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ZINC DEFICIENCY PRODUCES TIME-RELATED ULTRASTRUCTURAL CHANGES IN RAT CHEEK EPITHELIUM

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Abstract

The subject of this study was whether the ultrastructural changes in cheek epithelium of zinc-deficient rats are time related. Weanling male Sprague Dawley rats were fed a zinc-deficient diet containing 0.4 ppm zinc (ZD) *ad libitum* and controls were pair-fed zinc adequate diet containing 40 ppm zinc. After 9, 18, and 27 days of zinc deficiency, specimens from cheek epithelium of both groups were processed for transmission electron microscopy. Partial conversion of the orthokeratinized cheek epithelium to parakeratinized was seen as early 9 days. An electron-lucent band surrounding the nucleus was observed in ZD cells. Mitochondria, tonofilaments, keratohyalin granules and ribosomes seemed to be increased with the increase in time of zinc deficiency. There was a thickening of the stratum corneum as well as hyperplasia and widening of the intercellular spaces of the spinous layer cells. Retention of a few membrane coating granules (MCGs) in the parakeratinized layer was seen after 9 days. Parakeratinization was further increased after 18 days of zinc deficiency, and the number of MCG profiles also increased. The epithelium was fully parakeratinized following 27 days of zinc deficiency, and the number of MCG profiles was increased. It was concluded that zinc deficiency affected cell proliferation and differentiation of the epithelium as early as 9 days, and caused a delay in loss of nuclei and MCGs in parakeratinized cells.

Key Words: Rat cheek epithelium, basal cells, spinous cells, granular cells, cornified cells, parakeratinized cells, zinc deficiency, time related changes.

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Introduction

Zinc, as a trace element, is found in all biological systems. It is needed for growth and development, DNA synthesis, neurosensory functions and cell mediated immunity (Walsh *et al.*, 1992; Prasad *et al.*, 1993). It is required for the optimum functioning of over 300 enzymes (Vallee and Falchuk, 1993). Zinc deficiency is a worldwide problem. It is reflected in clinical syndromes which affect men and women of all ages and all socioeconomic and cultural classes in the United States. Subacute zinc deficiency is most common, and there are 4 million people in the United States with this syndrome, the initial symptom being dysfunction of taste and olfaction. It has long been known that zinc deficiency also causes hyperplasia in rat esophageal and oral epithelia, in contrast to the atrophic changes seen in most other organs (Follis *et al.*, 1941). A diet containing less than 1 ppm of zinc fed to weanling rats results 4 weeks later in lesions with marked premalignant features in buccal epithelium, including parakeratosis (Alvares and Meyer, 1968), hyperplasia (Meyer and Alvares, 1974), accelerated proliferation (Alvares and Meyer, 1973) and increase of lactic dehydrogenase activity (Gerson and Meyer, 1977). Osmanski and Meyer (1969) reported ultrastructural changes in the upper spinous, granular and keratin cell layers of buccal epithelium in rats after 27 days on a zinc-deficient diet; the changes included an increase in the concentration of ribosomes, mitochondria and endoplasmic reticulum, prominent desmosomes in the nucleated cell layers and persistence of nuclei in the keratin layer. Subsequent observations suggested changes in the distribution of membrane coating granules (MCGs) (Ashrafi *et al.*, 1979; 1980). Since these findings were based at 27 days of zinc deficiency, the present study was undertaken to investigate the time related ultrastructural changes specifically following 9, 18 and 27 days of zinc deficiency.

Materials and Methods

Thirty-six Sprague Dawley 21-day-old male weanling rats were used in this study. After arrival, all rats

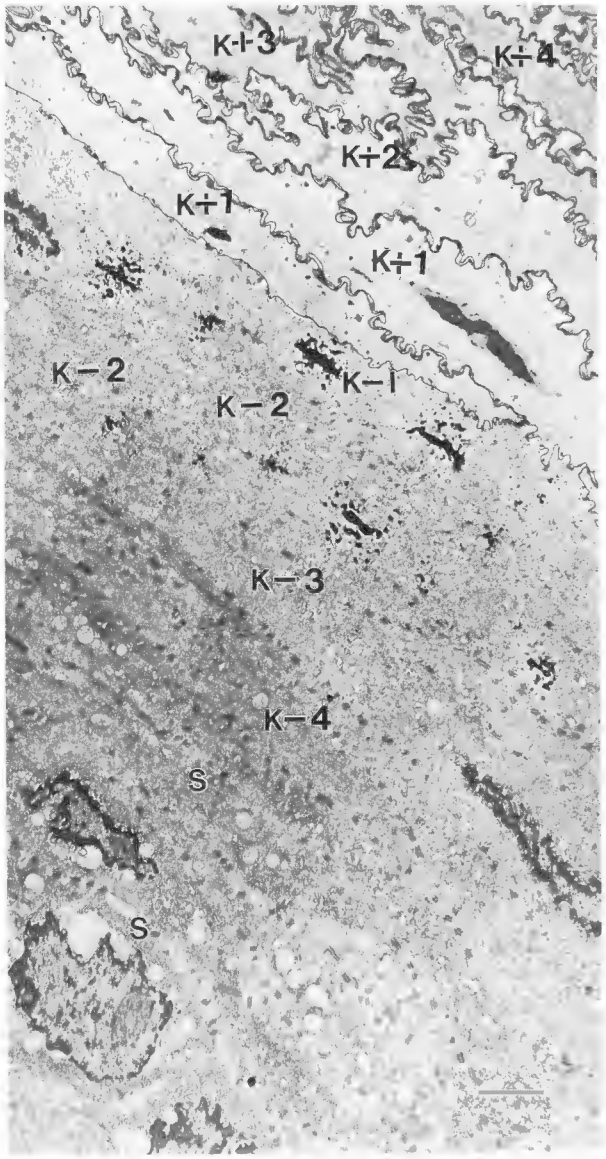

Figure 1. Electron micrograph of a 9 day PF rat cheek epithelium showing spinous cells (S), granular cells (K-) and cornified cells (K+). Bar = 2 μm.

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were fed *ad libitum* zinc adequate diet (40 ppm) for the first five days. Then animals were randomly divided into two groups: group (1) zinc-deficient (ZD) rats which were fed a diet containing 0.4-0.5 ppm zinc (Teklad Mills, Madison, WI) (N = 18) *ad libitum* and group (2) rats were pair-fed (PF) zinc adequate diet (40 ppm) at the same daily amount consumed by ZD rats. In order to prevent ingestion of extraneous zinc, the experimental animals were housed individually in hanging stainless steel cages with mesh bottoms, and double-distilled water was given in glass bottles with stainless steel nozzles. The rats were housed in a room which

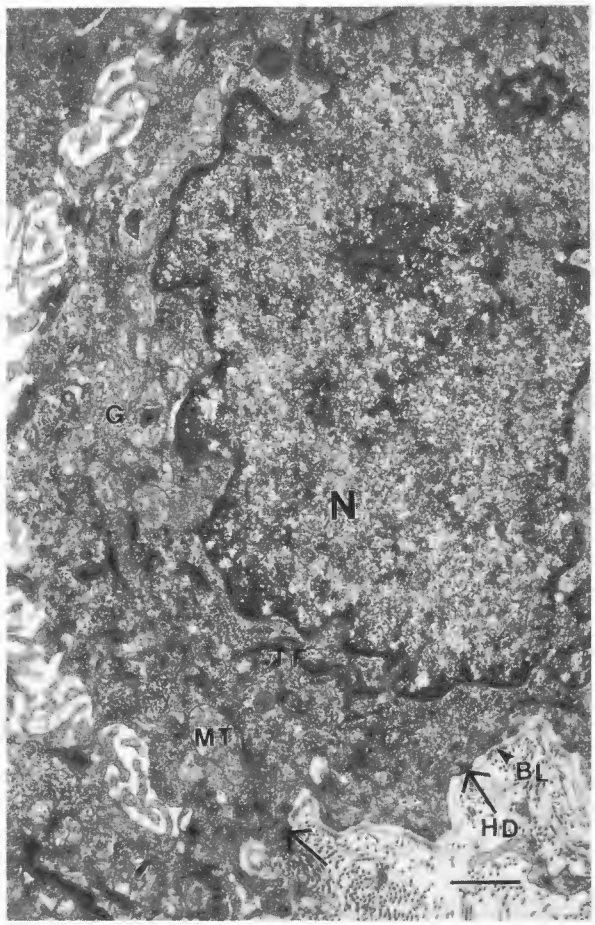

Figure 2. Electron micrograph showing a basal cell in a 9 day PF rat cheek epithelium. Basal lamina (BL) is continuous (arrowhead). HD: Hemidesmosomes (arrow); MT: mitochondria; TF: tonofilament bundles; G: Golgi; and N: prominent nucleus. Bar = 1 μm.

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was temperature controlled to 22-23°C with a 12-hour light-dark cycle.

Specimens of buccal mucosa were collected from ZD and PF rats under ether inhalation after 9, 18, and 27 days. One to two millimeter thick slices of buccal mucosa were cut perpendicular to the surface and were immediately placed for 2 hours in cold 4% glutaraldehyde made in 0.1 M cacodylate buffer at pH 7.4. The tissue blocks were then washed three times in cold 0.1 M cacodylate buffer and either stored overnight in fresh baths of the same buffer for other studies or immediately post-fixed for one hour in 1% OsO_4 in cacodylate buffer for the present study and subsequently washed twice in distilled water. They were dehydrated through a graded series of alcohols to absolute alcohol, placed in two changes of propylene oxide for 15 minutes each and embedded in Araldite for electron microscopy.

Semi-thin sections (0.5 μm thick) were cut from

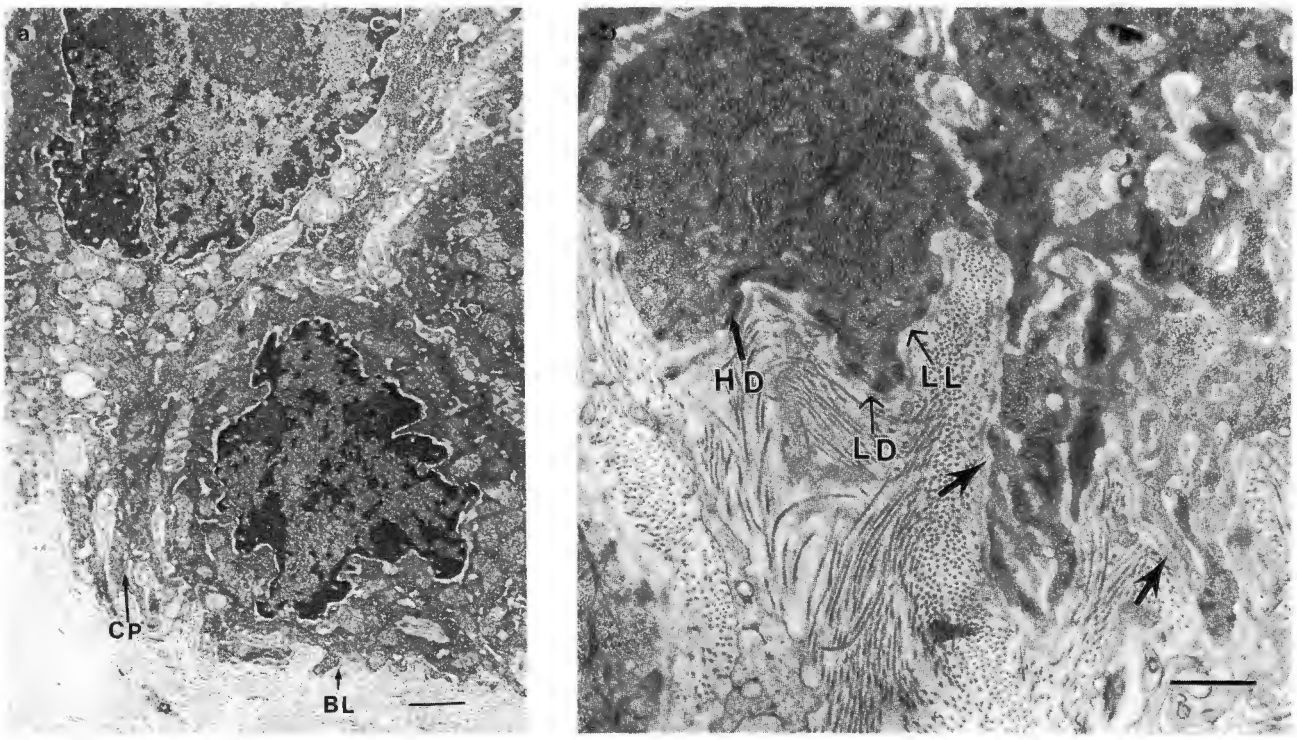

Figure 3. (a) Basal cells in 9 day zinc-deficient rat cheek epithelium. Note irregular basal lamina (BL) and basal cell processes (CP, magnified in Fig. 3b). (b) A higher magnification of a basal cell at 9 days zinc-deficient rat cheek epithelium showing basal cell process (CP, from Fig. 3a) extending deeper into connective tissue (arrows). Irregular basal lamina is consisting of lamina densa (LD) and lamina lucida (LL). Hemidesmosomes (HD) are present. Bars = 1 μm.

each block and stained with toluidine blue. The sections were surveyed by light microscopy to check the tissue orientation and verify the presence of epithelial layers to be analyzed for the presence of organelles. Accordingly, three to six properly oriented blocks were selected randomly from each tissue specimen. Only blocks of which sections showed that they contained the entire epithelium were selected for further study. The rationale was to be sure of the location of the keratinocytes to be examined. Thin sections (50-60 nm thick) were cut from the selected blocks, mounted on copper grids, stained with uranyl acetate and lead citrate and examined with a Philips 301A electron microscope (Philips Electronic Instruments, Mahwah, NJ). Montages of all layers of the oral epithelium were made from electron micrographs of low magnification to identify and select areas within the granular and cornified or parakeratinized cell layers for further examination at higher magnifications. The granular cell layer at the junction with the first cornified layer was designated as K-1, and the succeeding down deeper granular cell layers were designated as K-2, K-3 and K-4, respectively. Similarly, the cornified layer at the junction with the granular cell layer was designated at K+1, and the succeeding superficial cornified cell layers were named K+2, K+3, and K+4 (Fig. 1). The corresponding parakeratinized cells in ZD animals were designated PK+1, PK+2, PK+3 and PK+4, respectively.

Electron micrographs were made randomly at magnifications ranging from 9,100x to 15,000x for subjective qualitative observation of ribosomes, mitochondria, desmosomes, tonofilaments and keratohyalin granules and membrane coating granules. The coded electron micrographs were examined by three scientists and rated on the scale of 1 and 2 (**1 = low and 2 = high**). Evaluators were not made aware of which group (ZD or PF) electron micrographs were taken for their examination. Scores were averaged to obtain overall rating regarding the low or high presence of the organelles and narrow or widened intercellular spaces. Subsequently, the statements were made about low or high frequency of occurrence of organelles in ZD epithelial cells as compared with PF controls.

For quantitative analysis of MCGs, the randomness of observation was maintained by photographing sections at the right upper corner of the grid square. The primary magnification was 9,100x, and the negatives were enlarged 2.5 times to give a final magnification of

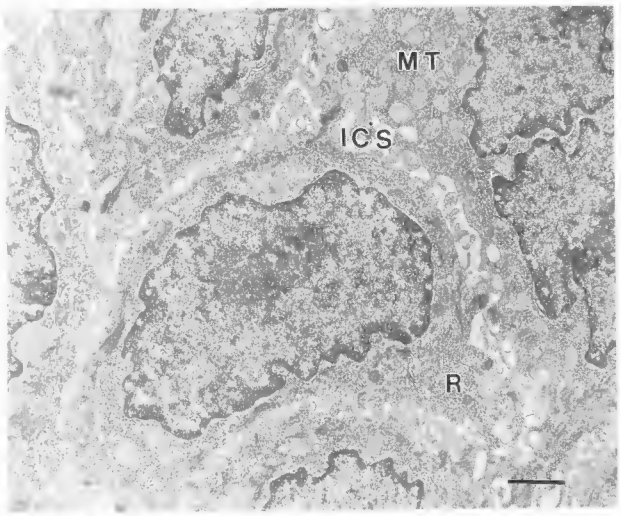

Figure 4. Intercellular spaces (ICS) in spinous layer of a 9 day PF rat cheek epithelium are narrower than in experimental epithelium. MT: mitochondria; R: ribosomes. Bar = 1 μm.

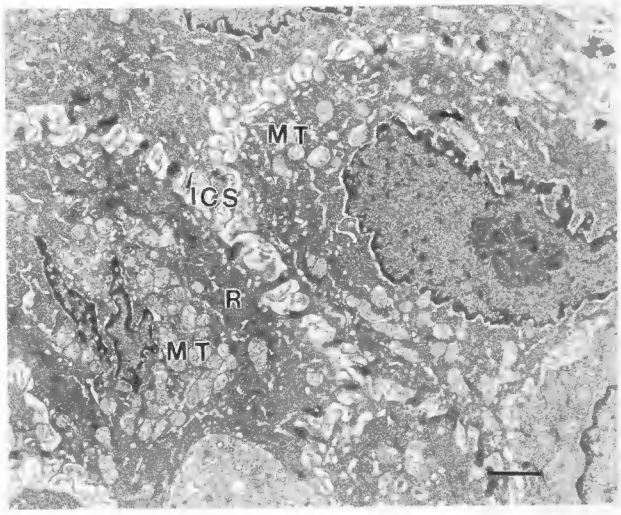

Figure 5. Intercellular spaces (ICS) in spinous layer of a zinc-deficient 9 day rat cheek epithelium are wider than in control epithelium. MT: mitochondria; R: ribosomes. Bar = 1 μm.

22,750x. At this magnification, the size of MCGs was 0.1 to 0.2 μm. The original area of the tissue photographed was 10 x 8 μm (80 μm^2), in order to collect data as the number of MCG profiles per 80 μm^2 of tissue. In PF controls, MCGs were counted in the granular cell layers K-1, K-2, K-3 and K-4, only as they were not present in cornified cells. In ZD cells, the number of MCGs were counted in granular K-1, K-2, K-3 and K-4, plus parakeratinized PK+1, PK+2, PK+3 and PK+4. The counts of MCGs were normalized to 100 μm^2 area for calculation purposes and presented in the form of means and standard errors of the means (SEM) based on the number of animals. The analysis of variance was applied by using the BMDP7D program (Dixon, 1985). Student's t-test was used to determine the significance of differences between the experimental and control groups. Differences were considered to be significant at $p \leq 0.05$.

Results

Gross observations

Zinc-deficient rats gained less weight than pair-fed controls. They gained 80%, 66% and 56% of the gain of control rats after 9, 18 and 27 days, respectively. They displayed the signs of zinc deficiency such as loss of hair and dermatitis of the paws, periocular area and snout. Control rats showed none of these signs.

Electron microscopic observations

Basal layer In PF, cells of the basal layer were separated from the connective tissue by a regular basal

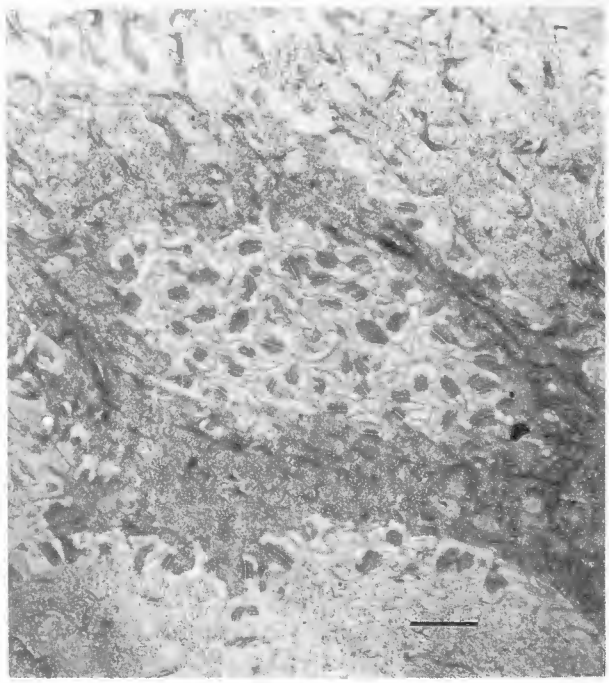

Figure 6. Spinous cells in zinc-deficient rat cheek epithelium showing intercellular space filled with numerous desmosomes. Bar = 1 μm.

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lamina (BL). The lamina lucida and lamina densa were parallel to the plasma membrane of the basal cells (Fig. 2). Hemidesmosomes (HD) and desmosomes were well formed. The epithelial changes were noticed as early as 9 days of zinc deficiency. The basement membrane was

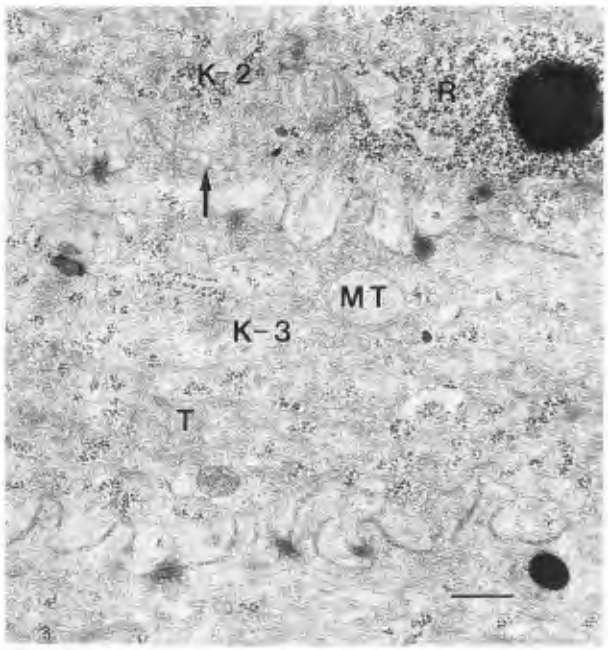

Figure 7. Granular layer cells in 18 day PF epithelium. Intercellular space between K-3 and K-2 cells contains extruded content membrane coating granules (MCG) (arrow). Keratohyalin granule surrounded by ribosomes (R), tonofilaments (T) and mitochondria (MT) are also seen in these cells. Bar = 0.5 μm.

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irregular because of cellular extensions (Fig. 3a). Basal lamina consisted of lamina densa (LD) and lamina lucida (LL) (Fig. 3b). Heterochromatin was condensed at the nuclear periphery in both cases, however, in the majority of cases, its presence was noticeable in ZD cell. Often an electron-lucent band surrounding the nucleus was observed in zinc-deficient cells (Fig. 3a). The presence of ribonuclear protein particles was prominent in ZD cells. The majority of mitochondria were grouped more in the basal aspect of the ZD cells as compared with PF basal cells (Figs. 2 and 3a).

Spinous layer The widening of the intercellular spaces in the lower spinous cell layers was noticed in 9, 18 and 27 days of ZD cheek epithelium as compared with PF (Figs. 4 and 5). Occasionally, intercellular spaces were filled with numerous desmosomes (Fig. 6). A few profiles of rough endoplasmic reticulum were seen in both PF and ZD tissues. Nuclear chromatin in the form of heterochromatin was noticeable adjacent to the inner membrane of the nuclear envelope in both ZD and PF cells. However, in the majority of cases, the presence of heterochromatin was more distinct in ZD cells. In the lower and upper spinous cells, the number of mitochondria seemed to be increased in ZD epithelium. They were regular in size and shape in both PF

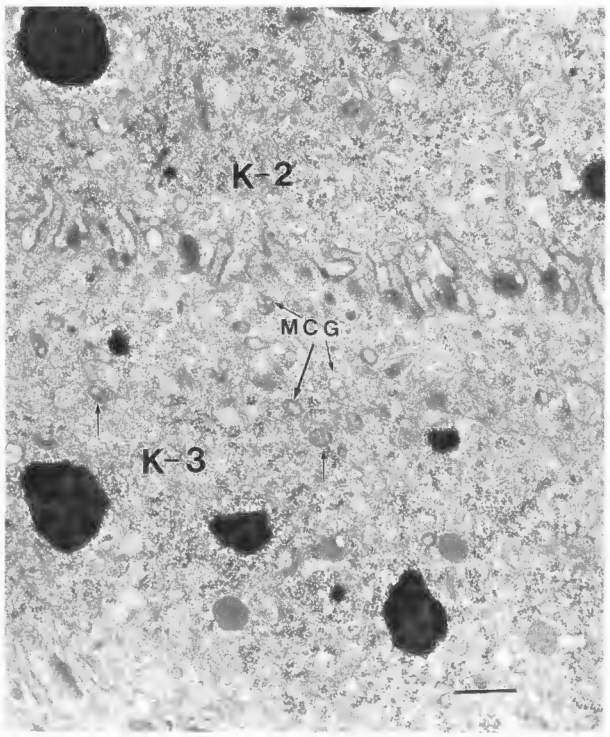

Figure 8. Granular layer cells in 18 day zinc-deficient rat cheek epithelium. Membrane coating granules (MCG, arrows) are present in K-3. Keratohyalin granules surrounded by ribosomes (R) are also seen. Bar = 0.5 μm.

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and ZD cells. The number of MCG profiles was increased in both PF and ZD tissues as the cells migrated superficially. However, this increase became more evident in ZD than in PF tissues. They were distributed throughout the cells, and some were oriented near the superficial plasma membrane of the cells. Tonofilament bundles were scattered to a greater extent throughout the cytoplasm in ZD epithelium as compared to PF (Fig. 6).

Granular layer Desmosomes were more numerous in 9, 18 and 27 day zinc-deficient cells than in 9, 18 and 27 day controls. Tonofilaments were uniformly arranged in bundles in the cytoplasm of PF and were scattered in the cytoplasm of the ZD cells. Keratohyalin granules were associated with ribosomes in both PF and ZD specimens (Fig. 7). They were oval to round in shape and were noticed more in upper granular cells of ZD epithelium as compared to PF (Fig. 7).

The number of MCG profiles was increased in zinc-deficient cells as compared to PF. The MCGs were distributed throughout the cells (Fig. 8). This distribution was more distinct in 18 and 27 day ZD cells than in 9 day ZD cells. In PF, the majority of MCGs extruded their contents into the intercellular spaces between K-1

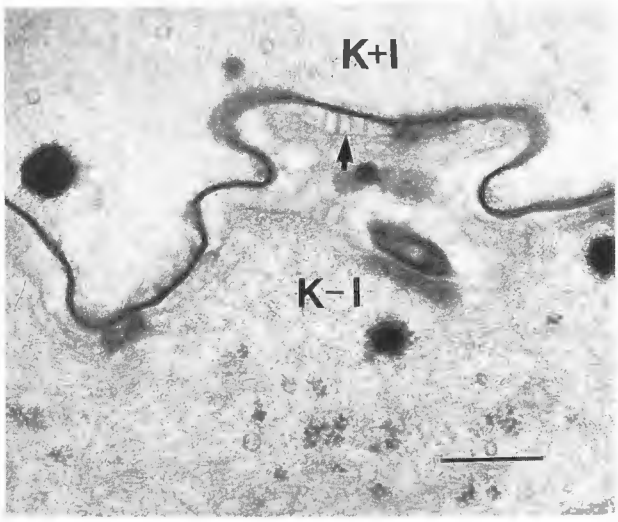


Figure 9. Lamellated material (arrow) extruded from MCG is present in the intercellular space between K-1 and K+1 cells in 9 day PF rat cheek epithelium. Note the extremely thickened plasma membrane in the K+1 cell. Bar = 0.5 μm.

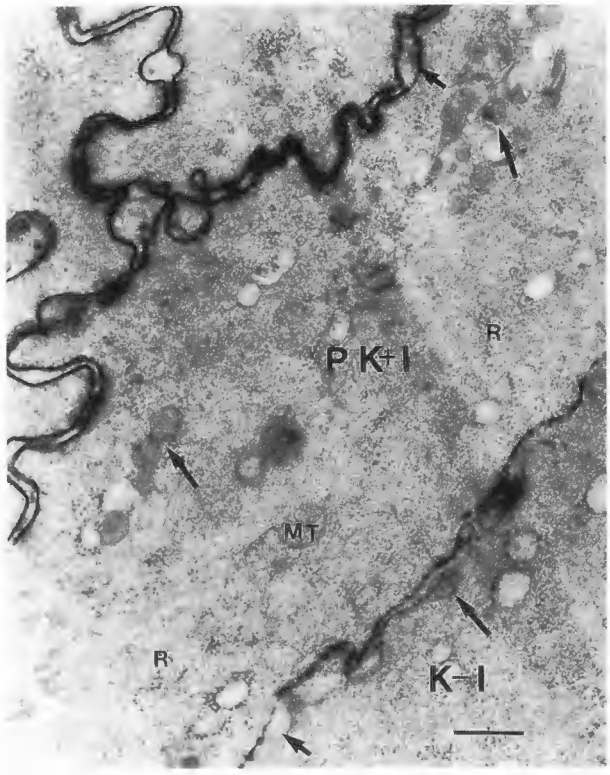


Figure 10. Last granular cell (K-1) and the first parakeratinized cells (PK+1) of a rat cheek epithelium following 27 days of zinc deficiency. Notice the presence of MCG (long arrows) and patchy filling of ICS at K-1 and PK+1 (two short arrows). The parakeratinized cell shows MCG and mitochondria (MT). R: ribosomes. Bar = 0.5 μm.

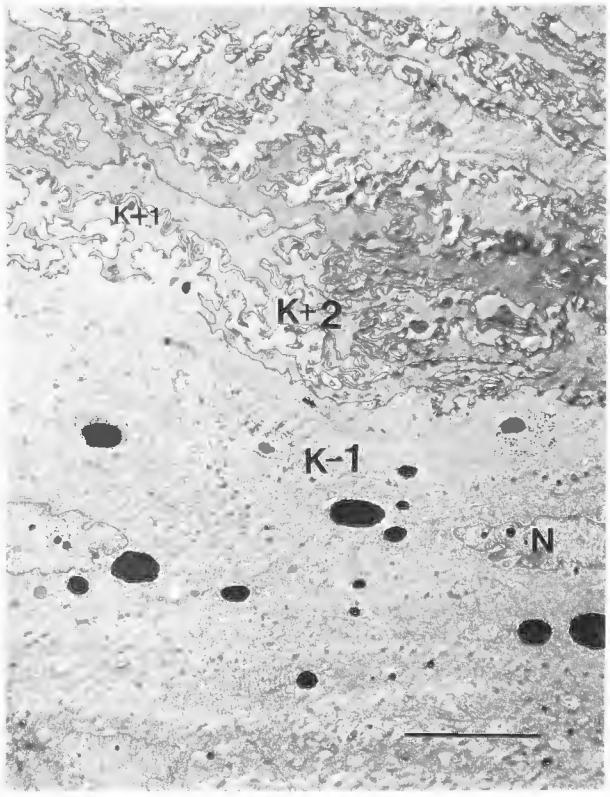
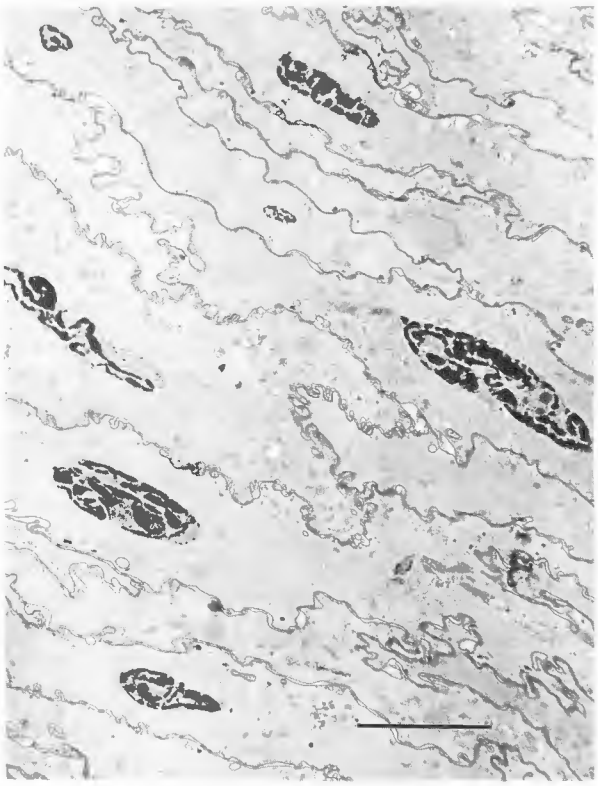


Figure 11. Rat cheek epithelium in 27 day PF showing cornified layer cell (K+1) and granular layer cells (K-1). Note absence of nuclei (N) in keratinized cells and presence of nuclei in granular cells. Bar = 5 μm.

and K+1 (Fig. 9). The extrusion of MCGs was retarded in the ZD group. In ZD epithelium, although some filling of the intercellular spaces with MCGs lamellated material was noticed between K-1 and K-2, as well as between K-2 and K-3, but it was patchy at the junction of K-1 and K+1. Several MCGs were distributed in ZD cells of the last granular layer (K-1, Fig. 10).

Cornified and parakeratinized layers In 9, 18 and 27 days of PF, the cornified cells were greatly flattened, and the density of the cytoplasm was increased as compared to granular cells (Fig. 11). Cell membranes were also thickened (Fig. 9). The cytoplasm consisted of a dense fibrillar network. Occasionally, nuclear remnants and keratohyalin granules were seen in the first transitional cornified layer.

The parakeratinized layer cells from 9, 18 and 27 days of ZD tissues were less flattened than PF cells. The cytoplasm was less opaque. Only a few cornified cell layers (20-35%) became parakeratinized at 9 days,



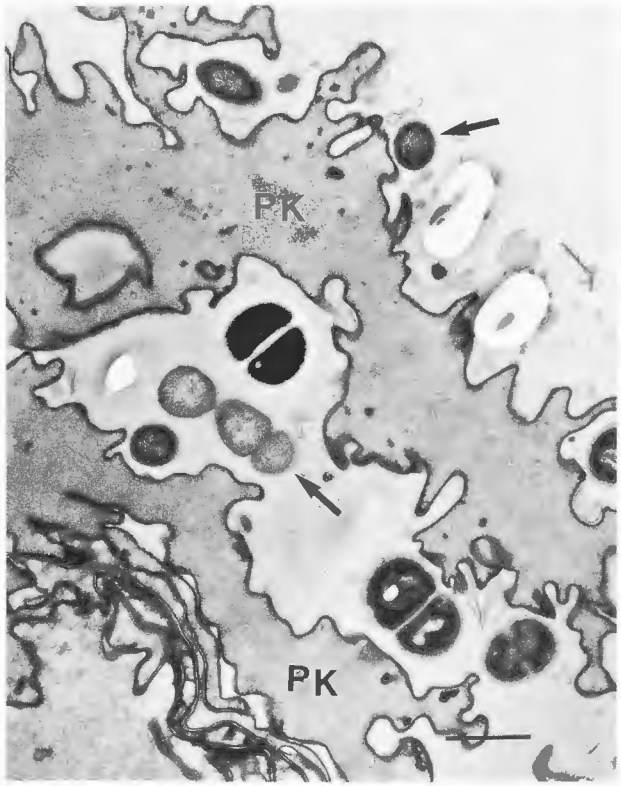


Figure 12 (at left). Parakeratinized stratum corneum in buccal epithelium after 27 days of zinc deficiency. Pyknotic nuclei are seen in cells of parakeratinized layer. Bar = 5 μm.

Figure 13 (at right). Eighteen day rat cheek epithelium in zinc deficiency showing cocci (arrows) on the surface and within the intercellular space between parakeratinized cells (PK). Bar = 1 μm.

Table 1. Sequence of changes in the distribution of MCGs in rat cheek epithelium during zinc deficiency.

Group	Days after start of experiment								
	N	C	9 Days	N	C	18 Days	N	C	27 Days
PF	5	25	10.5 ± 3.3	5	25	10.8 ± 3.6	6	25	16.8 ± 6.8
ZD	5	35	15.7 ± 4.1	5	35	17.8 ± 3.1*	6	35	31.8 ± 9.3†,‡

*Significantly different from PF **($p < 0.05$)**; †significantly different from PF **($p < 0.02$)**;

‡significantly different from zinc-deficient at 9 and 18 days **($p < 0.02$)**;

N = total number of animals; C = total number of cells in which MCG counting was performed.

and the majority of them were parakeratinized at 18 and 27 days of zinc deficiency (Fig. 12). Parakeratinized cells contained pyknotic nuclei after 9, 18 and 27 days of zinc deficiency. The chromatin was condensed along the inner aspect of the nuclear membrane. Dense packing of tonofilaments and lipid droplets was frequently seen. Intercellular spaces sometimes were filled with amorphous material. A small number of ribosomes and mitochondria were present (Fig. 10). MCGs were seen in the cytoplasm of the zinc-deficient parakeratinized epithelial cells and were not seen in controls.

The presence of numerous bacteria at the surface and within intercellular spaces of the superficial parakeratinized cells was noticed in 18 and 27 days zinc-deficient epithelium (Fig. 13).

Quantitative analysis (Table 1)

The number of MCG profiles in 9 days ZD appeared increased as compared with 9 day PF controls, but this increase was not statistically significant when compared with PF. The increase in number of MCG profiles in 18 and 27 days of ZD granular and parakeratinized cells combined as compared to 18 and 27 days PF was, however, statistically significant. Yet, the number of MCG profiles in the granular and parakeratinized cells in 27 day zinc-deficient epithelium was significantly increased ($p < 0.02$) over that in comparable 9, and 18 days cells (Table 1). The increase in MCG profiles can be due to an increased number of MCGs, an increased size of MCGs or both.

Discussion

The buccal epithelium of rats after 9, 18 and 27 days of zinc deficiency shows the features of hyperplasia (Giunta *et al.*, 1988; Hsu *et al.*, 1991). The time related ultrastructural changes occur as early as 9 days of zinc deficiency and increased at 18 and 27 days of zinc deficiency. The cheek epithelium of 9 day ZD rats shows sporadic parakeratosis (Hsu *et al.*, 1991). Disintegration of nuclei and cytoplasmic organelles takes place gradually in parakeratin cell layers of 9 day ZD rats and is decreased after 18 and 27 days of zinc deficiency.

Under normal circumstances as cells of stratified squamous epithelium move to the surface, MCGs are concentrated at the superficially oriented part of the cell, and then extrude their contents into the intercellular spaces (Ashrafi *et al.*, 1980). The extrusion products include acid hydrolytic enzymes, lipids and carbohydrate-containing material (Squier and Waterhouse, 1970; Weinstock and Wilgram, 1970; Menon *et al.*, 1992). Desquamation of superficial cells in stratified squamous epithelium is associated with the decomposition of desmosomes, which is effected, at least in part, by the acid hydrolytic enzymes (Waterhouse and Squier, 1966; Elias *et al.*, 1988). The time related increase in the concentration of intracellular MCGs (Table 1) is associated with the reported gradual increase in the thickness of the parakeratinized stratum corneum (Hsu *et al.*, 1991).

The presence of MCGs in parakeratinized layers indicates that they are not releasing their normal acid hydrolytic contents into the intercellular spaces to breakdown the desmosomal attachments. Therefore, accelerated passage of cells through the epithelium in zinc-deficient rat cheek mucosa (Alvares and Meyer, 1973) and the presence of MCGs in parakeratinized layers are contributing to the gradual increase in the thickness of parakeratin layers as the zinc deficiency period is increased.

An organ-specific sensitivity of many enzymes in zinc deficiency has been noticed (Vallee and Falchuk, 1993). Recently, Sondell *et al.* (1994) reported the presence of stratum corneum chymotryptic enzymes (SCCEs) in the uppermost granular cells, the level at which MCGs extrude their content into the intercellular spaces. SCCE is a prerequisite for the degradation of desmosomes leading to desquamation (Suzuki *et al.*, 1994). A decrease in the level of SCCE in the upper granular layer cells of ZD animals combined with the sporadic extrusion of MCG contents may be responsible for decreased desquamation (Menon *et al.*, 1992). Thus, reduced activity of these enzymes may result in abnormal desquamation and thickening of parakeratinized cell layer in zinc-deficient animals (Hsu *et al.*, 1991). Increased cell division and more rapid movement of cells towards the surface are features of ZD rat buccal epithelium (Alvares and Meyer, 1973).

The parakeratotic change could be due to a lack of time for hydrolysis of organelles to occur (Alvares and Meyer, 1973; Ashrafi *et al.*, 1980). In the hyperplastic buccal epithelium of ZD rats, there is a considerable decrease in the distribution of MCGs at the superficially oriented part of cells (Ashrafi *et al.*, 1980). The weakening mechanism of normal polarity may be one of the concomitant features of excessive cell proliferation. The increase in the number of keratohyalin granules (KHG) in the upper granular and lower parakeratin cells means that the heavily phosphorylated proteins did not undergo dephosphorylation to form filaggrin, a stratum corneum protein (Dale *et al.*, 1987). Filaggrin acts as a matrix and helps dense packing of keratin intermediate filaments into microfibrils. It may well be that parakeratin layers do not have enough stratum corneum protein to form a normal matrix as PF controls. Extrusion products of MCGs contribute to the permeability barrier in the upper layers of the cornified oral epithelium (Squier, 1991). The sporadic appearance of lamellated MCG material in the intercellular spaces may negatively influence the permeability barrier in zinc deficiency, as Healy *et al.* (1995) have reported that the presence of zinc protects the permeability of the oral mucosa.

It was concluded that epithelial changes taking place during zinc deficiency are time related. The increase in the number of MCGs, KHGs, nuclei and parakeratotic changes may be attributed to the decrease in the amount of intracellular hydrolytic zinc-metalloenzymes. The desquamation process does not accelerate to keep pace with accelerated cell proliferation during zinc deficiency.

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References

Alvares OF, Meyer J (1968) Regional differences in parakeratotic response to mild zinc deficiency. Arch Dermatol **98**: 191-201.

Alvares OF, Meyer J (1973) Thymidine uptake and cell migration in cheek epithelium of zinc-deficient rats. J Oral Pathol **2**: 86-94.

Ashrafi SH, Meyer J, Squier CA (1979) Quantitative study of microgranules in buccal mucosa of zinc-deficient rats. J Dent Res **58**: 418 (abstract).

Ashrafi SH, Meyer J, Squier CA (1980) Effects of zinc deficiency on the distribution of membrane-coating granules in rat buccal epithelium. J Invest Dermatol **74**: 425-432.

Dale BA, Gown AM, Fleckman P, Kimball JR, Resing KA (1987) Characterization of two monoclonal antibodies to human epidermal keratohyalin: Reactivity with filaggrin and related proteins. J Invest Dermatol **88**: 306-313.

Dixon W (1985) BMDP Statistical Software Manual. University of California Press, Berkeley, CA. pp. 345-427.

Elias PM, Menon GK, Grayson SG, Brown BE (1988) Membrane structural alterations in murine stratum corneum: Relationship to the localization of polar lipids and phospholipases. J Invest Dermatol **9**: 3-10.

Follis RA, Day HG, McCollum EV (1941) Histologic studies of the tissues of rats fed a diet extremely low in zinc. J Nutr **22**: 223-237.

Gerson SJ, Meyer L (1977) Increased lactic dehydrogenase activity in buccal epithelium of zinc-deficient rats. J Nutr **107**: 724-729.

Giunta JL, Hutchinson M, Wallwork JC (1988) Parakeratosis of the labial mucosa in zinc-deficient rats. J Oral Pathol **17**: 186-190.

Healy CM, Cruchley AT, Paterson MV, Thornhill LH, Williams DM (1995) Effect of SLS, zinc and triclosan on oral mucosa permeability. J Dental Res **74**: 476.

Hsu D, Meyer J, Gerson S (1991) Sequence of changes in rat buccal mucosa induced in zinc deficiency. J Oral Pathol Med **20**: 443-448.

Meyer J, Alvares OF (1974) Dry weight and size of cells in the buccal epithelium of zinc-deficient rats. A quantitative study. Arch Oral Biol **19**: 471-476.

Menon GK, Ghadially R, Williams ML, Elias PM (1992) Lamellar bodies as delivery systems of hydrolytic enzymes: Implications of normal and abnormal desquamation. Brit J Dermatol **126**: 337-345.

Osmanski CP, Meyer J (1969) Ultrastructural changes in buccal and palatal mucosa of zinc-deficient rats. J Invest Dermatol **53**: 14-28.

Prasad AS, Fitzgerald JT, Hess JW, Kaplan J, Pelen F, Dardenne M (1993) Zinc deficiency in elderly patients. Nutrition **9**: 218-224.

Sondell B, Thornell LE, Egelrud T (1994) Stratum corneum chymotryptic enzymes has a subcellular distribution compatible with a role in desquamation. J Invest Dermatol **103**: 405.

Squier CA (1991) The permeability of oral mucosa. Crit Rev Oral Biol Med **2**: 13-32.

Squier CA, Waterhouse JP (1970) Lysosomes in oral epithelium: The ultrastructural localization of acid phosphatase and non-specific esterase in keratinized oral epithelium in man and rat. Arch Oral Biol **15**: 153-168.

Suzuki Y, Nomura J, Koyama J, Hozii L (1994) The role of proteases in stratum corneum: Involvement in stratum corneum desquamation. Arch Dermatol Res **286**: 249-253.

Vallee BL, Falchuk KH (1993) The biochemical basis of zinc physiology. Physiol Rev **73**: 79-118.

Walsh CT, Sandstead HH, Prasad AS, Newberne PM, Fraker PJ (1992) Zinc: Health effects and research priorities for the 1990's. Environ Health Perspect **102**: 5-46.

Waterhouse JP, Squier CA (1966) Form and function of lysosomes in oral epithelium. Nature **211**: 420-421.

Weinstock M, Wilgram GF (1970) Fine-structural observations on the formation and enzymatic activity of keratinosomes in mouse tongue filiform papillae. J Ultrastruct Res **30**: 262-274.

Discussion with Reviewers

Reviewer III: How many layers were compared with each other?

Authors: The last four granular and first four parakeratinized layers of ZD were compared with the last four granular and first four cornified layers of PF for MCGs counts.

Reviewer III: How many layers were turned out to be parakeratinized?

Authors: Twenty to thirty-five percent cornified layers became parakeratinized in 9 days, 47-88% in 18 days and 75-98% in 27 days of zinc deficiency.

Reviewer III: Do you think sporadic appearance of MCG-lipids in the intercellular spaces after OsO_4 fixation is an evidence for the hypothetical discussed influence on the permeability barrier?
Authors: Our suggestion about the sporadic appearance of MCG extruded material in the intercellular spaces (ICS) influencing permeability barrier is based on the fact that a few MCGs were extruding their contents into ICS, and many of them were still present intracellularly. We agree with the reviewer that ruthenium tetraoxide fixation would be a better fixative to confirm our suggestion regarding zinc deficiency influencing permeability barrier.

B. Forslind: Though you refer to literature on epidermis, your discussion contains no critical assessment of differences occurring in oral epithelia as compared to epidermis in zinc deficiency.
Authors: The increase in thickness of the epithelium during zinc deficiency is similar to that of psoriatic epidermis; Hashimoto and Lever (1966) observed an abnormally large number of MCGs in psoriatic epidermis. Similarly, Werner *et al.* (1987) reported that the relative volume of MCGs in atopic dermatitis was significantly greater than in normal skin.

B. Forslind: In some instances, you have stored specimens overnight in buffer solutions after glutaraldehyde fixation. Have you assessed the protein extraction occurring due to this? To what extent does this procedure influence the size of the intercellular spaces?
Authors: Specimens used in this study were not stored overnight in a buffer. After glutaraldehyde fixation, they were simultaneously post-fixed in OsO_4 and immediately processed for electron microscopy. This procedure would have equally influenced the intercellular spaces in both cases (ZD and PF tissues).

W.H. Wilborn: Was there increase in the mitotic activity during zinc deficiency?
Authors: There was no significant difference in mitotic activity at 9 days of ZD and PF. At 18 days of zinc deficiency, it became twice as high as at 9 day ZD. It continued to increase from 9 to 18 to 27 days during zinc deficiency.

W.H. Wilborn: Did you consider that the quality of fixation and angle of sectioning could cause widened intercellular spaces?
Authors: The quality of fixation and angle of sectioning were maintained the same throughout the study. The coded electron micrographs were examined by three scientists. They confirmed the widened intercellular spaces in ZD as compared with PF at 9, 18 and 27 day zinc deficiency in the majority of pictures.

Additional References

Hashimoto K, Lever WF (1966) Elektronenmikroskopische Untersuchungen der Hautveränderungen bei Psoriasis (Electron microscopic investigations of the skin changes in psoriasis). Derm Wochenschr **152**: 713-722.

Werner Y, Lindberg M, Forslind B (1987) Membrane-coating granules in "dry" non-eczematous skin of patients with atopic dermatitis. Acta Derm Venereol (Stockholm) **67**: 385-390.