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P. G. Masse
Universite de Moncton

V. E. Colombo
F. Hoffmann-La Roche Ltd.

F. Gerber
F. Hoffmann-La Roche Ltd.

D. S. Howell
University of Miami School of Medicine

H. Weiser
F. Hoffmann-La Roche Ltd.

Utah State University
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MORPHOLOGICAL ABNORMALITIES IN VITAMIN B_6 DEFICIENT
TARSOMETATARSAL CHICK CARTILAGE

P.G. Masse[1,*], V.E. Colombo[2], F. Gerber[2], D.S. Howell[3], H. Weiser[2]

[1]Ecole de Nutrition et d'Etudes Familiales
Universite de Moncton, Moncton, New Brunswick, Canada E1A 3E9

[2]F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

[3]V. A. Medical Center, and University of Miami School of Medicine,
Department of Medicine (D26), P.O. Box 016960, Miami, FL 33101

Abstract

The aim of this study was to test the hypothesis that deficiency of vitamin B_6 would produce morphological characteristics of osteochondral lathyrism. To accomplish this goal, morphological characteristics of chick cartilage in which lathyrism was produced by two separate dietary regimens was compared to morphological changes encountered in vitamin B_6 deficiency. Vitamin B_6 deficiency should reduce activity of lysyloxidase needed for producing intermolecular cross-links. The question to be addressed was: would this latter deficiency impair collagen morphological features and secondarily other structures indirectly by reducing collagen molecular assembly? Failure of cross-linking of collagen in the positive controls was related to a lack of functional aldehyde cross-link intermediates which are blocked by homocysteine and aminoacetonitrile.

Day-old-male Lohmann chicks were fed adequate (6 mg/kg) or vitamin B_6-deficient diets. Cross-link defects were induced by homocysteine-rich diets (0.6 % w/w) or a diet containing aminoacetonitrile (0.1 % w/w). Animals were sacrificed at 6 weeks of age and Ossa tarsalia articular cartilage specimens, as well as the proximal end of tarsometatarsus were dissected from the tibial metatarsal joint, a major weight-bearing site. Light microscopic observations revealed reduction of subarticular trabecular bone formation, concurrent with overexpansion of the hypertrophic cell zone. Ultrastructural electron microscopy observations of articular fibro-cartilage indicated significant thickening of collagen fibers in vitamin B_6 deficient birds, as well as the positive controls in comparison to that of cage-matched control birds. It was concluded that vitamin B_6 deficient cross-linking may be responsible for the observed delay in bone development and aforementioned cartilage histological alterations.

*Address for Correspondence:
Priscille G. Masse,
Ecole de Nutrition et d'Etudes Familiales,
Universite de Moncton,
Moncton, New Brunswick, Canada E1A 3E9
Telephone: (506) 858-4285
FAX: (506) 858-4540

Introduction

The prime role of collagen is to provide the connective tissue with its structural integrity; the high tensile strength of collagen fibrils plays an essential supportive role. This property is due to its unique molecular conformation and a highly specific alignment and packing of the molecules in the fibrils (13, 22). The staggered configuration involves molecular hydrophobic and electrostatic interactions. The collagen molecules are then stabilized in the fibrils by covalent intermolecular cross-links. It has been proposed that in the extracellular matrix two supramolecular assemblies, collagen fibrils (defined arbitrarily here as fibers) and proteoglycans interact non-covalently to influence both the ultrastructure and the architecture of cartilage matrix. The amount and nature of proteoglycans associated with collagen will influence the morphology and overall structural properties of the connective tissue.

Special attention must be paid to pyridoxine, particularly in connection with bone and cartilage collagen. This vitamin plays a key role in the stabilization of the extracellular matrix, being a cofactor of the enzyme lysyloxidase (1). This important copper-dependent enzyme for cross-linking in connective tissue catalyzes the oxidative deamination of certain lysine (Lys) and OH-Lys residues. The resultant Lys and OH-Lys aldehydes from Schiff bases $(R_1-C=N-R_2)$ react with adjacent molecules in the fibrils. Cross-links of this variety give collagen fibrils most of their tensile and shearing strength. The importance of intermolecular cross-linking is apparent in lathyrism, a disease of animals caused by eating the seeds of sweet peas (Lathyrus odoratus). The seeds contain beta-aminopropionitrile (BAPN) which blocks the side chains of the collagen molecule where cross-linking normally takes place (9). As a consequence, the collagen of these animals is weak and fragile, giving rise to scoliosis, severe limb deformities and skin fragility. This disturbance, experimentally reproducible in growing animals with aminoacetonitrile and manifested in all connective-tissue structures, can also be induced by a complete withdrawal of vitamin B_6 (12).

The purpose of the present investigation was to study the role of vitamin B_6 in the molecular packing of cartilage collagen and the structural integrity of mineralized connective tissue in growing chick. The ultrastructural adverse effects of vitamin B_6 deficiency on collagen network has also been compared to those induced by a homocysteine-rich diet and a

Table 1: Biochemical indices pertinent to Vitamin B_6 status (nmole/L)

	B_6-adequate diet (n = 15)	B_6-deficient diet (n = 27)
Plasma PLP	18.6[a] ± 3.7	5.0 ± 1.8*
Erythrocyte[b] PLP	420 ± 50	133 ± 31*

[a]Values are means ± standard deviation
[b]erythrocyte suspension of 40% hematocrit
*p less than 0.001

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diet containing aminoacetonitrile (an analogue of BAPN). It is known that both chemical substances block the production of aldehyde cross-link intermediates (4, 7). Homocystinuria is a genetic defect which, in addition to mental deficiency, is characterized by symptoms related to defective connective tissue metabolism.

Materials and Methods

Animals and Diets
Day-old male Lohmann chicks were randomly assigned to an adequate (6 mg/kg) or a vitamin B_6-deficient diet containing a 22% protein level. They were weighed at the beginning of the experiment; each group had a similar mean body weight and weight distribution. The chicks were housed in an animal room in thermostatically control starter cages (31°C) for the first week and then allowed to grow up to 6 weeks of age in broader raised wire-bottom cages kept at a constant ambient temperature (28°C). A 12 hour constant light schedule was maintained daily. Feed and water were provided ad libitum throughout the experiment.

Vitamin B_6 deficiency was monitored by biochemical determinations of the pyridoxal phosphate (PLP) concentrations in plasma and in erythrocytes (using tyrosine apodecarboxylase, see ref. 6, 24). Homocystinuria has been induced by using a homocysteine-rich diet (0.6% w/w) corresponding to 690 mg/kg of body weight at the second week of age or 440 mg/kg of body weight at the sixth week of age. This dose represents twice the methionine requirement. Aminoacetonitrile, at a 0.1% w/w dose, has been employed instead of BAPN to produce lathyrism because of its lower toxicity.

Tissue Preparation
Immediately after the sacrifice of animals by cervical dislocation, slices of tissue for electron microscopic study were freshly dissected by a scalpel blade from the tangential zone of the Ossa tarsalia articular cartilage which is the most superficial and in immediate contact with the synovial fluid. For electron microscopy, each specimen was taken at the same distance from the surface on the left side of the right proximal end of tarsometatarsus. For light microscopic study, the left tarsometatarsus was removed and decalcified in 10% formic acid after formalin fixation. Histologic study was focussed on articular cartilage and secondary ossification centers of Ossa tarsalia.

Light microscopy
The proximal end of tarsometatarsus specimens were transferred to 10% buffered neutral formalin. They remained in formalin for 24 hours and were then transferred at daily intervals to 40%, 70%, 85%, and 100% ethanol at 4°C for dehydration. The samples were then embedded in paraffin and 2 μm sections were prepared. Paraffin sections were stained with alcian blue (pH 2.5) or safranin-O (in its orthochromatic form) as described by Rosenberg (17). Fast green was used in sequence with safranin-O to counterstain protein with a contrasting color according to Lillie's procedure (11).

Transmission Electron Microscopy (TEM)
Articular cartilage was cut into small pieces (less than 1 mm^3) and prepared according to the technique described by Reginato et al. (16). Immediately after dissection, the specimens were placed in Karnovsky's formaldehyde glutaraldehyde fixative diluted 1:1 with 0.1 M cacodylate buffer at pH 7.4. After fixation specimens were washed in the same buffer at 4°C overnight. They were then post-fixed in OsO_4 (2% in distilled water) for 2 hours. The specimens were dehydrated in ethanol, placed in a 50% propylene oxide and Epon mixture (50/50) for 2 hours and embedded in Epon 812. Thin sections were cut on an LBK ultramicrotome and double-stained with uranyl acetate and lead citrate and observed in a Philips EM 300 at an accelerating voltage of 80 kV. To observe matrix granules, each specimen was also stained with Ruthenium red.

Using glossy prints of 125,600 and 205,000 X, the width of collagen fibers at 3 points were measured on 6-17 fibers. Results were analyzed statistically using a Wilcoxon non-parametric test.

Scanning Electron Microscopy (SEM)
The same procedure as for TEM was applied until ethanol dehydration. The samples were dried according to the critical point drying method and cracked with a scalpel. The mounted samples were coated with about 20 nm of palladium/Gold (40/60) by means of diode sputtering. Specimens were examined with a JEOL JSM-840 operating at an acceleration voltage of 15 to 20 kV.

Results

Morphological Changes in Articular Cartilage
For all morphological studies, the light microphotographs and the magnified electron microscopy glossy prints were studied from normal control cage-matched animals, as well as positive control lathyritic and homocystinuric diet specimens against the B_6 deficient animals. Ultrastructural study was confined to the articular cartilage (Figs. 1 and 2). Vitamin B_6 deficiency was biochemically confirmed by a significant reduction of the coenzyme form (PLP) in plasma and in erythrocytes (Table 1).

In the superficial area, according to TEM observations, small collagen fibers running throughout the matrix align in a predominantly parallel orientation to the surface. It is an area high in collagen, (Fig. 1a). Collagen fibers in this zone of the Ossa tarsalia (hock joint) are normally slightly thickened compared to the hyaline cartilage and more characteristic of fibro-cartilage, such as found in menisci or margins of intervertebral discs (Fig. 1a). This finding confirms the observations of Seyer et al. (21) that the articular cartilage of postnatal chicken
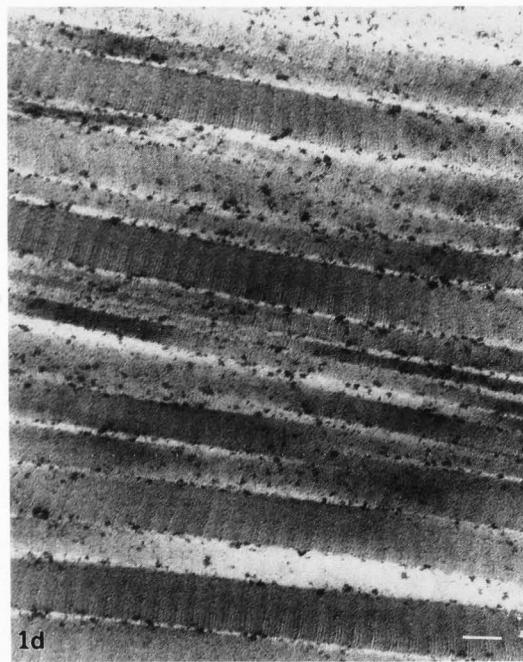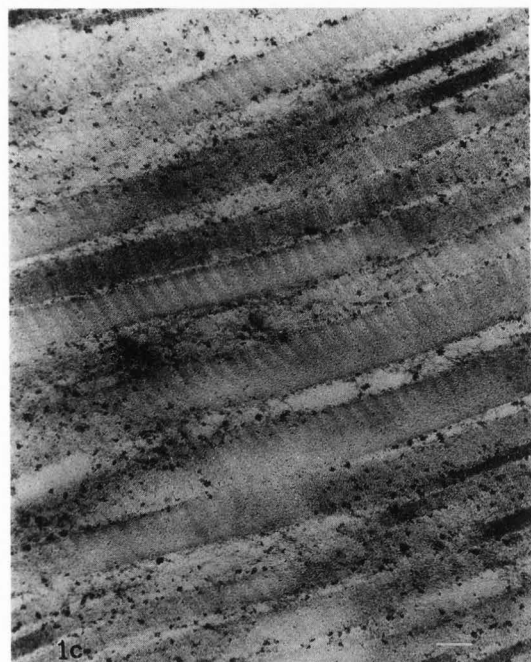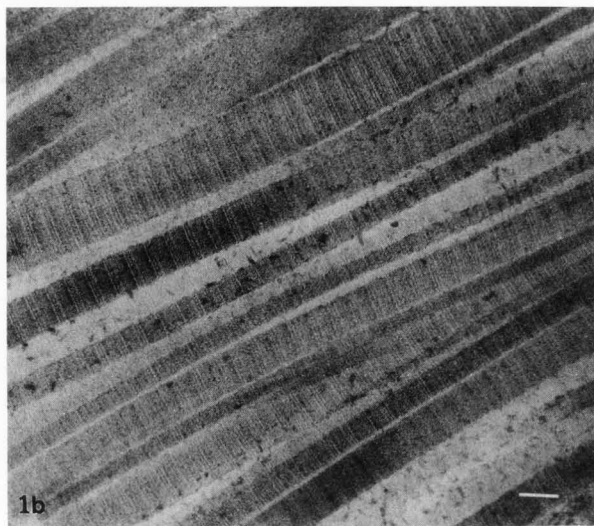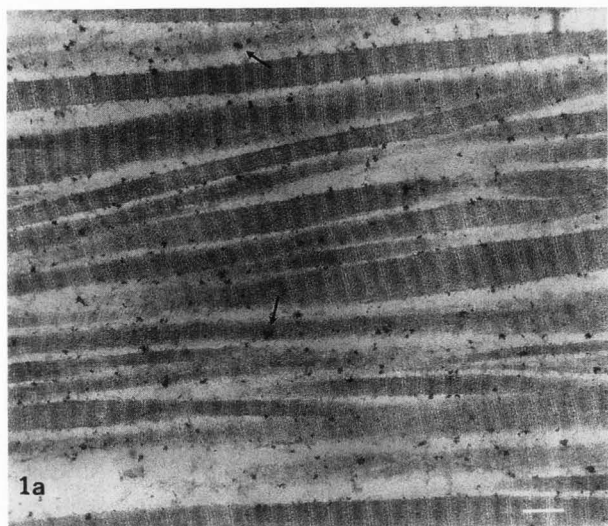
Fig. 1a. Transmission electron micrograph of articular cartilage collagen representative of Ossa tarsalia of control chicks fed an adequate diet. Note the axial periodicity of fibers and that thick and thin fibers are arranged side-by-side (arrow). Matrix granules appear random between fibers and in contact with them.

Fig. 1b. Collagen fibers from the corresponding site histographically in a vitamin B$_6$ deficient chick cartilage. Almost all of the fibers are thickened relative to normal controls.

Fig. 1c. Appearance of chick cartilage collagen when dietary homocysteine level is increased. Adverse effects due to the lack of functional cross-link aldehydes are more pronounced than with aminoacetonitrile. Fibers are irregular and swollen. The banding pattern is partly lost or reduced in clarity. Matrix granules are abundant in the interfibrous spaces.

Fig. 1d. Appearance of lathyritic cartilage collagen (aminoacetonitrile treatment). Fibers are swollen as in vitamin B$_6$ deficiency. However, the collagen banding pattern is severely altered; matrix granules are abundant and appear randomly distributed.

Bars = 100 nanometers on each figure.
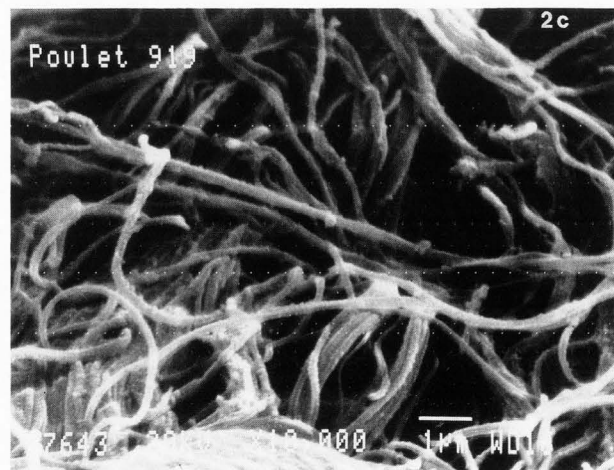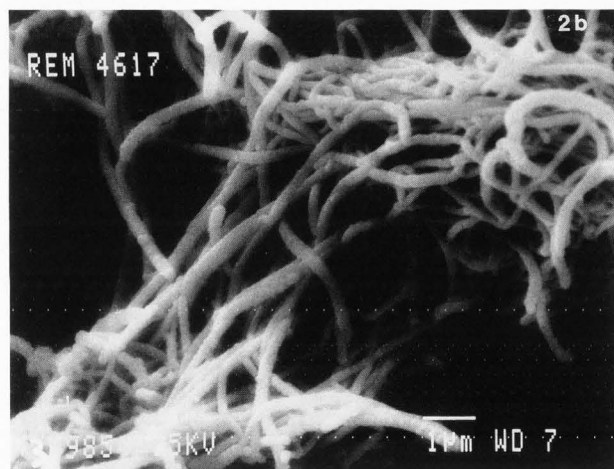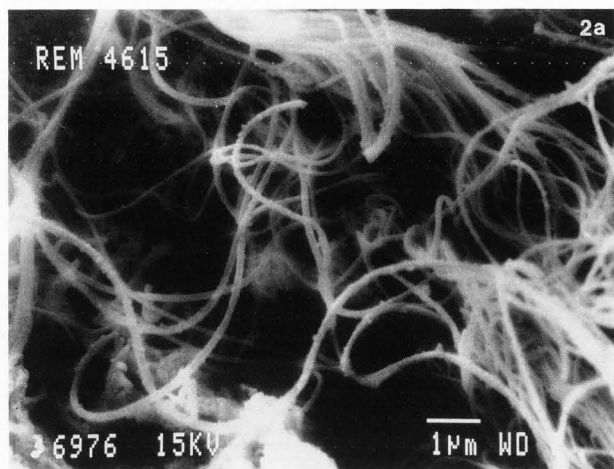
Bars = 1 micrometer on each figure.

Figures 3 and 4 (color plate of light micrographs on the facing page). PZ = proliferating cell zone; HZ = hypertrophic cell zone; HC = hypertrophic chondrocytes; Coll = Collagen.
Figs. 3a and 4a. Normal demineralized cartilage stained with safranin-O and counter-stained fast green (Fig. 3a, which marks the presence of collagen in the articular surface and in surrounding areas of growth centers; safranin-O stains intensely the matrix of the subarticular growth center corresponding to the proliferating cell zone; poor staining of articular cartilage with safranin-O is consistent with a fibro-cartilage component), and stained with alcian blue and safranin-O (Fig. 4a, where hypertrophic chondrocytes are seen with a normal safranin-O retention).
Fig. 3b and 4b. Vitamin B_6 deficiency cartilage sections from sample matched to control. In Fig. 3b, note the presence of hypertrophic cells and blood vessel invasion, the hypertrophic cells are expanded completely into the region where secondary ossification and a trabecular bone pattern should have been displayed; no evidence of enchondral ossification is seen. Fig. 4b shows chondrocytes in the hypertrophic cell zone stained intensely with safranin-O.

Table 2: Effects of vitamin B_6 deficiency and other dietary treatments on the size of collagen fibrils as observed by TEM

	Number of fibers	Size (nm) mean ± S.D.	% increase
Control	17	65.4 ± 21.7	
B_6-deficient	12	101.4 ± 37.9*	55
Aminoaceto-nitrile	10	115.4 ± 35.1*	77
Homocysteine	6	123 ± 29.1*	89

*p less than 0.001; S.D. = standard deviation.

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contains substantial amounts of type I collagen. The size of the collagen fibers for various groups is given in Table 2. Collagen banding patterns were not obscured in B_6 deficiency cartilage. In the aminoacetonitrile cartilage, the banding pattern of the collagen fibers in most images was indistinct and totally lost in areas of several fibers on their edges (Fig. 1d). In the homocystinuric cartilage the loss of the collagen banding pattern was slightly more severe (Fig. 1c).

The scanning electron micrographs of control articular cartilage showed an average array of thin intertwined fibers (Fig. 2a). In comparison, the B_6 deficient (Fig. 2b), as well as the positive control homocysteine articular cartilages (Fig. 2c), revealed thickened fibers in a loosened array. Slight nodularity on the fibrils was noted in all cases.

Fig. 2 (at left). SEM observations of collagen: **Fig. 2a.** Fiber-bundles from a normal cartilage; a close knit array of intertwined fibers is seen. **Fig. 2b.** Isolated from vitamin B_6 deficient cartilage; fibrils are swollen. **Fig. 2c.** Isolated from cartilage enriched in vivo with homocysteine; nodules seen between fibrils or in contact with them are more evident.
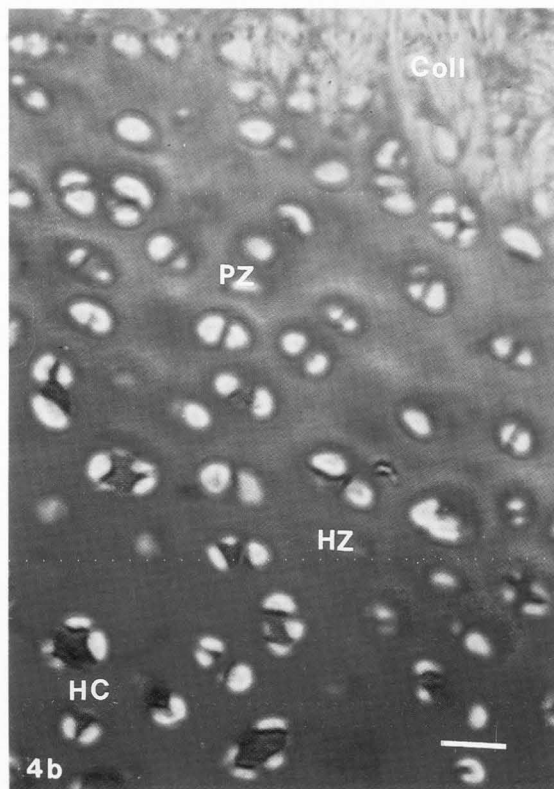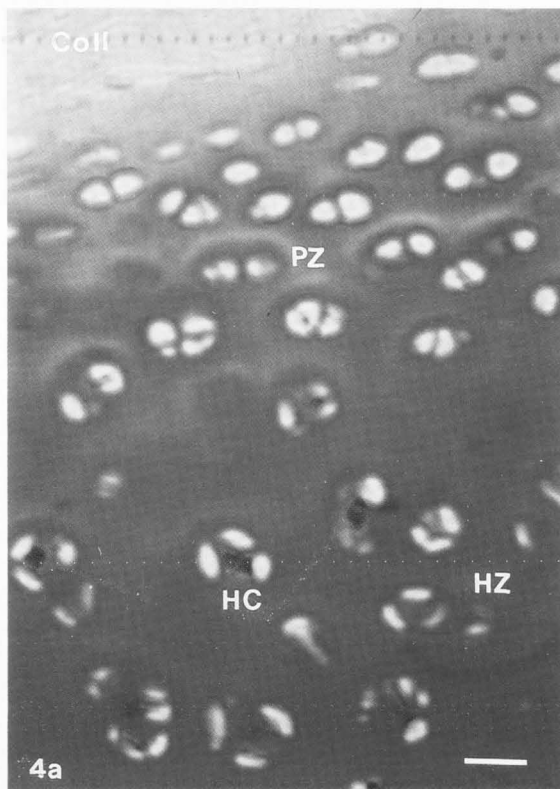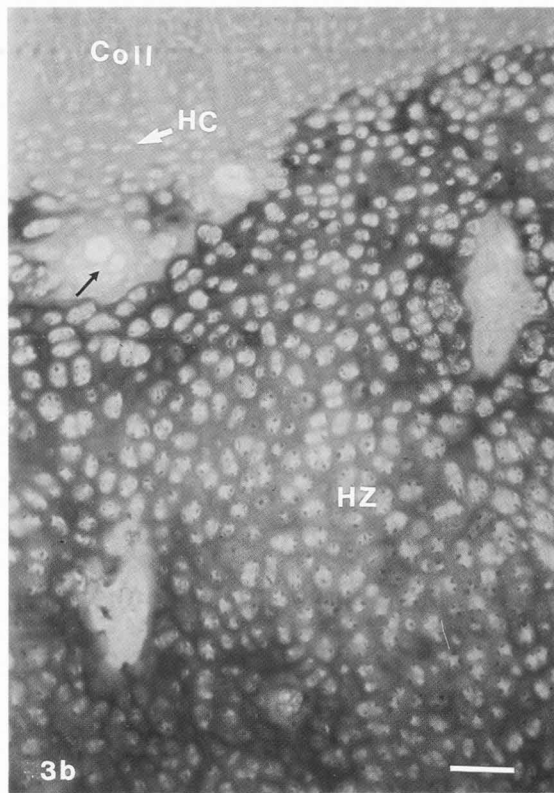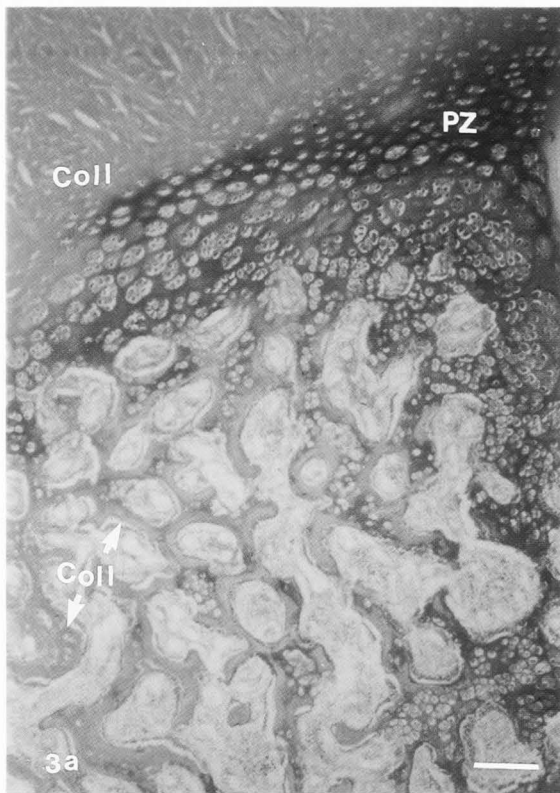
Bars = 6.25 micrometers on Figs. 3a and 3b; and 25 micrometers on Figs. 4a and 4b.

Ultrastructural Study of Matrix Granules

Matrix granules, probably containing proteoglycans according to many previous studies, and representing distribution of these important polymers in the cartilage (20), were observed in the TEM. In comparison to normal cage-matched controls (Fig. 1a), the matrix granule distribution in the dietary B_6 deficient cartilage (Fig. 1b) seemed altered in respect to a deficiency of granules and their loss from fibril surfaces. Most of the granules in the later animal tissues appear to be dispersed in the interfiber spaces. In the positive control cartilages there was also a loss of matrix granules from the periphery of collagen fibers and apparently, these granules are randomly distributed in the interfiber spaces (Figs. 1c and 1d). Ordinary fixation techniques were employed here. It has been claimed that extremely rapid low temperature freezing and/or freeze substitution with hydrophilic embedding media avoid artifacts in proteoglycan structural arrays. Nevertheless, with current older conventional fixation techniques, changes of matrix granule distribution can be seen (20). Also, as for the collagen fiber thickening, comparative effects of the dehydrating procedures on normal versus cross-link samples are negligeable, except in the direction which minimizes the observed treatment differences.

Morphologic Changes in the Subarticular Growth Center (Light Microscopic Study)

Figs. 3a and 4a depict a normal histological demineralized section through the articular and subarticular cartilage at the proximal end of the tarsometatarsus. Chondrocytes within the articular, intermediate, proliferating cell, and hypertrophic zones are displayed with a normal array of trabecular bone of the secondary ossification center shown in the lower half of the figure (stained fast green in this demineralized section). Poor staining of the fibro-cartilage (particularly in vitamin B_6 deficiency) like articular zone in the normal cartilage is seen. Adequate staining with safranin-O of the proliferating cell and hypertrophic cell zones is observed. In contrast, (Figs. 3b and 4b), vitamin B_6 deficient cartilages revealed histopathological changes: An irregular interface is seen between the growth center cartilage and the fibro-articular cartilage. In the subarticular region (Fig. 4b), there is a greatly overexpanded hypertrophic cell zone with upper and lower borders containing scattered hypertrophic cells with intensely staining proteoglycans in situ with safranin-O among profuse numbers of normal appearing hypertrophic cells. The extracellular matrix stained slightly with alcian blue. Fig. 3b depicts failure of the metaphyseal primary spongiosa to form and to mineralize, as well as deep penetration of capillaries, indicating that bone growth has not been initiated.

Discussion

The present light and electron microscopic evidence of disturbed collagen fiber size assembly in articular cartilage and failure of secondary ossification at a subarticular growth center, we believe, are the first observations made to substantiate a morphologic disturbance of cartilage in vitamin B_6 deficiency. The findings warrant focusing attention on the literature relevant to lathyritic collagen and collagen cross-linking. As the major physiological roles of collagen are accomplished by extracellularly assembled fibers from fibrils and microfibrils, the processing of fibril to fiber assembly is of paramount importance to the integrity of bone and cartilage. It is known that because lathyritic collagen or borohydride-reduced collagen lacks the Lys- and OH-Lys-derived aldehydes, allysyl and hydroxyallysyl, fibrils reconstituted from such preparations do not form covalent cross-links and disperse or dissolve when cooled to give clear solutions (8). The results from the cross-link studies as well as the specific radioactivity analysis of skin and bone collagens clearly showed that vitamin B_6 deficiency paralleled those obtained by other investigators with d-penicillamine and homocysteine intoxication (2, 6). Thiol-containing amino acids are elevated in rats fed vitamin B_6 deficient diets (18). Hence, in addition to a decrease in lysyloxidase activity and decreased allysine formation, there is a blockage by thiazolidine or thiazine complexes at aldehydic functions on side chains of the collagen molecules where cross-linking normally takes place. Fujii et al. (5) have demonstrated that skin and bone collagens from vitamin B_6 deficient rats is more soluble than those from control groups. Such hydrating effects on chick-embryo cartilage by lathyrogenic compounds have been demonstrated in chick-embryo cartilage by Levene (10). Skeletal lesions have been produced in weanling rats fed sweet pea seeds or aminonitrile (14, 15). One to two weeks after administration of the latter substance, the cartilage matrix began to disintegrate, particularly in the area of growth plates between the zone of hypertrophic cells and calcified cartilage zone; slippage of the epiphyses sometimes occurred at this level. Tongues of hypertrophic cell cartilage extended deep in the metaphyses, but eventually normal amounts of osteoid were visible. Ligamentous slippage, such as interspinous ligaments at the junction of the lumbar dorsal spine, was found with weakening of the ground substance of the vertebral growth plates. Although, the staining patterns for proteoglycans were more variable, ^{35}S sulfate uptake into the cartilage by audioradiography showed equal deposition in control and lathyritic rats, suggesting in Ponseti's study no overt error in sulfate metabolism of chondroitin sulfate turnover (15). It seems most likely that the impact of the loss of cross-linking is weakening of collagen networks and junctional fibril assemblies between metaphyseal and growth centers, among tendon attachments and within the articular cartilage per se.

The current morphological subarticular growth center findings and gross microscopic findings in the articular zone substantiate a similar response in vitamin B_6 deficiency; tissue responses induce collagen swelling and resultant collagen weakening defects (due to lack of cross-linking). Multiple physiological disturbances dependent on faulty collagen cross-linking can be conjectured, such as in deficiency of type IX collagen – type II collagen cross-links (3, 23); this deficiency could loosen a postulated tight network of type II fibers in cartilage destroying adequate compartmentalization and strength of the fiber network against the strong osmotic forces that result from contained proteoglycans. Also, the cartilage septal mineralizing apparatus could be disturbed by this same disorder. For example, bonding type X collagen into specifically located site in the mineralizing septa could be theoretically disturbed functionally by the failure of cross-linking (19). Since

this collagen makes up a substantial component of the bonding sites between the cartilage and metaphyseal bone, loosening at this region found to occur in lathyrism could prevent adequate mineralization and stabilization of the late hypertrophic cartilage-primary spongiosa junction and downward growth of unmineralized hypertrophic cell cartilage would ensue.

Finally, although it is possible that collagen swelling could result from lateral association of more collagen fibrils or altered packing with the changed amounts and composition of perifibrillar proteins, a failure of collagen cross-linking is by far the most likely explanation of our observed findings and explains the light microscopic findings completely.

Conclusions

If one prepares vitamin B$_6$ deficient chicks in a manner such as to avoid neurological deficits by a partial deficiency of the vitamin, an experimental model of "pseudolathyrism" has been hereby produced. To obtain evidence for production of this syndrome required study at an early stage before gross defects, such as lameness, scoliosis, etc., could be detected. Nevertheless, by refined histomorphological study of the most readily available tissue sites afflicted by defective collagen cross-linking, proof of the syndrome has been obtained. Significant increase of collagen fiber diameters in the upper articular fibrocartilage zone, as well as overgrowth of hypertrophic cell cartilage in the subarticular growth center, distorting the normal pattern of secondary ossification, characterized this model. Gross rents or hemorrhages at these sites were not detected here, but might have been in the course of longer development. Whether reduced patterns of proteoglycan on TEM staining reflect a disturbance of proteoglycan attachment sites on collagen network, or indicate an additional primary disturbance in proteoglycan metabolism remains unknown.

Acknowledgements

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Discussion with Reviewers

J. Wroblewski: It is evident from the electron micrographs (Figs. 1a - 1d) that the "diets" introduced in the present study cause changes in the diameter of the collagen fibers. Also the D-banding seems to be reduced. The authors are aware of the problems involved in specimen preparation. The conventional method of fixation, dehydration, and embedding causes severe alterations in the morphology of cartilage. It causes not only collapse of proteoglycans but also shrinkage of collagen fibers. Do you think that variation in hydration of cartilage could cause this change? And, if so, how does it effect the shrinkage of the specimens during dehydration prior to embedding?

Authors: Whether the d-banding patterns in these micrographs show important shrinkage is we think debatable. Any artifacts of shrinkage afflict control and experimental groups similarly.

J. Wroblewski: The light micrographs demonstrate that there is a prominent change in the proteoglycan content of the cartilage. Did you try by other biochemical methods to further investigate which component of the proteoglycans is directly affected by the vitamin B_6 deficiency?

Authors: A few sections of epiphyseal plate cartilage were also stained with safranin-O without a counterstain and indicated a loss of proteoglycans as suggested by a marked reduction in staining which was much more evident than in Fig. 3. The 4M Guanidine-HCL proteoglycan extract contents of hexuronic acid (HA) and sulphated glycosaminoglycans have been determined by the carbazole method and dimethylmethylene blue assay respectively to investigate whether the concentration of proteoglycans is decreased in vitamin B_6 deficient cartilage. These chemical studies confirmed histological findings and showed a significantly higher concentration of HA and chondroitin sulphate in the vitamin B_6 deficient extract.

J. Wroblewski: Did you study the distribution of alkaline phosphatase in the cartilage of normal and treated chicks?

Authors: The cartilage alkaline phosphatase has not been measured but the activity of bone isoenzyme of alkaline phosphatase in plasma was measured. This parameter was found to be significantly reduced in vitamin B_6 deficiency suggesting a decreased osteoblastic activity.

S. Doty: In SEM Figure 2c, there are areas along the fibrils which is described as "nodules"; do you have any interpretation for this appearance? Has it ever been described along collagen fibrils before?

Authors: The nodularity phenomenon could well be due to matrix interstitial proteins collapsing during dehydrating under the fibers and becoming coated with gold. This lumpy appearance in articular cartilage collagen bundles has also been observed by other investigators (Poole AR, et al., J Orthoped Res 6: 408, 1988). Note that a slight nodularity is also seen on normal articular collagen fibers.

C.I. Levene: I am very interested in this subject of vitamin B_6 deficiency; my MRC group and I have been working on the role of pyridoxal phosphate as an essential cofactor of the cross-linking enzyme lysyloxidase for very many years and the data presented by the authors are consistent with our own published work. The clinical implications of this deficiency are, in my view, of major significance in the etiology of many conditions, particularly that of coronary artery atherosclerosis and wound healing; a relevant reference is: Levene CI, Murray JC, "Hypothesis: the aetiological role of maternal vitamin B_6 deficiency in the development of atherosclerosis" Lancet, 1, 628-630 (1977).

Authors: Thank you for your comments.