Scanning Microscopy

Volume 9 | Number 4 Article 8

9-7-1995

Human Skin Physiology Studied by Particle Probe Microanalysis

B. Forslind
Karolinska Institute, bosse@mango.mef.ki.se

M. Lindberg
Academic Hospital, Uppsala

K. G. Malmqvist
Lund Institute of Technology

J. Pallon
Lund Institute of Technology

G. M. Roomans
University of Uppsala

See next page for additional authors

Human Skin Physiology Studied by Particle Probe Microanalysis

Authors

B. Forslind, M. Lindberg, K. G. Malmqvist, J. Pallon, G. M. Roomans, and Y. Werner-Linde

Scanning Microscopy Vol. 9, No. 4, 1995 (pages 1011-1026) 0892-953X/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

HUMAN SKIN PHYSIOLOGY STUDIED BY PARTICLE PROBE MICROANALYSIS

B. Forslind[1*], M. Lindberg[2], K.G. Malmqvist[3], J. Pallon[3], G.M. Roomans[4] and Y. Werner-Linde[5]

[1]Experimental Dermatology Research Group, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, [2]Department of Dermatology, Section of Occupational Dermatology, Academic Hospital, Uppsala, [3]Department of Nuclear Physics, Lund Institute of Technology, Lund, [4]Department of Human Anatomy, University of Uppsala, [5]Department of Dermatology, Danderyd Hospital, Stockholm, Sweden.

(Received for publication May 10, 1995, and in revised form September 7, 1995)

Abstract

Particle probe methods (electron probe and proton probe X-ray microanalysis) have been applied to investigate the distribution of elements and water over the different layers of the epidermis. For major elements, electron probe X-ray microanalysis (XRMA) provides the advantage of superior spatial resolution, but for trace element analysis the more sensitive proton probe (particle induced X-ray emission, PIXE) analysis has to be used. On a dry weight basis, the concentration of S is rather constant across the epidermis, whereas the concentrations of P, K, Cl and Na show gradients with high levels in stratum germinativum (basale) and stratum spinosum but low levels in the stratum granulosum and stratum corneum. Essentially, Fe and Zn are confined to the basal region in normal skin. The concentration of Ca, however, increased steadily from the basal region to the stratum corneum. The probe technique allows quantitative analysis of stratum-specific changes in elemental content in a variety of pathological conditions, e.g., changes induced by nickel, detergents and other chemicals, or in psoriatic skin. Of particular interest are findings of increased Fe and Zn in non-involved psoriatic skin. Since the different layers of the skin have different elemental concentrations and react differently under pathological conditions, the probe techniques are far superior to bulk chemical analysis in elucidating physiological and pathological processes in the skin.

Key Words: Skin, epidermis, apoptosis, trace elements, particle probe analysis, electron microprobe, scanning proton microprobe, energy dispersive X-ray microanalysis.

*Address for correspondence:
Bo Forslind
EDRG, Department of Medical Biophysics, MBB,
Karolinska Institute, 171 77 Stockholm, Sweden
Telephone number: +46 (8) 34 47 39
FAX number: +46 (8) 32 65 05
E.mail: bosse@mango.mef.ki.se

Introduction

The cellular part of the skin, the epidermis, is a very thin structure, approximately 120 μm thick, a fact which has been prohibitive to the exploration of the physiology of the epidermis in normal and pathological conditions [31]. Since the epidermis contains layers of cells at different stages of differentiation, it follows that conventional physiological capillary probes cannot be located with any satisfactory precision within a specified cell of a specified layer of the skin *in vivo*. Therefore, alternative ways for the exploration of skin physiology are required.

The distribution of ions in normal tissues serves as reference for pathological changes. In recent years, trace element analysis has been a subject of special interest as it becomes clear in many cases that these elements work as secondary messengers or regulatory substances. In a great variety of cellular systems, calcium (Ca^{2+}) has proven to be a very important signalling substance [1]. Studies of Ca^{2+} distributions using the transmission electron microscope (TEM) and histochemical methods have allowed an understanding of the role of Ca^{2+} in the differentiation process of the epidermis which eventually results in the formation of the physical barrier. The role of trace elements, such as, calcium, may also be extended to include direct effects on lipid aggregation in the barrier, but such effects remain to be unequivocally demonstrated.

The biological essentiality of zinc has been recognized already in the previous century. However, the exact role played by zinc in the skin and in the epidermal differentiation process is still an enigmatic question, in spite of the fact that zinc metallo-enzymes are involved at all biochemical levels from nucleic acid polymerase activity to specific roles in protein, fat, and carbohydrate metabolism **and** catabolism [30]. With the sensitivity of the proton probe, and present day good spatial resolution, we expect this technique to provide precise quantitative data that can be related to histochemical observations.

Table 1. The great variation in data on the elemental content of skin.

Element	Content (ppm)	Range (ppm)	Mean (ppm)*	Method
Ca	0.034	20	7.9 (0.18)	AES/XRF
Cl	2654	2654	2654	SAS
Cu	6.9	143	75.8	AAS/AES
Fe	34	1900	589 (58.1)	AAS/AES
K	0.846	118	30.8 (3.06)	AAS/FES
Mg	0.009	0.87	0.25 (0.049)	AES
Na	1016	4968	2248 (2612)	FES/misc
P	0.3	40	8.4 (0.5)	AES/SAS
S	1577	1850	1602	SAS/misc
Se	0.061	1.2	0.51	NAA
Zn (whole skin)	6.1	1000	261	AAS/NAA
Zn (epidermis)	39	83	62.5	NAA
Zn (dermis)	13	23	16.3	NAA

Data compiled from ref. [15]. *Mean value given here does not imply any biological significance. Mean values within brackets are recalculated excluding extreme values.

AAS: atomic absorption spectrometry; FES: flame emission spectrometry; NAA: neutron activation analysis; SAS: solution absorption spectrometry; XRF: X-ray fluorescence spectrometry; misc: miscellaneous techniques.

Apoptosis ("programmed cell death") is currently a "hot" subject for the obvious reason that the final differentiation step between the stratum granulosum level and the stratum corneum represents a particular aspect of "programmed cell death". The importance of calcium and zinc in apoptosis has been clearly demonstrated in a number of cellular systems [2]. There are old suggestions of the human skin's dependence on zinc (Zn) for normal function [13], and this is practically demonstrated by the topical use of zinc ointments. Obviously, this is a field of research that needs to be thoroughly explored in a near future.

Two decades ago substantial iron (Fe)-losses via psoriatic lesions were demonstrated [26, 27] but were given new meaning when we found that a more discrete loss in clinically normal looking psoriatic skin occurs [32]. No doubt, such findings stress the importance of understanding the relation between elemental content and normal and abnormal physiology.

In the past, analysis of the elemental content of skin has been done as bulk measurements. It can be demonstrated from the data compiled by Iyengar *et al.* [15] that surprisingly wide ranges of elemental content have been recorded (Table 1). Considering the precision of most of the methods used, the width of these ranges must be due to the sampling method rather than the sensitivity of the chosen method of analysis. In addition, the sampling method of such measurements does not discriminate between the different strata. Consequently, this old information provides little, if any, substance for a functional analysis of the processes involved in normal and pathological differentiation of the epidermis. The particle probes, however, have been able to bridge these methodological problems, and for such reasons, we have, in the past two decades, pursued PIXE (particle-induced X-ray emission) studies of normal human skin, normal looking paralesional skin from psoriatics, and skin from persons suffering from atopic dermatitis [5, 6, 7, 10, 11, 32, 45]. The aim of this paper is to give an overview of the state of the art in skin physiology made possible through the use of particle probes.

The ultimate goal of particle probe studies is to provide an understanding of the formation of a mature stratum corneum with a functional barrier [4, 8] reflected in physiological/biochemical mechanisms behind the properties of changed skin in patients afflicted with skin disorders of genetic and constitutional origin.

Particle Probes in the Study of the Integument

Analyses using particle probes are based on the fact that the impinging particles cause excitation of the elements contained within an irradiated volume resulting in emission of both characteristic and background X-ray

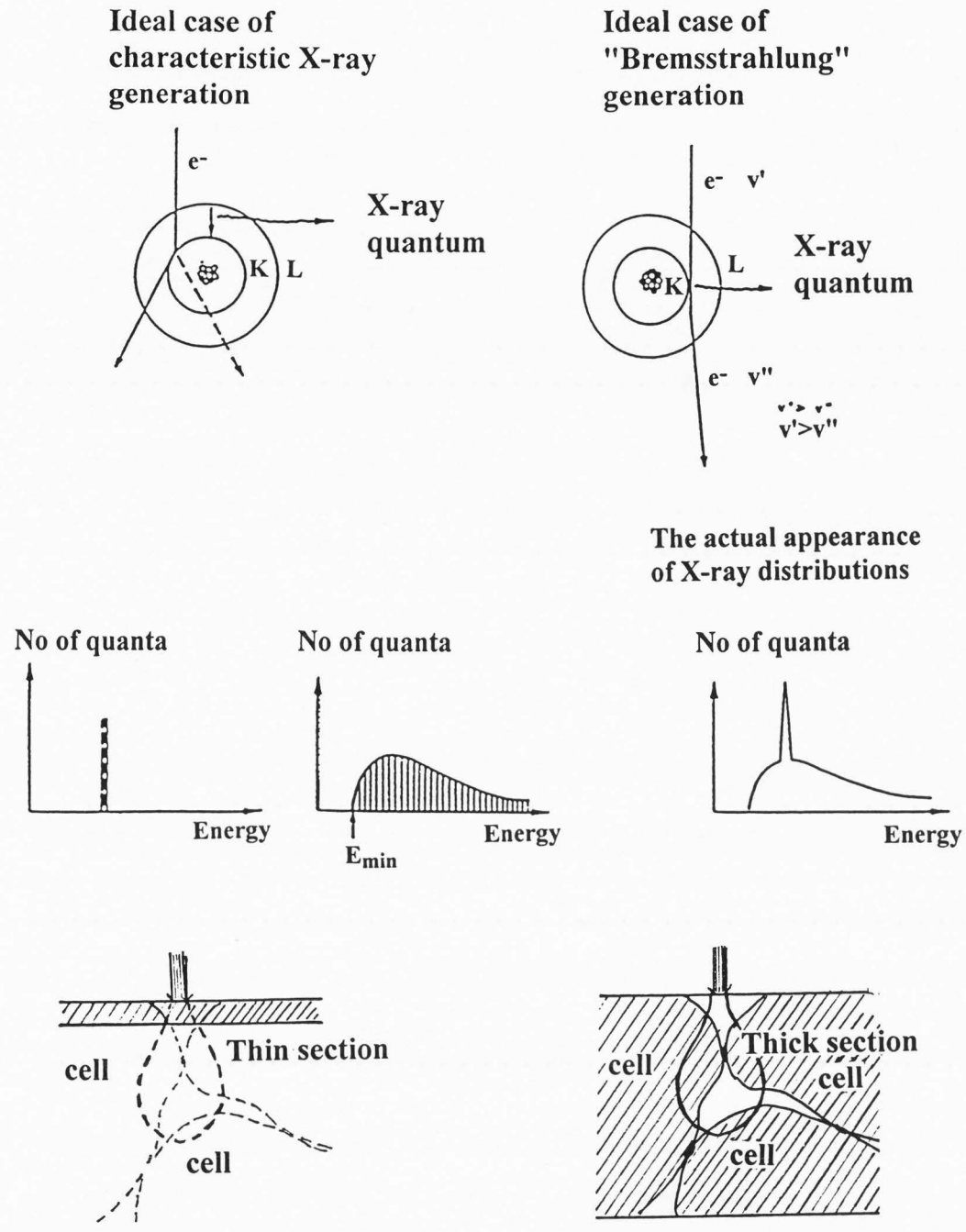


Figure 1. (**a**) Particle probe generation of X-rays utilizing an energy dispersive detector system. (**b**) The actual elemental distribution is represented in a compound diagram (right) representing the characteristic radiation (left) and the continuum (the "Bremsstrahlung") generated as a function of mass density in the specimen (middle). The heavy protons generated effectively no continuum due to low stopping power of (energy transfer to) the biological matrix. (**c**) The electron excitation volume is pear-shaped in a biological, organic matrix. This sets limits to the spatial resolution using bulk specimens (right) compared to thin section analysis (left). It should be noted that the diminished excitation volume requires longer exposure time for accumulation of the same number of quanta, hence sensitivity becomes reduced.

---

radiation (Fig. 1). The characteristic radiation can be used for assessing the elements present in the volume of analysis. In addition, the background radiation can provide information on the mass present in that same volume [6, 7]. A limitation of particle probe analysis is that the techniques only provide data on the elemental

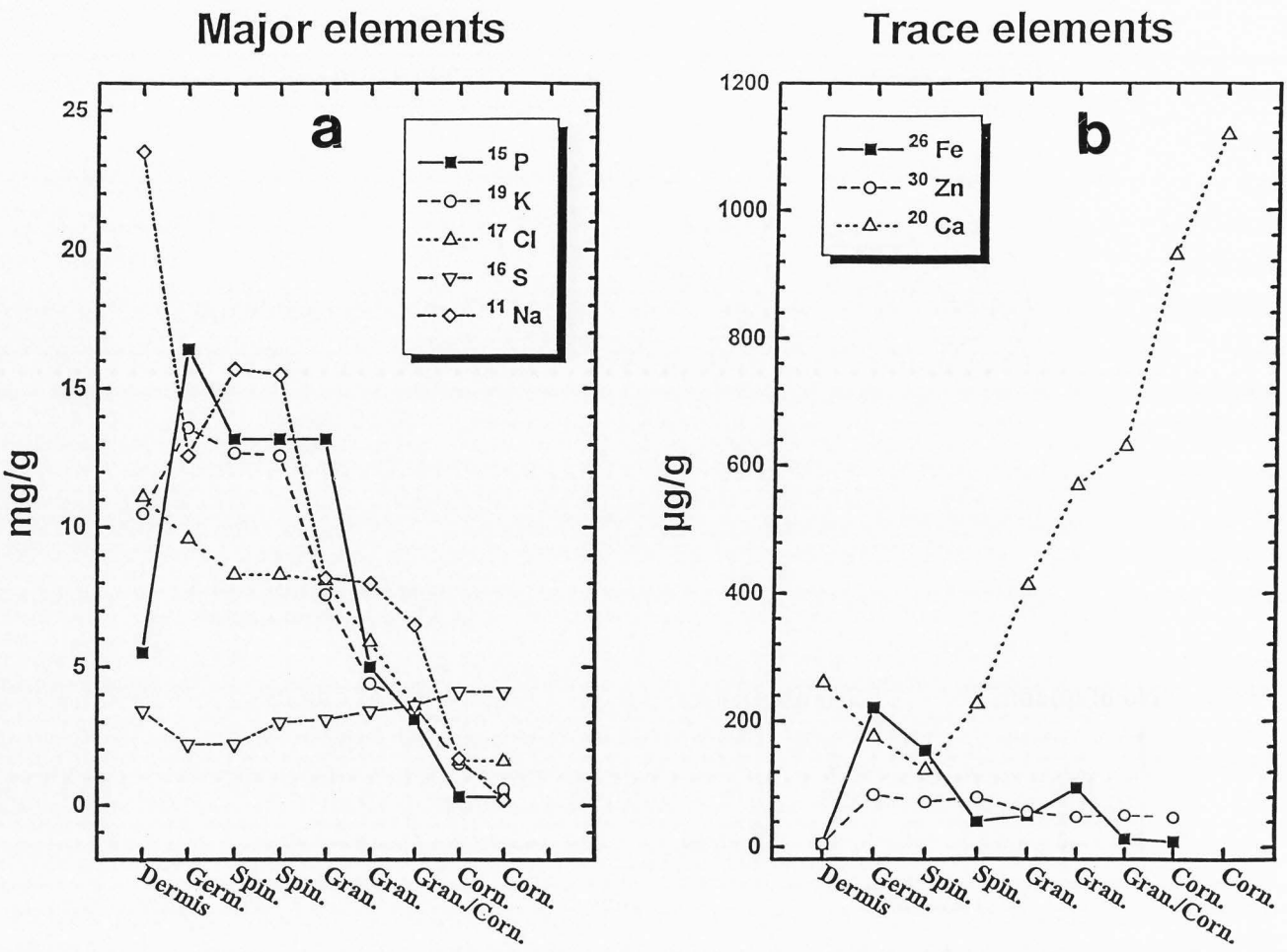


Figure 2. Generalized major elemental (**a**) and trace elemental (**b**) distribution over human skin cross-sections.

---

content irrespective of the chemical status of an element under analysis. A specified element covalently bound to any substance, e.g., a nucleic acid or a protein, cannot be differentiated from ions of the same element present in the volume of analysis [43].

Electron versus Proton Probes

In experimental dermatology electron probes in scanning (transmission) electron microscopes and proton probes generated from high energy particle accelerators have been used. The electron microprobe (EMP, also called XRMA, X-ray microanalysis) has a spatial resolution of approximately 200 Å (0.02 μm) under optimal conditions. The sensitivity of XRMA is approximately 200 ppm (μg/g dry weight), which allows the study of the common physiologically important elements such as Na, P, S, Cl, and K [5, 37, 44, 46] (Fig. 2). Elements that are present in concentrations just around or below this sensitivity threshold, e.g., Mg, Ca, Fe, Cu, and Zn, cannot be studied with this technique. These elements are important constituents of either messengers or of enzymes and these elements can be determined using the nuclear or proton microprobe (PMP) (Fig. 2) which has a spatial resolution of approximately 1-5 μm and a sensitivity down to about 1 ppm [31]. The PMP detector window which has to be adequately thick to prevent scattered protons from entering the detector, however, does not permit adequate passage of the very weak X-ray signal due to absorption and therefore does not give reliable X-ray data with PIXE (proton-induced X-ray emission) analysis. Nuclear reactions caused by the uptake of protons in the Na nucleus results in emission of γ-radiation with a yield that gives satisfactory sensitivity for Na quantitation when desired. Recently, the application of PIXE analysis technique to biological tissues [32] and to dermatological problems in particular was reviewed [6, 10].

An electron has a mass which is approximately 1/2000 of the mass of a proton. The retardation of the

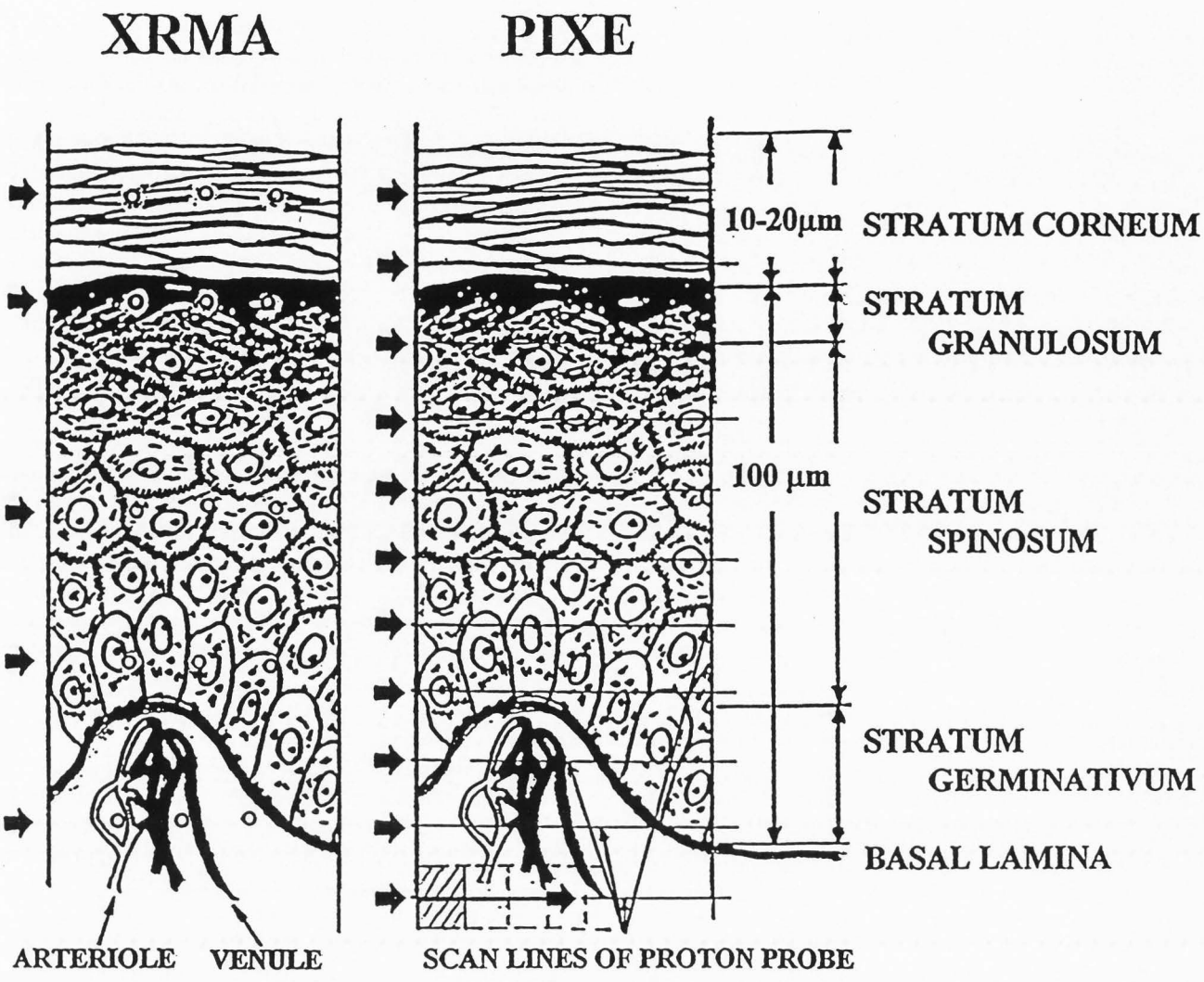


Figure 3. Analysis of a skin cross-section in the scanning transmission electron microscope involves localization of the probe in defined cell layers (left panel). With the proton probe scanning a cross-section of skin results in the build-up of a pixel maps which at present does not distinguish between the intra- and extracellular compartments due to probe size and section thickness (compare text).

---

electron and the energy transfer at this process generates the appreciable background of the electron probe spectra. Protons pass through biological sections virtually without any loss of speed, i.e., no appreciable energy transfer occurs, hence the very low background of the proton probe.

XRMA is usually performed in cells of different strata, i.e., the dermis, the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. To account for the biological and other possible variations between cells of the same stratum, three or more points are chosen in each stratum (Fig. 3). Today, PIXE analysis usually scans a chosen area of the section to produce a pixel map which contains spatial information as well as elemental intensity information of each pixel (Fig. 3). The scanning procedure has the advantage that the particle probe resides within a particular volume for only a fraction of time. This minimizes the thermal load on the specimen in contrast to the effect at constant irradiation of the specimen in a single spot which may carbonize the biological material, resulting in too high elemental concentrations due to loss of light elements (e.g., C, O, N) that constitute the bulk of mass in a biological specimen. Although it is possible to obtain pixel maps with the electron probe, the cross-section of the electron beam being about 50 times smaller than the proton beam cross-section, it follows that the acquisition time for corresponding sizes of pixel

maps at maximal resolution will practically be too long for the XRMA.

Requirements for Preparation

From the moment of biopsy, any redistribution of ions must be hindered or at least minimized. When specimens are processed at very low temperatures movement of diffusible components, e.g., physiologically important ions such as Na^+, K^+, Cl^-, and Ca^{2+} is satisfactorily minimized. Fresh biopsies are immediately quench-frozen at very low temperatures (from -120°C to -190°C), and subsequently sectioned and freeze-dried at low temperature. Through this process, it is possible to obtain satisfactory samples for elemental analysis [37, 38, 39].

Particle probe techniques are essentially spectrographic methods and therefore give data of relative contents of elements contained within the volume of analysis. Absolute quantitation can be obtained using standards based on crucial elements in stoichiometric amounts added to an organic matrix approximating the content of organic material in the cytosol [37, 38].

We perform particle probe analysis on skin-sections which are either thin sections (60-100 nm) or on bulk specimens, i.e., sections 1-15 μm thick. The stopping power of thin sections made for elemental quantitation using EMP in the scanning (SEM) or scanning transmission (STEM) electron microscope is too low for use with the proton probe. For PIXE analysis, we therefore, use bulk specimens which, even at a cross-section thickness > 15 μm, can still be regarded as "thin" sections.

The protocol usually adhered to in our investigations on human skin can be summarized as follows: Skin biopsies are obtained from the chosen area after subcutaneous infiltrations of the chosen area with 10 mg/ml lidocaine in 5 mg/ml adrenalin using 4 mm punches. Biopsies for controls are taken from a corresponding area from individuals with no records of skin disorder. The biopsies are immediately quench-frozen (and sometimes stored) in liquid nitrogen. Depending on the choice for subsequent preparation, i.e., thin sections or bulk sections, the appropriate measures are taken.

Specimens prepared by thin sectioning are glued to a specimen support using Tissue-Tek OCT compound (Miles, Elkhart, IN, USA). Sectioning should be performed at about 173 K (-100°C) and the sections can be mounted in "clam" grids that are folded to fix the section without the need of a support film, or the specimen can be mounted over a hole in a solid carbon support using a carbon based glue at the edge of the section where analysis will not take place. Prior to analysis, the sections are covered with a thin carbon film by evaporation in vacuum to render the surface conductive.

We store bulk specimens in a deep-freeze at -70°C until sectioning, which is performed at -25°C in a conventional cryostat with a nominal section thickness of 16 μm. The sections are subsequently transferred to specimen support rings covered with Kimfol® foil which gives virtually no contribution to the particle induced X-ray spectrum if the specimen cannot be accommodated within "clam"-grids with central openings of a diameter of approximately 1 mm. The mounted specimen is stored in sealed, preferably evacuated, vessels with drying material to prevent water uptake until analysis.

Freeze-Substitution - an Alternative Preparation Method for Particle Probe Analysis

The requirement that tissues processed for particle probe analysis should be prepared in accordance with the cryo-methods described in the previous paragraph involves problems related to thin sectioning. In practice, the outcome of thin sectioning of quench-frozen tissue is unsatisfactory with relation to the number of sections feasible for XRMA analysis, i.e., sections with constant thickness and free from artifactual cracks and folds. This is actually the main reason for the present day use of bulk sections (> 5 μm).

An attractive alternative preparation method is freeze-substitution. A quench-frozen tissue sample is immersed in a non-reactive solvent which replaces the diffusible water at low temperature. A new approach to this technique was recently elaborated by Pålsgård *et al.* [34, 35]. Using tetrahydrofurane (THF, Merck, Darmstadt, Germany) which is dried overnight by contact with a 0.4 nm molecular sieve, the quench-frozen sample is immersed in THF at -70°C for 48 to 72 hours. The substitution is then continued at -30°C for 48 hours and at -18°C for 36 hours. The samples are subsequently thawed at -3°C for 48 hours and at +8°C for another 22 hours. The freeze-substitution medium can be replaced by an embedding medium, e.g., Araldite, when the substitution has come to an end. Specimens prepared according to this protocol can be thin-sectioned on a dry knife and mounted on folding ("clam-") grids without support film. In the STEM, it is possible to recognize cells and subcellular compartments, which is generally the main obstacle when analyzing bulk samples.

The Lund Nuclear Scanning Proton Microprobe

The new Lund Nuclear Scanning Microprobe facility is representative of the modern generation of proton probes for elemental analysis. The system provides a 1 A, 2.5 MeV proton beam for irradiation of the specimens and the proton beam is focused by quadrupole electromagnets. For skin analysis, we use a beam of

about 1 nA with a beam cross-section of 5 μm x 5 μm. A rectangular beam scan is located over a selected area on each skin-section so as to cover a cross-section of epidermis and dermis down to the reticular dermis (c.f. Fig. 3) which corresponds to a total depth in the skin of about 200 μm. The probe irradiates each specimen volume (pixel) on the average 5 ms (milliseconds) in an iterative process. The pixel size is chosen to avoid overlap of the beam halo (8 μm in x- and y-directions, respectively). Typical acquisition times for a pixel map (64 x 64 pixels) are 30 minutes to 2 hours and the beam charge is collected in a Faraday cup positioned after, and in line with the sample. A Kevex energy dispersive system detects and quantifies the induced X-ray radiation, and the mass cross-section at a point (i.e., specimen sub-volume) of analysis is determined by the backscatter (BS) which is subsequently used for mass normalization of the samples [42]. Beam-scanning control and data collection are performed using a VME bus computer [24], while storage and sorting is done on a MicroVAX-II computer. The complete X-ray data (the relative elemental content) and BS (the mass cross-section) data for corresponding pixels are merged into a number of pixel spectra which can be evaluated by computer fitting using HEX [16]. Pixels representing similar structures (e.g., parallel band in the epidermis) can be chosen for the data on the quantitative elemental content of a specified cellular layer (Fig. 4) from the produced elemental maps.

Comparison between XRMA and PIXE Data

The basic principle of generating characteristic X-rays that denote the elemental content of the volume of analysis is essentially the same for XRMA and PIXE. However, it is not self-evident that the two methods give consistent data. We have used a reference system which contains the appropriate elements in known stoichiometric amounts and prepared according to the protocol used for specimen preparation. In this way, we were able to show that the two methods give data that fit with a correlation coefficient of 0.996 [2]. We are, therefore, able to evaluate the findings with either method within the same frame of reference.

Results from Particle Probe Analysis of Skin

Our initial studies were done on thin sections of guinea-pig skin in the scanning transmission electron microscope. All later data have been obtained from fresh biopsies from human subjects except for a few experimental studies [9, 19, 20, 21, 22, 23]. Here, we first outline the findings in normal skin using XRMA and then add more recent results obtained with PIXE analysis. A generalized representation of findings is presented in Figures 2a and 2b.

Sodium, potassium and chlorine gradients

Sodium (Na), potassium (K) and chlorine (Cl) are basically responsible for the ion gradient that creates the cell membrane potential which is essential for the physiological functions of the living cell. The initial experiments on guinea-pig skin showed that the content of Na, K and Cl varied with the degree of differentiation in the tissue [9, 48]. The basal cell layer of the epidermis was the only stratum that possessed a Na/K quotient compatible with the ion content of cells capable of renewing the tissue by cell division. In the next cellular layer of the differentiating epidermis, the stratum spinosum, there was a conspicuous increase in the Na content of the cells and a lowered content of K which is interpreted to mean that the Na^+-K^+ membrane pumps are no longer efficient in maintaining an ion gradient over the cell membrane and/or the cell membrane is leaking. The Cl distribution corresponds to the amount of the positively charged ions. The general outline of these elemental distributions have been verified in later studies and the data harmonize with the fact that, normally, no cell divisions are seen above the basal cell layer [10, 45].

The phosphorus gradient

The bulk of the phosphorus (P) resides in the nucleic acid and the phospholipids of the membranes of the Malpighian layers (stratum basale, stratum spinosum) [9, 14]. In addition, there are phosphorylated precursors to the fibrous protein, keratin, in these layers which contribute to the total P content to an unknown extent. Also, the phosphorylated precursor to filaggrin present in the keratohyalin granules of the stratum granulosum is another source of P in skin [36]. The P has a maximum in the basal cell layer and the lower stratum spinosum. The P content there successively declines to nil at the border between the stratum granulosum and the stratum corneum in harmony with the fact that no phospholipids or nucleic acids can be found in the stratum corneum.

Sulfur and mass distribution

Keratins characteristically contain high amounts of sulfur (S) located to the amino acid cyst(e)in. The mass (the keratin content) successively increases and the S follows this increase closely, as expected [9, 10, 32], as keratinocytes differentiate into corneocytes of the stratum corneum.

Water distribution profile of the skin

Skin samples prepared by freeze-drying methods allow determination of one important aspect of the physiology, namely the role of water. We used XRMA

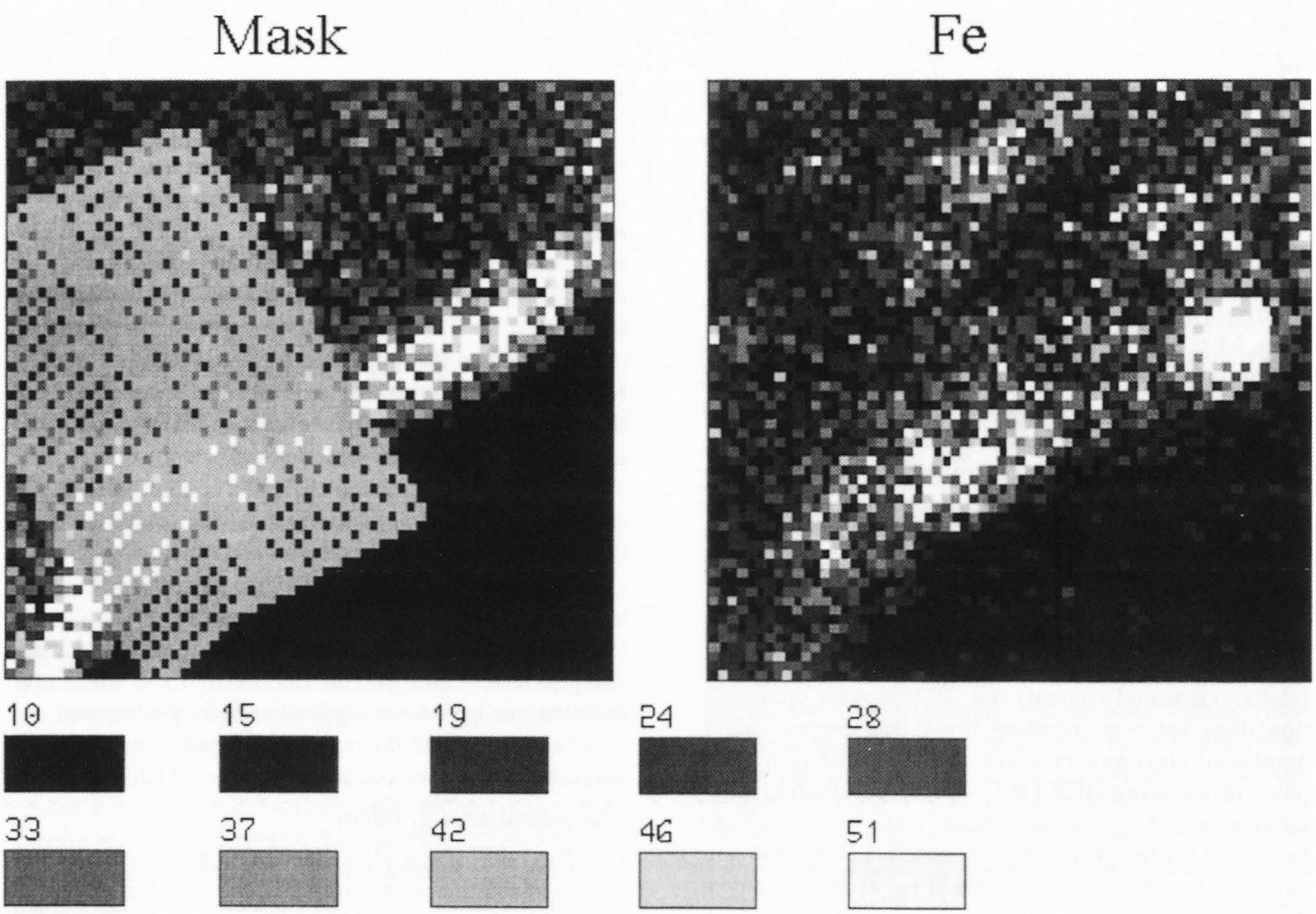
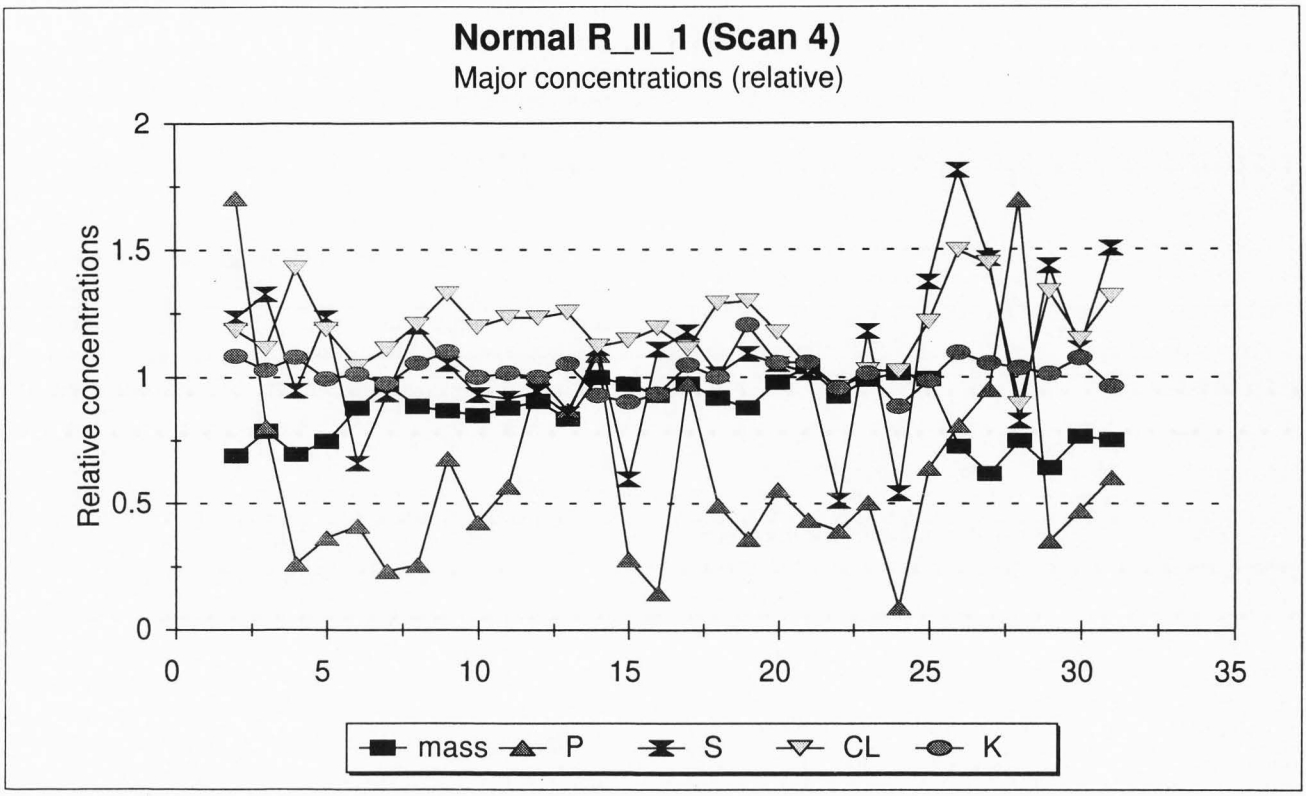


Figure 4. Pixel map of a normal skin cross-section showing the Fe distribution.

analysis of gluteal skin-sections and standards in the frozen hydrated state followed by freeze-drying in the STEM followed by a renewed analysis, to obtain data on the distribution of water over the skin cross-section [45]. It was shown that the distribution of Na, K, Cl, P and S followed the patterns previously established and served as control of validity for the measurements. The mass difference between the frozen-hydrated specimen and the freeze-dried specimen allowed calculation of the water profile. It was shown to give a constant value around 75% of wet mass from the reticular dermis to the stratum spinosum, i.e., the hydration level of the dermis is not significantly different from that of the cellular epidermis. In the stratum corneum, a substantial decrease to roughly 50% of wet mass, occurred and this value is slightly higher than what has been reported by other authors [47]. The difference may well be accounted for by the source of epidermis which, in the latter case, was lower leg epidermis and, therefore, thinner than gluteal epidermis. So far, only two sites, gluteal region and lower leg, have been investigated. In view of the fact that the permeability barrier has different properties in different locations of the skin, it is likely that the water distribution gradient over the skin cross-section also will vary. An interesting approach to the problem has been taken by Warner involving densitometric evaluation of transmission electron microscopic images of the stratum corneum (Ronald Warner, personal communication, 1995). Once correlation between such methods and XRMA have been established, a more extensive analysis of stratum corneum hydration will be possible.

In a tissue, a substantial part of the water is bound to diffusible ions and organic material. XRMA studies have shown that virtually no elements of the species important for the physiology of living tissues are detected in the stratum corneum [9, 46, 48] and the mass of keratin in the epidermal cells increase as they approach the stratum corneum. Thus, the corneocytes mainly contain fibrous protein and no cell organelles. Keratins bind substantial amounts of water and the amount available as a solvent for ions decreases towards the stratum corneum although the total amount of water is the same



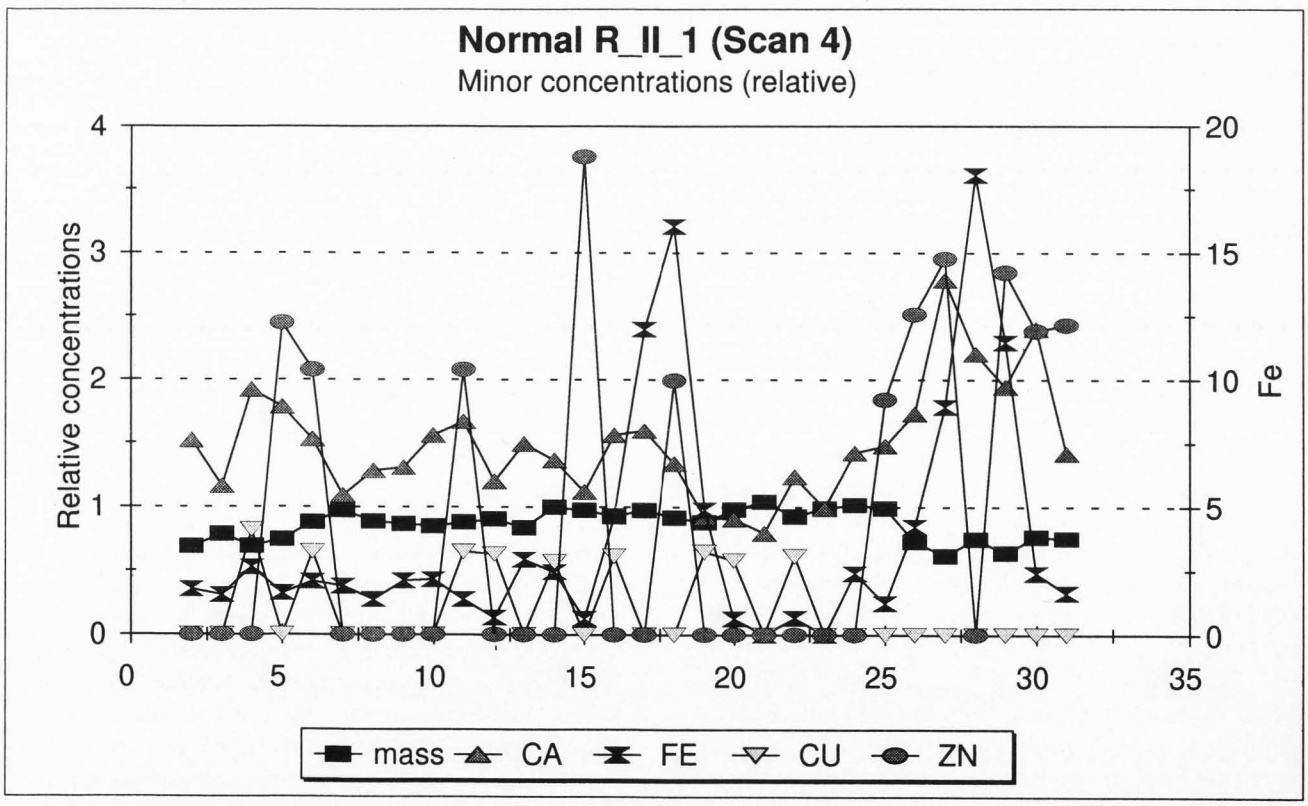


Figure 5. Compilation of data from a horizontal scan at the level of the basal epidermal cells extracted from a pixel map of a human skin cross-section: (**a**) major elements, and (**b**) trace elements.

over the epidermal cross-section. This creates a gradient for ions with a down-hill slope towards the dermis and, therefore, no active mechanism of ion transport is necessary to save precious ions.

PIXE elemental quantitation

The PIXE method has its strength in its sensitivity rather than its spatial resolution. In full agreement with the XRMA data, the mass distribution curves reach a peak in the stratum corneum region and flatten out in the basal cell region, again to rise in the dermis. The S distribution curves follow the mass curves in concert [10].

The P distribution peaks somewhat 50 μm below the maximum mass peak at a region corresponding to the stratum spinosum [6, 7, 9, 10].

Chlorine (Cl) has a weak minimum approximately where the P distribution has its peak followed by a conspicuous drop in the Cl content in the vicinity of the stratum corneum which is virtually free from Cl. Conversely, Cl content rises in the dermis well above the epidermal content. K reaches its highest levels in the Malpighian layers and drops to nil in the stratum corneum. In the dermis, relatively low values are recorded as is expected for the extracellular compartment [6].

Calcium (Ca) maintains a relatively constant level up to the stratum corneum and lingers somewhat closer to the skin surface than the trace elements Fe and Zn although very close to the detection limit so that true quantitation is not possible [7, 10, 32].

The trace element iron (Fe) has a high peak value in the basal cell region. The iron content then drops to values less than half the peak value in the uppermost epidermal layers and is not detectable in the stratum corneum region [10]. Zinc (Zn) is represented in the dermis by concentrations below or just at the detection level of the system [10, 32]. It shows a comparatively stable level over the Malpighian epidermis and disappears coincidentally with Fe in the stratum corneum region. Based on neutron activation analysis, it has been suggested that the ratio of epidermal to dermal Zn content should amount to 6:1 [28, 29]. Our PIXE studies so far suggest a more moderate difference, giving a ratio of 3:1. Copper (Cu) is just barely detectable in the Malpighian layers and, generally, no quantitation is possible within the acquisition times used in most of our experiments [32].

There are slight but obvious variations in the elemental distribution patterns from one section to another, although a general trend is clearly at hand. In a few cases, the Fe distribution has its center of gravity in the stratum spinosum/stratum granulosum area rather than in the basal cell region but in contrast Zn is confined to the basal layer.

Horizontal elemental distributions

The scanning procedure of modern PIXE analysis resulting in pixel maps allows additional analysis of data. One such set of data is represented by pixel "channels" that cover a specified stratum horizontally. The mass distribution along the basal lamina of normal skin varies somewhat along the horizontal scan (Fig. 5) but in contrast, remains approximately constant at the upper level (the level of the stratum spinosum/granulosum). We expect this as the population of cells in the basal lamina contains quiescent cells and cells in the process of entering or leaving the cell division cycle. In consequence with this reasoning, the K and Cl co-vary with mass in the basal region but the variation is more independent in the upper region. S co-varies to a great extent with the mass distribution but in both regions (upper and basal) there are more conspicuous variations in the P distribution as related to mass. Fe and Zn distributions vary extensively in the basal region and the Fe content is close to or below the detection limit in the upper region, whereas the Zn content shows some peaks above the detection limit. Single off-limit values in these trace elements were seen. It is of interest to note that Ca appears to stay rather constant within each horizontally scanned band which underlines the fact that the increase in Ca towards the stratum corneum is related to the physiological and regulatory effects of this ion.

The Physiology of Pathological Conditions

Experimentally induced irritative reactions

Non-sensitized guinea-pigs were injected intradermally with 0.05% potassium chromate ($K_2Cr_2O_7$) and 1% nickel sulphate ($NiSO_4$) dissolved in isotonic NaCl. XRMA on sections obtained after 24 hours showed that Ni had the most pronounced effect in the stratum germinativum [21]. This was revealed by an increase in the relative content of Na and Cl by almost 100% and Mg by approximately 50%, whereas the K content was diminished. It has been shown that increases in Na, Mg, Cl, P, and S occur under conditions corresponding to increased mitotic activity [19, 20]. The effects of $K_2Cr_2O_7$ were less conspicuous and an intracellular increase of Na and Cl and a decrease in K was recorded, compatible with effects seen in autolysis [18]. While $K_2Cr_2O_7$ is a potent oxidant and, therefore, has a high affinity to fibrous proteins such as the collagen of the dermis, whereas the Ni^{2+} ion does not have such properties. Therefore, the actual concentration of $Cr_2O_7^{2-}$ ions in the epidermis is likely to be much lower than that indicated by the concentration distributed intradermally.

Table 2. Elemental content of psoriatic epidermis.

Element	Normal		Psoriatic uninvolved		Psoriatic involved	
	mean ± SD	range	mean ± SD	range	mean ± SD	range
Fe	26.5 ± 6.7	16.5 - 30.5	30.6 ± 12.4	17.0 - 46.4	57.5 ± 26.3	27.5 - 97.8
Cu	3.8 ± 0.8	2.6 - 4.6	4.4 ± 0.6	3.7 - 5.1	4.3 ± 1.2	3.0 - 7.4
Zn	39.3 ± 12.4	28.7 - 59.2	41.0 ± 7.1	34.1 - 48.3	90.8 ± 48.2	46.6 - 198.7

Data in μg/g dry weight compiled from Molin and Wester [25, 26]. SD: standard deviation.

Sodium lauryl sulfate

In a study on non-sensitized female guinea-pigs, exposed to 5% sodium lauryl sulfate (SLS) for 24 and 48 hours, it was shown that Na, K and P increased, and there was also a rise in the Na/K ratio [22]. The increase in K and P is compatible with a mild proliferative activity comparable with the changes seen in involved psoriatic skin as opposed to the non-involved skin. An increased Na/K ratio suggests a membrane damage and experimental studies on the effect of dinitrochlorbenzen (DNCB) [20] have shown such effects.

Nickel penetration through guinea-pig skin

We used the guinea-pig skin model to study the enhancement effect of SLS on Ni^{2+} ion penetration [22]. Comparing the effect of a 5% $NiSO_4$ in distilled water and a 5% $NiSO_4$ in 5% SLS solution with untreated control animals, we could show that SLS enhanced the Ni^{2+} ion penetration manifold to allow a detection in all strata of the epidermis, i.e., at levels > 100 ppm. Pure $NiSO_4$, in distilled water only, allowed detection of Ni in the stratum corneum, corresponding to an accumulation of Ni^{2+} ions reported previously, using PIXE analysis on human skin *in vitro* (see below).

Metal ion penetration through human skin

After an 18 hour penetration of $Cr_2O_7^{2-}$ and Ni^{2+} ions through cadaverous human skin, PIXE analysis revealed an accumulation of both Ni^{2+} and $Cr_2O_7^{2-}$ in the stratum corneum, although the $Cr_2O_7^{2-}$ penetrated the stratum corneum barrier more effectively [11]. It must be admitted that, at the time of those studies, the spatial resolution of the system was low and the detection limit around 30 ppm (g/g). New studies on living human skin are in progress to clarify the matter.

Psoriasis

Uninvolved and involved (a stable plaque) psoriatic skin [14] was compared with the skin from healthy, normal persons in an XRMA study that revealed that Mg, P, and K were increased in the involved skin corresponding to what is recorded in highly proliferative, non-neoplastic cells [41]. A significant increase in P and Ca content in involved psoriatic skin compared to uninvolved skin from the same patients was previously seen by Burkhart and Burnham [3]. Kurtz *et al.* [17] reported corresponding findings from a PIXE study of psoriatic skin. These authors found no difference between the Zn content of control skin and uninvolved skin from psoriatic patients, but recorded a significant increase of Zn in pin-point lesions [17] as was the case in the study by Molin and Wester [26, 27] using neutron activation analysis (Table 2).

Our recent PIXE analysis of non-involved psoriatic skin showed a mass distribution that has the same general features as that of the normal skin, although generally at a lower level (absolute mass content). The P and S distributions are not conspicuously different from those of skin from healthy individuals (Y. Werner-Linde, J. Pallon, B. Forslind, submitted for publication).

This recent study of uninvolved psoriatic skin has revealed some interesting aspects on the elemental distribution. In contrast to the Ca distribution in normal skin, that of psoriasis follows the mass distribution more closely. Ca shows a two-fold or even higher increase in the stratum granulosum region compared to normal skin. In many sections, we observed an additional Ca peak in the vicinity of the basal cell layer but the full significance of this is not clear at the moment.

The uninvolved psoriatic skin has a Fe peak that appears closer to the mass distribution peak than in normal skin. There are obvious variations in the Fe content in different strata (cell layers) and it is of interest to note that the lowermost values are consistently at least twice as high as those in normal skin. The previously reported findings that psoriatic patients lose iron (Fe) through the shedding of stratum corneum cells in lesional areas [26] are, therefore, substantiated by the PIXE investigation which shows that clinically normal skin in psoriatic patients contains higher than normal amounts of Fe [32].

The Zn content of the uninvolved psoriatic skin is increased especially in the stratum spinosum except in one single section where the Zn closely follows the Fe distribution. Such variations are likely to occur as a function of the cell cycle position of a particular cell.

As a rule, the Cu concentrations are below the detection threshold and, therefore, not considered in this context (Y. Werner-Linde, J. Pallon, B. Forslind, submitted for publication).

Elemental distributions in different strata of psoriatic normal-looking skin - Horizontal scans

The mass distribution pattern essentially follows that which is seen in the normal control skin but with somewhat more variability in the upper layer. In comparison to the control skin, there are high mean values and prominent variations in the trace elements, notably Fe and Zn in the upper level of the epidermis. In comparison to the control skin, where copper (Cu) is a spurious finding, there are more spots of Cu above the detection limit in the psoriatic skin-sections both in the basal and the upper level. The reason for this finding is still unclear and the issue needs further exploration.

Bearing in mind that the spatial resolution of the PIXE method does not allow discrimination between the intra- and extracellular compartments, some crucial points concerning the physiology of normal-appearing psoriatic skin as opposed to the normal skin can be discerned. The Ca distribution profile is of special interest since it remains at an almost constant level over the skin cross-section in the normal skin but in certain specimens shows a slight increase in the stratum granulosum region. This finding differs to some extent from our earlier experience in a preliminary study [32] based on selected point measurements in different strata of skin-sections. These continuous changes seen in studies using quench-frozen specimens should be regarded as an effect of "snap-shots" depicting momentarily what are actually transient processes.

For a clearer indication of the background to these abnormal elemental distributions, further studies, including psoriatic plaques, will be needed. It may be noted that preliminary data from PIXE analysis of dry skin of atopics also present elemental distributions that vary conspicuously from those found in normal skin [32].

General Discussion

Multielement analysis is possible with particle probes

One obvious drawback of cytochemical methods is the difficulty of multielement analysis in the same section. A great advantage of the particle probes is the fact that virtually all elements of physiological interest can be measured **simultaneously** within one and the same section volume. This allows comparison of the relative elemental contents and formation of elemental ratios, e.g., Na/K, that provide sensitive markers for cellular function [39].

The Ca^{2+} signal

A full understanding of the influence of trace element content in the skin has not yet been reached. Elias and his group have contributed to the understanding of the role of calcium for epidermal differentiation in a series of elegant studies using ion capture cytochemistry at transmission electron microscopic resolution [25]. In the normal murine skin, the most prominent localization of Ca^{2+} was noted in the upper stratum granulosum and the dermis, whereas the basal region was virtually free from precipitates denoting the presence of Ca^{2+}. If the barrier was broken by acetone treatment, a redistribution of Ca^{2+} took place, with a conspicuous accumulation of precipitates in the extracellular space of the stratum corneum and loss of the stratum granulosum localization. Treatment of the barrier-disrupted skin with isoosmolar sucrose containing Ca^{2+} replenished the epidermal Ca^{2+} reservoir but impeded the secretion of lamellar bodies and hence barrier recovery. Conversely, treatment with isoosmolar sucrose only lead to barrier repair through lamellar body secretion in absence of the normal Ca^{2+} gradient. The authors [25] concluded that loss of the Ca^{2+} reservoir is an important signal for restoration of barrier function after damage. Obviously, there is a need for fully quantitative data to support these findings and such quantitative data can be obtained using PIXE analysis. In this context, it is of interest that melanin production in culture is successful only if Ca^{2+} levels of 1.5 mM are at hand [40].

Programmed cell death or apoptosis

Ca^{2+} has shown to be an important signal for various cell functions. Another aspect of the Ca^{2+} function is its property to promote "programmed cell death" or apoptosis; and Zn^{2+}, to inhibit this and the effects of Ca^{2+} ions, have therefore become a topic of interest [2]. No doubt, the sequences of cellular differentiation in the epidermis constitute a kind of "programmed cell death" which involves the complete dissolution of nucleic acid material in the stratum granulosum. One indication of the importance of increased Ca levels in this cellular stratum is related to the finding that a full differentiation of the epidermis does not occur in tissue culture unless the Ca content equals at least 0.1 mM [33]. Taken somewhat further (on a speculative basis as yet), these findings suggest an explanation to the sporadic occurrence of parakeratotic cells in the stratum corneum in the paralesional psoriatic epidermis where unusually high

Zn levels are recorded in the stratum granulosum zone. If this effect is directly coupled to an increased cellular activity in the germinative pool may be a matter of speculation but the actual high levels of Fe compared to normal skin suggest such an increased activity. More detailed analyses including particle probe studies are required before this question can be settled.

The skin seems to depend on an appropriate availability of Zn^{2+} for normal function [13, 29] but this subject has not been completely resolved. It is conceivable that these problems can be solved at least partially, using particle probe analysis.

Pixel maps provide information on the dynamics of tissue activity

One conspicuous feature of PIXE pixel maps is the variations of element and trace element distributions seen in different sections from the same tissue **block**. This is a finding occurring both in the normal skin and in the clinically normal psoriatic skin. Such variations in the distribution of elements and especially trace elements, indicate that there are obvious differences in the detailed cellular physiology of the differentiating keratinocytes. This underscores our previous experience from electron microscopic studies of contact irritative reactions which show that the correlation between the morphological image and the quantitative elemental data is not a direct one [19, 22]. Thus, certain stratum cells that morphologically appear to be similar, reveal their different stage of differentiation in the patterns of elemental distributions as suggested by the elemental maps. Future investigations using, e.g., immunological techniques to tag different markers of differentiation in parallel with elemental analysis should provide an additional detailed insight into the keratinization process programmed to develop a complete stratum corneum with a functional barrier.

Limitations of PIXE analysis

The spatial resolution of the PIXE analysis system is consequently low compared to the resolution obtainable with an XRMA system which allows subcellular compartment (< 1 μm) analysis. The nominal probe size used in our recent PIXE experiments was 5 μm x 5 μm to which must be added a halo of approximately 2 μm width. PIXE analysis thus superposes the intra- and extracellular compartment data during analysis, depending both on the comparatively low spatial resolution of the measuring system and on the thickness of the sections (16 μm), which of necessity must contain both compartments in a cross-section. This is the reason for the considerable smoothing of curves describing the elemental and mass distribution over the cellular layers of a differentiated epidermis (Fig. 4). Perhaps, this effect is most conspicuous in the very narrow stratum corneum region (a total width in a section of approximately 10 μm) where the mass curve is rather wide in these experiments.

The number of specimens investigated in a PIXE study may appear small in comparison with corresponding numbers used in different types of light microscopic investigations or biochemical studies. It must be realized that the typical acquisition time for a pixel map from a single section is generally at least 2 hours. Costs/benefits aspects of running an experiment will require optimization of data acquisition for evaluation. Cu, for example, was only occasionally detected above the threshold of sensitivity in our experiments. For a number of data that would allow statistical analysis by the algorithm used, the time allowed has not been sufficient so far.

Future Roles of Particle Probe Analysis

Modern microprobes, e.g., the new scanning nuclear microprobe in Lund, which collect data in the format of pixel maps, provide extensive possibilities of analysis of the primary data. Choosing interfollicular areas for the different strata of the skin it is possible to extract information on quantitative elemental contents of the skin cross-section, e.g., from stratum corneum down into the dermis from the maps in addition to the new information expressed in elemental maps. The PIXE maps can reveal the dynamics of the differentiating epidermis and give a more detailed image than morphology has hitherto provided. Future studies should help to relate the variations in elemental content of specific strata to the anabolic and catabolic processes that are the metabolic expressions of cellular differentiation in normal and diseased skin.

Acknowledgements

Our research on skin physiology has been possible through generous support from the Edvard Welander foundation (YWL, ML, BF), the funds of the Karolinska Institute (BF), the Swedish Work Environmental Fund (BF, ML), the Swedish Asthma and Allergy Foundation (ML), the Swedish Medical Research Council (grant no B94-39X-07897-08A), and the Crafoord Foundation (KM, JP).

References

[1]. Alberts B, Bray D, Lewis J, Raff, M, Roberts K, Watson JD (1994) Molecular Biology of the Cell; 3rd ed. Garland Publishing, New York. Chapters 9, 19.

[2]. Barr PJ, Tomei LD (1994) Apoptosis and its

role in human disease. Biotechnology **12**: 487-493.

[3]. Burkhart CG, Burnham JCV (1983) Elevated phosphorus in psoriatic skin determined by energy dispersive X-ray microanalysis. J Cutan Pathol **10**: 171-177.

[4]. Elias PM (1991) Epidermal barrier function: Intercellular lamellar lipid structures, origin, composition and metabolism. J Controlled Release **15**: 199-208.

[5]. Forslind B (1982) X-ray microanalysis in dermatology. Scanning Electron Microsc **1982**; IV: 1715-1724.

[6]. Forslind B (1986) Particle probe analysis in the study of skin physiology. Scanning Electron Microsc **1986**; III: 1007-1014.

[7]. Forslind B (1989) X-ray microanalysis of the integument. In: Microprobe Analysis in Medicine. Ingram P, Shelburne JD, Roggli VD (eds.). Hemisphere, Washington, DC. pp. 207-218.

[8]. Forslind B (1994) A domain mosaic model of the skin barrier. Acta Derm Venereol (Stockh) **74**: 1-6.

[9]. Forslind B, Wei X, Roomans GM (1983) Elemental distribution of guinea-pig epidermis: X-ray microanalysis in the electron microscope. In: Stratum Corneum. Marks R, Plewig G (eds). Springer, Berlin. pp. 217-220.

[10]. Forslind B, Roomans GM, Carlsson L-E, Malmqvist KG, Akselsson KR (1984) Elemental analysis on freeze-dried sections of human skin: Studies by electron microprobe and particle induced X-ray emission analysis. Scanning Electron Microsc **1984**; II: 755-759.

[11]. Forslind B, Grundin T, Lindberg M, Roomans GM, Werner Y (1985) Recent advances in X-ray microanalysis in dermatology. Scanning Electron Microsc **1985**; II: 687-695.

[12]. Forslind B, Kunst L, Malmqvist KG, Carlsson L-E, Roomans GM, (1985) Quantitative correlative proton and electron microprobe analysis of biological specimens. Histochemistry **82**: 425-427.

[13]. Goolamali SK, Comaish JS (1973) Zinc and the skin. Int J Dermatol **14**: 182-187.

[14]. Grundin TG, Roomans GM, Forslind B, Lindberg M, Werner Y (1985) X-ray microanalysis of psoriatic skin. J Invest Dermatol **85**: 378-380.

[15]. Iyengar GV, Kollmer WE, Bowen H (1978) The Elemental Composition of Human Body Fluids. A Compilation of Values for Adults. Verlag Chemie, Weinheim, Germany. pp. 51-54.

[16]. Johansson GI (1982) Modification of the HEX program for fast automatic resolution of PIXE spectra. X-ray Spectrom **11**: 194-200.

[17]. Kurtz K, Steuigleder GK, Bischof W, Gonsior B (1987) PIXE analysis in different stages of psoriatic skin. J Invest Dermatol **88**: 223-226.

[18]. Kuypers GAJ, Roomans GM (1980) Post-mortem elemental resditribution in rat studied by X-ray microanalysis and electron microscopy. Histochemistry **69**: 145-156.

[19]. Lindberg M (1991) Studies of irritant reactions on epidermis: The application of energy dispersive X-ray microanalysis. Scanning Microsc **5**: 229-238.

[20]. Lindberg M, Roomans GM (1983) Elemental redistributions and ultrastructural changes in guinea-pig epidermis after dinitrochlorobenzene (DNCB) exposure. J Invest Dermatol **81**: 303-308.

[21]. Lindberg M, Forslind B, Roomans GM (1983) Elemental changes at irritant reactions due to chromate and nickel in guinea-pig epidermis. Scanning Electron Microsc **1983**; III: 1243-1247.

[22]. Lindberg M, Sagström S, Roomans GM, Forslind B (1989) Sodium lauryl sulfate enhances nickel penetration through guinea-pig skin. Studies with energy dispersive X-ray microanalysis. Scanning Microsc **3**: 221-224.

[23]. Lindberg M, Forslind B, Sagström S, Roomans GM (1992) Elemental changes in guinea-pig epidermis at repeated exposure to sodium lauryl sulfate. Acta Derm Venereol (Stockh) **72**: 428-431.

[24]. Lövestam NEG (1989) Development of the Lund proton microprobe scanning and data acquisition system. Nucl Instrum Methods **B36**: 455-470.

[25]. Menon GK, Elias PM, Lee SH, Feingold KR (1992) Localization of calcium in murine epidermis following disruption and repair of the permeability barrier. Cell Tiss Res **270**: 503-512

[26]. Molin L, Wester P-O (1973) Iron content in normal and psoriatic epidermis. Acta Derm Venereol (Stockh) **53**: 473-476.

[27]. Molin L, Wester P-O (1973) Cobalt, copper and zinc in normal and psoriatic epidermis. Acta Derm Venereol (Stockh) **53**: 477-480.

[28]. Molokhia M, Portnoy B (1969) Neutron activation analysis of trace elements in the skin. I. Copper in normal skin. Br J Dermatol **81**: 110-114.

[29]. Molokhia M, Portnoy B (1969) Neutron activation analysis of trace elements in the skin. III. Zinc in normal skin. Br J Dermatol **81**: 759-763.

[30]. Nelder KH (1991) The biochemistry and physiology of zinc metabolism. In: Physiology, Biochemistry and Molecular Biology of the Skin, 2nd, ed. Goldsmith LA (ed.). Oxford University Press, Oxford, UK. pp. 1329-1350.

[31]. Odland G (1991) Structure of the skin. In: Physiology, Biochemistry and Molecular Biology of the Skin. Goldsmith LA (ed.). Oxford University Press, Oxford. pp. 3-62.

[32]. Pallon J, Knox J, Forslind B, Werner-Linde Y, Pinheiro T (1992) Applications in medicine using the

new Lund microprobe. Nucl Instrum Methods B **77**: 287-293.

[33]. Ponec M (1991) Lipid metabolism in cultured keratinocytes. Adv Lipid Res **24**: 119-139.

[34]. Pålsgård E (1994) Ion dynamics in insulin-producing cells. Elemental changes after momentary stimulation detected by electron and proton induced X-ray emission. Doctoral Thesis, Uppsala University, Sweden. pp. 1-55.

[35]. Pålsgård E, Lindh U, Roomans GM (1994) Comparative study of freeze-substitution techniques for X-ray microanalysis of biological tissue. Microsc Res Techn **28**: 254-258.

[36]. Resing KA, Dale BA (1991) Proteins of keratohyalin. In: Physiology, Biochemistry and Molecular Biology of the Skin, 2nd ed. Goldsmith LA (ed.). Oxford University Press, Oxford. pp. 148-167.

[37]. Roomans GM (1988) Quantitative X-ray microanalysis of biological specimens. J Electron Microsc Techn **9**: 19-44.

[38]. Roomans GM, Sevéus LA (1977) Preparation of thin cryo-sectioned standards for quantitative microprobe analysis. J Submicrosc Cytol **9**: 31-33.

[39]. Saubermann J, Heymann RV (1987) Quantitative digital X-ray imaging using frozen-hydrated and frozen-dried tissues. J Microsc **146**: 169-182.

[40]. Schallreuter KU, Wood JM (1991) EF hands calcium binding regulates the thioredoxin reductase/thioredoxin electron transfer in human keratinocytes and melanoma. In: Novel Calcium Binding Proteins. Heitzmann C (ed.). Springer Verlag, Berlin. pp. 339-360.

[41]. Smith NR, Sparks RL, Pool TB, Cameron IL (1978) Differences in the intracellular concentrations of elements in normal and cancerous liver cells as determined by X-ray microanalysis. Cancer Res **38**: 1952-1959.

[42]. Themner K, Malmqvist KG (1986) Determination of local area densities of heterogenous biological samples by elastically backscattered protons in PIXE microanalysis. Nucl Instrum Methods B **15**: 404-406.

[43]. von Zglinicki T, Ziervogel H, Bimmler M (1989) Binding of ions to nuclear chromatin. Scanning Microsc **3**: 1231-1239.

[44]. von Zglinicki T (1990) X-ray microanalysis - possibilities and methods. Acta Histochem Suppl **39**: 479-484.

[45]. von Zglinicki T, Lindberg M, Roomans GM, Forslind B (1993) Water and ion distribution profiles in human skin. Acta Derm Venereol (Stockh) **73**: 340-343.

[46]. Warner RR, Myers MC, Taylor DA (1988) Electron probe analysis of human skin. Element concentration profiles. J Invest Dermatol **90**: 78-85.

[47]. Warner RR, Myers MC, Taylor DA (1988) Electron probe analysis of human skin. Determination of the water concentration profiles. J Invest Dermatol **90**: 218-224.

[48]. Wei X, Roomans GM, Forslind B (1982) Elemental distribution in guinea-pig skin as revealed by X-ray microanalysis in the scanning transmission electron microscope. J Invest Dermatol **79**: 167-169.

Discussion with Reviewers

C.W. Kischer: I am fascinated by your assignment of apoptosis to the case of a renewal tissue such as epidermis. Normally, in "programmed cell death", a characteristic fine structural phenomenon is observed, that of paracrystalline arrays of the rough endoplasmic reticulum. In all of my observations by electron microscopy of epidermal cells, I never saw this illustrated. This is not to say that apoptotic cells must go through this event. What is your opinion about this?

Authors: The rough endoplasmic reticulum is not a very prominent part of the keratinocytes which could be one answer to the question. We like to stress that our interpretation of the Ca and Zn distributions as yet represent tentative assignments of the role of these trace elements in the differentiation process. The matter obviously needs to be substantiated with parallel studies using particle probe analysis and histochemical/morphological analysis of serial sections.

C.W. Kischer: If Ca promotes apoptosis and Zn tends to inhibit it, why would high levels of Zn occur only in the stratum granulosum? Does this mean that at the level of the stratum granulosum, the cell enter an obligatory phase? If so, would this mean that all levels below this stratum would not be programmed? What mechanism would tend to concentrate Zn at this level.

Authors: The particle probes allow only a quantitative assay of the elements per se. This means that we cannot differentiate elements bound to, e.g., proteins or nucleic acids from free ions. The elements present in ionic state will either be more or less free-floating in the intra- and extracellular compartments or bound to proteins or nucleic acids. We can only speculate that in the stratum granulosum cells, Zn is selectively bound to the thus inactivated nuclease, hence the given quantitative distribution patterns.

W.H. Wilburn: Did the quality of fixation deteriorate at deeper levels in the skin biopsies?

Authors: The deepest parts of punch biopsies are well cryofixed due to the comparatively loose and open architecture of the dermis which permits a rapid cooling rate if the tissue. In concord with its small cross-section (approximately 120 μm), the epidermis is, in our experi-

ence, always well fixed by quench freezing.

W.H. Wilburn: Do you expect to be able to detect differences in normal skin from one individual to another, between skin of males and females, and between skin from young and old individuals?
Authors: Particle probe analysis is a time consuming technique at the analysis stage and this has diminished our possibilities to study large enough population materials that would provide answers to your questions. There is no doubt about the fact that interindividual variations are recorded in normal and pathological tissues alike, hence, population studies may well reveal the other variations suggested by your question. So far, the difference between normal and pathological tissues have been conspicuous enough to allow interpretations of particle probe data.

E. Pålsgård: Do you have any idea why Fe and Zn are higher also in clinically normal psoriatic skin?
Authors: Knowing that psoriasis has a genetic cause and that at an outbreak of lesions the metabolic processes are tuned into abnormal expressions, the Fe and Zn distributions are further suggestions of inborn abnormalities in the psoriatic keratinocytes. These trace elements are precious to the body system and are therefore expected to be recirculated once they have performed their task bound to enzymes. We may speculate that, in the psoriatic skin, the break-down mechanism for such enzymes are deficient, hence the protein bound elements are not freed to an ionic form which would allow them to leave the epidermal compartment on a down-hill gradient similar to that postulated for Na^+ and K^+.

H. Sharata: Why should clinically quiescent uninvolved psoriatic skin contain Fe levels at least twice as high as normal in the stratum basale?
Authors: The higher than normal Fe level of the basal layer in psoriatic patients are likely to be related to a biochemical abnormality. Rather than speculating on the issue at this point, we may be able to get a better foundation for interpretation when we have pursued our studies of lesional psoriasis.

H. Sharata: I appreciate the comments regarding the limitations of PIXE but would like the authors to address the following points. Can the authors provide us with an idea of variability in their data. How many samples were examined, how many scans per sample, what were the ranges of data and the standard deviations associated with the mean values, etc. This is a seldom discussed point in electron microscopy and microanalysis, yet an important and obvious aspect to reaching meaningful quantitative results.
Authors: In the studies using the PIXE analysis, the typical experiments comprise a number of 6-10 individuals from which specimens are obtained. From each biopsy, the three best sections are chosen for PIXE analysis, two or three parallel sections are monochromatically stained for light microscope identification of structural features in the parallel sections. The microprobe PIXE-setup was calibrated using a procedure discussed by Johansson *et al.* [49] and Themner *et al.* [50]. The calibration is based on analyses of commercially available thin standard foils with elements covering the periodic system. The inaccuracy can then be estimated to be better than 5% using a focused non-scanning beam. The scanning model may add some percent to this, however, the main variability in the final data originates from the biological variability of the data.

Additional References

[49]. Johansson GI, Pallon J, Malmqvist KG, Akselsson KR (1981) Calibration and long-term stability of a PIXE set-up. Nucl Instrum Methods **181**: 81-88.

[50]. Themner K, Lövestam NEG, Tapper UAS, Malmqvist KG (1987) Calibration of a proton microprobe setup. Nucl Instrum Methods **B22**: 126-127.