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Synthesis and Characterization of Phosphocitric Acid, a Potent Inhibitor of Hydroxylapatite Crystal Growth†

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ABSTRACT: Human urine and extracts of rat liver mitochondria contain apparently identical agents capable of inhibiting the precipitation or crystallization of calcium phosphate. Its general properties, as well as ^{1}H NMR and mass spectra, have suggested that the agent is phosphocitric acid. This paper reports the synthesis of phosphocitric acid via the phosphorylation of triethyl citrate with *o*-phenylene phosphochloridate, hydrogenolysis of the product to yield triethyl phosphocitrate, hydrolytic removal of the blocking ethyl groups, and also chromatographic purification. An enzymatic assay of phosphocitrate is described. Synthetic phosphocitrate was found to be an exceedingly potent inhibitor of the growth of hydroxylapatite seed crystals in a medium supersaturated with respect to Ca^{2+} and phosphate. Comparative assays showed phosphocitrate to be much more potent than the most active precipitation–crystallization inhibitors previously reported, which include pyrophosphate and ATP. ^{14}C-Labeled phosphocitrate was bound very tightly to hydroxylapatite crystals. Such binding appeared to be essential for its inhibitory activity on crystal growth. Citrate added before, but not after, phosphocitrate greatly enhanced the inhibitory potency of the latter. This enhancement effect was not given by other tricarboxylic acids. The monoethyl ester of phosphocitrate had no inhibitory effect on hydroxylapatite crystal growth.

Biochemical agents capable of inhibiting the precipitation and/or crystallization of calcium phosphate have been detected in urine (Howard & Thomas, 1958; Howard, 1976; Fleisch & Bisaz, 1962), saliva (Gron & Hay, 1976), bile (Sutor & Percival, 1976), and other body fluids. Such inhibitory agents have also been detected (Tew & Mahle, 1977) in extracts of rat liver mitochondria, which can accumulate large amounts of Ca^{2+} and phosphate during respiration to form nondiffracting electron-dense deposits of "amorphous" calcium phosphate (Greenawalt et al., 1964). Similar inhibitors have also been detected in extracts of the hepatopancreas of the blue crab, *Callinectes sapidus*, in which large amounts of calcium phosphate are stored during ecdysis, also in a nondiffracting

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apparently amorphous form (Becker et al., 1976). Such agents have been suggested to assist in regulation of biological calcification processes [Howard (1976) and Lehninger (1970), inter alia].

Recent work in these laboratories has indicated that one of the major inhibitory agents in both normal human urine and rat liver mitochindria is relatively small in molecular size, highly acidic, and probably contains an organic moiety (Tew & Mahle, 1977; Lehninger, 1977). Concentrates of the urinary and mitochondrial factors, although still impure, have given ^{1}H NMR and mass spectra and other data consistent with only a limited number of possible structures. Among these is phosphocitric acid, which has not been reported to occur in biological material.

This paper describes the organic synthesis, purification, and properties of phosphocitric acid, as well as its powerful activity as an inhibitor of the growth of hydroxylapatite crystals (HA).[1] Some of the data recorded here have been briefly communicated (Tew & Mahle, 1977; Lehninger, 1977).

Experimental Details

Materials. *o*-Phenylene phosphochloridate was obtained from Aldrich Chemical Co., triethyl citrate was from Eastman Kodak Co., and platinum oxide was from Polyscience, Inc. *o*-Phenylene phosphochloridate and triethyl citrate were redistilled under vacuum. Alkaline phosphatase (calf intestine), citrate lyase, malate dehydrogenase, lactate dehydrogenase, and other enzymes used in this work were obtained from Sigma Chemical Co. All other compounds used were reagent grade. Solvents were dried by double distillation from CaH_2. Uniformly labeled [^{14}]citric acid was obtained from Amersham, Ltd.

Synthesis of Phosphocitric Acid. (1) A mixture of triethyl citrate (0.013 mol) and pyridine (0.013 mol) in 10 mL of dry benzene was added slowly to a solution of *o*-phenylene phosphochloridate (0.013 mol) in 10 mL of dry benzene. The mixture was sealed under N_2 and stirred for 5 min. The reaction mixture was then cooled to 5 °C and quickly filtered (Millipore, 0.22 μm) to remove the precipitated pyridine hydrochloride. The solvent was removed from the supernatant by vacuum evaporation at 45 °C, leaving a clear yellow oil. ^{1}H NMR spectra of this material showed it to be the *o*-phenylene phosphate ester of triethyl citrate. Yields by weight exceeded 90%.

(2) The *o*-phenylene group of the above product was removed by catalytic hydrogenolysis. One gram of the oil from step 1 was taken up in 50 mL of absolute ethanol and reduced with H_2 at atmospheric pressure with 200 mg of PtO_2. After 2 h the uptake of H_2 was 252 mL (97% of the calculated amount for reduction of the *o*-phenylene group to cyclohexane). ^{1}H NMR spectra of this material showed it to be the phosphate ester of triethyl citrate.

(3) The reduction product from step 2 was neutralized with 1 N NaOH, cooled to 5 °C, and filtered. The solvent was removed from the filtrate by vacuum evaporation at 45 °C to yield a clear oil. Hydrolytic removal of the ethyl groups was carried out at 4 °C by adding 1 g of the oil, dissolved in 25 mL of H_2O, to 165 mL of 0.1 NaOH, followed immediately by 10.5 mL of 1 M $CaCl_2$. The mixture was stirred for 4 h at 4 °C. Gradually a heavy, flocculent precipitate appeared. Elemental analysis of this precipitate, recovered by filtration, showed it to be the calcium salt of phosphocitric acid, containing small amounts of phosphate and citrate. If Ca^{2+} is omitted from the hydrolytic procedure, the major product is the monoethyl ester of phosphocitrate rather than phosphocitrate, as shown by chromatographic analysis.

Final purification was achieved by chromatographic separation of the hydrolysis products on DEAE-cellulose (Whatman DE 52). The calcium salt of phosphocitrate (0.5 g) was dissolved in dilute HCl and loaded on a 90 × 3 cm DEAE-cellulose column previously equilibrated with 50 mM triethylamine bicarbonate, pH 8.0 (Sy, 1977). Elution was carried out with a linear triethylamine bicarbonate gradient (50–333 mM), total volume 3 L. Collection of 25-mL fractions gave the following separations: fractions 20–25, phosphate; 60–65, citrate; 75–80, phosphocitrate. The fractions containing phosphocitrate were pooled and vacuum evaporated to dryness at 45 °C. The residue was dissolved in 20 mL of H_2O and phosphocitrate precipitated by addition of 5 mL of 1 M $CaCl_2$. The resulting precipitate was collected by centrifugation, washed with H_2O, and dried under vacuum at 45 °C. Elemental analysis of this material showed it to be the hydrated calcium salt of phosphocitrate. The overall yield of the essentially pure product was ~60%. Anal. Calcd for $C_6H_4O_{10}P\cdot 5H_2O$: C, 15.75; H, 3.06; P, 6.78; Ca, 21.88; C/PO_4, 0.76. Found: C, 15.80; H, 2.92; P, 6.59; Ca, 22.03; C/PO_4, 0.78.

The relatively insoluble calcium salt of phosphocitrate was converted into the soluble K^+ salt for many of the following experiments by treatment with the H^+ form of Dowex 50W cation-exchange resin, followed by neutralization.

^{1}H NMR spectra were obtained with a Varian A60 analytical spectrometer. Mass spectral measurements were made with a 21-110 Du Pont mass spectrometer at 70 eV with a source temperature of 200 °C.

Synthesis of [^{14}C]Phosphocitric Acid. For the preparation of [^{14}C]phosphocitrate, a small amount of [^{14}C]trimethyl citrate was added to the triethyl citrate solution in step 1 of the above procedure. The specific radioactivity of the final product could be controlled by the amount of [^{14}C]trimethyl citrate used. [^{14}C]Trimethyl citrate was prepared by treatment of uniformly labeled [^{14}C]citric acid (Amersham) with diazomethane (Arndt, 1943).

Hydroxylapatite Crystal Growth Assay. The activity of phosphocitrate and other substances in inhibiting the growth of hydroxylapatite crystals was assayed by a modification of the method of Meyer & Angino (1977). Hydroxylapatite crystal growth was initiated by the addition of 3.5 mg of HA seed crystals, prepared according to Nancollas & Mohan (1970), in an aqueous slurry (100 μL) to 75 mL of a freshly prepared supersaturated solution of Ca^{2+} and phosphate at pH 7.4 and 37 °C. The supersaturated solution was composed of 1.73 mM $CaCl_2$, 1.20 mM NaH_2PO_4, and 150 mM NaCl adjusted to pH 7.4 with 0.1 N NaOH. The solution was stirred continuously and purged of CO_2 with a stream of N_2. After addition of seed crystals, the pH of the solution was held constant by the addition of 0.01 N NaOH from a Metrohm E-473 pH stat/titrator. The rate of growth of the hydroxylapatite crystals was taken as proportional to the rate of uptake of OH, since the uptake of Ca^{2+} and P_i by the seed crystals results in acidification of the medium (Meyer & Angino, 1977).

Enzymatic Determination of Phosphocitric Acid. The enzymatic determination of phosphocitrate was carried out by adaptation of the method of Dagley (1974) for citrate. The cuvettes contained 2.5 mL of 0.2 M triethanolamine, pH 7.2, 30 units of malate dehydrogenase, 25 units of lactate dehydrogenase, and 3.5 mg of NADH. After addition of the

[1] Abbreviations used: HA, hydroxylapatite; LDH, lactate dehydrogenase; MDH, malate dehydrogenase.

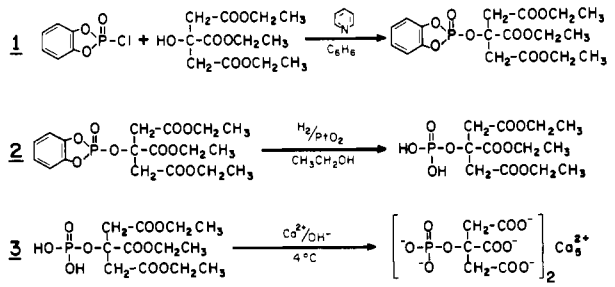

FIGURE 1: Synthesis of phosphocitric acid.

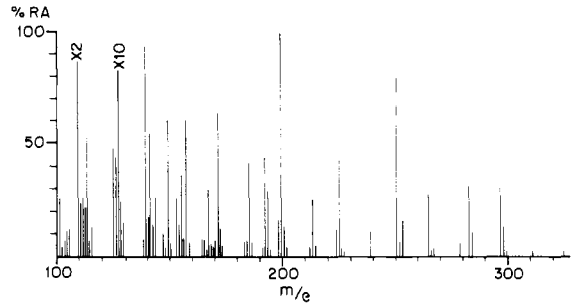

FIGURE 2: Mass spectrum of the methyl derivative of phosphocitric acid. The methyl derivative was prepared by treating phosphocitric acid with diazomethane in diethyl ether. The spectrum was obtained at 70 eV with a source temperature of 200 °C.

sample containing phosphocitrate, citrate lyase (2.5 units) was added and the absorbance monitored at 360 nm. Free citrate present in the sample was determined at this point by recording the decrease in A_{360}. After the absorbance reached a stable limit, 20 units of intestinal alkaline phosphatase was added and the change in A_{360} again was monitored. The amount of phosphocitrate present in the sample was calculated from the change in A_{360} after the addition of alkaline phosphatase.

Results

Synthesis and Properties of Phosphocitric Acid. Although a synthesis of phosphocitrate had been previously reported (Meyer et al., 1959), the procedure was lengthy and gave very poor yields in our hands. The new synthesis described here is based on the method of Khwaja et al. (1970) for the preparation of phosphate esters of simple alcohols. The synthesis involved three steps (Figure 1): (1) reaction of *o*-phenylene phosphochloridate with triethyl citrate to form the *o*-phenylene-protected phosphate ester of triethyl citrate; (2) removal of the phenylene protecting group by catalytic reduction; (3) hydrolytic removal of the protecting ethyl groups.

The first two steps in the reaction sequence gave greater than 90% yields. The final alkaline hydrolysis, however, was not always complete and gave yields between 30 and 85% due to formation of variable amounts of the monoethyl ester of phosphocitrate. Despite this difficulty, subsequent chromatographic purification and precipitation gave essentially pure calcium phosphocitrate.

The mass spectrum of the trimethyl derivative of phosphocitrate, prepared by treating phosphocitric acid with diazomethane (Arndt, 1943), is shown in Figure 2. Although no molecular ion was present, none of the fragments had an m/e ratio greater than 330, in agreement with the calculated molecular weight of 342 for the trimethyl derivative. Although structural assignments of the fragments are not given, the fragmentation pattern was consistent with the assumed structure.

The Ca^{2+} salt of phosphocitrate was relatively insoluble at pH values above 5, whereas the K^+ and Na^+ salts were quite

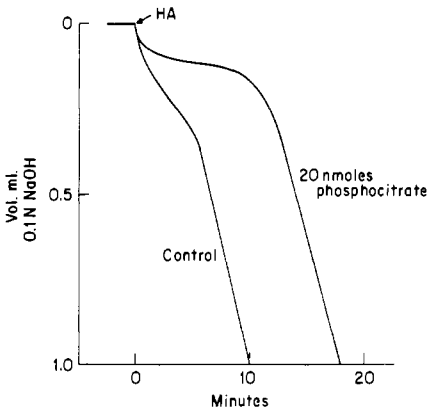

FIGURE 3: Effect of 20 nmol (0.266 μM) of phosphocitrate on the rate of hydroxylapatite crystal growth. Measurements were made in 75 mL of supersaturated calcium phosphate solution, pH 7.4, 37 °C. The pH of the solution was held constant by addition of 0.01 N NaOH from a pH stat. Crystal growth was initiated by the addition of 3.5 mg of seed crystals in 100 μL of aqueous slurry. Phosphocitrate was added to the medium prior to addition of crystals.

soluble between pH 1 and 12. Phosphocitrate was stable in 1 N HCl or 1 N NaOH for 1 h at temperatures up to 80 °C. Less than 5% of the phosphate was released in inorganic form upon boiling in 0.1 N NaOH for 1 h. However, in 1 N H_2SO_4 at 100 °C, hydrolysis of the phosphate group was complete in ~100 min.

Phosphocitrate (Na^+ or K^+ salt) was hydrolyzed at pH 5.5 by acid phosphatase (potato) and at pH 9.5 by alkaline phosphatase (calf intestine). The hydrolysis products from either phosphatase treatment were citric acid and inorganic phosphate.

Enzymatic Determination of Phosphocitrate. A sensitive enzymatic assay was developed in order to study the stability and other properties of phosphocitrate. It consisted of the enzymatic determination of citrate with citrate lyase and malate dehydrogenase (Dagley, 1974) before and after hydrolysis of phosphocitrate to citrate and phosphate by alkaline phosphatase. The difference in absorbance at 360 nm before and after the action of alkaline phosphatase is a measure of the phosphocitrate content of the sample. Pure synthetic phosphocitrate is not acted upon by citrate lyase. The response was linear in the range 10–50 nmol/3-mL test system. However, if free citrate is present in the test sample in a concentration significantly greater than 30 μM, the accuracy is diminished. Another restriction is that inorganic phosphate at concentrations above 100 μM severely inhibits the activity of alkaline phosphatase. The presence of 2 mM $ZnCl_2$ in the medium, as indicated by Dagley (1974) for maximum activity of citrate lyase, was found to greatly reduce the rate of hydrolysis of phosphocitrate by alkaline phosphatase. Zinc was therefore omitted from the medium without detrimental effects. Despite these limitations, the assay procedure was found valuable for accurate determination of small amounts of phosphocitrate.

Inhibition of Hydroxylapatite Crystal Growth by Phosphocitrate. In the studies reported here the rate and extent of crystal growth were measured by recording the volume of NaOH delivered by the pH stat, to maintain a constant pH, as a function of time. As is shown in Figure 3, addition of 3.5 mg of the seed crystals to 75 mL of supersaturated calcium phosphate solution was followed by very rapid growth of the HA crystals, reflected in the high rate of NaOH addition required to maintain the pH constant at 7.4. In contrast, the rate of base addition is reduced significantly when phosphocitrate is added to the medium prior to addition of HA crystals.

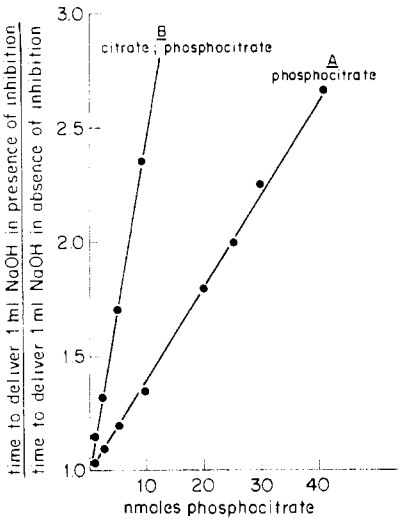

FIGURE 4: Effect of increasing amounts of phosphocitrate on the rate of hydroxylapatite crystal growth. Measurements were made in 75 mL of supersaturated calcium phosphate solution, pH 7.4, 37 °C. Crystal growth was initiated by addition of 3.5 mg of seed crystals in 100 μL of slurry. (A) Phosphocitrate was added to the solution prior to addition of seed crystals. (B) Seed crystals were added to the supersaturated solution containing 2 μmol of citrate and allowed to grow for 1 min prior to addition of phosphocitrate.

Table I[a]

	μmol added	concn (μM)	rel inhibn (1/μM)
phosphocitrate	0.025	0.333	3.0
2,3-diphosphoglycerate	0.050	0.666	1.5
pyrophosphate	0.090	1.20	0.83
1-ethane-1-diphosphonate	0.095	1.26	0.79
3-phosphoglycerate	1.0	13.3	0.07
ATP	1.5	20	0.05
Mg^{2+}	6.75	90	0.01
citrate	7.0	93	0.01
phosphoenolpyruvate	15	200	0.005
NAD^+	20	266	0.004
NADH	20	266	0.004

[a] Concentrations of various compounds required to halve the rate of hydroxylapatite crystal growth. Measurements were made in 75 mL of supersaturated calcium phosphate, pH 7.4, 37 °C, containing the amount of inhibitor indicated. Crystal growth was initiated by addition of 3.5 mg of crystals. The amount of inhibitor shown doubled the time required for the pH stat to deliver exactly 1.0 mL of 0.01 N NaOH.

Figure 4 shows the strongly inhibitory effect of increasing concentrations of phosphocitrate on the rate of HA crystal growth, which was linear in the range 1–40 nmol/75 mL. Higher concentrations are also active but resulted in a deviation from linearity.

Comparison of Phosphocitrate with Other Inhibitors of HA Crystal Growth. Table I compares the activity of several known inhibitors of HA crystal growth. The basis of comparison was the concentration of inhibitors required to halve the rate of HA crystal growth. For each compound tested the inhibition of crystal growth was measured over a range of concentrations. The relationship between inhibition and concentration in all cases was linear up to a twofold increase in the time required for the pH stat to deliver 1.0 mL of NaOH. Greater concentrations produced some deviations from linearity. For this reason all agents tested were compared at concentrations within the linear range.

The data in Table I show that phosphocitrate is the most potent inhibitor of the compounds tested. It was almost 4 times as active as inorganic pyrophosphate, one of the most potent

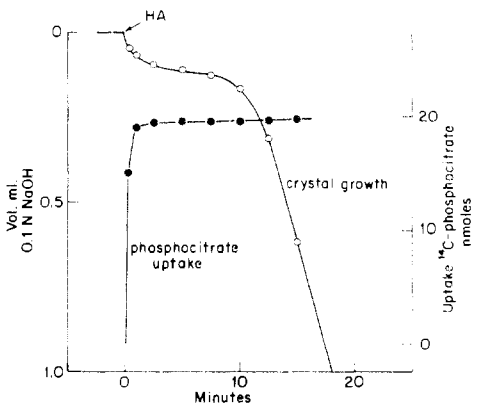

FIGURE 5: Uptake of [^{14}C]phosphocitrate by hydroxylapatite crystals. Measurements were made in 75 mL of supersaturated calcium phosphate solution containing 20 nmol of ^{14}C-labeled phosphocitrate. Crystal growth was initiated by addition of 3.5 mg of seed crystals. At the times indicated, 1.0-mL aliquots were withdrawn and rapidly filtered. The phosphocitrate remaining in the supernatant was determined by scintillation techniques.

naturally occurring agents reported to date (Fleisch et al., 1968a). Phosphocitrate was many times more active than ATP, citrate, or Mg^{2+}. ATP and Mg^{2+} in combination was earlier shown to be very effective in stabilizing amorphous calcium phosphate and retarding the formation of crystalline phases under several different concentrations (Blumental et al., 1977). However, when tested in the type of crystal growth assay used here, ATP and Mg^{2+} produced only additive effects. This may be due to a difference in assay conditions. Moreover, it is possible that the stabilization of amorphous crystals may have different structural requirements and be affected differently by compounds of different structures.

Binding of Phosphocitrate to HA Seed Crystals. With the aid of [^{14}C]phosphocitrate added to the reaction medium, it was found that phosphocitrate binds very tightly to hydroxylapatite crystals during the inhibition assay. After the addition of HA crystals, 1.0-mL samples of the reaction medium were withdrawn and rapidly filtered to remove the crystals. Determination of the [^{14}C]phosphocitrate remaining in the medium was made by scintillation counting. When 3.5 mg of HA crystals was added to 75 mL of supersaturated calcium phosphate solution containing 20 nmol of labeled phosphocitrate, ~18 nmol of the radioactive phosphocitrate was bound to the crystals within the first minute. The inhibitor remained bound to the crystals throughout the assay period. There was no release of the phosphocitrate even when the inhibition was overcome and crystal growth resumed (Figure 5). When higher concentrations of phosphocitrate were employed (20–50 nmol), with the seed crystals constant at 3.5 mg, there was a rapid uptake of 18–20 nmol of phosphocitrate within the first minute, followed by a slower uptake of the remainder. Within minutes most of the phosphocitrate became incorporated into the crystals. During the uptake of inhibitor and for some time thereafter there was no measurable crystal growth. With higher concentrations of phosphocitrate the inhibited state lasted proportionally longer. However, in all cases, crystal growth eventually resumed, ultimately reaching a rate identical with that of uninhibited crystal growth.

Such experiments were very reproducible so long as the measurements were made with seed crystals from the same batch. Crystals from other preparations gave slightly different values for phosphocitrate uptake but produced qualitatively the same effects.

Synergistic Effect of Citrate on Crystal Growth Inhibition by Phosphocitrate. The potency of phosphocitrate in pre-

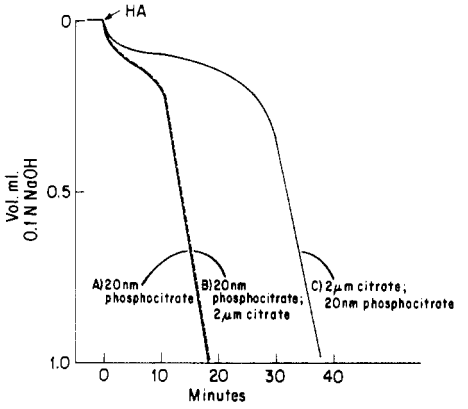

FIGURE 6: Synergistic effect of citrate on the inhibition of hydroxylapatite crystal growth by phosphocitrate. Measurements were made in 75 mL of supersaturated calcium phosphate solution, pH 7.4, 37 °C. Crystal growth was initiated by addition of 3.5 mg of seed crystals. (A) 20 nmol (0.0266 μM) of phosphocitrate added to solution prior to addition of crystals. (B) 20 nmol of phosphocitrate added to solution prior to addition of crystals, and then 1 min after addition of crystals 2 μmol of citrate was added. (C) 2 μmol of citrate added to solution prior to addition of crystals, and then 1 min after addition of crystals 200 nmol of phosphocitrate was added.

venting HA crystal growth was found to be greatly enhanced by the presence of citrate in the assay medium (Figure 4). The inhibition produced by both compounds together was much greater than the sum of their individual effects. Studies of this synergism between phosphocitrate and citrate showed that maximum inhibition is achieved if the HA seed crystals are allowed to grow for a few minutes in the presence of citrate prior to addition of phosphocitrate (Figure 6). The addition of citrate after preincubation of the crystals with phosphocitrate did not increase the inhibition of crystal growth above that observed for phosphocitrate alone.

Experiments with [^{14}C]citrate and [^{14}C]phosphocitrate indicate that the adsorption of citrate to the crystals prior to binding phosphocitrate is a prerequisite for maximum synergistic effect. A similar synergism by citric acid has been described for the inhibition of tendon calcification by pyrophosphate and inositol triphosphate (Thomas & Tilden, 1972).

The enhancement of phosphocitrate activity may be unique to citric acid since other similar compounds such as isocitric, aconitric, tricarballylic, and malic acids had no effect under identical conditions.

Discussion

Calcium phosphate crystallization proceeds through the initial separation from solution of an amorphous precipitate followed by an autocatalytic conversion of this precipitate into a crystalline phase (Termine & Posner, 1970). In time, the crystalline phase matures to the thermodynamically stable end product hydroxylapatite. At conditions of pH, temperature, and ionic strength near physiological, once precipitation occurs the amorphous → crystalline transformation is complete in a matter of minutes (Termine & Posner, 1970).

In contrast to the rapid conversion of amorphous calcium phosphate into crystalline phases observed in pure systems in vitro, some biological systems possess the ability to stabilize the amorphous phase and regulate the formation of crystalline calcium phosphates. Earlier work in this laboratory has demonstrated that isolated mitochondria can accumulate and maintain large amounts of amorphous calcium phosphate within the matrix in the form of nondiffracting granular deposits (Greenawalt et al., 1964). Similarly, normal human urine, although supersaturated with calcium and phosphate, does not normally allow precipitation and crystallization of calcium phosphate except under pathological conditions (Howard & Thomas, 1958; Fleisch & Bisaz, 1962).

Many different naturally occurring and synthetic compounds have been shown to inhibit the initial rate and extent of calcium phosphate precipitation and the subsequent formation of crystalline phases. Polyphosphates, diphosphonates, and pyrophosphate are all very potent inhibitors of HA crystal growth (Fleisch et al., 1968a; Francis, 1969). ATP, Mg^{2+}, and citrate also stabilize amorphous calcium phosphate; to a lesser extent they slow the rate of HA crystal growth (Blumenthal et al., 1977). Moreover, inhibition of soft tissue calcification has been reported in rats administered diphosphonates while on diets which induce vicarious calcification (Fleisch et al., 1968b). The relationship of these effects to the physiological regulation of calcium phosphate mineralization is not clear. Nevertheless, studies of the mechanism by which various inhibitors stabilize amorphous calcium phosphate and prevent the growth of crystalline phases may aid in elucidating the physiological regulation of calcification.

The growth of calcium phosphate in metastable solutions is thought to occur through the incorporation of ions from solutions at specific defects in the crystal lattice (Nancollas, 1976). Adsorption of lattice ions at these defects is energetically favored and results in rapid crystal growth (Nancollas, 1976). In contrast, deposition of lattice ions onto the edges or smooth faces of the crystals is less favored and may contribute only to slow growth (Nancollas, 1976). This view of HA crystal growth is supported by studies which have shown that the amount of inhibitor required to reduce substantially the rate of crystal growth is far less than the total surface area of the crystals (Nancollas, 1976). Measurements of the surface area of hydroxylapatite preparations similar to those used in this work have given values as large as 3800 $cm^2/100$ mg (Meyer et al., 1975). Only 25 nmol (0.333 μM) of phosphocitrate in a volume of 75 mL is required to reduce by one-half the rate of growth of 3.5 mg of seed crystals having a surface area of ~133 cm^2. This amount of phosphocitrate if packed in a monolayer could cover only a very small fraction of the total surface area of the crystals.

Support for these concepts of crystal growth and inhibition is demonstrated by the effect of phosphocitrate on hydroxylapatite crystals. The uptake of 20 nmol of phosphocitrate by 3.5 mg of crystals is essentially complete within the first few minutes of incubation, and further crystal growth does not resume for some time after adsorption of phosphocitrate. Larger amounts of phosphocitrate show a similar rapid uptake of 18–20 nmol of the compound, followed by a slower adsorption of the remaining inhibitor. Even with an initial phosphocitrate addition as large as 200 nmol, crystal growth eventually resumes and approaches a rate identical with uninhibited growth. These observations suggest that even in the presence of high concentrations of inhibitor, the HA crystals are continually forming new lattice defects. The newly formed growth sites, by binding inhibitor, can reduce the concentration of inhibitor in the medium sufficiently so that over a period of time crystal growth will begin again and proceed without inhibition.

The large variations in inhibitory activity demonstrated by the compounds tested in Table I suggest that there are very specific structural requirements for a molecule to be an effective inhibitor of hydroxylapatite crystal growth. Such factors as size, net electrical charge, charge spacing, and the presence of specific types of functional groups can be expected to determine inhibitory activity. None of these factors, taken

singly, appears to be of paramount importance in view of the comparative data in Table I. Some of the more potent inhibitors, 2,3-diphosphoglycerate, pyrophosphate, and 1-hydroxyethane-1-diphosphonate, contain closely spaced phosphate or phosphonate groups, as do condensed polyphosphates (Francis, 1969). On the other hand, ATP, ADP, NAD^+, and NADH, which also have closely spaced phosphate groups, are much less active. Phosphocitrate, with only one phosphate group, shows the highest activity of any compound studied here.

Similarly, negative charge density, whatever the contributing functional groups, also cannot alone be the paramount factor, since phosphoenolpyruvate, with a high density of negative charges, showed significant inhibition of HA crystal growth only at very high concentrations. Although phosphocitrate was the most active compound tested, the monoethyl ester of phosphocitrate produced no measurable inhibition at a 100-fold greater concentration.

Intuitively the inhibitory effect of a given molecule on crystal growth might be expected to be a function of how strongly it binds to the growth sites on the crystal. Presumably binding of an inhibitor is maximal when it contains properly spaced functional groups that insert easily into the crystal lattice. However, the remainder of the molecule also must play a role, since citrate at concentrations less than 1 μM binds readily to HA seed crystals but does not produce inhibition until concentrations above 5 μM are used. Thus, despite the ready adsorption of citric acid by HA crystals, it appears to lack the additional structure necessary to inhibit HA growth at low concentrations. The much greater potency of phosphocitrate, compared to citrate, may be related to the presence of two chemical structures, phosphate and citrate, either of which can be separately incorporated into the crystal lattice, while effectively blocking further crystal growth. Further investigations are under way in this laboratory to determine the structural requirements of inhibitors of hydroxylapatite growth and the mechanism of their action.

Since citrate is present in all body fluids and in calcified tissue, its synergistic action with naturally occurring inhibitors and with phosphocitrate may be of significance in the regulation of biological calcium phosphate mineralization. Because prior binding of citrate to the HA crystals was required for maximum enhancement of phosphocitrate activity, regulation of citrate concentration could provide some physiological control of inhibitor activity.

The crystal growth assay employed in this work cannot be considered as more than a model of biological calcium phosphate deposition and crystallization. However, it does provide a reproducible method for study of inhibition of crystal growth, which can supplement other methods for determining the effects of various compounds on the formation of amorphous calcium phosphate and the growth of crystalline phases (Fleisch et al., 1968a,b; Termine & Posner, 1970; Blumenthal et al., 1975; Termine et al., 1970). While all of these methods can provide data on inhibitory activity, it is important that comparisons of the inhibition produced by different compounds be made in identical systems. Biological calcium phosphate mineralization in all likelihood proceeds through the formation of multiple intermediate phases prior to formation of thermodynamically stable hydroxylapatite. It is therefore conceivable that the successive steps in this process may be inhibited by different kinds of biologically occurring compounds.

The properties of synthetic phosphocitrate described here will aid in carrying out a rigorous identification now under way of the structure of the naturally occurring inhibitor(s) in mitochondria, crab liver, and human urine.

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