Recent Advances in X-Ray Microanalysis in Dermatology

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RECENT ADVANCES IN X-RAY MICROANALYSIS IN DERMATOLOGY

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Abstract

Electron microprobe and proton microprobe X-ray analysis can be used in several areas of dermatological research. With a proton probe, the distribution of trace elements in human hair can be determined. In contrast to sulfur, which is homogeneously distributed, calcium, iron, and zinc appear to be non-homogeneously distributed over the hair cross-section.

Electron microprobe analysis on freeze-dried cryosections of guinea-pig and human epidermis shows a marked gradient of Na, P and K over the stratum granulosum. In sections of freeze-substituted human skin this gradient is less steep. This difference is likely to be due to a decrease in water content of the epidermis towards the stratum corneum.

Electron microprobe analysis of the epidermis can, for analysis of trace elements, be complemented by the proton microprobe. Quantitative agreement between the two techniques can be obtained by the use of a standard. Proton microprobe analysis was used to determine the distribution of Ni or Cr in human epidermis exposed to nickel or chromate ions.

Possible differences in water content between the stratum corneum of patients with atopic eczema and normal stratum corneum was investigated in skin freeze-substituted with Br-doped resin. No significant differences were observed.

Proliferative reactions in the epidermis appear to be associated with increased levels of the elements P and K. Such changes were found in guinea-pig skin after exposure to sodium lauryl sulfate, and in plaques of skin from patients with psoriasis.

KEY WORDS: skin, electron microprobe, proton microprobe, hair, nickel allergy, irritative reaction, occupational dermatology, water distribution, atopic eczema, psoriasis.

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Introduction

With the introduction of energy-dispersive X-ray microanalysis in dermatology (cf Forslind 1982) it became possible to study the physiology of the epidermis in normal and pathologic skin, at least in shock-frozen, freeze-sectioned and freeze-dried specimens. Until recently, particle probe analysis of skin was exclusively done with the electron microprobe (EMP). In 1983 PIXE (particle induced X-ray emission analysis with a proton probe) (Fig. 1) was introduced as a method for systematic exploration of the trace element content of epidermal strata in skin sections prepared by cryotechniques (Malmqvist et al. 1983). The feasibility of the method was previously noted by Gonsior et al. (1982). In view of the progress achieved in this field, an updated report on developments that have taken place since the subject of particle induced X-ray microanalysis was previously reviewed (Forslind 1982, 1984) appears relevant.

The present overview will only deal with the most recent papers. In addition, some new preliminary data demonstrating the potential of the particle probes as applied to dermatology will be presented. The review will be divided into a section on hair and a section on skin.

Particle induced X-ray analysis of hair

Although a number of papers have been devoted to PIXE analysis of hair, no systematic use of the method has been pursued until the recent theses by Li (1983) and Bos (1984). Li and collaborators developed a technique to obtain linear mass densities, longitudinal profiles and transverse distributions of trace elements in hair. It was shown that the geometric diameter of the hair strand is a suitable parameter in the quantification procedure (Li and Akselsson, 1985a,b), and that the distribution of elements over the cross section could be determined at perpendicular irradiation of the hair strand by using different proton energies. The spatial resolution obtained in this way is definitely inferior to that of a microbeam scan over a thin section (Malmqvist et al. 1983). However, the method offers a convenient procedure for routine analysis as it does
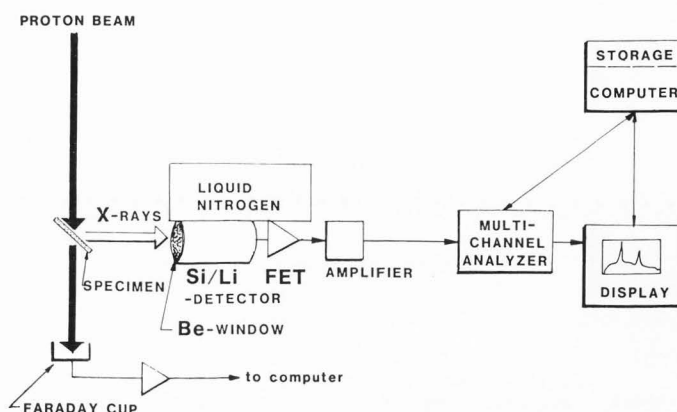
EMP – ANALYSIS
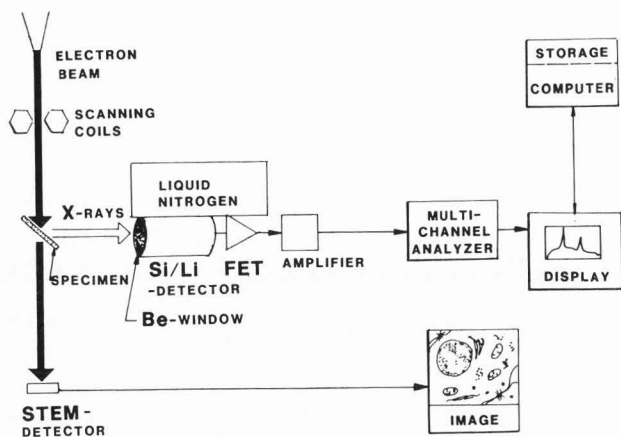
PMP – ANALYSIS

Fig. 1. Schematic comparison of the instrumentation for electron probe and proton probe X-ray microanalysis.

not require sectioning of the material. The diameter of the hair may be simultaneously determined by recording the backscattered proton signal (Li and Aksselson, 1985a,b). The experimental set-up in the Lund proton microprobe allows the analysis of 40 samples without breaking the vacuum (Li et al. 1984), and longitudinal scanning of single hair fibers is possible (Li and Malmqvist, 1985).

The developments in PIXE analysis led us to undertake a population study of sulfur, chlorine, calcium, and zinc in a normal, healthy caucasian population (Forslind et al. 1985a, Forslind et al., submitted for publication). The analysis was confined to anagen hairs taken from a region 1.5 - 2 cm above the right ear of the probands (n=103, 49 male, 54 female) to ensure that the samples (taken in triplicate) had been subject to the same (internal) conditions, and to reduce the influence of age differences in the area probed. Even with these strict criteria the mean values for sulfur and zinc content correspond to those found in the literature. There was, however, a considerable spread in the data, and this implies that sulfur is not a good internal standard for quantitative analysis.

In contrast to the experience with 'bulk' analysis of hairs (cf Brown and Crounse 1980) the study by Forslind et al. (1985a) suggests that rinsing does not significantly influence the quantitative results of PIXE analysis. This may to some extent be due to the fact that weathering effects have had little influence on the region 5-8 mm from the plucked hair fiber root end which was used for analysis.

Bos (1984) performed studies on the presence of trace elements in human hair cross sections taken from different heights of the hair follicle. He confirmed earlier observations by Cookson (1979) that the elemental distribution over the hair cross section is non-homogeneous for some of the elements. Thus, the cross section profiles of calcium, potassium, iron, copper, and zinc show high values at the periphery of the follicle

and low values in the center. This trend is constant from the bottom of the follicle up to a level some 500 μm from the follicle bottom, except for copper and zinc that are evenly distributed over the cross section at that level and upwards. It should be noted that these cross sections are situated below the level of the epidermis, so that these results do not reflect the effect of contamination. Sulfur shows an even distribution over the cross sections. The studies of Bos et al. (1984) were performed on bulk specimens embedded in plastic blocks which were sectioned step by step to obtain cross sections at different heights. In the study of Malmqvist et al. (1983) a different approach was used. Hair fibers were dipped into a carboxyl methyl cellulose gel and instantaneously frozen. Subsequently the fiber was freeze-sectioned at -100°C to a thickness less than 5 μm, transferred to a thin plastic supporting film and fixed to it by quick thawing. In this study the mature hair fiber cross section revealed high peaks of calcium, iron, and zinc about 20 μm inside the periphery of the hair and these elements decreased to very low levels 10 μm further towards the center of the hair fiber (Fig. 2). The sulfur content was constant over the cross section.

PIXE microbeam analysis can complement the corresponding macro-method when detailed information is desired. The most convenient preparation method appears to be cryo-sectioning which has the further advantage of allowing repeated analysis on the same section if desired; this is not possible with the bulk technique, where a new level is reached by cutting or grinding the entire block with the specimen. The advantage of sensitivity in the trace element region makes PIXE more attractive in hair analysis than the electron microprobe (EMP), for which the normal levels of e. g., iron, copper and zinc are at or below the detection limit.
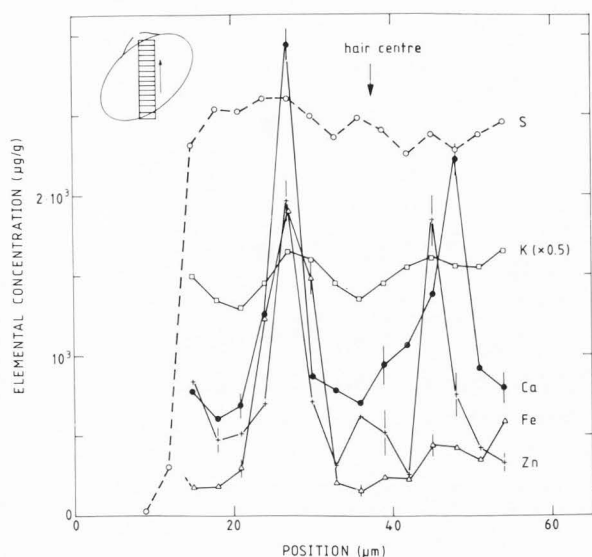
Fig. 2. Elemental distribution across a thin section of a human hair. The scanning was performed according to the drawing in the upper left corner. The broken line shows the sulfur distribution in arbitrary units and does not refer to the ordinate scale. The error bars show the uncertainty due to pulse statistics (one standard deviation). (From Malmqvist et al. 1983)

X-ray microanalysis of normal epidermis

The distribution of elements over the different epidermal strata was investigated in the guinea-pig (Wei et al. 1982, Forslind et al. 1983) and in man (Forslind et al 1984, Grundin et al. 1985). The data on guinea-pig skin were obtained on ultrathin cryosections, which allowed the analysis of a single epidermal layer in more detail. The thicker human epidermis was investigated using thick cryosections, which gives an inferior spatial resolution. Nevertheless, a comparison of the data (Fig. 3) shows a good qualitative and quantitative agreement for sodium, phosphorus and potassium distribution in guinea-pig and human skin.

In the first study on guinea-pig skin it was found that the Na/K ratio was lowest in the stratum germinativum (Wei et al. 1982, Forslind et al. 1983). This led us to speculate that the stratum germinativum was the outermost 'living' stratum of the skin, in the sense that already in the stratum spinosum the Na/K-pump was less efficient, which would result in a higher Na/K ratio in the stratum spinosum and other strata. This speculation seemed also to be in agreement with previous morphological observations that very early in the irritative and contact allergic reactions the morphological changes were confined to the stratum germinativum, whereas the other cell layers appeared morphologically unaffected (Lindberg et al. 1982).

In subsequent studies on guinea-pig epidermis (Lindberg and Roomans 1983, Lindberg et al. 1983) the difference in Na/K ratio between stratum germinativum and stratum spinosum did not appear to be significant, and also the data on human skin (Fig. 3) do not give any support for the idea of a substantial increase in Na/K ratio from stratum germinativum to stratum spinosum (Grundin et al. 1985). It should be taken into account that these studies (Lindberg et al. 1983, Lindberg and Roomans 1983, Grundin et al. 1985) were carried out on thick sections with inferior resolution compared to the thin cryosections used in the work of Wei et al. (1982). Small differences between strata might be obscured by partial overlap of the analyzed volumes.

A remarkable gradient for Na, P and K over the epidermis, particularly over the stratum granulosum, is observed in Fig. 3. It should be realized that in the curves of Fig. 3 the concentration data are related to the local dry weight of the tissue. However, there are good reasons to speculate that also the water content in the different strata is different, and will be much less in the stratum corneum than in the stratum germinativum. To investigate the distribution of elements over the different strata in relation to their wet-weight concentrations, analysis was carried out on sections of freeze-substituted skin. Skin biopsies were quench-frozen in Freon 22, freeze-substituted in diethyl ether at -80°C for 3 weeks, and finally embedded in Polarbed 812. Analysis was carried out on 0.4 μm thick dry-cut sections. Under the condition that the resin quantitatively replaces the tissue water, the concentrations obtained are related to tissue wet weight. The gradient over the stratum granulosum still exists (Fig. 4) but it is less steep, due to the fact that also the water content of the epidermis decreases towards the stratum corneum.

With the electron microprobe (EMP) the levels of calcium generally are at or below the detection limit. Also important trace elements such as iron and zinc are not detected by the EMP. Therefore it was of great interest to exploit the proton microprobe (PMP) and to compare the results obtained with this method with those obtained by the EMP. Paired freeze-dried cryosections were used for EMP and PMP analysis respectively.

The higher sensitivity of the PMP allowed the calcium profile of the epidermis to be determined. Above the stratum basale there was a slight decrease in the calcium curve which then increased to levels about six times the value at the stratum basale (Fig 5). The highest concentrations of iron and zinc were found in the stratum basale region (Fig 5) (Malmqvist et al. 1984).

Qualitatively, very good agreement between PMP and EMP concentration profiles for all elements that could be measured by both techniques (P, S, Cl, and K) was found. Quantitatively, however, there was a systematic difference between the EMP and PMP values; the EMP values being about 20% higher (Forslind et al. 1984). It was suggested that this difference could be due to two factors (1) difference in quantitation methods, and (2) difference in mass loss between EMP and PMP. In EMP analysis, quantitation was carried out with the help of a standard, in this
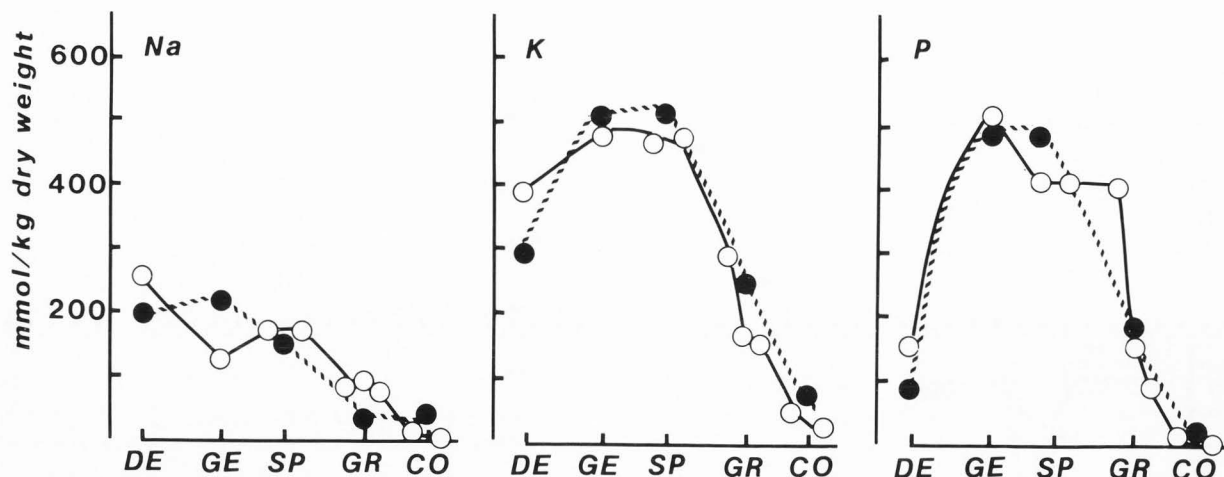
Fig. 3. Elemental profiles for Na, P and K in guinea-pig (O) and human (●) epidermis. The data for guinea-pigs were obtained on thin (Wei et al. 1982) and thick cryosections (Lindberg and Roomans 1983, Lindberg et al. 1983). Analysis of thin sections allowed measurements at different levels within strata. The data for human epidermis were obtained on thick cryosections (Grundin et al. 1985). The horizontal axis does not take into account the different thickness of the various epidermal strata in guinea-pig and human skin. DE: dermis, GE: stratum germinativum, SP: spinosum, GR: granulosum, CO: corneum.
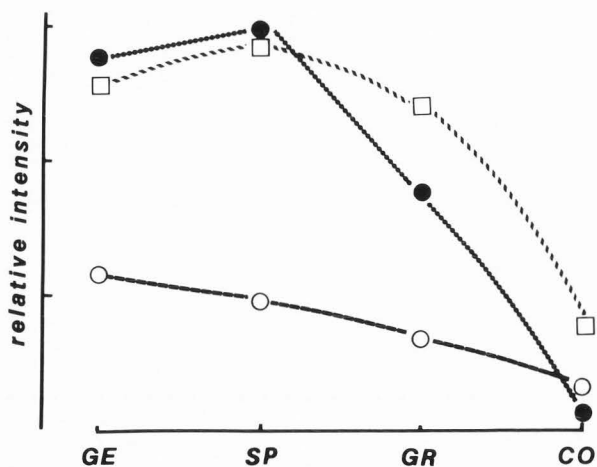

Fig. 4. Elemental concentration profiles for Na (O), P (●) and K (□) in human epidermis obtained from analysis of sections of freeze-substituted skin. The vertical axis shows the relative intensity corrected for the sensitivity of the detector for the different elements. The data represent mean values for 4 healthy adult volunteers; 6-12 measurements were carried out on each stratum.

case freeze-dried cryosections of a gelatin/glycerol matrix containing mineral salts (Roomans 1981). In PMP analysis, standardless quantitation was applied: sample mass thickness, determined from the continuum intensity, was used to calculate absolute concentrations.

It therefore appeared logical to attempt to use the same quantitation method in PMP and in EMP. As test specimens, a series of standards consisting of known concentrations of $NiCl_2$ in a 20% gelatin / 5% glycerol matrix was used. The standards were quench-frozen and 12-16 μm cryosections were cut, freeze-dried, and mounted for EMP or PMP respectively. When the signal was expressed as the ratio of characteristic intensity and continuum intensity, calibration curves were obtained that only showed deviation from linearity at very high $NiCl_2$ concentrations (500 mmol $NiCl_2$/kg dry weight) (Forslind et al. 1985b). A plot of the EMP versus the PMP signal gave a linear relation for Ni (Forslind et al. 1985b) and Cl (Fig. 6), with a correlation coefficient of 0.996 for both elements.

This indicates that quantitative correlative PMP and EMP analysis can be carried out using the same standard for both analytical techniques. This approach will be very useful for a complete understanding of the physiology of the skin in normal and pathological conditions. Elements occurring at sufficiently high concentrations can be routinely measured by EMP, whereas trace elements such as Ca, Fe, and Zn can be detected in adjacent serial sections by PMP. It should be noted that light elements (Na, Mg) cannot be very well measured in the PMP due to the thick mylar window shielding the detector from the high energy protons. PMP and EMP are thus truly complementary methods.

X-ray microanalytical studies on pathological skin conditions

Elemental changes in irritant reactions

Primary irritant contact reactions, caused by chemicals in the environment, are increasingly important in occupational dermatology.
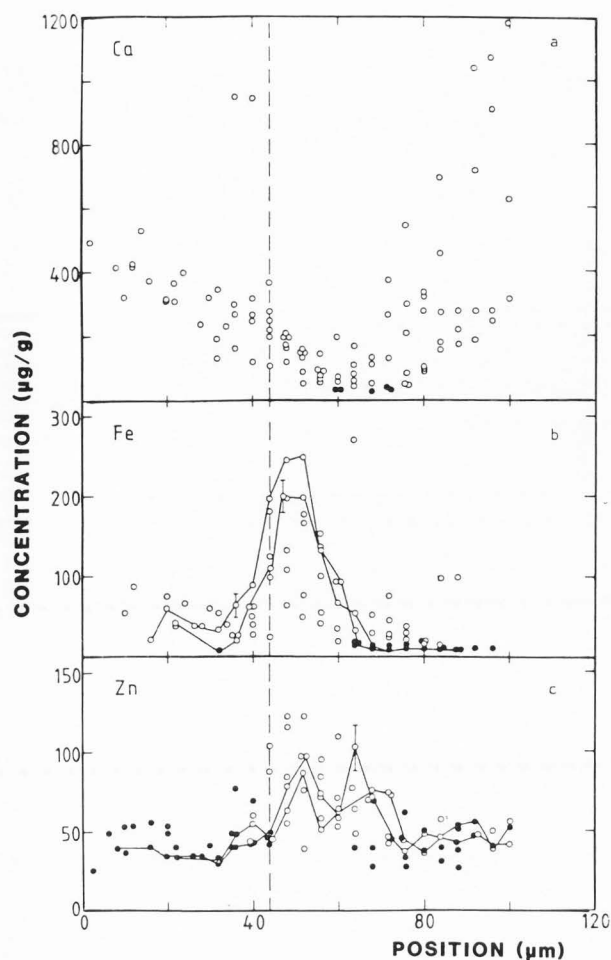
Fig. 5. Trace elemental distributions plotted for (a) calcium, (b) iron and (c) zinc, versus position for all sections with position related by matching the position of the steep phosphorus increase in each section to the position in one section (indicated by a broken line). Values below the detection limits are represented by full circles at the detection limits. The solid lines in (b) and (c) connect corresponding values from sections a and c of sample 1. The error bars show the statistical uncertainties ±1 SD. (Malmqvist et al. 1984)

In the present study, a mild irritant reaction was provoked by exposure to sodium lauryl sulfate (SDS). Female guinea-pigs were used for the experiments. Biopsy sites (on the flanks and the back) were clipped with an electric clipper prior to application of SDS (5% in distilled water). Biopsies were taken 24 or 48 h, respectively, after the application of SDS. In addition, biopsies were taken from untreated animals, to serve as controls. The biopsies were shock-frozen in liquid Freon 13 subcooled by liquid nitrogen. Electron probe X-ray microanalysis was performed on 16 μm cryosections cut at -25°C and freeze-dried in the cryostat.

The analysis shows an increase in the concentrations of P and K after SDS exposure, and an even stronger increase in Na concentrations.
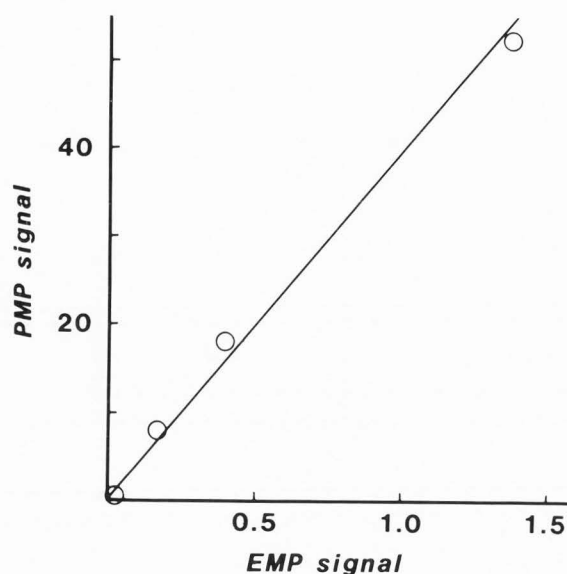

Fig. 6. Plot of the PMP signal versus the EMP signal (peak-to-background ratio) for Cl obtained from analysis of standards containing known concentration of $NiCl_2$ in a gelatin/glycerol matrix.
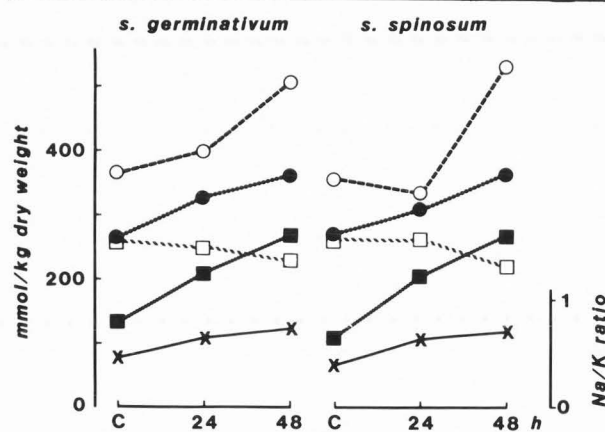

Fig. 7. Changes in the cellular concentrations of Na (■), P (O), S (□), K (●) and the Na/K ratio (X) in stratum germinativum and spinosum, 24 or 48 h, respectively, after SDS exposure, compared to control, unexposed, guinea-pig skin (c). The data points are arbitrarily connected by straight lines. Values represent mean of 5 animals; 8 measurements were carried out on each stratum.

This results in an increased Na/K ratio after exposure to SDS (Fig. 7). The stratum germinativum and the stratum spinosum react in a similar way to SDS.

The increase in P and K concentrations after SDS exposure could be related to a mild proliferative reaction. Similar changes are found in involved psoriatic skin as compared to non-involved psoriatic skin (Grundin et al. 1985) as discussed below. The increase in P content could be due to an increased level of nucleic acids (due to

Distribution of Ni
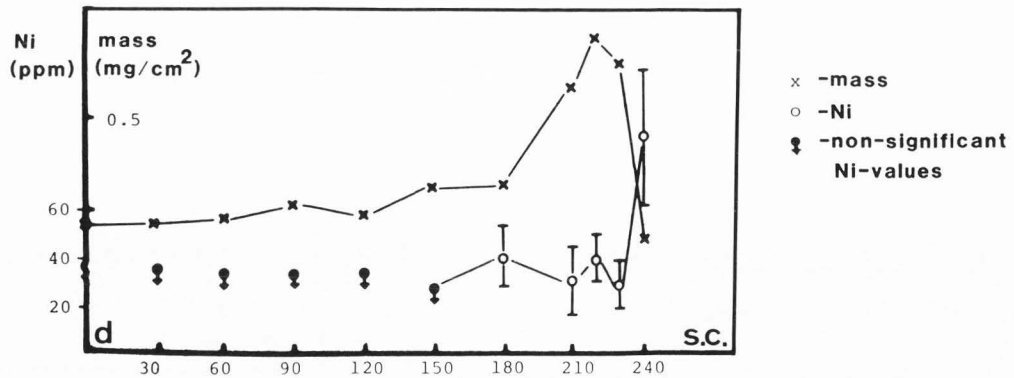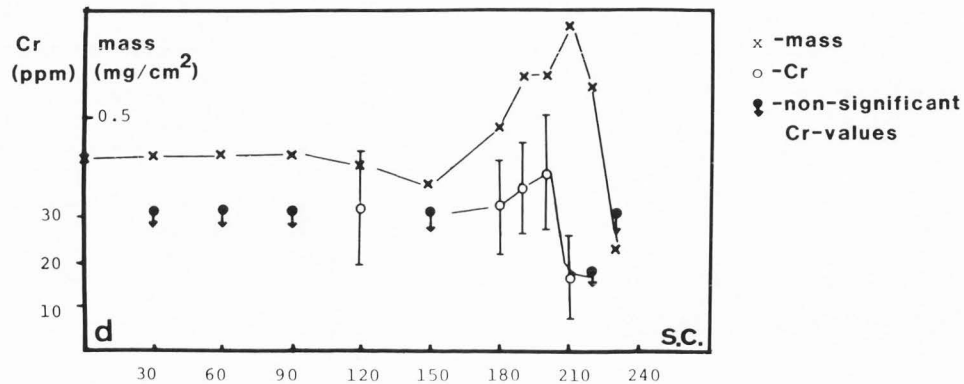

Distribution of Cr

Fig. 8. Elemental profiles for Ni and Cr in human skin after exposure to nickel or chromate ions, determined by proton microprobe analysis. Mass distribution is also shown. The dermis (d) is at the left side of the graph, the stratum corneum (s.c.) at the right side.

increased cell proliferation), and K ions are preferentially needed to stabilize nucleic acid structure. The increased Na/K ratio could point to membrane damage. Such an increase was also found after exposure of guinea-pig skin to dinitrochlorobenzene (DNCB) (Lindberg and Roomans 1983). The extra Na could, in part, be coming from the SDS solution.

Analysis of nickel and chromate in skin

The location of the barrier to nickel and chromate has long been an unsolved problem of great interest to occupational dermatologists.

Cadaverous human skin (not older than 24h post-mortem) dissected free of fat tissue and part of the reticular dermis was placed in an experimental chamber so that it spanned the opening between a donor compartment and a recipient compartment. The recipient compartment contained water, the donor compartment an aqueous solution of Ni^{2+} or $Cr_2O_7^{2-}$ ions. Diffusion was allowed to take place during 18 h at 4^oC to minimize

the effect of autolytic processes. The skin samples were shock-frozen and cryosections were analyzed by a proton microprobe. The Lund PMP used in this study has at present a spatial resolution of roughly 3 μm x 15 μm and the specimen was moved with a step motor so that the elemental distribution over the different strata could be determined.

The results indicate that the penetration mechanism of Ni^{2+} ions is different from that of chromate ions (Fig. 8). The chromate ions penetrate the skin more effectively than the nickel ions.

The concentrations of chromate and nickel in the epidermis are too low for EMP analysis. As shown and discussed previously (Lindberg et al. 1983, Forslind 1984) both chromate and nickel ions may induce changes in the cellular concentrations of Na, P, Cl, and K. These changes can be measured by EMP. The strategy of correlative EMP and PMP analysis, outlined above, would hence be very useful for this type of problem.

Analysis of skin in atopic eczema

The two most prominent features of atopic eczema are itching and dry skin. The presence of water is essential for the structure and the function of the stratum corneum. Unfortunately, there is no satisfactory method to determine the absolute water content of the stratum corneum.

Table 1

Relative Br concentration in stratum corneum of patients with atopic eczema and controls embedded in Br-doped resin.

	atopic eczema	control
1	4.5 ± 0.8	5.8 ± 0.5
2	9.9 ± 2.3	6.8 ± 2.0
3	13.0 ± 1.5	11.3 ± 4.3
mean	9.1 ± 2.5	8.0 ± 1.7

The data represent the ratio (in percent) of the Br signal in the stratum corneum and in the resin. The individual data are mean and standard error of 8 measurements. The difference between atopic eczema and control stratum corneum is not significant.

Based on desorption curves after full hydration, it was, however, found that water-binding capacity was reduced in patients with atopic eczema (Werner et al. 1982).

Recently, Ingram and Ingram (1983) suggested that microprobe analysis of samples embedded in Br-doped resin could be used to determine the local water content of the tissue. The method is based on the principle that the resin replaces the tissue water quantitatively, and that the local resin concentration can be accurately measured by adding an element not naturally occurring in the tissue to the resin. We have used a modification of this technique where the tissue was freeze-substituted rather than freeze-dried as done by Ingram and Ingram (1983).

For the present study, biopsies from the backs of 3 patients with atopic eczema and three controls were taken and immediately frozen in liquid Freon 13 subcooled by liquid nitrogen. The biopsies were freeze-substituted in diethyl ether at -80°C for 3 weeks and embedded in Spurr's epoxy resin to which dibromoacetophenone had been added to give a final Br concentration of 80 mmol Br/kg (Ingram and Ingram 1983). Electron probe X-ray microanalysis was carried out on 140 nm thick wet-cut sections.

The preliminary results (Table 1) do not indicate that the water content of the stratum corneum in patients with atopic eczema is lower than in controls. It should be noted that both inter- and intrasample variability are considerable, so that more samples are needed before a definitive conclusion can be reached.

X-ray microanalysis of psoriatic skin

Psoriasis may be considered as a model for an increased mitotic activity and this can be expected to involve changes in the elemental composition of the different strata of the skin. It was recently claimed that the stratum corneum of psoriatic skin would have a higher P and Ca content than non-involved skin (Burkhart and Burnham 1983). However, the preparative method used in that study carried a risk for loss or displacement of diffusible elements.
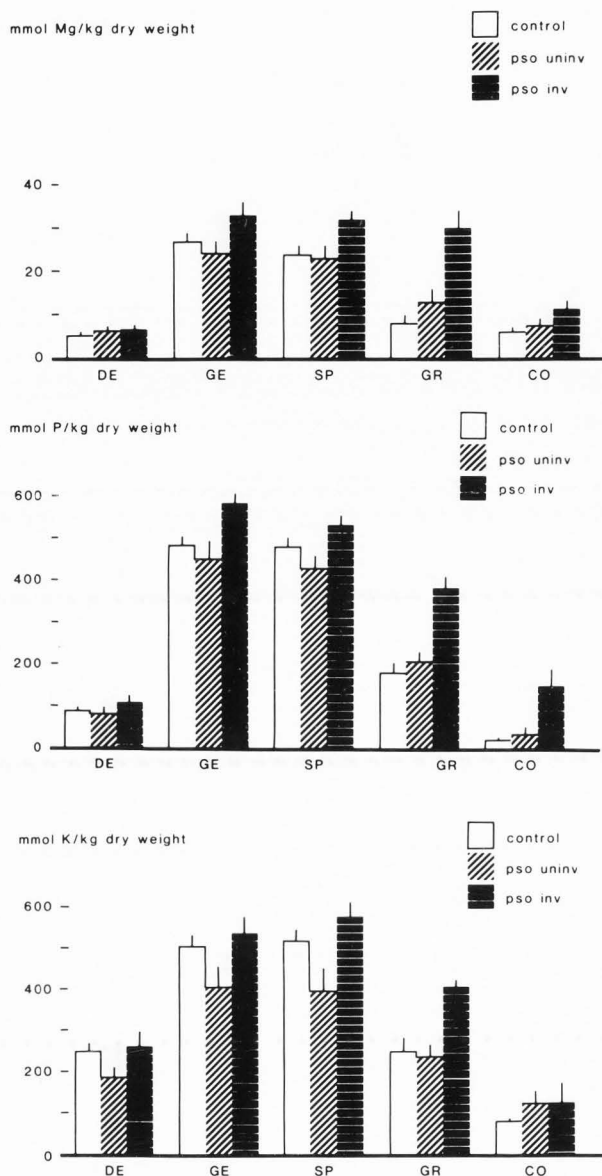

Fig. 9. Cellular concentrations of Mg, P and K in dermis and epidermal strata in involved and uninvolved psoriatic skin (mean of 10 patients) and in normal skin (8 controls). The data were obtained by analysis of thick cryosections. DE: dermis, GE: stratum germinativum, SP: spinosum, GR: granulosum, CO: corneum.

A more detailed study using cryotechniques was therefore carried out (Grundin et al. 1985). Skin biopsies were obtained from 10 male patients with stable plaque psoriasis and 8 male controls. From the patients one biopsy was obtained from a plaque (involved skin) and a second biopsy from paralesional uninvolved skin; all biopsies were taken from the vicinity of the right elbow. The samples were quench-frozen and electron probe analysis was carried out on thick cryosections.

Significant differences were found between the involved and the uninvolved skin of patients

with psoriasis. The concentrations of Mg, P, and K were higher in the stratum germinativum, spinosum and granulosum of the involved skin than in the corresponding strata of the uninvolved skin (Fig. 9).

The uninvolved skin from psoriatic patients shows the most clear-cut differences with the involved skin, not the skin from the controls. This may be caused by the high inter-subject variability of the results; the importance of this variability is less when each patient is compared with himself.

The biological significance of the differences between uninvolved and involved psoriatic skin is not completely clear. A high P content could be related to increased amounts of nuclear material, and Mg and K ions are preferentially needed to stabilize nucleic acid structure. The higher Mg, P, and K concentration in some of the strata in involved psoriatic skin could thus be a reflection of the increased proliferative activity.

Conclusions

With the introduction of inert preparation methods (i. e., methods preserving the in vivo elemental distribution) and the PMP technique, X-ray microanalytical techniques have proven to yield important information on physiological parameters expressed in the elemental distributions over the skin cross section. The EMP and PMP techniques complement each other and allow analysis of the elements involved in the regulation of cell homeostasis and control of cell division and differentiation. The recent application of these methods to pathological conditions suggests that new light can be shed on skin conditions through quantitative elemental analysis. We are at the threshold of the exciting field of quantitative dermatology where the different strata of the skin can be analyzed separately, and compared with the adjacent neighbouring layers.

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Discussion with Reviewers

C.W. Kischer: It appears that Ni and Cr are both at about 30 ppm except for the last two points (240 for Ni and 210 for Cr). On the basis of that one difference how do the authors conclude that Cr penetrates more effectively ?

Authors: The highest concentration of Ni is found at the skin surface, whereas for Cr the highest concentration is found about 30 μm below the surface (Fig. 8). We interpret this finding as a more efficient penetration of the skin by chromate as compared to Ni.

R.R. Warner: The authors conclude that more samples are needed to measure water content. I think they need a better technique instead and they might mention alternatives, such as the procedure described by us in Microbeam Analysis - 1984 (pp. 267-268). Our (unpublished) data does not support the preliminary conclusions of Table 1. Please comment.

Authors: The technique used in the present paper to measure local water content certainly has its problems. It is based on the assumption that cell water is quantitatively replaced by resin. Ingram and Ingram (1983) have, with freeze-dried specimens, shown that reliable quantitative results can be obtained on tissues such as muscle. It is possible, that freeze-substitution as used in our study gives rise to artifacts, or that the stratum corneum is a particularly difficult tissue in this respect. However, we feel that the method, even if not perfect, should be able to serve as a means to compare pathological and normal conditions.