A Cartilage Matrix Deficiency Experimentally Induced by Vitamin B_6 Deficiency (44210)

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Abstract. **A vitamin B_6-deficiency-induced disorder in avian articular cartilage resembling osteoarthritis has been further characterized. We measured several parameters of proteoglycan (PG) metabolism, i.e., fixed charge density and sulfated glycosaminoglycans (S-GAG) content in PN-deficient versus control articular cartilage and synovial fluid from the knee joint. Statistically significant changes were: 1) decreased content and increased extractability of total sulfated PGs from articular cartilage with guanidine HCl; 2) elevation of S-GAG concentration in synovial fluid; 3) increased plasma cystathionine (sulfur amino acid) levels. PG synthesis as assessed by $^{35}SO_4$ incorporation into S-GAGs was not impaired. A lack of cartilage swelling in 0.15 M saline and the normal water content indicated that although disturbed, the collagen network was not disrupted. This finding was in agreement with a previous microscopic study that revealed no fissures in the articular cartilage. Previous findings of a normal aggregating PG size-distribution and absence of elevated metalloproteases made a disturbance of aggregating PG metabolism unlikely. Escape into the synovial fluid of small PGs, normally bound to articular collagen, was believed to result from an alteration in collagen molecular organization that could be secondary to elevated circulating SH-compounds.** [P.S.E.B.M. 1998, Vol 217]

Proteoglycan (PG) aggregates, because of their very large size, are enmeshed and immobilized within the collagen network. These two components of the cartilage matrix have been studied extensively with respect to chemical structure and biological functions, but there has been a dearth of literature concerning the effect of nutritional deficiencies. Our previous investigation on avian cartilage and bone structure in vitamin B_6 deficiency has led to the demonstration of a matrix abnormality involving collagen.

The superficial layer of the avian articular cartilage is of particular interest because it contains predominantly Type I collagen as analysed by Eyre *et al.* (1). Relevant findings on collagen interactions with PGs are based on experiments using this collagen type and chick connective tissues (2). In B_6-deficiency, collagen fibers in the surface zone of articular cartilage were found to be larger in comparison to normal controls as evidenced by transmission and scanning (TEM/SEM) electron microscopy (3). Because of the vacuum used in this technique, this finding could not be attributed to osmotically induced water shifts (4). Therefore, biochemical disturbances were sought for fiber enlargement. Among such studies, a previous chemical analysis of B_6-deficient cartilage has revealed a more soluble collagen, indicated by higher proportions of neutral salt- and acid-

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soluble collagens (5). β-aminopropionitrile (BAPN)- and d-penicillamine (β,β dimethylcysteine)-treated cartilage defects share similar chemical changes in collagen properties (6–7).

In severely B_6-deficient chick epiphyseal cartilage, Murray *et al.* (8) observed a reduction of the activity of lysyl oxidase, believed important for collagen cross-link formation. In the Fujii *et al.* (9) radiolabelling study on severely B_6-deficient rat connective tissues, collagen cross-links in skin and bone were reduced. In contrast, collagen cross-link formation in articular cartilage from moderately B_6-deficient chicks was not found to be altered after the 8-week experiment (10). The main two reasons for this discrepancy are that, in our study, collagen cross-links were quantified (mol/mol of collagen). Moreover, a major reducible cross-link in soft connective tissue, dehydrohistidinohydroxymerodesmosine, or any of the nonreducible cross-links, were analysed. With regard to osteoarthritis, no proteolytic degradative process was detectable as opposed to prevalent findings of degradation in cartilage following injury in humans and other animal models (11). In addition, previous light microscopic studies repeatedly revealed that cartilage matrix was poorly stained with safranin O for sulfated PGs (glycosaminoglycans, S-GAGs) (3–9). The present study was aimed at probing for a disturbance in PG metabolism based on this histological finding.

On the premise that either severe weakening and disruption of the collagen network have occurred with leakage of interstitial large PGs into synovial fluid or that the fraction of PGs bonded to surface layer collagen fibers was disturbed, we examined B_6-deficient versus normal cartilage for: 1) evidence of collagen network disruption by saline swelling tests and water content; 2) evidence of leakage of PGs into synovial fluid by S-GAG analysis in the fluid; 3) evidence of reduced PG content of cartilage by determination of fixed charge density, a measure of the presence of the negatively charged groups (carboxyl and sulfate anions) of the component GAGs; 4) evidence of nutritional or other impairment of S-PG synthesis by a $^{35}SO_4$ incorporation rate study; and 5) evidence of weakened collagen-PG interactions by a cartilage PG extractability study. Because SO_4 is a substrate for the synthesis of connective tissue S-GAGs, and PG metabolism is responsive to growth factors such as the somatomedins, some further systemic measurements of SO_4 and IGF-I in plasma were performed, along with markers of SH-compound metabolism.

Materials and Methods

Animals and Experimental Procedures. Day-old-male, fast-growing broiler chicks from Truslow Farms (Chestertown, MD) were randomly assigned to a control group (n = 15) fed a pyridoxine (PN)-adequate diet (6 mg PN.HCl/kg) or to an experimental group (n = 15) fed a PN-deficient diet (0.4 mg/kg). Both diets were supplied by ICN Biochemicals (Cleveland, Ohio). The diet and the animal procedures have been fully described elsewhere (10, 12, 13). To assure that the central nervous system was not affected, as in severe vitamin B_6 deficiency, the PN-deficient diet was initiated only 2 weeks postnatally. Moreover, it contained yeast to compensate for the lack of dietary vitamin B_6 and to sustain normal growth of animals. Chicks were weighed at the beginning of the experiment; each group had a similar initial body weight distribution (average weight: 40g). They were provided feed and water *ad libitum* and allowed to grow until 8 weeks of age. All animals were examined once a week for characteristic B_6-deficiency symptoms, including loss of appetite, growth retardation, and neurological symptoms. Experimental procedures were reviewed and approved by the VA Medical Center animal care committee in accordance with current National Institutes of Health policy.

Blood Chemistry. Blood, collected in a 5-ml tube containing heparin at the end of the experiment, was immediately centrifuged at 4°C and at low speed (1000 rpm for 15 min) to obtain plasma. Aliquots of plasma were kept at –70°C until chemical analysis. Plasma sulfur (S)-amino acids were determined by an HPLC technique with pulsed integrated amperometry (14). Plasma total inorganic sulfate (iSO_4^{2-}) was measured by a microassay using controlled flow anion chromatography (15). Red blood cells were resuspended in an equal volume of isotonic saline and centrifuged for 10 min at 1000 rpm. The nutritional B_6-status was evaluated after the sixth week of depletion by the biochemical determination of pyridoxal 5′-phosphate (PLP, coenzyme form) concentration in erythrocytes (16).

Circulating insulin-like (IGF-I) growth factor. Plasma IGF-1 was measured by RIA as described by Zhao *et al.* (17). To separate IGF-I and -II from IGFBP (binding proteins), plasma samples (500 μl) were chromatographed in 0.25 mol/l formic acid on a 0.9 × 100-cm column containing Sephadex G-50 (Pharmacia LKB, Piscataway, NJ). Eluent containing free IGF-I and -II (between 47 and 72 ml) was collected, lyophilized, and resuspended to the original sample volume (500 μl) using RIA buffer (0.03 mol/l sodium phosphate, 2.5 g/l bovine serum albumin, 0.2 g/l sodium azide, pH 7.5). By spiking samples with known amounts of IGF-I, we determined a recovery of 90% for IGF-I (data not shown). IGF-I was measured using ^{125}I-IGF-I as radioligand and a polyclonal anti-rabbit somatomedin C-IGF-I antibody distributed through the Hormone Distribution Program of the NIDDK to the National Hormone and Pituitary Program.

Cartilage Analysis. *Fixed charge density (FCD) and physicochemical properties.* Immediately after sacrificing the animals by cervical dislocation, the full thickness of articular cartilages from the left medial femoral condyles were carefully dissected (with care to avoid entering the growth center) with an aseptic scalpel blade. Each slice was blotted, then placed in a pre-weighed vial, weighed and equilibrated in 0.15 mol/l NaCl for swelling determination. The minimum soaking volume was 100

times that of the slice, and the time of immersion was 1 hour (18–19). At the end of soaking, each slice was carefully blotted and re-weighed. Aliquots from the soaking solution were analyzed for sulfate to detect PG leaching. The amount of slice swelling was determined by the increase in weight of the slice relative to the original weight. Fixed charge density analysis was performed using the tracer-cation equilibration technique as described previously (20).

$^{35}SO_4$ uptake. The right femur was removed, and thin layers of articular cartilage were harvested from the medial condyle under sterile conditions. Immediately after removal, slices (50–75 mg wet weight) were immersed in 1 ml Hank's solution to assess PG synthesis by $^{35}SO_4$ incorporation into S-GAGs. Thereafter, they were weighed, minced, and incubated aseptically for 2 days at 37°C in a filtered HAM's F12 solution with 0.04 mol/l $NaHCO_3$, 0.02 mol/l HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Gibco BRL, Grand Island, NY), PEN (10 units/ml)-Strept (10/ml), CR-ITS (insulin 5 mg/l, transferrin 5 mg/l, selenious acid 5 μg/l) and ascorbic acid (50 mg/l), pH 7.4. The media were changed twice each day, and the label was added the third day for 6 hr as 10 μCi/ml $^{35}SO_4$ (Dupont, New Research Products, Boston, MA) in 0.5 ml medium per well. At the end of the incubation period, media were removed, and cartilage pieces were washed twice with HAM's F12 solution for 10 min, 1 ml per well. PGs synthesized by cultured tissue explants were extracted and isolated chromatographically in 4 mol/l guanidine HCl (21). For PG extraction, 0.5 ml, 4 mol/l guanidine HCl (Fluka, Buchs, Switzerland) was used in 1% CHAPS [3 (Cholamidopropyl)-dimethyammonio) propanesulfonate], 100 mmol/l sodium acetate, containing the following protease inhibitors:1mmol/l disodium-EDTA, 10 mmol/l dithiotreitol (DTT) (Calbiochem, San Diego, CA), PMSF (phenylmethylsulfonylfluoride, Sigma, St-Louis, MO), 15 mmol/l benzamidine HCl (Aldrich Chem, Milwaukee, WIS) at pH 5.8. The samples were shaken overnight at 4°C and centrifuged. The extracts were transferred to microfuge tubes and 0.5 ml 4 mol/l guanidine HCl was added to non-extractable residue. The tubes were shaken for 5 min, and the two extracts were pooled. ^{35}S-labelled PGs were isolated chromatographically with a PD10 column (Sephadex, Pharmacia), and eluted fractions were counted by 2200 CA liquid scintillation counter (Packard, Downwers Grove, ILL).

Collagen content and PG extractability. Other articular cartilage specimens were immediately immersed in 50 mmol/l sodium phosphate buffer and 0.15 mol/l NaCl (PBS), pH 7.4, supplemented with protease inhibitors as described above, and kept frozen at −70°C until chemical analysis. Aliquots were lyophilised and weighed prior to the collagen analysis. Hydroxyproline, used as a marker for the collagen content, was determined after 6N HCl hydrolysis according to Woessner's technique (22). Other frozen aliquots (average 32 mg) were diced into small cubes (≈ 1 mm^3). PGs were extracted with 10 volumes (v/w) of a solution of 4 mol/l guanidine HCl (Fluka, Buchs, Switzerland) in 1% CHAPS [3 (cholamidopropyl)-dimethyammonio) propanesulfonate], 100 mmol/l sodium acetate, containing the following protease inhibitors:1mmol/l disodium-EDTA, 10 mmol/l dithiotreitol (DTT) (Calbiochem, San Diego, CA), PMSF (phenylmethylsulfonylfluoride, Sigma, St-Louis, MO), and 15 mmol/l benzamidine HCl (Aldrich Chem, Milwaukee, WIS) at pH 5.8. The samples were shaken overnight at 4°C and centrifuged at 20,000 rpm for 15 min at 4°C to separate nonextractable material. Solubilized PG extracts were dialysed against H_2O. S-GAG, specifically chondroitin sulfate, and protein concentrations were determined according to specific standard techniques (23–24).

Synovial Fluid Analysis. Samples were obtained from the knee joint. Then 200 μl of sterile saline solution was injected into the joint, and synovial fluid was aspirated. All samples were immediately frozen at −70°C until use. The S-GAG content of the synovial fluid was determined using the alcian blue precipitation assay (23). Before samples were assayed, they were pre-treated with papain (100 μl of papain in buffer added to 100 μl of fluid; 550 U/l in 0.6 mol/l sodium acetate, pH 6.0, with 10 mmol/l EDTA and 50 mmol/l cysteine) and hyaluronidase (Bio-Rad, Cambridge, MA) as described by Carlson *et al.* (25). Bovine trachea chondroitin sulfate (Sigma Chemical) was used as a standard for the assay. A known volume of synovial fluid was lyophilised. The dried samples were hydrolysed with 12 N HCl in vacuo in an N_2 atmosphere for 24 hr at 110°C. The hydrolysates were evaporated, and the residues were dissolved in 50 μl of distilled water. Hydroxyproline content of each sample was directly quantified by an amino acid analyser (Varian 5500 liquid chromatography, AA911 column, Interaction) (26).

Statistical Analysis. All data are reported as means ± standard deviation (SD). The significance of differences between mean values was then determined by a two-tailed unpaired Student's *t* test or a Mann-Whitney test if the data were not normally distributed. For all statistical tests, values of $P \leq 0.05$ were considered to be significant.

Results

Evaluation of Systemic Factors Potentially Affecting Cartilage. The current B_6-deficient animals maintained a normal growth rate throughout the experimental period. Their body weight at the end of the experiment did not differ from that of the control group. Moderate vitamin B_6 deficiency was confirmed biochemically by a 48% reduction in erythrocyte PLP levels. At this stage, neurological symptoms, characteristic of severe deficiency, did not develop. However, the animals displayed a progressive lameness and swelling of the knee joints. Edema of the synovial membranes, slight widening of epiphyseal contours, and slight curvature of the tibial bones were observed during dissection at sacrifice. None of these findings were seen in normal age-matched controls. However, B_6-deficient joints were not unstable or subluxed, and bone

lengthening previously measured on x-rays was found normal (5).

No abnormality in S-amino acid metabolism was observed in the B_6-deficient group except for the significant ($P < 0.05$) elevation of plasma cystathionine, a homocysteine derivative (Table I). Plasma total inorganic SO_4 and IGF-1 levels were not affected by the nutritional depletion treatment.

Biochemical Findings in Cartilage and Synovial Fluid. Vitamin B_6 deficiency did not alter the water content and swelling properties of articular cartilage (Table II). Cartilage from the B_6-deficient animals swelled by 11% as compared to 10% in the control cartilage. However, fixed charge density, used as a chemical marker for PGs, was significantly reduced ($P < 0.001$), expressed either per dry or wet weight (Table II). The collagen content as estimated by hydroxyproline and PG synthesis as assessed by $^{35}SO_4$ incorporation rate were comparable in both groups (Fig. 1A,B). A slight trend upward of $^{35}SO_4$ incorporation (although not significant) was observed in B_6-deficient cartilage incubates at the end of the experimental period. The S-GAG content of 4 guanidine HCl extracts from B_6-deficient cartilage, as measured by chondroitin sulfate level, was significantly ($P < 0.01$) greater than those of controls (Fig. 2A). The protein concentration in final extracts was similar in both groups indicating that similar yields were obtained during extraction (Fig. 2B). The concentration of S-GAG in B_6-deficient synovial fluid was significantly ($P < 0.01$) higher in comparison to controls (Fig. 3A).

Discussion

In the early stages of osteoarthritis as reproduced in a dog model, the proportion of PGs firmly associated with collagen (hence not easily extractable) was reported to be diminished (27). The fate of PGs in other well-known connective tissue disorders involving either a collagen defect such as in lathyrism, homocystinuria, and d-penicillamine treatment is not well-documented. One study showed a decrease in the number of PG granules in the cartilages (as vizualized under electron microscope) of animals treated with d-penicillamine (28). PG alterations were restricted to regions with altered collagen structure. These authors speculated on the possibility that PG loss was a secondary process, and it was due to the damaged collagen structure that could no longer fix PG molecules, thereby facilitating their diffusion from the tissues. This is likely the same explanation for the observed loss of cartilage PGs in our animal model of osteoarthritis. To our knowledge, this is the first report on a PG disturbance consecutive to a collagen structural defect in a vitamin deficiency.

The present study showed that B_6-deficient articular cartilages extracted under dissociative conditions (4 mol/l guanidine HCl) contained more S-GAGs ($p < 0.01$), but whether this finding corresponds to a diminished proportion of PGs firmly associated (hence not easily extractable) as in McDevitt & Muir's dog model (27) or a higher extractability due to PGs not tightly bound to collagen is not clear. Transport ultracentrifugation profiles of normal avian cartilage PGs have shown two major peaks representing chick aggrecan 8–11 and small aggregates 22–39 Svedberg units; in vitamin B_6 deficiency the size distribution profile was approximately the same (29). The current analysis of the synovial fluid indicated that a greater proportion of PGs diffused out into the joint cavity. This enhanced release from articular cartilage was evidenced by the significantly higher synovial fluid S-GAG concentration in comparison to controls. This new finding supports the idea that the decreased fixed charge density (per g dry or wet tissue) observed in B_6-deficient cartilage and used as a chemical marker for PGs, reflected an actual loss of PGs from the tissue. First, in the presence of an intact collagen network, shown by normal swelling properties, large undegraded PGs could not easily migrate from the cartilage surfaces into the synovial fluid. Secondly, in comparison to mammalian hyaline cartilage, the proportion of large PGs in chick articular cartilage is low and from previous evidence, (9) they were probably not degraded because functional metalloprotease activity was not increased. Therefore, further studies on synovial fluid aggrecan ligands and chase experiments on cartilage to complement the current sulfate uptake data seemed unnecessary. The fixed charge density analysis is consistent with previous histological studies having revealed repeatedly that the B_6-deficient cartilage matrix was poorly stained with safranin O, used as a histochemical marker for PGs (3, 9).

The present study has also demonstrated that the thickening of collagen fibers previously observed in B_6-deficient

Table I. Blood Biochemistry

	Body weight	Erythrocyte PLP	Plasma IGF-1	Plasma cystathionine	Plasma total $_iSO_4$
	g	nmol/l blood	µg/l	µmol/l	mmol/l
$+B_6$	1004 ± 97 (15)	162.4 ± 29.3 (12)	16.9 ± 4.4 (11)	39.8 ± 9.1 (12)	2.18 ± 0.44 (10)
$-B_6$	965 ± 62 (15)	84.1 ± 15.2*** (11)	14.2 ± 3.3 (11)	47.3 ± 6.0* (11)	2.20 ± 0.32 (12)

Means ± SD; number in brackets represents sample size; PLP: pyridoxal phosphate; $_iSO_4$: total inorganic sulfate; IGF-1: insulin-like growth factor

*** $P < 0.001$

* $P = 0.03$

Table II. Physicochemical Properties of Articular Cartilage from Medial Femoral Condyle

	Fixed charge density[1]		Hydration		Swelling[4]
	Dry tissue	Wet tissue	%	Ratio[3]	%
$+B_6$	0.436 ± 0.065[2]	0.121 ± 0.017	73.0 ± 1.8	2.73 ± 0.26	10.4 ± 4.3
$-B_6$	0.357 ± 0.059***	0.101 ± 0.016***	72.2 ± 1.4	2.61 ± 0.17	11.3 ± 5.1

[1] after 18 hr incubation in 15 mmol/l NaCl; data are expressed as mol/g tissue.
[2] Means ± SD; $n = 15$ in each group; analysis was done on duplicates.
[3] wet to dry weight (mg).
[4] increase of initial weight after 150 mmol/l NaCl incubation.
*** $P \leq 0.001$.

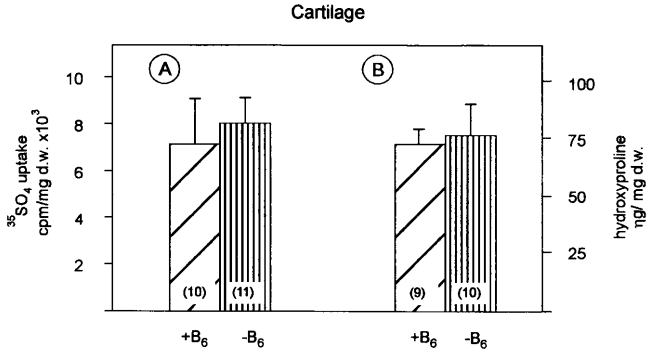

Figure 1. Characterization of articular cartilage from medial condyles in vitamin B_6 deficiency in comparison to age-matched normal controls: A) cartilage $^{35}SO_4$ uptake expressed as cpm x 10^3/mg dry weight tissue; B) cartilage hydroxyproline (µg/mg dry weight tissue) used as a marker for the collagen content. Data are expressed as mean ± SD.

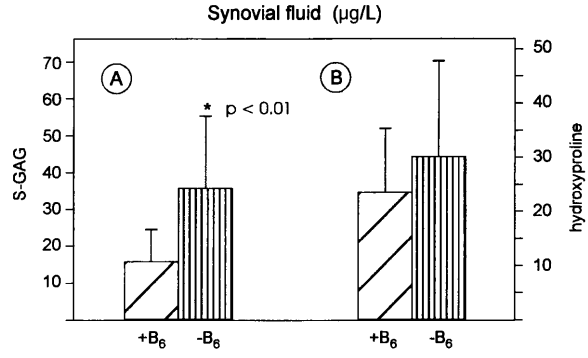

Figure 3. Characteristics of synovial fluid from the knee joint in vitamin B_6 deficiency in comparison to age-matched normal controls: A) sulfated glycosaminoglycan (S-GAG) concentration; B) hydroxyproline concentration (µg/l). Data are expressed as mean ± SD. ** $P \leq 0.01$

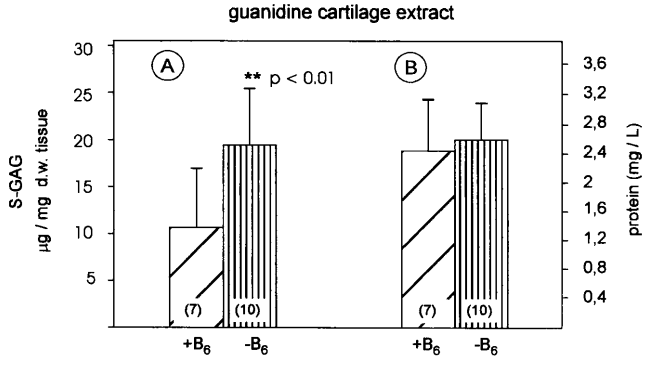

Figure 2. Characteristics of cartilage proteoglycan (PG) extract in 4 mol/l guanidine HCl from the medial femoral condyle in vitamin B_6 deficiency in comparison to normal controls: A) sulfated glycosaminoglycans (S-GAG) content (µg/mg dry weight tissue); B) protein concentration (mg/l). Data are expressed as mean ± SD. ** $P \leq 0.01$

articular cartilage by electron microscopy cannot be attributed to a hydrating effect as in lathyrism, a disorder due to a complete inhibition of lysyl oxidase activity by BAPN (6). In effect, this articular cartilage did not swell significantly in saline and was not hyperhydrated. These new observations combined with previous evidence that the formation of collagen cross-links was not impaired (9), implying that lysyl oxidase enzyme is normally active, underlie a collagen defect of a different origin. Electron microscopic studies on collagen assembly in various chick connective tissues support the view that some PGs act to control packing density of the fibrils and regulate their size (2). X-ray diffraction analysis of normal adult human femoral heads showed that a reduction of fixed charge density was inversely related to higher intermolecular spacing (d value), a situation explained by a lower molecular packing density (4). In the mutant cmd/cmd (cartilage matrix deficiency) mouse, which had genetically failed to synthesize PGs but was normal in terms of collagen synthesis, collagen fiber diameter increased (30). In view of these considerations as well as our previous TEM/SEM study (3), we postulated that the reduced PG content in B_6-deficient articular cartilage was functionally related to the thickening of collagen fibers.

The unchanged plasma inorganic iSO_4 concentrations indicated that the body pool as well as relevant renal functions were not impaired in our B_6-deficient chick model of osteoarthritis (28). This finding also indicates that the available pool of iSO_4 substrate for sulfation of PGs was not modified, suggesting an adequate chondrocyte PG synthesis, as revealed by a normal rate of incorporation of $^{35}SO_4$ into S-GAG. Sulfoconjugation reactions for which iSO_4 serves as a substrate are essential steps in PG biosynthetic pathway. An abnormality in the S-amino acids metabolism was observed which consisted of a significant ($P < 0.05$) elevation of cystathionine. This S-amino acid in excess and/or its sulfhydryl (-SH) derivatives offer a plausible etiopathogenesis for the collagen defect in vitamin B_6 deficiency, based on analogies that can be drawn from the collagen defect resulting from exposure to

homocysteine and d-penicillamine, conditions that have been well-characterized. This possibility needs further exploration and a brief review here.

The collagen defect in both pathologic conditions aforementioned has been attributed to the presence of a thiazine ring complex formed from the condensation of [-SH] groups from homocysteine in excess and aldehyde groups from collagen produced after oxidative deamination of lysine and [-OH]lysine residues under the action of lysyl oxidase (32–33). In moderate vitamin B_6 deficiency (as in cystathioninuria), the activity of PLP-dependent cystathionine-lyase (EC 4.2.1.15) is reduced (34). This explains the elevation of plasma cystathionine in the present study, a finding that is in agreement with other reports (35–36). Cystathionine and/or an SH-containing compound might be a key to the origin of the current syndrome. Data from Ricci *et al.* (37) strongly suggest that a cyclised form of the monoketo analog of cystathionine is produced under the action of L-amino acid oxidase. This cyclization is consistent with the behavior reported for the products of deamination of the lysyl residues of collagen side chains. The question remains as to whether the primary defect is caused by alteration of collagen structure or PG metabolism or both. An alteration involving PGs as a primary defect in PN-deficient articular cartilage due to excess cystathionine or other S-containing compounds cannot be ruled out. Recently, in a genetically engineered model of cystathioninuria by jointly overexpressing the metB gene coding for cystathionine synthase and disrupting the metC gene coding for cystathionine-lyase, it has been demonstrated that a cystathionine isomer (L-*allo*-) and another structurally equivalent chemical (*meso*-lanthionine), could be directly incorporated into the peptidoglycan structure of a bacterial cell wall (38). At this time, no report on such incorporation of similar S-containing compounds in mammalian or avian cartilage PG structure could be found.

As expected, when growth and anabolic activities proceed normally, circulating IGF-1 concentrations were not affected in our vitamin B_6-deficiency model. This parameter was measured for three reasons: 1) PG metabolism is responsive to growth factors such as somatomedins; 2) IGF-I is, in part, nutritionally regulated; a study has shown a decreased serum level in response to protein-energy malnutrition as well as zinc and vitamin C deficiencies (39); and 3) the effect of vitamin B_6 deficiency on circulating IGF-I had not been previously determined. Values found in the present study were comparable to previous reports on avian species (38–39).

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