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Research Article

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The Proteinpolysaccharides of Human Costal Cartilage

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ABSTRACT Water-soluble proteinpolysaccharides, called PPL, can be extracted from bovine nucleus pulposus in yields of 45%, and from bovine nasal cartilage in yields of 37% of the dry tissue weight. From human costal cartilage only 7% can be extracted. The method used to separate PPL from each of the first two tissues into four distinct fractions separates the PPL of human costal cartilage into four fractions called PPL 3, PPL 4, PPL 5, and PPL 6, which show an increase in protein content, a decrease in chondroitin sulfate content, a nearly constant keratan sulfate content, and an increase in ease of sedimentability and molecular weight. From each of the three tissues mentioned, PPL 3 has a similar amino acid profile and so does PPL 5, but PPL 5 differs from PPL 3 in having a lower content of serine and higher contents of aspartic acid, tyrosine, and arginine. A more extensive effort to characterize these products has been made by analytical ultracentrifugation, and this has led to a further fractionation of PPL 5.

Treatment of the cartilage residue or the water-insoluble protein polysaccharide called PPH, with neutral NH_2OH solution releases water-soluble protein polysaccharides which in composition resemble PPL 4. The water-insoluble residue left after NH_2OH treatment, when treated with collagenase, yields two soluble products, one resembling PPL 5 in composition, the other with a much lower chondroitin sulfate and much higher keratan sulfate content. The possibility is suggested that in human costal cartilage, binding of some forms of PPL to collagen may occur.

INTRODUCTION

Proteinpolysaccharides isolated from bovine nasal cartilage and bovine nucleus pulposus, after water extraction of these tissues, have been separated into a series of fractions by differential ultracentrifugation in high molarity salt solutions (1, 2). The fractions (defined by diagram 1 and called PPL 3, PPL 4, PPL 5, and PPL 6) from bovine nucleus pulposus all contained keratan sulfate in amounts almost equal to their chondroitin sulfate contents, while the fractions from bovine nasal cartilage contained very little keratan sulfate.

Proteinpolysaccharides from human costal cartilage had been separated previously, by a procedure using lanthanum, into a series of fractions each containing both chondroitin sulfate and keratan sulfate (3). However, as judged by analytical ultracentrifugation, this method gave poorly defined products. By ultracentrifugation in high molarity salt solutions, proteinpolysaccharides from human costal cartilage have been separated into a series of fractions similar to those from bovine nucleus pulposus, and the compositions and physical characteristics of the fractions are reported here.

Human costal cartilage yields only a small part of its proteinpolysaccharide by water extraction, behaving as if a large part were insoluble or were attached in some way to the collagen. The present work, in addition to the isolation and characterization of the forms of PPL extractable with water, examines the proteinpolysaccharides not directly extractable with water from human costal cartilage. These are rendered soluble, in part by treatment of the cartilage residue with NH_2OH, and finally by destruction of the remaining residue with collagenase.

METHODS

Human costal cartilage was collected at autopsy within less than 24 hr after death. In a cold room it was scraped clean of perichondrial tissue, cut into small pieces, and fragments grossly calcified were discarded. It was powdered and dehydrated in a VirTis-45 (Virtis Co. Inc., Gardiner, N. Y.) with ethanol, and after washing with ether, it was dried in a vacuum desiccator.

Extraction procedure. Two slightly different methods of extraction have been described in great detail. In one, water

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is used for extraction which yields a product called crude proteinpolysaccharide which can be separated into the fractions called PPL and PPH (1). In a second method, 0.15 N KCl is used for extraction which yields PPL directly, leaving PPH in the cartilage residue (2). Human costal cartilage extracted with water gives large yields of PPH (3). This offers the possibility of comparing the yields and compositions of the products called PPL 2 extracted from PPH or from the cartilage residue with a neutral solution of hydroxylamine (4). For this reason the human costal cartilage was extracted first with water (step 2 of diagram 1), and then the two products, PPH and CR, were each extracted separately with NH_2OH (steps 4 and 5). The insoluble residue now left, called CRR, still contains about 2% uronic acid, 4% hexosamine, and 7% hexose. Several procedures were tried to isolate additional water-soluble fractions containing polysaccharide. Autoclaving CRR in water, or treating it successively with pepsin and trypsin, rendered it practically completely soluble in water, but in each case, fractionation of the resulting solutions gave no clearly definable products. Much cleaner results were obtained when CRR was rendered soluble by the action of collagenase. From such solutions, two sharply separable products were obtained by fractionation as barium salts with ethanol, one precipitating at 25% ethanol, the other at 75% ethanol as indicated in step 9.

The entire procedure is outlined in diagram 1. This includes, in parentheses, average yields as grams per gram of dry cartilage, for each of the final products as well as for some of the intermediate products. Steps 1, 2, 3, 4, 6, 7, and 8 have been described in detail (1). Step 5, applied to the cartilage residue (CR) without drying it, is carried out just as step 4 (4).

Only step 9 will be described in detail by a specific example. To the residue from step 5 (CRR, 5.1 g) together with residue 1 from step 4 (0.9 g) are added 0.5 M Tris buffer (200 ml, pH 7.1) containing 0.01 M $CaCl_2$, and collagenase (200 mg, Worthington Biochemical Corp., Freehold, N. J.) dissolved in the same buffer (10 ml). The mixture is stirred slowly at 37°C for a day. A small undissolved residue (0.28 g) is removed by centrifuging (30 min, 60,000 *g*) and discarded. To the clear brown liquid, $CaCl_2$ (3 g) and ethanol (800 ml) are added, and after standing (5°C, 24 hr), the crude product is removed by centrifuging. It is washed with ethanol and ether and when dried weighs 2.23 g. It is dissolved in water (300 ml), and $BaCl_2$ (2.5 g) and ethanol (100 ml) are added. After standing (5°C, 24 hr), the crude precipitated product (CRRF 25) is removed by centrifuging. From the supernatant solution, the second product (CRRF 75) is similarly precipitated by addition of ethanol (800 ml). Each product was separately dissolved in water (20 ml), stirred with Dowex-50 in its potassium form to remove barium and calcium ions, and the products precipitated as potassium salts after addition of potassium acetate and ethanol. After a second precipitation from water with ethanol, the yields were 158 mg (CRRF 25) and 89 mg (CRRF 75).

Chemical and physical studies. Uronic acid was determined by Dische's carbazol method (5), hexose by the anthrone method (6), hexosamine by the Schloss modification of the Elson and Morgan procedure (7), and hydroxyproline by Woessner's method (8). Sialic acid was determined by the thiobarbituric acid method of Warren (9), sulfate by the method of Green and Robinson (10), and protein with the biuret reagent.

Sedimentation velocity experiments on proteinpolysaccharide fractions were carried out in a Spinco Model E analytical ultracentrifuge in the An-D rotor at 20°C, by use of the schlieren optical system. Double sector cells with a 12 mm Kel-F centerpiece were generally used. Schlieren patterns were recorded on Kodak metallographic plates and read with a Nikon two dimensional microcomparator. Sedimentation coefficients at various concentrations were calculated from values of the time and the distance of the maximum ordinate of the schlieren pattern from the axis of rotation. Plots of sedimentation coefficients against concentration were extrapolated to zero concentration, and this value was corrected for solvent density and viscosity to give $s^\circ_{20,w}$, the sedimentation constant in water at 20°C.

In sedimentation equilibrium experiments, the high speed procedure described by Yphantis was used (11). Three solution-solvent pairs were studied simultaneously using the six channeled 12 mm centerpiece. In a series of sedimentation equilibrium experiments, conditions were first established for satisfactory concentration distribution of solute, at initial solute concentrations of 0.02–0.05%, and an operating speed of 6000 rpm. Runs were continued at least 8 hr beyond reaching equilibrium to 48 hr. Interference patterns were read with care on the Nikon microcomparator.

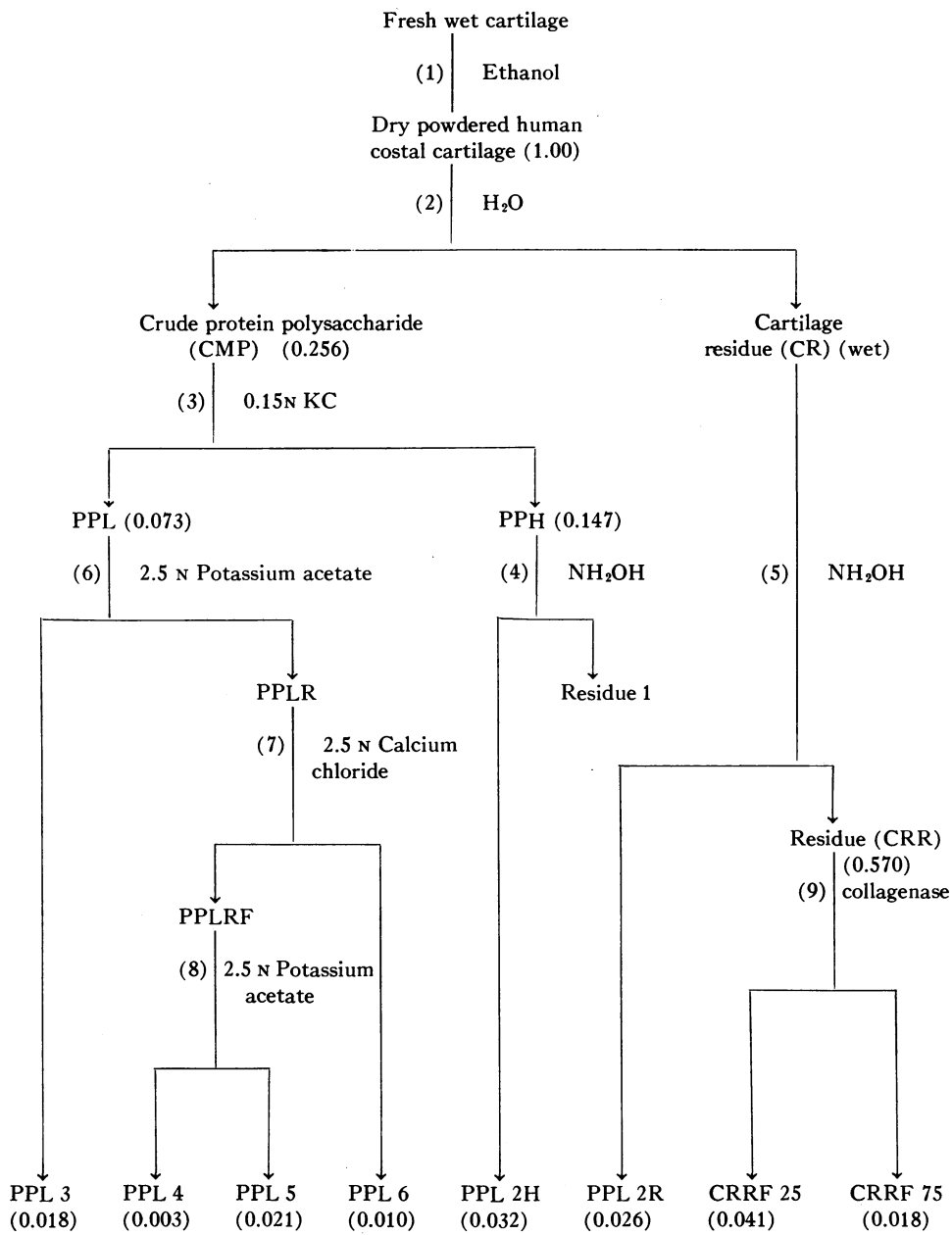
Partial specific volumes were calculated from solution densities determined at 20 ±0.005°C in a 10 ml Sprengel pycnometer. Additions of solvent to samples were made by weight, and solute concentrations are expressed as weight fractions. Viscosities were measured on the same solutions used for density determinations in an Ostwald viscometer in a constant temperature water bath at 20 ±0.005°C.

RESULTS

Table I gives the yields of eight products from each of nine samples of cartilage from individuals of ages from 22 to 79. There is often a considerable difference between yields even at closely similar ages. That yields of products from human costal cartilage are likely to be more erratic than those from bovine nasal cartilage was pointed out in earlier work (3). In the table, there seems to be a tendency for higher yields of all products to occur at younger ages. This becomes clearer by averaging the yields in groups of three as shown near the bottom of the table. For most of the products there is a progressive drop in these average yields with age, but for all products, the yields of the oldest group (46–69 yr) is less than the yields of the youngest group (22–33 yr), averaging 60% of the latter.

Table II is a summary of analytical data on the eight products. There were no significant differences in any of the analytical figures with age, and the values given are averages, usually of four or five samples. The set of products from human costal cartilage resembles that from bovine nucleus pulposus in having a high hexose content, both sets differing in this respect from the set of products derived from bovine nasal cartilage. This resemblance is made evident by including in the table data for the corresponding products from bovine nucleus pulposus where they exist. Considering first the set of products from human costal cartilage, PPL 3 to PPL 6 in this order, there are obvious regularities: the protein

Diagram 1



content increases, hexuronate and hexosamine decrease, hexose is constant, and hydroxyproline increases enormously. The corresponding set of products from bovine nucleus pulposus considered by itself shows qualitatively parallel changes. Individual comparison of corresponding members of the two sets shows that for the human products, hexuronate is always higher than, hexose is always lower than, hexosamine is always equal to, and protein nearly always equal to the values for products from bovine nucleus pulposus. Thus, there is a great similarity in the patterns of the analytical data on the four fractions of PPL from human costal cartilage and from bovine nucleus pulposus, although the yields from the human tissue, 7.3% before and 5.2% after fractionation, are so much lower than those from the bovine tissue, 45% before and 35% after fractionation.

Earlier work with bovine nasal cartilage had shown that not all of its polysaccharide could be extracted with

TABLE I

*Yields of the Final Products Isolated by the Method Outlined in Diagram 1 from Human Costal Cartilage of Individuals of Different Ages**

Age	Dry cartilage weight	PPL 3	PPL 4	PPL 5	PPL 6	PPL 2H	PPL 2R	CRRF 25	CRRF 75
yr	*g*								
22	11.1	0.0336	0.0048	0.0221	0.0048	0.0620	0.0711	0.0143	0.0080
29	24.0	0.0276	0.0039	0.0364	0.0112	0.0514	0.0198	0.0425	0.0234
33	18.5	0.0154	0.0023	0.0164	0.0183	0.0290	0.0183	0.0735	0.0372
36	20.0	0.0145	0.0028	0.0179	0.0098	0.0278	0.0246	0.0950	0.0202
37	25.0	0.0135	0.0042	0.0199	0.0068	0.0116	0.0221	0.0436	0.0142
40	30.2	0.0212	—	0.0195	0.0129	0.0226	0.0182	0.0523	0.0143
46	20.8	0.0128	0.0029	0.0262	0.0094	0.0403	0.0283	0.0078	0.0289
50	14.2	0.0106	0.0044	0.0176	0.0103	0.0293	0.0229	0.0112	0.0063
69	30.0	0.0176	0.0016	0.0098	0.0028	0.0123	0.0071	0.0293	0.0112
Average values									
22–33		0.0255	0.0037	0.0250	0.0114	0.0475	0.0364	0.0434	0.0229
33–40		0.0164	0.0035	0.0191	0.0098	0.0207	0.0216	0.0636	0.0162
46–69		0.0137	0.0030	0.0179	0.0075	0.0273	0.0194	0.0161	0.0155
Ratio of average yields at 46–69 to those at 22–33		0.54	0.81	0.71	0.66	0.57	0.53	0.37	0.68

* Yields as grams per gram of dry cartilage.

water or dilute salt solution in the form of the water-soluble PPL, part remained in the water-insoluble PPH, and part in the cartilage residue, CR. From either of these insoluble materials a water-soluble proteinpolysaccharide could be isolated by the action of dilute neutral aqueous hydroxylamine (4), which resembled PPL analytically and was called PPL 2. Treatment of the insoluble PPH and of the cartilage residue of human costal cartilage yields from each a water-soluble product called PPL 2H and PPL 2R. Table II shows that in composition they lie between PPL 3 and PPL 5.

The cartilage residue, CRR, which remains after extraction with both water and hydroxylamine, retains polysaccharide which is released in water-soluble form

TABLE II

Analytical Data on Products from Human Costal Cartilage and for Comparison Data on Similar Products from Bovine Nucleus Pulposus

Product	Source	Protein	Hexuronate	Hexose	Hexosamine	Sulfate	Sialate	Hydroxyproline
		%	%	%	%	%	%	%
PPL 3	HCC	10.2	18.2	11.2	20.0	18.0	1.0	0.03
	BNP	14.4	14.7	15.8	20.2	16.4	2.8	0.03
PPL 4	HCC	15.9	16.2	11.7	17.6	16.2	1.1	0.38
	BNP	18.9	9.0	15.8	18.3	16.5	3.4	0.07
PPL 5	HCC	32.2	8.9	12.4	15.6	13.4	1.6	1.02
	BNP	31.8	7.8	15.0	17.4	15.2	3.7	0.21
PPL 6	HCC	47.3	8.9	10.7	13.1	13.7	1.5	1.67
	BNP	48.0	6.6	12.5	14.0	13.6	3.0	0.18
PPL 2H	HCC	22.9	10.3	11.5	18.0	16.2	1.6	0.35
PPL 2R	HCC	16.7	15.9	11.7	18.5	20.6	1.1	0.61
CRRF 25	HCC	38.5	11.3	10.1	13.8	16.4	2.3	0.22
CRRF 75	HCC	32.0	3.9	23.1	15.8	13.4	1.1	0.27

HCC, human costal cartilage; BNP, bovine nucleus pulposus.

by the action of collagenase. The product is easily separated into the two fractions, CRRF 25 and CRRF 75. The first of these analytically most nearly resembles PPL 5 and the second differs mainly in its higher hexose content and lower hexuronate content.

Amino acid profiles. Table III gives the results of amino acid analyses for six of the products from human costal cartilage and, in addition, to facilitate direct comparison, results for PPL 3 and PPL 5 from bovine nasal cartilage and bovine nucleus pulposus. Preparations of PPL 3 from the three tissues show closely similar profiles, and so do preparations of PPL 5. Comparison of the three sets of figures for PPL 3 with the three sets for PPL 5 shows the clearest differences to be in the lower serine and in the higher aspartic acid, tyrosine, and arginine of PPL 5. For most other amino acids, the figures for PPL 3 and PPL 5 overlap. The figures for the products extracted with NH_2OH and those obtained after collagenase treatment also generally fall within the ranges of the figures for PPL 3 and PPL 5.

Physical characterization of the fractions. As a first step in the physical characterization of the proteinpolysaccharide fractions, analogous fractions from costal cartilages of several ages were studied in sedimentation velocity experiments. Samples were dissolved (0.5%) in an aqueous solvent containing 0.10 M KCl, 0.02 M KH_2PO_4, and 0.03 M K_2HPO_4 (pH 6.95). Solutions were examined in double sector cells at 56,000 rpm and 20°C in the Spinco Model E analytical ultracentrifuge. Photographs were taken with the schlieren optical system at 8-min intervals after reaching operating speed, usually for a duration of 80 min.

Sedimentation patterns were similar for analogous fractions from series of proteinpolysaccharides from each age studied. Examples of the sedimentation patterns for PPL 3, PPL 5, PPL 2H, PPL 2R, CRRF 25, and CRRF 75 from the costal cartilage of an individual aged 46 yr are shown in Figs. 1 and 2.

PPL 3. The schlieren pattern for PPL 3 in each case studied (ages 22, 29, 37, 46) showed a single major component (strip *a*, Fig. 1, age 46). Sedimentation coefficients were determined for PPL 3 from each of these costal cartilages over a concentration range from 0.2 to 0.6%, and the values for $s^\circ_{20,w}$, obtained by extrapolation of sedimentation coefficients to zero concentration and correction for solvent density and viscosity, are given in Table IV. The partial specific volume, $\bar{v}$, of PPL 3 (ages 22 and 29) was calculated from density determinations of solution whose concentration ranged from 0.3 to 0.7%. The dependence of partial specific volume on concentration was first evaluated according to the relationship

$$1 - \bar{v}\rho = (1 - w)\frac{1}{A}\frac{dA}{dw}$$

where A is the mass of the solution in the pycnometer, w is the weight fraction of solute, and ρ the solution density. A plot of A against w was linear and indicated

TABLE III

Amino Acid Profiles of Proteinpolysaccharides of Human Costal Cartilage and for Comparison Two from Bovine Nasal Cartilage and Two from Bovine Nucleus Pulposus

	PPL 3			PPL 5			PPL 2H	PPL 2R	CRRF 25	CRRF 25
Source...	HCC	BNC	BNP	HCC	BNC	BNP	HCC	HCC	HCC	HCC
Gly	142	126	124	102	148	87	107	133	114	137
Glu	128	132	133	118	119	119	136	133	109	105
Pro	87	89	90	81	92	100	94	95	68	121
Ser	123	127	128	70	72	76	82	106	70	72
Asp	62	68	71	85	89	82	80	69	109	76
Leu	88	84	82	82	76	81	84	86	67	56
Ala	68	83	65	77	77	66	79	72	66	67
Val	78	63	70	67	58	70	73	83	65	83
Thr	76	60	74	77	51	70	73	74	70	90
Arg	27	33	29	69	51	55	44	32	59	43
Ile	32	40	40	41	35	39	39	36	40	36
Phe	19	34	34	26	35	39	40	25	31	38
Lys	35	29	21	29	43	37	24	24	43	22
Tyr	27	11	11	48	25	31	24	20	38	30
His	7	19	8	18	14	19	11	8	13	17
Cys	Tr	Tr	13	Tr	8	22	Tr	Tr	26	Tr
Met	Tr	3	7	10	7	8	11	5	11	6

HCC, human costal; BNC, bovine nasal cartilage; BNP, bovine nucleus pulposus. Figures are amino acid residues per 1000 amino acid residues.

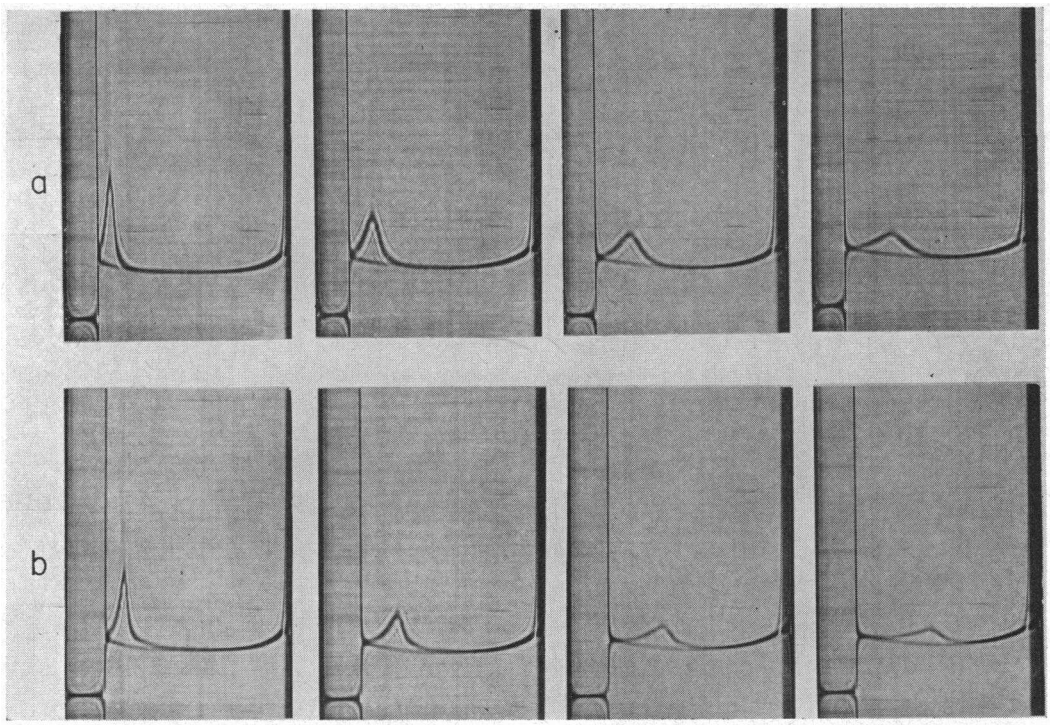


FIGURE 1 Schlieren patterns for PPL 3 (strip *a*) and PPL 5 (strip *b*). Sedimentation velocity experiment were carried out at 56,000 rpm at 20°C at concentrations of 5 mg/ml. Photographs are at 16-min intervals after reaching operating speed.

that partial specific volume was independent of concentration over this concentration range. Partial specific volumes, Table IV, were calculated from the average of seven apparent partial specific volume determinations. Viscosity measurements made on the same solutions used for density determinations gave intrinsic viscosities $[\eta]$ in Table IV.

Sedimentation equilibrium experiments were carried out at 6000 rpm, 20°C, and over a concentration range of 0.02–0.05%, in order to minimize effects of concentration dependence. These conditions represent a combination of the lowest operating speed and concentration range compatible with the requirements of the Yphantis technique, i.e., that the concentration of solute in the region of the meniscus at equilibrium be essentially zero. Plots of ln c, the natural logarithm of fringe displacement in centimeters, versus r^2, the distance from the axis of rotation in centimeters squared, are shown in Fig. 3. Plots *A, B,* and *C* represent the points (with net fringe displacement greater than 0.010 cm) from experiments at initial concentrations of 0.02, 0.04, and 0.05% PPL 3, respectively. At 0.05%, apparently due to the effect of concentration dependence, the upward curvature characteristic of polydispersity is slight. However, at lower concentrations of 0.02 and 0.04% the upward curvature of plots of ln c versus r^2 becomes pronounced, and the polydispersity of PPL 3 is clearly demonstrated. The straight lines are plotted from the averaged values of each slope (d ln c/dr^2) and *y* intercept calculated from a least squares treatment of the experimental points. Weight average molecular weights calculated from the averaged value of d ln c/dr^2 obtained by the least squares treatment as given in Fig. 4. The marked dependence of the apparent weight average molecular weight of PPL 3 on concentration in sedimentation equilibrium experiments is reflected in the large value of the negative slope displayed in Fig. 4. A weight average molecular weight of 959,000 for PPL 3 was obtained by extrapolation to zero concentration.

PPL 4. PPL 4 showed two major components corresponding in sedimentation velocity to PPL 3 and the more slowly sedimenting component of PPL 5 described below. Yields of PPL 4 were extremely small and insufficient for physical characterization.

PPL 5. Characteristic schlieren patterns for PPL 5 (0.5%) are shown in strip *a*, Fig. 2. Two main components are demonstrated. During acceleration to 56,000 rpm, a rapidly sedimenting component appears as a dark layer at the bottom of the cell, immediately followed by a hypersharp schlieren peak (age 46: strip *a* 1, 30,000 rpm; *a* 2, 40,000 rpm. On approaching 56,000 rpm, or in the first several minutes after reaching this operating

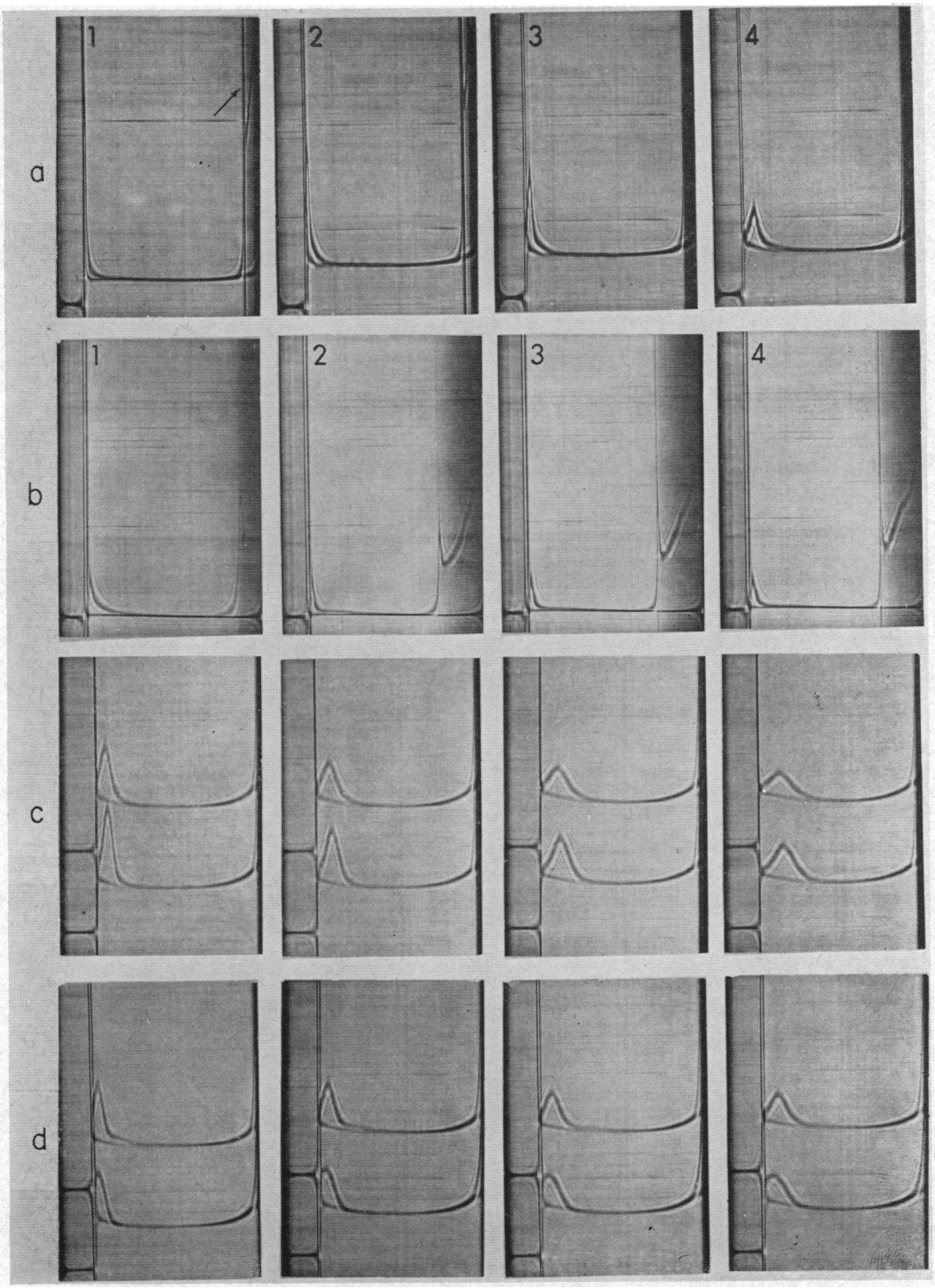


FIGURE 2 Strip *a:* Schlieren pattern of PPL 5 (5 mg/ml) before separation of slower (PPL 5α) and faster sedimenting components. *a 1:* 30,000 rpm, arrow points to hypersharp schlieren peak of the faster sedimenting component; *a 2:* 40,000 rpm; *a 3:* 56,000 rpm; *a 4:* 8 min after reaching 56,000 rpm. Strip *b:* Faster sedimenting component of PPL 5 (5 mg/ml) examined at 10,000 rpm; *b 1:* 2 min; *b 2:* 16 min; *b 3:* 24 min; *b 4:* 32 min. Strip *c:* Schlieren patterns of PPL 2H (top) and PPL 2R (bottom); photographs at 16, 24, 32, and 40 min after reaching operating speed (56,000 rpm). Strip *d:* Schlieren patterns of CRRF 25 (top) and CRRF 75 (bottom) at 16, 24, 32, and 40 min at 56,000 rpm.

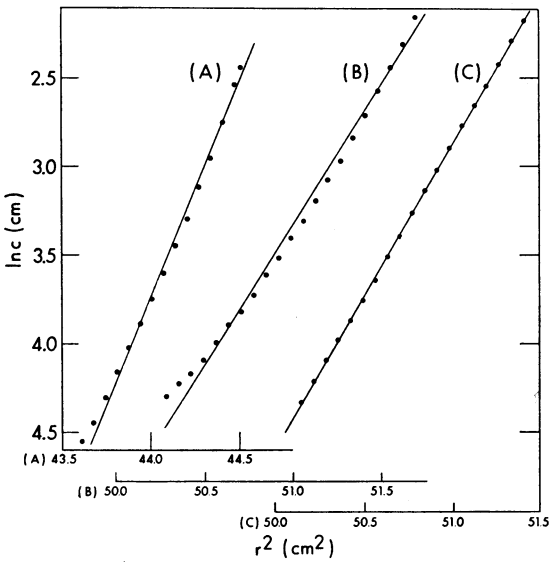


FIGURE 3 Results of sedimentation equilibrium study of human costal PPL 3 at three concentrations: *A*, 0.2; *B*, 0.4; *C*, 0.5 mg/ml. The abscissas show square of the distance from the axis of rotation (centimeters); ordinates, natural logarithm of the fringe displacement (centimeters).

speed, the hypersharp schlieren peak merges with the compacted layer of solute at the bottom of the cell, as the boundary of a second more slowly sedimenting component of PPL 5 moves away from the meniscus (age 46: *a* 3, 56,000 rpm; *a* 4, 8 min after reaching 56,000 rpm). This second major component of PPL 5, based on its faster sedimentation velocity as compared to PPL 3, appeared to be distinct from PPL 3.

Precise comparison of the slowly sedimenting component of PPL 5 with PPL 3 necessitated that this component be separated from the rapidly sedimenting component of PPL 5. In addition, satisfactory examination of the rapidly sedimenting component required that it be

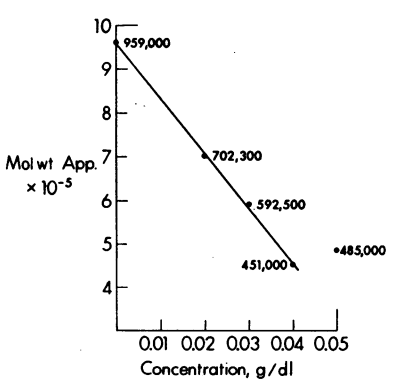


FIGURE 4 Concentration dependence of apparent weight average molecular weights from sedimentation equilibrium study of human costal PPL 3.

TABLE IV

Physical Characteristics Measured on PPL 3 and PPL 5α from Human Costal Cartilages of Four Ages

	PPL 3				PPL 5α
Age	$s^\circ_{20,w}$	$\bar{v}$	$[\eta]$	*Mol wt*	$s^\circ_{20,w}$
yr					
22	8.27	0.571	1.0	950,000	9.03
29	7.98	0.563	0.99		
37	7.51				8.91
46	7.57				9.13

$s^\circ_{20,w}$, sedimentation constant in water at 20°C; $\bar{v}$, partial specific volume; $[\eta]$, intrinsic viscosity.

studied in sedimentation velocity experiments at much lower speeds. The rapidly and slowly sedimenting components of PPL 5 were, therefore, separated from each other. Originally, both components of PPL 5 had sedimented in 1 hr at 40,000 rpm in 2.5 N potassium acetate. Based upon the differences in their sedimentation velocities at 56,000 rpm in the phosphate buffer, it seemed likely that a separation could be achieved in 0.15 M KCl. In a series of experiments in the analytical ultracentrifuge, it was found that at 40,000 rpm in either 0.15 M KCl or 0.15 M potassium acetate, the rapidly sedimenting component had sedimented to the base of the cell within a half hour while the boundary of the slowly sedimenting component had barely moved away from the meniscus. The two components were separated in a preparative ultracentrifuge at 40,000 rpm in 0.15 M potassium acetate for 30 min.

Strip *b*, in Fig. 1, shows the purified slowly sedimenting component of PPL 5 (age 46), which we call PPL 5α, examined in the analytical ultracentrifuge under exactly the same conditions as PPL 3 (age 46), shown in strip *a*. The concentration of solute in both cases is 0.5%, and photographs are at 16-min intervals after reaching 56,000. PPL 5α showed a higher sedimentation constant ($s^\circ_{20,w}$, Table IV) than PPL 3 in every case. On analysis, (Table V), PPL 5α showed more than double the protein content, double the keratan sulfate content, and half as much chondroitin sulfate as PPL 3.

The rapidly sedimenting component of PPL 5, which we call PPL 5γ, was examined at low speeds in the analytical ultracentrifuge after removal of all or most of PPL 5α from preparations of PPL 5. In a series of experiments at operating speeds ranging from 1000 to 10,000 rpm, no discrete visible boundary moved from the meniscus across the cell as the layer of compacted solute formed at the base of the cell. In Fig. 2, strip *b* shows the appearance of the heavy component of PPL 5 at 10,000 rpm. Under these conditions, its packed volume

TABLE V

Analytical Data on the Two Fractions, PPL 5α and PPL 5γ, Prepared from PPL 5 of Two Cases, 37 and 46 yr Old, Compared with PPL 3 from the Same Two Cases

	PPL 3		PPL 5g		PPL 5γ	
Age, yr...	37	46	37	46	37	46
% Uronate	16.4	15.0	7.5	7.2	6.7	5.9
% Galactosamine	16.6		7.5			
% Hexose	11.0	11.8	13.8	16.7	15.1	11.7
% Glucosamine	4.7		9.9			
% Sialate	1.6	2.1	2.9	3.3	2.7	2.8
% Protein	10.5	11.0	21.6	29.0	40.0	41.5

is now roughly one-fourth that of the solution volume in the cell. These results might indicate that PPL 5γ is an extremely polydisperse system of extremely high molecular weight.

PPL 6. PPL 6, after separation from the lower molecular weight fractions, is incompletely soluble.

PPL 2H, PPL 2R, CRRF 25, and CRRF 75. Sedimentation patterns for these fractions are shown in Fig. 2, strips *c* and *d*. Each fraction showed marked polydispersity, indicated by the spreading of the boundary with time. Their average sedimentation velocities are lower than that of PPL 3.

DISCUSSION

Bovine nucleus pulposus yields 45% of its dry weight in the form of water-soluble PPL, and bovine nasal cartilage yields 37%. Human costal cartilage yields only 7%, and even the action of dilute neutral NH_2OH yields only about 6% more as PPL 2H and PPL 2R of diagram 1. The proteinpolysaccharide of human costal cartilage which resists extraction with NH_2OH can be rendered soluble by destruction of the collagen fibers by autoclaving or by the action of proteases. Of the methods tried, the action of collagenase was found most favorable for the subsequent isolation of the remaining polysaccharide, yielding an additional 6% in the two fractions CRRF of diagram 1. The obstinate resistance to extraction of so large a part of the total proteinpolysaccharide contrasts with the easy solubility of the products once they have been extracted and suggests the possibility that in native human cartilage they were attached to the insoluble collagen framework of the tissue.

The part of the proteinpolysaccharide of human costal cartilage extractable with water as PPL can be fractionated by the method used earlier for the PPL of bovine nasal cartilage and bovine nucleus pulposus, giving a parallel series of fractions. As was pointed out with regard to the proteinpolysaccharides of bovine nucleus pulposus (1), so all human fractions contain hexose and sulfate beyond that expected from their chondroitin sulfate and keratan sulfate contents. Keratan sulfate of skeletal tissue can have molar ratios of sulfate to glucosamine lying between 1 and 2 (12). A marked feature of the human proteinpolysaccharides is the high content of hydroxyproline in some fractions (Table II). This has not been included in Table III, but in the most marked cases, PPL 5, PPL 6, and PPL 2R, this would amount to 30 residues/100. Could this be another aspect of the difficulty of separating the proteinpolysaccharides of human costal cartilage from collagen?

Although PPL 5α and PPL 5γ are of higher molecular weights than PPL 3, these larger species are not simply progressively higher polymers formed by the aggregation of a basic structural unit such as PPL 3, for then their compositions would be the same. In the series PPL 3 to PPL 6, there is a progressive rise in the protein content, a corresponding fall in the polysaccharide content, and a change in the proportions of arginine, aspartic acid, and serine (13). On this basis it was suggested that there could be two distinct proteins involved in forming the higher molecular weight members of the series. Partridge (14) suggested that two kinds of protein seemed to be associated in PPL, one of which he thought to be globular and removable by a chromatographic method.

PPL 3 behaves as a single component in sedimentation velocity studies. To obtain further information in regard to the homogeneity of PPL 3, sedimentation equilibrium experiments were carried out. Strictly homogeneous macromolecular species showing ideal behavior and no concentration dependence give linear plots of ln c versus r^2. Continuously polydisperse systems characteristically show plots of ln c versus r^2 with upward curvature of uniformly increasing slope, indicating a continuous distribution of weight average molecular weights. Concentration dependence or nonideal behavior in the absence of polydispersity results in plots of ln c versus r^2 with downward curvature. At initial solute concentrations frequently used in the Yphantis high speed technique (0.5 mg/ml), linear plots of ln c versus r^2 were obtained. These were apparently due to the balancing out of concentration dependence effects and polydispersity, for at solute concentrations close to the lower limits of resolution with the interference optical system (0.2–0.4 mg/ml), the smooth upward curvature characteristic of polydispersity appeared. PPL 3 is, therefore, a macrocolecular species which is both polydisperse and markedly concentration dependent (Fig. 4), and the value for molecular weight given should be considered a relative value useful for comparing PPL 3 with other protein polysaccharides under identical operating conditions, for example PPL 3 of bovine nucleus pulposus (1).

In the present work no significant differences in the compositions of the fractions were found with age, indicating no change in the proportions of chondroitin sulfate and keratan sulfate. Both Kaplan and Meyer (15) and Mathews and Glagov (16) have reported a large increase in keratan sulfate and a decrease in chondroitin sulfate in whole cartilage with age. However, most of this change occurs during the first three decades of life while most of our samples are from later decades.

Other methods of fractionation of cartilage proteinpolysaccharides have been described. Franek and Dunstone (17) used both rate zonal centrifugation and equilibrium density gradient centrifugation to fractionate those of bovine nasal cartilage. Their starting material for the fractionation was not PPL, but the product earlier called chondromucoprotein which is a mixture of PPL and PPH. They reported analytical values on three products called IIB, IIA, and IIIB which resemble values elsewhere reported (2) for PPL 3, PPL 5, and PPL 6, respectively. A third method of treatment of bovine nasal PPL, which depends on precipitation of calcium phosphate in its presence, also yields three fractions resembling PPL 3, PPL 5, and PPL 6 (18). A proteinpolysaccharide from pig laryngeal cartilage, described by Muir and Jacobs (19), on zone electrophoresis on glass fibers yielded two fractions differing in composition.

A report of Pedrini and Pedrini-Mille (20) described a method to separate the PPL of human costal cartilage by electrophoresis on cellulose acetate membranes into two fractions. The electrophoretically slower component was substantially richer in keratan sulfate and the faster component was richer in chondroitin sulfate.

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