EARLY CHANGES AT THE EPiphySIS OF RACHITIC CHICKS, FOLLOWING ADMINISTRATION OF VITAMIN D

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According to the classical concept expressed by Follis (1), the initial change in the process of repair of the rachitic lesion of bone consists in “the deposition of lime salts in the cartilage adjacent to the rachitic metaphysis.” A time lapse of 24 to 48 hours between the administration of vitamin D and the deposition of minerals has been reported by investigators who utilized histological methods for the recognition of salts (2, 3). Cohn and Greenberg (4) and also Morgareidge and Manly (5) have reported an increase in P32 uptake of rachitic bone, 54 to 72 hours after administration of vitamin D. Recent experiments by Dziewiatkowski (6) have revealed a time interval of 36 to 48 hours between the administration of vitamin D and the deposition of P32 in the femurs and tibiae of young rats. On the other hand, Ramalingaswami et al. (7) have recently studied the organic matrix of rat cartilage, noting changes in the glycogen metabolism, alkaline phosphatase, and some “nucleo-protein-like” material. Dziewiatkowski (6) and Carlsson (8) have also recently recognized that the uptake of S35 by cartilage was increased when the sulfur tracer was injected as early as 6 hours after administration of vitamin D. Furthermore, Dziewiatkowski has discovered that when S35-sulfate was given to rachitic rats without vitamin D, the rate of synthesis of chondroitin sulfate was comparable to that of animals treated at the same time with vitamin D. He postulated that vitamin D must stimulate the utilization of chondroitin sulfate so that the increased synthesis in the presence of this vitamin would be secondary to the stepped-up utilization.

These observations as well as the generally recognized delayed mineralization indicate that a series of events must take place in the growth centers of bones as part of the healing process previous to the appearance of calcification. The present study of normal and healing rachitic chicks has brought forward results which tend to reinforce this opinion and increase our understanding of the factors involved.

Materials and Methods

Animals.—At different times, two series of 15 and 18 one day old chicks were placed on A.O.A.C. (9) rachitogenic diet for 3 weeks. The first group were then given an oral dose of 10,000 units of vitamin D3; the second group received 5,000 units. Three chicks were sacrificed
from each group after intervals of 2, 6, 17, 24, and 48 hours, after vitamin D administration. In addition, three chicks were placed on vitamin D containing diet (100 units per cent for 1 week prior to sacrifice) and were killed along with the 48 hour animals, to serve as positive controls. Three non-treated rachitic birds were also sacrificed along with the second series, to serve as negative controls.

Histology.—The tibia along with the lower extremity of the femur was excised, roughly dissected, and placed in a mixture of 3 parts 95 per cent ethanol to 1 part 40 per cent neutral formaldehyde solution for 48 hours. These tissues were then demineralized in a mixture of 5 parts nitric acid to 95 parts 70 per cent alcohol for 1 week. The bones were subsequently split lengthwise and embedded in paraffin. Ten micra sections were stained with hematoxylin and eosin (H.E.), hematoxylin-phloxine B and orange (H.P.O.), toluidine blue, or by the McManus-Hotchkiss periodic acid–Schiff technique (P.A.S. (10)). Two sections from each specimen were incinerated by the technique of Policard-Scott (11) and subsequently studied by darkfield microscopy.

Ca\(^{45}\) in Vitro Uptake and Autoradiography.—Three sections from each specimen were de-paraffinized and soaked for 1 hour at room temperature in a weak solution of Ca\(^{45}\)Cl\(_2\) in distilled water (12). They were then washed in three changes of distilled water and prepared for integrated autoradiography by the coating technique (13, 14).

Röntgenography.—All the tibiae were x-rayed prior to treatment with vitamin D and also at time of sacrifice.

**OBSERVATIONS**

The Epiphyseal Cartilage of the Upper Extremity of the Tibia of Growing Chicks.—A description of the arrangement of the head portion of the tibia in the chick is necessary for an understanding of the transformations subsequently described.

The cartilaginous portion (Fig. 1) consists of three distinct parts. A large cap-like articular portion (Fig. 1, zone A) extends laterally over the underlying zone B and is attached to the fibrous periosteum. The matrix of zone A is generally acidophilic and weakly metachromatic. The cells are clustered in groups of 2 or 3 isogenic elements, each group widely dispersed except at the border of zone B where single, more flattened elements are closely gathered. There is irregular distribution of metachromatic material mostly intracellular but forming also diffuse "islands" in zone A. The border between zones A and B is distinctly and regularly non-metachromatic. The matrix of zone A is diffusely and intensely colored by the P.A.S. stain.

The matrix of zone B (Fig. 1) stains most intensely with hematoxylin (H.E., H.P.O.) and is also the most metachromatic when stained with toluidine blue. The cells at the border of zone A are small, flattened (Fig. 7) and closely gathered. They enlarge progressively showing at the border of B and C a distinct row of round cells with a small amount of highly metachromatic matrix in between. Zone B is weakly stained by the P.A.S. stain.

Zone C consists of large, round cells with relatively little matrix in between as compared to zone A. This matrix shows a P.A.S. stain reaction intermediate between that of zone A and zone B. Toluidine blue metachromasia is
apparently uniformly intense over the matrix of zones B and C, with a band of maximal intensity at the junction of these two portions of the epiphyseal cartilage. Corresponding to that of the P.A.S. stain (Fig. 1) zone C appears to be the most intensely nourished: large vessels penetrate it upwards from the metaphysis, occasionally passing into zone B. This zone also receives some of its blood supply from zone A.

In Vitro Uptake of Ca\textsuperscript{46}.—In previous reports (12, 15, 16), it has been shown that the uptake of Ca\textsuperscript{46} \textit{in vitro} by demineralized sections was related to organic constituents of cartilage, presumably to chondroitin sulfate acting as a cation binder. The autoradiographic record obtained from the epiphyseal cartilage of normal chicks (Fig. 2) after treatment \textit{in vitro} with Ca\textsuperscript{46}, has revealed constant differences between the three zones: in A, the picture was patchy with variable local intensity. The border of zone A was negative. Zone B was highly radioactive with maximal intensity at the AB and BC junctions. Zone C, also highly and uniformly radioactive near zone B, except in areas of blood vessel penetration, has shown a progressive decrease downward towards the metaphysis.

The epiphyseal cartilage in the rachitic chicks has shown a considerable hypertrophy of zone B. In one extreme case (Fig. 3), zone C was practically non-existent. In the two other animals of this group, a slightly larger zone C was present but in all cases, small, flattened cells were highly predominant. The P.A.S. staining was weakest over this enlarged zone B (Fig. 3) as compared to the apparently unchanged zone A where it was predominant and zone C where it was intermediate between A and B. The matrix of zone B has shown the largest general uptake of Ca\textsuperscript{46} (Fig. 4). Zone C has shown a much lesser calcium uptake.

The epiphyseal cartilage in healing chicks has revealed early changes related to structural distribution and Ca\textsuperscript{46} uptake. Two hours after the administration of vitamin D, there was already a remarkably larger zone C (Fig. 5), resulting probably from a rapid transformation of small cells into large cells. The limit between zones B and C was visible as a jagged line staining more intensely with the P.A.S. stain (Fig. 5) and toluidine blue. The Ca\textsuperscript{46} autoradiographs have shown a remarkable decrease of uptake in zone C (Fig. 6) while zone B was still intensely radioactive. The jagged limit between B and C was apparent by the sudden difference in calcium uptake. Zone A was apparently unchanged in its morphology, P.A.S. staining (Fig. 5), and Ca\textsuperscript{46} uptake (Fig. 6).

The 6 and 17 hour stages have revealed a progressively greater proportion of large cells in zone C (Fig. 7) which now was more extensive than zone B. These two zones were still segregated from each other by an irregular line which was intensely stained with P.A.S. stain (Fig. 7).

The autoradiographic record of the uptake of Ca\textsuperscript{46} \textit{in vitro} (Fig. 8) has
shown a progressively decreasing uptake involving zones C and B. Zone A remained apparently unchanged by all standards (Figs. 7 and 8).

These changes in the healing epiphyseal cartilage have progressed upwards at 24 and 48 hours. At these times, zone B (Fig. 9) has become reduced to approximately its normal size (Figs. 9 and 1). The zone of large cells (Fig. 9, C), separated from the zone of small cells by a jagged line, was still very wide but appeared largely invaded by blood vessels and cells from the metaphysis. The autoradiograms (Fig. 10) have revealed that the Ca uptake in zone C was now practically negligible; that of zone B was also much less than at 17 hours, with more intense bands at the AB and BC borders. Zone A apparently remained unchanged by all standards (Figs. 9 and 10).

While these progressive transformations from type B cartilage to type C were taking place, a change was also revealed in the B zone. In the early stages of healing (2 to 6 hours), isolated groups of small, flattened elements became larger and ovoid shaped, this increase in size being mostly the result of an accumulation of acidophilic cytoplasm. Later (17 to 24 hours) these cells became vacuolated and irregularly stained by the appearance of metaplastic constituents in the cytoplasm. At the same time, they became separated from one another by an increase of the residual intercellular matrix.

Toluidine blue metachromasia did not vary much in the rachitic animals and throughout the healing process.

The spodograms of demineralized sections revealed the presence of a blue-white dust in cartilage as previously described in man and other mammals (17). The ash was mostly extracellular with the exception of some cells in zone A which showed intracytoplasmic blue-white ash coincidental with intracellular metachromasia. In the normal animals, very little ash was seen in zones B and C. The rachitic animals showed an accumulation of ash in these two zones, B revealing the largest amount. The ash content decreased progressively in the 2 to 48 hour stages following administration of vitamin D.

Changes in the bone formation.—The treatment in vitro of demineralized sections of bone revealed practically no uptake of Ca by normal (Fig. 2) or rachitic (Fig. 4) bone spicules at any time during the process of healing (Figs. 6, 8, 10). In the P.A.S.-stained sections of the controls, the newly formed bone spicules under the epiphyseal plate were strongly P.A.S.-positive (Fig. 1). In the rachitic chicks 3 weeks old, spicules of a different type were observed in this location (Figs. 3 and 11). They were more numerous, larger, and weakly stained by P.A.S. stain, the stain revealing a fibrillar pattern (Fig. 11). Laterally, bone trabeculae, apparently of periosteal origin, were more intensely stained (Fig. 3). After administration of vitamin D, the number of osteoclasts increased locally and at 17 (Fig. 7) and 24 hours, the bone trabeculae formed during vitamin D deficiency disappeared progressively.
The 48 hour specimens revealed the reappearance of osteoblasts: thin trabeculae, staining intensely with the P.A.S. stain, were now growing at the epiphyseal plate (Figs. 9 and 12).

Comparative x-ray pictures of the bones made immediately prior to fixation have revealed no mineralization at the plate in rachitic animals or in vitamin D-treated animals until after 48 hours.

DISCUSSION

The facts here reported have revealed that after 3 weeks of vitamin D deficiency, chicks had severe rickets which was manifested by an absence of maturation and enlargement of the epiphyseal cartilage, by the formation of large fibrillar spicules (osteoid), and by the cessation of mineralization in both cartilage and newly formed bone. A substance capable of binding the calcium in vitro accumulated in the cartilage matrix as demonstrated by the uptake of Ca⁴⁺. This phenomenon had already been observed in cartilage of young children with the clinical manifestations of rickets (12).

The spodograms of demineralized sections have also revealed in the cartilage of rachitic chicks an accumulation of blue-white ash, predominantly in the zone of small cells, as in the case of the cartilage of vitamin D-deficient human beings and rats (18). It seems likely that the accumulation of blue-white ash and that of the metachromatic cation binder are related.

Two days after the administration of vitamin D, mineralization was resumed at the border of the epiphyseal cartilage, as revealed by the x-ray picture. In the meantime, rapid changes had occurred in the cartilage. As early as 2 hours after administration of the vitamin, morphological transformations indicative of an intensified and possibly new secretory activity were apparent. The zone of large round cells (zone C) reappeared (Fig. 5) and increased progressively in thickness at the expense of zone B subsequently (Figs. 7 and 9). Concurrently the substance capable of binding Ca⁴⁺ in vitro decreased progressively in concentration, as evidenced by the autoradiographic record (Figs. 6, 8, 10). It appears from comparison between the autoradiograms and the histological pictures that the decrease in the amount of cation binder present during all stages of recovery follows upon the maturation of the small, flat cells into large round elements (Figs. 5 to 10). The spodograms of demineralized sections also revealed a progressive loss of the accumulated blue-white ash, accompanying the renewed maturation.

The sections stained by the P.A.S. stain method or with toluidine blue have not shown any striking changes of coloration related to the maturation process; the rapidly maturing matrix was slightly more positive to the P.A.S. stain but not as much as that of normal animals. On the other hand, in all instances, the matrix of the articular cartilage was intensely P.A.S.-positive (zone A, Figs. 1, 3, 5, 7, 9). This was also the zone with the smallest concen-
tration of metachromatic substance, and the autoradiographic record of the intimate distribution of calcium binder matched the image of toluidine blue metachromasia.

This articular zone of the epiphyseal cartilage appeared little affected during the present nutritional deficiency or during recovery, while the underlying zones, which seem to contribute directly to the mechanism of bone growth, had undergone important disturbances. It would appear that the maturation of the cartilage cells which make way for bone formation is affected directly or indirectly by vitamin D, while the articular cartilage cells are apparently not influenced.

In the absence of vitamin D under the present experimental conditions, the cells which take part in the growth process apparently cannot mature; they accumulate, along with an immature matrix rich in metachromatic cation-binding substance. These findings as concern the properties of the immature matrix recall those of Neuman et al. (19), that chondroitin sulfate acts in vitro as a cation binder. They suggest that this substance in the matrix might well be chondroitin sulfate.

In the presence of vitamin D, the maturation process involving both cells and matrix was resumed. Dziewiatkowski (6, 20) has recently shown remarkable differences between the effects of vitamins A and D on the metabolism of chondroitin sulfate, while the former seeming to stimulate the synthesis of chondroitin sulfate directly whereas the latter does so only after the degradation process is stepped up.

The present series of experiments are perfectly in accord with this assumption: they point to an immediate increase in the degradation of the chondroitin sulfate accumulated during the period of nutritional deficiency. This renewed activity appears to be the earliest morphological change observed thus far that leads to recovery. Since this change is concurrent with the reappearance of large cells, it is possible that amongst the varied products of these cells (glycogen, neutral polysaccharides, chondroitin sulfate, alkaline phosphatase, etc.) there may also be an enzyme capable of hydrolyzing chondroitin sulfate. This step would appear a necessary event prior to the mineralization of cartilage and subsequent bone formation. Chondroitin sulfate acting as a cation binder can accumulate calcium locally and this calcium may then be released for combination with phosphate.

The maturation of the cartilage cells now observed appears to be possibly a direct effect of vitamin D. As against this, Migicovsky and Emslie (21) showed that a high calcium diet seemed to protect chicks against the accumulation of immature cartilage tissue (zone B) that was observed during the course of the present work in chicks on a low calcium diet. This raises the point whether vitamin D acts directly on the cartilage or indirectly through an alteration of the calcium metabolism. The present observations are an
added confirmation of all those which have already shown a considerable time lapse between the administration of vitamin D and the onset of normal mineralization. The interval of 2 days in the present experiments was occupied by changes occurring in the cartilage.

The abnormal spicules formed at the undersurface of the epiphyseal cartilage during the period of vitamin deficiency (Figs. 3 and 11) had to be removed (Fig. 7) before a new crop of normally mineralizable matrix (Figs. 9 and 12) could grow in their place. This new matrix, as well as that of the spicules of normal chicks (Fig. 1), was markedly and homogeneously colored by the P.A.S. stain, as compared to the spicules formed during the period of nutritional deficiency. This points to another phase of carbohydrate metabolism related to the neutral polysaccharide content of the bone matrix, that was apparently also influenced directly or indirectly by vitamin D.

**SUMMARY**

One day old chicks were fed a vitamin D-deficient diet for 3 weeks. Some were then given an oral dose of vitamin D and sacrificed at various times thereafter. The tibiae were x-rayed; demineralized sections were stained for neutral polysaccharides and sulfated mucopolysaccharides; other specimens were ashed; and others still were autoradiographed to determine their uptake of Ca\(^{46}\) in vitro.

Mineralization reappeared in the treated animals after 2 days, along with new P.A.S.-positive spicules.

Earlier, the immature cartilage rapidly matured morphologically. At the same time, the rachitic matrix, highly concentrated in cation binder, presumably chondroitin sulfate, lost this material progressively.

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**BIBLIOGRAPHY**


EXPLANATION OF PLATES

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Fig. 1. The upper extremity of the tibia of a 3 week old chick on normal diet, stained by the periodic acid–Schiff technique (P.A.S.). × 10. A, articular cartilage; B, young cartilage; C, mature and calcifying cartilage. The space between A and B is an artifact.

Fig. 2. Integrated unstained autoradiograph of a demineralized section adjacent to that in Fig. 1, treated in vitro with Ca^{45}. × 13.

Fig. 3. The upper extremity of the tibia of a 3 week old chick fed a vitamin D-deficient diet, P.A.S. stain. × 10.

Fig. 4. Integrated unstained autoradiograph of a demineralized section adjacent to that in Fig. 3 treated in vitro with Ca^{45}. × 10.
(Belanger and Migicovsky: Epiphysis following administration of vitamin D)
FIG. 5. The upper extremity of the tibia of a 3 week old chick fed a vitamin D-deficient diet and treated orally with vitamin D, 2 hours before sacrifice, P.A.S. stain. × 10. The dotted line indicates a portion of the limit between zones B and C.

Fig. 6. Integrated unstained autoradiograph of a demineralized section adjacent to that in Fig. 5 treated in vitro with Ca⁴⁵. × 10.

Fig. 7. The upper extremity of the tibia of a 3 week old chick fed a vitamin D-deficient diet and treated orally with vitamin D, 17 hours before sacrifice, P.A.S. stain. × 10.

Fig. 8. Integrated autoradiograph of a demineralized section adjacent to that in Fig. 7 treated in vitro with Ca⁴⁵. × 10.
(Belanger and Migicovsky: Epiphysis following administration of vitamin D)
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Fig. 9. The upper extremity of the tibia of a 3 week old chick fed a vitamin D-deficient diet and treated orally with vitamin D 48 hours before sacrifice, P.A.S. stain, × 10.

Fig. 10. Integrated autoradiograph of a demineralized section adjacent to that in Fig. 9, treated in vitro with Ca⁴⁵, × 13.

Fig. 11. A portion of cartilage and underlying osteoid in a section similar to that in Fig. 3, P.A.S. stain, × 156.

Fig. 12. A portion of cartilage and underlying newly formed spicules in a section similar to that in Fig. 9, P.A.S. stain, × 156.