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Visualizing drug transport across stratum corneum: cryotechniques, vapour fixation, autoradiography

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Summary

The potentials of a method for visualizing the transport of drugs across human stratum corneum at electron microscopic level were investigated. Possible extraction and/or dislocation of the drug during sample preparation for electron microscopy was reduced as much as possible. In this "dry" sample preparation drug-loaded human skin was rapidly frozen, freeze-dried, osmium tetroxide vapour fixed, and embedded in Epon under vacuum. Visualization of the drug was achieved by using electron microscope autoradiography (general applicability). The model compound in the autoradiography studies was tritiated hydrocortisone. No detectable extraction of drug was measured during the "dry" sample preparation. Furthermore, this method preserved the ultrastructure of stratum corneum very well. The combination of the "dry" sample preparation and electron microscope autoradiography was feasible and there were no problems regarding the detection limit; the only remaining problem was an unsatisfactory resolution. However, considering the dimensions of the inter- and intracellular domains and the resolution attainable for electron microscope autoradiography, it should be possible to discriminate between intercellular and transcellular drug transport across stratum corneum.

Introduction

The stratum corneum is recognized to be the most predominant diffusional barrier for transdermal drug delivery. Skin penetration enhancers, being compounds which increase drug transport across skin, usually interact with components of the stratum corneum (Barry, 1987; Cooper and Berner, 1987). Although many speculations have been made in this respect (e.g. Kurihara-Bergstrom et al., 1986; Morimoto et al., 1986; Barry and Bennett, 1987; Golden et al., 1987; Beastall et al., 1988; Hoelgaard et al., 1988; Sato et al., 1988) the pathways of diffusion across the stratum corneum (intercellular, transcellular) and the modes of action of skin penetration enhancers still have to be clarified for many cases.

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For that purpose, visualization of drug transport across the stratum corneum seems to be a logical and straightforward approach. However, usually this type of research is hampered by difficulties encountered in localizing diffusible substances on the electron microscopic level. These problems are mainly concerned with dislocation and/or extraction of the drug during sample preparation.

In the very few papers on this subject (e.g. Silberberg, 1968, 1971; Nemanic and Elias, 1980; Sharata and Burnette, 1988; Boddé et al., 1988) the problems regarding sample preparation were still not circumvented completely. The diffusible substances were localized by chemical precipitation and in some of these studies X-ray microanalysis (transmission electron microscope mode) was employed in conjunction with ultrastructural studies to determine the elemental composition of localized regions of the tissue. In this way Sharata and Burnette (1988) obtained a spatial resolution of 0.5–0.75 μm. As already stated by these authors, localization of diffusible compounds by freezing techniques is preferable to chemical precipitation. Several studies (e.g. Forslind, 1984; Forslind et al., 1985; Grundin et al., 1985) employed "cryo" techniques in conjunction with X-ray microanalysis for localization of native electrolytes within the epidermis or for localization of topically applied ions. Although satisfactory results were obtained, most of these studies employed X-ray microanalysis in the scanning electron microscope mode using thick specimens, which resulted in a poor spatial resolution; e.g. Grundin et al. (1985) reported a spatial resolution of 10–12 μm.

However, X-ray microanalysis is only applicable for a small range of compounds. At the present stage of technical development the method of choice for visualization of compounds diffusing across stratum corneum most likely is electron microscope autoradiography, since (almost) all drugs and penetration enhancers can be tritiated. The combination of "cryo" techniques (for excluding extraction during sample preparation) and autoradiography seems a rational approach.

At the light microscopic level several reliable techniques for the autoradiographic localization of diffusible substances are available. In these techniques a photographic emulsion is brought into contact with a frozen hydrated or freeze-dried cryosection (for review see Stumpf, 1976). However, on the electron microscopic level (our aim) severe practical problems exist (Tokuyasu, 1980; Robards and Sleytr, 1985). Although some authors (Christensen and Paavola, 1972; Baker and Appleton, 1976; Johnson and Bronk, 1979) have described the analysis of such ultrathin sections by autoradiography, still the risk of rehydration (and consequently dislocation of diffusible compounds) is substantial (e.g. during transfer to the electron microscope, during application of a photographic emulsion, during transfer to the carbon coating equipment).

A promising method for avoiding dislocation/extraction during sample preparation for electron microscope autoradiography can be derived from the work of Stirling and Kinter (1967). As starting material, these authors used rapidly frozen unfixed tissue (rings of hamster intestine), which was subsequently freeze-dried, fixed with osmium tetroxide vapour and embedded in Epon. Frederik and Klepper (1976) and Frederik et al. (1977) used essentially the same technique for localizing tritiated steroids in testes on the electron microscopic level.

The aim of this research is to investigate whether the combination of electron microscope autoradiography and "dry" sample preparation (rapid freezing, freeze-drying, osmium tetroxide vapour fixation, Epon embedding under vacuum) is suited for the localization of diffusible substances in the stratum corneum. Tritiated hydrocortisone is used as a model drug. Essential items to be considered are preservation of the ultrastructure of the stratum corneum, possible extraction/dislocation and the detection limit.

Materials and methods

Chemicals

Only distilled water was used. Sodium bromide used for separation of the epidermis from dermis, alcohol, dimethyl sulphoxide and all buffer salts were analytical grade. Freon 22 was purchased from Hoek Loos (Schiedam, The Netherlands).

For tissue fixation, osmium tetroxide (Merck, Darmstadt, F.R.G.), glutaraldehyde (Taab, Aldermaston, U.S.A.) and potassium ferrocyanide (analytical grade) were used. The epoxy resin components necessary for Epon embedding were supplied as LX-112 Resin kit by Ladd Research Industries (Burlington, U.S.A.). Propylene oxide (99.6%) was obtained from Janssen (Beerse, Belgium). All chemicals used in the development and fixation of the autoradiographs and the post-staining reagents were analytical grade. Hydrocortisone (Ph. Eur.) was supplied by OPG (Utrecht, The Netherlands). 3,3′,5′-Triiodo-D-thyronine (free acid) was obtained from Sigma (St. Louis, U.S.A.). [1,2-^{3}H]Hydrocortisone with a sp. act. of 51.9×10^3 Ci/mol was obtained from New England Nuclear Corporation (Boston, U.S.A.) in solution in ethanol 10% v/v and benzene 90% v/v. Before use, the radiochemical purity was tested by thin-layer chromatography.

Conventional "wet" sample preparation

Skin samples of 2 mm diameter were punched. Chemical fixation was performed in a 1.5% w/v glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) for 4 h at 4°C. After rinsing these samples 2 times (5 min each) in 0.1 M cacodylate buffer (pH 7.2) post-fixation was performed for 4 h at 4°C in a 1% w/v osmium tetroxide solution in 0.1 M cacodylate buffer (pH 7.2) containing 0.05 M potassium ferrocyanide. After again 2 rinses in the buffer, dehydration was carried out in graded ethanol series at room temperature. Then the specimens were immersed in propylene oxide for 30 min, followed by immersion in a mixture of propylene oxide and non-accelerated Epon (1:1 v/v) for 30 min. Further embedding was carried out with an Epon mixture according to Luft (1961). Polymerization took place for 48 h at 60°C.

"Dry" sample preparation

The method described by Frederik and Klepper (1976) was slightly adapted in the following manner. Skin samples of 2 mm diameter were punched, and rapidly frozen by plunging into solid/liquid Freon 22. These samples were transferred under liquid nitrogen into a specially designed sample-holder, which subsequently was mounted in a Balzers Freeze Etching system BAF 400 d (Balzers, Liechtenstein). The stage for the sample holder was thermostated at −80°C and the lid of the sample-holder hung on a pin which was attached to the knife of the Balzers, which was kept at < −150°C and served as a cold trap. After evacuation of the Balzers and positioning of the cold knife just above the sample-holder the freeze dry run was started. This run took 15 h under the following conditions: sample temperature was −80°C, cold trap temperature was < −150°C, the vacuum was $< 10^{-6}$ torr. Drying was completed by slowly increasing the sample temperature up to 30°C. Finally the sample-holder was covered with the lid by manipulation of the knife and dry nitrogen was admitted to the chamber.

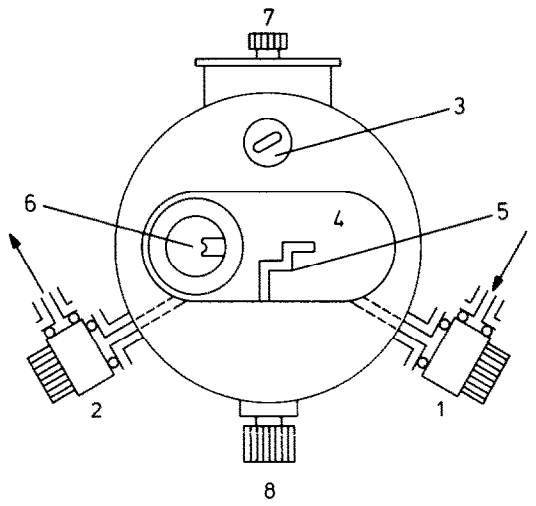

Fig. 1. Desiccator for vapour fixation and embedding under vacuum (schematically). Key: 1 = vacuum valve 1; 2 = vacuum valve 2; 3 = osmium chamber under screw; 4 = working chamber; 5 = mechanism for lifting lid of sample holder; 6 = mounting place for sample-holder; 7 = handle for controlling osmium inlet; 8 = handle for controlling lifting lid of sample-holder.

The sample-holder containing the dry samples in vacuo was dismounted and transferred to a specially designed desiccator where it was mounted again (see 6 in Fig. 1). The desiccator (see Fig. 1) was covered with a lid and the working chamber was evacuated (vacuum valve 2 opened and by using a vacuum pump). By using a manipulator (5

in Fig. 1) the lid of the sample-holder was lifted and removed. Osmium tetroxide vapour was admitted to the working chamber by opening a side port (using 7 in Fig. 1) between a chamber under a screw containing osmium tetroxide crystals (3 in Fig. 1) and the working chamber (4 in Fig. 1). Vapour fixation was carried out for 20 h at room temperature. Fixation was terminated by pumping away the osmium tetroxide vapour and the working chamber was evacuated again.

Non-accelerated Epon was injected through a septum in the desiccator lid onto the samples in the sample-holder using a syringe. This vacuum impregnation took 4 h. Subsequently the working chamber was flushed with dry air (vacuum valve 1 in Fig. 1 opened) and evacuated again several times to improve impregnation. Further embedding and polymerization were carried out in the same way as described for the conventional sample preparation after the propylene oxide/Epon immersion.

Essential backgrounds of the "dry" sample preparation

Plunging of small tissue blocks into solid/liquid Freon 22 causes vitrification of the samples over a range of about 10–20 μm (Elder et al., 1981), meaning that in this region water solidifies as an amorphous glass and the formation of ice crystals is avoided. During ice crystal formation solutes are separated from ice, causing dislocation of all solutes including diffusing drugs. Ice crystal formation may also cause ultrastructural artefacts. During freeze-drying the sample temperature is maintained at $-80\,^{\circ}C$; at higher temperatures growth of ice crystals may obscure the results, while at lower temperatures the freeze-drying is too slow. At $-80\,^{\circ}C$ the vapour pressure of water is 4×10^{-4} torr.

In the manipulations following a freeze-dry run extreme care is taken to prevent rehydration of the hygroscopic tissue and resulting artefacts (ultrastructure, dislocations). The subsequent dry osmium tetroxide vapour fixation fixes the tissue, renders contrast for microscopy in the specimen and anchors some diffusible substances (e.g. steroids) to the tissue.

TABLE 1

Relevant steps in the "dry" and the conventional sample preparation

"Dry" method	Conventional method
Rapid freezing (physical fixation)	Fixation in aqueous glutaraldehyde solution
Freeze-drying (physical dehydration)	Postfixation in aqueous osmium tetroxide solution
Osmium tetroxide vapour fixation	Dehydration in graded alcohol series
In vacuo impregnation in Epon	Impregnation in propylene oxide and propylene oxide/Epon
Epon embedding	Epon embedding

During sample preparation liquids may act as solvents and cause extraction or dislocation of the diffusing drug. From Table 1 it is evident that in the conventional sample preparation several "wet" steps, including the use of various excellent solvents, are involved. In the "dry" sample preparation the only remaining "wet" step, and so the only possibly extractive step, is the embedding in Epon. Compounds, soluble in Epon (a poor solvent) and not anchored to the tissue by osmium tetroxide vapour, may be extracted to some extent. When this would be the case two alternatives exist for reducing or preventing extraction: the use of another embedding agent (not or less extractive for the compound under study) and/or the use of another vapour fixative.

Microscopy

Except for the sections to be analyzed by X-ray microanalysis (described in a separate paragraph) all sections were cut on a Reichert Om U_2 microtome using dry glass knives and were examined in a Philips EM 201 electron microscope (about 100 nm thick sections) or under a Zeiss Photomicroscope I (about 1 μm thick sections for light microscopy).

Autoradiography

For light microscope autoradiography the sections were mounted on glass slides and covered with a layer of carbon (dark grey/brown). The

photographic emulsion was applied by dipping in Ilford L4 (Ilford, Mobberley, Cheshire, U.K.) prepared at a dilution of 1 : 1. After drying, the slides were stored at 4 °C in a light-tight box containing silica gel.

For electron microscope autoradiography the sections were collected on collodion-coated copper grids and covered with a light grey layer of carbon. The emulsion layer was applied using the loop method (Caro and Van Tubergen, 1962); the wire loop enclosed a fully gelled film of Ilford L4 prepared at a dilution of Ilford L4:water = 1 : 2 (v/v). Exposure took place at 4 °C in light-tight boxes containing silica gel. Development and fixation for both light and electron microscope autoradiography were performed at 20 °C as follows: developing in D19 developer (3 min), rinsing in water, fixation in sodium thiosulphate 24% (w/v), and rinsing 3 times in water.

The background grain density and the possible occurrence of negative chemography (latent image fading) and/or positive chemography were determined according to standard procedures (Williams, 1977).

X-Ray microanalysis

For X-ray microanalysis 80–100 nm sections were cut on a LKB microtome using a diamond knife. The sections were collected on collodion-coated copper grids. All specimens were investigated in a Philips EM 400 transmission electron microscope (twin objective lens system), fitted with a scanning attachment (STEM), a Tracor Northern X-ray detector (Be-window) and multichannel analyzer, and computer system DEC LS L/23. The specimen tilt angle was 18° towards the detector. Spectra were obtained at an accelerating voltage of 80 kV, a counting time of 100 s from a spot size of 400 nm.

Experiments for determining effects of the "dry" sample preparation on the ultrastructure of stratum corneum

Fresh human abdomen skin (female, 56 years of age), received from the hospital, was split with a dermatome (Padgett Electro Dermatome model B set) at 200 μm, and prepared for electron microscopy using both the described conventional "wet" and our "dry" method. The sections were post-stained: 10 min in 7% aqueous uranyl acetate solution and after washing with water 5 min in lead hydroxide (0.2% w/v lead citrate in 0.1 N NaOH).

In vitro diffusion experiment (X-ray microanalysis)

In vitro diffusion experiments were performed using fresh human abdomen skin, which was dermatomed to 300 μm thickness. The dermatomed skin was placed on the bottom of a PTFE holder (stratum corneum facing upwards). A glass tube (internal diameter: 1.0 cm) was pressed onto this skin and was clamped into the PTFE holder. This glass tube was filled with 2.0 ml of the donor solution, which was a 2.0% w/v triiodothyronine (model compound) solution in dimethyl sulphoxide:water (9 : 1 v/v). Diffusion was allowed to occur for 36 h at 32 °C, while the donor solution remained stationary.

After the diffusion experiment the skin was rinsed for 3 s in a 0. 1 M cacodylate buffer (pH 7.2), in this way substituting partly the dimethyl sulphoxide by water and enabling rapid freezing and freeze-drying. Subsequently the tissue was prepared for electron microscopy according to 2 alternatives: the conventional sample preparation and the "dry" sample preparation. Finally the samples were analyzed by X-ray microanalysis.

In vitro diffusion experiment (autoradiography)

Fresh human abdomen skin was immersed in a 2 N aqueous sodium bromide solution for 2 h at 37 °C in order to separate the epidermis from the underlying tissue. The epidermis was then sandwiched between 2 cellulose acetate 0.8 μm filters (Millipore, Molsheim, France), which served as support. This sandwich was clamped between the two halves of a PTFE diffusion cell. The stratum corneum side faced the donor solution (above the sandwich), which was 1.35 ml of a 5.4×10^{-4} M hydrocortisone solution in phosphate-buffered saline (pH 7.4) containing 36 μCi/ml of [1,2-^{3}H]hydrocortisone (determined with liquid scintillation counting). The acceptor compartment (beneath the sandwich) contained 1.35 ml of phosphate-buffered saline. The diffusion area was a

circle with a radius of 0.8 cm. Stationary diffusion was allowed to take place for 48 h at 32 °C.

Subsequently the epidermis was prepared for microscopy according to the "dry" sample preparation. The non-accelerated Epon used for the immersion and impregnation under vacuum of the rapidly frozen, freeze-dried and osmium tetroxide vapour fixed tissue was collected and the possible presence of extracted [1,2-^{3}H]hydrocortisone was determined by liquid scintillation counting, upon adding Dynagel scintillation fluid (Baker, Deventer, The Netherlands), using a Packard Tricarb 4640 Liquid Scintillation Counter. Quenching was corrected for by the external standard method.

Finally autoradiography was performed as described.

Results and Discussion

Experiments for determining effects of the "dry" sample preparation on the ultrastructure of stratum corneum

Fig. 2 shows an electron micrograph of the skin following the conventional sample preparation, while Fig. 3 shows the equivalent for our "dry" sample preparation. In comparing effects of both sample preparations on stratum corneum ultrastructure as regards the questions to be answered using autoradiography, the most striking features were:

- both methods allowed clear discrimination between inter- and intracellular domains. This is an absolute requirement for discriminating be-

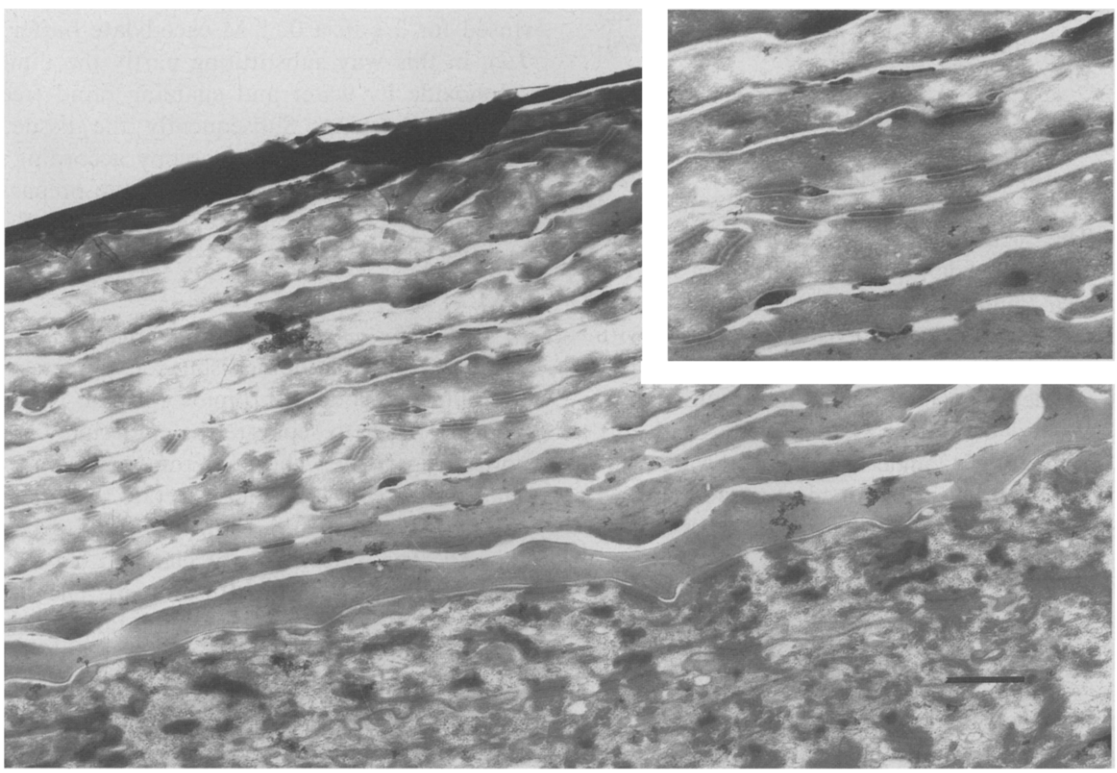

Fig. 2. Electron micrograph of human stratum corneum and some of the underlying viable epidermis obtained after the conventional sample preparation and post-staining with uranyl acetate and lead hydroxide; bar = 1 μm. Insert in right upper corner: 1.75 times magnification of central part of the micrograph.

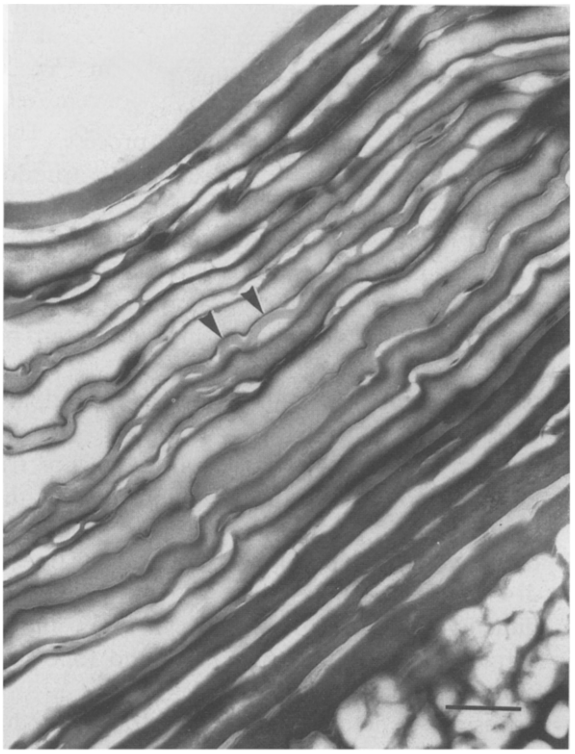

Fig. 3. Electron micrograph of human stratum corneum and some of the underlying viable epidermis obtained after the "dry" sample preparation. The arrowheads indicate intercellular material; bar = 1 μm.

tween intercellular and transcellular pathways for drug transport across stratum corneum;

- the "dry" method preserved more intercellular material than the conventional sample preparation. Lipids are very relevant constituents of the intercellular materials and probably are very essential to drug transport across stratum corneum (Elias, 1981; Bowser and White, 1985). From literature (Stein and Stein, 1971) it is known that conventional procedures for fixation, dehydration and embedding cause significant extraction of lipids and lipid like substances;
- the "dry" sample preparation preserved more layers of intact stratum corneum (average of 20) than did the conventional method (average of 13). The most logical explanation is that the "wet" steps in the conventional method extract intercellular material and "loosen" the stratum corneum layers; and
- the "dry" sample preparation completely disrupted the ultrastructure of the viable epidermis, while this was left virtually intact by the conventional method. Plunging into solid/liquid Freon 22 causes vitrification to occur only over a range of 10–20 μm (Elder et al., 1981); stratum corneum thickness is within this region. Stratum corneum has only a low water content, making it less vulnerable to the formation of ice crystals and other freezing artefacts than other tissue. In future experiments alternative (and better) methods for rapid freezing will be tested for improving the ultrastructural preservation of the viable epidermis.

From these results it can be concluded that the "dry" sample preparation preserved the ultrastructural features of the stratum corneum relevant to the questions to be answered by electron microscope autoradiography better than the conventional sample preparation, but had damaging effects on the viable epidermis.

In vitro diffusion experiment (X-ray microanalysis)

X-Ray spectra of intercellular domains in stratum corneum following the triiodothyronine diffusion experiment are shown in Figs. 4 and 5 for the

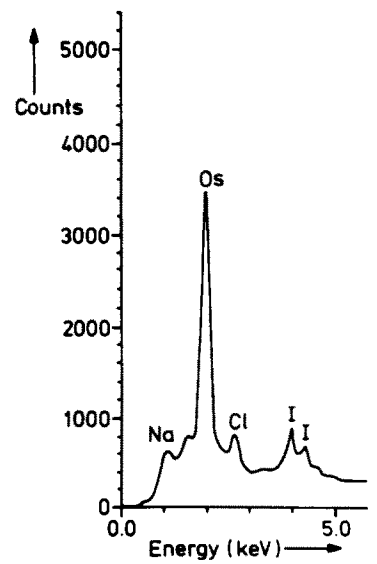

Fig. 4. X-Ray emission spectrum of intercellular domains in stratum corneum obtained after the triiodothyronine diffusion experiment and the conventional sample preparation.

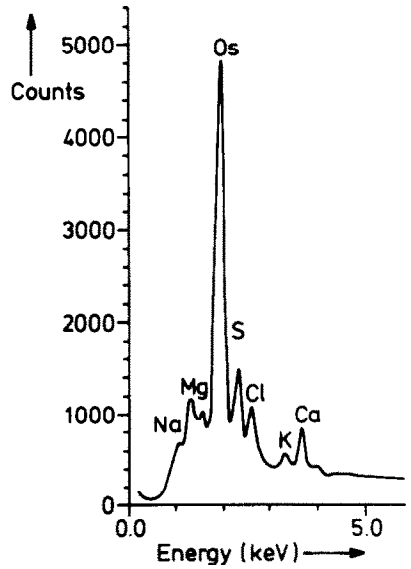

Fig. 5. X-Ray emission spectrum of intercellular domains in stratum corneum obtained after the triiodothyronine diffusion experiment and the "dry" sample preparation.

conventional "wet" and our "dry" sample preparation, respectively. The relevant peaks and their corresponding origins are presented in Table 2.

The results for the "wet" and "dry" sample preparation differ significantly. Obviously dimethyl sulphoxide is extracted completely during the "wet" sample preparation (no sulphur peak in Fig. 4). The prominent sulphur peak in Fig. 5 indicates that dimethyl sulphoxide is not removed (completely) by the freeze-drying process. The iodine peaks in Fig. 4 indicate that triiodothyronine is anchored to the tissue by glutaraldehyde ($-NH_2$ group crosslinking) in such a way that it is preserved in stratum corneum (at least partly) during the "wet" sample preparation. The absence of iodine peaks in Fig. 5 can be explained as follows:

- triiodothyronine is soluble in dimethyl sulphoxide;
- osmium tetroxide vapour does not anchor triiodothyronine nor dimethyl sulphoxide to the tissue;
- dimethyl sulphoxide is not removed by freeze-drying (see above);
- dimethyl sulphoxide is soluble in Epon and is extracted during the embedding and following polymerization; and
- diffusing dimethyl sulphoxide drags triiodothyronine into the Epon.

Dimethyl sulphoxide was used because the aqueous solubility of triiodothyronine was too low to obtain detectable amounts (X-ray microanalysis of iodine) in stratum corneum. It should be concluded that the use of 90% v/v dimethyl sulphoxide in the donor solution is an inappropriate alternative for visualizing triiodothyronine transport across stratum corneum by X-ray microanalysis in combination with our "dry" sample preparation.

The data in Table 2 indicate that native water-soluble Ca, K and Mg ions were extracted during the conventional sample preparation, while these ions were preserved (at least partly) during our "dry" sample preparation. The latter result implies that these water-soluble ions were not extracted by the lipophilic Epon, despite the fact that these ions are not fixed chemically by the osmium tetroxide vapour. This finding suggests that compounds that are insoluble in Epon are not extracted during the "dry" sample preparation, even when these compounds are not anchored to the tissue by osmium tetroxide vapour.

Again in these experiments it appeared that the

TABLE 2

Interpretation of X-ray emission spectra in Figs. 4 and 5

Energy (keV)	Element	Peak	Sample preparation		Origin
			"wet"	"dry"	
1.0	Na	Kα	present	present	stratum corneum
1.3	Mg	Kα	absent	present	stratum corneum
1.9	Os	Mα	present	present	osmium tetroxide
2.3	S	Kα	absent	present	dimethyl sulphoxide
2.7	Cl	Kα	present	present	stratum corneum
3.3	K	Kα	absent	present	stratum corneum
3.7	Ca	Kα	absent	present	stratum corneum
4.0	J	Lα	present	absent	triiodothyronine
4.3	J	Lβ	present	absent	triiodothyronine

"dry" sample preparation preserved more intact stratum corneum layers (average of 21) than did the conventional sample preparation (average of 12).

In vitro diffusion experiment (autoradiography)

Liquid scintillation counting of the non-accelerated Epon used for the impregnation of the [1,2-^{3}H]hydrocortisone loaded, rapidly frozen, freeze-dried and osmium tetroxide vapour fixed tissue showed that no detectable amount of radioactivity was present in the Epon. Following osmium tetroxide vapour fixation, apparently hydrocortisone is not extracted during the vacuum embedding in Epon. Osmium tetroxide vapour anchors steroids to the tissue and in this way prevents diffusion of hydrocortisone into the lipophilic Epon. Several other workers studied the extraction of steroids from rapidly frozen, freeze-dried and osmium tetroxide vapour fixed tissue during Epon embedding. Attramadal (1969) found for [^{3}H]oestradiol 0.25% extraction of label from liver tissue for 24 h infiltration in Epon at 50 °C and 1.53% extraction from uterus tissue for 48 h infiltration. In the same paper, for [^{3}H]testosterone 0.19–0.35% extraction of label from liver tissue for 48 h infiltration in non-accelerated Epon was reported. Frederik and Klepper (1976) reported that the loss of labelled steroids in Epon was lower than 1%; in their study they used testis tissue, oestradiol, testosterone and dehydroepiandrosterone.

Light microscope autoradiography showed that after 11 days of exposure silver grains could be developed. Separate experiments indicated that background grain density was very low, and that no positive or negative chemography had occurred. Apparently the applied carbon layer protects against positive chemography and negative chemography (osmium tetroxide). The carbon layer also prevents diffusion of label into the emulsion as the grain density over the Epon in the autoradiographs was identical to the background grain density.

Light microscopic autoradiographs showed that the silver grains were concentrated over the stratum corneum. These grains were numerous, and because only very few silver grains were recorded over the Epon at the margins of the tissue, it can be deduced that diffusion of [1,2-^{3}H]hydrocortisone from the tissue into the Epon during the polymerization did not occur (or occurred to an extremely low extent).

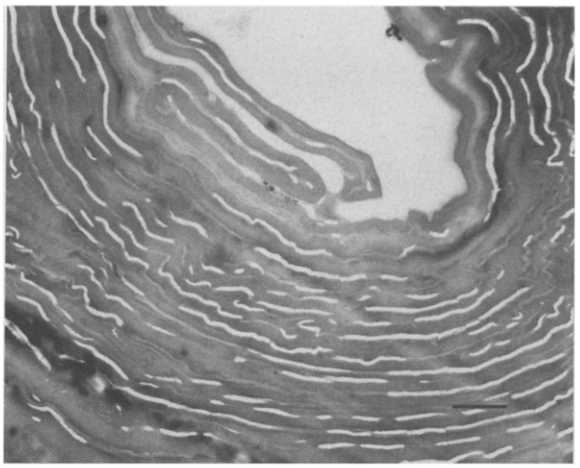

Fig. 6. Electron microscope autoradiograph obtained after a blank diffusion experiment, the "dry" sample preparation, using Ilford L4 emulsion, 10 weeks of exposure and using D19b developer; bar = 2 μm.

Fig. 6 shows an electron microscope autoradiograph obtained after a blank diffusion experiment (in the absence of tritiated hydrocortisone). It can be seen that the background grain density was extremely low and that positive chemography did not occur. Separate experiments indicated that negative chemography did not occur either.

Figs. 7 and 8 show electron microscope autoradiographs developed after 10 weeks of exposure at 4 °C. It can be seen that the ultrastructure of stratum corneum is clearly recognizable and that the developed grains are clearly projected on the underlying tissue. This indicates that from this point of view the combination of the "dry" sample preparation and electron microscope autoradiography is feasible.

Figs. 6–8 confirm the findings described earlier regarding the influences of the "dry" sample preparation on the ultrastructure of stratum corneum: inter- and intracellular domains can clearly be discriminated, intercellular material can be detected, and many intact layers of stratum corneum are preserved.

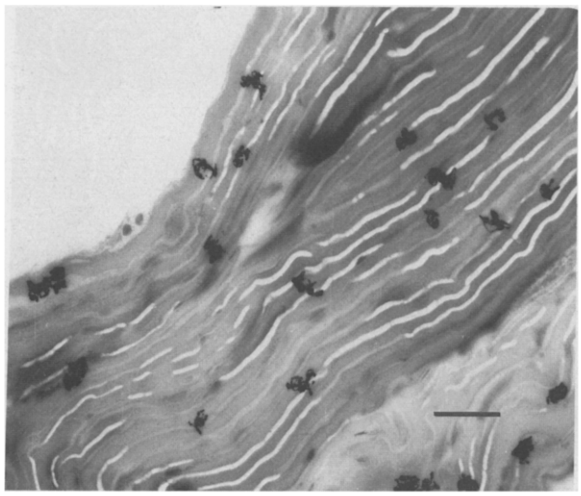

Fig. 7. Electron microscope autoradiograph obtained after the diffusion experiment with tritiated hydrocortisone, the "dry" sample preparation, using Ilford L4 emulsion, 10 weeks of exposure and using D19b developer; bar = 2 μm.

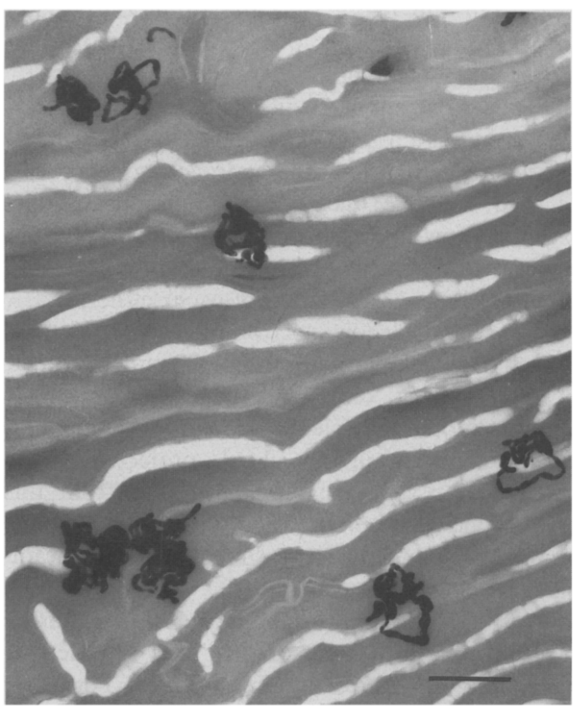

Fig. 8. Electron microscope autoradiograph obtained after the diffusion experiment with tritiated hydrocortisone, the "dry" sample preparation, using Ilford L4 emulsion, 10 weeks of exposure and using D19b developer; bar = 1 μm.

Figs. 7 and 8 show that a sufficient number of grains had developed, i.e. no problems exist regarding the detection limit. The donor solution contained only 36 μCi/ml of [1,2-^{3}H]hydrocortisone in phosphate-buffered saline. This latter concentration can easily be increased since the specific activity of the labelled compound is 51.9×10^3 Ci/mol.

In the electron microscope autoradiographs the grain density over the Epon was identical to the background grain density, indicating that diffusion of labelled compound into the photographic emulsion had not occurred. Furthermore these autoradiographs showed that (almost) no grains had developed over the Epon at the margins of the tissue (i.e. in close vicinity of the stratum corneum), suggesting that diffusion of drug from tissue into Epon had not occurred.

The only set-back in the obtained electron microscope autoradiographs can clearly be recognized in Figs. 7 and 8; the developed silver grains are too large and hence resolution is not satisfactory. Ilford L4 and D19 developer were used because in our laboratory for electron microscopy this is the standard routine procedure for autoradiography. Alternatives to be performed in future experiments are the use of another nuclear emulsion and/or the use of another developer. For the combination of Ilford L4 emulsion and a gold latensification–elon ascorbic acid developer, Ginsel et al. (1979) empirically determined the half-distance of resolution (HD) to be 115 nm (for 62 nm sections). Kopriwa et al. (1984) determined for sections of pale gold interference color the HD-value to be 76 nm for Ilford L4 emulsion and Agfa-Gevaert solution physical developer (SP) preceded by latensification in gold thiocyanate. Salpeter and Szabo (1976) described even better results for the combination of Kodak NTE-2 emulsion and Dektol developer.

The electron micrographs indicate the intracellular and intercellular domains to have thicknesses of 0.3–0.6 μm and 0.10–0.15 μm, respectively (see Figs. 2, 3, 6, 7 and 8). These dimensions are also obtained when the electron micrographs and the corresponding magnifications in the standard work of Odland and Reed (1967) are considered. Furthermore, these data are in good agreement with

those of Elias (1981), who indicated the volume fractions of the intracellular and intercellular domains to be 79% and 21%, respectively. Considering these dimensions and the resolution attainable for electron microscope autoradiography it should be possible to discriminate between intercellular and transcellular drug transport across stratum corneum.

Conclusion

The results suggest that the combination of the "dry" sample preparation and electron microscope autoradiography promises to be an excellent method for visualizing drug transport across stratum corneum:

- the described combination is feasible;
- hardly any (if any) extraction during sample preparation;
- preservation of ultrastructure of stratum corneum is excellent;
- no problems arise regarding detection limits; and
- almost all drugs can be visualized using tritium label.

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