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HISTOGENESIS OF EXPERIMENTAL CUTANEOUS CALCINOSIS*

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Experimental models of disease, as produced in animals, greatly facilitate the analysis of the factors that participate in the genesis of the morbid lesions seen in the clinical counterpart; they can also serve as convenient test objects for potential therapeutic procedures. This thought guided our earlier studies on the experimental production of nephrosclerosis, periarteritis nodosa, standardized inflammatory responses ("topical irritation arthritis", "granuloma pouch"), anaphylactoid inflammation, and stress-induced peptic ulcers or cardiac necroses [1—3].

There are few corresponding experimental models of dermatoses. The reactivity of the human skin differs so essentially from that of most animals that it is difficult to establish conditions under which the latter would react like the former. Besides, few animal experimenters are sufficiently familiar with the problems of practical dermatology and, conversely, few dermatologists are well versed in the techniques of experimentation on animals.

These were the considerations that led us to attach enough importance to observations on the experimental induction of cutaneous calcinosis to present them here, as a token of our admiration for the great Hungarian experimental morphologist, Professor IMRE TÖRŐ, whose jubilee this volume celebrates.

Soon after the discovery of vitamin D, one of us (while still a medical student) undertook systematic studies on the structural changes induced by excessive amounts of irradiated ergosterol. It was found that, in adult rats, this preparation produced widespread calcification in various tissues (particularly the cardiovascular system, kidneys, lungs, and intestine), but only minor changes in the skeleton. However, the young of rats so treated during pregnancy or lactation responded to vitamin D (received through the milk or placenta) with an altogether different syndrome. In them, soft-tissue calcification was absent or negligible, but the bones became so brittle that multiple spontaneous fractures occurred; simultaneously, the skin lost its elasticity and adhered to

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the subjacent tissues, so that the young rats became "hidebound" [4, 5]. Since these newborn animals also suffered from malnutrition and dehydration, we attached little importance to the skin lesions at that time and devoted our interest more to the skeletal changes.

A little later, we observed a patient in whom multiple organ calcifications — very similar to those induced in adult rats with vitamin D — developed presumably as a result of excessive endogenous parathyroid hormone secretion. As this man had a parathyroid adenoma, nephritis, and colitis, we thought that the parathyroid stimulation might be due to an adaptive hormonal reaction, an attempt to rectify calcium and phosphate metabolism when the organs normally concerned with the absorption and excretion of these materials are largely incapacitated. This interpretation could not be proven and other possibilities also had to be considered, but we thought our case might throw a new light upon VIRCHOW's classical concept of "metastatic calcification" in the presence of severe renal disease. In uremia, calcification may be largely mediated through the parathyroids as a consequence of a partly pathogenic adaptive reaction, that is, what we would now call a "disease of adaptation" [6].

Subsequent experiments showed that, in the rat, parathyroid hormone can produce a cutaneous calcinosis with sclerosis resembling a certain type of clinical scleroderma, the so-called "sclérodermie calcaire" [7]. This condition can also be produced in the same species with dihydrotachysterol (DHT), a vitamin-D derivative that closely imitates the actions of parathyroid hormone [8]. However, whether produced by parathyroid hormone or by DHT, these cutaneous lesions are not practical models of disease; they are accompanied by high mortality, develop only on the scalp and neck (not wherever the experimenter wants to induce the lesions), and occur only during the first few days of life, and even at that, irregularly. Still, these observations were repeatedly confirmed by others and eventually induced LERICHE and co-workers [9—11] to remove the parathyroids in patients with severe scleroderma. The results of this operation were rather inconstant, but in some cases, marked improvement did occur.

In view of what we have since learned about the importance of "conditioning factors" in the determination of adaptive hormonal reactions [1, 12], it seemed that further work on this experimental simile of calcareous scleroderma would be rewarding if the technique could be perfected. Continuation of this work revealed that topical trauma can cause local calcification in the internal organs of DHT-treated rats [13]. Clinical scleroderma also tends to develop at sites of local injury [14]. Therefore, we were particularly interested to note that cutaneous calcinosis with sclerosis can be produced at will in predetermined skin regions, even in adult rats, if the selected area is lightly traumatized (*e. g.*, by epilation) at a critical time of DHT-treatment (usually the fourth day) [15].

The present communication reports detailed studies on the histogenesis of this peculiar experimental dermatosis.

Materials and techniques

Twenty-four female Sprague-Dawley rats, with a mean initial body weight of 100 g (range: 96—104 g), were given 200 μg of DHT ("Calcamin," Wander) in 0.5 ml of corn oil by stomach tube, once daily, on four consecutive days. On the fourth day, a dual type of trauma was produced in each of these animals by epilation and mechanical compression of the skin. Epilation was performed by plucking the hair from an area of the back approximately 60 mm in length (craniocaudally) and 30 mm in width. The remaining dorsal hair was then shaved with electric clippers and compression trauma was applied by pinching the skin with a hemostat (at right angles to the longitudinal axis of the animal) twice on the epilated area and once below it on the shaved surface. These two types of trauma were applied because preliminary experiments had shown that, in DHT-sensitized animals, cutaneous calcinosis is most readily obtained by mild irritation (*e. g.*, epilation or rubbing with a hard brush), while strong trauma (*e. g.* prolonged squeezing with a hemostat or intracutaneous injection of strong irritants) is ineffective locally, although it may elicit a halo of calcinosis in the immediately surrounding skin area (Fig. 1).

For control purposes, similar trauma was applied in a series of rats not previously sensitized by DHT. However, in these, calcification never occurred and the reaction of the skin was limited to mild desquamation in the region injured with the hemostat.

Throughout the experiment all animals received only "Purina Fox Chow" (Ralston Purina Co., Ltd.) and tap water. For histologic study, three of the DHT-treated rats were killed immediately after trauma (on the fourth day of DHT treatment) and three more 2, 4, 6, 8, 10, 15, and 20 days later, respectively. Immediately after autopsy, one-half of the traumatized region was fixed in alcohol-formol (80% alcohol and 20% of the standard [10%] neutral formalin solution), which is particularly suitable for demonstrating traces of tissue calcium [16]. The other half of each tissue specimen was fixed in Susa solution saturated with picric acid, a fluid we found to be particularly suitable for connective-tissue studies, especially as related to the skin.

The material was subsequently embedded in paraffin and sectioned at 5 μ. Normally, rat skin so treated is very brittle and difficult to cut, but excellent thin sections can be obtained if the paraffin blocks are first cooled by contact with solid blocks of carbon dioxide snow.

Eleven staining techniques were used: (1) hematoxylin-phloxine, (2) PAS, (3) Van Gieson, (4) phosphotungstic acid fibrin, (5) fuchsin, (6) cresyl violet, (7) elastica, (8) mucicarmine, (9) von Kóssa, (10) celestin blue, (11) osmic acid. For the first seven of these techniques, we used material fixed in either alcohol-formol or Susa-picric, but only alcohol-formol-fixed slices were stained with mucicarmine (for the demonstration of mucoid material), von Kóssa, or celestin blue (for the demonstration of calcium). Osmication was employed for the detection of fats.

Most of these are standard techniques, so we may limit ourselves here to a description of the two procedures that were specially developed for this study.

Celestin-blue staining technique (for calcium). — (1) Fix for 48 hours in alcohol-formol solution; (2) Embed in paraffin; (3) Bring sections to water, as usual; (4) Mordant for 30 minutes in following solution: 90 ml aluminium-potassium sulfate 1% + 10 ml of 1/100 dilution of ammonium hydroxide 28%; (5) Rinse in running water, 2 minutes; (6) Stain 30 minutes in following solution, prepared on day of use: celestin blue B 0.02% in alcohol 50%; (7) Rinse in running water; (8) Counterstain lightly in phloxine or eosin; (9) Dehydrate and mount.

Fuchsin staining technique (for skin). — (1) Fix for 24 hours in Susa saturated with picric acid; (2) Embed in paraffin; (3) Deparaffinize and treat sections with iodine and thiosulfate in the usual manner; (4) Oxidize, 1 minute, in permanganate 0.25% + sulfuric acid 0.5%; (5) Rinse in running water; (6) One minute in oxalic acid 3%; (7) Rinse in running water, 10 minutes; (8) Fifteen seconds in alcohol 70%; (9) Stain (length of time depending upon age of solution: 10 minutes in 3—4 days-old solution) in Gomori's aldehyde fuchsin until elastic fibers stand out clearly; (10) Rinse in 3 changes of alcohol 70%; (11) Rinse in running water, 5 minutes; (12) Five minutes in 1 gm Wright's prepared powder in 2000 ml distilled water; (13) Rinse in running water, 1 minute; (14) Fifteen minutes in phosphotungstic acid 1%; (15) Rinse in running water, 1 minute; (16) Thirty minutes in following solution: 90 ml distilled water + 8 ml acid fuchsin 1% + 2 ml oxalic acid 1%; (17) Rinse rapidly in

one change of distilled water and 2 changes of absolute alcohol; (18) Fourty minutes in a saturated solution of saffron in absolute alcohol; (19) Rinse rapidly in absolute alcohol and in 3 changes of xylol, and mount.

Results

All the DHT-treated rats developed more or less intense cutaneous calcinosis at the site of trauma. The lesions were essentially of the same type throughout, so in our description we need not discuss the response to epilation and to compression, separately. It is noteworthy, however, that only epilation gave pronounced and constant results. Where epilated skin was compressed, a band corresponding to the thickness of the hemostat remained protected in the otherwise massively calcified patch. On the other hand, the same degree of skin compression, when applied to shaved skin, actually produced calcification. Apparently, the mild damage caused by epilation alone or pinching alone elicits calcium deposition, while the stronger injury that results from the combination of these agents inhibits it.

This interpretation was confirmed by a number of additional experiments (not described here in detail), in which we produced various degrees of skin trauma by applying the hemostat for varying lengths of time or by intracutaneously injecting varying concentrations of chemical irritants. It was found, for example, that if the hemostat is applied for more than 20 seconds to the shaved, not epilated, skin of a DHT-sensitized animal, the directly traumatized area does not calcify, but a halo of cutaneous calcinosis appears around it. If, on the other hand, a 10% aqueous solution of egg-yolk is injected intracutaneously, calcification does not appear in the central area (which was presumably exposed to the greatest amount of damage), but a ring of cutaneous calcinosis appears around the margin of the treated patch, so that a regular, circinate lesion is formed (Fig. 1).

For the sake of simplicity, we shall limit our description to changes observed in merely epilated skin regions as they appear when the most instructive staining techniques are employed. The first obvious signs of calcinosis were seen in skin specimens taken on the second and fourth days after trauma. Here, there was edema of the cutis and subcutis, with only very occasional inflammatory cells, but fine calcium deposits could be detected around the dermal collagen and elastic fibers with the von Kóssa and celestin blue stains (Figs. 2, 3 and 12). In other cases — and sometimes in other regions of the same skin specimen — the calcification commenced in the hair follicles, leaving the connective-tissue elements intact (Figs. 4 and 5). These differences in the early distribution of the calcium deposits may depend upon accidental variations in the intensity of the trauma inflicted on diverse structures.

There were some individual variations in the speed with which the skin

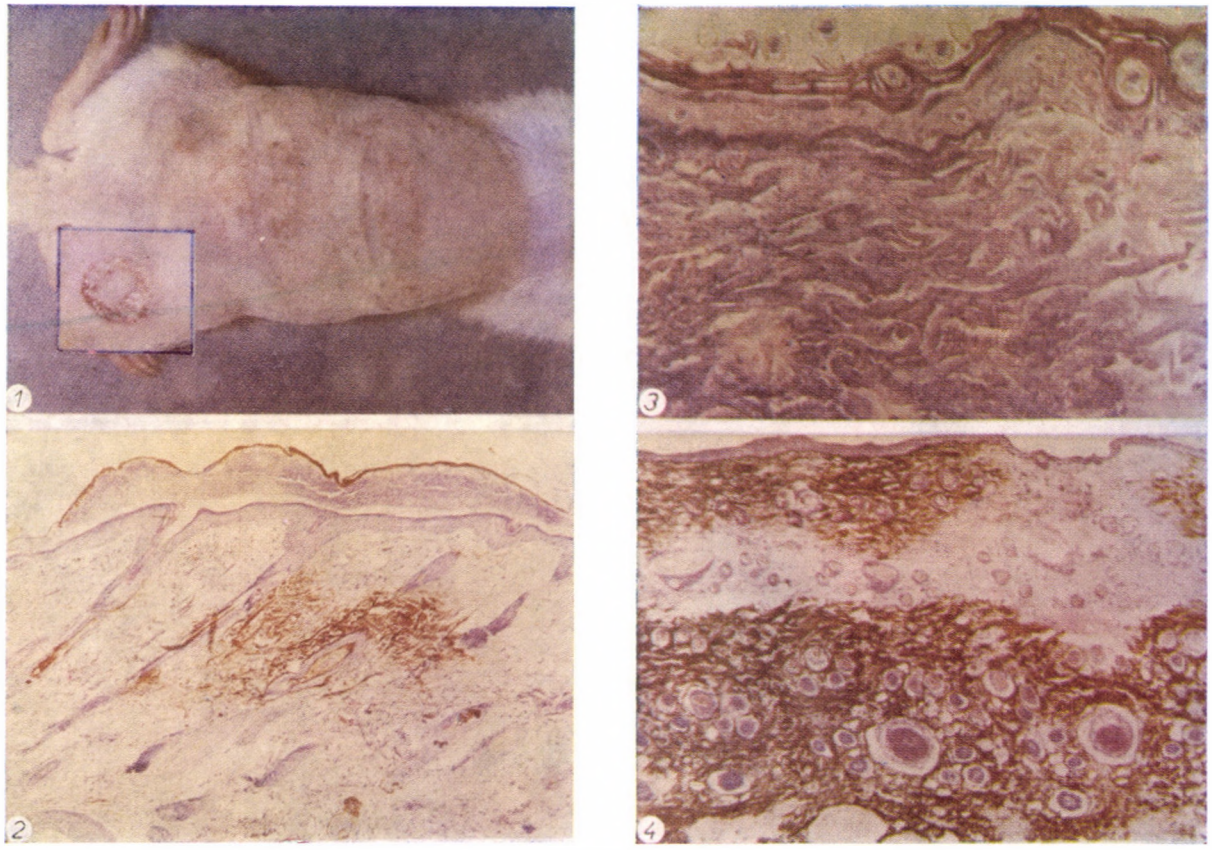

Fig. 1. — Halo of cutaneous calcinosis surrounding each of three linear hemostat pinches. The directly traumatized area itself is not affected. *Insert in lower left corner:* Circinate exulcerating cutaneous calcinosis, in another rat, on the margin of a skin patch infiltrated with 10% egg-yolk

Fig. 2. — Beginning calcification of slightly swollen dermal collagen fibres. Pronounced parakeratosis with calcification in the most superficial layer of the exfoliating skin (von Kóssa, ×110)

Fig. 3. — Beginning calcification of dermal collagen fibres. The celestine blue stain clearly shows that, in addition to diffuse calcium deposition, regular, round or oval granules of lime salts appear within the collagen fibres. Additional calcium deposits are seen along the basement membrane of the epidermis (Celestine blue, ×490)

Fig. 4. — Calcification of the dermal connective tissue is very pronounced and reaches deep into the derma, but avoids the hair follicles (von Kóssa, ×110)

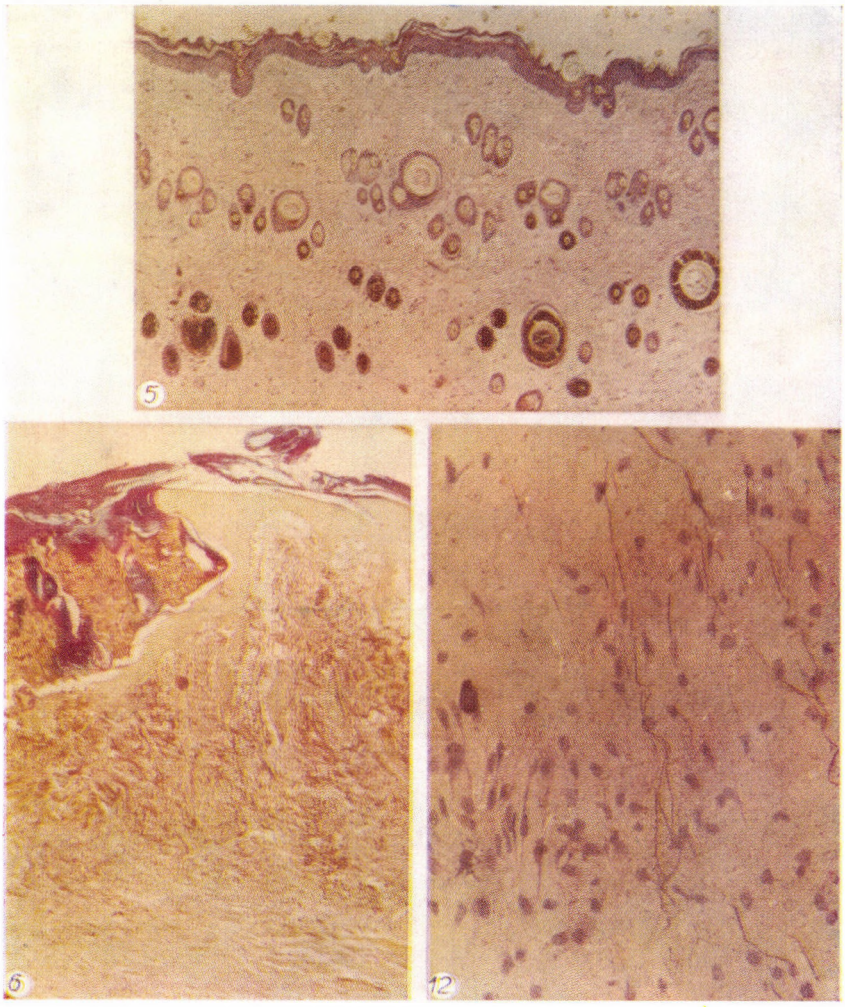

Fig. 5. — Skin region adjacent to that shown in Fig. 4. Here, there is selective calcification of the hair follicles, while the connective tissue is spared (von Kóssa, ×110)

Fig. 6. — Advanced exulceration with beginning epithelialization of the wound surface. The normal collagen fibers (center) stain red; the swollen, degenerating fibers (edge of the field) take on a yellowish tinge (Fuchsin stain, ×110)

Fig. 12. — One normal and one disintegrating mastocyte near lower edge of the field. Mastocyte granules are also diffusely distributed throughout the tissue in this region in which the elastic fibers have undergone selective calcification. The mastocyte granules (particularly in the mastocytes themselves) stain distinctly purple with the hematoxylin-phloxine counterstain. The mast cells and granules have been identified on adjacent sections by their metachromatic staining with cresyl violet and the elastic fibers by the phosphotungstic-elastica stain (von Kóssa, ×490)

lesions developed, but the changes just mentioned were fully apparent by the fourth day and gave way to more severe lesions between the sixth and fifteenth days. By then, the collagen fibers began to swell and often formed closely packed, solid masses within the derma; at the same time, their tinctorial properties changed. This was particularly obvious on sections stained with our fuchsin stain, which colors normal collagen fibers a bright red, while the affected swollen collagen takes on a yellowish tinge (Fig. 6). At the same time, the elastic fibers of the cutis become fragmented and eventually disappear, while large masses of calcium are being taken up by the damaged tissue.

This process progresses and eventually leads to complete necrosis in the more seriously affected regions, which subsequently exulcerate. Over the least markedly affected skin regions, there is merely intense desquamation with parakeratosis and hyperkeratosis, but when exulceration occurs, the epithelium tends to creep under the necrotic skin regions, so as to cover the underlying healthy derma (Fig. 6). If necrosis progresses farther into the deeper strata of the cutis, then on the surface, the epithelium grows down along the edge of the ulcer and tends to turn around following the borderline between the healthy and necrotic tissue. Thereby apparently, the epidermis attempts to cover the undersurface of the still healthy, superficial skin layer. Consequently, the strata of the newly-formed epithelium are reversed; the stratum malpighii is closest to the surface and in contact with the still healthy, superficial part of the derma, which, on the outside, is also covered by the original epidermis (Fig. 7). This tendency for the epidermis to creep down along the newly-created wound surfaces sometimes results in highly irregular, almost neoplasm-like, epithelial formations. However, these changes occur only where the dermal lesions exulcerate and, even there, they disappear after the necrotic parts are eliminated.

At the height of its development, cutaneous calcification may affect all layers of the derma and even result in the formation of large subcutaneous calcareous masses (Figs. 8 and 9). These resemble the calcium deposits seen in certain types of calcareous scleroderma or exulcerating acrosclerosis.

By the 15th or 20th day, all the heavily calcified, superficial regions are usually extruded, while isolated, deep deposits of calcium tend to become surrounded by a granuloma, which often contains foreign-body giant cells and gradually absorbs the mineral deposits. During this final stage of healing, there is intense proliferation of fibroblasts and connective-tissue fibers. The surface epithelium is greatly thickened and desquamating, but the cutaneous appendages are slow to regenerate. Hence, at this time, the skin consists of a thick epidermis and an underlying, rather homogeneous, dense layer of connective tissue, almost totally devoid of fat cells, hair, and sebaceous glands (even normal rat skin contains no sweat glands); cutaneous papillae are flattened or absent (Figs. 10 and 11).

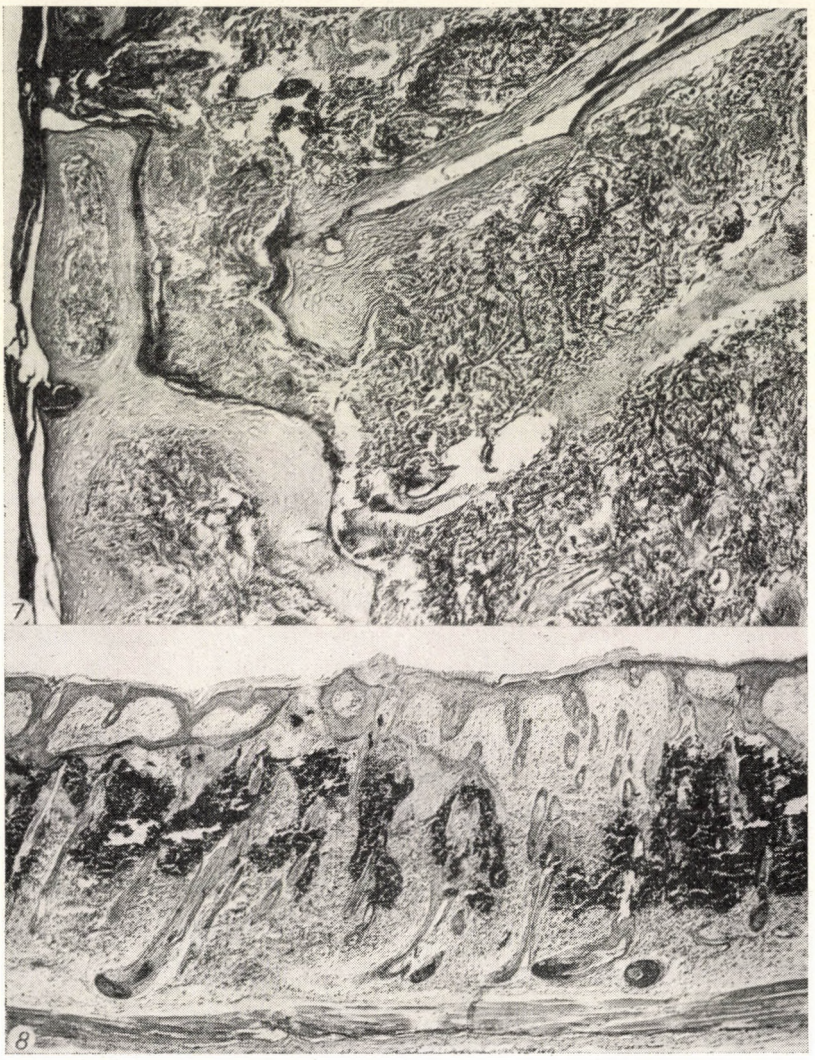

Fig. 7. — Here, the necrosis progresses farther in depth than in surface. The epidermis turns around the edge of the ulcer following the borderline between healthy (superficial) and necrotic (deep) tissue; consequently, the strata of the newly formed epithelium are reversed (Fuchsin stain, ×110)

Fig. 8. — Intense cutaneous calcification affects all layers. The process of "reverse epithelialization" is especially obvious (von Kóssa, ×30)

In comparing the experimental dermatosis with clinical scleroderma, it is important to bear in mind that, in man as in the rat, there may be calcification without sclerosis and *vice versa*. In the rat, the lesion roughly evolves in three stages: (1) edema with slight inflammatory in-

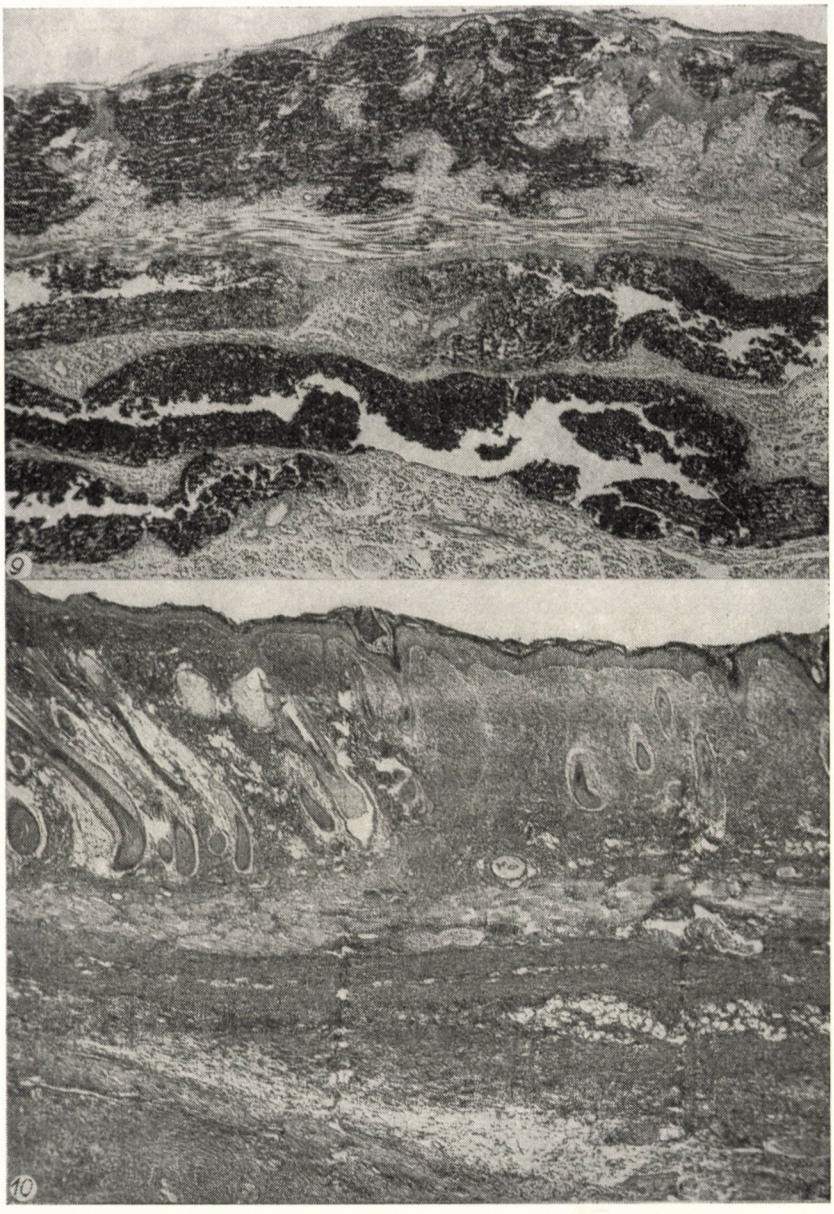

Fig. 9. — Extensive subcutaneous calcareous masses, separated from a calcified dermal patch by the skin muscle layer (von Kóssa, ×30)

Fig. 10. — Borderline between healthy and sclerosed skin during period of healing. In the affected region (left), there is dense connective tissue, sebaceous glands are absent, hair follicles rare, and the epidermis is thickened (Fuchsin stain, ×30)

filtration, (2) calcification and (3) sclerosis. Essentially, calcification appears to be an intermediate stage between the original tissue damage and the eventual healing. It remains to be seen whether minute calcium precipitates — histologically detectable only under perfect conditions of fixation and staining — or even still slighter increases in skin calcium that could only be revealed by chemical analysis, play a part in stimulating connective-tissue proliferation. Theoretically, this may be the case, not

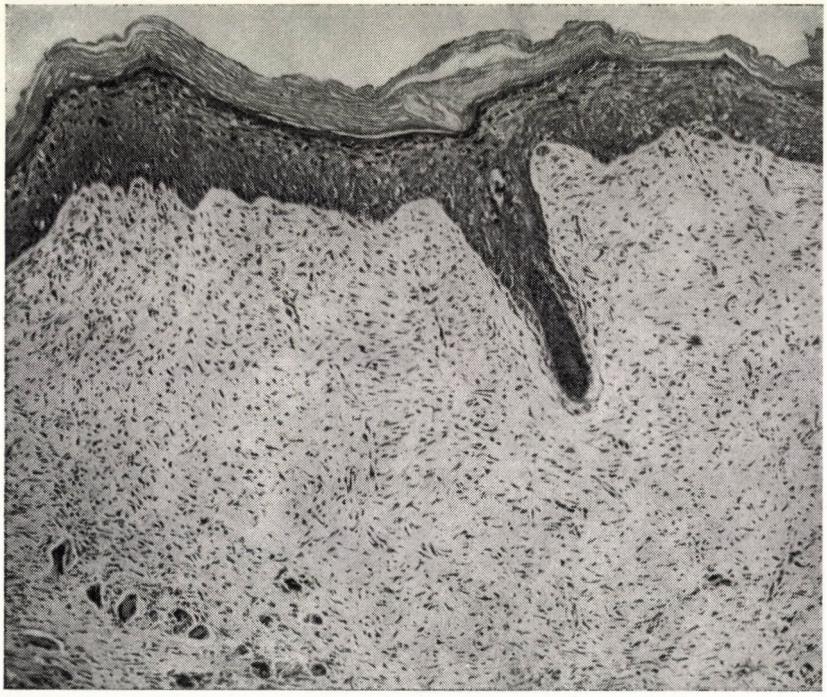

Fig. 11. — Calcium is no longer visible in the dense connective tissue of this healing patch. Only a few remaining calcified granules can be detected in the foreign-body giant cells near the lower left corner (von Kóssa, ×110)

only in scleroderma but also in other types of collagen diseases, particularly in systemic lupus erythematosus and dermatomyositis, where even grossly visible calcifications of the skin do occasionally occur.

Sections stained with hematoxylin-phloxine, PAS, Van Gieson and the elastica stain merely confirmed the previously mentioned findings. The fibrin stain colors only the calcified and necrotic areas intensely, while cresyl violet frequently reveals large numbers of often disintegrating mastocytes and free mastocyte granules in the vicinity of the damaged skin regions. Curiously, here

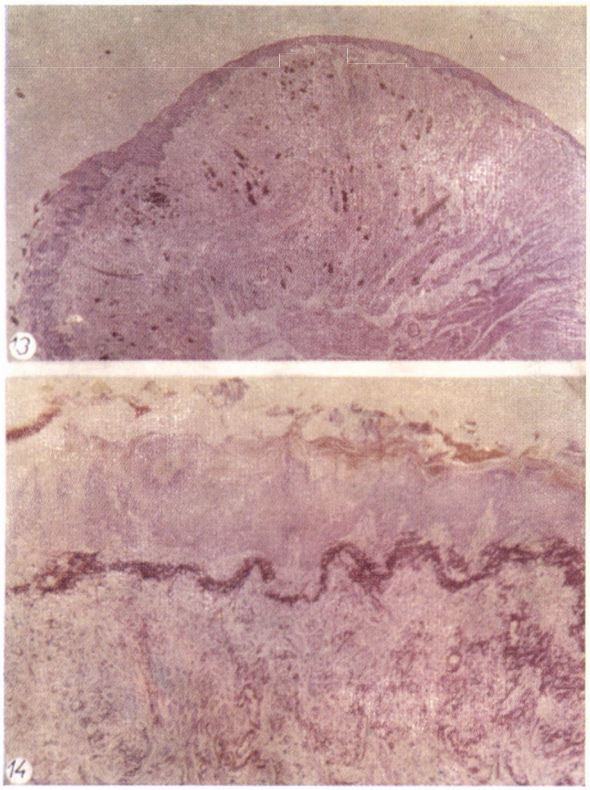

Fig. 13. — Typical lingual lesion. Some of the muscle cells are calcified, others involuted; there is marked connective-tissue proliferation and the lingual papillae are flattened (von Kóssa, ×30)

Fig. 14. — Lingual lesion in which calcification occurs predominantly underneath the epithelial surface, but to some extent, also in the proliferating intermuscular connective-tissue stroma (von Kóssa, ×110)

the mastocytes and the free mastocyte granules appear to stain slightly metachromatically even with hematoxylin (Fig. 12). Mucicarmine-tingible material was found particularly in severely damaged or necrotic skin regions. It will be recalled that mucoid substances are also found in many cases of clinical scleroderma. Osmication revealed no evidence of an initial fat deposition, preparatory to calcification.

Of all our techniques, the fuchsin stain proved to be the most informative: with it, the normal collagen fibers stain bright red (as with Van Gieson) and in addition, initial stages of degeneration show up in them by a change of the color towards yellow. Elastic fibers are stained as clearly with the elastica stain and mast cells are also quite evident. Incidentally, the technique gives excellent pictures of cross-striation in the cutaneous striated musculature.

The demonstration of calcium with the celestin blue technique deserves particular mention, since this stain does not tend to "bleed" into surrounding tissues and, hence, gives a much more precise definition than the von Kőssa stain. It is curious, however, that only the very recent calcium deposits and the borderline that delimits the calcified from normal tissue are tingible with celestine blue. Perhaps this dye is indicative, not of calcium itself, but of some chemical phenomena involved in the process of incipient calcification.

Perhaps the most noteworthy extracutaneous lesion in our rats was the rather frequent occurrence of calcification with sclerosis (and sometimes necrosis) along the lateral margins of the tongue and more or less diffusely in other parts of the skeletal musculature (Figs. 13 and 14). It remains to be seen whether there is any relationship between this change and the well-known predisposition for muscular lesions in patients with dermatomyositis and scleroderma.

Summary

In the rat, marked cutaneous calcinosis with sclerosis can be produced by topical skin trauma, following sensitization with dihydrotachysterol (DHT). The histogenesis of this lesion and its possible relationship to certain collagen diseases, especially the calcifying type of scleroderma, are discussed.

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ГИСТОГЕНЕЗ ЭКСПЕРИМЕНТАЛЬНОГО КАЛЬЦИНОЗА КОЖИ

Г. ШЕЛЬЕ и К. НИЛСЕН

При помощи кожной травмы можно на крысах после сенсибилизации дигидротахистеролом (ДГТ) вызвать значительный кальциноз кожи и склероз. Обсуждается гистогенез вызванного таким путем повреждения и его предположительная связь с определенными коллагенными заболеваниями, в частности с обызвествляющимся типом типом склеродермим.

HISTOGENESE DER EXPERIMENTELLEN HAUTKALZINOSE

H. SELYE und K. NIELSEN

An Ratten wurde nach Sensibilisierung mit Dihydrotachysterol mit Hilfe von lokaler Hautschädigung bedeutende Hautkalzinose und Sklerose hervorgerufen. Die Histogenese der auf diese Weise zustandegekommenen Hautschädigung und deren vermutlicher Zusammenhang mit gewissen Kollagenerkrankungen, insbesondere mit dem verkalkenden Typ der Sklerodermie werden erörtert.

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