Short Communications

The Nature of the Collagen Cross-Links in Bone in the Chronic Uraemic State

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The cross-links from NaB^3H_4 -reduced bone collagen of chronically uraemic rats and paired controls were compared. The ratio of the reduced cross-links $\delta\delta'$ -dihydroxylysinoxonorleucine to δ -hydroxylysinoxonorleucine was significantly increased in the uraemic animals. The observed increment in the dihydroxylysinoxonorleucine:hydroxylysinoxonorleucine ratio was accentuated as the uraemic state advanced. The data indicate that osteodystrophy of chronic renal insufficiency is characterized by an alteration of the quantitative relations between cross-links and aldehydic precursors of bone collagen.

After synthesis of the collagen molecule in the osteoblast and hydroxylation of specific proline and lysine residues, subsequent extracellular maturation of the nascent polypeptide includes formation of aldehydic precursors and their respective cross-links. Such cross-linking between adjacent microfibrils has been shown to stabilize the fibrillar macromolecular matrix of collagen, either by Schiff-base formation or aldol condensation (Börnstein, 1970). In the past, differences in types and relative abundance of borohydride-reducible cross-links have been demonstrated in tissues with different collagen architectures and in various states of maturation (Mechanic & Tanzer, 1970; Mechanic *et al.*, 1971; Mechanic, 1971). With this analytical tool, it has been shown that the most abundant reducible cross-links in bovine mineralized tissues are $\delta\delta'$ -dihydroxylysinoxonorleucine and δ -hydroxylysinoxonorleucine. The ratio of dihydroxylysinoxonorleucine:hydroxylysinoxonorleucine diminishes as bone collagen becomes more mature and mineralized (Mechanic *et al.*, 1971). Alternatively, disruption of normal maturation of collagen with lathyrin agents such as β -aminopropionitrile and D-penicillamine results in an increase in the ratio of dihydroxylysinoxonorleucine to hydroxylysinoxonorleucine, as well as in an accumulation of the soluble, immature, pool of collagenous protein and a diminution of the mature, insoluble, forms (Deshmukh *et al.*, 1973). Deprivation of chickens of cholecalciferol for as little as 7 days similarly results in defective collagen cross-linking (Mechanic *et al.*, 1972). The progressive accumulation of less dense, more immature, forms of bone collagen and mineral, which have been demonstrated to occur in the vitamin D-resistant phase of chronic renal insufficiency, by

bromoform-toluene density-gradient fractionation (Russell & Avioli, 1972) and X-ray-diffraction techniques (Russell *et al.*, 1972), is reminiscent of the actions of β -aminopropionitrile, penicillamine, and/or vitamin D deficiency on collagen maturation.

This investigation was consequently performed to study the nature of the NaB^3H_4 -reducible cross-links in compact diaphyseal rat bone during prolonged renal insufficiency. The accumulated data are consistent with an altered maturation of uraemic bone collagen, such that the chemically reduced cross-link pattern in uraemic bone of 15-week-old rats more closely resembles that of a young immature rat bone. The molecular defect is more analogous to that observed in lathyrin bone than in bone from vitamin D-deficient animals, however.

Materials and methods

Female Holtzman rats at 6 weeks of age were unilaterally nephrectomized with contralateral segmental renal infarction, resulting in an impairment of seven-eighths normal kidney function as previously described (Russell & Avioli, 1972). This ischaemic infarction of functioning renal mass persisted during the subsequent 2-, 5- or 9-week period of study. Control animals were pair-fed to maintain similar rates of growth between the two experimental groups. At the time of death, all animals were exsanguinated and plasma calcium (Willis, 1960), inorganic phosphorus (Chen *et al.*, 1956) and creatinine (*Auto-Analyzer Methodology*, 1965) determined for each. As reported in earlier studies (Russell & Avioli, 1972), the uraemic state was attended by elevation in plasma inorganic phosphate (controls, 7.58 ± 0.15 ,

Table 1. *Effect of chronic uraemia on dihydroxylysinoxorleucine and hydroxylysinoxorleucine content of bone in the rat*

Results are expressed as ratios of dihydroxylysinoxorleucine to hydroxylysinoxorleucine. Data are means $\pm$ S.E. obtained from group analysis with four or five animals per group.

Duration of uraemia (weeks)	Pair-fed control animals	Uraemic animals
2	2.41 $\pm$ 0.29	2.75 $\pm$ 0.23
5	1.97 $\pm$ 0.20	2.92 $\pm$ 0.26*
9	2.15 $\pm$ 0.17	3.13 $\pm$ 0.21*

* $P < 0.01$ according to Student's t test when compared with age-matched pair-fed control animals.

uraemics, 9.86 $\pm$ 0.23 mg/100 ml) and creatinine (controls, 0.56 $\pm$ 0.02, uraemics, 1.21 $\pm$ 0.05 mg/100 ml). Plasma pH and calcium values in the uraemic animals were 7.41 $\pm$ 0.02 and 10.13 $\pm$ 0.28 mg/100 ml respectively, and comparable with those obtained in the control group (7.40 $\pm$ 0.01 and 10.23 $\pm$ 0.34 mg/100 ml respectively).

The tibias from rats with uraemia of 2, 5 and 9 weeks duration and their pair-fed controls were immediately excised. The diaphysial portion of the bone was freed of all adhering connective tissue and marrow and demineralized with EDTA. Subsequent NaBH₄ reduction and analytical chromatography of the bone collagen was performed as previously described (Mechanic & Tanzer, 1970; Mechanic *et al.*, 1971; Mechanic, 1971).

Results and discussion

As illustrated in Table 1, there was a diminution in the ratio of dihydroxylysinoxorleucine to hydroxylysinoxorleucine in bones from pair-fed control animals during the 9-week experimental period, consistent with the progressive skeletal maturation that occurs with age. In contrast, those bones from the uraemic animals exhibited a progressive increase in the derived dihydroxylysinoxorleucine:hydroxylysinoxorleucine cross-link ratio, which was statistically elevated at 5 and 9 weeks of uraemia compared with the corresponding value obtained from the pair-fed control animals. These data reflect the altered maturation of the bone collagen, resulting in an accumulation of more immature collagen polypeptides, and as such are consistent with our previous observations with bromoform-toluene gradient techniques (Russell & Avioli, 1972).

The relationship of the uraemic maturational defect in bone to reports of defective collagen cross-linking in the vitamin D-deficient state (Mechanic *et al.*, 1972) bears consideration. In both instances there is a progressive increment in the dihydroxylysinoxorleucine:hydroxylysinoxorleucine ratio, suggesting

that insufficient quantities of α -amino adipic δ -semialdehyde are being synthesized to fulfill the cross-linking requirements of the tissue. However, the diminished ratio of hydroxylysinoxorleucine to an unknown peak 1 observed in the vitamin D-deficient state (Mechanic *et al.*, 1972) was not found in bones obtained from animals with chronic uraemia in our study. Thus it seems quite possible that, as in lathyrism, some factor may be blocking the aldehydic formation and/or cross-link condensation necessary for a normal, cohesive, mineralizing collagenous matrix.

This study does demonstrate therefore that, in addition to a specific defect in the maturation of bone mineral (Russell *et al.*, 1972), uraemia also results in a specific molecular defect in the collagenous component of the organic bone matrix. The premise that the overall changes in mineral metabolism observed in the chronically uraemic state may be secondary reflections of the changes in collagen metabolism are supported by the observations that altered maturational sequence of bone collagen attending experimental lathyrotic states is characterized by an increase in the immature amorphous calcium phosphate mineral fraction and alterations in the NaBH₄-reducible collagen cross-links (Deshmukh *et al.*, 1973), as well as previous results demonstrating that, in uraemia, defective collagen maturational profiles preceded alterations in the maturation and crystallinity of bone mineral (Russell & Avioli, 1972; Russell *et al.*, 1972). Sequential analyses on intra- and inter-molecular cross-linkages of bone in the experimentally induced chronic uraemic state together with diffractational analysis of bone mineral crystallinity and maturation may add further insight into the pathogenesis of the skeletal defects which attend the progression of renal insufficiency in man.

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