RACHITOMIMETIC EFFECTS OF FLUORIDE FEEDING ON THE SKELETAL TISSUES OF GROWING PIGS*

L. F. Bélanger, M.D.; W. J. Visek, Ph.D.; W. E. Lotz, Ph.D., and C. L. Comar, Ph.D.
From the Department of Histology and Embryology, University of Ottawa, Ottawa, Canada; and Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn.

In spite of the numerous approaches to the problem of fluorosis during the past 30 years,[1] the pathogenesis of the changes observed in skeletal tissues remains unclear. While some authors[2] consider the lesion to be a form of osteosclerosis, others attribute it to mineral deficiency characterized by an increase of osteoid formation.[3-5] Some consider the osseous condition a response to parathyroid hyperfunction[5] or intoxication;[6] others have reported the aggravating effects of a calcium deficient diet.[7] Studying young dogs, Kellner[8] recognized a gross similarity between the bony changes in fluorosis and rickets. Our present survey of growing pigs casts some light on this problem.

Materials and Techniques

In this study, tissues from 12 castrate male Hampshire pigs,† 6 weeks of age and averaging 65 lbs. in weight were used.[9] The animals, which had been raised on a normal farm ration, were paired, and one member of each pair was placed on a diet containing 1,000 parts per million of sodium fluoride. Thereafter the animals were pair-fed so as to insure equal intakes except, of course, for fluoride. After 30 days of feeding, each animal received an intravenous injection of 6 mc. of $S^{35}O_4$. Pairs were sacrificed at 10 minutes, 5 hours, 24 hours, 10 days, 30 days, 60 and 90 days.

Ribs and the heads of metatarsals were fixed in formaldehyde, demineralized in nitric acid and sectioned in paraffin. These sections were stained with hematoxylin and eosin, hematoxylin-phloxine B and orange G (HPO), Masson trichrome,[10] toluidine blue, the periodic acid-Schiff stain,[11] and the von Kossa stain. Some sections were examined unstained with phase contrast microscopy. Other sections were incinerated by the technique of Scott.[12]

A parallel histologic and histochemical study was made on the

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teeth.[13] Autoradiographic observations were carried out *in vivo*[14] and *in vitro*[15] in order to determine the uptake of radiosulfate and radiocalcium.

Observations

Distortion of the linear pattern of the epiphyseal plate chondrocytes, encroachment upon the hypertrophic zone by bone forming tissue, decrease in the size of the spicules and increased osteoid production have been reported in previous similar experiments.[16] In the present series, the bones were studied only after demineralization and embedding in paraffin. A marked difference was observed between fluorinated and control animals in respect to the diameter of the costochondral junction of the ribs. While the outer diameter of the diaphysis was comparable in both cases, the heads of the bones of fluorinated animals had a "beaded" appearance (Fig. 1) and were approximately twice the size of the controls at 90 days.

In the newly deposited osseous material, striking histologic differences were also observed. Osteoid, otherwise called prebone,[17] stained orange with the HPO stain and green with the Masson technique. The central portions of the trabeculae showed red acidophilic material which increased rapidly in thickness, so that in the more differentiated tissue (secondary spongiosa), only a narrow border of yellow or green prebone was visible.

The alveolar bone of fluorinated animals showed progressive changes comparable to those of dentine and cementum reported elsewhere[13] with gradual decrease of acidophilia and the formation of bright globular bodies. These appeared in the more mature trabeculae and were rather uniformly distributed. Subsequent changes consisted of irregular growth and enlargement of the trabeculae. The marrow cavities also became enlarged and contained distended blood vessels. The modified bone matrix as well as the cementum of fluorinated animals frequently had a fibrillar appearance, indicative of a decrease in the binding substance.

In the fluoride-treated animals at 60 and 90 days, there was evidence of increase in the number of osteoblasts in productive areas. On the other hand, osteoclasts were also more numerous. Some of them contained one or several bright intracytoplasmic globular masses.

Periosteal bone appeared to be affected more slowly. At the epiphyseal plate, the spicules did not show any globular material but appeared to grow progressively thinner and more fragile, becoming easily detached from the cartilaginous plate (Fig. 1).

The progressive hypertrophy of the cartilage head of long bones was

most impressive (Fig. 1). The stained sections indicated that this condition was due to an increase in the mitotic rate of cartilage cells and a state of immaturity associated with decrease in the formation of matrix. The outcome was a highly cellular pale-staining cartilage, invaded by blood vessels and apparently soft and fragile. The epiphyseal plate showed patchy degeneration with invasion by vascular bone-forming tissue from the shaft marrow (Fig. 2). The bone spicules grew over progressively thinner cores of cartilaginous tissue. There were no globular masses in cartilage at any time.

The cartilage of the head of long bones has been reported to show a decrease of metachromasia in the zone of mineralization.[18] In the present series, there was no apparent decrease in staining intensity in the epiphyseal plate of fluorinated animals. However, the color produced by toluidine blue at this level appeared to be violet-blue rather than magenta as in normal cartilage. When stained with toluidine blue, the sections of normal bone, particularly the newly formed trabeculae, were weakly metachromatic. The reaction was most apparent in the vicinity of the osteocytes. By comparison, the bone of the fluorinated animals did not stain metachromatically but exhibited a pale blue quality.

With the PAS stain[11] prebone appeared to be practically unstained. Normal bone showed a gradation from moderate to strong staining toward the center of the trabeculae. The bones of the fluorinated animals showed, as in the case of the dentine and cementum matrix,[12] a progressive decrease of PAS staining intensity.

With phase contrast microscopy, the normal cartilage matrix appeared dark under the conditions of observation. In fluorinated animals, fine bright granular material appeared in the matrix between the rows of hypertrophic cells (Fig. 3). These small bodies were increased in quantity, but not in size, near the region of bone growth. They were also more abundant in animals fed the fluoride diet for 60 and 90 days. Large, bright globular masses, comparable to those previously recognized in dentine and cementum,[12] were disseminated throughout the newly formed bone trabeculae (Fig. 4). These also appeared in the marrow spaces in the vicinity of growing bone.

All the tissues were received in demineralized state and failed to react to the von Kossa stain for lime salts after a maximal exposure of 60 minutes to a 1.5 per cent solution of silver nitrate.[19] The incinerated preparations of demineralized tissues were not expected, of course, to yield much ash. However, a recent series of experiments[20] revealed an intercellular distribution of blue-white ash in cartilage and other

soft tissues such as epidermis, hair, retina, etc., corresponding to regional Ca^{45} uptake.

The incinerated sections of demineralized normal pig cartilage contained a small proportion of intercellular blue-white dust and also relatively abundant localized residues of white ash reproducing the shape of cartilage cells. The demineralized sections of cartilage from the fluorinated animals yielded an increased amount of ash, comparable in appearance to normal cartilage ash and located between the rows of hypertrophic cells (Fig. 5). The newly formed bone trabeculae also produced a modified spodogram in which ash appeared more abundant in the outer portion of the trabeculae when compared to animals on the fluoride diet for 30 days (Fig. 7). At 60 days, the ash was more abundant and more uniformly distributed in the trabeculae which had developed during the period of fluoride feeding (Fig. 8).

For purposes of comparison, sections of the costochondral junctions of the ribs of 3 human children suffering from vitamin D deficiency rickets were also incinerated. The spodograms revealed an increased ash content, particularly abundant in the vascularized zone, characteristic of this disease (Fig. 6).[21]

It is possible to consider the ash content of the fluorinated bones to represent original deposits of calcium fluoride. This has been reported previously as a probable occurrence in fluorosis.[22] Calcium fluoride is known to be only slightly soluble in acids used in the usual techniques of demineralization. On the other hand, this substance is soluble in solutions of ammonium salts.[23]

Sections of bones of fluorinated animals were placed in a 2 per cent aqueous solution of ammonium acetate for 24 hours. They were then incinerated or mounted unstained for study by phase contrast microscopy. After this treatment, the majority of the globular masses were found to have disappeared from the bone trabeculae; some were actually observed to be partially dissolved. On the other hand, the substance which yielded the diffuse, fine ash did not seem to be affected by the ammonium acetate.

Discussion

Some of the manifestations of fluoride feeding reported here and in previous work[16] recall the classical rachitic syndrome: decreased growth, decreased and imperfect mineralization, hypertrophy of the costochondral junction, and overproduction of osteoid (Fig. 1). However, these may in part represent reactive, secondary changes. While the previously laid out portions of the skeleton appeared to be barely

involved, bone formed during the period of fluoride feeding was greatly modified in both its mineral and organic content.[16,24] Indeed, the epiphyseal cartilage increased in size and became softer so that weight bearing produced a distortion of the architecture.[16] On the other hand, autoradiographic records of tracer doses of $S^{35}O_4$ revealed that the tagged sulfate disappeared from fluorinated cartilage at a slower rate[14] and did not diffuse readily from predentine into dentine. The polysaccharide content of bone as well as that of dentine and cementum seemed to have decreased as evidenced by the toluidine blue and PAS reactions. However, in the fluorinated animals, the new trabeculae were larger and irregular.

The hypertrophy of the cartilage, bone and dentine is not conducive to growth of the animal and seems to be the result of accumulation and overproduction of an abnormal matrix. In bone and dentine, this substance appears immature, with staining affinities comparable to those of prebone and predentine. On the other hand, the bone and cartilage matrix of the fluorinated pigs contains some material which resists acid demineralization and yet may be a salt since it yields abundant ash in the process of micro-incineration. Deposition of CaF_2 in bone under similar conditions has long been suspected[2,8,22] and has recently been established by F^{18} autoradiography.[25]

The present experiments have revealed that only portions of the structural mass, presumably those containing salts, were dissolved in ammonium acetate, a solvent of CaF_2. These were the large globular masses observed in bone, dentine, cementum and some peripheral connective tissue structures, such as tendons and ligaments. They are, presumably, the *Kalkhörner* of Kellner,[8] and are considered responsible for the sclerosing character of the disease.[2]

In the areas of normal mineralization or premineralization, a large amount of blue-white ash, resistant to acid demineralization and to ammonium acetate, appeared in the spodograms. In cartilage, this pattern was comparable to that of vitamin D deficiency rickets in children (Fig. 6) and to unpublished observations on strontium rickets in the rat. As in these two hypertrophic disorders, here also there was an intense uptake of Ca^{45} *in vitro*.[15,26] It is thus possible to consider that this diffusely distributed material may represent not CaF_2 but an organic salt of calcium. The existence of an organic precursor to mineralization has been postulated by Sobel (the intrinsic factor).[27] On the other hand, Newman, Boyd, and Feldman[28] have shown that chondroitin sulfate can behave as a cation exchanger *in vitro*. It is possible that the blue-white ash deposits in fluoride intoxication may

represent an accumulation of chondroitin holding calcium as a loose combination or as a more stable, unnatural salt. Thus, calcium would be deposited in sites of growth, partly as CaF_2, partly as an organic salt, accumulating since normal mineralization cannot occur. The latter condition apparently also prevails in vitamin D deficiency and in strontium rickets.

Summary

Young pigs, fed for 30, 60 and 90 days on a diet containing 1,000 parts per million of sodium fluoride, have shown defective growth and mineralization of bones, costochondral beading, softened and deformed epiphyseal plates, and enlarged and malformed bone trabeculae.

Histochemical studies of demineralized sections have revealed a decrease in the stainable polysaccharides and an accumulation of salt, the solubility of which resembled that of calcium fluoride. The larger portion of the deposit observed in spodograms seemed related to an organic calcium combination, the significance of which is discussed in relation to the mechanism of mineralization and is compared with vitamin D deficiency and strontium rickets.

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[*Illustrations follow*]

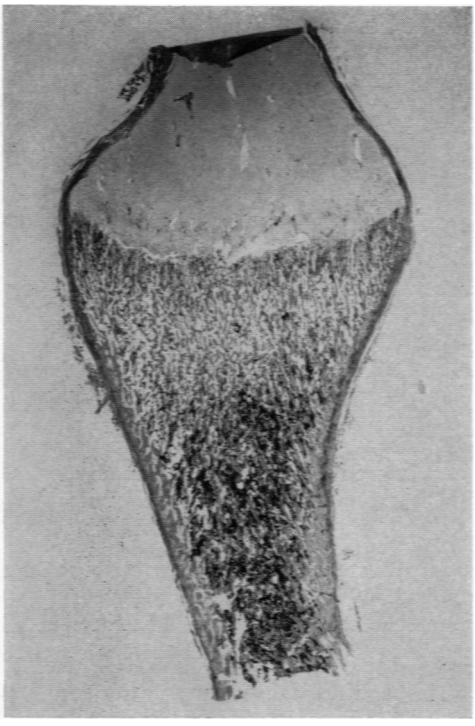
LEGENDS FOR FIGURES

FIG. 1. The costochondral junction of the rib of a pig 20 weeks of age on fluoride diet for 90 days. The diameter of the rib at the epiphyseal plate is approximately twice that of the shaft. In the control animal, these measurements are roughly identical. Hematoxylin-phloxine B and orange G (HPO) stain. × 3.

FIG. 2. The border of an epiphyseal plate modified by fluoride diet. Note the localized zone of cartilage destruction and, in the upper portion of the picture, deep penetration by marrow constituents. HPO. × 57.

FIG. 3. The epiphyseal cartilage of bone from a pig on fluoride diet for 60 days. In this demineralized section the bright dust in the matrix and the bright cytoplasm of the chondrocytes may be noted. Dark M phase contrast microscopy. × 200.

FIG. 4. A trabecula of alveolar bone from a pig on fluoride diet for 30 days. Note the bright globular masses in the bone and in lesser amount in the marrow spaces. There is no brilliant dust visible. Dark M phase contrast microscopy. × 200.



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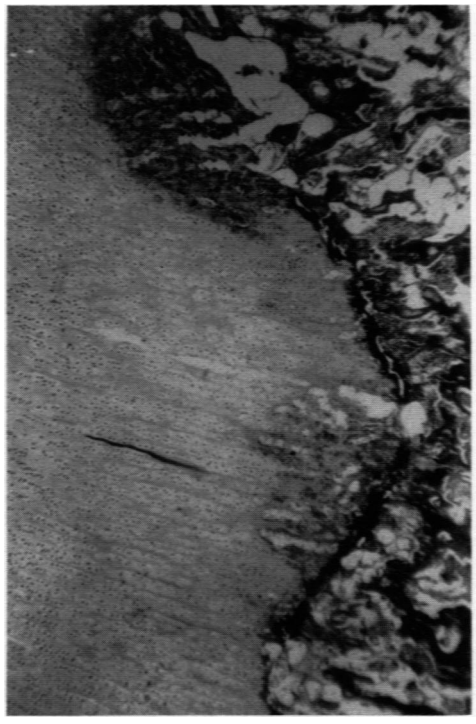

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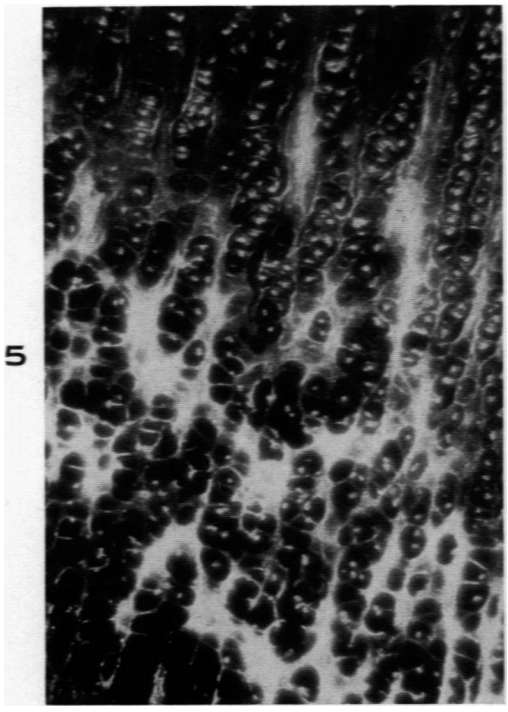
FIG. 5. Spodogram of epiphyseal cartilage of a pig on fluoride diet for 60 days. There is increase of matrix ash near the shaft margin of the plate. Phase contrast microscopy. × 100.

FIG. 6. Spodogram of a demineralized section of vascularized portion of rib at the costochondral junction. A 3-year-old child with vitamin D deficiency rickets. A heavy ash deposit is apparent in the cartilage matrix. Phase contrast microscopy. × 100.

FIG. 7. Patchy distribution of fine particles of blue-white ash in a newly formed bone trabecula of a pig on fluoride diet for 30 days. Spodogram. × 200.

FIG. 8. Homogeneous distribution of fine particles of blue-white ash in a bone trabecula of a pig. This has developed during 60 days of fluoride feeding. Note also the denser, ovoid masses corresponding to the globular bodies in Fig. 4. Spodogram. × 200.



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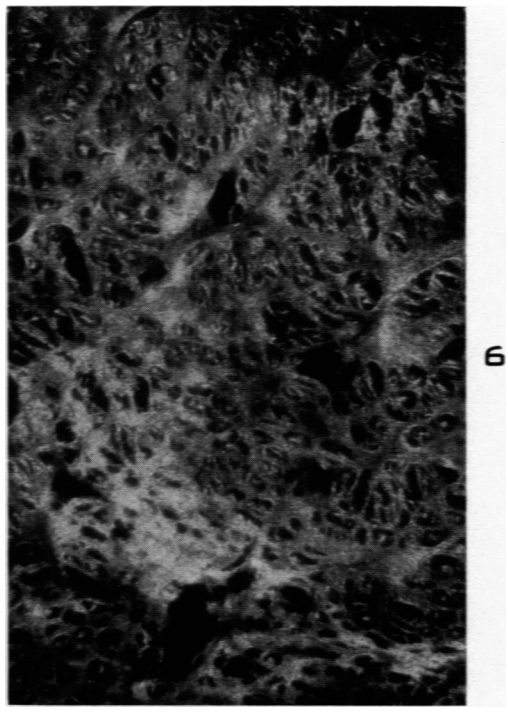

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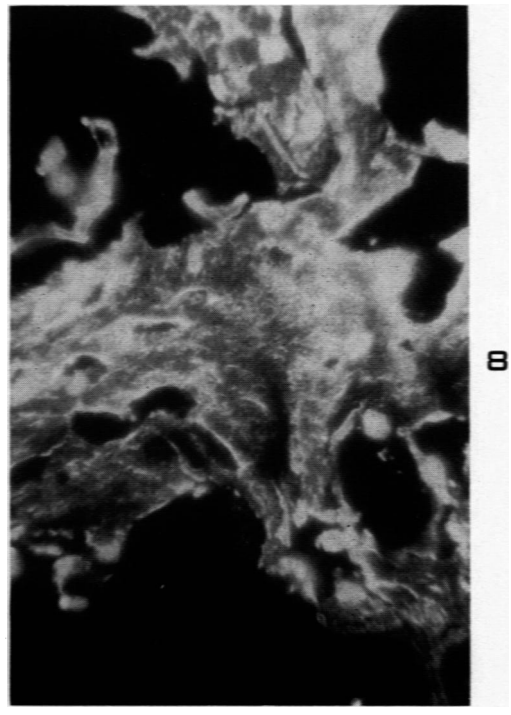

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