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The Effect of Papain on Epiphysial Cartilage in Rachitic Rats: Histologic, Autoradiographic and Microradiographic Studies

By

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With 4 Figures in the Text

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Papain has been found to have a very swift and powerful effect on epiphysial cartilage in growing animals of various species (HULTH 1958; HULTH and WESTERBORN 1959a; ENGFELDT, HULTH and WESTERBORN 1959; ENGFELDT and WESTERBORN 1960a; WESTERBORN 1961). A single dose of papain, injected intravenously, produces histologically detectable changes within ten minutes. The lesions conform to a specific pattern, a reparative stage beginning after approximately 24 hours and the cartilage showing a normal organization after 15—30 days. Various experimental studies employing histologic, autoradiographic, microradiographic and biochemical methods have demonstrated that papain releases chondroitin sulphate from cartilage matrix and concomitantly damages the cartilage cells. The effect of repeated injections of papain has also been studied: such treatment results in permanent lesions of the growth zones of long bones, sometimes followed by premature ossification of these zones and dwarfism (HULTH and WESTERBORN 1959b).

As regards rickets the lesions in the growth zones of the long bones occupy the forefront of interest. Although numerous workers have devoted attention to these pathologic changes, employing various morphologic, biophysical and biochemical methods, the mechanism of origin of the different lesions in the growth zones has not yet been fully elucidated (for references *vide* ENGFELDT and ZETTERSTRÖM 1955; FOLLIS 1958; BALL 1960; ENGFELDT and HJERTQUIST 1960).

The aim of the present investigation has been to study rachitic growth zones, utilizing knowledge of the action of papain upon normal epiphysial cartilage. The effects of both single and multiple doses of papain in rachitic rats were investigated by histologic, autoradiographic and microradiographic methods.

Material and Methods.

The experimental animals were rats six to seven weeks old. Some were normal and others had rickets induced by a rachitogenic diet *ad modum* BRUNIUS (1961) or HJERTQUIST (1961a), initiated at the age of three weeks.

Two different types of experiments were conducted, one type involving single doses of papain and the other repeated doses.

I. Rats Treated with a Single Injection of Papain

A 1 per cent solution of crude papain in NaCl was administered intraperitoneally in a single dose equivalent to 6,5 mg per rat. Radioactive sulphate (SJS2, Radiochemical Centre, Amersham, England) was injected intraperitoneally in a dose of 1 mC per rat.

A total of nine normal and 15 rachitic rats were injected with both papain and radiosulphate as shown in the Table. The controls comprised one group of three normal rats and another group of six rats with florid rachitis. These animals were injected solely with radiosulphate as described above and as in the papain-treated rats collected in the Table.

Table. *Relative times, in hours before death, for administration of papain and of radiosulphate*

	Experiment no.								
	1	2	3	4	5	6	7	8	9
A	6	6	6	48	48	48	168	168	168
B	2	24	168	2	24	168	2	24	168

A = number of hours before death for injection of papain.
B = number of hours before death for injection of radiosulphate.

The animals were killed with ether. Immediately after death the tibiae were dissected out and their proximal halves fixed in 10 per cent neutral formalin. After fixation the majority of specimens were decalcified for 24—48 hours in a solution of equal parts of 20 per cent formic acid and 44 per cent monosodium citrate, and then embedded in paraffin. A few specimens were similarly embedded without prior decalcification. Sections 5 micra thick were taken from each specimen and used for histologic or autoradiographic study. For histologic examination the sections were stained with hematoxylin and eosin or with azure A (KRAMER and WINDRUM 1955). The stripping film technique and Kodak Scientific Plates AR 10 were used in the autoradiographic studies.

II. Rats Treated with Repeated Injections of Papain

Ten of 52 rachitic rats were injected subcutaneously with 10 mg, 5 mg or 2.5 mg papain daily or every second day over the course of seven days. Twenty-four of the 52 received, in addition to this treatment, 3000 I.U. vitamin D by gastric tube either on the same day or the day following initiation of the papain injections. As from the time of vitamin D medication some of these animals were placed on a diet with a normal Ca/P ratio and adequate amount of vitamin D, and others continued on the original rachitogenic diet (with a high Ca/P ratio), which was supplemented by codliver oil in an amount of 10 per cent. Ten of the remaining rachitic animals received no papain injections but were otherwise treated in the same way as the above-mentioned 24 with papain and vitamin D. (In the following, the designation "vitamin D plus rachitogenic diet" signifies those rats which, prior to death, received vitamin D by gastric tube and were placed on a rachitogenic diet supplemented by vitamin D; the designation "vitamin D plus normal diet" refers to those which, before death, received vitamin D by gastric tube and were placed on a diet with a normal Ca/P ratio and an adequate amount of vitamin D.) The remaining eight rats with florid rachitis served as controls. Each of the 52 animals was killed on the eight day after initiation of the papain injections. One tibia from each rat was decalcified; the other tibiae were not. Sections stained with hematoxylin and eosin were then examined histologically as described above. For microradiographic examination of non-decalcified sections 5 micra thick a technique and an apparatus reported by ENGSTRÖM, LUNDBERG and BERGENDAHL (1957) were employed. In this way it was possible to identify sites of mineral salts in the tissues.

Results

I. Effect of a Single Injection of Papain

Histologic findings. The growth zones in the normally fed controls were, morphologically quite normal.

The histologic examinations of epiphysial cartilage from the normally fed rats which had received a single injection of papain yielded results consistent with those previously reported for rabbits (WESTERBORN 1961). Six hours after

the papain injection the basophilic reaction of cartilage matrix from the normal rats was either greatly diminished or absent. The cartilage cells were swollen, disintegrating, and less basophilic than normal, and those in some areas contained pyknotic nuclei. Forty-eight hours after the papain injection the cells, cell capsules, and adjacent parts of cartilage matrix in the proliferative zone had commenced to regain their basophilic characteristics. Seven days after the injection the cartilage matrix was once again basophilic except in the resting cartilage zone. The basophilic reaction had returned in the chondrocytes which, in general, showed a relatively normal appearance.

Those rats which were on a rachitogenic diet and had not received papain exhibited the classical picture of florid rickets, with thickened epiphysial plates and an abnormal number of hypertrophic cells. Some of these cells contained several vacuoles of varying size. Others consisted of shrunken cell remnants in otherwise empty cell lacunae, while yet others contained a large light-staining nucleus and minute streaks of cytoplasm. Certain cell lacunae were quite empty. The cartilage matrix showed largely similar tingibility in the proliferative and the hypertrophic zones. In some areas of cartilage, at the border of coarse bone trabeculae clothed with osteoid, were observed chondrocytes resembling those which DODDS and CAMERON (1939) term "rejuvenated cells". The metaphysis had an abundance of osteoid (Fig. 1 a).

In the rachitic animals which had received a single injection of papain the epiphysial cartilage matrix showed, six hours after the injection, conspicuously attenuated basophilic and metachromatic reactions. The cells in the resting cartilage zone and the proliferative zone were vacuolated and the cytoplasm was less basophilic and metachromatic than normal. Some of the nuclei were pyknotic. The cytoplasm in the hypertrophic zone exhibited granular degeneration and the nuclei were either pyknotic or large and light-staining. Numerous cell lacunae contained only the shrunken remains of a cell. In the metaphysis most of the cartilage matrix residue that was enclosed in bone and osteoid was basophilic (Fig. 1 b).

Forty-eight hours after the papain injection the rachitic animals exhibited a thickened epiphysial cartilage in which the matrix of the proliferative zone and an adjacent part of the hypertrophic zone had regained its basophilic reaction. The chondrocytes in the proliferative zone were basophilic and distinctly swollen, and their cytoplasm somewhat vacuolated. Large, light-staining cells with granular cytoplasm and large, slightly basophilic nuclei were observed in that part of the hypertrophic zone which adjoined the proliferative zone and had a basophilic matrix. In other parts of the hypertrophic zone the cells were either quite empty or contained only a markedly pyknotic nucleus (Fig. 1 c).

Seven days after papain administration the matrix in certain parts of the thickened cartilage was basophilic and metachromatic down to the metaphysis. In other areas, however, particularly where the cartilage was very thick, the matrix of a juxta-metaphysial area of the hypertrophic zone was neither basophilic nor metachromatic. The same was true of the cartilage tongues projecting into the metaphysis. The basophilic portion of the cartilage was now thicker than that observed two days after papain injection. The cells of the proliferative zone again manifested basophilic and metachromatic reactions, and had regained

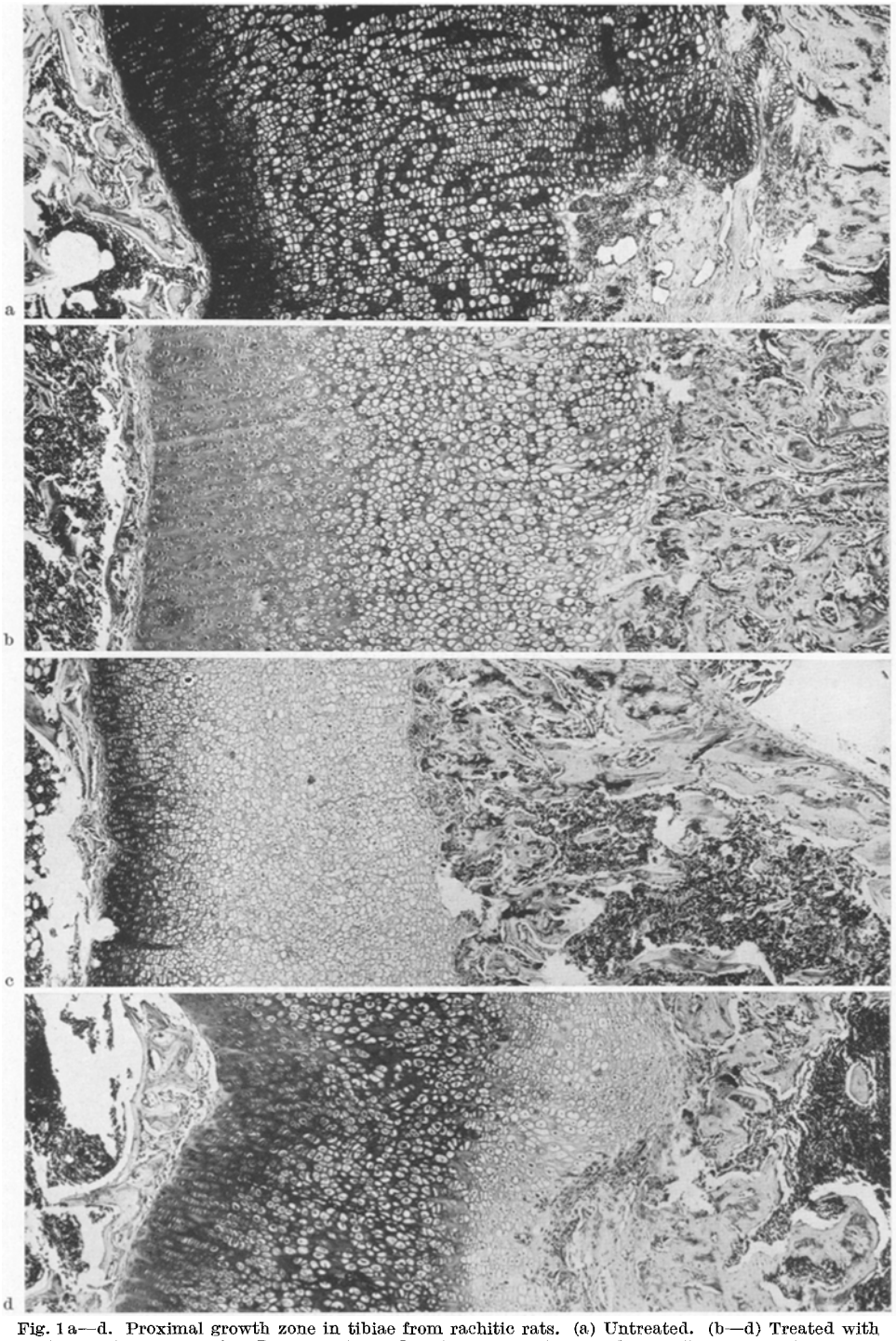


Fig. 1 a—d. Proximal growth zone in tibiae from rachitic rats. (a) Untreated. (b—d) Treated with single injection of papain. Hematoxylin and eosin. 43 ×. a Normal basophilic reaction throughout the epiphysial cartilage. b Six hours after papain: Very slight basophilic reaction of the entire cartilage zone. c Forty-eight hours after papain: Strongly basophilic reaction in the proliferative zone and the adjoining part of the hypertrophic cartilage. d Seven days after papain: Basophilic reaction pronounced in upper part of the epiphysial cartilage but very slight in the juxta-metaphysial part

their normal appearance. In the basophilic portion of the hypertrophic zone there were now "normal" rachitic hypertrophic cells, many of them containing small vacuoles without detectable papain damage. Many of the cell lacunae in the non-basophilic and non-metachromatic area of hypertrophic cartilage were flattened. The septa frequently showed folds. Some of the cell lacunae here contained cells with granular cytoplasm and pyknotic nuclei; others pyknotic nuclei alone. Many lacunae, moreover, were devoid of cellular contents (Fig. 1 d).

At each of the periodic examinations following injection of papain the cartilage was observed to be invaded by vessels and mesenchymal cells from the metaphysis precisely as in the rachitic controls.

Autoradiographic Findings. Both the normal controls and the controls with florid rickets exhibited, two hours after injection of radiosulphate, a primarily intracellular uptake of S^{35}; after 24 hours, labelling chiefly of the cell peripheries and/or of matrix adjoining the cartilage cells; and after seven days a largely extracellular labelling of the cartilage. Normally the entire epiphysial cartilage was labelled two and 24 hours after the injection, but in florid rickets there was, in general, little or no labelling of a juxta-metaphysial part of the hypertrophic zone (*cf.* HJERTQUIST 1961 a).

The autoradiographic findings in epiphysial cartilage from normal rats which had received radiosulphate and papain at varying times before death accorded with those previously reported for rabbits (ENGFELDT and WESTERBORN 1960 a).

Autoradiographic studies of papain-treated rachitic rats gave the following results:

Animals Killed Six Hours after Papain Injection. Two hours after radiosulphate injection (experiment 1) an abnormal diffuse uptake of S^{35} was found in the proliferative zone and a neighboring part of the hypertrophic zone, in conformity with the results for non-rachitic animals. As a rule no labelling was discernible in a juxta-metaphysial area of the hypertrophic zone (Fig. 2 a).

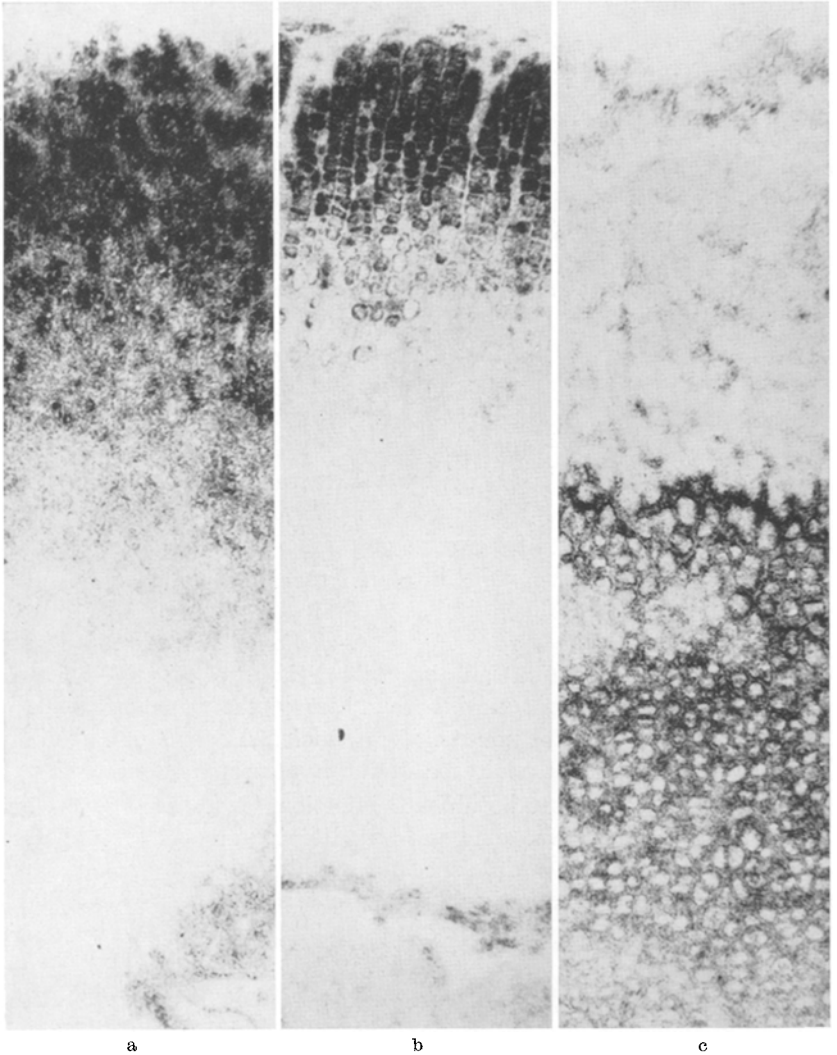
Twenty-four hours after radiosulphate injection (experiment 2) the resting cartilage zone, the proliferative zone and an adjoining area of the hypertrophic zone were diffusely labelled. Certain specimens exhibited, in a juxta-metaphysial part of the hypertrophic zone, either a very slight, diffuse uptake of S^{35} or none at all. Others showed relatively heavy, diffuse labelling of cells and matrix as far as the metaphysis.

Seven days after radiosulphate injection (experiment 3) the autoradiograms revealed diffuse labelling of the proliferative zone and a largely extracellular uptake in the hypertrophic zone. In some areas the uptake extended to the metaphysis; in others there persisted a narrow unlabelled zone of hypertrophic cartilage bordering on the metaphysis.

Animals Killed Forty-eight Hours after Papain Injection. Two hours after radiosulphate injection (experiment 4) the proliferative zone and the adjoining, basophilic portion of the hypertrophic zone showed labelling which was principally intracellular. In other parts of the cartilage, except for occasional "rejuvenated cells" bordering on the metaphysis, no uptake of S^{35} was detectable (Fig. 2 b).

Twenty-four hours after administration of radiosulphate (experiment 5) the S^{35} uptake was for the most part extracellular, though intracellular labelling was

also noted. Here and there the labelling extended as far as the metaphysis, though in certain areas a juxta-metaphysial part of the hypertrophic zone was unlabelled. The general uptake pattern was something intermediate between



a b c

Fig. 2a—c. Proximal growth zone in tibiae from rachitic rats treated with single injections of papain and of radiosulphate. Autoradiograms 80 ×. a Six hours after papain; two hours after radiosulphate. Diffuse uptake of S^{35} in the proliferative zone and adjoining part of the hypertrophic zone. b Forty-eight hours after papain; two hours after radiosulphate. Largely intracellular labelling of the proliferative zone and a contiguous part of the hypertrophic zone. Intracellular uptake by occasional chondrocytes near the borders of bone trabeculae. c Seven days after papain; seven days after radiosulphate. Fairly weak, largely extracellular labelling of the proliferative zone and an adjacent part of the hypertrophic zone. (This part of the cartilage was basophilic in histologic sections.) Heavier labelling of a subjacent portion of the hypertrophic cartilage (non-basophilic)

the patterns usually observed after, respectively, 24 hours and three days in a rat with florid rickets.

Seven days after the S^{35} injection (experiment 6) the autoradiograms revealed slight, chiefly extracellular labelling of the resting cartilage and proliferative

zone as well as of the basophilic and the non-basophilic portions of the hypertrophic zone. Greatly thickened parts of the epiphysial cartilage exhibited, in some places, a narrow unlabelled nonbasophilic zone next to the metaphysis.

Animals Killed Seven Days after Papain Injection. Two hours after radiosulphate administration (experiment 7) the thickened cartilage showed labelling identical to that commonly observed two hours after injection of radiosulphate in a rat with florid rickets alone. No S^{35} uptake was discernible in that area of hypertrophic cartilage which had lost its basophilic reaction.

Twenty-four hours after the radiosulphate injection (experiment 8) the uptake pattern accorded with that generally observed after a corresponding period in rats with florid rachitis alone. There was no labelling of non-basophilic areas near the metaphysis, but at the juncture with the metaphysis a heavy uptake was noted at the peripheries of "rejuvenated cells" and/or in the contiguous matrix.

Seven days after administration of radiosulphate (experiment 9) slight, somewhat diffuse though largely extracellular labelling of the proliferative zone and an adjoining, basophilic area of the hypertrophic cartilage was manifest. A nonbasophilic juxta-metaphysial part of the hypertrophic zone showed a heavier mostly extracellular uptake of S^{35}. In this non-basophilic region the labelling extended, in certain places, as far as the boundary between cartilage on the one side and connective-tissue cells, osteoblasts, vessels and osteoid on the other. Some areas of hypertrophic cartilage near this border, however, showed no appreciable uptake (Fig. 2c).

II. Effect of Multiple Papain Injection

Histologic Findings. Those controls which had received a rachitogenic diet but were otherwise untreated had growth zones similar to those found in florid rickets as described above (Fig. 3a).

Some of the non-papain-treated rachitic animals which, before death, had received vitamin D plus a rachitogenic diet showed incipient healing manifested in capillary and mesenchymal cell invasion of lateral areas of conspicuously thickened epiphysial cartilage. Other growth zones in similarly treated rats were also thicker than normal, but capillaries and mesenchymal cells had invaded the cartilage, cell column by cell column, along the whole of its junction with the metaphysis. In the metaphysis were observed slender, new-formed bone trabeculae with residue of cartilage matrix. — Those non-papain-treated rachitic rats which had received vitamin D plus a normal diet before death, showed even more advanced healing, with only slightly thickened cartilage, or complete healing, with cartilage of normal thickness (Fig. 3b).

In the rachitic animals treated with repeated papain injections but no vitaminD the rachitic growth zones had been severely damaged by papain. The greater part of the cartilaginous zone was thicker than normal, though in general not so thick as that observed in the non-papain-treated rats with florid rickets. The epiphysial cartilage consisted, in part, of short columns of rather large, vacuolated proliferative cells, some of them basophilic, and in part of severely damaged hypertrophic cells. The cartilage matrix of the proliferative zone was slightly basophilic, while that of the hypertrophic zone showed no basophilic reaction.

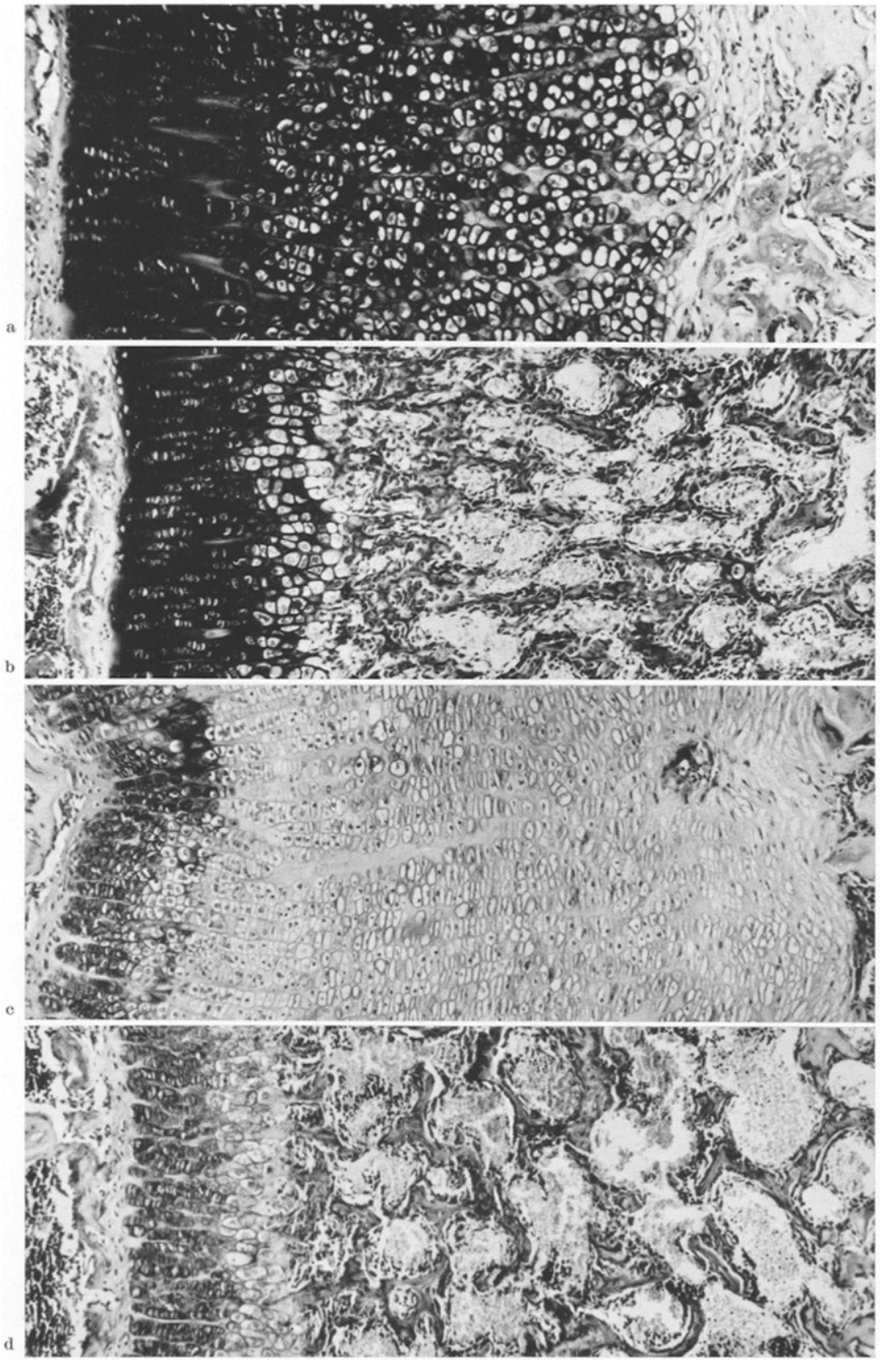


Fig. 3 a—d

The cartilage was of normal thickness in certain small areas of the growth zone, where it had been invaded from the metaphysis by vessels and mesenchymal cells which were destroying both chondrocytes and cartilage matrix. In no specimen was there any detectable healing in the form of normally invading capillaries — one to each cell column (Fig. 3c).

In general there were no manifest histologic differences between the growth zones of the rachitic rats which had received papain and vitamin D plus a rachitogenic diet before death, and those of the animals treated solely with papain. In a few specimens, however, mineralized bone trabeculae were observed between vessels which in some places had invaded the cartilage.

The papain-treated rachitic rats which had received vitamin D plus a normal diet shortly before death had papain-damaged growth zones, which, however, usually showed signs of healing. The bulk of the cartilage was of normal or even less than normal thickness. Here and there, however, cartilage tongues projected into the metaphysis. As regards tingibility and cellular picture the cartilage exhibited characteristics similar to those described above for rachitic cartilage treated solely with papain. The epiphysial cartilage as well as cartilage islets in the metaphysis had usually been intensively invaded by capillaries and mesenchymal cells, though not as regularly as in normal healing of rickets. In certain parts of the metaphysis somewhat irregular, new-formed bone trabeculae containing non-basophilic remains of cartilage matrix were visible, and incipient mineralization of the osteoid formed under the rachitogenic diet could be discerned (Fig. 3d).

Microradiographic Findings. Rats which had been on a rachitogenic diet but were otherwise untreated showed growth zone mineralization patterns similar to those in rats which had received an unfortified rachitogenic feed plus repeated papain injections. The thickened cartilage usually exhibited no mineral salt deposits, and osteoid was abundant in the metaphysis as well as in the compact bone tissue (Fig. 4a).

Those rachitic rats which, before death, had received vitamin D plus a rachitogenic diet, or vitamin D plus a normal diet, showed somewhat varying mineralization patterns. In the individual animals the pattern conformed to that usually observed in rachitic growth zones when healing is advanced or complete (HJERTQUIST 1961 b), Mineralization was initiated in the hypertrophic cartilage at its junction with the metaphysis, and subsequently extended well into the cartilage in advance of the invading capillaries (Fig. 4b).

In those papain-treated rachitic animals which had also received vitamin D before death the mineralization pattern of the growth zones differed somewhat from that found in animals treated with vitamin D but not with papain. The rats which had received vitamin D plus a rachitogenic diet before death showed

Fig. 3a—d. Proximal growth zone in tibiae from rachitic rats. Hematoxylin and eosin 90 ×. a Untreated. Normal basophilic reaction throughout the cartilage zone. b Vitamin D plus normal diet for seven days before death. Normal growth zone with regular capillary invasion. Healed rickets. c Papain daily for seven days before death. Basophilic reaction of the cells and cell capsules in the proliferative zone; remainder of the cartilage non-basophilic. Capillary invasion of the cartilage as in (a). d Papain daily and vitamin D plus normal diet for seven days before death. Cartilage zone of normal thickness. Cells and cell capsules in the proliferative zone are basophilic; the hypertrophic cartilage shows no basophilic reaction. Fairly irregular capillary invasion of the cartilage; coarse, irregular bone trabeculae in the metaphysis

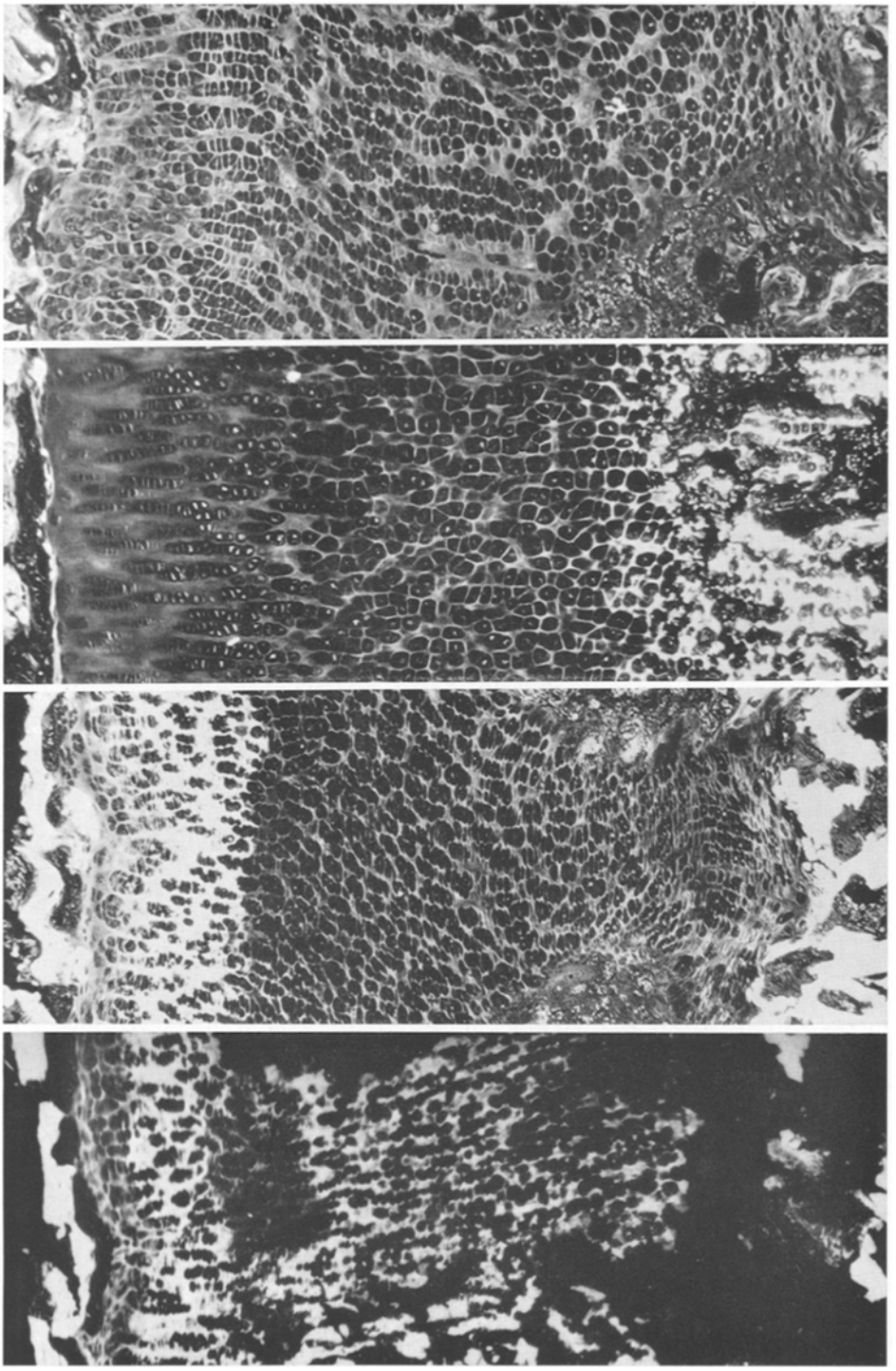


Fig. 4a—d. Proximal growth zone in tibiae from rachitic rats. Microradiograms, 8—20 Å, of non-decalcified sections. Mineralized areas have a high roentgen-ray absorption and are white. 85 ×. a One papain injection daily for seven days. No mineralization of the cartilage zone. b Vitamin D

mineralization of the longitudinal cartilage matrix in an area at the junction of the proliferative zone and the severely damaged hypertrophic zone. Other parts of the hypertrophic cartilage were not mineralized (Fig. 4c).

Microradiograms of the growth zones in papain-treated rachitic rats which had received vitamin D plus a normal diet before death revealed, in cartilage areas of normal or less than normal thickness, mineralization of the longitudinal septa between the hypertrophic cells as well as somewhat irregular invasion by capillaries and mesenchymal cells. In the metaphysis below these areas were observed fairly irregular mineralized bone trabeculae. The thickened areas of epiphysial cartilage which occurred here and there were either completely or as described above for the papain-treated rachitic rats given vitamin D plus rachitogenic diet partially mineralized. Fully mineralized, thickened cartilage invariably showed intensive though somewhat irregular invasion by vessels and mesenchymal cells, but in the non-mineralized areas there was no demonstrably increased resorption (Fig. 4d).

Discussion

The histologic and autoradiographic studies on the effect of a single dose of papain in normal rats demonstrated that the observed lesions are identical with those previously reported for growing rabbits.

Histologic examination six hours after single administration of papain in rachitic rats showed that the enzyme had produced, in both the cells and the matrix of the epiphysial cartilage, morphologic changes similar to those observed in normal animals. The lesions involved the whole of the rachitic epiphysial cartilage irrespective of thickness. The general development of these lesions largely coincided with that in non-rachitic animals. Six hours after papain injection the cartilage cells were manifestly damaged and the matrix had lost its basophilic and metachromatic staining reactions. Forty-eight hours after the injection the cells and cartilage matrix in the proliferative zone and the adjoining portion of the hypertrophic zone had regained their normal tingibility, and the cells appeared to be active. After the lapse of seven days the cartilage in the proliferative zone and the bulk of the hypertrophic zone exhibited the usual rachitic characteristics. After forty-eight hours and, in some instances, even after seven days a non-basophilic, non-metachromatic zone of hypertrophic cartilage with damaged cells was still present near the metaphysis.

Previous investigations indicate that in papain experiments conducted as above, loss and reappearance of the basophilic and metachromatic reactions signify, respectively, loss and re-formation of chondroitin sulphate. It has also been shown that chondroitin sulphate is synthesized in the cartilage cells, then delivered to the matrix (Belanger 1954; Engfeldt and Westerborn 1960b; Hjertquist 1961a; Westerborn 1961). The mechanism underlying the above-mentioned phenomena in rachitic cartilage may well involve gradual resumption

plus rachitogenic diet for seven days before death. Mineralization of cartilage matrix adjoining the metaphysis, and of new-formed bone trabeculae. Regular capillary invasion of the cartilage. c Papain daily and vitamin D plus rachitogenic diet for seven days before death. Mineralization of cartilage matrix in the proliferative zone and a contiguous part of the hypertrophic zone. d Papain daily and vitamin D plus normal diet for seven days before death. Mineralization of lower portion of the proliferative zone and of the hypertrophic zone. Note the mineralized cartilage tongue and mineralized bone trabeculae in the metaphysis

of chondroitin sulphate production by the cartilage cells in the proliferative zone and perhaps by certain initially damaged hypertrophic cells adjacent to that zone as well as restoration of proliferation. Concomitantly there is some resorption of damaged cartilage from the metaphysis. In contrast to the behavior in normal animals, not all of the damaged hypertrophic cells have time to become fully resorbed, since, in the first place, the cartilage is greatly thickened and, secondly, resorption of rachitic cartilage may be comparatively inhibited.

Evaluation of the autoradiographic findings is based on earlier conclusions that the S^{35} uptake by cartilage following radiosulphate administration reflects the distribution and metabolism of chondroitin sulphate (DZIEWIATKOWSKI 1951a, b, 1952; BOSTRÖM 1952, 1953; BELANGER 1954; ENGFELDT and WESTERBORN 1960b; HJERTQUIST 1961a). The present autoradiographic results indicate that rachitic cartilage reacts to papain principally in the same way as does epiphysial cartilage in normal animals. Six hours after a papain injection there is cell damage and probably a change in permeability (experiment 1), but after a day or so recovery of the cell function is observed in the proliferative zone and parts of the hypertrophic cartilage (experiments 4, 5, 7 and 8). During the course of the experiment much of the hypertrophic cartilage seems incapable of taking up S^{35}. This observation, like the histologic findings, lends credence to the view that the relevant cells are permanently damaged.

Investigations employing both histologic and autoradiographic techniques thus show that after papain injection rachitic epiphysial cartilage undergoes changes similar on the whole to those in normal animals: release of chondroitin sulphate from the cartilage matrix, cell damage, reduced or arrested synthesis of chondroitin sulphate and, after a day or so, transition to a reparative stage.

These results suggest that insofar as the above-discussed functions are concerned, the proliferative zone and the adjoining portion of the hypertrophic cartilage do not differ fundamentally from normal epiphysial cartilage.

The cause of the deficient calcification of epiphysial cartilage has been discussed (*vide* ENGFELDT and ZETTERSTRÖM 1955; FOLLIS 1958; BALL 1960; ENGFELDT and HJERTQUIST 1960). It has been questioned whether the arrested mineralization might not be due in part to local changes in the cartilage structure and function and whether, accordingly, rachitic cartilage might not differ from normal epiphysial cartilage in other respects than in its defective mineralization and its abundance of hypertrophic cells. HJERTQUIST (1961a) has shown that in the juxta-metaphysial part of the hypertrophic zone there is a defect of the chondroitin sulphate production, which, however, does not appear to constitute a fundamental cause of the arrested mineralization. The present investigation has demonstrated that this juxta-metaphysial area of the cartilage reacts to papain injection. In this particular respect, therefore, it does not differ from the corresponding area in normal animals.

In the other section of the investigation rachitic rats were given repeated injections of papain and, in some instances, vitamin D plus a rachitogenic diet or vitamin D plus a normal diet before death.

No healing phenomena were observed in the rachitic animals which had received papain but not vitamin D.

All rachitic rats in which vitamin D but not papain had been administered showed healing phenomena, and in some of those which had been transferred to a normal diet the rachitic lesions healed. Microradiographic examination revealed a zone of calcified matrix in the cartilage bordering upon the metaphysis.

Those rats which had received papain and vitamin D plus a rachitogenic diet before death, had an area of mineralized cartilage matrix at the junction of proliferative and hypertrophic cartilage inside the cartilaginous zone. This mineralization of the growth zone did not extend to the metaphysis. The papain-treated rachitic rats which had been given vitamin D plus a normal diet, showed, in some areas, a mineralization pattern similar to that last mentioned. In other areas, however, these animals showed mineralization throughout the hypertrophic cartilage and even quite deep into the proliferative zone.

These observations suggest that mineralization of the cartilage matrix may occur if the blood has a sufficiently high content of calcium, phosphate and vitamin D, even when there are no blood vessels in the immediate neighborhood. Certain authors, however, consider the presence of such vessels to be essential (TRUETA and AMATO 1960).

The calcified areas observed in the matrix of severely damaged hypertrophic cartilage which apparently had a greatly reduced chondroitin sulphate content, suggest that matrix calcification may still occur when the chondroitin sulphate content is not normal and damaged hypertrophic cells are present.

The mechanism of origin of rachitic thickening of epiphysial cartilage has frequently been discussed (for references *vide* FOLLIS 1958; BALL 1960; ENGFELDT and HJERTQUIST 1960). SHOHL and WOLBACH (1936) and WOLBACH and BESSEY (1942) have expressed the view that the hypertrophic cartilage cells in rickets fail to complete their cytomorphosis and do not degenerate sufficiently. Thus no empty or almost empty cell lacunae arise. This accounts for the failure of capillaries to invade the cartilage and for the thickening of the epiphysial cartilage. In the opinion of Park (1938/39) the thickening is primarily due to the arrested mineralization, and the capillaries are prevented from absorbing the cartilage cells partially because of mechanical factors. In subsequent papers PARK (1954a and b) seems inclined to emphasize the importance of completion of cytomorphosis.

The present histologic and autoradiographic studies have shown that both after single and after multiple doses of papain large juxta-metaphysial areas contain damaged hypertrophic cells and even quite empty cell lacunae. This notwithstanding, there is only sparse capillary invasion of the rachitic cartilage.

From these results it is plausible to assume that cell degeneration associated with empty or almost empty cell lacunae does not in itself have the significance which SHOHL and WOLBACH (1936) and WOLBACH and BESSEY (1942) have imputed to it.

In this investigation one group of rachitic animals received, before death, repeated papain injections as well as vitamin D plus a rachitogenic diet, and another group of papain-treated rachitic rats were put on vitamin D plus a normal diet.

In the experiments in which papain-treated rachitic animals received vitamin D plus a rachitogenic diet, a zone of non-calcified cartilage was noted in the vicinity

of the metaphysis. There was neither increased resorption of cartilage nor demonstrably increased capillary invasion. — When, on the other hand, papain-treated rachitic rats had been put on vitamin D plus a normal diet the greater part of the cartilage was observed to be of normal or less than normal thickness, and cartilage tongues occasionally projected into the metaphysis. In these animals the cartilage showed, for the most part, intensive though somewhat irregular invasion by capillaries and mesenchymal cells. Such areas of cartilage were seen, on microradiograms, to be mineralized as far as the metaphysis. These findings, like previously reported results of microradiographic examination (HJERTQUIST 1961 b), indicate that mineralization of the epiphysial cartilage is a determinant factor in resorption of the latter.

Summary

Epiphysial cartilage from rachitic and that from nonrachitic rats showed largely similar reactions to single administration of papain, as was demonstrated both histologically and by autoradiographic examination following injection of radiosulphate.

Rachitic rats which had received multiple injections of papain exhibited severe, perhaps irreversible lesions of the epiphysial cartilage zones. Healing of rickets, was not observed in rats which had received vitamin D and a rachitogenic diet in conjunction with papain. Intensive capillary invasion of the epiphysial cartilage was noted, however, in rats which had been transferred to a normal diet plus vitamin D in conjunction with papain administration.

Rachitic rats treated solely with papain showed no mineralization in the cartilage zone. In those rats which received vitamin D and a continued rachitogenic diet, on the other hand, mineralization was regularly observed in that part of the cartilage zone which comprised both proliferative cells and neighboring hypertrophic cells; the remainder of the hypertrophic cartilage was not mineralized. The animals which had been transferred to a normal diet supplemented by vitamin D showed, in some areas, a mineralization pattern similar to that, described above. In other areas, mineralization was observed throughout the hypertrophic cartilage and even quite deep into the proliferative zone.

Mineralization of rachitic cartilage may occur despite a greatly reduced chondroitin sulphate content and the presence of severely damaged hypertrophic cells. The findings suggest that mineralization of the epiphysial cartilage is a determinant factor in its resorption.

Zusammenfassung

Der Epiphysenknorpel von experimentell rachitisch gemachten und nichtrachitischen Ratten zeigte eine weitgehend ähnliche Reaktion gegenüber einer einmaligen Injektion von Papain, und zwar sowohl histologisch als auch bei autoradiographischer Untersuchung nach Injektion von S^{35} markiertem Sulfat.

Jene rachitischen Ratten, die wiederholte Injektionen von Papain erhalten hatten, zeigten schwere, ja vielleicht irreversible Veränderungen des Epiphysenknorpels. Eine Heilung der Rachitis wurde nicht beobachtet bei Ratten, die Vitamin D und eine Rachitis-Diät zusammen mit Papain erhalten hatten; jedoch wurde eine intensive Invasion des Epiphysenknorpels durch Capillaren festgestellt bei

Ratten, die danach auf eine normale Diät gesetzt wurden bei gleichzeitigen Vitamin D- und Papain-Gaben.

Die nur mit Papain behandelten Ratten zeigen keine Mineralisierung der Knorpelzone. Andererseits zeigten Tiere, die außer mit Vitamin D eine rachitische Diät erhalten hatten, regelmäßig eine Mineralisierung derjenigen Knorpelzonen, welche die proliferierenden Zellen und die benachbarten hypertrophischen Zellen umfaßten; der übrige hypertrophische Knorpel war nicht mineralisiert. Diejenigen Tiere, die dann auf normale Diät und Vitamin D gesetzt wurden, zeigten in manchen Gebieten eine Mineralisierung, entsprechend der oben erwähnten. In anderen Gebieten konnte eine Mineralisierung über den ganzen hypertrophischen Knorpel und sogar in der Proliferationszone beobachtet werden.

Die Mineralisierung des rachitischen Knorpels findet trotz eines stark verringerten Gehaltes von Chondroitinsulfat und in dem Vorhandensein von schwergeschädigten hypertrophischen Knorpelzellen statt. Die Befunde lassen annehmen, daß die Mineralisierung einen bestimmenden Faktor für die Resorption des Epiphysenknorpels darstellt.

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