Effect of Experimental Chronic Renal Insufficiency on Bone Mineral and Collagen Maturation

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Abstract The effect of chronic renal disease on bone matrix and mineral maturation was evaluated in rats with experimental renal insufficiency of 2–11 wk duration utilizing bromoform-toluene gradient fractionation of bone powder, pulse labeling experiments with \(^{40}\)Ca and proline-\(^{3}H\) differential extraction, and X-ray diffraction techniques.

Maturation defects in both collagen and mineral (\(^{40}\)Ca) metabolism were documented as early as 2 wk after the induction of uremia, when total bone calcium, inorganic phosphate, and hydroxyproline content were unchanged. The maturation defect progressed with advancing uremia despite insignificant changes in plasma pH and calcium, and normal bone carbonate levels.

Although circulating levels of 25-hydroxycholecalciferol were significantly lower than normal in the uremic animals, pretreatment with either this vitamin D metabolite or vitamin D\(_{3}\) itself failed to alter the observed changes in skeletal maturation.

Introduction

Chronic renal failure is attended by a metabolic acidosis, intestinal malabsorption of amino acids (1) and minerals, alterations in vitamin D\(_{3}\) metabolism as well as increased circulating levels of parathyroid hormone, and uremic toxins such as indoles and guanidines. These biological derangements either singularly or in concert result in a form of osteopenia which because of its varied histological manifestation is termed “renal osteodystrophy” (2, 3). Pellegrino and Blitz (4) and Kaye, Frueh, and Silverman (5) have reported alterations in the chemical composition and “exchangability” of bone mineral obtained from uremic subjects. Recently, Nichols et al. demonstrated that both the metabolic activity of bone cells and collagen turnover are increased in virtually all patients with chronic renal insufficiency despite absence of bone disease by routine radiographic analysis (6). Preliminary observations from this laboratory have revealed an increase in the soluble, immature bone collagen pool and a decreased concentration of mature, insoluble bone collagen (7).

Clearly multiple factors are operative in chronic renal disease which contribute to the derangement in bone metabolism. Osteitis fibrosa, osteosclerosis, and osteomalacia have been well documented histologically in patients with end-stage renal failure (8, 9) and variably attributed to relative degrees of parathyroid hyperfunction (10, 11), defective renal synthesis of biologically active vitamin D\(_{3}\) metabolites (12, 13), chronic acidosis (14), and calcium malabsorption. To date however, the sequence of biochemical events which initiate and perpetuate the derangements in skeletal metabolism are still ill-defined. This study was undertaken to more fully delineate the defect in collagen metabolism in the chronic uremic state and to relate these changes to alterations in matrix mineralization.

Methods

Female Holtzman rats at 6 wk of age were unilaterally nephrectomized with contralateral segmental renal infarction under light ether anesthesia. The surgery resulted in a \(^{1}\) ischemic infarction of functioning renal mass which persisted during the subsequent 2, 5, or 11 wk period and was accompanied by an intestinal malabsorption of calcium (15). During this time, all rats were maintained on Purina laboratory chow ad lib. (Ralston Purina Co., St. Louis, Mo.) and tap water supplemented with 56 mM glucose and 50 mM sodium chloride. Concomitantly nonoperated littermates were pair-fed to the uremic rats so that both groups were of similar weight.

At the time of sacrifice all animals were bled via the abdominal aorta and the serum analyzed for calcium (16), inorganic phosphate (17), blood urea nitrogen (BUN) \(^{1}\)

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1 Abbreviations used in this paper: ACP, amorphous calcium phosphate; BUN, blood urea nitrogen; 25HCC, 25-

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and creatinine (18). Serum samples from both 5-wk uremic and pair-fed control groups were additionally analyzed for levels of 25-hydroxycholecalciferol (25DHC) by the method of Haddad and Chyu (19). Tibiae were excised, freed of adhering connective tissue, and washed with cold isotonic saline to remove all marrow constituents. After mincing, the bone fragments were air-dried and processed as described below to determine the bone density-gradient distribution of the mineral and collagenous components.

After 4 wk of renal insufficiency, two groups of animals were given either 500 IU of 25HC or 1000 IU of vitamin D3 per day orally in cottonseed oil, for 7 days. After such therapy the rats were sacrificed as described above and the tibiae analyzed as outlined below. The fragmented air-dried bone was reduced to a fine powder (particle size 10 μ or less) in a Spex bone mixer-mill (model 5000, Spex Industries, Inc., Metuchen, N. J.), a weighed amount then layered on top of a continuous bromoform-toluene density gradient (sp gr, 1.7–2.3). After 30 min of centrifugation at 10,000 rpm, measured portions were pipetted from the tube, each corresponding to a particular density fraction of 1.7–2.3 sp gr. Bone powders were distributed throughout the gradient, the less dense localized to the 1.7 to 2.0-sp gr fractions and more dense in the 2.1 to 2.3 sp gr fractions. Since the particles were 10 μ or less in size, the probability that one particle was representative of more than one osteon was slight, the diameter of an osteon being estimated as 200 μ (20). That this density-gradient profile of bone reflects states of mineral-collagen maturation has been well substantiated previously by Lapiere (21).

Each density fraction was then evaporated to dryness in air and analyzed for the concentration of calcium (22) and hydroxyproline (23). Bone carbonate levels were also measured in 5-wk uremic animals and their pair-fed controls. The bone mineral and matrix-gradient profile was obtained in animals with uremia of 2, 5, and 11 wk duration, as well as in those who had been previously treated with either vitamin D3 or 25HC for 1 wk.

To further substantiate that the density-gradient technique was representative of varying maturational states of bone collagen and mineral, bones from 5-wk uremic and pair-fed control rats were extracted with neutral salt, dilute-acid, and ethanol-ether solutions. Jackson and Bentley (24) have equated the degree of extractability of collagenous protein with its state of maturation or polymerization, and Mills and Bavetta (25) have demonstrated that this premise is valid for bone collagen and mineral. Thus the most easily extractable or neutral salt-soluble collagen represents the newly synthesized, least polymerized collagen polypeptides whereas the collagen extracted by dilute acid and ethanol-ether represents more polymerized moieties. The remaining insoluble collagen is the most mature, highly cross-linked component of the bone matrix. In these experiments the bones were progressively extracted with 0.5 M sodium chloride, 5% trichloroacetic acid and ethanol-ether (1:1:1) for 1-day periods each, and the calcium and collagen hydroxyproline contents of each fraction determined as described above. The remaining insoluble bone collagen was similarly analyzed.

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Measurements of Calcium, Phosphate, BUN, Creatinine, and pH in Rats with Chronic Renal Insufficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uremic rats (41)</td>
</tr>
<tr>
<td>Calcium, mg/100 ml</td>
<td>10.23 ± 0.39</td>
</tr>
<tr>
<td>Inorganic phosphate, mg/100 ml</td>
<td>9.66 ± 0.33</td>
</tr>
<tr>
<td>BUN, mg/100 ml</td>
<td>56.00 ± 2.59</td>
</tr>
<tr>
<td>Creatinine, mg/100 ml</td>
<td>1.24 ± 0.05</td>
</tr>
<tr>
<td>pH</td>
<td>7.40 ± 0.35</td>
</tr>
</tbody>
</table>

* The numbers in parentheses indicate the number of rats used in each experimental group. All values represent the mean ±1 se; statistical comparisons were made according to the Students’ t test.

Since Richelle (30) had demonstrated that fractions of increasing density corresponded to more mature bone mineral and matrix components, a series of pulse-labeling studies were also designed to distinguish impaired matura-

RESULTS

The arterial concentrations of calcium, inorganic phosphate, BUN, and creatinine in the rats at the time of sacrifice as well as the pH are listed in Table I. Inasmuch as no significant difference was noted in these values between the 2, 5, or 11-wk uremic animals, Table I is a composite of all data. Although no change in serum calcium or pH was noted there was a significant elevation in inorganic phosphate. Whereas the histological picture of the bones obtained from 2-wk uremic animals was normal, the histological pattern of 5- and 11-wk uremic animals was characterized by an increase in osteoid tissue and osteitis fibrosa.

Despite evidence of parathyroid overactivity as demonstrated by twofold increments in gland weights and cellular hyperplasia (as well as osteitis fibrosa and osteo-

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malacia at 5 wk) analysis of the total calcium, phosphate, and hydroxyproline content (Table II) showed comparatively little change at this time. Bone carbonate levels were also normal in these 5-wk uremic animals. No differences in the calcium and phosphate concentrations per gram of bone were noted until the uremia had progressed for 11 wk. Despite histological evidence of progressive increments in osteoid tissue, the bone hydroxyproline content of 11-wk uremic animals although lower than that of control animals was not significantly different (Table II).

The density distribution of the mineral and matrix moieties of bone obtained from the uremic animals is illustrated in Figs. 1 and 2. An analysis of the normal maturational sequence of bone revealed that with increasing age a greater proportion of the mineral and collagen matrix of bone is normally distributed in the more dense or mature fractions (a, b, c, Figs. 1 and 2). A minimal proportion is located in the immature fractions of 1.7-2.0 sp gr. In the bones obtained from animals with uremia of 2 wk duration, there was a slight increase in the mineral and collagen of less dense, immature fractions (Figs 1 and 2; a and a'). As the uremic state advanced, a greater proportion of the calcium and hydroxyproline was recovered in the less dense, more immature bone density fractions (Figs. 1 and 2; b, b' and c,c'). These findings were substantiated by the results of the salt-fractionation experiments which demonstrated a significant increase in the neutral-salt and dilute-acid soluble or immature collagen moieties in the 5 wk uremic animal (Table III).

Whether these changes in the distribution of the bone mineral and matrix were a consequence of diminished maturation and/or increased degradation of pre-existing bone could not be ascertained from an analysis of the density-gradient profiles. Consequently, a group of rats were injected sequentially with a pulse label of 40Ca and proline-4H from 24 hr to 4 days before sacrifice, during the 5th wk of renal insufficiency. As illustrated in Fig. 3, by 24 hr most of the proline-4H and 40Ca label in the bones from pair-fed control animals was located in the 2.2 and 2.3, most dense, fractions. In contrast, the distribution of radioactivity in the bones from the uremic rats

**Table II**

<p>| Mineral and Hydroxyproline Levels in Bone Obtained from Normal and Uremic Animals* |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Animal weight</th>
<th>Calcium (mg/g bone)</th>
<th>Phosphate (mg/g bone)</th>
<th>Hydroxyproline (mg/g bone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair-fed control (6)</td>
<td>150.7±2.5</td>
<td>206.09±1.15</td>
<td>97.42±3.22</td>
</tr>
<tr>
<td>2 wk uremic (6)</td>
<td>149.7±12.4</td>
<td>203.34±1.04</td>
<td>95.77±7.66</td>
</tr>
<tr>
<td>Pair-fed control (20)</td>
<td>3074±J. E.</td>
<td>201.94±2.23</td>
<td>98.98±5.96</td>
</tr>
<tr>
<td>5 wk uremic (16)</td>
<td>3074±J. E.</td>
<td>199.77±7.23</td>
<td>101.75±6.66</td>
</tr>
<tr>
<td>Pair-fed control (4)</td>
<td>3074±J. E.</td>
<td>210.05±2.19</td>
<td>92.24±2.21</td>
</tr>
<tr>
<td>11 wk uremic (4)</td>
<td>3074±J. E.</td>
<td>230.98±14.00</td>
<td>97.84±0.15</td>
</tr>
<tr>
<td>Pair-fed control (2)</td>
<td>3074±J. E.</td>
<td>230.98±14.00</td>
<td>97.84±0.15</td>
</tr>
</tbody>
</table>

*All values represent the mean ±1 se; statistical comparisons were made according to the Students’ t test.
The numbers in parentheses represent the numbers of animals in each group.

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FIGURE 1 Effect of progressive renal failure on bone calcium distribution, according to the bromoform-toluene fractionation technique of Quinaux and Richelle (30). a', b', c', represent results obtained from animals with uremia of 2, 5, and 11 wk duration, respectively; a, b, c refer to the appropriate pair-fed control population. The (*) in each instance signifies statistical significance between uremic and control animals with P < 0.001 according to the Student’s t test. The number of animals in each group are as noted in Table II.

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TABLE III
Bone Mineral and Hydroxyproline Content of Neutral Salt, Acid, Alcoholic and Insoluble Collagen Fractions*

<table>
<thead>
<tr>
<th></th>
<th>0.5 M NaCl</th>
<th>Dilute acid</th>
<th>Ethanol-ether</th>
<th>Insoluble collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total calcium, mg/g bone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-wk uremic (13)</td>
<td>2.32±0.35</td>
<td>195.0±11.2</td>
<td>1.49±0.84</td>
<td>2.40±0.24</td>
</tr>
<tr>
<td>Pair-fed control (12)</td>
<td>1.28±0.07</td>
<td>195.5±34.1</td>
<td>0.26±0.13</td>
<td>3.41±0.48</td>
</tr>
<tr>
<td><strong>Total OH-proline, mg/g bone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-wk uremic (13)</td>
<td>0.25±0.01</td>
<td>0.27±0.02</td>
<td>0.057±0.011</td>
<td>5.64±1.24</td>
</tr>
<tr>
<td>Pair-fed control (12)</td>
<td>0.18±0.01</td>
<td>0.24±0.01</td>
<td>0.028±0.002</td>
<td>6.67±0.70</td>
</tr>
</tbody>
</table>

* According to a modification of the methods of Jackson and Bentley (24) and Mills and Bavetta (25); statistical comparisons were made according to the Students' t test. All values represent the mean ±1 SE. The numbers in parentheses indicate the number of animals in each experimental group.

was shifted to the left, into the more immature mineral and matrix moieties. 4 days after the administration of the radioactivity, a minimal proportion of the label was located in the 1.7 to 2.1-immature fractions in the control animals, whereas the concentration of tritiated or 46Ca radioactivity in the density gradients of bones obtained from uremic animals was the greatest in these same low density fractions. The decreased radioactivity content of the higher density, more mature bone fractions (2.2 and 2.3) observed in this study could have resulted from a combination of decreased maturation and increased resorption, the latter reflecting the attendant increment in circulating parathyroid hormone which characterizes the chronic uremic state. The accumulation of radioactivity in the less mature 2.0 and 2.1 density fractions is consistent with the interpretation that renal failure imposes maturational defects in bone mineral and matrix metabolism.

The effect of chronic uremia on circulating 25HCC levels is illustrated in Table IV. Although the chronic uremic state was associated with a significant decrease in circulating 25HCC, pretreatment with either vitamin D₃ or 25HCC in daily doses of 1000 or 500 IU respectively failed to normalize the distorted density-gradient patterns of the mineral or collagen moieties of bone described above for the untreated uremic animals (Figs. 1 and 2; b and b').

DISCUSSION
The studies described herein demonstrate that progressive derangements in mineral and collagen maturation of skeletal tissue attend the experimental chronic uremic state. These changes were associated with normocalcemia, hyperphosphatemia, and normal blood pH values. The maturational defects obtained despite normal bone content of collagen (i.e. hydroxyproline), calcium, inorganic phosphate, and carbonate, and also proved resistant to vitamin D₃ or 25HCC therapy. To date it has not been ascertained whether the defect in mineral maturation represents an independent and coincidental abnormality or the necessary result of an intrinsic defect in collagen maturation. That the defect in mineral maturation uncovered by the density gradient (Fig. 1) and 46Ca experiments (Fig. 3) is a true representation of bone mineral maturation has been further substantiated by measurements of "bone crystallinity" with X-ray diffraction analysis. The results demonstrate a significant decrease...
Bone mineral has the low Richards anid 4 days obtained in uremic signifi-
cances < 0.001 according to the Students' t test.

In mature hydroxyapatite crystals of 5-wk uremic ani-
mals (5.7% ± 1.4) when compared with age-matched, pair-fed controls (64.4% ± 1.6) as well as smaller av-
erage crystalline dimensions. These findings represent an increase in the immature, amorphous calcium phos-
phate (ACP) mineral fraction. This ACP fraction of bone mineral has been previously identified as a separate immature phase distinct from the more mature crystal-
line bone hydroxyapatite (27). Electron microscopic analysis (28) and in vitro physicochemical studies (29) indi-
cate that the ACP fraction is metastable with respect to the crystalline apatite and serves as its immediate precursor. It has also been demonstrated by Quinaux and Richelle that the ACP concentration is greatest in the low density fractions illustrated in Figs. 1 and 2, decreasing progressively in more dense, mature fractions (30).

Table IV
Effect of Chronic Uremia on Plasma 25-Hydroxycolecalciferol Levels

<table>
<thead>
<tr>
<th></th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair-fed control rats</td>
<td>7.40±0.59</td>
</tr>
<tr>
<td>5-wk uremic rats</td>
<td>3.46±0.70 (P &lt; 0.005)</td>
</tr>
</tbody>
</table>

The numbers in parentheses indicate the number of rats used in each experimental group; all values represent the mean ± 1 S.E.; statistical comparisons were made according to the Students' t test.

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Although collagen represents only 23% of the total dry weight of bone, it comprises almost 90% of the organic matrix. The normal sequence which results in a mature collagen fibril begins with the enzymatic hydroxylation of appropriate proline and lysine residues of a polypeptide "protocollagen" precursor of collagen. Cofactors essential for this hydroxylation include oxygen, Fe++, ascorbic acid (or suitable reducing compounds), and a-ketoglu-
tate (31). Since the resultant hydroxyproline accounts for 15% of the total amino acids in the collagen molecule and is not found in noncollagenous proteins, it provides a useful index of collagen content and can be equated with the collagenous protein content of skeletal tissues. Before the immature collagen molecule is extruded from the osteoblast, hydroxylysine residues may also be gly-
cosylated with glucose and/or galactose by an enzym-
atic system which requires Mn++ as a cofactor. Su-
sequent extracellular maturation of the immature "tropo-
collagen" molecule includes the formation of covalent bonds or "cross-links" between adjacent microfibrils. This "cross-linking" initially involves the conversion of the ε-amino group of lysine or hydroxylysine of the collagen peptide to an aldehyde by a copper containing, pyridoxine-dependent enzyme, amine oxidase (32). The aldehydes subsequently form the inter- or intracollagen cross-links either by Schiff-base formation or Adol condensation (32). This phase of collagen maturation can be inhibited by lathyritic agents such as β-amino- propionirile (β-APN), and D-penicillamine. Whereas β-APN inhibits amine oxidase activity (33), D-penicil-
lamine acts by reacting with the aldehydes generated by the amine oxidase reaction so that they are unavailable for cross-linking (34). In both instances the induced defects in collagen metabolism result in an accumulation of the soluble, immature pool of collagenous protein and a diminution of the mature, insoluble forms.

The progressive accumulation of less dense, more im-
mature forms of bone collagen observed in this study during the course of chronic renal failure is reminiscent of the actions of β-APN and penicillamine on collagen maturation. Uremic toxins (35) may function as in-
hibitors of the enzyme, amine oxidase, and/or chelators of ionic moieties essential for maximal enzymatic activity. They may also act as thiosemicarbazides which inhibit.
the formation of aldehydes in collagen and bind to preformed aldehydes (36, 37). In either instance the toxins would interrupt the normal progressive polymerization of the bone matrix into more mature insoluble forms. Collagen-mineral maturational defects observed in long-standing uremia may also result from alterations in bone cell carbohydrate metabolism. The hydroxylation of lysyl residues in the tropocollagen polypeptide is required to insure adequate collagen cross-linkage (32). As noted earlier, this hydroxylation step requires \( \alpha \)-ketoglutarate, a Kreb's cycle intermediate, and is accompanied by a stoichiometric decarboxylation of \( \alpha \)-ketoglutarate. Although it has not yet been demonstrated that either the intracellular hydroxylations or glycosylations of the immature collagen molecule are rate-limiting, it is worth noting that preliminary observations in this laboratory demonstrate a 50% reduction in the Kreb's-cycle activity of cartilage cells of chronically uremic animals (38). If the cytoplasmic concentrations of \( \alpha \)-ketoglutarate in uremic animals prove to be rate-limiting for the enzymatic hydroxylation of lysine, collagen deficient in hydroxylated lysine residues would be extruded from bone cells and the maturational sequence compromised. Additionally, one could link the observed derangements in bone collagen maturation to alterations in copper metabolism which apparently attend the proteinuria of chronic renal disease (39). It has been well established in animal models that copper deficiency results in bone fragility and defects in collagen maturation (40). The defect has been ascribed to an interference with the formation of lysine-derived cross-linkages between collagen polypeptides because the amine oxidase which modifies these residues is copper dependent (41).

Vitamin \( D_3 \) and its active metabolites are instrumental in affecting bone metabolism and recent observations of Canas, Brand, Neuman, and Terepka demonstrate a stimulatory effect of the vitamin on bone collagen synthesis (42). It is obvious that neither vitamin \( D_3 \) nor 25HCC deficiency (Table IV) could account for the maturational defect of skeletal tissue observed in this study since in separate experiments replacement therapy with either agent failed to reverse the biochemical lesion. Chronic uremia has been considered to represent a state of relative vitamin \( D \) deficiency because of the attendant osteomalacia, calcium malabsorption (43, 44), and documented defects in vitamin D metabolism (26, 45) which includes a decreased synthesis of 1,25-dihydroxycholecalciferol (1,25DHCC). It is possible therefore that the decrease in functioning renal mass in the uremic rats leads to a decrease in 1,25DHCC synthesis and that this metabolite is of fundamental importance in maintaining the integrity of bone collagen. In this regard, it is noteworthy that Oliver, Leavers, and Scott have reported that diets deficient in both vitamin D and calcium lead to a decrease in the rate of synthesis of soft tissue mature collagen (46), Weber, Pons, and Kodicek cite a preferential accumulation of 1,25DHCC in bone (47) and Termine and Posner observed a skeletal accumulation of immature ACP mineral in the vitamin D-deficient state (27).

In this study all the uremic animals evidenced parathyroid hyperplasia and those with long-standing disease manifested the histological changes of osteitis fibrosa. It has been demonstrated that exogenous administration of parathyroid hormone (PTH) leads to a biphasic response in collagen metabolism. After short-term PTH treatment, collagen synthesis diminishes whereas prolonged therapy leads to an elevation in the rate of collagen synthesis (48). Flanagan and Nichols have subsequently demonstrated that a similar phenomenon obtains in patients with intermittent and continually elevated PTH secretory rates (49). Additionally, prolonged periods of excessive PTH secretion are attended by increased degradation of pre-existing bone (50). Neither of these aforementioned phenomena would however, account for the maturational changes in bone which were documented in these animals with chronic uremia and progressive secondary hyperparathyroidism. Moreover, Bergstrom, Jacobs, Jones, and Prior have demonstrated that in the chronically uremic rat, ablation of the parathyroid glands reduced but did not eliminate the skeletal defects (51). Clearly therefore, factors other than elevated circulating PTH levels are operative in establishing and perpetuating the maturational derangement of mineralized tissue accompanying the chronic uremic state.

Although the biochemical changes attending the chronic uremic state may exert independent and unrelated effects on the mineral and collagen components of skeletal tissue, it is tempting to postulate that the overall changes in mineral metabolism observed in the uremic animals are actually secondary reflections of the changes in collagen metabolism. The unique physical properties of collagen distinguish it from all other proteins and as such the oriented pattern of mature skeletal collagen serves as a nidxus for the deposition of mineral (52). The sites for nucleation and mineral deposition within the protein matrix of bone are geometric features intrinsic to the 640 A repeat crystal lattice of collagen (53). This "nucleation theory" is strengthened by the demonstration that maturation and increased cross-linking of newly synthesized collagen preceeds calcification (25, 54). It is also supported by the observation that the altered maturational sequence of bone collagen attending experimental lathrytic states is characterized by an increase in the immature ACP mineral fraction. The present studies do demonstrate that in chronic renal insufficiency decreased maturation of both matrix and mineral components of osseous tissue: (a) preceed any detectable al-

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terations in total bone mineral and collagen content; (b) are not directly related to circulating levels of vitamin D3 or 25HCC, alterations in bone carbonate or plasma pH; and (c) become progressively severe as the uremic state advances. The relationship of these findings to 1,25DHCC metabolism, PTH, and circulating uremic toxins is still to be determined.

ACKNOWLEDGMENTS

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