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Effect of Proteoglycans on in Vitro Hydroxyapatite Formation

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Summary. Well-characterized bovine nasal proteoglycan A1 fraction (aggregate) and proteoglycan D1 fraction (subunit) have been shown to be effective inhibitors of hydroxyapatite (HA) formation in two in vitro test systems: (a) the transformation of amorphous calcium phosphate (ACP) to crystalline HA, and, (b) the direct precipitation of HA from low-concentration calcium phosphate solutions. A1 or D1 in solution slowed the transformation kinetics in system (a) without affecting the time to the onset of conversion. In system (b), A1 or D1 in solution increased the time to the onset of HA formation without affecting the HA formation kinetics. In both test systems A1 was a more effective inhibitor than D1, although the difference was not great. In both systems the inhibitory effect was proportional to the A1 or D1 solution concentration. The action of solutions of low and high molecular weight neutral dextrans on both test systems showed that high molecular weight and/or extended spatial molecular conformation has a much stronger correlation with inhibitory ability than solution viscosity. Proteoglycans have been implicated as playing a role in regulating biological mineralization particularly in the epiphyseal growth plate. Our study suggests that just enzymatic cleavage of aggregate into subunit is not sufficient to allow mineralization to occur, since we find that D1 itself is a potent inhibitor of HA formation. Further degradation and/or removal of D1 appears to be necessary for calcification to take place.

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Key words: Proteoglycans — Hydroxyapatite — Amorphous calcium phosphate — Nucleation — Calcification.

Introduction

Chemical and histological studies of the epiphyseal growth plate in past years have led many workers to conclude that proteoglycans play a regulatory role in biological calcification [1]. This hypothesis is supported by the decrease in proteoglycan concentration and size in moving from the resting cartilage to the calcified cartilage zone of the growth plate. When ruthenium red staining is used to visualize the proteoglycan matrix granules, electron microscopic studies have shown a decrease in proteoglycan molecular size [2] in going from the uncalcified cartilage of the growth plate to the calcified zone. Another study [3], using high-resolution X-ray spectrophotometry, showed a sharp drop in sulfur concentration in the epiphyseal growth plate in the region where an abrupt rise in phosphorus was observed. Since proteoglycans contain sulfated polysaccharides, this experiment showed that a decrease in proteoglycan content accompanied the deposition of mineral in cartilage. Several studies suggest that the decrease in proteoglycan content in the calcifying regions of the growth plate results from proteolytic degradation of proteoglycans. Studies of extracellular fluid aspirated from growth plate cartilage have provided insight into the role of

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proteoglycans in the mineralization process [4]. Chemical analysis of this fluid showed a proteoglycan concentration of about 9.6 mg/ml and the presence of antigenic determinants for antibody against proteoglycans [5]. Further, ultramicro analytical ultracentrifugation of the proteoglycan fraction of this aspirated fluid revealed an S value profile roughly comparable to that obtained from bovine nasal cartilage proteoglycan [5]. When isolated, the high S value proteoglycans from micropuncture were shown to have a strong inhibitory effect on mineral growth in synthetic test systems. On the other hand, the low S value subunits from the same micropuncture fluid were ineffective inhibitors of hydroxyapatite (HA) formation in the same test systems [6]. When the micropuncture fluid containing proteoglycan at approximately 10 mg/ml was diluted to 2 mg/ml, the ability to inhibit mineral growth was lost [5].

All of the above results suggest an important role for the proteoglycans in biological mineralization. The exact mechanism of action of proteoglycans in retarding biological mineralization is yet to be elucidated.

This paper describes the inhibitory effect of chemically and physically characterized proteoglycans on in vitro HA formation. The effects of proteoglycans are also compared to the action of several different high molecular weight dextrans. Two systems of HA formation were used: (a) the transformation of amorphous calcium phosphate (ACP) to HA; and (b) the direct precipitation of HA from solution. Both of these processes may be involved in biological mineralization.

Experimental Procedures

Proteoglycans

Proteoglycan A1 and D1 fractions were prepared from bovine nasal cartilage and chemically and physically characterized by methods previously described [7]. Sedimentation velocity studies were carried out as previously described [7]. Runs were made at 48,000 rpm and 20° C in 0.15 M NaCl, 0.01 M 2-(N-morpholino) ethanol sulfonic acid (MES-Calbiochem), pH 7.0 ($\rho = 1.0061$ g/ml, $N_{rel} = 1.0001$).

Effect of Proteoglycans on HA Formation

Preparation of Amorphous Calcium Phosphate (ACP). ACP was prepared by a previously published procedure at pH 7.4 and room temperature [8] and was stored in a vacuum desiccator in order to prevent conversion to hydroxyapatite by atmospheric moisture. Prior to use the ACP was ground and passed through a 325 mesh sieve in an air-conditioned low-humidity room.

Effect of Proteoglycans on the Conversion of ACP to HA. When ACP converts to HA, hydroxyl ions are removed from solution and incorporated in the crystalline solid. As a result the kinetics of this transformation can be followed by measuring the amount of base needed to maintain nonbuffered slurries of ACP in water at constant pH. The conversion rates observed by this method have been shown to agree with results obtained by X-ray diffraction analyses of aliquots of the solid in the system removed as a function of time [9]. Further, the ACP to HA transformation rates in systems buffered with Tris-HCl at pH 7.4, studied in a similar manner by X-ray diffraction, were found to agree with pH-Stat experiments [9]. In all cases the only phase detected in the conversion of ACP was HA. The kinetics of the conversions reported in this paper were followed by recording continuously with time the volume of 0.1 N NaOH added automatically by a Mettler pH-Stat to keep the pH constant at 7.4 at 30° C.

Proteoglycan solutions were prepared by dissolving the macromolecule in 0.15 M NaCl with stirring at 4° C for 24 h. The A1 and D1 fractions from bovine nasal cartilage were used in these preparations. The NaCl solution increased the ionic strength, which resulted in a considerable decrease in viscosity. We found that without the salt the solution viscosities were too high to obtain adequate stirring in the pH-Stat, especially with the 3 mg/ml and 5 mg/ml proteoglycan concentrations. A control experiment showed that 0.15 M NaCl solution, with no proteoglycan present, had no effect on the induction time or transformation kinetics of the ACP to HA conversion. To 5 ml of each proteoglycan (A1 or D1) solution 4.0 mg of ACP (325 mesh powder) was added and the transformation was followed automatically on the pH-Stat. The slurry in the reaction vessel of the pH-Stat was vigorously stirred with a Teflon-covered magnetic bar to insure rapid mixing of added titrant, and the system was purged of air by a stream of nitrogen.

Effect of Proteoglycans on HA Formation from Calcium Phosphate Solutions. At low Ca and PO_4 concentrations it has been shown that HA precipitates directly from calcium phosphate solutions without first forming ACP as an intermediate precursor phase [10]. Under these conditions the solutions are supersaturated with respect to HA but slightly undersaturated with respect to ACP. The time from initial mixing of the reagents to the begin-

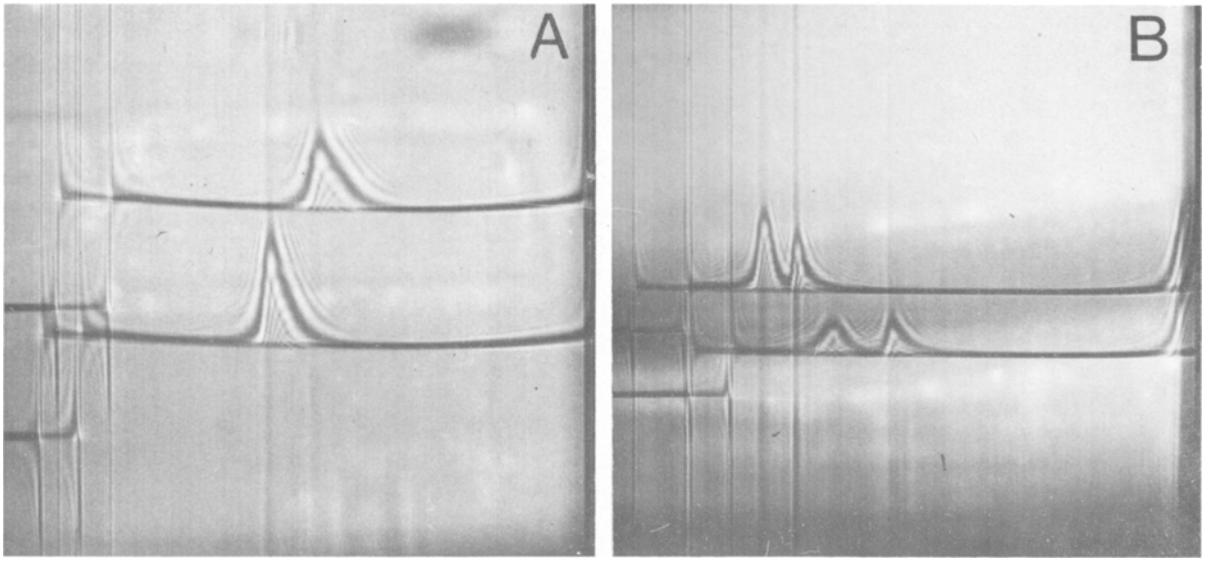

Fig. 1. Sedimentation velocity patterns of the proteoglycan subunit and aggregate species present in fractions D1 and A1. Runs were made at 44,000 rpm and 20° C in 0.15 M NaCl, 0.01 M MES, pH 2.0. The direction of sedimentation is from left to right. (A) Proteoglycan subunit in fraction D1 at 1.46 mg/ml (top) and 1.29 mg/ml (bottom). (B) Proteoglycan aggregate and subunit species present in fraction A1 at 2.09 mg/ml (top) and 1.66 mg/ml (bottom)

ning of HA formation is called the induction time (t_i) and varies inversely with Ca and PO_4 concentration. For example, with 1.64 mM Ca and 1.1 mM PO_4, t_i is about 1 h, and with 1.2 mM Ca and 0.8 mM PO_4, t_i is about 5 h. In this study we used the system with 1.64 mM Ca and 1.1 mM PO_4 as a control to test for the effect of proteoglycans on HA formation. Inhibition of HA formation was noted by an increase in t_i relative to the control system and/or by a change in the HA formation kinetics. In these calcium phosphate solution experiments the HA formation kinetics were also followed by continuously recording OH uptake as a function of time in the pH-Stat.

Proteoglycan A1 and D1 fractions from bovine nasal cartilage were dissolved by stirring in an appropriate volume of deionized water at 4° C for 24 h. In contrast to the experiments where ACP converts to HA, the proteoglycans for these solution studies were not dissolved in 0.15 M NaCl because lower concentrations were used and the resulting viscosities were not excessive. We added 0.5 ml of 16.4 mM $CaCl_2$ and 0.5 ml of 11 mM Na_2HPO_4 solution to 4 ml of each proteoglycan solution. When HA formation began, the amount of 0.1 N NaOH added automatically to maintain the pH at 7.4 at 30° C was continuously recorded. The contents of the reaction vessel were vigorously stirred and purged of air by a stream of nitrogen throughout each experiment.

Dextran Experiments. We studied the effect of two neutral dextrans of 80,700 MW (Sigma Chemical Co.) and 16×10^6 MW on the ACP to HA transformation and on the direct precipitation of HA. The dextrans were dissolved in deionized water and the solutions were then used with these two test systems in the pH-Stat as described above. Changes in t_i and HA formation rates caused by the dextrans were examined.

Viscosity Measurements. Relative viscosities of proteoglycan and dextran solutions were determined in a Cannon-Fenske viscometer in a constant temperature bath at 30 ± 0.1° C. Proteoglycan solutions were adjusted to 1.64 mM Ca and 1.1 mM PO_4 in order to correspond to the ionic concentrations found in solution in the pH-Stat experiments. These calcium and phosphate concentrations were found to have no effect on the viscosities of dextran solutions.

Results

Proteoglycan fractions A1 and D1 were isolated from bovine nasal cartilage by previously described procedures [7]. Sedimentation coefficients of the proteoglycan species present in A1 and D1 were then determined in sedimentation velocity studies in the analytical ultracentrifuge. Figure 1 shows sedimentation velocity patterns of proteoglycan fraction D1 (Fig. 1A) and of proteoglycan A1 fraction (Fig. 1B). Proteoglycan A1 fraction contains a proteoglycan aggregate species with a sedimentation coefficient ($S°_{20}$, solvent) of 89.1 *S* and a proteoglycan subunit species of 30.5 *S*. Proteoglycan D1 fraction is free of aggregate and contains only a proteoglycan subunit with a sedimentation coeffi-

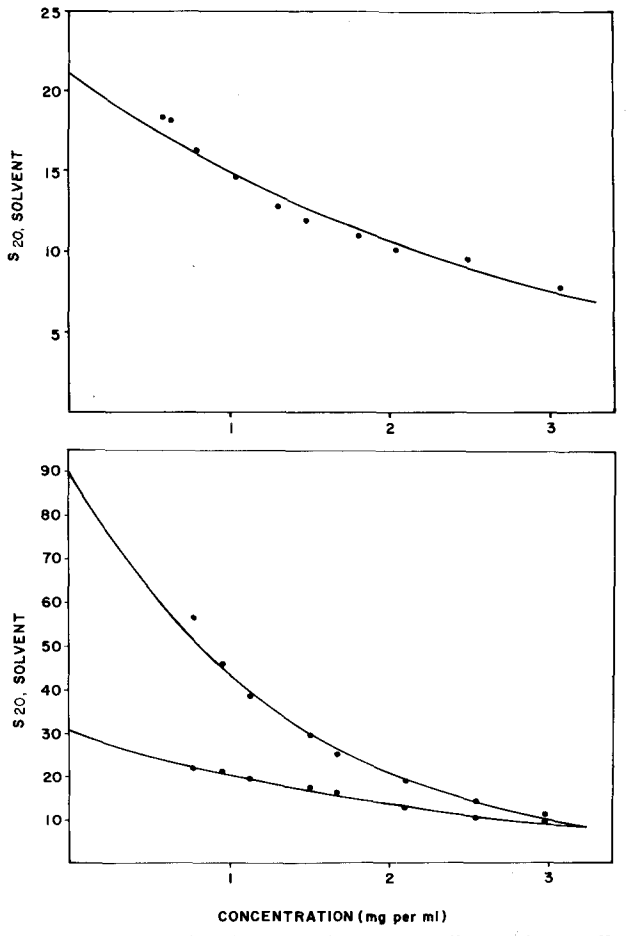

Fig. 2. Concentration dependencies of the sedimentation coefficients of the proteoglycan subunit and aggregate species present in fractions D1 (top) and A1 (bottom)

cient of 21.1 *S*. The concentration dependence of sedimentation coefficients of these species is shown in Fig. 2.

Figure 3 shows the slowing effect of proteoglycan A1 fraction and proteoglycan D1 fraction on the rate of the ACP to HA conversion. Fraction A1 has a greater inhibitory effect than the fraction D1. Fraction A1 (No. 1) had been stored for several months, whereas fraction A1 (No. 2) was freshly prepared. Figure 4 displays the effect of increasing the A1 concentration on the ACP to HA conversion kinetics. The rate of ACP conversion decreases as the A1 concentration is increased.

It is important to note in both Figs. 3 and 4 that the time to the beginning of HA formation for all systems stabilized with proteoglycans was close to the corresponding time for unstabilized ACP, i.e., in the 20 to 40 min range. The proteoglycans acted only to decrease the HA formation rate, as a function of concentration, in the ACP to HA conversions, but they had a negligible effect on the time at which conversion began.

Figure 5 shows the effect of A1 on the direct precipitation of HA from low-concentration calcium phosphate solutions. With increasing concentration the aggregate increased the time to the onset of HA formation (i.e., t_i) as compared to the proteoglycan-free control system. Not shown on Fig. 5, but as will be seen in Fig. 6, D1 had a similar but smaller effect on delaying apatite formation. The differences in the shapes of the OH-uptake curves in Fig. 5 represent minor variations (not understood at present) that one observes from experiment to experiment. It should be noted that one should not compare the shapes of the OH-uptake curves of the direct HA formation experiments (Fig. 5) with the OH-uptake curves of the ACP to HA transformations (Figs. 3 and 4) because they are completely different chemical systems.

The action of proteoglycans on direct apatite formation from solution is in marked contrast to their effect on the ACP to HA conversion. In direct precipitation from solution the proteoglycans cause an increase in the time to the onset of HA production but produce only small changes in the HA formation kinetics. However, as previously mentioned, proteoglycans have the opposite effect on the ACP to HA transformation.

Figure 6 shows the change in induction time, measured from mixing of reagents to onset of HA formation, with increasing A1 and D1 concentrations. The induction time of the control with no proteoglycan present (marked with an X) is a little less than 1 h; the induction time for HA formation rapidly goes up as the proteoglycan concentration is increased. The system used for the data of Fig. 6 is the same as used in Fig. 5 where HA is directly precipitated from low-concentration calcium phosphate solutions. As in the ACP to HA system, A1 is more effective on a weight basis than D1 in inhibiting HA formation. At the times of the two points marked "no HA formation," the experiments were terminated because of a concern for possible proteoglycan deterioration.

Figure 7 shows the effect of two dextrans of widely different molecular weights on the conversion of ACP to HA. The dextran of 80,700 MW, at a concentration of 80 mg/ml (relative viscosity = 5.48), had a considerably smaller effect on slowing the transformation than a solution of the 16×10^6 MW dextran, at a concentration of only 6 mg/ml (relative viscosity = 1.79). In fact, the high molecular weight dextran slowed the transformation to about the same extent as BN-A1 at a solution concentration of 5 mg/ml (relative viscosity = 7.97) (Fig. 4). Both dextrans had an effect on the ACP conversion, at the concentrations reported here, similar to that of proteoglycan aggregate and subunit, i.e., the transformation rate was decreased with no change in induction time.

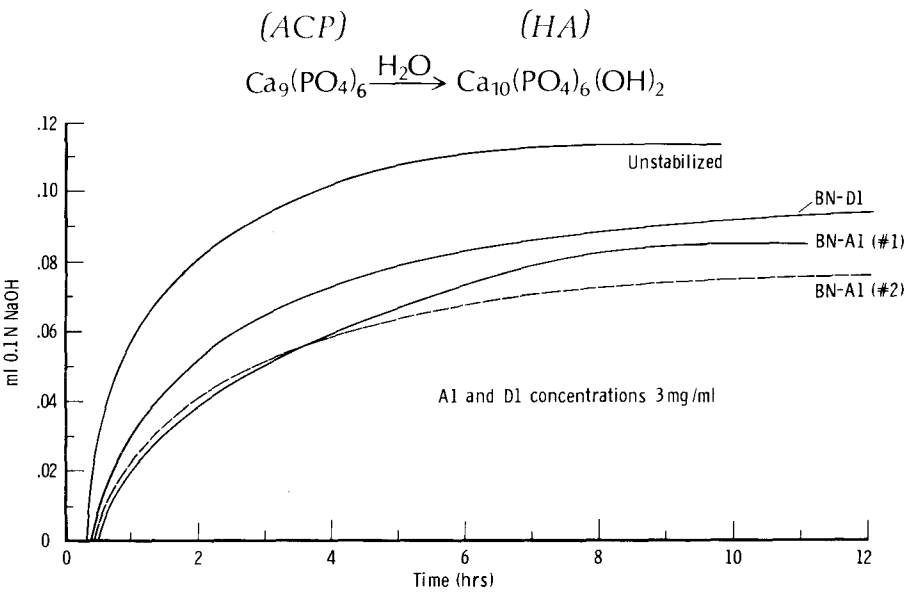

Fig. 3. The effect of certain proteoglycan A1 and D1 fractions on the decrease in the transformation rate of amorphous calcium phosphate (ACP) to crystalline hydroxyapatite (HA). The extent of conversion, as a function of time, was followed by continuously recording the uptake of 0.1 N NaOH in a pH-Stat at pH 7.4 and 30° C. The proteoglycan fractions were obtained from noncalcifying bovine nasal (BN) cartilage

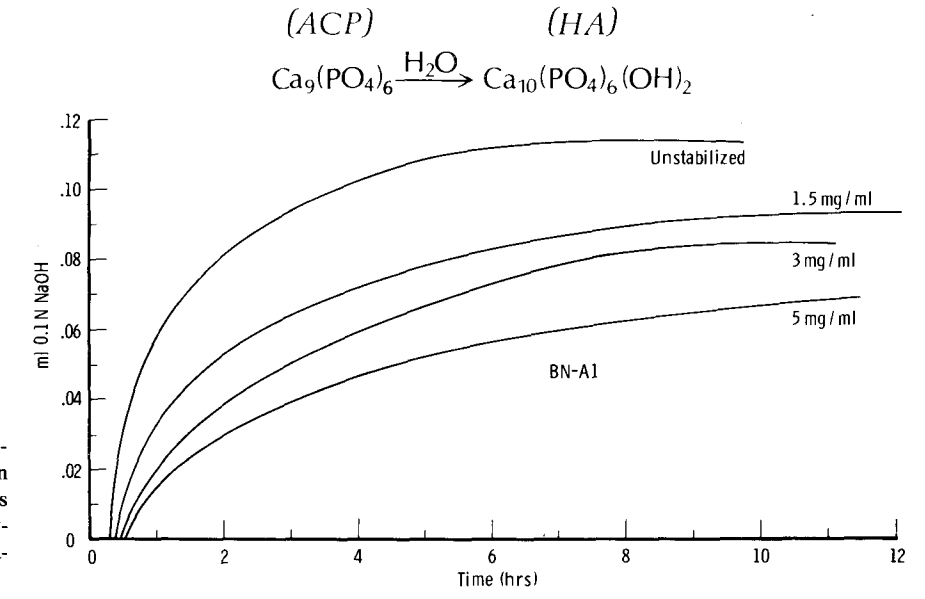

Fig. 4. Effect of the concentration of bovine nasal A1 proteoglycan fraction on the rate of transformation of amorphous calcium phosphate to crystalline hydroxyapatite, at pH 7.4 and 30° C, measured in a pH-Stat as in Fig. 3.

Table 1 shows the effect of the two dextrans on the direct precipitation of HA. The 16×10^6 MW dextran had a considerably greater effect on HA formation than the 80,700 MW dextran when compared on the basis of solution concentrations or relative viscosities. Both macromolecules acted to increase the induction time for HA precipitation with only small changes in the shapes of the HA formation curves, as found in the proteoglycan experiments.

Discussion

Both the proteoglycan A1 and D1 fractions were effective inhibitors of HA formation in the two in vitro test systems used in this study. The D1 fraction, which consists of free proteoglycan subunits unattached to a hyaluronic acid backbone, was surprisingly effective in its inhibitory action. The A1 fraction, which contains proteoglycan aggregate, was substantially more effective. However, the difference in the inhibitory effect of the subunit and aggregate-containing fractions was not as striking as one might have expected from the work of Cuervo, Howell, and Pita [6]. They examined the in vitro inhibitory effect on HA formation of proteoglycans present in micropuncture fluids aspirated from cartilage. Their HA formation studies were done in a synthetic lymph system which differed from both of our test systems in several important respects, in-

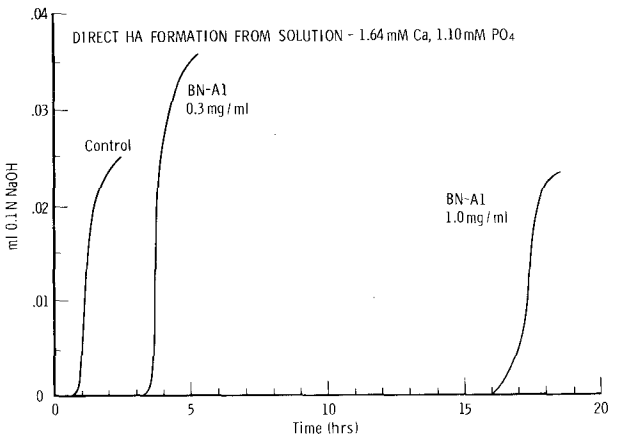

Fig. 5. The increase in the time to the onset of crystalline hydroxyapatite (HA) formation (i.e., induction time) resulting from the presence of bovine nasal proteoglycan A1 fraction in calcium phosphate solution. Formation curves were obtained by continuously recording, as a function of time, the uptake of 0.1N NaOH in a pH-Stat at pH 7.4 and 30° C

cluding solution calcium phosphate concentrations and the presence of $Na_2B_4O_7$, NaCl, and HCl. In addition, Cuervo, Howell, and Pita seeded their supersaturated calcium phosphate solutions, which was not done in either system used in the present study. Since nucleation experiments in solution are sensitive to a host of experimental variables, it is difficult to make direct quantitative comparisons between different test systems. It is sufficient to say that under our experimental conditions proteoglycan subunit has a potent inhibitory effect on HA formation.

The relatively small apparent difference in the inhibitory effect of the proteoglycan aggregate (A1) and proteoglycan subunit (D1) fractions is due to another factor. As shown in Fig. 1B, the A1 fraction

Table 1. Comparison of the effect of proteoglycans and dextrans on the direct precipitation of hydroxyapatite

Macromolecule	Concentration (mg/ml)	Relative viscosity	Induction time (h)
Control	0	1.00	0.8
BN - Al	1.0	1.75	15.8
"	0.60	1.37	6.8
"	0.30	1.16	3.8
"	0.17	1.07	1.7
BN - Dl	1.50	2.19	19.3
"	1.35	1.96	9.0
"	1.20	1.79	7.3
"	0.60	1.27	2.5
"	0.30	1.12	3.3
Dextran 80,700 MW	70.0	3.44	2.4
Dextran 16×10^6 MW	7.5	1.97	6.3

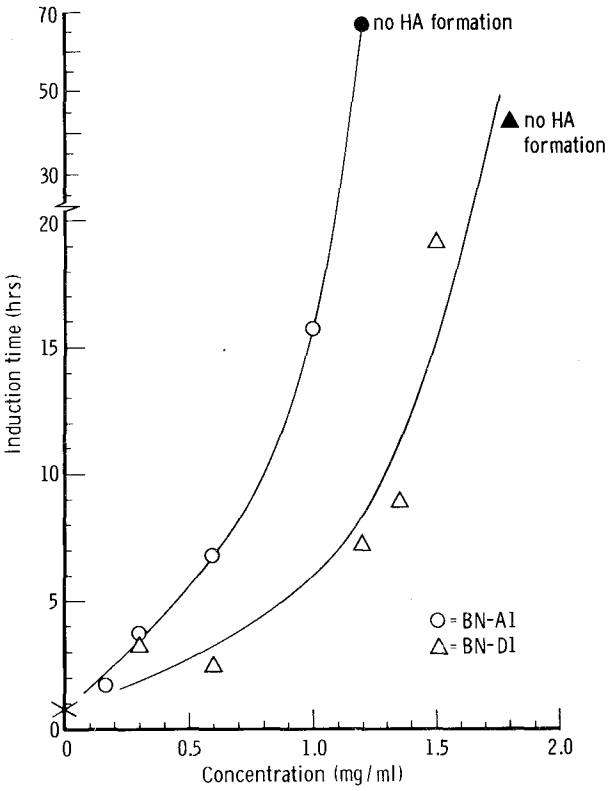

Fig. 6. Effect of changing the concentration of bovine nasal A1 and D1 proteoglycan fractions on the induction time for direct precipitation of crystalline hydroxyapatite (HA) from calcium phosphate solutions (1.64 mM Ca, 1.10 mM PO_4). The X on the ordinate represents the induction time for HA formation in the absence of proteoglycans (control)

consists of approximately 50% proteoglycan aggregate and 50% proteoglycan subunit. If the proportion of aggregate in a fraction were increased, for example, by agarose chromatography, the difference in HA inhibition between the proteoglycan aggregate and proteoglycan subunit preparations should be further increased. Nevertheless, pure proteoglycan subunit (D1) appears to be a potent inhibitor of hydroxyapatite formation.

The neutral dextran molecules had the same qualitative effect on inhibiting HA formation in the two test systems as did the proteoglycan fractions. The dextrans slowed the ACP to HA conversion rate without affecting the induction time, t_i, and, in the direct precipitation of HA, the dextrans increased t_i without affecting the HA formation kinetics. This suggests a similarity in the chemical mechanism of action of dextrans and proteoglycans in affecting the formation of HA. We studied the dextrans in order to sort out the effect of solution viscosity and molecular weight and/or size on HA formation.

From our results it is clear that there is no correlation between the relative viscosity of the solution

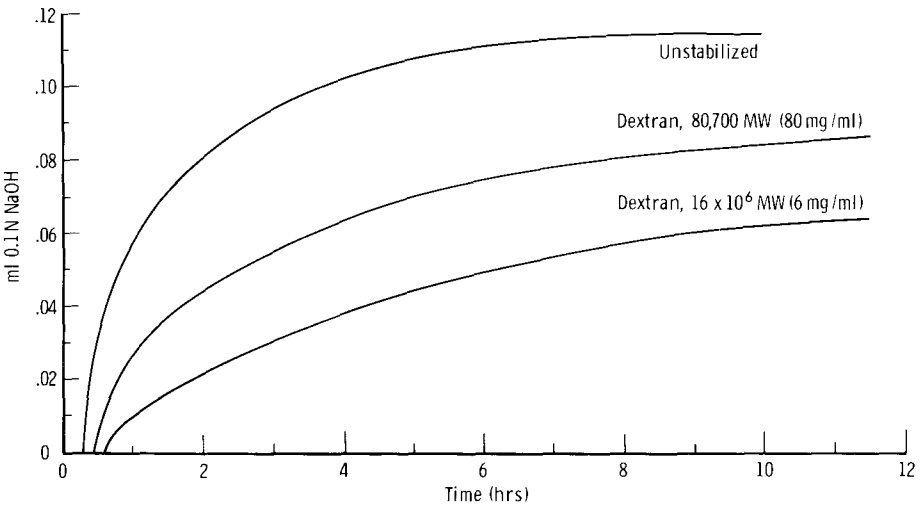

Fig. 7. The effect of two dextrans, of widely different molecular weights, on the transformation of amorphous calcium phosphate to crystalline hydroxyapatite, at pH 7.4 and 30° C as measured in a pH-Stat as in Figs. 3 and 4.

in either test system and the effect of a given macromolecule on HA formation. This is consistent with the well-established observation that ion diffusion rates in high-polymer solutions are essentially independent of viscosity. On the other hand, we have shown that there is a good correlation between molecular weight and the action of these macromolecules on HA production in both test systems. The dextran of 16×10^6 molecular weight was much closer in its action to A1 and D1 than to the action of a dextran of 80,700 molecular weight. The molecular weights of proteoglycan D1 fractions from bovine nasal cartilage range from 3×10^6 to 4×10^6; the molecular weights of proteoglycan A1 fractions are in the order of 200×10^6.

An understanding of the effect of such variables as proteoglycan molecular weight and solution viscosity on HA formation must take into account the ionic processes involved in HA production in both synthetic test systems. The first step in the conversion of ACP to HA is the dissolution of the ACP to provide calcium and phosphate ions in solution [11]. Then calcium, phosphate, and hydroxyl ions must aggregate, heterogeneously and/or homogeneously, to form embryonic HA nuclei which can grow into critical HA nuclei and then into HA crystals by accretion of more ions. If the growth of an embryonic HA nucleus is interferred with in some way it will redissolve, since it has not yet reached the minimum size necessary to maintain its integrity, i.e., the critical nucleus size. In the direct precipitation of HA the calcium and phosphate ions are initially in solution, which eliminates the ACP dissolution step.

It is likely that the physical size and extended conformation of the proteoglycan aggregate and subunit, and the 16×10^6 molecular weight dextran, all highly branched molecules, interfere sterically with the growth of HA crystals. In this regard, Rosenberg et al. [12] showed by dark-field electron microscopy that the hyaluronic acid backbone of the proteoglycan aggregate was as long as 4200 nm with about 140 attached proteoglycan subunits which were each several hundred nanometers in length. The reduced interference with HA formation by the smaller (80,700 MW) dextran molecules may be related to a reduction in this steric effect.

Recent work has shown that many macromolecules can order large domains of water in their vicinity [13]. This concept has been developed from nuclear magnetic resonance (NMR) studies of water proton relaxation times and/or line widths in aqueous solutions and suspensions of various macromolecules such as gelatin, agar, and collagen [13]. The possible creation of ordered water domains by proteoglycan and dextran molecules may result in a barrier to HA nucleation and growth. However, preliminary NMR studies in our hands have indicated that at the concentrations used in this study there was no evidence of ordered water in proteoglycan aggregate solutions. We were able to duplicate the work showing the broadening of the water proton line in a 2% agarose solution, which was interpreted as indicating the presence of ordered water [13]. Solutions of proteoglycan aggregate up to 30 mg/ml (the highest concentration used in our study was 5 mg/ml) failed to show any water proton NMR line broadening. Our NMR results are consistent with a previous study [14] which found no water proton line broadening in a 20 mg/ml solution of hyaluronic acid of 3×10^6 MW.

Aside from the above effects which could interfere with HA formation in either test system, there are several possible interactions of proteoglycans and dextrans which might specifically interfere with the ACP to HA transformation. If these macromole-

cules bind to the ACP surface and partially coat it, the ACP dissolution rate could be decreased. Since the dissolution of ACP provides the calcium and phosphate ions required for HA formation, any interference with ACP solubilization could be a rate-limiting step in HA production. Serum proteins slow the ACP to HA transformation by such an effect [15]. Further, it has been suggested that the surface of the yet-undissolved ACP may act as a substrate for HA nucleation. If proteoglycans and dextrans bind specifically to active HA nucleation sites on ACP, this could result in making these sites unavailable for apatite formation resulting in a decrease in the rate of conversion of ACP to HA.

The previous discussion is an enumeration of some of the possible interactions that may be occurring when proteoglycan or dextran molecules perturb HA formation in the two test systems used in this study. At the present time the exact combination of chemical processes that actually occur to effect HA production is not known either in vitro or in vivo. Since nuclei formation and crystal growth from solution are complex, poorly understood processes, considerably more work will be required to delineate the precise mode of action of proteoglycans on the ACP to HA transformation and on the system where HA is directly precipitated from solution.

The concentration of proteoglycans in cartilage was found to be in the 15 to 60 mg/ml range by Maroudas [16]. This is consistent with a proteoglycan concentration of about 25 mg/ml that we find in bovine fetal epiphyseal cartilage (unpublished results). These values are considerably higher than the concentrations used here. From our study we can predict that such high A1 or D1 concentrations will have a potent inhibitory effect on bone mineral deposition, whether bone apatite is deposited directly or by means of an amorphous precursor phase. Breakdown of aggregate into subunit is apparently not sufficient to allow calcification to occur. Further degradation and/or removal of subunit seems necessary for biological mineralization to proceed.

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