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The Effect of β-Aminopropionitrile (BAPN) on Bone Mineralization (39660)[1]

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There is evidence that collagen soluble in neutral salt solutions represents recently deposited collagen, and that more mature collagen is insoluble by virtue of intermolecular crosslinks (1). In bone, about 98% of collagen is insoluble (2). Very little increase has been found in the amount of collagen extracted by neutral salt solution after demineralization in EDTA (3). These findings suggest that the majority of soluble collagen is located in unmineralized osteoid which consists of recently deposited collagen, and that the mineral phase is located in insoluble collagen. Moreover, it has been suggested that the formation of intermolecular crosslinks in collagen may precede mineral deposition in bone matrix and may, thus, be a prerequisite for appropriate bone matrix mineralization (4).

If collagen crosslinking of bone matrix is somehow involved in bone mineralization, one would expect to find impairment of bone mineralization in response to lathyrogens, such as β-aminopropionitrile (BAPN), which are strong inhibitors of lysyl oxidase and, thus, of collagen crosslinking (5). The present study was undertaken to evaluate the effect of BAPN treatment on bone mineralization in rats.

Material and methods. Thirty growing male Holtzman rats weighing 66.2 ± 1.9 g (SD) were divided into control and experimental groups. The rats were housed separately in wire cages, and the control group was pair-fed a semisynthetic diet containing 0.6% calcium and 0.6% phosphorus, dry weight (6). For the experimental group, β-aminopropionitrile fumarate, 0.1% dry weight, was added to this diet. The animals were treated for 15 days. Five days prior to sacrifice, they were injected ip with 20 mg of tetracycline per kg body weight.

Just prior to sacrifice, blood was obtained by cardiac puncture under light ether anaesthesia. The animals were then killed by exsanguination. The tibiae were removed and stored in gauze moistened with a 10% buffered formalin solution, pH 7.4. This treatment has a negligible effect on calcium and phosphorus concentrations in young as well as mature bone, as determined by the electron microprobe (unpublished observation). The left femora were removed and stored at −20° for subsequent bone ash analyses.

Serum analyses. Serum calcium was analyzed by atomic absorption spectrophotometry (7), and phosphorus by a conventional Technicon autoanalyzer technique.

Bone ash analyses. The left femora were thawed, dissected free of soft tissue, and sawed at each end to secure diaphyseal bone (middle third) from which the bone marrow was removed by a jet of air. After rinsing in distilled water, the diaphyses were dehydrated and defatted in acetone, changed daily for 3 days. Subsequently, the bones were air dried at room temperature, weighed, and then ashed at 600° for 16 hr. The ashed bones were then cooled for 2 hr and weighed. The ash was analyzed for calcium (7) and phosphorus content.

Bone measurements. From each tibia, two 60-μm-thick sections were sawed immediately proximal to the tibio-fibular synostosis with a Gillings-Hamco thin sectioning machine (Hamco Machine, Inc., Rochester, N.Y.). The bone specimens were mounted in a goniometer to facilitate sawing sections perpendicular to the long axis of the tibial diaphysis. The sections were then hand

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ground to a final thickness of about 25 μm. All sections were labeled *in vitro* with tetracycline by placing them for 1 min in a 0.2% aqueous solution of Achromycin.[4] After rinsing in distilled water, the distal sections were mounted unstained in Abopon (Valnor Co., Brooklyn, N. Y.) and the proximal sections were stained with nuclear fast red, dehydrated in acetone, cleared in xylene, and mounted in Fluormount (E. Gurr Ltd., London S. W. 14, England). The unstained sections were used for measurements of total bone area, periosteal surface length, area encompassed by the periosteal tetracycline label, the length of this label (given 5 days prior to sacrifice), and width of the *in vitro* tetracycline label. The stained sections were used for osteoid width measurements.

These measurements were performed using a model 1010A Grafacon tablet (Compunetics Inc., Monroeville, Penn.) interfaced with a computer (PDP-8, Digital Equipment Co., Manor, Mass.). The calculations of the bone parameters from the above measurements have been described earlier (8) and are only defined below.

(i) Periosteal bone formation rate (mm^3/day) is the amount of bone or matrix formed at the periosteal surface per day. Dividing this rate by the length of the forming surface gives the bone or matrix apposition rate (μm/day).

(ii) Periosteal osteoid maturation rate (%/hr) is a measure of the onset of mineralization. The time elapsing between the deposition of osteoid and the onset of mineralization in this osteoid can be calculated by dividing the width of the osteoid by the matrix apposition rate. If one assumes the osteoid to be 0% mature when first deposited and 100% mature at the onset of mineralization, the osteoid maturation rate can be calculated simply by dividing 100% by the above time.

(iii) Periosteal initial mineralization rate (% of maximum/hr): It has been shown that tetracycline is only incorporated into bone that has 20% or less of its maximum mineral concentration (8). The time required to reach 20% of maximum mineralization is determined by converting the width of the *in vitro* tetracycline band to time, using the bone apposition rate. Dividing 20% (mineral concentration) by this time gives the rate. The initial mineralization rate and the osteoid maturation rate measure two different aspects of mineralization. The initial mineralization rate is the rate at which mineral concentration increases (up to 20% of maximum) once mineralization is initiated at the mineralizing front. In contrast, the osteoid maturation rate is a measure of the onset of mineralization, which is a function of the time elapsing between the deposition of osteoid matrix and the onset of mineralization in this matrix.

Results. Two of the 15 BAPN-treated rats had final weights of 85 and 75 g, compared with 135.9 ± 15.6 g (2 SD) for the other BAPN-treated rats, and were therefore excluded. In the remaining test animals, physical activity and appearance were similar to the control rats throughout the experiment. As indicated by the final body weight (Table I) and total cross-section area (bone and medullary area) of the tibial diaphysis (Table II), growth was similar in the two groups.

As can be seen in Table II, there was a significant decrease of 31% ($P < 0.001$) in the initial mineralization rate in the BAPN group, compared with the pair-fed control rats. However, BAPN treatment did not alter the onset of mineralization as indicated by the normal osteoid maturation rate. The ash weight of the diaphyseal (cortical) bone was significantly reduced by 5% ($P < 0.001$), whereas the ratio of Ca/P in bone ash was unchanged by BAPN treatment.

Discussion. Our results showed that, in rats, BAPN treatment caused a highly significant 31% decrease in the initial mineralization rate of diaphyseal bone. However, BAPN treatment did not inhibit the osteoid maturation rate and, thus, did not inhibit the onset of mineralization. In past studies, we consistently found that a change in mineralization rate was accompanied by a similar change in the onset of mineralization (8–10). Therefore, it is noteworthy that BAPN treatment impaired only one aspect of the mineralization process, which suggests that these two aspects of mineralization may be, to some extent, controlled independently.

Consistent with impaired mineralization

[4] Generously supplied by Lederle Laboratory Division, American Cyanamide Co., Pearl River, N.Y.

TABLE I. BODY WEIGHT, SERUM CONCENTRATIONS, AND BONE ASH WEIGHT (CORTICAL DIAPHYSEAL BONE) IN CONTROL AND BAPN-TREATED RATS.[a]

Paramater	Control (n = 15)	BAPN-treated (n = 13)	P^b
Final body weight (g)	133.6 ± 9.6[c]	135.9 ± 7.5	NS[d]
Serum Ca (mg/100 ml)	11.4 ± 0.3	11.2 ± 0.3	NS
Serum P (mg/100 ml)	8.8 ± 0.5	8.6 ± 0.5	NS
Bone ash weight (% of dry weight)	60.6 ± 1.3	57.3 ± 2.5	<0.001
Ca/P of bone ash	1.69 ± 0.06	1.67 ± 0.05	NS

[a] Diet: 0.1% BAPN.
[b] Probability estimated by Student's t-test.
[c] Mean ± SD.
[d] Not statistically significant at the 0.05 probability level.

TABLE II. PERIOSTEAL FORMATION AND MINERALIZATION IN CORTICAL BONE OF CONTROL AND BAPN-TREATED RATS.[a]

Parameter	Control (n = 15)	BAPN-treated (n = 13)	P^b
Total cross-section area (mm^2)	3.197 ± 0.245[c]	3.236 ± 0.165	NS[d]
Periosteal bone formation rate (mm^3/day)	0.067 ± 0.006	0.065 ± 0.005	NS
Periosteal bone apposition rate (μm/day)	10.3 ± 0.8	10.0 ± 0.9	NS
Periosteal initial mineralization rate (%/hr)	2.6 ± 0.4	1.8 ± 0.3	<0.001
Periosteal osteoid width (μm)	7.4 ± 1.4	6.7 ± 0.9	NS
Periosteal osteoid maturation rate (%/hr)	5.9 ± 1.5	6.3 ± 1.0	NS

[a] Diet: 0.1% BAPN.
[b] Probability estimated by Student's t-test.
[c] Mean ± SD.
[d] Not statistically significant at the 0.05 probability level.

was a 5% decrease in the percentage of bone ash in diaphyseal bone of the BAPN-treated group (Table I). Although a decrease in the percentage of bone ash has been previously observed in response to BAPN treatment, it is not clear whether or not the bone samples contained epiphyseal cartilage and/or marrow (11).

It seems doubtful, however, that a 31% decrease in the mineralization rate could have accounted for a 5% decrease in the percentage of total bone ash since, in normal diaphyseal bone from rats of the age and size used in this study, the volume of bone undergoing mineralization is only about 10% of the total bone volume (9, 13). Moreover, if, as seems likely, the decrease in bone ash was limited to the volume of lathyritic bone formed during BAPN treatment, which was about 36% of the bone sample (9, 13), the decrement in the percentage of ash in the lathyritic bone would have been much greater than the 5% decrease observed with the total diaphyseal bone sample. Thus, there may have been a decrease in maximum mineralization as well as a decrease in mineralization rate. Definitive assessment of this possibility will require electron microprobe measurements of calcium and phosphorus concentrations in lathyritic bone.

This study does not establish the mechanism whereby BAPN treatment inhibits the process of bone mineralization. Because BAPN treatment at the dose used in the present study increases the fraction of soluble collagen in bone and, thus, presumably decreases collagen crosslinking, it is possible that the latter is involved in the observed mineralization defect. Alternatively, it is possible that impaired bone mineralization and impaired bone collagen crosslinking are independent effects of BAPN treatment. If impaired collagen crosslinking inhibits the process of bone mineralization, this mechanism could be operative in vitamin D deficiency since, in bones from vitamin D-deficient animals, there is some evidence of decreased lysyl oxidase activity (15) and impaired collagen crosslinking (16).

Summary. The process of bone mineralization in the tibial diaphysis, in response to treatment with a lathyrogen, β-aminopropionitrile (BAPN), at a dose known to inhibit collagen crosslinking, was studied in growing rats. BAPN treatment impaired the

initial rate of mineralization but not its onset. In addition, in the BAPN treated group, there was a 5% decrease in diaphyseal ash weight, a decrement estimated to be greater than expected from the decreased rate of mineralization. This suggests that the maximal level of mineral deposition was also decreased as a result of BAPN treatment.

1. Jackson, D. S., and Bentley, J. P., J. Biophys. Biochem. Cytol. **7,** 37 (1960).
2. Henneman, D. H., Clin. Orthop. **83,** 245 (1972).
3. Mills, B. G., and Bavetta, L. A., Clin. Orthop. **57,** 267 (1968).
4. Avioli, L. V., Kidney Int. **4,** 105 (1973).
5. Pinnell, S. R., and Martin, G. R., Proc. Nat. Acad. Sci. USA **61,** 708 (1968).
6. Wergedal, J., Calcif. Tissue Res. **3,** 55 (1969).
7. Willis, J. B., Spectrochim. Acta **16,** 259 (1960).
8. Baylink, D., Stauffer, M., Wergedal, J., and Rich, C., J. Clin. Invest. **49,** 1122 (1970).
9. Stauffer, M., Baylink, D., Wergedal, J., and Rich, C., Amer. J. Physiol. **225,** 269 (1973).
10. Wergedal, J., Stauffer, M., Baylink, D., and Rich, C., J. Clin. Invest. **52,** 1052 (1973).
11. Bar, A., and Hurwitz, S., Comp. Biochem. Physiol. **47A,** 1257 (1974).
12. Wergedal, J. E., and Baylink, D. J., Amer. J. Physiol. **226,** 345 (1974).
13. Baylink, D. J., Morey, E., and Rich, C., Endocrinology **84,** 261 (1969).
14. Golub, L., Stern, B., Glimcher, M., and Goldhaber, P., Proc. Soc. Exp. Biol. Med. **129,** 465 (1968).
15. Siegel, R. C., Tsai, H. C., and Morris, R. C., Jr., Clin. Res. **23,** 136A (1975).
16. Mechanic, G. L., Toverud, S. U., Ramp, W. K., and Gonnerman, W. A., Biochim. Biophys. Acta **393,** 419 (1975).

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