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Visualization of percutaneous ^{3}H-estradiol and ^{3}H-norethindrone acetate transport across human epidermis as a function of time

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Developing transdermal therapeutic systems for estradiol and norethindrone acetate raised questions about the steroids penetration pathway across and retention in the skin. This paper describes the distribution of ^{3}H-estradiol and ^{3}H-norethindrone acetate in human stratum corneum after topical application to dermatomed skin *in vitro*. The study involved (a) permeation experiments to determine the steroid flux, (b) autoradiographical visualization of the steroid distribution in the same skin samples, and (c) a correlation between flux and skin distribution in time.

On correlating the steroid flux with intraepidermal steroid distribution, it was concluded that both permeants were bound in the skin tissue. The steroids were preferentially located in or close to the intercellular lipids of the stratum corneum, indicating that both transport and binding occurred via this domain of the stratum corneum.

This study demonstrated the importance of correlating drug flux with intraepidermal drug distribution as a function of time.
Key words: Autoradiography; Microscopy; Skin penetration.

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INTRODUCTION

It is common to study percutaneous steroid permeation by comparing drug fluxes across the skin (1–4). Thus only limited information can be obtained on the transport kinetics across the skin. However, by simultaneously evaluating the percutaneous drug flux and the intraepidermal drug distribution in the same skin samples as a function of time, it is possible to clarify the relationship between the kinetics of percutaneous transport and the kinetics of intracutaneous accumulation, distribution and binding. Furthermore, this approach permits visualization of the actual pathways of transport.

Although extensive literature exists on transdermal delivery of steroids, data on visualization of the penetration route across the stratum corneum at a cellular level are scarce. Using a freeze-drying/electron microscopic autoradiography method, Squier & Lesch (5) demonstrated that ^{14}C-cholesterol appeared to be located predominantly in the intercellular compartment of porcine skin stratum corneum. De Haan et al. (6) investigated the potentials of the same method to localize the pathways of ^{3}H-hydrocortisone in human skin stratum corneum. Bidmon et al. (7) were the first to visualize the effect of enhancers on the distribution of ^{3}H-estradiol (^{3}H-E2) in human skin. They also demonstrated the importance of time-resolved visualization studies. The applied dry-mount autoradiography method, however, did not allow cellular distribution studies in the stratum corneum.

We optimized and validated the freeze-drying method to visualize the distribution of ^{3}H-estradiol in human stratum corneum by autoradiography at the light and electron microscopical level (8). We also demonstrated that this visualization method has its limitations regarding the hydration level of the skin (9). In the latter study a matrix type patch appeared to be very suited for localization studies of steroids in human stratum corneum.

The present study was undertaken with a twofold purpose: (a) to elucidate the relationship between percutaneous steroid transport and intracutaneous steroid distribution, and (b) to visualize the transepidermal steroid pathway(s). Unique in this approach was that after the steroid permeation kinetics across a skin sample was assessed, the same sample was processed for the autoradiographical localization of the steroid.

MATERIALS AND METHODS

Materials

[2,4,6,7-^{3}H]Estradiol (SA 14.6 GBq/mg) and [2,6-^{3}H]norethindrone acetate (SA 3.24 GBq/mg) were purchased from Amersham (Buckinghamshire, U.K.); Freon 22 from Hoek Loos (Schiedam, The Netherlands); Osmium tetroxide from Agar Scientific LTD (Stansted, U.K.); Ria Luma from Lumac LSC (Groningen, The Netherlands); Soluene 350 and Toluene Scint from Packard (Meriden, CT, USA); Spurr resin from Bio-Rad/Polaron (Cambridge, MA, USA); 4% Collodion in diethyl ether from Merck (Darmstadt, Germany); Ilford L4 from Ilford Limited (Mobberley, Cheshire, England). All other chemicals used were of analytical grade. All solutions were prepared with purified water (Milli-Q UF Plus Water System, Millipore, Etten-Leur, The Netherlands); Phosphate-buffered saline (PBS) had the following composition: sodium chloride (NaCl) 8 g/l, potassium chloride (KCl) 0.19 g/l, disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) 2.86 g/l, potassium hydrogenphosphate (KH_2PO_4) 0.20 g/l, pH was set to 7.4 with 0.1 N sodium hydroxide (NaOH).

Fresh human abdominal skin was obtained from female donors (51 and 56 years old) after cosmetic surgery. Following prior removal of the subcutaneous fat, the skin was dermatomed at 0.275 mm (Padgett Electro Dermatome model B, Kansas City, Mo, USA). The dermal side of the skin was spread on parafilm. Disks with a diameter of 16 mm were punched and stored at 4 °C in a petri dish on filter paper soaked with PBS. The skin was used the next day for permeation experiments.

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Preparation of the transdermal therapeutic systems

The ^{3}H-E2 and ^{3}H-norethindrone acetate (^{3}H-NETA) containing adhesive solutions for the preparation of the transdermal therapeutic systems were provided by Schwarz Pharma AG (Monheim, Germany), with solid mass fractions (mass of matrix polymer + active steroid) of 39.63% (E2) and 41.50% (^{3}H-NETA). The ^{3}H-E2 and ^{3}H-NETA patches were prepared as follows: 0.6 g of the steroid adhesive solution was diluted with 900 µl toluene spiked with ^{3}H-steroid and poured on backing foil (20 µm, Schwarz Pharma AG) adhering to a glass plate from a "patch-making device" (Erichsen model 509/1, Hemer-Sundwig, Germany). A thin film was made at 6 mm/s with the knife height set at 200 µm (Erichsen knife model 411/220). After overnight air drying the film was covered by a release liner (100 µm, Schwarz Pharma AG). The drug containing matrix with a thickness of 24 ± 1 µm in the dry state (Tesa Micromaster® 0.002 mm, Switzerland), contained either 2.49% (w/w) estradiol (E2) with a molecular ratio ^{3}H-E2:E2 of approximately 1:2660, or 6.15% (w/w) norethindrone acetate (NETA) with a molecular ratio ^{3}H-NETA:NETA of approximately 1:1500. Non-radioactive patches were made with the same specifications.

Permeation experiments

A flow-through permeation setup was designed in such a way that the apparent flux profile would resemble the intrinsic flux profile. This requires minimizing the residence time of ^{3}H-steroids in the permeation cell and fraction collector tube by using a high receptor fluid flow rate, short collecting intervals, a small receiver cell volume, a small collector tubing volume and reporting of the apparent flux from collector vial concentration data at the average time over which it was collected (10). This approach is a prerequisite to be able to accurately compare steroid flux profiles with ^{3}H-steroid distributions in human stratum corneum.

Flow-through polytetrafluoroethylene (PTFE) permeation cells (Fig. 1), modified from Tiemessen et al. (11), with a permeation area of 0.79 cm^2 and a receiver chamber volume of 79 µl, were kept at 32 °C (Haake, model E52, Berlin, Germany). A PTFE tube with a volume of 0.45 ml was used to connect the receiver chamber of the cells to a fraction collector (Retriever II, ISCO), that was set to a sampling frequency of 15 minutes. Fractions were collected in pico "hang in" vials (Packard, Meriden, CT, USA). A peristaltic pump (Ismatec SA, Glattburg-Zürich, Switzerland) fitted with Tygon® pump tubes (type ENE 09, Ismatec SA) was set to a continuous flow of 5.4 ml/hr.

The ^{3}H-steroid-patches were applied to the stratum corneum side of the skin and mounted in the permeation cells while degassed PBS receiver solution (32 °C) was pumped through. The duration of the permeation run was varied between 2, 4, 8, 16 and 24 hours (each permeation experiment was carried out in duplicate). In the latter case, i.e. 24 hours of permeation, the first 16 hours of permeation were with the patch, followed by 8 more hours without the patch. The cells had to be disassembled to pull the patch off. Blank permeation runs were performed with non-radioactive steroid patches (8 hours). The collected receiver solutions were dissolved in Ria Luma and assayed by liquid scintillation counting (Packard Tri-Carb 4640 Liquid Scintillation Counter, Tilburg, The Netherlands).

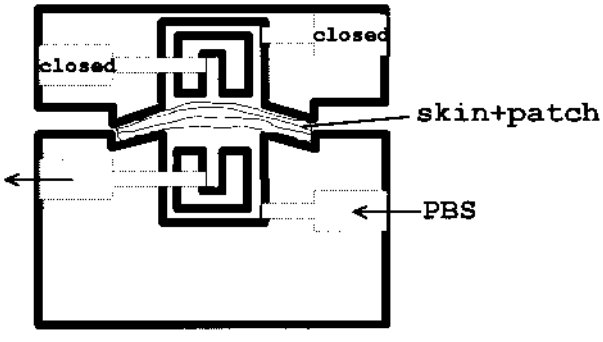

Fig. 1. PTFE flow-through permeation cell.

SAMPLE PREPARATION

Following skin permeation, the patches were carefully removed from the skin. The skin sample was cut into small pieces ($\pm 1 - 1.5 \times 2 - 3$ mm) with a razor blade, immediately cryo-fixed in liquid nitrogen cooled Freon 22 and stored in liquid nitrogen. Seven randomly chosen skin pieces per cell were not frozen, but instead analyzed for total accumulated radioactivity determinations. First, the exposed stratum corneum surface area of the skin pieces was estimated with a microscope equipped with an eyepiece micrometer scale. Then, the samples were dissolved separately in Soluene 350 (3 days at 37 °C), diluted with Ria Luma and assayed by liquid scintillation counting. Thus the surface related activity (defined as the total radioactivity taken up in the skin per unit exposed surface area) of the skin was determined (in dpm/mm^2, see *'Calculation of the autoradiography exposure time'*).

The frozen skin pieces were freeze-dried and resin embedded as follows: Two frozen skin pieces of each permeation cell were transferred into a multi-chamber sample holder and freeze-dried at −80 °C and a vacuum $< 10^{-4}$ Pa. After 16 hours, the sample holder was heated (10 °C/15 minutes) to 0 °C, allowed to reach room temperature (22 hr), closed with a lid while still under vacuum and moved to a desiccator. After evacuation of the desiccator (about 1 Pa), the sample holder lid was removed and the skin pieces were exposed to osmium tetroxide vapor for 3 hours. Residual osmium tetroxide vapor was removed cautiously and freshly prepared complete Spurr medium was dropped on the skin pieces while flushing them with dried nitrogen gas. Following an infiltration under vacuum (30 min), the resin was replaced twice for an infiltration at ambient conditions (2×30 min). Polymerization took place at 60 °C for 48 hours. Diffusion of ^{3}H-steroid into the Spurr medium was verified by liquid scintillation counting, using Toluene Scint cocktail.

AUTORADIOGRAPHY

Both light and electron microscopical (LM and EM) autoradiography were performed according to the "flat substrate" procedure as described by Salpeter (12). Thin sections (± 100 nm) were cut perpendicularly to the skin surface with a diamond knife, picked up and placed on cleaned glass slides or collodion coated glass slides. All different skin pieces under study were placed on the same slide to ensure exactly the same autoradiographical conditions. A thin carbon layer was evaporated on the surface of the sections to prevent interactions with the photographic emulsion (positive chemography and extraction of ^{3}H-steroids). Slides were dipped in a solution of Ilford L4 in water (1:2.5 v/v) at 32 °C and withdrawn at a speed of 84 mm/min (13, 14). After vertical drying at ambient conditions for 1 hour, the slides were placed in black slide boxes containing silica gel and stored at 4 °C. The slides were developed at 20 °C in Kodak D19b (2 min) or Gold-Elon Ascorbic acid developer (GEA, gold latensification 5 min, elon ascorbic acid

5 min) and fixed in a non-hardening fixative (20% $Na_2S_2O_3 \cdot 5H_2O$/2.5% $K_2S_2O_5/H_2O$). For LM autoradiography the slides were examined with a Leitz Orthoplan microscope (Leitz, Wetzlar, Germany) equipped for reflection contrast microscopy (RCM, for details see (15)). For EM autoradiography the ultrathin sections with the accompanying films were floated on water, covered with grids, picked up with parafilm and examined with a JEOL JEM-100S transmission electron microscope at an accelerating voltage of 60 kV.

CALCULATION OF THE AUTORADIOGRAPHY EXPOSURE TIME

In Neelissen et al. (8) an equation was introduced to calculate the autoradiography exposure time for cross sections of the skin. Because the stratum corneum is the region of prime interest, as it is the main barrier to steroid permeation, it became desirable to expose the autoradiographs such that grain densities in the stratum corneum were optimal. To compensate for the higher grain densities in the stratum corneum compared to the rest of the skin, a distribution factor was introduced (8). Thus, the equation became:

$$t_{sc} = \frac{n_{sc} \cdot d_{sk}}{f_{sc} \cdot a_s \cdot \left(1 - \frac{\alpha}{100}\right) \cdot 1440 \cdot d_{se} \cdot s \cdot \sigma} \quad (1)$$

with t_{sc} the calculated autoradiograph exposure time for the stratum corneum (days), n_{sc} the desired grain density over the stratum corneum (grains/mm^2), d_{sk} the thickness of the skin sample (mm), f_{sc} the steroid distribution factor in the stratum corneum, a_s the surface related activity (defined as the total radioactivity taken up in the skin, per unit exposed surface area, in dpm/mm^2), α the isotope loss during sample preparation, 1440 the conversion from minutes to days, d_{se} the section thickness (mm), s the sensitivity of the emulsion/developer combination (grains/disintegration), and σ the relative sensitivity factor, to compensate for ^{3}H-isotope absorption in the sections).

In (9), an E2 distribution factor was determined for human stratum corneum after topical application of the same E2 patch to dermatomed skin *in vitro*: $f_{sc\ E2} = 4.0$. According to Salpeter (12), the optimal (i.e. countable) grain density n_{sc} lies between 10^5 and 5×10^5 grains/mm^2. For $d_{sk} = 0.275$ mm, $d_{se} = 0.0001$ mm, $s = 0.25$ (the Ilford L4 monolayer/Kodak D19b or GEA combination has a sensitivity of 1/3 – 1/5, (12) and $\sigma = 1$ (for 0.0001 mm sections the relative sensitivity is 1, (12)), equation (1) becomes:

$$t_{scE2} = \frac{(1.9 \sim 9.5) \cdot 10^5}{a_s \cdot \left(1 - \frac{\alpha}{100}\right)} \quad (2a)$$

and

$$t_{scNETA} = \frac{(7.6 \sim 38) \cdot 10^5}{f_{scNETA} \cdot a_s \cdot \left(1 - \frac{\alpha}{100}\right)} \quad (2b)$$

Although the E2 distribution factor for stratum corneum was determined under different permeation conditions (Franz-type permeation cells, large stationary acceptor phase), equation (2a) still gives an indication when to develop the autoradiographs. No stratum corneum distribution factors have been determined yet for human skin samples containing ^{3}H-NETA.

RESULTS

Fluxes

The transepidermal fluxes of E2 and NETA are plotted versus time in Fig. 2A + B. Maximum E2 flux was reached after 2 to 4 hours of permeation. After removal of the patch the E2 flux decreased slightly. Maximal NETA flux was reached after approximately 4 hours. After removal of the patch the NETA flux decreased fast. In the first hour after removal of the patch no accurate receiver sample could be collected, because the skin had to be removed from the permeation cell in order to pull of the patch. The cumulative penetrated masses of E2 and NETA are drawn in Fig. 2C + D (note that the same Y-axis range was used).

SURFACE RELATED ACTIVITY AND CALCULATION OF AUTORADIOGRAPHY EXPOSURE TIMES

The surface related activities (dpm/mm^2) of the skin samples at the five predetermined permeation times are shown in Fig. 2C + D, expressed as surface related steroid contents (μg/cm^2). Skin from E2 permeation experiments had taken up much more steroid than "NETA skin", however, the cumulative penetrated E2 mass was lower.

In Table I ^{3}H-E2 autoradiography calculated exposure times are compiled. The optimal exposure time for ^{3}H-E2 autoradiographs can be expected between 56 (2 hr permeation, minimal grain density 10^5 grains/mm^2) and 95 days (16 hr permeation, maximal grain density 5×10^5 grains/

Table I. *Surface specific activities and calculation of autoradiography exposure times for stratum corneum after ^{3}H-E2 and ^{3}H-NETA permeation*

Permeation time (hr)	^{3}H-E2 $a_s \cdot (1-\alpha/100)$[(a)] (dpm/mm^2)	$t_{sc\ E2}$[(b)] (days)	^{3}H-NETA $a_s \cdot (1-\alpha/100)$[(a)] (dpm/mm^2)	$t_{sc\ NETA}$[(c)] (days)
2	3402 ± 607	56~279	704 ± 127	(1085~5398) f
4	4188 ± 743	46~227	723 ± 131	(1057~5256) f
8	9192 ± 1853	21~103	1267 ± 278	(603~2999) f
16	9989 ± 1561	19~95	1704 ± 406	(448~2230) f
24[(d)]	6473 ± 801	30~147	838 ± 257	(912~4535) f

[(a)] surface related activity a_s corrected for isotope loss α (1%); means ± SEM (n = 7). [(b)] and [(c)] see equations (2a) and (2b) respectively; the calculated exposure times predict grain densities in the stratum corneum between 10^5 and $5 \cdot 10^5$ grains/mm^2. [(d)] 16 hours permeation with patch followed by 8 hours without.

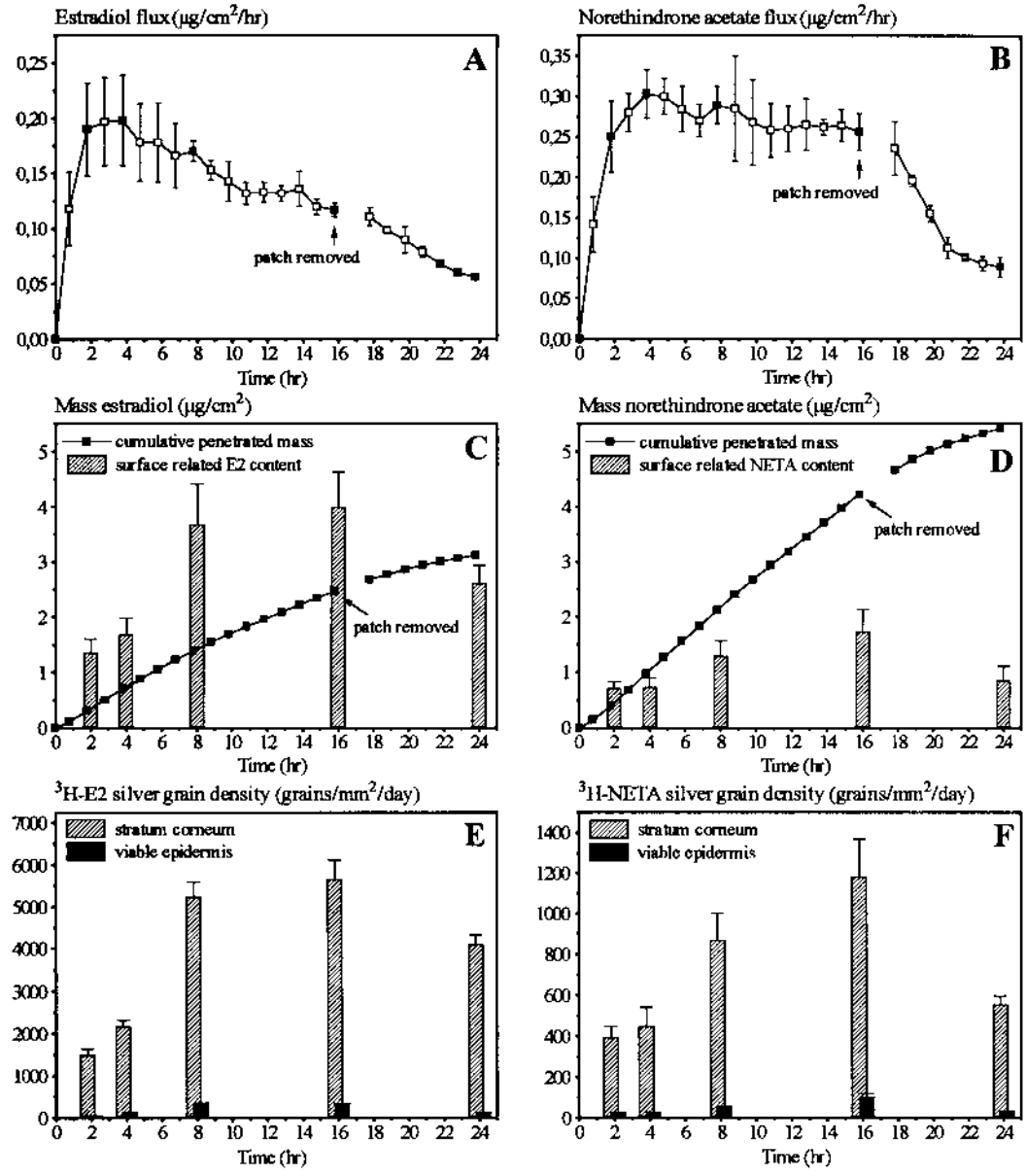

Fig. 2. E2 (A) and NETA flux (B). The curves contain the average flux data from all ten permeation cells (2, 4, 8, 16 and 24 hr, in duplicate). Black squares mark the predetermined hours where each time two permeation cells were disassembled to prepare the skin samples for visualization studies. E2 (C) and NETA (D) cumulative penetrated mass and surface related steroid contents. ^{3}H-E2 (E) and ^{3}H-NETA (F) silver grain densities (divided by the autoradiography exposure time) over stratum corneum and viable epidermis.

mm^2). Assuming that the stratum corneum distribution factor for NETA is of the same order as for E2, optimal ^{3}H-NETA autoradiography exposure times can be expected between 271 (2 hr permeation, minimal grain density 10^5 grains/mm^2) and 558 days (16 hr permeation, maximal grain density 5×10^5 grains/mm^2).

AUTORADIOGRAPHS

LM autoradiographs from ^{3}H-E2 and ^{3}H-NETA permeation experiments are shown in Fig. 3. To support the visual impression of the autoradiographs, the silver grains in stratum corneum and viable epidermis were counted (Fig. 2E+F), showing a good correlation with absolute levels of steroid in the skin (Fig. 2C+D). The number of silver grains in the autoradiographs developed with Kodak D19b did not differ significantly from GEA developed autoradiographs. Only grain counts from EM autoradiographs were used, because grain counts from LM autoradiographs were always lower than from EM autoradiographs, especially in regions with high grain densities. This was expected because grains lying closer to each other than can be optically resolved, will be seen as one grain.

For ^{3}H-E2 autoradiographs, the number of silver grains in the stratum corneum and viable epidermis increased until 8 hours of permeation (Fig. 2E, Fig. 3A–C), and remained more or less constant up to 16 hours (Fig. 2E, Fig. 3C+D). After an elimination phase of 8 hours a considerable amount of silver grains was still present in the stratum corneum, while the silver grain density in the viable epidermis had decreased

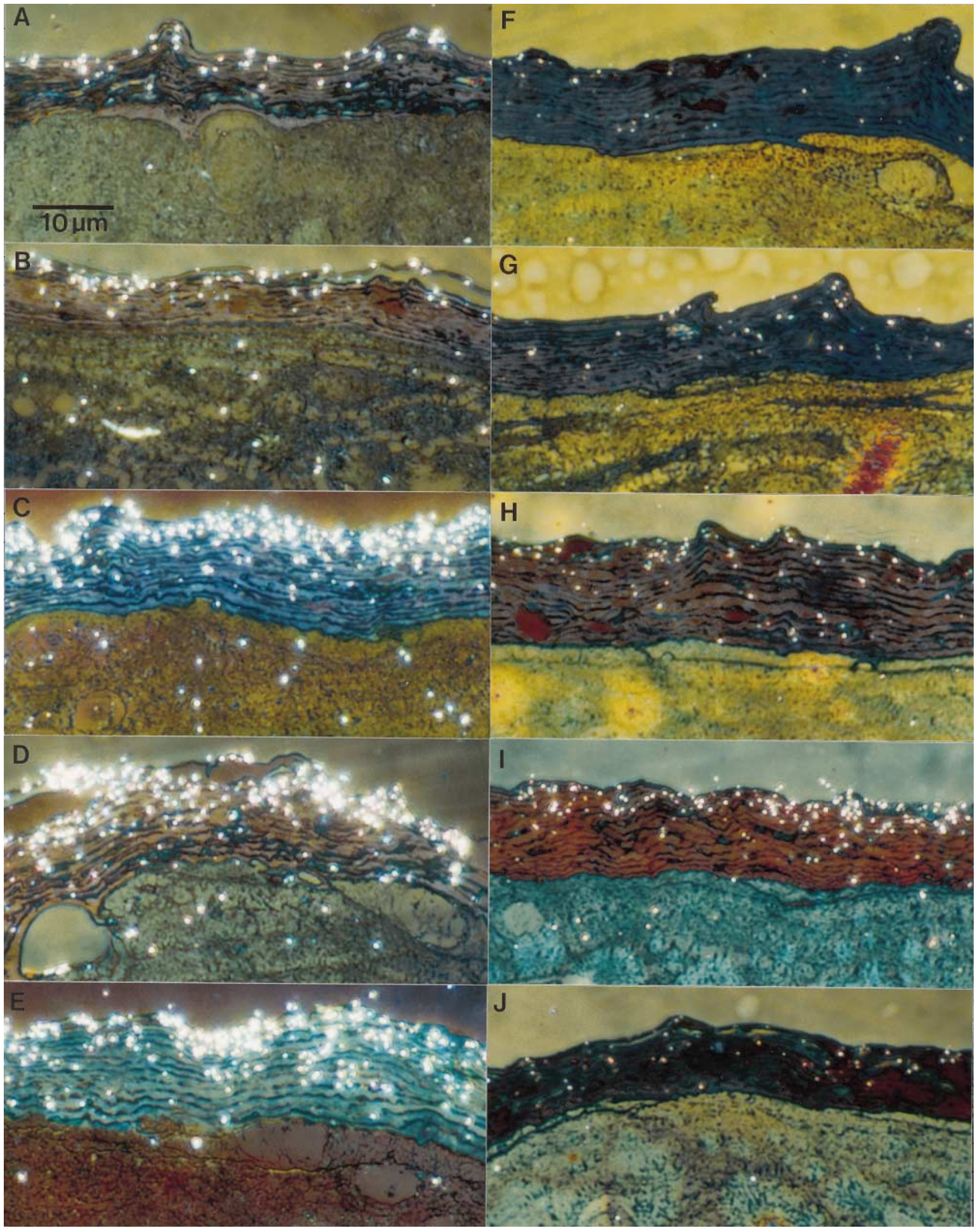

Fig. 3. RCM images of LM autoradiographs of human stratum corneum after ^{3}H-E2 (A – E) and ^{3}H-NETA (F – J) patch permeation experiments, representing intracutaneous distribution of the steroid molecules after 2 (A + F), 4 (B + G), 8 (C + H), 16 (D + I) and 24 hours (16 hours with patch followed by 8 hours without, E + J). ^{3}H-E2 autoradiographs: 103 days of exposure, Kodak D19b development, ^{3}H-NETA autoradiographs: 342 days of exposure, GEA development (1420 ×).

(Fig. 2E + 3E). It is apparent that in all five autoradiographs a gradient of silver grains existed, with the highest grain densities in the superficial layers of the stratum corneum, and the lowest in the basal part of the stratum corneum (Fig. 3A – E). In the viable epidermis, the grain density was always more or less homogeneous. When comparing the autoradiographs with the flux curve (Fig. 2A), it was striking to see that although maximal flux was reached around 2 – 4 hours after patch application, the silver grain density continued to increase considerably up to 8 hours (Fig. 2E). At the cellular level, many of the grains present in the basal part of the stratum corneum were located over or directly adjacent to the intercellular domain. In the superficial part of the stratum corneum the grains were located both over the corneocytes as well as over the intercellular domain. These LM autoradiographical observations were confirmed with EM autoradiography (Fig. 4A + B).

For ^{3}H-NETA autoradiographs, the number of silver grains in the stratum corneum and viable epidermis increased at least until 16 hours of permeation (Fig. 2F, 3F – I). 8 Hours after removal of the patch, the amount of silver grains in both stratum corneum and viable epidermis was greatly reduced (Fig. 2F + 3J). Although steady state was reached around 4 hours after patch application (Fig. 2B), stratum corneum grain accumulation continued up to 16 hours (and possibly longer). A gradient in silver grains, as seen with E2 permeation, developed between 8 and 16 hours of permeation. At the cellular level, the silver grains over the basal part of the stratum corneum were located predominantly over or near the intercellular domain, while over the superficial part silver grains were more or less randomly distributed. EM autoradiographs confirmed these observations (Fig. 4C + D).

The graphs in Fig. 2E + F give the impression that, due to the low grain densities in the viable epidermis, nearly all of the grains were located in the stratum corneum. However, when the thickness' of both layers (stratum corneum 8 – 13 μm, viable epidermis 79 – 113 μm) are taken into account, the amount of steroid in the viable epidermis can add up to 50% of the amount in the stratum corneum.

Grain counts were not performed for the dermis, however its grain density was slightly less than that of the viable epidermis. The total number of grains residing in the dermis, however, may exceed that of stratum corneum and viable epidermis together, because the dermis had swollen considerably during the permeation experiments.

DISCUSSION

The highest steroid fluxes were already observed after 2 – 4 hours (for E2) and 4 hours (for NETA) of permeation, while the highest steroid concentrations in the stratum corneum were found after 8 hours (for E2) and 16 hours or more (for NETA), respectively. A possible cause for this "mismatch" is that the silver grains reflected both transepidermal steroid transport and binding of steroid molecules, while the flux curves resulted from transepidermal steroid transport only. It was apparent from the elimination phase in the permeation experiments that both ^{3}H-E2 and ^{3}H-NETA were retained by the stratum corneum. Several papers mention the existence of a reservoir or depot in the stratum corneum for topically applied steroids (e.g.7, 16,17). This merely refers to the observation often made that the stratum corneum, due to its high content of absorbed steroids, is able to release steroid molecules during an extended period of time after removal of the donor phase. Although this so-called reservoir function can often be explained on the basis of the diffusive and solubility properties of the drug in the stratum corneum alone, drug binding to stratum corneum components may also be involved, especially with steroids. Surber et al. (18) studied the partitioning of drugs, including E2, into isolated human stratum corneum. When the E2 concentration in the donor phase was increased, the stratum corneum/water partition coefficient decreased. This was explained using a "dual sorption model" (19), which postulates the existence of "bound" and "free diffusible" molecules within the stratum corneum. Hence, it is possible that while the unbound steroid fraction in the stratum corneum reached maximum values (corresponding to highest fluxes), binding of the steroids in the stratum corneum still continued until saturation was complete (corresponding to highest silver grain densities).

The amount of steroid retained by skin tissue (stratum corneum, viable epidermis and dermis) was considerable compared to the amount of permeant collected from the receiver chamber. Thus a significant impact on the apparent flux can be expected. A comparable situation was assessed by Liu et al. (20) for E2 applied to hairless mouse skin *in vitro*. They approached this phenomenon, what they called "error in flux measurement caused by permeant retention in skin tissue", with physical models. In our experiments the gradual, continuous decrease in E2 flux, observed after reaching a maximum, may have been caused by continuous withdrawal of free steroids due to ongoing binding to skin components. If we assume that the steroid binding sites were not saturated, steroids could indeed be withdrawn from the free diffusing steroid pool. Because E2 was bound to a much higher extent than NETA, the binding effect on the E2 flux profile was more pronounced. If on the contrary the E2 binding sites were saturated after 8 hours of permeation (i.e. maximal silver grain densities), the decrease between 8 and 16 hours of permeation must have been caused by another factor, e.g. a steroid depletion of the patch. It is apparent that E2 was absorbed to a much greater extend than NETA. As a result the concentration in the E2 patch (2.49% w/w) decreased faster than in the NETA patch (6.15% w/w). After 16 hours of permeation the total E2 concentration in the patch was reduced by 10.9%, while in the NETA patch only by 3.9% (determined with liquid scintillation counting).

The difference between E2 and NETA elimination from the skin samples after removal of the patch is explained by the fact that less free NETA was present in the skin resulting in a steep decline in flux. After 22 hours, the flux is then only due to the slow release of bound steroid molecules. Especially for E2 skin samples, a long elimination phase can be expected due to its highly bound E2 content.

The important question to solve is which part of the silver grains in the autoradiographs derived from unbound steroids and which part from free diffusing steroids. Both E2 and NETA were bound in great quantities, indicating that a significant part of the silver grains must have originated from bound ^{3}H-steroid molecules. Because for NETA both a higher flux and lower steroid concentration in the skin were found, the highest ratio unbound:bound molecules can be expected, especially in the early phase of permeation (after 2 and

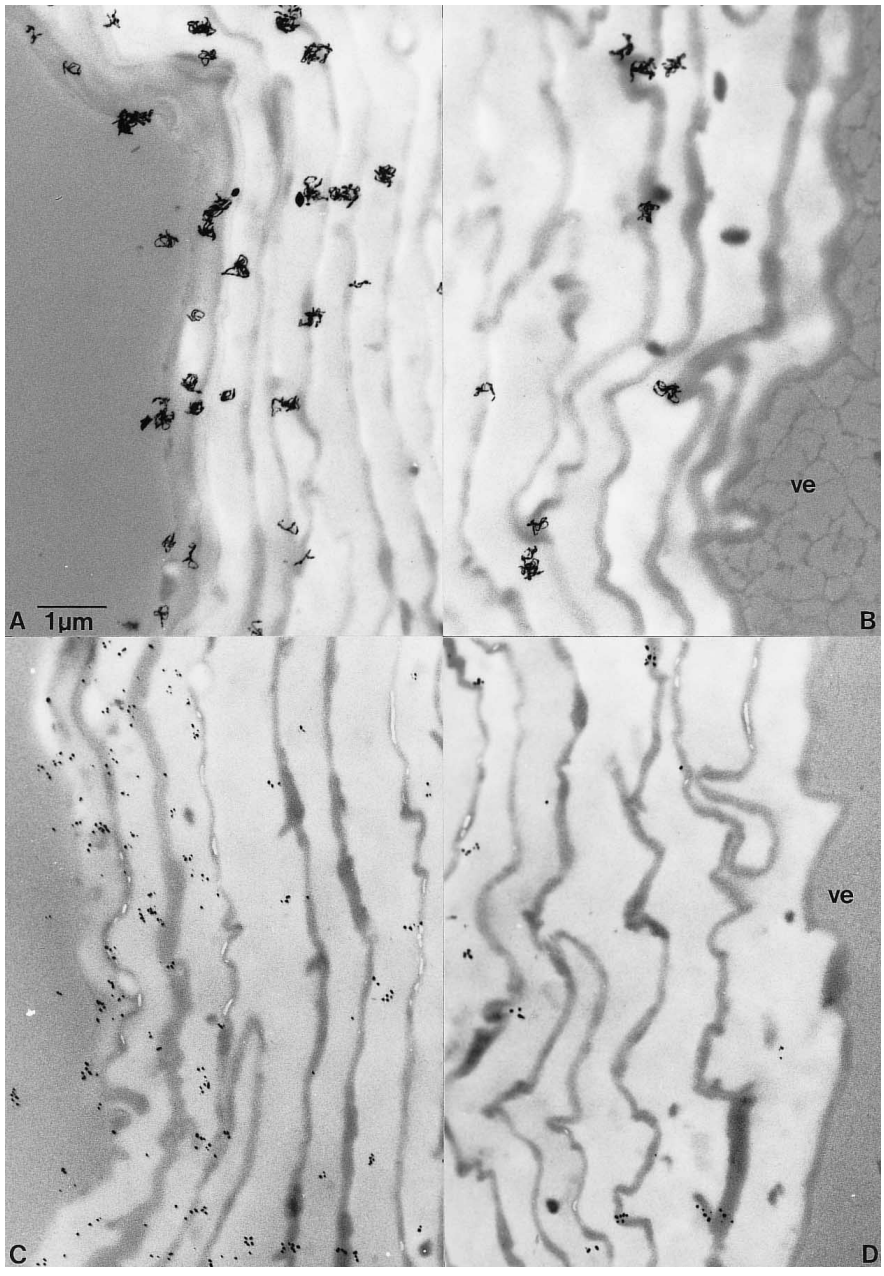

Fig. 4. Electron microscopical autoradiographs of human stratum corneum after 8 hours absorption from a ^{3}H-steroid patch. ^{3}H-E2 distribution in the superficial (A) and basal part (B) of the stratum corneum (103 days of exposure, Kodak D19b development), and ^{3}H-NETA distribution in the superficial (C) and basal part (D) of the stratum corneum (342 days of exposure, GEA development); ve = viable epidermis.

4 hours). However, as long as this ratio is not known, the autoradiographs have to be interpreted with reservation. A possible way to solve this problem would be an experiment, where a non-radioactive patch is applied to the skin for 16 hours, then replaced with a radioactive patch for several hours, followed by visualization. Despite these uncertainties, the localization of the silver grains in the basal part of the stratum corneum, i.e. mainly over or close to the intercellular lipid domain, indicated that both transport and binding took place in the intercellular lipids.

Because so much steroid is bound in the skin tissue, a penetration enhancing effect may be achieved by just inhibiting steroid binding sites. It makes you wonder if the effect of steroid penetration enhancers may (partly) be derived from interactions with steroid binding sites.

The use of immunohistochemistry may be an alternative to autoradiography. The better resolution in immunolabeling as compared to autoradiography can be taken advantage of thus allowing a more precise detection of steroids in the intercellular spaces of the stratum corneum. Another advantage is that the immunolabeling procedure is a faster and easier procedure than autoradiography. The compatibility between tissue freeze-drying, osmium tetroxide fixation, resin embedding and estradiol antibody reactivity is being investigated.

CONCLUSION

Permeation and visualization studies were performed on the same skin samples, to elucidate the ^{3}H-E2 and ^{3}H-NETA penetration route across human stratum corneum *in vitro*. The confrontation between cumulative penetrated steroid mass and total steroid skin content after different times of permeation revealed that considerable amounts of steroid were bound to the skin tissue, i.e. stratum corneum, viable epidermis and dermis. Hence, the silver grains in the autoradiographs originated from both free and bound steroids. Nevertheless, an accurate localization of the steroids was found in the basal part of the stratum corneum, i.e. preferentially over or close to the intercellular lipid domain, indicating an intercellular penetration route across the stratum corneum. Hence, this approach is useful to study the effect of penetration enhancers on the distribution of the steroids.

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