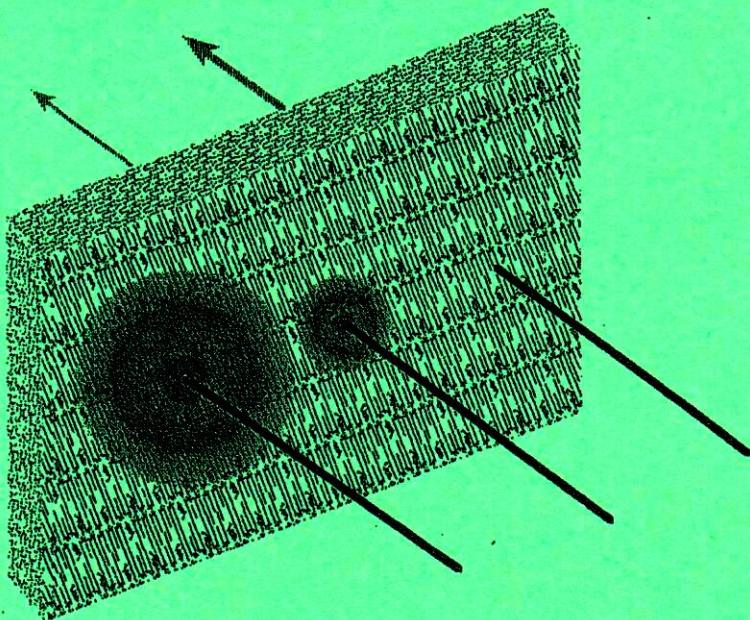


# **Perspectives in Percutaneous Penetration**

**Volume 8a**



**Edited by K.R. Brain and K.A. Walters**

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## DIFFERENT FIXATION AND FREEZING METHODS FOR THE MICROSCOPIC STUDY OF STRATUM CORNEUM

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The main function of the stratum corneum (SC), the barrier function, depends strongly on the specific structure of this tissue. Thus, a number of skin studies are based on visualization techniques<sup>1,2</sup>. Cryotechniques have now proved to be the best method to prevent drying artefacts in the study of biological materials. For very thin samples with low water content (such as the SC) the chemical fixation followed by propane freezing has resulted appropriate<sup>3</sup>. Otherwise, high-pressure freezing (HPF) allows immobilizing thicker samples without chemical fixation. In this technique the freezing quality is variable and depends critically on the water content, this fact could be a reason of the low use of HPF in the SC study.

In this work a comparison between HPF of chemically unfixed SC samples and propane-jet freezing of chemically fixed SC samples was established. The frozen samples were freeze-fractured, coated with Pt/C and a) transferred to a scanning electron microscope under liquid nitrogen and imaged on a cryo-holder at low temperatures<sup>4</sup> or b) digested and observed in a transmission electron microscope. Our results indicate that although both freezing techniques were appropriate for the study of the SC ultrastructure, the plane of freeze-fracture was different depending on the fixation and freezing methodology used. In the samples frozen by HPF without chemical fixation, the fracture plane laid mainly between the lipid lamellae. As a consequence, the micrographs showed in most cases flat and smooth surfaces corresponding to the fracture between the lipid lamellae. However, when chemical fixation and propane-jet freezing was used, the fracture plane did not show preference to a specific way. These micrographs showed flat and smooth surfaces (fractures along the lipid lamellae), sharps steps (fractures across lipid lamellae) and granular surfaces, characteristic of the keratin filaments due to fractures across the corneocytes. These results seem to indicate that the HPF preserves the natural behaviour of the SC, which has a tendency to be fractured along the "weaker areas", that is, along the lipid lamellae. Propane-jet freezing of chemically fixed samples, on the other hand, provides a more homogeneous fracture behaviour. Thus, depending on the methodology used, we can favour a visualization of either protein domain (using chemical fixation and propane-jet freezing) or lipid domain (using HPF). These results could be very useful in future ultrastructural studies in order to facilitate the microscopical visualization of specific domains in the SC.

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# DIFFERENT FIXATION METHODS FOR THE MICROSCOPIC STUDY OF STRATUM CORNEUM STRUCTURE

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## INTRODUCTION

The main function of the stratum corneum (SC), the barrier function, depends strongly on the specific structure of this tissue. Thus, a number of skin studies are based on visualization techniques<sup>1</sup>. Cryo-sections have now proved to be the best method to prevent drying artifacts in the study of biological materials. However, a control of the freezing is necessary to minimize the formation of ice crystal that could damage the samples. In order to avoid this problem, sufficiently high cooling rates have to be used. For very thin samples with low water content such as the SC, the chemical fixation followed by propane freezing has resulted appropriate<sup>2</sup>. Otherwise, high-pressure freezing (HPF) allows immobilizing thicker samples without chemical fixation<sup>3</sup>. In addition to this, the use of immobilization methods avoiding post-fixation with RuO<sub>4</sub> and OsO<sub>4</sub> allow us to reduce staining artifacts. In this sense, freeze-fracture has turned to be very useful<sup>4</sup>.

In this work a comparison between two fixation methods for freeze-fracture of the SC was established. HPF of chemically fixed samples and propane freezing of chemically fixed samples.

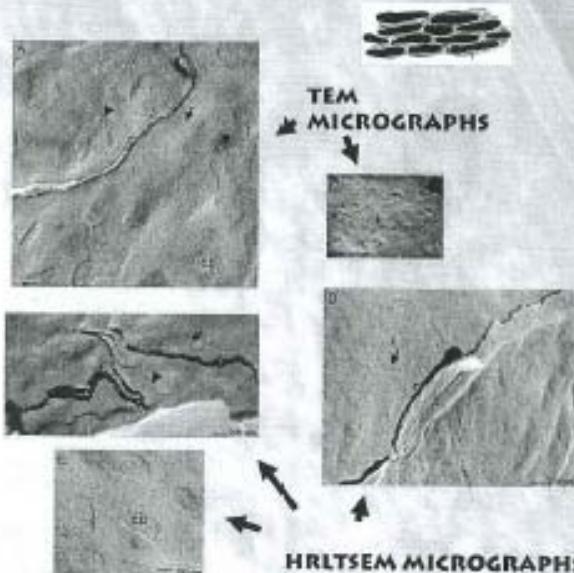
## RESULTS AND DISCUSSION

Our results indicate that although both freezing techniques were appropriate for the study of the SC ultrastructure, the plane of freeze-fracture was different depending on the fixation and freezing methodology used.

### HIGH PRESSURE FREEZING FIXATION

These images show predominantly flat and smooth surfaces corresponding to the fracture between the lipid lamellae (arrow 1). In some cases granular surfaces associated with the cornosomes (G) and sharp steps related to the fracture across lipid lamellae were also observed (arrowheads 1). In addition, it is noteworthy the presence of canals/voids in the interlamellar spaces (C). These last indicate that the fracture plane runs mainly between the lipid lamellae. This phenomenon was observed in TEM observations (micrographs A-B), where replicas have been completely cleaned and in HRLTSEM observations, without removal of the biological material (micrographs C-E).

In the following cartoon, a possible fracture plane is indicated (red line).



## CONCLUSIONS

These results seem indicate that the HPF preserves the natural behaviour of the SC, which has a tendency to be fractured along the weaker areas, that is, along the lipid lamellae. Propane freezing of chemically fixed samples, on the other hand, provides a more homogeneous fracture behaviour. Thus, depending on the methodology used, we can favour a visualization of either lipid (using HPF) or protein domains (using chemical fixation and propane freezing) of the SC. These results could be very useful in future ultrastructural studies in order to facilitate the microscopic visualization of specific domains in the SC.

## MATERIALS AND METHODS

The epidermis was removed from pig skin by incubation with water at 35°C for 4.5 min and then was stored in 0.5% glycerol in PBS at 4°C overnight, and 2 h more at 25°C in fresh 0.5% glycerol. After several washes the SC pieces were fixed and frozen by two different methods.

### Fraction and Freezing Methods

**High pressure freezing fixation (HPF)** without chemical fixation using a Bal-Tec equipment (temperature: -200°C., pressure: 2100 MPa).

**Chemical Fixation and Propane Freezing** using 2% glutaraldehyde in 0.1% cacodylate, overnight at 4°C, followed by sucrose gradient with 30% glycerol in 0.1% cacodylate, 1 h at 22°C. Then, the samples were frozen using liquid propane immerser at -196°C.

All frozen samples were freeze-dried and coated with platinum-carbon using a freeze-drying unit (BDF 000 (BALTEC, Liechtenstein)). Fracturing was carried out at -150°C with the microtome method, at a 10<sup>-6</sup> mbar of vacuum. An unidirectional shearing of the fractured surface was made by suspending 2 nm platinum carbon at an angle of 45° followed by 30 nm of carbon evaporated at a 0° angle.

### Microscopy Methods

#### High Resolution Low Temperature Scanning Electron Microscopy (HRLTSEM)

The replicas were immediately cryo-transferred on a Gatan cryo-holder (in a Hitachi B-200, in low field emission scanning electron microscope equipped with a highly sensitive annular XEG-detector for back-scattered electrons (BSE)). Specimens were investigated at -150°C without any sputter, directly. The beam current was 1.3x10<sup>11</sup> pA measured with a Faraday cage. The primary accelerating voltage was 10 kV. Images were obtained with the back-scattered electron signal and recorded digitally with a Gatan Digital-SIS connected to an Agfa Quasar 950.

#### Scanning Transmission Electron Microscope (TEM)

The replicas for transmission electron microscope were cleaned with an acid mixture (acetic acid, HNO<sub>3</sub> acid and orthophosphoric acid), for 3 days, followed by 8% sodium hypochlorite for 1 day and several washes in distilled water and three picking up on Formvar-coated grids. The replicas were observed at room temperature using a Hitachi B200 MT at 70 kV.

## CHEMICAL FIXATION - PROpane FREEZING



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