THE ANTIGENIC DETERMINANTS OF THE PROTEIN-POLYSACCHARIDES OF CARTILAGE*, ‡

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The early immunologic studies of the polysaccharides present in cartilage were performed on chondroitin sulfate that had been treated by methods designed to remove as much protein as possible (1, 2). Antigenicity of chondroitin sulfate could not be demonstrated. More recently it has been shown that the anionic polysaccharides of cartilage are firmly bound to noncollagenous protein to form compounds called protein-polysaccharides (3). Cartilage protein-polysaccharides can be fractionated by ultracentrifugation into two products, a light fraction, PP-L, and a heavy fraction, PP-H (4). Both PP-L and PP-H contain chondroitin sulfate and keratan sulfate firmly bound to protein.

Several investigators have reported that the protein-polysaccharides of cartilage are antigenic. Sanders, Mathews, and Dorfman (5) in 1962 reported skin sensitivity in rats immunized with bovine cartilage protein-polysaccharide. In 1963 Di Ferrante (6, 7) described precipitating antibodies to bovine PP-L. White et al. (8, 9) produced both precipitating and agglutinating antibodies to human cartilage protein-polysaccharide, and showed that the antigenicity was closely associated with the protein moiety. Di Ferrante and Pauling (10) in 1964 reported the production of agglutinating antibodies to bovine PP-L that cross-reacted with human and porcine PP-L. The antigenic determinant responsible for the cross-reactions was thought to be chondroitin sulfate A. Loewi (11) produced precipitating and agglutinating antibodies to porcine cartilage protein-polysaccharides, and recently Loewi and Muir (12) reported that the cross-reacting antigenic determinant in porcine cartilage protein-polysaccharide was not chondroitin sulfate.

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In each of the above studies animals were immunized with cartilage protein-polysaccharide obtained from a single species. To evaluate further the antigenic determinants of PP-L, antisera were prepared in rabbits to bovine, human, and porcine PP-L. Antisera to PP-L from each species were studied by both precipitation and agglutination techniques with PP-L from all three species. The results indicate that PP-L contains a minimum of two antigenic determinants; one is species specific, and the other is present in all three species. Both antigenic determinants appear to be in, or closely associated with, the protein moiety of PP-L.

Methods and Materials

Materials.—Samples of normal bovine, human, and porcine cartilage were obtained immediately after death. The nasal septum was used as a source of bovine and porcine cartilage while both human articular and costal cartilages were employed. Cartilage protein-polysaccharides were isolated by rapid homogenization in water (2) and fractionated into PP-L and PP-H by ultracentrifugation (3). Chondroitin sulfate and keratan sulfate were prepared by ethanol precipitation of bovine PP-L that had been digested first with pepsin (pH 1.0, 3 hr, 43°C), then with trypsin (pH 7.2, 16 hr, 37°C) and finally with papain (pH 5.3, 20 hr, 53°C). These products were dried and stored as powders at 20°C.

Immunization.—Rabbits were immunized with 2.5 mg (1 mg/ml) of either bovine PP-L, human PP-L, human PP-H, or porcine PP-L,[1] mixed with an equal volume of Freund's complete adjuvant. Booster injections of 1 mg (1 mg/ml) were given 4 wk after the start of immunization and approximately every 4 wk thereafter. Blood was drawn 10 to 14 days after booster injections, the serum separated, and stored at −20°C.

Skin Tests.—The immunized rabbits were injected intradermally 8 wk after the start of immunization with 0.1 ml (1 mg/ml) of the product used for immunization. Some rabbits were also tested with chondroitin sulfate, keratan sulfate, and with PP-L from all three species.

Immunoelectrophoresis and Agar Diffusion Studies.—Immunoelectrophoresis was performed as described elsewhere (13, 14). The plates were stained with either buffalo-blue black or toluidine blue. Agar double diffusion studies were also performed (15). In the above studies, the concentration of protein-polysaccharide used was 10 mg/ml.

Agglutination Studies.—Sheep red blood cells[2] were tanned according to the method of Boyden (16) and coated with PP-L. 1.5 ml of PP-L (10 mg/ml) was digested with testicular hyaluronidase[3] (see below) and used to coat 10 ml of 3% suspension of tanned sheep red blood cells. All antisera were absorbed with sheep red blood cells, inactivated at 56°C for 30 min, and then serially diluted. An aliquot (0.07 ml) of the suspension of coated cells were added to each tube. The tubes were then shaken and the test was read after the cells had settled for 16 hr at 4°C. Appropriate controls with coated and uncoated tanned sheep cells were performed.

Precipitation Tests.—A quantitative precipitin study was performed as described by Kabat and Mayer (17). PP-L that had been labelled with Iodine^{131} (I^{131}), dialyzed, and purified by zone electrophoresis at pH 7.4 (to remove denatured PP-L I^{131}) was used as the antigen. 2.0

[1] Porcine PP-L was a generous gift of Dr. Maxwell Schubert, New York University, New York.

[2] Probio, Inc., Nyack, New York.

[3] Wyeth Laboratories, Philadelphia.

ml of antiserum was added to increasing amounts of PP-L-I^{131}; the contents of the tubes were mixed, incubated for 30 min at 37°C and then placed at 4°C. After 21 days at 4°C, the precipitate was removed from each tube by centrifugation, washed (× 2), and the amount of I^{131} present in the precipitate was determined. When PP-L-I^{131} from the three different species were used to study the same antiserum, the specific activity (CPM/mg protein) of each preparation was the same. Appropriate controls with normal rabbit serum and with antigen alone were performed.

Absorption of Antiserum.—Lyophilized normal serum was added to antiserum, incubated at 37°C for 30 min, allowed to stand for 10 hr at 4°C, and the precipitate removed by centrifugation. This process was repeated until no additional precipitate formed. Thoroughness of absorption was checked by hemagglutination tests with sheep red blood cells coated with human serum. Titers of <1:10 indicated adequate absorption. All antisera used in the agar and agglutination studies were absorbed with serum from the homologous species.

Absorption of certain antisera was also similarly performed with human blood group substances, calf thymus nucleoprotein, human collagen, human normal hyaluronateprotein, bovine testicular hyaluronidase, chondroitin sulfate, keratan sulfate, heparin, polystyrene sulfate, and PP-L from each species. 1 ml of antiserum was incubated with 10 mg of each of the above products. A second absorption with 5 mg of product was then performed.

Enzymatic Digestions.—10 mg of PP-L or PP-H was dissolved in 0.9 ml of water. 0.1 ml of buffer (1.5 M NaCl, 0.075 M NaAc, pH 4.7) containing 1 mg testicular hyaluronidase (1 mg contained 500 turbidity reducing units) was added and incubation performed at 37°C for 18 hr.

2 mg of trypsin[4] was added to 1 ml (10 mg/ml) of PP-L in buffer (0.04 M phosphate, 0.1 M NaCl, pH 7.2) and incubated at 37°C for 16 hr.

Chemical Analysis.—The protein-polysaccharides used in these studies were analyzed for protein (18), hexuronic acid (19), hexosamine (20), hexose (21, 22), and sialic acid (23).

TABLE I

Chemical Analysis of Polysaccharides Isolated from Cartilage Per Cent of Dry Weight

	Human PP-L		Human PP-H		Bovine PP-L	Porcine PP-L	Chondroitin sulfate	Keratan sulfate
	Articular	Costal	Articular	Costal				
Protein	30.2	31.5	67.5	70.3	15.1	20.6	0.32	4.1
Hexosamine	15.3	15.7	10.6	10.8	26.7	17.8	1.0	17.0
Hexuronic acid	9.6	9.3	3.9	3.3	25.1	19.3	22.4	2.3
Hexose	11.2	11.8	9.3	9.0	5.94	6.0	5.8	22.3
Sialic acid	3.2	2.6	1.7	1.4	0.80	*	*	*

* Not determined.

RESULTS

Analytical Data.—The chemical composition of the products used in these studies is shown in Table I.

Skin Tests.—Each rabbit developed positive skin tests when injected intra-

[4] Tryptar, Armour Pharmaceutical Co., Chicago.

dermally with the protein-polysaccharide used in its immunization. Erythema started after 4 hr and reached peak intensity at 24 hr. Sections of skin obtained

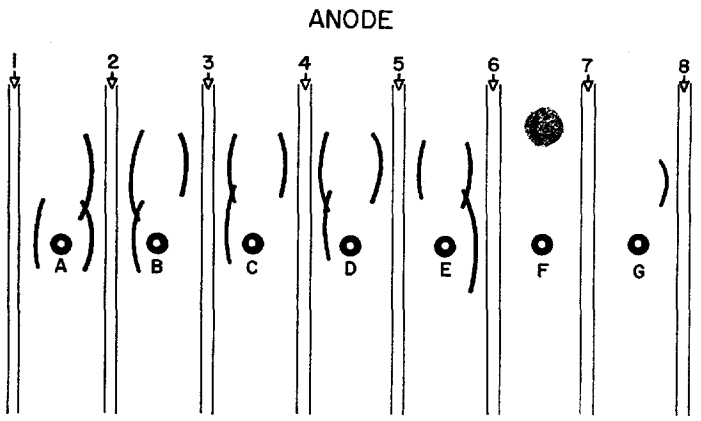

FIG. 1. Composite diagram of immunoelectrophoretic studies. Wells A and B, human PP-L; C, bovine PP-L; D, human PP-H; E, porcine PP-L; F, chondroitin sulfate, and G, human serum albumin. Trough 1, antiserum to human PP-L absorbed with bovine PP-L; 2, antiserum to human PP-L; 3, 5, and 7, antiserum to bovine PP-L absorbed with testicular hyaluronidase; 4, antiserum to human PP-H; 6, antiserum to porcine PP-L; and 8, antiserum to human serum albumin.

All protein-polysaccharides were digested with testicular hyaluronidase prior to electrophoresis. All antisera were absorbed with normal serum from the homologous species. After electrophoresis chondroitin sulfate (stippled area) was localized by staining with toluidine blue.

TABLE II

Antigenic Determinants of Polysaccharides isolated from Cartilage

	Common determinant(s)	Species specific determinant(s)
Bovine PP-L	F	S_{bovine}
Human PP-L	F	S_{human}
Human PP-H	F	S_{human}
Porcine PP-L	F	$S_{porcine}$
Chondrotin sulfate	None	None
Keratan sulfate	None	None

by biopsy showed edema, vasculitis, and infiltration with predominantly polymorphonuclear cells. Prior digestion of the protein-polysaccharide with testicular hyaluronidase neither lessened nor intensified the positive skin tests. A rabbit immunized with human PP-L also developed positive skin tests with either bovine or porcine PP-L. The same rabbit had a negative skin test with either chondroitin sulfate or keratan sulfate.

Immunoelectrophoresis and Agar Diffusion.—The results obtained on immunoelectrophoresis of bovine, human, and porcine PP-L were similar. When human PP-L was dissolved in water forming highly viscous solutions, the PP-L did not migrate in agar and no precipitin arcs were obtained with antiserum to human PP-L. When viscosity was decreased by dissolving the PP-L in 0.15 M

TABLE III

Precipitin Arcs Obtained on Immunoelectrophoresis

Antiserum to	Antigen (digested with hyaluronidase)					
	Bovine PP-L	Human PP-L	Human PP-H	Porcine PP-L	Chondroitin sulfate	Keratan sulfate
Bovine PP-L	F S_{bovine}	F —	F —	F —	None	None
Human PP-L	F —	F S_{human}	F S_{human}	F —	None	None
Porcine PP-L	F —	F —	F —	F $S_{porcine}$	None	None
Human PP-L absorbed with bovine PP-L	None	— S_{human}	— S_{human}	None	None	None
Human PP-L absorbed with porcine PP-L	None	— S_{human}	— S_{human}	None	None	None
Human PP-L absorbed with human PP-L	None	None	None	None	None	None
Human PP-L absorbed with human PP-H	None	None	None	None	None	None
Human PP-L absorbed with chondrotin sulfate	F —	F S_{human}	F S_{human}	F —	None	None
Human PP-L absorbed with keratan sulfate	F —	F S_{human}	F S_{human}	F —	None	None

NaCl instead of water, a long faint arc was obtained with antiserum to human PP-L which extended from the center well toward the anode. When hyaluronidase-digested human PP-L was used, two arcs were formed with antiserum to human PP-L (Fig. 1, Tables II and III), one (F) that migrated slightly more rapidly (than albumin) and one (S) more slowly with a mobility in the alpha$_2$ globulin zone. When hyaluronidase-digested PP-L was digested with trypsin no arcs were obtained. Identical results were obtained with human PP-H.

When hyaluronidase-digested human PP-L was studied with antiserum to either bovine or porcine PP-L only the F arc was obtained (Fig. 1, Table III). If hyaluronidase-digested human PP-L was studied with antiserum to human PP-L that had been absorbed with either bovine or porcine PP-L only the S arc was obtained. When hyaluronidase-digested human PP-L was studied with antiserum to human PP-L absorbed with chondroitin sulfate both F and S were still formed. Identical studies using either hyaluronidase-digested bovine or

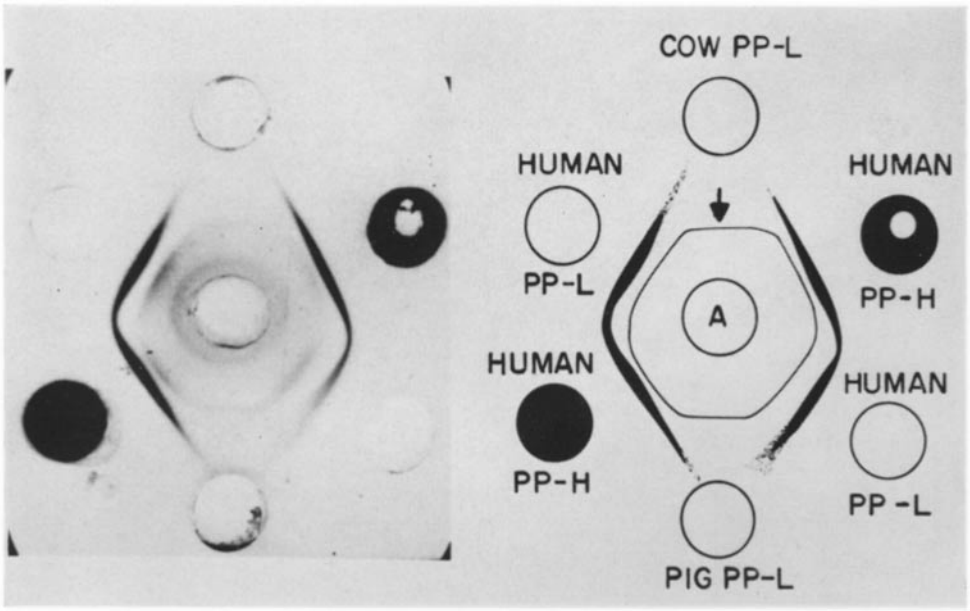

FIG. 2. Agar double diffusion study. A, antiserum to human PP-L absorbed with human serum. All protein-polysaccharides were digested with testicular hyaluronidase. The inner circle of precipitin lines (arrow) is due to the common antigenic determinant F. The outer precipitin lines in the human PP-L and PP-H are due to the species specific determinant S_{human}.

porcine PP-L yielded qualitatively identical results (Fig. 1, Tables II and III). Since the S component was immunologically distinct in each species, the designations S_{bovine}, S_{human}, and $S_{porcine}$ were employed. Chondroitin sulfate and keratan sulfate formed no precipitin arcs with antisera produced to PP-L from any of these 3 species (Table III). Chondroitin sulfate could be located after electrophoresis in agar by staining with toluidine blue. It migrated more rapidly than F.

Bovine testicular hyaluronidase formed no precipitin arcs with antiserum to either human or porcine PP-L but formed a precipitin arc with antisera to

bovine PP-L which migrated slightly more slowly than S_{bovine}. It was, therefore, necessary to absorb the antisera to bovine PP-L with testicular hyaluronidase in all the agar and agglutination studies.

TABLE IV

Agglutination Studies with PP-L

Antiserum	Antigen used to coat tanned sheep RBC	Titer
Anti-human PP-L	Human PP-L	1:10,240
	Bovine PP-L	1:5,120
	Porcine PP-L	1:2,560
	Chondroitin Sulfate	<1:10
Anti-bovine PP-L	Bovine PP-L	1:20,480
	Porcine PP-L	1:1,280
	Human PP-L	1:2,560
	Chondroitin Sulfate	<1:10
Anti-porcine PP-L	Porcine PP-L	1:10,240
	Bovine PP-L	1:1,280
	Human PP-L	1:5,120
	Chondroitin Sulfate	<1:10
Anti-human PP-L absorbed with human PP-L	Human PP-L	<1:10
	Bovine PP-L	<1:10
	Porcine PP-L	<1:10
Anti-human PP-L absorbed with bovine PP-L	Human PP-L	1:5,120
	Bovine PP-L	<1:10
	Porcine PP-L	<1:10
Anti-human PP-L absorbed with porcine PP-L	Human PP-L	1:2,560
	Bovine PP-L	<1:10
	Porcine PP-L	<1:10
Anti-human PP-L absorbed with chondroitin sulfate	Human PP-L	1:10,240
	Bovine PP-L	1:5,120
	Porcine PP-L	1:2,560
Anti-human PP-L absorbed with keratan sulfate	Human PP-L	1:10,240
	Bovine PP-L	1:5,120
	Porcine PP-L	1:2,560

When agar double diffusion was used to compare hyaluronidase-digested bovine PP-L, human PP-L, human PP-H, and porcine PP-L with an antiserum produced to either human PP-L or human PP-H the results shown in Fig. 2

were obtained. Human PP-L formed two lines which fused completely with the 2 lines obtained with human PP-H. Porcine and bovine PP-L each formed only

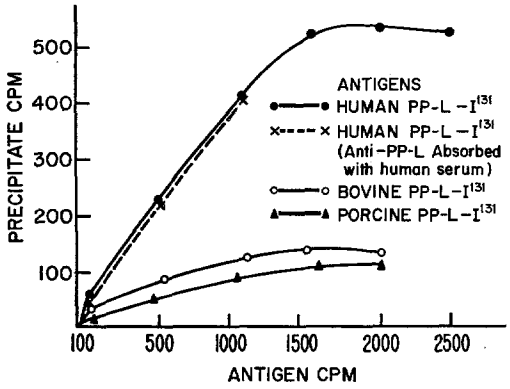

FIG. 3. Precipitin studies using antiserum to human PP-L and PP-L labeled with I^{131}. CPM/mg protein were the same in each of the above studies (10,000 CPM = 0.2 mg protein). For details see text.

TABLE V

Effect of Prior Absorption of Antiserum to Human PP-L on Agglutination of Cells Coated with Human PP-L (Hyaluronidase-Treated)

Material used to absorb antiserum	Titer
None	1:10,240
Human PP-L	<1:10
Human serum	1:10,240
Human blood group substance (A and B)	1:10,240
Human normal hyaluronateprotein	1:5,120
Human collagen	1:10,240
Bovine testicular hyaluronidase	1:10,240
Bovine thymus nucleoprotein	1:10,240
Keratan sulfate	1:10,240
Chondroitin sulfate	1:10,240
Heparin	1:10,240
Polystyrene sulfate	1:10,240

one line (F) which fused completely with the inner line (see arrow, Fig. 2) formed by the human products.

Agglutination Studies.—Antisera produced to PP-L from any of the 3 species agglutinated tanned sheep red blood cells coated with either bovine, human, or porcine PP-L. Absorption of antiserum to human PP-L with either bovine or porcine PP-L only slightly reduced the agglutination titer obtained with red

blood cells coated with human PP-L (Table IV). However, absorption of antisera to human PP-L with porcine PP-L reduced the titer obtained with red blood cells coated with bovine PP-L to 1:10 (Table IV). When antiserum to human PP-L was absorbed with a human blood group substance, calf thymus nucleoprotein, human collagen, human hyaluronateprotein, bovine testicular hyaluronidase, chondroitin sulfate, keratan sulfate, or polystyrene sulfate the titer obtained with red blood cells coated with human PP-L was not significantly reduced (Table V).

Precipitin Tests.—The results of a precipitin study with PP-L-I^{131} are shown in Fig. 3. The curves obtained are of interest in several respects: (*a*) absorption of anti-human PP-L with human serum did not remove significant amounts of the antibody to human PP-L-I^{131}; (*b*) the amount of PP-L-I^{131} precipitated was approximately proportional to the amount of antigen added throughout the region of antibody excess; and (*c*) considerably more human PP-L-I^{131} was precipitated with anti-human PP-L than either bovine or porcine PP-L-I^{131}. It was not possible to precipitate 100% of the PP-L-I^{131} in these studies. At the point of equivalence 35% of the added human PP-L-I^{131} was precipitated. The PP-L-I^{131} that remained in the supernatant could not be precipitated either by adding additional anti-human PP-L or anti-rabbit gamma globulin (sheep) to the supernatant. Controls using normal rabbit serum and PP-L-I^{131} were negative.

DISCUSSION

Several technical difficulties were encountered in the demonstration of antibodies to cartilage protein-polysaccharides. The protein-polysaccharide molecules are too large to diffuse well through agar and do not readily coat tanned red blood cells. After the protein-polysaccharides were depolymerized by digestion with testicular hyaluronidase, immunoelectrophoresis, agar double diffusion, and tanned red blood cell agglutination techniques were then used to demonstrate both agglutinating and precipitating antibodies to PP-L from all three species. Because of the presence of rather large amounts of protein in the precipitates, neither hexuronic acid, hexosamine, nor hexose determinations could be reliably used as analytical methods in the precipitation studies. Isotopic techniques were successfully employed for this analysis and since the antigenicity seemed to reside in the protein moiety, I^{131} was used to label the protein-polysaccharide.

The data obtained by immunoelectrophoresis, agar double diffusion, and tanned red blood cell agglutination tests indicated that there were at least two antigenic determinants in bovine PP-L, human PP-L, human PP-H, and porcine PP-L (Table II). On the basis of electrophoretic mobility in agar these determinants have been designated as F (fast) and S (slow). F in each species was immunologically identical and responsible for the cross-reactions between

PP-L from these three species. S in each of these three species was immunologically distinct. Both F and S appear to be closely related to the protein moiety of the protein-polysaccharide for after tryptic digestion neither F nor S could be detected on immunoelectrophoresis. The failure of Di Ferrante to obtain precipitin lines in agar after hyaluronidase digestion of bovine PP-L is not clear. He first digested the PP-L and then attempted to reisolate the products of digestion (7). Since recovery was low (30%), the antigenic determinants may not have been present in the isolated material.

In contrast to the findings of Di Ferrante and Pauling (10), the cross-reacting determinant (i.e. F) in these studies was not chondroitin sulfate. Chondroitin sulfate did not form precipitin arcs with the antisera. Agglutination studies using chondroitin sulfate to coat the red blood cells were consistently negative and absorption of the antiserum with chondroitin sulfate did not remove antibody to F (Fig. 1, Tables III and IV). On agar electrophoresis F migrated slightly slower than chondroitin sulfate (Fig. 1). The fact that the chondroitin sulfate used in the present study was subjected to more rigorous proteolysis than the chondroitin sulfate employed by Di Ferrante and Pauling may explain the differences in the findings. Our results are in agreement with the observations of Loewi and Muir (12) who reported that the cross-reacting determinant of porcine PP-L was not chondroitin sulfate. They speculated that the cross-reacting determinant might be located near the point of attachment of the protein and polysaccharide.

It is not clear whether F and S are both present in the same molecule and are two antigenic determinants on the same molecule, or whether F and S are actually present in two different protein-polysaccharides. The fact that F and S can be separated after hyaluronidase (i.e. nonproteolytic) digestion suggests that F and S may be on different protein-polysaccharides. These protein-polysaccharides may be so entangled and so similar chemically that by most analytical procedures they appear to be a single compound.

The inability to precipitate 100% of PP-L-I^{131} with specific antiserum is not completely clear. Di Ferrante (6) was able to precipitate only 25% of bovine PP-L with anti-bovine PP-L. In the present study at the point of equivalence 35% of the added human PP-L-I^{131} was precipitated with antiserum to human PP-L. It is possible that some of the PP-L was altered by labeling with I^{131} and was no longer reactive with the antiserum. Another possibility is that PP-L is not a single chemical compound but is indeed heterogeneous, and that one (or more) of its constituents was not antigenic in the rabbit.

The most provocative finding in this study is the common antigenic determinant (F) present in bovine, huma, and porcine PP-L. Previous workers (10, 12) have shown that antisera to either bovine or porcine PP-L cross-react with PP-L isolated from a number of heterologous species, but the present study is the first which clearly demonstrates that PP-L from three different species con-

tain the same common antigenic determinant (F). Work is planned to determine whether this determinant is also present in PP-L from other species of vertebrates and in the polysaccharides of microorganisms. The extent to which this common determinant is distributed in living organisms should prove to be of considerable interest from the phylogenetic point of view. It is intriguing to speculate whether this common antigenic determinant may be involved (i.e. through cross-reactions with exogenous antigens) in some of the immunological phenomena observed in connective tissue diseases (24, 25).

SUMMARY

1. Protein-polysaccharides isolated from bovine, human, and porcine cartilage are antigenic in the rabbit.

2. PP-L from each of these species contains a minimum of two antigenic determinants; one is species specific, and one is common to all three species. Human PP-H also contains these two determinants.

3. Both antigenic determinants are in, or closely associated with, the protein moiety of the compound. Chondroitin sulfate and keratan sulfate were not demonstrated to be antigenic in these studies.

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