Water Distribution and Related Morphology in Human Stratum Corneum at Different Hydration Levels

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This study focused on the water distribution in human stratum corneum and on the swelling of the corneocytes. For this purpose stratum corneum was hydrated to various levels and used either for Fourier transform infrared spectroscopy or for cryo-scanning electron microscopy. The images were analyzed with respect to water localization and cell shape. The Fourier transform infrared spectra were measured to study the water–lipid interactions. The results show that water only slightly changes the lipid transitions in the stratum corneum even at a hydration level of 300% wt/wt compared to stratum corneum and that water is inhomogeneously distributed in the stratum corneum. No gradual increase in water level was observed in depth. At 57%–87% wt/wt water content the hydration level in the central part of stratum corneum is higher than in the superficial and deeper cell layers. Water domains are mainly present within the corneocytes and not in the intercellular regions. At a very high hydration level (300% wt/wt), the corneocytes are strongly swollen except for the deepest cell layers adjacent to the viable epidermis. The corneocytes in these layers are not swollen. At 300% wt/wt hydration level water domains are also present in intercellular regions. Between 17% wt/wt and 300% wt/wt the cell thickness increases linearly with the hydration level suggesting that swelling of cells mainly occurs in the direction perpendicular to the skin surface. At an increased hydration level, the corneocyte envelope more efficiently surrounds the cell content compensating for the increased cell volume. The changes in stratum corneum morphology with increasing water level have also been observed in dermatomed skin. ***Key words: swelling/water/stratum corneum/distribution. J Invest Dermatol 120:750–758, 2003***

The rate-limiting step for diffusion across the skin is the upper layer of the skin, the stratum corneum (SC). The SC is composed of corneocytes embedded in hydrophobic lipid domains. The majority of these lipids form crystalline lamellar phases. The corneocytes are filled with crosslinked soft keratin. It has now been established that most drugs applied onto the skin permeate across the intercellular lipid lamellar regions. This has been particularly illustrated by confocal studies (Simonetti *et al*, 1995), which revealed that fluorescent probes mainly diffuse along the intercellular tortuous pathway. In the 1960s it was already established that the lipid domains in SC play an important role in the skin barrier function (Matolsky *et al*, 1968). Besides a proper lipid organization, the level of hydration of the SC is one of the key elements for the skin barrier function. The level of hydration is a function of the humidity of the environment and the hygroscopic properties of the SC. The hygroscopic properties of the SC are strongly affected by the presence of natural moisturizing factor. For example, a reduced level in natural moisturizing factor (Rawlings *et al*, 1993) is one of the reasons why in dry skin the hydration level is lower than in normal skin. The skin hydration level not only affects the skin barrier, but also affects the enzymatic activity in SC and in turn the desquamation process and natural moisturizing factor level (Rawlings *et al*, 1994). Recently it has been established that the natural hydration level of SC *in vivo* is around 30%–50% (Caspers *et al*, 2001) of its dry weight. *In vitro* the SC hydration level can vary widely. When SC is equilibrated over water, the SC increases to 300%–400% of its dry weight. Water is a very effective natural penetration enhancer in increasing the flux of hydrophilic as well as of lipophilic substances. It has been reported (Scheuplein and Blank, 1971; Blank *et al*, 1984) that the penetration of water increased by a factor of 2–3 when the hydration level increased. Even after an uptake of 300% wt/wt water, however, a reduced skin barrier remains. Furthermore, whereas at low hydration levels the SC is brittle and fragile, as soon as the water level exceeds a value of around 10%–15% wt/wt, the SC softens markedly. Most probably, water acts as a plasticizer similarly as reported in hydrophilic polymers (Yates, 1996). It has been reported that only if the amount of water exceeds 20%–30% of SC dry weight will free water (having the same properties as pure water) be present in the skin (Bulgin and Vinson, 1967; Hansen and Yellin, 1972). Although the hydration level under normal conditions is 30%–50% wt/wt of SC dry weight (Caspers *et al*, 2001), the hydration level of SC *in vivo* can increase substantially under occlusion (Graves *et al*, 1995; Held and Jorgensen, 1999) or in a very humid environment. Therefore the effect of high hydration levels on SC structure are also of interest for the *in vivo* situation.

Several studies have been published in which the interactions between water and SC lipids have been addressed. In one of the first studies the effect of water on lipid transitions was studied by

Manuscript received August 20, 2002; revised October 20, 2002; accepted for publication December 17, 2002

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Abbreviation: SC, stratum corneum.

differential thermal analysis. Van Duzee (1975) reported a slight reduction in lipid phase transition temperatures when the water content was increased, indicating that water affects the skin lipid organization. When the water content exceeded 20% wt/wt, however, a further increase in water content did not further reduce the SC lipid transition temperatures (Cornwell *et al*, 1996). In another study Fourier transform infrared spectroscopy was used to examine the effect of water on the mobility of the SC lipids (Mak *et al*, 1991; Gay *et al*, 1994). These studies reported almost no alterations in lipid mobility, but a linear relation between water transport and chain mobility at elevated temperatures (Potts and Francoeur, 1990). In contrast, electron spin resonance studies showed a slight increase in lipid mobility with increasing water content (Alonso *et al*, 1995). The swelling of the lipid lamellae in SC has also been studied. The X-ray diffraction technique revealed almost no swelling of the lamellae (Bouwstra *et al*, 1991) when hydration increased. In addition no changes in the lateral packing of the lipids were reported either (Bouwstra *et al*, 1992). From these results it is obvious that almost no water is located in the lamellar domains in the intercellular regions. Fourier transform infrared, X-ray diffraction, and electron spin resonance are all bulk techniques, however, which are not able to detect local changes in the lipid organization. Norlén *et al* (1997) studied the swelling of the SC on a light microscopy level and reported that swelling mainly occurs in the direction perpendicular to the skin surface. Recently, the swelling of isolated corneocytes has been studied by Richter *et al* (2001) by scanning force microscopy. In order to obtain more detailed information about the localization of free water in the SC, freeze fracture electron microscopy has been explored (Van Hal *et al*, 1996). This study revealed that at high SC hydration levels water is located in the interior of the corneocytes and in the intercellular regions. In the intercellular regions water domains were frequently visualized. The almost complete absence of swelling of the lamellae, the insensitivity of the lipids to the hydration level, and the presence of water domains led us to conclude that water and SC lipids most probably phase-separate and that the level of water in the head group regions is very limited. The cleaning of the replicas, however, which cannot be avoided with freeze fracture electron microscopy, results in fragmentation of the replicas. For this reason it is almost impossible to visualize the ultrastructure of the SC as a function of depth or localization. An additional problem is that the fracture is created along the pathway of least resistance. The absence of a flat transverse fracture surface makes it impossible to study the shape of cells as a function of hydration level.

As SC is the protecting layer between the highly hydrated viable epidermis and the relatively dry environment, it has been suggested that a water gradient exists in the SC. This water gradient has even been measured *in vivo* in humans (Bommannan *et al*, 1990; Caspers *et al*, 2001). Until now the water has not been localized as a function of depth in the SC, however. In addition, a key issue is to understand the mechanism of swelling of the corneocytes, while the cells are entirely surrounded by bound lipids chemically linked to the cornified envelope. These bound lipids are arranged in crystalline packing and act as a template during the formation of the intercellular lamellae. Due to this tight packing, it is expected that a swelling of the corneocytes has to occur without inducing a reduction in packing density of these lipids and thus without a marked change in the total surface of the corneocytes.

The aim of this study was to reexamine the interactions of water and SC lipids using Fourier transform infrared spectroscopy and to provide information on the water localization in the SC. The gradient and the swelling mechanisms of the cells after uptake of water were studied using cryo-scanning electron microscopy in combination with cryo-planing. Compared to freeze fracture electron microscopy this method has the advantage that flat surfaces are created that allow the shape of transverse sections of the corneocytes to be studied. Furthermore, as the planed surface is intact it is possible to examine the swelling of corneocytes and the localization of water domains as a function of depth in one plane. The studies presented in this paper reveal a small reduction in temperature of the lipid transition at 35°C. Furthermore, no gradual increase in water content from the superficial cell layers to the deepest corneocyte layer adjacent to the viable epidermis has been observed. Even after uptake of 300% wt/wt of water, the corneocytes in the deepest cell regions do not swell compared to dry SC, which is in agreement with the localization of natural moisturizing factor (Rawlings *et al*, 1993; 1994).

MATERIALS AND METHODS

Isolation and hydration of human dermatomed skin and SC Human abdomen or mammae skin was obtained after cosmetic surgery and processed the same day. The subcutaneous fat was removed and the skin was dermatomed to a thickness of approximately 250–300 μm (Padgett Dermatome, Kansas City, KS). If dermatomed skin was used, it was immediately equilibrated over either a saturated Na_2CO_3 solution or pure water for 24 h as described below for SC. To obtain SC, the dermatomed skin was incubated with its dermal side on Whatman paper soaked in a solution of 0.1% (wt/vol) trypsin (Sigma, Zwijndrecht, The Netherlands) in 0.15 M phosphate-buffered saline (PBS: NaCl 8 g per l, KCl 0.19 g per l, KH_2PO_4 0.2 g per l, Na_2HPO_4 2.4 g per l) overnight at 4°C and for 1 h at 37°C. After this the SC was peeled from the epidermis. Then the SC was equilibrated at 37°C over either silica gel, a 27 wt/wt% NaCl solution, a saturated Na_2CO_3 solution, or pure water for 24 h. After that the SC was cut into small pieces and processed for either weighing or cryo-planing followed by cryo-scanning electron microscopy. All experiments were carried out with SC of at least three donors.

Weighing of SC In order to determine the water content, SC was cut into four sheets each of 2.5 × 8 mm. Prior to hydration the SC was dried over silica gel and weighed on a microbalance (Mettler 3000 system), after which the sheets were put on a wire mesh. Then the SC was equilibrated at 37°C for a period of 24 h over the various solutions described above and weighed again. The hydration level was calculated as follows: wt/wt% = 100 × (weight hydrated SC − weight dry SC)/weight dry SC.

Fourier transform infrared spectroscopy Prior to Fourier transform infrared spectroscopy measurements the SC sheets were hydrated for 24 h over salt solutions prepared with deuterated water for 24 h (see above). Then the sheets were clamped between AgCl windows. The Fourier transform infrared spectra were collected at 2 cm^{-1} resolution at 2.5°C intervals from 20°C to 50°C and at 5°C intervals between 50°C and 90°C on a Biorad spectrometer equipped with a broad band MCT detector. Then the spectra were analyzed with the WIN-IR Pro 2.5 software.

Cryo-scanning electron microscopy

Cryo-planing The method of cryo-planing and cryo-scanning microscopy is schematically presented in **Fig 1**(***A***). SC was cut into sheets of 8 × 8 mm^2 (dermatomed skin was cut in sheets of 2 × 3 mm^2) and equilibrated as described above. Then the equilibrated SC sheets were folded and fixed in a small copper "shoe-nail" using tissue-freezing medium (TBS, Durham, NC) as embedding medium, except for SC equilibrated over pure water and dermatomed skin, which did not require additional embedding material. Tissue-freezing medium is a mixture of polyvinyl alcohol, carbowax, and nonreactive ingredients.

When embedded in tissue-freezing medium the SC surface side cannot be distinguished from the epidermal side. In the case of fully hydrated SC, however, a very dense stacking of SC sheets is achieved with alternately the superficial SC layers and deepest SC layers of successive SC sheets facing each other. This offers the possibility of localizing the surface and epidermal sides of the SC. After folding and embedding, the SC samples were immediately rapidly frozen in liquid propane (KF80, Reichert Jung, Vienna, Austria). The cryofixed samples were placed in a sample holder in a cryo ultramicrotome (Reichert Ultracut E/FC4D). Then the samples were planed with a specimen temperature of −90°C and a knife temperature of −100°C. The samples were first cut with a glass knife, after which the surface was planed with a diamond knife (Histo no trough, 8 mm 45°C, Drukker International, The Netherlands). For each donor at least five images were taken per hydration level.

Cryo-scanning electron microscopy After planing the samples were placed in a cryo-transfer unit (CT 1500 HF, Oxford Instruments, U.K.). The samples were freeze dried for 3 min at −90°C at 0.1 Pa to obtain contrast, after

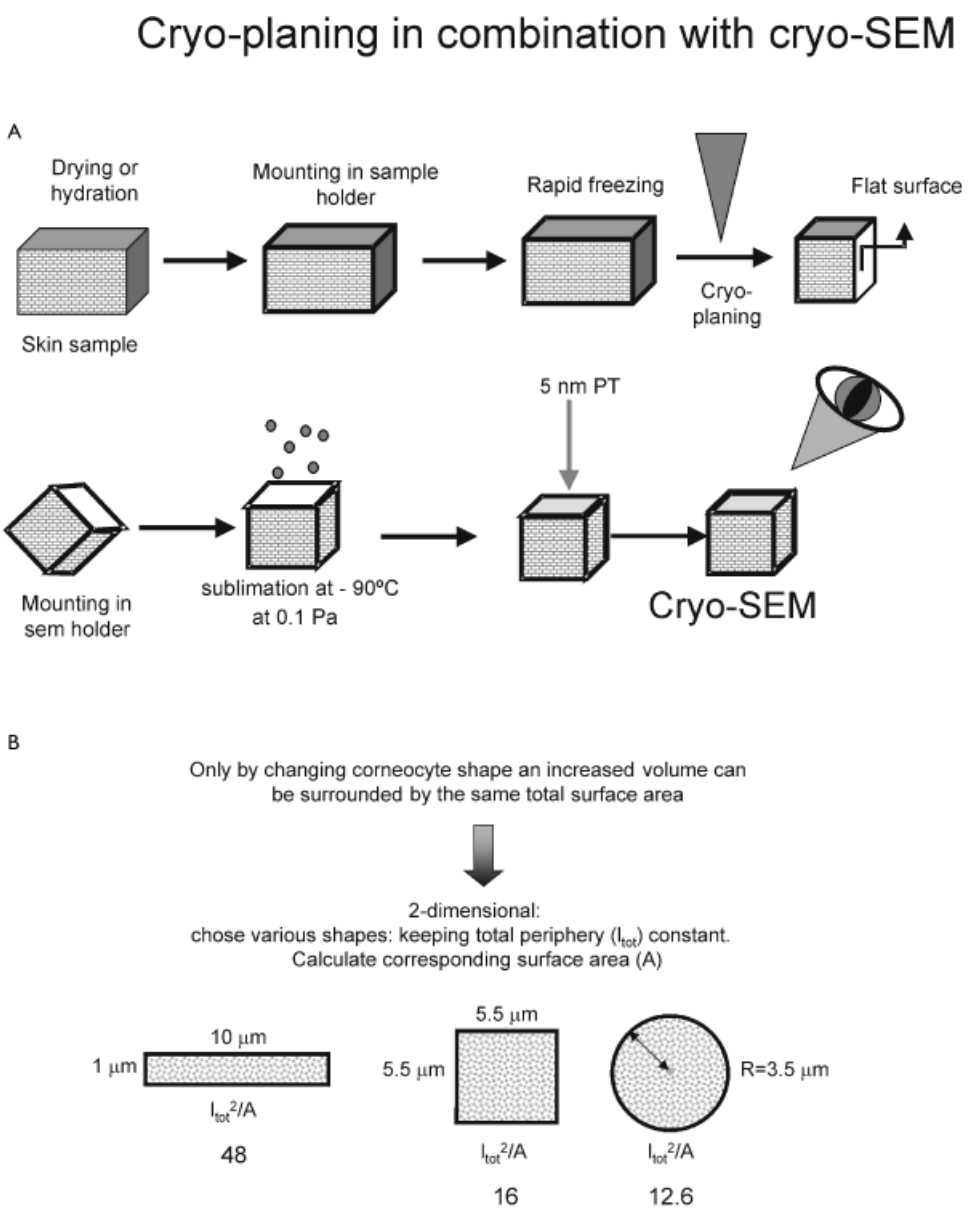


Figure 1. Cryo-scanning electron microscopy. (*A*) Schematic presentation of the sequential events of the cryo-scanning electron microscopy technique. (*B*) The change in the $(l_{tot})^2/A$ ratio when the shape of the selected area is changed from a rectangular to a circle. In the calculations l_{tot} (the total length of the periphery) was kept constant. *A* is the surface area of the selected shape. This parameter varies between the various shapes.

which the samples were sputtered with a layer of 5 nm platinum. Then the samples were transferred into the field emission scanning electron microscope (Jeol 6400F, Japan). The samples were analyzed at −190°C and the micrographs were digitally recorded.

Numerical cell parameters For each donor and hydration level three micrographs were selected. In the upper, central, and lower part of the SC a cell cross-section was selected. Only those cells were selected for which the entire cell contour was visible in the images. With an adjusted version of the program SCILImage 1.3 (Faculty of Mathematics and Computer Science, University of Amsterdam, Amsterdam, The Netherlands) the cell thickness perpendicular to the longest axis was determined for each pixel along the longest axis of the cross-section, after which the mean thickness of the cell was calculated. Furthermore the cross-sectional surface of the selected cell (*A*) and the total length of the cell envelope (l_{tot}) surrounding the cross-sectional surface were determined. The mean value of the dimensionless ratio $(l_{tot})^2/A$ was calculated. This dimensionless parameter is strongly dependent on the shape of the cell: a reduction in $(l_{tot})^2/A$ indicates that the periphery (l_{tot}) more efficiently surrounds the enclosed area (*A*). An example is provided in **Fig 1**(***B***), in which the value is calculated for a rectangle, a square, and a circle, all having the same l_{tot} enclosing a different area *A*. The highest value of these three samples is obtained in the case of the rectangle, and the lowest $(l_{tot})^2/A$ value is obtained when considering a circle.

RESULTS

Level of hydration The level of hydration in SC after hydration over NaBr varied between 18% and 26% wt/wt. The equilibration over Na_2CO_3 results in a substantial variation in the hydration level between 57% and 87% wt/wt. The level of hydration after hydration over pure water varied between 292% and 332% wt/wt. The variations in hydration level are most probably either caused by a high sensitivity of the SC sheets for small changes in relative humidity of the environment or due to a variation in the quantities of natural moisturizing factor between the various donors.

Orthorhombic–hexaganol phase transition is slightly affected by the hydration level In **Fig 2**(***A***) a typical example is shown of the CH_2 rocking frequency of the lipids in

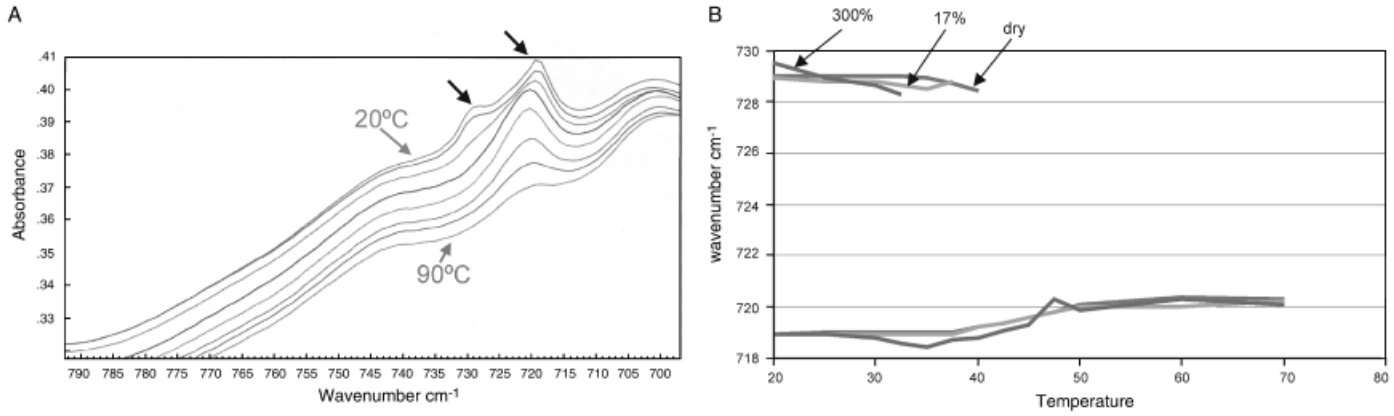


Figure 2. The orthorhombic–hexagonal transition as detected by Fourier transform infrared spectroscopy. (*A*) A typical example is shown of the CH_2 rocking frequencies as a function of temperature in dry SC. Below 30°C the mode splits into two bands (group splitting) characteristic for orthorhombic packing. Above 40°C only one single band is observed indicating hexagonal lateral packing. (*B*) The temperature dependence of the rocking frequencies as a function of temperature for SC of one donor. A similar frequency dependence as a function of temperature was observed for the other donors as well (SC from three donors was used).

SC as a function of temperature. The orthorhombic phase is characterized by group splitting, which results in a doublet at a wavenumber of approximately 730 cm^{-1}. Increasing the temperature results at around 35°C in disappearance of the group splitting. This is characteristic for a transition from orthorhombic to hexagonal lateral packing. When considering the hydration dependence of this transition, a small decrease in the temperature of transition is observed when the hydration level increases (see **Fig 2*B***). This is observed for the SC of all three donors indicating a small effect of water on lipid organization. The infrared spectrum also permits the detection of the intramolecular chain conformational order. Transition from fully extended all-*trans* to disordered chains results in a frequency increase of the CH_2 asymmetric and symmetric stretching modes localized at around 2850 and 2920 cm^{-1}. In the spectra obtained between 20°C and 95°C no hydration level dependence on the all-*trans* to disordered mode (monitored at around 60°C–70°C) could be detected (spectra not shown).

Cryofixation and slicing results in flat surfaces After cryofixation, the SC was cryo-planed approximately perpendicular to the skin surface in order to create a flat surface. A low magnification image of the planed surface of stacked layers of SC embedded in tissue-freezing medium is depicted in **Fig 3(*A*)**. In the embedding material thin sheets of SC cross-sections are observed. This image demonstrates that planing of SC in tissue-freezing medium results in a flat surface. In **Fig 3(*B*)** 300% wt/wt hydrated SC is shown. The SC is very densely stacked, and no tissue-freezing medium was required. Again a flat slicing surface is observed, which is needed in order to study the corneocyte contours and cell thickness. The number of cell layers in SC varied between 12 and 20; the mean number of cell layers was 15.

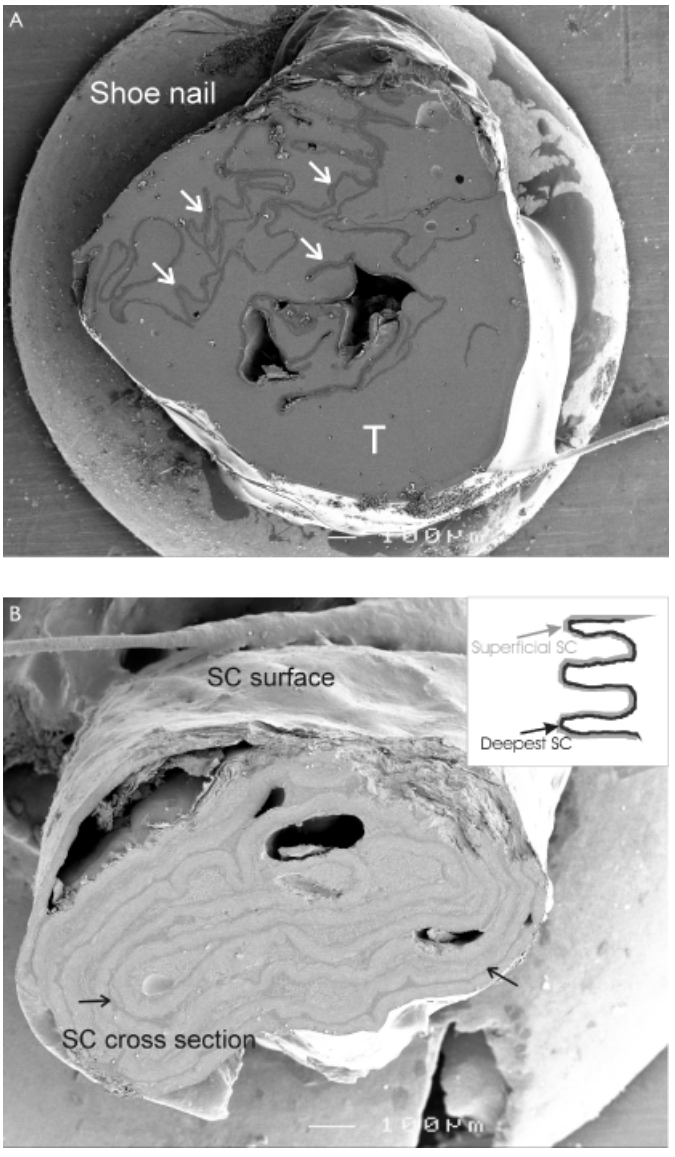


Figure 3. Low magnification images of cryo-planed SC. In both images flat planes are present. (*A*) A cross-section of SC sheets hydrated to 17% wt/wt embedded in tissue-freezing medium (T). The *arrows* indicate the SC transverse planes. (*B*) A cross-section of SC sheets hydrated to 300% wt/wt. The SC is densely stacked. No tissue-freezing medium was required. The *arrow* indicates the nonhydrating cell layers in the SC (see **Fig 4** for more details). The SC surface is clearly visible. As depicted in the *inset*, the SC is folded in such a way that the deepest SC layers and superficial SC layers alternately face each other. This allows us to distinguish the superficial and deepest layers in SC in the cryo image.

Interpretation of images obtained by cryo-scanning electron microscopy Contrast in an image of unstained tissue is related to the relief of the sample surface. A low contrast image is indicative of a very flat surface created by planing the cryofixed samples. A higher contrast results from the freeze drying procedure of water, which increases the relief of the sample surface. In a higher contrast image the dark regions correspond to holes (lower regions) in the surface. The bright regions correspond to the higher regions of the sample surface. This contrast allows the localization of holes in which, prior to the cryofixation, the free water was located in the tissue (see **Fig 1*A***). Therefore, in the text below a higher water content in the tissue is related to a higher contrast in the images caused by the presence of holes. No bound water can be detected by this method.

The water in SC is not homogeneously distributed and does not change gradually with depth dry SC A high magnification image of dry SC is depicted in **Fig 4(*A*)**. On both sides of the SC the embedding material is shown, which is characterized by a rough surface. At strongly modulating cell boundaries the cells are interdigitating. This was particularly observed at cell ends (**Fig 4*A*, *B***). In dry SC undulations of cell contours were quite frequently observed. These undulations were not homogeneously distributed in the SC. No gradual change in shape between the corneocytes in the superficial, central, and deeper SC regions was observed. The mean cell thickness was 360 ± 27 nm. SC hydrated over NaBr (18%–26% wt/wt). The images obtained showed almost no contrast. In some SC regions undulations and interdigitation are observed (see **Fig 4*B***, *arrows*). The mean cell thickness is 370 ± 21 nm. The low contrast appearance indicates the absence of regions with free water. SC hydrated over saturated Na_2CO_3 (57%–87% wt/wt). The mean cell thickness in the SC increases to 750 ± 100 nm. As depicted in **Fig 4(*C*)**, **(*D*)**, nonhomogeneous appearance of the SC is observed. In the deepest SC regions and in the superficial regions of the SC the appearance is almost similar to that of 17% hydrated SC. In the central SC region the images reveal a higher contrast, however, indicating the presence of free water in the corneocytes in these regions. The presence of water is also demonstrated by the increased cell thickness in the central SC regions compared to the cell thickness in the superficial and lower part of the SC. The images reveal no indication of water-rich domains in the intercellular regions of the SC. This strongly suggests that most of the free water is taken up into the corneocytes. In the low contrast regions undulation can still be observed (see **Fig 4*C***, *arrow*). SC hydrated over pure water (292%–332% wt/wt). In **Fig 4(*E*)** cross-sections of four fully hydrated sheets of swollen SC are shown, indicated by the roman numerals I, II, III, and IV. SC I and SC II are folded in such a way that in the cross-section the deepest SC regions of sheets I and II are in contact (see **Fig 3*B*** for an explanation of folding SC). **Figure 4(*E*)** clearly shows a nonswollen (low contrast) region. This region contains the two to three deepest corneocyte layers of each SC sheet having a small thickness (see *asterisks*) and a low contrast appearance, both indicating that these cells do not contain free water. The cells located adjacent to this region are considerably swollen. The swollen cells are characterized by a high contrast. The appearance indicates the presence of many water domains between the keratin filaments. In some regions there is even an alternating sequence of swollen and nonswollen cells (see **Fig 4*E***, *small arrows*). Furthermore, even within one corneocyte differences in swelling are observed between the various regions as demonstrated by a variation in density of the holes caused by the water domains and the slightly smaller contrast in appearance of these cells. The mean thickness of the swollen corneocytes is approximately 2970 ± 260 nm. In fully hydrated SC water is not only taken up by the corneocytes, but also present in domains in the intercellular regions. The image in **Fig 4(*F*)** indicates clearly the presence of large and many small water pools. The intercellular water domains have an oval shape, most probably minimizing the interface between the hydrophilic water and lipophilic lipid domains. The contours of the cells in fully hydrated SC are still slightly undulating but less undulation is shown than observed in dry skin. Furthermore, the cell ends are more rounded. Finally we often noticed that the central part of the cells was more swollen than the regions close to the cell ends.

A linear relationship is observed between the cell thickness and hydration level in SC In **Fig 5(*A*)** the mean cell thickness for each donor as calculated by the SCILImage 1.3 program is plotted against the hydration level of the SC. An approximately linear relationship exists between the hydration level and the cell thickness. This obviously shows that the corneocytes mainly swell in the direction perpendicular to the skin surface (increase in thickness). This is in agreement with the findings of Norlén *et al* (1997). **Figure 5(*B*)** shows that the parameter $(l_{tot})^2/A$ decreases as a function of the SC hydration level. This indicates that at higher water content the corneocyte envelope more efficiently surrounds the cell content compared to the efficiency at low water content.

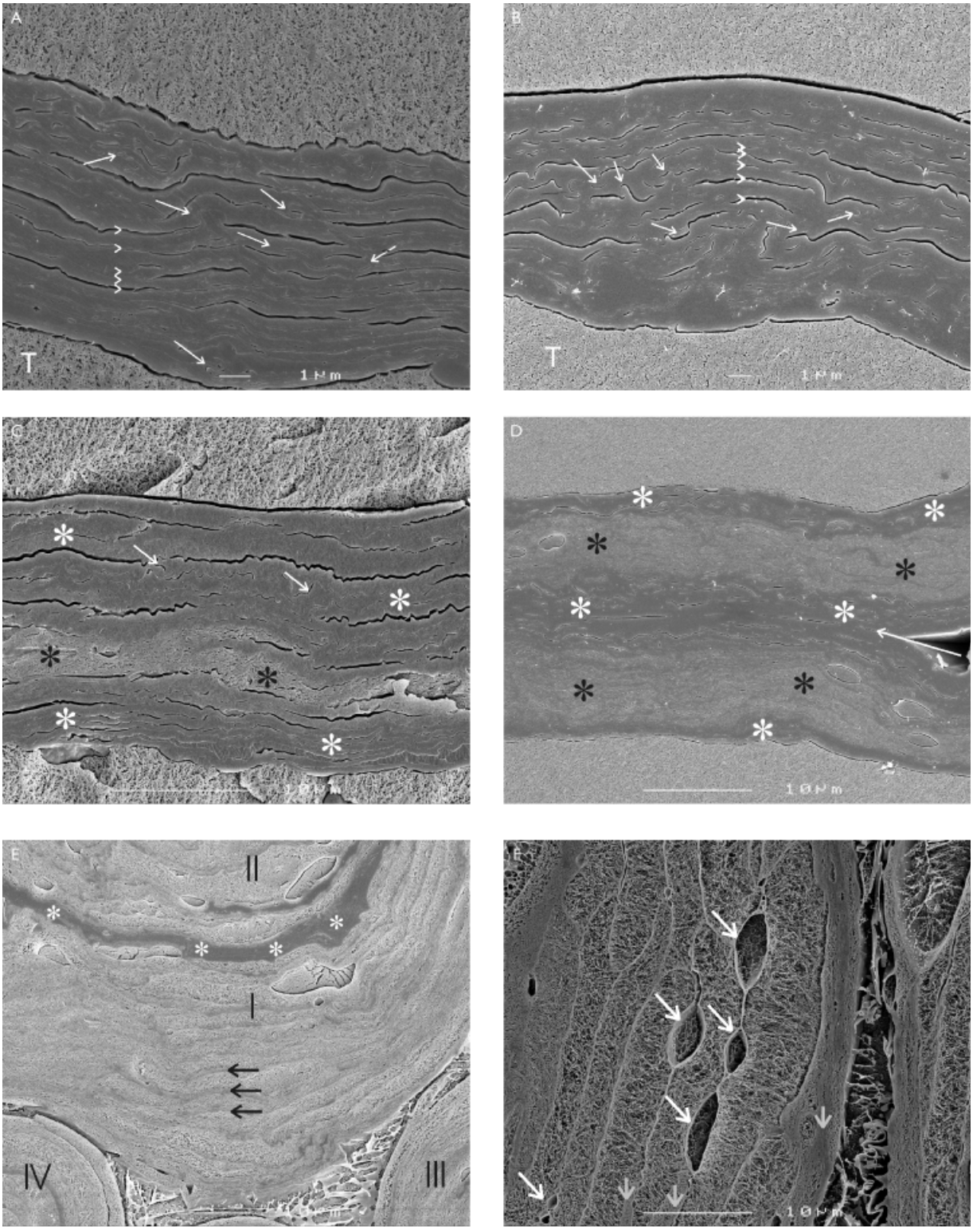
DISCUSSION

As is shown in **Fig 2(*B*)**, only a slight dependence of the orthorhombichexagonal lipid phase transition has been observed, which is in agreement with previous studies (Gay *et al*, 1994). This indicates that only a very small amount of water is located in

Figure 4. High magnification cryo-scanning electron microscopy images of SC hydrated to various levels. (*A*) Dry SC is characterized by low contrast images. Note that (see *arrows*) undulations and interdigitations of cells are observed in this image. Undulations are often observed close to the corneocyte cell ends. The cells are approximately 360 nm in thickness. *Arrowheads* indicate the cell boundaries. T, tissue-freezing medium. The spaces between the corneocytes in the dry and 17% wt/wt hydrated SC (see *B*) is mostly air-filled spaces. The spaces might be caused by drying of the SC required for low hydration levels. (*B*) SC hydrated to 17% wt/wt reveals a low contrast image, similar to that observed for dry skin. This indicates the absence of water pools. *Arrows* indicate undulations or interdigitations of cells. *Arrowheads* indicate the cell boundaries. (*C*) SC hydrated to 70% wt/wt reveals in the central part slightly swollen cells with a higher contrast (see *black asterisks*) indicating the presence of water. In the upper and lower part the appearance of the SC is similar to that of dry skin (*white asterisks*). The *white arrows* indicate undulations and interdigitations. (*D*) Two SC sheets hydrated to 90% wt/wt. The *white arrow* indicates the interface between the sheets. Both sheets show an increased hydration level in their central regions (higher contrast, see *black asterisks*) and a low contrast in the superficial and lowest part of the SC (*white asterisks*). Note that the cells in the central SC region are also strongly swollen. (*E*) Four SC sheets (I to IV) hydrated to 300% wt/wt. Most corneocytes are very markedly swollen and are characterized by a network of keratin filaments with water. Occasionally it seems that there is an alternating sequence of strongly swollen and less swollen cells (see *black arrows*). Even within one cell a large variation in cell thickness exists. Although difficult to quantify, the undulations seem to be fewer compared to dry SC. The lowest SC regions of sheets I and II are in contact; see **Fig 3(*B*)** for the explanation of the folding. Remarkably the corneocytes in these regions are not swollen (low contrast regions indicated by *white asterisks*). (*F*) A high magnification of a fully hydrated SC sheet. The keratin network is clearly depicted. This network is surrounded by the cornified envelope. Between the cells frequently large and very small water pools are observed (see *white arrows*). If no water pools are present a close cell to cell contact is observed. The cell ends are round and fewer undulations are observed compared to dry SC or SC hydrated to approximately 20% wt/wt. Again a difference in the degree of swelling between the various cells is noticed. The cells that are less swollen are indicated by *short arrows*.

between the lipid lamellae. In a previous study, however, the orthorhombichexagonal phase transition as detected by the factor group splitting of the CH_2 scissoring mode was reported to occur between 55°C and 65°C (Gay *et al*, 1994). In the present study, the onset of the transition is clearly observed between 30°C and 40°C. X-ray diffraction (Bouwstra *et al*, 1992) and electron diffraction (Pilgram *et al*, 1999) studies revealed also an orthorhombichexagonal phase transition at around 35°C, confirming the results of this study. A small reduction in the temperature of transition and only little water in the head group regions might partly explain the enhancing effect of water on skin permeation. In our view, however, it most probably does not fully explain the strong penetration enhancing effect of water.

Until now only a few publications have reported on the natural hydration level as a function of depth in SC. In a publication by Bommannan *et al* (1990) a hydration gradient has been reported from the relatively low humidity of the environment to the relatively wet viable epidermis. As Bommannan used the stripping



method (maximum number of strips is 14), however, most probably the low water level in the deepest SC regions was not measured. Warner *et al* (1988) reported that the deepest layers in the SC close to the epidermis are less hydrated than the central part in the SC. Very recently the hydration level *in vivo* was measured by an elegant new method, confocal Raman microspectroscopy (Caspers *et al*, 2001). These authors observed a gradual increase in the hydration level as a function of depth in human skin. The resolution in depth of this technique, however, is approximately 5 μm, which makes it more difficult to detect very local and abrupt changes in the hydration level. In addition most of their results were obtained *in vivo* with epidermis from the palm. In the same paper natural moisturizing factor as a function of depth was measured and appeared to decrease in level close to the skin surface and close to the SC–viable epidermis interface, which is in agreement with earlier reports (Rawlings *et al*, 1994). Recently the skin has been visualized carefully by high pressure freezing followed by freeze substitution (Pfeiffer *et al*, 2000). Although in this study the corneocytes were excellently preserved, no difference in corneocyte swelling was reported between the deepest SC regions and the central region of the SC. To interpret this study in terms of corneocyte swelling is difficult, however, as the SC water content is not known and during sample preparation the water was exchanged for an organic solvent and the embedding material, HM-20. It cannot be excluded that the embedding procedure changes the corneocyte swelling.

In this study the cryo-scanning electron microscopy method has been used to provide information about the water distribution in the SC. In order to determine the water distribution, the SC was equilibrated in a conditioned environment, after which the SC was folded and embedded in tissue-freezing medium (if required). After embedding, the SC was immediately cryofixed to minimize interactions between the embedding medium and SC. To check whether the tissue-freezing medium, containing only small amounts of water, extracted water from SC, SC equilibrated over Na_2CO_3 was also embedded in water. SC in this procedure revealed a similar appearance (not shown), namely a high contrast appearance in the central region of the SC sandwiched between regions with a low contrast, confirming that the uneven distribution of water in SC was not caused by the embedding medium. The cryofixed sample was planed perpendicular to the SC surface and subsequently dehydrated to create holes in the tissue. The holes either are caused by the presence of free water accumulated in water pools in the SC or are a consequence of the segregation of free water during the cryofixation procedure. A minimal segregation of water during fixation is even required to facilitate the detection of free water in tissue. Segregation only occurs with free water.

In this study the water distribution in the SC as a function of depth has been examined. In addition the corneocyte thickness and shape were accurately determined. As the resolution of the cryo-scanning electron microscopy equipment used in this study is approximately 20 nm, only holes larger than 20 nm in diameter can be detected. Furthermore, we chose SC and not dermatomed skin, as we wanted to study the relation between the water content and the mean thickness of the corneocytes, which is not possible using dermatomed skin. Only in the case of isolated SC can the water content in SC be determined by weighing. As can be observed in **Fig 4**, water domains are not observed at a hydration level of approximately 18%–26% wt/wt confirming the findings that at this level only bound water is present in SC (Bulgin and Vinson, 1967; Hansen and Yellin, 1972). At a hydration level of 57%–87% wt/wt, the water level in the central region in SC is higher than in the superficial and deeper regions in the SC and water domains are only observed in the intracellular regions. Both observations are in excellent agreement with the concentration of natural moisturizing factor, which appears to be maximal in the central regions of the SC (Rawlings *et al*, 1994). A further increase in the water content to 300% wt/wt increased the swelling of the cells tremendously. Only in the deepest layers of the SC close to the stratum granulosum was no swelling observed.

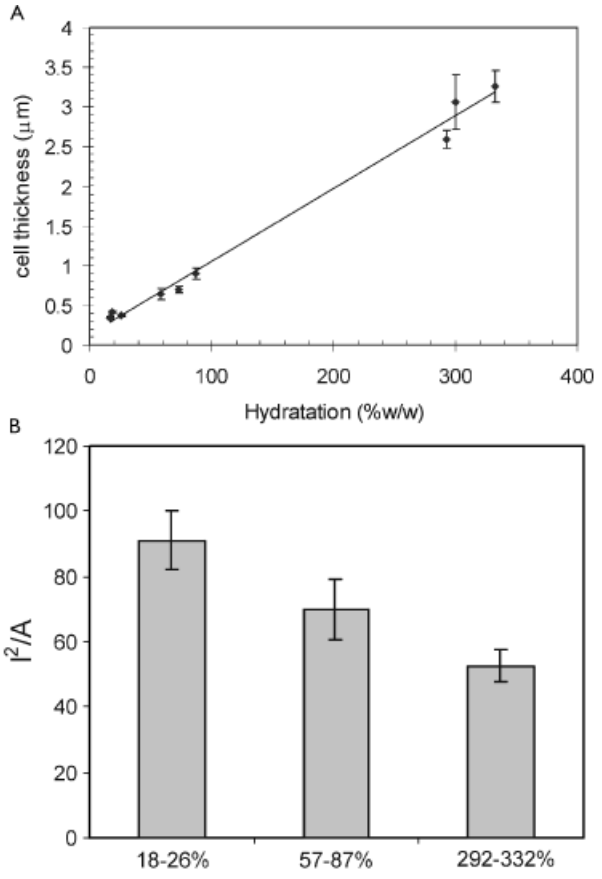


Figure 5. Numerical cell parameters as function of water content. (*A*) The mean cell thickness calculated with an adjusted version of the program SCILImage 1.3 is plotted as a function of the hydration level. A linear relation is observed. (*B*) $(l_{tot})^2/A$ is plotted as a function of the hydration level. A decrease is observed at higher water contents indicating an increase in efficiency of the cornified envelope to surround the cell content.

This is a very remarkable observation indicating that in these layers no free water is present. Most probably these layers prevent a strong increase in the water flux across the SC at very high hydration levels and thus prevent dehydration of the viable epidermis. Whether this is due only to the absence of natural moisturizing factor or because the corneocyte envelope is also less permeable for water in this region is not clear. In order to establish whether the water distribution is not an artefact induced by the separation of the SC by trypsin digestion, dermatomed skin hydrated either over Na_2CO_3 or over pure water was also visualized by cryo-scanning electron microscopy. The results are presented in **Fig 6**. When hydrated over water the absence of swelling in the lowest layers is clearly present in dermatomed skin confirming the results obtained with SC. After hydration over Na_2CO_3 a similar distribution was observed as in isolated SC: an increased hydration in the central SC layers. This clearly demonstrates that the water distribution in SC is not an artifact due to the isolation of human SC by trypsin digestion.

A final question arises that, if water is mainly taken up by the corneocytes, the volume of the corneocytes is expected to increase. As the corneocytes are surrounded by lipids in a crystalline or gel state, however, the increase in total surface area of the cells is expected to be minimal or even absent during water uptake. As the diameter of the corneocytes is approximately 20–30 μm and the cell thickness at low hydration level is approximately 300 nm, a minimum increase in total cell surface after uptake of water is only achieved when the volume increase

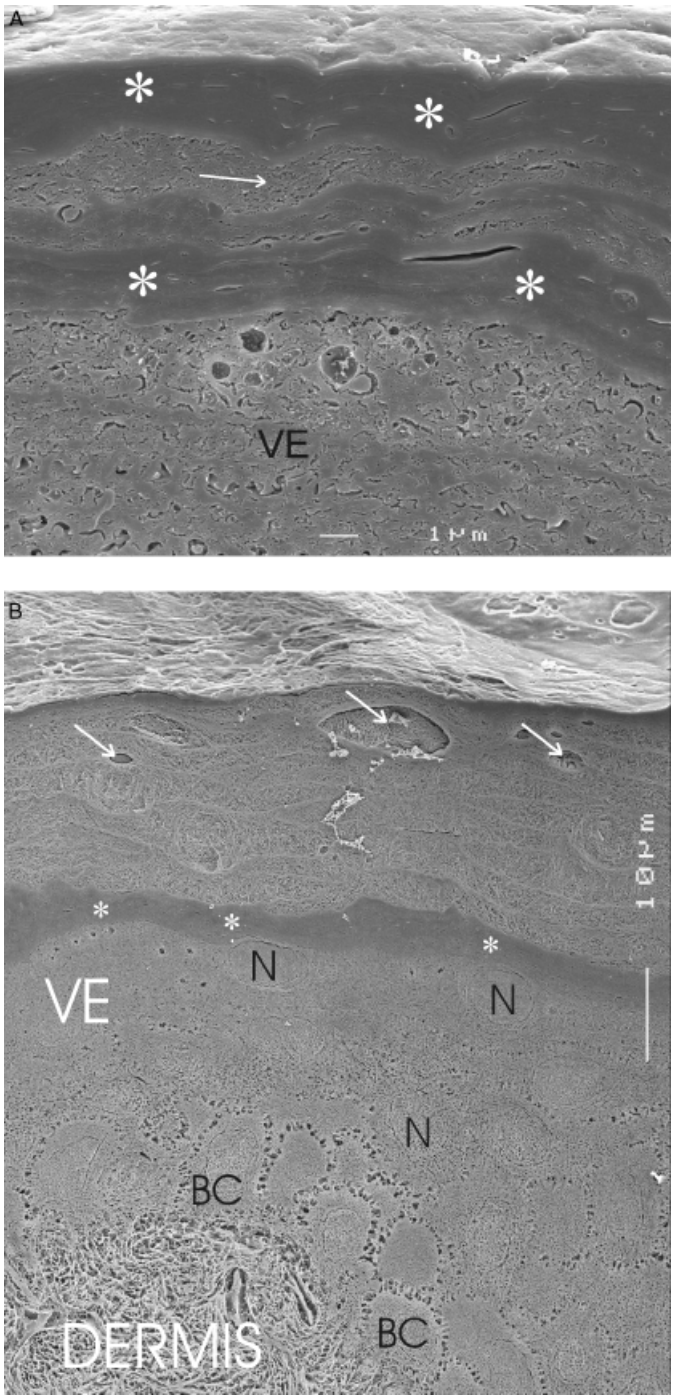


Figure 6. Water distribution in dermatomed skin. (*A*) An image of epidermis hydrated over Na_2CO_3. The SC and viable epidermis (VE) can clearly be distinguished. In the SC only the central region is swollen (see *arrow*) and shows more contrast, as observed in **Fig 4**(***C***, ***D***). The nonswollen regions are indicated by *white asterisks*. (*B*) An image of fully hydrated dermatomed skin. In this image the SC, viable epidermis (VE), and dermis are clearly depicted. At the interface between SC and stratum granulosum a low contrast region is observed representing the nonswollen cells, as observed in isolated SC (see *white asterisks*). Some large water pools (*white arrows*) in the intercellular regions are shown. In the epidermis the cell nuclei (N) and basal cells (BC) are visible.

mainly occurs by an increase in cell thickness. An approximate linear relationship as observed in **Fig 5**(***A***) indeed supports the hypothesis that swelling of the corneocytes mainly occurs by an increase in cell thickness. Furthermore, as can also be inferred from **Fig 5**(***B***), the ratio $(l_{tot})^2/A$ decreases as a function of the SC hydration level. This reduction in ratio suggests that the cornified envelope increases its efficiency to surround the cell content. This might be caused by either a change in the cell shape, by which the cell cross-section changes from a flat shape to a more inflated one, or a reduction in undulation of the cornified envelope. The images in **Fig 4** suggest that both mechanisms play a role. It is not possible to quantify the contribution of each of the mechanisms, however. Richter *et al* (1997) studied the swelling of isolated corneocytes by scanning force microscopy. Upon increasing the water content by soaking corneocytes in distilled water not only was an increase in corneocyte thickness was observed, but also a lateral swelling. This might be because isolated corneocytes were used instead of intact SC as used in this study.

As is clearly shown in **Fig 4**(***E***), (***F***) even after 24 h hydration over water the cells in the SC are very cohesive, although in the intercellular region holes representing the presence of water are quite frequently observed. The close cell to cell contact is in contrast with a recent paper by Warner *et al* (2000), who noted that *in vivo* after 6 h of occlusion with water intercellular spaces were often filled with water and that after 24 h there is almost no cell to cell contact. Indeed, also in our studies after 24 h occasionally large water pools are present. In most SC regions, however, a cell to cell contact is still observed, indicating that cohesion is still largely present. Another study (Van Hal *et al*, 1996), in which the lipid organization was studied by freeze fracture electron microscopy, also revealed that the majority of the lipid organization after extensive hydration is similar to that observed at lower hydration levels. Finally very recently we studied the SC structure after equilibration for 15 h in a phosphate saline solution. These studies confirmed the results of the previous studies, namely only occasionally the presence of water pools in the intercellular regions and a close cell to cell contact. This cell to cell contact even at high hydration levels may explain the remaining skin barrier even after uptake of 300% wt/wt water, whereas the lack of free water in the deepest SC at all hydration levels prevents a continuous dehydration of the bulk of the body.

In conclusion this new combination of techniques provides detailed insights into the localization of free water in the SC. In future we shall use this technique to study the effect of additives and of iontophoresis on the SC swelling in dermatomed skin.

We would like to thank ICI for financing the study. We wish to thank Dr. A. Burgess (ICI) for the suggestions and discussions made during our meetings.

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