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PARTICLE PROBE ANALYSIS IN THE STUDY OF SKIN PHYSIOLOGY

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Abstract

The basic function of the epidermis is to provide a barrier which will separate the body compartment from the environment thus protecting the organism from excessive loss of water and to hinder the entrance of noxious agents. A continuous renewal of the actual barrier makes it possible to fulfil these requirements. Using particle probe analysis, electron microprobe (EMP) and proton microprobe (PMP) analysis we have demonstrated the feasibility of these techniques in the study of skin physiology. The results reported here have been obtained on quench frozen skin specimens inertly prepared by cryotechniques to produce freeze-dried sections presenting cross sections of the skin. The distribution of Na and K is compatible with the idea that the Na/K pump of the cell membranes is dysfunctional above the basal cell layer. The phosphorus distribution over the epidermal cross section coincides with a previously shown phospholipid distribution. S and mass distributions correspond to the results of the keratin synthesis of the epidermis. Calcium displays a profile over the epidermis which is compatible with recent data obtained on the calcium dependence of the differentiation of epidermal cells in culture. Also this distribution corresponds to recent data obtained by histochemical methods at transmission electron microscope resolution. Zn and Fe have been shown to reside mainly in the basal cell layer of the normal epidermis but are found in high amounts in the outer cell layers of the epidermis in hyperproliferative paralesional psoriasis. The penetration of Ni and Cr ($Cr_2O_7^{2-}$) through human epidermis was studied in vitro and it was found necessary to employ the PMP for the analysis of these substances due to the low amounts present (<100 ppm). It appears that chromate penetrates more readily than nickel at neutral pH.

KEY WORDS: skin, epidermis, cryotechniques, electron probe X-ray microanalysis, proton microprobe analysis, calcium, trace elements, pathology, allergy, psoriasis.

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Introduction

The basic function of the epidermis is to provide a barrier which separates the interior of the organism from the environment. This barrier function of the skin is necessary to keep up the internal homeostasis with special relation to the control of water loss. Uncontrolled fluctuations of the body water content would put an immense strain on the physiological homeostasis mechanisms. Also, the barrier should prevent noxious substances from getting entrance to the inner compartments of the body. The continuous wear and tear on the body surface caused by physical and chemical agents requires that the barrier is constantly renewed to ensure optimal function.

Since the main task of the skin is to continuously provide a fresh barrier, the study of skin physiology in normal and pathological conditions becomes of great interest. Progress in this field has been greatly hampered by the following facts:

1. The human epidermis is an extremely thin tissue, roughly 100-150 μm in thickness (Fig 1).

2. The epidermis is a differentiated tissue which may be subdivided into four main strata, the stratum basale (or germinativum), the stratum spinosum, the stratum granulosum, and the stratum corneum.

3. The cell divisions only take place in the lowermost layer, the stratum basale, which in principle is a single layer.

4. The barrier of the skin is located to the stratum corneum which is roughly 10-20 μm thick on the average. The actual barrier preventing the water loss is likely to comprise the lowermost part of the stratum corneum.

From these facts it is clear that the conventional physiological probes are inadequate for the study of ion distributions over the various layers of the epidermis due to the conspicuous difficulties of a precise localisation of the actual probe in the living tissue.

The application of particle probes in biological microanalysis is of comparatively recent

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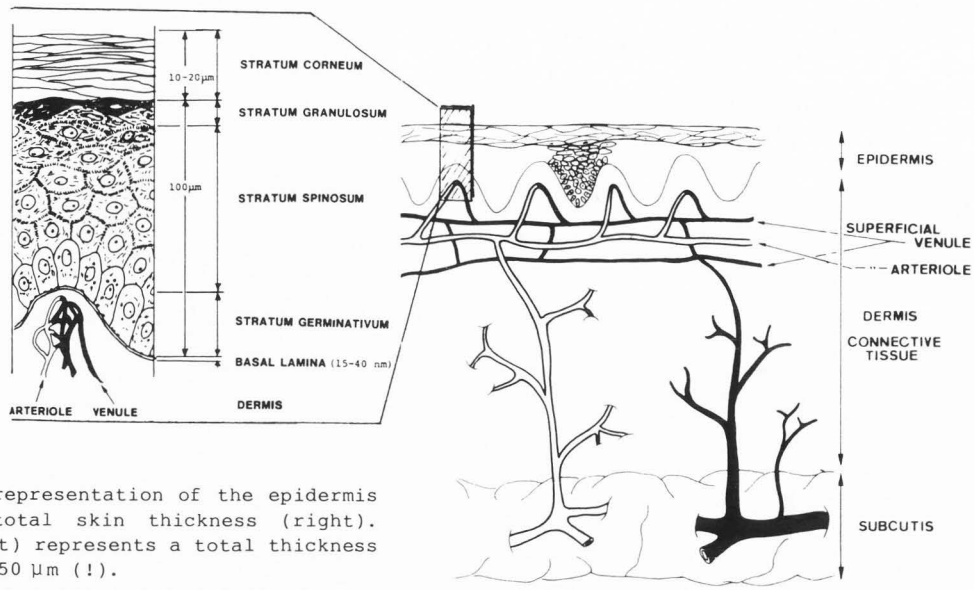


Fig 1: Schematic representation of the epidermis (left) and the total skin thickness (right). The epidermis (left) represents a total thickness of approximately 150 µm (!).

origin. During the seventies the application of the electron microprobe (EMP) to biological problems appeared in the literature and applications of proton microprobe (PMP) analysis on inertly prepared specimens was not presented until the beginning of the present decade.

The energy-dispersive detection systems of particle probes have as a special advantage the property of a simultaneous recording of all elements in the analyzed volume. With the introduction of these probes, a detailed study of epidermal physiology with regard to electrolyte content and electrolyte shifts became possible in inertly prepared skin specimens. This presentation will highlight our recent results obtained with the particle probes using human skin and experimental animal systems in the study of the physiology of skin in normal and pathological conditions.

Principle of the specimen preparation for particle probe analysis

Since the particle probes require a vacuum, living tissue cannot be directly analyzed. Using inert preparation techniques we have come close to the situation in the living tissue by avoiding treatments which result in dislocation of elements. Such rigid requirements on the preparation procedure are probably the reason for the comparatively small number of publications in the field.

Immediately upon biopsy the specimen is quench frozen in freon subcooled with liquid nitrogen (-190°C). Thin cryosections are prepared in a cryoultramicrotome at a knife and specimen temperature of below -100°C. Thick sections (12-16 µm) are sectioned in a conventional cryostat at -20°C. The sections are freeze-dried in the sectioning chamber and are subsequently stored over molecular sieve under vacuum until analysis.

We have also prepared thin sections from freeze-substituted tissue. For this purpose, the tissue was kept for 21 days in dry diethylether (-80°C). After slowly warming the tissue to room temperature it was embedded in epoxy resin. Sections were cut on a dry glass knife on an ultramicrotome at room temperature.

For analysis in the electron microprobe we mount the thick specimens on spectrographically pure carbon and for proton microanalysis the specimens are mounted between two thin plastic foils (Kimfol®) which do not contribute characteristic signals to the spectra obtained. Thin sections (cryosections or material embedded in plastic) for electron microprobe analysis were placed on Formvar film covered copper or nickel grids.

The particle probes

Basically the same principle governs the generation of characteristic X-ray quanta at the application of the electron microprobe (EMP) and the proton microprobe (PMP). When charged particles such as electrons or protons impinge on the specimen material, they collide with inner orbital electrons of atoms in the specimen. The orbital electron is ejected, leaving a vacancy behind which in a fraction of time is filled by an outer shell electron. Since the outer shell electrons have higher energies than inner shell electrons, the surplus energy is emitted as a characteristic X-ray quantum, the energy of which corresponds to the specific energy difference between the electron shells of that particular atom (Fig 2).

Typically all X-ray quanta produced are detected in an energy dispersive detector system which is sensitive to elements down to sodium (Z=11) for the EMP and aluminium (Z=13) for our present PMP instrument. The X-ray quanta are detected by an energy dispersive detector system (Fig 3), the characteristic feature of which is that all elements in the analyzed volume

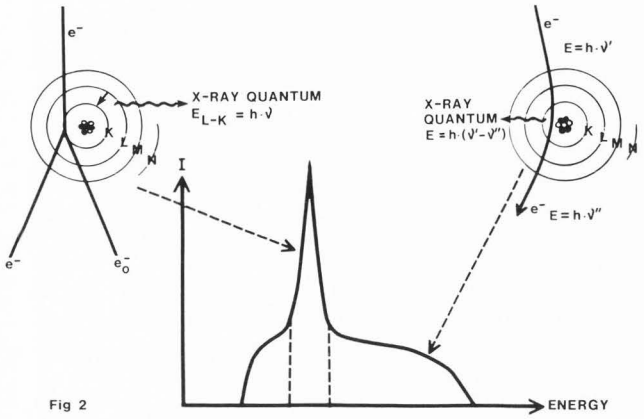
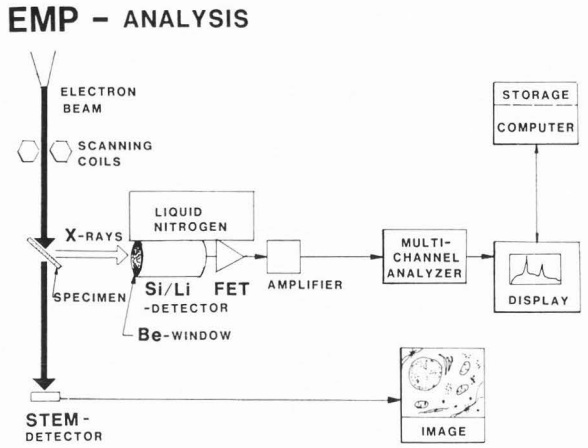


Fig 2: The production of characteristic X-rays (left) and the background continuum (right) results in peaks overriding a background (mid-panel).



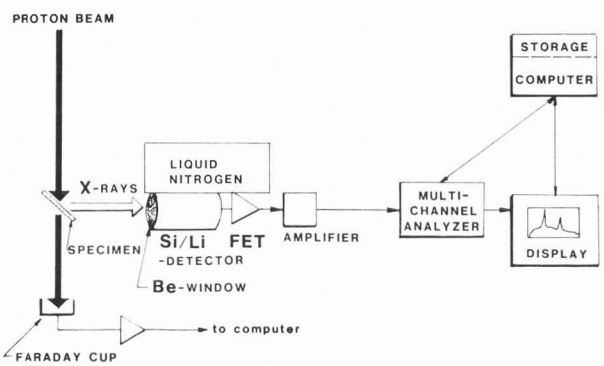


Fig 3: The equipment for electron microprobe analysis (EMP) and proton microprobe analysis essentially are built up according to the same pattern. The heart of the analyses system is the energy dispersive detector and the multi-channel analyser.

are recorded simultaneously. This makes it possible to determine ratios of elements present, e.g., Na/K ratios which may give valuable information on the condition of the cell or cell layer under investigation.

The sensitivity of the particle probes is to a considerable extent dependent on the production of background radiation. In addition to their specific interaction with inner orbital electrons of the object matter the light particles, e.g., electrons, are also retarded in the object in a non-specific way. The energy loss caused by this retardation also gives rise to X-ray quanta, which, however, are unrelated to specific elements in the specimen but represent a spectrum (a continuum) of energies (Fig 2). This continuum appears as a substantial background which prevents the detection of elements in trace amounts. The lower limit for the EMP sensitivity can thus be set to at best roughly 200 ppm.

The heavy protons in principle cause no background. However, a background originating from the retardation of the ejected electrons produces a small continuum in PMP analysis. The sensitivity of the PMP can thus easily reach 10 ppm. Hence, trace elements of importance in skin biology such as iron (Z = 26) and zinc (Z = 30) are readily detected by PMP which also detects calcium (Z = 20), which often is close to the detection limit of the EMP. In contrast, our PMP instrument cannot detect Na (Z = 11) and Mg (Z = 12), because the window shielding the detector is thicker than in an EMP instrument, causing absorption of low energy X-ray quanta. It thus appears that the EMP and PMP are complementary in their sensitivities. This complementarity can be used to investigate the same specimen by EMP and PMP. Using the same standard for both types of analysis we obtain matching quantitative results as was shown in a recent investigation (Forslind et al. 1985) where we analysed nickel-containing specimens prepared as standards for quantitative EMP analysis. We found a correlation coefficient for EMP versus PMP of 0.996 and hence we can safely state that the two methods are indeed complementary.

The spatial cross section is mainly dependent on the probe cross section, the particle retardation capacity of the object and the thickness of the object. In very thin specimens, e.g., 100 nm, the resolution of the EMP is roughly equal to the specimen thickness at optimal probe diameter. This allows detection of elements within volumes as small as cell organelles (Fig 4). In thick specimens, the resolution may be no better than about 10 µm. The probe diameter is essentially the determining factor for the spatial resolution of our PMP instrument which presently can be brought down to 3 µm. This means that in our case the spatial resolution of PMP analysis corresponds to a cell or a cell layer. In practice, the task of producing 100 nm cryosections for particle probe analysis is such a demanding task that most of our work

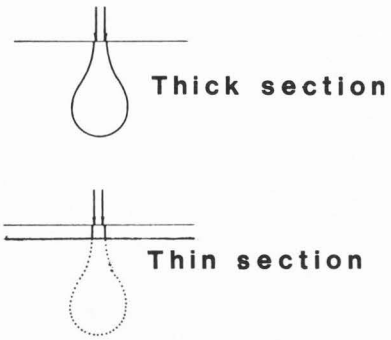


Fig 4: When electrons in EMP hit the target (the section) the electrons are scattered in the material of the object to form a tear-shaped 'volume of excitation'. The width of this volume determines the spatial resolution of the set-up (top panel). To improve the spatial resolution, thin sections which only allow the neck of the volume of excitation to be present (lower panel) can be used. The increase in spatial resolution is payed for by lower count rates and worse statistics (less good P/B ratios).

has been confined to thick specimens (roughly 12 μm). The spatial resolution of EMP is then the same as that of the PMP.

Elemental distribution in normal skin

The first particle probe analyses on inertly prepared skin sections were performed on guinea-pig epidermis. For this study we used thin sections (Wei et al. 1982, Forslind et al. 1982). The most conspicuous finding was the significant change in the sodium/ potassium (Na/K) ratio when the probe moved from the basal cell layer up towards the stratum spinosum (Fig 5). We interpreted this to be an effect of suboptimal function in the Na/K pumps of the cell membranes of the stratum spinosum cells. There is no direct blood vessel supply to the epidermal cells and obviously the oxygen supply at the stratum spinosum is limited due to the long diffusion path and the fact that the basal cell layer is intervening in that path. This suggests that the energy metabolism in the different strata of the epidermis must rely on different mechanisms. Indeed, studies by Long (1970) show a steep glucose gradient with the upper strata cells finding themselves in a virtually glucose free environment. Coupled to the finite low values of the glucose gradient is a decrease in phospholipids (Long 1970). In a glucose free culture medium epidermal cells degrade endogenous phospholipids (Long and Yardley 1970). A reasonable presumption is thus that the energy requiring reactions in the upper strata of the epidermis are driven by oxidation of endogenous fatty acids derived from membrane phospholipids. Deprived of oxygen and possibly having their membrane lipids exchanged, stratum spinosum cells are prevented from going into mitosis. This could obviously be one of the means of confining cell division to the basal cell layer of the epidermis.

The phosphorus content is high in the basal cell layer and declines towards the stratum

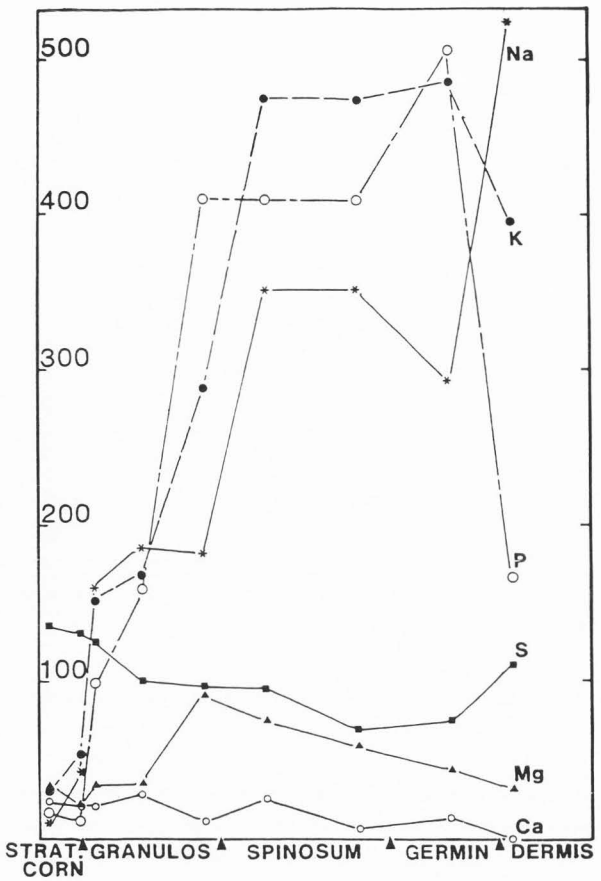


Fig 5: The elemental distribution over a cross section of guinea-pig epidermis. Na: sodium, K: potassium, P: phosphorus, S: sulfur, Mg: magnesium, and Ca: calcium. Vertical axis in mmol kg^{-1} dry weight.

corneum in a manner conspicuously similar to the phospholipid distribution presented by Long (1970) (Fig 6). Phospholipids have been shown to be replaced by ceramides during the differentiation process (Yardley and Goldstein 1976). As there are other sources for phosphorus such as keratin precursors,nucleic acids, ATP, etc, this remarkable correlation remains to be further investigated.

As expected in a keratinized tissue the sulfur content increases towards the stratum corneum.

The fact that the proton microprobe has a greater sensitivity than the electron microprobe prompted us to investigate its capacity to detect zinc and other trace elements in the skin (Fig 7) and to compare its analytical results with the EMP data. We found close agreement in the elemental profiles of skin cross sections from the same specimens studied with the two techniques (Malmqvist et al. 1984, Forslind et al. 1984) with reference to phosphorus, chlorine, sulfur, and potassium. An interesting finding was that zinc and iron were confined to the basal region of the epidermis reaching levels of 100 ppm and 200 ppm respectively. Another interesting finding was a conspicuous increase in the calcium profile towards the

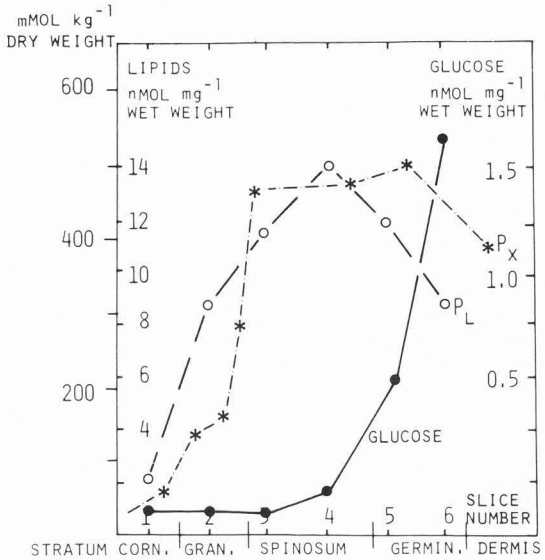


Fig 6: Distribution of phosphorus (P_X) as analyzed by X-ray microanalysis, plotted in relation to data on phospholipid (P_L) distribution and glucose distribution according to Long (1970).

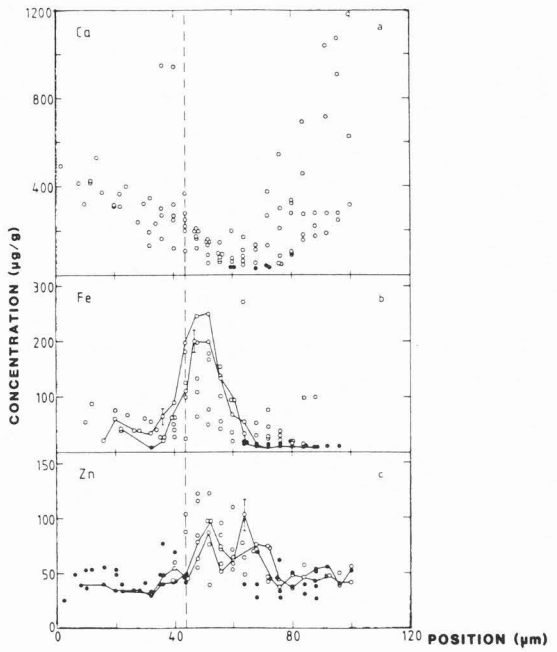


Fig 7: PMP analysis of calcium and trace elements, Fe and Zn in normal human epidermis. The Ca profile with a remarkable increase towards the stratum corneum is likely to express the conditions at hand for differentiation (cf text). The Fe and Zn data are mainly below 200 ppm and are therefore not resolved by EMP. Note that the significant content of these two metals are confined to the region of the basal cells (dotted vertical line represents border between dermis and epidermis).

stratum corneum (Malmqvist et al. 1984, Forslind et al. 1985). This corresponds to recent cytochemistry findings and experience from keratinocyte cell cultures which do not differentiate until a critical level of 1mM Ca is reached the support medium (Hennings et al. 1980, Boyce and Ham 1983, Ponec and Kempenaar 1985).

Elemental distribution in pathologically altered skin

One main focus of our research is the study of the effects of nickel and chromium on normal and sensitized skin. To study the possible elementary changes caused by such metal solutions we injected guinea-pigs intradermally in the right flank with 0.05% $K_2Cr_2O_7$ or 1% $NiSO_4$ in isotonic saline (Lindberg et al. 1983). Thick sections were analysed with the EMP and our results showed that the two metal ion solutions had different effects on the epidermal tissue in vivo. Chromate solutions which are potent oxidative agents caused cell damage characterized by decreased potassium and phosphorus levels and increased sodium and chlorine levels. This is in agreement with a previous study on DNCB effects on the epidermis in vivo (Lindberg and Roomans 1983). In contrast, in the nickel-exposed skin a conspicuous increase in sodium, magnesium, phosphorus and chlorine was recorded which suggests a stimulation of the germinative cell population (Lindberg et al. 1983).

These experimental findings made it interesting to study the effect in a hyperproliferative condition. We therefore chose psoriatic skin as a model. Skin from stable lesions, paralesional skin and skin from subjects not afflicted by psoriasis was analysed with the EMP. We found increased levels of magnesium, phosphorus, and potassium in the involved skin compared to uninvolved, paralesional skin. The elemental composition of the involved psoriatic skin compared to the uninvolved shows a pattern typical for a high proliferative, non-neoplastic tissue (Grundin et al. 1985).

Recently we made an additional PMP study of paralesional psoriatic skin. The phosphorus and calcium profiles correspond to those obtained with the EMP. The iron and zinc profiles which cannot be obtained with the EMP, extend out to the stratum corneum in contrast to what was found in normal skin. (Fig 8). The iron loss (anemia) observed in extensive exfoliative psoriasis agrees with this finding. It may be speculated that these elemental profiles are due to a defect in recirculation of the essential trace elements in the psoriatic skin (Cavill et al. 1969).

The barrier function of the skin is crucial at exposure to substances which may cause damage to the skin in particular and also to the internal organs of the body. In an in vitro system we have started studies on the penetration of nickel and chromate solution through the epidermal barrier. Our first experiments were performed on cadaverous human skin obtained less than 24 h post-mortem. Whole skin preparations freed from most of the reticular dermis were mounted so as to separate two vessels. The vessel facing

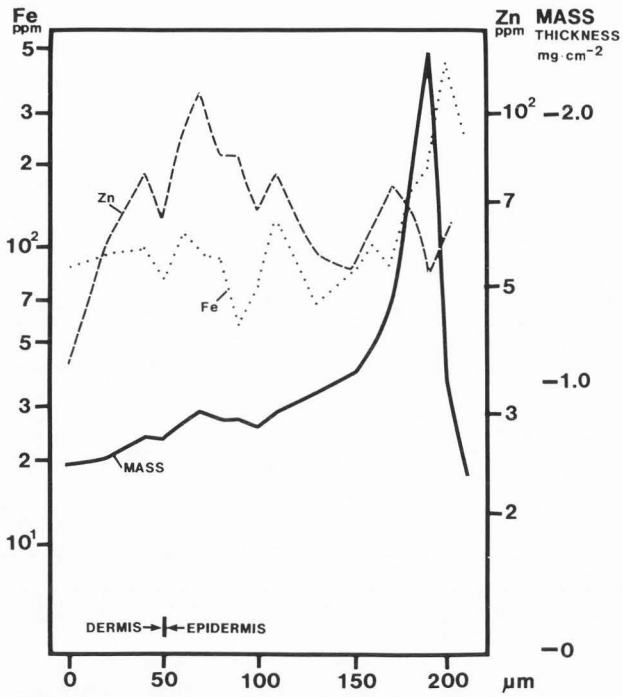


Fig 8: PMP analysis of paralesional (normal looking) psoriatic skin. The iron distribution peaks at approximately 500 ppm in the stratum corneum. The zinc distribution appears rather high even in the cell layers close to the stratum corneum. The mass curve denotes the dry weight distribution.

the stratum corneum contained the metal salt aqueous solution. Permeation was allowed to take place during 18 h at 4°C to minimize effects of autolytic degradation of the tissue. After the usual inert preparation the specimens were subjected to analysis. The penetration of nickel was less efficient than that of chromate. The amounts present in the tissue was too low for EMP analysis and PMP analysis showed the concentrations to be below 60 ppm in the skin. The advantage of PMP analysis in this kind of work was thus clearly demonstrated (Forslind et al. 1985). Judging from these preliminary studies chromate penetrated the skin more freely than nickel ions. In layers below the stratum corneum/ granulosum the metal content was not significantly above the detection limit.

The future. Further applications of particle probes

Although we have confined ourselves to dermatological problems, we have convincingly shown the feasibility of PMP analysis in conjunction with EMP analysis in normal and pathological conditions. The methodological problems are essentially solved and the methods may now be applied to a broader spectrum of experimental approaches.

Barrier penetration even of organic molecules may in the future be possible to analyze if these molecules can be 'tagged' with elements usually not present in the epidermis. A number of cytochemical applications in this vein may be realized in the future underlining the fact that the particle probes provide us with powerful qualitative and quantitative tools for the study of tissue physiology.

Acknowledgements

The work presented in this review was done in cooperation with research units headed by Dr G.M. Roomans, University of Stockholm, and Dr K. Malmqvist, University of Lund, Sweden, whose enthusiastic support of our projects is gratefully acknowledged. I am indebted to Ms Elsbeth Scholtes for her patience and her excellent typing of many versions of the manuscript. Financial support was granted by the Swedish Work Environmental Fund (ASF 79/92; 82/80; 85/0175).

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Discussion with Reviewers

B.A. Afzelius: You report an increase in the calcium level towards the stratum corneum of the human skin. Could this be an effect of washing the body with soap which normally contains calcium?
Author: The calcium profile is not likely to be due to Ca from soaps as these would cause saponification (Ca-soaps) at contact with skin lipids. The Ca would thus be trapped in the outer part of the stratum corneum and a step gradient would thus be expected. On the other hand, recent data (Boyce and Ham 1983, Ponec and Kempenaar 1985) also indicate that high Ca levels (>0.1 mmol) are required for the differentiation of the epidermis. Hence the Ca gradient seen in the PMP analyses should represent a physiological situation in a maturely developed skin.

B.A. Afzelius: The decrease in phosphorus content during the cell passage from stratum basale toward stratum corneum is interpreted as possibly due to replacement of the phospholipids by ceramides. How do you visualize the disappearance of phosphorus? As a resorption back through the stratum basale to the dermis or as a loss to the environment due to for instance washing?
Author: The phosphorus (P) of the epidermis not only represents the P of phospholipids but also that of phosphorylated proteins. As the P is released from the phospholipids thus providing energy for anabolic and catabolic reactions in the upper (anaerobic) strata of the epidermis, the ion moiety will diffuse down a gradient in the direction of the dermis. Under normal conditions this represents an economical recovery of essential ions (including the P of the said proteins), a principle which also is likely to encompass trace elements such as iron and zinc.

G.M. Roomans: When you refer to elemental gradients in the epidermis, your data give elemental concentrations related to dry weight. Shouldn't one take into account changes in cell water content, to arrive at physiologically more relevant data?
Author: The water gradient of the epidermis has not been satisfactorily determined yet. Hence direct physiological concentrations are not easily obtained. In principle it should be possible to obtain the water content from the mass profile data as these represent dry mass. Total mass - dry mass = water content, after corrections for shrinkage etc.

G.M. Roomans: If Na and K are lost when the cells pass through the stratum spinosum to the stratum corneum, are these ions lost to the outside of the skin (e.g., washed away by water) or are they reabsorbed into stratum basale or dermis?
Author: Na and K are likely to diffuse down a concentration gradient caused by the low water content of the uppermost strata, e.g., the stratum granulosum and the upper stratum spinosum. The high mass values obtained in this region of the epidermis with the aid of PMP (Forslind et al. 1984) suggest a corresponding low content of free water which is available for solutes. As larger volumes of free water are available in the lower parts of the epidermis and in the dermis, a concentration gradient with downhill direction towards the dermis will be created. From an economical point of view this type of gradient would be most beneficial especially with relation to the saving of essential elements such as Fe and Zn.

G.M: Roomans: The increased levels of Ca, Fe and Zn in the psoriatic skin must reflect an increase in bound rather than free elements. Are there any specific macromolecules or cellular structures that you suspect to be responsible for this increase?
Author: Fe and Zn are expected to be incorporated into proteins with enzymatic functions especially important for the production of new cell generations. Ca has been shown to be important for the differentiation of keratinocytes in cell culture and the Ca gradient is most likely to be a reflection of this fact in the normal skin. The exact nature of the macromolecules that bind Ca is not yet known.

G.M. Roomans: Zinc is known to be of importance in tissue regeneration. Could the increased zinc levels in psoriatic skin be connected with increased production of cellular material?
Author: The increased Zn levels could well be connected to an increased cell turnover of the (non-lesional) psoriatic skin.

J. Wroblewski: Have you attempted to use conventionally fixed and (paraffin) embedded tissue for PMP analysis of Fe, Zn, S, Ni, and Cr? To what extent are these elements lost by fixation?
Author: We have not tried PMP analysis on fixed tissues yet. Our experience of Ni analyses in fixed tissue has indicated that glutaraldehyde extracts Ni present in the tissue.

J. Wroblewski: Could you provide a table with reference values for the elemental concentrations in normal skin?

Author: So far we have not found data on the elemental content of the different strata of the skin in the literature. In a few cases the elements of the stratum corneum are given separately from those of the rest of the epidermis. Generally data have been obtained with spectroscopic methods such as AAS and not with EMP or PMP. Whole skin elemental data are found in Ilyar et al. (1978). Data on the elemental composition of the different elemental strata in normal human skin as determined by electron probe X-ray microanalysis have recently been published by us (Forslind et al. 1985, Grundin et al. 1985).

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