Transport of β-Estradiol in Freshly Excised Human Skin *in Vitro*: Diffusion and Metabolism in Each Skin Layer

Puchun Liu,[1,2] William I. Higuchi,[1,4] Abdel-Halim Ghanem,[1] and William R. Good[3]

Received November 8, 1993; accepted July 15, 1994

This paper describes an experimental and theoretical evaluation of β-estradiol (E2) transport in post-surgery fresh human skin *in vitro*. Necessary auxiliary experimental methods were newly developed for these studies. The experimental fluxes of E2 and the metabolite, estrone (E1), using the dermis, stripped skin, and split-thickness skin were consistent with a model considering the human skin as a three-layer (stratum corneum, viable epidermis, and dermis) membrane with the enzyme activity mainly residing in the basal layer of the viable epidermis. The diffusion and metabolism parameters for each skin layer were determined in the overall transdermal transport of E2. Compared to fresh hairless mouse skin, fresh human skin appears more resistant to the stratum corneum diffusion of E2 and is much less capable of metabolizing E2 to E1. These *in vitro* results have been extrapolated to the possible *in vivo* human skin situation with blood vessels directly beneath the viable epidermis providing "sink" conditions a short distance from the dermo-epidermal junction. The model analysis has demonstrated that there would be less metabolism and that a much smaller amount of the transdermal metabolite (E1) would be taken up by the blood capillary due to the shorter dermis path length for permeants *in vivo* than in the *in vitro* case using dermatomed split-thickness skin.

KEY WORDS: freshly excised human skin; β-estradiol; diffusion and metabolism in skin; methodology; biophysical models; dermis thickness effect.

INTRODUCTION

Recently, simultaneous diffusion and metabolism of β-estradiol (E2) in fresh hairless mouse skin was systematically investigated (1). The procedures combined experiments and biophysical models in a unique way, providing the means for factoring out and quantifying the diffusion and metabolism of E2 in the overall skin transport process. E2 can be bioconverted into estrone (E1) in skin by 17β-hydroxysteroid dehydrogenase, an enzyme which is located in the microsomal fraction of skin tissue and has a preference for NADP as cofactor (2–4). A "best" model has been deduced in which the mouse skin is considered to be a three-layer membrane (stratum corneum, viable epidermis, and dermis) with the enzyme activity totally residing in the basal cell layer of viable epidermis (1).

The transport studies with human skin are expected to be most informative using fresh viable human skin (5,6). Because of limitations in the availability of fresh human skin, however, most *in vitro* studies are currently performed using human cadaver epidermal membrane consisting of excessively hydrated stratum corneum and epidermis lacking *in vivo* enzymatic activity (7). Skin metabolism studies with human cadaver skin preparations are therefore of questionable value. There is also a need to know whether permeant diffusion in the viable epidermis is dependent upon the viability of the tissue.

The objective of this research was to assess the diffusion and metabolism of E2 in each layer (stratum corneum, viable epidermis, and dermis) of fresh human skin obtained from a surgical procedure. Necessary experimental methods have been developed for the *in vitro* study of fresh human skin. The *in vitro* results have then been extrapolated to predict outcomes for the *in vivo* human skin situation with blood flow beneath the viable epidermis.

MATERIALS AND METHODS

Materials

E2 and E1 (SIGMA Chemical Co., St Louis, MO), normal saline (McGaw, Irvine, CA), analytical grade acetonitrile (Baker Chemical Co., Phillipsburg, NJ), and liquid scintillation fluid, Ready-Solv CP (Beckman Institute, San Ramon, CA) were used in this study. [6,7-^{3}H]-E2 (60.0 Ci/mmol) and [6,7-^{3}H]-E1 (51.8 Ci/mmol) were obtained in their ethanol solutions (New England Nuclear, Boston, MA). The ethanol was evaporated with the aid of a nitrogen stream before ^{3}H-E2 or ^{3}H-E1 was used in the diffusion/metabolism experiments.

Human skin was freshly removed from two plastic surgery skin donors (40- and 47-year-old females). For a comparison study, the skin of hairless mouse (male, strain SKH-HR1, Temple University, Pittsburgh, PA) at age of 13 weeks old was also obtained (1).

Human Skin Preparations

The following sequence of steps, from surgical excision to storage, was involved in the preparation of the human skin: (a) the abdominal skin was scrubbed with betadine solution (10% povidone-iodine, The Purdue Frederick Co., Norwalk, CT) to disinfect the skin before surgery; (b) after surgical excision the skin was stored at 4°C in phosphate buffered saline (pH 7.4) containing penicillin to prevent degradation; (c) the split-thickness component (stratum corneum + viable epidermis + part of dermis) was obtained by dermatoming and the dermis component was obtained by a second slicing of the dermis after removal of the split-thickness portion; and (d) the membranes were then stored in a tissue culture media (Dulbecca's modified Eagles me-

[1] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah 84112.
[2] Present address: Pharmaceuticals Division, Ciba-Geigy Corporation, Ardsley, New York 10502.
[3] TheraTech, Inc., Salt Lake City, Utah 84108.
[4] To whom correspondence should be addressed at Department of Pharmaceutics and Pharmaceutical Chemistry, Skaggs Hall 301, University of Utah, Salt Lake City, Utah 84112.

0724-8741/94/1200-1777$07.00/0

dium with 5% fetal calf serum, Flow Laboratories, McLean, VA) at 4°C and used within 4 to 24 hr postsurgery in this study.

To prepare stripped skin (viable epidermis + part of dermis), the stratum corneum of the split-thickness skin was removed by tape-stripping 35 times (fresh tape for each stripping) using a 2-inch cellophane package sealing tape (3M Co., St. Paul, MN). The efficiency of the tape-stripping was checked by trypsin-digestion (1% trypsin solution for 12 hr at room temperature) of the stripped skin (8). Also, in some instances, the dermis was separated from the split-thickness skin or stripped skin by exposure to 60°C water for 2 min (8,9). The dermis thicknesses of the split-thickness skin, stripped skin, and dermis were measured with a micrometer, by sandwiching the hydrated skin membrane between two glass slides.

Skin Diffusion/Metabolism Experiments

Two-chamber horizontal diffusion cells were used for diffusion/metabolism experiments with dermis, stripped skin, and split-thickness skin. The diffusion cell was made up of two halves, each chamber having a volume of 2 ml and diffusional area of about 0.7 cm^2. In each chamber, a stainless steel stirrer with a small stainless steel propeller was driven by a 150 r.p.m. constant speed motor. A skin membrane was mounted between the two chambers of the diffusion cell with the dermis-side facing the receiver chamber, clamped and excess skin trimmed. The assembled diffusion cells were then immersed in a water bath at 37°C.

A tracer level of ^{3}H-E2 was added into the donor chamber and aliquots were withdrawn at predetermined time intervals (15, 15, and 40 min for the dermis, stripped skin, and split-thickness skin experiments, respectively). In most of the experiments, 100-μl aliquots were taken from the receiver chamber and 5-μl aliquots from the donor chamber. The same volume of saline solution was added back to the receiver chamber; solution was not added back to the donor chamber. In the experiments with dermis and stripped skin, the donor solution was replaced at 1 hr intervals to keep the donor E2 concentration constant. The duration of the experiment was 2, 2, or 4 hours for the dermis, stripped skin, or split-thickness skin, respectively. A limited number of ^{3}H-E1 transport experiments with the dermis and stripped skin was also conducted.

Assay Methods and Data Analysis

Analysis of ^{3}H-E2 or ^{3}H-E1 involved interfacing the HPLC (high-performance liquid chromatograph) with a fraction collector (1). E2 and E1 were separated by reverse-phase HPLC at wavelength 280 nm with a C18 column and acetonitrile-water (40:60) as the mobile phase. The radioactive samples were mixed with the non-radioactive methanol solution of E2 and E1 to give the HPLC chromatograms. The fraction collector was programmed to give fraction sizes based on the peak signals with a built-in peak separator. Each fraction was analyzed by liquid scintillation counting.

For the experiment in which E2 is added into the donor chamber at zero time, the steady-state fluxes were calculated using the following equations:

$$J_{2,f} = \frac{\left(\frac{dA_{2,R}}{dt}\right)}{S} \quad (1)$$

$$J_{1,f} = \frac{\left(\frac{dA_{1,R}}{dt}\right)}{S} \quad (2)$$

$$J_{1,b} = \frac{\left(\frac{dA_{1,D}}{dt}\right)}{S} \quad (3)$$

Here $J_{2,f}$ and $J_{1,f}$ are the forward fluxes of E2 and E1, and $J_{1,b}$ is the back flux of E1, S is the effective diffusion area, $A_{2,R}$ is the amount of E2 transported into the receiver chamber, $A_{1,R}$ and $A_{1,D}$ are the amounts of E1 transported into the receiver and the donor chambers, respectively, and t is time. Normalized fluxes are defined as the fluxes given by Eqs. 1–3 divided by the donor permeant (e.g., E2) concentration. The flux values were also corrected for possibly significant amount of permeant retained in the epidermis/dermis (1). In the case of no metabolism, the normalized flux obtained from Eq. 1 is simply the permeability coefficient of E2.

RESULTS AND DISCUSSION

Methodology Development for *in Vitro* Study of Fresh Human Skin

In designing this study, normal saline was selected as the aqueous medium because some chemical reactions took place between the stainless steel stirrer and phosphate ions when phosphate-buffer saline (PBS) was employed, and identical results were obtained using either PBS or normal saline in hairless mouse skin metabolism studies. Also, PBS has been reported (5) to have the same ability as Hepes-buffered Hank's balanced salt solution (HHBSS) to maintain E2 metabolism in fuzzy rat skin during 0–6 hr period (17.2 ± 2.0 vs 20.8 ± 1.3 metabolite formation as % of sample radioactivity). Thus, we believe that normal saline is a satisfactory solvent for these short duration (0–4 hr) experiments.

In the present study, necessary auxiliary experimental methods were developed for the diffusion/metabolism studies with the freshly excised human skin *in vitro*. All of the procedures are summarized in Fig. 1 and highlights are discussed as follows.

A matter of significant importance was that of assessing the skin metabolism of E2 as a function of time after surgery. The earliest time after surgery for conducting a transport/metabolism experiment was approximately 4 hr. For comparison purposes, similar experiments with hairless mouse skin were also carried out, including some immediately after surgery (t = 0). Diffusion/metabolism experiments were conducted using a split-thickness human skin with dermis thickness of around 200 μm and with a sample of full-thickness hairless mouse skin. The enzyme stability during the experimental period (<4 hr) was consistent with the fact

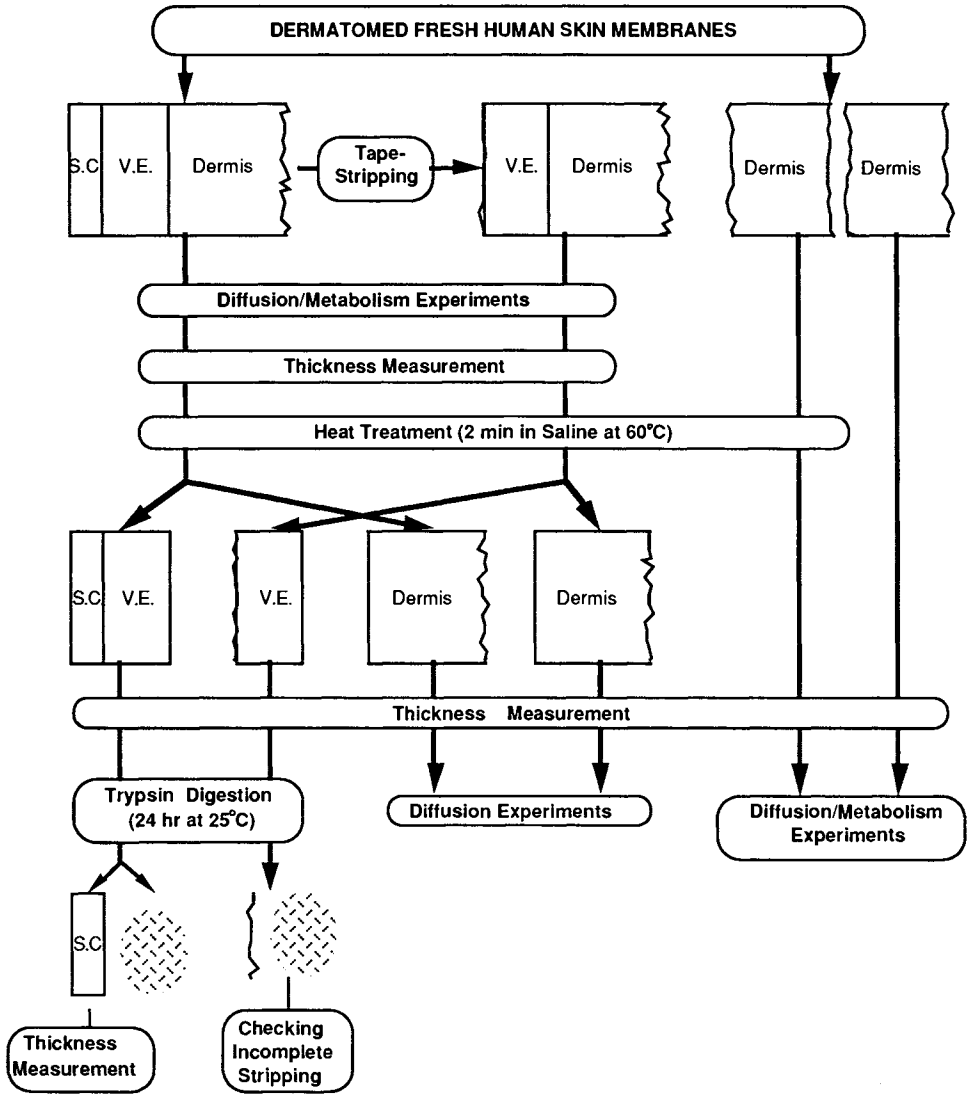

Fig. 1. Flow chart for methodology development and experimental procedures for *in vitro* diffusion/metabolism studies with the fresh human skin.

that the concentration of E2 and E1 increased linearly with time after steady state was attained. Figure 2 summarizes the normalized fluxes for both the human skin and the hairless mouse skin studies. An effect of post-surgery time on transdermal metabolism of E2 was observed. Both the human skin and the mouse skin showed a slow decrease of E1 flux under the storage conditions. For the 4-hr stored skin samples, the fluxes of the metabolite (E1) were about 10% of the total E1 plus E2 fluxes for human skin and about 70% for hairless mouse skin.

With regard to another matter, the dermis thickness and the apparent permeability coefficient (normalized flux) of the dermis were determined in each experiment because dermatoming was highly variable. Also, because the dermis for the permeability experiment was obtained by heat-treatment removal after each diffusion/metabolism experiment, it was necessary to show that the heat-treatment did not alter the dermis permeability coefficient. Table 1 presents the dermis thickness (h_d), the apparent dermis permeability coefficient (P_d), and the product ($P_d h_d$) for several preparation conditions: freshly dermatomed, fresh heat-treated, and heated-treated after storage. The heat-treatment and the storage time did not significantly alter the permeant diffusion across the dermis with a very small statistical coefficient of variation for the ($P_d h_d$)-values, $(2.6 \pm 0.5) \times 10^{-6}$ cm^2/s.

Finally, as the wet surface of the split-thickness skin occasionally compromised the effectiveness of the tape-stripping method for preparing stripped skin, a novel procedure was developed to demonstrate incomplete stripping of the stratum corneum when this occurred. Incomplete stripping was checked by (a) determining whether there was a residue of stratum corneum after the trypsin digestion of the stripped skin and, if there was, (b) determining whether the skin fluxes were low in that instance. This procedure worked well for checking the tape-stripping efficiency. As can be seen in Table 2, when the efficiency of the tape-stripping was not good (i.e., when there was a residue after the trypsin digestion), the apparent permeability coefficient (total forward normalized flux) was low ($\leq 3 \times 10^{-5}$ cm/s); when the tape-stripping was effective, the apparent permeability coef-

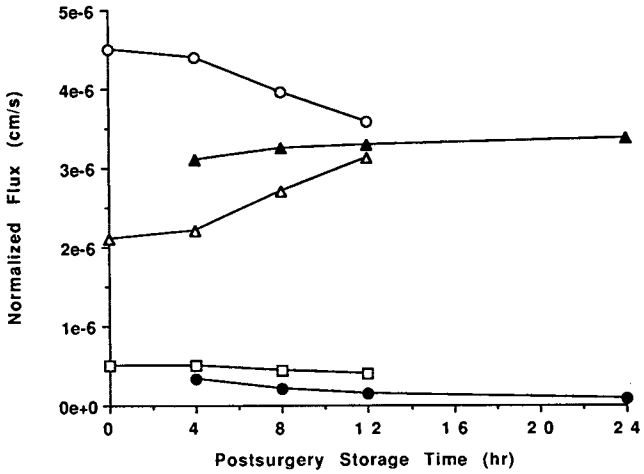

Fig. 2. Effect of the storage time on the normalized forward flux of E2 (▲, △) and on the normalized forward (●, ○) and back fluxes (■, □) of its transdermal metabolite, E1. Filled symbols are for the split-thickness human skin (the 40-year old skin donor with dermis thickness of 150 μm). Open symbols are for the full-thickness hairless mouse skin. The normalized back flux of E1 is below the detection limit ($<1 \times 10^{-8}$ cm/s) for split-thickness human skin.

ficients were always in the range of $7 - 11 \times 10^{-5}$ cm/s. The incomplete stripping data were not used in further analysis.

Diffusion/Metabolism of E2 in Each Skin Layer

The fresh (4-hr post-surgery) human skin preparations were used in E2 diffusion/metabolism experiments. Table 3 summarizes the normalized fluxes determined with dermis, stripped skin, and split-thickness skin. No significant metabolism of E2 was found in the dermis experiment, indicating that dermis is not a site for the enzymatic reaction. Results from the stripped skin experiments (h_d = 190–350 μm) showed 4–6% of both E1 forward and back fluxes. About 10% of forward flux of E1 is determined with the split-thickness skin (h_d = 150–350 μm). Both the stripped skin and the split-thickness skin produce the same percentage of E1 (about 10% in the total E1 flux) metabolized from E2. It is therefore concluded that the viable epidermis is the main site for the enzyme reaction. From a limited number of experiments using E1 as the substrate/permeant, no significant metabolism of E1 was determined with both dermis and stripped skin.

Also shown in Table 3, the approximately same values of forward and back fluxes of E1 (metabolite) from the stripped skin experiments indicate that the enzyme is not located in the upper layer of the viable epidermis (i.e., immediately beneath the stratum corneum). For fresh hairless mouse skin, the enzyme activity was determined to reside in the basal layer of the viable epidermis (near the dermo-epidermal junction) (1). Thus, the same enzyme distribution profile for the viable epidermis is suggested for human skin. More work would be of interest to examine this further. In the following analysis, therefore, we consider the fresh human split-thickness skin as a three-layer (stratum corneum, viable epidermis, and part of the dermis) membrane with the enzyme activity in the basal layer of the viable epidermis. For the skin samples tested, the measured average thicknesses for stratum corneum and viable epidermis were 20 and 70 μm, respectively. The basal layer is assigned a thickness equal to 40% of that for the viable epidermis (10).

Based on a quantitative biophysical model previously developed with fresh hairless mouse skin (1), the calculated first-order enzyme rate constant values are presented in Fig. 3. Compared to the hairless mouse skin ($k \approx 0.7\ s^{-1}$), the human skin is much less capable of metabolizing E2 ($k \approx 0.07\ s^{-1}$). The possible explanation of the differences may be related to: (a) the concentrations of 17β-hydroxysteroid de-

Table 1. Influence of Preparation Procedures on Transport Parameters of Human Dermis

Skin[a]	Dermis[b]	h_d (cm)	Permeant	P_d (cm/s)[c]	$P_d h_d$ (cm²/s)
47/4	D/FD	2.2×10^{-2}	E2	1.1×10^{-4}	2.5×10^{-6}
47/4	D/FD	1.9×10^{-2}	E2	1.2×10^{-4}	2.3×10^{-6}
47/4	D/FD + HT	1.5×10^{-2}	E2	1.4×10^{-4}	2.0×10^{-6}
47/4	D/FD + HT	1.7×10^{-2}	E2	1.5×10^{-4}	2.6×10^{-6}
40/4	D/FD	3.1×10^{-3}	E2	5.2×10^{-4}	1.6×10^{-6}
40/4	S/HT	4.9×10^{-2}	E2	4.1×10^{-5}	2.0×10^{-6}
40/4	Sp/HT	3.1×10^{-2}	E2	8.4×10^{-5}	2.6×10^{-6}
40/4	Sp/HT	7.0×10^{-2}	E2	5.6×10^{-5}	3.9×10^{-6}
40/8	Sp/HT	2.7×10^{-2}	E2	1.0×10^{-4}	2.7×10^{-6}
40/12	Sp/HT	2.1×10^{-1}	E2	1.4×10^{-5}	2.9×10^{-6}
40/24	Sp/HT	2.1×10^{-2}	E2	1.3×10^{-4}	2.8×10^{-6}
40/4	S/HT	3.8×10^{-2}	E1	7.1×10^{-5}	2.7×10^{-6}
40/4	S/HT	3.4×10^{-2}	E1	9.4×10^{-5}	3.2×10^{-6}
40/4	Sp/HT	2.2×10^{-2}	E1	1.3×10^{-4}	2.9×10^{-6}
				X ± S.D.	$(2.6 \pm 0.5) \times 10^{-6}$

[a] Female abdominal skin: age (year)/postsurgery time (hour).
[b] Dermis preparation: original membrane (Sp: split-thickness skin, S: stripped skin, or D: dermis)/separation method (FD: fresh dermatoming, and/or HT: heat treatment).
[c] Apparent permeability coefficient (normalized flux).

Table 2. Tape-Stripping Efficiency for Removing Stratum Corneum and Stripped Skin Permeability Coefficients

Skin[a]	h_d (cm)	Permeant	P (cm/s)[b]	Residue[c]
47/4	1.9×10^{-2}	E2	8.3×10^{-5}	No
47/4	1.9×10^{-2}	E2	3.1×10^{-5}	Yes
40/4	3.5×10^{-3}	E2	9.1×10^{-5}	No
40/4	3.1×10^{-3}	E2	9.5×10^{-5}	No
40/8	3.2×10^{-2}	E2	1.8×10^{-5}	Yes
40/8	3.0×10^{-2}	E2	2.1×10^{-5}	Yes
40/12	3.9×10^{-2}	E2	9.7×10^{-6}	Yes
40/12	2.1×10^{-2}	E2	7.9×10^{-5}	No
40/4	3.2×10^{-3}	E1	1.2×10^{-4}	No
40/4	1.4×10^{-2}	E1	7.5×10^{-5}	No

[a] Female abdominal skin: age (year)/postsurgery time (hour).
[b] Apparent permeability coefficient (total forward normalized flux).
[c] Incomplete stripping was checked by observing the residue of stratum corneum after trypsin digestion of the stripped skin.

hydrogenase and/or NADP in the mouse skin are intrinsically higher than in human skin (19), (b) the 4-hr post-surgery period in case of human skin may be sufficient to cause substantial loss of enzyme activity or NADP cofactor, and (c) the human enzymes may have different kinetic constants than mouse enzymes.

The dermis thickness (h_d) and the permeability coefficient (P_d) were directly determined for each dermis membrane. While both the thickness and the permeability coefficient varied by more than 70-fold, the dermis ($P_d h_d$)-value was demonstrated as essentially constant of 2.6×10^{-6} cm^2/s (Table 1). Not surprisingly, this value is not different for E2 and E1 and this is also essentially the same for fresh hairless mouse dermis (1). The thickness independence indicates that the diffusion barrier for the permeant is relatively constant with respect to position in the dermis.

The permeability coefficients for the viable epidermis (P_{VE}) and for the stratum corneum (P_{SC}) can be determined by best-fitting the experimental data to the diffusion/metabolism model (1). For the case of human skin in which the percent metabolism to E1 is small (as is the case here) and for which E2 and E1 have essentially the same values of the viable epidermis and dermis permeability coefficients, the values for P_{VE} and P_{SC} may be simply calculated with <5% error, according to Eqs. 4 and 5, respectively:

$$\frac{1}{P_{VE}} = \frac{1}{P_S} - \frac{h_d}{(P_d h_d)} \tag{4}$$

$$\frac{1}{P_{SC}} = \frac{1}{P_{Sp}} - \frac{1}{P_S} \tag{5}$$

where P_S and P_{Sp} are the apparent permeability coefficients (total normalized forward fluxes) determined in the stripped skin and the split-thickness skin, respectively, and ($P_d h_d$) is the constant (Table 1). The data suggest that the P_{VE} value was not altered with storage time and that the P_{VE} values for E2 and E1 were not greatly different. Compared to the hairless mouse stratum corneum with $P_{SC} = 8.9 \times 10^{-6}$ cm/s (1), human stratum corneum is more resistant to E2 diffusion. In the present study, both the diffusion and metabolism parameters for fresh hairless mouse skin (t = 0) are essentially the same as those previously reported (1).

Table 3. Transdermal Metabolism of E2 in Human Skin[a]

Skin membrane	h_d (cm)	Normalized flux (cm/s)[b] E2 forward	E1 forward	E1 back
Dermatomed dermis	2.2×10^{-2}	1.1×10^{-4} (98.1%)	2.2×10^{-6} (1.8%)	$<1 \times 10^{-8}$ (~0%)
	1.9×10^{-2}	1.2×10^{-4} (98.3%)	2.1×10^{-6} (1.6%)	$<1 \times 10^{-8}$ (~0%)
	3.1×10^{-3}	5.1×10^{-4} (99.0%)	5.2×10^{-6} (1.0%)	$<1 \times 10^{-8}$ (~0%)
Stripped skin	1.9×10^{-2}	7.8×10^{-5} (88.6%)	4.9×10^{-6} (5.5%)	5.2×10^{-6} (5.9%)
	3.1×10^{-3}	9.1×10^{-5} (90.7%)	4.1×10^{-6} (4.0%)	5.3×10^{-6} (5.3%)
	3.5×10^{-3}	8.6×10^{-5} (89.7%)	4.2×10^{-6} (4.3%)	5.8×10^{-6} (6.0%)
Split-thickness skin	1.5×10^{-2}	1.6×10^{-6} (91.2%)	1.6×10^{-7} (8.8%)	$<1 \times 10^{-8}$ (~0%)
	3.1×10^{-2}	3.1×10^{-6} (90.1%)	3.4×10^{-7} (9.9%)	$<1 \times 10^{-8}$ (~0%)
	3.5×10^{-2}	2.4×10^{-6} (89.5%)	2.9×10^{-7} (10.5%)	$<1 \times 10^{-8}$ (~0%)

[a] E2 diffusion/metabolism experiments (dermis facing the receiver chamber) with 4 hour postsurgery abdominal skin from a 40-year old and a 47-year old female.
[b] Normalized fluxes are defined as the fluxes divided by donor E2 concentration. The numbers (%) in brackets are the fluxes divided by the total flux. There was 1–2% of ^{3}H-E1 impurities in the ^{3}H-E2 donor solution.

Table 4. Permeability Coefficients for Viable Epidermis and Stratum Corneum[a]

Skin[b]	Permeant	P_{VE} (cm/s)	P_{SC} (cm/s)
47/4	E2	2.1×10^{-4}	
40/4	E2	1.1×10^{-4}	
40/4	E2	1.0×10^{-4}	
40/12	E2	2.1×10^{-4}	
40/4	E1	1.4×10^{-4}	
40/4	E1	1.2×10^{-4}	
47/4	E2		1.8×10^{-6}
40/4	E2		3.6×10^{-6}
40/4	E2		2.8×10^{-6}
	X ± S.D.	$(1.5 \pm 0.5) \times 10^{-4}$	$(2.7 \pm 0.9) \times 10^{-6}$

[a] Permeability coefficients in the viable epidermis (P_{VE}) and stratum corneum (P_{SC}) calculated from Tables 2 and 3, respectively.
[b] Female abdominal skin: age (year)/postsurgery time (hour).

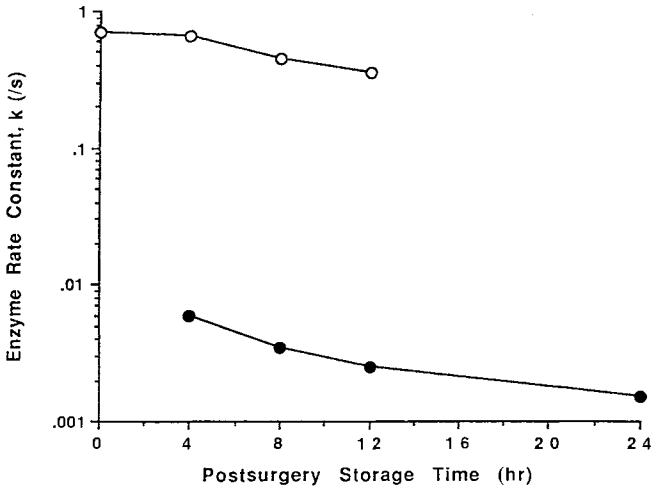

Fig. 3. Storage-time dependence of the first-order enzyme rate constant of the reaction of E2 → E1 in human skin (●) and in hairless mouse skin (○).

Extrapolation of Results to the *in Vivo* Human Skin Situation

The present *in vitro* results may be extrapolated via the theoretical physical model to determine the possible significance of simultaneous diffusion and metabolism of E2 in human skin *in vivo*. Permeants, after passing the stratum corneum and the viable epidermis, are easily taken up by blood capillary vessels directly beneath the viable epidermis. Blood circulating through the capillary loops in the upper papillary dermis carries away much of the absorbed drug (i.e., sink conditions) as a result of the mean capillary blood flow velocity of 7.5×10^{-2} cm/s (11,12). Therefore, the *in vitro* transdermal path length across the dermatomed split-thickness human skin (typically 300–450 μm or 0.012–0.018 inch thick) to the receiver is much longer than the *in vivo* distance from the human skin surface to the microcirculation (typically 100–200 μm) (13,14). The effect of dermis on transdermal diffusion, especially for hydrophobic compounds, has been well recognized (7,15). However, alteration of transdermal drug metabolism due to a significantly shorter dermis path length *in vivo* has generally been ignored. In a recent study (16), the metabolism of di- and monoester of salicylic acid in post-surgery fresh split-thickness human skin was found higher in the *in vivo* (grafted to athymic mice) than in the *in vitro* (diffusion cell) experiments, but with a uniform 300-μm thick skin for both *in vivo* and *in vitro* experiments.

In the following, a theoretical model analysis is carried out determining the effect of dermis thickness on the transdermal metabolism of E2. We take h_d = 200 μm for the *in vitro* dermatomed split-thickness human skin and h_d = 20 μm for the *in vivo* skin case. It should be noted that h_d = 20 μm for the *in vivo* case is taken as a reasonable, effective value for the present calculations and not as an exactly documented value. Table 5 presents the normalized steady-state fluxes of E2 and E1 for both *in vitro* and *in vivo* cases. As can be seen, the *in vitro* fluxes are consistent with experimental results in Table 3. Due to the stratum corneum being rate-limiting for both cases, the total fluxes (sum of the three fluxes) are essentially the same and the back fluxes of E1 are negligible (i.e., below the experimental detection limit). The most significant outcome here is that the transdermal metabolism of E2 to E1 are 3.1 and 12.7% for skin with dermis thicknesses of 20 and 200 μm, respectively. These results strongly suggest that transdermal metabolism (percentage) may be overestimated *in vitro* using the split-thickness skin.

Table 5. Calculated Fluxes and Dermo-Epidermal Interfacial Concentrations of E2 and E1

Case[a] (h_d)	Normalized flux (cm/s)[b]			Dermo-epidermal interface fractional concentration[c]	
	E2 forward	E1 forward	E1 back	E2	E1
In Vitro skin (h_d = 200 μm)	2.21×10^{-6} (87.0%)	3.22×10^{-7} (12.7%)	7.78×10^{-9} (0.3%)	1.70×10^{-2} (87.3%)	2.48×10^{-3} (12.7%)
In Vivo skin (h_d = 20 μm)	2.55×10^{-6} (96.9%)	8.05×10^{-8} (3.1%)	5.07×10^{-10} (0.02%)	1.96×10^{-3} (96.9%)	6.19×10^{-5} (3.1%)

[a] Same as Fig. 4; h_d = 200 μm for the *in vitro* dermatomed split-thickness human skin case and h_d = 20 μm for the *in vivo* human skin case with capillary flow.
[b] Normalized fluxes are defined as the fluxes divided by donor E2 concentration. The numbers (%) in brackets are the fluxes divided by the total flux.
[c] Fractional concentrations are defined as the concentrations divided by donor E2 concentration. The numbers (%) in brackets are the concentrations divided by the total concentration.

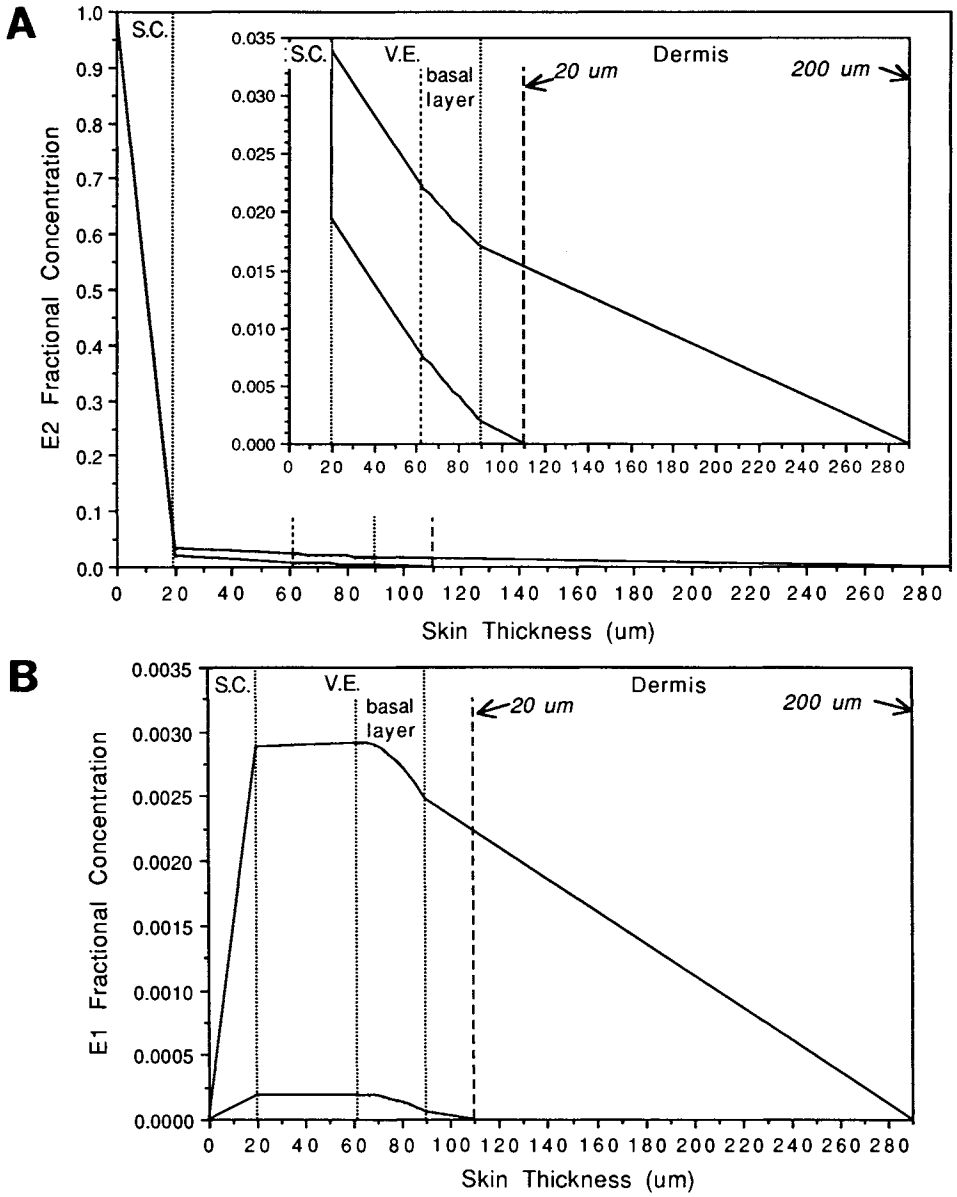

Fig. 4. Steady-state concentration gradients of E2 and E1 across skin during the simultaneous diffusion and metabolism of E2 in human skin with dermis thicknesses of 200 μm (for the *in vitro* dermatomed split-thickness human skin case) and 20 μm (for the *in vivo* human skin case with capillary blood flow). The parameters used in the calculation (1): $P_S = 2.7 \times 10^{-6}$ cm/s, $P_{VE} = 1.5 \times 10^{-4}$ cm/s, $P_d h_d = 2.6 \times 10^{-6}$ cm^2/s, $h_{basal\ layer} = 28$ μm, and $k_{basal\ layer} = 0.0065$ s^{-1}. (A) The E2 fractional concentration gradients. Inset gives the fractional concentration range between 0 to 0.035. (B) The E1 fractional concentration gradients.

Therapeutic doses of E2 may result in even less metabolism of E2 to E1 in the skin due to possible saturation of the enzyme and/or cofactor. It is interesting to note that the rather low extent of the predicted transdermal E2 metabolism in human skin *in vivo* is consistent with the results of clinical studies: the systemically converted E1 percentages were 27 ± 7 (n = 7) after intravenous E2 (17) and 29 ± 11 (n = 8) after transdermal E2 (18). It should be also noted that the difference between 3.1% and 12.7% E2 metabolism would be too small to have clinical consequences in transdermal estradiol delivery.

Also shown in Table 5, the model-deduced ratios of E2 and E1 forward fluxes are the same as those of E2 and E1 concentrations at dermo-epidermal interface. Figure 4 gives the steady-state fractional concentration profiles of E2 and E1 across skin for the two cases. These informative plots reflect the overall result of skin metabolism of E2 to E1 and diffusion of both E2 (forward) and E1 (forward and back). Linear concentration-distance profiles are shown except in the basal layer region where nonlinearity arises because of the metabolism reaction in this layer. Although the main concentration drop for E2 occurs across stratum corneum, increasing the thickness of the dermis from 20 μm to 200 μm results in a 3–9 time increase of E2 levels in the basal layer

(Fig. 4A) and a 15–40 times higher concentration of E1 in the basal layer (Fig. 4B). The important point here is that there is a significant alteration of transdermal metabolism due to a change in the dermis thickness even when the dermis layer has a negligible diffusion resistance compared to stratum corneum.

In conclusion, transport of E2 in the freshly excised human skin was quantitatively evaluated *in vitro* using a newly developed experimental approach. The diffusion and metabolism parameters in each skin layer (stratum corneum, viable epidermis, and dermis) were determined with the fluxes of E2 and the metabolite (E1) involving simultaneous diffusion and metabolism of E2 across the three skin membranes (dermis, stripped skin, and split-thickness skin). Compared to fresh hairless mouse skin, fresh human skin appears to be more resistant to the stratum corneum diffusion of E2 and less capable of metabolizing E2 in the basal layer of the viable epidermis. Finally, due to a shorter dermis path length for both E2 and E1 *in vivo*, the amount of the transdermal metabolite (E1) taken up by the blood capillary is expected to be much less than that typically measured *in vitro* with split-thickness fresh human skin.

ACKNOWLEDGMENTS

This research was supported by the Ciba-Geigy Corporation (Ardsley, NY). The authors express their thanks to Dr. Lynn K. Pershing (University of Utah, Salt Lake City, UT) for her valuable suggestions on preparation of fresh human skin. Helpful discussions with Professor Gerald G. Krueger (University of Utah, Salt Lake City, UT) and Dr. Tamie Kurihara-Bergstrom (Wyeth-Ayerst Laboratories, Philadelphia, PA) are also acknowledged.

REFERENCES

1. P. Liu, W. I. Higuchi, A-H. Ghanem, T. Kurihara-Bergstrom, and W. R. Good. Quantitation of simultaneous diffusion and metabolism of β-estradiol in hairless mouse skin: Enzyme distribution and intrinsic diffusion/metabolism parameters. *Int. J. Pharm.* 64:7–25 (1990).
2. B. P. Davis, E. Rampini, and S. L. Hsia. 17β-Hydroxysteroid dehydrogenase of rat skin: Substrate specificity and inhibitors. *J. Bio. Chem.* 247:1407–1413 (1972).
3. M. J. Finnen, M. L. Herdman, and S. Shuster. Distribution and subcellular localization of drug metabolizing enzymes in the skin. *Br. J. Dermatol.* 113:713–721 (1985).
4. C. Longcope. The metabolism of estrogens by human skin. In P. Mauvais-Jarvis, C. F. H. Vickers, and J. Wepierre (eds.), *Percutaneous Absorption of Steroids,* Academic Press, New York, 1980, pp. 89–98.
5. S. W. Collier, N. M. Sheikh, A. Saker, J. L. Lichtin, R. F. Stewart, and R. L. Bronaugh. Maintenance of skin viability during *in vitro* percutaneous absorption/metabolism studies. *Toxicol. Appl. Pharmacol.* 99:522–533 (1989).
6. D. R. Friend. *In vitro* skin permeation techniques. *J. Contr. Rel.* 18:235–248 (1992).
7. R. L. Bronaugh. Determination of percutaneous absorption by *in vitro* techniques. In R. L. Bronaugh and H. I. Maibach (eds.) *Percutaneous Absorption: Mechanisms—Methodology—Drug Delivery.* 2nd ed., Marcel Dekker, Inc., New York, 1989, pp. 239–258.
8. A. M. Kligman and E. Christophers. Preparation of isolated sheets of human stratum corneum. *Arch. Dermatol.* 88:702–705 (1963).
9. R. J. Scheuplein. Mechanism of percutaneous absorption I. Routes of penetration and influence of solubility. *J. Invest Dermatol.* 45:334–370 (1965).
10. W. Montagna. *The Structure and Function of Skin.* 3rd ed., Academic Press, New York, 1974, p. 46.
11. M. Asano and P. I. Branemark. Cardiovascular and microvascular responses to smoking in man. *Adv. Microcirc.* 3:125–158 (1970).
12. B. Fagrell, A. Fronek, and M. Intaglietts. A microscope-television system for studying flow velocity in human skin capillaries. *Am. J. Physiol.* 233:H318–H321 (1977).
13. R. J. Scheuplein. Mechanism of percutaneous absorption II. Transient diffusion and the relative importance of various routes of skin penetration. *J. Invest. Dermatol.* 48:79–88 (1967).
14. R. L. Bronaugh and S. W. Collier. Preparation of human and animal skin. In R. L. Bronaugh and H. I. Maibach (eds.) *In Vitro Percutaneous Absorption: Principles, Fundamentals, and Applications.* CRC Press, Boca Raton, Florida, 1991, pp. 2–6.
15. E. R. Cooper and B. Berner. Skin permeability. In D. Skerrow and C. J. Skerrow (eds.) *Methods in Skin Research.* John Wiley & Sons Ltd., New York, 1985, pp. 407–432.
16. D. B. Guzek, A. H. Kennedy, S. C. McNeill, E. Wakshull, and R. O. Potts. Transdermal drug transport and metabolism. I. Comparison of *in vitro* and *in vivo* results. *Pharm. Res.* 6:33–39 (1989).
17. C. Longcope and K. I. H. Williams. The metabolism of estrogens in normal women after pulse injection of ^{3}H-estradiol and ^{3}H-estrone. *J. Clin. Endocrinol. Metab.* 38:602–607 (1974).
18. J. A. Balfour and R. C. Heel. Transdermal estradiol. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in the treatment of menopausal complaints. *Drugs* 40:561–582 (1990).
19. J. Kao, F. K. Patterson, and J. Hall. Skin penetration and metabolism of topical applied chemicals in six mammalian species, including man: An *in vitro* study with benzo[a]pyrene and testosterone. *Toxicol. Appl. Pharmacol.* 81:502–516 (1985).