INTRODUCTION

The stratum corneum of stratified squamous epithelia, composed of multiple layers of flattened dead cells, forms a tough, impenetrable barrier that shields the underlying living cells from hazards of the surrounding environment. Corneocytes are the end-product of the terminal differentiation pathway of keratinocytes in these tissues. Their organelles having broken down, they consist simply of a keratin filament matrix encased within the cornified cell envelope (CE). The CE is assembled late in the pathway when it replaces the cytoplasmic membrane, and consists of a layer of cross-linked protein coated with covalently bound lipids (Swartzendruber et al., 1987). It is a resilient material that is thought to be a major contributor to the protective role of the stratum corneum. This resilience is due primarily to crosslinking of the constituent proteins by ε-(γ-glutamyl)-lysine isopeptide bonds (Rice and Green, 1977; Thacher and Rice, 1985), reinforced by disulfide bridges. Indeed, it has been proposed that the biomechanical properties of the CE, considered as a ‘composite’ biomaterial, may be modulated by altering the frequency and nature of the cross links (Jarnik et al., 1996).

Despite its functional importance, the structure and assembly of the CE remain scantily understood. A number of proteins have been identified as CE precursors (Table 1; for reviews see Watt, 1989; Hohl, 1990; Reichert et al., 1993; Simon 1994), and it appears from analysis of peptides from proteolytic digests of isolated CEs (Steinert, 1995; Steinert and Marekov, 1995; 1997) that the major constituents are now known. Several lines of evidence support the notion that CE assembly is a multi-stage process (Reichert et al., 1993; Eckert et al., 1993; Steven and Steinert, 1994). First, a backing layer of such proteins as involucrin and cystatin-A is established by the transglutaminase cross-linking enzymes (Rice et al., 1994), possibly initiating at desmosomal sites (Ishida-Yamamoto et al., 1996; Steinert and Marekov, 1997), and continuing by processive attachment of substrate proteins to each other and to putative membrane-anchoring proteins (Reichert et al., 1993) such as enoplakin (Rührbeg et al., 1996). This layer serves as a substrate for deposition of loricrin together with other proteins, notably the SPRs (small proline-rich proteins: Kartasova and van den Putte, 1988; Jarnik et al., 1996). Loricrin is the major protein of nearly all native, i.e. tissue-

SUMMARY

In stratifying squamous epithelia, the cornified cell envelope (CE), a peripheral layer of crosslinked protein, is assembled sequentially from precursor proteins initially dispersed in the cytoplasm. Its major component is loricrin (37 kDa in mouse), which contributes from approx. 60% to >80% of the protein mass in different tissues. Despite its importance to the mechanical resilience and impenetrability of these tissues, detailed information has not been obtained on CE structure, even on such basic properties as its thickness or uniformity across a given CE or from tissue to tissue. To address this issue, we have studied CEs isolated from three murine epithelia, namely epidermis, forestomach and footpad, by electron microscopy of metal-shadowed specimens and scanning transmission electron microscopy (STEM) of unstained specimens. The former data reveal that the cytoplasmic surface is smoothly textured whereas the extracellular surface is corrugated, and that the average thickness is 15.3±1.2 nm, and strikingly uniform. Measurements of mass-per-unit-area from the STEM images yielded values of approx. 7.0±0.8 kDa/nm², which were remarkably consistent over all three tissues. These data imply that the mature CE has a uniquely defined thickness. To explain its uniformity, we postulate that loricrin forms a molecular monolayer, not a variable number of multiple layers. In this scenario, the packing density is one loricrin monomer per 7 nm², and loricrin should have an elongated shape, 2.5-3.0 nm wide by approx. 11 nm long. Moreover, we anticipate that any inter-tissue variations in the mechanical properties of CEs should depend more on protein composition and cross-linking pattern than on the thickness of the protein layer deposited.

Key words: Terminal differentiation, Cornified cell envelope, Keratinocyte, Covalent cross-linking, Transglutaminase, Loricrin, Electron microscopy
derived, CEs characterized to date (Hohl et al., 1993), accounting for approx. 60% to >80% of their protein mass (Tables 1, 2).

Beyond this broad outline, however, little is known about the detailed structure or modes of packing of molecules in the CE, or even whether these properties are uniform over the entire CE or exhibit local variations. So far, the basic property of thickness has been defined only in terms of measurements from electron micrographs of transverse thin sections. According to Matoltsy (1977), the CE consists of two electron-dense layers, one approx. 10 nm, the other approx. 2 nm thick, separated by a thin electron-translucent layer, for a total of approx. 15 nm: other observers have reported values of 15-20 nm (e.g. Hashimoto, 1969; Steven et al., 1990). However, these values are subject to considerable uncertainty. Fixation and dehydration may be accompanied by substantial shrinkage; staining may not be stoichiometric; in situ, the CE may be coated with additional material that is not detected in sections; and departures from exactly transverse sectioning geometry may result in an artifactual increase in perceived thickness (Leapman et al., 1997).

As a step towards achieving a more detailed account of their molecular architecture, we have studied the structures of isolated CEs by electron microscopy, with particular attention to thickness. The methods used, namely freeze-drying/metal shadowing (Abermann et al., 1972; Nermut, 1977; Kistler et al., 1977), and dark-field scanning transmission electron microscopy (STEM) of unstained specimens (Crewe and Wall, 1970; Wall et al., 1974), are unaffected by the shortcomings listed above. The shadowed specimens characterize the physical thickness and surface relief of the CEs, while the STEM data yield local measurements of mass-per-unit area.

**MATERIALS AND METHODS**

**Isolation of cell envelopes**

CEs were isolated from newborn mouse epidermis and from the forestomach and footpad of adult BALB/c mice, essentially as described by Mehrer et al. (1990). The epidermis was separated from the dermis after heating skin in PBS at 65°C for 30 seconds. Entire forestomachs and footpads were taken from killed mice. These tissues were thoroughly rinsed in PBS, and extracted for 10 minutes in 2% SDS-extraction buffer (EB) (100 mM Tris, pH 8.5, 2% SDS, 20 mM DTT, 5 mM EDTA), 5 ml per epidermis or corresponding amounts for the other isolates, on a boiling water bath with vigorous stirring. The suspension was centrifuged for 10 minutes at 12,000 g, the supernatant was discarded and the pellet re-extracted under the same conditions. This pellet was washed twice with 0.2% SDS-EB; (100 mM Tris pH 8.5, 0.2% SDS, 20 mM DTT, 5 mM EDTA), resuspended in 0.2% SDS-EB, and centrifuged for 10 minutes through 3% Ficoll (M, 400x10^6; Sigma, St Louis, MO) in 0.2% SDS-EB at 12,000 g. The pellet containing tissue debris and disrupted corneocytes was discarded. The suspension overlying the Ficoll cushion was checked by phase-contrast microscopy to confirm the presence of intact corneocytes. The forestomach preparation was contaminated with bacteria, which were removed by repeated centrifugation over a Ficoll cushion. The resulting isolated corneocytes were washed twice with 0.2% SDS-EB, disrupted by sonication for 3x10 seconds at 70% of maximum power on a Model 911001 sonicator (Kontes, Vineland, NJ), and collected by centrifugation for 10 minutes at 12,000 g. The pellet was resuspended and washed twice with 0.2% SDS-EB. CE fragments were separated from residual intact corneocytes by centrifuging through 3% Ficoll and washing 4x with 0.2% SDS-EB.

After the last wash, the pellet was resuspended to a final protein concentration of 0.5-5 mg/ml in 0.2% SDS-EB, and stored at 4°C for up to several months.

**Transmission electron microscopy**

Replicas of freeze dried/metal shadowed samples (Henderson and Griffiths, 1972) were prepared as follows: typically, 10 μl of isolated CE suspension were pelleted by centrifugation for 3 minutes at 3000 g, the supernatant was carefully removed, and the pellet resuspended in 40 μl of PBS with brief sonication. CEs in 10 μl drops were adsorbed for 5 minutes on 5x5 mm squares of freshly cleaved mica, the squares were washed 3x with PBS and 6x with doubly distilled water (DDW) and, after blotting off excess water, rapidly frozen in liquid nitrogen. The specimens were then placed on a metal block precooled in liquid nitrogen, transferred into a BAF 060 freeze-fracturing apparatus (Bal-Tec AG, Lichtenstein), and freeze dried at −100°C for 2 hours, at −80°C for 1 hour and at −60°C for 1 hour. Unidirectional shadowing with 0.7 nm of tantalum-tungsten was performed at −100°C at an elevation angle of 30°, and overlaid by approx. 15 nm of carbon, applied from above (i.e. 90°). Replicas were floated on DDW, cleaned with a 25% solution of sodium hypochlorite (bleach), washed 10x with DDW, and picked up on SPI EM grids (Athena, UK).

For antibody labeling, CEs were adsorbed to freshly glow-discharged, formvar/carbon-coated, 400 mesh EM grids as described above for mica. After three washes with PBS, nonspecific binding was blocked by incubation with 2% fetal calf serum in PBS. The specimens were incubated for 45-60 minutes with the primary antibody, washed 3x with PBS, and incubated on a drop of protein A/gold complex (30 μg/ml of protein, BBInternational, UK) for 30 minutes. After washing 3x with PBS and 6x with DDW, the samples were freeze-dried and shadowed with tantalum-tungsten as described above, except that only 3-5 nm of carbon were applied as a protective film.

Freeze-dried/shadowed samples and replicas were viewed on a Zeiss EM902 electron microscope, and micrographs recorded at a primary nominal magnification of 30,000×. Thickness measurements were made from enlarged prints at approx. 203,000×, with the magnification calibrated using negatively stained catalase crystals (Wrigley, 1968). As the thickness of the shadowing metal was very low (about 0.7 nm), no correction was necessary for the thickness of the metal layer (Moor, 1959).

**Scanning transmission electron microscopy (STEM)**

STEM was performed on the Brookhaven Biotechnology Resource instrument (Wall, 1979; Wall and Hainfeld, 1986). Freeze-dried specimens were prepared essentially according to Wall and Hainfeld (1986). Briefly, 20 μl of isolated CEs were pelleted, the supernatant removed, and the CEs resuspended in 20 μl of DDW by brief sonication. A 3 μl drop of tobacco mosaic virus (TMV) suspension in DDW (100 μg/ml) was placed on a thin carbon film mounted on a titanium grid, and adsorbed for 1 minute. After four washes with DDW, a 3 μl drop of CE suspension was adsorbed for 1 minute, and washed 10x with DDW. Excess water was blotted off, the grid frozen by plunging into liquid nitrogen, and dried overnight by gradually warming to ~80°C under vacuum. The grid was then transferred under vacuum into the microscope for viewing. Digital images of 512x512 pixels were recorded. In the images used for mass measurements, the pixel step was either 1 or 2 nm.

**Image processing**

Image processing was performed using the PIC-III program (Trus et al., 1996). The mass measurements represent averages over circular areas, 40 pixels in diameter. A mouse was used to designate the centers of selected areas on monitor-displayed images. Background subtractions were effected and the calculations completed according...
to standard procedures (Steven et al., 1983; Thomas et al., 1994). Mass measurements for images from a given grid were calibrated with reference to a set of TMV particles from the same grid. Line-scans of density (e.g. Fig. 5) represent lateral averages over areas 10 pixels (10 or 20 nm) across.

**Amino acid analysis and mathematical modeling of protein compositions**

Amino acid analysis was performed on an analyzer System 6300 (Beckman, Palo Alto, CA) using the ninhydrin detection method as described previously (Jarnik et al., 1996). The amino acid compositions of isolated CEs were estimated in terms of candidate proteins as described by Jarnik et al. (1996), using a least-squares program written for this purpose by P. Gryzenia, running on an Alpha 3000-500 workstation (Digital Equipment Corp., Maynard, MA).

**RESULTS**

**Surface relief and sidedness of isolated CEs**

To examine the molecular topography of isolated CEs, they were prepared for electron microscopy by freeze-drying and unidirectional shadowing with tantalum-tungsten (Ta-W). CEs usually adhered well to substrates of freshly cleaved mica or glow-discharged collodion/carbon films. Typical specimens from newborn mouse skin are shown in Fig. 1. The CEs appear as flakes of variable size up to several tens of μm across, with two clearly distinct surfaces, one smooth (s) and the other coarse (c). The coarse side exhibits shallow bumps, spaced approximately 10 nm apart, but without regular ordering. The relief of the smooth side appears to be as featureless as the supporting film at this shadowing angle of 30°, as well as the other angles tried (25°, 35°). Essentially the same results were obtained when platinum/carbon (Abermann et al., 1972) was substituted as the shadowing medium, except that the metal grain size was slightly larger. Shadowed CEs isolated from forestomach (Fig. 3B) and footpad (data not shown) yielded the same two topographies, smooth and coarse.

On the smooth surface, we occasionally observed patches, 0.2-0.5 μm across (e.g. Fig. 1, arrowheads), which have a different texture and presumably represent locally

### Table 1. CE major protein components

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Molecular mass (kDa)</th>
<th>Mass % in CE (1,2)</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loricrin</td>
<td>Mouse</td>
<td>37(3)</td>
<td>65-80%</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>25(4)</td>
<td></td>
</tr>
<tr>
<td>Involutin</td>
<td>Mouse</td>
<td>55(9)</td>
<td>&lt;5%</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>68(10)</td>
<td></td>
</tr>
<tr>
<td>SPRs</td>
<td>14±1(16)</td>
<td>6-18%</td>
<td>Crosslinked to loricrin and other proteins (17), Possibly</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>modulation of CE mechanical characteristics (1)</td>
</tr>
<tr>
<td>Cystatin A</td>
<td>14(18)</td>
<td>&lt;5%</td>
<td>Crosslinked to involucrin (2)</td>
</tr>
<tr>
<td>Keratins</td>
<td>50-58(19)</td>
<td>approx. 10%</td>
<td>Adhesion of cytoskeleton to CE (20)</td>
</tr>
<tr>
<td>Envoplakin</td>
<td>195(21)</td>
<td>&lt;5%</td>
<td>Anchoring protein for CE initiation (7,2)</td>
</tr>
<tr>
<td>Elafin (Human)</td>
<td>12±2.23(23)</td>
<td>&lt;5%</td>
<td>Crosslinked to loricrin (7)</td>
</tr>
</tbody>
</table>

References: (1) Jarnik et al., 1996; (2) Steinert and Marekov, 1997; (3) Mehrel et al., 1990; (4) Hohl et al., 1991; (5) Hohl et al., 1993; (6) Steven and Steinert, 1994; (7) Steinert, 1995; (8) Steinert and Marekov, 1995; (9) Djian et al., 1993; (10) Rice and Green, 1979; (11) Eckert et al., 1993; (12) Downing, 1992; (13) Marekov and Steinert, 1996; (14) Kartasova and van den Putte, 1988; (15) Gibbs et al., 1993; (16) Kartasova et al., 1996; (17) P. M. Steinert, personal communication, (18) Takahashi et al., 1992; (19) Blumenberg, 1993; (20) Steinert et al., 1995; (21) Rührberg et al., 1996; (22) Schalkwijk et al. 1993; (23) Molhuizen et al., 1993.

*Unless stated otherwise, murine proteins are described.

**Table 2. Protein compositions of CEs from mouse epidermis, forestomach and footpad**

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Molecular mass (kDa)</th>
<th>Mass % in CE (1,2)</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loricrin</td>
<td>Mouse</td>
<td>81.1±0.6</td>
<td>65-80%</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>25(4)</td>
<td></td>
</tr>
<tr>
<td>Filagrin</td>
<td>Mouse</td>
<td>5.5±0.9</td>
<td></td>
</tr>
<tr>
<td>Keratins</td>
<td>Mouse</td>
<td>5.6±1.4</td>
<td></td>
</tr>
<tr>
<td>Elafin (human)</td>
<td>12±2.23(23)</td>
<td>&lt;5%</td>
<td>Crosslinked to loricrin (7)</td>
</tr>
<tr>
<td>SPRs</td>
<td>14±1(16)</td>
<td>6-18%</td>
<td>Crosslinked to loricrin (7)</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>MD</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The protein compositions were estimated by mathematical modeling of the experimentally determined amino acid compositions of the respective CEs in terms of those of candidate proteins (Steven and Steinert, 1994; Jarnik et al., 1996). An average of 3 independent amino acid analyses was used for each sample. The compositions are given in percentages (w/w). The quoted uncertainties follow from statistical analysis of multiple calculations (1000 trials) in which random errors (s.d. 0.4%) were applied to the experimental data (see Jarnik et al., 1996). (→) The protein was concluded not to be present in detectable amounts (i.e. a negative or zero coefficient was given by the calculation). Other proteins, presumably present, are inferred not to account for more than a few percent, given the likely sensitivity of this method (see Jarnik et al., 1996). The quality of fit is gauged by the values of two residuals: MD, median discrepancy, and RD, root-mean-square discrepancy; and how close the unconstrained Sum of coefficients is to 100%.

†The epidermal and forestomach data are reproduced from Jarnik et al. (1996); SPRs, median of SPR1 and SPR2.

*The amount of keratin estimated here is higher than previously (Steven and Steinert, 1993) and is somewhat variable, in our experience. Owing to the very large amount of keratin in the cytoplasmic matrix of corneocytes, contamination of CE preparations with even a tiny fraction of this material would make keratin appear as a significant fraction of total CE protein. However, evidence for the covalent attachment of at least some keratin molecules has been documented in the recovery of covalent cross-links between keratin and loricrin, albeit at very low incidence (Steinert and Marekov, 1995). On account of these uncertainties, our current model (Fig. 6) does not specify the involvement of keratin with the CE.
differentiated structures. Since these patches are integral to normally textured CEs, we conclude that they are not contaminating structures (e.g. bacterial envelopes). They are observed on CEs from all three source tissues studied, accounting for approx. 5% of the net surface area of epidermal and forestomach CEs, and somewhat more, approx. 15%, for footpad CEs.

### CEs isolated from skin, forestomach and footpad have the same uniform thickness

At places where the CE lies flat on the substrate and its edge is perpendicular to the direction of shadowing (e.g. Fig. 1, inset), one may measure its thickness from the length of shadow cast, given the elevation angle of the shadowing source (Henderson and Griffiths, 1972). Our thickness data are shown in Fig. 2 and Table 3. The CEs average 15.5 nm (newborn mouse skin), 14.9 nm (forestomach) and 15.2 nm (footpad), respectively. For all three specimens the thickness was quite uniform, as documented by the narrow unimodal distributions (Fig. 2A-C). In no case did the standard deviation exceed 8% of the average thickness, and no significant difference was recorded between CEs from the three different source tissues.

The coarse and smooth surface topographies correspond to opposing sides of the CE, as demonstrated by specimens that are folded over and present both topographies (e.g. Fig. 1). To identify which side represents the cytoplasmic surface, we labeled CEs with antibodies specific for loricrin and SPR1. These proteins are known to be deposited on the cytoplasmic surface of CEs from newborn mouse skin (loricrin: Mehrel et al., 1990) and forestomach (SPR1: Jarnik et al., 1996). Isolated CEs were adsorbed to EM grids and then subjected to antibody labeling with detection by Protein A and colloidal gold (see Materials and Methods). Finally, the specimens were prepared for observation by freeze drying and metal shadowing. Typical results for newborn mouse skin CEs labeled with anti-loricrin antibodies are shown in Fig. 3A, and for forestomach CEs labeled with anti-SPR1 antibodies in Fig. 3B. In all such experiments, we found that the labeling was confined to the smooth side, thus demonstrating that it represents the cytoplasmic surface.

The differentiated patches visible on the smooth surface of the CE label less densely, if at all, with anti-loricrin and anti-SPR1 antibodies, further suggesting that they have a different molecular composition from the bulk of the CE.

### Table 3. Physical thickness and mass-per-unit-area of CEs from three source tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Physical thickness±s.d. (nm)</th>
<th>Mass-per-unit-area (kDa/nm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn mouse skin</td>
<td>15.5±1.1 (n=159)</td>
<td>7.0±0.8 (n=234)</td>
</tr>
<tr>
<td>Mouse forestomach</td>
<td>14.9±1.1 (n=63)</td>
<td>6.8±0.8 (n=278)</td>
</tr>
<tr>
<td>Mouse footpad</td>
<td>15.2±1.3 (n=54)</td>
<td>7.3±0.8* (n=233)</td>
</tr>
</tbody>
</table>

*These data apply to the major peak in this distribution (see Fig. 4F). n, number of measurements.
Uniformity in mass-per-unit-area of isolated CEs

Freeze-dried CEs, prepared without metal shadowing, were imaged by dark-field STEM (Fig. 4A–C). Except for local folds and rumpled areas, CEs from all three sources spread uniformly on the support film and appeared as sheets of constant density. Their edges are sharply demarcated, but otherwise they show few structural features. The differentiated patches seen in the shadowed data were not evident in the STEM images, implying that their mass-per-unit area is very similar to that of the rest of the CE.

Because we intended to use these images for mass measurements, the specimens were comprehensively washed after being adsorbed to the grids, to eliminate non-volatile salts and other deposits that might bias the measurements. In this respect, we were particularly concerned about the possible retention of SDS, which is needed in the CE storage buffer to prevent clumping: tightly bound SDS might not be removed, even by extensive washing. To assess the SDS retained by CEs after grid preparation, the molar ratio of sulfur:nitrogen was measured by electron spectroscopy in a STEM (R. D. Leapman, personal communication). The sulfur signal emanates from the SDS as well as from Cys and Met residues in CE proteins, while the nitrogen signal reflects the protein content. These calculations indicated that the mass fraction of SDS on CEs after our grid-washing protocol is less than 4%.

Local measurements of mass-per-unit-area were made directly from these images. Large data sets (>240 measurements) were obtained for each type of CE. The resulting distributions are presented in Fig. 4D–F and Table 3. The data for epidermal CEs and forestomach CEs observe similar Gaussian-like distributions, averaging 7.0 kDa/nm² and 6.8 kDa/nm², respectively. Footpad CEs contain a major component (average 7.3 kDa/nm²) that is very similar if not identical to the two other kinds of CEs. Taken together with the physical thickness measurements, these data imply that their structures are essentially the same, despite some differences among their respective protein compositions (Table 2, see also Jarnik et al., 1996).

Footpad CEs also contained a minor component at 5.2±0.5 kDa/nm², accounting for approx. 15% of the data. These CEs may represent a distinct, less mature population (see Discussion). We do not ascribe any significance to the fact that similarly low density CEs were not observed in our epidermal and forestomach preparations since, even in footpad preparations, CEs of this kind were rare.

The apparent constancy in mass density of the CEs was examined more closely in line-scans across the STEM micrographs (Fig. 5A–I). The scale of local fluctuations, as assessed from these scans, is notably small. Some variability is encountered at the edges, where one sometimes sees a narrow zone of higher density (e.g. Fig. 5A,D), which presumably represents rumpling. However, the predominant impression given by these data is of constancy of mass density along a scan-line; from scan to scan in different parts of a given CE; from one CE to another for a given specimen (e.g. forestomach); and for CEs from all three source tissues.

DISCUSSION

In this study, we used electron microscopy to compare CEs isolated from three different murine tissues. Their respective protein compositions differ somewhat (Table 2; Steven and Steinert, 1994; Jarnik et al., 1996). Footpad CEs appear to contain less loricrin (although at approx. 60% it is still by far the most abundant protein) and more filaggrin, and thus resemble forestomach CEs more closely than epidermal CEs. However, these compositional differences are not sufficiently pronounced to register as structural differences. The most striking observation to emerge from these experiments is the remarkable uniformity of these specimens in both physical thickness (approx. 15.3 nm) and projected mass thickness (approx. 7.0 kDa/nm²).

CE thickness and laminar organization

Thin section micrographs have yielded values of 15-20 nm for the thickness of the CE (Hashimoto, 1969; Matoltsy, 1977). Despite the abundant reasons why these values should be treated with caution (see Introduction), the average value of 15.2 nm that we have obtained by quantitative analysis of isolated CEs is remarkably similar. We qualify this
concordance by noting that isolation involved the harsh procedure of boiling in SDS, which would be expected to denature most proteins, so that the thickness of native CEs may be altered by this treatment. However, their networks of covalent cross-links are not disrupted by this treatment and impose constraints that may allow the CEs to revert to something like its original state. Also, it is possible that additional proteins may be non-covalently associated with the CE in situ and would contribute to the stained layer seen in thin sections, although they would be detached by our isolation procedure.

The total thickness of the CE places an upper limit on the thickness of its protein layer, for some of this space must be occupied by the attached lipids (Swartzendruber et al., 1987). In transverse sections, the CE has a trilaminar appearance (Matoltsy, 1977; Steinert and Idler, 1979; Swartzendruber et al., 1989; Steven et al., 1990). The thicker electron-dense layer (10 nm) is on the cytoplasmic side and presumably represents positive staining of CE protein. Interpretation of the other two layers (the 2 nm electron-dense and electron-translucent layers, respectively) is less evident: they may represent coating lipids, in which case the protein layer would be only 10-11 nm thick; alternatively, they may represent the putative backing layer of protein with attached lipids.

**CE assembly: implications of uniformity of thickness**

CE precursor proteins are synthesized in the cytoplasm and then incorporated into the CE by transglutaminase action (Reichert et al., 1993; Rice et al., 1994). In granulocytes of some tissues (e.g. mouse forestomach), loricrin condenses into spherical L-granules (Steven et al., 1990; Jarnik et al., 1996), presumably reflecting unusually high levels of synthesis. Because there is no sign of these granules in corneocytes, it appears that they are redistributed into the nascent CE. In this context, the remarkable uniformity in thickness of the CE requires explanation, i.e. it appears that some specific and widely applicable mechanism ensures this uniformity. Otherwise, if precursor proteins were simply incorporated where they first make contact with the nascent CE, one would expect significant variations in thickness, and in particular, a substantial thickening around sites where L-granules were dismantled, providing locally rich sources of precursors. A further argument in favor of such a mechanism follows from the consideration that the synthesis of precursors in granulocytes is apparently regulated to provide just enough material to cover the cytoplasmic surface of corneocytes to a precisely specified thickness, with little if any protein left over: e.g. little soluble loricrin may be extracted from the epidermis (Mehrel et al., 1990).

**Specialized regions of the CE**

Structurally differentiated patches, 0.2-0.5 μm in diameter, are detected in our shadowed micrographs (e.g. Fig. 1), accounting for 5-15% of the net surface area of CEs in our preparations. The patches are not visualized in the STEM micrographs, whence we conclude that their projected density is similar to that of bulk phase CE. Their different texture and immunoreactivity suggest that their protein composition differs from that of the bulk phase. Possibly, they may represent the remnants of desmosomal sites. (We note that 5% of total surface area is equivalent to about 20% in linear extent, given random sampling in transverse sections).

The only other specimens with different structural properties that we have observed were the rare low-density CEs seen in footpad preparations. Our tentative interpretation is that they may represent partially matured CEs, since their average density, approx. 5 kDa/nm², is higher than we would expect for
Molecular architecture of cornified cell envelope

...a loricrin-less backing layer (approx. 2 kDa/nm², see below). However, the amount of data available does not warrant further discussion at this stage. An investigation of immature CEs (Reichert et al., 1993) prepared as described (Steinert, 1995; Steinert and Marekov, 1995) is being undertaken.

**A molecular model of the CE**

The simplest explanation that we have been able to devise for the remarkable uniformity in thickness and projected density of mature CEs is that they should contain a monolayer of loricrin. Our current model, incorporating elements of earlier proposals, is shown in Fig. 6. A surface density of 7.1 kDa/nm² for the mature CE corresponds to 6.4 kDa/nm² of protein, since covalently bound ceramides are estimated to account for 10% of its mass (Swartzendruber et al., 1988). If loricrin and SPRs account together for 80-90% of the protein mass (Table 2), then the scaffold can account for no more than 10-20%, i.e. 0.6-1.2 kDa/nm². This is a rather low density and implies that the scaffold should consist of a sparse web of involucrin rods (Eckert et al., 1993), inter-connected with other proteins (Steinert and Marekov, 1997). In view of its low density, such a structure would not be readily visualized directly in thin sections, although it may well be already assembled in the sub-granular layers and detectable by immuno-gold labeling (Ishida-Yamamoto et al., 1996).

The remainder of the CE protein mass is mainly loricrin,
accounting for approx. 5.2 kDa/nm² in epidermis. In the postulated loricrin monolayer, this corresponds to an average footprint of approx. 7 nm² per monomer. Thus close-packed molecules should have a diameter of approx. 2.8 nm.

Approximating loricrin as an ellipsoid with this cross-section, it should be about 11 nm long to accommodate a mass of 37 kDa, taking a typical partial specific volume for protein (0.73 ml/g; Perkins, 1986). This calculated length fits well with our CE thickness measurements, i.e. an 11 nm rod projecting from a layer approx. 4 nm thick of ceramide plus scaffolding proteins. The inferred closeness of packing (average intermolecular spacing approx. 2.8 nm) is consistent with the fine-grained texture of the cytoplasmic face of the CE, portrayed by shadowing (Fig. 1).

This model envisages loricrin as an elongated molecule with an axial ratio of approx. 4. However, even if it were spherical (axial ratio=1.0), a 37 kDa monomer would have a diameter of 4.5-5.0 nm, so that the 11 nm-thick loricrin layer could not be stacked more than 2-3 molecules deep (cf. Fig. 3 of Steinert and Marekov, 1995). While our hypothesis of a loricrin monolayer clearly needs further testing, we find it more plausible than the alternative of an indeterminate number of multiple layers because it explains, in a straightforward way, the remarkable uniformity in CE thickness that we observe. In contrast, the multi-layer scenario does not readily account for a uniformly thick CE unless one invokes some additional and indirect mechanism, such as limited reach of a membrane-bound transglutaminase that fills in the loricrin layer behind it.

Fig. 5. Line-scans across-dark-field STEM images of CEs isolated from newborn mouse skin (A-C), mouse forestomach (D-F) and mouse footpad (G-I). The scans represent lateral averages over areas 10 pixels (20 nm) wide. Their density scale was calibrated relative to TMV, our internal mass standard (see Materials and methods). The arrowhead on each scan indicates the average value of mass-per-unit-area calculated for the respective type of CE.

Fig. 6. Molecular model of the mature CE. Loricrin is depicted as an elongated molecule (red), approx. 11 nm long and 2.5-3.0 nm in cross section. The length assigned to involucrin (44 nm) is from Yaffe et al. (1992), whence we infer that it should lie in the plane of the CE. Loricrin molecules engage in side-by-side interactions, probably in both parallel and antiparallel relative orientations (see Discussion). The crosslinking molecules in the part (purple ellipses) should be mainly SPRs, whereas those deeper in the CE (i.e. in the part furthest from the cytoplasm) should be mainly elafin (yellow spheres), if mouse CEs resemble human ones in this respect (Steinert and Marekov, 1995). The inference of underlying anchoring proteins is from Reichert (1993).
as it retreats in a processive manner. While such a mechanism is not ruled out, we find it relatively contrived.

The foregoing discussion is somewhat over-simplified in the sense that at least two modes of interaction between neighboring loricrin molecules are likely to be in effect (Steinert and Marekov, 1995, 1997), i.e. direct loricrin-loricrin contacts, and linkage via the SPRs (Fig. 6). The above calculations apply to an average over all such arrangements. We note that the oligomeric status of loricrin as a building block of the native CE has not been determined, but essentially the same arguments apply if loricrin were to be an oligomer of laterally associated, 11 nm-long, subunits.

A further point in favor of the proposed arrangement of an in-register, side-by-side packing of elongated loricrin molecules is that it accounts readily for many of the loricrin-loricrin and loricrin-SPR cross-links detected by Steinert and Marekov (1995), particularly if the loricrins may be oriented either in parallel or antiparallel (Fig. 6).

Finally, the present observations have implications for our hypothesis of the CE as a composite biomaterial (Jarnik et al., 1996), whose mechanical properties are tailored to the requirements of a given cornified epithelium via the structure of the CE. Taking into account the observed uniformity of thickness, we may further specify that such modulation should be accomplished by adjusting the molecular composition of the composite and/or the frequency of cross-links, not by simply changing the thickness of the CE, e.g. by enhanced expression of loricrin.

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