Water-Impermeable Occlusion Effect to Intercorneocyte Lipid Layers in Hairless Mice

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Background : Stratum corneum lipids are arranged as intercellular membrane bilayers presumed to mediate the epidermal permeability barrier. Acute disruption in barrier function will initiate epidermal lipid synthesis, which can be prevented by occlusive membrane. Whereas, occlusion of the skin is known to cause an increased transepidermal water loss(TEWL) and enhanced percutaneous absorption of a variety of compounds.

Objective : Previous reports with electron microscopy showed varying sizes of lacunae and disorganized intercorneocyte lipids after tape stripping and occlusion with a water impermeable membrane on the murine skin. Hence we studied the effects on stratum corneum lipids and changes in barrier function after occlusion with a water-impermeable membrane.

Methods : Male hairless mice were occluded with one finger of a Latex glove for 24, 48 and 60 hours. After occlusion, TEWL was measured and biopsy specimens were taken from skin. For electron microscopic examination the samples were treated with osmium tetroxide, ruthenitum tetroxide, and tracer (lanthanum) and infrared spectroscopy were also applied.

Results : Occlusion with a water-impermeable membrane on the skin induced higher TEWL Values and greater penetration of the tracer than normal. Alterations of the lipid bilayer membrane and lacunae formation in the stratum corneum interstices were also induced after 24 hours of occlusion. However, the orderness of the lipid alkyl chain in the stratum corneum was not changed until 60 hours of occlusion.

Conclusion : These studies indicate that the increased epidermal permeability after occlusion may be due to the abnormal lipid membrane structures and volume expansion of existing lacunar domains in the stratum corneum interstices.(Ann Dermatol 9:(2) 116~125, 1997).

The primary function of the epidermis is to provide terrestrial animals with a barrier to water permeation. This permeability barrier is localized to the stratum corneum (SC) interstices, where it is medi-

ated by lamellar bilayers composed of a mixture of cholesterol, ceramides and free fatty acids. These lipids are derived from secreted lamellar body contents from upper stratum granulosum (SG) cells deployed to the extracellular spaces of the stratum corneum as foreshortened membrane sheets, which subsequently undergo reorganization into an elongated bilayer structure[1,2].

Recent studies have shown that when the barrier is disrupted with solvents or tape stripping, a repair sequence is triggered that leads to normalization of barrier function over 24 to 48 hours[3,4]. Moreover, when the defect in barrier frunction, in-
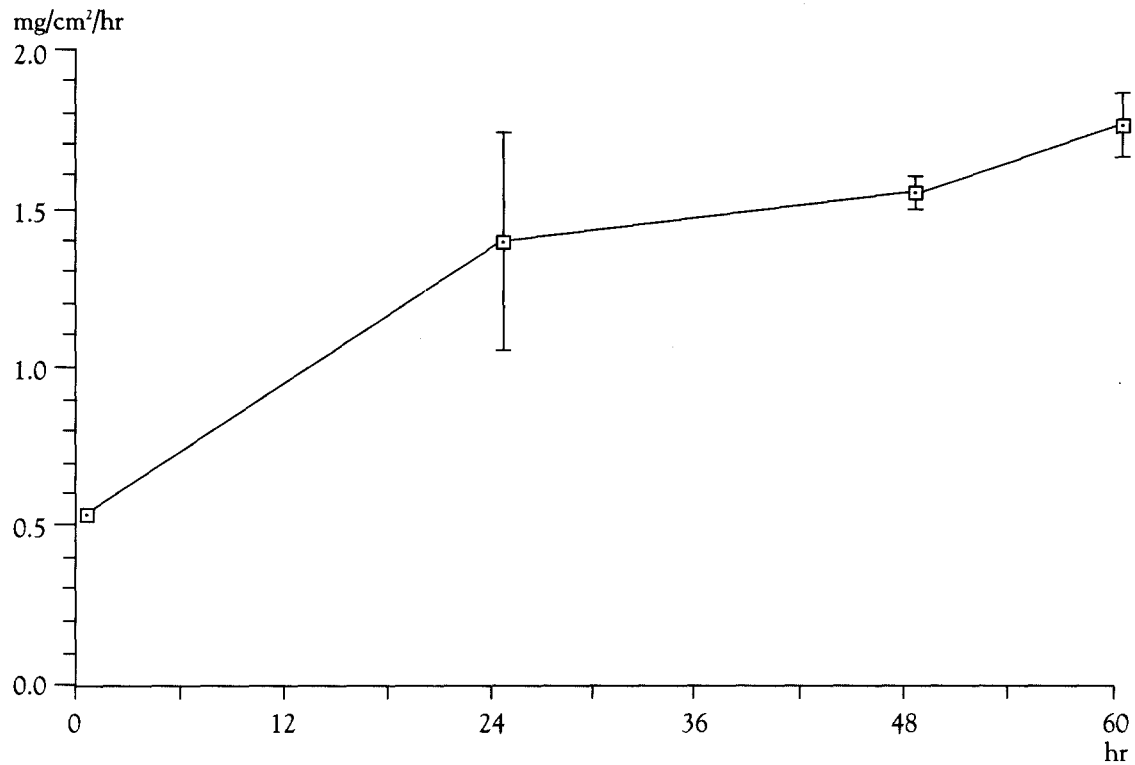
mg/cm²/hr

Fig. 1. TEWL after occlusion in normal epidermis.

Fig. 2. Normal stratum corneum intercellular bilayer structures, the electron -lucent lamellae that alternate with electron-dense lamella. Inset, the intercellular membranes which comprise nine electron-lucent lamellae alternating with eight electron-dense lamellae. The single arrow indicates the continous electron-lucent lamella and the double arrows indicate the interrupted electron-lucent lamella. Magnification × 240,630; inset, magnification × 202,100).

duced by either acetone or essential fatty acid deficiency, is corrected by application of a water-impermeable membrane, the characteristic increase in epidermal lipid synthesis is prevented, whereas epidermal lipid synthesis is increased in animals covered with a water permeable membrane[3]. These studies described the effects of occlusion of the skin with a defect in barrier function and raised the question that after occlusion with a water-impermeable membrane the barrier function of the stratum corneum might be affected. Furthermore, prior studies have demonstrated that after only a few days of occlusion, ultrastructural injury to keratinocytes, Langerhans cells, fibroblasts and the endothelium may be seen without clinically evident changes, and such changes may progress with repeated occlusion[6]. To delineate this issue, we employed ruthenium tetroxide post-fixation to examine the morphological changes on the skin, a lanthanum tracer study to see the tracer permeability and infrared spectroscopy to examine the hydrocarbon-chain disorderness of the SC lipids after occlusion with a water-impermeable membrane on the skin.

Fig. 3. 24 hours occlusion in normal epidermis. Note the disorted membrane structure, and the fragmentary membrane (arrow). Magnification × 105,470.

Fig. 4. Occlusion in normal epidermis for 48 hours, the intercellular lipid lamellae were separated by lacunae (asterisks) and loss of normal structure in foci (arrows). Magnifcation × 160,420.

Fig. 5. After 60 hours occlusion, variability in the intercellular lamellar organization is apparent, in some sites and absence of all electron-lucent and electron-dense lamellae is evident(arrows), and interspersed with extensive clefts containing flocculent electron-dense material (stars). Magnification × 133,680

MATERIALS AND METHODS

Materials

Hairless male mice were obtained from the animal laboratory at Yonsei University College of Medicine and were fed a regular mouse diet and water ad libitum. They were 8-12 weeks old at the time of this study. Ruthenium tetroxide (RuO_4), osmium tetroxide (OsO_4), lanthanum nitrate$(LaNO_3)$ and embedding resins were from polyscience Inc (Warrington PA).

Experimental protocols and electron microscopy

While under Following an anesthesia with intramuscular injection of ketamine hydrochloride, the occlusion of the skin was accomplished by sliding a mouse into one finger of a Latex glove so that the entire torso was covered tightly with a water-impermeable membrane for 24, 48, 60 hours. Transepidermal water loss (TEWL) was measured with an electrolytic water analyzer (Meeco Inc., Warrington, PA) about 5 minutes after a removal of occlusion. Samples were taken from the treated and untreated animals for electron microscopy. Samples were briefly minced $0.5mm^3$ and fixed in

modified Karnovsky's fixative overnight, washed in 0.1M of cacodylate buffer, and post-fixed in 0.25% ruthenium tetroxide (RuO4) in 0.1M of cacodylate buffer for 45 min at room temperature in a dark place. After fixation, all samples were rinsed in buffer solution, dehydrated in graded ethanol solutions and embedded in an Epon-epoxy mixture. Ultrathin 60-to 80-nm sections were examined after further contrast with uranyl acetate-lead citrate in a H-500 electron microscope, operating at 75KV.

Lanthanum tracer studies

Lanthanum nitrate was used as a tracer to delineate the pathways and extent of water permeation through the epidermis. Samples were taken after 24 hours of occlusion following tape stripping as well as occlusion for 24, 48 and 60 hours in normal skin, and then minced and incubated in equal parts modified with Karnovsky's fixative and 0.05M Tris buffer containing 4% (wt/vol) sucrose and 8% (wt/vol) lanthanum nitrate, pH 7.6, for 1 hour at room temperature. The samples were then rinsed in fixative, placed in fresh fixative for 1 hour at room temperature, and incubated in fixative at 4 ℃ overnight. After rinsing in 0.1M cacodylate buffer, the samples were post-fixed in 1% osmium

Fig. 6. Ultrastructure of lanthanum-treated epidermis. In normal epidermis no lanthanum tracer is found in the intercellular spaces of the stratum corneum, it can only be seen in a patchy distribution in the intercellular spaces of the granular layer (arrows, A). By 24 hours of occlusion, the tracer penetrates the SG-SC interface and lower part of SC interstices (arrows, B). C, in Samples taken after occlusion for 60 hours in normal skin, the tracer percolates through the SC-SG interface, and it can be seen in the intercellular spaces within SC (arrows). Whereas, in samples taken from tape stripped specinens that had been occluded for 24 hours the tracer percolates thoughout all the spaces of SC (arrows, D). A, magnification × 20,425; B, magnification × 26,360; C, magnification × 25,830; D, magnification x 20,420.

tetroxide (OsO4) rinsed, dehydrated, embedded and sectioned as discribed above.

Infrared spectroscopy

After the occlusion for 24, 48 and 60 hours, a section of the occluded area was subjected to ATR-FTR(attenuated total reflectance - Fourier transform infrared) spectroscopy (Bio-Rad, Digilab FTS-80, Boston, MA, U.S.A.). The sample was isolated from the mouse after sacrifice and sealed be-
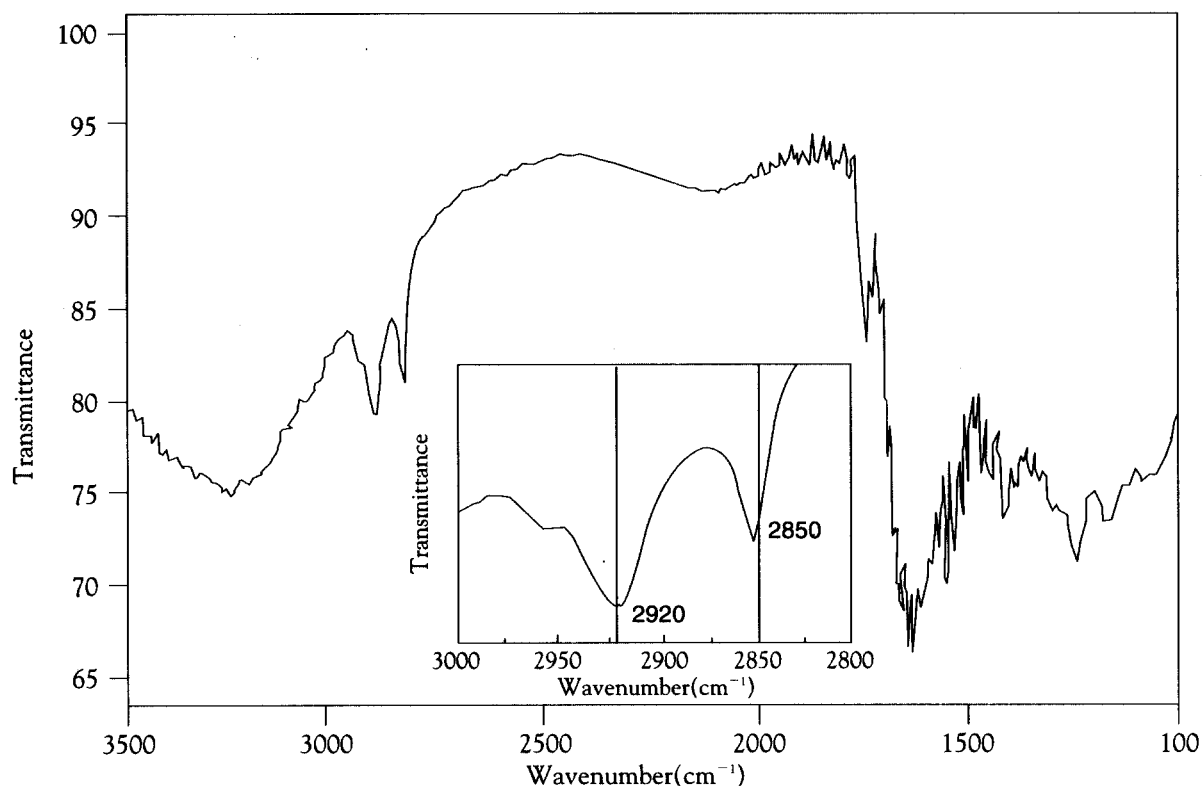
Fig. 7. IR spectra of untreated hairless mouse skin. The inset is the enlarged view of the wavenumber range where the absorption maxima of the C-H asymmetric (2920 cm^{-1}) and symmetric (2850 cm^{-1}) stretching vibration occur.

tween two IR-transparent, ZnS windows. This ensemble was then mounted in the path of the IR beam and the spectra at 25 °C were obtained. The spectra obtained were analyzed using WIN-IR software of Bio-RAD, and the effect of hydration was evaluated by the wavenumber shift in the absorption maxima (transmittance minima) of the C-H asymmetric and symmetric stretching vibration.

RESULTS

Transepidermal water loss (TEWL)

To determine whether the occlusion affected the permeability barrier function, we first checked the TEWL after 24, 48 and 60 hours of occlusion. As seen in Fig. 1, the normal range of TEWL is about 0.5mg/cm^2/h. and the TEWL gradually increased with the time of the occlusion. This result demonstrates that occlusion with a water-impermeable membrane may cause damage to the skin barrier.

Effects of intercellular lipid structure in stratum corneum

The structure of the intercellular lamellae of the normal epidermis comprises multiple lipid bilayers that are fully preserved in the intercellular spaces of the stratum corneum (SC). From the corneocyte envelope outward, the electron-lucent lamellae comprises first a continuous sheet immediately exterior to the cornified envelope. External to the first electron-lucent lamella lies the second type of electron-lucent sheet, which is characterized by frequent interruptions along its long axis, resulting in a fenestrated appearance (Fig. 2). The typical intercellular lamellae is comprised of six electron-lucent lamellae alternating with five electron-dense lamellae or nine electron-lucent lamellae alternating with eight electron-dense lamellae (Fig. 2 inset). Moreover, more than nine electron-lucent intercellular lamellar structures can be seen occasionally

Whereas, in samples taken after 24 hours occlusion, alterations in the intercellular lamellar bilayers are comparable with normal stratum corneum
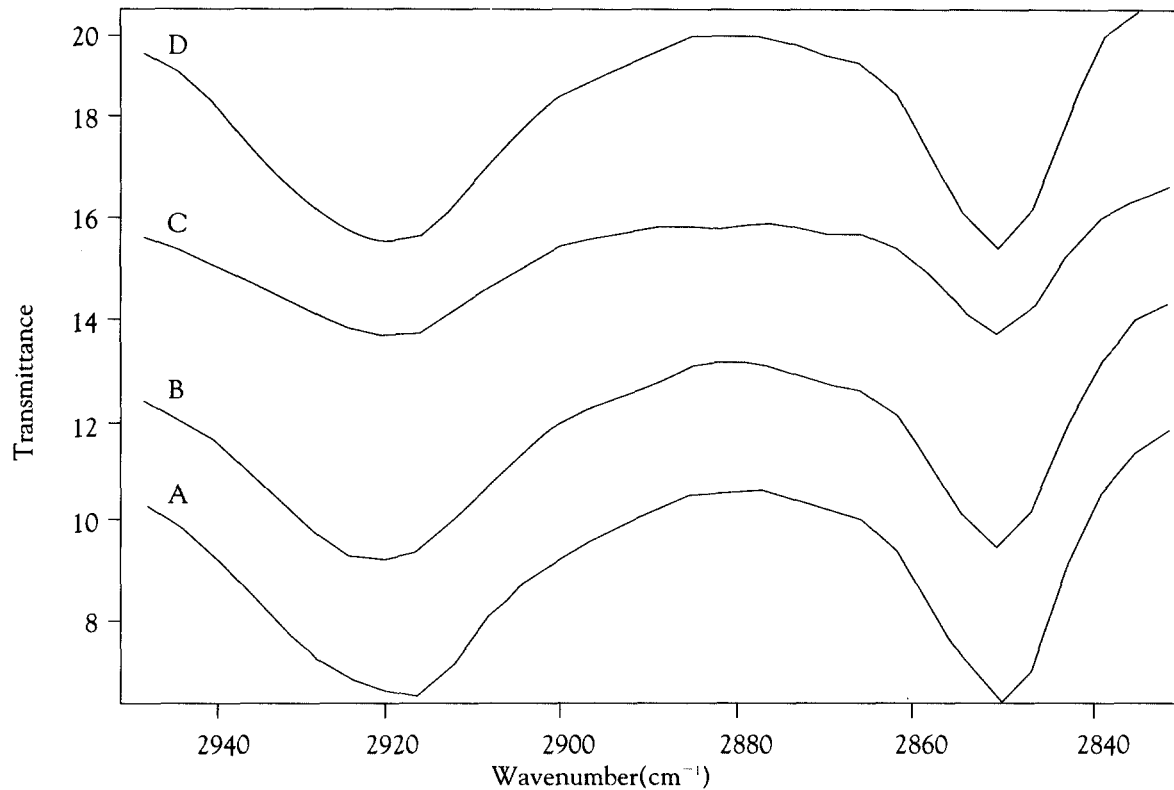
Fig. 8. IR spectra of hairless mouse skin occluded for 24, 48 and 60 hours. The spectra obtained from control skin is also shown [(A) control; (B) 24 hours, (C) 48 hoursm, (D) 60 hours].

lamellar bilayers shown in Fig 2, where the tightly packed structure of the intercellular lamellar bilayer system is distorted, and short fragmentary membrane structures are produced. Moreover, the lipid bilayers are separated by electron-dense material (Fig. 3). By 48 hours of occlusion, the intercellular domains of the stratum corneum exhibit electron-lucent dilatations or lacunae within the intercellular bilayers, separating lipid lamellae which olbiterated the normal structures (Fig. 4). Furthermore, after 60 hours of occlusion, extensive disorganization is evident within the intercellular domains of the stratum corneum, with distorted membrane configurations, loss of the basic unit membrane structure, foci were filled with flocculent, amorphous electron-lucent material, and clefts were present throughout interstices(Fig. 5). These results indicate that occlusion with a water-impermeable membrane in normal epidermis may induce abnormalities of lipid bilayer structures in the stratum corneum.

Tracer studies

To insure the failure of barrier function after occlusion, we also assesed barrier competence in the epidermis after occlusion by examining the extent of penetration of lanthanum. The water-soluble tracer, lanthanum nitrate normally does not penetrate intact stratum corneum[5]. As shown in Fig. 6A, in normal epidermis with complete barrier function, the tracer is seen in a patchy distribution within the intercellular spaces of stratum granulosum. Wher-eas, after 24 hours occlusion, the tracer is penetrated through the stratum corneum-stratum granulosum junction and lower stratum corneum interface(Fig. 6B). However, in samples taken from the animals after 48 and 60 hours of occlusion, the tracer could also be found in the lower part of the stratum corneum interstices and the stratum corneum-stratum granulosum junction, but not in the upper part of stratum corneum (Fig. 6C). Furthermore, in samples taken after 24 hours of occlusion following tape stripping with more defects in barrier function, the tracer penetrated all the stratum

corneum spaces(Fig. 6D). These results demonstrate that the increased TEWL that occurs with occlusion is due to alterations with the intercellular spaces of the statum corneum.

Spectral analysis

Fig. 7 shows the IR-spectra of skin obtained from an untreated hairless mouse. The inset in Fig. 7 is the enlarged view of the wavenumber range between 3000 and 2800 cm^{-1}, where the absorption maxima of the C-H asymmetric (2920 cm^{-1}) and symmetric (2850 cm^{-1}) stretching vibration occur. The stratum corneum consists of keratinized dead cells and intercellular lamellar lipids. In normal skin, most of the C-H conformers of the intercellular lipid alkyl chains are in the trans conformation and the packing of the alkyl chains are very efficient (highly ordered state). If there is an increased number of gauche conformers in the lipid alkyl chain by any treatment, and thus reducing the efficiency of packing, the energy needed to vibrate the C-H bonds will increase. This means that the absorption maxima will shift to a higher wavenumber[7]. Fig. 2 shows the spectra obtained from skin occluded for 24, 48 and 60 hours, together with the spectra from control skin. In all cases, the absorption maxima of symmetric stretching were observed at 2850 cm^{-1}. This indicates that there is no change in the orderness of the lipid alkyl chain domain. In the case of asymmetric stretching, it was rather difficult to assign the exact wavenumber where the absorption maxima occurred. In order to find the exact wavenumber, the spectra were analyzed by a second derivative method, using the WIN-IR software. In all cases, the absorption maxima were observed at 2850 cm^{-1}. These results show that the orderness of the intercellular lipid alkyl chain in the stratum corneum is not affected by the occlusion.

DISCUSSION

Ample circumstantial evidence supports a role for stratum corneum lipid in mammalian cutaneous barrier function[8,9]. Prior studies have shown that epidermal lipid synthesis is regulated by barrier requirements[10]. Acute insults, such as organic solvent treatment, cellophane tape stripping and surfactant treatment, induce an increase in cholesterol, ceramides and fatty acid synthesis, limited to the underlying epidermis[11,12]. Furthermore, the increase in lipid synthesis is attributable to an antecedent increase in mRNA expression, enzyme content, enzyme activity, as well as alterations in the phosphorylation state of some of the key enzymes of lipid synthesis[13]. Barrier requirements not only regulate epidermal lipid synthesis, but in addition, regulate mRNA for certain proteins involved in lipid transport, such as apoprotein E and LDL receptors[14]. Therefore, while all of the nucleated cell layers retain the capacity to synthesize lipid with various types of acute and chronic barrier disruption, distinctive changes in lipid synthesis and enzyme activity occur within specific epidermal cell layers[15]. Finally, the burst in lipid synthesis results in a return of stratum corneum lipid, previously removed during barrier disruption, to the stratum corneum in parallel with barrier restoration[5]. Additionally, the extent of the burst in lipid synthesis following barrier abrogation is proportional to the extent of barrier disruption, and most importantly, when the barrier is disrupted in the acute model, such as in acetone treatment, and then is artificially restored with a water-impermeable membrane, the burst in epidermal lipid synthesis that occurs in response to perturbations in barrier function is prevented[5]. Occlusion cannot prevent the epidermal hyperplasia and also cannot block the increased TNF-α and IL-1α that is induced by repeated barrier disruption[16]. Occlusion enhances the percutaneous absorption of a variety of compounds and psoriasis is also often improved by occlusion[17]. Occlusion blocks TEWL, this leads to an increase in the water content of the SC.

The present study demonstrates that occlusion with a water-impermeable membrane not only delays the barrier recovery in the acute insult model[5,18], but also induces the abnormal structures of the stratum corneum in the normal epidermis. By 24 hours of occlusion, as seen in Fig. 3, the membrane-fragmented region would be seen as "pores" due to their lack of normal membrane structure. Because these fragmented domains are quite comparable with normal lipid bilayers, they presumably account for the overall increase in permeability of the stratum corneum in occlusion with normal epidermis. In addition, it is also possible that the presence of lacunae and the separated lamellae which differ from the normal substructure and organization caused abnormality in their

permeability properties(Fig. 4). However, our prolonged occlusion (60 hours) shows that it increases the number of lacunae locally and causes the foci to expand laterally, until they become continuous, forming broad clefts within the interstices of the stratum corneum(Fig. 5). These horizontal clefts split the lamellar membrane structures along electron-dense lamellae. Thus, the lacunar domains represent the site of preferential permeability within the stratum corneum intercellular spaces. In addition, established lesions show abnormal lamellar membrane structures (Fig. 5, arrows). These morphologic observations are consistent with the functional abnormality that occurs in occlusion with a water-impermeable membrane in normal epidermis.

Yet these examinations point to structural abnormalities in the interstices of the stratum corneum. The junction of stratum corneum-stratum granulosum and the lower stratum corneum are also an important determinant of the barrier abnormality. The localization of the critical defect to the lower stratum corneum and stratum corneum-stratum granulosum junction is supported by the lanthanum tracer studies. Because the intact tight junctions are impermeable and therefore exclude lanthanum, their indentification indicates the presence of an impermeable barrier. As shown in Fig. 6, the tracer percolating through these regions is in occluded, but not in the control epidermis. A similar situation occurs in essential fatty-acid deficiency and lovastatin treated skin, in which abnormal lamellar body contents and incomplete secretion result in membrane abnormalities that correlated with enhanced tracer permeability through these regions and defective barrier function[19].

The technique of infrared (IR) spectroscopy, and most notably Fourier transform infrared(FTIR) spectroscopy, has been used extensively to study the phase behavior of lipid membranes. In general, it is believed that hydration increases the rate of percutaneous absorption, though the exact mechanism remains unclear. One reason suggested is the decrease in alkyl chain orderness of the intercellular lamellar lipid in stratum cornuem, which is known to be the major penetration route for most chemicals. This suggestion is based on the report that the penetration of water is highly dependent on the orderness of the lipid alkyl chain[20]. It seems

that the results of Gay et al.[21], which are obtained at physiological temperatures using human skin, support this suggestion. However, the results of current work indicate that the reason for the increase in percutaneous absorption rate after hydration is not by the decrease in alkyl chain orderness, but by some other mechanism. Moreover, according Mak et al[22], Fourier transform infrared spectroscopy also showed that hydration does not alter SC lipid organization, and that permeation enhancement with hydration does not result from increased alkyl chain disorders. Instead, they suggested that lipid phase separation occurs, creating interfacial defects thereby reducing diffusional resistance.

Our observation in the lacunar system following prolonged occlusion (Fig. 5) provides a structural basis for these views, and explains the mechanism by which superhydration enhances penetration. Furthermore, our previous study in occlusion following tape stripping demonstrated that after 48 hours of occlusion, electron-dense material appeared in the stratum corneum interstices[23]. This also appeared in acetone-treated mouse skin plus latex occlusion for 24 hours[11], fluindostatin treated mouse skin[19], type 2 Gaucher neonate skin[24], and psoriasis[25]. Our present observation demonstrates that occlusion also caused abnormal lipid membrane structures(Fig. 5). Thus we suggest that volume expansion of existing lacunar domains alone can account for the hydrated or "pore" pathway. Together with the disorganization of the lipid membrane of SC, both would provide a pathway for solute movement and explain the elevated transepidermal water loss characteristic of hydrated stratum corneum. Furthermore, this morphologic change depends on whether it reflects a large disordering of a small subset of SC lipids, or a small disordering of a larger subset. With that in mind, it is easy to conceive a number of situations where significant disordering of a small population of SC lipids might readily increase epidermal permeability. However, it is obvious that more work needs to be done to fully characterize the relationship between lipids and permeability in the stratum corneum.

In summary, we have described the effects of occlusion on the skin, TEWL increased and the tracer penetrated more than the control. The characteristics could be ascribed to both lipid bilayer structure alterations as well as clefts or lacunae formation in the stratum corneum interstices. These factors

therefore have a direct effect on epidermal permeability.

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