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Inhibition of hydroxyapatite formation in collagen gels by chondroitin sulphate

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Crystal growth in native collagen gels has been used to determine the role of extracellular matrix macromolecules in biological calcification phenomena. In this system, type I collagen gels containing sodium phosphate and buffered at pH 7.4 are overlayed with a solution containing $CaCl_2$. Crystals form in the collagen gel adjacent to the gel–solution interface. Conditions were determined which permit the growth of crystals of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. At a Ca/P molar ratio of 2:1, the minimum concentrations of calcium and phosphate necessary for precipitation of hydroxyapatite are 10mM and 5mM, respectively. Under these conditions, precipitation is initiated at 18–24h, and is maximal between 24h and 6 days. Addition of high concentrations of chondroitin 4-sulphate inhibits the formation of hydroxyapatite in collagen gels; initiation of precipitation is delayed, and the final (equilibrium) amount of precipitation is decreased. Inhibition of hydroxyapatite formation requires concentrations of chondroitin sulphate higher than those required to inhibit calcium pyrophosphate crystal formation.

Proteoglycans have long been suspected to be involved in the regulation of cartilage calcification (for review see Buckwalter, 1983). At the epiphyseal plate of long bones, calcification of cartilage by deposition of hydroxyapatite occurs continuously during the period of skeletal growth. Vascular invasion of the calcified cartilage from the adjacent subchondral bone is associated with the removal of hypertrophic chondrocytes and the deposition of a newly-synthesized bone matrix, resulting in longitudinal bone growth. The fate of the pre-existing cartilage matrix is still the subject of controversy. Early ultrastructural studies indicated that the matrix (in particular the proteoglycan) was degraded prior to calcification of the cartilage (Thyberg, 1974). More recent work, however, suggested that there is no loss of proteoglycan from epiphyseal cartilage during calcification; the cartilage matrix is entombed by nascent bone trabeculae and subsequently eroded by osteoclast-type cells (Poole *et al.*, 1982).

Nonetheless, the putative association of proteoglycan with inhibition of calcification in cartilage is supported by a variety of experimental studies. Fluid removed from cartilage by micropuncture inhibits the formation of hydroxyapatite in a 'synthetic lymph' system (Cuervo *et al.*, 1973). Chondroitin sulphate (CS) was shown to retard the transformation of amorphous calcium phosphate to hydroxyapatite (Termine & Posner, 1970). Highly-purified cartilage proteoglycan inhibited the formation of hydroxyapatite both directly and by transformation of amorphous calcium phosphate (Blumenthal *et al.*, 1979), and also inhibited the growth of pre-existing hydroxyapatite seed crystals in metastable calcium phosphate solutions (Chen *et al.*, 1984).

In the present study, a new system has been developed to investigate the role of matrix components in biological calcification phenomena. Hydroxyapatite crystals were grown in native collagen gels in an attempt to simulate the extracellular matrix of connective tissues. Using this system, it was shown that CS is only a weak inhibitor of hydroxyapatite formation.

Materials and methods

Materials

Chondroitin 4-sulphate (type A, from whale cartilage) was obtained from Sigma. Antibiotic mixture (penicillin/streptomycin/amphotericin B) was obtained from Gibco. $^{45}CaCl_2$ (CES-3, 10–40mCi/mg) was obtained from Amersham International.

Abbreviation used: CS, chondroitin sulphate.

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Preparation of collagen gels

Type I collagen was purified from rat tail tendon (Miller & Rhodes, 1982). In brief, tendons were extracted with 0.5M-acetic acid, and an insoluble residue was removed by centrifugation. Collagen was precipitated with 0.7M-NaCl, redissolved in 1M-NaCl/50mM-Tris/HCl, pH7.4, and re-precipitated with 2.6M-NaCl. The final precipitate was redissolved in and dialysed against 10mM-HCl, and lyophilized.

Purified type I collagen was dissolved at 5mg/ml in 20mM-HCl. Appropriate volumes of 1M-Tris/HCl, pH7.4, 5M-NaCl, 0.2M-sodium phosphate, pH7.4, and antibiotic solution (100× concentrate) were added, and the solution was diluted to a collagen concentration of 2mg/ml. Neutralized collagen solutions were maintained at 4°C with constant stirring to prevent premature gelling. After degassing, 5ml portions of gel solution were poured in 17mm × 100mm polystyrene tubes (Falcon) and allowed to set overnight at 37°C. Final concentrations were as follows: 100mM-Tris/HCl, 50mM-NaCl, 100units of penicillin G/ml, 100μg of streptomycin sulphate/ml and 0.25μg of amphotericin B/ml. For inhibitor studies, appropriate volumes of chondroitin 4-sulphate (100mg/ml) were added to neutralized collagen solutions immediately prior to pouring. After gels had set, 5ml of a solution containing 50mM-NaCl/100mM-Tris/HCl, pH7.4, plus antibiotics (as above) and $CaCl_2$ was carefully layered over the gel phase, and tubes were incubated at 37°C. Except where noted below, gels contained 5mM-sodium phosphate and overlay solution contained 10mM-$CaCl_2$.

Precipitates were harvested from gels after removal of the solution phase and freezing at −20°C. Tubes were cracked open and the crystal-bearing region of the gel (the top 5mm) was cut out. After thawing, which resulted in collapse of the gel, the excess aqueous phase was removed, and the gel was dried at 37°C and weighed. Powder X-ray diffraction of precipitates was performed with a Debye–Scherrer camera using Cu–K_α radiation (0.15418nm) at 40kV and 25mA with an exposure time of 2h. Ashing of precipitates was performed in hot $HNO_3/HClO_4$ (4:1, v/v) for 2h. Calcium was determined by using an Instrumentation Laboratory IL 151 atomic absorption spectrometer, and phosphate was determined by the method of Chen *et al.* (1956).

Kinetic analysis of hydroxyapatite formation in gels

Collagen gels were set up containing 5mM-sodium phosphate. The overlay solution contained 10mM-$CaCl_2$ plus 0.2μCi of $^{45}CaCl_2$/ml. Gels were incubated at 37°C, and 50μl samples of overlay solution were removed at various times for scintillation counting. Control gels contained no phosphate. All experiments were performed in quadruplicate.

Effect of chondroitin sulphate on hydroxyapatite formation in collagen gels

The effects of CS on the kinetics of hydroxyapatite formation and on the final amount of precipitate produced were determined separately. For kinetic studies, ^{45}Ca was included in the overlay solution as described above. Gels contained 0, 10 or 30mg of CS/ml (each in triplicate). Because the presence of CS affects the rate of calcium diffusion into gels, it was necessary to use control gels (containing no phosphate) at each CS concentration.

To determine absolute amounts of hydroxyapatite formed, collagen gels were set up containing 0, 5, 10, 20 or 30mg of CS/ml (each in triplicate). After 14 days incubation, the top 5mm of the gel was harvested, ashed, and calcium was quantified by atomic absorption spectroscopy.

Results

Growth of hydroxyapatite crystals in native collagen gels

In initial studies, conditions were determined which permit the formation of hydroxyapatite crystals in collagen gels. Type I collagen gels containing sodium phosphate and buffered at pH7.4 were overlayed with solutions containing $CaCl_2$ and incubated at 37°C. To determine the minimum $Ca \times PO_4$ activity product for precipitation in gels, the phosphate concentration was varied in the range 3–10mM and the $CaCl_2$ concentration in the range 6–20mM. The concentrations in physiological fluids are approximately 2mM-calcium and 1mM-phosphate. A Ca/P ratio of 2:1 was therefore used in all gels (the Ca/P ratio in hydroxyapatite is 1.67). Gels were incubated at 37°C for 14 days. At that time, the top 5mm of gel, which contained all visible precipitate, was harvested, dried and weighed. Dry weight of precipitate is plotted as a function of phosphate concentration in Fig. 1.

The plot shows a discontinuity between 4 and 5mM-phosphate. Above 5mM, the curve is approximately linear up to 10mM-phosphate. The best-fitting straight line was applied to this region of the curve, and gave a correlation coefficient of 0.98 and a gradient of 0.94. The latter value indicates that, between 5 and 10mM-phosphate (10–20mM-calcium), the amount of precipitation is directly proportional to the phosphate concentra-

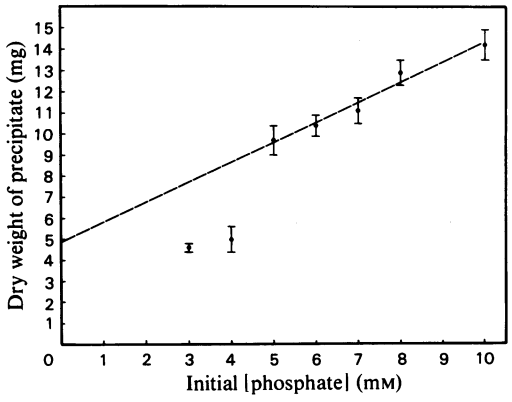

Fig. 1. *Effect of phosphate concentration on hydroxyapatite formation in collagen gels*
Dry weight of final precipitate is plotted as a function of initial phosphate concentration. The broken line represents the best-fitting straight line through the linear part of the curve (5–10mM). The calcium concentration was double the phosphate concentration in all cases.

tion; a doubling of phosphate concentration results in an approximate doubling in the weight of the precipitate. The regression line has a *y*-axis intercept of 4.87 (Fig. 1). Therefore, extrapolation to zero phosphate gives a precipitate weight of approx. 5mg. This value represents the weight of the collagen in the region of the gel harvested, as shown by weighing the equivalent region of phosphate-free gels. Since the samples from 3mM- and 4mM-phosphate gels weighed approx. 5mg, this indicates that, under the conditions used, essentially no calcium phosphate precipitation occurs below 5mM-phosphate. Therefore, at an initial Ca/P ratio of 2:1, the minimum concentrations for precipitation in collagen gels are 5mM-phosphate and 10mM-calcium. These minimum concentrations were used in all experiments described below.

Precipitates formed in collagen gels under these conditions were analysed by powder X-ray diffraction (Fig. 2). After 14 days incubation, by which time precipitation was complete (see below), the precipitate formed was identified, by comparison with standards, as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$].

The precipitates formed in gels containing 5–10mM-phosphate were ashed in $HNO_3/HClO_4$ for quantification of calcium and phosphate. As shown in Table 1, the Ca/P molar ratios of these precipitates were in the range 1.66–1.70, very close to the theoretical hydroxyapatite ratio of 1.67. These data are therefore in agreement with the powder X-ray diffraction analysis (Fig. 2).

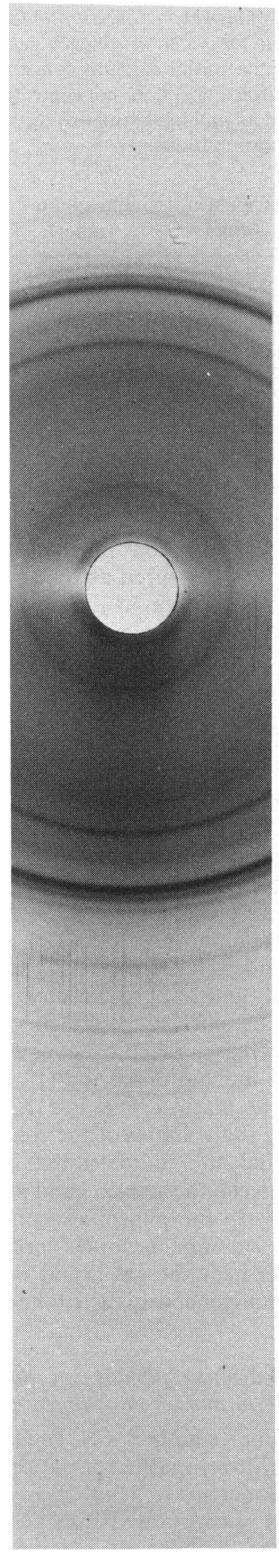

Fig. 2. *Powder X-ray diffraction of precipitate formed in collagen gel*

Table 1. *Effect of phosphate concentration on hydroxyapatite formation in collagen gels*

In all cases, the initial calcium concentration was twice the initial phosphate concentration. Results are expressed as μmol/gel, and are the means ± S.D. for three determinations.

Initial [phosphate] (mM)	Calcium (μmol)	Phosphate (μmol)	Ca/P molar ratio
5	33.6 ± 0.529	19.9 ± 0.361	1.69 ± 0.014
6	41.8 ± 0.252	24.8 ± 0.252	1.69 ± 0.007
7	48.6 ± 0.529	28.7 ± 0.173	1.69 ± 0.009
8	55.6 ± 0.346	32.8 ± 0	1.70 ± 0.011
10	68.4 ± 1.20	41.1 ± 0.141	1.66 ± 0.019

Kinetic analysis of hydroxyapatite formation in collagen gels

The rate of formation of hydroxyapatite in collagen gels was measured as the loss of calcium from the overlay solution as a function of incubation time, using tracer amounts of $^{45}CaCl_2$ in the overlay solution. To correct for the loss of calcium due to diffusion into the gel phase, control gels containing no phosphate were set up. Hydroxyapatite formation was therefore expressed as the difference between control and experimental gels.

Gels were incubated at 37°C and samples of overlay solution were removed at various times for scintillation counting. Results are shown in Fig. 3. The experimental and control curves are not significantly different until 18h, indicating that calcium phosphate precipitation is initiated between 18 and 24h. Thereafter, the two curves diverge sharply until approx. 6 days, after which they tend to be parallel. Both curves exhibit a hyperbolic decrease, reaching a plateau level by the end of the incubation period (13 days). In the control gels, the plateau level is 51% of the zero-time value. As the volumes of the solution and gel phases are equal, this indicates that equilibration of calcium between the solution and gel phases has been achieved. In the phosphate-containing gels, most of the calcium becomes precipitated as hydroxyapatite near the gel–liquid interface, and thus is non-homogeneously distributed throughout the system.

Effect of chondroitin sulphate on the amount of hydroxyapatite formation in collagen gels

To determine whether CS functions as an inhibitor of hydroxyapatite formation in collagen gels, various concentrations of CS in the range 5–30mg/ml were incorporated into gels. Following incubation, the amount of calcium phosphate precipitation was determined by atomic absorption spectroscopy.

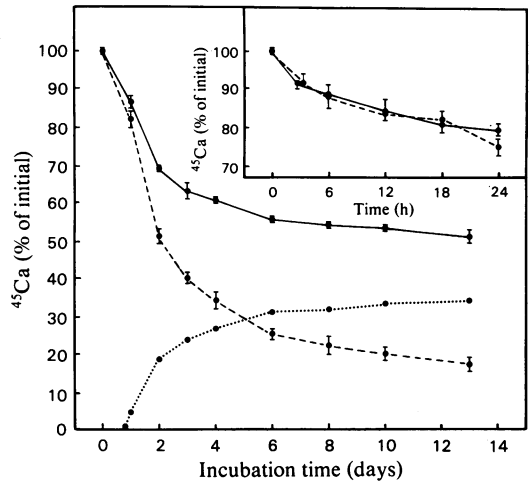

Fig. 3. *Kinetics of hydroxyapatite formation in collagen gels*

Precipitation of calcium phosphate in gels was quantified by measuring the loss of ^{45}Ca from the overlay solution. Results are expressed as means ± S.D. (four determinations). The inset represents an expanded scale to show the earliest time-points. ●——●, control (minus phosphate); ●---●, experimental (plus phosphate); ●·····●, control minus experimental.

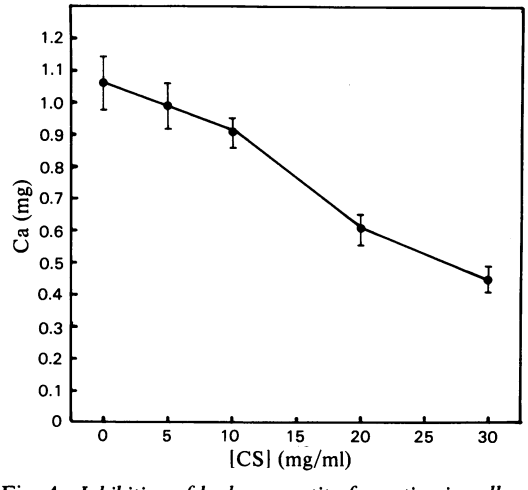

Fig. 4. *Inhibition of hydroxyapatite formation in collagen gels by chondroitin sulphate*

The calcium content of precipitates formed in collagen gels was determined by atomic absorption spectroscopy. Values represent the means ± S.D. for three determinations.

As shown in Fig. 4, CS inhibited calcium phosphate precipitation in an approximately linear fashion. A CS concentration of 30mg/ml resulted in 56% inhibition of precipitation.

Effect of chondroitin sulphate on rate of hydroxyapatite formation in collagen gels

The effect of CS on the kinetics of hydroxyapatite formation in gels was determined by measuring the loss of ^{45}Ca from the overlay solution, using collagen gels containing 0, 10 or 30mg of CS/ml. As preliminary studies indicated that CS affects the rate of diffusion of ^{45}Ca into collagen gels, control gels containing no phosphate were set up for each CS concentration. Results are shown in Fig. 5. In the absence of CS, precipitation of calcium phosphate is initiated between 12 and 24h, as indicated by the divergence of control and experimental curves (Fig. 5*a*). At a CS concentration of 10mg/ml, no significant difference between experimental and control curves is observed until 2 days (Fig. 5*b*), and at 30mg of CS/ml, initiation of precipitation is further delayed, until 8 days (Fig. 5*c*). By plotting the difference between control and experimental values, it was shown that, in the presence of CS, not only was initiation of precipitation delayed, but the final (equilibrium) amount of hydroxyapatite formation was lower (Fig. 5*d*). This is in agreement with the results shown in Fig. 4.

Two further aspects of this experiment are worthy of note. First, the variation between replicates in the experimental series at the highest CS concentration is greater than the corresponding control (no phosphate) series, particularly when the experimental and control curves first begin to diverge (Fig. 5*c*). This is apparently due to precipitation commencing at slightly different times in different replicate gels. This may indicate that calcium phosphate precipitation is, to some extent, a random process; however, it is not clear

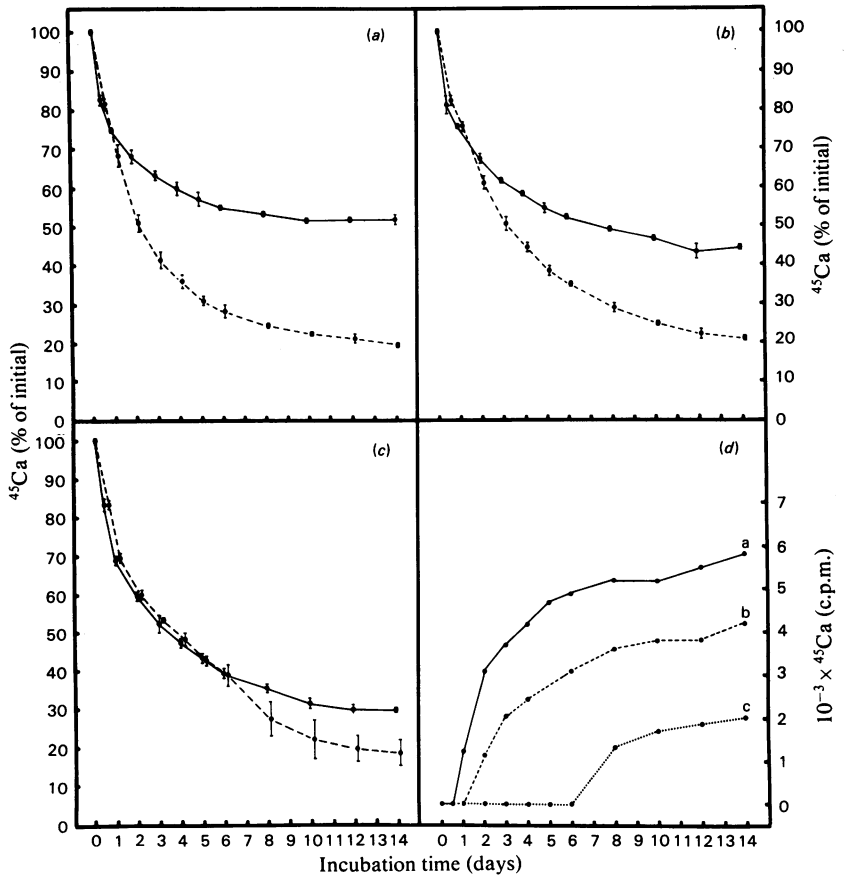

Fig. 5. *Effect of chondroitin sulphate on rate of hydroxyapatite formation in collagen gels*
Rates of hydroxyapatite formation in gels were measured as loss of ^{45}Ca from overlay solution. (*a*) No CS; (*b*) 10mg of CS/ml; (*c*) 30mg of CS/ml. In (*a*)–(*c*), the solid line represents control gels and the broken line represents experimental gels. Values are shown as means ± S.D. for three determinations. (*d*) The data from (*a*)–(*c*) are replotted as the difference between control and experimental gels: ●——●, no CS; ●----●, 10mg of CS/ml; ●·····●, 30mg of CS/ml.

why this effect should be more apparent in the presence of CS.

Secondly, it should be noted that incorporation of CS into collagen gels increases the loss of ^{45}Ca from the overlay solution. In the absence of CS, the ^{45}Ca values reaches a plateau at approx. 50% of the initial value (Fig. 5*a*). At 10mg of CS/ml, the plateau level is 46% (Fig. 5*b*), and at 30mg of CS/ml, 37% (Fig. 5*c*) of the initial value. This presumably means that Ca^{2+} ions are being bound to CS in the gel phase, resulting in an unequal partition of calcium between solution and gel phases.

Discussion

Growth of hydroxyapatite crystals from metastable calcium phosphate solutions has been extensively used to study factors affecting biological mineral formation. Using this approach, the work of several laboratories has shown that cartilage proteoglycan acts as an inhibitor of hydroxyapatite nucleation and growth. Specifically, proteoglycan at concentrations of 1.5–5.0mg/ml partially inhibited the transformation of amorphous calcium phosphate to crystalline hydroxyapatite, and delayed or prevented the precipitation of hydroxyapatite from physiological calcium phosphate solutions (Blumenthal *et al.*, 1979). This inhibitory effect apparently correlates with hydrodynamic volume; proteoglycan aggregates were more effective than monomers, which were in turn more effective than free CS chains. Proteoglycan also inhibited the further growth of hydroxyapatite seed crystals in metastable solutions. At a nominal physiological concentration of 10mg/ml, aggregated proteoglycan decreased the amount of hydroxyapatite formed over an 8h incubation (Chen *et al.*, 1984). Desulphation of CS chains decreased the inhibition of hydroxyapatite crystal growth.

In the present study, an alternative system for the analysis of hydroxyapatite formation is described. Hydroxyapatite crystals are grown in native collagen gels, into which other matrix macromolecules may be incorporated. This system represents a logical development of earlier gel systems, in which calcium phosphate and other crystals were grown in non-physiological semi-solid media, including gelatin (heat-denatured collagen), polyacrylamide and sodium metasilicate (Le Geros & Le Geros, 1972; Pritzker *et al.*, 1978; de Jong *et al.*, 1980). Deposition of calcium phosphate in native collagen gels has previously been reported, but the crystal phases formed were brushite ($CaHPO_4,2H_2O$) and octacalcium phosphate [$Ca_8H_2(PO_4)_6,5H_2O$] rather than hydroxyapatite (Pokric & Pucar, 1979).

The collagen gel system described here therefore has the advantage that crystals form in a collagenous matrix which is similar to that of mineralizing connective tissues. However, a disadvantage is that crystal formation has not yet been possible at physiological calcium and phosphate concentrations. Nonetheless, collagen gels represent a complementary experimental approach to the classical solution-chemistry system.

Conditions have been determined which permit the growth of hydroxyapatite crystals in gels composed of native type I collagen. The minimum concentrations of calcium and phosphate necessary for precipitation are 10mM and 5mM respectively. Kinetic analysis of hydroxyapatite in collagen gels was performed by measuring the loss of calcium from the overlay solution as a function of incubation time, using ^{45}Ca as a tracer. Since the gel initially contains no calcium, phosphate-free gels are a necessary control to differentiate loss of calcium due to diffusion from that due to precipitation. In control (phosphate-free) gels, approx. 13 days is necessary for complete equilibration of calcium between the solution and gel phases, indicating that diffusion into the gel is very slow. This is apparently due to 'structuring' of solvent molecules in semi-solid media (Maquet *et al.*, 1984). Precipitation of calcium phosphate commences between 18 and 24h, and reaches a plateau level after 6 days.

Incorporation of CS into collagen gels resulted in inhibition of hydroxyapatite formation, but only at high concentrations. This inhibition apparently involves both kinetic and thermodynamic factors, as both the initiation time and final amount of precipitation are altered. These results indicate that CS is a relatively weak inhibitor of hydroxyapatite formation. In comparison, CS and cartilage proteoglycan inhibit formation of calcium pyrophosphate dihydrate crystals at concentrations lower than those required to inhibit hydroxyapatite formation (C. K. Hunter, M. D. Grynpas, P.-T. Cheng & K. P. H. Pritzker, unpublished work). Although caution must be exercised in comparing crystals of different structures and solubility products, such a difference in inhibitory potential suggests that distinct mechanisms may be involved.

Incorporation of CS into collagen gels in the absence of phosphate results in preferential uptake of calcium into the gel phase, presumably due to binding of Ca^{2+} ions by negatively-charged groups on CS chains. At 30mg of CS/ml, this effect results in a calcium concentration 26% higher in the gel phase than in the overlay solution. As the CS-bound calcium is presumably unavailable for precipitation, this phenomenon could in itself reduce the available calcium below the concentration necessary for precipitation. Therefore, inhibi-

tion of hydroxyapatite formation in collagen gels by CS may simply be due to binding of Ca^{2+} ions. In any case, the large amounts of CS necessary to cause inhibition in the present study appear to rule out a specific mechanism of inhibition, such as binding of CS to growth sites on nascent crystallites.

Steric hindrance has been suggested as a possible mechanism of inhibition of hydroxyapatite formation by cartilage proteoglycan in solution, as proteoglycans occupy large hydrodynamic domains (Blumenthal *et al.*, 1979). However, steric effects are less likely to be important in gels as the solvent phase is already ordered. As shown in the present study, diffusion through collagen gels is very slow; the calcium concentrations in the overlay solution and gel phase reach equilibrium only after approx. 13 days. Therefore, diffusion of proteoglycan molecules within collagen gels would be extremely restricted. Mechanisms of inhibition which obtain in free solution may not be applicable to the semi-solid media of connective tisues *in vivo*.

In view of the possibility of different inhibitory mechanisms in solution and semi-solid media, it would be of interest to determine whether proteoglycan aggregates are more potent inhibitors than monomers in collagen gels. We have shown that cartilage proteoglycan aggregates (A1 fraction) and monomers (A1D1 fraction) at 5mg/ml do not inhibit hydroxyapatite formation in gels (G. K. Hunter, B. L. Allen, M. D. Grynpas & P.-T. Cheng, unpublished work). Experiments are now required to compare the inhibition of aggregates and monomers at higher proteoglycan concentrations.

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References

Blumenthal, N. C., Posner, A. S., Silverman, L. D. & Rosenberg, L. C. (1979) *Calcif. Tissue Int.* **27**, 75–82

Buckwalter, J. A. (1983) *Clin. Orthop. Relat. Res.* **172**, 207–232

Chen, C.-C. (1984) Ph.D. Thesis, Cornell University

Chen, C.-C., Boskey, A. L. & Rosenberg, L. C. (1984) *Calcif. Tissue Int.* **36**, 285–290

Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1762

Cuervo, L. A., Pita, J. C. & Howell, D. S. (1973) *Calcif. Tissue Res.* **13**, 1–10

de Jong, A. S. H., Hak, T. J. & van Duijn, P. (1980) *Connect. Tissue Res.* **7**, 73–79

Le Geros, R. Z. & Le Geros, J. P. (1972) *J. Crystal Growth* **13/14**, 476–480

Maquet, J. L., Theveneau, H., Djabourov, M. & Papon, P. (1984) *Int. J. Biol. Macromol.* **6**, 162–163

Miller, E. J. & Rhodes, R. K. (1982) *Methods Enzymol.* **82**, 769–800

Pokric, B. & Pucar, Z. (1979) *Calcif. Tissue Int.* **27**, 171–176

Poole, A. R., Pidoux, I. & Rosenberg, L. (1982) *J. Cell Biol.* **92**, 249–260

Pritzker, K. P. H., Cheng, P.-T., Adams, M. E. & Nyburg, S. C. (1978) *J. Rheumatol.* **5**, 469–473

Termine, J. D. & Posner, A. S. (1970) *Arch. Biochem. Biophys.* **140**, 307–317

Thyberg, J. (1974) *J. Ultrastruct. Res.* **46**, 206–218