Acta Derm Venereol 2013; 93: 138–143

INVESTIGATIVE REPORT

Stratum Corneum Drying Drives Vertical Compression and Lipid Organization and Improves Barrier Function *In vitro*

Ichiro IWAI[1], Naomi KUNIZAWA[1], Eiichiro YAGI[1], Tetsuji HIRAO[1] and Ichiro HATTA[2]
[1]*Shiseido Research Center, Yokohama, and* [2]*Japan Synchrotron Radiation Research Institute/Spring-8, Hyogo, Japan*

The stratum corneum dehydrates after exogenous hydration due to skincare or bathing. In this study, sheets of stratum corneum were isolated from reconstructed human epidermis and the barrier function and structure of these sheets were assessed during drying with the aim of improving our understanding of skincare. Water diffusion through the sheets of stratum corneum decreased with drying, accompanied by decreased thickness and increased visible light transmission through the sheets. Electron paramagnetic resonance revealed that the order parameter values of stratum corneum lipids increased with drying. X-ray diffraction analysis revealed increases in the diffraction intensity of lamellar structures, with an 11–12 nm periodicity and spacing of 0.42 nm for lattice structures with drying. These results suggest that the drying process improves the barrier function of the stratum corneum by organizing the intercellular lipids in a vertically compressed arrangement. ***Key words: electron paramagnetic resonance; reconstructed human epidermis; stratum corneum; skin barrier; transepidermal water loss; X-ray diffraction.***

Accepted May 9, 2012; Epub ahead of print Nov 20, 2012

Acta Derm Venereol 2013; 93: 138–143.

Ichiro Iwai, Shiseido Research Center, 2-2-1 Hayabuchi, Tsuzuki-ku, Yokohama 224-8558, Japan. E-mail: ichiro.iwai@to.shiseido.co.jp

In daily life, the stratum corneum (SC) dehydrates after exogenous hydration during skincare, bathing or sweating. Water plays important roles in the structure and characteristics of the SC because it is the major component of the SC. Many studies have described the relationship between water content and various properties of the SC, including mechanical properties (1–3), morphology (4–7), optical properties (8), and intercellular lipid organization (9–13). The water barrier function of human SC, which is dependent on intercellular lipid organization, decreases as a result of hydration (14). However, previous functional and structural studies have been performed independently, using different kinds of SC as experimental materials. Furthermore, appendages of human skin critically influence water diffusion through the SC, making stable measurement of transepidermal water loss *in vitro* difficult (14). Reconstructed human epidermis (RHE), which is free of appendages, has been developed as an alternative to animal tests; its use is recommended for *in vitro* dermatological research because it shares many characteristics with normal human epidermis (15–17). To understand the process of dehydration from an exogenously hydrated state, both the barrier function and the structure of SC isolated from RHE were investigated *in vitro*. X-ray diffraction and electron paramagnetic resonance (EPR) analyses were used to investigate the effect of dehydration on the organization of SC lipids. Because fine structural changes in the SC are reflected in its optical properties, the effect of dehydration on visible light transmission, and the thickness of the SC sheet, were investigated as morphological features. Taken together, the effects of the drying process on SC were investigated in order to understand the basis for improving skin conditions in daily life.

MATERIALS AND METHODS

Stratum corneum sheets

RHE (12-well LabCyte, 5-week cultured model; Japan Tissue Engineering Co. Ltd, Aichi, Japan) was floated on 0.1% trypsin in phosphate-buffered saline (PBS), with the epidermal side down, for 30 min at 37ºC in order to separate the SC sheet from the lower epidermis. The SC sheet was washed with deionized water (DW) filtered through a water purification system (MilliQ Advantage A10 system; Millipore, Billerica, MA, USA). Isolated SC sheets approximately 9 mm in diameter were placed on glass slides in a fully hydrated state. They were then dried in a chamber (MTH-2200; Sanyo Electric Co. Ltd, Tokyo, Japan) at a constant temperature of 34.0ºC and 60% relative humidity (RH) to various hydration levels.

Water content of stratum corneum sheets

Wet SC sheets were weighed with a microbalance combined with an ionizer in order to reduce the static charge and obtain stable measurements. Weights were recorded every second, and the weight of the wet SC was calculated as the mean of the measurements collected over 1 min. To determine the dry weight of the SC, the wet SC was heated for 2 h at 120ºC and dried under a dry nitrogen gas flow for 2 h in a moisture sorption analyser (IGAsorp; Hiden Isochema Ltd, Warrington, UK). The weight of the SC in the sorption analyser at 0% RH was measured as the dry weight of the SC. The water contents of wet SC sheets were determined according to the following formula: [(weight of wet SC–weight of dry SC)/(weight of wet SC)]×100(%).

In vitro *evaluation of barrier function*

A SC sheet 9 mm in diameter was placed on the air–water boundary, using a Franz cell (orifice diameter, 5 mm; receptor and sampling port volume, 5 ml; PermeGear Inc., Hellertown, PA, USA), and the water diffusion rate from the lower chamber, which was filled with DW, to the air of the upper chamber was evaluated semiquantitatively at 22.0ºC and 45.0% RH with a VapoMeter in the nail mode (Delfin Technologies Ltd, Kuopio, Finland). In order to prevent leakage at the junction of the upper chamber and VapoMeter, parafilm (Pechiney Plastic Packaging Inc., Chicago, IL, USA) was wrapped around the upper chamber (Fig. 1). Room temperature and RH were controlled at 22.0ºC and 45.0%, respectively; monitored air ranged from 22.0ºC to 22.1ºC and 44.5% to 45.5% RH. The water diffusion rate was evaluated every 30 min for 4 h after a SC sheet was set into a Franz cell. All equipment was acclimatized to the conditioned air before the experiments. The DW level in the Franz cell was adjusted every 30 min. Six measurements were made in each experiment, and the mean of 4 measurements, excluding the maximum and minimum values, was adopted as the result of a single experiment. The obtained values are expressed as arbitrary units (AU).

Stratum corneum sheet thickness

The Z-position of the upper surface of the SC sheet on the glass slide was determined optically (mercury emission lamp $\lambda_{peak=}$546 nm; CCD detector quantum efficiency peak, 550 nm) in a 6×6-mm area with a confocal laser scanning microscope (HD100D; Lasertec Corporation, Yokohama, Japan). The thickness of the SC sheet was measured as the mean Z-position of the SC surface minus that of the upper surface of the glass slide.

Optical properties of stratum corneum sheet

On a glass slide, a SC sheet was irradiated with incandescent light emitted from the Commission Internationale de l'Eclairage (CIE) standard illuminant C. Total light transmission, including the scattered transmission that passed through the SC sheet on the glass slide, was measured using a hazemeter equipped with an integrating sphere (HR100; Murakami Color Research Laboratory Co. Ltd, Tokyo, Japan) as described previously (18). The ratio of the total light transmission through the SC sheet on the glass slide to that without the SC was calculated as the total transmittance.

Analysis of structural ordering of stratum corneum lipids with electron paramagnetic resonance

The order parameter values indicating SC lipid fluidity and organization were measured by EPR, as described previously (19). Briefly, isolated SC sheets were incubated in spin probe solution (0.1% ethanol, 0.001% 5-doxyl-stearic acid (5-DSA), Sigma-Aldrich Co., St Louis, MO, USA) for 60 min at 37ºC. After incubation, the excess spin probe was removed with DW. SC sheets were dried at 34.0ºC and 60% RH to various hydration levels on glass slides. They were then weighed and mounted in the EPR cavity. The EPR spectra of the 5-DSA, which were obtained with an EPR spectrometer (JEOL JES-RE1X X-band 9 GHz; JEOL Ltd, Tokyo, Japan), were analysed using the conventional method of the order parameter (*S*) (20). After EPR analysis, the dry SC sheets were weighed in order to determine their water content, as described above.

Small-angle X-ray diffraction and wide-angle X-ray diffraction study of stratum corneum lipids

Individual SC sheets were dried at 34.0ºC and 60% RH to various hydration levels, and half of a SC sheet from one well was used for X-ray diffraction study. The X-ray diffraction study was performed with synchrotron radiation at BL40B2 (Structural Biology II Beamline) of SPring-8 (Hyogo, Japan) at a controlled air temperature (25.9–26.1ºC). X-ray diffraction profiles were recorded using an imaging plate system (R-AXIS IV; Rigaku Corporation, Tokyo, Japan) with a 30×30-cm area. The X-ray wavelength was 0.083 nm, and the sample-to-detector distance was approximately 500 mm. The reciprocal spacing [$S=(2/\lambda)\times sin\theta$] was calibrated from the lattice spacing (d=5.838 nm, where d is the lamellar repeat distance) of a silver behenate crystal at room temperature, where 2θ is the scattering angle. The exposure time was 30 s. The diffraction pattern was circular averaged in order to obtain a radical intensity profile. The wet and dry SC sheets were weighed before and after X-ray diffraction analysis in order to determine their water content, as described above.

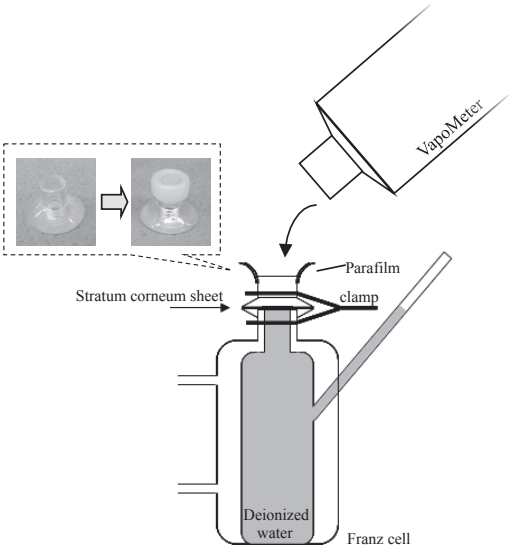

Fig. 1. Parafilm was wrapped around the upper chamber of a Franz cell to prevent leakage at the junction of the upper chamber and VapoMeter.

Statistical analysis

Analysis of variance (ANOVA) was used to detect significant differences with respect to the water diffusion rate between groups, then a *post-hoc* analysis using the Tukey–Kramer test was conducted to further determine the significance of differences in the water diffusion rate from 0.5 to 4 h after the start of measurement.

RESULTS

Decrease in water contents and water diffusion rates of stratum corneum during drying

Water content decreased almost linearly from 80% to 30% during the first 3 h and levelled off thereafter, reaching a minimum of 10% at 18 h (Fig. 2).

After drying for 0, 1, 2, 3, 5, or 18 h, the SC sheets were divided into 2 groups: 1 group was used to estimate water content, and the other was used to evaluate water diffusion rate from the SC (Fig. 3). Except at the start of the measurement period, the water diffusion rate from non-dried SC sheets was significantly lower than that without SC, the diffusion rate from non-dried SC sheets

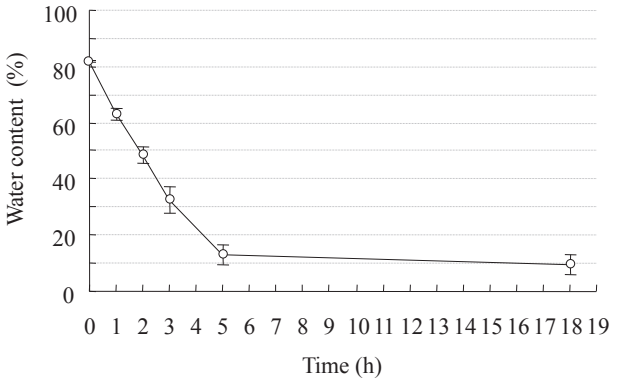

Fig. 2. Changes in the water content of stratum corneum (SC) sheets with drying. SC sheets isolated from reconstructed human epidermis were dried at 34.0ºC and 60% relative humidity for 18 h. The drying SC sheets were weighed in order to evaluate the water content during the drying process. Data are expressed as means ± standard deviations (n = 4).

was significantly higher than that of SC sheets dried for 1 or 2 h, and SC sheets dried for 1 or 2 h had significantly greater water diffusion rates than sheets dried for 3, 5, or 18 h. The water diffusion rate from SC sheets dried for 3 h was the same as those dried for 1 and 2 h at the start of the measurement; the diffusion rate then decreased to the same level as those from the SC sheets dried for 5 and 18 h. SC sheets with lower estimated water contents tended to have lower water diffusion rates during the measurement period, except at the start of the measurement.

Decreased stratum corneum thickness and increased light transmission with drying

Weight, thickness, and total light transmission were measured during the drying process. The thickness of the SC sheets decreased almost 90% with drying for 18 h (Fig. 4a). The total light transmission of the sheets increased from 65% to 90% within 3 h (Fig. 4b). When the water content was below 30%, the total light transmission tended to decrease further with more drying.

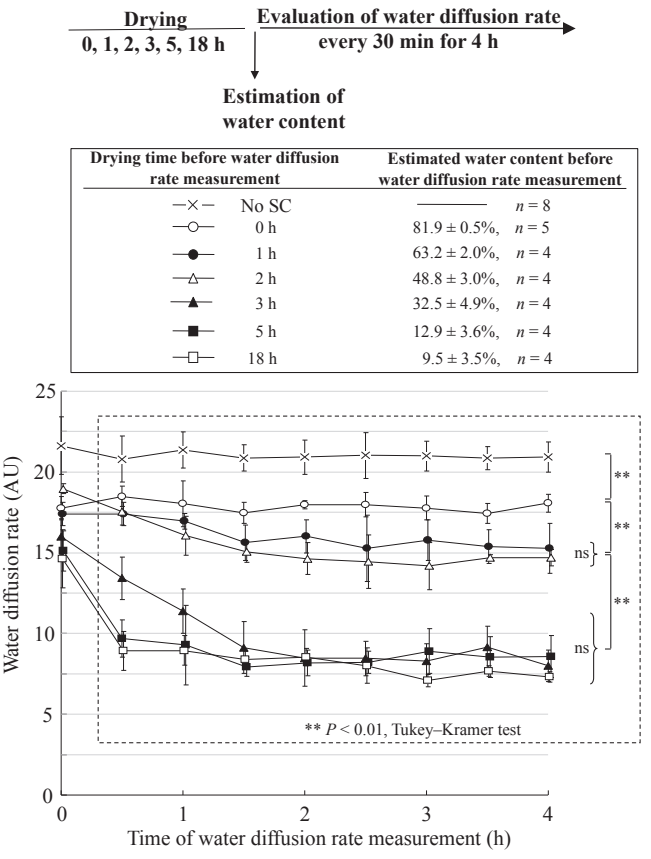

Fig. 3. Water diffusion rate from stratum corneum (SC) sheets with various hydration levels after SC sheets were placed into Franz cells. SC sheets isolated from reconstructed human epidermis were dried at 34.0ºC and 60% relative humidity for 0, 1, 2, 3, 5, or 18 h to different hydration levels. The diffusion rate of the water evaporating from the SC sheet was evaluated every 30 min for 4 h after the sheets were set in the Franz cells. Data are expressed as means ± standard deviation (n = 4–8). Water diffusion rate data after 0.5–4 h were analysed statistically.

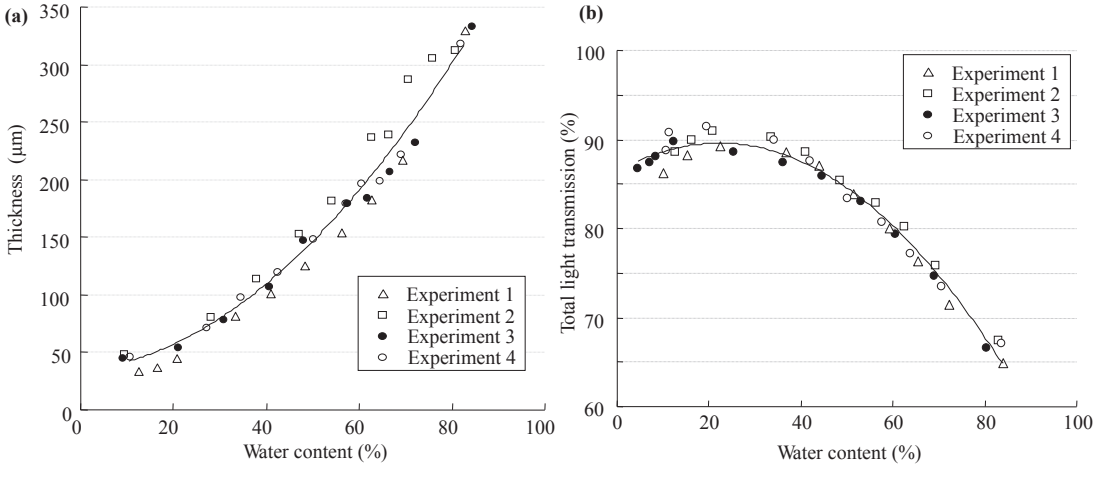

Fig. 4. Changes in (a) thickness and (b) light transmission of the stratum corneum (SC) sheets with drying. SC sheets isolated from reconstructed human epidermis were dried at 34.0ºC and 60% relative humidity for 18 h. Data for 4 independent experiments are shown.

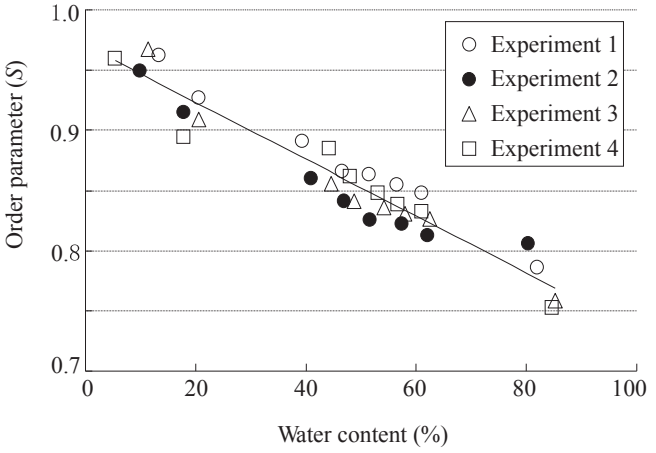

Fig. 5. Changes in the order parameter (*S*) of stratum corneum (SC) lipids with drying. SC sheets isolated from reconstructed human epidermis were dried at 34.0ºC and 60% relative humidity for 18 h. The order parameter of SC lipids was measured by electron paramagnetic resonance (EPR) analysis during the drying process. Data were obtained after drying for 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, or 18.0 h. Data for 4 independent experiments are shown. The water content at each hydration level is indicated.

The order parameter values of stratum corneum lipids increased with drying

Wet SC sheets were weighed, and the order parameter values of the SC lipids were evaluated by EPR during drying. EPR was performed after 18 h of drying, and the dry weights of the SCs were determined. Lipid order parameter values increased linearly as the water content decreased with drying (Fig. 5).

Changes in the intensity of X-ray diffraction with drying

SC sheets of various hydration levels were prepared, weighed, and analysed according to X-ray diffraction measurements. Small-angle X-ray diffraction (SAXD) and wide-angle X-ray diffraction (WAXD) measurements were conducted simultaneously in this study. SAXD revealed an intensity of a multiple-order diffraction of approximately 11–12 nm (S=0.083–0.089 nm^{-1}) periodicity, which tended to increase with drying (Fig. 6a). WAXD revealed an intensity of 0.42 nm (S=2.43 nm^{-1}) spacing, which tended to increase with drying from the fully hydrated state (Fig. 6b). The intensity of 0.42 nm spacing did not change significantly with a reduction in water content from 37.4% to 11.2%.

DISCUSSION

Diffusion of water vapour through the SC *in vitro* has been measured previously (14, 21). However, *in vivo*, water evaporates at the SC. Elkeeb et al. (22) measured the diffusion rate of water evaporating through whole human skin pieces. In the present study, SC sheets isolated from RHE were used in order to evaluate the rate of water diffusion evaporating from the SC. RHE is a reproducible material because it has no appendages that might influence water diffusion, while still possessing many characteristics of normal human epidermis (15–17). The use of RHE enabled us to investigate the relationship between the barrier function and various properties of the SC during drying from an exogenously hydrated state. The results indicate that the rate of diffusion of water through the SC decreased with drying. This result is similar to that of a previous study showing that hydrated SC is more water-permeable than dry SC (14). The rate of diffusion of water from SC sheets dried for 3 h before the measurement decreased after the sheets were placed in Franz cells. This suggests that initial drying for 3 h leads to further dehydration, with structural and functional changes, during measurement.

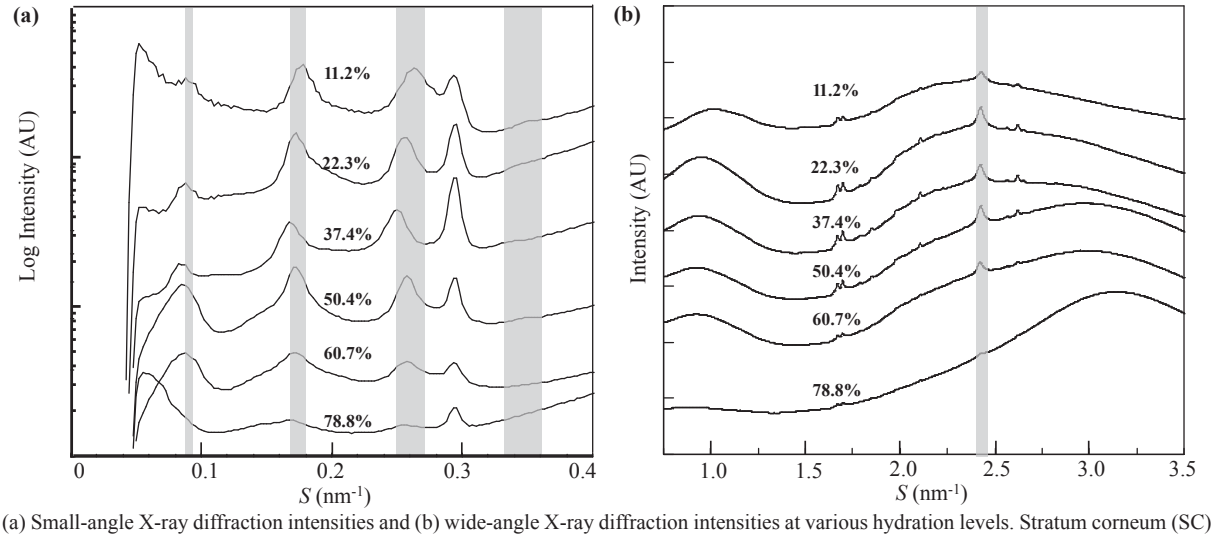

Fig. 6. (a) Small-angle X-ray diffraction intensities and (b) wide-angle X-ray diffraction intensities at various hydration levels. Stratum corneum (SC) sheets isolated from reconstructed human epidermis were dried at 34.0ºC and 60% relative humidity for 0.5, 1.5, 2.0, 2.5, 3.0, or 5.0 h. Each SC sheet with different hydration level was subjected to X-ray diffraction. The intensities of the diffractions are expressed as arbitrary units (AU). The water content at each hydration level is indicated. In the small-angle region (S<0.04 nm^{-1}), the intensities were masked by a beam stopper.

The drying process also resulted in thinner SC sheets with increased transmission of visible light. Similar changes in thickness have been described previously in a study on swelling (7). Higher light transmission suggests lower back-scattered light as a result of a tightly packed SC. Vertical packing, the increase in light transmission of hydrated biological materials, and the curve shape in Fig. 2 suggest a role for bound water in the observed functional changes in addition to morphological changes occurring during the drying process (23, 24). Hence, the drying process results in SC sheets with vertically compact and dense layers, accompanied by enhanced barrier function.

Barrier function is known to be closely related to intercellular lipids. Previous studies (25, 26) suggest that the peak of a multiple-order diffraction of approximately 11–12 nm (S=0.083–0.089 nm^{-1}) periodicity is a periodic structure of lamellae in the intercellular space, while the peak at 0.42 nm (S=2.43 nm^{-1}) is the lattice structure assigned to the hydrocarbon chain packing of the hexagonal phase. In this study, the peak at 0.38 nm (S=2.6 nm^{-1}), which was previously assigned to the orthorhombic phase (25, 26), was not reproducibly observed. Other reflections observed in this X-ray diffraction study might be a result of anhydrous crystalline cholesterol (S approximately=0.3 nm^{-1}) and keratin (S approximately=1.0 and 2.3 nm^{-1}) (25, 27). The peaks at 11–12 and 0.42 nm in X-ray diffraction and order parameters in EPR tended to increase with drying from the fully hydrated state, suggesting that the intercellular lipids are organized during drying, as reported in previous studies (9–11). During drying, from a water content of 37.4% to 11.2%, the 0.42-nm peak in the X-ray study did not change markedly. However, in the EPR analysis, the order parameters of the lipids continued to increase linearly with drying as a function of water content. In addition to their organization, the mobility of lipids is reflected in the order parameters in EPR studies. Thus, it is assumed that the results of the EPR analysis in this study were strongly influenced by the change in lipid mobility.

In conclusion, the diffusion rate of water evaporating through the SC decreased with drying, and this was accompanied by vertical compression of the SC sheets and the organization of intercellular lipids. These results suggest that the drying process itself plays a role in the restoration of barrier function after a period of exogenous hydration. A recent study using cryo-electron microscopy of vitreous sections did not reveal any marked changes in the periodicity of lamellae of exogenously hydrated SC (12). However, this does not preclude a possible interaction between water and extracellular lipids. Indeed, X-ray diffraction studies suggest slight swelling of the lamellar lipid structure as a result of hydration (13). Although the relationship between the drying process and lipid organization remains unclear, the results of the present study suggest that vertical compression, driven by the drying process, induces re-organization of the intercellular lipids, resulting in improved barrier function. Moreover, the results suggest that the drying process itself somehow allows the skin to self-regulate its condition.

ACKNOWLEDGEMENTS

The synchrotron radiation experiments were performed at the BL40B2 in the SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) (proposal no. 2007B1808).

The authors declare no conflicts of interest.

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