PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 321–327 (1982)

Trace Metal–Citric Acid Complexes as Inhibitors of Calcification and Crystal Formation[1] (41437)

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Abstract. At high citrate:metal ratios the small polyvalent metals Fe^{3+}, Be^{2+}, Al^{3+}, and Cr^{3+} interact with citrate to form potent inhibitors of both calcium uptake by a calcifiable matrix and crystal formation from a metastable solution of calcium phosphate. These unique metal–citrate complexes are effective inhibitors at $0.2-30 \times 10^{-6}$ *M*, with iron (III) citrate being the most potent. The inhibitor effects of mixtures of the metal–citrate complexes are additive. These observations define a new role for citrate and indicate how certain trace elements may, by interaction with citrate, be important determinants in the control of calcium salt deposition *in vivo*. The possible pertinence of these observations to the deposition of calcium salts within the kidney and to the prevalence of idiopathic calculi in various geographic areas is now under investigation.

A number of substances are known to inhibit formation and growth of calcium phosphate crystals *in vitro*, and some of these are believed to be involved in the regulation of various processes of mineralization *in vivo* (1). Certain metals, such as zinc, aluminum, manganese, and copper, are among those substances that may inhibit crystal growth, whereas other metals may promote formation of calcium phosphate crystals from metastable solutions (2–4). For example, Schiffman *et al.* (5) observed that addition of iron (III) to the incubation media enhanced mineralization of "elastin." Similarly, we observed that microgram concentrations of iron (III) reduced the concentration of calcium required to mineralize rachitic rat cartilage *in vitro* and also increased calcium uptake during mineralization of collagen. In attempting to counteract this latter enhancement of calcium uptake by adding an appropriate small amount of citrate, we obtained a surprising result—calcium uptake by collagen was completely inhibited. This unexpected effect is possibly explained by observations made some years ago by Spiro *et al.* (6, 7). They reported that in the presence of a high concentration ratio of citrate to iron (III) such as that we fortuitously used, a low-molecular-weight iron-citrate complex, probably a ferric dicitrate, was formed. Conceivably, such a complex would be an excellent inhibitor of calcium deposition, which, if present, could account for the observed inhibition of calcium uptake.

The foregoing observations served as the stimulus for us to examine in more detail the effect of various concentrations of citrate and iron (III) on calcium uptake by mineralizable collagen and also on the growth of calcium phosphate crystals. Furthermore, Dr. R. C. Stoufer (Dept. of Chemistry, University of Florida, Gainesville, personal communication) suggested that other metals in which the ratio of charge to radius is large, such as beryllium, aluminum, or chromium, should react with citrate in a manner similar to that of iron (III). If so, these metal–citrate complexes might also be inhibitors of calcium deposi-

[1] This research was supported by the Research Service of the Veterans Administration and by NIH SCOR Grant Urolithiasis AM 20586.

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0037-9727/82/070321-07$01.00/0

tion. Our preliminary observations supported this suggestion (8), and we report here our investigations with those trace metals that apparently do interact with citrate to form potent inhibitors of mineralization and crystal formation.

Methods. *Mineralization of collagen.* The source of collagen was fresh tendon obtained from the forefeet of cows not injected with "tenderizer" before slaughter and prepared for mineralization as reported previously (9). Fifty milligrams of shredded tendon were used for each incubation because this amount provided a reproducible (±5%) uptake of calcium (10, 11). For the mineralization procedures, shredded tendon was incubated at 37°C in 50-ml volumes of a 0.14 *M* salt solution buffered with bicarbonate to pH 7.4 and containing 1.75 m*M* Ca and 1.61 m*M* inorganic P (9). A tracer of ^{45}Ca (Amersham Corp, Arlington Heights, Ill.) was added to each solution, and the decrease in radioactivity (cpm, counted sufficiently to ensure 98% reliability) during the 24-hr period of incubation was used as the index of calcium uptake by the tendon.

Analytical reagent grade chemicals were used throughout this experiment, and all solutions were prepared with deionized, glass-distilled water. To minimize hydrolysis the stock solutions of the various metals were prepared fresh on the day of use in 0.001 *M* HNO_3. When only a single concentration of the metal was to be tested, we found that the results were the same whether the metal salt was dissolved in the HNO_3 or directly in 0.005 *M* citric acid. The appropriate amounts of the compounds to be tested were not added to the incubation mixture until after the pH of this mixture had been brought to 7.4 by aeration with a certified 5% CO_2–95% O_2 gas mixture (9). All observations were conducted in duplicate, and shredded tendon was not added until after the incubation mixtures had been completely constituted and a 1-ml aliquot obtained for a time "0" measure of counts per minute.

Studies of crystal formation. By use of a modification of the method of Solomons and Neuman (12) those metal–citrate combinations that inhibited calcium uptake by the shredded tendon were tested for their ability to prevent formation of crystals from a supersaturated solution of calcium and phosphorus. The basal incubating solution was similar to that used to assess mineralization of tendon except that it was necessary to increase the calcium and phosphorus to 2.5 m*M* and 3.0 m*M*, respectively, so that crystals would form within 72 hr. Duplicate 50-ml volumes containing a range of concentrations for the various metals were incubated at 37°C in a Dubnoff shaker for 96 hr. Reproducible results required the use of new or siliconized glassware. The solutions were inspected daily for the presence of visible crystals. At 96 hr, even if no crystals were visible, a portion of each solution was filtered rapidly under pressure with a 5% CO_2–95% O_2 gas mixture through a Millipore membrane that excluded molecules of greater than 10,000 daltons, and the filtrate was promptly acidified and subsequently analyzed for calcium by atomic absorption.

Results. *Effect of iron salts on mineralization of tendon.* When the standard incubation mixture was modified by the inclusion of iron (III) at concentrations of $1-20 \times 10^{-6}$ *M*, calcium uptake by the tendon was augmented by 7 to 10%. Conversely, when the incubation mixture contained 0.3 m*M* citrate, iron (III) at concentrations of 1.8×10^{-6} *M* or greater caused complete inhibition of calcium uptake. A detectable inhibitory effect of iron (III) was evident with concentrations of only 0.2×10^{-6} *M* (Fig. 1). The slight reduction in calcium uptake (15% or less) induced by citrate per se was the amount expected on the basis of chelation of calcium and accounted for very little of the total inhibition achieved when both iron (III) and citrate were in the incubation medium.

Iron (II) in the presence of 0.3 m*M* citrate was one-third to one-half as effective as iron (III) in causing inhibition of calcium uptake by shredded tendon. Possibly much of this observed effect with iron (II) was actually caused by iron (III) derived from

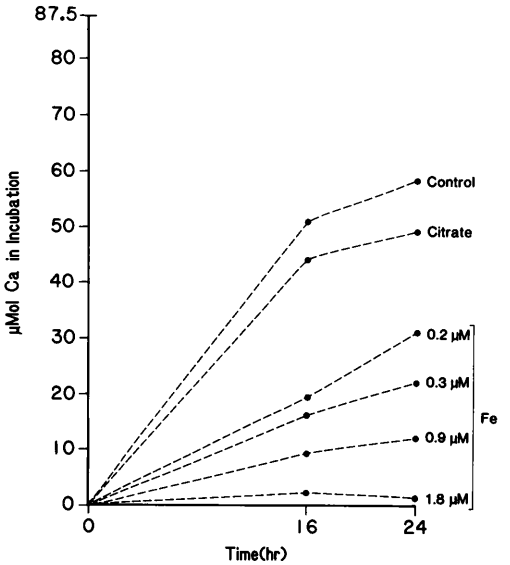

FIG. 1. Effect of Fe^{3+} + citrate on Ca uptake by tendon. The basic 50-ml incubation solution contained a total of 87.5 μmol of calcium. Plotted are uptakes of calcium by 50 mg of shredded tendon during 24 hr of incubation in the basic solution (labeled Control), in the basic solution supplemented with citrate (0.3 m*M*) (labeled Citrate) and in solutions supplemented with citrate plus the designated concentrations of metal. This same format was used for Figs. 2–4.

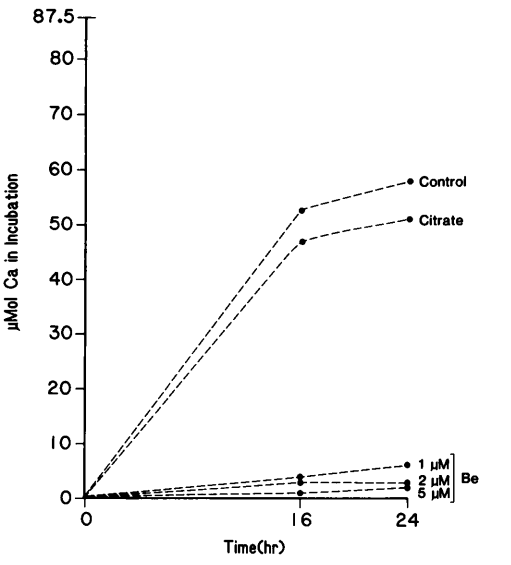

FIG. 2. Effect of Be^{2+} + citrate on Ca uptake by tendon. See legend to Fig. 1 for details.

the oxidation of iron (II) during the 24-hr incubation in the presence of increased tension of oxygen (13).

When the citric acid in the incubation mixture was replaced by equimolar concentrations of isocitric or lactic acid, the addition of iron (III) did not cause inhibition of calcium uptake by the tendon. Furthermore, when the source of iron (III) used in the incubation with citrate was from an aqueous solution of ferric chloride in which the iron had hydrolized, the calcium uptake was not inhibited. When ferric citrate ($FeC_6H_5O_7 \cdot 3H_2O$) was the source of iron (III), uptake was not inhibited unless sufficient additional citrate was added to provide a citrate concentration of approximately 0.3 m*M*.

Effects of beryllium, aluminum, and chromium salts on mineralization of tendon. The addition of small amounts of Be^{2+}, Al^{3+}, or Cr^{3+} to an incubation mixture containing 0.3 m*M* citrate caused complete inhibition of calcium uptake by shredded tendon. Beryllium was effective at much lower concentrations than were required for comparable degrees of inhibition by Al^{3+} or Cr^{3+} (Figs. 2–4). When citrate was deleted from the incubation mixture, some inhibition of calcium uptake was demonstrable with each of these three metals. However, with the exception of Cr^{3+}, the magnitude of this inhibition was small. For example, with citrate in the incubation media, essentially complete inhibition was induced by Be^{2+}, Al^{3+}, and Cr^{3+} at concentrations of 2, 20, and 30 μM, respectively; whereas in the absence of citrate, the same concentrations of these metals reduced calcium uptake by only 5, 10, and 40%, respectively.

When incubated in the presence of citrate, combinations of Fe^{3+} with Be^{2+}, Al^{3+}, or Cr^{3+} had an additive effect in reducing calcium uptake. For comparative purposes, we also determined the effectiveness of two other well-defined inhibitors of calcium uptake—phosphocitrate and inorganic pyrophosphate. The results of this evaluation are recorded in Table I. Phosphocitrate

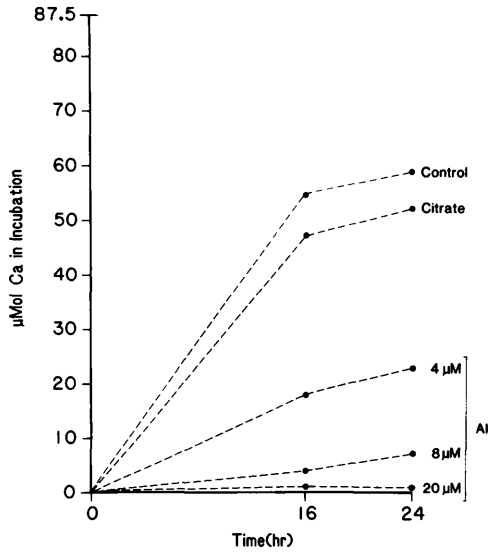

FIG. 3. Effect of Al^{3+} + citrate on Ca uptake by tendon. See legend to Fig. 1 for details.

was the most potent inhibitor (14), but it is noteworthy that based on iron concentration the iron (III)–citrate complex was a more potent inhibitor than inorganic pyrophosphate.

To gain some idea of the affinity of citrate for the particular metals being investi-

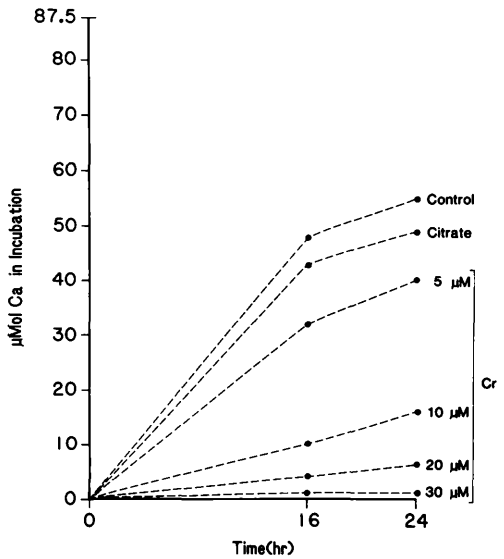

FIG. 4. Effect of Cr^{3+} + citrate on Ca uptake by tendon. See legend to Fig. 1 for details.

TABLE I. COMPARATIVE POTENCY OF INHIBITORS OF CALCIFICATION IN THE PRESENCE OF CITRATE[a]

Inhibitor	Concentration (M) for 100% inhibition
Phosphocitrate[b]	0.5×10^{-6}
Fe^{3+}	1.8×10^{-6}
Be^{2+}	2.0×10^{-6}
Pyrophosphate	7.0×10^{-6}
Al^{3+}	20.0×10^{-6}
Cr^{3+}	30.0×10^{-6}

[a] Each incubating solution contained Ca, P, and citrate at the following concentrations: 1.75, 1.61, and 0.3 mM, respectively.

[b] Provided by Dr. W. P. Tew of The Johns Hopkins University School of Medicine.

gated, we tested several compounds for their ability to prevent the formation of citrate–metal inhibitor complexes. The sodium salt of ethylenediaminetetraacetic acid (EDTA) at 5×10^{-6} M blocked completely the inhibitory effect to be expected with iron (III)–citrate. This concentration of EDTA did not modify the inhibition induced by mixtures of aluminum or chromium and citrate. Sodium silicate at a concentration of 2 mg/dl (as SiO_2) almost completely nullified the inhibition of calcium uptake expected with iron (III) and citrate, but a silicate concentration of 10 mg/dl was required to prevent the inhibition induced by complexes of citrate with aluminum. The inhibitory effect of chromium (III) in the presence of citrate was not modified by either of these concentrations of silicate. The effects of silicate on the incubation mixtures containing iron (III) or aluminum are consistent with the known binding affinity of silicate for these two metals (15).

Effects of variation in citrate:metal ratio. Observations were conducted to determine if a high citrate:metal ratio was required for development of inhibitor activity. In the presence of 300 μM citrate the inhibition of calcium uptake induced by iron (III) at 1.8 μM was unchanged on reducing the citrate concentrations by one-half, i.e., to 150 μM. However, a marked reduction in inhibitory effect occurred when the citrate concentration was reduced to 75 μM or less. Mainte-

nance of chromium in the incubation media at 30 μM during serial reductions in citrate concentration was accompanied by progressive lessening of the inhibitory effect on calcium uptake, but the changes were not so striking as with iron (III). However, quite different results were obtained on reducing the citrate concentration in incubation mixtures containing citrate and aluminum. As illustrated in Fig. 3, almost complete inhibition of calcium uptake by tendon was achieved with a citrate:aluminum ratio of 300 μM:20 μM. When the aluminum concentrations were reduced, e.g., to 5 or 10 μM, so that only partial inhibition of uptake would be expected, twice the inhibition was obtained with citrate concentrations of 150 and 75 μM as with a concentration of 300 μM.

Effects of other trace elements of calcium uptake by tendon. Zinc, manganese, cobalt, and cupric ions were tested for their effect on calcium uptake during incubation of the tendon matrix in the presence and absence of citrate. Cobalt and cupric ions caused little demonstrable inhibition (15% or less) of calcium uptake when added to the incubation solution at concentrations up to 15 μM, and there was no synergistic effect when citrate (0.3 mM) was present. Both manganese (10 μM) and zinc (15 μM) have been previously noted to have inhibitory effects on mineralization *in vitro* (2), and these metals did considerably reduce (approximately 50%) calcium uptake by the tendon, but just as with cobalt and cupric ions, there were no synergistic increases in inhibition when these metals were included with citrate during the incubations. However, the inhibitory effect of zinc or manganese on calcium uptake was additive to that induced by partially blocking concentrations of citrate complexes with iron, aluminum, or chromium.

Effect of Fe^{3+}, Al^{3+}, and Cr^{3+}, on crystal formation. The solution used to assess crystal formation was sufficiently metastable that crystals of calcium phosphate regularly formed after 36 to 72 hr of incubation. In the presence of citrate, Fe^{3+}, Al^{3+}, or Cr^{3+} retarded crystal formation at concentrations less than those required to block calcium uptake by tendon (Table II). Also, the inhibitor effect of mixtures of these metals on crystal formation was shown to be additive.

Discussion. Various investigators have used different matrices to evaluate particular aspects of mineralization. Each such matrix usually has certian unique properties that affect the initiation or extent of mineralization. For example, mineralization of the elastin-containing nuchal ligament may depend on the presence of sulfhydryl groups for binding metal ions (5). The orthophosphate-saturated collagen matrix that we used has been shown to reflect changes in the availability of calcium or the presence of inhibitors of mineralization in a manner analogous to that of rachitic cartilage matrix (9, 16). Furthermore, preparation of the tendon by exposure to multiple changes of a 3% solution of orthophosphate effectively removes soluble proteins thought to inhibit apatite crystal deposition (17). Whether such proteins are similar to the soluble phosphoprotein inhibitors extractable from dentin is unknown (18). Also it will be important to determine the effect of the metal–citrate mixtures defined in the pres-

TABLE II. INHIBITION OF CRYSTAL FORMATION BY METALS IN THE PRESENCE OF CITRATE[a]

Additives	Visible occurrence of crystals (hr)	Ca concentration in supernatant (mM)
Control	72	0.8
Fe^{3+} 0.4×10^{-6} M	96	2.2
Al^{3+} 15×10^{-6} M	96	2.1
Cr^{3+} 15×10^{-6} M	>96	2.3

[a] Incubation of 0.14 M bicarbonate-buffered salt solution containing Ca, 2.5 mM; P, 3.0 mM; and citrate, 0.3 mM. Because of potential toxicity, beryllium salts were not tested.

ent experiment on calcium binding by other matrices such as carboxyglutamic acid-containing osteocalcin (19) and the complex of Type 1 collagen with osteonectin (20). The latter complex possibly constitutes the initial locus for apatite crystal formation in normal bone.

The results obtained in this experiment provide convincing evidence that certain metals—Fe^{3+}, Be^{3+}, Al^{2+}, and Cr^{3+}—interact with citrate to form unique complexes capable of interfering with the availability of calcium either for mineralization of a matrix or for the formation of crystalline calcium phosphate. The inhibitory effect of these citrate complexes on rates of apatite crystal formation is sufficient to account for the observed inhibition of calcium uptake by the tendon matrix. Moreover, in an extension of the present observations, these metal–citrate complexes have also been found to inhibit growth of calcium phosphate seed crystals *in vitro* (21). The propensity with which these polyvalent metals hydrolyze undoubtedly accounts for their unique interaction with citrate to form inhibitors of calcium uptake and formation, or growth, of apatite crystals. Interestingly, there was no evidence of inhibitor formation by iron (III) when another hydroxylated carboxylic acid, isocitric acid, was substituted for citric acid in the incubation mixtures. Thus conformational properties must be critically important to the inhibitor effect of the metal–citrate complexes.

An investigation into the composition and structure of the complexes formed between citrate and iron (III), aluminum, and chromium (III) has been initiated. Spiro and associates (7) found that the large ferric citrate polymer formed in 1:1 mixtures of citrate and iron (III) did not develop when there was a 20:1, or greater, excess of citrate to iron in solution, and their data indicated that a ferric dicitrate was formed at such high ratios. However, we found that almost none of the iron from a 30:1 citrate–iron (III) mixture labeled with radioactive iron was filterable through a filter with a 1000-dalton exclusion limit, whereas approximately 70% of the iron from such a mixture was filterable through a filter with a 5000-dalton exclusion limit; and all of the iron was filterable when the exclusion limit was increased to 10,000 daltons. These observations indicate that the complex formed between iron (III) and citrate is not simple ferric dicitrate and is probably a small polymer. However, initial attempts at characterization of the anionic iron–citrate complex separated by column chromatography did reveal that the iron: citrate ratio in the complex was approximately 1:2.4. Further studies on the structure of these metal–citrate complexes are in progress.

The present experiment was conducted as part of a long-range investigation into possible causes of kidney stone formation. The marked affinity of citrate for iron (III), aluminum, and chromium (III) and the fact that these metal–citrate complexes form at an acid pH (22–24) suggest the possibility that these complexes may occur *in vivo*, especially in urine. For example, at pH 7.4 the transferrin in plasma has a greater affinity for iron than does citrate. However, at a pH of less than 6.6, as is usual in urine, citrate will remove iron from transferrin (22). The stability constants for aluminum and chromium binding to citrate under physiologic conditions have not been so precisely defined as those for iron (III) but are sufficiently high to indicate the likelihood of metal:citrate interaction *in vivo* wherever unusual concentrations of the metals might occur (23, 24). In studies in progress we have found sufficient amounts of iron and aluminum in ultrafiltrates of urine that if present as complexes with citrate, would have an appreciable effect in modifying calcium phosphate stability in urine. Investigations are now underway to determine whether such metal–citrate complexes do exist in urine and whether there is any correlation between the amounts of these metals excreted and susceptibility to renal calculus formation. The potential of silicates to prevent formation of iron and aluminum citrates may also be relevant to renal calculus disease, in that silicates are present in urine, often in considerable concentrations.

Independent of the foregoing speculations, however, the observations in the present experiment certainly provide evidence of new aspects of citrate chemistry and the means whereby citrate and certain polyvalent cations may participate to a greater degree than previously recognized in the control of bone mineral deposition.

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Received November 9, 1981. P.S.E.B.M. 1982, Vol. 170.