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Change in the electrochemical properties of skin and the lipid packing in stratum corneum by ultrasonic irradiation

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Abstract

Effect of ultrasound on the skin permeation of benzoate anion (BA), a model compound, through excised hairless rat skin was investigated using electrochemical techniques. When the skin surface was sonicated at 150 kHz frequency and 111 mW/cm^2 intensity, skin impedance measured by alternative current with 10 Hz frequency was decreased and skin permeation rate (flux) of BA and deuterium oxide was correspondingly increased. A constant current iontophoresis with 0.1 mA/cm^2 after pretreatment of skin by the ultrasound for 60 min significantly increased the BA flux through the skin compared to that without the pretreatment. In contrast, electric potential difference across the skin during iontophoresis with the ultrasonic pretreatment was one-third lower than that without the pretreatment. Analysis of these results using the Nernst-Planck equation suggests that the ultrasound increased aqueous region in the stratum corneum (s.c.) as well as effective diffusivity of BA in the skin as a result of a structural disorder in the stratum corneum lipids. The ultrasound significantly increased leaching of sterols and ceramides, typical lipids, from the skin when 0.1% Tween 20 was used as a donor solution, and thus disordered the lipid packing of s.c. to lower the skin impedance and to increase the diffusivity in the s.c. We concluded that ultrasound acts on the s.c. lipids and increases the diffusivity of polar molecules in the skin barrier.

Keywords: Percutaneous absorption; Skin penetration-enhancement; Phonophoresis; Ultrasound; Iontophoresis; Skin impedance; Stratum corneum lipids

1. Introduction

Several skin penetration-enhancing methods including the use of chemical enhancers (Walters and Hadgraft, 1993), iontophoresis (Banga and Chien, 1988), and electroporation (Powell et al., 1989) have been investigated to improve low skin permeability of drugs. Ultrasound can also enhance skin permeation (Skauen and Zentner, 1984), and is feasible for use in skin penetration-enhancement (phonophoresis) by controlling irradiating frequency, intensity, and period. Optimum

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conditions for efficient enhancement of skin permeation have been investigated: Bommannan et al. (1992) reported that high frequency ultrasound was beneficial because of the high focusing of the ultrasonic energy into the stratum corneum (s.c.) in spite of its low intensity, and Benson et al. (1988) described that 1.5 or 3 MHz ultrasound was effective for percutaneous absorption of lidocaine and prilocaine. They also suggested that the effect of ultrasound was dependent on the molecular polarity (Benson et al., 1991). The enhancing mechanism of phonophoresis, however, has not been identified.

We previously investigated the effect of ultrasound with 150 kHz frequency and 111 mW/cm^2 intensity on the skin permeability of nine drugs with different polarities through excised hairless rat skin (Ueda et al., 1995). The 150 kHz frequency was selected, because low frequency ultrasound (90–250 kHz) was effective for the in vivo phonophoresis (Skauen and Zentner, 1984) and easily induced acoustic cavitation which caused several biological effects (Suslick, 1988). The ultrasound lowered the barrier function of s.c. and markedly enhanced polar molecules. Drug diffusion through the s.c. lipid region would be a significant step in overall skin permeation, and ultrasound is assumed to act on this region and to lower skin permeation resistance to a drug. It is believed, therefore, that ultrasound changes the electrochemical properties of skin.

The objective in the present study was to confirm the lowering of barrier function of s.c. and to obtain mechanistic information related to the skin penetration-enhancing effect by phono-phoresis. Change in skin impedance was measured by 10 Hz alternative current to estimate the effect of ultrasound on skin permeation resistance to drugs. The in vitro permeation rate (flux) of a model drug, benzoate anion (BA), and electric potential difference across the excised skin during iontophoresis with ultrasonic pretreatment were measured, and changes in the electrochemical properties of the skin by ultrasound were evaluated using the Nernst-Planck equation. The influence of ultrasound on the leaching of lipids (sterols and ceramides) from s.c. to diffusion cell was determined.

2. Materials and methods

2.1. Equipment

A continuous ultrasound generator (Dai-Ichi High-Frequency Co., Ltd., Tokyo, Japan) connected to an ultrasonic transducer with 150 kHz frequency and an effective irradiation area of 3.14 cm^2 was used. Power of the ultrasound from the transducer measured by a radiation force balance method was 111 mW/cm^2 (Rooney, 1973).

2.2. Materials

Sodium benzoate (Japanese Pharmacopeia grade) was purchased from Yamada Pharmaceutical Co., Ltd. (Ibaraki, Japan), and deuterium oxide (D_2O) from Merck Co. (Darmstadt, Germany). Silver potassium cyanide and cholesterol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and ceramide IV from Sigma Chemical Co. (St. Louis, USA). Other chemicals and solvents were of reagent grade and obtained commercially.

2.3. Animals

Male hairless rats (WBN/ILA-Ht strain) weighing 160–180 g (7–8 weeks old), supplied by Life Science Research Center, Josai University (Saitama, Japan) were used in all animal experiments.

2.4. Measurement of skin impedance, and BA and D_2O flux

Excised abdominal hairless rat skin was mounted on a vertical diffusion cell (donor and receiver volume, 5 and 12.5 ml; effective diffusion area, 4.91 cm^2) with a water jacket connected to a water bath at 32°C. BA solution at a concentration of 0.021 M (in 0.9% NaCl in H_2O or D_2O) was added to the donor compartment (s.c. side), and 0.9% NaCl with H_2O solution to the receiver compartment (dermis side). The receiver compartment was stirred with a star-head magnetic bar driven by a constant speed motor (MC-301, Scinics, Tokyo) at 1200 revs. min^{-1}. Platinum

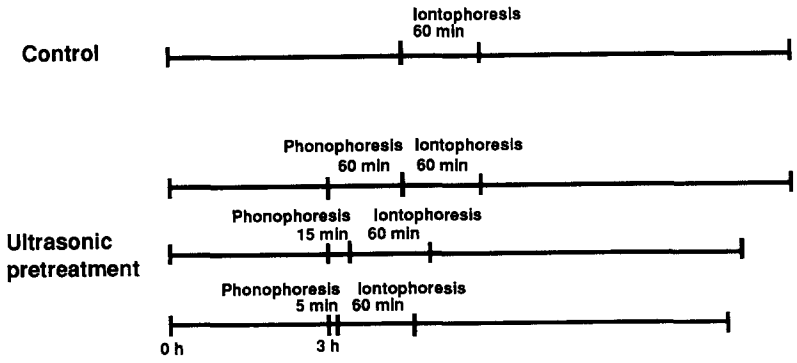

Fig. 1. Time schedules of iontophoretic experiments on the ultrasonic pretreated skin.

wires as electrodes were placed in both compartments and connected to an impedance meter (Advance Co., Ltd., Tokyo). The voltage from the output terminal of the impedance meter was measured to be 2.5 V generating a 10 Hz sine wave by oscilloscope. The skin impedance was corrected by an impedance for the whole cell system without skin. After achieving pseudo steady-state impedance of the excised skin (3 h after beginning of the experiment), ultrasound was applied to the donor compartment for 1 h to compare the skin impedance and the flux of BA and D_2O through the skin before, during, and after ultrasonic irradiation. The skin impedance was measured at predetermined times, and an adequate amount of sample was withdrawn from the receiver compartment to simultaneously measure the concentration of BA and D_2O. The same volume of 0.9% NaCl solution was added after sampling to keep the volume constant.

2.5. Iontophoretic study with ultrasonically pretreated skin

Ag/AgCl was used as electrodes. A cathode was plated with silver in silver potassium cyanide solution, and an anode was electrolyzed in 0.1 N HCl after silver plating.

Iontophoretic study was carried out under the time schedules shown in Fig. 1. Excised abdominal hairless rat skin was mounted on the vertical diffusion cell, as described above, then 0.021 M BA and 0.9% NaCl solutions were added to the donor and receiver compartments, respectively. Cathode and anode were placed in the donor and receiver compartment, respectively, and connected to a DC power source, Phoresor® (PM 600, IOMED Inc., Salt Lake City, UT, USA). The donor compartment was irradiated by ultrasound for 5, 15, or 60 min from 3 h after the beginning of the experiment, and 0.1 mA/cm^2 of direct current was applied to the skin. A sample was withdrawn at predetermined times from the receiver compartment to measure BA concentration. The same volume of 0.9% NaCl solution was added after sampling to keep the volume constant.

Potential difference during the permeation experiment was measured between two salt bridges (3% agar in 3 M KCl solution) connected via each calomel electrode to a digital multimeter (TR 6843, Takeda Riken, Tokyo).

2.6. Measurement of lipids (sterols and ceramides) leaching from stratum corneum

Excised abdominal hairless rat skin was mounted on the vertical diffusion cell, as described above, then 0.1% Tween 20 and water were added to the donor and receiver compartments, respectively. After 3 h, the donor compartment was irradiated by ultrasound for 5, 15, or 60 min. Donor and receiver samples (3 ml) were withdrawn after ultrasonication to measure the amounts of sterols and ceramides leached from s.c. For the control experiments, the donor and receiver solutions were withdrawn after 4 h.

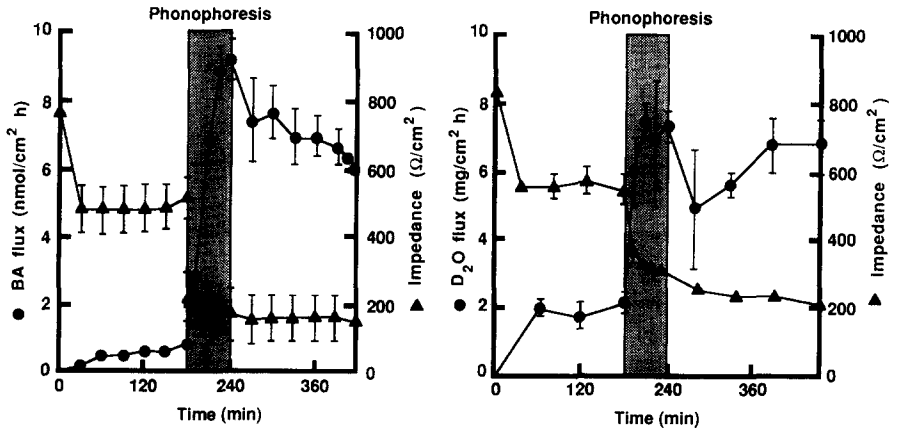

Fig. 2. Time courses of BA and D_2O fluxes through excised hairless rat skin and skin impedance measured at a frequency of 10 Hz. Each data point represents the mean ± S.E. of three experiments.

2.7. Analysis

A high liquid performance chromatography system equipped with a pump (LC-6A, Shimadzu, Kyoto, Japan), a UV spectrophotometric detector (SPD-6A, Shimadzu) and an integrator (C-R 6A, Shimadzu) was used to analyze benzoate anion. Acetonitrile/0.05 M phosphate buffer solution (pH 2.5) (1:1, v/v) was used as a mobile phase at a flow rate of 1.0 ml/min. Benzoate anion was resolved using a 4.6 mm × 250 mm stainless steel column packed with Nucleosil® $5C_{18}$ (Macherey Nagel, Germany) and detected at a wavelength of 230 nm. Five μg/ml of *p*-ethyl benzoate in acetonitrile solution was used as an internal standard.

D_2O was quantified from the intensity of the O-D stretching vibrational band at 2512 cm^{-1} (Hatanaka et al., 1993). The absorbance of the sample in a calcium fluoride cell (0.025 mm thick) was determined with an infrared spectrophotometer (260-30, Hitachi, Tokyo).

Amount of sterols was measured by the method of Sugibayashi et al. (1991) with slight modifications. The donor and receiver solutions (3 ml) collected in a test tube were evaporated, and 6 ml of 0.1% $FeCl_3$ in acetic acid was added to dissolve the lipids. After centrifugation (2000 revs. min^{-1}, 10°C, 5 min), 3 ml of supernatant was mixed with 2 ml of sulfuric acid. After 30 min, the resulting solution was colorimetrically determined at 565 nm by a spectrophotometer (model UV-160A, Shimadzu). The amount of sterols was normalized to that of cholesterol.

Ceramides were measured by the method of Lauter and Trams (1962) with slight modifications. After evaporating the donor and receiver solutions (3 ml), 1 ml of 2 N HCl in methanol were added to the tube and the solutions were refluxed at 80°C for 5 h. By alkalizing with 0.5 ml of 7 N NaOH and distilled water, 5 ml of ethyl acetate was added to the tube. The resulting ethyl acetate phase was washed twice with 2 ml of distilled water, and 2 ml of acetic acid-sodium acetate buffer (pH 3.65) and 0.1 ml of 0.5% methyl orange were added. After mixing and centrifugation, the ethyl acetate phase was withdrawn. The resulting solution was colorimetrically determined at 415 nm by a spectrophotometer, and the amount of ceramides was normalized to that of ceramide IV.

3. Results

3.1. Effects of ultrasound on the skin impedance and BA and D_2O fluxes

Fig. 2 shows the time course of BA or D_2O flux through excised hairless rat skin and skin

impedance measured at 10 Hz frequency. Skin impedance before ultrasonic irradiation was nearly constant at 550–600 Ω/cm^2. The impedance was greatly decreased by ultrasound, and remained lower (about 150–200 Ω/cm^2) than that before the irradiation. BA and D_2O fluxes prior to the irradiation were low, but were increased by ultrasound. The reduction in skin impedance thus corresponded to increases in the BA and D_2O fluxes. These results indicate that decreases in the electrical resistance of skin by ultrasound directly relate to the enhancement of polar molecules.

3.2. Effects of iontophoresis on the ultrasonic pretreated skin

Fig. 3 shows the time course of the cumulative amount of BA permeated through excised hairless rat skin when iontophoresis was applied to ultrasonic pretreated skin. For iontophoresis alone (control), BA flux (slope in Fig. 3) during iontophoresis was greatly increased, but decreased thereafter. For iontophoresis after ultrasonic pretreatment, the permeation behavior of BA was similar to control, although the flux during iontophoresis was dependent on the duration of ultrasonic pretreatment. Replotting the data in Fig. 3 to compare the BA flux during iontophoresis

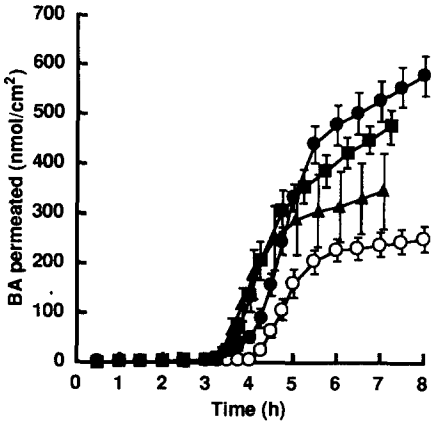

Fig. 3. Effect of iontophoresis on the ultrasonic pretreated skin. The symbols represent iontophoresis with ultrasonic pretreatment for 0 (○), 5 (▲), 15 (■), and 60 min (●). Each data point represents the mean ± S.E. of three experiments.

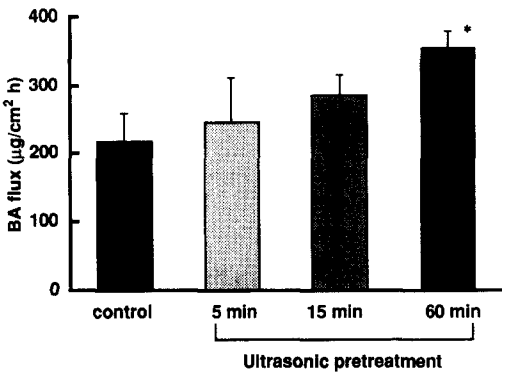

Fig. 4. Comparison of maximum flux of BA during iontophoresis on the ultrasonic pretreated skin. $*P < 0.05$ (compared to control). Each value represents the mean ± S.E. of three experiments.

(Fig. 4), the maximum flux was increased according to the pretreatment duration. On the other hand, potential difference (voltage drop) through the skin during iontophoresis with the ultrasonic pretreatment for 60 min was decreased about 30% against that of control (Fig. 5). Ultrasound increased the BA flux during iontophoresis, although it decreased the potential difference which is the driving force of iontophoresis.

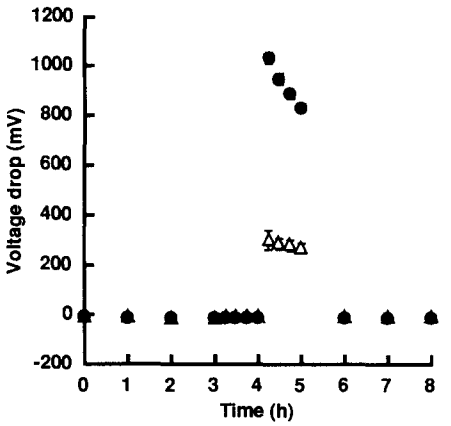

Fig. 5. Effect of ultrasonic pretreatment on the voltage drop during iontophoretic experiments. The symbols represent iontophoresis with ultrasonic pretreatment for 0 (●) and 60 min (△). Each data point represents the mean ± S.E. of three experiments.

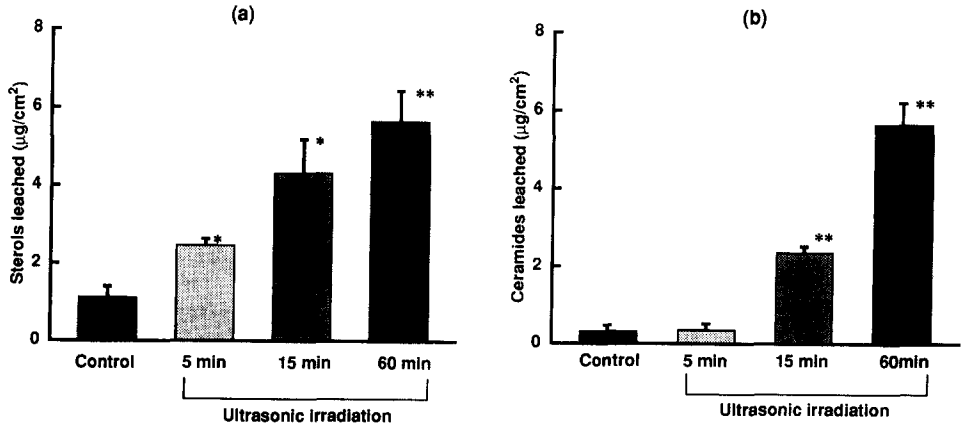

Fig. 6. Effect of ultrasound on the leaching of (a) sterols and (b) ceramides from hairless rat skin. $*P < 0.05$ (compared to control); $**P < 0.01$ (compared to control). Each value represents the mean ± S.E. of three experiments.

3.3. Effect of ultrasound on the lipid packing of s.c.

The leaching of lipids (sterols and ceramides) from s.c. after ultrasonic irradiation was measured to estimate the effect of the ultrasound on the lipid packing of the s.c., the main barrier of skin permeation for polar molecules. Fig. 6a and b show the amount of sterols and ceramides, respectively, leached from the skin into 0.1% Tween 20 solution (donor solution) after ultrasonic irradiation. The amounts leached were significantly increased by ultrasonic irradiation, with the extent greater with longer irradiation. When distilled water was used as a donor solution, leaching of lipids was not detectable due to their low solubility in water (data not shown). In addition, the leaching the lipids into the receiver compartment was not found.

4. Discussion

Our previous study showed that ultrasound increased the skin permeability of polar molecules (Ueda et al., 1995). To obtain mechanistic information on the skin penetration-enhancing effect of phonophoresis, change in electrical properties by ultrasonic irradiation was measured at a frequency of 10 Hz alternative current. The skin impedance after irradiation was clearly lower than that before the irradiation, and BA and D_2O fluxes were correspondingly increased (Fig. 2). Assuming that the skin is constituted by an equivalent circuit which consists of resistive and capacitive components, it was reported that the skin impedance measured at low frequency alternative current primarily reflected the s.c. resistance (Yamamoto and Yamamoto, 1976). In addition, when measured at low frequency, hydrated skin had low impedance (Kohli et al., 1985). Consequently, skin impedance measured at 10 Hz frequency reflects the s.c. resistance against polar molecules more than that against lipophilic ones. Results of the present experiments suggest that ultrasound enhances the extent of hydration in the s.c., and decreases the permeation resistance to polar molecules.

In order to confirm the increase in the extent of hydration in the s.c., effect of ultrasound on the skin permeation of BA was evaluated using the iontophoretic technique. In general, the flux of i species through membrane, J_i ($mol \cdot cm^{-2} \cdot h^{-1}$) is described by the Nernst-Planck equation (Schultz, 1980; Banga and Chien, 1988). Because the driving forces of the species during iontophoresis in this experiment are their concentration and potential gradients across the membrane, the flux during iontophoresis can be expressed by the following equation:

$$J_i = -u_i \cdot [R \cdot T \cdot (dc_i/dx) + z_i \cdot F \cdot c_i \cdot (d\psi/dx)] \quad (1)$$

where $(c_i)_{x = \text{skin surface to donor solution}} = \beta_i \cdot c_d$. In these equations, β_i, u_i, c_i and c_d, and z_i are partition coefficient between the skin and aqueous donor solution, mobility in the membrane ($mol \cdot cm^2 \cdot cal^{-1} \cdot h^{-1}$), concentration in the skin and donor solution ($mol \cdot cm^{-3}$), and charge of i species, respectively. Ψ, x, F, R, and T are potential difference across the membrane (V), thickness of the membrane (cm), Faraday constant (96485 $C \cdot mol^{-1}$), gas constant (1.9872 $cal \cdot mol^{-1} \cdot K^{-1}$), and absolute temperature (K), respectively. Because of a slight potential difference arising from the concentration difference under the passive condition, the driving force during iontophoresis was originated only from the current applied. Ionic flow should be kept constant in the diffusion cell system, because the current applied to the system is constant (Morimoto et al., 1991). Concentration of BA in donor compartment was also almost constant. Therefore, Eq. (1) suggests that ultrasound reduces $d\Psi/dx$ during iontophoresis, but increases β_i or u_i more than the extent of this decrease. β_i it is a correlation factor for partitioning of ionic species to skin. It may be related to a volume or area fraction of aqueous porosity in lipoidal skin barrier, because no ions generally are able to penetrate into the lipid phase of the membrane. β_i may be also related to diffusivity of ionic species in skin barrier, because water content of s.c. affects an effective diffusion coefficient (Blank et al., 1984). On the other hand, the μi is generally expressed by the following Eq. (2) (Bockris and Reddy, 1973):

$$D_i = u_i \cdot R \cdot T \quad (2)$$

where D_i is diffusion coefficient of i species in the membrane. Ultrasound may affect the volume or area fraction of aqueous porosity or β_i as well as effective diffusivity of BA in the membrane, D_i. An increase in the aqueous porosity by ultrasound was confirmed by a hydrodynamic pore theory (Ueda et al., 1995). These findings collectively suggest that ultrasound increase the fraction of aqueous region in the s.c. to increase the effective diffusivity of drugs.

A decrease in permeation resistance for polar compounds by ultrasound may occur due to conformational changes of s.c. lipids, because the lipid region serves as a penetration barrier to drugs, especially polar compounds. In this study, sterols and ceramides leached from the s.c. into donor solution were measured to estimate the effect of ultrasound on the lipid region of s.c. Sterols might be contained both in s.c. and sebum, but ceramides were specific lipids in s.c. (Barry, 1983; Downing, 1992). We assumed that leaching of these lipids mainly reflected the lipid inorganization in the s.c. We reported that the s.c. of hairless rat contained about 240 and 170 $\mu g/cm^2$ of sterols and ceramides, respectively (Hatanaka et al., 1993). When distilled water was applied to the donor compartment in the in vitro permeation study, leaching of these lipids was not detectable because of their insolubility in water. DSC and FTIR studies of sonicated s.c. and skin also did not exhibit significant effects of phonophoresis on the s.c. lipids (Ueda et al., 1994). When 0.1% Tween 20 was added to the donor compartment, however, these lipids were significantly leached by ultrasound compared to control (Fig. 6), which indicates that ultrasonic irradiation significantly influenced the s.c. lipids. The amount of lipids leached from the s.c. after ultrasonic irradiation for 60 min was about 2.5–3.5% of the total amount in the s.c. Mak et al. (1991) reported that, by FTIR measurement, hydration of s.c. did not influence s.c. lipid organization, although it did increase the skin permeability of drugs. They also described that hydration induced lipid phase separation (permeable defects) (Ongpipattanakul et al., 1991) in the s.c. and/or potential action of water on other structural domains of the s.c. such as the intercellular keratin phase. The same phenomena, therefore, may have occurred in the sonicated skin when water was applied as a vehicle.

It was found from the present study that 150 kHz ultrasound decreased the permeation resistance of polar molecules, BA and D_2O, through the s.c. This is probably due to formation of a permeable defect in the s.c. lipids, resulting from the disorder of lipid packing of s.c. It is difficult to focus ultrasonic energy of 150 kHz into several tens of μm thicknesses of s.c., because the wave-

length is relatively long. Ultrasound, however, can have a cavitational effect on and exert shearing stress to the skin and vehicle, even though the frequency is low (Suslick, 1988). These actions affect the relatively soft region in the s.c., i.e. the lipid region, and aid the hydration of the s.c.

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