobility and a reduction in adhesion between scales, leading to beta-delta failure. Therefore, the degradation of 18-MEA between scales as occurs in chemical (81) or photochemical bleaching (81) or even permanent waving treatments (82) leads to further weakening of this structure and more rapid beta-delta failure, leading to faster cuticle fragmentation and cuticle loss.

WET STATE VS DRY STATE FAILURE

Deformations such as stretching (4,83) (including extension cycling (32), bending, or twisting in the wet state) are very different from deformations in the dry state. This is because failure in the wet state generally involves fractures or breaking bonds in hydrophilic layers, e.g., the endocuticle or the central contact zone of the CMC, whereas failure in the dry state generally involves fractures in or between hydrophobic layers, e.g., beta-delta failure (4,33). Failure in the wet state generally involves hydrophilic regions because when a layer or region is completely swollen, less mechanical stress is required to distort that layer and to produce a fracture. On the other hand, the lower the relative humidity or swelling condition of a hydrophilic layer, the more mechanical stress and strain required to distort hydrophilic vs hydrophobic layers, and therefore fractures are generally produced in hydrophobic layers.

Extension of undamaged hair-to-break generally produces smooth fractures (32). However, as the hair becomes more damaged or as the relative humidity is decreased, and especially at lower humidities, more step fractures are produced (32), and step fractures involve extensive fracturing in the cortex–cortex CMC, most likely in the beta layers. Kamath and Weigmann (83) have shown, for human hair at low moisture content, that crack initiation occurs most often in the cortex, whereas at high moisture content, fractures almost always initiate at or near the surface of the fiber because of the high pressure of the swollen cortex against the cuticle. Step fractures involve the axial propagation of cracks either through the cortex–cortex CMC or the medulla (83) and therefore occur more frequently in the dry state than when hair fibers are wet (83). Kamath and Weigmann (83) also concluded that the CMC seems to "play an important role in stress transfer and axial splitting" of human hair fibers.

The abrasion resistance of human hair is decreased by most chemical treatments and photo-oxidation, as shown by the "protein loss" test of Sandhu and Robbins (84) or by the release of labile and eluted proteins as described by Inoue *et al*. (85). These tests are both wet-state methods. The inside of cuticle and cortical cells is degraded by alkaline peroxide, thereby weakening the cuticle and cortex cells internally, and the cuticle–cuticle CMC is degraded, weakening the cellular cohesion or the resistance of scales to break apart. Cuticle fragmentation in the dry state is caused primarily by the rupture between cuticle cells

through beta-delta failure (32,33) and the resultant chipping of cuticle from the hair via abrasive actions. Cuticle loss in the wet state is primarily caused by the rupturing of cuticle cells internally and is greater in chemically damaged hair such as alkaline peroxide-treated or permanent-waved hair than in chemically untreated hair (84).

Fatigue testing, a method developed at TRI-Princeton by Ruetsch, Kamath and, Weigmann (involving attaching a weight to a hair and dropping the weight multiple times to continuously shock or jar the fiber), shows that alkaline peroxide treatment of human hair fibers when fatigued produces numerous scale-edge fractures with scale-edge chipping. Ruetsch (86) fatigue tested peroxide-treated hair, followed by extension, and found extensive fracturing in the CMC between the scales due to a weakened cuticle–cuticle CMC. This effect is most likely due to peroxide attack on thioester linkages that disrupts the beta layers of the cuticle–cuticle CMC.

Takahashi *et al*. (87) have provided evidence that wet cuticle wear in Asian hair is due more to CMC failure (possibly involving the central hydrophilic "contact zone" of the delta layer) rather than failure inside cuticle cells as in Caucasian hair (most likely endocuticular failure). Takahashi *et al*. (87) showed that wet cuticle wear on Asian hair occurs at a faster rate than on Caucasian hair because of differences in elasticity of the different layers inside cuticle scales. These scientists showed that the scales of Asian hair are removed faster by wet sonication after extension to 35% or by bleaching the hair followed by shampooing and combing the hair over a large number of cycles. In the latter case, after 90 times for four cycles, fewer scales were found on Asian hair relative to Caucasian hair (3.2 vs 1.3 scales, respectively).

On further examination of the hair using an atomic force microscopic probe, these scientists found a greater difference in elasticity as a function of depth for the Caucasian hair (1.41 vs 1.26), and they concluded that the scales of Asian hair are more uniform inside and that therefore the intracellular matter of Asian hair cuticle is more resistant to fracturing. Therefore, they concluded that the scales of Asian hair are removed more by fracturing in the cuticle–cuticle CMC (even in the wet state), while the scales of Caucasian hair fractures inside the scales are most likely in the swollen endocuticle. It is interesting to note here that Nakamura *et al*. (8) by staining reactions has concluded that the composition of the proteins of the delta layer of the cuticle–cuticle CMC is very much like that of the very hydrophilic endocuticle.

CMC LIPIDS DEGRADED BY VISIBLE LIGHT MORE THAN BY UV

Hoting and Zimmerman (88) have studied radiation damage as a function of wavelength and have shown that the CMC lipids of hair fibers are degraded most by visible light, but also by UV-A and UV-B light, helping to explain the weakened CMC (of cuticle and cortex) and the multiple-step fractures that result from the axial propagation of cracks through the cortex–cortex CMC in sunlight-oxidized hair. Obvious weak links to photochemical attack on lipid structures are the tertiary hydrogen atoms of 18-methyl eicosanoic acid (89) and cholesterol and cholesterol sulfate. The allylic hydrogen atoms of oleic and palmitoleic acids and of cholesterol and cholesterol sulfate in the cortex–cortex CMC are also vulnerable to photo-oxidative reactions.

Long-term irradiation does not provide for clean breakage between structural components of human hair, as was observed for peroxide-oxidized hair, but leads to cross-linking

or fusion reactions similar to long-term irradiation effects on wool fiber, as explained in the next section of this review (90). For hair damaged by sunlight, in most cases, the lipids of the cuticle–cuticle CMC sometimes appear altered to a greater extent than the more susceptible areas of the cortex–cortex CMC because the outer layers of the fiber receive higher intensities of radiation.

SHORT-TERM IRRADIATION ATTACKS CMC LIPIDS, PRODUCING INTERNAL STEP FRACTURES

Fracturing of wool fiber exposed to simulated sunlight has been studied microscopically by Zimmermann and Hocker (90). Electron micrographs of human hair fibers exposed to simulated sunlight and then fractured, showing that human hair provides similar effects to those of wool fiber, have been provided to this author by Sigrid Ruetsch. Zimmerman and Hocker demonstrated that stretching nonirradiated control wool fibers in water provided primarily smooth fractures, while short and intermediate times of simulated sunlight exposure caused the fibers to break mainly as step fractures. These scientists suggested that short- and intermediate-term irradiation damages the lipids of the CMC (all three types of CMC) and thereby provides many internal step-type fractures by axial propagation of cracks through the photochemically damaged cortex–cortex CMC. Longer-term irradiation creates crosslinks in the hair, fusing it and creating amorphous fractures.

Thus, short- and intermediate-term irradiation attacks CMC lipids, providing for increased beta-delta failure in the cuticle–cuticle CMC and multiple step fractures in the cortex–cortex CMC. Longer-term irradiation produces amorphous fractures by fusion reactions through the creation of carbonyl groups that are cross-linked through lysine groups, analogous to the oxidative damage to proteins and mitochondrial decay associated with aging, as described by Dean *et al*. (91). These fusion reactions start in the periphery of the fiber, where it receives higher intensities of radiation than the core, providing a smooth fracture at the periphery and multiple step fractures in the interior of the fibers and, if exposed long enough to light radiation, amorphous fractures are produced across the entire fiber.

Short- and intermediate-term exposures to radiation are propagated by abstraction of hydrogen atoms from tertiary carbon atoms of 18-MEA (89) and of allylic hydrogen atoms of oleic and palmitoleic acids in free lipids of the cuticle–cuticle CMC. The cortex–cortex CMC contains tertiary hydrogen atoms on cholesterol and cholesterol sulfate and allylic hydrogen atoms on oleic and palmitoleic acids, and on cholesterol and cholesterol sulfate, which react similarly.

The abstraction of hydrogen atoms from tertiary carbon atoms on amino acid side chains (analogous to the tertiary carbon atoms on 18-MEA and cholesterols) has been shown by Goshe *et al*. (92) to predominate over the abstraction of hydrogen atoms at the alpha carbon atom of amino acids in polypeptides. This fact helps to explain why the beta layers are degraded faster by photo-oxidation than the hair proteins via the cross-linking fusion reactions and why step fractures are produced on short- and intermediate-term radiation exposures, while amorphous fractures are produced only after longer-term exposures.

Hoting and Zimmermann (88) have also demonstrated that the CMC lipids of the cortex of hair, previously bleached with peroxide-persulfate, are more readily degraded by radiation

than the lipids of chemically unaltered hair or the lipids of hair dyed with a red oxidation dye. This conclusion was reached by analysis of the cholesterol-containing lipids of hair that reside primarily in the cortex–cortex CMC (Figure 3). Peroxide-persulfate oxidation of hair is primarily a free-radical oxidative process and it leaves hydroperoxide groups in the hair in the CMC and in other regions. Thus the action of sunlight on peroxide-persulfate bleached hair (containing hydroperoxides) makes the hair more vulnerable to cuticle fragmentation and to splitting effects that the CMC plays a significant role in. In this same paper these scientists demonstrated that one red oxidation dye provides photoprotection to both UV-A and visible light but not to UV-B light when compared to chemically untreated hair, and therefore retards the degradation of the CMC lipids, most likely by the dye acting as a radical scavanger.

LIPIDS REMOVED FROM HAIR BY PERMANENT WAVING

Hilterhaus-Bong and Zahn (93) and Mahrle *et al*. (94) independently showed that part of the lipid components of the CMC were removed from hair by permanent waving. Hilterhaus-Bong and Zahn examined internal hair lipids from permanent-waved hair versus non-waved hair by extraction with chloroform/methanol (internal lipids of the cortex–cortex CMC) and found significantly less internal lipid in permanent-waved hair permed at neutral pH and even less internal lipid in hair permed at pH 9.

PENETRATION INTO HAIR AND THE CMC

In 1983, Leeder and Rippon (95) described the effects of formic acid on the dyeing of wool fiber and concluded that formic acid removes labile lipid and non-keratin proteins from the CMC. These scientists described that formic acid is an excellent swelling medium for keratin fibers and applied Zahn's swelling-factor calculations (96) to the amino acid analysis of the CMC, from which they estimated that the CMC is swollen to a very large degree by formic acid (97). They also described that formic acid modifies the CMC and has a greater effect on the sorption of n-propanol than surface degradation treatments, and they therefore concluded that the CMC is an "alternative to the cuticle" for the penetration of dyes into keratin fibers. Naito *et al*. (98), in 1992 suggested that the delta layer provides a pathway for hydrophilic ingredients to penetrate into hair. Swift (99) and Inoue (52) have provided additional evidence that the CMC and endocuticle are pathways for diffusion of molecules into hair. Kreplak *et al*. (100) have shown by microbeam X-ray diffraction that the delta layer of the cuticle–cuticle CMC swells about 10–15% in water, and therefore, although it is hydrophilic, it is not as hydrophilic as originally thought; nevertheless, it still can serve as a pathway for diffusion of hydrophilic ingredients into hair. In addition, when the CMC or endocuticle have been weakened or damaged, the hair is even more penetrable to dyes and other chemicals through the CMC (52).

THE CMC OF WOOL FIBER VS HUMAN HAIR

To date, I could not find any references comparing the CMC of wool fiber versus human hair wherein significant structural or reactivity differences have been cited. As of this writing it would appear that the primary differences lie in the number of cuticle layers,

which relates to the amount of cuticle-to-cuticle overlap and to the relative amounts of cuticle–cuticle CMC versus cuticle–cortex CMC versus cortex–cortex CMC.

The Allworden reaction can be produced on both wool fiber and human hair (29). Although the reaction appears different on human hair than wool fiber, Bradbury and Leeder (15) have shown that this is because of much greater scale overlap on human hair, where only about $1/5^{th}$ to $1/6^{th}$ of each cuticle cell shows on the surface of hair fibers, and they explain that Merino wool contains only a single layer of cuticle scales, with approximately $5/6^{th}$ of each scale in the surface and only about $1/6^{th}$ scale overlap (29) on the surface. Individual cuticle cells have also been isolated from wool, human hair, and several other keratin fibers, and have been shown to behave similarly to chlorine water (29). 18-MEA accounts for 40–50% of the covalently bound fatty acids in human hair and wool fiber (35) and it is attached to the top surface of cuticle cells in both fibers (11). Table I shows that the covalently bound fatty acids are similar in both human hair and wool fiber and that covalently bound fatty acids are in the cuticle–cuticle CMC but not in the cortex in both of these fibers.

The compositions of the solvent-extractable lipids of both fibers are similar, consisting primarily of fatty acids, cholesterol, cholesterol sulfate, and ceramides (34,35). Furthermore, these lipids are extractable from both human hair and wool fiber with chloroform/methanol/aqueous potassium chloride, and liposomes can be generated from these extracts (48). Furthermore, these extracted lipids represent the main ingredients in the cortex–cortex CMC, and they are very similar in both fibers. And last, but not least, the effects of chemical and photochemical reactions and physical stresses are similar for the CMCs of both of these fibers, as shown in "Chemical and Physical Actions on the CMC of Hair," in this review. Perhaps, with additional research, significant structural and/or reactivity differences between the CMCs of human hair and wool fiber will become more apparent, but they are not apparent today.

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