Effect of Calcipenia on Proteoglycan Metabolism and Aggregation in Normal Articular Cartilage *in vitro*

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Glycosaminoglycan synthesis in normal adult dog knee cartilage cultured in medium containing 0, 0.3 mM- and 0.9 mM- Ca^{2+} was 52, 67 and 78%, respectively, of that in cartilage from the same joints cultured in a normal concentration of Ca^{2+} , i.e. 1.8 mM. Pulse-chase experiments indicated that the rate of degradation of glycosaminoglycans in cartilage cultured in the absence of Ca^{2+} was similar to that of glycosaminoglycans in cartilage cultured in 1.8 mM- Ca^{2+} . Although [^{35}S]sulphate incorporation into glycosaminoglycans was decreased in the presence of calcipenia, [^3H]leucine incorporation into protein was unaffected. The average hydrodynamic size of newly synthesized proteoglycan aggregates and purified disaggregated proteoglycans from cartilage cultured in the absence of Ca^{2+} was similar to that of aggregates and disaggregated proteoglycans from cartilage cultured in 1.8 mM- Ca^{2+} .

Intra- and extra-cellular concentrations of Ca^{2+} have been implicated in the control of many biological processes (Rasmussen, 1977). In cartilage, Ca^{2+} has been shown to play an important regulatory role in the biosynthesis of the two major constituents of the extracellular ground substance, namely collagen and proteoglycans. Thus in suspension culture chondrocytes from articular cartilage of mature rabbits synthesize Type-II collagen when the medium does not contain Ca^{2+} , but Type-I collagen in the presence of Ca^{2+} (Deshmukh *et al.*, 1976). Chick vertebral cartilage has been shown to synthesize less proteoglycan than normal when the Ca^{2+} concentration in the culture medium is decreased below a normal value (Shulman & Opler, 1974).

Since its effects on the macromolecular organization of normal adult articular cartilage had not heretofore been systematically examined, the present study was designed to determine whether changes in concentration of Ca^{2+} alter proteoglycan metabolism or aggregation in that tissue.

Experimental

Tissues and culture conditions

Knee joints were removed from three adult mongrel dogs (weighing 15–20 kg, aged 4–6 years) immediately after the animals were killed with sodium pentothal. Cartilage slices less than 0.5 mm thick, down to but excluding subchondral bone, were shaved with a scalpel from the articular surfaces of the lateral and medial femoral condyles and posterior surfaces of the patellas. The shavings from

each animal were pooled separately and placed in Falcon tissue-culture dishes (60 mm $\times$ 16 mm) containing 6 ml of Ca^{2+} -free Dulbecco's Modified Eagle's Medium, pH 7.4, supplemented with 10% dialysed foetal calf serum, streptomycin (50 $\mu\text{g}/\text{ml}$), penicillin ($\geq 20 \mu\text{g}/\text{mol}$) and various concentrations of CaCl_2 ; all components of the tissue-culture medium were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. The Ca^{2+} concentration of the dialysed serum, determined with a Perkin-Elmer model 303 atomic absorption spectrophotometer, was less than 0.1 mM. The cartilage slices were incubated with gentle rocking at 37°C under air/ CO_2 (19:1) for 18 h, after which $\text{Na}_2^{35}\text{SO}_4$ (20 $\mu\text{Ci}/\text{ml}$; 825.9 mCi/mmol) (New England Nuclear Corp., Boston, MA, U.S.A.) was added and the incubation was continued for an additional 18 h. After incubation the medium was removed with a Pasteur pipette and the cartilage was washed with 0.9% NaCl. Medium and wash were combined and placed in Spectrapor membrane tubing no. 3 (approx. mol.wt. cut-off 3500; Spectrum Medical Industries, Los Angeles, CA, U.S.A.). The samples were dialysed at 4°C for 24 h against 200 vol. of 0.05 M-sodium acetate, pH 6.8, and then for 24 h against running tap water.

Determination of net glycosaminoglycan synthesis

The effect of various concentrations of Ca^{2+} on net glycosaminoglycan synthesis was determined on triplicate samples of cartilage (approx. 10 mg each) from each dog. After incubation of the tissue with $\text{Na}_2^{35}\text{SO}_4$ and recovery of the medium, as described

above, the washed cartilage was suspended in 3 ml of 0.1M-sodium borate buffer, pH 8.0, containing 0.02M-CaCl₂. Then 1mg of Pronase (Calbiochem, San Diego, CA, U.S.A.) was added and the sample was digested under toluene for 24h at 55°C. The digest was then dialysed overnight at 4°C against 200vol. of water, after which the sacs were washed with water and the material remaining in the sacs and washes were combined. Net glycosaminoglycan synthesis was determined from the sum of the non-diffusible ³⁵S radioactivity in 0.1ml samples of medium and the Pronase digest, which were mixed with 10ml of Ready-Solve HP 60 (Beckman Instruments, Irvine, CA, U.S.A.) and counted for radioactivity in a model LS 230 Beckman liquid-scintillation spectrometer. Results (c.p.m.) were adjusted for differences in wet weight of the cartilage.

Extraction of proteoglycans from the cartilage

Approx. 40mg of cartilage was stirred for 48h at 4°C in 10ml of 4M-guanidinium chloride in 0.05M-sodium acetate, pH 5.8, containing 0.01M-EDTA, 0.1M-6-aminoheptanoic acid and 0.05M-benzamidine hydrochloride (Oegema *et al.*, 1975). The extract was filtered and the residue was washed with fresh 4M-guanidinium chloride containing the above proteinase inhibitors. Filtrate and washings were combined and dialysed against 100vol. of 0.05M-sodium acetate, pH 5.8, also containing the proteinase inhibitors. The cartilage residue was digested completely with Pronase as described above, after which radioactivities of the medium, the 4M-guanidinium chloride extract and the Pronase digest were determined as described above, and adjusted for differences in the wet weight of the tissues.

Isolation and purification of aggregated and disaggregated proteoglycans

Proteoglycans in the medium and 4M-guanidinium chloride extract were isolated by equilibrium density-gradient centrifugation under associative conditions in CsCl in 0.4M-guanidinium chloride (initial density 1.69g/ml) by the method of Hascall & Sajdera (1969). The bottom two-fifths of the gradients ($\rho \geq 1.76$ g/ml) was recovered and dialysed against several changes of 0.05M-sodium acetate, pH 6.9, to yield fractions designated A_{Med} and A_{Gu} from the medium and the guanidinium chloride extract, respectively.

To obtain purified disaggregated proteoglycans, portions of fraction A_{Gu} were made 4M with respect to guanidinium chloride and subjected to a second CsCl-density-gradient centrifugation (initial density 1.50g/ml). The bottom two-fifths of this gradient ($\rho \geq 1.51$ g/ml), which contains disaggregated proteoglycans (Hascall & Sajdera, 1969), was dialysed exhaustively at 4°C against 0.05M-sodium acetate, pH 6.9, and designated fraction D_{Gu}.

Pulse-chase experiments

Triplicate samples of cartilage slices from dog 3 (approx. 10mg each) were incubated with Na₂³⁵SO₄, as described above, in medium to which various concentrations of CaCl₂ had been added. The tissue was then washed three times with 5ml portions of sterile Hanks' solution (Balanced Salt Solution; Grand Island Biological Co.) (free of Ca²⁺) and transferred to culture dishes containing 5ml of fresh medium without Na₂³⁵SO₄. After incubation for an additional 1, 2, 6 or 24h the medium was dialysed against 0.05M-sodium acetate, pH 6.8, and the tissue was digested with Pronase as described above. The very small amount of insoluble material that remained after Pronase digestion was removed by centrifugation at 2000g for 10min and, after the supernatant was dialysed as described above, the [³⁵S]glycosaminoglycan contents of the Pronase digest and the medium were determined.

Determination of net protein synthesis

Triplicate samples (approx. 10mg each) of the cartilage from dog 1 were incubated, as described above, in medium containing various concentrations of Ca²⁺, to which nominally labelled L-[4,5-³H]leucine (5μCi/ml; 57.1 Ci/mmol; New England Nuclear Corp.) had been added. At the end of the incubation period the medium was removed and the cartilage slices were washed with cold saline and suspended in 3ml of 0.1M-EDTA in 0.1M-sodium acetate, pH 5.1, containing 0.1% sodium dodecyl sulphate. The samples were then immersed in ice and homogenized at top speed in a Polytron homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.) for three 1-min intervals, during which the temperature of the suspension did not exceed 5°C. The homogenate was made 1% with respect to sodium dodecyl sulphate and rotated overnight at room temperature in a stoppered flask on a multi-purpose rotator (Scientific Industries, Springfield, MA, U.S.A.), after which the small amount of tissue that remained was removed by centrifugation at 2000g for 10min and digested with Pronase as described above. Analysis of the Pronase digest indicated that the homogenization had extracted 95% of the total [³H]leucine that had been taken up by the tissue. To precipitate the [³H]leucine-labelled protein, 5mg of bovine serum albumin, as unlabelled carrier, was dissolved in 0.5ml of the homogenate, after which an equal volume of 10% (w/v) trichloroacetic acid was added. The precipitate was left at 4°C for 1h, after which it was washed three times with 2ml portions of cold 10% trichloroacetic acid and finally solubilized at 37°C in 0.5ml of 0.01M-NaOH. The radioactivities of the solutions were determined as described above, and c.p.m. values were converted into d.p.m. by the external standardization method and adjusted for differences in the wet weights of the tissues.

Gel chromatography

Samples (0.5 ml) of proteoglycans in 0.5M-sodium acetate, pH 6.9, were applied to a column (95 cm $\times$ 1.0 cm) of Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and eluted with the same buffer at 2 ml/h. Radioactivities (^{35}S) of 1 ml effluent fractions were determined. For purposes of comparison, a sample of purified chondroitin sulphate (2 mg in 0.5 ml of the acetate buffer) was chromatographed under the same conditions. The partition coefficients (K_D) of the proteoglycan samples and of the chondroitin sulphate were calculated from the formula $K_D = (V_e - V_0)/(V_t - V_0)$. V_e was taken as the peak fraction in the elution diagram, V_0 as the void volume and V_t as the total column volume.

Enzymic dissociation of fraction A_{Gu} by hyaluronic acid β -1 $\rightarrow$ 3-hydrolase

Portions (1 ml) of fraction A_{Gu} were mixed with

1 ml of 0.2M-citric acid/0.4M- $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, pH 5.6. Then 1.5 μg of hyaluronic acid β -1 $\rightarrow$ 3-hydrolase (EC 3.2.1.36) prepared from leeches (Biotrics, Arlington, MA, U.S.A.) was added in five equal portions over a 5 h incubation period at 37°C (Perricone *et al.*, 1977). The digests were chromatographed on the Sepharose 2B column and elution profiles of digested and undigested samples were compared. The hyaluronidase had no activity against disaggregated proteoglycans, since the elution profile of fraction D_{Gu} was unaltered after incubation with the enzyme.

Results

On the basis of the mean non-diffusible radioactivity in the medium and Pronase digest of cartilage samples from three dogs, glycosaminoglycan synthesis in the presence of 0, 0.3 mM- and 0.9 mM- Ca^{2+} was 52, 67 and 78%, respectively, of that in cartilage cultured in the presence of a Ca^{2+} concentration similar to that found in normal serum (1.8 mM) (Fig. 1).

The rate of [^{35}S]sulphate incorporation into glycosaminoglycans in cartilage cultured in Ca^{2+} -free medium (Fig. 2), although lower than that in cartilage

Fig. 1. Net synthesis of [^{35}S]glycosaminoglycans by normal cartilage from three dogs, cultured in various concentrations of Ca^{2+}

Results are determined from the sum of non-diffusible ^{35}S radioactivity in 0.1 ml samples of medium and Pronase digests of the tissue (see the text for details). $\square$, Dog 1; $\blacksquare$, dog 2; $\blacksquare$, dog 3. Results are expressed as a percentage of glycosaminoglycan synthesis by cartilage from the same animal cultured in the presence of a normal concentration of Ca^{2+} (1.8 mM). The 100% value for each experiment represents the mean of triplicate samples of cartilage cultured in medium containing 1.8 mM- Ca^{2+} . Data are means $\pm$ S.E.M. for triplicate cultures of cartilage from each dog.

Fig. 2. Rate of [^{35}S]sulphate incorporation into glycosaminoglycans in cartilage from dog 1

Radioactivity is the non-diffusible ^{35}S in medium plus Pronase digest of the tissue (see the text for details). Results depict means $\pm$ S.E.M. of triplicate samples. $\circ$, Cartilage cultured in 1.8 mM- Ca^{2+} ; Δ , cartilage cultured in the absence of Ca^{2+} .

Fig. 3. Pulse-chase experiment showing percentage decrease in ³⁵S-labelled material in medium and Pronase digests of cartilage from dog 3

For details see the text. ○, Cartilage incubated in 1.8 mM-Ca²⁺; △, cartilage incubated in the absence of Ca²⁺. Results represent means ± S.E.M. of triplicate samples.

cultured in 1.8 mM-Ca²⁺, remained linear with time, suggesting that the lack of Ca²⁺ did not affect the viability of the chondrocytes over the 18 h period of the experiment. Furthermore, the pulse-chase experiments indicated that the rate of degradation of newly synthesized glycosaminoglycans in cartilage cultured in the absence of Ca²⁺ was no different from that of glycosaminoglycans in cartilage incubated in the presence of 1.8 mM-Ca²⁺ (Fig. 3). About 10% of the total non-diffusible ³⁵S radioactivity was lost during the initial 1–2 h of chase and no further decrement occurred during the remainder of the 24 h period of the experiment.

Although, as noted above, [³⁵S]sulphate incorporation into glycosaminoglycans was substantially diminished by a lack of Ca²⁺, [³H]leucine incorporation into protein was unaffected. Thus, in triplicate samples cultured in the absence of Ca²⁺ or in 0.3 mM-Ca²⁺, [³H]leucine incorporation into material that was precipitable by 10% trichloroacetic acid was about 110% and 90%, respectively, of that exhibited by cartilage cultured in 1.8 mM-Ca²⁺ (Fig. 4).

Table 1 shows the relative proportions of ³⁵S-labelled proteoglycans in the culture medium, 4 M-guanidinium chloride extract and Pronase digest of the cartilage residue in two experiments, with cartilage from dogs 2 and 3. The medium contained 7% and 22%, respectively, of the total newly synthesized proteoglycans, whereas in each case the 4 M-guanidinium chloride extract contained about 57%. The concentration of Ca²⁺ in the culture system had no apparent effect on the distribution of ³⁵S-labelled

Fig. 4. Net synthesis of [³⁵S]glycosaminoglycans and [³H]leucine-labelled protein by cartilage from dog 1 cultured in the presence of 0.3 mM-Ca²⁺ and in the absence of Ca²⁺

Results represent means ± S.E.M. of triplicate samples. The 100% value represents the results obtained with tissue cultured in medium containing a normal concentration of Ca²⁺ (1.8 mM) (see the text for details). □, ³⁵S radioactivity; ■, ³H radioactivity.

proteoglycans between the medium, guanidinium chloride extract and tissue residue.

Fraction A_{Med} accounted for only 20–30% of the non-diffusible ³⁵S radioactivity contained in the medium, regardless of the Ca²⁺ concentration in the culture system. Since the non-diffusible radioactivity in fraction A_{Med} in no instance represented more than 5% of the total newly synthesized proteoglycans, fraction A_{Med} was not analysed further.

Fraction A_{Gu}, on the other hand, invariably contained 80–90% of the non-diffusible ³⁵S radioactivity in the 4 M-guanidinium chloride extracts. Sepharose 2B-elution profiles of fraction A_{Gu} from dog 2 cultured in Ca²⁺ concentrations ranging from 0 to 1.8 mM are shown in Fig. 5. In all cases 15–18%

Table 1. Distribution of proteoglycans (non-diffusible ³⁵S radioactivity) between culture medium, 4M-guanidinium chloride extract and Pronase digest of normal dog articular cartilage incubated at 37°C in 6 ml of Dulbecco's Modified Eagle's Medium, pH 7.4, containing various concentrations of CaCl₂. After 18 h Na₂³⁵SO₄ (20 µCi/ml) was added and the incubation was continued for an additional 18 h. Radioactivity in the various fractions was determined as described in the text. Radioactivity values are ³⁵S c.p.m./10mg wet wt. of cartilage.

Source of cartilage	Ca ²⁺ concn. ...	Fraction	1.8 mM			0.9 mM			0.3 mM			0		
			Radio-activity	(% of total)		Radio-activity	(% of total)		Radio-activity	(% of total)		Radio-activity	(% of total)	
Dog 2	Medium	4M-Guanidinium chloride	6063	7		5420	7		5563	9		4034	8	
		Pronase digest	51238	57		43640	58		34176	52		27031	52	
			32042	36		26408	35		25667	39		21253	40	
		Total	89343	100		75468	100		65406	100		52318	100	
Dog 3	Medium	4M-Guanidinium chloride	19434	22		13860	21		6087	11		7482	16	
		Pronase digest	49074	57		35619	54		35934	64		27125	57	
			18339	21		16239	25		14316	25		12750	27	
		Total	86847	100		65718	100		56337	100		47358	100

Fig. 5. Gel chromatography of ³⁵S-labelled proteoglycans extracted with 4M-guanidinium hydrochloride from cultured cartilage of dog 2 and purified under associative conditions (fraction A_{Gu}: —), and of the same fraction after digestion with 1.5 µg of hyaluronic acid β-1→3-hydrolase (----) for conditions. Samples (0.5 ml) of the proteoglycan fractions in 0.5M-sodium acetate, pH 6.9, were applied to a column of Sepharose 2B (95cm×1.0cm) and eluted at 2 ml/h with the same buffer. Radioactivities (³⁵S) of 1 ml effluent fractions were determined. Cartilage was incubated (a) in 1.8 mM-Ca²⁺, (b) in 0.9 mM-Ca²⁺, (c) in 0.3 mM-Ca²⁺ or (d) in the absence of Ca²⁺.

of the sample was eluted at V₀, and those proteoglycans that were sufficiently small in hydrodynamic size as to be retarded by the gel were eluted in a single broad peak (K_D = 0.33) (Table 2). Purified chondroitin sulphate, in comparison, was eluted with K_D 0.90. Sepharose 2B-elution profiles of fraction A_{Gu} from cartilage of a second animal (dog 3) also appeared to be unaffected by the Ca²⁺ concentration of the culture medium, but fraction A_{Gu} from dog 3 differed from the corresponding fraction for dog 2 with respect to hydrodynamic size, since a larger

Table 2. *Effect of Ca^{2+} on the Sepharose 2B elution profiles of proteoglycans extracted with 4M-guanidinium chloride from normal dog articular cartilage*

[^{35}S]Sulphate-labelled proteoglycans were extracted with 4M-guanidinium hydrochloride and purified under associative (fraction A_{Gu}) and dissociative (fraction D_{Gu}) conditions as described in the text. Portions (1 ml) of fraction A_{Gu} were digested with 1.5 μg of hyaluronic acid β -1 $\rightarrow$ 3-hydrolase (see the text for conditions). Samples (0.5 ml) of the various proteoglycan fractions in 0.5M-sodium acetate, pH 6.9, were applied to a column of Sepharose 2B (95cm $\times$ 1.0cm) and eluted at 2ml/h with the same buffer. Radioactivities (^{35}S) of 1 ml effluent fractions were determined. The percentage of total ^{35}S radioactivity eluted in the void volume (V_0) and the partition coefficients (K_D) of the retarded ^{35}S radioactivity were calculated as described in the text.

Source of cartilage	Ca^{2+} (mM)	Fraction A_{Gu}		Fraction A_{Gu} + hyaluronic acid β -1 $\rightarrow$ 3-hydrolase		Fraction D_{Gu}	
		(% at V_0)	K_D	(% at V_0)	K_D	(% at V_0)	K_D
Dog 2	0	15	0.29	0	0.50	0	0.53
	0.3	16	0.31	0	0.45	—	—
	0.9	20	0.38	0	0.45	—	—
	1.8	18	0.37	0	0.45	0	0.50
Dog 3	0	50	0.66	0	0.65	0	0.65
	0.3	38	0.63	0	0.65	—	—
	0.9	—	—	—	—	—	—
	1.8	44	0.70	0	0.68	0	0.68

proportion of the former (38–50%) was eluted at V_0 , whereas the K_D of the retarded material was greater (about 0.65).

Digestion with hyaluronic acid β -1 $\rightarrow$ 3-hydrolase, which specifically degrades hyaluronic acid, markedly affected the average hydrodynamic size of all A_{Gu} samples (Fig. 5). Treatment with the leech hyaluronidase abolished the Sepharose 2B void-volume peaks of fraction A_{Gu} from dogs 2 and 3. The proteoglycans from dog 2 that were retarded by the gel were also decreased in hydrodynamic size, as indicated by a shift in their K_D from 0.33 to 0.45 (Table 2). In contrast, the hydrodynamic size of those proteoglycans from fraction A_{Gu} of dog 3 that were small enough to be retarded by Sepharose 2B was not changed by treatment with the hyaluronidase ($K_D \leq 0.65$).

In every case fraction D_{Gu} contained about 85–90% of the ^{35}S radioactivity present in the corresponding fraction A_{Gu} from which it was derived. The hydrodynamic size of the proteoglycans in fraction D_{Gu} from cartilage cultured in the presence of Ca^{2+} was similar to that of fraction D_{Gu} from cartilage cultured in the absence of Ca^{2+} (Table 2). Fraction D_{Gu} was wholly retarded by Sepharose 2B and eluted in a broad unimodal peak. Fraction D_{Gu} from dog 2 had a K_D of about 0.50, whereas that from dog 3 was somewhat smaller in average hydrodynamic size ($K_D = 0.65$). Both fraction- D_{Gu} samples were similar in hydrodynamic size, therefore, to the proteoglycans obtained after digestion with hyaluronic acid β -1 $\rightarrow$ 3-hydrolase of the fraction- A_{Gu} samples from which they were derived.

Discussion

The above results show that, when the concentration of Ca^{2+} is less than normal, proteoglycan synthesis in normal adult dog articular cartilage is inhibited. This is in agreement with a previous study which reported that decreasing concentrations of Ca^{2+} caused a decrease in proteoglycan synthesis in chick embryonic vertebral cartilage (Shulman & Opler, 1974). Although the streptomycin employed in the culture system is a tervalent cation and hence may have acted in competition with Ca^{2+} , the results of the present study indicate clearly that the degree of inhibition of proteoglycan synthesis was inversely proportional to the Ca^{2+} concentration.

The decrease in proteoglycan synthesis that occurred in the face of calcipenia in the culture system appeared to be specific and could not be attributed to a general inhibition of chondrocyte function or to cell death. Thus, although [^{35}S]sulphate incorporation into glycosaminoglycans was inhibited by a deficiency of Ca^{2+} , [^3H]leucine incorporation into protein was unaffected (Fig. 4). Furthermore, [^{35}S]sulphate incorporation into glycosaminoglycans in the absence of Ca^{2+} increased throughout the incubation period (Fig. 2) and the rate of catabolism of newly synthesized glycosaminoglycans was similar to that which occurred when the Ca^{2+} concentration of the culture medium was normal (1.8 mM) (Fig. 3). The pulse-chase data suggested that two populations of ^{35}S -labelled material were being synthesized. One, representing about 10% of the total ^{35}S -labelled non-diffusible material, exhibited a rapid turnover (within 1–2h). The other, which accounted for about

90%, was metabolically much more stable and showed no evidence of turnover during a 24 h chase period (Fig. 3).

The distribution of ^{35}S -labelled proteoglycans between the medium and the tissue was similar at all concentrations of Ca^{2+} studied (Table 1), suggesting that the organizational integrity of the cartilage had not been compromised by the absence of Ca^{2+} . If that had been the case, a greater than normal proportion of the total proteoglycans might have diffused into the medium.

Although the Ca^{2+} concentration had no effect on the distribution of ^{35}S -labelled proteoglycans between the medium, the 4M-guanidinium chloride extract and the cartilage residue, Table 1 indicates that marked differences existed between the tissue from dog 2 and those from dog 3 in this respect. Thus, regardless of the Ca^{2+} concentration, the medium in which dog 3's cartilage was cultured contained three times as much ^{35}S -labelled proteoglycans as the medium from the studies with dog 2's cartilage. Since the cartilage from each animal was cultured under identical conditions, this variation presumably indicates inherent differences in individual tissue metabolism or in collagen-proteoglycan interaction.

Fraction A_{Med} represented only about 20% of the non-diffusible ^{35}S radioactivity in the medium, whereas fraction A_{Gu} accounted for over 80% of the non-diffusible ^{35}S radioactivity in the 4M-guanidinium chloride extract. This implies that the proteoglycans in the medium did not have as high a glycosaminoglycan content, relative to protein, as those in fraction A_{Gu} . Possibly fraction A_{Med} contained degraded proteoglycans. Since it constituted only 5%, or less, of the total newly synthesized proteoglycans it was not analysed in detail. However, Sepharose 2B-elution profiles of analogous fraction- A_{Med} samples from normal adult dog cartilage, cultured in this laboratory under conditions identical with those used in the present study, have indicated that this fraction does indeed contain large-sized proteoglycans, and not free glycosaminoglycans (Palmoski & Brandt, 1978). Alternatively, proteoglycans in fraction A_{Med} may have interacted with proteins in the foetal calf serum, forming a complex with a relatively high protein/glycosaminoglycan ratio, which was relatively low in buoyant density and equilibrated in the upper portions of the associative CsCl gradient.

The Sepharose 2B-elution profiles of fraction A_{Gu} show that proteoglycan aggregation was not affected by the Ca^{2+} concentration in the culture medium (Fig. 5). Thus, even in the absence of Ca^{2+} , the hyaluronate-binding region of the proteoglycan core protein was functional and sufficient high-molecular-weight hyaluronic acid was present to permit extensive proteoglycan-hyaluronate interaction. This high-molecular-weight hyaluronic acid, which is syn-

thesized by the chondrocyte, comprises about 1% of the proteoglycan aggregate (Hascall & Heinegård, 1974). Its turnover rate, however, and the effects of Ca^{2+} on the metabolism of cartilage hyaluronic acid are unknown.

The difference between the A_{Gu} fractions from dogs 2 and 3, with respect to the proportion of each eluted in the void volume (approx. 20 and 45% respectively), presumably reflects individual variation in the extent of aggregation within the tissue, since culture conditions, and the procedures used to extract the proteoglycans and to prepare fraction A_{Gu} from tissues of the two animals, were identical.

The average hydrodynamic size of the dissociated proteoglycans, i.e. fraction D_{Gu} , was unaffected by calcipenia and they were nearly identical in size with proteoglycans obtained after digestion of fraction A_{Gu} with hyaluronic acid β -1 $\rightarrow$ 3-hydrolase. The elimination, after incubation with the enzyme, of that portion of fraction A_{Gu} eluted in the Sepharose 2B void volume was not due to digestion of chondroitin sulphate chains, since the enzyme had no effect on the hydrodynamic size of fraction D_{Gu} . Furthermore, the proteoglycans obtained after digestion of fraction A_{Gu} were much larger than purified chondroitin sulphate, indicating that the hyaluronidase was not contaminated with proteolytic enzymes which extensively degraded the proteoglycan core protein.

Notably, the average hydrodynamic size of the proteoglycans in fraction A_{Gu} from dog 2 which were retarded by Sepharose 2B, as well as those in V_0 , was diminished after incubation with hyaluronic acid β -1 $\rightarrow$ 3-hydrolase (Table 2), when their K_D was essentially the same as that of the proteoglycans exposed to the dissociating conditions of the second CsCl gradient. This suggests strongly that these proteoglycans were aggregated via association with hyaluronic acid, even though such aggregates are characteristically eluted in the void volume on Sepharose 2B chromatography. The size of aggregates from normal nasal and tracheal cartilage has been shown to be proportional to the length of the hyaluronic acid polymer in the complex (Hascall & Heinegård, 1974). Since the purified proteoglycan subunits (fraction D_{Gu}) were essentially unimodal with respect to hydrodynamic-size distribution (Table 2), the existence of two distinct populations of aggregates excluded and retarded by Sepharose 2B suggests that two populations of hyaluronic acid, which differed in hydrodynamic size, were extracted by 4M-guanidinium chloride.

Whatever the molecular basis for the effect, our results indicate that Ca^{2+} plays an important regulatory role in proteoglycan metabolism in normal articular cartilage. The decrease in proteoglycan synthesis seen with calcipenia could eventually lead to a net decrease in proteoglycan content of the cartilage, which would affect its biomechanical prop-

erties, such as resistance to compression in weight-bearing.

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