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Discovery of a High Molecular Weight Complex of Calcium, Phosphate, Fetuin, and Matrix γ-Carboxyglutamic Acid Protein in the Serum of Etidronate-treated Rats*

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In the present study we report the discovery of a novel protein-mineral complex in the serum of rats treated with doses of the bone-active bisphosphonate etidronate that inhibit normal bone mineralization. The composition of this high molecular mass protein-mineral complex consists of about 18% mineral, 80% fetuin, and 2% matrix Gla protein (MGP) by weight, and the presence of the complex in serum after an injection of 8 mg etidronate/100 g of body weight elevates calcium by 1.8-fold (to 4.3 mM), phosphate by 1.6-fold (to 5.6 mM), and MGP by 25-fold (to 12 μg/ml). The serum mineral complex reaches maximal levels at 6 h after subcutaneous injection of etidronate and is subsequently cleared from serum by 24 h. This highly specific complex of fetuin, MGP, and mineral prevents the growth, aggregation, and precipitation of the mineral component, which indicates that the previously reported calcification inhibitory activities of fetuin and MGP may be related to their ability to form stable complexes with nascent mineral nuclei. Treatment with the vitamin K-antagonist warfarin prevents the increase in serum MGP after etidronate injection, which shows that the increase in serum MGP is due to new synthesis and that the γ-carboxylation of MGP is necessary for its binding to the serum mineral complex.

The initial objective of the present investigations was to understand how matrix Gla protein (MGP)[1] inhibits the abnormal calcification of arteries and other soft tissues. Recent genetic and biochemical studies have established MGP as the first protein known to act as a calcification inhibitor *in vivo*. In humans, defects in the MGP gene that predict a non-functional MGP protein have been shown to be responsible for Keutel syndrome (1), a rare inherited disease characterized by multiple peripheral pulmonary artery stenoses, by abnormal calcification of cartilages, including costal, nasal, auricle, tracheal, and growth plate cartilage, and by nasal hypoplasia and brachytelephalangia (2, 3). In mice, targeted deletion of the MGP gene causes rapid calcification of the elastic lamellae of the arterial media, which begins at birth and is sufficiently extensive by 3–6 weeks of age that the arteries become rigid tubes that fracture, causing death by exsanguination in most of the affected mice by 6 weeks of age (4). MGP-deficient mice also display abnormal calcification of growth plate and tracheal ring cartilage. Finally, treatment of rats with the vitamin K antagonist warfarin at doses that inhibit the γ-carboxylation of MGP causes rapid calcification of elastic lamellae of arteries and of aortic heart valves and increased expression of MGP mRNA in the calcifying artery (5, 6).

Matrix Gla protein is a 10-kDa secreted protein that was originally discovered in demineralization extracts of bone but is now known to be expressed by a wide variety of tissues and cell types. The rat tissues with the highest levels of MGP mRNA are cartilage, heart, kidney, and lung (7, 8), and cells known to express MGP mRNA include osteoblasts, chondrocytes, vascular smooth muscle cells, pneumocytes, kidney cells, and fibroblasts (7–12). Although several noncalcified tissues do express MGP mRNA at a higher level than bone, significant levels of the protein itself have only been found in bone and calcified cartilage (8, 13). This observation suggests that the protein may accumulate at sites of calcification and that much of the protein secreted by noncalcified tissues probably escapes to plasma, where MGP is found at 0.3–1 μg/ml depending on the species. MGP contains five residues of the vitamin K-dependent calcium binding amino acid, γ-carboxyglutamic acid (Gla) (14, 15) and three residues of phosphoserine located at conserved N-terminal sequence positions in MGP from shark, rat, cow, and human tissues (16). Specific proteolytic cleavage at a conserved dibasic site in the C-terminal region has also been observed for MGP isolated from human, bovine, and shark tissues (13, 17).

The original objective of the present study was to use the geminal bisphosphonate etidronate (ethylhydroxybisphosphonate) to probe the function of MGP in bone metabolism. These studies were prompted by the fact that etidronate and MGP both bind strongly to bone mineral and by previous studies that showed that etidronate competes with bone Gla protein (BGP; osteocalcin), a related vitamin K-dependent protein, for binding to hydroxyapatite *in vitro*, and that etidronate injection into a rat produced a transient 3-fold elevation in serum BGP levels (18). We report here that etidronate produced a greater than 25-fold elevation in serum levels of MGP within 6 h and that this elevation is caused by the unexpected appearance of a novel complex of calcium, phosphate, fetuin, and MGP in serum after etidronate injection. The structure and properties of this complex have direct relevance to an understanding of how MGP normally inhibits calcification *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Simonsen albino male rats (Sprague-Dawley-derived) were purchased from Simonsen labs (Gilroy, Ca). Etidronate was a gift

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[1] The abbreviations used are: MGP, matrix γ-carboxyglutamic acid protein; BGP, bone Gla protein (osteocalcin); fetuin, α2-HS-glycoprotein; Gla, γ-carboxyglutamic acid.

from Proctor and Gamble (Cincinnati, OH). Sephacryl S-300 HR gel filtration media was purchased from Amersham Biosciences, Inc. Ultrafree CL filtration devices were purchased from Millipore Corp. Warfarin was purchased from Sigma. Stock solutions of sodium warfarin were prepared at 50 mg/ml in 0.15 M NaCl and stored in sterile foil-wrapped containers at 4 °C. MGP was purified from rat bone as described (17). Rabbit antibodies against rat fetuin were prepared as described (19). All other reagents used were reagent grade or better.

Maintenance of Animals—Rats were fed rodent diet 5001 (Purina Mills Inc., St. Louis, MO), a diet that is 0.67% phosphorus and 0.95% calcium by weight. Etidronate was dissolved with 0.15 M NaCl, titrated to pH 7.4 with dilute NaOH, and administered subcutaneously to 40-day-old male rats. Control animals received injections of 0.15 M NaCl only. In the initial time course experiment (Fig. 1), 5 40-day-old male rats were injected with 8 mg of etidronate/100 g of body weight at $t = 0$. At the times indicated in Fig. 1, each rat was anesthetized with metofane, and a 400-μl sample of blood was withdrawn from the jugular vein. Blood samples were allowed to clot for 15 min at room temperature, and serum was obtained after centrifugation in a clinical centrifuge and then frozen rapidly on dry ice and stored at −70 °C until later analysis. The same procedure was followed for the second time course experiment (Fig. 2), with the difference that the etidronate dose was increased to 32 mg/100 g of body weight.

To determine the effect of warfarin on the generation of the serum protein-mineral complex, 4 rats received subcutaneous injections of 15.4 mg of warfarin/100 g of body weight, and 4 rats received injections of saline vehicle. Two hours later, all rats were injected with etidronate at a dose of 8 mg/100 g ($t = 0$), and 400 μl of blood were removed from each animal at the indicated times for biochemical analyses. Control experiments were also carried out in which four animals received warfarin but not etidronate and four animals were injected with saline vehicle only. In neither control group could we detect a significant change in serum levels of calcium, phosphate, or MGP at any time point.

To examine the possible linkage between the generation of the serum mineral complex and inhibition of bone mineralization, 12 100-g rats were injected with 4 mg of etidronate at $t = 0$ and were then divided into 2 groups of 6 rats each. 0.5-ml blood samples were obtained from the group 1 rats at $t = 0$, 2, 6, and 12 h, and the rats were exsanguinated at $t = 24$ h. The group 2 rats were injected with a second dose of etidronate at $t = 24$ h, and 0.5-ml blood samples were obtained at $t = 24$, 26, 30, and 36 h. These rats were exsanguinated at $t = 48$ h. Serum was obtained at each time point and stored at −20 °C until later analysis. Tibias from the rats killed at 48 h as well as from age-matched controls were removed, cleaned of adhering tissue, and fixed in 70% ethanol. Tibia samples were embedded in plastic and cut into sections of 500 μm by Pathology Associates International (Frederick, MD). The resulting sections were microradiographed using a Hewlett-Packard model 4380N Faxitron x-ray machine. All animal experiments were approved by the University of California at San Diego animal subjects committee.

Biochemical Characterization of the Complex between Calcium, Phosphate, Fetuin, and MGP—The serum mineral complex was characterized by filtration using Ultrafree CL filtration devices with a 300-kDa molecular mass cut-off membrane. In a typical experiment, blood was obtained from 2 control rats and from 2 rats 6 h after a dose of 8 mg of etidronate/100 g of body weight, and each blood sample was immediately placed into a 2.5-ml gold top Vacutainer tube (SST gel and clot activator tube, Becton Dickinson). Thirty minutes later 1-ml aliquots of the serum were removed, placed into the filtration device, and centrifuged for 80 min at 2500 × g to force the sample through the membrane. The filtrate and retentate were then analyzed to determine calcium, phosphate, and volume. The typical filtrate volume recovered was 0.70 ml, and the typical retentate volume recovered was 0.26 ml.

The effect of centrifugation on the serum mineral complex was investigated in serum samples obtained 6 h after administration of etidronate at doses of 8 mg/100 g and 32 mg/100 g of body weight. At the lower dose, no significant amount of calcium, phosphate, or MGP was sedimented after centrifugation for 30 min at 16,000 × g. At the higher dose, a well defined translucent pellet was obtained after centrifugation for 30 min at 16,000 × g. This pellet was dissolved in 1 ml of 50 mM HCl and analyzed for calcium, phosphate, and MGP.

The serum mineral complex was characterized by gel filtration using 25-ml columns of Sephacryl S-300 HR that were prepared in disposable plastic pipettes and were equilibrated with 20 mM HEPES, pH 7.4, 0.15 M NaCl, and 10 mM $CaCl_2$ at room temperature. Serum was obtained from rats 6 h after administration of etidronate at a dose of 8 mg/100 g, and 1-ml aliquots were immediately applied to the Sephacryl column. Fractions of 0.5 ml were then collected and analyzed to determine the level of phosphate and MGP.

Analytical Methods—For determination of MGP and BGP, aliquots of fractions and serum samples were diluted into diluent and assayed in triplicate using radioimmunoassay procedures previously described (12, 13). Calcium levels in serum and other samples were determined colorimetrically using cresolphthalein complexone (Sigma), and phosphate levels in serum, effluent fractions, and other samples were determined colorimetrically as described (20). For determination of ionic calcium, freshly obtained blood samples were immediately placed into a 2.5-ml gold top Vacutainer tube to avoid out-gassing of CO_2 and the associated shift in pH. Clotted blood was centrifuged for 10 min in a clinical centrifuge, and serum was analyzed for ionic calcium at the University of California, San Diego Medical Center Chemistry Laboratory. Electrophoresis was carried out using 4–20% polyacrylamide gels (Novex, Inc., San Diego, CA) run in Tris-glycine buffer containing SDS. In the Western blot experiment shown in Fig. 4*B*, 100 μl of etidronate serum obtained as described in Table II was centrifuged at 16,000 × g for 30 min at room temperature. The supernatant was then removed, and the pellet was dissolved with 100 μl of SDS loading buffer containing 60 mM EDTA. Dilutions were made with the same SDS/EDTA loading buffer so that the 20 μl loaded onto the gel contained 0.1 μl of serum from a control rat, 0.1 μl of serum from the etidronate-treated rat before centrifugation, or 0.1 μl of the supernatant or dissolved pellet obtained after centrifugation. After electrophoresis on a 4–20% polyacrylamide gel, proteins were transferred to nitrocellulose, incubated overnight with rabbit antibody against rat fetuin (1:500 dilution in TBST), and detected using alkaline phosphatase-conjugated second antibody (Sigma). Protein sequencing was carried out on bands transferred to polyvinylidene difluoride membranes using a Procise 494 Sequencer (ABI division, PE Biosystems, Foster City, CA).

RESULTS

Effect of High Etidronate Doses on Serum Levels of Phosphate, Total Calcium, Ionic Calcium, and Matrix Gla Protein—The initial study was carried out to determine the effect of etidronate on serum MGP levels using a subcutaneous dose that, in a previous study, was found to elicit an elevation in serum levels of BGP (18), a vitamin K-dependent bone protein related in sequence to MGP. Serum was obtained from rats at different times after the subcutaneous administration of etidronate at a dose of 8 mg/100 g of body weight, and each serum sample was then analyzed for levels of MGP and BGP by radioimmunoassay. In agreement with the earlier study (18), serum levels of BGP were elevated by a maximum of 3-fold, and the peak serum BGP level was observed at 1 h (data not shown). In marked contrast, serum MGP levels were increased by 25-fold rather than 3-fold, and the peak level of MGP was observed at 6 h rather than at 1 h.

Because MGP is known to inhibit the calcification of arteries and other soft tissues (5, 6), we suspected that the dramatic serum MGP response to etidronate could be associated with the appearance of a calcium phosphate mineral complex in serum. We accordingly carried out a second experiment in which we measured serum levels of MGP, calcium, and phosphate at different times after the administration of etidronate. As shown in Fig. 1, serum calcium and phosphate levels increased rapidly after subcutaneous injection of etidronate, and by 6 h the levels of total calcium and phosphate were 76 and 59% above control levels, respectively. Serum levels of MGP increased more slowly than total serum calcium and phosphate levels, with half of the maximal levels at 3 h for MGP compared with 1 h for calcium and phosphate.

To determine whether the rise in total calcium after etidronate administration is due to an increase in ionic calcium or to an increase in a calcium complex, in a follow up experiment 4 rats were exsanguinated at $t = 0$, 1, 2, and 6 h after injection of 8 mg of etidronate/100 g of body weight, and the level of ionic and total calcium were measured in each serum sample. Ionic calcium values were 1.46 ± 0.03 mM at $t = 0$ h, 1.47 ± 0.04 mM at 1 h, 1.51 ± 0.04 mM at 2 h, and 1.46 ± 0.03

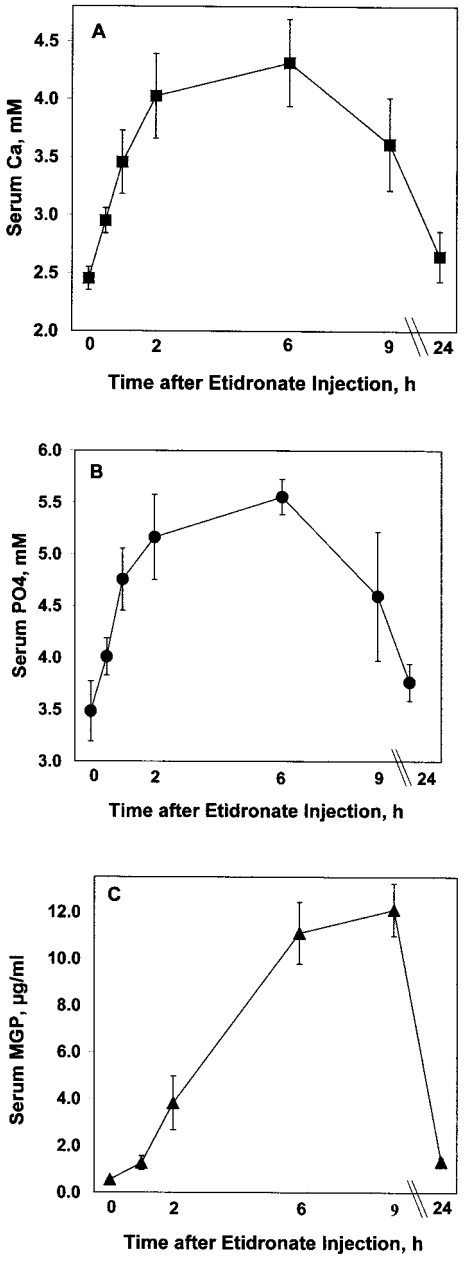

FIG. 1. **Effect of an 8-mg/100-g etidronate dose on serum levels of calcium, phosphate, and matrix Gla protein.** Five forty-day-old male Sprague-Dawley rats were given subcutaneous injections of etidronate at a dose of 8 mg/100 g body weight at $t = 0$. Blood was removed from each animal at the indicated times and analyzed to determine the levels of calcium, phosphate, and MGP (see "Experimental Procedures"). Each data point is the average of the individually determined levels in the 5 experimental animals, and the *error bars* denote the S.D. *Panel A*, serum calcium, mM; *panel B*, serum phosphate, mM; *panel C*, serum MGP, µg/ml.

mM at 6 h, whereas total calcium levels were 2.45 ± 0.11 mM at $t = 0$ h, 3.45 ± 0.34 mM at 1 h, 4.05 ± 0.44 mM at 2 h, and 4.41 ± 0.43 mM at 6 h. The failure of etidronate to cause a rise in ionic calcium levels indicates that the increase observed in total calcium levels must be due to the appearance of a non-ionic form of calcium in serum. Because there is a parallel increase in serum phosphate and total calcium levels after etidronate injection (Fig. 1), it seemed likely that this non-ionic form of serum calcium is a complex of calcium and phosphate.

As seen in Fig. 2, a 4-fold higher subcutaneous dose of etidronate produced a greater elevation in serum total calcium, phosphate, and MGP. The time course of the response to the

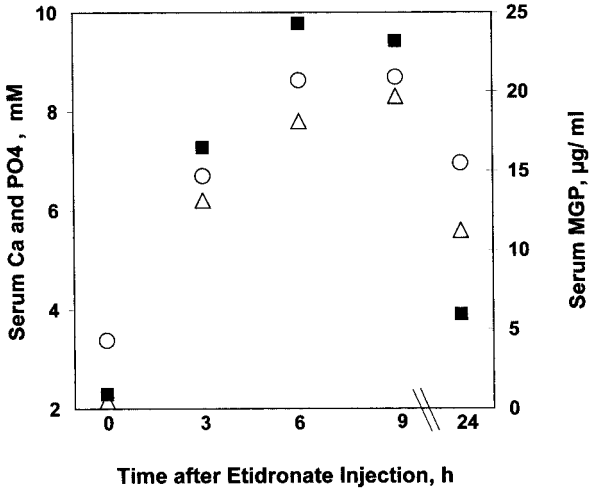

FIG. 2. **Effect of a 32-mg/100-g etidronate dose on serum levels of calcium, phosphate, and matrix Gla protein.** Three forty-day-old male Sprague-Dawley rats were given subcutaneous injections of etidronate at a dose of 32 mg/100 g body weight at $t = 0$. Blood was removed from each animal at the indicated times and analyzed to determine the levels of calcium, phosphate, and MGP (see "Experimental Procedures"). Each data point is the average of the individually determined levels in the three experimental animals. ■, serum calcium, mM; ○, serum phosphate, mM; △, serum MGP, µg/ml.

32-mg/100-g etidronate dose was similar to the response seen with the 8 mg/100 g dose over the first 9 h, with a maximal level in serum total calcium, phosphate, and MGP at 6–9 h after treatment with 32 mg/100 g etidronate. Serum levels of total calcium, phosphate, and MGP remained substantially elevated at 24 h after the 32 mg/100 g dose of etidronate, however, suggesting that the higher dose prolongs the serum response. Despite the 4.5-fold increase in serum total calcium at 6 h after treatment with 32 mg/100 g of etidronate, there was no increase in the level of serum-ionized calcium at 6 h, which further supports the conclusion that the increase in serum calcium is due to the appearance of a calcium complex. A 2-fold lower etidronate dose of 4 mg/100 g of body weight produced a smaller increase in serum calcium, phosphate, and MGP at 6 h than the 8 mg/100 g dose (data not shown). In contrast, the amino bisphosphonate alendronate had no effect on serum calcium, phosphate, and MGP levels measured at 1, 2, 6, 12, and 24 h after administration of a 4 mg/100 g dose of the drug (data not shown).

Filtration Evidence for a High Molecular Weight Complex of Calcium, Phosphate, and MGP in the Serum of Etidronate-treated Rats—To further characterize the calcium complex that is responsible for the rise in total calcium but not ionic calcium after etidronate injection, serum from etidronate-treated and control rats was filtered through 300-kDa molecular mass cut off membranes using an Ultrafree CL filtration device, and the filtrate and retentate fractions were separately analyzed for calcium, phosphate, and MGP. As can be seen in Table I, the increase in total serum calcium, phosphate, and MGP levels produced by the 8 mg/100 g dose of etidronate proved to be due to an increase in the levels of calcium, phosphate, and MGP in the high molecular mass retentate fraction, and the filtrate levels of calcium and phosphate were the same for control and etidronate-treated rats. This result indicates that the increases in total calcium, phosphate, and MGP seen in the serum of rats treated with etidronate is probably due to the appearance of a high molecular mass serum complex of calcium, phosphate, and MGP.

To determine the stability of the putative serum mineral complex, another 1-ml aliquot of serum from the etidronate-

TABLE I

Effect of filtration through a 300-kDa membrane on serum calcium, phosphate, and matrix Gla protein

Serum was obtained from 2 40-day-old male rats 6 h after subcutaneous injection with etidronate at a dose of 8 mg/100 g of body weight and from 2 age-matched control rats. One-ml aliquots of the 4 serum samples were filtered through a 300-kDa molecular mass cut-off membrane using a Ultrafree CL filtration device, and the levels of calcium, phosphate, MGP, and volume were measured for the filtrate and the retentate fractions (see "Experimental Procedures" for details). The data show the number of μmol of calcium, phosphate, and MGP in 1 ml of serum before filtration and in the 0.7-ml filtrate and 0.26-ml retentate volumes recovered after filtration. The data are the average of the values for the two control rats and the two etidronate-treated rats.

	Calcium		Phosphate		MGP	
	Control	Etidronate	Control	Etidronate	Control	Etidronate
	μmol		*μmol*		*ng*	
Initial serum	2.68	4.30	3.56	5.10	454	14,800
Filtrate	1.20	1.18	2.72	2.98	42	291
Retentate	1.28	2.72	0.98	1.84	284	11,630

treated rats (see Table I legend) was placed into a sealed tube, incubated at room temperature for 1 day, and then filtered through a 300-kDa molecular mass cut off membrane using the method described in Table I. The levels of calcium, phosphate, and MGP in the resulting retentate and filtrate fractions were comparable with the values found for serum filtered immediately after serum was obtained (shown in Table I), with retentate calcium levels of 2.83 μmol, phosphate levels of 2.02 μmol, and MGP levels of 12,127 ng, and with filtrate calcium levels of 1.21 μmol, phosphate levels of 2.79 μmol, and MGP levels of 320 ng. This result shows that the putative calcium phosphate mineral phase found in the serum of an etidronate-treated rat does not grow at the expense of free calcium and phosphate levels in serum. In another stability test, a 1-ml aliquot of serum from the etidronate-treated rat was frozen on dry ice and thawed 5 times and then subjected to filtration. Retentate and filtrate levels of calcium, phosphate, and MGP levels were again comparable with the values shown in Table I, which demonstrates that the amount of the putative serum mineral complex is not affected by repetitive freezing of the serum sample.

Centrifugational Evidence for a Complex of Calcium, Phosphate, Fetuin, and MGP in the Serum of Etidronate-treated Rats—We next examined the possible sedimentation of the serum calcium phosphate complex during centrifugation, a property that might be anticipated for the complex based on the fact that calcium phosphate mineral phases typically have densities about 3-fold greater than serum. As shown in Table II, centrifugation of serum from rats treated with the 32-mg dose of etidronate resulted in a pellet containing calcium, phosphate, and MGP. When the pellet was dissolved in acid and analyzed by SDS-PAGE, a major band was found at 59 kDa that accounted for at least 80% of the Coomassie staining (Fig. 3). When this component was electrophoretically transferred to polyvinylidene difluoride and subjected to N-terminal protein sequencing, one sequence was obtained, APQGAGLGFR, which matches the N-terminal sequence of rat fetuin (21). The other major band in the gel had an apparent molecular mass of 66 kDa and accounted for about 10% of the total Coomassie staining; this band was identified as rat serum albumin by N-terminal sequence analysis. Based on the recovery of fetuin in the pellet, we estimate the weight ratio of fetuin to mineral phosphate in the pellet to be 3.4 mg/mg. Western blot analyses, an example of which is shown in Fig. 3*B*, revealed that formation of the protein mineral complex did not significantly increase the total levels of serum fetuin and that formation of the serum mineral complex consumed about half of the fetuin initially found in serum.

Because it is conceivable that the nature of the protein mineral complex could be affected by the blood coagulation needed for serum collection, the filtration and centrifugation experiments described above were repeated using heparinized plasma samples. In the filtration experiment, filtrate and retentate levels of calcium, phosphate, and MGP in plasma from control rats and from the etidronate-treated rats were each within 5% of the values reported in Table I. In the centrifugation experiment, the amount of calcium, phosphate, and MGP in the supernatant and pellet fractions obtained by centrifuging plasma from control and etidronate-treated rats were each within 7% of the values reported in Table II, and the SDS gel of the proteins in the dissolved pellet was indistinguishable from that shown in Fig. 3. These experiments show that the nature of the mineral complex does not appear to be affected by the process of blood coagulation.

TABLE II

Effect of centrifugation on serum calcium, phosphate, and matrix Gla protein

Serum was obtained from 3 40-day-old male rats 6 h after subcutaneous injection with etidronate at a dose of 32 mg/100 gm of body weight and was pooled. A one-ml aliquot of pooled serum was immediately centrifuged at 16,000 × *g* for 30 min at room temperature. The supernatant was removed, and the pellet was rinsed once with 1 ml of 0.15 M NaCl and then dissolved with 1 ml of 50 mM HCl. The data show the number of μmol of calcium, phosphate, and MGP in 1 ml of serum before centrifugation and in the supernatant and dissolved pellet.

	Calcium	Phosphate	MGP
	μmol	*μmol*	*ng*
Initial serum	9.64	7.49	16,200
Supernatant	6.88	5.84	800
Dissolved pellet	3.27	1.68	14,400

In experiments using serum obtained 6 h after administration of 8 mg of etidronate/100 g, we were unable to demonstrate the sedimentation of calcium, phosphate, or MGP after 30 min of centrifugation at 16,000 × *g*. This result indicates that the putative complex of a calcium phosphate mineral phase and protein found after the 8 mg/100 g etidronate dose has different sedimentation properties than the complex found after the 32 mg/100 g dose.

Gel Filtration Evidence for a High Molecular Weight Complex of Calcium, Phosphate, Fetuin, and MGP in the Serum of Etidronate-treated Rats—To further characterize the calcium phosphate complex found in the serum of rats treated with the 8 mg/100 g dose of etidronate, we sought to partially purify this complex by gel filtration over a column of Sephacryl S300 using 10 mM calcium in the buffer to stabilize the putative complex. As seen in Fig. 4, there is a peak of MGP and phosphate in the excluded volume position of the chromatogram of serum from the etidronate-treated rat that is not found in the chromatogram of serum from an untreated rat. The MGP antigen recovered in this excluded volume peak accounts for the amount of MGP antigen in the serum sample applied to the column, and there was no detectable MGP antigen in the elution position of the 10-kDa MGP monomer, which is about fraction 40. In contrast, no BGP antigen could be detected in the high molec-

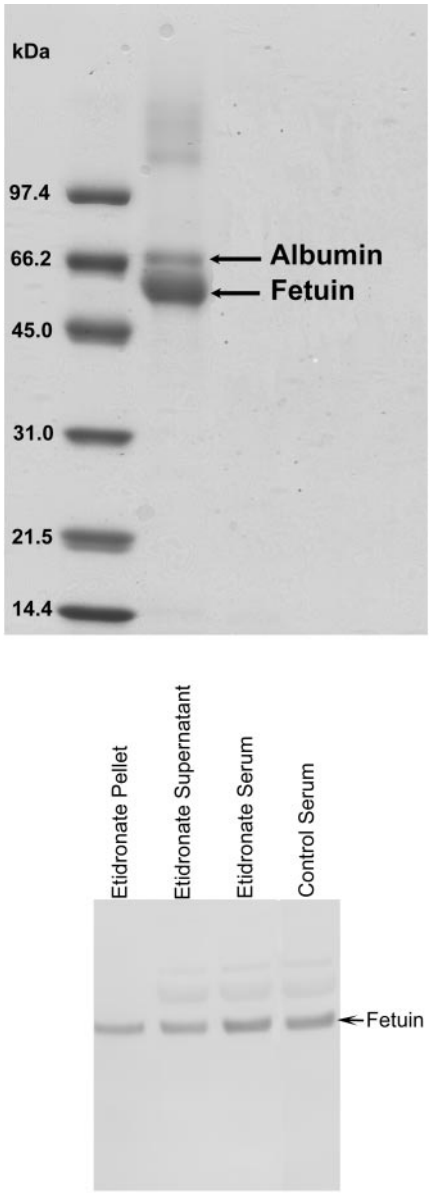

FIG. 3. **SDS-polyacrylamide gel electrophoresis of the proteins associated with the serum mineral complex.** *Panel A*, a 7-μg aliquot of the dissolved pellet from the centrifugation experiment described in Table II was dried, dissolved in loading buffer, and then electrophoresed on a 4–20% polyacrylamide gel and stained with Coomassie Brilliant Blue (*right lane*). Bio-Rad low molecular mass markers are in the left lane. *Panel B*, aliquots of the supernatant and dissolved pellet from a repeat of the experiment described in Table II and aliquots of serum from the etidronate-treated rat before centrifugation and of serum from a control rat were electrophoresed on a 4–20% polyacrylamide gel. Proteins were transferred to nitrocellulose, incubated with rabbit antibody against rat fetuin, and detected using alkaline phosphatase-conjugated second antibody. Each lane in the gel contains exactly the same proportion of the original serum sample, and the intensities of the bands therefore reflect the relative amount of fetuin antigen in the different fractions examined (see "Experimental Procedures").

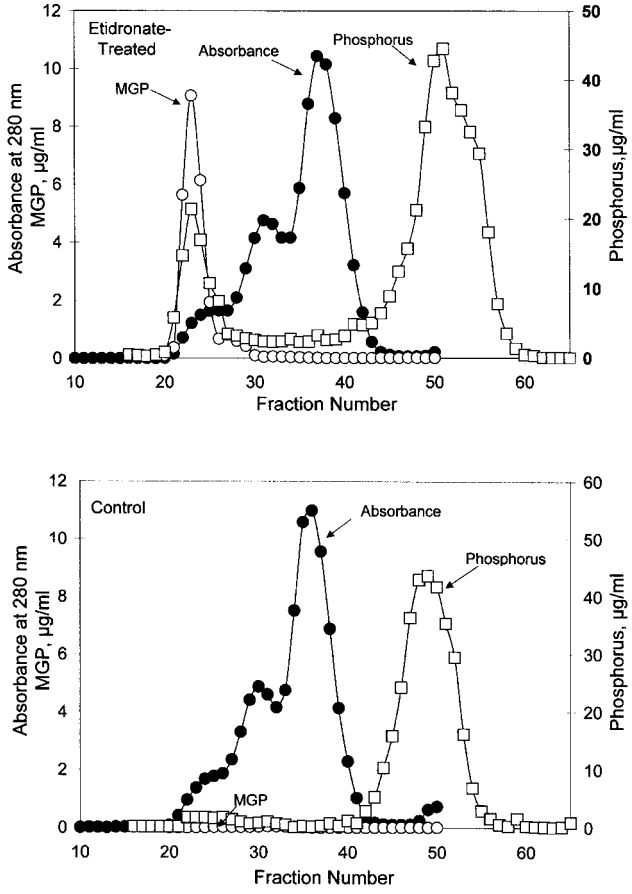

FIG. 4. **Sephacryl S-300 HR filtration of serum from etidronate-treated and control rats.** Forty-day-old male rats received a single subcutaneous dose of 8 mg of etidronate/100 g of body weight or of vehicle alone, and blood was collected 9 h later. One-ml serum samples were then immediately applied to a 25-ml column of Sephacryl S-300 HR equilibrated with 20 mM HEPES, pH 7.4, 0.15 M NaCl, and 10 mM $CaCl_2$. Temperature, 22 °C; fraction size, ~0.5 ml. ●, absorbance at 280 nm; ○, μg/ml MGP as determined by radioimmunoassay; □ μg/ml phosphorus. *Upper panel*, serum from an etidronate-treated rat; *lower panel*, serum from a control rat.

ular mass position in the chromatogram, and all BGP antigen was recovered in a single peak in the fraction 45 position expected for the 6-kDa BGP monomer (data not shown). This result indicates that the association of MGP with the serum mineral complex is highly specific, since it is well established that BGP binds strongly to hydroxyapatite *in vitro* and in serum (18, 22).

To evaluate the possible presence of other proteins associated with the serum mineral complex, the Sephacryl S300 fractions corresponding to the high molecular mass phosphate peak from an etidronate-treated rat (Fig. 4, *upper panel*) and the corresponding fractions from a normal rat (Fig. 4, *lower panel*) were separately combined with EDTA and fractionated by SDS-PAGE. As shown in Fig. 5, there is a prominent Coomassie-stained protein band in the lanes for the etidronate-treated animal that is not seen in the lanes for the control animal, a band with an apparent molecular mass of about 59 kDa. To identify this protein constituent, fraction 23 from the high molecular mass phosphate peak in the chromatogram of etidronate-treated rat (Fig. 4, *upper panel*) was fractionated by SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride. N-terminal protein sequencing of this 59-kDa band revealed that its sequence matched the N-terminal sequence of rat fetuin (21). Comparison of the SDS-PAGE for fraction 23 from the Sephacryl S300 gel filtration of etidronate and control rat serum using a more sensitive colloidal Coomassie stain revealed the presence of a band in the 14-kDa position expected for purified MGP in the lanes from the etidronate-treated rat but not in the lanes from the control rat (figure not shown). No other band could be detected in the SDS-PAGE of fraction 23 from the etidronate-treated rat chromatogram that was not also found at comparable levels in fraction 23 from the control rat chromatogram.

To estimate the amount of fetuin in the high molecular mass

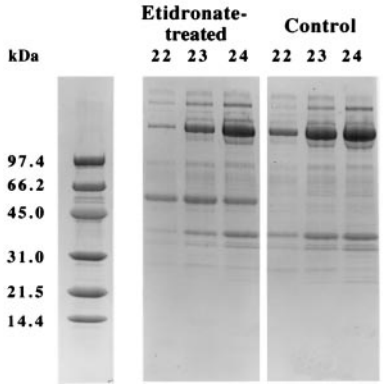

FIG. 5. **Electrophoresis of the high molecular mass fractions from the Sephacryl S300 fractionation of serum from etidronate-treated and control rats.** Ten μl of fractions 22, 23, and 24 from the chromatograms shown in Fig. 4, *upper* and *lower panels*, were each first mixed with 10 μl of 2× loading buffer containing 60 mM EDTA and then electrophoresed on a 4–20% polyacrylamide gel and stained with Coomassie Brilliant Blue.

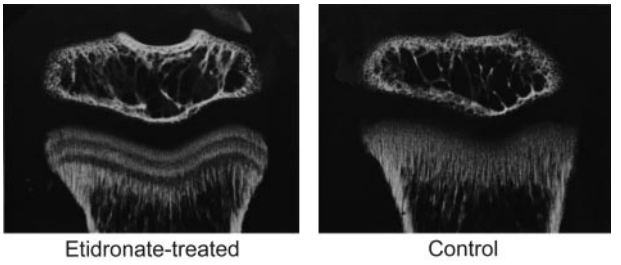

FIG. 6. **Effect of two 4-mg/100-g doses of etidronate spaced 24 h apart on the microradiograph of the proximal tibia.** One hundred gram rats were injected with 4 mg of etidronate at $t = 0$ and 24 h, and killed at $t = 48$ h. Tibias were removed from the etidronate-treated rats and from age-matched control rats, fixed in 70% ethanol, embedded in plastic, cut into 500-μm sections, and radiographed. Note the alternating bands of mineralized and non-mineralized matrix in the microradiograph of the proximal tibia from the etidronate-treated rat.

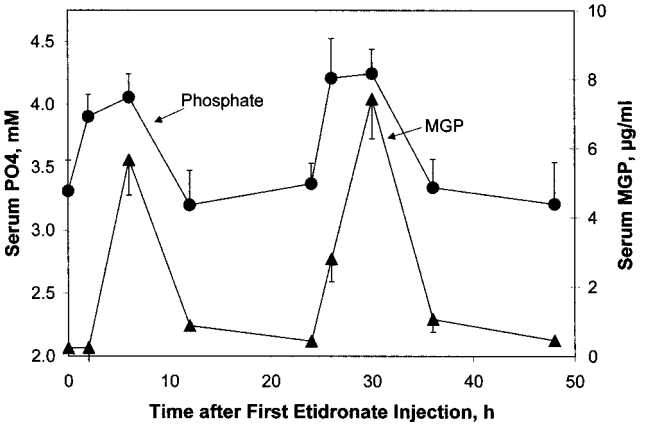

FIG. 7. **Effect of two 4-mg/100-g doses of etidronate spaced 24 h apart on serum levels of phosphate and matrix Gla protein.** Serum was obtained at the indicated times from the rats described in the legend to Fig. 6, and each serum sample was analyzed to determine the levels of phosphate and matrix Gla protein. Each data point is the average of the individually determined levels in six experimental animals, and the *error bars* denote the S.D. ●, serum phosphate, mM; ▲, serum MGP, μg/ml.

phosphate peak fractions, we performed two repeat SDS-PAGE analyses of fractions 22–24 of Fig. 4, *upper panel*, together with lanes containing known amounts of pure fetuin. Quantitative analysis of the amount of Coomassie staining in these fetuin bands using a densitometer yielded an estimate of 630 μg of fetuin in fractions 22–24. The phosphate content of these fractions is 83 μg of phosphate, and the weight ratio of fetuin to phosphate is 7.6 mg/mg. The total MGP content of fractions 22–24 is 11 μg (Fig. 4), and the calculated molar ratio of MGP to fetuin in these fractions is 1:8.

The rise in serum levels of calcium and phosphate after etidronate injection is very rapid, with a 21% rise in serum calcium at 30 min (Fig. 1). To see if this rapid rise in serum calcium and phosphate is due to the appearance of the fetuin mineral complex in serum, aliquots of serum obtained 30 min after the injection of the 8 mg/100 g etidronate dose were also subjected to gel filtration over the column of Sephacryl S300. This experiment showed that there is a peak of phosphate in the excluded volume position of the 30-min serum sample that is about 25% the amount seen in a 6-h serum sample. SDS gel electrophoresis further showed that the excluded volume fractions of the 30 min serum sample contained fetuin at a ratio of fetuin to phosphate of about 7 mg/mg.

Evidence That Etidronate Generates the Serum Mineral Complex by Inhibiting Bone Mineralization—Previous studies show that the doses of etidronate used here to cause the appearance of the complex of calcium, phosphate, fetuin, and MGP in serum also cause the inhibition of the normal calcification of bone and cartilage, resulting in the formation of unmineralized osteoid in bone and of unmineralized cartilage in the growth plate (23). When 100-g rats are given a dose of 4 mg of etidronate per day, this inhibition of mineralization is discontinuous and results in the appearance of alternating bands of calcification and no calcification in the proximal tibia. In the present studies we sought to determine whether the timing of the appearance of the calcium-phosphate-fetuin-MGP complex in serum correlates with the inhibition of growth plate cartilage mineralization. As seen in Fig. 6, microradiographs of the proximal tibial metaphysis of 100-g rats given 4 mg of etidronate at $t = 0$ and 24 h and killed at 48 h revealed alternating bands of calcification and no calcification that are identical to those reported in the earlier study (see Fig. 6 in Schenk et al. (23)), with inhibition of calcification from ~0 to 12 h, calcification from 12 to 24, inhibition of calcification from 24 to 36 h, and calcification from 36 to 48 h. As shown in Fig. 7, the 0–12- and 24–36-h intervals during which calcification was inhibited are the intervals during which serum levels of calcium, phosphate, and MGP became elevated, whereas the 12–24- and 36–48-h intervals during which cartilage calcification returned to normal are the intervals in which serum levels of calcium, phosphate, and MGP also returned to normal values. These results show that the appearance of the serum mineral complex after etidronate injection correlates with the timing of the inhibition of growth plate cartilage mineralization.

Effect of Warfarin Treatment on the Mineral Complex Found in the Serum of Etidronate-treated Rats—We have previously shown that the vitamin K antagonist warfarin inhibits the γ-carboxylation of MGP and thereby inactivates the calcification inhibitory activity of the protein and causes extensive calcification of arteries and heart valves (5, 6). To determine whether the γ-carboxylation of MGP is necessary for the accumulation of the protein in the serum complex of calcium, phosphate, and fetuin, rats were injected with warfarin 2 h before the administration of etidronate to ensure that all MGP synthesized from the time of etidronate administration is non-γ-carboxylated. Blood samples were then obtained at suitable times after etidronate injection and analyzed to determine serum levels of MGP, calcium, and phosphate. As shown in Fig. 8*C*, warfarin blocked the increase in serum MGP after etidronate administration but did not affect the time course of the elevation in serum calcium and phosphate (Fig. 8, *A* and *B*).

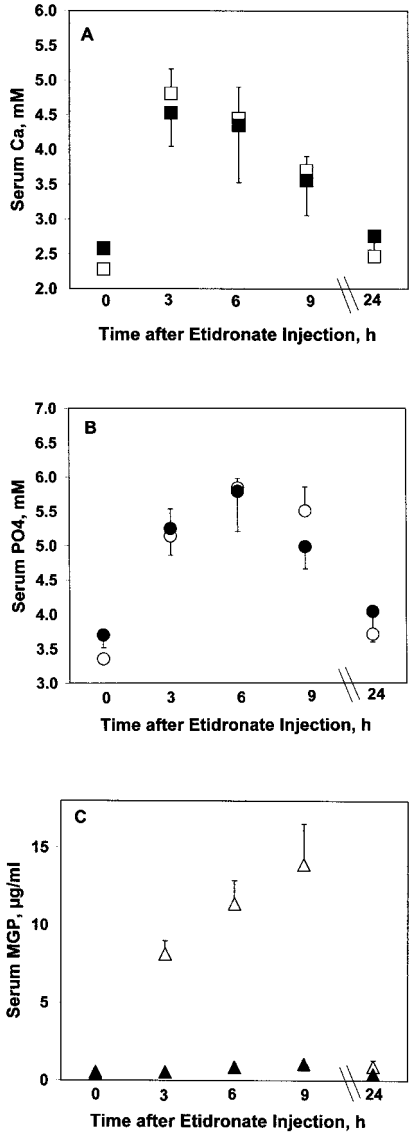

FIG. 8. **Effect of warfarin on serum levels of calcium, phosphate, and matrix Gla protein in animals treated with a 8-mg/100-g dose of etidronate.** Eight forty-day-old male Sprague-Dawley rats were given subcutaneous injections of 8 mg of etidronate per 100 g at $t = 0$. Four animals also received an injection of 15 mg of warfarin per 100 g 2 h before the etidronate injection. Blood was removed from each animal at the indicated times and analyzed to determine the levels of calcium, phosphate, and MGP. Each data point is the average of the individually determined levels in the four experimental animals in each treatment group, and the *error bars* denote the S.D. *Panel A*, ■, serum calcium, mM, in warfarin-treated rats; □, serum calcium, mM, in control rats. *Panel B*, ●, serum phosphate, mM, in warfarin-treated rats; ○, serum phosphate, mM, in control rats. *Panel C*, ▲, serum MGP, µg/ml, in warfarin-treated rats; △, serum MGP, µg/ml, in control rats.

Since warfarin treatment had no effect on serum levels of MGP in animals that did not receive etidronate, warfarin does not inhibit the synthesis of MGP *per se*, only the accumulation of MGP in serum after etidronate treatment. To further examine the effects of warfarin on the etidronate response, serum was obtained at 6 h after etidronate treatment from rats that were treated concurrently with warfarin. Analysis by Sephacryl S300 chromatography revealed phosphate levels in the high molecular mass, excluded volume position that were comparable with phosphate levels in vitamin K-replete, etidronate-treated rats and MGP levels in the high molecular mass position that were only 5% of the level seen in vitamin K-replete, etidronate-treated rats (chromatogram not shown). These results demonstrate that the γ-carboxylation of MGP is critical for its incorporation into the serum mineral complex and also show that the MGP that accumulates in this complex must arise from new MGP synthesis. Because serum calcium and phosphate levels are not affected by warfarin treatment, the absence of MGP in the serum mineral complex does not affect the magnitude of the serum mineral response to etidronate or the subsequent clearance of the mineral complex from serum. SDS gel electrophoresis of the high molecular mass phosphate-containing peak from the Sephacryl S300 chromatogram (data not shown) demonstrated the presence of fetuin at the level found in previous experiments (see Fig. 5), which indicates that the incorporation of fetuin into the serum mineral complex is independent of the presence of MGP.

DISCUSSION

The present study is the first to report the presence of a complex of calcium, phosphate, and protein in serum and the first to isolate this complex and to determine its structure. This protein mineral complex appears in serum shortly after the administration of the bisphosphonate etidronate, and within 6 h of injection with a 32 mg/100 g dose of etidronate, the presence of this complex in serum increases total serum calcium levels by more than 4-fold (to 8.8 mM calcium), phosphate levels by 2.5-fold (to 8.6 mM phosphate), and MGP levels by 36-fold (to 18 µg/ml). Because free calcium and phosphate are not elevated by etidronate treatment (see Table I), the protein mineral complex cannot be formed in serum in a physicochemical process driven by the enhanced supersaturation of serum with respect to calcium phosphate mineral phases. In fact when enhanced supersaturated conditions are created in serum by a vitamin D treatment that elevates ionic and total serum calcium by 40%, there is no detectable level of the protein mineral complex in serum. It is therefore probable that the protein mineral complex is formed outside of the vascular system as a consequence of etidronate treatment and subsequently travels to blood. This model does not rule out the possibility that changes in the initial mineral complex may occur after its appearance in serum, and the delayed appearance of MGP in the complex indeed suggests that the MGP content of the complex does change after the initial appearance of the complex in blood.

We believe that the serum mineral complex is probably generated as a consequence of the inhibition of bone mineralization by etidronate rather than as a consequence of the inhibition of bone resorption. Several arguments support this hypothesis as follows. 1) The appearance of the serum mineral complex and the inhibition of bone mineralization both occur within an hour after etidronate administration (Fig. 1 and Schenk *et al.* (23)). In contrast, the inhibition of bone resorption by etidronate and other bisphosphonates can only be detected 1–2 days after injection of the drug (24). 2) There is good agreement between the timing of the inhibition of bone mineralization and the appearance of the serum mineral complex after etidronate treatments spaced 24 h apart (Figs. 6 and 7). 3) The amino bisphosphonate alendronate does not generate the serum mineral complex even though the dose tested here is more than 1000-fold above those needed to inhibit bone resorption in rats of this age. It has been shown previously that these alendronate doses do not inhibit normal bone mineralization (25).

Although the size of the complex cannot be established from these studies, the filtration experiments suggest that the complex must be large enough to be retained by a 300-kDa molecular mass cut-off membrane, which supports a size of 300 kDa or larger, and the gel filtration studies indicate that the complex must be large enough to be in the excluded volume position of the Sephacryl S300 column, which is consistent with a size of

250 kDa or larger. The complex may in fact have a range of sizes, since half of the complex formed by the 32 mg/100 g etidronate dose sediments, whereas half of the complex does not (Table II). The size of the complex may also vary with the etidronate dose, since the protein mineral complex found in serum the 8 mg/100 g dose does not sediment upon centrifugation. Fetuin is the major protein component of the serum mineral complex, with an estimated weight ratio of fetuin to mineral of 4.4 for the complex found in serum at the 8 mg/100 g etidronate dose and an estimated ratio of fetuin to mineral of 1.9 at the 32 mg/100 g dose of etidronate. The MGP content of the serum mineral complex increases with time after etidronate injection, reaching a molar ratio of MGP to fetuin of 1:8. If the average molecular mass of the serum mineral complex were 550,000 daltons, the complex found in serum 6 h after treatment with the 8 mg/100 g dose of etidronate would consist of ~8 fetuin molecules, 1 MGP molecule, 790 atoms of calcium, and 580 molecules of phosphate. It should be noted that these calculations are based on the assumption that the only protein constituents of the complex are fetuin and MGP and that the SDS gel shown in Fig. 3 indicates that higher molecular mass proteins could in fact be present in the complex. Future studies will be needed to identify these components and to establish their possible role in the serum complex.

Because free calcium and phosphate levels remain at control values when serum containing the protein mineral complex is incubated at room temperature for 24 h, a primary function of the protein components of the complex may be to inhibit the growth of the mineral phase component. The protein components may also inhibit the aggregation and precipitation of the mineral phase component, because there is no evidence of the aggregation and precipitation of a mineral phase after 24 h of incubation at room temperature. Because the serum complex is cleared from serum within 6 h of attaining its peak value (Figs. 1 and 7), a secondary function of the protein components may be to target the complex for clearance from blood.

Role of Fetuin in the Serum Complex—The most abundant component of the serum complex is fetuin, not mineral or MGP, and it seems probable that the properties of the complex largely reflect the presence of fetuin in it. It is our hypothesis that fetuin molecules aggregate on the surface of the mineral nuclei and thereby prevent growth of the mineral phase and the generation of additional crystal nuclei. We believe that the most likely role for the protein component of fetuin is to mediate the binding of fetuin to mineral and to associate laterally with other fetuin molecules on the mineral surface to inhibit crystal growth. We further speculate that the 5 oligosaccharide moieties of fetuin, which account for 25% of its weight, project away from the mineral and into the surrounding aqueous phase. The functions of oligosaccharides in fetuin would be to lower the density of the mineral complex so that it will not sediment in serum and to prevent aggregation of one complex with another.

Previous studies demonstrate that fetuin inhibits the sedimentation of calcium from supersaturated solutions of calcium and phosphate after centrifugation for 5 min at $15{,}000 \times g$ (26). Fetuin in fact accounts for roughly half of the inhibitory activity found in serum. Although the mechanism by which fetuin inhibits calcium precipitation was not identified in these studies, the inhibitory activity was shown to be mediated by acidic amino acids clustered in the D1 cystatin-like domain of fetuin. Our present results are consistent with the putative calcification inhibitor activity of fetuin identified in these earlier studies and suggest that this action of the protein could be associated with its ability to form stable, soluble complexes with mineral nuclei that inhibit nuclei growth and precipitation.

The present studies indicate that most of the fetuin found in the serum mineral complex originates in the endogenous fetuin pool found in serum before the injection of etidronate rather than from the fetuin found in bone. Because the normal concentration of fetuin in rats is ~1 mg/ml (27) and the amount of fetuin found in the serum mineral complex is 0.5–0.6 mg/ml, formation of the complex from the endogenous serum fetuin pool would be predicted to significantly deplete the serum pool of monomeric fetuin. Comparison of the amount of fetuin remaining in the supernatant and pellet fractions after sedimentation of the complex by centrifugation shows that formation of the complex at the 32-mg etidronate dose does indeed consume about half of the fetuin initially present in serum. Direct comparison of total fetuin levels present in serum at different times after a 32-mg etidronate dose also failed to reveal a significant increase in the serum fetuin pool, as would have been expected had the fetuin found in the serum mineral complex originated in bone rather than serum.

Because the mineral component of the serum mineral complex clearly arises from the inhibition of bone mineralization and fetuin is an abundant constituent of the extracellular bone matrix, it is possible that some of the fetuin found on the complex does originate in the bone, perhaps through a direct effect of etidronate on fetuin synthesis by osteoblasts. This possibility is supported by the observation that fetuin is synthesized by osteoblasts in fetal rat bone (28, 29) and by fetal rat osteoblasts in cell culture (28). A number of studies have, however, failed to find evidence for the synthesis of fetuin or for the expression of mRNA for fetuin in the bones of adult rats (19, 29–31). Although these observations indicate that basal expression of fetuin in the adult rat is undetectable, it remains possible that fetuin expression by osteoblasts in adult rat bone could be induced by etidronate treatment and that bone synthesis of the protein could contribute to the formation of the fetuin mineral complex found in serum after etidronate injection.

Role of MGP in the Serum Mineral Complex—The present studies suggest that MGP accumulates in serum after etidronate injection by virtue of its binding to the serum complex. Because the vitamin K antagonist warfarin completely blocks the accumulation of MGP in the complex, it is clear that the MGP that accumulates in the complex arises from new synthesis and that accumulation of MGP in the complex requires the vitamin K-dependent γ-carboxylation of the protein. We believe that the dramatic increase in the total level of serum MGP after etidronate administration is caused by a reduced rate of MGP clearance from blood rather than by an increased rate of MGP synthesis. Serum proteins the size of the 10-kDa MGP molecule are cleared rapidly by kidney filtration ($t_{1/2}$ = 5 min), and the 0.5 μg/ml level of MGP found in normal rat serum consequently reflects a dynamic balance between new synthesis and clearance, with 0.25 μg/ml/5 min of new MGP synthesis compensating for the amount of MGP lost by kidney filtration. MGP bound to the much larger serum mineral complex would evade this clearance mechanism and, therefore, accumulate in serum. This hypothesis accounts for the approximately linear accumulation of MGP in serum during the first 6 h after etidronate injection (Fig. 1) as well as the total increase in serum MGP found at 6 h.[2] The alternative hypothesis for the 30-fold increase in serum MGP after etidronate administration is that the presence of the fetuin mineral complex in serum could

[2] In 6 h the amount of MGP that could accumulate in serum by evading kidney clearance would be the rate of appearance of newly synthesized MGP in serum, 0.25 μg/ml/5 min, ×360 min, which is 18 μg/ml. This number is in good agreement with the actual level of the protein in serum at this time (Fig. 2).

stimulate a dramatic increase in the rate of MGP synthesis by tissues that contribute MGP to blood. Although we cannot rule out this hypothesis entirely, investigations of the level of MGP mRNA in several tissues have failed to reveal a significant increase at 6 h after etidronate treatment.

The present studies demonstrate that MGP binds to the fetuin mineral complex with considerable strength and specificity. The gel filtration analysis of the elution position of MGP antigen (Fig. 4, *upper panel*) failed to detect the presence of any MGP in the elution position of the MGP monomer, which indicates that the concentration of monomeric serum MGP in equilibrium with MGP bound to the complex must be very low. The binding of MGP to the fetuin mineral complex must also be highly specific, since we could detect no other Coomassie-stained proteins associated with the complex other than fetuin and MGP (see Fig. 5). The specificity of this interaction is further supported by the observation that the structurally related vitamin K-dependent protein, BGP, fails to accumulate in the complex despite its known high affinity for hydroxyapatite (22).

The ability of MGP to bind with great avidity to the mineral complex despite the presence of fetuin suggests that MGP could in fact have a greater affinity for mineral than fetuin and so could be the stronger inhibitor of crystal growth. This possibility is supported by the observation that targeted deletion of the MGP gene in the mouse causes rapid and extensive calcification of the elastic lamellae of arteries beginning at birth (4), whereas fetuin-deficient mice have no evidence of soft tissue calcification except for the specialized case of occasional microcalcifications in a few muscles of some female retired breeder mice (32). We speculate that the failure of soft tissues to calcify in the fetuin-deficient mouse could be due in part to the ability of MGP to inhibit calcification and that the capacity of serum MGP to inhibit calcification is adequate to prevent soft tissue calcification under normal physiological circumstances. One prediction of this hypothesis is that a high capacity stress on the ability to inhibit calcification in serum, such as is imposed by a high dose of etidronate, will cause fetuin-deficient mice to experience a massive rate of mineral formation, a mineralization that cannot be retarded by the low capacity inhibitory function of serum MGP. A second prediction of this hypothesis is that warfarin treatment and the fetuin gene deletion should act synergistically to produce more rapid ectopic calcification than is found with either condition alone.

Although we have focused here on the ability of fetuin and MGP to prevent the growth of the mineral component of the serum complex, it is important to note that both proteins have other important biological activities. Fetuin binds transforming growth factor-β and bone morphogenic protein-2 (BMP-2) and blocks the osteogenic activity of these cytokines in cell culture assays (33, 34). MGP also binds BMP-2 and blocks the activity of BMP-2 on cells in culture (35). An important goal of future studies will be to determine whether fetuin and MGP retain their ability to block the activity of cytokines when they are part of the serum complex.

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