

The skin barrier: analysis of physiologically important elements and trace elements

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Changes in the properties of the skin barrier should have correlates in the physiological status of the differentiating epidermal cells. However, the quantitative distributions of physiologically important elements and trace elements of the skin has been a neglected area of research for lack of tools to investigate this highly differentiated tissue. With the event of the particle probes, the electron microprobe and the scanning proton microprobe, it has become possible to investigate different aspects of normal skin physiology as well as pathophysiological processes. In addition penetration profiles of allergenic metals can be demonstrated with the trace element sensitive proton probe. Future approaches to the study of skin physiology in normal and pathological conditions should incorporate other techniques including immunological and biochemical tagging of particular cells to achieve a broad basis for interpretations of data. *Key words: Elemental; trace elemental distributions; skin physiology; particle probes; electron microprobe; proton microprobe; atopic dry skin; nickel allergy.*

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INTRODUCTION

Recent insights into the structure-function relationships of the human skin barrier suggests that interindividual differences in the composition of various lipid fractions may not be negligible (1,2). Such variations still allow a normal barrier function. Although we still lack truly quantitative data on lipid compositions in diseased skins it seems logical to look for correlations in the physiology of normal and pathological skin conditions. Thus it is relevant in the context of discussions over human skin barrier problems to present some of the basic data on skin physiology that have been obtained in the past two decades.

PREREQUISITES FOR A PHYSIOLOGICAL ANALYSIS OF EPIDERMAL DIFFERENTIATION

The epidermis is a highly differentiated tissue which is covered by corneocytes emanating from matrix cells at the border to the dermis. The cornification is the result of a differentiation process which starts roughly 120 µm below the corneocytes. The minute dimensions of the epidermis precludes physiological investigations using classical instrumentation such as glass or other types of electrodes. The optical properties of a viable epidermis with a stratum

corneum, e.g. light scattering properties, makes it impossible to secure the exact position of such an electrode even if this is monitored with micromanipulators under a high power light microscope.

An alternative way of approaching the problem is to use particle probe analysis, either an electron microprobe (a.k.a. XRMA – X-ray microanalysis) in the scanning transmission microscope (STEM) or a scanning proton microprobe (a.k.a. PIXE – particle induced X-ray emission). Such an analysis will capture the physiological status of cell as reflected in its elemental content at the particular time when a biopsy is taken.

SAMPLE PREPARATION

The sample to be analysed. The object intended for the analysis must retain the distribution of ions that corresponds to the living, functional state of the cells of the tissue. The object of the preparation must therefore be to obtain an essentially unperturbed skin section. This is generally achieved through quench-freezing of the biopsy in isopentane cooled by liquid nitrogen at temperatures around -190°C (Fig. 1). Sectioning is then performed at low temperature; -100°C for thin section of a thickness of 60–100 nm, at -20°C for thick sections approximately 6–16 µm thick (3). The sections are subsequently freeze-dried and stored over water absorbing media and/or in an evacuated exicator.

CHARACTERISTICS OF THE PARTICLE PROBES

Spatial resolution. Basically the the electron microprobe offers high spatial resolution, better than 20 nm, i.e. works at

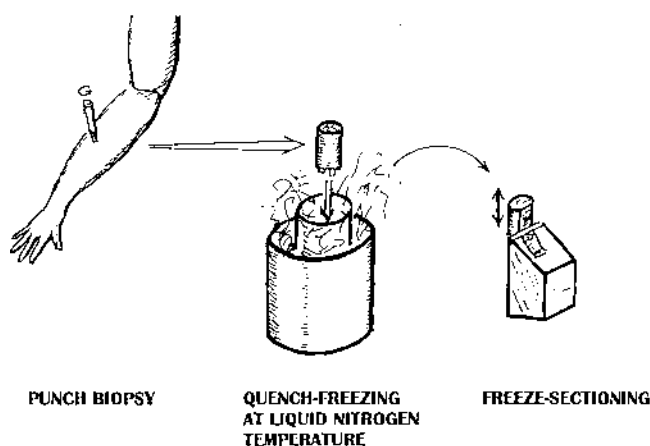
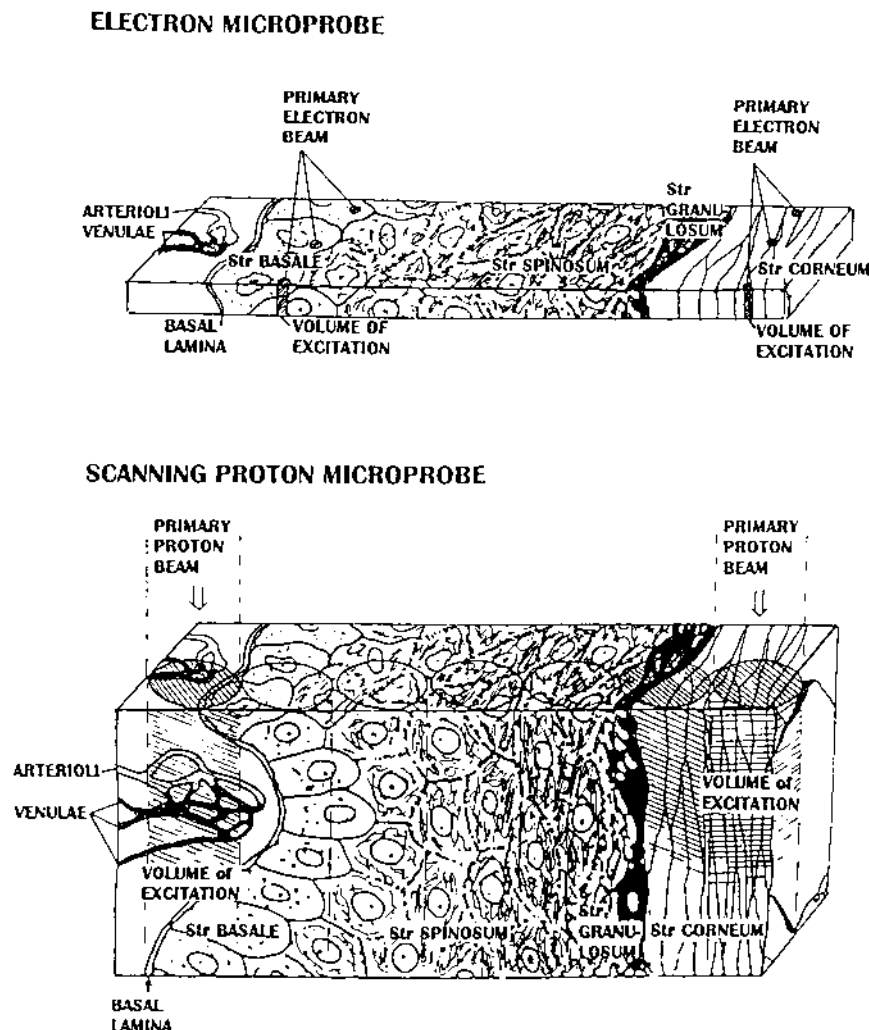


Fig. 1. The principle of cryo-preparation for elemental analysis by particle probes. After sectioning the punch biopsy is stored over a water absorbing medium until the analysis.

Fig. 2. The spatial resolution of the particle probes is to a certain extent dependent on section thickness as illustrated. Top panel represents a thin, approx. 100 nm section for electron microprobe analysis which allows assessment of elements in subcellular volumes. The lower panel represents a thick section, ~15 µm, for proton probe analysis. Several cells in the depth of the section may be included in the excitation volume. Hence, the spatial resolution is confined to strata rather than to single cells.



subcellular resolution, but it is insensitive to trace elements like calcium (Ca), iron (Fe), and zinc (Zn), since its detection limit is around 200 ppm ($\mu\text{g/g}$). The proton probe offers a high sensitivity, 1 ppm or even better, but the spatial resolution is lower, around 5 μm (4,5). Basically this means that in a tissue like the epidermis the resolution of the proton probe is practically limited to strata (Fig. 2).

To a certain extent the resolution is related to section thickness. The sections used in electron probe analysis are generally approximately 100 nm thick and there is essentially no overlapping of cells in the depth of the tissue section. Hence, data will emanate from a single cell. Since the stopping power of the biological specimen material is conspicuously lower for protons than for electrons, thicker specimens are needed in the latter case. A consequence is that there will be an overlap of cells in the depth of the tissue specimen (Fig. 2) in the approximately 15 μm thick specimens used for PIXE analysis, hence the low spatial resolution.

Sensitivity. Another factor related to particle probe sensitivity is the number of quanta reaching the detector per unit time. Actually a very small proportion of all quanta generated reach the detector (Fig. 3). The closer the detector can approach the specimen the larger the effective angle will

be, i.e. the number of quanta per unit time will increase proportionally.

METHODS OF ANALYSIS

In electron microprobe analysis the probe is commonly directed to a section area selected by the operator in the electron microscope transmission mode. In PIXE analysis a rectangular beam scan (nominal beam cross section of 5 $\mu\text{m} \times 5 \mu\text{m}$) is located over a selected area on each skin section so as to cover a cross section of epidermis down into the reticular dermis. This usually corresponds to a total skin depth of about 200 μm . The thermal load on the specimen is also minimised when using a scanning mode for specimen excitation and data acquisition. Thus the probe irradiates each specimen volume (pixel) on the average 5 ms (milliseconds) in an iterate process. The pixel size is chosen so as to avoid overlap of the beam penumbra (8 μm in X- and Y-direction respectively). Typical acquisition times for a pixel map (64 $\times$ 64 pixels) was about 1.5–3 h¹ (6).

¹The numbers given in this paragraph refer to the Lund scanning nuclear microprobe, Lund University, Sweden, as of 1999.

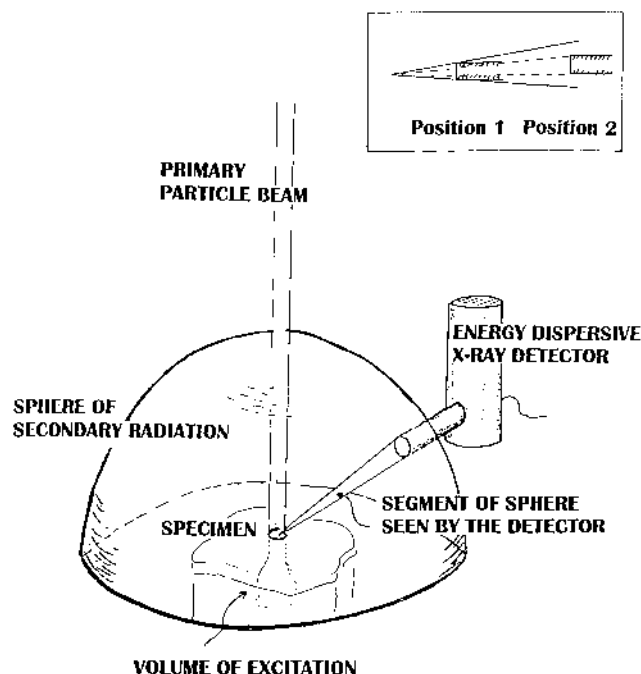


Fig. 3. The impinging primary beam excites a volume of the specimen causing secondary X-ray ray emission in all directions – the sphere of secondary radiation. The efficiency of the detector will increase by increasing the angle it “sees” as it moves closer to the specimen, cf. inset (top right).

RESULTS GAINED BY THE ELECTRON MICROPROBE

The first attempt to analyse the distribution of elements over a skin cross section was done on guinea-pig skin inertly prepared by cryo-technique (7). The most interesting data concerned the shift in sodium (Na) and potassium (K) content that occurred on the transition from the basal layer into the spinous layer. Here the lowered K and the increased Na precludes a cell division activity in the stratum spinosum and that suggests that this helps to regulate the cell division rate so that cell turn over of the progeny within each proliferative unit is approximately constant per unit time under normal conditions (Fig. 4). The keratinocytes on the basal lamina are in mutual functional contact via gap junctions which obviously is another means of controlling the cell division events (8,9). The phosphorous (P) distribution (Fig. 4) closely resembles the phospholipid distribution given by Long (10) and is in full agreement with other data that have demonstrated that there are no phospholipids or nucleic acids in the stratum corneum. Using the electron microprobe Warner et al. have demonstrated corresponding findings in human skin (11).

The water profile over the entire human epidermis was established by analysing frozen hydrated specimens before and after freeze-drying (12).

ELECTRON PROBE ANALYSIS OF PATHOLOGICAL CONDITIONS

The mechanisms behind contact reactions have been extensively investigated within the realm of immunology. However, only few reports on physiological changes have so far been

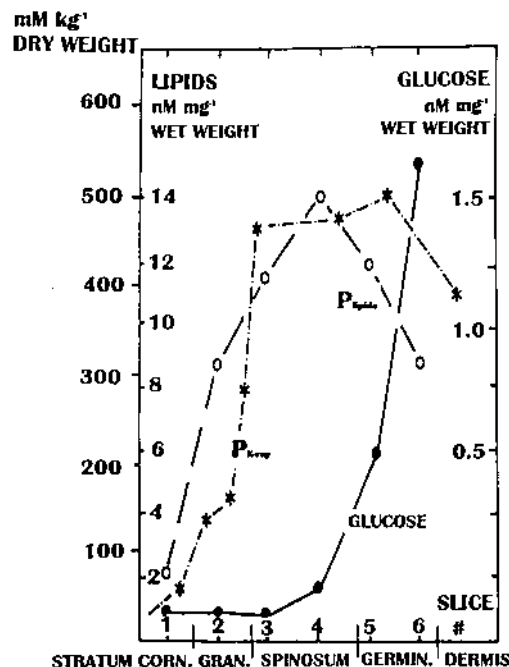


Fig. 4. Comparison between data from electron probe analysis of phosphorus (P_{x-ray}) distribution over skin cross section (ref. 7) and Long's data on phospholipid distribution (P_{lipids}) and glucose distribution over cow's snout (ref. 10) epidermis. The glucose distribution suggests at least partial anaerobic metabolism in the upper part of the viable epidermis.

published. Lindberg has studied irritant reactions in the experimental guinea-pig model (13,14). A time dependence was recorded for an irritation reaction elicited by a 10% DNCB solution which was expressed as lowered phosphorus (P) and potassium (K) contents at 6 h post exposure. Clearcut morphological changes were not recorded until 24 h had passed and at this time the calcium (Ca) level had increased and the magnesium (Mg) decreased (13). The irritancy effects of chromate ($K_2Cr_2O_7$) and nickel sulfate ($NiSO_4$) was studied in the animal model and it was demonstrated that chromate caused the same signs of epidermal cell injury after intradermal injection as had been recorded for DNCB. In the case of $NiSO_4$ it was found that increase Mg, P and sodium (Na) suggested a stimulation of the germinative epidermal cell layer (14).

Sodium lauryl sulfate (SLS) has been a model substance for the study of experimentally induced irritation reactions. To evaluate if the sodium/potassium (Na/K) ration can be used as an indicator of cell proliferation iterative SLS-induced contact reactions in guinea-pig skin was studied with the electron microprobe at different time intervals after the exposures (15). The results demonstrated that repeated SLS exposure induced a hyperplasia after 24 hours which was persisting after 84 hours. A decrease in the Na/K was recorded mainly as a function of increase in the K content. A corresponding finding was previously seen at exposure to n-hexadecane (16).

Recently Gränsjö et al. (17) have published data supporting the idea that the physiological influences of non-ionic detergents differs of those from ionic detergents, e.g. SLS. The effects were time dependent as differences were found at 6 hours after application of the substance but not at 24 hours.

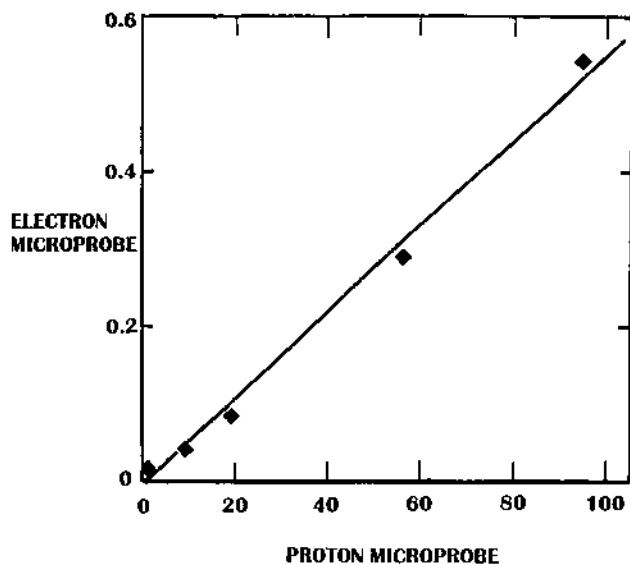


Fig. 5. Correlation between data obtained with the electron probe to those obtained by the proton probe from parallel sections of elemental standards (ref. 5).

A study on psoriasis demonstrated that in the involved psoriatic skin the concentrations of Mg, P and K were higher in the viable epidermis than in the non-involved skin. Comparing the non-involved psoriatic skin to normal control skin only a somewhat lower K was recorded in the former. In general terms the involved psoriatic skin showed a pattern of elemental distributions typical for a highly proliferative cell (18).

PROTON PROBE ANALYSIS

At the time of our first studies it was not possible to delineate a complete calcium (Ca) profile over the entire normal skin cross section by means of the electron microprobe. Turning to the more sensitive proton probe we could demonstrate that the proton probe and the electron microprobe actually produced results from nearly identical sections and standards that had a correlation coefficient of 0.996, i.e. were completely overlapping (19) (Fig. 5). Using the proton probe (PIXE) to study trace element distributions we were able to demonstrate the presence of iron (Fe) and zinc (Zn) (Fig. 6) (20). Interestingly the Ca content showed a dramatic increase in the upper stratum spinosum and granulosum to drop below the detection limit in the stratum corneum. Seen from a functional point of view this corresponds to the need for a Ca signal to trigger nucleases that start the degradation of keratinocyte nucleic acids – a programmed cell death much like apoptosis (21–25). The absence of Ca in the stratum corneum is also deemed functional with respect to the fact that divalent ions will have a heavy influence on the structural organisation of the barrier lipids (26). Essentially these Ca data demonstrated by proton probe analysis correspond to the findings of a precipitation method used for histochemical demonstration of Ca in the transmission electron microscope by Menon et al. (27).

Ca has been shown to be of fundamental importance to the development of a fully differentiated epidermis with a stratum corneum (28). Recently Vicanova et al. has demonstrated the

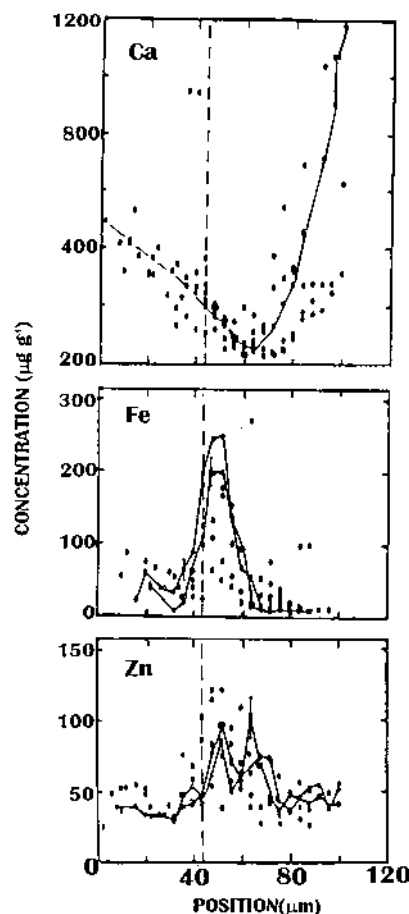


Fig. 6. Distribution of calcium (Ca), iron (Fe), and zinc (Zn) over the human skin cross section assessed by proton probe analysis (ref.16).

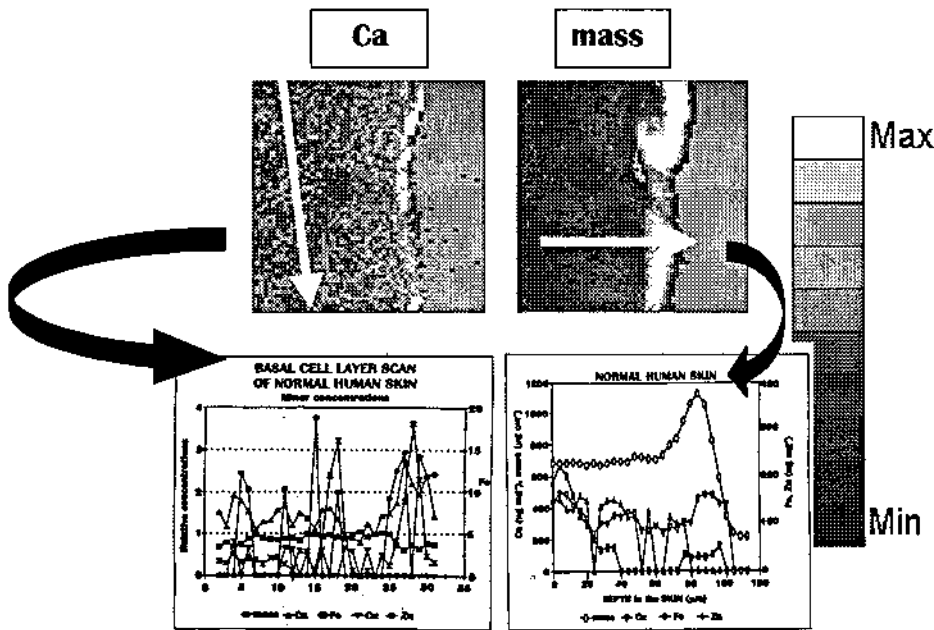
importance of the Ca level in culture using a number of analytical methods including PIXE analysis (29).

As stated above the scanning proton probe (PIXE) allows mapping of mass and elemental distributions (Fig. 7). The scanning type of data retrieval not only results in elemental maps but also diminishes the thermal load on the specimens. In this way the risk of a burn-out of the organic matrix harbouring the elements is minimized and the elemental data will be representative for the actual living distributions. A matrix burn-out will yield too high values (element concentration over background) or even cause an evaporation of light elements resulting in too low values.

From a pixel map of elemental data cross section distributions can be extracted but also horizontal distributions, e.g., along the basal lamina (Fig. 7). Horizontal distributions should enable us to understand the physiological changes brought about when a cell enters the cell division cycle and its fate within this process (30).

PIXE ANALYSIS OF PATHOPHYSIOLOGICAL CONDITIONS

Comparing data from normal skin with those obtained from dry atopic skin we found remarkable differences in trace element contents and distributions. Zn is a trace element the



t- Fig. 7. The scanning proton probe allows mapping of element distributions over a chosen skin cross section. From such maps “channels” of data can be chosen to represent the cross section distribution of elements or a horizontal distribution of elements within a stratum. The two left panels demonstrate a horizontal “channel”, the right panels represent the cross section “channel”. Not that the “channels” include all data not only the Ca distribution that is seen in top left panel, and mass seen in top right panel.

role of which in the differentiation process of the epidermis is still enigmatic (31,32). In the normal skin the Zn peak is generally confined to the lowermost stratum spinosum and at concentration levels around 150 ppm. In the dry atopic skin we found Zn levels up to 300 ppm and the center of gravity located to the stratum granulosum/ corneum (33). Even in psoriasis the Zn levels in the upper epidermis are higher than in normal skin (34). Ca has been shown to trigger the apoptosis process via nucleases (24) and in a recent book Whitefield (34) has suggested that in the epidermis this process should be nametaged as diffpoptosis. Considering the fact that Zn has been shown to be antagonistic to Ca in the apoptotic process the immaturity of the dry atopic skin thus may have a clear physiological indicator.

It is interesting to speculate over a possible inactivation effect of Zn^{2+} on the putative defensins of the atopic skin. Zn-binding to histidin and cystein residues may cause conformational changes resulting in inactivation of the epidermal anti-microbial peptides (defensins) (cf. 36). This is one possible explanation to the increased tendency to skin infections recorded in atopics.

One of the problems of data retrieval from pixel maps was that only a fraction of the data were used when cross section distributions were extracted. Using SIMCA (Fig. 8), a multidimensional statistical analysis method (37) which allows us to identify correlations in a multidimensional space, e.g., between skin types, strata of the epidermis, elements and trace elements etc., it became possible to demonstrate that the atopic skin actually has the physiological characteristics of immaturity (Fig. 9) (38).

PIXE AS A PROBE FOR ENVIRONMENTAL EFFECTS ON SKIN AND SKIN PHYSIOLOGY

In occupational dermatology nickel (Ni) allergy has become increasingly important in recent years.

However, the penetration mode of this metal ion has not been thoroughly investigated, although we have data to show

that lauryl sulfate increases the permeation of the metal about fourfold (39). Using PIXE analysis on biopsies from occupational Ni-tests it was possible to demonstrate that Ni exposure under occlusion resulted in an accumulation of the metal ion in the stratum corneum and that the Ni levels in the viable epidermis was below the detection level for Ni (40). Such findings suggest that the amount of Ni needed for eliciting an allergic reaction in a sensitized individual amounts to a few ions.

CONCLUSIONS

We have demonstrated that PIXE analysis of human skin yields valuable information about skin physiology in normal and pathological skins. However, it becomes clear that to gain a clear understanding of what changes in the elemental and

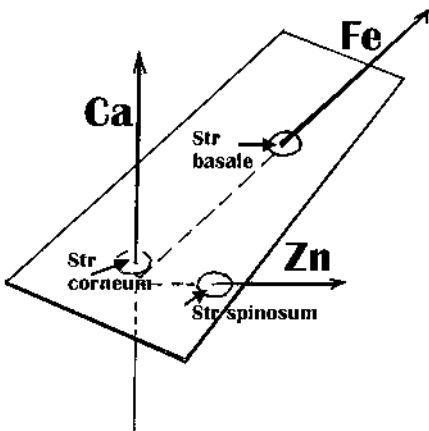


Fig. 8. The multivariate method of SIMCA finds correlations in a chosen *n*-dimensional space, e.g. strata of normal skin versus strata of atopic and/or psoriatic skin as related to content of elements and trace elements. Here the principle demonstrates that Ca correlates with Str. corneum/granulosum, Zn with the spinous layer, and Fe with the basal cell layer in the normal skin.

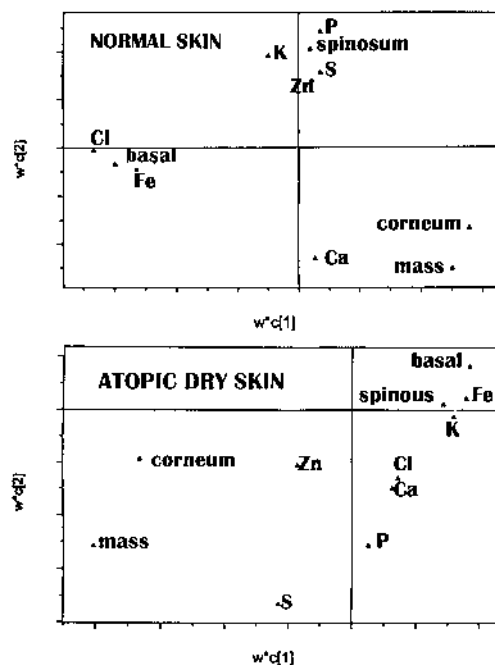


Fig. 9. Scatter plot based on multivariate statistical analysis demonstrating a) the co-variation shown in Fig. 8 for normal skin and b) that the spinous layer and the basal layer cannot be distinguished in the dry atopic skin.

trace elemental contents represent the PIXE data should be correlated to data assessed by other methods, e.g., autoradiography, immunological labeling etc. The development of this physiologically oriented approach to experimental and clinical dermatology should allow identification of cells in the process of cell division, cells with diverging physiology etc. Such information should enable us to understand the development of a normal, functional skin barrier and the mechanisms that are involved when barrier formation is incomplete.

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