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# CLINICAL APPLICATIONS OF SCANNING ELECTRON MICROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSIS IN DERMATOLOGY - AN UP-DATE

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## Abstract

Dermatological papers comprising scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analysis data published 1983 through 1986 in international journals are reviewed, as an update to our 1984 paper on Clinical applications of scanning electron microscopy and X-ray microanalysis in dermatology. The present paper not only deals with a review of recent publications in this area but also presents the application of microincineration to hair and cryosectioned freeze-dried skin specimens. Examples of the increased contrast obtained in hair cross sections are presented and a discussion on the feasibility of micro-incineration at analysis of hair and skin cross sections is given.

Particle probe analysis (EDX: energy dispersive X-ray analysis and PMP: proton microprobe analysis) as applied to hair and skin samples are presented with stress put on the proton probe analysis. The complementarity of EDX and PMP is demonstrated and future applications are suggested.

Key Words: Dermatology, skin, hair, nail morphology, normal, pathological, scanning electron microscopy, transmission electron microscopy, energy dispersive X-ray microanalysis, electron, proton (micro)probe, particle induced X-ray emission.

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## Introduction

Hair analysis as a diagnostic tool has met with rising interest in the past few years. Commercially operating analyst companies associated with "the new green wave" dietic movements invite the public to have a "complete trace element analysis" of their hair.The proposed aim is to reveal deficits in the oral intake of the customers and thereby to "know" what to compensate in their oral intake. This is actually an international problem concerned with the misuse of hair as a diagnostic tool which has been looked upon with concern by the medical society (c.f. 41). The need for a sound knowledge of hair structure and composition is therefore invaluable even for the general practitioner in order to be able to advise patients on this topic. Techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive X-ray emission (EDX) analysis have contributed to and can provide such knowledge.

In the past ten years a number of reviews on the application of SEM to dermatological problems have been published (3,6,52). Recently the EDX (energy dispersive X-ray) technique in the electron microscope has been used to advantage in clinical as well as in experimental dermatology as witnessed by the reviews written especially with this topic in mind (3,59). As a further development of particle probe analysis the introduction of PMP (Proton Micro Probe) has shown the advantage of physical techniques in the study of skin physiology in normal and pathological conditions (3,61) and in the analysis of the elemental composition of hair.

As we have recently reviewed these topics the intention of the present paper is to provide an up-date of the previous ones (3,60). We will not include papers published in "The Integument" (6) in this presentation. A discussion of specific topics, e.g. microincineration is included to highlight a technique which presently may be overlooked by scientists in the field.

## Scanning Electron Microscopy

It is a remarkable fact that in the past two years the number of publications of SEM investigations as applied to dermatological problems is small compared to the number of TEM

publications. At present, the interest in structure-function relationships which are accessible with the SEM seem to attract little interest. The reason for this is obviously not to to be found in the technique **per se** as the preparation procedure for SEM analysis of skin specimens is in no way more complicated than any other microscopic technique.

In reading the literature it is interesting to note that a conservative attitude towards the preparation procedures is at hand. Thus, as a rule dermatological specimens are chemically fixed by fixation solutions which contain a physiologically equivalent vehicle. In the rinsing procedures the same type of buffer is utilized not taking into account that fixation by definition excludes a physiological condition in the tissue and that salt buffers are more or less potent as protein extraction media. Especially when dealing with outgrown hair, nail and with elastic tissue a need for reevaluation of the preparation technique may provide easier and more accurate preparation. This means that there is still a place for methodological work in this field.

**SEM studies on hair**

Both normal hair and hair in pathological conditions have been at the focus of interest.The plucking of hair fibres for subsequent biochemical or other type of analysis often requires that anagen hairs are selected. The spectrum of hairs encompasses actively growing fibres (anagen) to fibres in the resting phase of the hair cycle (telogen). For studies in man the classical plucking with the aid of a pair of rubber-coated tweezers seems to be the most advantageous method. However, in experiments involving animal hairs other methods with higher yields may be considered. Green and co-workers (7) have introduced a method where the follicles from skin biopsies are freed from the surrounding dermal tissue by shearing. Hair fibres are then collected individually under liquid using gentle aspiration. The success of their method is demonstrated in the SEM micrographs accompanying the paper.

The practitioner seldom has a direct access to a SEM and alternative methods for analysing the surface topography which utilize the more common light microscope are therefore welcomed. van Neste & Houbion (31) in a short report suggest that **replication techniques for the light microscope** may give some of the information available in the SEM. This is exemplified with two cases of bleached hair suffering from hair breakage as a result of the chemical treatment. From their documentation of SEM vs light micrographs it is fairly obvious that the resolution of the SEM is far better than that of the light microscope replicae. Undoubtedly the latter method presents an easy and fairly reliable way in clinical practice for a preliminary diagnosis of the surface condition of a damaged hair fibre.

Morphological changes in the hair follicle and the hair shaft structure may indicate abnormalities which can include the integument as such. Ortonne et al. (33) reported on a case of **familial rolled and spiral body hairs** with **palmoplantar keratoderma** where the hairs proved to be low in sulfur, e.g., cysteine. Many hair fibres rolled up in hyperkeratotic follicles had a smaller dimension of the shaft than normal straight fibres. The authors also report on a spiral arrangement of the rolled hairs but their histological investigation and the published SEM micrograph did not reveal if these spirals were composed of several individual fine fibers or due to abnormalities in the hair follicle.

Diagnosis of defects of the hair shaft whether congenital or acquired are usually supported by SEM investigation. Recently a study on **Pili bifurcati** which is an unusual condition was reported by Sala et al (37). There was no clear inheritage found and laboratory investigations were negative. The hair fibres were characterized by cavities within the cortex, irregular hair shaft diameter along the fibre and the hair fibres were bifurcated to produce a deep longitudinal groove. The hair shafts were partially twisted and the structure of the cuticle was irregular.

Two cases of **spun glass hair** (synonyms: **pili canaliculi et triangulati, cheveux incoiffable/ uncombable hair**) were reported by Forslind et al. (5) The characteristic changes in the fibre diameter were recorded in both cases with the SEM. TEM investigation revealed completely normal cross section characteristics and the study underlines the findings of previous reports that state that this is a condition which is localized to the root sheath only and does not involve the rest of the integument or other tissues of ectodermal origin.

Chanwichitrana et al. (2) have reported recently on a case of **spun glass hair** in a five year old Thailandian girl. Except for her hair disorder no physical abnormalities were recognized. A conspicuous feature of this case was the unusual involvement of the eyebrows and eyelashes. The SEM investigation revealed features found in previously reported cases, i.e., no abnormalities except for the cross section shape of the hair fibres.

In the past several studies on **monilethrix** have been published. A recent contribution using the SEM for thickness determinations of the nodes and internodes for a comparison with normal hair shafts showed that the thickness of nodes can be reduced to nearly 50% of the node thickness. Ito et al.(12) also give data for the length of the intervals between nodes which on the average amounts to about 700 $\mu$m. Although the authors do not give information on the way primary data are obtained to allow an estimation of the accuracy of their measurements the accompanying TEM micrographs provide new and interesting information of the morphology of the monilethrix hair fibres. Thus the keratin pattern in cortical cells was lost and replaced by highly dense areas where no filaments could be seen. The size of the cortical cells was apparently the same in node- and internode sections of the monilethrix hairs. The dimension of the keratin filaments was normal (roughly 8 nm) in both regions of the hair fibres.

The **trichorhinophalangeal syndrome (TRP)** is characterized mainly by the sparse and fine, slow

growing hair, a conspicuous pear-shaped nose, and skeletal anomalies. Prens et al. (35) reported on a solitary case of the **TRP type I** where the SEM study of the hair revealed the cuticle cells to have highly irregular distances between scales. Longitudinal pleating and denudement of the cortex through the complete loss of cuticle cells was also observed in places.

The chemical-physical wear and tear on hair fibres named weathering is expected to have greater impact on diseased hairs, e.g., **trichothiodystrofic hair fibres**, than on normal fibres. Venning et al. (51) studied fibres from three affected individuals. In the SEM partial as well as complete **Trichorrhexis nodosa**-type of breaks were observed. At such sites the cuticle was severely damaged or lost, the cortical cells were showing longitudinal splitting, all these effects giving rise to the brush type of breaks in the end. In two of the patients the cortex was more or less completely denuded of the cuticle. In relation to the sulfur deficiency the authors argue that the gross abnormalities seen in trichothiodystrophy are primarily to be attributed to weathering effects.

Dimension changes of the hair fibre cross section may indicate abnormalities in the hair formation or even in the function of the integument as such. Artifacts may of course be involved, e.g., self inflicted changes due to cosmetic treatment(s), due to thrichotillomania or habitual tics, etc. Also the sample collection technique may produce artifacts, e.g., unskilful pulling of hair fibres may produce root ends which have the appearance of dysplastic ones. Zitelli (56) has pointed out that **pseudo-monilethrix** type of artifactual changes may be produced when overlapping hair fibres are pressed between glass slides used in light microscopy. He demonstrates the production of such "pseudo"- swellings in the light microscope as well as in the SEM. The solution to the problem is obviously not to use cover glasses at light microscopy of hair fibres.

**Bamboo hair** of **Netherton's syndrome** has been described by means of the SEM previously. Recently hair fibres of this type have been the subject of a study into the pathogenesis of the condition using the SEM, TEM and the SH-group stain DACM (N-(7-dimethylamino-4-methyl-3-coumaryl)maleimide). Ito et al. (13) conclude that the defect of invagination may be the result of softness of the cortex in the zone of keratinization which may be the result of an incomplete conversion of -SH-groups into -S-S-bonds of the cortex proteins.

**Hair casts**, which were first described by Kligman in 1957, often occur in association with parakeratotic types of scalp disorder. Such conditions are consequently termed parakeratotic hair casts.These changes are the consequence of scalp conditions such as seborrhoic skin involvement, psoriasis, etc. The casts may surround more than one hair fibre and consist of loosely adhering and overlapping squamae surrounding an apparently normal hair fibre.

Another type of hair cast is the **peripilar cast** which may contain parts of the root sheaths. In addition, hair casts may be composed of non-keratin material such as bacteria, fungus organisms and artifactual material, e.g., hair spray material, etc. Taieb et al. (43) have made a clinical and SEM/TEM study of two preadolescent girls with idiopathic hair casts. In these cases both inner and outer root sheath components were found in the casts. The casts consisted of white movable cylinders encompassing the fibre and appeared as strings of beads. There was no other involvement of the integument and treatment initially only consisted in brushing of the hair. A 0.025% tretioin lotion was applied to the scalp twice weekly which resulted in an improvement. The authors discuss the origin of different types of hair casts differentiating between two main groups. The first one consists solely of young girls (2 - 8 years) with no involvement of the integument otherwise. As follow-up is very seldom reported for this group, the time course, development and regression of the condition is unknown. Generally the treatment has been mechanical removal of the hairs casts through brushing. In the second group which is more heterogeneous with respect to sex and age scalp disease is always present. At differential diagnosis white piedra, nits of pediculi, trichonodosis, etc, should be observed. In addition to informative SEM and TEM micrographs the authors have compiled a comprehensive table of reported cases in the literature from 1957 to 1985, and discuss the mechanisms behind the phenomenon as presented in the current literature (43). In a recent review of the subject Keipert (18) has discussed the details of hair casts and illustrated his viewpoints with light micrographs.

Unusual hair surface structures can be the result of a pathological process in the vicinity of the hair follicle. A report on **uncombable hairs from localized areas of neurofibromas** on the scalp was published by Henkes et al. (8). The hair fibres had a wider diameter than the normal hairs of that scalp and at high resolution SEM micrographs showed the cuticle surface to be covered with tiny hemispherical structures. Unfortunately the findings are not supported by corresponding TEM micrographs so the internal morphology of these fibres are unknown as is the actual cause of the changes.

In recent years **pediculosis** has increased in most civilized countries presumably due to the long hair fashion endeared by young people of both sexes. Meinking et al (25) reported on the ovicidal activity in freshly collected hairs from cases of pediculosis in a population with endemic pediculosis capitis The study site was an isolated mainland village with no prior history of pediculicide or pesticide usage.The reason for this choice of patient material is the lack of an **in vitro** system for the study of ovicidal activity. The study evaluates different methods of killing procedures using light microscopy and SEM and the authors conclude that "a single-treatment, fast-acting, completely ovicidal and cosmetically elegant pediculicide has yet to be developed".

In clinical practice the differential diagnosis of the **Thrichophyton mentagrophytes** and

**Thrichophyton rubrum** relies on their different modes of attacking virgin hair fibres from preadolescents in a suitable fungus medium. In the light microscope the **T. mentagrophytes** produces what appears like the mark after a blow with an axe at a right angle to the fibre axis but the **T. rubrum** does not cause this type of effect. The actual change caused by these organisms has been studied in the SEM for the first time by Kaaman & Forslind (15). Using the TEM the study revealed that both organisms penetrate the cuticular layer damaging the endocuticle which causes the cuticle to be lifted up from the underlying cortex. This change is conspicuous also in the SEM micrographs where folds mainly parallel to the longitudinal axis of the fibre are visualized. **T. mentagrophytes** in addition penetrated the cortex of the fibre and the "axe blow" change is likely to be an optical illusion as the SEM micrographs showed vertical holes in the fibre attacked by this organism. Although the cuticle was attacked corresponding changes in the cortex are not found in the case of the **T. rubrum**. The study nicely demonstrates the advantage of using both SEM and TEM at morphological analysis.

A case study on hairs from an adult patient suffering from **osteogenesis imperfecta** using the SEM and TEM prompted Forslind & Kaaman (4) to investigate the possibility of detecting biochemical change in hair fibres by growing T. mentagrophytes on such fibres. In the actual case the hair fibres were readily infected by the organism as seen in both SEM and TEM micrographs. The study suggested that fungus growth on hair fibres from adult individuals may reveal a biochemical defect.

We have made a follow-up study on hair from 14 cases of disorders comprising macroscopic changes of the hair (Forslind B, Kaaman, T, Miyasaka S, Yoshino M, Sato H, Seta S, unpublished data). Hair fibres were collected from individuals preferentially from the area 1.5-2 cm above the right ear. The fibres were incubated on a Sabouraud agar in the presence of T. mentagrophytes organisms for 7 days or more at 25°C. Subsequently after rinsing in a phosphate buffer the hairs were fixed in glutaraldehyde in the same buffer for 2 days or more prior to rinsing and dehydration on graded ethanol. After mounting on spectrographically pure carbon stubs the specimens were sputter coated with carbon. They were observed in a JEOL JSM U3 at 25 kV. The results suggest that the connotation proposed by Forslind & Kaaman (4) is a valid one (Fig.1). We find that further studies with unrelated methods are needed, however, and that positive fungal culture experiments are indicative of rather than conclusively proving the presence of a biochemical disorder.

The method may be used by clinicians for a screening which may indicate that more expensive techniques are needed in the clinical investigation for a diagnosis. An interesting observation in this context was that in a case of a young boy born in 1983 with highly abnormal, brittle hair which was readily attacked by the fungus the hair fibres of both parent were also attacked by the fungus organism. In two sisters (age 13 & 15) afflicted by epidermolysis bullosa Weber-Cockayne who on elemental analysis showed normal sulfur values but dramatically abnormal Cu/Zn quotients (64) no fungal invasion of the hairs was observed. The mycologists' observation that hair fibres to be used for the differentiation of T.mentagrophytes from T. rubrum is most effectively done on hairs from youngsters not more than 5 years old is supported by the absence of fungal invasion in hairs from one of our cases. This girl, 7 years of age, is suffering from a sparse hair growth but with a normally appearing hair in the SEM and a normal elemental composition at PIXE analysis (64).

A comprehensive overview of structural abnormalities of the hair shaft suggesting a classification into four basic types was presented by Whiting (50) in 1987. The types of hair shaft abnormalities are given as fractures, irregularities, twisting and extraneous matter affecting the hair shaft. The paper is a didactically excellent introduction to the subject and illustrated with a number of excellent SEM micrographs.

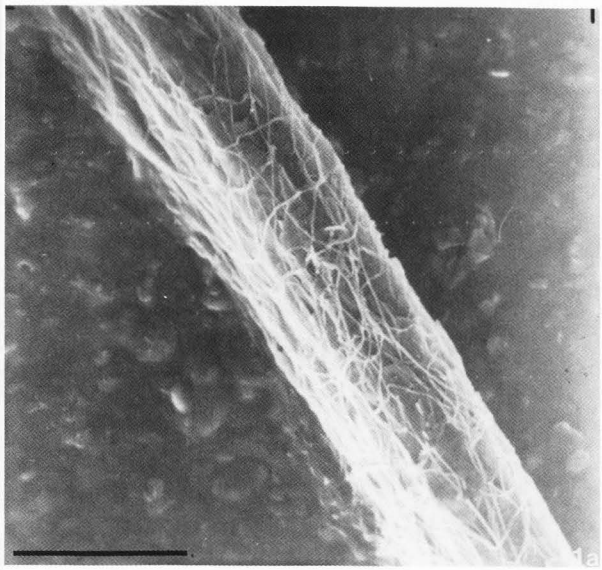
**SEM studies on nails**

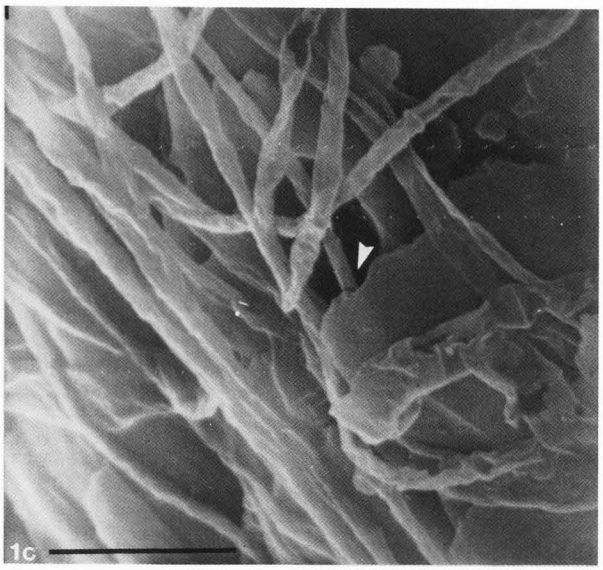
Although nails are ideal objects for replication little interest has been focussed on nails. Generally nail biopsies from living subjects are rarely used in the analysis of pathological conditions. A study comprising 7 healthy toes from male individuals by Meyer & Grundmann (26) provides additional SEM information on morphological features of the normal nail most of which have been described in detail at light and TEM resolution.

With the direct clinical approach characteristic of Shelley & Shelley (39) four cases of **onychoschizia** were studied with the SEM. Lamellar splitting between the cell layers of the nail plate were detected. Further, the detailed analysis of a large fault exposing the undamaged undulating folds characteristic of the nail cell surface indicated the loss of the cementing material. Hence, evidence has been given by the authors that onychoschizia is caused by a loss of the cementing substance binding the nail plate cells together. The cause(s) of this loss remain(s) to be elucidated.

**SEM studies on skin**

In general, few SEM studies in recent years have involved laboratory experimental data Rather, the material used emanates from clinical cases. It thus appears that the main use of the SEM in the study of skin (as is the case for hair investigations) is that of giving additional basic morphological data as a complement to light microscopy and TEM. In this vein Bavinton et al (1) reported on **dermatospraxis in sheep** after investigating the condition with the aid of TEM and SEM. This pathological condition was characterized by a loose arrangement of the collagen fibrils which allow the fibrils a more convoluted path through the dermis than is found in the normal skin. Also a sheet-like arrangement of the fibrillar bundles parallel to the skin surface explained the fragility of the sheep skin. It was found that the mechanical resistance of the skin was so low that a finger could accidentally penetrate the skin causing it to





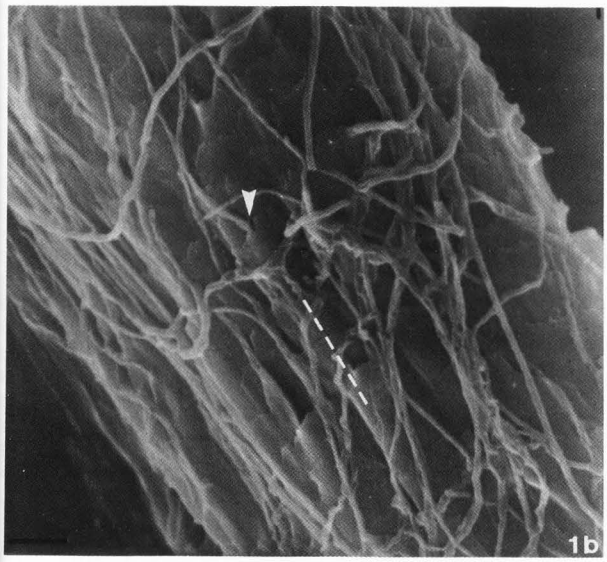


**Fig.1 a**. Hair fibre from an adult woman, age 33 years whose son is suffering from an non-diagnosed genodermatosis. Note longitudinal ridges of the cuticle and the fine meshwork of the fungus **T. mentagrophytes** (bar 100 μm).
**Fig.1 b**. Same fibre at higher magnification. Note the fungus hyphae penetrating the cuticle at the free edge of the cuticle cell.(arrow). The ridging of the cuticle cell due to the penetration of the hyphae is clearly seen (dashed line)(bar 10 μm).
**Fig.1 c**. The entrance of the hyphae appears as an etched hole in the cuticle cell (arrow) (bar 10 μm).

separate in the mid-dermis. Additional TEM analysis of the collagen showed that the reticular dermis contained the characteristically distorted collagen fibrils of dermatospraxis. Previous finding of an increased fibroblast population was confirmed in the light microscope.

Meyer & Neurand (27) recently published a comparative study on the integument of pig, dog, cat, horse, cattle and goat. The particular features of the epidermis, the pilary system and the fibrous parts of the dermis are well demonstrated in the different species.

An advanced method of skin preparation for the demonstration of **dermal elastin** has been exploited by Tsuji in a number of recent studies. Abnormal elastic fibers were thus found in a case of **Marfan's syndrome** (47) exposing varying thicknesses of the fine cylindrical fibres with knobby or lumpy appearance in places. These fine fibres appeared to be continuous with normal elastic fibres. The presence of the fine fibres suggest that the structural abnormality underlying the defects of the Marfan syndrome is the inability of the fine elastic fibres to fuse into elastic units.

**Solar elastosis** (46) was characterized by the SEM depending on three degrees of change corresponding to three different depths of the dermis. The most conspicuous changes were located to the shallowest part of the skin where the elastin component of the dermis was described as a large mass composed of fine net-like and cord-like elements. There is a transition from this shallow region to the middle layer of the dermis where thick, cylindrical fibres dominate the picture. In the depth of the dermis the elastin has a normal configuration.

**Pseudoxantoma elasticum** was investigated by

Tsuji (45) who found that the clumped material in the depth of the dermis in this condition consisted of clumped elastin material which on EDX analysis proved to contain calcium and phosphorus. In this investigation the specimens were rinsed in phosphate buffer a fact which may infer that at least some of the phosphorus had a preparation origin.

The **wrinkles in the aged persons' skin** have been studied by Tsuji et al. (48). Deep and shallow wrinkles were studied, a deep wrinkle defined as one which did not disappear on transverse stretching of the skin, the opposite being valid for a shallow wrinkle. The deep wrinkles appear to have less elastotic changes in the actual wrinkle fold area than in the surroundings of the fold where the elastic fibres are thick and tortuous. In the shallow wrinkles there is no obvious difference in the wrinkle area and its surroundings, the elastic component of the dermis being composed of fine and curly fibres in the upper part of the dermis. It is probably their fine cross sections which prevents them from being seen in the light microscope preparations. The deep wrinkles are most probably due to mechanical causes related to muscular action on the skin which produces permanent folds rather than being due to pure histological change.

The **basal lamina** undergoes characteristic changes during the ageing of the skin. Hull & Warfel (11) have demonstrated the changes occurring in the topography of the basal lamina on split skin specimens. By treating skin biopsies with 2 N sodium bromide at 4°C for 14 h the epidermis splits in the region of the lamina lucida allowing the epidermal side of the basal lamina to be exploited for SEM investigation. The results show that the mature epidermal-dermal junction in young and middle-aged individuals showed distinct tall domeshaped dermal papillae and that the basal lamina was arranged in prominent corrugations. These characteristics were smoother in the skin from subjects in their 7th decade or later. In a sequel paper (49) these authors have studied the migration of lymphocytes through the cutaneous basal lamina of normal human skin. Using 2 N sodium bromide the dermal and epidermal layers could be completely separated leaving the the surface facing the epidermis clean for observation in the SEM. The authors had taken care to control the efficiency of their preparation procedure by TEM sections taken perpendicular to the skin surface. A number of structural deformations are described which are correlated to the passage of lymphocytes through the basal lamina. The authors also argue that their excellent SEM micrographs provide support for an idea that the basal lamina should havethixotropic properties. In this reviewers opinion this idea cannot be substantiated even with the highly dynamic interpretation that the authors give their morphological findings. Nevertheless, from a morphological point of view this is an interesting paper concerning the effective preparation method and the parallel use of the SEM and TEM.

The dermal surface relating to the human **dermatoglyphics** have been investigated by Misumi & Akiyoshi (28). Using a technique where the skin specimens were initially formalin fixed the specimens were subsequently incubated in 3% KOH for 12 h at 37°C after which the separation of the epidermis from the dermis could be performed mechanically under a dissection microscope. This technique, however, does not allow a positive evidence for the basal lamina to be obtained in the TEM. Also the basal lamina continuity is broken in places, a situation the authors take advantage of in their description of the reticular fiber organization below the basal lamina. The pattern on the dermal surface reflects the finger print in a negative image. The dermal part of the dermatoglyphics is shown to be composed of furrows, grooves and a double row of papillae. The grooves form continuous valleys between furrows which are continuous ridges with papillae on each side. Some morphometric, age related, data on the frequency of papillae per 1 $mm^2$ of dermal surface are also given.

The **thick skin of the palmar and plantar region** in man is highly specialized to resist friction. Kawabe et al. (17) have studied the fine structure configurations of these integumental areas using EDTA separated basal lamina preparations. The fine surface architecture of the basal lamina reflects the differences between areas specialized in cell attachment and regions providing space for the epidermal stem cells producing new generations of keratinocytes.

The changes occurring in **the ageing skin** not only cause a change in the skin surface texture to make it less attractive but also involves changes both in the cellular and fibrous components of the skin. A well written paper by Lavker et al. (21) demonstrates the changes occurring at light and electron microscopic levels. Especially the interpretation of the TEM and SEM findings should provoke any dermatologist with interests in structure-function relationships.

It is a well-known clinical observation that corticosteroid treatment eventually will result in a thinning of the skin. The **steroid atrophy** induced by clobetasol proprionate was studied in 3 volunteers by continuous occlusive application during 6 weeks by Lehmann et al. (23). The SEM showed the dermis to be strikingly reorganized during the treatment. Decreased spaces between the collagen and elastin fibers seen in the TEM and SEM proved to be caused by the loss of ground substance visualized by the progressive dimunition of Hale's stain for acid mucopolysaccharides in light microscopic preparations. During this process the papillary and the reticular dermis became more compacted and the fibrous components became reoriented. No differences in the collagen or elastin fine structure was seen, however. There was also a continuous loss of Mast cells whereas the fibroblasts were shrunken but not reduced in density. The thinning of the epidermis and the smoothing out of the papillary profile was a conspicuous feature at the termination of the treatment.

**Restrictive dermopathy** is a fatal genetic disorder which is expressed in a hyperplastic,

hyperkeratotic and parakeratotic epidermis. In a model study of problem oriented research including the use of the SEM Holbrook et al. (10) were able to demonstrate that the dermis in these cases was thin and that the connective tissue appeared stretched with an orientation much like that of a tendon. The dermal-epidermal junction was conspicuously flat and a thick hypodermis of adipose and fibrous connective tissue comprised almost 75% of the thickness of the skin. In addition to the morphological investigations biochemical and immunohistochemical investigations were used to characterize the condition.

The **cellular adhesion** is an interesting parameter to understand in relation to the mechanical properties of normal and pathological skin. One way of obtaining viable skin has been the formation of suction blisters introduced by Kiistala in 1967 (19), a method which also provides possibilities for the study of mediators in blister fluid. It thus renders itself to experimental procedures on human skin without causing ethical problems as the lesions induced heal without scars as a rule. Juhlin et al. (14) intrigued by the ease by which suction induced blisters ruptured on too high suction pressures, investigated the morphology of the dermal side of the **suction blister roof in psoriatic lesions** before, during and after treatment. The authors were able to separate the epidermis at the epidermal-dermal junction. After a conventional glutaraldehyde fixation and dehydration on graded ethanol the specimens were critical point dried and gold sputtered. In the normal appearing skin basal cells of the separated epidermis showed a characteristic stellate form, the cytoplasmic extensions of which formed intercellular bridges. The same picture was presented by the almost healed psoriatic lesions. In the non-treated active psoriatic lesion cells were rounded and the cellular contact points in the form of intercellular bridges were not clearly seen. The possible contribution of the chosen preparation procedure is not discussed by the authors. It would be most interesting to elucidate the effect of different osmolarity in the fixative for the resulting morphology in the normal-looking and the lesional psoriatic skin.

Isolation of cells from suction induced blisters has been used to study the cell surface characteristics in normal skin and diseased skin. Hietanen et al. (9) used this experimental model to study the **effect of pemphigus sera on cells derived from normal, healthy skin**. The epidermal cells were isolated from the blister roofs by trypsination. The pemphigus serum was shown to induce a loss of cellular microvilli at high levels of significance ($p<0.001$). This effect of pemphigus serum was likewise significantly inhibited by ovomucoid. The authors conclude that the microvilli loss induced by pemphigus sera irrespective of the presence of complement may play a significant role in the acantholysis of pemphigus.

Using pan-leucocyte monoclonal antibodies Wood et al. (53) succeeded in enriching murine and human Langerhans cells from cell suspensions of normal skin. In the SEM the isolated LC showed characteristic dendrites and a relatively smooth cell contour.

The epithelial tissue response to **vitamin A** varies with the site of tissue, the age of the individual and the species. Exposing tissue **explants from chick embryonic skin** to different concentrations of **retinoic acid** (low, moderate and high doses) the tissue effect was studied by TEM and SEM by Peck et al. (34). Reversible metaplastic changes could be noted to be dose dependent. Particularly it was observed that mucin-containing epidermal cells did not revert to keratin production through redifferentiation at withdrawal of the retinoic acid but were gradually replaced by keratinizing cells. The retinoic acid treatment of the chick embryonic skin produces hyperplastic changes leading to acanthosis and loss of horizontal stratification. Keratohyalin granules did not form in contrast to the development in control cultures. Individual epidermal cells were variably affected and microvilli with axial filaments and surface glycocalyx developed. Desmosomal cleavage is another observed characteristic of the cultured and treated cells. Tight junctions appeared between superficial epidermal cells and elongated gap junctions could be discerned three to four cells deep into the epidermis. Also glycogen was found in the cytoplasm of superficial epidermal cells. The SEM revealed the premature sloughing of the periderm caused by the retinoic acid and the upwards bulging of the epidermal cells. The development of surface villi could be seen to occur to different degrees in individual cells as a response to the drug. The complementarity of SEM and TEM was clearly documented in this study.

The effects of a specified treatment is sometimes possible to follow with the SEM. In an experimental study of **artificially induced comedones** in the rabbit ear epidermis the isotretinoin effect was investigated by Lee et al (22). Comedones were produced by smearing the ear epidermis with insoluble cutting oil daily for two weeks. **Isotretinoin** suspended in soybean oil in two different doses, high (20 mg $kg^{-1}$) and low (2 mg $kg^{-2}$), was administered orally through a feeding tube daily for 4 weeks. The histological biopsies were fixed in formalin for the histoplanimetry investigation and in glutaraldehyde for the SEM investigation. The high power SEM micrographs show the comedones as "chrysanthemum" structures with loosely adhering squamae. The authors only present the effects after 4 weeks of treatment where masses of desquamating cells still were observed around the hair follicle openings. The histoplanimetry investigation revealed the success of the high dose treatment which was statistically significant compared to the control group. However, the lack of documentation of skin surface topography at later stages makes the final evaluation of the treatment difficult.

The topography of skin tumors provides insight into the relation between normal and abnormal tissue. A study on 10 lesions of **cherry hemangiomas** by Sala et al. (36) showed them to be well differentiated from the surrounding tissue. The endothelium observed at high magnification had projections out in the lumen most likely to correspond to pedunculate processes observed in

the TEM by other authors.

**Pilomatricoma** or **calcifying epithelioma of Malherbe (CEM)** is a benign tumor originating or differentiating towards pilar structures. Zina et al. (55) have examined five cases of CEM. The oval or multilobulate macroscopic form of the tumor showed small protuberances and when cut the mineral content grated the cutting blade. The SEM study showed the tumor to consist of irregular islands of mummified cells separated by strands of connective tissue. Multinucleate foreign body-type giant cells were seen in association with the epithelial islands. X-ray mapping showed an abundance of calcium around the epithelial islands

The comparison of the surface characteristics of **benign dermatofibromas, dermatofibrosarcomata protuberans, fibroxantomas** and a **malignant fibrous histocytoma** in explant tissue culture revealed according to Oku et al. (32) that culture behaviour of the cells from the tumour group correlated well with **in vivo** growth and histological features. The method thus may prove useful in arriving at a correct diagnosis in a clinical situation.

In the past **fungal invasion** of the integument and its appendices have been successfully investigated using the SEM (c.f. 2). Recently Montes & Wilborn (29) have contributed with a review on the subject of **candidosis** using the resources of SEM and TEM in illustrating the paper. They contribute with new data on the morphology of the organism revealing that the cell wall, plasma membrane and intracellular organelles are more complex than the description in classical books on mycology suggest. The value of SEM in the diagnosis of candidosis was underlined by the fact that SEM can demonstrate the presence of chlamydospores, blastospores and pseudohyphae which identifies **C. albicans**. The authors maintain that since specimen preparation for SEM only requires a maximum of six hours the diagnosis can be ascertained before the results given by conventional culture.

**Geographic tongue** is a finding in psoriatics and in atopic children. Clinically it is characterized by atrophic areas mostly on the dorsum of the tongue often surrounded by white margins. The etiology of the conditions is not known. The lesions seen can be divided into three categories according to a SEM study made by Kullaa-Mikkonen (20), i.e., the atrophic area, the white margin and areas of macroscopically normal appearance. The normal mucosa was smooth with some desquamating cells and filiform "hairs" which were covered by extensive plaques of microorganisms. "Hairs" were lacking in the atrophic areas and the mucosa formed low elevations. In the white margin areas desquamating cells abound. Light microscopy indicated moderate inflammatory reaction in the dermis and inflammatory infiltrate in the epidermis. In the normal appearing areas the desquamation of the mucosa on the papillary bodies was more pronounced than in normal tongue tissue. All specimens revealed a fissure. On the walls of this fissure the superficial cells had broken microplicae and knob-like structures. Candida hyphae was seen in the area of the fissure. The author does not consider the presence of **candida** to be definitively etiological as the loss of cohesion between cells may provide for an environment suitable for the organism.

The **moluscum virus** is harboured in a sack-like structure within the infected epidermal cell as seen in the SEM according to a study which derived its material from 14 victims of the disease. The cell as such is, according to Shelley & Burmeister (40), atrophic and the tumour is thus the product of a rare form of intracellular viral colonization rather than hypertrophy. Their finding corresponds to an old histopathological finding of a sack-like structure obtained at microdissection. The unique qualities of the SEM and the preparation procedures involved are underlined by the authors' findings.

**SEM studies on parasites of the skin**

Using a non-invasive replication technique Kairinen & Kaszynski (16) have been able to demonstrate details of **demodex** infestation of the pilosebaceous follicle.

Another parasite is the **mite (Sarcoptes scabiei)** which after removal with a forceps and needle from infested individuals and processed routinely (including critical point drying) for SEM analysis by Nonaka et al. (30). After inspection in the SEM the mite was stripped off the mounting tape on the stub, remounted with other side up and again inspected after a new vacuum coating of the previously hidden side. By this means sex identification was possible in addition to a thorough anatomical study of the animal.

## Microincineration

**Introduction**

In **SEM analysis** of tissue cross sections the all important factor is that of contrast. Biological materials generally have approximately the same elemental composition notwithstanding a few elements specific for a certain tissue or organelle. Sectioning of cellular tissues thus produces a comparatively smooth surface with little contrast in the SEM. This is especially so if the tissue has been embedded in a plastic medium which fills the "gaps" in the tissue and thus decreases contrast additionally.

At **EDX analysis** one of the main problems is related to the background of the energy spectrum. This background which is proportional to the mass, i.e., the stopping power, of the specimen material in the spot of analysis will produce an unfavourable peak-to-background relation in elemental analysis. Thus, the reduction of the background is one way to obtain a higher sensitivity of the EDX analysis. That this is an important aspect is readily demonstrated by the fact that sodium X-rays which in addition are partially absorbed in the detector window have a low yield in normal EDX preparations. Furthermore, in many cases calcium is found in concentrations barely above the detection limit (59). Hence, preparation methods which allow elements close to the detection limit of the EDX technique to be recorded with a higher degree of accuracy are strongly desired.

**Microincineration** (44) is a technique which allows a comparatively inert way of reducing the mass of a specimen through **flameless combustion** of material with a low melting point. In a biological specimen this kind of material is represented by the organic matrix, the composition of which is mainly H, O, C, N, P, S, At microincineration specimens are introduced to a moderate vacuum (0.01 torr) in a chamber through which oxygen gas is streamed at a controlled flow rate. Radiofrequent (RF) energy of some 50 Watts at roughly 13 MHz creates an oxygen plasma which etches the specimen(s) in the radiofrequent field of the chamber. When the plasma reacts with organic molecules in the sample gases are produced and removed by the vacuum pump of the incineration system. The oxygen molecules are relatively immobile and attain a gas temperature under these conditions which typically is only a few tens or hundreds of degrees centigrade. The plasma etching is therefore intrinsically a very gentle treatment as the molecules of the plasma hits the object at velocities corresponding only to some 10 electron volts. The oxygen plasma cannot form volatile oxides of heavy metals or mineral substances which therefore are retained within the carbon matrix residue of the object after microincineration. Depending on the type of specimen the time required varies between seconds to minutes. The resulting specimen which is deprived of lesser or greater amounts of the organic material leaving the inorganic part in situ of the remaining ash matrix. It will now reveal enhanced contrast in morphological detail in the SEM in relation to the amount of material which has been etched away.(c.f. below). At EDX analysis the improvement in the peak/background (P/B) ratio may be improved by a factor 5-10.

In biological specimens the physiologically interesting ions are mostly diffusible ones. It is thus necessary to precede the analysis with an inert fixation. At EDX analysis freeze-fixation and freeze-drying procedures are utilized (cf 72) in order to prevent the ions/elements to diffuse through the specimen during the preparation and the subsequent analysis. To our knowledge the application of microincineration to the freeze-dried skin specimens has not been done so far. We have thus made some preliminary studies on freeze-fixed and freeze-dried guinea-pig skin to assess the value of such a procedure. Also we have revisited the subject of hair morphology at microincineration/SEM for this review.

**The morphology of hair at microincineration**

Microincineration of hair was introduced by Swift (42) as a means of enhancing contrast in hair fibre cross sections. The method was further developed by Sato et al.(38) for the forensic analysis of plastic embedded cross sections. By this means it was possible to unambiguously record the number of cuticula cell layers and to map the outlines of the cuticular cells with a high resolution in the SEM. It was also possible to localize the melanin granules and to get a semiquantitative appreciation of their number with this technique.

An example of a Caucasian hair cross section is shown in Fig 2. This hair was fixed in 2.5% glutaraldehyde and embedded in Spurr epoxy resin after conventional dehydration in ethanol gradients. Semi-thin sections in the range 0.5 to 1 μm were cut on an LKB Ultrotome with a glass knife. The sections were transferred to a spectrographically pure and highly polished carbon support and fixed onto this with the aid of a carbon glue. The specimens were transferred to a reaction chamber of a Yamato Plasma Reactor Type PR-503 and surface etched for 5 minutes in an oxygen plasma produced at 13.56 MHz RF with an energy output of 50 watts. The oxygen flow through the chamber was monitored to 20 ml $min^{-1}$.

The result of this treatment reveals that the morphology of the fibre cross section is readily discernible at low magnification (Fig. 2a). At higher magnification it is easy to locate the remains of pigment granules (Fig. 2b) of this essentially blond hair. The arrangement of cuticle cells and their numbers is clearly visualized (Fig. 2d). Also the profiles of the cortex cells are nicely visualized by this technique (Fig. 2c).

**Microincineration of skin sections**

Not only hair has been studied with the means of microincineration. Thus Yoshino et. al (54) have demonstrated the applicability of the technique to exocrine and endocrine glands in the rat pancreas. To our knowledge no studies on skin have been performed.

Referring to the background problem in EDX analysis discussed above we have made preliminary studies on the effect of microincineration on guinea pig skin.(Forslind B, Lindberg M, Miyasaka S, Seta S, Sato H, Yoshino M , unpublished data). The investigation included normal skin and skin exposed to nickel solutions in an additional attempt to evaluate the feasibility of the microincineration at detection of ion penetration through the skin barrier (Fig 3).

The normal skin was obtained from the shaved back of albino guinea-pigs. In a second set of the investigation guinea-pigs were exposed to a 5% solution of nickel sulfate for 6 and 24 h without or with the addition of 5 % SLS (sodium lauryl sulfate).The solution was contained within plastic chambers glued to the shaved back of the animals. After sacrifice of the animals small pieces of the skin specimens were quench frozen in Freon supercooled with liquid nitrogen to -190°C and subsequently freeze-sectioned at -20°C to a nominal section thickness of 15 μm. Subsequently the sections were freeze-dried in the cryostat overnight and mounted on spectrographically pure and polished carbon planchettes.

Microincineration was perfomed as described for the hair sections in a previous paragraph. The sections were subsequently covered by evaporation with carbon to achieve conductivity and thus preventing local charging of the specimen.For reasons not fully understood this procedure also promotes the preservation of the specimens in the time between preparation and analysis.

In these sections coarse details of the skin could be discerned in the SEM (Fig 3.a). Rough profiles of nuclei could be seen and the border between the epidermis and dermis was relatively well seen. In our specimens the dermal fibrous

components appeared clumped together. In thicker sections the dermis appeared more or less like a solid mat of material although the border between the dermis and the epidermis could be clearly observed. In the thick sections the effect of the microincineration was remarkable. The weave of interlacing fibrous bundles was now seen and the border between the non-cellular dermis and the cellular epidermis was conspicuous. As is generally the case in SEM and TEM the differences in contrast were much enhanced in the photographic recordings compared to the contrast of the viewing screen. However, the microincinerated thick sections (>15 μm) were remarkably easy to study in detail even on the microscope screen.

Our study suggests that on optimization of section thickness and the parameters for the microincineration procedure thick cryosections may be studied in detail in the scanning microscope.

At EDX analysis the Ni peaks were increased roughly 2-5 times in microincinerated sections compared to parallel sections which were analysed without previous microincineration (Fig 3 b). The gain at microincineration according to this pilot study was a function of the success at the incineration process. This is dependent on the parameters of section thickness and the completeness of the microincineration. We believe that an optimization of the section thickness and the conditions at microincineration thus will produce reproducible results with a gain of at least five times in the peak-to-background (P/B) ratio for cryosections.

**Particle probe analysis**

In recent years new techniques for elemental analysis have been applied to dermatological problems (59,69,70). Initially the EDX (**e**nergy **d**ispersive **X**-ray) analysis technique in the electron microscope dominated the field, but recently proton probes (PIXE: **p**article/**p**roton **i**nduced **X**-ray **e**mission and PMP: **p**roton **m**icro**p**robe) have proved to be of great value in dermatological research. Both types of particle probes have the great advantage of a simultaneous recording of all elements (above a certain energy threshold) in the spot of analysis. This allows effects of physiological change to be recorded with high sensitivity, e.g., study of the Na/K quotient may give information on the membrane function of a specific cell type under normal or experimental conditions.

The EDX systems are generally attached to a SEM or a TEM/STEM and this allows analysis of thick (bulk-) specimens and thin sections to be done with a spatial resolution down to subcellular dimensions. The main drawback of the EDX analysis system is the background produced which decreases the sensitivity of the analysis. Also the signals from elements below phosphorus (Z:15) are partially absorbed in the detector window. The PMP offers greater sensitivity (< 10 ppm) than the EDX (>200 ppm) as the background is very low due to the low proton stopping power of the specimen (70). On the other hand the spatial resolution is generally restricted to cellular levels although certain groups have achieved resolutions down to one or a few microns

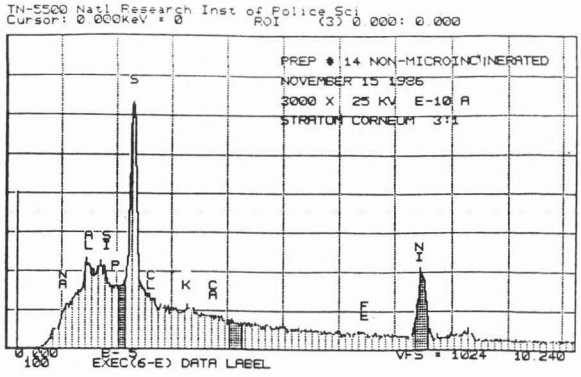



**Fig.2 a**. Microincinerated cross section of a Caucasian hair embedded in Epon (bar 100 μm).
**Fig.2 b**. The remains of the pigment granules can easily be identified (arrow) This individual is a redhead ! (bar 10 μm).
**Fig.2 c**. A region close to the medulla. The outlines of the cortex cells and pigment granules can be seen (arrow) (bar 10μm).
**Fig.2 d**. The endocuticle part of the cuticle cells have suffered the greatest mass loss at microincineration. Cell boundaries are clearly demarcated and it is easy to determine the number of cuticle cells (bar 10 μm).
(M. Yoshino is acknowledged for the microincineration and SEM.)

**3b**

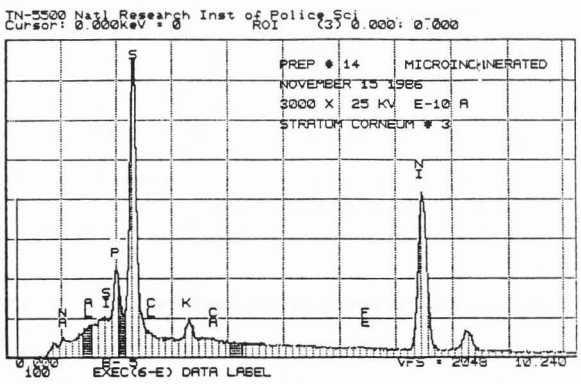
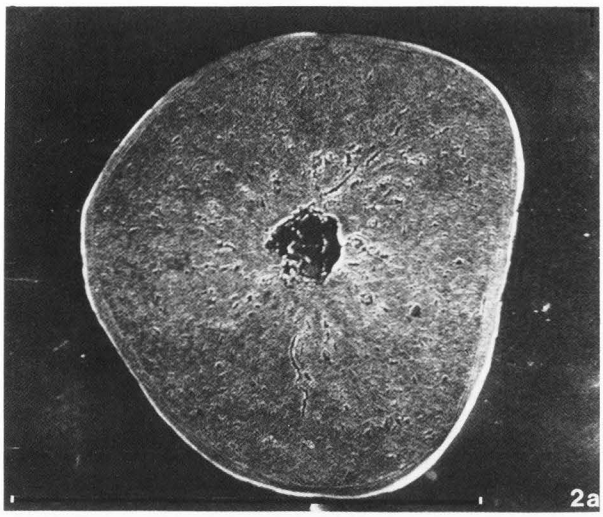



**Fig.3 a.** Microincinerated guinea-pig skin section. Oblique hair follicles cross sections are seen in the dermis. The epidermis is denoted by opposing arrowheads.
**Fig.3 b.** Guinea-pig skin exposed to Ni-solution and detergent (SLS) for 24 h. Two parallel cryosections were studied, one of them microincinerated (lower panel). Note the improved peak-to-background situation in the microincinerated section compared to the untreated one.

**Fig.4 a.** 1-2 mm high hair cross sections cut with a razor blade for cross section analysis (bar 100 μm).
**Fig.4 b.** Hair cross section. The probe locations are seen as depressions in the surface caused by superficial mass loss (arrows)(bar 10 μm).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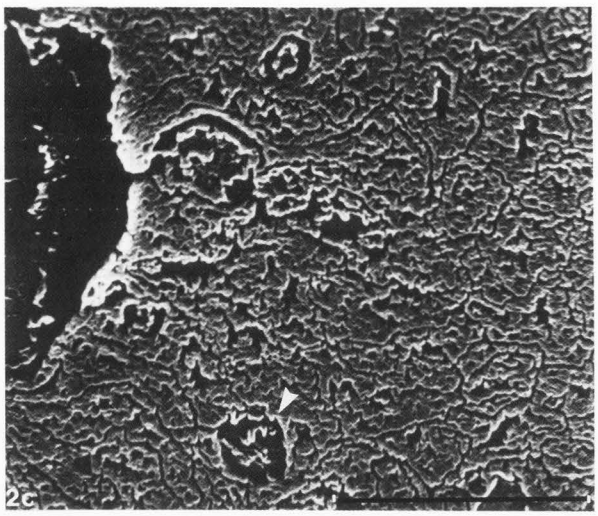
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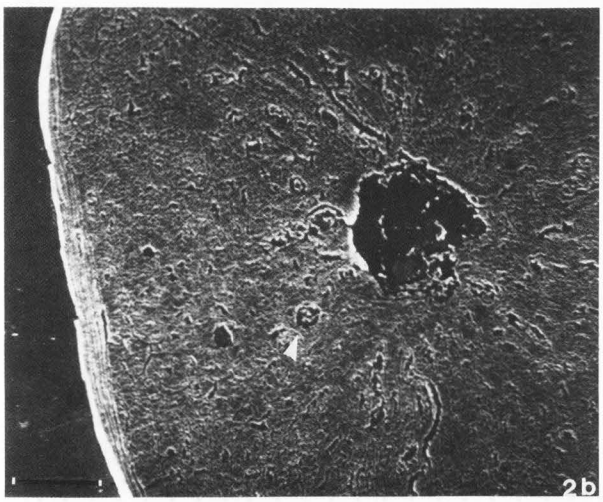
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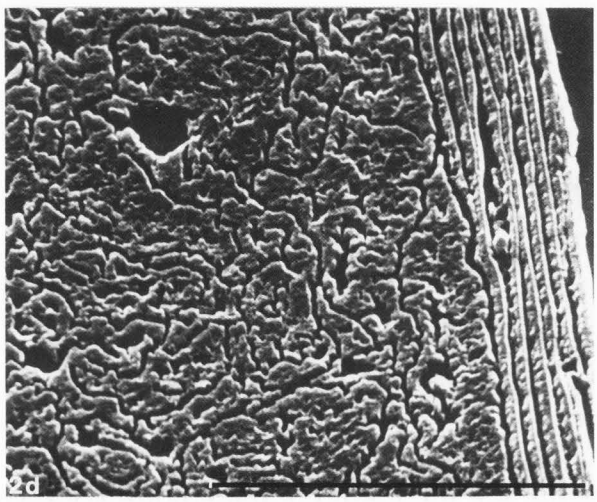
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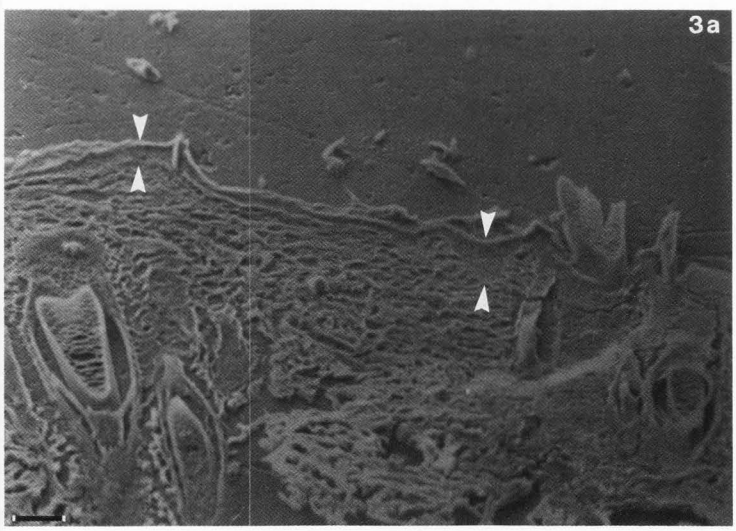
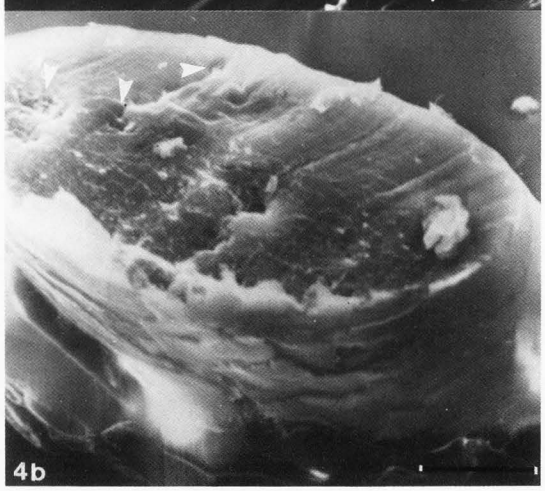

3a


4a


4b

square($\mu m^2$). Thus the PMP is excellent for analysis of trace elements which "drown" in the background of the EDX spectra under conditions where cellular resolution is satisfactory.

EDX systems are to-day common and thus their availability is no problem to the interested scientist who wants to avail himself of this facility. PIXE/PMP systems are much more rare things since they require accelerators for producing the proton beam.

As mentioned under the heading of micro-incineration the main problem in particle probe analysis is the question of inert specimen preparation if the interesting elements are diffusible (ions, small molecules, etc). In case the element of interest is firmly bound to a tissue/cell constituent normal preparation methods can be used. In the latter case thin sectioning is possible which allows a high degree of spatial resolution down to organelle levels. As a "rule of thumb" one may state that the spatial resolution at EDX is roughly of the same order as the section thickness and for PMP the spatial resolution is roughly that of the proton beam cross section. The EDX can be used for qualitative as well as for quantitative analysis. In the latter case standards have to be produced and prepared according to the same criteria as the specimen itself (cf 75).

**EDX analysis of hair**

It is a rare experience to read a paper devoted to elemental EDX analysis of hair. Using bulk specimens prepared by cutting cross sections of the fibre with a clean razor blade we have studied hairs from uremic patients to find out if there may be a correlation between aluminum content and "uremic" itch (Fig.4). In two out of five dialyzed patients we found Al contents appreciably higher than is seen in normal individuals (Forslind B, Bäckdahl M, Hägermark Ö, Sato S, Yoshino M, Miyasaka S, Seta S, unpublished data). At this stage the correlation between Al content of the hair and "uremic" itch remains an open question.

**PIXE analysis of hair**

In spite of the (commercial) interest in hair analysis as a means to record if an individual is healthy or not few scientific papers have been published in this area in the last few years. It is also a fact that PMP baseline data obtained from larger populations have not been available until recently. Forslind et al. (63) have studied a Caucasian population comprising both sexes in the age region 10 - 69 years. In order to minimize influences from the sampling area, e.g., effects of a non-obvious male pattern alopecia, etc, hair specimens were plucked from an area 1.5-2 cm above the right ear on all probands using rubber coated tweezers. Three anagen hairs were selected from each proband. In the initial phase of the investigation two sets of three hairs were obtained, one set being rinsed in a chloroform: ethanol (1:1) mixture to remove surface contaminants, the other set of three fibres was taken to analysis without prior treatment. The specimens were mounted on conventional slide frames for automatic analysis. This allowed 2 mm of the fibre 5-8 mm from the root to be irradiated by the proton beam.

The analysis of the 3 x 123 anagen hairs from 103 individuals (M:49/F:54) revealed that the sulfur content varies to a great extent in hairs from a given individual although the mean value, 4.9 + 0.8 % (0.049 + 0.008 g/g) corresponds nicely with data from the literature. However, the difference in the sulfur content of hair fibres from a given individual could amount to more than 1 %.

Zinc distributions were restricted around a mean of 170 + 50 μg/g. Hence, this study confirms previous reports stating that the Zn content of hair fibres is amazingly constant.The variation in Zn content from hair to hair in a given individual appears to be much less than that of sulfur. Chlorine and calcium gave widely scattered data and the analysis of these elements

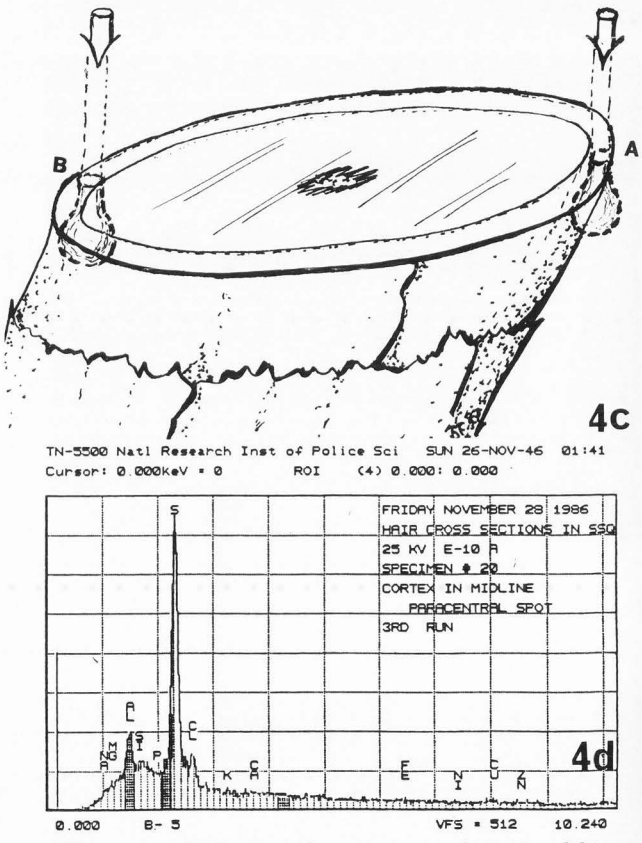



**Fig.4 c.** Diagram illustrating how an oblique bulk section can be utilized for isolated analysis of the cuticle with minimal contribution from the cortex. **A)** only the neck of the excitation volume in the cuticle, no contribution from the cortex. **B)** Neck of the excitation volume in the cuticle, but body of excitation volume is located in the cortex the composition of which will contribute to the elemental spectrum.

**Fig.4 d.** X-ray spectrum from hair cross section obtained from a patient undergoing dialysis due to kidney function failure. Note the aluminum peak (AL). Corresponding Al-peaks were not found in normal hair cross sections analysed in this experimental series.

is most likely to be suffering from contamination. The effect of the chloroform: methanol rinsing was completely negligible in this study. In conclusion we suggest that Zn rather than S be used as an internal standard when such is needed due to lesser variation of Zn content in a given individual (63).

Technical aspects of the PIXE technique at analysis of hair has been presented in the referred paper (63). Extending the interest to pathological hairs Forslind et al. (64) investigated 12 cases of (suspected) genetic disease with the SEM and PIXE. One of the main problems encountered in this investigation was related to the algorithm used for quantitative assessment of elements in the irradiated section of the hair fibre. Many of the hair fibres in the investigation had a very irregular and/or flat cross section. The algorithm used surmises that the cross section is approximately circular or elliptical, neither of which conditions were fullfilled by some of the specimens. It therefore appears necessary to make PMP analyses of cross sections of such pathological hairs to assess their elemental composition. Such studies are now underway. In two cases of **epidermolysis bullosa Weber-Cockayne** abnormally high Cu/Zn ratios indicated a disturbance. A study of the diseased skin would therefore be of great interest. A case of a 7 year old boy with a Zn deficiency and with an **acrodermatitis** type of lesion was cured by oral intake of **Zn.** Hair fibres from this patient taken before and after treatment revealed that the abnormal **Cu/Zn ratio** before treatment was normalized after treatment, as was the **S** content of the hair fibres. There were no morphological findings in the SEM micrographs before or after treatment which indicated the existence of an abnormality in this case. In conclusion the PIXE elemental analysis of single hair fibres which may be successfully applied to hairs with circular or elliptical cross sections may be complicated by abnormal cross section forms. PMP analysis of cross sectioned hair fibres may solve this problem.

**EDX on nails.**

Except for case reports nails are seldom the subject of particular interest in clinical and experimental dermatology. Djaldetti et al. (57) have investigated the elemental content of fingernail clippings from 13 patients with liver cirrhosis and compared these data with those obtained from 50 normal individuals. The percentage of Na, Mg, and P showed a significant increase and there was also a significant decrease in the S and Cl values. However, the authors do not offer any functional interpretation of their data in relation to other laboratory findings or clinical symptoms.

**EDX on conventionally prepared skin**

Often the location of an inclusion bound to a tissue component will be the problem of interest in a clinical diagnostic analysis. This case represents a qualitative question rather than a quantitative one, and often the included material will be visible in light microscope sections or even in thin TEM sections. The identification by EDX then confirms the presence of foreign material in the tissue/cell. This elemental identification in a spectrum can be done in a spot region of the specimen or the element distribution over a defined specimen area can be represented in a dot density map.

A case of herpetic erosive balanoposthitis which had been successfully treated with a commercial ointment containing titanium was reported by Dupre et al (58) using a semiquantitative EDX analysis set-up. Titanium was identified in biopsies taken from previously eroded areas and TEM verified that the deposits were located in the dermis. This was judged to be due to the extensive erosive process at hand when the ointment was topically applied.

Argyria has been the topic of many previous qualitative clinical reports (c.f. 59). A recent report indicates that acupuncture needles may deposit silver in the tissue. The actual case reported by Tanita et al. (71) was concerning a lady who 20 years previously had been treated with acupuncture in the antecubital fossa where later blueish maculae appeared. Xeroradiographic depicting of the legs revealed linear marks corresponding to areas where the acupuncture treatment was concentrated. In the TEM granular deposits were found under the basal lamina of the epidermis in such regions. The qualitative EDX analysis showed granules mainly to be composed of silver but some mercury granules were also found. The elemental content of the needles used verified the presence of Ag, Cu, Zn, and Cd whereas no Hg was found.

Minocycline treatment has previously (cf 59) been reported to cause hyperpigmentation and it was revealed that this change of the skin was accompanied by cutaneous inclusion of hemosiderin X-ray microanalysis proved them to contain iron, sulfur, chlorine and calcium in cases of focal discoloration whereas the more diffuse and generalized pigmentation appears to be associated with increased melanization. In a recent contribution Gordon et al. (65) have studied discoloured finger nail and skin biopsies by light and electron microscopy and in addition performed X-ray microanalysis studies on their samples which were processed as conventional TEM preparations including post-osmication after glutaraldehyde fixation. Only the finger clippings were analysed in their "virgin" state. The morphological study showed that pigment loaded macrophages were found in normal as well as in pigmented areas of the skin the difference being that the macrophages of the pigmented regions contained a greater number of pigment loaded granules. The elemental analyses confirmed that the pigments contained iron and calcium. It is interesting to point out that even the stratum corneum of the pigmented specimens were containing iron and calcium in two of the minocycline treated patients. It is suggested that air oxidation of chelated minocycline to coloured quinones might occur in the superficial epidermis. This was also the case for the pigmented nail specimens. However it is to be noted that nail discolouration only was at hand in female subjects. Hence, nail polish may be the source of iron in these cases as well as air oxidated products. In summary these results

suggested that the discolouration is possibly due to an iron-chelate of minocycline and/or quinoid derivates of minocycline.

**EDX on inertly prepared skin**

In spite of the fact that EDX is now an established technique in dermatological research few investigations are performed on inertly prepared material. Supposedly the amount of labour involved especially in the sectioning procedure (with a comparative low output) is prohibitive. However, with this technique there is a lot of interesting information to gather both from a clinical as well as an experimental point of view.

A comparison between the results of EDX analysis on thick sections of human epidermis and guinea-pig skin by Forslind et al. (60) revealed that the elemental distributions were close. It must be realized that the water content of the different strata of the human epidermis varies to a considerable degree and that data presented are given as mass fractions (weight of element to dry weight of tissue) at the point of analysis. A preliminary attempt to estimate the water content by freeze-substitution of human epidermis shows that the gradients of physiological elements still pertain although they are less steep, as expected, in the freeze substituted specimens.

This analysis of the influence of the water content on the elemental distribution data was challenged by Warner who recently has given an account on the problems involved in the determination of tissue water by X-ray microanalysis techniques (73). As can be recognized from Warner's paper one of the main problems in the determination of the water content of a skin (or even an epidermal) cross section is the considerable heterogeneity in the mass distribution of the different strata. Warner gives an equation for solving this problem which allows for variations in the density of proteins without serious variation in the determination of the water content. In a sequel paper Warner et al. (74) have investigated the water concentration profile of normal human skin. An interesting aspect of this paper is the discussion of the conspicuously steep water gradient at the stratum granulosum-stratum corneum junction which may offer a number of functional advantages in the protection from intruding substances and conservation of substances within the body.

The elemental profile of psoriatic skin was studied by Grundin et al. (66) using skin from stable plaque areas and paralesional uninvolved skin. Significant differences were found between involved and uninvolved skin in these psoriatics. The levels of Mg, P and K were higher in the epidermal cross section of plaque skin compared to paralesional skin. Neither involved nor non-involved psoriatic stratum germinativum differed markedly from non-psoriatic control stratum germinativum. In comparison with the non-involved psoriatic skin the elemental composition of the various strata of psoriatic skin showed a pattern typical for highly proliferative, non-neoplastic cells.

Irritant reactions in the skin represent a type of condition where the barrier function of the skin is severed to a greater or lesser degree. In a study performed to analyse the elemental changes occurring at irritant reaction Lindberg & Grundin (68) used the guinea-pig model. A mild irritant reaction was obtained by applying sodium lauryl sulfate (SDS) to the skin of the back of shaved guinea-pigs. 24 and 48 h after the application of the SDS the animals were sacrificed and inertly processed for EDX analysis and in addition specimens were processed for conventional TEM. A hyperplastic response was elicited by the treatment and this phenomenon was accompanied by significantly increased levels of intracellular Na and Cl. The number of keratinocytes was greatest at 48 h. However the most conspicuous change in the elemental composition was recorded already at 24 h. The morphological pattern and elemental patterns are compatible with an initial membrane dysfunction which is followed by an increase in proliferative activity.

**Proton microprobe analysis of skin**

The elemental composition of the skin reveals information about the physiology in normal and pathological conditions. However, the main drawback of the EDX technique, as mentioned above, is its insensitivity to elements present in low concentrations, e.g., Ca, Fe, and Zn. The proton probes (PMP) on the other hand may provide information on the concentration and distribution of these elements. The preparative conditions for PMP analysis, of course, requires that inert techniques are used in these cases.

One important question is to what degree there is a correspondence between EDX and PMP when sections from the same specimen are analysed. Forslind et al. have undertaken a comparative study of EDX and PMP analysis of soft biological tissue using freeze-dried sections of gelatin containing known concentrations of nickel chloride (62). With both techniques calibration curves (signal versus the known Ni concentration) were obtained that showed deviation from linearity only at high Ni concentrations. However, plotting the EDX signal versus the PMP signal a linear relation was obtained with a correlation coefficient of 0.996, an indication of the high complementarity of the two techniques. It may be noted that the plot of the characteristic signals of Ni and S (which is endogenous to the collagen matrix) versus the known concentrations of the specimens yielded a correlation coefficient of 0.999.

For quantitative analysis the peak-to background method used in EDX analysis has little relevance in PMP analysis since protons are not scattered by the biological material under analysis to a significant degree. In PMP analysis peak-to-continuum normalization using an interval free of peaks has been employed to estimate sample thickness at the area of analysis. In spectra with many peaks an interval which is large enough can be hard to find and other techniques have been sought for. To this end Lövestam et al. (69) have investigated the feasibility of using backscattered protons for mass thickness determination by studying a series of plastic foils constituting specimens of varying thicknesses and of an approximately

"biological" composition. The results show that the mass thickness of the specimen at the point of analysis can be accurately determined with this technique. Gelatin glycerol standards (c.f. 62) with different concentrations of Ni showed very good correspondence between the measured data and the known Ni concentrations of the samples. Additional investigation of the feasibility of commercial standards showed that standard deviations were indeed very small (<1% of which 0.3-0.7% is due to statistical fluctuations) and such items can be used for the calibration of PMP systems with a focussed beam.

In a preliminary experimental study of nickel sulfate and potassium chromate penetration through autopsy human skin (60) PMP analysis was shown to detect the penetration profiles of the $Ni^{2}+$ and the $Cr_2O_7^{2}-$ even at concentration levels in the range 30 - 60 ppm (parts per million).

Elements present in low concentration and trace elements, e.g. Ca, Fe and Zn play crucial roles in the physiology of skin. Ca which has been shown to actively influence the differentiation of epidermal cells into a final stratum corneum was shown to reveal a profile of increasing amounts towards the stratum corneum in the normal skin and this is also the case in paralesional psoriatic skin. In the normal skin the Fe and Zn is essentially confined to the basal cell layers. However it is interesting to note that in the paralesional psoriatic skin we found that the iron levels increased towards the stratum corneum reaching levels of 500 ppm compared to the roughly 250 ppm maximum found in the normal skin. The Zn levels of paralesional psoriatic skin were approximately the same as in the normal skin, roughly 100 ppm, but did not level off to levels below significant values in the upper epidermis (str. granulosum & corneum) as is the case in normal skin (60).

Using PMP analysis at cellular resolution Kurz et al. (67) have investigated elemental distributions in psoriatic skin. The results are to a great extent confirming previous EDX data (c.f. 66) including the observation that the striking differences were found between involved an uninvolved psoriatic skin. However it is interesting to note that their Fe data from paralesional psoriatic skin differ from the preliminary results that was previously reported (61) which sustained an old neutron activation analysis study of iron loss via psoriatic skin (cf 67). The obvious difference between the two PMP investigations is that in contrast to our study Kurz et al. (67) accepted that the lesions were treated with petrolatum. The possible influence of an increased hydration of the skin due to this treatment is difficult to evaluate at present. It is therefore of interest to further investigate different stages of psoriatic lesions in a larger population of patients suffering from this disease with the PMP technique to settle the question of trace element distributions over the psoriatic epidermis.

## Conclusions

### SEM

In completing this review the general impression is that neither SEM nor the particle probe methods have been used as extensively in dermatological research as they merit in view of the wealth of information which is obviously attainable with these means.

In the analysis of pathological hair fibres much of the topographical information provided by the SEM is unattainable by light microscopy due to the (partial) transparency (or total light absorption) and light reflexions of the specimens. The single preparative procedure which is needed for topographical analysis in hair preparation is defattening. As seen by the SEM papers cited above,detailed information is made available from hairs which have allowed precise diagnosis in several cases.

The application of the SEM to the study of the integument in normal and pathological conditions is not a matter of basic science only. This fact has obviously been neglected by clinicians who are not educated to see it as a tool in practical diagnosis. It is a fact, which obviously has not reached the general mind, that all that is needed for a cooperative work between a dermatologist and a microscopist is a vessel containing glutaraldehyde in a physiological saline solution for instant fixation of biopsies. Once the specimen is in the glutaraldehyde it can be stored for very prolonged periods, weeks and months, in the cold ($+4^{o}C$) without appreciable morphological changes.This has been documented in a study at TEM resolution on the preservation of skin biopsies in glutaraldehyde for prolonged periods (24). In my opinion, the between 20 to 30 SEM papers on skin conditions published in the wake of my past and present review illustrate that microscopists have done little to make dermatologists share their enthusiasm for this versatile instrument.The lack of papers introducing new techniques for preparation of integument specimens is obviously a symptom of neglect as well.

### Particle probes

The problem of preparation for particle probe analysis is far more complex than that involved in the SEM preparations. If tissues are to be analysed under "physiological conditions" inert freezing techniques are indispensible as was discussed above. However, it is quite obvious that when one deals with problems concerning deposition of foreign material in the skin conventional preparation techniques (for SEM/TEM) are often sufficient. The restricted availability of the particle probe instruments and laboratories also explains the comparatively few papers which have utilized this technique. It is my opinion that in the next few years an expansion in this field will not be obvious but as the number of instruments will increase and the initial preparative and other techniqual problems have been solved the study of the physiology of the skin in normal and pathological conditions will require the use of this tool.

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**Discussion with Reviewers**

**S.G. Ronan:** I would like to know the author's opinion on the use of SEM in classifying the various types of Ehlers-Danlos syndrome ?

**Author:** Not being a pathologist my impression obtained at reviewing the literature is that the SEM has provided us with useful information on the collagen arrangement in these disorders and that this kind of topographic information is complementary to histochemical analysis providing a more definite and thorough understanding of the biochemical disturbances which influences on the integument architecture.

**S.G. Ronan:** How practical is it to use SEM compared to ordinary light microscopy of tumour tissue and if necessary, TEM ?

**Author:** A generalized answer to this question is that the SEM is the instrument of choice if the main question involves topographical (3-D) information.

In addition, a bulk specimen which has been fixed so as to allow preparation for TEM, e.g. fixed,rinsed, dehydrated on an ethanol gradient and (critical point) dried may successfully be scrutinized by the SEM .Subsequently such a specimen may be embedded in plastic for thin sectioning necessary for TEM investigation.

**GM Roomans:** The outcome of the microincineration experiments results in some increase in the EDX sensitivity, which however is not too impressive. This suggests to me that the stopping power of the scaffold of ashes left after incineration is low enough to allow the excitation volume to include the carbon support of your specimens.

**Author:** Yes, you are probably right. The section thickness chosen was roughly 15 μm and the microincineration was proceeded until the entire organic matrix was carbonized. Our results thus suggests that thicker sections should be used. Alternatively the microincinerated sections should be mounted on a support film with minimal stopping power.