Evidence for a Role of Proteinpolysaccharides in Regulation of Mineral Phase Separation in Calcifying Cartilage

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ABSTRACT Our previous studies have indicated the presence of a macromolecular inhibitor of in vitro mineral growth, as well as a mineral nucleational agent in extracellular matrix fluid aspirated by micropuncture methods from epiphyseal hypertrophic cell cartilage. In this report, new miniaturized methods were used to extract proteinpolysaccharide complexes (PPC) from cartilage, to isolate a light fraction (PPL-C), and further, to separate it into R1, R2, and SR2 subfractions. These methods were applied to PPL-C complexes separated from microdissected epiphyseal cartilages and to cetylpyridinium chloride (CPC) precipitates of extracellular matrix fluid aspirated from similar cartilages. Most of all of the inhibitory action on an in vitro system of mineral growth shown by whole cartilage PPL-C and by cartilage fluid PPC obtained from noncalcifying sites was contained in the R2 fraction which represented $\frac{1}{4}$–$\frac{1}{3}$ of the total hexuronate.

The R2 fraction was diminished or absent from calcified cartilage fluids and from whole calcified epiphyseal septa. The ratio R1 + R2: SR2 ranged from 0.37 to 0.71 in the fluids and whole tissue samples of noncalcified cartilages. The R2 fraction was distinguished from SR2 by a 2- to 3-fold higher protein: hexuronate ratio. These data are interpreted to indicate that the inhibitory R2 fraction was degraded or otherwise inactivated at the zone of provisional calcification and that this inhibitor participates in the physiological mechanism that regulates endochondral calcification.

INTRODUCTION

Various hypotheses proposed for the biochemical events which cause mineral formation in growing cartilage have been difficult to test for lack of methods to sample and quantitate components of in vivo calcifying systems. Recently, an extracellular fluid phase was demonstrated by micropuncture techniques in the hypertrophic cell zone of calcifying epiphyseal cartilage[1] and was characterized in respect to some parameters of calcification (1, 2). Starting samples of only 20 nl of cartilage fluid compelled miniaturization of several classical methods for the separation and identification of relevant parameters including nucleotide, hemoglobin, protein, Ca, phosphate, hexuronate, and an immunofluorescent antibody method for whole proteinpolysaccharide complexes (PPC) identification (1–3). Partition by ultracentrifugation and enzymic treatment of protein-containing fractions as well as development of a microscopic system for measurement of mineral formation permitted study of factors in this cartilage fluid which initiate or inhibit mineral growth in vitro (1, 2).

Earlier findings (1, 2) important to the current report were (*a*) hypertrophic cell cartilage and extracellular fluid of normal rats or its dilutions in synthetic lymph up to 1:4 completely prevented mineral growth in vitro, (*b*) the inhibitory action on mineral growth was confined to the sediment after ultracentrifugation and was abolished by prior incubation with trypsin or hyaluronidase, (*c*) the sediment of this extracellular cartilage fluid contained about 2 mg of hexuronate per ml of cartilage fluid and yielded a positive reaction of identification for proteinpolysaccharide with fluorescent antibodies to rat PPL-C,[2] and (*d*) there was evidence of a

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[1] Calcifying cartilage—zone of provisional calcification of normal rats and the expanded juxtametaphyseal cartilage in the healing stage of rickets.

[2] PPL-C—proteinpolysaccharide complex light fraction as characterized by Pal, Doganges, and Schubert (4) isolated from whole PP-C in various cartilages.

mineral phase and/or organic nucleational agent for mineral production in hypertrophic cell cartilage fluid of normal rats and rats with healing rickets but not in cartilage fluid of untreated rachitic rats. These results were interpreted to indicate that certain PPC functioned as inhibitors of crystal growth at extracellular sites premonitory to calcification (2).

The current report represents a continuation of these studies in which the microscopic samples of various cartilage extracellular fluids as well as whole cartilage were fractionated according to schemes which separate proteinpolysaccharides. These subfractions were studied for their hexuronate, protein, Ca,[3] and phosphate contents as well as for their capacity to inhibit mineral growth. As will be shown, (*a*) an inhibitory effect on mineral growth was confined to a single PPC fraction obtained from the extracellular fluid and solid phase of cartilages of various noncalcifying sites, and (*b*) PPC from calcified cartilage and calcified fluids were shown to have either severely reduced content or absence of this inhibitory fraction.

METHODS

Animal preparations and collection of extracellular cartilage fluids. Female Sprague-Dawley rats of the Holtzman strain were assigned at age 3 wk to a regimen of normal Purina laboratory chow or to USP rachitogenic diet No. 12 as described previously (1). After 23–35 days on these diets, the rachitic animals received a massive oral dose of vitamin D_3 (Drisdol 2000 IU), along with a normal diet for 48 hr. On the 24–26th day of the experiments, all animals received intraperitoneal injections of Nembutal and underwent collection of 20–60 nl of clear viscous fluid from the hypertrophic cell zone, immediately adjacent to calcified cartilage zone of the exposed upper tibial epiphyseal cartilage.

In this report, for the first time, the collection of fluids by micropuncture was accomplished from sites of overtly calcified septa, as demonstrated histologically. For this purpose, micropipets were advanced so that the tips were located about 50 μm caudal to the border between uncalcified hypertrophic cell cartilage and newly calcified cartilage of healing rachitic epiphyses. Fluid was obtained in 5- to 8-nl amounts from this calcifying zone. Such fluids collected from calcifying cartilage were examined by phase microscopy and fluids which uncommonly revealed red cells or fibers were discarded. After 15–20 min of fluid collection, the animals were sacrificed and some tissues, including the collection site, was isolated for histologic studies. All cartilage fluids were delivered under mineral oil in concavity slides, promptly taken up in calibrated micropipets, and diluted for procedures described below. Exactly the same technique was applied for micropuncture collection of fluid from rat nasal cartilage in vivo. For this procedure, the nasal septum of anesthetized rats was exposed and the lining membranes peeled downward. After the surgical field was rendered bloodless with fibrin foam, the micropipets were introduced through a thin layer of mineral oil placed on the surface into the anterior portion of the nasal septal cartilage. 20–30 nl of clear fluid was aspirated within 10–15 min at 300 mm Hg negative pressure. About 250 mm of this pressure was required to overcome tip resistance of the micropipets. In a similar manner, female rabbits (weight 2–3 kg) were anesthetized with Nembutal and placed supine on a similar operating table. The upper tibial condylar articular cartilage was exposed through a horizontal skin and synovial incision. Synovial fluid was removed and mineral oil was replaced on the cartilage surface. Articular cartilage fluid collection by micropipets was made from points approximately 100 μm below the surface and about 0.5 cm medial to the lateral synovial lining attachments.

Preparation of cartilage whole tissue samples, "calcified" and "uncalcified." Animals similar to those prepared for micropuncture were sacrificed for solid tissue analysis. In order to isolate uncalcified cartilage, 25- to 100-mg samples of tissue were sliced in serial sections about 1 mm thick. Each section was then subjected to microdissection at 50 × magnification under a dissecting microscope at 5°C. Fragments of "uncalcified cartilage" for biochemical analysis contained only (*a*) the resting cell zone, the proliferating cell zone, and the proximal half of the hypertrophic cell zone of epiphyseal cartilage; (*b*) the surface layer, the tangential, and intermediate zones of articular cartilage; and (*c*) the anterior two-thirds of nasal cartilages. The "calcified cartilage" included (*a*) healing rachitic epiphyseal cartilage of rats with avoidance of proximal uncalcified cartilaginous zones and metaphyseal granulation, and (*b*) upper tibial plate calcified cartilages dissected from normal 4–6 wk old calves which were obtained at a local abattoir. The layers of primary and secondary spongiosa were dissected free and processed separately from the uncalcified cartilage. After these dissections, most of the like tissues described above were pooled and were carried through biochemical preparation of PPC fractionation, as described below, on fresh tissue samples.

PPL-C preparation. The samples were tared wet and carried through the procedure of Pal, Doganges, and Schubert (4) for isolation of a light fraction of proteinpolysaccharides (PPL-C). The steps of this purification were modified slightly for miniaturization, including suspension of supernatant fractions with a microultrasonicator and homogenization in a microchamber Virtis homogenizer (Fig. 1). Also, a step including 2 M HCl wash, dilution and dialysis was introduced for PPL-C preparation from calcifying tissues according to DiSalvo and Schubert (5). A small sample of homogenized tissue was saved for wet ashing and analysis of Ca and P as previously described (1). Average yield of cartilage powder per unit dry weight of whole tissue for rat articular, growth, and nasal cartilages were 17, 23, and 25%, respectively. Average yields of PPL-C per unit dry weight of cartilage powder for these three cartilages were 15, 18, and 21%.

Partition of PPL-C. Purified PPL-C was carried through a modified fractionation procedure of DiSalvo and Schubert (5) (Fig. 2). By the original method, precipitation of Ca-phosphate salts in the presence of PPL was first used to separate three fractions, R1, R2, and S (herein labeled SR2, Table I).[4] Later, to facilitate miniaturization of this method, it was necessary to make the following modifications: purified PPL-C extracted from various cartilages was dispersed by low power sonication in a bicarbonate-buffered synthetic lymph at pH 7.6. Aliquots of 20–40 nl

[3] Data on Ca not presented; Ca : phosphate ratios (Tables IV and V) were for R1 and R2, 1.1–1.2.

[4] R1, R2, and SR2 are fractions of PPL-C separated on the basis of calcium phosphate binding in differential centrifugation as performed by DiSalvo and Schubert (1967) and the current slightly modified procedure.

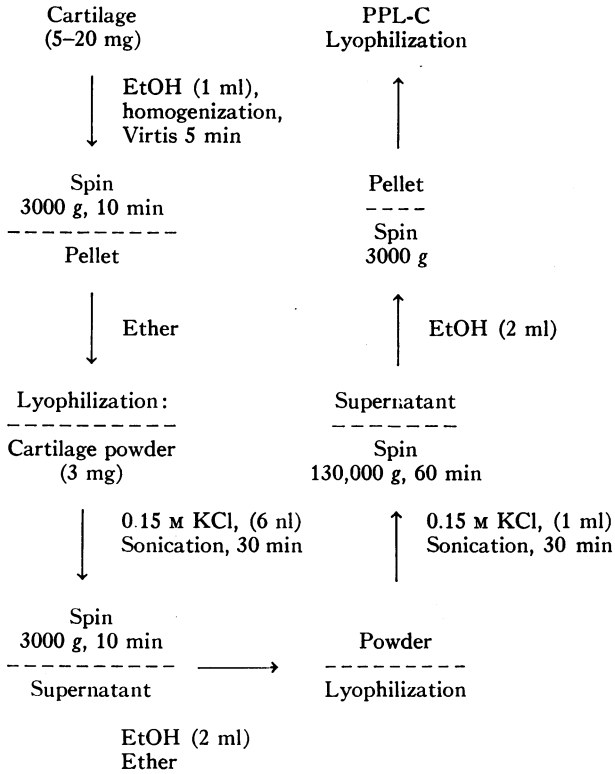

FIGURE 1 Miniaturized scheme for PPL-C preparation.

of these dispersions (or of aspirated cartilage fluids) were mixed with equal volumes of a suspension of amorphous calcium phosphate (M_{IV}) containing 0.04 mg of mineral per ml; and after 1 hr incubation, centrifugation was performed as indicated (Fig. 2). The average recovery yields of R1 + R2 + SR2 ranged from 91 to 96% of the total PPL-C in the samples. Due to the shortness of the sedimentation path (less than 1.3 mm), it was necessary to include a control by ultracentrifugating C_{f1} samples diluted 1 to 1 with synthetic lymph (Table I).

Cetylpyridinium chloride (CPC) microprecipitation procedure. It was not possible to apply the aforementioned purification steps for PPL-C to aspirated cartilage fluids

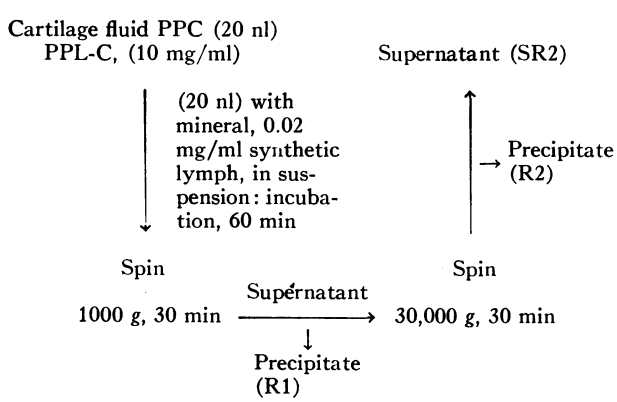

FIGURE 2 PPL partition by mineral phase.

TABLE I

Behavior of Various Proteinpolysaccharide Preparations after Partition on an Ultramicro Scale—With Ca-P Mineral Phase

Sample	Comment	Proportion of protein-polysaccharide fractions		
		R1	R2	SR2
		% of total starting sample		
PPL-C	DiSalvo and Schubert methods (4)	11	27	53
PPL-C	Data of Campo, R, 1969 on PPL3, PPL5, PPL6 recalculated from his yields (10)	8	37.6	54.0
PPL-C	Current miniaturized version*	14	31	56
PPC-f1	Current miniaturized version‡		<5	>95
PPC-f1	Current miniaturized version‡§		26‖	65
PPL5	Current miniaturized version		>95	<5
PPL3	Current miniaturized version		<5	93
PPL5	Same except dispersed in mineral phase, weight ratio, MIV/PPL5, 10:1		93	<5
PPL-C	Same except incubated with testicular hyaluronidase 750 TRU/ml, for 4 hr, at 37°C		<5	94

* See text for details of methods. Demineralized with 2 M HCl before fractionation.

‡ Sedimentation control lacking Ca-P mineral addition.

§ Hypertrophic cell cartilage extracellular fluid diluted 1:1 with synthetic lymph.

‖ R2 fraction of PPC-P (micropuncture fluid) contains R1.

and a cetylpyridinium chloride precipitate from these fluids was used as a substitute. A CPC method (6) was miniaturized to obtain a crude PPC fraction from cartilage fluids (Fig. 3). Precipitation was carried out by addition of CPC, centrifugation for 15 min at 30,000 *g*, and discarding the supernatant to eliminate extraneous proteins. The CPC–

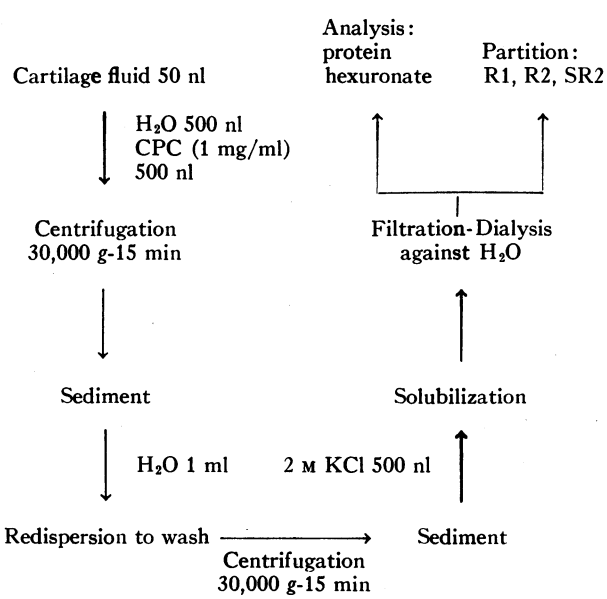

FIGURE 3 Cetylpyridinium chloride (CPC) precipitation of cartilage fluid PPC.

TABLE II

Distribution of Protein and Hexuronate in Cetylpyridinium Chloride (CPC) Precipitates of Cartilage Fluid (50 nl) and a Control Solution of Similar Composition

Sample	No. of samples	Protein*		Hexuronate*	
		Total	CPC precipitate	Total	CPC precipitate
		mg/ml		*mg/ml*	
Bovine nasal PPL-C (10 mg/ml) + serum albumin	4	26	1.6	2.0	2.2
		24–29	1.5–1.8	1.8–2.2	1.8–2.5
Hypertrophic cell cartilage fluid	8	29	1.7	2.5	2.3
		25–32	1.3–2.0	1.8–2.7	2.0–2.5

* Mean and range.

PPL-C precipitates were washed thoroughly with distilled water, redissolved in 500 nl of 2 N KCl, and transferred to the microfiltration-dialysis system.

This system consisted of a set of capillary tubes (2.5 cm length, I.D. 0.5 mm). The top of each capillary tube was covered with a disc of Millipore filter (0.22 μm pore size) (Millipore Corp., Bedford, Mass.) cemented with Acryloid B-44[5] to the rim. Each capillary was half filled with dialyzing solution in contact with the bottom surface of the filter, and the entire set was submerged in a bath of mineral oil. With a micromanipulator the sample was transferred from the micropipet to the top surface of the filter. By cooling the air phase in the bottom of the capillaries with a cold temperature plate, several portions of distilled water can be sucked through the filters. Finally, the residues on the filters were resuspended in 0.9% saline by sonication, analyzed for hexuronate and protein (Table II), and partitioned into R1, R2, and SR2 subfractions (Tables I and IV).

Spectrophotometric determinations. The R1, R2, and SR2 subfractions of PPL-C from cartilages and from CPC precipitates of aspirated cartilage fluids were analyzed for protein and hexuronate content by methods of Lowry, Rosebrough, Farr, and Randall (7)[6] and Dische (8), respectively, as previously miniaturized (1, 2). Ca was measured by a chlorophosphanazo dye method and phosphate by the method of Kuttner and Cohen (9), both also miniaturized (1–3).

Behavior of purified PPL-C in the ultramicro scheme for separation of R1, R2 and SR2 subfractions. Separatory comparisons were made on well characterized bovine nasal cartilage PPL fractions (Table I). The starting whole PPL-C for these studies contained 23% protein, 21% hexuronic acid, 21% hexosamine, and 3.5% hexoses. Characterization of PPL5 prepared according to Pal, Doganges, and Schubert was 27% protein, 20% hexuronate, 23% hexosamine, 17% galactosamine, 1% sialate, 0.2% hydroxyproline, and chain weight x 10^{-6}, 109.0 (4). Sedimentation of the PPL5 fraction performed in a Spinco ultracentrifuge at 56,100 rpm from a solution of 0.05 M phosphate, pH 7.0, and 0.10 M KCl at 20°C revealed a single sharp peak. The PPL3 bovine nasal cartilage contained 27% hexuronate, 22% hexosamine, and 7% protein.

The characterized PPL-C, PPL3, and PPL5 (Table I) were first carried through both the macroscale and the modified microscale system for separating PPL into R1, R2, and SR2 fractions of DiSalvo and Schubert (5). In order to facilitate comparisons with their data (5) hexuronate data herein (Tables I, IV, V) have been multiplied by factors (4, 5) which convert hexuronate to the appropriate PPL fraction (Table I). The starting samples (Table I) were 25 mg for the macroscale method and 10^{-6} g for the ultramicro method. It can be seen that the R1, R2, and SR2 fractions were virtually identical by the two methods despite a difference of weight ratio for starting samples of 2.5×10^4 (Table I). Purified PPL3 appeared entirely in the SR2 fraction and PPL5 in R2 fraction (Table I), indicating for present purposes their close respective similarities. A large quantity of calcium phosphate mixed with purified PPL-R2 could be removed by the same acid wash step employed for preparation of PPL-C samples from calcified cartilage, with almost complete recovery of the R2 fractions (Table I). In contrast, 0.4 mg/ml of PPL-C incubated with 750 TRU/ml of testicular hyaluronidase[7] for 4 hr and then carried through this fractionation showed displacement of hexuronate from R1 and R2 into the SR2 fraction (Table I). These methodological results obtained with the ultramicro system of partition indicate their equivalence to those originally reported for the macroscale system (5).

It was next necessary to determine whether extraneous proteins in the cartilage fluid were effectively removed by the CPC microprecipitation system (Table II). A control consisting of 10 mg/ml of purified PPL mixed with bovine serum albumin at the level of 26 mg/ml as well as whole cartilage fluid from normal rat growth cartilages were precipitated with CPC. The protein: hexuronate ratio of the PPL-C CPC precipitate in the control samples was not altered by the presence of albumin (Table II). Also, evidence that most of the hexuronate in whole cartilage fluids was contained in PPC and not in free polysaccharide was indicated by the finding of the same narrow range of values of protein and hexuronate in CPC precipitates of cartilage fluids as that of purified PPL-C (Table II). However, since free chondroitin sulfate as well as hyaluronic acid could still be unspecifically precipitated by CPC, a further check was made in this respect by diffusing the sample fluid through a Millipore filter of convenient size (0.22 μ) using the above mentioned microdialysing system. No uronic-containing material was found on the other side of the filters.

[5] Rohm and Haas Co., Philadelphia, Pa.

[6] To improve relative values for protein, purified rat nasal PPC was employed for standards.

[7] Worthington Biochemical Corp., Freehold, N. J.

TABLE III

Effect of PPL Fractions on Mineral Accretion In Vitro

Source	No. of samples	Fraction tested	Δ Pi	Inhibitory action
			mmoles/liter	
Rat hypertrophic	5	R1 + R2	−0.06	Positive
Cell cartilage fluid	6	SR2	−0.60 ±0.04*	Negative
Same after treatment with hyaluronidase‡	10	SR2	−0.86 ±0.04	Negative
Rat hypertrophic	5	R2	−0.05	Positive
Cell cartilage PPL	6	SR2	−0.69 ±0.05	Negative
Calf hypertrophic cell cartilage‡	10	SR2	−0.87 ±0.02	Negative

* Mean ±SD.

‡ No detectable R1 + R2 fraction.

In contrast, trypsin-treated samples showed 30% uronic acid diffusion.

Finally, since the partition into R1, R2, and SR2 of purified PPL-C and of deproteinized cartilage fluid CPC precipitates followed along the same lines as those native whole cartilage fluids (Table IV), it was considered sufficient to add directly the Ca-phosphate mineral for this purpose without the preliminary CPC precipitation step.

Determination of inhibitory properties on mineral accretion.[8] Serial histologic sections were made on blocks of cartilage after injection of carbowax and ferric chloride, as previously described (1). A Prussian blue reaction, carried out on these sections, localized the site of the micropipet tip. Adequacy of rickets in the various cartilages were also checked through examination of histologic sections stained by hematoxylin and eosin, toluidine blue and von Kossa techniques.

RESULTS

Effect on mineral accretion in vitro of PPL fractions. The (R1 + R2) PPC-containing fraction from extracellular fluids of hypertrophic cell cartilages uniformly inhibited in vitro mineral accretion, whereas the SR2 fraction had no inhibitory action (Table III). The pooling of R1 with R2 fraction necessitated by micromethods on fluid phases had no detectable deterrent effect on the inhibitory action of the R2 fraction (Table III). There was uniformly positive inhibitory action on the in vitro system of mineral accretion by the R2 fractions of purified PPL-C from the various epiphyseal cartilages examined (Table III). In contrast, there was a consistent lack of inhibitory action on mineral accretion by the individual SR2 fractions of the same cartilage PPL-C.

Partition of proteinpolysaccharides in cartilage fluids. Theoretical total PPL-C content ranged from 8.7 to 10.3 mg/ml of various cartilage fluids and the proportion of R1 + R2 fraction in total theoretical PPL-C ranged from 27 to 38% (Table IV). Protein: hexuronate ratios of hypertrophic cell cartilage fluid CPC precipitates ranged from 0.60 to 0.76. In uncalcified cartilage fluids, SR2 ranged from 53 to 60% of the total, with whole cartilage fluid and CPC precipitates from these fluids providing almost identical partitions (Table IV). The highest proportion of R2 fraction was in fluid from nasal cartilage. The capacity of the ultramicro partition method to detect an alteration in the profile as a result of enzymic degradation is shown by the data on incubations of rat growth, nasal, and articular cartilage fluids separately with hyaluronidase and trypsin. In these instances, there was virtually total abolishment of the R1 + R2 fraction with displacement of the degraded fragments into the SR2 fraction. No effect of rickets on behavior of untreated or enzymically degraded fluids from uncalcified cartilages was observed (Table IV). In an additional experiment, centrifugation of the hypertrophic cell cartilage sediments after hyaluronidase treatment at 3000 *g* for 10 min was used to synthesize a "native fluid" mineral phase as previously reported (2). When this crude mineral sediment was resuspended at a level of 0.02 mg of phosphate per ml, in fresh cartilage fluid, there resulted an R1 + R2, SR partition not detectably different from that caused by MIV mineral phase (Table IV).

In contrast to the uncalcified or hypertrophic cell cartilage fluids, six fluid samples pooled from 12 animals of healing rachitic rat "calcified" growth cartilage revealed absence of the R2 fraction. This finding constitutes direct evidence for the removal or alteration of PPL-C inhibitory to calcification within microscopic sites undergoing mineral growth.

Partition of proteinpolysaccharides in whole cartilages. The R2 fraction varied from 22 to 33% of the total PPL-C in whole uncalcified nasal, articular, and epiphyseal cartilage. The highest values for R2 were in the nasal cartilages, as noted in our cartilage fluid samples

[8] Mineral accretion is used here to mean growth of a mineral particle fraction without implications in respect to an increase in size of mineral particles versus number of new particles.

TABLE IV

Partition of Proteinpolysaccharides Directly from Cartilage Fluids and CPC Precipitates of Cartilage Fluids ·

Cartilage source of fluid sample	Experimental treatment	No. of samples	Starting sample volume	Theoretical PPL-C		Proteinpolysaccharide partition			
				Hexuronate*‡ × 4.55	Protein/ hexuronate‡	(R1 + R2)		(SR2)	
						Hexuronate	(R1 + R2)/PPL-C	Hexuronate	SR2/PPL-C
			nl	*mg/ml*		*mg/ml*	%		%
Normal rat									
HC Cell	Control, whole fluid	20	100	9.6	0.60	0.56	29	1.4	56
HC Cell	Cetylpyridinium chloride precipitate	6	100	9.7	—	0.55	27	1.3	55
HC Cell	Trypsin, hyaluronidase	6§	100	—	—	<0.05	—	2.0	95
Nasal	Control	8	60	8.7	0.72	0.66	38	1.2	53
Nasal	Trypsin, hyaluronidase	6§	100	—	—	<0.05	—	1.7	96
HC Cell	Mineral containing sediment from HC cell fluid	4	100	9.5	—	0.65	34	1.5	62
Rachitic rat									
HC Cell	Control	10	100	10.3	0.74	0.67	32	1.6	59
HC Cell	Trypsin, hyaluronidase	6§	100	—	—	<0.05	—	2.2	96
HC Cell	Healing, 48 hr, restoration of normal diet vitamin D	6‖¶	75	—	—	<0.05	—	2.5	—
Rachitic rabbit articular	Control, whole fluid	16	100	9.5	0.68	0.64	34	1.5	60

* Represents concentration in unaltered native samples.

‡ SDS of all of the hexuronate and protein values were <10% of the mean.

§ Trypsin, 0.4 mg/ml or testicular hyaluronidase 750 TRU/ml, incubated with PPL fractions in synthetic lymph (see Methods).

‖ <0.05 indicates here and elsewhere that the value is below the minimum level of detection of this fraction.

¶ Each sample pooled from aspirations of five or six animals, with healing rickets, in the distal calcified HC (hypertrophic cell) cartilage.

TABLE V

Partition of Purified PPL-C Extracted from Various Cartilages, R2 Fraction and Absence of R2

Description of cartilage	Sample PPL-C		Proteinpolysaccharide R1		
	Hexuronate × 4.55	Protein/ hexuronate	Mineral precipitated P	Hexuronate × 4.25	R1/PPL-C
	mg/ml		*mg/100 ml*	*mg/ml*	*%*
Uncalcified, nasal, calf	10.1	0.52–0.73	1.3 ±0.15	0.44	4.3
Uncalcified, nasal, calf	10.2	0.60–0.75	1.6 ±0.20	0.41	4.0
Uncalcified, growth, rat	10.0	0.50–0.68	1.3 ±0.20	0.41	4.1
Uncalcified, articular, rat	9.9	0.45–0.62	1.5 ±0.12	0.30	3.0
Calcified growth, calf§	10.0	0.30–0.35	1.8 ±0.20	<0.01	—
Calcified growth, rat	10.1	0.26–0.33	1.8 ±0.20	<0.01	—
Healing rachitic§					

* For each of these six experimental groups, samples of cartilage from eight animals were analyzed. Results are expressed per unit volume of starting PPL-C solution. Distribution of mineral = mean ±SD; range of protein:hexuronate ratios are shown. Factors 4.55, 4.25, 5.00, and 3.70 convert hexuronate to appropriate theoretical PP-L fraction value according to DiSalvo and Schubert (5). SD of hexuronate and protein determinations were <12% of the mean.

‡ 10 mg/ml of purified PPL-C and 0.02 mg/ml of MIV mineral suspended in synthetic lymph comprised starting solutions and standard of reference.

§ *P* content of "Calcified" cartilages was >6 g/100 g dry weight and of "Uncalcified" cartilages was <0.3 g/100 g.

‖ Tissue obtained from rachitic rats 48 hr after restoring the normal diet and vitamin D.

(Table IV), and observed previously in a composite survey of similar PPL partitions of articular, epiphyseal, and nasal cartilages (10). Range of protein content was 6.8–10% dry weight of SR2, whereas the range of protein was 14–28% for R2 (see protein: hexuronate ratios, Table V). Of the total mineral added in the precipitation scheme, about three-fourths precipitated in R1 and the rest was in the R2 fraction.

Calcified normal bovine and healing rachitic cartilages (Table V), when carried through the same procedure for PPL-C purification, developed a distinctively different profile. The ratio of protein: hexuronate was reduced as described for calcified epiphyseal cartilage by Campo and Tourtellotte (11). As with fluid samples, there was a substantial reduction or disappearance of the R2 fraction from these calcified cartilages. The R1 fraction was not measurable and the hexuronate in the SR2 fraction was increased. Distribution of Ca-phosphate among the three fractions of PPL was 98% in R1 and none was measurable in R2 and SR2.

The final experiments indicated that there was a stoichiometric relationship between sedimentad hexuronate in the R2 fraction separated directly for hypertrophic cell cartilage fluid and stepwise increments of mineral used for precipitation (Fig. 4). For this purpose, 20-nl samples of fresh nasal cartilage fluid were carried through the R1, R2, and SR2 separation. When in successive experiments 0.25, 0.5, 0.9, and 1.3 μmoles of $CaHPO_4$ (MIV) mineral per ml of final solution were added to hypertrophic cell cartilage fluid containing 10 mg of PPC per ml of final solution, hexuronate in the R2 fraction increased in a curvilinear manner up to the level of mineral addition, 0.9 μmoles/ml, and no further increment of R2 fraction occurred thereafter. Above this concentration of added mineral, moreover, all the mineral increments precipitated in the R1 fraction. The maximum hypothetical molar ratio of MIV mineral to PPC is severalfold less than that 13:1 ratio found suspended by R2 fraction by DiSalvo and Schubert (5) probably due to differences in composition of their mineral forming systems. It is noteworthy that for each fragment of PPL-R2 of weight 10^6 approximately 30,000–40,000 moles of MIV mineral (represented as $CaHPO_4$) were held in suspension at 700 *g* (Fig. 4).

DISCUSSION

Our earlier findings that fluid aspirated by micropuncture techniques from hypertrophic cell epiphyseal cartilage contains both a nucleational agent and a macromolecular inhibitor of in vitro mineral growth consistent with PPC (2) have been extended in this report to add new interest in the following respects: a scheme for subfractionation of proteinpolysaccharide by centrifugation in the presence of calcium phosphates (5) could be

with Demonstration of a High Protein: Hexuronate Ratio in the Fraction in Calcified Cartilage

partition

Mineral precipitated P	R2 Hexuronate × 5.00	R2 Protein/ hexuronate	R2/PPL-C	SR2 Hexuronate × 3.70	SR2 Protein/ hexuronate	SR2/PPL-C
mg/100 ml	*mg/ml*		%	*mg/ml*		%
0.7 ±0.20	3.2	1.1–1.4	31	5.6	0.27–0.35	56
0.7 ±0.15	3.3	1.0–1.3	33	5.3	0.30–0.38	52
0.6 ±0.18	2.8	1.1–1.2	28	6.1	0.25–0.35	61
0.7 ±0.15	2.9	1.0–1.3	22	6.9	0.29–0.33	68
<0.01	<0.01	—	—	9.3	0.30–0.35	96
<0.01	<0.01	—	—	9.6	0.28–0.35	96

successfully miniaturized for starting quantities of 10^{-6} g of proteinpolysaccharide, contained in 100 nl of fluid obtained by micropuncture from various cartilaginous sites. A fairly constant proportion of total PPC could be separated as the R2 fraction from extracellular fluids of several different noncalcified cartilages. The R2, but not the SR2, fraction functioned as an inhibitor of mineral growth in vitro, and this R2 fraction was absent from whole calcified cartilage and from extracellular fluid aspirated from calcified cartilage in vivo.

In regard to physiological relevance of present findings, the likelihood that polysaccharides "shield" reactive sites on collagen fibrils and block mineral nucleation was suggested by Glimcher (12) in 1958. The first direct evidence for an inhibitory action of proteinpolysaccharides on calcification was obtained by Weinstein, Sachs, and Schubert who observed that sedimentation of calcium phosphates at low centrifugal forces was prevented by these compounds (13). Their observations were extended by DiSalvo and Schubert, who noted that proteinpolysaccharides, particularly those contained in the PPL5 fraction, combined stoichiometrically with calcium phosphates (5).

In a previous report we confirmed those findings concerning PPL5 and, in addition, demonstrated arrest of mineral growth with only slight removal of calcium and phosphate from the solutions in the presence of the PPL5 fraction. The conditions in our experiments differed from those employed by DiSalvo and Schubert (5) in respect, not only to the presence of magnesium, bicarbonate buffer, and a lower $\alpha\ Ca^{++} \times \alpha\ HPO_4^{=}$ in the incu-

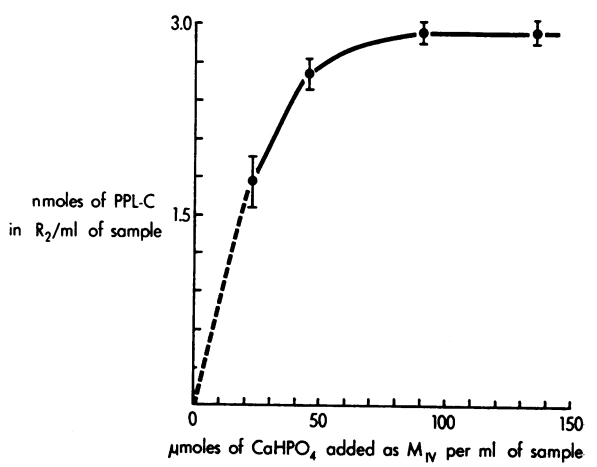

FIGURE 4 Stoichiometric molar relationship between MIV mineral expressed as di-calcium phosphate and theoretical amount of PPL-C sedimented in R2 fractions. Here PPL-C = hexuronate × 5.0/10^6. Actual molecular weight of R2 component is > 10^6 (unpublished observations) but is expressed here as a fragment of 10^6 in order to indicate approximate capacity for mineral binding.

bating lymph, but also of our much smaller sedimentation system. For both of these experimental calcifying systems, intact whole PPL and PPL5 were effective inhibitors, whereas PPL3, chondroitin sulfate, or PPL degraded by trypsin and hyaluronidase were ineffective. This latter finding fit well with the hypothesis advanced in 1962 by Campo and Dziewiatkowski that enzymic degradation of proteinpolysaccharides at calcifying sites occurred and might be part of a mechanism of calcification (14). Subsequently, enzymes active in degrading the protein moeity of proteinpolysaccharides have been found in cartilage (15, 16), and evidence from immunochemical studies (17) as well as X-ray elemental analysis (18) indicate disappearance of protein relative to sulfated polysaccharides in endochondral calcifying cartilage septa. Originated on the data in the current report, we postulate that a component of the R2 fraction PPC or protein linked to PPC is a physiological inhibitor of growth and multiplication of mineral particles, which growth should otherwise occur spontaneously along the margins of growing septal mineral deposits during calcification of cartilages in vivo. Perhaps the aforementioned enzymic degradations assist in initiation of mineral formation at nucleational sites in extracellular septal matrix (2) by degrading R2 fraction at calcified sites (Tables IV and V).

Biochemical identification of the R2 and SR2 fractions isolated by these microtechniques is only in a preliminary stage, but their properties in common with PPL5 and PPL3 respectively provide a starting point for characterization. PPL5 and R2 fraction share in common a similar high protein:hexuronate ratio, share the property of suspending calcium phosphate at low sedimentation forces, and inhibit mineral phase growth in metastable synthetic lymphs. Our current data do not indicate (Table III) whether the component(s) of the R2 fraction which enables suspension of mineral particles (5, 13) also is responsible for inhibition of mineral growth, but in those fluids so far studied, all SR fractions lacking capacity to suspend mineral were also unable to inhibit mineral growth. From the stoichiometric data concerning calcium phosphate added and R2 fraction precipitated (Fig. 4), a close similarity of this interaction of mineral and R2 fraction to that described by DiSalvo and Schubert (5) seems likely. They provided experiments which indicated that the binding to R2 fraction was not an adsorption phenomenon. In the first place, dinitrophenylation of their R2 fractions prevented adsorption of mineral phase but did not prevent mineral suspending properties. Also, calcium phosphate added to PPC fractions at high concentrations required to demonstrate adsorption did not show any preferential uptake of R1, R2, and S fractions in a manner which would explain selective partitioning. Another important feature of their study (5) was the fact that R2 fraction bound to mineral phase could be removed by acidification and recycling whereas R2 fraction which had been degraded with trypsin or hyaluronidase failed to respond to the partitioning procedure. Thus, failure of the acidification step to recover R2 fraction from the current calcified tissues (Table I, IV, V) provides strong evidence that this particular inhibitory fraction is degraded or otherwise altered within the calcifying sites of our experimental preparations.

Recent studies of Sajdera and Hascall have raised a question concerning the polydispersity of the PPL-C extract (19). By use of hypertonic potassium or magnesium chloride extraction of diced tissue, these authors were able to extract 85% of total PPC in a manner not requiring high shear extraction of the cartilages. After purification by cesium chloride density gradient ultracentrifugation, purified fractions provided Schlieren patterns of fast moving components similar to PPL5 which disappeared under dissociative conditions. This finding indicates that the fast moving component might be simply an aggregation of proteoglycan subunits present in PPC linked to glycoprotein fraction also found in PPC extracts (20). It seems plausible that these aggregates are contained in the R2 fractions shown to be present in native extracellular cartilage fluids.

Certainly, the finding that the mineral generated in a native cartilage fluid (Table IV) partitions PPC in a fresh native cartilage fluid is evidence that chemical binding required for partition probably can occur in vivo when matrix fluid containing PPC contacts newly-growing native mineral particles (Table IV).

A final point of interest is that only about 6% of the soluble proteins in cartilage extracellular fluids examined in the current study can be accounted for by proteinpolysaccharides (Table II). The virtual exclusion of serum proteins from such cartilages was postulated by Gerber and Schubert (21); thus the nature and function of this new pool of interstitial proteins is being investigated.

ADDENDUM

Additional support to the conclusion reached in this paper was found in analyses of well-characterized proteinpolysaccharides provided us recently by Dr. Lawrence Rosenberg. In his preparations, PPC of bovine cartilage was isolated by a low shear dissociative extraction technique (20) using 3 M $MgCl_2$. Subsequent fractionation by ultracentrifugation in high molarity salt solutions provided sharp discreet macromolecular species labeled PPC3 and PPC5.

This PPC3 showed, upon analytical ultracentrifugation, a single major component with $S = 19.5$, and PPC5 two components, one with $S = 59$ and the other $S = > 100$. Samples of this PPC5 ($n = 3$) inhibited mineral growth and partitioned similarly into R2 and SR2 fractions. PPC3 was not partioned by the MIV mineral phase and failed to inhibit mineral growth in vitro.

These findings reinforce our data on solid tissue and cartilage fluids, which indicate that the Schubert PPL separation schemes have not created an artifactual property either in respect to mineral inhibition or partitioning into the R2 and SR2 fractions.

However, the PPC5 of Rosenberg revealed a lower protein: hexuronate ratio, 11–12% vs. 20% in R2. Protein content of R2 fractions in excess of 8% has not been found critical either to inhibition of mineral growth in vitro or to partitioning in our experiments on similar PPC preparations (unpublished observation) using the Sajdera and Hascall dissociative extraction techniques.

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