Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5528–5532, September 1981
Biochemistry

Phosphocitrate inhibits mitochondrial and cytosolic accumulation of calcium in kidney cells *in vivo*

(nephrocalcinosis/crystallization)

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ABSTRACT **Synthetic 3-phosphocitrate, an extremely potent inhibitor of calcium phosphate crystallization as determined in a nonbiological physical-chemical assay, has many similarities to a mitochondrial factor that inhibits crystallization of nondiffracting amorphous calcium phosphate. In order to determine whether phosphocitrate can prevent uptake and crystallization of calcium phosphate in mitochondria *in vivo*, it was administered intraperitoneally to animals given large daily doses of calcium gluconate or parathyroid hormone, a regimen that causes massive accumulation and crystallization of calcium phosphate in the mitochondria and cytosol of renal tubule cells *in vivo*. Administration of phosphocitrate greatly reduced the net uptake of Ca^{2+} by the kidneys and prevented the appearance of apatite-like crystalline structures within the mitochondrial matrix and cytosol of renal tubule cells. Phosphocitrate, which is a poor chelator of Ca^{2+}, did not reduce the hypercalcemia induced by either agent. These *in vivo* observations therefore indicate that phosphocitrate acts primarily at the cellular level to prevent the extensive accumulation of calcium phosphate in kidney cells by inhibiting the mitochondrial accumulation or crystallization of calcium phosphate.**

Previous reports from this laboratory have described the occurrence of a factor in rat liver mitochondria that inhibits the *in vitro* conversion of amorphous calcium phosphate to crystalline forms such as hydroxyapatite (1–6). A factor or factors with similar properties also are present in the hepatopancreas of the blue crab *Callinectes sapidus* which salvages and stores large amounts of calcium phosphate in a nondiffracting form during ecdysis (7, 8). Other factors that inhibit precipitation or crystallization of calcium salts have also been demonstrated in urine, saliva, bile, and other body fluids (9–14), where they have been proposed to maintain high concentrations of Ca^{2+} and phosphate in solutions in apparent excess of the solubility product of calcium phosphate and other relatively insoluble calcium salts. The mitochondrial factor has many similarities with 3-phosphocitrate, prepared synthetically in this laboratory, with respect to molecular weight, chromatographic behavior, and mass spectrum (15). Moreover, synthetic phosphocitrate was found to be the most potent known inhibitor of the growth of hydroxyapatite crystals from supersaturated calcium phosphate solutions at pH 7.4, as determined in an *in vitro* crystal growth assay system (15).

This communication reports experiments designed to determine whether phosphocitrate administration can inhibit mitochondrial calcium phosphate deposition or crystallization in intact tissues *in vivo* in two well-studied animal models in which acute calcification of the kidney was induced by administration of calcium gluconate to rats (16, 17) and of excess parathyroid hormone (PTH) to mice (16, 17). Both agents have been shown to cause hypercalcemia, a large increase in the calcium content of the kidneys, and deposition of electron-dense granules of insoluble and often crystalline calcium salts within the mitochondria of tubule cells, followed by extensive extramitochondrial precipitation of calcium salts, a chain of events that eventually results in cell necrosis.

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MATERIALS AND METHODS

The two experimental models of kidney calcification used in this investigation were chosen because they have been extensively studied and yield severe nephrocalcinosis in a few days. Kidney calcification was induced in young male CD rats (50–60 g, Charles River Breeding Laboratories) by administration of calcium gluconate (16, 17). The animals were fed standard chow and water ad lib throughout the experimental period. They were divided into two groups, a control group receiving daily intraperitoneal injections of isotonic saline and an experimental group receiving daily intraperitoneal injections of phosphocitrate. One hour after each of these injections, all animals were given 1.0 ml of 10% calcium gluconate intraperitoneally. The amounts of phosphocitrate administered are given in the figure legends. Twenty-four hours after the 3rd, 5th, 7th and 10th injections of calcium gluconate, animals from both groups were anesthetized with sodium pentobarbital, blood was withdrawn from the inferior vena cava for analysis, and both kidneys were removed for microscopic examination and determination of their calcium content.

Renal calcification was induced in young male Swiss mice (15–18 g, Buckberg Laboratories, Tomkins Cove, NY) by administration of excess parathyroid hormone. The mice were divided into two groups that received daily intraperitoneal injections of either isotonic saline (controls) or phosphocitrate (experimental group), followed 1 hr later by injection of 50 units (USP) of the hormone (Eli Lilly). Details of the phosphocitrate dosage are given in the figure legends. Animals from both groups were sacrificed 24 hr after the first, second, third, and fourth hormone injections, blood was withdrawn from the inferior vena cava for calcium analysis, and both kidneys were removed for measurement of calcium accumulation and microscopic examination.

For determination of calcium content of the kidneys, midcoronal sections were weighed, homogenized by sonication in 5.0 ml of a butanol/HCl/La^{3+} mixture (5.85 g of La_2O_3, 25 ml of concentrated HCl, and 40 ml of *n*-butanol, diluted to 1 liter with deionized water), and centrifuged (10,000 × *g* for 10 min) to remove insoluble matter (18). Calcium in the supernatant fraction was determined with a model 251 Instrumentation Laboratory atomic absorption spectrophotometer. Total serum calcium was measured by atomic absorption in 10-μl serum samples diluted to 1.0 ml with the butanol/HCl/La^{3+} mixture (19).

Abbreviation: PTH, parathyroid hormone.

Representative kidney sections were retained for microscopic verification of calcification and ultrastructural examination with a Zeiss 10B electron microscope. Details of tissue preparation are given in figure legends.

Phosphocitric acid was synthesized from triethylcitrate and o-phenylene phosphorochloridate as described and purified by anion exchange chromatography (15). Stock solutions of phosphocitric acid were converted to the sodium salt before use by neutralization with NaOH. The concentration of phosphocitrate was determined enzymatically (15).

RESULTS

Effect of Phosphocitrate Administration on Kidney Calcification Induced by Calcium Gluconate and PTH. Intraperitoneal administration of small quantities of phosphocitrate effectively inhibited the severe kidney calcification produced by administration of calcium gluconate in rats and by PTH in mice. In the calcium gluconate animals, the calcium content of the kidneys increased 70-fold, from a normal level of 2 μmol/g tissue to a mean (±SD) of 140 ± 32 μmol/g over the 10-day period (Fig. 1). The kidney calcification produced in mice by excess PTH administration was qualitatively similar to that produced by calcium gluconate, although it became manifest over a shorter time period. Kidney calcium levels increased from 2 μmol/g tissue to 60 ± 10 μmol/g by day 4 of hormone administration. Kidney calcification induced by these agents progressed as described (10, 11).

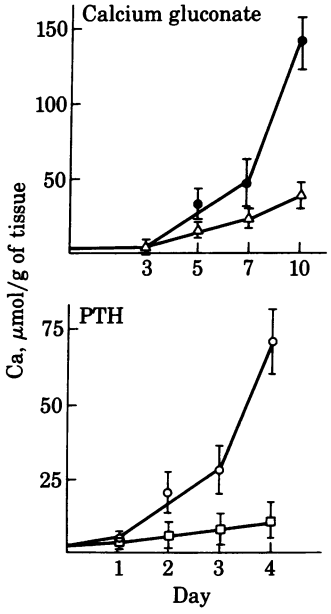


FIG. 1. Effect of phosphocitrate on kidney calcification induced by calcium gluconate and PTH. (*Upper*) Two groups of 20 rats (50–60 g) were given daily intraperitoneal injections of 1.0 ml of 10% calcium gluconate for 10 days. The treated group (△) also received 3.0 μmol of phosphocitrate in 0.5 ml of isotonic saline (60 μmol/kg) intraperitoneally 1 hr prior to each calcium gluconate injection. The control group (●) received an equal volume of isotonic saline prior to each calcium gluconate injection. Five rats from each group were sacrificed on the days indicated and the calcium content of both kidneys was determined. Each point represents the mean ± SD of the data from 10 kidneys (five rats) for each point. (*Lower*) Two groups of 20 mice were given daily intraperitoneal injections of 50 units of PTH for 4 days. One hour preceding each PTH injection, one group (□) of mice received 1.0 μmol of phosphocitrate in 0.25 ml of isotonic saline (60 μmol/kg) and the control group (○) received an equal volume of saline. Five mice from each group were sacrificed on the days indicated and the calcium content of both kidneys was determined. Each point represents the mean ± SD of the data from 10 kidneys (five mice).

In contrast, the kidneys from animals receiving intraperitoneal injections of phosphocitrate (60 μmol/kg) prior to administration of calcium gluconate or PTH showed a delayed and greatly decreased calcium uptake over the same period (Fig. 1). The mean calcium content of the kidneys of animals receiving phosphocitrate was only 10 ± 5 μmol/g tissue by day 4 of the PTH treatment and 37 ± 13 μmol/g tissue by day 10 of the calcium gluconate treatment. In both groups these values represent a significant reduction in tissue calcium content relative to the control animals. In identical control experiments, intraperitoneal administration of sodium citrate or sodium phosphate (60 μmol/kg) in place of phosphocitrate had no detectable effect on the development of kidney calcification produced by calcium gluconate or PTH.

Serum Calcium Levels. Measurements of serum calcium levels showed that hypercalcemia prevailed throughout the experimental period in both the calcium gluconate- and PTH-treated animals, but in neither group was the hypercalcemia diminished by phosphocitrate administration. Total serum calcium increased from a normal level of 1.8 mM to > 3.0 mM and remained high throughout the experimental period in all groups, with no significant differences upon phosphocitrate administration.

Direct measurement of free ionized Ca^{2+} in the serum samples with a calibrated Ca^{2+}-selective electrode showed increased but essentially equal Ca^{2+} activities in all groups. These measurements thus indicate that phosphocitrate administration does not result in sequestration of the increased serum Ca^{2+} in the form of unionized complexes or chelates. Other experiments (unpublished data) have shown that phosphocitrate is only weakly active as a chelating agent for Ca^{2+} at pH 7.4. The apparent formation constant of Ca^{2+}·phosphocitrate complex is not high enough to affect significantly the serum levels of free Ca^{2+} at the doses of phosphocitrate administered.

Response to Phosphocitrate Dose. Inhibition of PTH-induced renal calcification was observed at all levels of phosphocitrate tested (Fig. 2), from 0.075 to 3.0 μmol/day. Although the higher doses were more effective in preventing nephrocal-

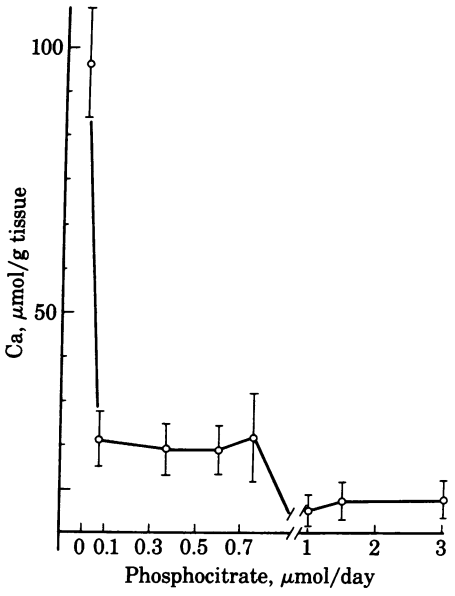


FIG. 2. Response to phosphocitrate. Eight groups of five mice each were given various doses of phosphocitrate followed 1 hr later by 50 units of PTH over 4 consecutive days. The mice were sacrificed 24 hr after the fourth PTH injection and the calcium content of both kidneys was determined. The data plotted represent the mean ± SD of 10 kidneys (5 mice) at each dose level.

cinosis, the lowest dose inhibited Ca^{2+} accumulation by the kidneys by 80% relative to controls.

Light Microscopy. Microscopic examination of kidney sections from control animals receiving calcium gluconate or PTH showed a progressive increase in deposition of calcium phosphate over the experimental period, as indicated by the von Kossa stain (20). Although the lesions progressed more rapidly in the PTH treated group than in the calcium gluconate group, the overall patterns of mineralization were qualitatively similar in the two models. In the control group receiving calcium gluconate, no deposition of calcium salts was observed in the proximal tubule areas through day 5 of treatment. On day 7, small areas of calcification were observed along the basement membrane of the proximal tubules, and by day 10 all sections examined showed extensive mineralization in the proximal tubule area similar to that described in earlier reports of acute calcium gluconate nephrotoxicity (21). In some areas the calcification was so extensive that no cellular detail could be discerned. Much cellular debris and mineralized material was also present in the tubular lumens.

A similar course of calcification was observed in the control animals treated with PTH. No calcium deposits were observed in the kidneys of the mice 1 day after the first PTH injection. Twenty-four hours after the second PTH injection, small sites of mineralization were evident in the proximal tubule area. Large areas of calcification were observed after the fourth PTH injection, along with many necrotic cells along the lumen of the proximal tubules (Fig. 3), in agreement with previous reports (16, 17).

Kidney sections from animals receiving phosphocitrate together with calcium gluconate or PTH were remarkably free from obvious calcium deposits. The von Kossa stain (20) for calcium salts was completely negative in the tubular area of the kidneys of all animals treated with calcium gluconate and phosphocitrate through day 7. Only a few sections contained small areas of calcium deposition by day 10. Similarly, no calcium deposits appeared in the PTH/phosphocitrate group until day 4, and then only a few very small deposits were found (Fig. 3).

Electron Microscopic Examination. In confirmation of the observations by light microscopy, the kidneys of the control animals receiving calcium gluconate or PTH showed calcification changes on electron microscopic examination that were in close agreement with earlier reports (16, 17) on ultrastructural changes in nephrocalcinosis induced by these agents. The earliest evidence of calcium accumulation in the kidneys of the control animals was the appearance of very dense granules within the mitochondria of the proximal tubular cells; these were first observed on day 3 with calcium gluconate and on day 2 with PTH. As the lesions progressed, the number of dense granular deposits within the mitochondria increased in approximate proportion to the increase in total calcium content of the kidneys. By day 7 of the calcium gluconate regimen and day 3 of PTH, the mitochondria were filled with dense mineral deposits, the intracristal spaces were dilated, and many tubule

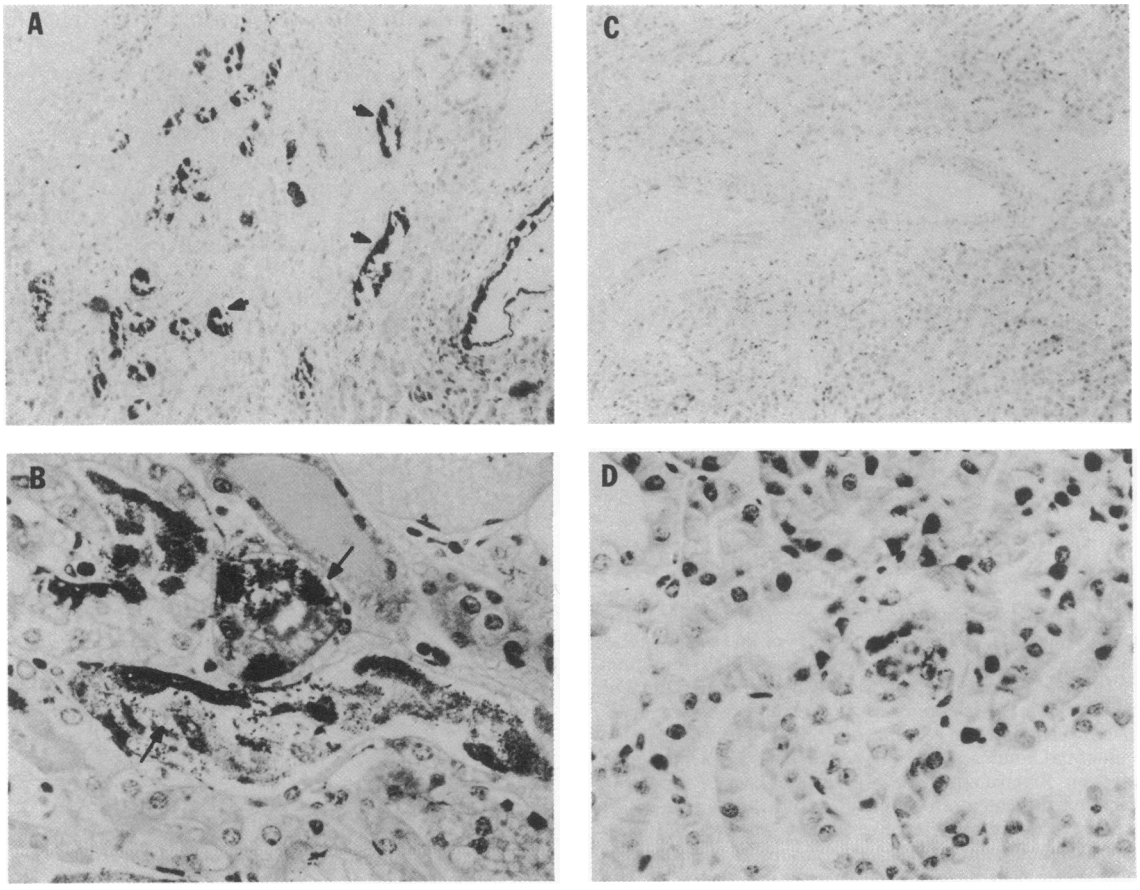


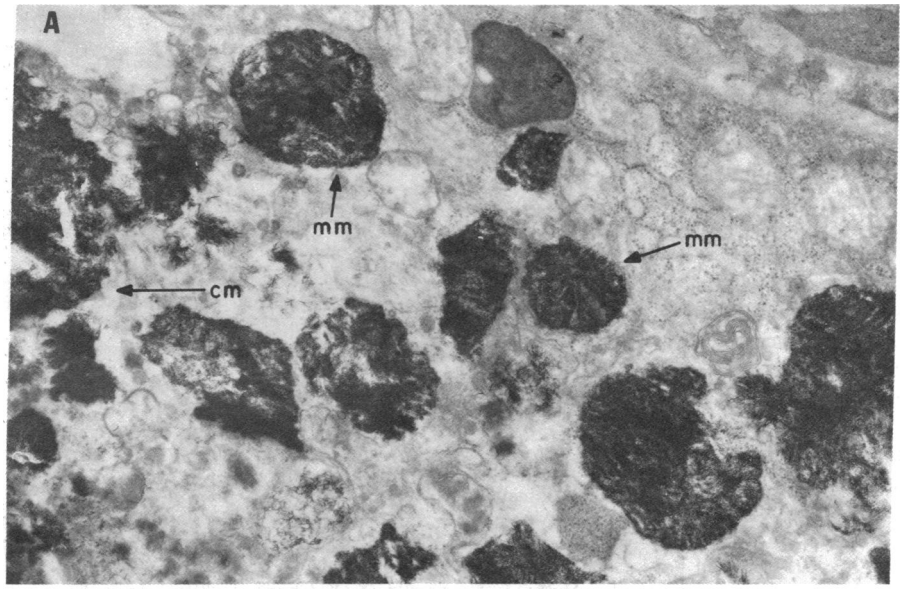
FIG. 3. Light microscopic study of the effects of phosphocitrate on PTH induced nephrocalcinosis. Kidney sections from mice treated with saline and PTH or with phosphocitrate and PTH for 4 days were fixed with 10% buffered formalin, stained for calcium salts by the von Kossa technique, and counterstained with hematoxylin. The control group (*A*, ×120; *B*, ×250), which received saline and PTH, showed extensive deposition of calcium salts (arrows) within the tubular lumen and adjacent cells. Similar sections from the phosphocitrate and PTH group (*C*, ×120; *D*, ×250) were substantially free of mineral deposits.

cells were noticeably swollen. In the most severely damaged cells the mitochondria were rounded and swollen, and contained numerous granular dense bodies within the matrix; there was extensive cytoplasmic swelling and apparent rupture of plasma membranes. In some cells the mitochondria contained apatite-like spicules, usually associated with the cristae. On day 10 of calcium gluconate or day 4 of PTH administration the mitochondria were heavily calcified, and extensive mineral deposits appeared in the extramitochondrial cytoplasm (Fig. 4). At this stage, many of the mitochondria had lost their characteristic shape and appeared as rounded bodies full of apatite-like crystalline structures.

Similar examination of kidney tissue from animals given phosphocitrate prior to calcium gluconate or PTH revealed few intracellular abnormalities within the proximal tubular area. In most of the tissue sections examined at the end of the experimental periods, there was slight mitochondrial swelling (Fig. 4). In only a few highly localized areas were intramitochondrial or cytosolic mineral deposits observed. Tissue sections from the phosphocitrate-treated animals were otherwise unremarkable.

DISCUSSION

Considerable evidence suggests that pathological calcification of kidney cells, and possibly other mammalian cell types, may begin with the net entry of Ca^{2+} into the cells because of increased plasma Ca^{2+} concentration or because of damage to the selective permeability of the plasma membrane. Because the plasma Ca^{2+} concentration is almost 10^4 times higher than the cytosolic level, any breach of the membrane may lead to net influx of Ca^{2+}. The increased concentration of Ca^{2+} in the cytosol accelerates the transport of Ca^{2+} and phosphate into the mitochondria via the Ca^{2+} and phosphate transport systems, at the expense of energy delivered by electron transport (reviewed in refs. 22, 23). Such an influx of Ca^{2+} and phosphate into the mitochondrial matrix is a reflection of the normal capacity of animal cells to maintain cytosolic Ca^{2+} homeostasis at concentrations in the neighborhood of 0.2 μM. Mitochondria of all mammalian cells tested have an extremely high rate of Ca^{2+} accumulation, which may exceed 1000 nmol/mg of protein per min (22, 23), and a large capacity, 3000 nmol/mg of protein with



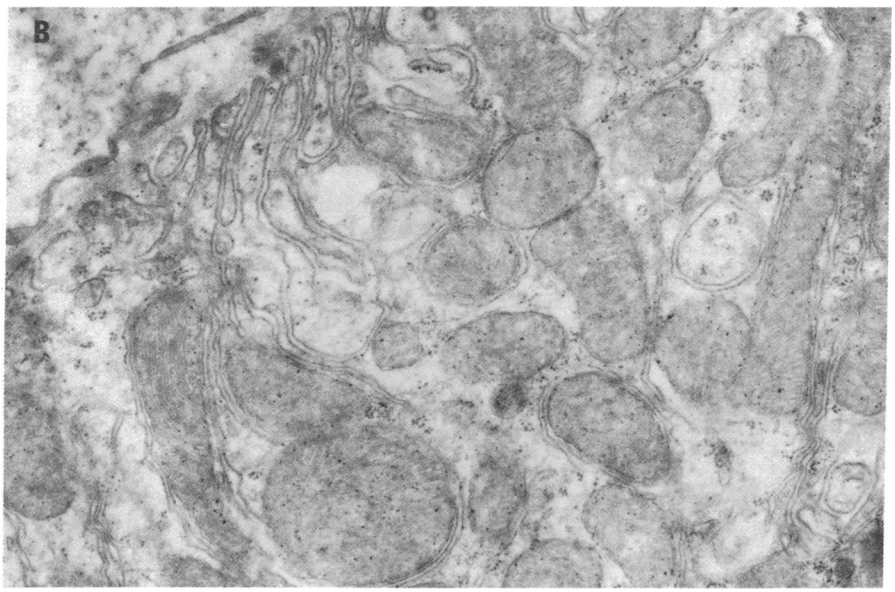


FIG. 4. Electron microscopic study of the effects of phosphocitrate on PTH-induced nephrocalcinosis. Thin sections from mice treated with phosphocitrate and PTH or saline and PTH for 4 days were fixed with 3% glutaraldehyde/0.1 M phosphate-buffered saline, pH 7.4, and stained with uranyl acetate and lead citrate. (×12,400.) The control (saline/PTH) sections (*A*) contained numerous heavily mineralized mitochondria (mm) as well as extensive areas of calcification within the cytoplasm (cm). Mineral deposits were not observed in sections from those animals given phosphocitrate prior to PTH (*B*).

uptake of equivalent amounts of phosphate (22, 23). Endoplasmic reticulum of the liver, on the other hand, has the capacity to segregate only a few nanomoles of Ca^{2+} per mg of protein (24). Because accumulation of even small amounts of Ca^{2+} and phosphate within the small volume of the mitochondrial matrix will exceed the solubility product of brushite ($CaHPO_4$) or tricalcium phosphate $Ca_3(PO_4)_2$, insoluble deposits of one or both of these salts is likely to result.

Calcium phosphate deposits in normal rat liver mitochondria loaded *in vitro* are amorphous and show no crystalline x-ray diffraction pattern (24, 25). On microincineration at 900°C, however, they are converted into residues that show the characteristic diffraction pattern of hydroxyapatite (26, 27). Because amorphous precipitates of calcium phosphate spontaneously undergo crystallization at pH 7.4 and 37°C within minutes, it has been postulated (1–6) that the amorphous calcium phosphate deposits in mitochondria are kept from crystallizing into apatite or other crystalline forms by the presence of endogenous inhibitors within the mitochondrial matrix.

It is possible that the capacity of mitochondrial inhibitors to prevent crystallization of matrix deposits of amorphous calcium phosphate may be exceeded when abnormally large amounts of calcium and phosphate pass into the mitochondrial matrix, as occurs during administration of excess calcium gluconate or PTH. Once such deposits become crystalline they also become less soluble, dissolve more slowly, and serve as nuclei for further growth. The growth and accumulation of such crystals could result in irreversible damage to the mitochondrial inner membrane and its Ca^{2+}-concentrating capacity. Crystallization of hydroxyapatite in the extramitochondrial cytoplasm then may ensue as the plasma membrane becomes more permeable and allows Ca^{2+} and phosphate to pass into the cell more freely.

It appears possible that phosphocitrate administered as described here prevents or greatly retards such a sequence of events by gaining entrance into the renal tubule cells and, further, into their mitochondria to supplement the action of endogenous mitochondrial factors in retarding crystallization. It is also possible that phosphocitrate may act primarily in the cytosol or at the plasma membrane. Examination of the organ and intracellular distribution of administered phosphocitrate should yield information as to the site of its action in suppressing renal calcification. In any case, its action is other than as a Ca^{2+}-chelating agent; this is also shown by data in Fig. 3; 0.1 μmol of phosphocitrate per mouse, equivalent to 6 μM in total body water, prevented calcification induced by 3 mM Ca^{2+} in the blood plasma.

Other substances have been found to prevent nephrocalcinosis under similar conditions, in particular several different synthetic diphosphonates (28), which are less active than phosphocitrate in preventing calcium phosphate crystallization in nonbiological crystal growth assays (15). Although diphosphonates were found to be effective in preventing clinical nephrocalcinosis (29), they are toxic to normal calcification processes in the periosteum and epiphyseal disks of long bones (30). Comparative studies with ethane-1-hydroxy-1,1-diphosphonate as a control have shown that daily administration of phosphocitrate (60 μmol/kg) for up to 14 days to rats and mice had no apparent effect on normal bone development and growth plate structure, in contrast to the inhibition of normal calcification produced by the diphosphonate over the same period (unpublished data). Other studies have shown that phosphocitrate has no effect on *in vivo* calcification in a matrix-induced model of bone formation (31). Further investigation is necessary, but it appears possible that phosphocitrate functions primarily to inhibit ectopic or pathological calcification selectively.

The authors thank Jane Foard for her expert technical assistance and Glenn Decker for the electron microscopy. This work was supported by grants from the National Science Foundation (PCM-7818190), the National Institutes of Health (GM05919), the Harris Foundation, and the Blum Foundation. C.D.M. was supported by National Institutes of Health Training Grant 5-T-32-AM-07145.

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