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Interaction of Cadmium Ions with Calcium Hydroxyapatite Crystals: A Possible Mechanism Contributing to the Pathogenesis of Cadmium-Induced Bone Diseases

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Summary Cadmium ions adsorb onto calcium hydroxyapatite crystals (HA) and are as effective as inorganic pyrophosphate and aluminum ions in retarding the rate of *in vitro* dissolution of HA. In contrast, cadmium ions have no important retarding effect on the growth of HA, but are built into the crystals, thus making them very resistant to subsequent dissolution. These effects could interfere with bone remodeling, with cadmium protecting normal sites of resorption and thus causing resorption at pathological sites.

Key words: Cadmium — Hydroxyapatite — Itai-Itai — Osteoporosis — Osteomalacia — Osteosclerosis — Pseudofractures.

Chronic cadmium exposure may result in development of a variety of bone lesions, osteomalacia, osteoporosis, osteosclerosis, and pseudofractures, all caused by occupational exposure to cadmium [1–4]. The most serious cases of cadmium-induced osteopathy have occurred among inhabitants of the Toyama prefecture in Japan, where the rice was heavily polluted by cadmium. The disease, called "Itai-Itai byo" (Ouch-Ouch disease) is characterized by severe bone pain, several spontaneous fractures (often of the spine), severe osteomalacia with extensive bone decalcification, and many pseudofractures [5, 6].

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Although a direct effect of cadmium on the skeleton was possible, most Itai-Itai patients also suffered from renal dysfunction, as indicated by proteinuria [7], often of the tubular type with increased urinary excretion of β-2-microglobulin [8], retinol-binding protein [9] and metallothionein [10]. Many patients also had glucosuria [7, 11] and aminoaciduria [12].

In laboratory animals, the same type of bone lesions [13–15] have been experimentally induced. Elevated cadmium levels, in bones of both Itai-Itai patients and alkaline battery factory workers suffering from bone diseases, have been reported [16, 17].

Although the exact mechanism in cadmium-induced osteopathy is still unknown, it is clearly related to the calcium metabolism. Plasma calcium is maintained at a constant level by a complex homeostatic mechanism involving 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$)-mediated stimulation of intestinal calcium absorption as well as bone resorption, bone mineralization and renal excretion of calcium, controlled by parathyroid hormone (PTH), $1,25(OH)_2D_3$, and calcitonin. These processes depend to a large extent on the availability, dissolution, and growth of calcium hydroxyapatite in the skeletal tissues.

The rate of dissolution of synthetic calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals (HA) has been studied extensively [18–23] and is controlled by a surface nucleation process. The effects of inhibitors depend on the type, concentration, and amount of the inhibitor and on the degree of supersaturation with respect to HA, pH, and on the surface area of the crystals, whose variation can be represented by m/m_o, with m_o being the initial mass

of crystals and m being the actual mass of crystals. Thus, plots of rate or degree of reaction against time are not suited for description of effects of inhibitors because of the strong dependence on the above-mentioned parameters. Instead, the rate for prechosen values of the parameters influencing the rate of dissolution of HA can be compared for systems with or without an inhibitor [20, 23–27]. With J_o and J_L being the rates of dissolution per gram HA without and with an inhibitor present, respectively, other parameters being constant, the effect of the inhibitor can be described by plots of J_L/J_o or J_o/J_L against the concentration of the inhibitor. Although this method is tedious and requires a high accuracy of the experiments, each experiment contributing only one point to such a plot, this approach was deemed crucial for a better understanding of the possible interactions of cadmium with bone tissue metabolism.

Growth of HA has not been investigated to nearly the same extent as the dissolution process, but work yet to be published shows that the rate of growth can also be described by the same type of mechanism as the dissolution process.

The aims of the present paper are to clarify the effects of cadmium ions on the rate of dissolution and growth of calcium hydroxyapatite crystals, the main inorganic component of bones and teeth.

Materials and Methods

All chemicals were of analytical grade. The pyrophosphate content of phosphate solutions was reduced by treatment with alkaline phosphatase (Boehringer Mannheim, 108146), the enzyme subsequently being removed by pressure filtering through a Diaflo PM10 ultrafiltration membrane. Deionized water, further purified by a Millipore Milli-Q analytical water system, was used. Care was taken to avoid contamination by carbon dioxide [18]. The HA crystals were prepared as described by Christoffersen et al. in [18]. These crystals have a Ca/P mole ratio of 1.67 ± 0.01 and a specific surface area of 33 m^2/g, determined by a single point BET (Brunauer, Emmett, and Teller) method with a Quantasorb apparatus. All experiments were made at 37.0 ± 0.1°C.

For the adsorption measurements, 10 mg HA crystals were added to 1,000 ml aqueous solution, slightly undersaturated with respect to HA at 37°C and pH = 7.2 ± 0.2 ([HA]~5 μM) and containing 1–3 μM cadmium nitrate. In additional experiments, the solvent also contained 0.1 M sodium chloride in order to simulate a biological system more closely. In these cases, the solvent contained [HA]~10 μM, due to the higher solubility of HA at higher ionic strength. At various times samples were removed, filtered through a 0.2 μm Millipore filter, and the cadmium concentration in the filtrate was then measured by means of a Perkin-Elmer model 4000 atomic absorption spectrometer with an HGA-400 graphite furnace. Concentrations greater than 0.1 μM could be measured with an accuracy of ± 5%. Finally, the mass of crystals in an experiment was determined by acidifying the remainder of the mixture in the reaction vessel along with the filters and measuring the calcium and phosphate content of this solution. Calcium concentrations were determined by atomic absorption spectrometry. Phosphate concentrations were determined by spectrophotometric measurement of the molybdenum blue complex, using a Carl Zeiss PMQII spectrophotometer.

The rate of dissolution at pH 7.00 ± 0.02 was studied, as previously described [18], by measuring the rate of addition of 2 mM nitric acid in order to keep pH constant. About 10 mg HA crystals were added to 900 ml water or 0.1 M sodium chloride, containing 0–3 μM cadmium nitrate. After approximately 2 hours, a portion of the suspension was filtered and the cadmium concentration of the filtrate was measured. The remainder of the reaction system was acidified to determine the mass of crystals from calcium and phosphate measurements.

The rate of growth of HA was studied in a similar way, except that the initial solution was either 10 times or 4.5 times supersaturated with respect to HA, and the pH was kept constant at 7.2 ± 0.02 by titration with 2 mM potassium hydroxide. In these experiments, 15 mg HA was added to 900 ml solution containing 0–5 μM cadmium nitrate. Further, in one case, crystals, which had grown to 1.4 times their original mass from a 10 times supersaturated solution containing 5 μM cadmium nitrate, were recovered from the suspension and transferred to 900 ml water for assessment of their dissolution rate.

Results

Adsorption of Cadmium Ions onto HA

The adsorption of cadmium ions onto HA is most appropriately described by the simple Langmuir adsorption equation

$$K_L = x/(1 - x)C = (B/B_{max})/(1 - B/B_{max})C \quad (1)$$

where K_L is the adsorption constant, C the concentration of free Cd^{2+} ions in the solution, and x is the

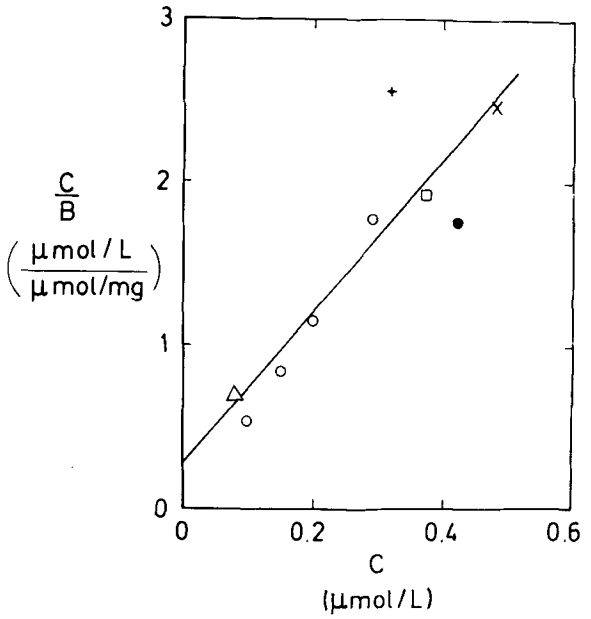

Fig. 1. Adsorption of cadmium ions onto HA crystals, pH = 7.2, 37°C, solution slightly undersaturated with respect to HA, plotted according to the Langmuir adsorption isotherm, equation (2). C is the concentration of free Cd^{2+} ions in solution, B is the amount of cadmium adsorbed per unit mass of HA. The symbols are explained in Table 1. From the regression line drawn in this figure we obtain $K_L = 1.7 \times 10^7$ liter/mol and $B_{max} = 2.1 \times 10^{-4}$ mol/g, where K_L is the Langmuir adsorption constant and B_{max} is the binding capacity of HA for cadmium ions.

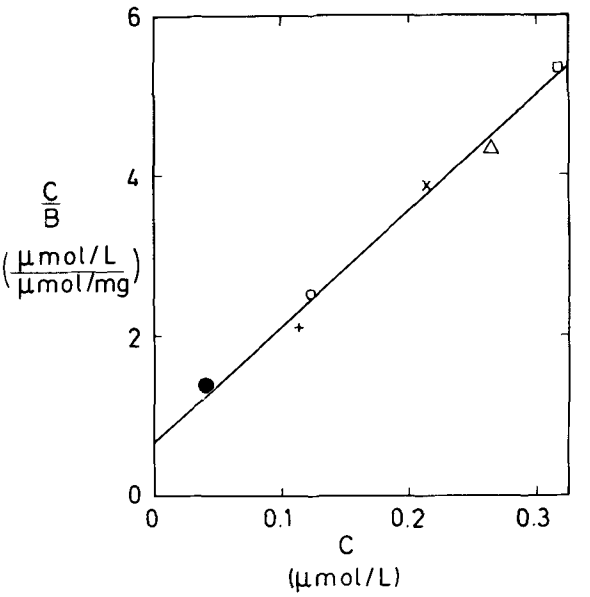

Fig. 2. Adsorption of cadmium ions onto HA crystals, 0.1 M NaCl, pH = 7.2, 37°C, solution slightly undersaturated with respect to HA in this medium, plotted according to the Langmuir adsorption isotherm, equation (2). The symbols are explained in Table 2. From the regression line drawn in this figure, we obtain $K_L = 2.1 \times 10^7$ liter/mol and $B_{max} = 0.7 \times 10^{-4}$ mol/g.

fraction of adsorption sites occupied by cadmium. This fraction is equal to the amount of cadmium bound per unit mass of HA, B, divided by the binding capacity (B_{max}). Equation (1) can also be expressed as

$$C/B = C/B_{max} + 1/K_L B_{max} \qquad (2)$$

The important assumptions leading to the Langmuir adsorption equation are that the number of adsorption sites per unit surface area is constant, and that adsorbed ions or molecules do not interact [28]. In the present case, cadmium ions probably adsorb onto vacant calcium sites and thus interact mainly with the phosphate groups in the crystal surface. In equations (1) and (2), concentrations rather than activities are used, mainly because activity coefficients for vacant sites and for adsorbed species are unknown.

If equation (1) can be used to describe the adsorption, a plot of C/B against C should give a straight line with the slope $1/B_{max}$ and an intercept with the C/B axis equal to $1/K_L B_{max}$. The results of the adsorption experiments without and with sodium chloride in the solution are given in Figures 1 and 2 and in Tables 1 and 2. From the latter, our data can be reinterpreted in terms of other models than the one used here. In Table 1 and Figure 1 it is seen that the amount of cadmium absorbed onto HA in systems not containing sodium chloride increases with time. Nevertheless, the data represent a linear increase of C/B with increasing C (Fig. 1). From this line, we obtain $B_{max} = 2.1 \times 10^{-4}$ mol/g and $K_L = 1.7 \times 10^7$ liter/mol. Taking the specific surface area, $A_{sp} = 33$ m²/g into account, B_{max} can also be expressed as $B_{max} = 6.4 \times 10^{-6}$ mol/m².

In Figure 2 and Table 2, results are given for adsorption experiments with sodium chloride present. Formation of cadmium-chloride complexes of the type $CdCl^+$ has to be taken into account when calculating the concentration of free cadmium ions. We have adopted the association constant

$$K = (CdCl^+)/(Cd^{2+})(Cl^-) = 10^2 \text{ liter/mol} \qquad (3)$$

a typical value from Stability Constants [29]. In equation (3), round brackets denote activities. Using the expression

$$\log y_z = -0.524 z^2 I^{\frac{1}{2}}/(1 + zI^{\frac{1}{2}}) \qquad (4)$$

for activity coefficients, in which z is the charge number of the ion, I the ionic strength, and y_z the activity coefficient of an ion with charge number z, we obtain for the ionic strength I = 0.1 mol/liter, $y_1 = 0.75$ and $y_2 = 0.39$ at 37°C. From the concentration of total cadmium in solution ($[Cd]_{aq}$) as mea-

Table 1. Adsorption of cadmium ions onto HA

Symbol (Fig. 1)	$[Cd]_t$ (μmol/liter)	m_{HA} (mg)	$[Cd]_{aq}$ (μmol/liter)	Time (d)
△	1.09	8.8	0.08	1
+	1.63	10.5	0.32	1
○	1.64	8.3	0.29	1
			0.20	2
			0.15	3
			0.10	6&7
□	2.18	9.4	0.37	1
X	2.45	10.2	0.48	1
●	2.73	9.7	0.42	3

[NaCl] = 0
$[Cd]_t$ is the total concentration of cadmium in the system. $[Cd]_{aq}$ is the measured concentration of cadmium in solution and is equal to the concentration of free Cd^{2+} ions (C) in these experiments. C/B is plotted against C in Figure 1
$B = ([Cd]_t - [Cd]_{aq})V/m_{HA}$ V = 1 liter

Table 2. Adsorption of cadmium ions onto HA

Symbol (Fig. 2)	$[Cd]_t$ (μmol/liter)	m_{HA} (mg)	$[Cd]_{aq}$ (μmol/liter)	Time (d)
●	0.54	10.6	0.21	1, 5
+	1.09	9.6	0.57	1, 2
○	1.09	9.9	0.61	2, 3
X	1.63	10.1	1.07	1, 2, 3
△	1.90	9.3	1.33	0, 3
□	2.18	9.9	1.59	2, 3

[NaCl] = 0.1 mol/liter
In these experiments $C = [Cd]_{aq}/5$, due to the formation of $CdCl^+$ ion pairs. C/B is plotted against C in Figure 2.
$B = ([Cd]_t - [Cd]_{aq})V/m_{HA}$ V = 1 liter

sured by atomic absorption, the concentration (C) of free cadmium ions in the solution can be calculated using the above expressions for activity coefficients and the cadmium chloride association constant.

The adsorption equilibrium was obtained faster when sodium chloride was present and no time dependence was observed after 1 day. From Figure 2, we obtain $B_{max} = 6.9 \times 10^{-5}$ mol/g $= 2.1 \times 10^{-6}$ mol/m^2 and $K_L = 2.1 \times 10^7$ liter/mol. The adsorption experiments in which sodium chloride was present appear to be more accurate than experiments without sodium chloride. This observation may be related to the reduced time effect, and probably results from the initial concentration of free cadmium ions being less in the chloride-buffered than in the unbuffered solutions, as experiments with the same final concentration of free cadmium ions are compared.

Effect of Cadmium Ions on Dissolution of HA

Results of HA dissolution experiments with cadmium ions present without and with sodium chloride are given in Figures 3 and 4, respectively. In these experiments, the degree of saturation varies, as only pH is kept constant. For each experiment, the rate of dissolution was determined for $Ca/Ca_s = 0.23$ and $Ca/Ca_s = 0.30$, with Ca being the concentration of calcium at the time the rate is measured, and Ca_s the saturation concentration of calcium at the actual value of pH. In Figures 3 and 4, the rate of HA dissolution, J_L, with cadmium present, divided by the rate of dissolution, J_o, without cadmium, but otherwise under identical conditions, is plotted against the concentration of free Cd^{2+} ions. In Figure 3, the concentration of free Cd^{2+} ions is equal to the total concentration of cadmium in the system minus the amount bound per unit volume.

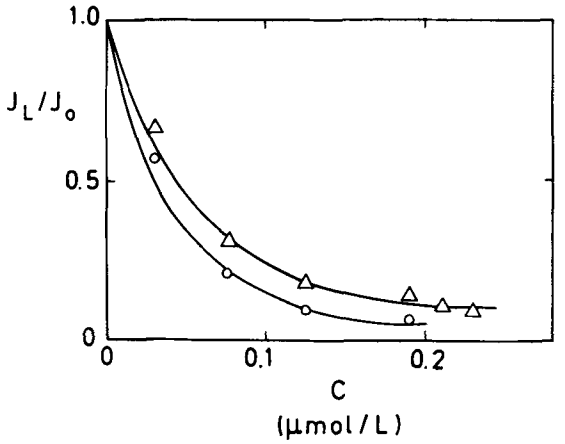

Fig. 3. Ratio of the rate of HA dissolution in systems containing cadmium (J_L) to the rate of dissolution without cadmium (J_o) plotted against the concentration of free Cd^{2+} ions, pH = 7.0, 37°C. △: Ca/Ca_s = 0.23; ○: Ca/Ca_s = 0.30.

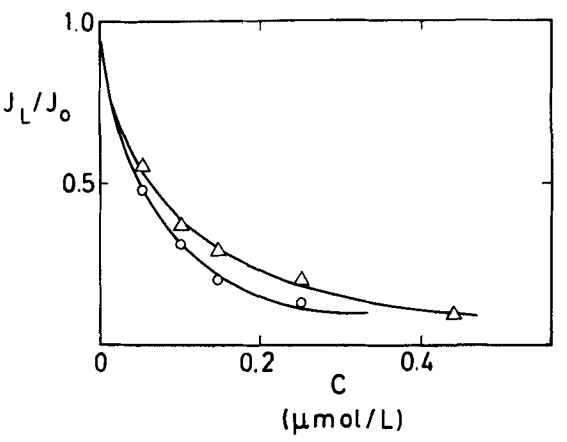

Fig. 4. Ratio of the rate of HA dissolution in systems containing cadmium (J_L) to the rate of dissolution without cadmium (J_o) plotted against the concentration of free Cd^{2+} ions, 0.1 M NaCl, pH = 7.0, 37°C. △: Ca/Ca_s = 0.23; ○: Ca/Ca_s = 0.30.

In Figure 4, the concentration of cadmium ions is further corrected for complex formation with chloride ions according to equations (3) and (4). From these plots it can be seen that free cadmium ions in concentrations as low as 0.1 μM severely retard the rate of dissolution of HA and that the inhibitory effect increases with the degree of saturation.

Effect of Cadmium Ions on growth of HA

Experiments were conducted with HA at pH = 7.2 and Ca_o/Ca_s = 4.5 and Ca_o/Ca_s = 10 without and with cadmium ions present. The results are shown in Figures 5 and 6 and in Table 3. In Figure 5, the volume of 1.84 mM potassium hydroxide required to maintain pH = 7.2 is plotted against time. From this figure it can be seen that the presence of cadmium ions in concentrations up to 5 μM has little effect on the rate of HA growth. Analysis of the solution after the experiments were terminated showed that the concentration of cadmium ions in the solution was drastically reduced to a level below the detection limit of 0.05 μM. From Table 3 it can be seen that under the present experimental conditions, the presence of cadmium ions causes a slight reduction in the initial rate of growth and a slight increase in the rate of growth after the cadmium ions have been incorporated in the solid material. Figure 6 clearly shows that the crystals initially added have grown.

The rate of dissolution of crystals recovered from a growth experiment, in which 15 mg HA grew to 1.4 times the initial mass at pH = 7.2, Ca/Ca_s = 10 and $[Cd]_t$ = 5 μM, was below the detection limit.

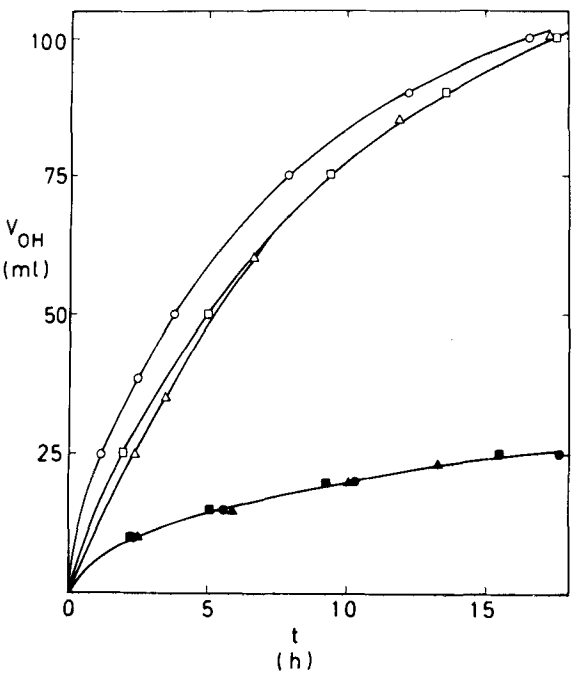

Fig. 5. Volume of 1.8 mM KOH added in order to maintain pH = 7.2 during the growth of HA crystals, ~16 mg in 900 ml, at initial supersaturations Ca_o/Ca_s = 10 (open symbols, upper curves) and Ca_o/Ca_s = 4.5 (closed symbols, lower curves), plotted as a function of time. ○ and ● no cadmium present; □ and ■: $[Cd]_t$ = 3 μM; △ and ▲: $[Cd]_t$ = 5 μM. Detailed results of these experiments are given in Table 3.

Discussion

From the adsorption study it can be concluded that cadmium ions can adsorb onto HA. The adsorption can be described by a Langmuir adsorption

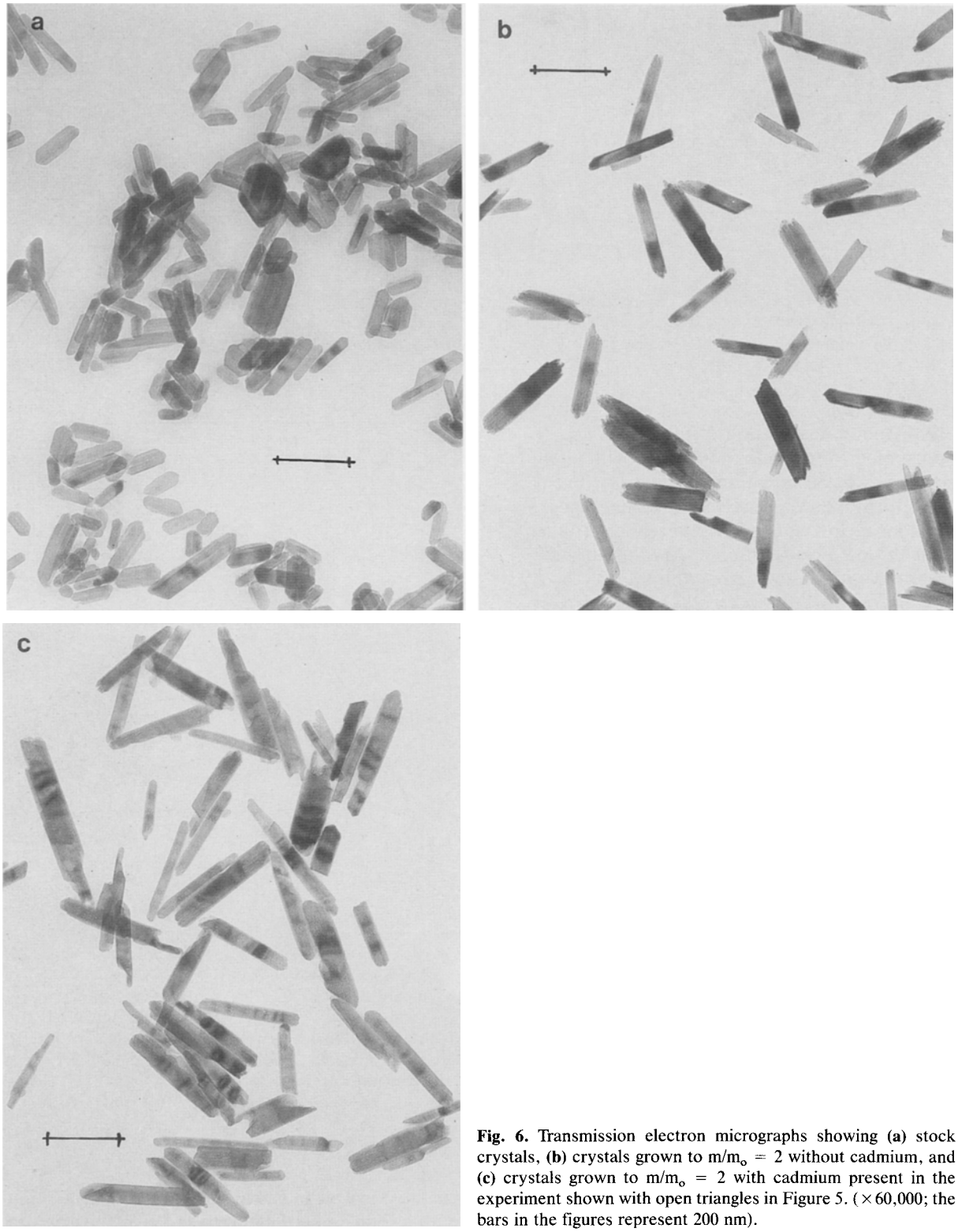

Fig. 6. Transmission electron micrographs showing **(a)** stock crystals, **(b)** crystals grown to $m/m_o = 2$ without cadmium, and **(c)** crystals grown to $m/m_o = 2$ with cadmium present in the experiment shown with open triangles in Figure 5. ($\times 60{,}000$; the bars in the figures represent 200 nm).

Table 3. The ratio of the rate of growth of HA crystals in the presence of cadmium to the rate in the absence of cadmium at various supersaturations for the experiments shown in Figure 5

Ca_o/Ca_s	Ca/Ca_s	m/m_0	J_L/J_o ($[Cd]_t$ = 3 μM)	J_L/J_o ($[Cd]_t$ = 5 μM)
10	9	1.2	0.8	0.8
	8	1.4	1.0	1.2
	7	1.6	1.1	1.2
4.5	4	1.1	0.9	0.6
	3.5	1.2	1.0	0.9

J_L = rate in presence of cadmium; J_o = rate in absence of cadmium; Ca/Ca_s = supersaturation; m/m_0 is the ratio of the mass of HA crystals to the initial mass of the crystals

isotherm. The value determined for the adsorption constant, $K_L \approx 2 \times 10^7$ liter/mol is comparable to that obtained for adsorption of methylene diphosphonate onto HA [23]. The value $B_{max} = 2.1 \times 10_{-4}$ mol/g in systems of low ionic strength is comparable to the amount of calcium ions, 3×10^{-4} mol/g, situated in the surface of the HA, assuming the crystal surface area to be dominated by ac-faces [20]. At high ionic strength, B_{max} is reduced to 0.7×10^{-4} mol/g. This reduction is most likely due to the interaction between ions, not only in solution but also in the crystal surface, being different in aqueous solution with or without a relatively high concentration of sodium chloride. The dissolution experiments show that free cadmium ions at a concentration of 0.1 μM severely inhibit the dissolution process. However, cadmium concentrations up to 5 μM in the solution have no important inhibitory effect on HA growth; cadmium ions can be built into the crystal lattice and thereby cause these crystals to be highly resistant towards subsequent dissolution.

Based on these *in vitro* studies, cadmium ions adsorbed onto or built into bone crystals are likely to result in a high resistance towards resorption of these crystals. The amount of cadmium in bone crystals will depend on many parameters; the most important ones are probably the concentration of cadmium in the solution wetting the bone crystals, the time the crystals are exposed to the cadmium-containing solution and, in the case of *in vitro* systems, the fluid dynamics. Although cadmium levels in extracellular fluid within bone tissue are unknown, a concentration of 0.1 μM would correspond to about 11 μg/liter, a level frequently exceeded in whole blood and urine in cases of excess cadmium exposure. As the fluid exchange is expected to be higher in regions where normal bone turnover is high, the inhibition of bone crystal dissolution can be expected to be greater in these regions. At normal sites for resorption this process will take place with a reduced rate if the crystals at these locations have adsorbed cadmium. Acid produced for resorption may thus penetrate deeper into the bone structure before reacting with the bone mineral. This could cause development of a porous bone structure, with mineral dissolved at pathological locations (cf. formation of subsurface lesions in dental enamel).

The main factors for development of cadmium-induced osteoporosis and osteomalacia are presently believed to be cadmium-induced renal damage leading to urinary calcium loss in combination with nutritional deficiency with regard to calcium and vitamin D [30]. In short, cadmium inhibits the synthesis of $1,25(OH)_2D_3$ from 25-hydroxycholecalciferol ($25OHD_3$) by the renal tubular cells [31, 32]. This effect may be related to the inhibition by cadmium of PTH stimulation of renal tubular cell adenylcyclase [33]; this inhibition may further lead to increased plasma PTH levels and thus accelerated bone resorption [34] and, at the same time, reduced intestinal calcium absorption due to $1,25(OH)_2D_3$ depletion [35]. Further cadmium may interact directly with intestinal calcium absorption, as cadmium in mucosal bathing fluid inhibited calcium transport in $1,25(OH)_2D_3$-stimulated rat duodenum, as measured by the everted sac technique [36]. Finally, cadmium increases the renal calcium loss due to induction of renal tubular damage [37].

Cadmium may also directly interfere with deposition and resorption of mineral in the organic bone matrix. So far, very little experimental support for this mechanism has been published. Cadmium has been shown to cause an increase in calcium release from embryonic chick bone when exposed to cadmium concentrations of about 10 μM and to cause inhibition in the calcium release at cadmium concentrations of about 0.3 mM [38]. The stimulation in the calcium release was suggested to be due partly to an increased production of lactic acid in connection to the glycolysis [38]. Adsorption studies in which cadmium ions adsorbed onto HA have previously been reported [39]. The amount adsorbed per unit mass of HA increased with decreasing pH and decreased with increasing particle size of HA. The solubility of HA with calcium ions partly substituted by cadmium ions showed a minimum at the molar fraction of cadmium of the order 0.4. Except for this paper, we are not aware of other published evidence of direct cadmium interference with the bone mineralization process at the level of HA metabolism.

The present results were obtained in an experimental *in vitro* system where indirect effects due to perturbation of hormone or serum calcium balance are excluded. However, both direct and indirect ef-

fects may contribute to the pathological changes in the bone tissue. The effects of cadmium *in vitro* are similar to those recently seen with aluminum in parallel studies where aluminum ions adsorbed onto HA and bone crystals and inhibited HA dissolution [40]; the pathogenesis of aluminum-related osteomalacia in dialysis patients is also unclear. Thus, the *in vitro* studies with both aluminum and cadmium suggest that direct effects on HA may well be of importance in metal-induced bone disease.

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