The Effect of Magnesium and Fluoride on Bone Pyrophosphatase Activity[1] (34262)

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(Introduced by W. R. Featherston)
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Several workers (1–5) have presented evidence for an inhibitory role of inorganic pyrophosphate in the calcification process. Fleisch *et al.* (1) demonstrated that vitamin D-induced aortic calcification could be prevented by injections of either pyrophosphate or Graham salt, a long chain polyphosphate. Similarly, the calcification in rat skin induced by $KMnO_4$ injection could be checked by a simultaneous injection of pyrophosphate (2). Fleisch and Bisaz reported the isolation from plasma (3) and urine (4) of a phosphorus compound which strongly inhibits hydroxyapatite precipitation. Its chromatographic, infrared and X-ray diffraction characteristics were similar to inorganic pyrophosphate. It has been suggested (4) that plasma pyrophosphate protects soft tissue collagen from calcification. Bone collagen mineralizes, presumably, after the hydrolysis of pyrophosphate by pyrophosphatase which was demonstrated to be present in bone (6).

Gardiner *et al.* (7) reported inhibition of bone mineralization in chicks fed diets containing elevated levels of magnesium (Mg) and fluoride (F), and Elliott (8) demonstrated inhibition of guinea pig microsomal pyrophosphatase by Mg ions in the presence, but not in the absence, of F ions. The purpose of the present work was to study the effect of dietary Mg and F treatment on femur pyrophosphatase activity and its possible relation to the bone calcification process.

Materials and Methods. Day-old Hubbard White Mountain male chicks were randomly assigned to four groups and placed in electrically heated, wire-floored batteries. The four diets (control; 0.08% supplemental F; 0.45% supplemental Mg; and 0.08% supplemental F + 0.45% supplemental Mg) as described by Griffith *et al.* (9) and deionized water were supplied *ad libitum* during the experimental period. The control diet was found by analyses to contain 0.058% magnesium and 0.0001% fluoride.

A crude extraction of pyrophosphatase from bones was performed as follows. The chicks were sacrificed and the right femur was removed and freed of adhering tissue. The epiphyses were sliced off and bone marrow was removed from the diaphysis with a stream of an ice-cold solution of 0.25 *M* sucrose in 0.0025 *M* Tris–0.0017 *M* acetate buffer, pH 7.5. Both epiphyses and the diaphyses from two animals were then homogenized in 40 ml of the Tris–sucrose solution for 2 min in a Virtis homogenizer with the container immersed in ice water. The homogenate was centrifuged at 10,000*g* for 30 min in a refrigerated centrifuge and the resulting clear supernatant was removed and stored at −20° until used. Enzyme activity was not reduced by storage for as long as 5 weeks.

Enzyme analysis was initiated by adding 1 ml of the femur supernatant to 2 ml of a buffered solution of ^{32}P-labeled sodium pyrophosphate. The final concentration in 3 ml of solution was: 0.333 m*M* sodium pyrophosphate; 17 m*M* tris(hydroxymethyl)aminomethane(Tris); 21 m*M* sodium acetate; and 10^4–10^5 cpm of ^{32}P labeled sodium pyrophosphate. The pH before incubation was 7.5. A 60-min incubation time was chosen since the

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reaction was shown to be linear with time for at least 90 min. After incubation the reaction was stopped by adding 1 ml of cold 1 *N* $HClO_4$. The tubes were shaken and the precipitated proteins removed by centrifugation. One ml of the clear supernatant was added to 3 ml of an ice-cold solution of ammonium molybdate (0.330 *M* in Mo) in 2.4 *N* HCl. The ^{32}P-labeled phosphomolybdate was separated from ^{32}P-pyrophosphate by adding 3 ml of a 1:1 mixture of isobutanol–benzene and extracting. Two ml of the organic layer were removed and added to 10 ml of a scintillation solution (10) and counted by liquid scintillation spectrometry.[3] Appropriate standards were also prepared and similarly counted.

A similar procedure was followed to determine plasma pyrophosphatase activity. For this assay 1 ml of chick plasma was added to 0.1 ml of the buffer solution containing ^{32}P-labeled pyrophosphate. The final concentrations of pyrophosphate and Tris–acetate were the same as above, and 8.9 ml of 1 *N* $HClO_4$ was added to terminate the reaction. Protein analysis was performed according to a modification of the Lowry method (11) using bovine serum albumin as the standard.

Results and discussion. The effect of Mg on femur pyrophosphatase activity in the presence and absence of F is presented in Fig. 1. As shown, pyrophosphatase was strongly inhibited by Mg in the presence of F. However, similar to the results reported by Elliott (8) with liver microsomal pyrophosphatase, in the absence of F, Mg stimulated femur pyrophosphatase activity.

These findings were consistent with the hypothesis that high Mg–high F treatment inhibits bone mineralization by inhibiting bone pyrophosphatase. However, when femur pyrophosphatase activity was measured in chicks fed each of the four diets, opposite effects were observed. As demonstrated in Table I, femur pyrophosphatase activity was increased in chicks fed diets containing high levels of both Mg and F for 3 or 6 days as compared with chicks fed diets containing identical levels of either element alone. Diets containing high levels of either Mg or F alone did not appear to influence femur pyrophosphatase activity at 3 days, but at 6 days, pyrophosphatase activity was somewhat lower in the control group as compared with either the high Mg or high F group. Chemical analysis of the femur supernates showed that the Mg concentration was less than 20 μ *M* in all four groups. Thus, these results suggest an actual increase in the levels of pyrophosphatase in bones of chicks fed high Mg–high F diets rather than increased enzyme activity due to differences in Mg concentrations. This increase in femur pyrophosphatase activity may be related to the increased plasma alkaline phosphatase levels in chicks fed high Mg–high F diets as reported by Griffith *et al.* (9). Although plasma pyrophosphatase activity appeared to be decreased in 3-day-old chicks fed high Mg–high F diets, no such differences were observed after 6 days on treatment.

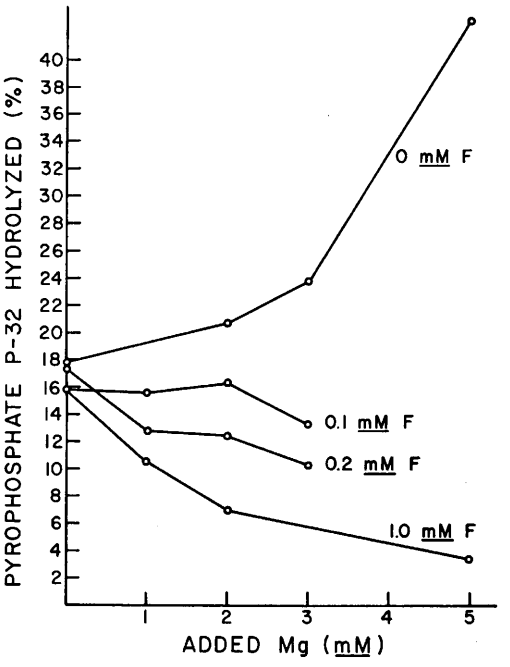

FIG. 1. Effect of added Mg on femur pyrophosphatase activity in the presence and absence of F. The incubation medium contained in a volume of 3 ml: 1 ml of femur supernatant from the control group containing 160 μg of protein, 51 μmoles of Tris, 63 μmoles of acetate, 1 μmole of sodium pyrophosphate labeled with 10^5 cpm of ^{32}P. Incubation was at 30° and pH 7.5 for 60 min.

[3] Model 314 EX, Packard Instrument Company, Downers Grove, Illinois.

TABLE I. The Effect of Dietary Treatment on Pyrophosphatase Activity in Femurs and Plasma.

Days on treatment	Treatment			
	Controls	High Mg	High F	High Mg–high F
	Femur activity[a]			
3	518 ± 21 (9)[c]	540 ± 26 (9)	477 ± 42 (9)	669 ± 43 (9)
6	321 ± 35 (3)	595 ± 60 (3)	597 ±150 (3)	1473 ±479 (3)
	Plasma activity[b]			
3	40.3 ± 6.1 (5)	36.6 ± 6.1 (5)	38.3 ± 6.4 (5)	28.0 ± 2.8 (5)
7	52.1 ± 7.2 (5)	43.7 ± 2.4 (5)	33.8 ± 2.9 (5)	46.4 ± 2.9 (5)

[a] μmoles of pyrophosphate hydrolyzed/hr/g of protein.

[b] μmoles of pyrophosphate hydrolyzed/hr/liter of plasma.

[c] The mean and standard error for the number of determinations in parentheses. Each determination represented femurs from two chicks.

The results of these studies suggest that the Mg × F inhibition of bone mineralization cannot be explained by an inhibition of femur pyrophosphatase activity. The observed increase in enzyme activity is probably an indirect effect resulting from altered bone metabolism. However, it should be mentioned that enzyme activity measured *in vitro* may or may not be a reflection of *in vivo* conditions, particularly in respect to Mg and F concentrations at the site of calcification.

Summary. The addition of Mg ions to bone homogenates stimulated pyrophosphatase activity in the absence of F ions, but inhibited pyrophosphatase activity in the presence of F ions. However, femur pyrophosphatase levels were increased in chicks fed diets containing high levels of both Mg and F for 3 or 6 days as compared with chicks fed diets containing identical levels of either element alone.

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