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LONG-TERM SKIN PERMEATION KINETICS OF ESTRADIOL: (II) KINETICS OF SKIN UPTAKE, BINDING, AND METABOLISM

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ABSTRACT

The *in vitro* skin permeation system developed in this laboratory was utilized to investigate the kinetics of uptake, binding, and metabolism of estradiol, the female hormone, by the hairless mouse skin. The kinetics of uptake of estradiol and its subsequent metabolism to estrone by the skin were examined

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by exposing one side of a freshly excised skin to an estradiol solution, while the other side of the skin was protected with an impermeable aluminum foil.

The results concluded that the stratum corneum plays a rate-limiting role in the uptake and binding of estradiol and its metabolism to estrone by the skin. The mechanisms of the uptake and binding of estradiol to whole skin tissue (with stratum corneum) showed a sex dependence, with the rate higher in the female than in the male mouse. On the other hand, no sex-dependent difference was observed in the mechanism and rate of metabolism to estrone.

After stripping off the rate-limiting stratum corneum, the mechanisms of the uptake, binding and metabolism of estradiol all became sex dependent with identical rates observed between male and female mice in the initial 14 hrs of the experiment; Between 14 to 21 hrs, the rates increased substantially with the female skin, but not with the male skin. The increase in the rate of uptake of estradiol by the female skin was related primarily to the increased rate of metabolism of estradiol to estrone.

INTRODUCTION

In the past years, studies completed in several laboratories have contributed towards the understanding of steroid metabolism in human skin. Not only have transformations of several steroidal hormones been demonstrated in this organ, but also there is an

increasing evidence that these transformations are of endocrinological importance. It has been demonstrated that human skin is capable of transforming testosterone into 5 α - dihydrotestosterone, which, by certain criteria, is a more potent androgen than testosterone itself (1,2). It has also been reported (2) that the skin of young men (but not of women) is capable of converting dehyroepiandrosterone into testosterone, i.e. transforming a biologically inactive precursor into a potent androgen. Therefore, skin appears to participate not only in the catabolism of steroids, but also in the formation of active hormones from the inert steroidal precursors supplied through the blood circulation.

Extensive metabolism of progesterone and dehydroepiandrosterone was reported to occur in the skin (2,3) as well as the interconversion of hydrocortisone and cortisone (4) and of estradiol to estrone (5). These findings suggested that the skin may play a significant part in the regulation of the activity of steroidal hormones.

Histochemical studies by Baillie et al (6) indicated that estradiol undergoes dehydrogenation in human skin. In the in vitro studies (7, 8), using radiolabeled estradiol and estrone, the interconversion of these two estrogens was demonstrated in the neonatal foreskin, in the abdominal skin of adult male and female, and in the vaginal mucosa. The major metabolism found in the foreskin and the abdominal skin was the oxidation of estradiol to estrone, while in vaginal mucosa the reverse reaction predominated.

In the past, for the hormonal deficiency treatment the steroids were administered mostly by the oral route; for example, the micronized estradiol or conjugated estrogens were found to be effective in the management of menopausal changes at a daily dose as low as in milligram range. However, the major portion of the oral estrogen dose was reportedly converted to less active estrone and estriol and became conjugated on its passage through the liver, resulting in a highly unphysiologic pattern of estrogen metabolites and in the increased production of hepatic proteins, such as renin substrate.

Optimization of estrogen replacement treatment was recently achieved by the controlled administration of estradiol through a transdermal therapeutic system (9), called TTS-estradiol. It is designed to provide a continuous delivery of the natural estradiol through the intact skin at a rate sufficient to maintain the plasma level in the range similar to that observed in the early follicular phase of the menstrual cycle.

Recently, Goerz et al. (10) investigated a new animal model for studying the cutaneous drug-metabolizing enzymes and found that the Ng/- mouse (also a hairless mouse) has the highest activities of both cutaneous and hepatic drug-metabolizing enzymes ever found in various animal species. Furthermore, the female Ng/- mouse showed a 5- to 7- fold greater enzyme activity than the male. In other mouse strains, the sex-dependent differences in the drug-metabolizing enzyme level were minimal (11).

In this investigation, the kinetics involved in the uptake

of estradiol by the hairless mouse skin, male and female, and its subsequent metabolism by the 17 β-hydroxysteroid dehydrogenases to estrone will be analyzed.

EXPERIMENTAL

Materials:

Estradiol[1], estrone[2], sodium chloride[3], and acetonitrile[4] (distilled-in-glass HPLC grade) were used as obtained. HPLC grade water was prepared freshly in the laboratory[5].

Analytical Methods:

A liquid chromatograph[6] equipped with a reciprocating pump (model 6000A), an injector (model U6K), an UV detector (model 440, with a cell volume of 15.6 µl), a reverse-phase µBondapak C_{18} column with a guard column containing 37-50 µm Bondapak C_{18}/Corasil packing material, and an Omniscribe recorder was used in this investigation. The UV detector was operated at the wavelength of 280 nm to detect estradiol at a sensitivity of 0.005 AUFS. Another UV detector[8] (model 773, with a cell volume of 12 µl) was also used at 205 nm to detect estrone and estriol at a sensitivity of 0.005 AUFS. A combination of acetonitrile and water, at a ratio of 50:50, was used as the mobile phase. At ambient condition, a flow rate of 1.5 ml/min was used, yielding an operating pressure of 2000 psi.

Determination of estradiol and estrone concentrations in the sample solution was carried out by first measuring the peak height of estradiol and estrone peaks at a retention time of 4.9 and 6.1 minutes, respectively; and then computing the

concentration (μg/ml) from the calibration curves constructed from standard solutions of both drugs.

Skin Permeation Cell:

The same *in vitro* skin permeation system[9] reported earlier (12, 13) was used in this investigation.

Skin Preparation:

For this study, a full-thickness skin sample was freshly excised from a 5-7 weeks old hairless mouse (HRS/J strain)[10]. The hairless mouse was sacrificed just prior to the experiment by snapping the spinal cord at the neck. A square section of the abdominal skin (~3 x 3 cm) was surgically removed from the animal and the subcutaneous tissue and blood vessels were cleaned (14, 15).

In some of the studies the stratum corneum side of the hairless mouse skin was stripped with a cellophane tape[11] for 25 times to eliminate the stratum corneum layers. It was carried out by securing the animal on a surgical glass plate and the abdominal skin was stripped by placing the tape on the stratum corneum surface and moving the thumb back and forth a few times, with a pressure as uniform as was possible (16). A fresh piece of the tape was used for each stripping.

Skin Uptake/Metabolism Studies

A full-thickness skin or a stripped skin sample was mounted between the two compartments of the Valia-Chien skin permeation cell (12) in such a way that either the stratum corneum or the dermis side facing a bulk of drug solution and the other side

of the skin was protected with an impermeable aluminum foil (Figure 1, A & B). A saturated saline solution of estradiol (3.5 ml) was then introduced into the compartment with the uncovered skin surface and the compartment with the aluminum foil-covered skin surface remained empty. A 50 µl sample was withdrawn from the drug solution at predetermined time intervals and assayed for estradiol and any possible metabolites by the HPLC method outlined above.

RESULTS AND DISCUSSION

The enzymatic interconversion of estradiol and estrone was known to occur in a variety of human tissues (5). Frost et al. (7) shown that when neonatal foreskin (finely minced) was used, an average of 47% of the incubated estradiol dose was oxidized to estrone by 200 mg of the tissue in 5 hr. However, in the experiments with estrone only 3% of the incubated hormone was found to be converted to estradiol. The fresh abdominal skin obtained from either adult male or females yielded only 9-10% conversion of estradiol to estrone for every 200 mg of the tissue. The activity in the vaginal mucosa was even less, with only 3% of estradiol converted to estrone.

In the skin uptake/metabolism studies, only the stratum corneum or the dermis was allowed to be in contact with the saline solution of estradiol, while the other side of the skin was sealed with a sheet of impermeable aluminum foil (Figure 1, A & B). Under this experimental design, the estradiol was allowed to penetrate the skin from either the stratum corneum side or the

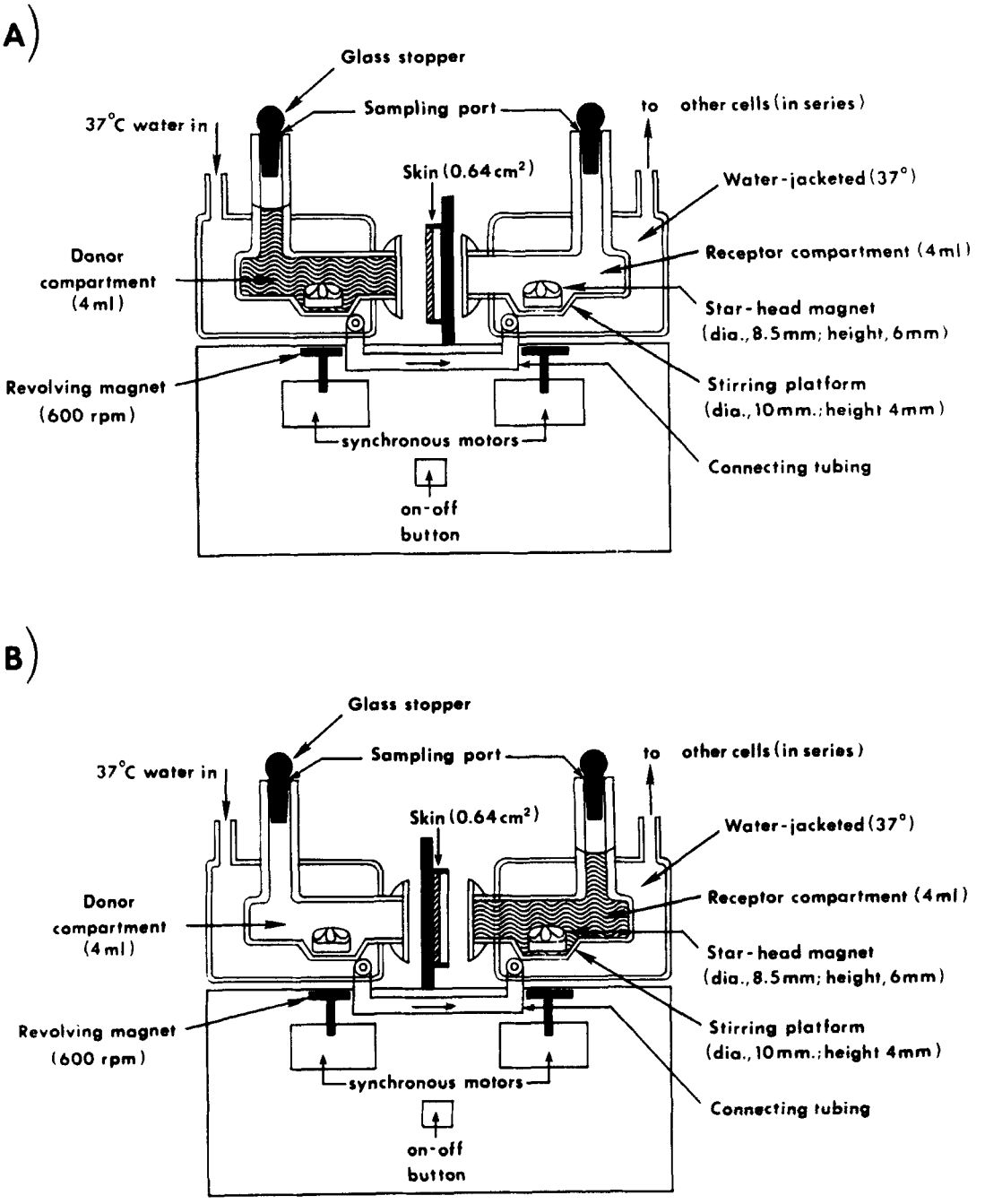


Figure 1: Diagrammatic illustration of the skin permeation setup used in the skin uptake/metabolism experiments; A) - from stratum corneum side, B) - from dermis side.

dermis side. After metabolized by the cutaneous enzymes, the matabolites could then diffuse back into the bulk solution. By analyzing the change in the concentrations of estradiol and metabolites, such as estrone, in the solution, the rates of skin uptake, binding and cutaneous metabolism in epidermis and dermis of the hairless mouse skin could be determined from the rate profile for the disappearance of estradiol from the solution and the rate profile for the appearance of metabolite in the solution.

Skin Uptake/Metabolism of Estradiol from Stratum Corneum Side

Figures 2 and 3 show the time course for the disappearance of estradiol and the appearance of its major metabolite, estrone, in the saline solution which is in contact with the stratum corneum of the female and male hairless mouse abdominal skin.

In the case of female skin (Figure 2), the estradiol disappeared from the solution at zero-order kinetics with a rate constant of 0.1079 (±0.0154) μM/hr. In view of the fact that no degradation products of estradiol could be detected during the course of study, so the disappearance of estradiol could only be attributed to the uptake of estradiol by the skin. This zero-order kinetics of skin uptake was observed to stop at the 24-hr point, and resume at a slightly slower rate (0.0707 ± 0.0281 μM/hr) after the rupture of stratum corneum at 54-hr point. In the meantime, the appearance of estrone in the solution began after a lag time of 38.5 hrs with a zero-order rate constant of 0.0505 (±0.0059) μM/hr.

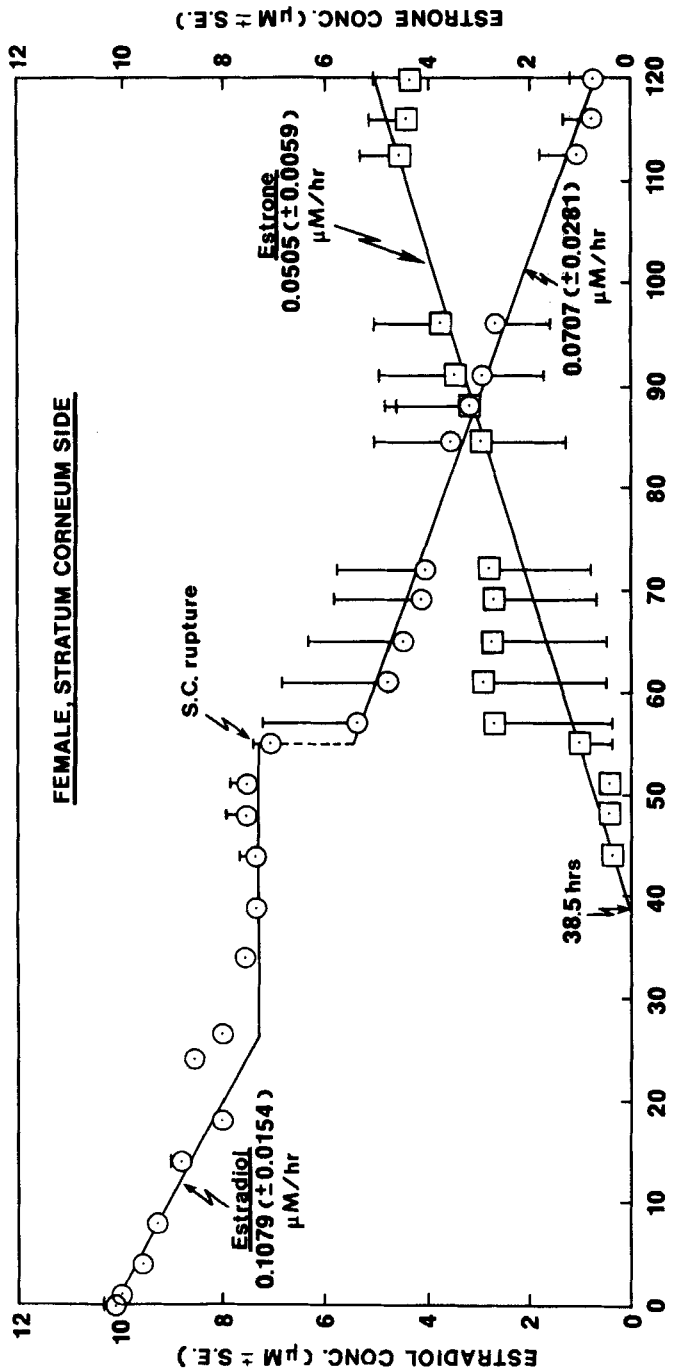


Figure 2: The time course for the uptake of estradiol by the stratum corneum of the female hairless mouse skin and the formation of estrone. Each data point represents the mean and standard error of 3 determinations.

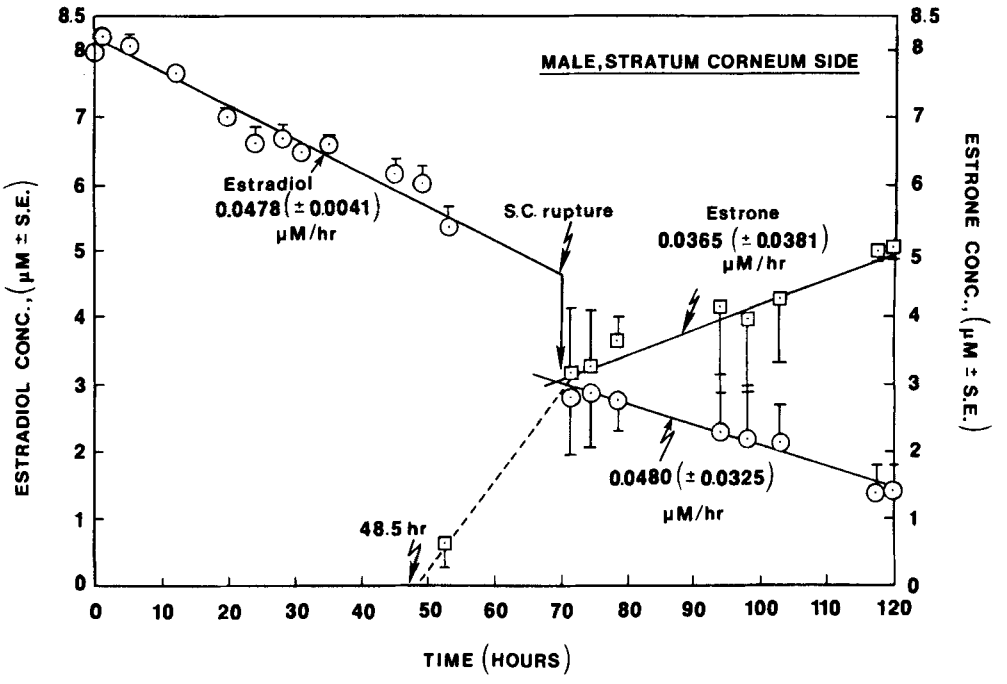


Figure 3: The time course for the uptake of estradiol by the stratum corneum of the male hairless mouse skin and the formation of estrone. Each data point represents the mean and standard error of 3 determinations.

In the case of male abdominal skin (Figure 3), the estradiol disappeared from the solution also at zero-order kinetics, but with a rate constant (0.0478 ± 0.0041 μM/hr) which is 2.3-fold slower than in the female skin (0.1079 ± 0.0154 μM/hr). Interestingly, this zero-order kinetics of uptake was found to continue until the stratum corneum ruptured at 68 hrs after the initiation of experiment. After the rupture of the stratum corneum, the concentration of estrone in the solution was increased

substantially due to the dumping of the estrone which was formed and accumulated inside the skin tissue. After the release of estrone, uptake and metabolism of estradiol continued, also at zero-order kinetic fashion, with rate constants of 0.0480 (±0.0325) and 0.0365 (±0.0381) μM/hr, respectively. There appears no statistical difference between the rate constants for the disappearance of estradiol before and after the rupture of stratum corneum in the same sex as well as between the rate constant for estradiol disappearance and the rate constant for estrone formation following the rupture of stratum corneum.

Effect of Stripping on Skin Uptake/Metabolism of Estradiol

By stripping the skin with a cellophane adhesive tape for 25 times (16), the stratum corneum layers can be completely removed and the viable epidermis is thus exposed. This stripping treatment was noted to enhance substantially the rates of estradiol disappearance and of estrone formation (Figures 4 & 5).

In the case of female hairless mouse abdominal skin (Figure 4), stripping off the rate-limiting stratum corneum was observed to modify the initial 24-hr period of the estradiol disappearance profile from monophasic to biphasic pattern. For the first 12 hrs, the rate of estradiol disappearance was increased by 2.1 folds from 0.1079 (±0.0154) μM/hr with the presence of stratum corneum to 0.2280 (±0.0150) μM/hr without the stratum corneum. During the period of 12-21 hrs, the rate of disappearance was further increased by another 3.1 folds to 0.6967 (±0.1341) μM/hr. After 21 hrs of experiment, approximately 90% of the estradiol

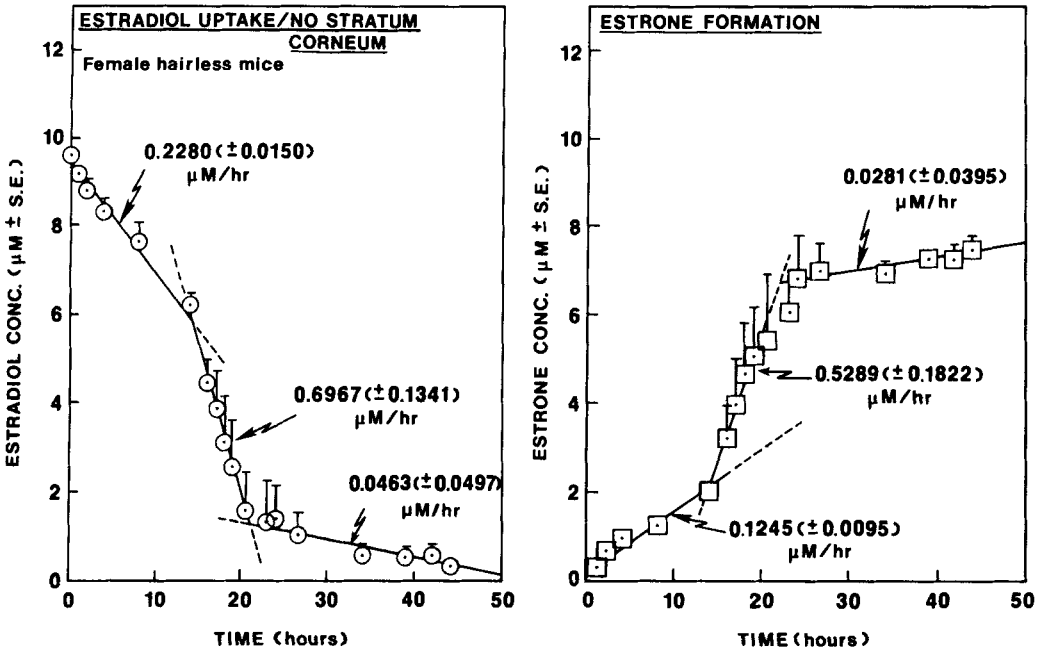


Figure 4: The time course for the uptake of estradiol by the stripped female hairless mouse skin and formation of estrone. Each data point represents the mean and standard error of 3 determinations.

dose had disappeared from the solution; so, the rate of disappearance decreased to 0.0463 (±0.0497) μM/hr. The formation of estrone also showed the same biphasic kinetic profile. With the removal of the rate-limiting stratum corneum, the formation of estrone was detected as early as 30 minutes, as compared to the 38.5 hrs observed in the presence of stratum corneum (Figure 2); and the rate of appearance of estrone was also enhanced by 2.5 folds from 0.0505 (±0.0059) μM/hr to 0.1245 (±0.0095) μM/hr during the first 14 hrs and increased for another 4.2 folds to

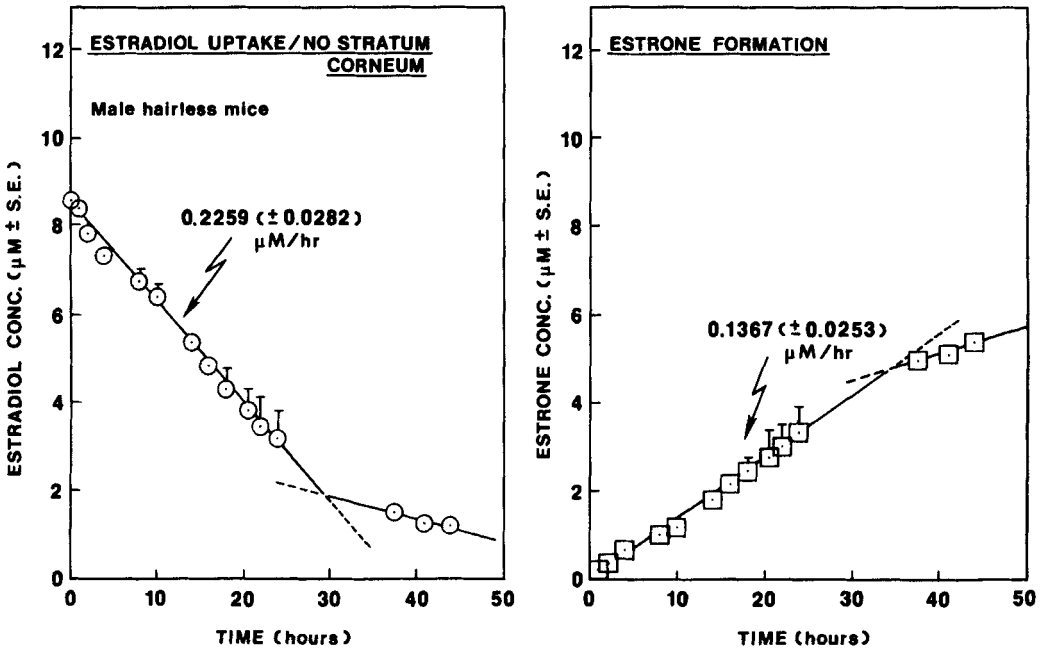


Figure 5: The time course for the uptake of estradiol by the stripped male hairless mouse skin and the formation of estrone. Each data point represents the mean and standard error of 3 determinations.

0.5289 (±0.1822) μM/hr from 14 to 21 hrs. After 21 hrs,, the rate of estrone formation reduced to 0.0281 (±0.0395), which may be due to the significant reduction in estradiol concentration in the solution.

In the case of male skin (Figure 5), stripping off the rate-limiting stratum corneum did not affect the mechanism of estradiol disappearance. The disappearance of estradiol still followed the monophasic zero-order kinetics, but the rate of estradiol disappearance was increased by 4.6 folds from 0.0478

(±0.0041) μM/hr in the presence of stratum corneum to 0.2259 (±0.0282) μM/hr without the stratum corneum. The formation of estrone was detectable also as early as 30 minutes, as compared to the 48.5 hrs observed in the presence of stratum corneum (Figure 3). The appearance of estrone also followed the same monophasic zero-order kinetics and the rate of estrone appearance was increased by 3.7 folds from 0.0365 (±0.0381) μM/hr to 0.1367 (±0.0253) μM/hr.

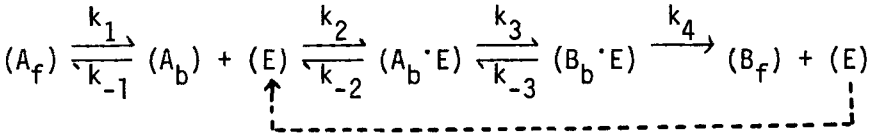
Comparison of the results in Figures 4 and 5 with the data in Figures 2 and 3 suggests that the long lag time required for the appearance of estrone from the intact skin appears to be related to the impermeability of stratum corneum, which limits the back diffusion of estrone, after metabolism, into the solution from the skin tissue.

The rate profiles for the skin uptake of estradiol and the formation of estrone are summarized and compared in Tables 1 and 2.

Kinetics of Skin Binding of Estradiol

Due to the continuous metabolism of estradiol to estrone in the skin, it is difficult, although not impossible, to measure the extent and concentration profile of estradiol bound to the skin tissues. Therefore, it is desirable to monitor some measurable quantities in the solution, such as the changes in the concentration profiles of estradiol and estrone, in a hope that the mechanism and rate profile of binding to the skin can be characterized.

The uptake and metabolism of estradiol by skin tissues can be considered to follow this sequence of kinetic pathways:



where (A_f) and (A_b) are the free and bound estradiol concentrations, respectively; (B_f) and (B_b) are the free and bound estrone concentrations, respectively; $(A_b \cdot E)$ and $(B_b \cdot E)$ are the concentrations of estradiol-enzyme (substrate-enzyme) and estrone-enzyme (product-enzyme) complexes, respectively; k_1, k_2, k_3 and k_4 are the rate constants for the forward reactions; k_{-1}, k_{-2} and k_{-3} are the rate constants for the backward reactions, respectively.

The rate of disappearance of estradiol from the saline solution is:

$$-\frac{d(A_f)}{dt} = k_1(A_f) - k_{-1}(A_b) \quad (1)$$

The rate of binding of estradiol to the skin can be expressed by:

$$\frac{d(A_b)}{dt} = [k_1(A_f) - k_{-1}(A_b)] - [k_2(A_b)(E) - k_{-2}(A_b \cdot E)] \quad (2)$$

The rate of formation of substrate-enzyme complex is:

$$\frac{d(A_b \cdot E)}{dt} = [k_2(A_b)(E) - k_{-2}(A_b \cdot E)] - [k_3(A_b \cdot E) - k_{-3}(B_b \cdot E)] \quad (3)$$

The rate of formation of product-enzyme complex is:

$$\frac{d(B_b \cdot E)}{dt} = [k_3(A_b \cdot E) - k_{-3}(B_b \cdot E)] - k_4(B_b \cdot E) \quad (4)$$

The rate of appearance of estrone is:

$$\frac{d(B_f)}{dt} = k_4(B_b \cdot E) \quad (5)$$

If the concentrations of the complex $(A_b \cdot E)$ and $(B_b \cdot E)$ are constant at the steady state and are always much less than the concentration of A_f and B_f, then:

$$\frac{d(A_b \cdot E)}{dt} = 0 \quad (6)$$

and

$$\frac{d(B_b \cdot E)}{dt} = 0 \quad (7)$$

Therefore, Equation (4) is reduced to:

$$k_4(B_b E) = k_3(A_b \cdot E) - k_{-3}(B_b \cdot E) \quad (8)$$

and Equation (3) is reduced to:

$$k_2(A_b)(E) - k_{-2}(A_b \cdot E) = k_3(A_b \cdot E) - k_{-3}(B_b \cdot E) \quad (9)$$

Combining Equations (8) and (9) to give:

$$k_2(A_b)(E) - k_{-2}(A_b{}^{\cdot}E) = k_4(B_bE) \quad (10)$$

Substituting Equation (10) into Equation (2) gives:

$$\frac{d(A_b)}{dt} = k_1(A_f) - k_{-1}(A_b) - k_4(B_bE) \quad (11)$$

Substituting Equations (1) and (5) into Equation (11) produces:

$$\frac{d(A_b)}{dt} = \left(-\frac{d(A_f)}{dt}\right) - \left(\frac{d(B_f)}{dt}\right) \quad (12)$$

That means:

$$\begin{bmatrix}\text{The rate of binding}\\ \text{of estradiol}\end{bmatrix} = \begin{bmatrix}\text{The rate of}\\ \text{disappearance}\\ \text{of estradiol}\end{bmatrix} - \begin{bmatrix}\text{The rate of}\\ \text{formation of}\\ \text{estrone}\end{bmatrix}$$

Equation (12) suggests that one can determine the rate of binding of estradiol from the rate of disappearance of estradiol and the rate of formation of estrone.

Using equation (12), the rates of estradiol binding by the skin were calculated from the data in Table 1 and 2 and summarized in Table 3. Results indicated that the rates of skin binding of estradiol by hairless mouse was found to be dependent upon the sex and the stratum corneum. The rate of binding by female skin (0.0574 μM/hr) was 5 times greater than that by male skin (0.0113 μM/hr). Removal of stratum corneum was found to

Table 1

Effect of Stratum Corneum and Sex on Skin Uptake Rate of Estradiol*

Sex	Rate of Skin Uptake (μM/hr ± S.E.)	
	Whole Skin	Viable Skin
Male	0.0478 (0.0041) (< 53 hr.)	0.2259 (0.0282) (< 24 hr.)
Female	1) 0.1079 (0.0154) (< 18 hr.)	0.2280 (0.0150) (< 14 hr.)
	2) 0.0000 (0.0000) (18-55 hr.)	0.6947 (0.1341) (14-21 hr.)
	3) 0.0707 (0.0281) (57-120 hr.)	0.0463 (0.0497) (23-44 hr.)

*Hairless mouse

Table 2

Effect of Stratum Corneum and Sex on Estrone Formation Rate

Sex	Rate of Estrone Formation (μM/hr ± S.E.)	
	Whole Skin	Viable Skin
Male	0.0365 (0.0381) (70-120 hr.)	0.1367 (0.0253) (1-24 hr.)
Female	1) 0.0505 (0.0059) (44-120 hr.)	0.1245 (0.0095) (1-14 hr.)
	2) —	0.5289 (0.1822) (14-21 hr.)
	3) —	0.0281 (0.0395) (24-44 hr.)

Table 3

Effect of Stratum Corneum and Sex on Skin Binding Rate of Estradiol[a)]

Sex	Rate of Skin Binding[b)] (μM/hr ± S.E.)	
	Whole Skin	Viable Skin
Male	0.0113 (< 53 hr.)	0.0892 (< 24 hr.)
Female	0.0574 (< 18 hr.)	0.1035 (< 14 hr.)
	—	0.1658 (14-21 hr.)
	0.0202 (57-120 hr.)	0.0182 (23-44 hr.)

a) Hairless mouse

b) Rate of skin binding = Rate of skin uptake − Rate of skin metabolism

increase the rate of skin binding by 8 times in male and by 2-3 times in female.

Skin Uptake/Metabolism of Estradiol from Dermal Side

When the estradiol solution was added to the compartment facing dermis, the drug was taken up by the dermis, diffused through the epidermis, and during the course of diffusion, it was metabolized by the enzyme called 17 - hydroxysteroid dehydrogenases to form estrone, which then diffused back into the compartment. Hsia (1) reported that the epidermis has a greater enzymatic activity in converting estradiol into estrone than the dermis.

The time courses for the uptake of estradiol by the dermis in the male hairless mouse abdominal skin and for the formation of estrone for up to 120 hr are shown in Figure 6. After a rapid initial uptake, the estradiol was found to disappear by a

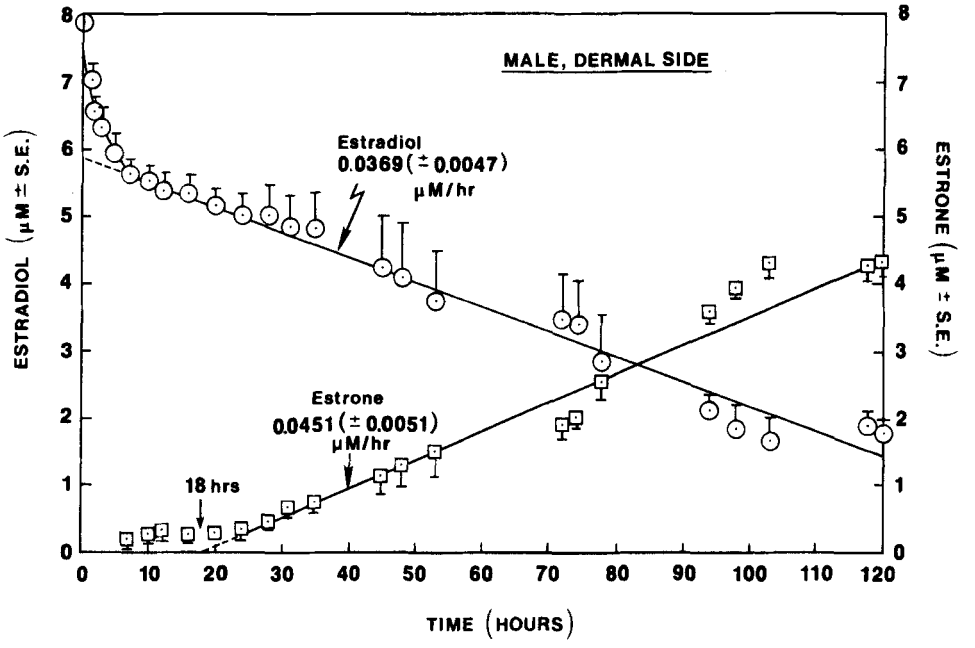


Figure 6: The time course for the uptake of estradiol by the dermis of the male hairless mouse skin and the formation of estrone. Each data point represents the mean and standard error of 3 determinations.

zero-order kinetics with a rate constant of 0.0369 (±0.0047) μM/hr. Meanwhile, after a lag time of 18 hr, the metabolite estrone, appeared in the solution also by a zero-order kinetics with a rate constant of 0.0451 (±0.0051) μM/hr. The rate of disappearance of estradiol (0.0369 ± 0.0047 μM/hr) and the rate of appearance of estrone (0.0451 ± 0.0051 μM/hr) are not statistically different. The observation suggested that the decrease in estradiol concentration in the solution is primarily the result of the formation of estrone from estradiol by metabolism.

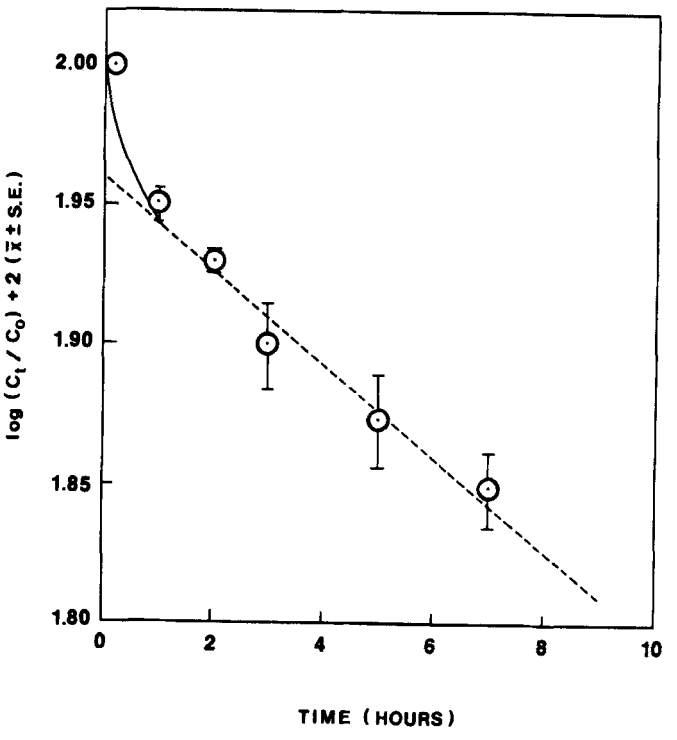


Figure 7: A first-order kinetic plot for the initial phase of the disappearance of estradiol from the dermal solution. Each data point represents the mean and standard error of 3 determinations. A rate constant of 0.0389 hr^{-1} was obtained.

The initial phase for the disappearance of estradiol was observed to follow a first-order kinetics with a rate constant of 0.0389 $hr.^{-1}$ (Figure 7), which may be attributed to the uptake of estradiol by the dermis.

Conclusions

In conclusion, the results indicated that the stratum corneum plays a rate-limiting role in the uptake and binding of estradiol

and the subsequent metabolism of estradiol to estrone by the skin. The mechanisms of the uptake and binding of estradiol to whole skin tissue (with stratum corneum) showed a sex dependence, with the rate higher in the female than in the male mouse. On the other hand, no sex-dependent difference was observed in the mechanism and the rate of metabolism to estrone.

After stripping off the rate-limiting stratum corneum, the mechanisms of the uptake, binding and metabolism of estradiol all became sex dependent, with identical rates observed between male and female mice in the initial 14 hrs of the experiment; Between 14 to 21 hrs, the rates increased substantially with the female skin, but not with the male skin. The increase in the rate of uptake of estradiol by the female skin was related primarily to the increased rate of metabolism of estradiol to estrone.

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FOOTNOTES

1. Roussel-UCLAF, Paris, France.
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