Effects of Chondroitin Sulphates on Mineralization *in vitro*

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1. Soluble sodium chondroitin sulphate, from bovine ribs or puppy epiphyseal plate, at a concentration 80 mM in terms of glucuronate, decreased the amounts of calcium and phosphate precipitated from a solution 6.9 mM in phosphate and 6.9 or 13.8 mM in calcium, buffered in the pH range 6.6–8.2 with 20–25 mM-collidine and 5–20 mM-hydrochloric acid when incubated for 2 hr. at 30°, and for 0.25–24 hr. when buffered at pH 7.0. 2. An insoluble fraction of puppy epiphyseal plate, containing chondroitin sulphate and collagen, was found to have the same effect at lower concentrations of chondroitin sulphate and a higher calcium/chondroitin sulphate glucuronate ratio, but the formation of calcium phosphate from calcium bound to this material appeared to proceed more rapidly than from calcium in solution, when both were present in the same system. 3. The implications of these results are discussed in relation to the calcium/chondroitin sulphate glucuronate ratios found in different parts of non-calcifying and calcifying cartilage *in vivo* and to the calcium and phosphate gradients between blood and calcified tissue.

The ion-exchange uptake of calcium and other cations by cartilage slices was shown by Boyd & Neuman (1951) to be closely correlated with their sulphate content. Dunstone (1962) showed that the CS† of cartilage bound calcium and other cations in the form of counterions to the polyelectrolytes, and Mathews (1964) has studied the factors involved in this binding. Radioisotope studies with calcium *in vivo* (e.g. Lacroix, 1960) have shown that the uptake of calcium in growing puppies is fastest in the epiphyseal plate and that calcium from this area is subsequently laid down as solid mineral matter in the growing bone. It has often been suggested that CS is a necessary factor in calcification, but the exact role proposed has varied greatly. For instance, Sobel (1955) proposed that it was responsible, together with collagen, for initiating the process of crystal formation, whereas Glimcher (1959) proposed inhibition of calcification as the main function of CS. The CS of rib cartilage (Gross, Mathews & Dorfman, 1960; Bowness, 1961; Davidson & Small, 1963) and of epiphyseal plate (Bowness, 1963) consists of at least two metabolically distinct fractions. The fraction that is most difficult to extract, with the lowest turnover rate, has the greatest affinity for calcium, being able to retain the

major part of the non-precipitated calcium in cartilage even in the presence of a much greater quantity of the soluble CS fraction (Bowness, 1962). Glimcher & Krane (1964) have shown that collagen alone will bind inorganic phosphate. The question then arises whether bound calcium and phosphate can react together and thus play a part in calcification *in vivo* or whether they are so tightly bound that they cannot react, thus producing inhibition of calcification.

The present work investigates the role of water-soluble and -insoluble CS fractions in calcification through a study of a model system in which other components of calcifying tissues, apart from calcium, inorganic phosphate, sodium and a buffer were omitted and in which CS fractions were used at concentrations as close as possible to those present in the epiphyseal plate of puppies (Bowness, 1962; Cipera, 1962).

MATERIALS AND METHODS

Epiphyseal CS. This was isolated from the epiphyseal cartilage of stray puppies of ages approx. 8–16 weeks. The long bones were cleaned of adhering tissues and their ends broken off with bone-nibbling forceps. The epiphyseal cartilage was removed with a scalpel and homogenized with 0.05 M-EDTA (disodium salt) solution (10 ml./g. of cartilage) in a corrugated tube for 20 min. at 4°. The homogenate was centrifuged at 22000 *g* and 4° for 30 min. The process was repeated twice. The EDTA supernatants were pooled.

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† Abbreviation: CS, chondroitin sulphate.

Protein was removed by shaking with pentan-1-ol-chloroform mixture (3:1, v/v). The aqueous solution was then dialysed at 4° in a Visking tube for 50 hr. with four or five changes of distilled water. The non-diffusible residue was concentrated in a rotary evaporator at 38–40°. The epiphyseal CS was precipitated from the concentrated solution with sodium acetate (1g./100ml.) and 3 vol. of redistilled 95% (v/v) ethanol. After standing overnight at 4° the CS was centrifuged down at 2000 *g* for 5 min. and washed four times with the redistilled 95% ethanol. It was finally dried in a vacuum desiccator over anhydrous CaCl₂. Analytical data ($\mu\text{g./100}\mu\text{g.}$ of preparation) were: glucuronolactone, 30.5; sulphate, 8.9; protein, 2.2; rotation ($[\alpha]_D^{20}$), –22.6°.

Commercial bovine tracheal CS. This was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and was reprecipitated with sodium acetate (1g./100ml.) and 95% ethanol before use. Analytical data ($\mu\text{g./100}\mu\text{g.}$ of preparation) were: glucuronolactone, 30.7–31.5; sulphate, 13.9–14.7; protein, 4.2–4.3; rotation ($[\alpha]_D^{20}$), –27.0° to –29.1°.

Epiphyseal collagen-CS complex. The insoluble residue after extracting homogenized epiphyseal cartilage with EDTA, as described above, was washed three times with cold distilled water by homogenization and centrifugation as described for the extraction of epiphyseal CS, but with water replacing EDTA solution. The washed insoluble residue was stirred up with water and the large particles (containing less than 4% of the total CS of the epiphyseal plate) were removed by passage through a 100-mesh sieve. The viscous sieved suspension was dialysed against distilled water in a Visking tube for 70–72 hr. with five changes of water. Chloroform (0.5–1 ml.) was added to the suspension to prevent bacterial infection. The dialysed material was adjusted to the required pH with NaOH and then freeze-dried. It contained 6–10% of water, 14–20 mg. of CS and 33–40 mg. of collagen (from hydroxyproline estimates)/100 mg. of freeze-dried material.

Preparation of CS solutions for precipitation experiments. A concentrated solution of CS (approx. 1g. in 30ml.) was made in aqueous NaCl so that the quantity of NaCl was more than 20 times the glucuronic acid content. It was then dialysed against distilled water at 4° for 48–50 hr. or until the non-diffusible residue attained pH 5.3–5.5. This step was undertaken to convert the counterions of CS into Na⁺. At the end of the dialysis the solution was adjusted to pH 7.0 with NaOH and concentrated in a rotary evaporator at 38–40° to a concentration greater than 150 μmoles of glucuronic acid/ml. The concentrated solution was centrifuged at 22000 *g* and 4° for 1 hr. to remove any insoluble particles. The clear solution was then adjusted to the appropriate pH with 0.05N-NaOH or 0.05N-HCl. CS solutions of pH 6.6, 7.0, 7.4, 7.8 and 8.2 were prepared. The glucuronic acid and sodium contents of the solutions were determined.

0.2M-Calcium chloride solution labelled with ⁴⁵Ca. A CaCl₂ solution of concentration slightly greater than 0.2M was prepared. An amount of ⁴⁵CaCl₂ solution of high specific activity (from The Radiochemical Centre, Amersham, Bucks.) was added such that the resulting solution would have a specific activity of 7000–8000 counts/min./ $\mu\text{g.}$ atom of Ca. The pH of the ⁴⁵CaCl₂ solution was adjusted to that required and the concentration of calcium then determined. The solution was diluted to exactly 0.2M and the specific activity determined. On

subsequent days the specific activity was obtained from a decay curve.

Precipitation conditions. (a) Controls. A known volume of labelled 0.2M-CaCl₂, 0.1 ml. of buffer (0.2M-collidine and N-HCl to give the required pH) and sufficient distilled water to make the volume 0.70 ml. were mixed in a glass centrifuge tube of capacity 2.0 ml. To this was added 5 μmoles of phosphate (0.025 ml. of 0.2M-KH₂PO₄-Na₂HPO₄ buffer). The solution was again mixed, and the tube stoppered and stood in a water bath at 30±0.1°.

(b) Tests. In experiments with the polysaccharides in solution, an amount of dissolved CS equivalent to 58.0 μmoles of glucuronic acid was mixed with the ⁴⁵CaCl₂ solution and collidine buffer before the addition of phosphate. An appropriate volume of M-NaCl was added to the mixture to make the molar ratio Na⁺/glucuronic acid to 2.0. With the dry collagen-CS complex, 10.0 mg. of the material was first soaked and then mixed with 0.3 ml. of water or the required amount of CS before it was mixed with the other solutions. In all cases the volume of the solution was made up to 0.70 ml. and mixed before the addition of phosphate. At all stages thorough mixing was done with a fine drawn glass rod.

Separation and treatment of precipitate. After standing the solution was centrifuged at 13000 *g* and 4° for 40 min. The supernatant (containing soluble CS, calcium and phosphate) was fraction S₁. The precipitate was washed successively with 1.0 ml. each of distilled water and 3% (w/v) KCl solution, the precipitate being mixed thoroughly with the solution each time. The supernatants, centrifuged exactly as fraction S₁, were fractions S₂ and S₃ respectively. The precipitate was finally mixed with 1 ml. of 0.05N-HCl to dissolve the solid calcium phosphate. The supernatant after centrifuging as before was fraction S₄. When collagen-CS had been added the precipitate contained about 90% of the original collagen, as estimated by hydroxyproline analyses. The working of the method is illustrated in Table 1, where analyses were obtained for fractions S₁, S₂, S₃ and S₄. In most experiments quantitative analyses for calcium and phosphate were only performed on fractions S₃ and S₄.

Radiochemical analyses. Samples were dried on aluminium planchets under an overhead lamp. Samples (0.05 ml.) of fractions S₁, S₂ and S₃ were dried alone, and 0.05 ml. of fraction S₄ was mixed with an equal volume of

Table 1. *Calcium and phosphate in the supernatants obtained in single experiments by the procedure described under 'Precipitation conditions'*

All values are in μmoles /supernatant. Where tracheal CS was added incubation was for 8 hr. at pH 7.8. Where collagen-CS was added incubation was for 2 hr. at pH 7.0. Calcium was assayed by radiochemical analysis as described in the text.

Experiment	Assay	S ₁	S ₂	S ₃	S ₄
Tracheal-CS added	Calcium	3.34	0.80	0.37	6.39
	Phosphate	0.52	0.51	0.32	4.05
Collagen-CS added	Calcium	2.73	0.88	1.50	5.51
	Phosphate	0.52	0.52	0.53	2.98
Collagen-CS added with no phosphate	Calcium	6.55	0.94	2.32	0.51

0.05N-NH₃ before drying. The radioactivities were measured with a Nuclear-Chicago Gas-Flow D-47 Counter and 181A Scaler. Duplicate counts were taken. The quantity of calcium was calculated from the specific activity of the labelled 0.2M-CaCl₂, as obtained from the decay curve. In preparing the labelled 0.2M-CaCl₂ the activity was measured with 0.05ml. of a 50-fold diluted solution.

Comparison of rates of precipitation of calcium in solution and bound to collagen-CS. A 20.0mg. sample of freeze-dried collagen-CS was stirred in a centrifuge tube with 20 μ moles of ⁴⁵CaCl₂ in 2ml. of water. After 5min. the mixture was centrifuged and the supernatant poured off. The precipitate was then washed twice with 2ml. of water by stirring, centrifuging and discarding the supernatant each time. The amounts of ⁴⁵Ca that remained attached to the collagen-CS after this procedure varied considerably, but were in the range 1–2.5 μ g.atoms. The final wet precipitate (volume about 0.3 ml.), containing ⁴⁵Ca, was mixed with collidine buffer, CS solution (containing 58 μ moles of glucuronate) and 8 μ moles of unlabelled CaCl₂. After diluting to 0.70 ml., adding phosphate and mixing, the tube was incubated at 30° for 1 hr. and the precipitate separated and treated as described above except that the washing with 1 ml. of water was omitted, so that only three extracts, fractions S₁, S_{KCl} and S_{HCl}, were obtained. Fraction S_{HCl} was analysed as fraction S₄ above.

Other analytical procedures. Glucuronic acid was estimated by the carbazole reaction (Bowness, 1957) with glucuronolactone as a standard. Chemical estimation of calcium was by titration with EDTA with calcein as indicator (Wallach & Steck, 1963). For each titration 20 μ l. of a 25 mg./100ml. solution of calcein in 0.25N-KOH was used, together with a solution of 0.45g. of EDTA/l. Inorganic phosphate was estimated by the Berenblum & Chain (1938) method as modified by Martin & Doty (Lindberg & Ernster, 1956). Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951) and hydroxyproline by the method of Leach (1960) after the collagenous materials had been hydrolysed in a sealed tube with 6N-HCl for 16 hr. at 105°. Sulphate was determined by the method of Dodgson & Price (1962) after hydrolysis of the carbohydrate esters.

RESULTS

The results in Table 1 show the working of the main procedure. The bulk of the soluble calcium was found in fraction S₁, and the bulk of the precipitated calcium phosphate in the hydrochloric acid extract, fraction S₄. When collagen-CS was present the calcium content of fraction S₃ was much higher, both in the presence and absence of phosphate. This was presumed to be due to binding of calcium to the insoluble collagen-CS material. In the absence of phosphate the number of μ g.atoms of calcium removed from dilute solutions by collagen-CS and released again by treatment with 3% potassium chloride (0.4M) is approximately equivalent to the CS glucuronate extractable from the collagen-CS by alkali (Bowness, 1962).

Because of the impossibility of removing all fluid from the centrifuge tubes all supernatants except fraction S₁ had added to them a small amount of the

previous supernatant; the effect of this on the volume of each supernatant was ignored in calculating the results, which, in the absence of collagen-CS, show values calculated on assumed volumes of 0.725 ml. for fraction S₁ and 1.0 ml. for fractions S₂, S₃ and S₄. In the presence of collagen-CS the volume of fraction S₁ was measured and the calcium and P_i contents were calculated accordingly. The recoveries obtained with calcium estimated either by radioactivity or by EDTA titration and with phosphate were within 10% of the added amount, as determined by estimation at the same time.

Table 2 shows that in the presence of tracheal CS in solution there was a significant decrease in the precipitation of calcium at all the pH values tested ($P < 0.01$ in the 't' test with the two samples at each pH). In all but one experiment there was a decrease of similar significance in the phosphate precipitated. There was some variation in the ratios of calcium to P_i, but this was not as great as has been observed with bone salts (Neuman & Neuman, 1958). With epiphyseal CS at the same concentration as tracheal CS in Table 2 and under the same conditions the calcium precipitated at pH 7.0 was $3.16 \pm 0.07 \mu$ g.atoms

Fig. 1. Variation in amount of calcium precipitated (estimated in fraction S₄) with amount added in solution. The total volume for each precipitation experiment was 0.725 ml. Each mixture was incubated for 2 hr. at pH 7.0 and 30°. O, Blank; 17.5 μ moles of collidine, 12.3 μ moles of HCl, 5 μ moles of phosphate, calcium as shown. ●, Blank with 58 μ moles of tracheal CS glucuronate. The symbols represent the mean and the bars above and below the standard deviation (6–12 replicates for each point).

(5 μ g.atoms added) and $5.37 \pm 0.08 \mu$ g.atoms (10 μ g.-atoms added); the corresponding values for phosphate were $2.02 \pm 0.05 \mu$ moles and $3.34 \pm 0.07 \mu$ moles. At 30° and a concentration of CS glucuronate of 7.5 μ moles/ml. the viscosity of epiphyseal CS was 1.03 centipoise, as compared with 1.00 centipoise for tracheal CS and 0.80 for water.

Table 3 shows that there was also a decreased precipitation of calcium or phosphate in the presence of collagen-CS. The differences were of the same order of significance as in Table 2.

Figs. 2 and 3 show that the shape of the time curves for the precipitation of calcium phosphate in the presence and absence of tracheal CS in solution were very

Table 2. *Effects of pH on the amounts of precipitated calcium and phosphate in fraction S₄*

In each case 5 μ moles of phosphate were added and the materials were incubated for 2hr. Numbers in parentheses show the numbers of replicates. Calcium assay, by radiochemical analysis, and other procedures were as described in the Materials and Methods section.

		Mean $\pm$ s.d.					
		Ca in S ₄ (μ g.atoms)		Phosphate in S ₄ (μ moles)		Ca/P ratio (means)	
pH	Ca added (μ g.atoms)	Tracheal CS		Tracheal CS		Tracheal CS	
		Control	present	Control	present	Control	present
6.6	10.0	5.31 $\pm$ 0.14	4.38 $\pm$ 0.14	3.43 $\pm$ 0.07	2.83 $\pm$ 0.25	1.55	1.52
		(5)	(5)	(5)	(5)		
7.0	5.0	3.95 $\pm$ 0.17	2.83 $\pm$ 0.25	2.56 $\pm$ 0.13	1.96 $\pm$ 0.12	1.54	1.44
		(12)	(8)	(12)	(8)		
	10.0	5.66 $\pm$ 0.12	4.64 $\pm$ 0.15	3.56 $\pm$ 0.19	3.11 $\pm$ 0.14	1.59	1.49
		(10)	(10)	(10)	(10)		
7.4	5.0	4.11 $\pm$ 0.15	3.23 $\pm$ 0.11	2.72 $\pm$ 0.08	2.01 $\pm$ 0.09	1.51	1.61
		(10)	(10)	(10)	(10)		
	10.0	6.49 $\pm$ 0.42	5.43 $\pm$ 0.22	4.16 $\pm$ 0.07	3.27 $\pm$ 0.11	1.56	1.66
		(10)	(10)	(10)	(10)		
7.8	5.0	4.64 $\pm$ 0.09	4.03 $\pm$ 0.02	2.81 $\pm$ 0.07	2.63 $\pm$ 0.08	1.65	1.53
		(10)	(10)	(10)	(10)		
	10.0	6.89 $\pm$ 0.18	6.20 $\pm$ 0.08	4.08 $\pm$ 0.07	3.95 $\pm$ 0.12	1.69	1.57
		(10)	(10)	(10)	(10)		
8.2	5.0	4.70 $\pm$ 0.10	4.10 $\pm$ 0.20	3.07 $\pm$ 0.09	2.76 $\pm$ 0.07	1.53	1.49
		(6)	(6)	(6)	(6)		
	10.0	6.77 $\pm$ 0.27	6.05 $\pm$ 0.24	4.45 $\pm$ 0.28	3.82 $\pm$ 0.13	1.52	1.58
		(6)	(6)	(6)	(6)		

Table 3. *Effect of collagen-CS and tracheal CS on amounts of calcium and phosphate found in fractions S₃ and S₄*

In each case 5 μ moles of phosphate were added and the materials were incubated for 2hr. at pH 7.0. All values are means for the numbers of replicates shown in parentheses. Standard deviations were similar to those in Table 2. Calcium assay, by radiochemical analysis, and other procedures were as described in the Materials and Methods section. Values for calcium are in μ g.atoms and for P_i in μ moles.

Ca added (μ g.atoms)	Assay	Supernatant	Blank	Blank + 10mg. of collagen-CS	Blank + 10mg. of collagen-CS + tracheal CS (58 μ moles of glucuronate)
5.0	Ca	S ₃	0.42 (4)	1.03 (4)	0.50 (4)
		S ₄	3.95 (12)	2.74 (5)	1.75 (5)
	P _i	S ₃	0.40 (4)	0.47 (4)	0.30 (4)
		S ₄	2.56 (12)	1.59 (5)	1.01 (5)
10.0	Ca	S ₃	0.47 (4)	1.33 (8)	0.81 (8)
		S ₄	5.66 (10)	5.40 (8)	4.27 (8)
	P _i	S ₃	0.40 (4)	0.41 (8)	0.38 (8)
		S ₄	3.56 (10)	2.98 (8)	2.38 (8)

similar. In the presence of collagen-CS the time-course of precipitation was quite distinct (Fig. 3).

In Table 4 the mean specific activity of the S_{HCl} extracts was significantly higher than that of the S_{KCl} extracts. In this system ^{45}Ca originally bound to collagen-CS might be lost in one of two ways: (i) exchange with ^{40}Ca in solution (estimated in fraction S_1); (ii) conversion into solid calcium

Fig. 2. Effect of time on precipitation of calcium (estimated in fraction S_4). Conditions were as given in Fig. 1 except that each solution contained $5 \mu g.$ atoms of calcium and the time of incubation was as shown. $\circ$, Blank. $\bullet$, Blank with $58 \mu moles$ of tracheal CS glucuronate.

Fig. 3. Effect of time on precipitation of calcium in the presence of various materials. Conditions were as given in Fig. 1 except that each solution contained $10 \mu g.$ atoms of calcium and the time of incubation was as shown. $\circ$, Blank. $\square$, Blank + $10 mg.$ of collagen-CS. $\bullet$, Blank with $58 \mu moles$ of tracheal CS glucuronate. $\blacksquare$, Blank with $58 \mu moles$ of tracheal CS glucuronate + $10 mg.$ of collagen-CS.

phosphate (estimated in fraction S_{HCl}); the loss here may then be replaced by ^{40}Ca from solution; any ^{45}Ca not lost in these two ways would be estimated in fraction S_{KCl} , which contains ^{45}Ca and ^{40}Ca bound to collagen-CS at the end of the experiments. To estimate the relative rates of the exchange reaction and calcium phosphate formation from bound and soluble calcium it is necessary to carry out a kinetic analysis of readings taken at a series of times. This has not been done in the present work. However, the finding that there is a small difference in the specific activities of fraction S_1 and S_{KCl} indicates that the exchange is not at equilibrium at 1 hr. but is probably not far from it. The finding that the specific activity in fraction S_{HCl} is higher than that in fraction S_{KCl} indicates that considerable conversion into phosphate of ^{45}Ca bound to collagen-CS occurred before exchange. If the exchange reaction (1 in Scheme 1) was very rapid there should be little or no difference in specific activities in the three supernatants; if the exchange reaction was not rapid there would be differences in the specific activities of the three extracts which reflect differences in the relative rates of the reactions shown in Scheme 1. A small carry-over of material from one extract to the subsequent one is to be expected, but it is unlikely that the carry-over would differ in specific activity from the extract preceding it. Also, the amount of ^{45}Ca carry-over would not be correlated with the amount of phosphate precipitated. The variability in amount of phosphate precipitation is

Table 4. Specific activities in supernatant fractions formed after incubating phosphate with labelled calcium bound to collagen-CS and unlabelled calcium in solution

Each experiment (N , total number of each type) was started with $1-2.5 \mu g.$ atoms of labelled Ca (assigned 100% specific activity) bound to $20 mg.$ of insoluble collagen-CS, which was mixed with collidine buffer, pH 7.0. Then $8 \mu moles$ of unlabelled $CaCl_2$ were added with or without CS in solution. Finally $5 \mu moles$ of phosphate were added. The final volume was $0.725 ml.$ The incubation and separation procedures are described in the Materials and Methods section. Calcium was assayed in each supernatant by radiochemical analysis and by EDTA titration.

	Specific activities ($\frac{\text{Labelled Ca in supernatant}}{\text{Total Ca in same supernatant}} \times 100$)	
	No CS in solution	CS in solution
S_1	18.5 ± 2.0	11.1 ± 4.2
S_{KCl}	19.9 ± 2.3	13.3 ± 6.5
S_{HCl}	24.0 ± 3.9	18.1 ± 8.5
t test for $S_{HCl}-S_{KCl}$	$P \approx 0.01$ ($N=5$)	$P < 0.01$ ($N=13$)

Scheme 1.

Fig. 4. Percentage precipitation of labelled calcium (from collagen-CS) and unlabelled calcium (from solution) in relation to amount of phosphate precipitated. The total volume for each precipitation experiment was 0.725 ml.; the mixtures were incubated for 1 hr. at pH 7.0 and 30°. The procedure described in Table 4 was used, except that in some cases (specified below) no phosphate was added. The individual results for calcium (except where no phosphate was added) are grouped and presented in a different form in Table 4 (fraction S_{HCl}). ${}^{45}\text{Ca}$ in fraction S_{HCl} , as percentage of total label added with collagen-CS: ▲, 5 μmoles of phosphate, no CS in solution; ●, 5 μmoles of phosphate, 58 μmoles of tracheal CS glucuronate in solution; ■, no phosphate, 58 μmoles of tracheal CS glucuronate in solution. ${}^{40}\text{Ca}$ in fraction S_{HCl} , as percentage of total ${}^{40}\text{Ca}$ in solution: Δ, 5 μmoles of phosphate, no CS in solution; ○, 5 μmoles of phosphate, 58 μmoles of tracheal CS glucuronate in solution; □, no phosphate, 58 μmoles of tracheal CS glucuronate in solution.

shown in Fig. 4. The percentages of both the labelled and unlabelled calcium added that were found in fraction S_{HCl} are evidently related to the degree of phosphate precipitation. When no phosphate was added 5–11% of the total unlabelled calcium and 8–14% of the labelled calcium were present in

fraction S_{HCl} . These amounts are the carry-over from the S_{KCl} extraction. When phosphate was added the percentages of both labelled and unlabelled calcium present in fraction S_{HCl} increased, the increases being related to the amount of phosphate precipitated. Also, in each experiment, except one, the percentage of the labelled calcium added that was found in fraction S_{HCl} was greater than that of the unlabelled calcium added. This, together with the finding (Table 4) of a higher specific activity in fraction S_{HCl} than in fraction S_{KCl} or S_1 in each experiment, despite the varying amounts of phosphate precipitated, indicates that the rate of conversion into phosphate of calcium bound to collagen-CS was greater than that of calcium in solution, either in the presence or absence of CS in solution. The great variability in amounts of calcium and phosphate in fraction S_{HCl} in these experiments may be due in part to differences in the collagen-CS material, of which several different batches were used. There appeared to be a tendency for the results obtained with a single batch to cluster together. The possibility of a small number of pre-existing calcium phosphate nuclei in some of the batches is difficult to exclude. In view of the vigorous extraction with EDTA and the fact that no calcium or phosphate was detected in hydrochloric acid extracts of 10 mg. of collagen-CS, with the procedures described in the Materials and Methods section, the amounts involved could only be very small and certainly could not account for the binding of calcium or phosphate in the quantities observed.

DISCUSSION

Except for experiments carried out with cartilage slices, calcium binding has been studied with much lower concentrations of CS than are present in epiphyseal or costal cartilage. In the present work the concentrations (80 μmoles of soluble CS glucuronate/ml.) were higher, but were still not quite as high as the concentration of water-soluble CS in the puppy epiphyseal plate (100 μmoles of water-extractable CS glucuronate/g. of fresh tissue) (Bowness, 1962). The ionic strength of the medium has been shown to have a considerable effect on the cation binding of CS (Mathews, 1964). In epiphyseal and costal cartilage the ratio of total cationic strength to CS glucuronate molarity is 2.8–3.2 (Iob & Swanson, 1938; Eichelberger, Brower & Roma, 1951; Bowness, 1962; Cipera, 1962). In the present experiments the corresponding ratio was 2.8 or 3.14, depending on the amount of calcium added. The nature of the ions, however, was not exactly the same as in cartilage: the main omission was magnesium and the main addition collidine. Carbonate is a minor constituent in uncalcified cartilage

(Eichelberger & Roma, 1954) and seems unlikely to be involved in the first stage of calcification; the presence of concentrations of phosphate below those required for precipitation of calcium phosphates prevents the precipitation of calcium carbonate (Bachra, Trautz & Simon, 1963). Heteroionic exchange with phosphate of apatite crystals can account for accumulation of carbonate at a later stage in calcification (Neuman & Neuman, 1958).

Under the conditions of the present work it is clear that both soluble CS and insoluble collagen-CS decrease the precipitation of calcium phosphates from a solution containing fixed amounts of calcium and phosphate (Tables 2 and 3, Figs. 1-3). That the effect of CS in solution was not due mainly to a decreased rate of movement of ions is shown in Fig. 2, where the difference between the two curves is as great at 24 hr. (when precipitation is almost complete) as at 2 hr. (when precipitation is far from complete).

Using a much higher molar ratio (1-5) of calcium to CS glucuronate, Weinstein, Sachs & Schubert (1963) found a decrease in the sedimentation rate of calcium phosphate in the presence of chondromucoprotein (PP-L), but no significant difference in the total amount precipitated. Fig. 1 shows that the effect of CS decreases as the molar ratio of calcium to CS glucuronate increases from 0.06 to 0.25. It seems probable that at a ratio of 1 or higher the effect of CS in solution would be barely noticeable. The operative ratio of calcium to CS glucuronate is difficult to assess in calcifying tissues such as the epiphyseal plate, where precipitated calcium phosphate is present; in uncalcified costal cartilage it is 0.3-0.4, but it appears likely that most of this is bound to a fraction of the CS that is not extracted with water and has a lower turnover rate than the water-extractable CS (Bowness, 1962, 1963). The ratio of calcium to CS glucuronate in water extracts of costal cartilage is 0.12-0.15 and that of P_i to CS glucuronate is 0.05-0.08 (Bowness, 1962), as opposed to 0.086 in the present experimental system. If these extracts correspond to an aqueous ground substance compartment of cartilage it is apparent that an effect on precipitation of calcium phosphate is to be expected in this part of the tissue *in vivo*. However, CS in cartilage is covalently linked in the form of one or more chondromucoproteins whose complete composition and structure are not yet determined (Schubert, 1965; Partridge, Whiting & Davis, 1965; Meyer, Anderson, Seno & Hoffman, 1965). Although titration curves show that the acid groups in chondromucoprotein are largely free (Gilbert & Myers, 1960) it is possible that steric influences of the protein affect calcium binding.

With the insoluble collagen-CS a decrease in precipitation of calcium phosphate at 2 hr. was

found with a ratio of calcium to CS glucuronate of 2 and a smaller decrease with a ratio of 4 (Table 3). This may be explained partly by phosphate binding to collagen (Glimcher & Krane, 1964) and partly by a greater affinity of collagen-CS for calcium than that possessed by CS alone. Such a difference is implied by the observation that the water-insoluble fraction of cartilage, containing collagen and a smaller fraction of the CS, binds more calcium than the much larger fraction of CS, which is extracted with water (Bowness, 1962), and which must be in contact with the collagen material either *in vivo* or during the homogenization and extraction procedure, or both. In uncalcified rib cartilage the ratio of calcium to CS glucuronate is 0.45-0.6 in the fibrous collagen compartment whereas in calcifying epiphyseal plate it is probably 1 or higher (Bowness, 1962), with a ratio of P_i to CS glucuronate of 0.3-0.4.

Despite the stronger binding of the calcium attached to the collagen-CS, equilibrium is reached faster in systems containing this material (Fig. 3) and calcium bound to it appears to be converted into calcium phosphate faster than calcium in solution (Table 4 and Fig. 4), either alone or with bovine CS in solution as well, when both forms of calcium are present together in the same system. This effect may be the same as that observed in nucleation of metastable calcium phosphate solutions by collagen fibrils (Glimcher, 1959) and may be explained in part by the localized concentration of phosphate brought about by binding to collagen (Glimcher & Krane, 1964). It seems unlikely that the difference in the time-course of precipitation between systems with and without collagen (Fig. 3) can be explained by the existence of pre-existing calcium phosphate nuclei in the collagen (Sobel, 1965), as such nuclei must be formed very soon after the start of incubation in the solutions without collagen.

Although interactions between collagen and CS have been demonstrated *in vitro* (Mathews, 1956), it is not known whether the insolubility of CS in the collagen-CS used in the present work is due to association with collagen or with non-collagenous protein, which may also be present in the portion of the material that remains unaccounted for by estimates of the CS and collagen content.

These results suggest that there are steps or compartments in the concentration gradients of calcium and phosphate, in calcifying cartilage or epiphyseal plate, between blood on the one hand and solid mineral on the other (Scheme 2). The suggested steps in the calcium gradient are associated with chondroitin sulphates differing in their turnover rate, attachment to fibrils containing collagen and strength of calcium binding. It is further suggested that the decreased reactivity to phosphate that would be associated with the increasing strength of binding of calcium is offset in

Scheme 2. Suggested compartments and components in concentration gradients of calcium and inorganic phosphate (P_i). P_o , Organic phosphate; ~ represents an interaction of unspecified type. Solid lines are the suggested major pathways of movement.

the fibrous compartment by the proximity of a high phosphate concentration brought about by attachment to collagen, or perhaps by other factors that aid the epitactic growth of solid mineral.

The present results indicate that known differences in the ratios of calcium to CS glucuronate and of P_i to CS glucuronate, between calcifying and non-calcifying types of cartilage and between one compartment of a tissue and another, would affect the precipitation of calcium phosphates *in vivo*. The initiating factor in producing these differences remains unknown; both water-extractable and non-extractable CS may be present in both calcifying and non-calcifying tissues (Bowness, 1962). That there may be differences in calcium binding between one form of water-soluble CS and another is indicated in the present work by the finding of a difference between the effects of CS from tracheal cartilage and a fraction of epiphyseal-plate CS. The smaller effect of the latter was not due to any difference in viscosity, but may perhaps be associated with the lower sulphate content found in it; Mathews (1964) found that desulphation of CS decreased the calcium binding. It should be noted, however, that the preparation of epiphyseal CS probably represents only a portion of the total epiphyseal-plate CS, namely that which is not precipitated with protein by shaking with pentan-1-ol-chloroform. These differences, between various

CS and chondromucoprotein preparations, together with the chemical and physical nature of the insoluble material termed 'collagen-CS' in the present paper, require further investigation.

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