Inhibition of Hydroxyapatite Crystal Growth by Bone-Specific and Other Calcium-Binding Proteins†

Robert W. Romberg,‡ Peter G. Werness,§ B. Lawrence Riggs,‖ and Kenneth G. Mann*,‡

Section of Hematology Research, Nephrology Research Unit, and Endocrine Research Unit, Departments of Internal Medicine and Laboratory Medicine, Mayo Clinic/Foundation, Rochester, Minnesota 55905, and Department of Biochemistry, University of Vermont, Burlington, Vermont 05405

Received June 13, 1985

ABSTRACT: Mineralization of bone matrix may be influenced by the presence of specific, noncollagenous bone proteins. The quantitative influence of two bone-specific proteins—bone γ-carboxyglutamic acid (Gla) protein and osteonectin—and other proteins that decreased the rate of crystal growth was measured by adding seed crystals of hydroxyapatite to a solution of $CaCl_2$ and KH_2PO_4, pH 7.4 at 37 °C. The molar concentrations of proteins needed to inhibit the rate of crystal growth by 50% were as follows: osteonectin, 0.15 μM; bone Gla protein, 0.8 μM; prothrombin, 0.9 μM; prothrombin fragment 1, 1.0 μM; soybean trypsin inhibitor, 3 μM; prethrombin 1, 9 μM; cytochrome *c*, 30 μM. Calmodulin and parvalbumin were found to be less active than prothrombin fragment 1 and had no activity in the micromolar range. The combination of two inhibitors resulted in a mixture with an inhibitory activity that was the sum of the two inhibitors. Decarboxylation of bone Gla protein significantly reduced its inhibitory activity. These results indicate that the inhibitory activity of a protein does not correlate with Ca^{2+}-binding affinity under these conditions, that the mixture of inhibitors has an additive effect, and that γ-carboxyglutamic acid residues enhance the ability of a protein to inhibit hydroxyapatite-seeded crystal growth.

Bone mineralization occurs in two stages: primary mineralization, which is a rapid increase in mineral to about 70% of its final density due to nucleation and crystal multiplication, and secondary mineralization, which is a more gradual completion of mineralization due to crystal growth (Glimcher, 1981). The regulation of bone mineralization is a complicated process controlled by many factors including serum calcium and phosphate concentrations, hormones, enzymes, and the structure of the organic matrix (Vaughan, 1981; Boskey & Posner, 1984). The macromolecular organization of type I collagen is a factor facilitating bone mineralization. Initially, calcium phosphate is deposited in the holes of the collagen fibrils and later fills in the pores and the rest of the space within the collagen fibrils (Glimcher, 1981). Although collagen comprises 90% of the organic constituents of bone (Triffit, 1980), type I collagen is present in many connective tissues that do not mineralize. This suggests that the remaining non-collagenous bone proteins may regulate bone mineralization.

One of the most abundant non-collagenous bone proteins, bone Gla protein (BGP),[1] also called osteocalcin, has generated considerable interest (Haushka et al., 1975; Price et al., 1976; Poser et al., 1980; Haushka & Carr, 1982; Delmas et al., 1984; Menanteau et al., 1982). Bone Gla protein contains three residues of γ-carboxyglutamic acid (Gla), which are formed by a posttranslational carboxylation of three glutamic acid residues in the protein. Prior to the discovery of BGP, only one class of proteins, the blood coagulation factors, were known to contain Gla (Fernlund et al., 1975; Stenflo & Suttie, 1977). Six coagulation proteins are known to contain Gla: prothrombin, factor IX, factor X, factor VII, protein C, and protein S. In experiments where the Gla was not incorporated or thermally decarboxylated to Glu, the blood coagulation factors lost their coagulation activity and their ability to bind calcium and lipid (Esmon et al., 1975; Tuhy et al., 1979). In addition, another Gla-containing bone matrix protein has been recently described (Price et al., 1983); its function is not as yet known.

Gla has been found to be responsible for the binding of BGP to hydroxyapatite and for its affinity for Ca^{2+} (K_d = 1.75 × 10^{-3} M) (Poser & Price, 1979; Price et al., 1977; Poser et al., 1980; Delmas, 1984). Since Gla affects the physical properties of BGP, it is possible that the presence of Gla in non-collagenous bone proteins may be essential for some aspect of bone metabolism. Studies in rats treated long term with warfarin (an inhibitor of Gla formation) suggest that BGP may be responsible for preventing excessive mineralization of the bone growth plate (Price et al., 1982). Also, the rate of resorption of BGP and Gla-depleted rat bone was found to be reduced when implanted into normal rats (Lian et al., 1984).

While Gla may be important for the functional role of BGP, most of the proteins found in bone do not contain Gla. One of these proteins, osteonectin, isolated from guanidine hydrochloride extracts of fetal bone by Termine et al. (1981) and isolated under nondenaturing conditions from adult bone by this laboratory (Romberg et al., 1985), was found to have a molecular weight of 29 000 by sedimentation equilibrium, a p*I* of 5.5, and a high affinity for hydroxyapatite (K_d = 8 × 10^{-8} M) and collagen. It has been suggested that osteonectin may provide the link between collagen and the apatite mineral phase (Termine et al., 1981).

We have evaluated the mineralization-inhibiting properties of bone Gla protein and osteonectin by measuring their effect on hydroxyapatite-seeded crystal growth. To put these findings

† This research was supported in part by National Institutes of Health Grants AG04875, AM07147, and AM20605 and the Mayo Clinic/Foundation.

* Address correspondence to this author at the Department of Biochemistry, University of Vermont.

‡ Section of Hematology Research.

§ Nephrology Research Unit.

‖ Endocrine Research Unit.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Gla, γ-carboxyglutamic acid; $NaDodSO_4$, sodium dodecyl sulfate; BGP, bone Gla protein; IU, inhibitory unit.

0006-2960/86/0425-1176$01.50/0

in perspective, we have made similar measurements on several Gla-containing coagulation proteins and on other non-bone proteins with Ca^{2+}-binding properties.

Experimental Procedures

Materials

Bone Gla protein and osteonectin were isolated as previously described (Price et al., 1976; Romberg et al., 1985). Prothrombin was isolated from bovine plasma (Bajaj & Mann, 1973). Prothrombin fragment 1 and prethrombin 1 were obtained by thrombin cleavage of prothrombin and isolated by ion exchange and gel filtration (Heldebrandt et al., 1973). Calmodulin, soybean trypsin inhibitor, parvalbumin, and cytochrome *c* were purchased from Sigma Chemical Co. Additional calmodulin was a gift from Dr. John Penniston (Mayo Clinic). All protein solutions were dialyzed vs. 0.15 M NaCl, pH 7.4, prior to use. Seeded crystal growth experiments were performed in carbonate-free water. Seed crystals of hydroxyapatite were prepared by previously described methods (Meyer & Nancollas, 1972).

Methods

The effect of proteins on hydroxyapatite-seeded crystal growth was measured by the method of Werness et al. (1981). A beaker containing 75 mL of 1.67 mM $CaCl_2$ and 1.00 mM KH_2PO_4 was equilibrated at 37 °C and adjusted to pH 7.4 by the addition of 0.086 M NaOH with a pH stat (Metrohm, Combitrator-3D). For control experiments, 0.100 mL of a hydroxyapatite seed crystal slurry (20 mg/mL) was added to the reaction mixture, and the volume of NaOH added to keep the pH constant for 60 min was recorded. For experiments in the presence of proteins, the proteins were added to the reaction mixture prior to the addition of the seed crystals.

For every mole of hydroxyapatite grown, 8.3 mol of protons is released in converting the protonated forms of phosphate to PO_4^{3-} as shown in eq 1a. In addition, 2 mol of OH^- is

$$3.7HPO_4^{2-} + 2.3H_2PO_4^- \rightleftarrows 6PO_4^{3-} + 8.3H^+ \text{ (pH 7.4)} \quad (1a)$$

incorporated per mole of hydroxyapatite as shown by the stoichiometry of the precipitation reaction as shown in eq 1b.

$$2OH^- + 10Ca^{2+} + 6PO_4^{3-} \rightleftarrows Ca_{10}(PO_4)_6(OH)_2 \quad (1b)$$

Thus, from the volume of NaOH added to the reaction mixture, the moles of hydroxyapatite can be determined. The concentration of inhibitor needed to reduce the rate of crystallization by 50% was defined as 1 inhibitor unit (IU) and calculated with the Langmuir adsorption isotherm (Davies & Nancollas, 1955):

$$\frac{\kappa}{\kappa - \kappa_a} + \frac{1}{(1-b)} = \frac{k_2}{(1-b)k_1[A]} \quad (2)$$

where κ is the rate of crystallization without inhibitors, κ_a is the rate of crystallization with a particular inhibitor, [A] is the concentration of the inhibitor, k_1 is the rate constant for inhibitor adsorption, k_2 is the rate constant for inhibitor desorption, and b is a limiting rate constant. Once the concentration of protein needed for 1 inhibitor unit was experimentally determined, experiments with mixtures of proteins were designed where each inhibitor contributed an equal amount of inhibition.

The amino acid composition of the isolated proteins were determined by 24-h acid hydrolysis of the protein. Gla content was determined by alkaline hydrolysis (Hauschka, 1977).

The surface area of the hydroxyapatite crystals was measured on a dynamic flow surface area analyzer (Monsorb, Quantachrome Corp.), with the modified Brunauer Emmett Teller equation for single-point determinations (Brunauer et al., 1938; Gregg & Sing, 1967).

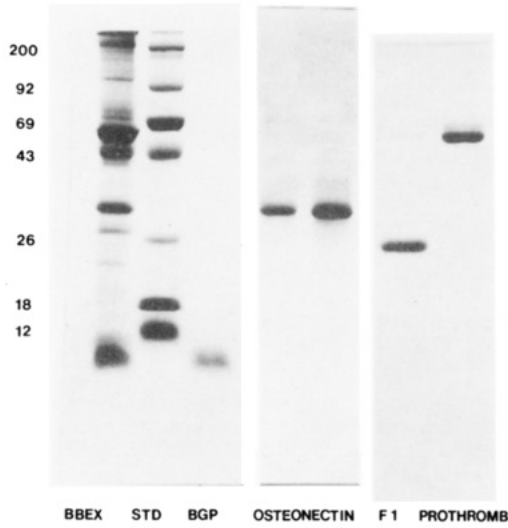

FIGURE 1: Polyacrylamide–$NaDodSO_4$ slab gel of hydroxyapatite growth inhibitory proteins. Electrophoresis was performed on an 8–18% gradient sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide slab gel. The molecular weight in thousands is listed on the far left as determined by the molecular weight standards in lane 2. Lane 1 contains EDTA extracts of bovine bone; lane 3 contains bovine bone Gla protein (BGP); lanes 4 and 5 are bovine osteonectin; lane 6 is bovine prothrombin fragment 1; lane 7 is bovine prothrombin.

It should be pointed out that these experiments do not distinguish between crystal growth and nucleation. However, comparison of the hydroxyapatite crystal size by scanning electron microscopy has shown that the crystals have increased in size over the duration of the experiment. Also, uptake of hydroxide ions is not observed without the addition of seed crystals, indicating that the proteins by themselves do not induce nucleation.

Results

Initial experiments showed tht EDTA extracts of bovine bone (1 IU = 4.3 μg/mL) and EDTA extracts of human bone (1 IU = 6.2 μg/mL) had significant inhibitory effects on hydroxyapatite-seeded crystal growth. Two proteins, osteonectin and BGP, were isolated from the EDTA extract of bovine bone and were tested for their inhibitory activity in a hydroxyapatite-seeded crystal growth system along with the blood coagulation factors prothrombin and prothrombin fragment 1 and several other proteins. Figure 1 shows an 8–18% polyacrylamide–SDS gel of osteonectin, BGP, the total EDTA extract of bovine bone, prothrombin, and prothrombin fragment 1.

The rate of hydroxyapatite crystallization was determined by measuring the uptake of NaOH over a 1-h period after the addition of hydroxyapatite seed crystals with a surface area of 36 m^2/g. Figure 2 shows the growth curve of hydroxyapatite crystals with no added inhibitors and in the presence of the most potent inhibitor, osteonectin. The rate of crystallization κ_a was calculated for each of the inhibitors at three different concentrations of protein or protein mixtures. The inhibitory activity of each protein was then determined by plotting $\kappa/(\kappa - \kappa_a)$ vs. concentration^{-1} according to the Langmiur adsorption isotherm (eq 2) (Davies & Nancollas, 1955). For comparison, Figure 3 shows the linear plots of $\kappa/(\kappa - \kappa_a)$ vs. concentration^{-1} for the four most potent inhibitors; the point at which each line crosses $\kappa/(\kappa - \kappa_a) = 2$ represents the inverse of the concentration needed for 1 IU for each of

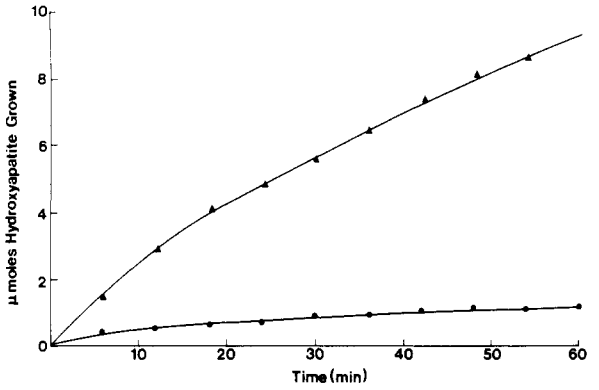

FIGURE 2: Micromoles of hydroxyapatite grown in the presence and absence of osteonectin. Seed crystals of hydroxyapatite were added to a solution of 1.67 mM $CaCl_2$, 1.00 mM KH_2PO_4, and 0.15 M NaCl, at 37 °C, pH 7.4. The micromoles of hydroxyapatite grown was calculated from the amount of NaOH required to maintain the pH at 7.4. The upper curve represents a control experiment (▲), and the lower curve represents the rate of crystal growth in the presence of 1×10^{-6} M osteonectin (●).

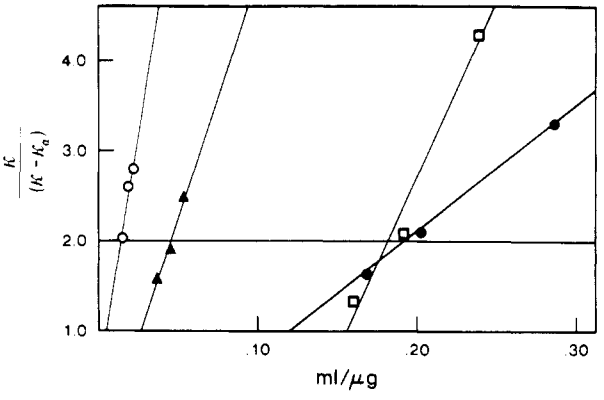

FIGURE 3: Quantitation of inhibitory activity of proteins on hydroxyapatite-seeded crystal growth. Seed crystals of hydroxyapatite were added to 70 mL of 1.67 mM $CaCl_2$, 1 mM Na_2HPO_4, and 0.15 M NaCl at 37 °C, pH 7.4, with varying concentrations of proteins. The results are plotted according to the Langmuir adsorption isotherm (Davies & Nancollas, 1955) (eq 2): (○) prothrombin; (▲) prothrombin fragment 1; (□) bone Gla protein; (●) osteonectin.

the respective proteins. Table I lists the inhibitory activity in μg/mL and in molarity for the bone matrix proteins, the blood coagulations factors, selected calcium-binding proteins, and other proteins and inhibitors. In addition, Table I lists the dissociation constant for calcium for the calcium-binding proteins.

The bone matrix proteins bone Gla protein and osteonectin were the most potent inhibitors of hydroxyapatite-seeded crystal growth of any of the proteins measured. On a molar basis, osteonectin was more than 5 times as potent as bone Gla protein. Prothrombin and prothrombin fragment 1, both of which contain 10 Gla residues, were potent inhibitors in this system. On a molar basis, prothrombin, prothrombin fragment 1, and bone Gla protein had approximately equal inhibitory activity (1×10^{-6} M). Since all of these inhibitory proteins bind Ca^{2+}, two other Ca^{2+}-binding proteins, with high affinity for this metal ions, were tested. Both calmodulin and parvalbumin showed no inhibitory activity in this hydroxyapatite-seeded growth system at concentrations in the micromolar range.

Both acidic and basic groups on proteins have been found to be responsible for the binding of proteins to hydroxyapatite. Soybean trypsin inhibitor, a protein that binds to hydroxyapatite by virtue of its acidic properties (Gorbunoff, 1984), was found to inhibit hydroxyapatite-seeded crystal growth but

Table I: Hydroxyapatite Crystal Growth Inhibitors

inhibitor	IU (μg/mL)	IU/L (M)	$K_d(Ca^{2+})$ (M)
bone Gla protein	5	9×10^{-7}	1.8×10^{-3} [a]
decarboxylated bone Gla protein	32	6×10^{-6}	
osteonectin	5	1.5×10^{-7}	$\sim 3 \times 10^{-7}$ [b]
prothrombin	70	1×10^{-6}	2×10^{-4} [c]
prothrombin fragment 1	20	9×10^{-7}	2×10^{-4} [c]
prethrombin 1	400[d]	9×10^{-6}	
soybean trypsin inhibitor	75	3×10^{-6}	
cytochrome *c*	400[d]	3×10^{-5}	
calmodulin	>100[e]	$>5 \times 10^{-6}$	1×10^{-7} [f]
parvalbumin	>100[e]	$>8 \times 10^{-6}$	$\sim 1 \times 10^{-8}$ [f]
pyrophosphate	0.18	1.04×10^{-6} [g]	
citrate	0.65	1.23×10^{-5} [g]	
magnesium	4.08	1.7×10^{-4} [g]	

[a] Delmas et al., 1984. [b] Romberg et al., 1985. [c] Bajaj et al., 1974. [d] Extrapolated to 1 IU. [e] No inhibition observed at 25 μg/mL. [f] Levine & Dalgarno, 1983. [g] Werness et al., 1981.

Table II: Effect of Inhibitor Mixtures on Crystal Growth

inhibitor	obsd IU (μg/mL)	theoretical IU (μg/mL)
prothrombin fragment 1	20	
bone Gla protein	5.1 ± 0.6 (n = 4)	
osteonectin	4.8 ± 0.5 (n = 3)	
osteonectin + bone Gla protein	5.1	4.95
osteonectin + fragment 1	12	12.4
bone Gla protein + fragment 1	11	12.5

was less than one-third as potent as any of the Gla-containing proteins. Cytochrome *c*, which is known to bind to hydroxyapatite because of its basic properties (Gorbunoff, 1984), was a very weak inhibitor of hydroxyapatite-seeded crystal growth.

When the γ-carboxyglutamic acid residues of BGP were thermally decarboxylated by the method of Bajaj et al. (1981), the inhibitory activity of BGP decreased. BGP that was 48% carboxylated had an inhibitory activity of 1 IU = 12 μg/mL, while BGP that was 4% carboxylated had an inhibitory activity of 32 μg/mL.

Prethrombin 1, the non-Gla-containing fragment of prothrombin, was found to be a very weak inhibitor when compared to its Gla-containing counterpart prothrombin fragment 1. These data are summarized in Table I. For comparison, Table I also lists the inhibitory activities of other types of hydroxyapatite crystallization inhibitors: citrate, pyrophosphate, and Mg^{2+}.

The effect of more than one inhibitor on crystal growth was investigated by preparing mixtures in which each inhibitor component might contribute an equal amount of inhibition on the basis of single inhibition runs. The predicted, additive, theoretical inhibitory capacity was determined by the addition of 0.5 inhibitor unit of the first inhibitor to 0.5 inhibitor unit of the second inhibitor. The measured inhibitory activity of the mixtures was found to be additive in each mixture tested as shown in Table II. This indicates that osteonectin and BGP do not undergo any synergestic interaction nor is there any preferential, competitive, binding to hydroxyapatite.

Discussion

Substances that promote or inhibit hydroxyapatite seeded crystal growth have the potential for being regulators of bone mineralization. The study of the mechanism of crystal growth inhibition is important in identifying drugs and regulating factors that may effect bone growth such as disphosphonates (Fleisch et al., 1969; Francis et al., 1969). Thus, the effect of bone proteins, such as BGP and osteonectin, on in vitro

hydroxyapatite crystal growth may shed light on the in vivo effect of these proteins.

The Gla containing protein BGP has been shown to be the seventh most abundant protein in vertebrae (Hauschka et al., 1975; Price et al., 1976) and appears in newly developing bone about the same time as the initial mineral phase matures into hydroxyapatite (Price et al., 1981). Cultured human bone cells have been shown to synthesize BGP when exposed to 1,25-dihydroxyvitamin D_3, suggesting that BGP may mediate the direct action of 1,25-dihydroxyvitamin D_3 on bone metabolism (Beresford et al., 1984). Studies using radioimmunoassay have shown that increases in serum BGP levels correlate with increases in alkaline phosphatase (Price et al., 1980; Delmas et al., 1983), that serum BGP is dramatically increased in many patients with metabolic bone diseases (Price et al., 1980), and that BGP may be a specific marker for bone formation (Brown et al., 1984). The bone matrix protein osteonectin has been found to bind to hydroxyapatite and to collagen (Termine et al., 1981; Romberg et al., 1985).

The results presented here indicate that γ-carboxyglutamic acid is responsible for the potent inhibitory action of BGP, prothrombin, and prothrombin fragment 1. Both decarboxylated BGP and the non-Gla-containing fragment of prothrombin prethrombin 1 had significantly reduced inhibition of hydroxyapatite crystal growth compared to that of the Gla-containing species. The physical interaction between BGP and hydroxyapatite has previously been interpreted as an interaction between appropriately spaced Gla residues on the protein with the hydroxyapatite crystal. Hauschka and Carr (1982) have suggested a predicted structure for BGP that places all three Gla residues on the same face of one α helix at 5.4-Å intervals. This organization is similar to the spacing of Ca^{2+} in the hydroxyapatite crystal.

The absence of γ-carboxyglutamic acid does not exclude a protein from being a potent inhibitor. Osteonectin, which does not contain Gla, was the most potent of any of the inhibitors of hydroxyapatite crystallization that we tested. Preliminary sequence data indicate that osteonectin has a very acidic NH_2 terminus containing glutamic acid and aspartic acid residues. Thus, it is likely that the inhibitory activity of osteonectin is due to a binding interaction between appropriately spaced clusters of acidic residues on osteonectin and the hydroxyapatite crystal lattice.

To examine if these effects were general properties of proteins or were unique to bone matrix proteins, a basic and an acidic protein were tested. As shown in Table I, the activity of the acidic protein soybean trypsin inhibitor (pI = 4.5) was found to be several times more potent than the basic protein cytochrome c (pI = 10.6), suggesting that carboxyl groups are more important for inhibiting hydroxyapatite crystal seeded growth than are basic groups. While osteonectin (pI = 5.5) is less acidic than soybean trypsin inhibitor, it was a much better inhibitor of hydroxyapatite crystal seeded growth, suggesting that it was not only the charge on osteonectin but also the orientation of acidic residues that can easily interact with the hydroxyapatite crystal lattice.

It has been suggested that Ca^{2+}-binding proteins may bind to the Ca^{2+} on the hydroxyapatite surface or that a protein with a bound Ca^{2+} may bind to hydroxyapatite by presenting a Ca^{2+} to a vacant position in the crystal lattice (Hauschka & Carr, 1982). The results in Table I suggest that the Ca^{2+}-binding ability of a protein does not correlate well with its ability to inhibit hydroxyapatite-seeded crystal growth. However, it is possible that binding sites for Ca^{2+} in proteins such as calmodulin or parvalbumin do not have a favorable conformation to interact with the hydroxyapatite surface. The exposure of several hydrophobic side chains on calmodulin induced by Ca^{2+} binding (Dalgarno et al., 1983) would not favor protein–mineral interaction. Besides proteins, other organic and inorganic molecules have the ability to affect the rate of calcium phosphate growth. Both citrate and pyrophosphate are endogenous compounds that have been shown to prevent calcium oxalate and hydroxyapatite crystal growth and may partially control mineralization in vivo (Werness et al., 1981). It is not surprising that compounds such as citrate have an inhibitory activity on crystal growth similar to the Gla-containing proteins as shown in Table I, since both citrate and Gla-containing proteins have similarly spaced carboxyl groups that can interact with the hydroxyapatite lattice. Pyrophosphate and diphosphonates both have been shown to inhibit the growth and dissolution of hydroxyapatite crystals in vitro (Frances et al., 1969; Fleisch et al., 1969). Diphosphonates, which have been used in treating bone disorders, have a P–C–P bond that is resistant to enzymatic hydrolysis. The similarity of these compounds to phosphate and the clusters of negatively charged oxygen atoms on the molecules probably account for their strong interaction with hydroxyapatite.

Besides pyrophosphate, organic molecules, and proteins, several inorganic ions have been found to affect the crystallization rates of hydroxyapatite and octacalcium phosphate [$Ca_8(PO_4)_5(OH)$], which is a precursor to hydroxyapatite in mineralizing bone in vivo. Mg^{2+}, Sr^{2+}, Be^{2+}, and F^- have been found to be inhibitors of nucleation and of hydroxyapatite crystal growth (Bachra & Fischer, 1966). However, unlike the proteins studied in this paper, these elemental ions do not possess carboxyl groups or negatively charged oxygen atoms that could effectively interact with the hydroxyapatite lattice. Rather, ions such as Be^{2+}, Mg^{2+}, and Sr^{2+} are capable of interacting with the lattice by competing with Ca^{2+} for the phosphate ions on the surface of the crystal lattice. Although Mg^{2+} has a smaller ionic diameter than Ca^{2+}, magnesium ions do not effectively penetrate the apatite lattice (Trautz, 1955). However, Mg^{2+}, reduces the rate of transformation of octacalcium phosphate into apatite and inhibits the growth of hydroxyapatite by interfering with the formation of the appropriate nuclei (Brown et al., 1962). On the other hand, Sr^{2+} ions easily enter the hydroxyapatite lattice where they compete with Ca^{2+} and slow down crystal growth because of their larger size. Uptake of Mg^{2+} and Sr^{2+} in the hydroxyapatite crystal lattice makes the crystals imperfect and more soluble, while uptake of the anion F^- makes the mineral crystals larger and less soluble (Boskey & Posner, 1984). The anion F^- has been shown to suppress octacalcium phosphate formation and to facilitate the transformation of octacalcium phosphate into hydroxyapatite (Brown et al., 1962). Sodium fluoride has been shown to be a promising therapeutic agent for osteoporosis. Larger doses, however, may be associated with impaired mineralization of bone (Riggs, 1984).

The extent of crystal inhibition for any substance depends upon the mechanism of inhibition, the concentration of the inhibitor, the pH, the temperature, the ionic strength of the media, and the individual properties of the inhibitor. Small inorganic ions that are able to enter the hydroxyapatite lattice are probably able to inhibit crystal growth by distorting the crystal lattice, while larger molecules and proteins are inhibitory because they bind to the surface where crystal growth is taking place. On a mass basis, the most potent inhibitors are small compounds that bind to the surface of the lattice, such as pyrophosphate and citrate. Proteins, that have a large

molecular weight but only a limited number of sites that can bind to the crystal at one time are less potent inhibitors on a mass basis than small ions. However, comparison of all of the inhibitors on a molar basis, as shown in Table I, shows that the protein osteonectin is significantly more potent than pyrophosphate, citrate, and magnesium.

Registry No. Mg, 7439-95-4; hydroxyapatite, 1306-06-5; prothrombin fragment 1, 72270-84-9; prethrombin 1, 69866-47-3; soybean trypsin inhibitor, 9078-38-0; cytochrome *c*, 9007-43-6; pyrophosphate, 14000-31-8; citric acid, 77-92-9.

REFERENCES

Bachra, B. N., & Fischer, H. R. A. (1966) *Calcif. Tissue Res. 3*, 348–357.

Bajaj, S. P., & Mann, K. G. (1973) *J. Biol. Chem. 248*, 7729–7749.

Bajaj, S. P., Butkowski, R. J., & Mann, K. G. (1974) *J. Biol. Chem. 250*, 2150–2156.

Bajaj, S. P., Price, P. A., & Russell, W. A. (1981) *J. Biol. Chem. 257*, 3726–3731.

Beresford, J. N., Gallegher, J. A., Poser, J. W., & Russell, R. G. G. (1984) *Metab. Bone Dis. Relat. Res. 5*, 229–234.

Boskey, A. L., & Posner, A. S. (1984) *Orthop. Clin. North Am. Oct. 15*, 597–612.

Brown, J. P., Delmas, P. D., Malaval, L., Edouard, C., Chapuy, M. C., & Meunier, P. J. (1984) *Lancet 1*, 1091–1093.

Brown, W. E., Smith, J. P., Lehr, J. R., & Frazer, A. W. (1962) *Nature (London) 196*, 1055–1055.

Brunauer, S., Emmett, P. H., & Teller, E. (1938) *J. Am. Chem. Soc. 60*, 309.

Dalgarno, D. C., Drabikowski, W., Klevit, R. E., Levine, B. A., & Williams, R. J. P. (1983) in *Calcium-Binding Proteins* (Carofoli, E., Ed.) Elsevier/North-Holland, Amsterdam.

Davies, C. W., & Nancollas, G. H. (1955) *Trans. Faraday Soc. 51*, 823.

Delmas, P. D., Stenner, D., Wahner, H. W., Mann, K. G., & Riggs, B. L. (1983) *J. Clin. Invest. 71*, 1316–1321.

Delmas, P. D., Stenner, D. S., Romberg, R. W., Riggs, B. L., & Mann, K. G. (1984) *Biochemistry 23*, 4720–4725.

Esmon, C. T., Suttie, J. W., & Jackson, C. M. (1975) *J. Biol. Chem. 250*, 4095–4099.

Fernlund, P., Stenflo, J., Roepstorff, P., & Thomen, J. (1975) *J. Biol. Chem. 250*, 6125–6133.

Fleisch, H., Russell, R. G. G., & Francis, M. D. (1969) *Science (Washington, D.C.) 165*, 1262.

Frances, M. D., Russell, R. G. G., & Fleisch, H. (1969) *Science (Washington, D.C.) 165*, 1264.

Glimcher, M. J. (1981) *The Chemistry and Biology of Mineralized Connective Tissues* (Veis, A., Ed.) pp 617–673, Elsevier/North-Holland, Amsterdam.

Gorbunoff, M. J. (1984) *Anal. Biochem. 136*, 425–432.

Gregg, S. J., & Sing, K. S. W. (1967) in *Adsorption, Surface Area and Porosity*, Academic Press, New York.

Hauschka, P. V. (1977) *Anal. Biochem. 801*, 212–223.

Hauschka, P. V., & Carr, S. A. (1982) *Biochemistry 21*, 2538–2547.

Hauschka, P. V., Lian, J. B., & Gallop, P. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3925–3929.

Heldebrant, J. C. M., Butkowski, R. J., Bajaj, S. P., & Mann, K. G. (1973) *J. Biol. Chem. 248*, 7149–7163.

Levine, B. A., & Dalgarno, D. C. (1983) *Biochim. Biophys. Acta 726*, 182–204.

Lian, J. B., Trassinari, M., & Glowacki, J. (1984) *J. Clin. Invest. 73*, 1223–1226.

Menanteau, J., Neuman, W. F., & Neuman, M. W. (1982) *Metab. Bone Dis. Relat. Res. 4*, 157–162.

Meyer, J. L., & Nancollas, G. H. (1972) *Arch. Oral Biol. 17*, 1623–1627.

Poser, J. W., & Price, P. A. (1979) *J. Biol. Chem. 254*, 431–436.

Poser, J. W., Esch, F. S., Ling, N. C., & Price, P. A. (1980) *J. Biol. Chem. 255*, 8685–8691.

Price, P. A., Otsuka, A. S., Poser, J. P., Kristanponis, J. A., & Raman, N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1447–1451.

Price, P. A., Otsuka, A. S., & Poser, J. W. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., Ed.) pp 333–337, Elsevier, Amsterdam.

Price, P. A., Parthemore, J. G., & Deftos, L. J. (1980) *J. Clin. Invest. 66*, 878–883.

Price, P. A., Lothringer, J. W., Baukol, S. A., & Reddi, A. H. (1981) *J. Biol. Chem. 256*, 3781–3784.

Price, P. A., Williamson, M. K., Haba, T., Dell, R. B., & Jee, W. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7734–7738.

Price, P. A., Urist, M. R., & Otawara, Y. (1983) *Biophys. Res. Commun. 117*, 756–771.

Riggs, B. L. (1984) *Bone Miner. Res. 2*, 366–373.

Romberg, R. W., Werness, P. G., Lollar, P., Riggs, B. L., & Mann, K. G. (1985) *J. Biol. Chem. 260*, 2728–2736.

Stenflo, J., & Suttie, J. W. (1977) *Annu. Rev. Biochem. 46*, 157–172.

Termine, J. D., Belcort, A. B., Conn, K. M., & Kleinman, H. K. (1981) *J. Biol. Chem. 256*, 10403–10408.

Trautz, O. R. (1955) *Ann. N.Y. Acad. Sci. 60*, 696–712.

Triffit, J. T. (1980) in *Fundamental and Clinical Bone Physiology* (Urist, M. R., Ed.) pp 45–82, Lippincott, Philadelphia, PA.

Tuhy, P. M., Bloom, J. W., & Mann, K. G. (1979) *Biochemistry 18*, 5842–5848.

Vaughan, J. (1981) in *The Physiology of Bone*, Oxford University Press, New York.

Werness, P. G., Bergert, J. H., & Lee, K. E. (1981) *Clin. Sci. 61*, 487–491.