Analysis of the Rat Skin Permeation of Hydrophilic Compounds Using the Renkin Function

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The Renkin function was applied to characterize the penetration pathways through rat skin following different pretreatments. Nonmetabolic oligosaccharides and sugar alcohols, as model hydrophilic compounds, were applied simultaneously to the excised skin to obtain the equivalent cylindrical pore radius (R) and pore occupancy/length ratio (ε/L) for each skin piece. The R and ε/L values obtained were used to construct the simulation curves of the permeability coefficient (P_a)–molecular weight (MW). In the case of full-stripped skin, the P_a of the model compounds and separately obtained P_a of 5(6)-carboxyfluorescein (CF) showed good agreement with the simulation curve based on the Renkin function, suggesting that the viable epidermis and dermis in the full-stripped skin contained permeation pathways for hydrophilic compounds like aqueous channels. On the other hand, there was poor agreement of P_a with the simulation curve for skin pretreated with an ethanol-menthol mixed enhancer system and the observed P_a of CF in the pretreated skin was twice that calculated. The enhancer system might not be able to create aqueous channels in the lipid layer of the stratum corneum and could increase the permeation of CF in the layer in a different way. The analysis presented here will be useful not only for quantitative evaluation of drug permeation through aqueous channels in treated skins but also for investigation of the mechanism of skin-permeation enhancing techniques.

Key words Renkin function; skin permeation; stripped skin; penetration pathway; oligosaccharide

Although skin is recognized as a good application site for small lipophilic drugs acting topically and systemically, development of transdermal delivery systems for large hydrophilic drugs such as peptide drugs has been considered difficult because of the nature of skin as a barrier. However, some studies of physical penetration-enhancing techniques suggest the possibility of transdermal peptide delivery.[1] Such techniques, *e.g.* iontophoresis,[2] electroporation,[3] sonophoresis[4,5] and application of a microneedle array,[6] may assist penetration and/or create permeation routes in the skin barrier. These permeation routes could involve water and work like channels for hydrophilic drugs. Although chemical penetration-enhancers also enhance the skin permeation of hydrophilic drugs, the mechanism might not involve forming such channels. While electroporation and sonophoresis could create permeation routes in the intercellular lipids of the stratum corneum,[7,8] a microneedle array allows perforation of the whole stratum corneum.[9] The size of channels formed by electroporation or sonophoresis determines the molecular weight (MW)-dependence of the permeation of hydrophilic drugs. Stratum corneum subjected to electroporation or sonophoresis could work as a molecular "sieving" membrane. In the case of microneedle treatments, the MW-dependence of the permeation of hydrophilic drugs could be related to the structure of the viable epidermis and/or dermis. In both cases, characterization of the penetration routes for large hydrophilic drugs is required to develop novel transdermal delivery systems for peptide drugs.[10]

In our previous studies, the Renkin molecular sieving function, which characterizes the porous permeation pathways in terms of the equivalent cylindrical pore radius (R) and pore occupancy/length ratio (ε/L), was used to evaluate the paracellular permeation pathways of the epithelial membranes.[11,12] When cationized polymers were applied as absorption enhancers to the epithelial membranes and the apparent permeability of paracellular markers was fitted to the Renkin function, the ε/L of the membranes increased while the R value did not, suggesting that the number of pathways for hydrophilic molecules in the membranes could be increased by the addition of the enhancers.[13] Such methodology using the Renkin function can be applied to evaluation of the permeation of hydrophilic drugs through skin treated with physical penetration-enhancing techniques.

In the present report, an analysis using the Renkin function was applied to excised rat skins subjected to a variety of pretreatments. Nonmetabolic oligosaccharides and sugar alcohols were used as model hydrophilic compounds. These compounds were applied simultaneously to the excised skin to obtain ε/L and R values for each skin sample. A difference in the ε/L and R values could be related to the effects of the pretreatments on the penetration pathways. In order to validate the analysis, 5(6)-carboxyfluorescein (CF), another model hydrophilic compound, was applied separately to the excised skins.

MATERIALS AND METHODS

Materials D-(+)-Melezitose (ML, MW 504.4), D-mannitol (MN, MW 182.1), D-glucose (GL, MW 180.2) and meso-erythritol (ET, MW 122.1) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Isomaltose (IM, MW 342.3) was obtained from Tokyo Chemical Industry (Tokyo, Japan). CF was purchased from Sigma-Aldrich Japan (Tokyo, Japan). All other chemicals were of reagent grade or HPLC grade, and used as received.

Pretreatments of Excised Rat Skin Animal studies were performed according to the guidelines for animal use approved by the Life Science Research Center, Josai University. Male WBN/ILS-Ht strain hairless rats (240—260 g, the Life Science Research Center, Josai University, Saitama,

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Japan) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the full thickness skin was excised from the abdomen. Three different pretreatments were applied to the excised skin. (1) 5SP: Stratum corneum was treated by tape-stripping five times using CELLOTAPE® (NICHIBAN, Tokyo, Japan), and then the pretreated skin was mounted on a two-chamber diffusion cell (0.98 cm^2, 2.6 ml in each cell). (2) FSP: Tape-stripping (full-stripping) was carried out twenty times to remove the stratum corneum completely from the skin surface, and then the skin was mounted on the diffusion cell. (3) EMT: Ethanol–menthol mixed system (40% ethanol, 5% L-menthol/water) is a typical skin-penetration enhancing system and this was used to pretreat the skin.[14] The full thickness skin was mounted on the diffusion cell, and then the enhancing system and a receptor medium were applied for 1 h to the stratum corneum and dermis side of the cell, respectively. The receptor medium consisted of 1.0 mM GL, 242 mM MN, 10 mM tris(hydroxymethyl)aminomethane and hydrochloric acid (pH=7.4, 280 mOsm).

Metabolism of ET, IM and ML by Skin Tissues In order to ensure nonmetabolic properties of ET, IM and ML during the skin permeation process, their stability in the receptor cell was examined. Full thickness skin was mounted on the diffusion cell, and then ET (0.093 mM), IM (0.093 mM) and ML (0.093 mM) in the receptor medium were applied to the receptor cell. The cell was kept at 37 °C and samples of the receptor medium were withdrawn at predetermined times.

Permeation Experiments on Full Thickness Skin In our preliminary experiments, the intact full thickness hairless rat skin penetration of ET could be determined in our experimental system, but this was not the case for ML and IM. Therefore, 280 mM ET solution was applied to the stratum corneum side of the full thickness skin, while the analysis using the Renkin function was abandoned. The receptor medium was applied to the dermis side of the cell, and the whole cell set was kept at 37 °C. At predetermined times (1, 2, 4, 6, 8, 10, 12 h), 1.0 ml of the receptor solution was withdrawn to determine the ET concentration and replaced with the same volume of medium to maintain a constant volume.

Permeation Experiments on Pretreated Skin The donor solution containing 93 mM ET, 93 mM IM and 93 mM ML was applied to the epidermis side of the treated skin mounted on the diffusion cell and the receptor medium was applied to the dermis side. The whole cell set was kept at 37 °C and 1.0 ml of the receptor solution was withdrawn to determine the concentration of the markers at predetermined times (1, 2, 4, 6, 8, 10, 12 h). The same volume of the medium was added to maintain a constant volume at each sampling time.

Analysis Using the Renkin Function The Renkin function (Eq. 1) has been used for characterization of the paracellular absorption pathways.[15]

$$F\left(\frac{r_i}{R}\right)=\left(1-\left(\frac{r_i}{R}\right)\right)^2\left[1-2.104\left(\frac{r_i}{R}\right)+2.09\left(\frac{r_i}{R}\right)^3-0.95\left(\frac{r_i}{R}\right)^5\right] \quad (1)$$

where r_i is the molecular radius of penetrant i. Although the r_i values can be calculated from the diffusion coefficient (D_i) as the Stokes–Einstein radius (r_{SE}), for small molecules (MW<272.5), the hydrodynamic radius (r_{HYD}) can be calculated from the following equation (Eq. 2) and used for the analysis.[16]

$$r_{HYD}=\left(0.92+\frac{21.8}{MW}\right)\cdot r_{SE} \quad (2)$$

The apparent permeability coefficient (P_a) of ET and IM ($P_{a,ET}$ and $P_{a,IM}$) was used to obtain R and ε/L as the characteristic parameters for the absorption pathway using the following equations (Eq. 3) and the diffusion parameters in Table 1.

$$P_{a,ET\text{ or }IM}=(\varepsilon/L)\cdot D_{ET\text{ or }IM}\cdot F\left(\frac{r_{ET\text{ or }IM}}{R}\right) \quad (3)$$

The P_a value of ML ($P_{a,ML}$) observed was compared with that calculated from the R and ε/L values of each membrane and the D value of ML. The simulation curves of P_a–MW were constructed using the obtained parameters (R and ε/L) and the following relationship between MW and D_i.[17]

$$\log D_i=-0.4145\cdot\log MW-4.111 \quad (4)$$

Concentration Determination of ET, IM and ML in Samples A dual-pump HPLC system (LC10, Shimadzu, Kyoto, Japan) with a Charged Aerosol Detector (CAD, ESA Biosciences, MA, U.S.A.) was used. A Shodex SC1011 column (SHOWA DENKO, Tokyo, Japan) was used for the separation at 80 °C. A pump provided water at 0.5 ml/min as the mobile phase passing through the injector and column. Another pump was used to provide acetonitrile at 0.5 ml/min to increase the sensitivity of the CAD. The water from the column and the acetonitrile were introduced into a mixer and the outlet of the mixer was connected to the CAD. Solution samples (20 μl) were injected.

CF Permeation Experiments through Pretreated Skin The permeation of CF through FSP and EMT skin was examined separately to validate the analysis using the Renkin function. CF solution (0.50 mg/ml) in phosphate buffered saline (PBS, pH 7.4) was applied to the epidermis side of the intact or pretreated skin mounted on the diffusion cell. CF-free PBS was used as the receptor medium. The procedure for the permeation experiments was similar to that described above. The concentration of CF was determined using a fluorescence spectrophotometer (Ex. 490 nm, Em. 520 nm, RF-5300, Shimadzu).

RESULTS AND DISCUSSION

As a preliminary experiment, the stability of the marker compounds was examined to investigate the nonmetabolic properties. A solution containing ET, IM and ML (0.093 mM each) was applied to the dermis side of the excised rat skin in

Table 1. Diffusion Parameters of Marker Compounds Used

	MW	D_i (cm^2/s) at 37 °C $\times 10^{-6}$	r_i (nm)
Erythritol (ET)	122.1	11.4	0.320[a]
Isomaltose (IM)	342.3	7.11	0.459[b]
Meletytose (ML)	504.4	6.51	0.501[b]

a) Calculated hydrodynamic radius using Eq. 2 from the Stokes–Einstein radius. *b*) Stokes–Einstein radius.

the diffusion cell for 8 h. Each marker compound could diffuse into the dermis and viable epidermis during this period. The concentrations relative to the initial (%) of ET, IM and ML at 8 h were 100.0±2.1, 100.8±0.7 and 100.8±0.7, respectively, suggesting that the marker compounds were stable during permeation through the excised skin samples.

Since the permeation of ML and IM through the intact full thickness hairless rat skin could not be detected in our experimental system in preliminary experiments, ET solution was applied to the stratum corneum side of the full thickness skin and then the steady-state permeation rate of ET was determined. The permeation rate was divided by the initial ET concentration in the donor phase to obtain P_a. The P_a of ET through the full thickness skin was very low (4.71×10^{-8} cm/s), suggesting that the stratum corneum presented a barrier for hydrophilic compounds in the intact skin (Table 2).

When ET, IM and ML were applied simultaneously to the treated skin samples, they could be detected in the receptor cell, suggesting that the pretreatments enhanced the permeation of hydrophilic compounds through skin (Table 2). Since their P_a values through FSP skin were higher than those through 5SP skin, a certain amount of stratum corneum remained in 5SP skin as a permeation barrier. Although their P_a values were also enhanced by EMT, the MW-dependence of the enhancement was different from tape stripping. The ET permeation through EMT skin was the highest, while the permeation rates of IM and ML were lower than those through FSP skin.

$P_{a,ET}$ and $P_{a,IM}$ were used to obtain R and ε/L as the characteristic parameters for the absorption pathway of treated skins based on the Renkin function. The obtained parameters are shown in Table 3. These parameters were not directly related to the real pores and space in the skin because of the complex skin structure. Therefore, the obtained parameters should be discussed in relative ways. The obtained R and ε/L for FSP skin were 1.50±0.05 nm and 0.60±0.08 cm^{-1}, respectively. Assuming that the viable epidermis with a thickness of 0.1 mm was a rate-limiting barrier for their entire permeation processes, the porosity ε was calculated from the ε/L value 0.60 cm^{-1} as 6.0×10^{-3}. The R and ε/L values for 5SP skin were lower than those for FSP. On the other hand, the R value for EMT was lower than that for FSP, while the ε/L value was higher. This result could be due to a difference in the penetration-enhancing mechanism. While tape-stripping removes the stratum corneum and changes the rate-limiting barrier from the stratum corneum to the viable epidermis and dermis, chemical enhancer treatments such as EMT affect the properties of the stratum corneum. The enhancing mechanism of ethanol–menthol mixed systems has been reported to be an increased perturbation of intercellular lipid in the stratum corneum.[18,19] Small ET might be able to diffuse easy in the modified lipid layer, but not IM and ML. Although these three compounds are very hydrophilic (log $K_{octanol/pH\ 7.4}<-3.8$), a large hydrophilic surface area of IM and ML related with a lot of hydroxyl groups may reduce their distribution to the modified lipid layer. The relatively high ε/L value for EMT might mean that a larger area could be used for ET permeation in EMT skin. Since the $P_{a,ET}$ of EMT was higher than that of FSP, EMT could act not only on the stratum corneum but also on the viable epidermis.

The simulation curves of P_a–MW based on the Renkin function (Eq. 1) using the parameters in Table 3 are shown in Fig. 1. Relationships between MW–D_i (Eq. 4) and between D_i-r_i (the Stokes–Einstein equation and Eq. 2 for MW<272.5) can also be used to construct the simulation curves.[20] Such simulation curves will be useful for prediction of the permeability of hydrophilic compounds through membranes with aqueous channels. In the addition, comparison between observed and predicted permeability will give some information about the permeation mechanism of the compounds. The observed P_a of ET and IM, which were used to obtain the parameters in Table 3, and the observed P_a of ML for the validation are also shown in Fig. 1. The P_a ratio (observed/calculated) of ML (MW=504.4) was 1.38, 1.84 and 2.30 for FSP, 5SP and EMT skin, respectively, suggest-

Table 2. Permeability Coefficient of the Marker Compounds through Pretreated Hairless Rat Skin

	P_a (cm/s) $\times10^{-7}$		
	ET	IM	ML
Without pretreatment	0.471±0.229	—	—
5SP	11.4±3.4	3.02±1.16	2.30±0.84
FSP	24.3±4.1	8.56±1.65	6.10±1.48
EMT	35.7±5.9	5.80±1.13	3.79±1.01

Each value is the mean±S.D. (n=3).

Table 3. Characteristic Parameters for the Absorption Pathway in Pretreated Skin

	R (nm)	ε/L (cm^{-1})	$\varepsilon^{a)}\times10^{-3}$
5SP	1.14±0.12	0.44±0.14	4.4
FSP	1.50±0.05	0.60±0.08	6.0
EMT	0.85±0.03	2.62±0.44	26.2

a) Assuming a viable epidermis with a thickness of 0.1 mm. Each value is the mean±S.D. (n=3).

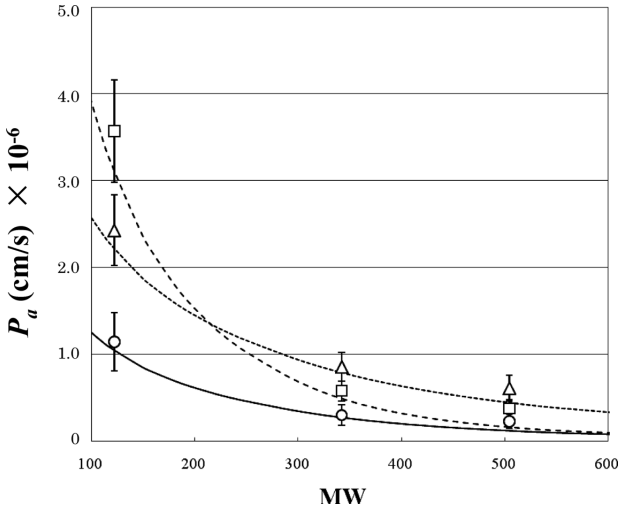

Fig. 1. Relationship between MW and P_a

The lines are simulation curves based on Eq. 1 and Eq. 4. Parameters in Table 3 and the hydrodynamic radius calculated from diffusivity based on Eq. 4 were used for the calculation. The Stokes–Einstein equation was used for the radius calculation, while correction was made for small molecular weight compounds (MW<272.5) using Eq. 2. The circles and solid line (○, ——) are for 5SP; the triangles and dotted line (△, ------) are for FSP; the squares and broken line (□, - - - - -) are for EMT. The plotted data points are shown with their S.D. values (n=3).

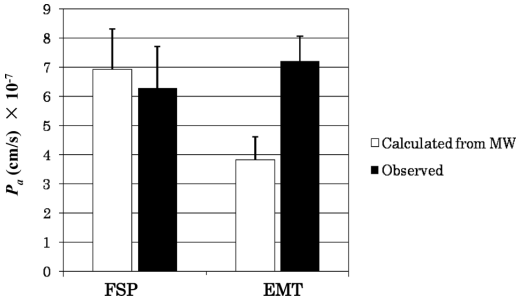

Fig. 2. Predicted and Observed Permeability Coefficient of CF

The parameters in Table 3 and Eq. 4 were used for the calculation. Each value is the mean±S.D.: calculated values, $n=3$; observed value for FSP, $n=5$; observed value for EMT, $n=4$.

ing that a better fit to the simulation curve was obtained in FSP skin. The extracellular space in the viable epidermis and dermis could work as an aqueous channel, resulting in a good fit to the Renkin function in the FSP skin. On the other hand, since EMT never produces pores in the stratum corneum,[21] the conformity to the Renkin function could be low in EMT skin. The consistency of the MW-dependence of P_a with the Renkin function might be associated with the effects of different skin treatments. If a type of skin treatment creates aqueous channels in the skin barrier, a better fit to the Renkin function could be obtained, and the simulation curve will be able to predict the P_a of hydrophilic drugs with different MW through the treated skin. Possible skin treatments having such effects are physical penetration-enhancing techniques, *e.g.* electroporation, sonophoresis and application of a microneedle array.

Figure 1 also suggests low permeability of peptides drugs having a higher MW (1000<) across the viable epidermis. Since tight junctions could be formed in epidermis, the structures might limit permeation of higher MW molecules.[22] The effects of the physical penetration-enhancing techniques on the structures will be investigated by us next for the development of transdermal peptide delivery systems.

Permeation experiments of CF through FSP and EMT skin were carried out separately to confirm the above possibilities. CF is a hydrophilic compound (MW=376.3, $\log K_{\text{octanol/pH 7.4}}=-3.3$) and has been used as a marker compound for paracellular permeation pathways. The P_a of CF through intact rat skin was $2.4\times10^{-9}\pm0.9\times10^{-9}$ (cm/s), suggesting that little CF can cross the lipophilic barrier of the stratum corneum. The P_a of CF through the treated skins was calculated from the MW using Eqs. 1 and 5, and the parameters in Table 3. In the case of FSP skin, a good agreement between the calculated and observed P_a of CF was obtained, suggesting that the aqueous channels in the viable epidermis and dermis were common permeation pathways for those compounds (Fig. 2). On the other hand, the observed P_a of CF in EMT skin was twice that calculated. CF might distribute and diffuse in the intercellular lipids of EMT skin, but not in intact skin. The behavior of CF in such a lipid layer could be different from that of IM and ML, although they are all good aqueous channel markers in intact skin. Differences in the $\log K_{\text{octanol/pH 7.4}}$ values, hydrophilic surface area of molecules or the presence of a charge might affect the permeability of the compounds through EMT skin. These results suggest that our analysis is useful not only for quantitative evaluation of drug permeation through aqueous channels in treated skins but also for consideration of the mechanism of skin-permeation enhancing techniques. As the next step, we will apply this methodology to an examination of the physical penetration-enhancing techniques in transdermal drug delivery systems.

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