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QUANTITATIVE AND COMPUTER ASSISTED ELECTRON MICROSCOPIC AND MICROPROBE STUDIES IN DERMATOLOGY

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Abstract

Electron microscopes are not yet routine instruments in modern dermatological pathology even though they have provided detailed data about pathological changes in the skin for more than three decades. At present, dermatopathology is still dominated by light microscopy and especially so since the introduction of immunological techniques such as the use of monoclonal antibodies. These tools applied at electron microscopic resolution, however, may provide the ultimate identification of cells and subcellular components. In addition, electron microscopes have no peers in areas of quantitative investigation at subcellular levels, e.g. morphometry.

The electron microprobe provides a unique tool in elemental analysis and may be used for the analysis of conventonally prepared specimens when foreign matter, not soluble in water, is deposited in the tissue. On the other hand, with water soluble substances the technique is most effective when freeze sections are utilized.

This paper gives a selected review of the present day status of quantitative skin research as analysed with electron microscopy and the related technique of electron microprobe analysis.

Key Words: Dermatology, dermatopathology; quantitation; electron microscopy, electron microprobe analysis.

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Introduction

The tendency of biological sciences to orientate towards molecular techniques has had an enormous impact on the status of modern dermatologic science and dermatopathology. Since the introduction of new immunological techniques such as the use of monoclonal antibodies, present day dermatopathology has remained dominated by light microscopy. In this context, it is noteworthy that the electron microscopes are not yet regarded as routine instruments in these fields of research, even though they have provided detailed data about pathological changes in the skin for more than three decades. To a certain extent this is related to the cost of setting up an electron microscopic research unit.

There are, however, areas of investigation where electron microscopes have no peers. When high resolution of subcellular morphology is needed (< 0.1 um), e.g., for the identification of Langerhans' granules (27,29) and corresponding subcellular particles, light microscopic staining or immunological labelling can only indicate the possibility of specificity, whereas the transmission microscope (TEM) provides direct identification of the morphological entity. In addition to the information obtained by the transmission electron microscope, the scanning electron microscope (SEM) can reveal topographical details at the cellular and subcellular level which are impossible to discern with the light microscope.

In the past, electron microscopes have been used mainly for descriptive analysis of morphological details with normal and pathological tissues. In clinical and experimental dermatology, quantitative aspects of morphology have been reported only to a very small extent. How can electron microscopy be used in order to extract an optimal amount of data from normal or pathological skin biopsies ? There are essentially two main roads to choose from. One is concerned with conventionally prepared tissue, the other requires inert preparation procedures. In the first case, morphology is evaluated qualitatively by ocular inspection or quantitatively by means of morphometry. In the second case, elemental analysis is at focus of interest.We shall look into these topics in the light of their application to dermatology and dermatopathology.

In the last decade, a demand for quantitative data has evolved among electron microscopists. Morphometry, or to

use a synonym, stereology, has been found to represent an attractive means of analysing electron micrographs to obtain relative or absolute quantitative information. Using these techniques quantitative data on subcellular compartments of the skin can be assessed using computer programs designed to give stereological data from electron micrographs. A further development along these lines is provided by image analysis systems.

In addition to the image forming properties of electron microscopes, their special derivative, the electron microprobe (EMP), provides a unique and very sensitive tool for elemental analysis with a subcellular resolution. In the EMP, the characteristic X-rays generated from the elements in the sample volume when hit by the impinging electron beam are detected. The EMP is generally an electron microscope provided with an energy dispersive (EDX: energy dispersive X-ray) analysis system, which allows a simultaneous qualitative and quantitative recording of all elements present in the sample. It may be used for the analysis of conventionally prepared specimens when foreign matter, not soluble in water, is deposited in the tissue. On the other hand, for water-soluble substances, e.g. physiologically interesting elements such as the electrolytes, the technique is most effective when cryo-sections are used.

This paper will present some of the recent developments in skin research performed with the electron microscope and the related technique of electron microprobe analysis, rather than giving a complete review of this literature in dermatopathology. Reviews on clinical dermatological applications of electron microscopy and energy dispersive X-ray microanalysis have been recently published (7-11). This paper is divided into three main parts, dealing with transmission electron microscopy (TEM) including stereology, scanning electron microscopy (SEM), and electron microprobe (EMP) analysis of skin and its appendages.

Quantitative Transmission Electron Microscopy

Studies on skin

Although dermatopathology is at present dominated by immunological techniques, transmission electron microscopy plays an important role in the analysis of skin diseases. The field is too vast to be condensed within this paper. We shall therefore give a few examples of areas where quantitative analysis may be developed and then concentrate on outlining some of the recently developed techniques for quantitative evaluation of different compartments of the skin and its appendages.

First, let us consider tissue culture, the ultimate aim of which is to mimic the conditions of the tissue *in vivo*. For epidermal cultures the goal of culture is to obtain a differentiation of cells to form all the different strata of an epidermis including a stratum corneum in the normal case. One of the morphological details seen in the process of differentiation is the formation of contact points between the epidermal cells, mainly desmosomes. Using the TEM, Hino *et al.*,(19) have studied the process of desmosome formation in the culture of human epidermal keratinocytes. They were able to discern six different stages of cell adhesion, the last stage being the formation of the complete desmosome. The analysis of the time sequence of desmosome formation suggests that it takes at least 24 h for a desmosome structure to form. An over-looked consequence of this paper is the possibility of analysis of cell proliferation when it is not possible to use autoradiography. Thus, it is permissible that TEM identification of cells with "non-mature" contact points would actually allow a quantitative analysis of rapidly dividing epidermal (and epithelial ?) tissue.

Stereology

Stereology is a technique that was originally developed among mineralogists as a tool for the estimation of different mineral components of ores (c.f. 1). As early as in the 1940's, stereology was introduced into the biological sciences (3) but real interest in quantitative biology, e.g. stereology, was not evoked until electron microscopy had been established as a routine technique in biology and medicine. One of the first applications of this method in the field of dermatology was presented in work on the keratinization process in the hair follicle from our laboratory (12). The real break-through in biological stereology came with the excellent work of Weibel who developed the theory for biological applications (40). In recent years comprehensive reviews on and development of the topic have been presented by Gundersen and co-workers (17,18).

Stereology is based on the demonstration that a quantitative measurement of areas within a given two-dimensional image (e.g. an electron micrograph) faithfully corresponds to the volumes of the three-dimensional entities represented by these areas in the two-dimensional picture. When applied in a dermatopathological context, stereological theory thus allows estimation of changes in subcellular compartments not only in area cross-sections but also in volumes.

In the field of dermatology, the use of quantitative morphometric methods at electron microscopic resolution has been extremely sparse. Klein-Szanto (22) made an attempt to provide base-line data for the normal human epidermis, but unfortunately no corresponding or comparative work has been done using modern fixation techniques including inert fixation. However, stereological methods have been used for the analysis of fixation effects on tissues. Lindberg (23) has shown that changes in the osmolarity of the fixation buffer cause appreciable changes in cell volume and intercellular spaces of human epidermis. Corresponding studies on human normal oral epithelium were made by Squire *et al.*(38). Lindberg *et al.*(24) have also shown that simple occlusion, which results in an increased hydration of the stratum corneum, also causes increases of the intercellular spaces of the epidermis. The dilatation of the intercellular space has a biphasic time-dependent course.

The practical use of stereological methods has been greatly advanced with modern measurement devices such as digitizing tablets connected to a personal computer (PC). A digitizing tablet allows simple computing of areas which are encircled on the micrograph. Checking the magnification against a standard grid allows the measuring system (digitizing tablet + PC) to be calibrated by conversion numbers for magnification. The actual dimensions may thus be presented directly by the computer program. In order to study a

dimension such as the thickness of the epidermis it is possible to obtain useful data on such a parameter by measuring the area of the epidermis between two parallel linesat a fixed distance perpendicular to the epidermal surface. The area, divided by this fixed distance gives the mean value of epidermal thickness in the area selected (Fig 1).

The greater transepidermal water loss (TEWL) of atopic skin compared to that of normal human skin was tentatively attributed to a difference in the lipid composition of the stratum corneum barrier lipids. Werner *et al.* have made a quantitative morphometric study of the lamellar granulae volume in normal and atopic skin on randomly mixed electron micrographs from atopic and control specimens (41). The results show that the relative volume of the lamellar bodies in the atopic skin was greater than that of the normal skin. This indicates a lack of maturation in the atopic skin lipid processing (42).

Whereas digitizing tablets can be regarded as the "poor man's aid in stereology" in general, a number of very advanced measuring systems have been introduced on the market lately. Many of these systems are based on the principle of digital image analysis which allows electronic modification of the digitized image data. Background reduction may provide a clearer view and definition of the profiles under investigation. It must be realized, however, that in spite of the objectivity of the computer, an element of subjectivity always edges its way into the measurements, e.g., when the operator makes a decision about the degree of background reduction. The background reduction process may eliminate fringes on the objects and such fringes may have a biological significance. The results obtained with these advanced systems will therefore be no better than the subjective judgement of the scientist. In addition, it is unfortunate that, in general, the statisticians responsible for the software of these computerized systems have not created them in intimate cooperation with biologists familiar with stereological problems. In the experience of these authors, this is the explanation why there are many biological problems that are difficult to solve using the present day image analysis systems.

The mechanical properties of the skin, i.e. the dermis, are to a major extent dependent on the properties of its main constituent, the collagen. Changes in the normal architecture of the connective tissue of the dermis which alter the mechanical properties generally involve this fibrous protein. Ageing, simple scarring after a mechanical trauma, damage by irradiation (UV and X-ray), and congenital disorders such as Ehler Danlos syndrome provide a spectrum of morphological changes in this connective tissue constituent. The distributions of collagen fibril diameters are therefore of particular interest to research in dermal function and morphology.

Collagen fibril diameter is related to the species. Within a species it is also related to the functional unit it is part of, e.g., connective tissue, fasciae, tendons, etc. The diameter of normal human dermaately 50 nm (4, 21,33). Even in the same TEM section, it is possible to see variations in the fibrillar diameter at magnifications of 7,000 - 10,000 or larger. The reason for these diameter variations has not been unambiguously settled but it seems plausible that factors such as the content of glucosaminoglycans, closely associated with the collagen, have an influence on the diameter (5,32). Since

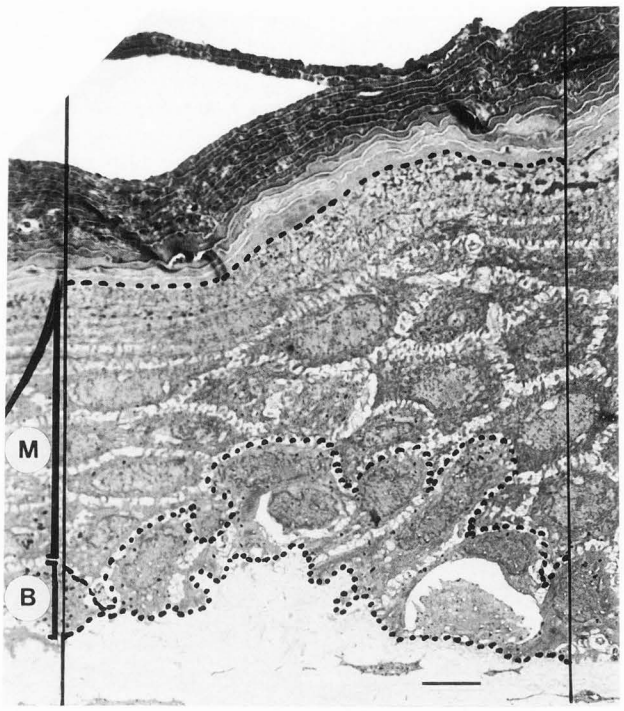

Fig 1. Vertical section through human skin. The area outlined is the area measured on a digitizing table by the PC-based program for area estimatimation, here used for determination of epidermal thickness between the parallel vertical lines. Bar denotes 25 μm.

these polymers have a great affinity for water, they are also likely to be influenced by the preparation procedures used for TEM. Support for this idea can be drawn from the study by Craig et al. (4), which showed that the differences in collagen fibril diameters occur as a function of the fixation technique employed.

The influence on diameter values by the preparation technique may explain seemingly controversial data on the collagen fibril diameters. Barton and Marks (2), using a Quantimet 720 image analysis system, have reported that in normal individuals the mean collagen fibril diameter of papillary dermis (the superficial part) is about 56 nm whereas that of the reticular dermis (the deep part) is approximately 63 nm. In addition, they tentatively suggest that short term steroid treatment of the skin does not effectively change these data.

Using the Zeiss Kontron IBAS system, we have studied the collagen fibril cross-section areas in skin which had been subjected to expansion in an attempt to gain skin for plastic reconstruction of the breast. The system gives data from a large number of entities, i.e., collagen cross-sections ($n > 50$), which allows statistical analysis for a differentiation between normal and possible scar type of collagen. The analysis of skin collagen before, during, and six months after expansion showed that collagen fibrils in the newly formed skin have normal diameters. Hence, the contraction of the breast tissue, sometimes subjectively experienced by the patient and objectively recorded by the surgeon as a "hard" breast, is not likely to be due to scar formation but possibly to a contraction

of normal tissue when the permanent prosthesis has been in place for a prolonged time (31).

Studies on hair

Transmission electron microscopy of hair fibres has to a great extent relied on the thioglycollate-osmium tetroxide method for high resolution imaging of the keratin filaments of the cortex cells developed by Rogers (36). Only recently, an alternative way of preparation was proposed (16). The new method is based on a direct embedding of the degreased fibre in plastic (Vestopal R) without any previous fixation. The fibre is dehydrated in alcohol, introduced into the plastic solvent and embedded in the plastic. Thin ultratome sections, approx. 60 nm, are contrasted with a saturated uranyl acetate solution and lead citrate. At first sight, the result is comparable with the thioglycollate method. A comparative analysis of the resolution obtainable with the two preparation techniques using optical diffraction is under way.

Studies on nails

Reports on nail disorders analysed with the transmission electron microscope are sparse. Studies on the normal finger nail have not been made since the late 1960's (6) and only few reports include TEM information.

Scanning Electron Microscopy

Studies on skin

SEM analysis of skin was recently reviewed (7,11). Undoubtedly the greatest advantage of the SEM analysis of skin is the information on topographical relations between the fibrillar and cellular components in the dermal part of the skin, including the dermal vessels. In addition to this, epidermal cellular adhesion relations are beautifully shown by the SEM. In general, skin biopsies are used in such studies. There is, however, an attractive alternative in replication techniques which faithfully reproduce the surface topography of the stratum corneum (11). Dental silicone plastics used for the modelling of dental work are ideal in this respect since they cure rapidly without heat production. Such hydrophobic silicone material adheres closely to the surface of the stratum corneum cells and reproduces not only the major and minor grooves of the skin surface but also the small, fine details of cellular surfaces such as knobs of previous attachment points between the cells. The most attractive advantage of the silicone plastic replication technique is the possibility to revisit the same site of the skin on unlimited occasions as the surface structures are not deteriorated by the technique. An example of the practical use of the replica technique is the monitoring of progressive change of the stratum corneum cells in an area treated with local ointments which can be recorded by the replication technique (Fig 2).

Studies on hair

Human hair fibre form, i.e. the straight fibres of Oriental individuals, the curly hair of Negroid races and the straight to slightly wavy hair of Caucasians, has been attributed to the hair fibre cross-section. Starting from old observations of sheep follicle form and the peculiar spun glass hair follicle defect (39) we tentatively surmised that the hair fibre form was determined by the follicle form rather than the cross-section of the hair shaft. Using serial sections of hair follicles at light microscopic resolution from individuals of the three

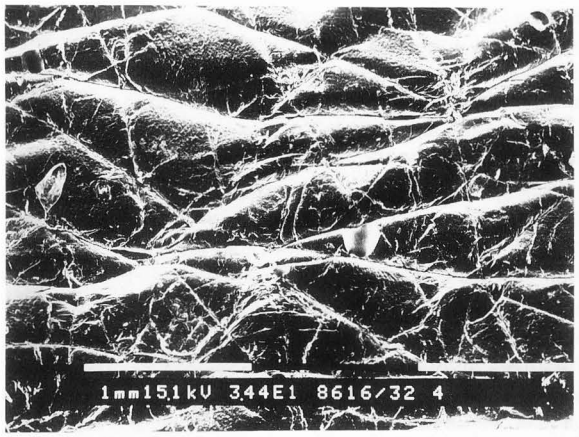

Fig 2. Positive replica of a lower arm skin surface (gold-sputtered Araldite (R)) obtained from a (dental) silicone plastic negative imprint of the skin.

dominating races, Lindelöf and co-workers made computerized reconstructions of the follicles (28). The results showed clearly that the hair follicles of Negroid hairs represented a helical structure, i.e. a three-dimensional spiral, whereas those of Oriental origin were absolutely straight. The Caucasian hair follicles fell between the two former groups having a slightly curved overall form in just two dimensions.

In addition to the image reconstruction work, scanning electron microscopy was performed on very short stubs (<1 mm) cut perpendicularly from the hair shafts from the aforementioned individuals and viewed end on. The hair fibre form was also revealed by knots tied on hair shafts (28). The investigation showed that the hair fibre cross-section did not give an obvious correlation between the hair shaft cross-section and hair form although the old generalizations of hair fibre cross section and form seem to be roughly valid. It is obvious, however, that the gross hair follicle form is the responsible factor for the hair shaft form rather than the cross-section appearance (28).

Studies on nails

There have been very few reports on SEM analysis of nail morphology in normal and pathological conditions in recent years. Either cadaverous specimens have been chosen for the investigation of the normal nail morphology (30) or distal clippings in pathological cases (37). It seems that this is mainly due to the fact that a whole nail biopsy may be painful or is awkward to obtain. The possibility of using silicone plastic replication techniques seems to have escaped many dermatologists (c.f. above).

Qualitative and Quantitative Elemental Analysis with Energy Dispersive X-ray (EDX) Microanalysis

Studies on skin

Elemental analysis of conventional preparations of skin may provide information on the nature of inclusions. These may be of external or internal origin and are often seen as dense bodies or crystals in electron microscopic sections. The sensitivity of the analysis in such sections is usually hampered

by two main factors, namely the overlap of specific peaks and a conspicuous background. Concerning the first issue, L- and M-peaks of the heavy elements Os, Pb, and U, originating in the osmium post-fixation and the section contrasting by uranyl acetate and lead compounds will overlap some of the specific K-peaks from physiologically interesting elements . The second issue relates to the fact that the heavy metals provide a high stopping power to the area of analysis, hence producing an important Brehmsstrahlung background which will lower the sensitivity, especially in weak characteristic peaks.

Examples where EDX analysis of conventionally prepared skin sections has been used sucessfully are the identification of foreign material such as Fe-particles etc in granulomatous skin lesions (7,11).

EDX and skin physiology

Skin physiology is hampered by the fact that the epidermis is approximately 0.1 mm thick. Conventional physiological techniques using cellular probes are therefore virtually impossible to use as there is no way to know exactly which cell layer is studied. EDX analysis of quench frozen, freeze-dried cryosections will allow determination of physiologically important elements such as Na,P, S, Cl, K, and to a certain extent Ca and Mg.

It has been shown that the Na/K concentrations change as the cells move from the basal layer towards the stratum spinosum. The change in the Na/K ratios may be interpreted as a means of restricting cell division to the basal cell layer (8). The P distribution changes from high levels at the basal and the stratum spinosum layers to extremely low levels in the upper stratum granulosum and stratum corneum. This distribution coincides with the loss of phospholipids and nucleic acids (13)

In more recent applications, the penetration of Ni ions through the skin barrier has been studied with the EDX technique. Concomitant exposure of the skin to 2% sodium lauryl sulfate (SLS) increases the Ni penetration manyfold indicating that the detergent makes the barrier deficient (26).

Proton Probe Analysis

The problem of normal reference data on the elemental content of skin and its appendages

Unfortunately there is no reliable source of the normal elemental content of skin and hair. The obvious discrepancies in the ranges of "normal" elemental contents of hair fibres shown in the otherwise excellent compilation of elemental data given by Iyengar *et al.* (20) clearly indicate the need for standardized sampling methods (Table I). An attempt in this direction was recently published (15). The data obtained with a proton probe show that there are important inter- and intraindividual variations in sulfur and zinc content in a normal Caucasian population. It cannot be completely ruled out that this is in part due to instrumental problems and further studies with other techniques such as EDX and X-ray fluorescent techniques are needed (15)

Studies on skin

In this context, proton probe analysis should be mentioned. The technique is essentially similar to EDX except that the much heavier proton particles generate much less much less background radiation, allowing a higher sensitivity.

Table I. The great variation in elemental content of hair. Data compiled from Iyengar et al. (20)

ELEMENT	CONTENT ppm	MEAN * ppm	METHOD
Al	4.2 -29.3	9.3 (4.4)	NAA
As	0.13 - 3.71	0.86	NAA
Au	0.0017-1.25	0.39 (0.06)	NAA
Br	0.65 - 53.3	21.5	NAA
Ca	146 - 3190	1100	NAA/AAS
Cd	0.24 -2.7	1.3	AAS
Cl	950 - 4805	2316	NAA
Co	0.2 - 1.05	0.41	NAA
Cr	0.13 - 3.65	1.95	NAA/AAS
Cu	11 - 34	18.8	NAA/AAS
Fe	5 - 44.7	24.9	AAS/NAA
Hg	1.25 - 7.6	4.95	NAA/AAS
I	0.085 - 15.1	3.46	NAA
K	150 - 663	323	AAS/FES
Mg	19 - 163	70	AAS
Mn	0.25 - 5.7	2.0	NAA
Mo	0.064 - 2.83	0.58	NAA
N	140 -157	148	CAT/XRF
Na	18 - 1720	698,5	NAA
Ni	0.6 - 6.5	2.82	AAS/NAA
P	83 - 165	168.6	SAS
Pb	3 - 70	22.4(16.5)	AAS
S	695 -47 000	44 533	SAS
Sb	0.09 - 3	1.64(0.84)	NAA
Se	0.64 - 2.53	2.4(1.6)	NAA
Sn	1	1	MS
Sr	0.15 - 14.7	3.3(0.44)	misc
Ti	0.048 - 14	8.8(13.2)	misc
U	0-00013	0.00013	misc
Zn	99 - 450	180	AAS/NAA

AAS: atomic absorption spectrometry
CAT: catalytic method
FES: flame emission spectrometry
NAA: neutron activation analysis
MS: mass spectrometry
SAS: solution absorption spectrometry
XRF: X-ray fluorescence spectrometry

*Note: The mean value given here does not imply any biological significance. Mean values within brackets are calculated excluding extreme values.

The proton probe has a sensitivity of approximately 1-10 ppm compared to the 200 ppm of the EMP technique. This means that elements in trace element concentrations, which generally cannot be determined by EMP, such as Mg, Ca, Fe, and Zn, are readily detected at significant levels. PMP analysis of normal skin thus demonstrated that the Ca gradient is increasing from the basal cell layer towards the stratum corneum of the epidermis corresponding to later data indicating that a 0.1 mM Ca level is needed for the stratum corneum to cornify in tissue culture (34). Also, it has been

shown on parallel sections that the EMP and PMP analysis techniques give highly correlated data and can be used in parallel investigations (14). Trace levels of foreign elements such as Cr and Ni have been studied with the PMP technique. Preliminary data show that Cr in the form of chromate penetrates the barrier more rapidly than Ni (13,25).

Studies on hair

The EMP has a great advantage over conventional chemical and biochemical methods in that the amount of material present in one or a few single strands of hair is sufficient for an analysis of elements that are important in the analysis of pathological hairs. Price (35) used EDX for sulfur analysis in trichothiodystrophy which allowed her to pinpoint the sulfur deficiency without harvesting a lot of hair.

Studies on nails

To our knowledge, there have been no reports on qualitative or quantitative analysis of nails in the last decade.

Conclusions

The study of skin physiology and pathophysiology has been hampered by the fact that the epidermis is a very thin tissue in addition to its being composed of a number of different cell populations which constitute the differentiation products of the matrix cells. Moreover, the epidermis contains immunocompetent cells and other cell types with a capacity to move freely in the tissue. These facts make it easy to understand why physiological studies of the skin have hitherto been extremely sparse. With the recent developments of quantitative techniques for application to electron microscopy, scientists in dermatology have been provided with new tools which will make it possible to understand skin physiology and pathophysiology at subcellular levels.

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Discussion with Reviewers

R Warner: You note that the Na/K changes as the cells move from the basal layer towards the stratum spinosum, and that this may be interpreted as a means of restructing cell division to the basal layer. In contrast we observed rather uniform Na/K ratios across the viable tissue (43). How confident are you that the Na/K ratio changes across the epithelium, and do you have any explanation why our results differ ?

Author: In analysis of bulk specimens with the EMP technique, it is true that the Na/K ratio appears more constant than in thin section analysis. The most probable reason for the discrepancy between thin section and bulk section EMP analysis results is surely to be found in the fact that bulk analysis will encompass the cellular as well as the intracellular compartments. In contrast, thin section analysis allows the probe to be located exclusively to the cellular compartment with high precision. However, the results from thin sections suggested a possible functional scheme for control of the cell division pattern that will ensure a smooth skin surface. Thus the data suggest that in the epidermal cells, divisions can be confined to the basal layer through a lateral control of cell division regulated through a transcellular system of gap junctions (44). With higher than normal Na concentrations, intracellularly mitosis can be prevented at low energy costs by anihilating the effects of the Na-K-pump function which demands production of ATP for its work.

M. Scourfield: What is the distribution of sulphur through the skin? Is there any distinctive pattern detected?

Author: The sulfur distribution follows the increase in mass over the epidermal cross section (7,8). As the EMP gives the total amount of elements present in the analysed volume and not information about its chemical status no conspicuous patterns were revealed.

M. Scourfield: Do you think it would be possible to "normal-ize" the elemental contents of hair? Would you expect the variation to be significant either inter- or intra-individually, or neither?

Author: A data base on normal elemental content of hair is highly desirable in order to give researchers in the domains of

medicine and forensic sciences a possibility to define what levels of an element that can be considered abnormal/pathological in a given sample. Particle probe analysis (EMP and PMP) of single hair fibres are important tools in these contexts (15) but unfortunately the costs for erecting a data base through these means have not yet been met. Recently we have used a new approach to single fibre analysis using an energy dispersive X-ray fluorescent technique which has a sensitivity comparable to that of PMP (Stocklassa B, *et al.* [1992] Submitted to Scanning Microscopy). In this work we have found that most elemental distributions are centered within a relatively narrow range around a mean value. There are, however, single fibres in this normal population that give data for particular elements >200 % out of range compared to the standard error of mean, in spite of the fact that only virgin parts of the hair fibres were subject to analysis. No co-variance between any pairs of elements were detected in such individual cases. The variation between hair fibres from a single subject is sometimes considerable (15). If this is related to the mass cross section of the cuticle, cortex and medulla, and for certain elements, to the degree of pigmentation, remains to be investigated.

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