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The Inhibition of Calcium Phosphate Precipitation by Fetuin Is Accompanied by the Formation of a Fetuin-Mineral Complex*

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The present studies show that the previously reported ability of fetuin to inhibit the precipitation of hydroxyapatite from supersaturated solutions of calcium and phosphate *in vitro* is accompanied by the formation of the fetuin-mineral complex, a high molecular mass complex of calcium phosphate mineral and the proteins fetuin and matrix Gla protein that was initially discovered in the serum of rats treated with etidronate and that appears to play a critical role in inhibiting calcification *in vivo*. Rat serum potently inhibited the precipitation of calcium phosphate mineral when the concentration of calcium and phosphate were increased by 10 mM each, and the modified serum was incubated at 37 °C for 9 days; in the absence of serum, precipitation occurred in seconds. Large amounts of the fetuin-mineral complex were generated in the first 3 h of this incubation and remained throughout the 9-day incubation. Purified bovine fetuin inhibited the precipitation of mineral for over 14 days in a solution containing 5 mM calcium and phosphate at pH 7.4 at 22 °C, whereas precipitation occurred in minutes without fetuin. There was a biphasic drop in ionic calcium in the fetuin solution, however, from 5 to 3 mM in the first hour and from 3 to 0.9 mM between 20 and 24 h; these changes in ionic calcium are due to the formation of complexes of calcium, phosphate, and fetuin. The complex found at 24 h to 14 days is identical to the fetuin-mineral complex found in the serum of etidronate-treated rats, whereas the complex found between 1 and 20 h is less stable.

The present studies were carried out to better understand the mechanisms responsible for the generation of the serum fetuin-mineral[1] complex in the rat. The fetuin-mineral complex is a complex of a calcium phosphate mineral phase and the proteins fetuin and matrix Gla protein (MGP) that appears in the blood of rats treated with doses of etidronate that acutely inhibit bone mineralization (1). The appearance of this complex in serum following etidronate injection causes a 4-fold increase in total serum calcium without any increase in ionic calcium levels. The fetuin-mineral complex is quite stable in serum at 37 °C, with no evidence for the growth, aggregation, and precipitation of the mineral component of the complex, which suggests that the previously reported calcification inhibitory[2] activities of fetuin (2, 3) and MGP (4–7) may be related to their ability to form stable complexes with nascent mineral nuclei.

The dominant protein component of the fetuin-mineral complex, fetuin, is a 59-kDa glycoprotein that consists of two N-terminal cystatin domains and a smaller C-terminal domain. Fetuin is synthesized in the liver and is found at high concentrations in mammalian serum (8, 9) and bone (10–15). The serum fetuin concentration in adult mammals ranges from 0.5 to 1.5 mg/ml, whereas the serum fetuin concentration in the fetus and neonate is typically far higher (9). Fetuin is one of the most abundant noncollagenous proteins found in bone (10–15), with a concentration of about 1 mg of fetuin/g of bone in rat (14), bovine (10), and human (12) bone. Despite the abundance of fetuin in bone, however, it has not been possible to demonstrate the synthesis of fetuin in calcified tissues, and it is therefore presently thought that the fetuin found in bone arises from hepatic synthesis via serum (13, 15).

Our working hypothesis is that the serum fetuin-mineral complex is formed spontaneously in bone metabolism under conditions in which fetuin inhibits the spontaneous precipitation of a calcium phosphate mineral phase (16). Two experimental systems have been used to test this hypothesis. Previous studies have shown that fetuin is the major inhibitor of the precipitation of calcium phosphate mineral found in blood (2), and the first test accordingly examined the possible generation of the fetuin-mineral complex in serum to which sufficient calcium and phosphate had been added to exceed the spontaneous precipitation of a calcium phosphate mineral phase in serum. Purified bovine fetuin has also been shown to inhibit the precipitation of a calcium phosphate mineral phase from solutions containing supersaturating amounts of calcium and phosphate (2), and the second test therefore examined the possible generation of the fetuin-mineral complex in the course of this inhibition. The results of these experiments show that a fetuin-mineral complex apparently identical to that found in serum following etidronate administration is indeed generated during the course of the inhibition of calcium phosphate precipitation in serum containing supersaturating amounts of calcium and phosphate and during the course of inhibition of calcium phosphate precipitation from supersaturated solutions containing purified bovine fetuin.

EXPERIMENTAL PROCEDURES

Materials—Simonsen albino male rats (Sprague-Dawley-derived) were purchased from Simonsen Labs (Gilroy, CA). Sephacryl S300HR gel filtration media was purchased from Amersham Biosciences. ^{45}Ca was purchased from PerkinElmer Life Sciences. Microcon YM-10 and

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

[2] "Calcification inhibitory" refers to the ability of fetuin and MGP to inhibit the formation of insoluble calcium phosphate complexes *in vitro* and at ectopic calcification sites in an animal. Neither protein has been shown yet to inhibit calcification in bone, dentine, or other sites of normal mineralization.

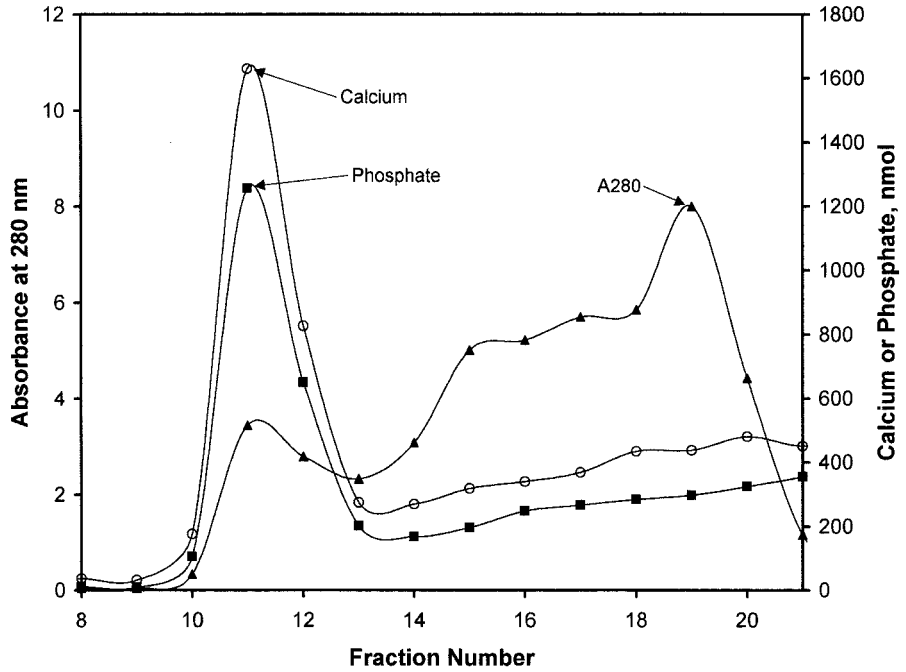

FIG. 1. **Gel filtration evidence for the generation of the serum fetuin-mineral complex when rat serum is first increased by 10 mM in calcium and phosphate and then incubated for 3 h at 37 °C.** Sufficient phosphate and calcium labeled with ^{45}Ca were added to serum from 42-day-old male Sprague-Dawley rats to increase total levels of each by 10 mM, and the resulting modified serum was incubated for 3 h in a humidified incubator at 37 °C and 7.5% CO_2. A 1-ml aliquot of incubated serum was immediately applied to a 25-ml column of Sephacryl S300HR filtration media equilibrated at room temperature with 20 mM HEPES, pH 7.4, 0.15 M NaCl, and 10 mM $CaCl_2$. The figure shows the amounts of phosphate and incorporated calcium (calculated from the ^{45}Ca label) in each 1-ml fraction and the absorbance at 280 nm for each fraction.

Ultrafree CL filtration units were purchased from Millipore. Bovine fetuin (Sigma) was further purified before use by gel filtration over a 2 × 140-cm column of Sephacryl S300HR equilibrated with 5 mM ammonium bicarbonate.

Formation of the Fetuin-Mineral Complex in Rat Serum—Blood was obtained from 42-day-old male Sprague-Dawley rats. The blood was allowed to clot for 30 min at room temperature, and serum was collected by centrifugation at 1,400 × *g* for 10 min in a clinical centrifuge and was stored in 1-ml aliquots at −70 °C until use. To increase the concentrations of calcium and phosphate in serum, an aliquot of 0.5 M $CaCl_2$ containing 700,000 cpm of ^{45}Ca label was added rapidly to a 1-ml serum aliquot to achieve twice the desired final level of added calcium, and an aliquot of 0.5 M sodium phosphate, pH 7.4, was added rapidly to a second 1-ml serum aliquot to achieve twice the desired final level of added phosphate. The calcium-boosted serum aliquot was then drawn up in a disposable pipette and expelled rapidly into the phosphate-boosted aliquot. The serum samples were placed in 35-mm culture dishes (Falcon 3001) and incubated for 3 h or 9 days in a humidified incubator at 37 °C and 7.5% CO_2. The serum maintained a stable pH value of 7.4 throughout the duration of the incubation.

Formation of the Fetuin-Mineral Complex during the Inhibition of Calcium Phosphate Mineral Formation by Pure Fetuin—The formation of calcium phosphate mineral was investigated at room temperature in solutions containing 0.2 M HEPES buffer at pH 7.4. In some experiments, 5 mg/ml of bovine fetuin was also added to this buffer. In a typical experiment, equal volumes of buffer were first prepared that contained either 10 mM phosphate, pH 7.4, or 10 mM calcium containing ^{45}Ca label. These solutions were then mixed rapidly to obtain a homogenous solution containing 5 mM in each ionic component. The samples of 100 μl were withdrawn at the appropriate time after mixing and centrifuged for 10 s in an Epifuge. The supernatants were immediately diluted 1:4 with HEPES buffer to arrest further mineral formation and stored at −70 °C until analysis.

Biochemical Analyses—The calcium levels were determined colorimetrically using cresolphthalein complexone (Sigma), and the phosphate levels were determined colorimetrically as described (17). Ionic calcium levels were measured at the indicated times using a calcium-specific electrode. The filtration experiments were carried out using 0.5-ml aliquots with a Microcon YM-10 filtration unit and using 2-ml aliquots with an Ultrafree CL filtration unit. The concentration of rat MGP was determined by radioimmunoassay, as described (18).

The amount of the high molecular mass fetuin-mineral complex in samples was determined by gel filtration using 25-ml columns of Sephacryl S300HR equilibrated with 20 mM HEPES, pH 7.4, 0.15 M NaCl, and 10 mM $CaCl_2$ at room temperature as described previously (1). Fractions of 1 ml were collected and analyzed to determine the absorbance at 280 nm and the level of phosphate and ^{45}Ca; the concentration of incorporated calcium was calculated from the specific activity of the ^{45}Ca label. The amount of the high molecular mass fetuin-mineral complex was also investigated by analysis of the pellets formed after centrifugation of 0.25-ml aliquots for 2 h at 16,000 × *g*. After removal of the supernatants, each tube was rinsed briefly with 0.25 ml of ice-cold 0.15 M NaCl. For chemical analysis, the pellets were dissolved in 125 μl of 50 mM HCl and analyzed for calcium, phosphate, and MGP. For electrophoresis, the pellets were dissolved in SDS gel loading buffer containing 60 mM EDTA, pH 7.4, and electrophoresed using 4–12% polyacrylamide gels (Novex, Inc., San Diego, CA). To see whether the presence of albumin in the pellet is due to nonspecific contamination of the pellet with serum, the initial pellet was resuspended in 125 μl of HEPES buffer (20 mM HEPES buffer, pH 7.4, containing 1 mM $CaCl_2$, 2 mM Na_2HPO_4, 0.02% azide, and 0.15 M NaCl) and centrifuged for 30 min at 16,000 × *g*; the resulting pellet was then analyzed as described above.

RESULTS

Formation of the Fetuin-Mineral Complex in Serum Following the Elevation of Calcium and Phosphate Levels—The first experiments were carried out to determine whether the fetuin-mineral complex is formed spontaneously when the calcium and phosphate levels in rat serum are increased above normal, and the modified serum is then incubated at 37 °C. The possible presence of the fetuin-mineral complex was initially assessed by gel filtration over Sephacryl S300HR, a method that has been shown previously to separate the high molecular mass fetuin-mineral complex from most other proteins in serum (1, 16). Calcium and phosphate were added to serum to increase the total levels of each by 10 mM, and the resulting modified serum was incubated at 37 °C for 3 h, a time comparable with that required for the generation of the fetuin-mineral complex *in vivo* (1). As seen in Fig. 1, Sephacryl S300 analysis of this incubated serum sample revealed the presence of a large peak of calcium and phosphate in the high molecular mass position expected for the fetuin-mineral complex. Additional Sephacryl S300 experiments showed that no detectable high molecular mass peak of calcium and phosphate was formed after incubation for 3 h at 37 °C of serum alone or of serum with added 1, 2, or 5 mM calcium and phosphate (data not shown).

The presence of the fetuin-mineral complex in serum was also assessed by centrifugation for 2 h at 16,000 × *g*, a procedure shown previously to sediment the fetuin-mineral complex found in the serum of etidronate-treated rats (1). Calcium and

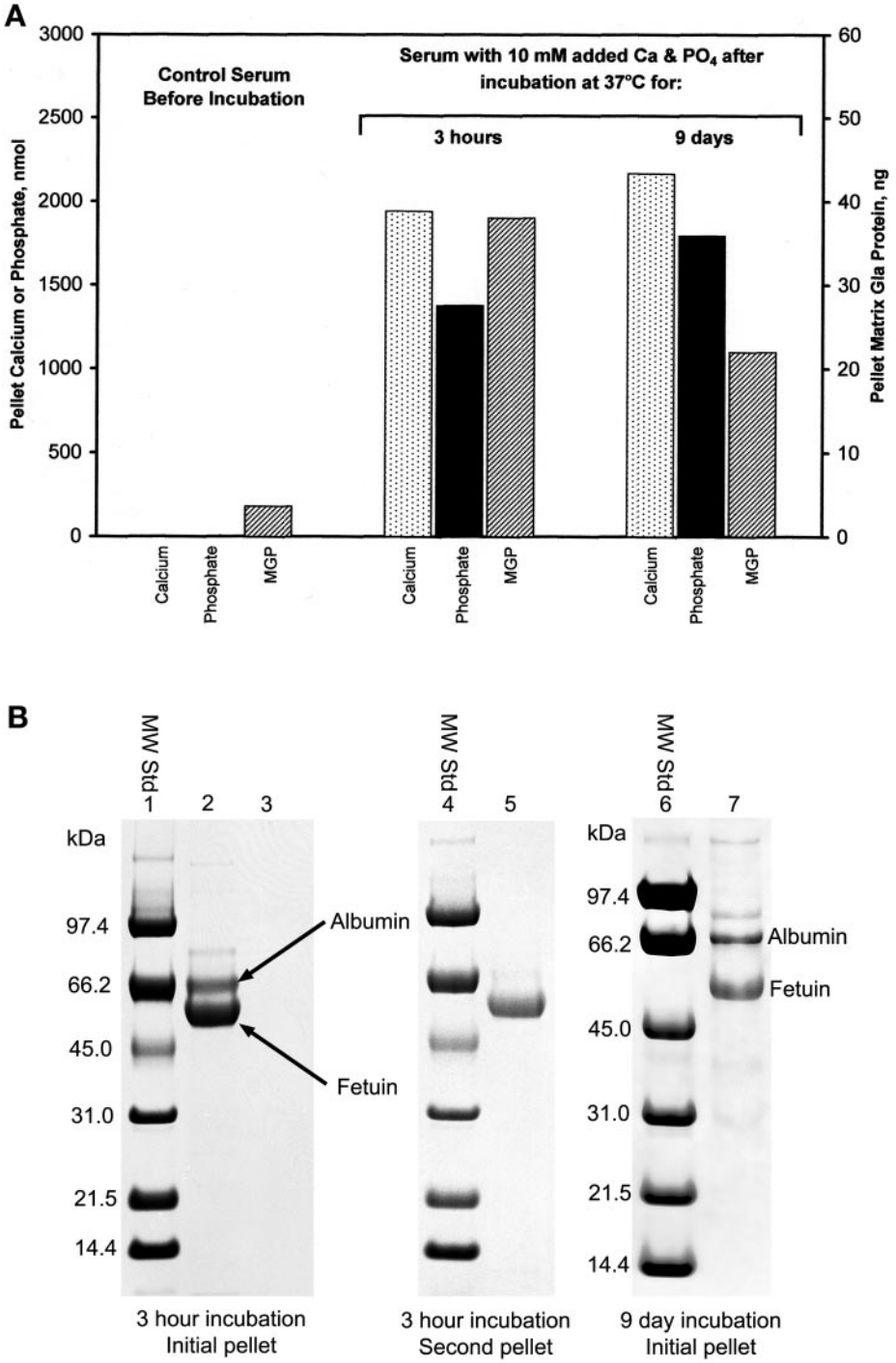

FIG. 2. **Centrifugation evidence for the generation of the serum fetuin-mineral complex when rat serum is first increased by 10 mM in calcium and phosphate and then incubated at 37 °C.** Sufficient calcium and phosphate were added to rat serum to increase total levels of each by 10 mM, and the resulting modified serum was incubated at 37 °C for 3 h or 9 days. Aliquots of 250 μl were centrifuged for 2 h at 16,000 $\times$ *g*, the supernatant was removed, and the pellets were washed once with the same volume of ice-cold 0.15 M NaCl. Some pellets were then resuspended in 125 μl of HEPES buffer and repelleted by centrifugation for an additional 30 min. *A*, pellets were dissolved in 100 μl of 150 mM HCl, and the solutions were analyzed to determine the level of calcium, phosphate, and MGP. *B*, pellets were dissolved in SDS gel loading buffer containing 60 mM EDTA, electrophoresed on a 4–12% polyacrylamide gel, and stained with Coomassie Brilliant Blue. *Lanes 1*, *4*, and *6*, Bio-Rad low molecular mass markers (*MW Std*); *lane 2*, initial pellet formed by centrifugation of serum that was modified by the addition of 10 mM calcium and phosphate and incubated for 3 h at 37 °C; *lane 3*, pellet fraction formed by centrifugation of control serum; *lane 5*, pellet obtained after resuspending the initial pellet in HEPES buffer and then centrifuging a second time; *lane 7*, initial pellet formed by centrifugation of serum that was modified by the addition of 10 mM calcium and phosphate and incubated for 9 days at 37 °C (see "Experimental Procedures").

phosphate were again added to increase total serum levels of each by 10 mM, and the resulting modified serum was incubated at 37 °C for 3 h. Centrifugation of this modified serum produced a translucent, compact pellet that was visually identical to the translucent fetuin-mineral complex pellet formed by centrifuging serum from etidronate-treated rats (1). Chemical analysis of this pellet showed that it contained calcium, phosphate, and MGP (Fig. 2*A*), and SDS gel electrophoresis of this pellet revealed a protein composition essentially identical to that found in the fetuin-mineral complex pellet formed by centrifugation of serum from an etidronate-treated rat (1), with a major band at 59 kDa and a minor band at 66 kDa (Fig. 2*B*, *lane 2*). N-terminal protein sequencing demonstrated that the 59-kDa band is rat fetuin and the 66-kDa band is rat albumin

Because albumin is the major protein found in rat serum, with an abundance about 100-fold greater than fetuin, it is possible that the presence of albumin in the pellet fraction could reflect a small contamination of serum within the pellet rather than the specific association of albumin with the fetuin-mineral complex. To test this hypothesis, the initial pellet was resuspended in HEPES buffer and repelleted by centrifugation. This stratagem removed essentially all of the albumin contaminant with good recovery of fetuin (Fig. 2*B*, *lane 5*), which shows that the presence of albumin in the pellet is probably due to contamination of the pellet with serum rather than to the specific association of albumin with the fetuin-mineral complex.

To further test the ability of serum fetuin to prevent the

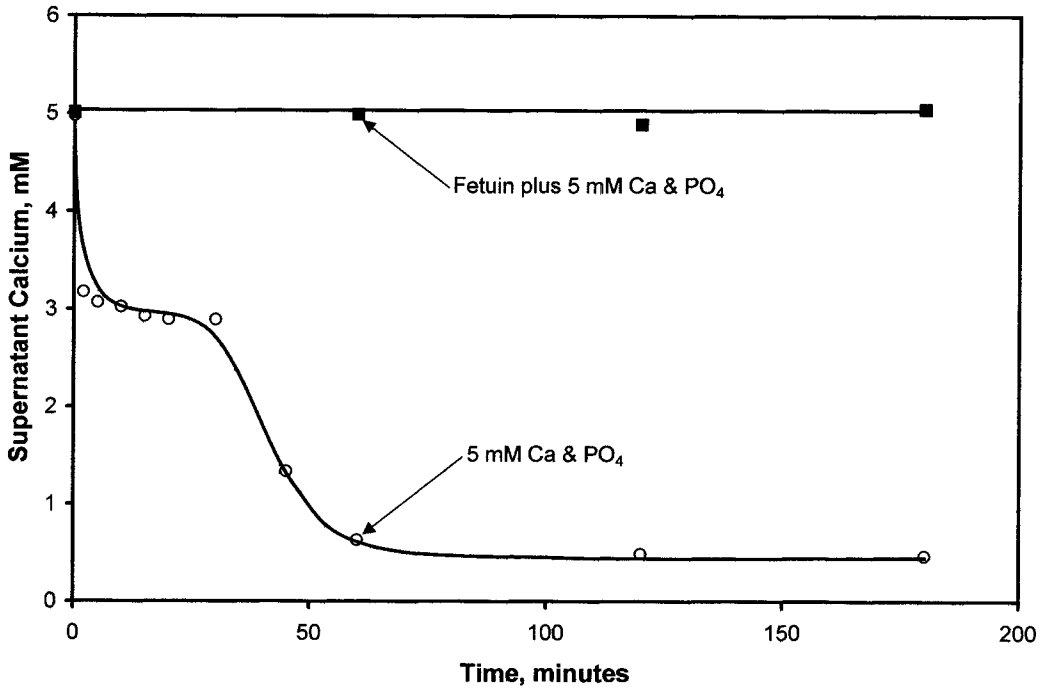

FIG. 3. **The precipitation of a calcium phosphate mineral in solutions containing 5 mM calcium and phosphate is biphasic, and is inhibited by fetuin.** The formation of calcium phosphate mineral was investigated at room temperature in solutions containing 0.2 M HEPES buffer at pH 7.4. 1-ml aliquots of buffer were first prepared that contained 10 mM phosphate (pH 7.4) or 10 mM calcium. These solutions were then mixed rapidly to obtain a homogenous solution containing 5 mM in each ionic component. Samples of 100 μl were withdrawn at the indicated times and centrifuged for 10 s in an Epifuge. The supernatants were immediately diluted 1:4 with HEPES buffer to arrest further mineral formation. The effect of fetuin was investigated by addition of 5 mg/ml bovine fetuin to the buffer prior to the addition of calcium or phosphate. The data show the concentration of calcium measured in the supernatant (see "Experimental Procedures").

growth and precipitation of the mineral component of the complex, the levels of calcium and phosphate in rat serum were again increased by 10 mM each, and the resulting modified serum was incubated for 9 days at 37 °C. Centrifugation of this modified serum sample showed that a translucent pellet was formed that contained calcium and phosphate in amounts somewhat greater than the amounts seen after 3 h of incubation and MGP levels that were significantly lower (Fig. 2*A*). SDS gel electrophoresis of this pellet revealed a protein composition that is essentially identical to that found for the pellet formed after 3 h of incubation of the modified serum sample (Fig. 2*B*, *lane 7*), with a major band at the 59-kDa position of fetuin and a minor band at the 66-kDa position of albumin. These results show that the fetuin-mineral complex formed by incubation of modified serum *in vitro* is exceptionally stable and further support the hypothesis that one function of the fetuin component of this complex is to arrest growth of the mineral component.

Formation of the Fetuin-Mineral Complex during the Inhibition of Calcium Phosphate Mineral Formation by Pure Fetuin at 5 mM Calcium and Phosphate—Previous studies have shown that bovine fetuin is a potent inhibitor of calcium phosphate mineral precipitation (2), and experiments were accordingly carried out to determine whether the fetuin-mineral complex might be formed in the course of this inhibition. The first experiment monitored the detailed time course of the precipitation of calcium phosphate mineral in the presence and absence of bovine fetuin using 5 mM calcium and phosphate at pH 7.4, which are conditions that are essentially identical to those used in previous inhibition studies with bone Gla protein (19) and fetuin (2). As can be seen in Fig. 3, in the absence of fetuin the precipitation of a calcium phosphate mineral phase is rapid and biphasic, with an initial drop in solution calcium to ~3 mM within 1 min, a plateau until 30 min, and a subsequent rapid decline to about 0.5 mM by 1 h. The biphasic nature of calcium phosphate precipitation was not noted in earlier studies because the first time points measured were 1 h or later (2, 19). In agreement with the earlier fetuin study (2), fetuin completely prevented the precipitation of a calcium phosphate mineral phase (Fig. 3), and the solution remained clear over the 3-h period of observation.

To see whether the ability of fetuin to prevent the formation of a calcium phosphate precipitate is due to the formation of complexes between fetuin and nascent crystal nuclei, the level of ionic calcium was measured with an ion-specific electrode over the period in which fetuin inhibits the precipitation of a calcium phosphate mineral phase. As can be seen in Fig. 4, ionic calcium levels fell to about 3 mM within 1 h and then remained at this value for 20 h. There was then a transition to 0.9 mM calcium at 24 h and a slower decrease to less than 0.5 mM calcium at 48 h. The biphasic nature of these changes closely parallels the biphasic precipitation of calcium in the absence of fetuin shown in Fig. 3, and the concentration of calcium in the first and second plateau regions is similar. The solution of fetuin and 5 mM calcium and phosphate remained completely clear over the initial 16-h period of observation. It then became distinctly opalescent by 24 h and remained comparably opalescent over a 14-day period of observation, with no evidence for the precipitation of a calcium phosphate mineral phase.

This experiment was repeated using a filtration procedure to separate free calcium from calcium associated with higher molecular mass complexes. As seen in Fig. 5, filtration of the solution of fetuin and 5 mM calcium and phosphate through a membrane with a 10-kDa molecular mass cut-off revealed a biphasic decrease in filtrate calcium essentially identical to that seen for ionic calcium, with a decrease to 3 mM calcium within 1 h, a plateau until 16 h, and then a decline to below 1 mM by 24 h. The solution was clear until 16 h and again became distinctly opalescent by 24 h.

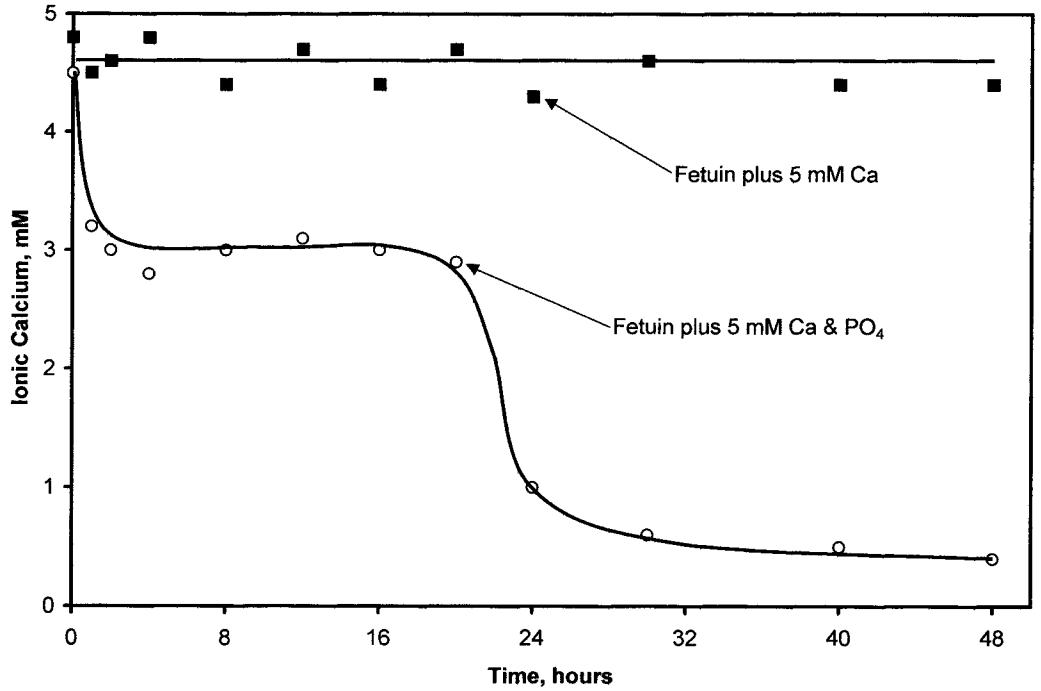

FIG. 4. **There is a biphasic change in ionic calcium levels in solutions containing 5 mM calcium and phosphate when the precipitation of mineral is inhibited by fetuin.** The changes in ionic calcium levels were investigated at room temperature in solutions containing 0.2 M HEPES buffer at pH 7.4 and 5 mg/ml bovine fetuin. 2.5-ml aliquots of buffer were first prepared that contained 10 mM phosphate (pH 7.4) or 10 mM calcium. These solutions were then mixed rapidly to obtain a homogenous solution containing 5 mM in each ionic component. The control solution contained the same 0.2 M HEPES buffer, 5 mg/ml fetuin, and 5 mM calcium but did not contain phosphate. Ionic calcium levels were measured at the indicated times using a calcium-specific electrode (see "Experimental Procedures").

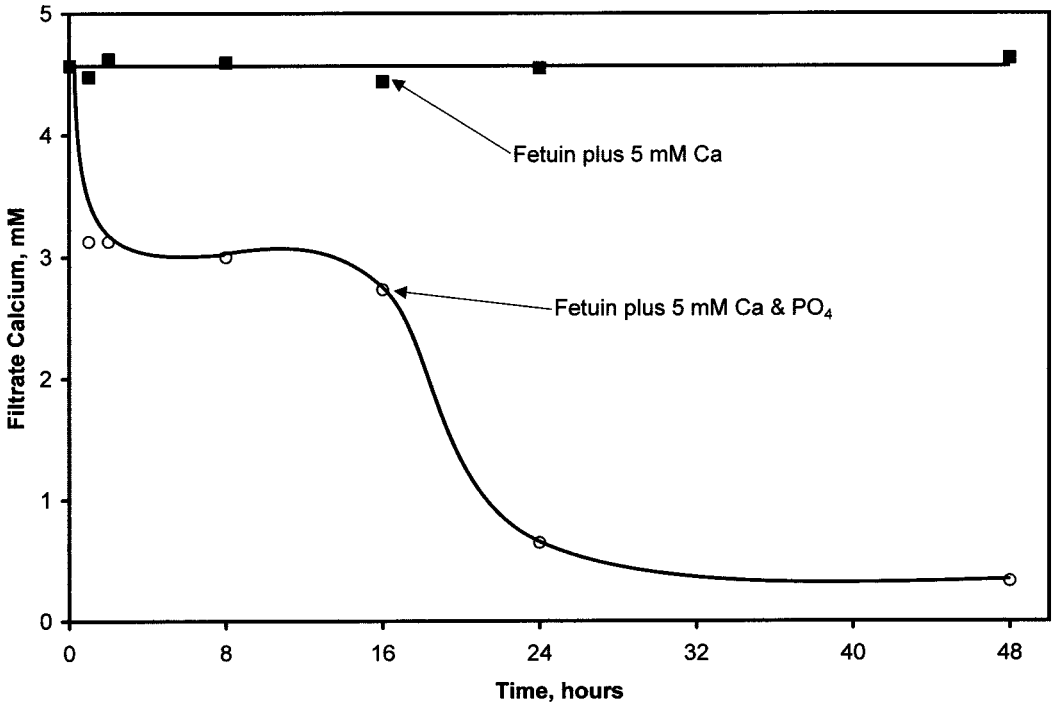

FIG. 5. **There is a biphasic change in filtrate calcium levels in solutions containing 5 mM calcium and phosphate when the precipitation of mineral is inhibited by fetuin.** The changes in filtrate calcium levels were investigated at room temperature in solutions containing 0.2 M HEPES buffer at pH 7.4 and 5 mg/ml bovine fetuin. 3-ml aliquots of buffer were first prepared that contained 10 mM phosphate (pH 7.4) or 10 mM calcium. These solutions were then mixed rapidly to obtain a homogenous solution containing 5 mM in each ionic component. The control solution contained the same 0.2 M HEPES buffer, 5 mg/ml fetuin, and 5 mM calcium but did not contain phosphate. 0.5-ml aliquots were removed at the indicated times and immediately filtered through a 10-kDa membrane using an YM-10 filtration unit. (see "Experimental Procedures").

This filtration procedure was repeated to obtain quantitative data for the calcium and phosphate content of the solution retained by the filter and the solution that passed through the filter. As seen in Table I, a substantial amount of calcium and phosphate was retained during the filtration of the solution obtained at 2 h following mixing, and the molar ratio of calcium

TABLE I

Centrifugation evidence for fetuin-mineral complexes at 2 and 72 h after mixing to achieve 5 mM calcium and phosphate

A solution containing 0.2 M HEPES buffer, pH 7.4, 5mg/ml fetuin, and 5 mM calcium and phosphate was prepared as described in the Fig. 5 legend. 0.5-ml aliquots were removed at the indicated times and immediately filtered through a 10-kDa membrane using a YM-10 filtration unit. For 2 and 72 h phosphate and 72 h calcium, the values are the total amounts of calcium and phosphate retained by the filter. For 2 h calcium, the net calcium in the complex was obtained by subtracting calcium bound to fetuin (200 nmol) from total retentate calcium (950 nmol) (see "Experimental Procedures").

Time	Amount retained by filter		Molar calcium/PO_4 ratio	Concentration in filtrate		Calcium phosphate ion product
	Calcium	Phosphate		Calcium	Phosphate	
h	*nmol*			*mM*		
2	750	450	1.67	3.1	4.1	12.71
72	2450	1530	1.60	0.1	1.9	0.19

to phosphate in the retained fraction was 1.67. Control experiments using the same amount of fetuin and 5 mM phosphate alone failed to demonstrate retention of phosphate by the filter. Control experiments with fetuin and 5 mM calcium alone did reveal retention of some calcium by the filter because of calcium binding to fetuin (20), but the amount was substantially less than the calcium retention observed in the solution of fetuin with 5 mM of both calcium and phosphate (data not shown). As seen in Table I, there was a further increase in the amount of calcium and phosphate retained by the filter at the 72-h time point, and the molar calcium phosphate ratio at this time point was 1.60. The concentration of calcium and phosphate in the filtrate at 2 h is much higher than the concentration at 72 h (Table I), which shows that there is a very large difference in the apparent solubility between the calcium phosphate mineral phases found at these two times.

Additional experiments were carried out to determine whether the failure of calcium and phosphate to pass through the filter was due to the formation of a high molecular mass complex of fetuin and a calcium phosphate mineral phase similar to the fetuin-mineral complex recently discovered in the serum of rats treated with etidronate. The possible presence of the fetuin-mineral complex in solutions of fetuin and 5 mM calcium and phosphate was assessed by gel filtration over Sephacryl S300HR. As seen in Fig. 6, there is a massive peak of calcium, phosphate, and fetuin in the sample at 24 h after mixing to attain the final 5 mM in calcium and phosphate, but no evidence for this peak in the sample at 12 h after mixing. The high molecular mass peak of fetuin, calcium, and phosphate found in the 24-h sample is in the same elution position as the fetuin-mineral complex found in the serum of etidronate treated rats and accounts for 74% of the calcium applied to the column. Gel filtration chromatograms obtained for samples at 4 and 14 days after mixing to attain 5 mM calcium and phosphate had high molecular mass peaks of fetuin, calcium, and phosphate that were identical in elution position and composition to the fetuin-mineral complex found at 24 h (data not shown). The possible presence of the fetuin-mineral complex in solutions of fetuin and 5 mM calcium and phosphate was also assessed by centrifugation for 2 h at 16,000 $\times$ *g*. Centrifugation of the solution obtained 24 h after mixing produced a translucent, compact pellet, and chemical analysis of this pellet showed that it contained calcium, phosphate, and fetuin in amounts comparable with those found in the high molecular mass peak of the Sephacryl S300 column at this time point (data not shown). In contrast, centrifugation of the solution obtained 2 h after mixing did not produce a detectable pellet, and no calcium, phosphate, or fetuin could be detected at the bottom of the centrifuge tube.

The fetuin-mineral complex found in the serum of etidronate-treated rats binds MGP with high affinity, as shown by the observation that over 95% of the MGP in serum is associated with this complex (1, 16). MGP is also found in the fetuin-mineral complex formed in serum containing 10 mM added calcium and phosphate (Fig. 2*A*). Additional experiments were accordingly carried out to determine whether MGP binds strongly to the fetuin-mineral complex formed *in vitro* with purified fetuin. In the initial experiment, 100,000 cpm of ^{125}I-labeled rat MGP were added to a 1-ml solution of fetuin and 5 mM calcium and phosphate at 24 h after mixing, and the solution was then filtered over a column of Sephacryl S300 as described in the Fig. 6 legend. Over 95% of the MGP label eluted in the same high molecular mass position as the fetuin-mineral complex (data not shown). In contrast, when this experiment was repeated with a solution of fetuin and 5 mM calcium and phosphate at 2 h after mixing, there was no detectable MGP label in the elution position of the fetuin-mineral complex, and all of the MGP label emerged in the 10-kDa elution position expected for MGP. These results indicate that the fetuin-mineral complex found in solutions of fetuin and 5 mM calcium and phosphate at 24 h after mixing has the ability to bind MGP strongly.

Although there is no centrifugation or gel filtration evidence for the presence of the fetuin-mineral complex at 2 or 12 h after mixing to achieve 5 mM in calcium and phosphate, the data in Table I show that there is filtration evidence for the presence of nonfilterable calcium and phosphate. To see whether fetuin is associated with this nonfilterable calcium phosphate component, the experiment was repeated using an ultrafree CL filter with a 300-kDa molecular mass cut-off, a cut-off that allows passage of the 59-kDa fetuin monomer through the membrane. Analysis of filtrates showed that there was again a nonfilterable calcium and phosphate component in the solution containing 5 mM calcium and phosphate but not in the solution containing 5 mM calcium alone and that the presence of this nonfilterable calcium and phosphate component was associated with a 37% reduction in the amount of fetuin in the filtrate (data not shown). This reduction in filtrate fetuin suggests that fetuin could be bound to the nonfilterable calcium phosphate component and that the amount of bound fetuin may be as much as 37% of the fetuin in the original solution.

Formation of the Fetuin-Mineral Complex during the Inhibition of Calcium Phosphate Mineral Formation by Pure Fetuin at 4 mM Calcium and Phosphate—Experiments were carried out to determine whether a fetuin-mineral complex is also formed at 4 mM calcium and phosphate. The presence of the fetuin-mineral complex at different times after mixing to achieve 4 mM calcium and phosphate was initially assessed by gel filtration over Sephacryl S300HR. These experiments showed that there is no evidence for a high molecular mass peak of fetuin, calcium, and phosphate immediately after mixing and at 4 days after mixing and a massive high molecular mass peak of calcium, phosphate, and fetuin at 5 days after mixing (Fig. 7). When these experiments were repeated using filtration through a 10-kDa membrane to separate free calcium from calcium associated with higher molecular mass complexes, filtrate calcium was found to remain close to initial levels for 4 days and then to decrease to 0.5 mM between days

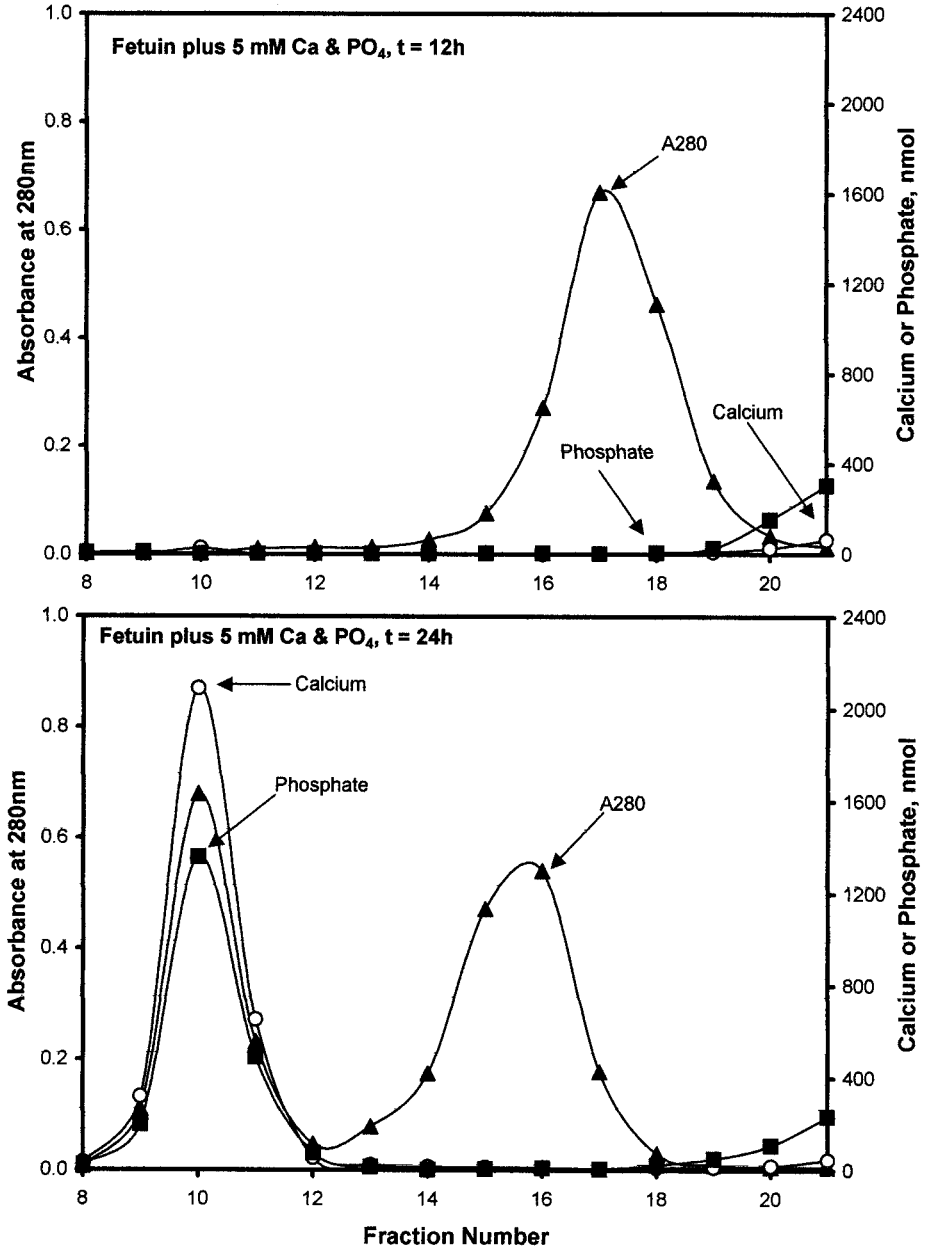

FIG. 6. **Gel filtration evidence for the formation of a fetuin-mineral complex during the inhibition of calcium phosphate mineral precipitation by fetuin at 5 mM calcium and phosphate.** 3-ml aliquots of buffer were first prepared that contained 0.2 M HEPES buffer, pH 7.4, 5 mg/ml fetuin, and either 10 mM phosphate (pH 7.4) or 10 mM calcium labeled with ^{45}Ca. These solutions were then mixed rapidly to obtain a homogenous solution containing 5 mM in each ionic component. 1-ml aliquots were removed at 12 and 24 h and immediately applied to a 25-ml column of Sephacryl S300HR filtration media equilibrated at room temperature with 20 mM HEPES, pH 7.4, 0.15 M NaCl, and 10 mM$CaCl_2$. The figure shows the amount of phosphate and incorporated calcium (calculated from the ^{45}Ca label) in each 1-ml fraction and the absorbance at 280 nm for each fraction.

4 and 5 (data not shown). The solution of fetuin and 4 mM calcium and phosphate was completely clear for the first 4 days and then became opalescent at day 5 and remained comparably opalescent over a 14-day period of observation. These observations show that the fetuin-mineral complex is also formed at 4 mM calcium and phosphate, but the timing of its formation occurs at 4–5 days after mixing rather than at the 20–24 h after mixing found at the 5 mM calcium and phosphate conditions.

DISCUSSION

Formation of the Fetuin-Mineral Complex in Serum—The present studies show that a complex of the proteins fetuin and matrix Gla protein and a calcium phosphate mineral phase forms within 3 h when the concentrations of calcium and phosphate in serum are raised by 10 mM each and the resulting solution is incubated at 37 °C and pH 7.4. Because the presence of the fetuin-mineral complex was detected using the same methods devised earlier for the detection of the fetuin-mineral complex in the serum of etidronate-treated rats, namely gel filtration and sedimentation by centrifugation, it seems probable that the structure of the fetuin-mineral complex formed during the 3-h incubation of modified serum is essentially identical to that of the fetuin-mineral complex in the serum of etidronate-treated rats. The observation that fetuin is the major protein that is strongly associated with the fetuin-mineral complex formed during the 3-h incubation of modified serum (Fig. 2*B*, *lane 5*) attests to the extraordinary specificity of fetuin for the interaction with the mineral phase of the complex.

The major difference between the fetuin-mineral complex formed during 3 h of incubation of serum containing 10 mM added calcium and phosphate and the complex found in the serum of etidronate-treated rats is the content of MGP, which is 40 ng MGP/ml for the complex formed after 3 h of incubation compared with 20 μg/ml for the complex found in the serum of etidronate-treated rats. This difference is explained by the fact that the amount of MGP that can associate with the complex *in vitro* is limited by the amount of MGP in 1 ml of rat serum, whereas the amount of MGP that associates in the 9 h after

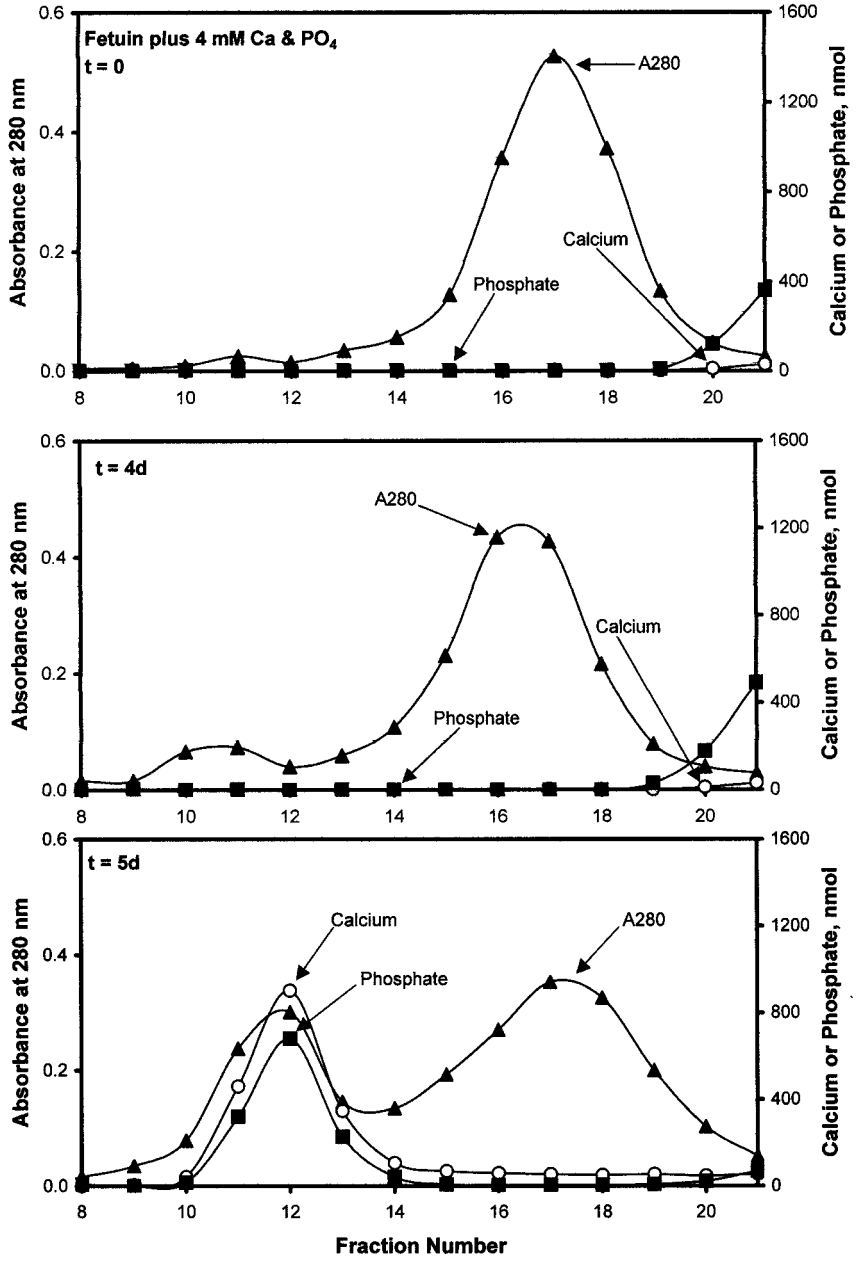

FIG. 7. **Gel filtration evidence for the formation of a fetuin-mineral complex during the inhibition of calcium phosphate mineral precipitation by fetuin at 4 mM calcium and phosphate.** 3-ml aliquots of buffer were first prepared that contained 0.2 M HEPES buffer, pH 7.4, 5 mg/ml fetuin, and either 8 mM phosphate (pH 7.4) or 8 mM calcium labeled with ^{45}Ca. These solutions were then mixed rapidly to obtain a homogenous solution containing 4 mM in each ionic component. 1-ml aliquots were removed at $t = 0$, 4 days, and 5 days and immediately applied to a 25-ml column of Sephacryl S300HR filtration media equilibrated at room temperature with 20 mM HEPES, pH 7.4, 0.15 M NaCl, and 10 mM$CaCl_2$. The figure shows the amount of phosphate and incorporated calcium (calculated from the ^{45}Ca label) in each 1-ml fraction and the absorbance at 280 nm for each fraction.

etidronate injection is not. In the etidronate-treated rat, the association of MGP with the fetuin-mineral complex in fact raises total serum MGP levels by over 40-fold over the first 9 h after etidronate injection because of the incorporation of new MGP synthesis over this period (1).

The present studies further support the role of fetuin as a major calcification inhibitor[2] in serum and show that this activity of the protein may be in part be due to the formation of the fetuin-mineral complex. The conditions used here to form the complex within 3 h entail raising the concentrations of calcium and phosphate in serum by 10 mM each. These concentrations of calcium and phosphate are far above those that form a spontaneous mineral phase at 37 °C and pH 7.4 in the absence of proteins, and indeed the formation of a calcium phosphate precipitate occurs within seconds of mixing solutions to achieve a final 10 mM calcium and phosphate at pH 7.4.[3] The fact that no precipitate formed even after incubation of serum containing 10 mM calcium and phosphate for a period of 9 days at 37 °C is therefore evidence for the remarkable ability of fetuin to arrest the growth and precipitation of the mineral component of the fetuin-mineral complex.

Formation of the Fetuin-Mineral Complex with Pure Bovine Fetuin—The present studies show that solutions of purified bovine fetuin also inhibit the growth and precipitation of a calcium phosphate mineral phase by forming complexes with mineral. The inhibition of mineral formation occurs in two stages at 5 mM calcium and phosphate, and it is useful to consider each separately.

In the absence of fetuin, the first calcium phosphate mineral phase that forms at a concentration of 5 mM calcium and phosphate and a pH of 7.4 is transient and soluble. This phase forms a white precipitate immediately upon mixing calcium and phosphate and persists for about 30 min in apparent equilibrium with a solution calcium concentration of 3.1 mM (Fig. 3). Between 30 and 60 min this phase is replaced by a more stable phase with a solution calcium concentration of about 0.8 mM. The initial, less stable calcium phosphate mineral phase was not noted in earlier studies at 5 mM calcium and phos-

[3] P. A. Price and J. E. Lim, personal observations.

phate, probably because the first time points measured were at 1 h or later (2, 19).

When fetuin is present, the immediate precipitation of calcium and phosphate does not occur, but there is nevertheless evidence that an initial mineral phase is formed in the presence of fetuin that has the same apparent solubility as the initial phase formed in the absence of fetuin. This evidence includes the fact that ionic calcium levels fall to 3.1 mM within 30 min of mixing calcium and phosphate at final concentrations of 5 mM each and stay at this level for 20 h before falling to 0.9 mM at 24 h and to 0.6 mM at 30 h. Filtration through a 10-kDa membrane reveals essentially identical results, with filtrate calcium levels falling to 3.1 mM within 30 min, staying at this level for over 16 h and then falling to less than 1 mM calcium at 24 h. It seems likely that fetuin forms a specific complex with this initial mineral phase, one that inhibits its growth to a size that causes visual opalescence in the solution, that inhibits its precipitation from solution, and that delays its transformation to the final, stable mineral phase from 30 min in the absence of fetuin to 20 h in the presence of fetuin. The only direct evidence that fetuin does form a complex with this initial mineral phase, however, is the reduction in the passage of fetuin through a 300-kDa filter that occurs in association with the presence of this calcium phosphate complex in solution. It should be noted that the putative complex formed between fetuin and the initial, soluble mineral phase is not identical to the fetuin-mineral complex seen in the serum of rats treated with etidronate, because it cannot be detected by gel filtration over a column of Sephacryl S300 or by centrifugation at 16,000 $\times$ *g*.

Fetuin does form a more typical fetuin-mineral complex with the final, less soluble calcium phosphate mineral phase that forms at 5 mM calcium and phosphate, the phase in apparent equilibrium with a solution calcium concentration of about 0.5 mM. Because this complex binds MGP and can be detected using the same methods devised earlier for the detection of the fetuin-mineral complex in the serum of etidronate-treated rats, gel filtration, and sedimentation by centrifugation, it seems probable that the structure of this fetuin-mineral complex is essentially identical to the structure of the complex found in the serum of etidronate-treated rats. Solutions containing this complex of fetuin and the more stable calcium phosphate mineral phase are extraordinarily stable and persist in solution for at least 14 days at room temperature without evidence for aggregation, growth, or precipitation.

The present studies also show that a typical fetuin-mineral complex, one essentially identical to that found in the serum of etidronate-treated rats, is formed in solutions containing purified bovine fetuin and 4 mM calcium and phosphate. This complex is formed between 4 and 5 days after mixing to achieve 4 mM calcium and phosphate, however, which is considerably longer than the between 20 and 24 h found for the 5 mM calcium and phosphate conditions. The timing of fetuin-mineral complex formation at 4 mM calcium and phosphate is quite reproducible and, in 10 independent experiments that were carried out over a period of 6 months, always occurred between 4 and 5 days after mixing. The reproducibility of the timing of this transition suggests that there may be time-dependent chemical changes that occur in the mineral phase over the first 4 days after mixing that lead to the formation of the complex between days 4 and 5.

Possible Mechanisms for the Generation of the Fetuin-Mineral Complex in Etidronate-treated Rats—We have previously proposed that the fetuin-mineral complex forms in etidronate-treated rats when the continuing action of osteoclasts and the acute inhibition of mineralization with etidronate combine to cause a sharp rise in the concentrations of calcium and phosphate in the bone remodeling compartment (16). This rise would be expected to exceed the threshold for the spontaneous precipitation of a calcium phosphate mineral phase in a solution that, because of the vascular nature of the bone-remodeling compartment, is presumably identical to plasma. Fetuin in plasma would then form a specific complex with calcium phosphate crystal nuclei that would inhibit the growth and precipitation of this mineral phase. The present studies support this model by showing that the fetuin-mineral complex does in fact form rapidly in serum containing 10 mM added calcium and phosphate and by showing that the fetuin-mineral complex also forms when the precipitation of calcium phosphate is inhibited by solutions of pure fetuin. What is not yet clear, however, is whether the magnitude of the putative increase in calcium and phosphate within the bone remodeling compartment in etidronate-treated rats indeed approaches a 10 mM increase in calcium and phosphate needed to rapidly form the fetuin-mineral complex in serum *in vitro*.

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