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INFLUENCE OF PHOSPHOCITRATE, A POTENT INHIBITOR OF HYDROXYAPATITE CRYSTAL GROWTH, ON MINERALIZATION OF CARTILAGE AND BONE.

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SUMMARY: An in vivo bone induction system was used to study the effect of phosphocitrate, a potent inhibitor of calcium phosphate crystallization, on the initial stages of mineralization. Subcutaneous transplantation of coarse powders of demineralized rat diaphyseal bone matrix into allogenic recipients results in a well defined sequence of mineralization steps involving cartilage formation, calcification and bone formation. Phosphocitrate when administered locally or systemically had no effect on the mineralization of either cartilage or bone. Dose levels were chosen to be consistent with that of a previous study which demonstrated that ethane-1-hydroxy-1, 1-diphosphonate(EHDP), a similarly effective in vitro inhibitor of hydroxyapatite crystallization, has a strong inhibitory effect on the initial mineralization steps. Since EHDP is not metabolized in vivo, the lack of effect of phosphocitrate in this study is suggestive of possible enzymatic hydrolysis at the calcification site which may regulate the level of phosphocitrate.

INTRODUCTION: There is a growing realization of the importance of naturally occurring inhibitors of mineralization as possible modulators of normal calcification in skeletal tissue (1-3). Recent work has tentatively identified phosphocitrate as one such agent present in human urine and rat liver mitochondrial extracts which is inhibitory to precipitation and crystallization of calcium phosphate (4). The chemical synthesis and properties of phosphocitric acid have been recently described (5). It was therefore of interest to examine the influence of synthetic phosphocitric acid on mineralization of cartilage and bone. In order to characterize the influence of phosphocitrate on de novo mineralization we have employed the matrix-induced endochondral bone development system (6-8) and this communication describes these findings.

MATERIALS AND METHODS: Demineralized bone matrix prepared from rat diaphyses was implanted subcutaneously in the thoracic region of 28 day old (110-120 g) male rats of the Long-Evans strain in bilateral sites (6). The day of implantation was

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designated as day 0 and the rats were utilized on designated days as indicated in the Tables.

The local effect of phosphocitrate was determined by injecting a solution (pH 6.8) around the implant site at a dose of 1.6 μmoles/site on days 7-10 or 9-14. The rationale for the first protocol is to assess the effect of phosphocitrate on early bone mineralization and the second schedule is to evaluate bone remodeling. The present experiments were performed on day 10 (cartilage mineralization and early bone formation) and day 14 (bone mineralization and remodeling). The acute systemic influence of phosphocitrate was assessed by an injection of 4.0 μmoles intravenously via the caudal vein on day 14, 4 hours prior to termination of the experiment.

Acid and Alkaline Phosphatases and ^{45}Ca Incorporation: $^{45}CaCl_2$ was injected intravenously via the caudal vein in saline at a dose of 1 μCi/g body wt 2 hr prior to the termination of the experiment. The plaques were dissected out and homogenized in ice-cold 0.15 M MaCl containing 3 mM $NaHCO_3$ and centrifuged at 20,000 g for 15 min at 4°. Acid and alkaline phosphatase activity in the supernatant was determined as described previously (8, 9). The acid phosphatase (0.1 M sodium acetate buffer, pH 5.0) activity is an index of bone remodeling and the alkaline phosphatase (0.1 M sodium barbital buffer, pH 9.3) activity indicates bone formation. The saline-insoluble sediment representing the mineral fraction was suspended in 5 ml 0.1 M $CaCl_2$ in 50 mM tris-HCl, pH 7.4 at 25°C and stirred for 30 min. After centrifugation at 3000 g the supernatant fractions were discarded. The sediment fractions were washed twice in 10 ml of 5 mM tris-HCl, pH 7.4, at 25°C and the pellets were stirred for 2 hr in 10 ml 0.5 M HCl and centrifuged at 3000 g for 5 min. Radioactivity in the aliquots of the supernatant acid extract were determined in a Beckman liquid scintillation counter. The ^{45}Ca incorporation was expressed as cpm/mg tissue and represented the rate of mineralization during the 2-hr pulse of $^{45}CaCl_2$. Acid and alkaline phosphatase activity was expressed as units/mg protein. The statistical significance was evaluated by means of the Student's t test.

Hydroxyapatite Crystal Growth Assay. The in vitro calcium phosphate crystal growth inhibitory ability of the phosphocitrate was tested in an experimental system previously described (10). Briefly, 2.0 mg hydroxyapatite (HA) seed in slurry form was added to 75 ml of a metastable supersaturated solution of calcium phosphate with a total initial calcium and phosphate concentration of 2.0 and 1.2 mM, respectively. This solution, at 37°C, pH 7.40, and an ionic strength of 0.15 (NaCl), is stable with respect to spontaneous nucleation, in the absence of HA seed, for a period of at least 24 hours. The addition of HA seed results in immediate crystal growth on the seed material in the absence of crystallization inhibitors. The precipitation reaction is characterized by the release of protons into the medium as the protonated phosphate species are incorporated into the growing crystal phase as the deprotonated phosphate ion (approximate composition of the crystal phase is $Ca_3(PO_4)_2$ (11)). The kinetics of the crystal growth reaction were monitored by recording the amount of base, added via a pH-stat (Metrohm Combititrator, 3-D), required to maintain constant pH. Crystal growth inhibitors were added to the metastable solution prior to the HA seed.

RESULTS: Influence of Phosphocitrate on Mineralization of Bone: Implantation of demineralized bone matrix in subcutaneous sites results in induction of de novo bone formation and mineralization (6-8). This model was chosen so that it would permit distinction of the possible effects of phosphocitrate on initiation of mineralization versus maintenance of previously initiated mineral formation. Table 1 summarizes the results of local administration of phos-

TABLE 1

INFLUENCE OF LOCAL PHOSPHOCITRATE ON MINERALIZATION OF MATRIX-INDUCED BONE

Day	Group	Phosphatase U/mg Protein Acid	Alkaline	^{45}Ca Incorporation cpm/mg tissue
10	Control	1.6 ± 0.1*	6.0 ± 1.0	5150 ± 836
	Phosphocitrate	1.3 ± 0.1	3.9 ± 0.4	4104 ± 575**
14	Control	1.6 ± 0.1	2.3 + 0.1	1570 ± 258
	Phosphocitrate	1.9 ± 0.3	2.6 ± 0.4	1960 ± 206**

* Mean ± SE of 8 observations.

** Difference not significant in relation to control.

phocitrate on mineralization. In the first experiment the phosphocitrate was injected on days 7 through 9 so that the tissue was exposed to phosphocitrate before the initiation of mineralization (chondrogenesis is initiated on day 7). The results revealed that there was no consistent influence of phosphocitrate on phosphatase activity or on the initiation of mineralization in vivo, as indicated by the similarity in the levels of uptake of radiolabeled calcium into the plaques. In the next set of experiments the phosphocitrate was administered starting on day 9 through day 13 after the initial mineralization had occurred. ^{45}Ca incorporation and phosphatase activities were similar to controls indicating no discernible effect of phosphocitrate on bone mineralization and remodeling (Table 1).

It was of interest then to investigate the systemic effect of phosphocitrate after administration intravenously on mineralization of matrix-induced bone and the tibial metaphyses. As seen from Table 2, under the conditions employed phosphocitrate is devoid of any inhibitory influence on mineralization of both matrix-induced bone and in the proximal tibial metaphyses, as indicated by ^{45}Ca incorporation into newly formed bone mineral.

Inhibition of Hydroxyapatite Crystal Growth. The ability of phosphocitrate to inhibit the crystal growth is shown in Figure 1. It is apparent that the

TABLE 2

INFLUENCE OF SYSTEMIC PHOSPHOCITRATE ON MINERALIZATION OF BONE ON DAY 14

Tissue	Group	Phosphatases U/mg Protein Acid	Alkaline	^{45}Ca Incorporation cpm/mg tissue
Matrix-induced bone	Control	1.4 ± 0.1	3.4 ± 0.5*	2490 ± 1180
	Phosphocitrate	1.5 ± 0.2	3.9 ± 0.3	3790 ± 660
Tibial Metaphyses	Control	4.4 ± 0.3	6.9 ± 0.8	9880 ± 4140
	Phosphocitrate	3.6 ± 0.4	8.0 ± 0.5	12030 ± 2700

* Mean ± SE of 8 observations.

phosphocitrate sample used in this investigation was an effective crystal growth inhibitor since at a concentration of 1 μM it completely blocked the initial stages of the growth of calcium phosphate on the HA seed as shown by the relative rates of uptake of KOH solution in the presence and absence of inhibitor. Crystal

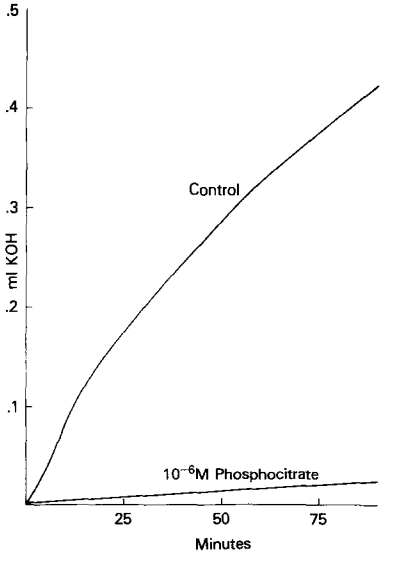

Figure 1: Uptake of KOH solution with time for metastable supersaturated solutions seeded with crystals of HA either in the absence (Control) or presence of 10^{-6} M phosphocitrate. The KOH solution was required to maintain a constant pH of 7.40 and the amount of base is directly proportional to the extent of calcium phosphate crystal growth.

growth did occur in the phosphocitrate experiment, however, after an induction period of 130 minutes.

DISCUSSION: The synthesis and characterization of phosphocitrate was described recently and it was shown to be a potent inhibitor of hydroxyapatite crystal growth (5). It was therefore of interest to assess the in vivo effects of this powerful in vitro inhibitor. The inhibitory activity of the sample of phosphocitrate used in the in vivo bone induction experiments was verified in an in vitro seeded crystal growth system which demonstrated that 10^{-6} M phosphocitrate was sufficient to completely inhibit calcium phosphate crystal growth (Figure 1). The fact that crystal growth was observed in the inhibited system after a reproducible induction period is consistent with the phenomena observed with other phosphate containing crystal growth inhibitors which are effective at micromolar concentrations such as pyrophosphate (12), the nucleoside triphosphates (12) and the multidentate organic phosphonate (13). It is important to note that phosphocitrate appears to have about the same calcium phosphate inhibitory activity as ethane-1-hydroxy-1, 1-diphosphonate (EHDP) when tested in a similar in vitro system (13). EHDP, which is not metabolized in physiological fluids (14), has been shown in another study, however, to have a marked effect on the early mineralization steps when administered at comparable doses in the in vivo bone induction system used for the present study (15). Our in vivo results were uniformly unsuccessful in demonstrating any effects in the living organism both by local and intravenous administration. In this regard phosphocitrate may be similar to pyrophosphate (1) which is effective in in vitro inhibition of crystal growth but lacks any in vivo inhibitory effects. It is possible that the administered phosphocitrate may have undergone hydrolysis by the action of endogenous phosphatases. It is noteworthy that developing bone is a rich source of phosphomonesterases catalyzing the hydrolysis of ester phosphates. In view of this it would be of interest in the future to investigate the synthesis and actions of analogues of phosphocitrate that are resistant to enzymatic hydrolysis.

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