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PHYSICOCHEMICAL FEATURES  
OF  
HUMAN SKIN STRATUM CORNEUM.

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## Introduction

The stratum corneum(SC) of terrestrial mammals is a flattened anucleate cornified portion of the outermost layer of the epidermis (Fig.1). This layer is generally 10-20  $\mu\text{m}$  thick and plays an important role in the function of human skin as a barrier to water loss which protects the living organism from dehydration and environmental insults. Barrier disruption is observed in variously induced scaly skin(1), and is known to cause changes in epidermal biochemical processes, including lipid biosynthesis(2), DNA synthesis(3), calcium localization(4) and cytokine production(5). Because a decline of SC barrier function might be related to many types of skin abnormalities, the role of the SC barrier function has recently become the focus of intense research. However, there are only a few reports of the relationship of the morphology or biochemistry of the SC to its barrier function.

From the view point of a physical chemist, the SC has a very interesting property. A recent report suggested that the SC has a water permeability about 1000 times lower than most other biomembranes(6). The SC also has a very unique morphology analogized as " brick and mortar"(7)(Fig.2). Its protein enriched corneocytes are embedded in an intercellular matrix which is rich in non-polar lipids. These intercellular lipids include sphingolipids, cholesterol and fatty acids and are important to the SC barrier function (8). These lipids form multilamellar lipid sheets in the SC intercellular spaces (9), where permeability through the SC is

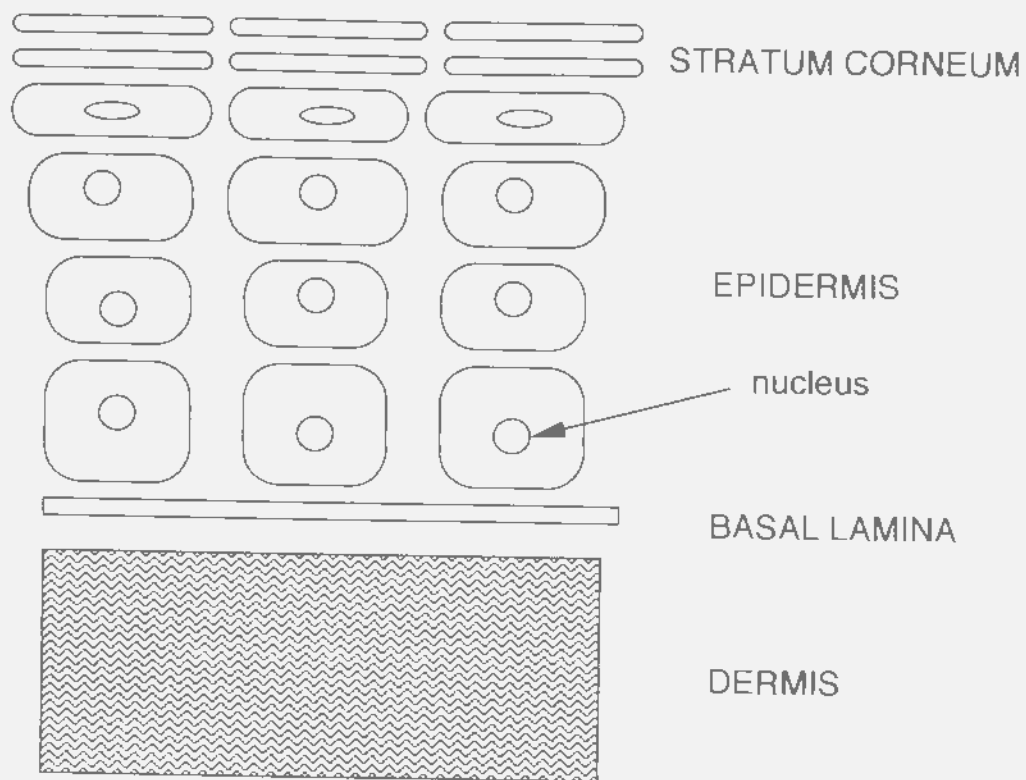


Figure 1. Schematic model of human skin.

related to this unique morphology of the lipid membrane(10)(Fig 3). In the SC, the marginal band called the "cornified envelope" is formed on the surface of each anucleate cell. A special group of long chain,  $\omega$ -hydroxyacid-containing lipid molecules are covalently bonded to the envelope. The bonded lipids exist in ester linkage with protein on the surface of the cornified envelope. The tightly packed hydroxyl acylsphingosine molecules thus form a lipid envelope for each corneocytes. Swartendruber et al. suggested (11) that the corneocyte membrane has physical properties that control the architecture of the intercellular lipid domains. Using X-ray diffraction, Hou et al. reported (12) lamellar spacing of approximately 13 Å. This repeat distance is more than twice the thickness of typical bilayers and White et al. (13)suggested that each lamellar repeating unit consists of two opposing bilayers. With X-ray diffraction study at various temperature, they also reported that at 25°C, two sharp and one broad diffuse band in intercellular lipid were detected and interpreted this result as an indication that at this temperature, there are crystalline and liquid alkyl chains coexisting in the structure. At 45°C, the two sharp lines were replaced by a single one. They interpret this phenomena as a transition from a crystalline to a gel state in which alkyl chains were free to rotate on their axes. However, lipid extracted from the SC and dispersed in excess water does not produce a lamellar diffraction pattern, so that, they suggested another component might sustain the lipid architecture. This special structure of the lipid molecules is currently attracting great interest (8).

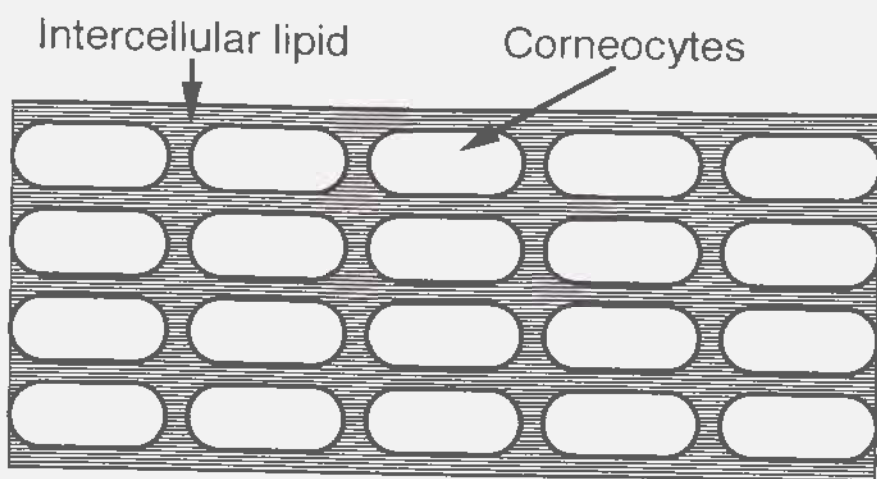


Figure 2. "Brick and Mortar" model of stratum corneum. Original description in ref. 8.

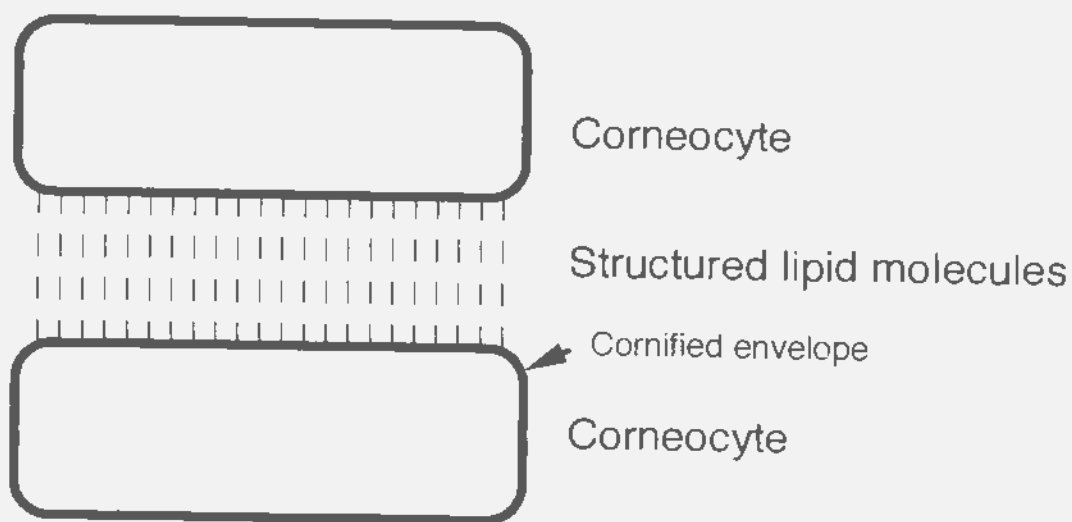


Figure 3. Schematic model of intercellular lipid molecules.

In this thesis, we present three topics related to the SC function from the standpoint of physical chemistry. In Chapter 1, we describe the relation between the intercellular lipid morphology and the barrier function of the SC in normal skin(14). Stratum corneum lipid morphology was evaluated using attenuated total reflectance infrared spectroscopy (ATR-IR) in normal skin. To evaluate the order of the intercellular lipid alkyl chain conformation, we measured the wavenumbers (frequency shifts) of the symmetrical and asymmetrical C-H stretching vibrations. There was a correlation between the wavenumbers and transepidermal water loss in normal skin suggesting that in normal skin, lipid morphology plays an important role in the barrier function of the stratum corneum.

In Chapter 2, we discuss the differences between scaly skin and normal skin with regard to substances that exist in the SC(15)(16). Stratum corneum sphingolipids are of particular importance in maintaining the water permeability barrier of mammalian epidermis(4). Free amino acids also play an important role in water retention in the SC(15). To clarify the way in which these substances affect scaly skin, stratum corneum sphingolipids and free amino acids collected from artificially-induced scaly skin were analyzed. Scaly skin was induced by tape stripping and treatment with surfactant. The total amount of sphingolipids was quantified by gas chromatography and five sphingolipid fractions were isolated and quantified by thin-layer chromatography. Free amino acids were analyzed using a high-speed amino analyzer.

The total amount of sphingolipid in scaly skin did not differ statistically from that in healthy controls. However, a significant change in the distribution of the five sphingolipid variants was observed in scaly skin and the total amount of amino acids was decreased in scaly skin. The SC lipid morphology in scaly skin was also evaluated using ATR-IR and the result compared with that of Chapter 1. The decline of barrier function in surfactant-induced scaly skin was not due to conformational disorder of the lipid alkyl chain.

Finally in Chapter3, we investigated skin aging using ultrasound measurement(17) and lipid analysis(18). We describe the age-related changes in skin thickness of the forehead and cheek for healthy male and female subjects. With age, skin thickness decreased in the forehead and cheek in both genders and in each age group, it was thicker in males than in females. By measuring the percent composition of five stratum corneum sphingolipid species (ceramide 1-6) in 26 males and 27 females, we found a significant change in their composition only among female subjects in various age groups. There was a significant increase in ceramide 1 and 2 with a corresponding decrease in ceramide 3 and 6 from prepubertal age to adulthood. Thereafter, the ratio of ceramide 2 to total sphingolipid composition decreased with aging in contrast to ceramide 3 which showed an increase. Such a pattern of change in the senile population is different from that observed in scaly skin experimentally-induced by tape stripping. The present results suggest that female hormones greatly influence the composition



of stratum corneum sphingolipids. Moreover, the different patterns of change in sphingolipid composition of stratum corneum lipids between scales from inflammatory skin and these from aged skin also suggest that epidermal biosynthesis of sphingolipids is under the influence of epidermal proliferative activity.

These investigations were conducted to increase our understanding of the property and the function of the stratum corneum and helped to clarify the physicochemical features of the SC.

## Reference

1. Tagami H, Yoshikuni K (1985) Interrelationship between water-barrier and reservoir functions of pathologic stratum corneum. *Arch Dermatol* 121: 642-645
2. Grubauer G, Elias PM, Feingold KR (1989) Transepidermal water loss: the signal for recovery of barrier structure and function. *J Lipid Res* 30: 323-333
3. Proksch E, Feingold KR, Man MQ, Elias PM (1991) Barrier function regulate epidermal DNA synthesis. *J Clin Invest* 87: 1668-1673
4. Menon GK, Elias PM, Lee SH, Feingold KR (1992) Localization of calcium in murine epidermis following disruption and repair of the permeability barrier. *Cell Tissu Res* 270: 503-512
5. Wood LC, Jackson SM, Elias PM, Grunfeld G, Feingold KR (1992) Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest* 90: 482-487
6. Potts RO, Francoeur ML (1991) The influence of stratum corneum morphology on water permeability. *J Invest Dermatol* 96 : 495-499

7. Elias PM (1983) Epidermal lipids, Barrier function and desquamation. J Invest Dermatol 86 : 044s-049s
8. Elias PM, Menon GK (1991) Structural and lipid biochemical correlates of the epidermal permeability barrier. In: Elias PM (ed.) Skin lipid (Advances in lipid research, Vol.24) Academic Press, San Diego, California, pp.-26
9. Landmann L (1986) Epidermal permeability barrier. Transform of lamellar granule-disks into cellular sheets by a membrane-fusion process, a freeze-fracture study. J Invest Dermatol 87 : 202-209
10. Schurer NY, Elias PM (1991) The biochemistry and function of stratum corneum lipids. In: Elias PM (ed.) Skin lipid (Advances in lipid research, Vol.24) Academic Press, San Diego, California, pp.-26
11. Swartzendruber DC, Wertz PW, Madison KC, Downing DT (1987) Evidence that the corneocyte has a chemically bound lipid envelope. J Invest Dermatol 88 : 709-713

12. Hou SYE, Mitra AK, White SH, Menon GK, Ghadially R, Elias PM (1991) Membrane structures in normal and essential fatty acid-deficient stratum corneum : characterization ruthenium tetroxide staining and x-ray diffraction. *J Invest Dermatol* 96 : 215-223
13. White SH, Mirejovsky D, King GI (1988) Structure of lamellar lipid domains and corneocyte envelope of murine stratum corneum. *Biochemistry* 27 : 3725-3732
14. Denda M, Koyama J, Namba R, Horii I (1994) Stratum corneum lipid morphology and transepidermal water loss in normal skin and surfactant-induced scaly skin. *Arch Dermatol Res* 286 : 41-46
15. Denda M, Hori J, Koyama J, Yoshida S, Namba R, Takahashi M, Horii I, Yamamoto A (1992) Stratum corneum sphingolipid and free amino acid in experimentally induced scaly skin. *Arch Dermatol Res* 284 : 363-367.
16. Denda M, Koyama J, Takahashi M, Horii I (1994) Changes of sphingolipids and free amino acids in surfactant induced scaly skin. *J Soc Cosmet Chem Jpn* 27 : 55-62.
17. Denda M, Takahashi M (1990) Measurement of facial skin thickness by ultrasound method. *J Soc Cosmet Chem Jpn* 23 :2. 316-319

18. Denda M, Koyama J, Hori J, Horii I, Takahashi M, Hara M, Tagami H (1993) Age- and sex dependent change in stratum corneum sphingolipids. Arch Dermatol Res 285 : 415-417

## Chapter 1

### Stratum Corneum Lipid Morphology and Transepidermal Water Loss in Normal Skin.

It has been reported that in most case, penetration pathway in the stratum corneum is via the intercellular lipid domain(1). This lipid plays a crucial role in SC water barrier function as demonstrated that treatment of the skin with lipid extractants resulted in drastic increase of water flux (2). Elias (1) reported that regional variation of SC water flux appears to be related to the amount of lipids in each site, with flux and lipid content varying inversely.

Intercellular lipid molecules are highly organized and form multilamellar lipid sheets(3). With increasing temperature, this intercellular lipid hydrocarbon chain is disordered and at the same time the permeability of water in SC increases(4). Golden(5) reported the relationship between stratum corneum lipid structure and water barrier function by using differential scanning calorimeter (DSC), infrared (IR) spectroscopy and water permeability techniques. They presented that the SC lipids undergo thermal transitions between 60 and 80 °C. At this temperature, water flux is characterized by an activation energy only slightly higher than that of free diffusion, suggesting that the SC offers little diffusional resistance under these conditions. So, there is a correlation between water flux and the order of intercellular lipid

molecules. Some kind of permeability enhancer like oleic acid, also make the lipid structure disordered(6). In this part, I report the relationship between the order of intercellular lipid molecules and the amount of trans epidermal water loss in healthy skin(7). Potts et al.(8) reported that frequency shifts of the symmetrical and asymmetrical C-H stretching absorbance observed at approximately  $2850\text{ cm}^{-1}$  and  $2920\text{ cm}^{-1}$ , respectively, provide a measure of conformational disorder of the lipid alkyl chain in the SC. In the present study, the absorption of these two frequencies was selected as measure of lipid morphology.

#### Materials and methods

Of the 18 healthy male volunteers ranging in age from 26 to 37 years who were selected for this study.

#### Measurement of transepidermal water loss and skin surface conductance

Transepidermal water loss (TEWL) was measured with an evaporimeter (EP1 Servomed, Stockholm, Sweden) (9). The probe of this instrument is constructed with two humidity sensors. The value of the vapour-pressure gradient close to the surface of the skin is constant, so that the water exchange is approximately proportional to the difference between the vapour pressure measured at two separate fixed points situated on a line perpendicular to the surface and in the zone of the diffusion (Fig.1).

The relation between the water evaporation and vapor pressure is given by this formula;

$$\frac{1}{A} \frac{dm}{dt} = -D \frac{dp}{dx}$$

A is the area of the measurement, m is the amount of water, t is time, D is the diffusion coefficient for the water-vapour-air, p is the vapour pressure of the water in the air, and x is the distance from the surface of the skin. With using the two probes, the value of dp/dx (vapour pressure gradient) is measured and then the value of dm/dx : amount of water evaporation from the surface of the skin per unit time and area is calculated(10).

Skin surface conductance was measured with a skin surface hydrometer (Skicon 200, IBS, Hamamatsu, Japan) (11). The probe of the instrument is composed of 2 concentrically arranged brass electrodes separated by a cylindrical synthetic resin. The conductance of the stratum corneum was estimated with high frequency current at 3.5 MHz. These measurements were carried out under constant temperature (22°C) and relative humidity (50%) conditions.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

The method of application of ATR-IR to the study of lipid morphology used has previously been reported (8). In ATR-IR, skin



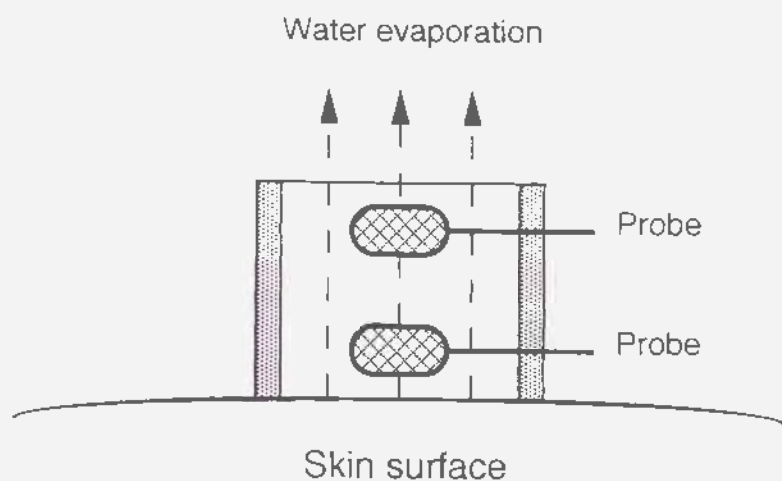


Figure 1. Schema of the probes of the evaporimeter.

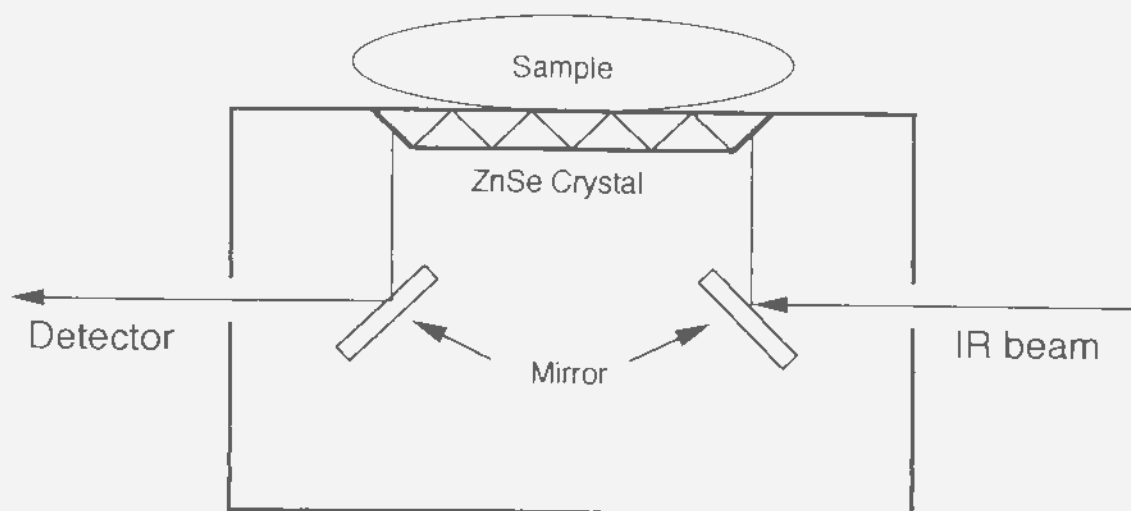


Figure 2. Model of optic system of ATR-IR

of each subject is placed on IR transparent crystal. The IR beam directed to the crystal from which an evanescent wave exist and penetrates into the stratum corneum. The symmetric, trapezoidal geometry of the optics then guides the beam back to the detector of the spectrophotometer(Fig.2). A Fourier-transform infrared spectrometer (Bio Rad, FTS-40, Cambridge, MA,USA) was used to record the measurements. The spectrophotometer was equipped with an ATR accessory (Contact Sampler TM, Spectra-Tech, Stanford, CT, USA) which supports a zinc selenide crystal as the ATR prism. We tested 3 prisms which have different incident angle each of them and selected the prism which has 40° incident angle. Each spectrum obtained was the average of 64 scans. The maximum IR peak frequency was determined to within  $0.1\text{ cm}^{-1}$  using a software for Fourier manipulation( Bio Rad, Digilabo Division). FT-IR spectra were collected in the frequency range  $400\text{-}4000\text{ cm}^{-1}$ , and frequency shifts observed near  $2850\text{ cm}^{-1}$  and  $2920\text{ cm}^{-1}$  were examined. As the degree of the disorder of the lipid alkyl chains increases, these absorbances undergo shift to higher wave number(8). All data represent the average of 5 trials.

### Statistical analysis

Student's t-test was employed to evaluate differences between the two groups, and a P value of less than 0.05 was taken to indicate

### Results

A typical ATR-IR spectrum of human SC is shown in Fig. 3. In this

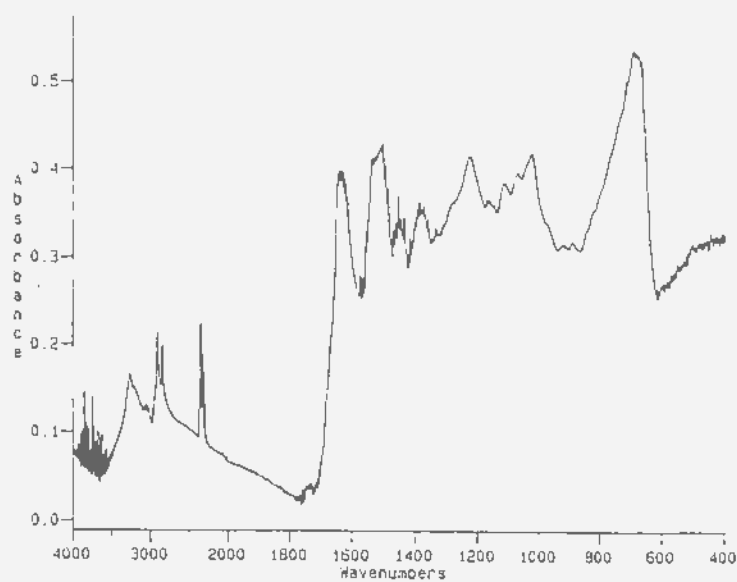


Figure 3. The ATR-IR spectrum of human SC.

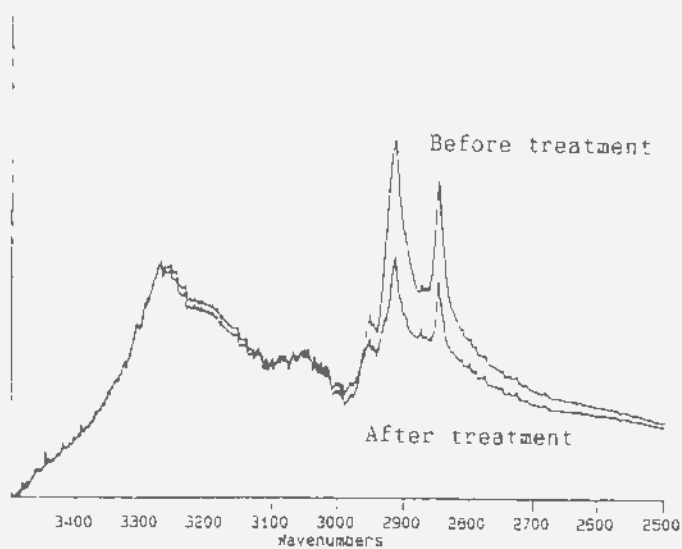


Figure 4. The change in the C-H stretching frequency before and after acetone treatment.

spectrum, there are several peaks due to lipids, proteins, and water. The two peaks between 2800 and 300  $\text{cm}^{-1}$  due to C-H stretching vibrations. We observed these peaks before and after acetone treatment and found that those peaks dramatically reduces after acetone treatment (Fig 4). This result suggests that these two peaks are mainly associated with lipid alkyl chain.

Before the measurement of the subjects, we evaluate the difference of the results of each IR study when we change the incident angle of the ATR prism to define the adequate condition. We measured healthy volunteer's forearm skin using three types of the prism; incident angle 40°, 45°, and 60°. The depth to which the IR beam penetrates the skin varies from the refractive index between the prism and skin and the angle of incident beam. This relation is given by the formula:

$$d = \frac{\lambda}{2 \pi (\sin^2\theta - n_2/n_1)^{1/2}}$$

In this formula,  $d$  is the depth,  $\lambda$  is wave length, and  $n_2/n_1$  is the ratio of the skin refraction index to that of prism crystal. In this present experiments, we observed two IR peaks which has approximately 2900 $\text{cm}^{-1}$  wavenumber. The refraction index of ZnSe is 2.4. About the SC refraction index, 3 decades ago, Scheuplein reported (12) the value 1.55, however, the outermost layer of the SC contains water which refraction index is 1.33 and fatty material

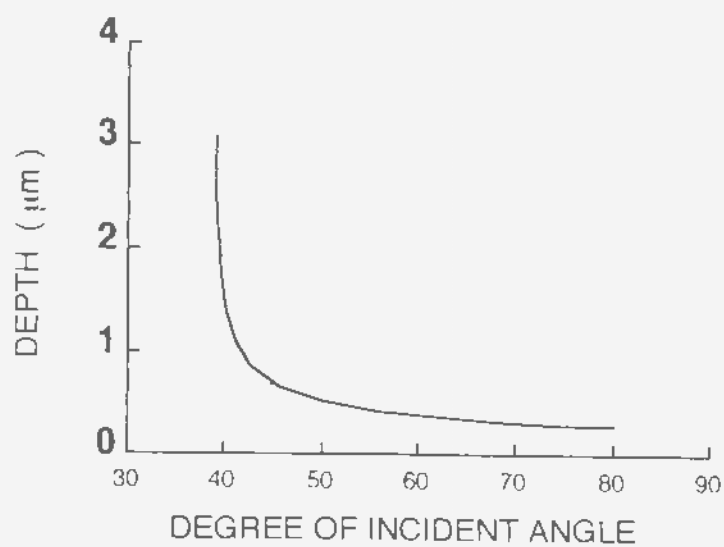


Figure 5. The relationship between incident angle of the prism and depth of the IR beam penetration.

which index 1.46, so that the value of the SC surface might be lower than 1.55. The relation between the depth of the IR beam penetration and the incident angle is shown in figure 5. In this figure, I use refraction index 1.50 as the value of SC surface. The depth decreases with the increasing incident angle. The value of the IR beam absorbance also relates to the incident angle of the prism, because the number of the reflection decreases with the increasing of the angle (Table 1).

Table 1. Number of reflections as a function of the incident angle of prism.

|                       |     |     |     |
|-----------------------|-----|-----|-----|
| Incident angle        | 40° | 45° | 60° |
| Number of reflections | 14  | 12  | 7   |

The results of the SC measurement with using 3 different prism is shown in Fig. 6. The strongest absorbance was observed when we used 40° incident angle prism as we expected. The symmetric(S) and asymmetric wave number shift to higher number with increasing the incident angle. These results suggest that in the deeper portion of the SC, the lipid molecules were more tightly organized than that of in the outer layer. Bommannan reported (13)

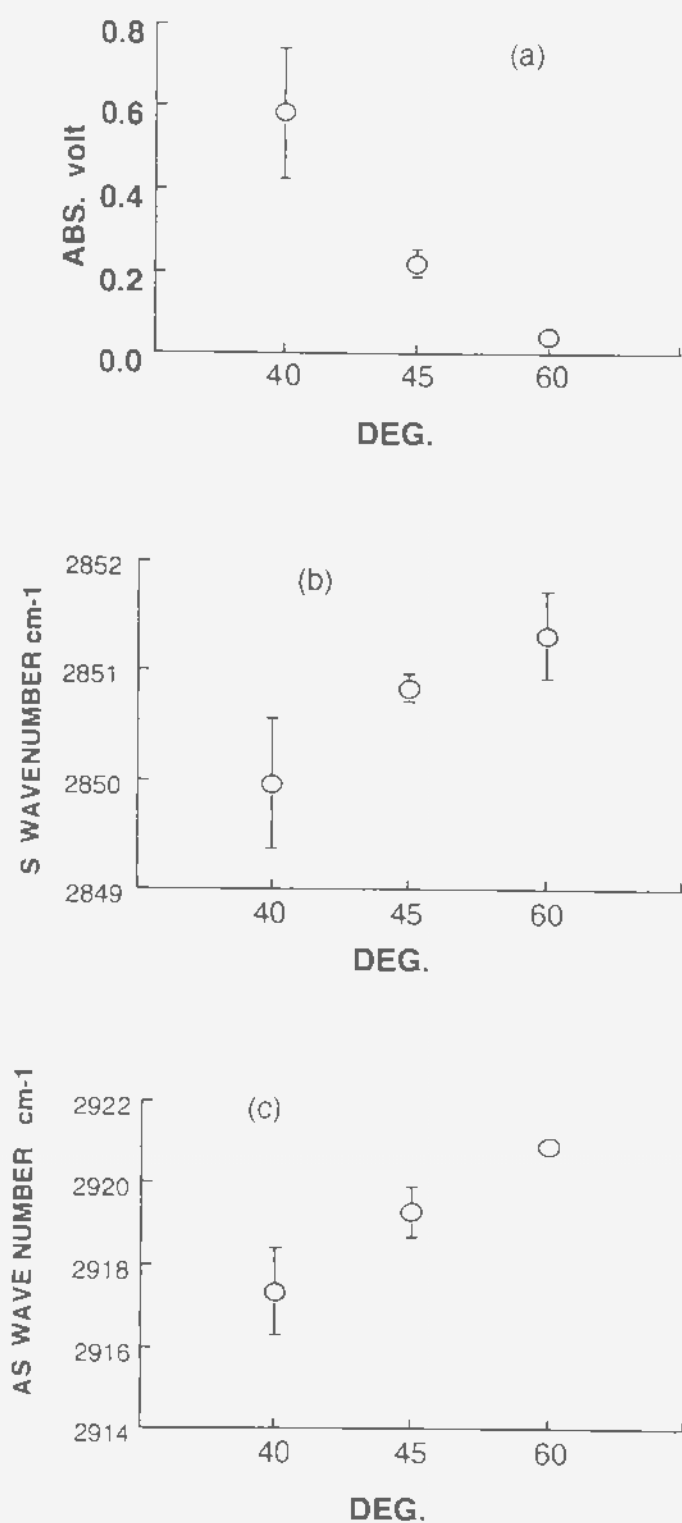


Figure 6. Effect of ATR prism incident angle. (a): absorbance, (b): S wave number, (c): AS wave number.

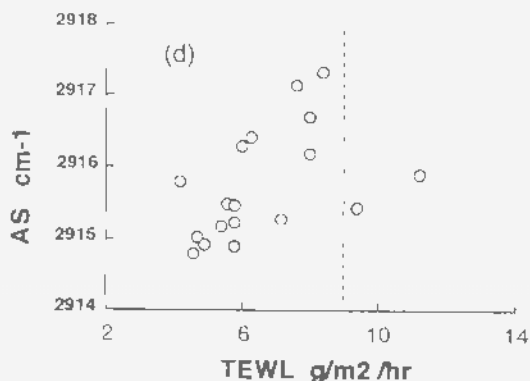
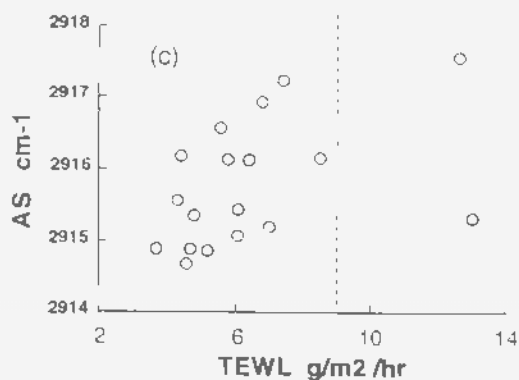
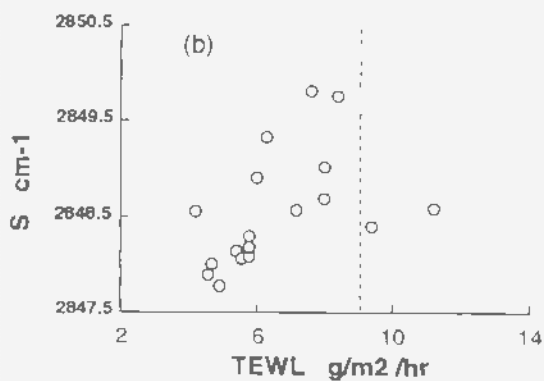
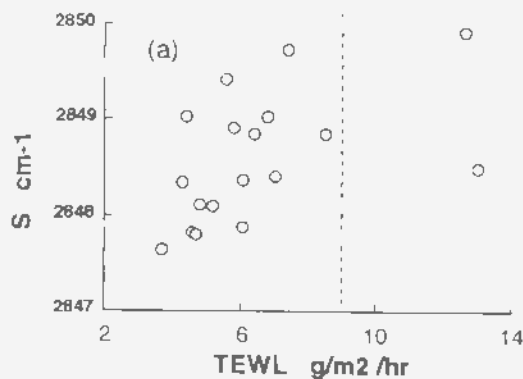


Figure. 7 The relationship of TEWL to symmetric (S) and asymmetric (AS) stretching frequency on the left (a and c) and right (b and d) forearm in normal subjects. In each case there was a stronger correlation when the TEWL value was under 9 g/m<sup>2</sup>/hr (indicated by a broken line).



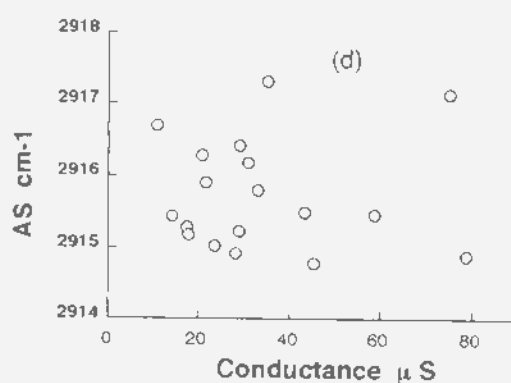
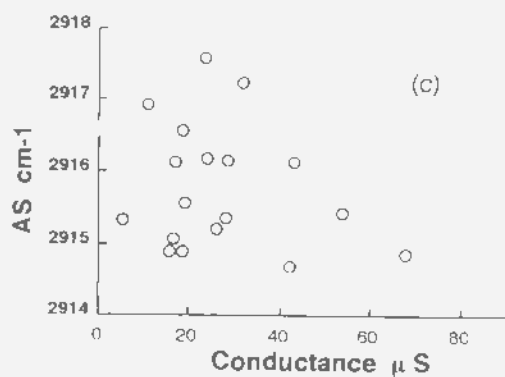
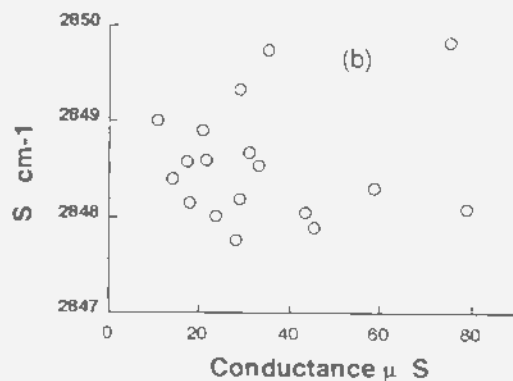
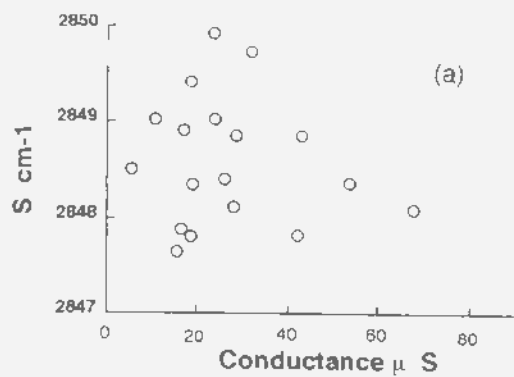


Figure. 8 The relationship of conductance to symmetric(S) and asymmetric(AS) stretching frequency on the left (a and c) and right (b and d) forearm in normal subjects.

that the degree of disorder of the SC intercellular lipids has been found to decrease over the outer cell layers. Our results are a good agreement with their report. Our purpose of this present study is to evaluate whole SC properties, so that , the IR beam should be penetrated in deeper part of the SC. Because of these reason, we selected 40° incident angle prism to our measurement.

In normal skin, a correlation between TEWL and IR-frequencies was observed. Figure 7 shows the relationship between symmetric and asymmetric frequency on right and left forearms in normal subjects. In each case there was a stronger correlation when the TEWL value was under 9 g/m<sup>2</sup>/hr (indicated by broken line). There was no correlation between skin surface conductance and either IR frequency in normal skin (Fig. 8). Table 1 shows the correlation coefficient of frequency with TEWL, TEWL < 9 g/m<sup>2</sup>/hr, and conductance in normal subjects.

Table 2. Pearson's correlation coefficient for IR frequency and skin surface parameters.

| Parameters Compared         | Left forearm |        | Right forearm |        |
|-----------------------------|--------------|--------|---------------|--------|
|                             | S            | AS     | S             | AS     |
| IR Frequency with:          |              |        |               |        |
| TEWL                        | 0.512*       | 0.449  | 0.478*        | 0.492* |
| TEWL< 9g/m <sup>2</sup> /hr | 0.542*       | 0.541* | 0.598*        | 0.586* |
| Conductance                 | 0.129        | 0.214  | 0.078         | 0.009  |

\* : P < 0.05

## Discussion

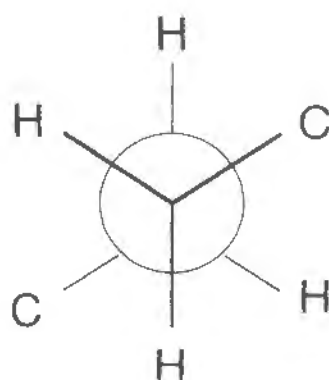
The SC intercellular lipid plays a important role in maintaining the skin barrier function. Elias and Menon (14) suggested that the sequestration of lipids to intercellular domains and their organization into a unique multilamellar system have broad implications for permeability barrier function, water retention, desquamation, and percutaneous drug delivery. In this chapter, I describe how the multilamellar structure relates to TEWL and water content in the SC of healthy normal skin.

The frequencies of the C-H symmetric and asymmetric stretching absorbance are conformational-sensitive and respond to temperature-induced changes of the trans/gauche ratio in alkyl chains(5)(15). When the alkyl chains adopt a gauche conformation, these bands shift to higher frequencies. In the minimum free energy state, all of alkyl chains are in all-trans conformation and in this state, each methylene group experiences minimal steric hindrars from neighboring groups (8) (Fig.9). In this condition, lipid molecules form highly organized crystal like structure. As the temperature is increased, bulkier gauche conformers begin to occur, C-H stretching is sterically hindered and more energy is required to stretch this bond. As the result of that, the vibration of the C-H bond occurs at higher frequency. Thus, the IR spectroscopy has been used to study the lipid membrane structure. Crowe (16) reported the gel-liquid crystalline phase transition in pollen membrane phospholipids accompanying rehydration. They estimated the phase transition by using Fourier transform infrared

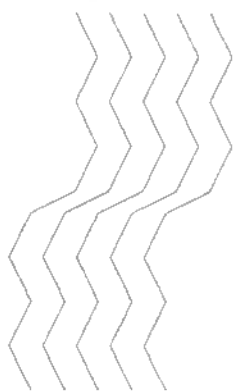
All trans



Trans



2 gauche



Gauche

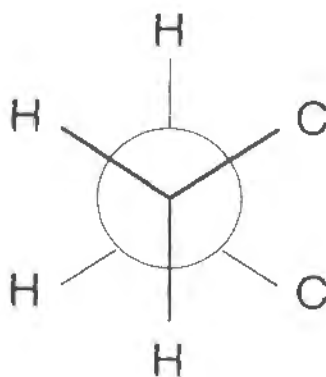


Figure 9. Schematic model of the trans and gauche conformers in alkyl chains. Original description in ref.8.

spectroscopy. By using same method, Cameron (17) presented the gel to liquid-crystal phase transition in live microprasma cells. About human SC intercellular lipids, Potts and his co-workers has reported several important works (8) as I described before. Prompted by these reports, I initiated present study.

In normal skin, there was a significant correlation between TEWL value and the IR-frequencies in bilateral forearms. These results suggest that the intercellular lipid lamellar structure is related to the SC barrier function in normal skin. As Potts et al.(8) reported, permeability to water increases with disorder of the lipid structure. The lipid morphology is affected by skin surface temperature and the lipid composition of the SC(4)(13). However, the finding that the correlation between TEWL and IR wavenumbers was weaker at TEWL level above  $9\text{g}/\text{m}^2/\text{hr}$ . suggests the existence of other factors which regulate the TEWL value.

Golden et al reported(5) that the integrity of the intercellular lipid lamellar structure is related to the permeability of the SC, and that disorder of the hydrophobic domain of the lipid structure is associated with increase of the flux of water through the SC. Potts and Francoeur reported(18) the influence of SC morphology on water permeability. In this report, they suggested that the low permeability of the SC is not necessary due to the unique lipid composition, rather the unique morphology of the SC results in a highly convoluted and tortuous lipid pathway for water diffusion. At TEWL level above  $9\text{g}/\text{m}^2/\text{hr}$ , the crucial factor which regulate the TEWL may not the lipid lamellar structure but the morphology of

the SC itself.

Imokawa et al. suggested(19) that the importance of sphingolipids in the water retention capacity of the SC, however, there is no correlation between lipid morphology and water content in SC. The intercellular lipid order is influenced by water. So, our present results suggest that lipid domain itself does not have the water-holding capacity in healthy skin. Potts and Francoeur reported (18) that the SC lipids are maximally hydrated at less than one water molecule per lipid and in the comparison with phospholipid bilayer, they have less affinity for water. Their result has a good agreement with our present study. In the water retention capacity, much more hydrophilic molecules such as free amino acid might play a crucial role in the SC. I discuss this topic again in the next chapter.

In conclusion, the intercellular lipid structure is related to the water flux via the SC, however there are other factors which relate to the barrier function of the SC. Intercellular lipid domain it self does not have a capacity to hold water molecule.

## Reference

1. Elias PM, Cooper ER, Korr A, Brown BA (1981) Percutaneous transport in relation to stratum corneum structure and lipid composition. *J Invest Dermatol* 67: 291-301
2. Grubauer G, Feingold KR, Elias PM (1989) Lipid content and lipid type as determinants of the epidermal permeability barrier. *J Lipid Res* 30 : 89-96
3. Landman L (1986) Epidermal permeability barrier: Transformation of lamellar granule-disks into inter cellular sheets by a membrane-fusion process, a freeze-fracture study. *J Invest Dermatol* 87 : 202-209
4. Golden GM, Guzek DB, Harris RR, McKie JE, Potts RO (1986) Lipid thermotropic transitions in human stratum corneum. *J Invest Dermatol* 86: 255-259
5. Golden GM, Guzek DB, Kennedy AH, McKie JE, Potts RO (1989) Stratum corneum lipid phase transitions and water barrier properties. *Biochemistry* 26: 2382-2388

6. Mak VHW, Potts RO, Guy RH (1990) Oleic acid concentration and effect in human stratum corneum : non-invasive determination by attenuated total reflectance infrared spectroscopy in vivo. *J Controlled Release* 12 : 67-75
7. Denda M, Koyama J, Namba R, Horii I (1994) Stratum corneum lipid morphology and transepidermal water loss in normal skin and surfactant-induced scaly skin. *Arch Dermatol Res* 286 : 41-46
8. Potts RO, Mak VHW, Guy RH, Francoeur ML (1991) Strategies to enhance permeability via stratum corneum lipid pathways. In: Elias PM (ed.) *Skin lipid (Advances in lipid research Vol.24)* Academic Press, San Diego, California, pp173-210
9. Nilsson GE (1977) Measurement of water exchange through skin . *Med Biol Eng* 15 : 209-218
10. Sugai T (1981) Clinical significance of transepidermal water loss. *Nihon Hihukagakukaishi*, 93 : 1551-1560
11. Tagami H, Yoshikuni K (1985) Interrelationship between water-barrier and reservoir function of pathologic stratum corneum. *Arch Dermatol* 121 : 642-645



12. Scheuplein RJ (1964) A survey of some fundamental aspects of the absorption and reflection of light by tissue. *J Soc Cosmet Chem* 15 : 111-122
13. Bomannan D, Potts RO, Guy RH (1990) Examination of stratum corneum barrier function in vivo by infrared spectroscopy. *J Invest Dermatol* 95: 403-408
14. Elias PM, Menon GK (1991) Structural and lipid biochemical correlates of the epidermal permeability barrier. In: Elias PM (ed.) *Skin lipid (Advances in lipid research, Vol.24)* Academic Press, San Diego, California, pp.-26
15. Casal HL, Mantsch HH (1984) Polymorphic phase behavior of phospholipid membrane studied by infrared spectroscopy. *Biochim Biophys Acta* 779 : 381-401
16. Crowe JH, Hoekstra FA, Crowe LM (1989) Membrane phase transitions are responsible for imbibitional damage in dry pollen. *Proc Natl Acad Sci USA* 86 : 520-523
17. Cameron DG, Martin A, Moffat DJ, Mantsch HH (1985) Infrared spectroscopic study of the gel to liquid-crystal phase transition in live *Acholeplasma laidlawii* cells. *Biochemistry* 24 : 4355-4357

18. Potts RO, Francoeur ML (1991) The influence of stratum corneum morphology on water permeability. *J Invest Dermatol* 96: 495-499
19. Imokawa G, Hattori M (1985) A possible function of structural lipids in the water-holding properties of the stratum corneum. *J Invest Dermatol* 84: 282-284

## Chapter 2.

Stratum corneum lipids and amino acids in experimentally-induced scaly skin.

Scaly skin is characterized by deterioration of the barrier function and decrease in the water retention capacity of the SC(1). This study is an attempt to identify the substances that contribute to these functions. Sphingolipids, such as ceramide and acylceramide, are functionally important in the water permeability barrier of human epidermis, so they are currently attracting great interest(2) . These substances construct intercellular multilamellar lipid sheets in the SC with free fatty acids and cholesterol(3). The composition of each lipid species is shown in table 1 (4)(5). Free amino acids also play an important role in the water retention properties of this part the epidermis (6), and indeed, a significant correlation has been found between the hydration state of the stratum corneum and its amino acid content(7). The diagram of the metabolism on each substances in the epidermis is shown in Fig. 1. The intercellular lipid bilayers originate from small organelles, termed lamellar bodies, in the spinous and granular cells in the epidermis(2). This organelle contains parallels stacks of lipid containing disks. In the outer granular layer, the lamellar bodies secretes the lipid-rich contents. The secreted contents fill the intercellular space and form broad lamellar bilayers. The origin of the free amino acid is keratohyaline granule in the granular

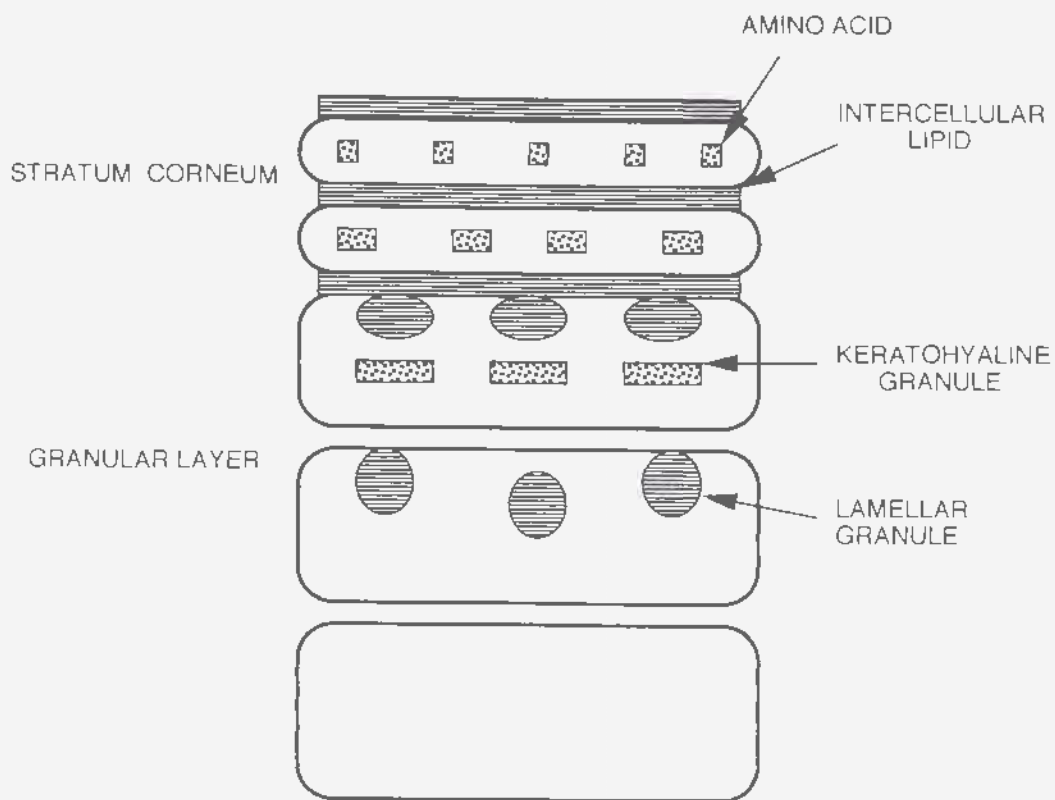


Figure 1. Diagrammatic representation of the epidermis. Intercellular lipids and free amino acid synthesis.

Table 1. Strarum corneum lipid composition

| Ref. 4              |          | Ref.5               |     |
|---------------------|----------|---------------------|-----|
| Phospholipids       | 6.6      |                     |     |
| Cholesterol sulfate | 2.0      | Cholesterol sulfate | 1.9 |
| Neutral lipids      |          |                     |     |
| Free sterols        | 18.9     | Free sterols        | 27  |
| Free fatty acids    | 26.0     | Free fatty acids    | 9   |
| Triglycerides       | Variable |                     |     |
| Sterol/wax ester    | 7.3      | Sterol ester        | 10  |
| Squalene            | 6.5      | Sterol diester      | 0.9 |
| n-Alkanes           | 8.2      |                     |     |
| Sphingolipids       |          |                     |     |
| Glucosylceramides   | Trace    | Ceramide ester      | 3.8 |
| Ceramides           | 24.4     | Ceramide            | 41  |

Values given as wt % of total lipid.

layer(8). This granule contains special protein termed profilagline. In the upper part of the granular layer, this protein changes into histidine-rich protein, filagline. The filagline finally degrades and produces the free amino acids in the cornified cells.

Both sphingolipids and free amino acids are closely related to skin surface conditions but little is known of the relationship between sphingolipids and amino acids in scaly skin. To increase our understanding of the way in which these substances affect scaly skin, we investigated the change of sphingolipids and free amino acids in scaly skin induced by a surfactant, sodium lauryl sulfate (SLS).

In this part, first, I report the differences between scaly skin and normal skin with regard to sphingolipids and amino acids and then present the relation between intercellular morphology and transepidermal water loss in scaly skin.

## Material and method.

### Measurement of SC sphingolipids and amino acids.

Scaly skin was induced by sodium lauryl sulfate (SLS). Nine healthy male volunteers, between 25 and 42 years old, were selected for the study. An area of 20 x 10 cm<sup>2</sup> on their back was treated with a 5 % aqueous solution of SLS under an occlusive dressing for 4 hours and the changes in sphingolipids and amino acids in the SC were examined 1 week after treatment. To evaluate the degree of disorder of the SC intercellular lipids, the forearm skin of another

ten healthy male volunteers was treated similarly. To qualify the amount and the composition of sphingolipids and amino acids, we collected SC from each subject by tape stripping 1 week after treatment. The specimen from which the sphingolipids were to be extracted was removed from the adhesive tape by washing with n-hexane and the other samples from which the free amino acids were to be extracted was removed from tape by washing with toluene. The solvent used for washing was then evaporated off. After evaporation, each sample was weighed.

#### Lipid analysis

Sphingolipids in SC samples were extracted with chloroform/methanol (2:1) by the method of Folch et al(9). The extracts were then washed with 1 M aqueous KCl solution, after which the solvent was evaporated and diethylether was added. The lipid sample was dispersed in diethylether using ultrasonic vibration. A silica-gel column (Bond Elut SI, Analytichem International,U.S.A) was used to separate the sphingolipids. The samples were washed successively with 10 ml of diethylether, then loaded onto the column and washed again with 10 ml of diethylether to remove the non-polar lipids. Sphingolipids were eluted from the column with chloroform/methanol (2:1) and collected into test tubes. High-performance thin-layer chromatography (HPTLC: Merck 20 x 10cm<sup>2</sup> HPTLC plate, Darmstadt, Germany) was used to separate the lipids, and chloroform / methanol (19:1) was eluted once up the plate. After

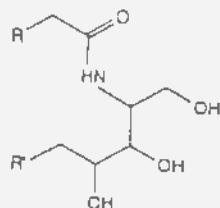
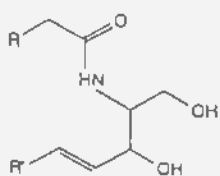
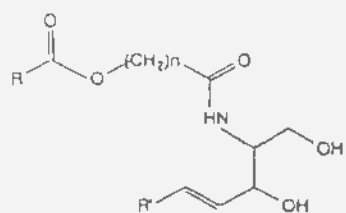
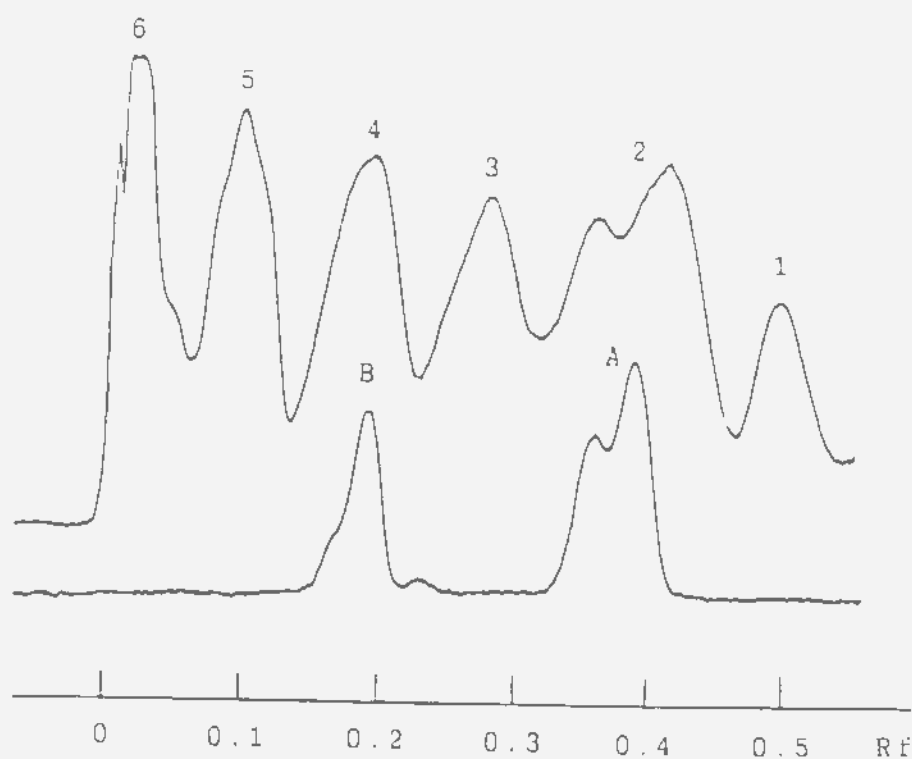
diethylether to remove the non-polar lipids. Sphingolipids were eluted from the column with chloroform:methanol (2:1) and collected into test tubes. High-performance thin-layer chromatography (HPTLC: Merck 20 x 10cm<sup>2</sup> HPTLC plate, Darmstadt, Germany) was used to separate the lipids, and chloroform/methanol(19:1) was eluted once up the plate. After development, the HPTLC plate was dried, sprayed with 50% H<sub>2</sub>SO<sub>4</sub>, and heated at 170 °C for 10 minutes to char the lipid. The HPTLC plate was then cooled and scanned on a recording photodensitometer (Shimazu TLC Scanner CS 930, Kyoto, Japan) which provides automatic peak integration and calculation of percentage composition. The ceramide species were identified by the GC-MS method, and acylceramide was identified by the method of Wertz et.al.(10), as we have previously described (11)(12). Atypical profile is shown in Fig. 2. The total amount of sphingolipids, determined by GC, was expressed as µg/mg SC.

#### Analysis of free amino acid

Exactly 1mg of SC was homogenized for 1 min. with 10% sulphosalicylic acid solution. The solution was then centrifuged and the amino acid content in the supernatant was determined using a high-speed amino-acid analyzer (Hitachi, Tokyo, Japan, Model 835)(7). The amino-acid content was expressed as nmol/mg SC.

Measurement of transepidermal water loss (TEWL) and skin surface conductance.

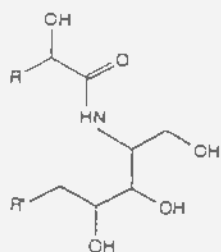
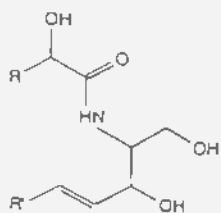




1:ceramide 1

2:ceramide 2

3:ceramide 3



4:ceramide 4/5

5:ceramide 6

6:other polar lipids

A: Ceramide 2 standard, B: Ceramide 4/5 standard

Figure 2. Densitometric profiles of TLC separation of SC sphingolipids and ceramide standards.

Before, and 1 week after SLS treatment, TEWL was measured using an evaporimeter (EP1, Servomed Stockholm, Schweden)(13) and skin surface conductance using a skin surface hydrometer(14) (Skicon 100, IBS, Hamamatsu, Japan). These measurements were carried out under the fixed conditions of 22°C and 50% relative humidity (RH).

#### Measurement of lipid morphology with ATR-IR.

The method of application of ATR-IR to the study of lipid morphology used has previously been described in the Chapter 1. A Fourier-transform infrared spectrometer (Bio Rad, FTS-40, Cambridge, MA,USA) was used to record the measurements. The spectrophotometer was equipped with an ATR accessory (Contact Sampler TM, Spectra-Tech, Stanford, CT, USA) which supports a zinc selenide crystal with a 40° incident angle as the ATR prism. Each spectrum obtained was the average of 64 scans. The maximum IR peak frequency was determined to within 0.1  $\text{cm}^{-1}$  using a software for Fourier manipulation( Bio Rad, Digilabo Division). FT-IR spectra were collected in the frequency range 400-4000  $\text{cm}^{-1}$ , and frequency shifts observed near 2850  $\text{cm}^{-1}$  and 2920  $\text{cm}^{-1}$  were examined. All data represent the average of 5 trials.

10 healthy male volunteers were selected for the induction of scaly skin. The forearm skin was treated with a 5 % aqueous solution of sodium lauryl sulfate (SLS) and an occlusive dressing applied. The dressing was removed 4 hours later, and the treated

region gently washed with water. Transepidermal water loss (TEWL), skin surface conductance, and attenuated total reflectance infrared spectroscopy (ATR-IR) were evaluated just before the treatment, and one hour and 1, 3, 7, 10 and 14 days after the treatment. Before the measurements, the test region was cleaned by pure water gently and waited for 15 minutes. The subjects were asked not to apply any drugs or cosmetic products or rub the forearm skin during the 2-week measurement period.

Results

Distribution of sphingolipids in normal skin

We measured the total amount of sphingolipids and also their proportions in each layer of normal skin, i.e. the outermost, the middle and the deepest layers. There were no changes in any layers of the SC with regard to either the total amount or the proportion of each type of sphingolipid (Table 2).

Table 2. Relative proportions of sphingolipids in each layer of the SC.

|                 | Total (mg/mg) | Ceramide (%) |          |          |          |          |
|-----------------|---------------|--------------|----------|----------|----------|----------|
|                 |               | 1            | 2        | 3        | 4/5      | 6        |
| Outermost layer | 8.24±1.99     | 8.2±1.9      | 15.1±2.9 | 23.6±2.4 | 28.4±2.1 | 24.6±3.2 |
| Middle layer    | 8.63±4.37     | 8.5±1.5      | 13.9±2.4 | 23.2±2.1 | 29.1±1.7 | 25.4±2.9 |
| Deepest layer   | 6.58±2.99     | 8.7±1.4      | 15.0±2.2 | 22.3±2.2 | 28.8±1.7 | 25.3±2.3 |

Values are the mean plus standard deviation from nine subjects.

There were no significant differences between any layer of the SC.

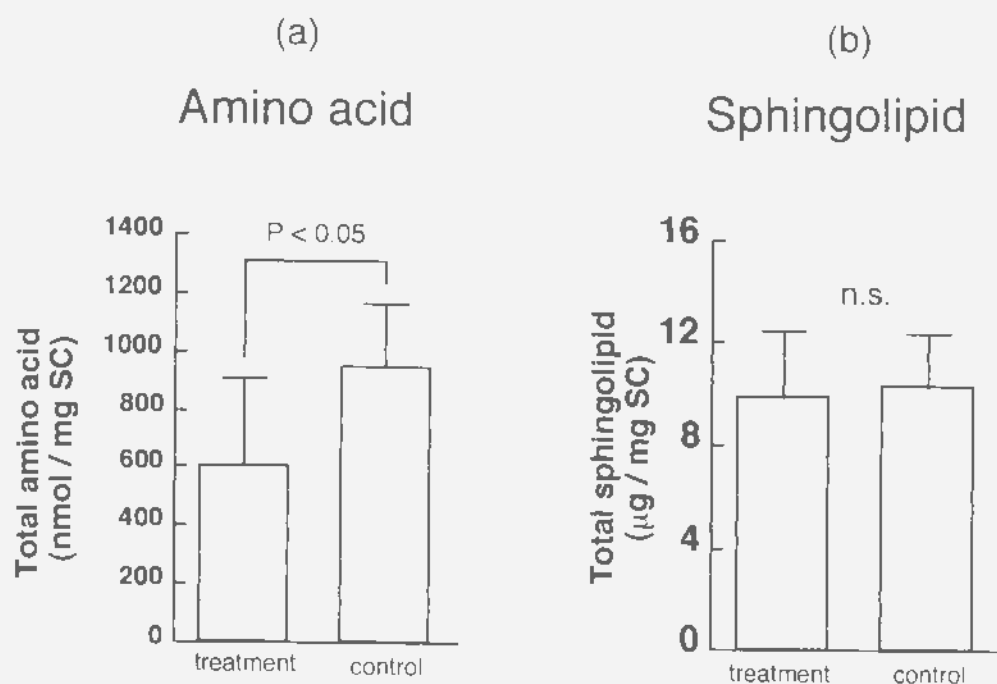


Figure 3. Change in total amino acid and total sphingolipids in SC 1 week after SLS treatment.

Table 3. Relative proportion of sphingolipids in normal skin and scaly skin induced by SLS treatment.

| Ceramide (%) |           |              |            |              |            |
|--------------|-----------|--------------|------------|--------------|------------|
|              | 1         | 2            | 3          | 4/5          | 6          |
| Normal skin  | 5.6 ± 3.4 | 13.7 ± 1.8   | 22.4 ± 4.7 | 26.7 ± 3.0   | 31.4 ± 3.6 |
| Scaly skin   | 6.0 ± 1.8 | 16.8 ± 2.0 * | 23.9 ± 3.0 | 21.9 ± 2.0 * | 31.5 ± 4.3 |

Values are the mean plus standard deviation from nine subjects.

\* $p < 0.05$ , significance of difference between normal and scaly skin.

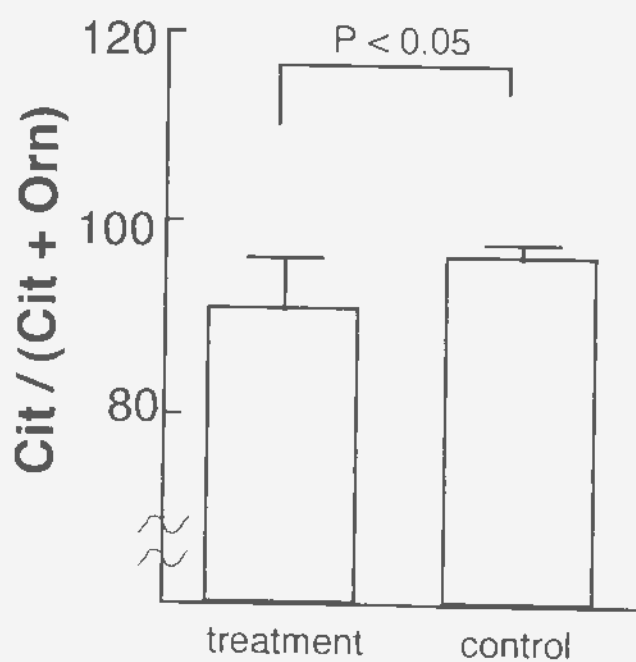


Figure. 4 Change in ratio of citrulline/(citrulline+ornithine) in normal skin and SLS induced scaly skin.

## Sphingolipids and free amino acids in treated skin

Total sphingolipid levels were unaffected by SLS treatment (Fig. 3b). However, we found that the distribution of the five types of sphingolipid species had changes significantly (Table 3). The free amino acid level was significantly decreased (Fig. 3a). The ratio of citrulline/(citrulline + ornithine) which is known as a good parameter indicating the degree of hyperkeratosis (7), in normal skin and treated skin in Fig. 4. In the scaly skin, the ratio decreased significantly. These results suggest an abnormality in amino acid metabolism in SLS-induced scaly skin.

TEWL, skin surface conductance, and IR-frequency in the SLS treated subjects.

Figure 5 shows the difference of TEWL from the baseline value before and after SLS treatment. Upon SLS treatment, TEWL value increased and remained significantly higher than that of controls for 2 weeks thereafter. Figure 6 shows the difference of skin surface conductance value from baseline after SLS treatment. Upon SLS treatment, the conductance decreased and remained significantly lower than that of controls for 2 weeks thereafter.

Analysis of the difference from baseline of symmetric(Fig. 7a) and asymmetric(Fig. 7b) stretching absorbance (frequency shift) after SLS treatment revealed a marked change immediately after the treatment. The values increased in 8 of the 10 subjects and decreased in the other two. Three days after the treatment, the

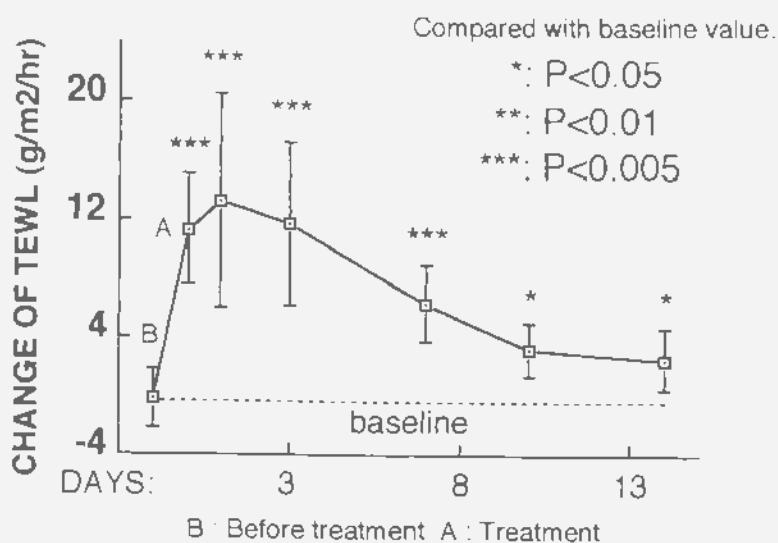


Figure.5 Difference of TEWL from baseline value after SLS treatment.

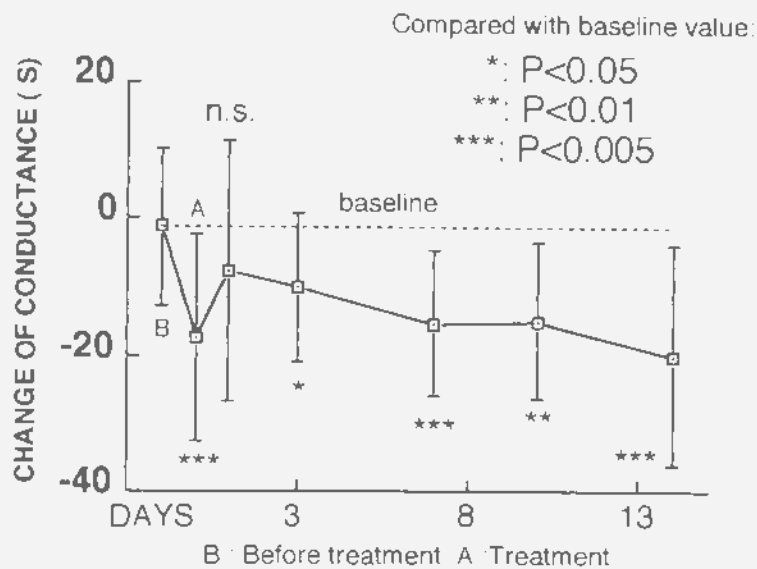


Figure.6 Difference of skin surface conductance value from baseline after SLS treatment.

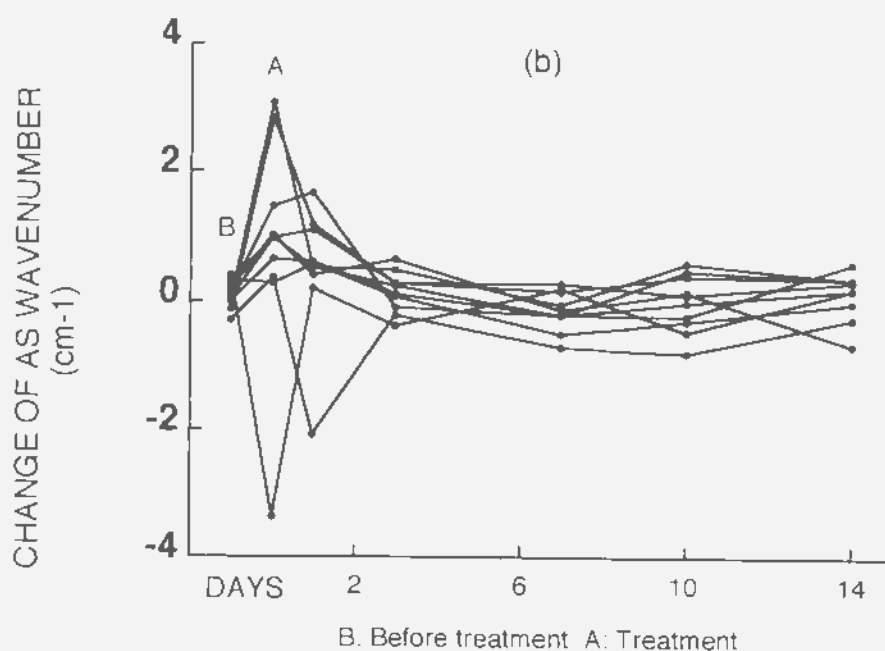
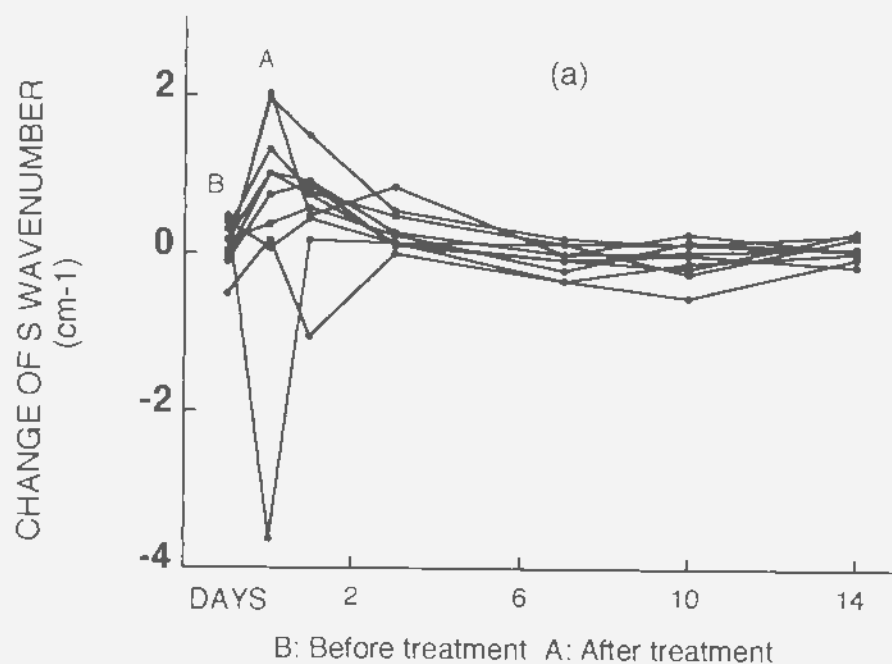


Figure 7. Deference from baseline (frequency shift) of symmetric (a) and asymmetric (b) frequency before and after SLS treatment.



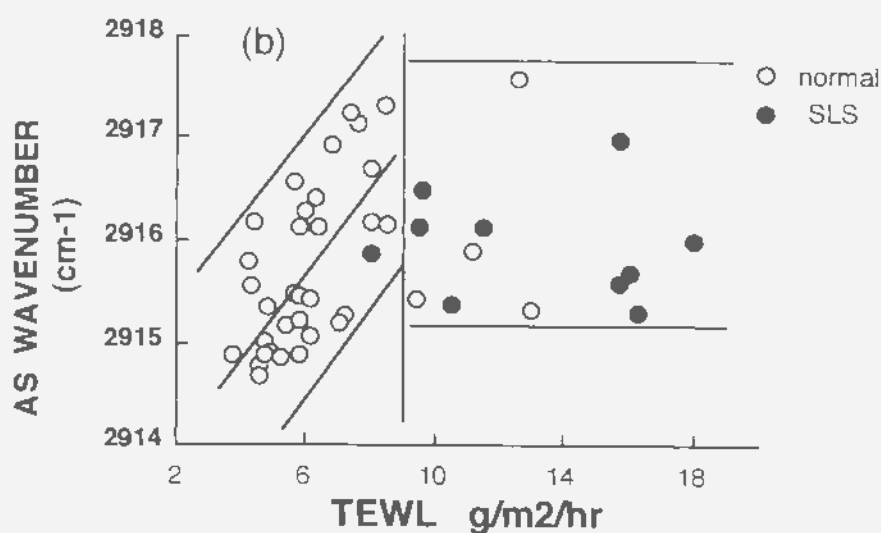
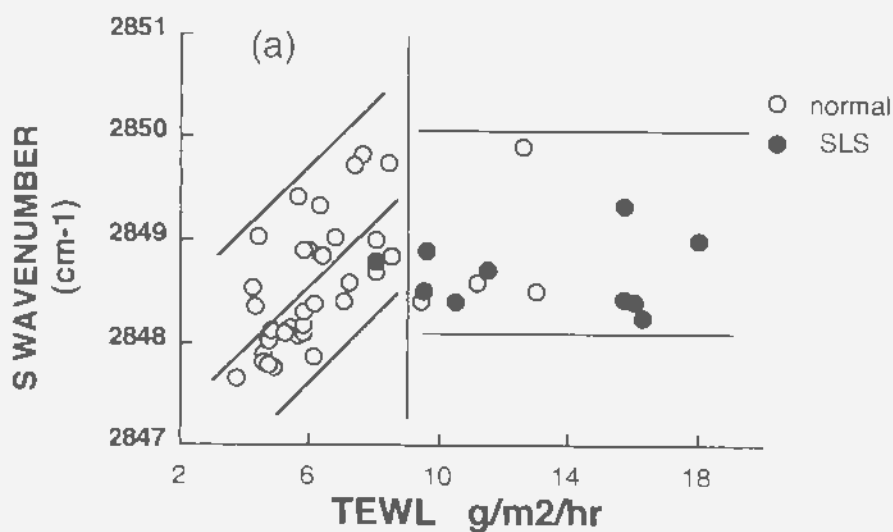


Figure 8. The relationship of TEWL to symmetric frequency(a) and to asymmetric frequency(b) in normal and SLS treated subjects.

values returned to their original levels. Figure 8 shows the relationship of TEWL value to each IR wavenumber in each normal subject and each SLS-treated subject 7 days after treatment. There was a stronger relationship between the two parameters when the TEWL value was less than 9 g/m<sup>2</sup>/hr. of the normal subjects had TEWL value below this level.

## Discussion

Scaly skin might be induced by many factors(1). In this study, we investigated epidermal hyperproliferation which we induced by SLS treatment. We observed scaling on the surface of the skin at this time. These changes were the same as those in typical scaly skin, including atopic xerosis. Watanabe et al. (15) reported that the barrier function and water-retaining capacity were reduced in atopic SC, and that the amount of amino acids was decreased. With regard to these characteristics, the scaly skin we report here was quite similar to atopic scaly skin. With regard to the sphingolipid findings, however, our results differ from those in atopic xerosis. Melnik (16) and Imokawa (17) reported decreased ceramides in the SC of atopic patients. In the scaly skin induced by SLS treatment in our study , however, the sphingolipids did not change in amount, only in their relative proportions.

To investigate scaly skin induced by a different method, we treated the skin of another nine volunteers with tape stripping and examined changes in sphingolipids and amino acids in the SC 1 week after the treatment. The results of this experiment were

almost the same as those of SLS treatment(Fig. 9). The amount of amino acids was lower than that in the control skin(Fig. 9b), but the total amount of sphingolipids did not differ from that in the control skin(Fig.9a). In tape-stripping scaly skin, the composition of the sphingolipids changed significantly (Table 4).

Table 4. Proportions of sphingolipids in normal and scaly skin in the tape stripping experiments.

|            |             | Ceramide (%) |             |           |           |             |
|------------|-------------|--------------|-------------|-----------|-----------|-------------|
|            |             | 1            | 2           | 3         | 4/5       | 6           |
| Outer part |             |              |             |           |           |             |
|            | Normal skin | 8.2±1.9      | 15.1±2.9    | 23.6±2.4  | 28.4±2.1  | 24.6±3.2    |
|            | Scaly skin  | 9.5±1.3      | 16.1±2.7    | 23.3±1.4  | 26.7±1.6* | 24.5±2.7    |
| Inner part |             |              |             |           |           |             |
|            | Normal skin | 8.5±1.5      | 13.9±2.4    | 23.2±2.1  | 29.1±1.7  | 25.4±2.9    |
|            | Scaly skin  | 10.4±1.1*    | 17.8±2.6*** | 21.5±1.4* | 26.7±1.9* | 23.7±2.2*** |

Values are the mean plus standard deviation from nine subjects.

\*  $p < 0.05$  and \*\*\*  $p < 0.001$ , significance of difference between normal and scaly skin.

Fulmer and Kramer reported (18) that in surfactant-induced SC, surfactant-induced perturbation of keratinization led to abnormal ceramide biosynthesis and that the proportions of ceramide species was significantly altered, but the total amount of ceramides did not change. These results suggest that quantitative changes of sphingolipids do not always contribute to changes in skin surface conditions in scaly skin. The reduction in sphingolipids in the SC of atopic patients is not a general tendency in other types of scaly skin conditions.

The mechanism underlying the biosynthesis of epidermal sphingolipids is poorly understood. The pathway of sphingolipid synthesis in the epidermis has been described by Madison et al.

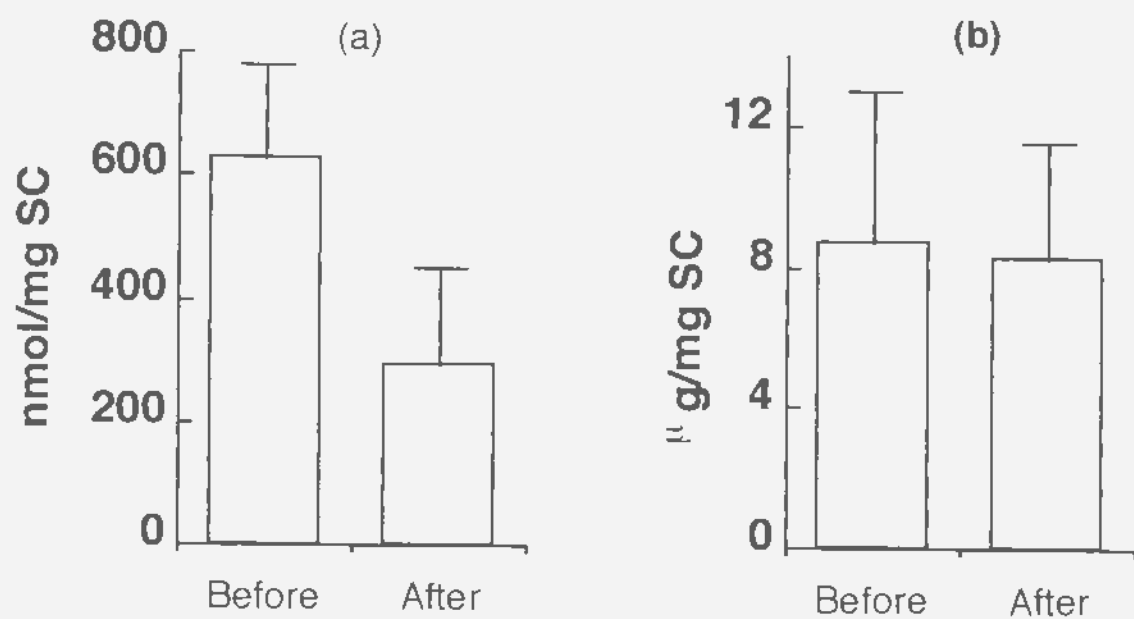


Figure 9. Change in total amino acids (a) and total sphingolipid (b) in SC 1 week after tape stripping. A statistically significant decrease in the total amount of amino acids was noted ( $p < 0.05$ ).

(19). However, little is known of the mechanism underlying the relative proportions of different types of ceramides. Yamamoto et al. showed that there is a low proportions of acylceramide (ceramide 1) in the SC of atopic patients(20). Wefers et al. reported that changes in the proportion of certain species are induced by UV radiation (21). The changed proportions of sphingolipids might be due to many factors, and the results of these changes are variable. Further work is needed in this area.

In normal skin, neither the total amount of sphingolipid nor the proportion of any species changed at any depth in the SC. In scaly skin induced by tape stripping, the outer part of the SC showed no change in the proportion of any sphingolipids species compared with normal skin. However differences were found in the proportions in the inner part of the SC. This indicates that the changes originated in a deeper part of the epidermis. These results suggest that the tape stripping affected a viable part of the epidermis and that, as a result, these were perturbations in the biosynthesis of each of the sphingolipid species.

Upon treatment with SLS solution, the TEWL value increased significantly and remained higher than that of normal skin for 2 weeks thereafter. On the other hand, the IR-frequency peaks shifted to higher wavenumbers in 8 of the 10 subjects immediately after treatment. In these subjects, the intercellular lipid structure may have been disrupted by the SLS solution. In the other 2 subjects, the peaks shifted to lower wavenumbers. Bommannan et al. (22) reported that the intercellular lipid structure is more

compact in the deeper portion of the SC. We also got same results as we described in the previous chapter. We interpret our results for these two subjects to indicate that the outermost layer of the SC was desquamated by the SLS treatment so that the deeper layer with its more tightly arranged lipid structure was exposed. The discrepant results obtained in the 10 subjects may have been due to individual differences in the intercellular connective function.

Three days after SLS treatment, the frequencies on each treated area recovered to the original level, and one week after treatment, it recovered completely. This result suggests that because of accelerated SC turnover, the damaged SC surface desquamated quickly. At that time, however, the disruption of barrier function and water content on SC had not recovered. These results demonstrate that in the SLS-induced scaly skin, the changes of skin surface condition such as decline of barrier function and decreased water content on SC one week after treatment is not due to the abnormalities of intercellular lipid morphology. Our findings for the relationship of TEWL value to each IR wavenumber indicate that the order of the lipid alkyl chain is the principal factor in barrier function when the TEWL value is less than  $9 \text{ g/m}^2/\text{hr}$ . However, when TEWL value exceeded this level, as it did in most of the treated subjects, no correlation with wavenumber was observed. At this TEWL value, the other factors might have affected the decline of barrier function. Recently, Fartasch et al. (23) reported that transformation of the lamellar bodies in human

epidermis takes place within a hemispherical saccular area of dilatation in the intercellular space at the stratum granulosum/stratum corneum interface and the lower stratum corneum. In normal skin, the lamellar body-derived lipid sheets appear prior to the lamellar lipid structures. This process can be disturbed by several causes. In atopic patients, although the total volume of lamellar bodies in the upper portion of the stratum granulosum does not differ from that of controls, the percentage of fused lamellar bodies is lower(24). Menon et al. (25) reported that, in barrier disruption induced by the inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme-A reductase in murine epidermis, the deposition of abnormal lamellar body contents resulted in the formation of clefts in the intercellular spaces at the stratum granulosum/stratum corneum interface, resulting in increased permeability through these domains. However, according to this report, membrane bilayer structures in the mid-to-outer stratum corneum of inhibitor-treated specimens appeared normal. These results suggest that the abnormal secretion of lamellar bodies might cause the barrier disruption not because of the abnormal membrane lipid bilayer structures but because of the abnormal clefts which appear in the intercellular region. Potts et al.(26) reported that the low permeability through the SC is not necessarily due to the unique lipid composition but rather to the unique morphology of the SC, as demonstrated by Elias et al.(27). Christophers et al. (28) and Menton et al.(29) reported that the arrangement of the SC cells became disordered with high mitotic

activity. In our previous report(11)(12), we demonstrated that, in experimentally-induced scaly skin, the SC cell area was decreased. This result suggests that the keratinocyte mitosis was accelerated in the scaly skin. We interpret our present results to indicate that there are at least two factors affecting SC barrier function, one being the morphology of the lipid lamellar structure and the other the morphology of the SC itself. In normal skin, the order of the lipid alkyl chain is the crucial factor in the barrier function. In the case of hyper-proliferation, e.g., surfactant-induced scaly skin, the clefts in the intercellular region caused by abnormal secretion of lamellar bodies and/or the disorder of the stacked arrangement of the SC cells might be the principal cause of the barrier disruption.

The importance of sphingolipids in the water retention capacity of the SC has been reported(30)(31). In our present study, however, we demonstrated that, in experimentally-induced scaly skin, the concentration of SC sphingolipids, which are the principal intercellular lipids of the SC, did not differ from that of healthy controls, while that of amino acids was significantly decreased(11). Bouwstra et al. (32) reported that, in the stratum corneum intercellular lipid domain, the incorporation of water in the head group regions of the lipids results in an increase in the mean interfacial area per lipid without changing the repeat distance. Thus, if the water is contained only in the lipid domain, the IR frequency of the lipid alkyl chain would be affected by the water content. In this study, however, we demonstrated that the intercellular lipid structure is not changed in SLS-induced scaly



skin, in spite of the decline of the value of skin surface conductance. These results suggest that the decrease of water content in SLS-induced scaly skin is not caused by abnormalities of intercellular lipid structure but by abnormalities of the production of the amino acids and/or proteins which are cornified cells. Even in normal skin, there was no correlation between the degree of order of the lipid alkyl chain and skin surface conductance. The intercellular lipids might not be directly related to the water retention capacity of the SC. Horii et al. reported (6) that a decreased amount of amino acids may be the results of low profilaggrin biosynthesis in the xerotic skin epidermis. They also indicated that the treatment with the urea-containing cream was effective in improving the defective water-holding ability of the SC. Their report suggests that hydrophilic molecules like free amino acid plays a crucial role for the water retention capacity of the SC.

In conclusion, the degree of the order of the intercellular lipid structure in healthy skin is related to the TEWL. However, neither the decline of barrier function nor the decreased water content in SLS-induced scaly skin is directly due to intercellular lipid structural abnormalities but rather to the abnormal SC morphology and abnormalities of amino acids and/or proteins in cornified cells.

## Reference

1. Tagami H, Yoshikuni K (1985) Interrelationship between water-barrier and reservoir function of pathologic stratum corneum. Arch Dermatol 121 : 642-645
2. Elias PM, Menon GK (1991) Structural and lipid biochemical correlates of the epidermal permeability barrier. In: Elias PM (ed.) Skin lipid (Advances in lipid research, Vol.24) Academic Press, San Diego, California, pp.-26
3. Landmann L (1986) Epidermal permeability barrier. Transform of lamellar granule-disks into cellular sheets by a membrane-fusion process, a freeze-fracture study. J Invest Dermatol 87 : 202-209
4. Lampe MA, Burlingame AL, Whitney J, Williams ML, Brown BE, Rottman E, Elias PM (1983) Human stratum corneum lipids ; characterization and regional variations. J Lipid Res 24 : 120-130
5. Wertz PW, Swartzendruber DC, Madison KC, Downing DT (1987) Composition and morphology of epidermal cyst lipids. J Invest Dermatol 89 : 419-425

6. Horii I, Nakayama Y, Obata M, Tagami H (1989) Stratum corneum hydration and amino acid content in xerotic skin. Br J Dermatol 121 : 587-592
7. Koyama J, Horii I, Kawasaki K, Nakayama Y, Morikawa Y, Mitsui T (1984) Free amino acids of stratum corneum as a biochemical marker to evaluate dry skin. J Soc Cosmet Chem 35 : 183-195
8. Horii I, Kawasaki K, Koyama J, Nakayama Y, Okazaki K, Seiji M (1983) Histidine-rich protein as a possible origin of free amino acids of stratum corneum. J Dermatol 10 : 25-33
9. Folch J, McElroy WD, Glass B (1952) Phosphorus Metabolism 2 John Hopkins, Baltimore p 195
10. Wertz PW, Downing DT (1983) Ceramides of pig epidermis ; Structure determination. J Lipid Res 24: 759-765
11. Denda M, Hori J, Koyama J, Yoshida S, Nanba R, Takahashi M, Horii I, Yamamoto A (1992) Stratum corneum sphingolipid and free amino acid in experimentally induced scaly skin. Arch Dermatol Res 284 : 363-367.
12. Denda M, Koyama J, Takahashi M, Horii I (1994) Change of sphingolipids and free amino acid in surfactant induced scaly skin. J Soc Cosmet Chem Jpn 27 : 589-596

13. Nilsson GE (1977) Measurement of water exchange through skin . Med Biol Eng 15 : 209-218
14. Tagami H, Yoshikuni K (1985) Interrelationship between water-barrier and reservoir function of pathologic stratum corneum. Arch Dermatol 121 : 642-645
15. Watanabe M, Tagami H, Horii I, Takahashi M, Kligmann AM (1991) Functional analyses of the superficial stratum corneum in atopic xerosis. Arch Dermatol 127 : 1689-1692
16. Melnik B, Hollmann J, Hofmann U, Yuh M-S, Plewig G (1990) Lipid composition of outer stratum corneum and nails in atopic and control subjects. Arch Dermatol Res 282 : 549-551
17. Imokawa G, Abe A, Jin K, Higaki Y, Kawashima M, Hidana A (1991) Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiological factor in atopic dry skin? J Invest Dermatol 96 : 523-526
18. Fulmer AW, Kramer GJ (1986) Stratum corneum lipid abnormalities in surfactant induced dry scaly skin. J Invest Dermatol 86 : 598-602

19. Madison KC, Swarztendruber DC, Wertz PW, Downing DT  
(1989) Murine keratinocyte cultures grown at the air/medium interface synthesized stratum corneum lipid and 'recycle' linoleate during differentiation. *J Invest Dermatol* 94 : 10-17
20. Yamamoto A, Serizawa S, Ito M, Sato Y (1991) Stratum corneum lipid abnormalities in atopic dermatitis. *Arch Dermatol Res* 283 : 219-223
21. Welfers H, Melnik RC, Flur M, Bluhm C, Lehmm P, Plewig G  
(1991) Influence of UV irradiation on the composition of human stratum corneum lipids. *J Invest Dermatol* 96 : 959-962
22. Bomannan D, Potts RO, Guy RH (1990) Examination of stratum corneum barrier function in vivo by infrared spectroscopy. *J Invest Dermatol* 95: 403-408
23. Fartasch M, Bassukas ID, Diepgen TL (1993) Structural relationship between epidermal lipid lamellae, lamellar bodies and desmosomes in human epidermis: an ultrastructural study. *British J Dermatol* 128: 1-9
24. Fartasch M, Bassukas ID, Diepgen TL (1992) Disturbed extruding mechanism of lamellar bodies in dry non-eczematous skin of atopics. *Britisch J Dermatol* 127: 221-227

25. Menon GK, Feingold KR, Man MQ, Schande M, Elias PM  
(1992) Structural basis for the barrier abnormality following inhibition of HMG CoA reductase in murine epidermis. *J Invest Dermatol* 98: 209-219
26. Potts RO, Francoeur ML (1991) The influence of stratum corneum morphology on water permeability. *J Invest Dermatol* 96: 495-499
27. Elias PM (1983) Epidermal lipids, Barrier function and desquamation. *J Invest Dermatol* 80: 044s-049s
28. Christophers E, Wolff HH, Laurence EB (1974) The formation of epidermal cell columns. *J Invest Dermatol* 62: 555-559
29. Menton DN, Eisen AZ (1971) Structural organization of the stratum corneum in certain scaling disorders of the skin. *J Invest Dermatol* 57: 295-307
30. Imokawa G, Hattori M (1985) A possible function of structural lipids in the water-holding properties of the stratum corneum. *J Invest Dermatol* 84: 282-284
31. Imokawa G, Akasaki S, Minematsu Y, Kawai M (1989) Importance of intercellular lipids in water-retention properties of the stratum corneum: induction and recovery study of surfactant dry skin. *Arch Dermatol Res* 281: 45-51

32. Bouwstra JA, de Vries MA, Gooris GS, Bras W, Brusse J, Ponc M(1991) Thermodynamic and structural aspects of the skin barrier. J Controlled Release 15: 209-220

## Chapter 3.

### Age dependent change of the skin.

Aging is strongly associated with a loss of homeostasis in our body. In the histologic features of skin with age, there is a flattening of the dermal-epidermal junction, loss of dermal and subcutaneous mass, shortened capillary loops with corresponding reduction in area of the superficial vascular flexus, and reduced numbers of Langerhans cells, melanocytes, and mastcells (1). Functional properties as well as the appearance of the skin surface show change with aging (2,3); aged skin is characterized by decreased water contents in the stratum corneum (3) associated with a decreased value of transepidermal water loss (2). In the previous chapter, I presented the results of our study how the intercellular lipids and free amino acids affect the SC functions. I also reported that the proportions of these sphingolipid species are altered in artificially-induced scaly skin. However, the information about their distribution in normal individuals has been poor and it is not clear whether such altered composition is common to all the types of scaly skin conditions.

The present study was designed to examine the influence of age and sex on epidermal lipogenetic metabolism (4). We also studied the change in skin thickness with age for healthy male and female subjects using ultrasound method (5) and compared the both results to discuss about skin aging.



## Materials and methods

### Measurement of sphingolipid composition in stratum corneum

#### Subjects

Twenty-six healthy male volunteers ranging in from 10 to 77 years in age and 27 healthy female volunteers aged between 10 and 79 years were selected for the study. They were arbitrarily grouped into 4 age groups; i.e. group 1 with prepubertal age bracket consisting of 7 males (mean age, 10 years) and 5 females (mean age, 10 years), group 2 with age bracket between 20 and 39 years consisting of 7 males (mean age, 31 years) and 8 females (mean age, 30 years), group 3 with ages between 40 and 59 consisting of 6 males (mean age, 50 years) and 6 females (mean age, 50 years) and group 4 with ages between 60 and 79 consisting of 7 males (mean age, 70 years) and 7 females (mean age, 70 years). There were no menopausal subjects in our panel who were under oestrogen substitution.

#### Collection of stratum corneum lipids

Stratum corneum lipids were collected from the flexor aspect of the forearm by gently rinsing with 250 ml of 99.5% ethanol(6). The ethanolic extracts were concentrated in a rotary evaporator and then evaporated until dry.

#### Lipid analysis

The lipid sample was dispersed in diethylether using ultrasonic vibration. A silica-gel column (Bond Elut SI, Analytichem International,U.S.A) was used to separate the sphingolipids. The samples were washed successively with 10 ml of diethylether, then loaded onto the column and washed again with 10ml of diethylether to remove the non-polar lipids. Sphingolipids were eluted from the column with chloroform/methanol (2:1) and collected into test tubes. High-performance thin-layer chromatography (HPTLC; Merck 20 x 10cm<sup>2</sup> HPTLC plate, Darmstadt, Germany) was used to separate lipid classes, and chloroform/methanol(19:1) was eluted once up the plate. After development, the HPTLC plate was dried, sprayed with 50% H<sub>2</sub>SO<sub>4</sub>, and heated at 170 °C for 10 minutes to char the lipid. The HPTLC plate was then cooled and scanned on a recording photodensitometer (Shimazu TLC Scanner CS 930, Kyoto, Japan) which provides automatic peak integration and calculation of percentage composition. The ceramide species were identified by the GC-MS method, and acylceramide was identified by the method of Wertz et.al.(7), as we have previously described in previous chapter.

Measurement of facial skin thickness by ultrasound method.

We studied the change in skin thickness of forehead and cheek with age for 63 healthy male volunteers ranging in age from 23 to 82 years and 111 healthy female volunteers aged between 19 and 76 years. Ocuscan 400 (Sonometrics) and Derascan A (Cortex Technology) was used for the measurement of skin thickness.

## Statistical analysis

Student's t-test was employed to evaluate the results.

## Results

The sphingolipid composition of male and female subjects in each age group is shown in Figure 1. There were some changes from prepubertal age to adulthood in male subjects, but no significant difference was observed because of wide variations in obtained values in the prepubertal age group. In contrast in female subjects, there was a significant increase in the proportion of ceramide 1 (acylceramide) and ceramide 2 to total sphingolipids from the prepubertal age (Group 1) to early adult (Group 2) (Fig. 1-A and 1-B). In accordance to these increases, the relative compositions of ceramide 3 and of ceramide 6 showed a significant decrease with the growth from the prepubertal to adult age (Fig. 1-C and 1-E). After reaching maturity, the percentage of ceramide 2 showed a decrease with age in contrast to ceramide 3 whose percentage increased with age only in females.

The variation of skin thickness in both male and female group with aging are shown in figure 2. In the case of male forehead, there is no significant correlation between skin thickness and age (Fig 2-a), however skin thickness decreases significantly in male cheek skin (Fig.2-b) and both parts of female skin (Fig 2- c,d). Both group subjects were arbitrarily divided into seven age groups: group 10s aged younger than 20 years; group 20s aged 20-29

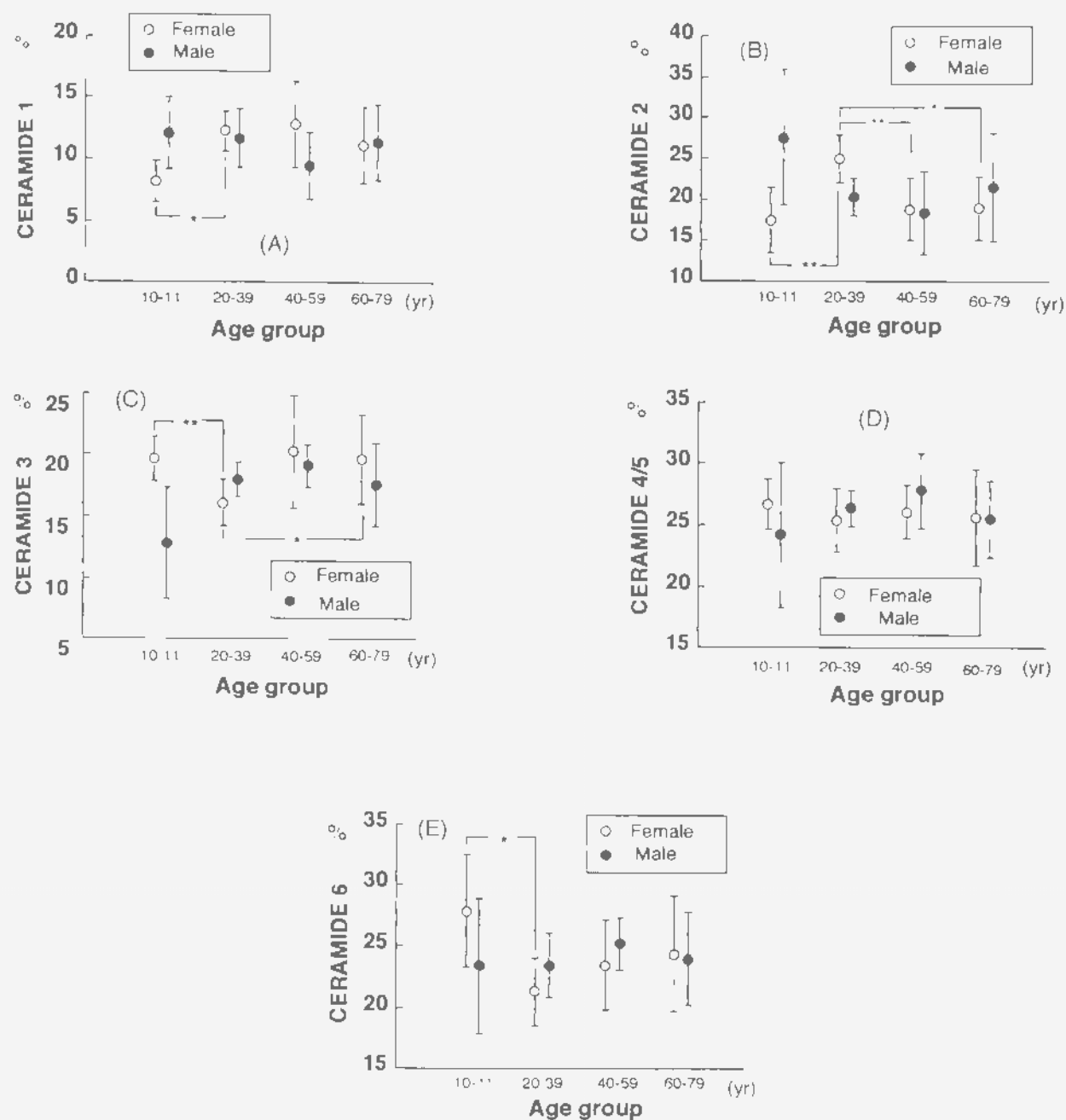


Figure. 1A-E. Sphingolipid composition by age group.  
 ○ , Female; ● , Male; (\*  $p < 0.05$ , \*\*  $p < 0.01$ )

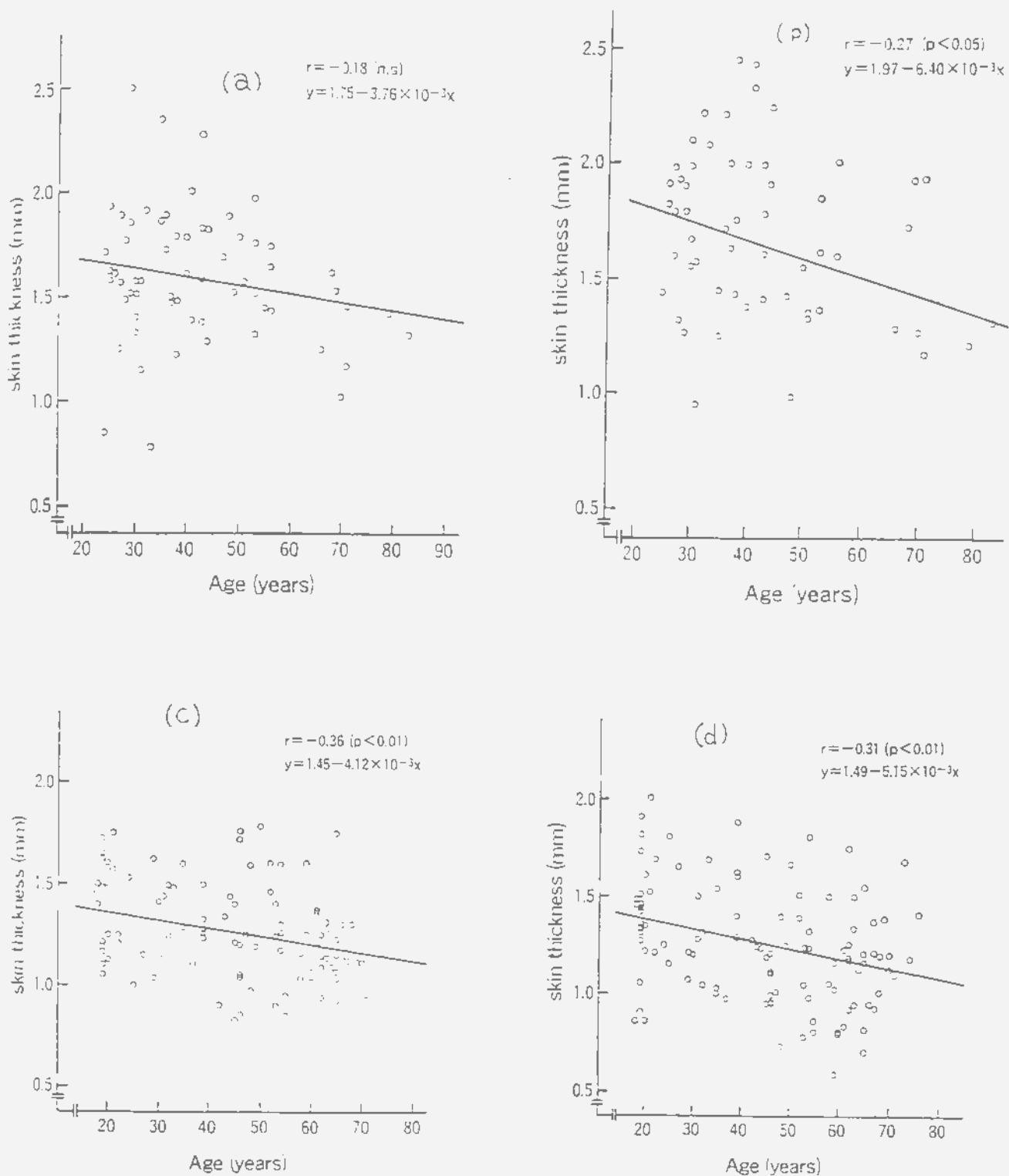


Figure 2. Variation of skin thickness in male forehead (a), male cheek (b), female forehead (c), and female cheek (d).

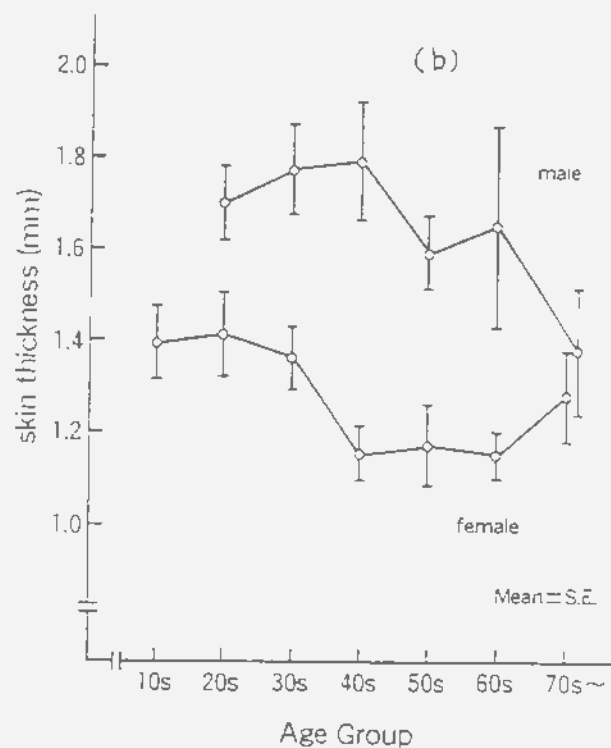
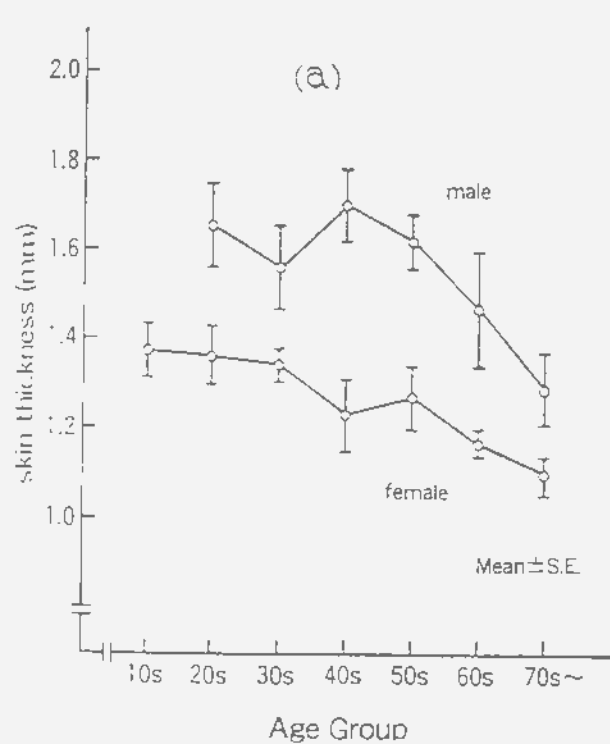


Figure 3. Variation of skin thickness in relation to age ; forehead (a) and cheek (b).

years; group 30s aged 30-39 years; group 40s aged 40-49 years; group 50s aged 50-59 years; group 60s aged 60-69 years; group 70s aged elder than 70 years. The average skin thickness of male and female subjects in each age group is shown in Fig. 3 a,b. Skin thickness was thicker at every age group in males than in females. In male groups, skin thickness decreases from there age of 50s, however in female groups it decreases from their age younger than that of male groups.

## Discussion

We previously found that, although the total amount of sphingolipid in experimentally induced scaly skin did not differ from that of normal skin, the percent composition of the six sphingolipid species was significantly altered (8,9). Although the appearance of the skin surface shows a remarkable change between different age groups, in our preliminary study, in which we evaluated the weight of sphingolipids on 10 young subjects ranging from 16 to 30 years in age (mean age, 24 years) and 10 elderly subjects aged between 68 and 81 years (mean age, 72 years), we found only a slight but not significant difference between these two groups;  $53.4 \pm 18.3 \mu\text{g}/\text{mg SC}$  vs.  $39.7 \pm 19.1 \mu\text{g}/\text{mg SC}$  (10). Therefore in this study, we measured the percent composition of each of the six sphingolipid species on the flexor surface of healthy individuals of various ages. The obtained results showed no significant difference among various age groups in male subjects. However,

there was a biphasic rather than a linear change with age in females. These phases seem to correspond to the developmental stage from the childhood to adult and to the stage of aging.

Age-associated skin changes have been reported in skin thickness (5,11) and activity of sebaceous gland (12). Because both of these changes are also more pronounced in females than in males, the influence of the endocrine system has been suggested for them (13,14,15). Interestingly these changes also take a biphasic pattern corresponding to maturity and aging. For example, the activity of sebaceous gland shows an increase with maturation and a decrease thereafter, especially after menopause (12). Similarly, changes suggesting the influence of sex hormones were observed in skin thickness (13,15). Tan et al. reported (16) the thickness of forearm skin decreases with age. Leveque et al. also presented the change of forearm skin thickness with age. They reported that in female subjects, skin thickness begin to decrease of their younger age than that of male subjects. Our results agree with these previous reports. Shuster suggested the collagen density in dermis determines the skin thickness and androgen relates the collagen density. Brincat also reported oestrogen or testosterone replacement affect the collagen density and skin thickness. Recently, Urano et al. (17) reported that the in vitro induction and proliferation of normal human epidermal keratinocytes were thought to be due, at least in part, to the action of female hormone through its receptor. Our present results suggest that sphingolipid lipogenesis in the epidermal cell might also be affected by the changes in endocrine



activity, although precise mechanisms underlying the biosynthesis of epidermal sphingolipids is still poorly understood. Although there also seem to be changes in male subjects between the prepubertal and adult age groups, the wide variations in obtained values in the former made it impossible to make any definite conclusion. Further studies are required with much larger numbers of subjects.

Our previous studies demonstrated a decrease in the proportion of more polar groups of sphingolipids, ceramides 3,4/5 and 6 in stratum corneum lipids in experimentally-induced scaly skin. However, we could not find any tendency in the proportions of polar species in the present study when a comparison was made between those younger than age 39 years and those older than 39 years, although dry skin surface is more prevalent in the latter (Table). Tape-stripping or surfactant irritation of the skin induces dry scaly skin surface together with enhanced epidermal proliferation. Although aged skin also takes a dry scaly appearance, the turnover rate of its stratum corneum is rather prolonged (18). Thus, it is reasonable to presume that a change in the sphingolipid percent composition might reflect the epidermal proliferative activity. Despite the interesting attempt made by Imokawa et al. (19) in regard to relation between the water content of the stratum corneum and the change of sphingolipid species composition, our so far obtained findings indicating differences in sphingolipid composition of the stratum corneum between the dry skin in the experimentally induced scaly inflammatory skin and that in aged

Table 1. Comparison of sphingolipid composition in the present study subjects with that in scaly skin condition.

| Sphingolipid type                  | CER.1 | CER.2 | CER.3 | CER.4/5 | CER.6 |
|------------------------------------|-------|-------|-------|---------|-------|
| Present study subjects:            |       |       |       |         |       |
| Prepubertal to young adult females | △     | △     | ▼     |         | ▼     |
| Young adult to elderly females     |       | ▼     | △     |         |       |
| Scaly skin*                        |       |       |       |         |       |
| Tape stripping                     | △     | △     | ▼     | ▼       | ▼     |
| Surfactant                         |       | △     |       | ▼       |       |

\*: Data from the reference(7)

▼ : Significant decrease      △ : Significant increase

skin suggest that the relationship of sphingolipids to skin surface dryness may not be so straightforward. The relationship of the lipid composition of the stratum corneum to its structure and function waits further investigation and the results of our present study will definitely lay the groundwork for such future functional studies. Saint leger et al. reported (20) the decrease in sterol esters and tryglycerids and also continuously increase skin surface dryness with aging. They suggested that the activity of the sebaceous gland is associated with aging and the decline of the glycerol produced by the degradation of the triglyceride is related to the skin dryness. Hara et al. reported the morphological change of the SC with aging(10). These factors might also relate the age associated change of the SC functional properties.

## Reference

1. Gilchrest BA (1991) Physiology and Pathophysiology of aging skin. in : Physiology, Biochemistry and Molecular Biology of the skin. 2nd ed. Goldsmith LA ed. Oxford University Press. New York
2. Kligman AT (1979) Perspective and problems in cutaneous gerontology. J Invest Dermatol 73 : 39-46
3. Potts RO, Boras EM, Chrisman DA (1984) Changes with age in the moisture content of human skin. J Invest Dermatol 82: 97-100
4. Denda M, Koyama J, Hori J, Horii I, Takahashi M, Hara M, Tagami H (1993) Age- and sex dependent change in stratum corneum sphingolipids. Arch Dermatol Res 285 : 415-417.
5. Denda M, Takahashi M (1990) Measurement of facial skin thickness by ultrasound method. J Soc Cosmet Chem Jpn 23 :2. 316-319
6. Yamamoto A, Serizawa S, Ito M, Sato Y (1991) Stratum corneum lipid abnormalities in atopic dermatitis. Arch Dermatol Res 283:219-223

7. Wertz PW, Downing DT (1983) Ceramides of pig epidermis ; Structure determination. J Lipid Res 24: 759-765
8. Denda M, Hori J, Koyama J, Yoshida S, Namba R, Takahashi M, Horii I, Yamamoto A Stratum corneum sphingolipid and free amino acid in experimentally induced scaly skin. Arch Dermatol Res 284 : 363-367.
9. Denda M, Koyama J, Takahashi M, Horii I (1993) Changes of sphingolipids and free amino acids in surfactant induced scaly skin. J Soc Cosmet Chem Jpn in press.
10. Hara M, Kikuchi K, Watanabe M, Denda M, Koyama J, Nomura J, Horii I, Tagami H (1993) Senile xerosis. Functional, Morphological and Biochemical studies. J Geriatr Dermatol 1: 111-120
11. Leveque JL, Corcuff P, de Rigal J, Agache P (1984) In vivo studies of evolution of physical properties of the human skin with age. Int J Dermatol 23: 322-329
12. Pochi PE, Strauss JS (1974) Endocrinologic control of the development and activity of the human sebaceous gland. J Invest Dermatol 62: 191-201

13. Brincat M, Moniz CF, Studd JWW, Darby AJ, Magos A, Cooper D (1983) Sex hormones and skin collagen content in postmenopausal women. *Br Med J* 287:1337-1338
14. Cunliffe WJ (1989) Control of the sebaceous gland. In : *Acne* Martin Dunitz Ltd.
15. Shuster S, Black MM, McVitie E (1975) The influence of age and sex on skin thickness, skin collagen and density. *Br J Dermatol* 93: 639 - 643
16. Tan CY, Statham B, Marks R, Payne PA (1982) Skin thickness measurement by pulsed ultrasound: its reproducibility, validation and variability. *Br J Dermatol* 106 : 657-667
17. Urano R, Sakabe K, Kawashima I, Ohkido M, Seiki K (1992) Effects of estrogen and progesterone on induction and proliferation of normal human keratinocytes. *J Dermatological Science* 4: 141 (Abstract)
18. Grove GL, Kligman AM (1983) Age-associated change in human epidermal cell renewal. *J Gerontol* 38: 137-142  
the moisture content of human skin. *J Invest Dermatol* 82:97-100

19. Imokawa G, Akasaki S, Minematsu Y, Kawai M (1989)  
Importance of intercellular lipids in water-retention  
properties of the stratum corneum: induction and recovery  
study of surfactant dry skin. Arch Dermatol Res 281: 45-51
20. Saint Léger D, François AM, Lévêque JL, Stondemayer TJ,  
Grove GL, Kligman AM (1988) Age-associated changes in  
stratum corneum lipids and their relation to dryness.  
Dermatologica 177 : 159-164

## Concluding remarks

In normal skin, there was a significant correlation between transepidermal water loss and IR-frequencies of SC intercellular lipid. There was a stronger correlation when the TEWL value was under  $9 \text{ g/m}^2\cdot\text{hr}$ . The stratum intercellular lipid structure plays a crucial role in the barrier function of healthy normal skin.

However, the degree of disorder of this structure did not change in SLS-induced scaly skin. There was no correlation between the degree of the lipid alkyl chain order and skin surface conductance in both normal and SLS-induced scaly skin. The total amount of sphingolipids which are functionally important in the water permeability were not altered in scaly skin. Only the composition of the five sphingolipids species and the amounts of amino acids changed significantly. Neither the decline of barrier function nor the decreased water content in SLS-induced scaly skin is directly due to intercellular lipid structural abnormalities.

In aged skin, which is also dry and scaly, the changes in sphingolipid species composition differed from that in experimentally induced scaly skin. This result suggests that epidermal biosynthesis of sphingolipids is influenced by epidermal proliferative activity. Both skin thickness and sphingolipid composition take a biphasic pattern corresponding to maturity and ageing. We interpret our results to indicate that sphingolipid lipogenesis in the epidermal cell might also be affected by the changes in endocrine activity.



These findings suggest that the special morphology of the stratum corneum and abnormalities of amino acids and/or proteins in cornified cells are responsible for the dryness of the SLS-induced scaly skin.

Our present study lays the groundwork for future functional studies of the stratum corneum. These will help to clarify the physicochemical and biochemical features of scaly skin in general, and will lead to clinical improvement in stratum corneum disorders.

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