Rat Epiphyseal Cartilage: I. Chemical Analysis and Isolation of Crude Protein-Polysaccharide.* (29929)

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The mucopolysaccharides in cartilage exist as protein complexes which have been designated collectively as protein-polysaccharides (PP)(1). Although the biochemical and physical properties of cartilage PP of various types and species have been studied, the metabolism of these compounds has received limited investigation(2-5). It would appear that isolation and characterization of PP from epiphyseal cartilage might prove quite useful because of the suitability of this tissue for *in vitro* studies and because of the possibility that, with proper application, the metabolism of these compounds in relation to calcification might also be studied(5). This paper reports the chemical analysis of rat epiphyseal cartilage and the isolation of a crude protein-polysaccharide from this same tissue.

Materials and methods. Male Sprague-Dawley rats (Charles River Lab.), 21 to 28 days old and maintained on Purina Chow and water *ad libitum,* were used during these experiments. The animals, usually 6 to 8/experiment, were sacrificed by cervical traction and the lower extremities quickly isolated. The proximal end of the tibia and the distal end of the femora were scraped clean of adherent tissue and the epiphyses separated from their shafts. Although the entire epiphyseal apparatus could be easily isolated from the tibia by a transverse section through the metaphyseal junction of the epiphyseal plate, the irregularity of the femoral epiphyseal plate necessitated a transverse section through the metaphysis and separation of the underlying metaphyseal fragments from the distal femoral plate. The epiphyseal segments from individual animals were weighed, placed in acetone overnight, and dried *in vacuo* to constant weight.

The PP was isolated by the method of Schubert(2) as modified by Bollet(3). All procedures were carried out at 4°C. The acetone dried cartilage segments were rehydrated in 5.0 cc distilled water for 16 hours, followed by homogenation in a Virtis 45 apparatus operating at maximum speed (45,000 rpm) for 30 minutes in a total volume of 25.0 cc of water. The homogenate was centrifuged for 10 minutes at 34,800 × *g* and the supernatant was removed and made 75% alcohol (v/v) by addition of 3 volumes of absolute alcohol. After equilibration in the cold for 30 minutes, a gelatinous precipitate was removed by centrifugation and 0.1 volumes of 20% potassium acetate were added to the total volume of the remaining supernatant. The mixture was incubated in the cold for 30 minutes and the crude PP collected by centrifugation. The crude PP was washed with several volumes of 95% ethanol, ether and dried *in vacuo.*

Uronic acid was determined by the method of Dische(6) on the cartilage hydrolysates after hydrolysis for 2 hours at 100°C in 2 N HCl(3) and on the isolated PP directly. Other analyses were nitrogen (micro-Kjeldahl); hexosamine(7) after hydrolysis of the cartilage in 4 N HCl for 15 hours at 100°C; hydroxyproline(8) after hydrolysis in 6 N HCl for 16 hours at 100°C; and DNA and RNA by the method of Schneider(9). The ash weight of the cartilage was determined after treatment in a muffle furnace at 600°C overnight.

Results. The mean body weight of animals employed in these studies was 55.1 ± 5.7 g. In *Table I* data on the composition of epiphyses isolated from individual animals are presented. An estimate of the major constituents of this connective tissue was derived with suitable calculations as demonstrated in *Table II.* Comparison of these data with similar data on analyses of hyaline

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TABLE I. Data on Analysis of Rat Epiphyseal Cartilage.

	"Cellular"			"Matrix"		
	DNA	RNA	RNA/DNA ratio	Uronic acid	Hexosamine	Hydroxy-proline
	(mg*)	(mg*)		(mg*)	(mg*)	(mg*)
Mean	.435	.383	.930	.643	.905	.696
S.D.	.032	.035		.046	.045	.050
(N)	21	13	13	36	20	12

* mg per 100 mg of wet cartilage.
Animals between ages of 23 to 25 days old were used and analyses were conducted on the cartilage segments isolated from individual animals. Mean wet and dry weight of cartilage segments was 259.0 ± 22.7 and 70.3 ± 5.9 mg respectively.

cartilage(10) suggests a tendency towards a loss of water, increased ash, decreased hexosamine with a reciprocal change in hydroxyproline/hexosamine ratio occurring in epiphyseal cartilage. These changes are apparently indicative of calcification(11) and are in accord with the presence of quite visible secondary ossification centers in this age epiphyseal cartilage.

The extensive endoplasmic reticulum observed histologically in this tissue(12) correlates well with the RNA determination. Although the DNA concentration found is higher than previously reported(13), this difference may be attributable to the immaturity of the epiphyses employed in these studies. The relatively high RNA/DNA ratio is consistent with the active metabolism and rapid turnover of matrix occurring in rat epiphyses (12).

Data on yield and analysis of crude PP are presented in *Table III*. In animals between 24 to 25 days old the yield of crude PP averaged 13 mg/100 mg dry cartilage. This weight represented approximately 20% of the organic weight of the cartilage and on analysis was composed of 6.9% nitrogen and 10.9% uronic acid (U.A.). At least 3 reprecipitations were necessary to obtain a PP of constant protein (nitrogen) to polysaccharide (U.A.) ratio(3,4). The analysis of crude PP treated in this manner is presented in *Table IV*. Recovery of U.A. in PP, as calculated from cartilage U.A., was 72% in these 24-day-old animals. Electrophoresis of this reprecipitated PP on cellulose acetate strips in veronal buffer (pH = 8.6)(3) revealed both protein and polysaccharide in one band, while the molar ratio of hexosa-

TABLE II. Chemical Anatomy of Rat Epiphyseal Cartilage.

Component	Wet cartilage (mg/g)
Water*	708
Ash	97
Organic†	185
Collagen‡	56 (30%)§
Crude protein-polysaccharide	37 (20%)§

* Wet wt—dry wt.
† Wet wt—water + ash wt.
‡ mg hydroxyproline/13.8% = mg collagen.
§ % of organic weight.

TABLE III. Analysis of Crude Protein-Polysaccharide from Rat Epiphyseal Cartilage.

Group No.*	Animal age	Cartilage dry wt	Crude protein-polysaccharide Wt	Uronic acid	Nitrogen
	(days)	(mg)	(mg†)	(%)	(%)
A	25	72.5	12.99	10.44	7.25
B	24	75.4	12.36	11.03	6.94
C	25	72.3	13.42	11.13	6.62
D	28	99.7	13.34	8.51‡	6.63

* Each group consisted of 6 animals (except A—7 animals).
† mg isolated per 100 mg dry cartilage.
‡ Statistically significant $P < 0.05$.

TABLE IV. Data on Reprecipitated Protein-Polysaccharide.

Group No.	No. of animals	Cartilage dry wt	Protein-polysaccharide Uronic acid	Nitrogen
		(mg)	(mg*)	(mg*)
E	6	71.4	1.653 ± .088	.850 ± .058
D	6	99.7	1.127 ± .078†	.811 ± .071

* mg/100 mg dry wt.
† Statistically significant $P < 0.01$.
Crude PP was isolated from 2 groups of animals, gr E (24 days old) and gr D (28 days old), and reprecipitated 3×, followed by analysis for total PP uronic acid and nitrogen.

mine/uronic acid/nitrogen was 1.0/1.2/6.9 respectively.

While isolation of PP from animals between 24 to 25 days old was found fairly reproducible, significant differences were observed when attempts to isolate a similar material from the cartilage of older animals were conducted. Analysis of crude PP (Table III) and reprecipitated PP (Table IV) from one such group of animals 28 days old is presented. Associated with an increase in dry weight of the cartilage was a decrease in concentration (%) of U.A. in crude PP and in total PP U.A. recovered after reprecipitation, while no appreciable difference was noted in PP nitrogen. Above this age group, polysaccharide (U.A.) containing material was observed to precipitate with alcohol prior to addition of potassium acetate.

Discussion. While these studies are preliminary, it would appear that the material isolated from animals of 24 to 25 days old represents a crude PP. In support of this are the similarities in method of isolation, in representation of total cartilage matrix and polysaccharides, in analysis, and in electrophoresis when compared to other PP(2-4). The relatively high yield of this material in conjunction with the known rapid synthesis of sulfated mucopolysaccharides in this tissue (5) suggests that this may be a suitable tissue for study of the biosynthesis of these complex compounds *in vitro*.

The crude nature of this PP limits interpretation of the observed protein to polysaccharide ratio and the suggested change occurring with age. However, from the fact that this tissue, which is initially cartilage, is rapidly being replaced by bone with onset of the secondary ossification centers, one would expect a simultaneous change in polysaccharide content both qualitatively and quantitatively(14,15). The relatively high protein content of epiphyseal PP and its suggested increase with age (and calcification) may imply that alteration in the character of PP may be associated with this transition. Such an occurrence would not be totally inconsistent with previous observations suggesting alterations in these complexes in calcification(5); the known correlation between keratosulfate and increased protein content of PP(1); and the most recent implication of keratosulfate in calcification(16).

Summary. 1. The epiphyseal cartilage of 3- to 4-week-old rats has been analyzed for its constituents, DNA, RNA, hexosamine, uronic acid and hydroxyproline. An estimation of the total chemical nature of this cartilage is presented which suggests changes consistent with calcification. 2. Isolation of a crude PP from the epiphyses of 24-25-day-old animals is reported, which represents 20% of cartilage organic matrix and contains 72% of total cartilage uronic acid. Analysis of the reprecipitated PP reveals a molar ratio of hexosamine/uronic acid/nitrogen of 1.0/1.2/6.9, respectively. 3. The changes observed in PP type material isolated from older animals may be indicative of alterations occurring in PP in association with calcification.

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