The Separation of New Forms of the Proteinpolysaccharides of Bovine Nasal Cartilage*

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SUMMARY

The principal proteinpolysaccharide, called PP-L, of bovine nasal cartilage has been fractionated centrifugally to give four products. The method requires low concentration of PP-L, high concentration of salt, and makes use of a remarkable difference between the effects of salts with monovalent and divalent cations on the sedimentation behavior of these proteinpolysaccharides. A fifth product, PP-L2, has previously been isolated from cartilage residue. Of these five, three are almost identical analytically, but differ in the stage of the whole procedure at which their separation becomes possible. The protein contents of the five lie in the range 9 to 36%, but the amino acid profiles of all five are very similar. Of the five forms, the one called PP-L3 behaves in the analytical ultracentrifuge as a single component; the others do not. The two forms with high protein contents sediment most easily and have the highest limiting viscosity numbers. These five products account for 40% of the original dry cartilage weight and 85% of its uronic acid content.

With a high speed homogenizer, 60% of the total chondroitin sulfate of bovine nasal cartilage and 40% of its dry weight can be extracted with water as a product called crude proteinpolysaccharide, PP, separable by centrifugation at 100,000 X g into two fractions; one sedimenting readily, called PP-H; one not sedimenting, called PP-L (1). Both fractions contain protein, chondroitin sulfate, and a small amount of keratan sulfate and sialic acid.

In this earlier work, PP-L (0.6% in 0.15 M KCl), in the analytical ultracentrifuge seemed to sediment as a single peak (1). On free electrophoresis at pH 5 to 9, it migrated as a single peak with nearly the same speed as chondroitin sulfate. On paper electrophoresis it did not move at all, although chondroitin sulfate moves rapidly. On addition of lanthanum chloride to a cold solution of PP-L it is practically completely precipitated as a lanthanum salt which can be converted to a potassium salt with the same composition as the initial PP-L (2). The use of cationic cellulose derivatives in columns seemed to yield no significantly distinct fractions. These methods gave no evidence that PP-L could be fractionated.

Yet it was sometimes observed that solutions of PP-L centrifuged at 100,000 X g yielded more than traces of sediment. This sediment, at first thought to be PP-H that had been incompletely removed, proved, however, to be easily soluble in water. Then it was found that PP-L dissolved in solutions of KCl over 1 M yielded very opalescent solutions which gave large sediments when centrifuged at high speeds. The possibility that this might represent a separation of PP-L into significantly different fractions was further explored, and the results are here reported. The failure to recognize this possibility in earlier sedimentation studies was due to our usual habit of working at too high a concentration of PP-L (>0.5%) and too low a concentration of salt (0.15 M). Separations became marked at lower concentrations (0.2 to 0.05%) of PP-L and higher concentrations of salt (1.0 to 2.5 M). In addition a totally different behavior of PP-L on sedimentation was found when CaCl2 was used in place of KCl. On the basis of such observations a method has been worked out by which bovine nasal PP-L can easily be separated into four fractions.

EXPERIMENTAL PROCEDURE

A simpler method for preparing PP-L from bovine nasal cartilage has been adopted because it was found (3) that both PP-H and the cartilage residue (CR) on treatment with NH2OH (0.2 M, pH 7) yielded the same form of PP, called PP-L2. Since there is no advantage to preparing PP-H as a product separate from CR, the new procedure is designed so that PP-H is included in the cartilage residue (CR of Diagram 1).

Dry Powdered Cartilage—Bovine nasal cartilage fresh from the slaughter house was cleaned in a cold room (5°C) and cut into small pieces. It was powdered and dried simultaneously by running in the VirTis-45 homogenizer (with a double set of blades) with ethanol (2 g with 200 ml) at top speed for 5 min, centrifuging, washing with ether, and drying in a vacuum. The product is a fine white powder, weighs 0.5 g, and can be stored indefinitely.

Diagram 1 shows schematically the procedure for the prep.
The residue left after extraction with NH₄OH is washed with ethanol and ether and dried in a vacuum. It is referred to as CR and weighs 1.80 g.

**Fractionation of PP-L**—Exploratory experiments showed the possibility of fractionating PP-L by stirring it with salt solutions and then centrifuging. The method finally adopted was to add the dry PP-L as a potassium salt to the salt solution and stir for 16 hours at 5°C. The resulting solutions were more or less opalescent depending on the kind and concentration of salt. On centrifuging at 100,000 X g, a part of the PP-L was sedimented and a part stayed in the supernatant solution which was now clear. To estimate the amounts sedimented, the uronic acid contents were measured by the method of Dische (4) in the initial opalescent solution and in the clear solution after centrifuging. Table I summarizes results with different salts at several concentrations of PP-L and of salt. Potassium acetate and calcium chloride were studied over a wider range of PP-L concentrations because these salts were incorporated in the method finally adopted. They were preferred because they are soluble in ethanol and, in using them, precipitation of products with ethanol did not require preliminary dialysis of solutions. At any one concentration of potassium acetate the fraction of PP-L sedimented decreases with rising PP-L concentration, that is, as more PP-L is added less of it sediments. Only at the highest potassium acetate concentration is PP-L completely sedimented at all PP-L concentrations. At any one concentration of PP-L, the fraction of PP-L sedimented increases with the potassium acetate concentration. There are concentration ranges of particular interest because in those the fraction of PP-L sedimented seems to be nearly constant, suggesting that possibly a more significant fractionation occurs in these ranges than elsewhere. From 1.0 to 2.5 N potassium acetate and from 0.2 to 2.0 mg of PP-L per ml the PP-L sedimented lies in the range 60 to 70%. This plateau decided the conditions for the first step in the fractionation of PP-L, Step 3 of Diagram 1.

**Step 3**—PP-L as its potassium salt (1.0 g) from Step 1 is extracted with NH₄OH (250 ml), and the mixture is again centrifuged at 100,000 X g, a part of the PP-L was sedimented and a part stayed in the supernatant solution which was now clear. To estimate the amounts sedimented, the uronic acid contents were measured by the method of Dische (4) in the initial opalescent solution and in the clear solution after centrifuging. Table I summarizes results with different salts at several concentrations of PP-L and of salt. Potassium acetate and calcium chloride were studied over a wider range of PP-L concentrations because these salts were incorporated in the method finally adopted. They were preferred because they are soluble in ethanol and, in using them, precipitation of products with ethanol did not require preliminary dialysis of solutions. At any one concentration of potassium acetate the fraction of PP-L sedimented decreases with rising PP-L concentration, that is, as more PP-L is added less of it sediments. Only at the highest potassium acetate concentration is PP-L completely sedimented at all PP-L concentrations. At any one concentration of PP-L, the fraction of PP-L sedimented increases with the potassium acetate concentration. There are concentration ranges of particular interest because in those the fraction of PP-L sedimented seems to be nearly constant, suggesting that possibly a more significant fractionation occurs in these ranges than elsewhere. From 1.0 to 2.5 N potassium acetate and from 0.2 to 2.0 mg of PP-L per ml the PP-L sedimented lies in the range 60 to 70%. This plateau decided the conditions for the first step in the fractionation of PP-L, Step 3 of Diagram 1.

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**Percentage of uronic acid sedimented at 100,000 X g, at varied PP-L concentration, and varied concentration of several salts**

<table>
<thead>
<tr>
<th>Conc. PP-L</th>
<th>Kind of salt</th>
<th>Uronic acid sedimented at salt concentrations of</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td></td>
<td>0.05 N</td>
</tr>
<tr>
<td>0.2</td>
<td>Potassium acetate</td>
<td>56.3</td>
</tr>
<tr>
<td>0.5</td>
<td>Potassium acetate</td>
<td>49.1</td>
</tr>
<tr>
<td>2.0</td>
<td>Potassium acetate</td>
<td>13.4</td>
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<td>5.0</td>
<td>Potassium acetate</td>
<td>7.2</td>
</tr>
<tr>
<td>10.0</td>
<td>Potassium acetate</td>
<td>0.2</td>
</tr>
<tr>
<td>0.5</td>
<td>Potassium chloride</td>
<td>62.3</td>
</tr>
<tr>
<td>0.5</td>
<td>Potassium sulfate</td>
<td>29.5</td>
</tr>
<tr>
<td>0.5</td>
<td>Lithium acetate</td>
<td>35.6</td>
</tr>
<tr>
<td>0.2</td>
<td>Calcium chloride</td>
<td>72.8</td>
</tr>
<tr>
<td>0.5</td>
<td>Calcium chloride</td>
<td>66.4</td>
</tr>
<tr>
<td>2.0</td>
<td>Calcium chloride</td>
<td>56.4</td>
</tr>
<tr>
<td>0.5</td>
<td>Magnesium chloride</td>
<td>67.0</td>
</tr>
<tr>
<td>0.5</td>
<td>Zinc acetate</td>
<td>67.0</td>
</tr>
</tbody>
</table>
stirred with potassium acetate solution (500 ml, 2.5 N) at 5° for 16 hours. The opalescent solution is centrifuged at 40,000 rpm (100,000 × g) for 1 hour and the clear solution is separated from the well packed residue. The residue is stirred again with potassium acetate solution (500 ml, 2.5 N, 5°, 16 hours) and centrifuged as before. Addition of ethanol (2000 ml) to the combined clear solution causes precipitation of the product, PP-L3, as its potassium salt. Separated, washed, and dried, it weighs 0.207 g. The residue in the centrifuge cups if further extracted with potassium acetate would give very little additional PP-L3. This residue is rinsed out with small amounts of potassium acetate solution, precipitated with ethanol, separated, washed, and dried. It weighs 0.650 g and is called PP-LR and will be further fractionated in Step 4.

A possibility for a second stage of fractionation of PP-L was suggested by the contrasting behavior of the salts with monovalent cations compared with those having divalent cations, shown in Table I. At any one concentration of PP-L, more PP-L was sedimanted the higher the concentration of salts with monovalent cations, and less PP-L was sedimented the higher the concentration of salts with divalent cations. In particular with calcium chloride, over the concentration range of 1 to 4 N, there is a sharp drop in the amount of uronic acid sedimented. A similar experiment with PP-LR also showed a sharp drop in the amount sedimented. Guided by such exploratory experiments, we subjected the product PP-LR to Step 4 of Diagram 1 with calcium chloride at a high concentration; no significant difference was found between 2.5 and 4.0 N CaCl2.

Step 4—PP-LR (0.650 g) is stirred with CaCl2 (325 ml, 2.5 N) at 5° for 16 hours, and the opalescent solution is centrifuged at 40,000 rpm for 1 hour. The clear supernatant solution is removed, ethanol (650 ml) is added to it, and the precipitated product is collected by centrifuging. It is dissolved in water (250 ml) and stirred with Dowex-50 (2.5 g) in its potassium form, and after addition of potassium acetate (2.5 g) to the clear solution the product is precipitated by addition of ethanol (500 ml). It is the potassium salt of PP-LRF, and after drying it weighs 0.467 g.

The residue in the centrifuge cups from the high speed centrifuging is dissolved in water (100 ml); the solution is stirred with Dowex 50 in the potassium form, and after addition of potassium acetate (1 g), the product is precipitated with ethanol (200 ml). It is the potassium salt of PP-LR, and after drying it weighs 0.063 g.

The products so far prepared (PP-L2, PP-L3, PP-LRF, and PP-L6) were studied for sedimentability at a series of salt concentrations (potassium acetate and CaCl2) by the procedure described for Table I and the results are given in Fig. 1. PP-L2 and PP-L3 behave alike; in potassium acetate up to 2.5 N, they are only slightly sedimented, at 4 N they are nearly completely sedimented; in calcium chloride they are scarcely sedimented at any concentration. PP-L6 is nearly completely sedimented at all concentrations of either potassium acetate or calcium chloride. Thus PP-L2, PP-L3, and PP-L6 seem not to be further fractionable at any salt concentration. With PP-LRF the situation is different. With potassium acetate (0.1 to 2.5 N) a considerable fraction remains unsedimented. This is surprising since PP-LRF was made from PP-LR which was made by repeated sedimentation in potassium acetate (2.5 N) until no further significant amount remained in the supernatant solution. A further step in fractionation thus seems possible. This is Step 5 in which potassium acetate at 2.5 N was again chosen.

Step 5—PP-LRF (0.467 g) is stirred with potassium acetate solution (250 ml, 2.5 N) and fractionated exactly as in Step 4 and yields two products, PP-L4 is recovered from the supernatant solution in a yield of 0.124 g and PP-L5 is recovered from the sediment in a yield of 0.267 g.

The whole procedure outlined in Diagram 1 results in the separation of the bovine nasal cartilage powder into an insoluble residue (called CRR) and a series of five products, all called PP-L because of their close relation to the product originally called PP-L. These five protein polysaccharides are all easily soluble in water. Their combined actual yields account for 40% of the dry weight of the cartilage. In Diagram 1 are given in parentheses the yields of products, at each step, in gram per g of dry cartilage; all are calculated from the data given in the description of the separate steps. The total recovery as gram per g cartilage, after each step, calculated from the data of Diagram 1 is as follows: after Steps 1 and 2, 0.967; after Step 3, 0.947; after Step 4, 0.902; after Step 5, 0.882. Since chondroitin sulfate is the dominant polysaccharide in this cartilage, the fraction of total polysaccharide recovered in each of the products can be estimated.

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield of each fraction</th>
<th>Hexurionate</th>
<th>Hexurionate recovered in each fraction</th>
<th>Total hexurionate</th>
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<tr>
<td>Original cartilage</td>
<td>1.0</td>
<td>11.2</td>
<td>0.112</td>
<td>4.4</td>
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<tr>
<td>CRR</td>
<td>0.450</td>
<td>1.1</td>
<td>0.0049</td>
<td>4.4</td>
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<tr>
<td>PP-L2</td>
<td>0.120</td>
<td>24.8</td>
<td>0.0298</td>
<td>28.0</td>
</tr>
<tr>
<td>PP-L3</td>
<td>0.110</td>
<td>27.0</td>
<td>0.0298</td>
<td>20.0</td>
</tr>
<tr>
<td>PP-L4</td>
<td>0.046</td>
<td>25.8</td>
<td>0.0119</td>
<td>10.6</td>
</tr>
<tr>
<td>PP-L5</td>
<td>0.107</td>
<td>19.9</td>
<td>0.0213</td>
<td>19.0</td>
</tr>
<tr>
<td>PP-L6</td>
<td>0.023</td>
<td>15.5</td>
<td>0.0035</td>
<td>3.2</td>
</tr>
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</table>

*Fig. 1. Percentage of uronic acid sedimented after centrifuging (at 40,000 rpm, 60 min) solutions made by stirring each form of PP-L (6 mg) with a salt solution of the indicated normality (12 ml, stirred 16 hours at 5°). O, PP-L2; Δ, PP-L3; V, PP-LR; and □, PP-L6.*
Absorbance (O.la/), of dinitro-
Per cent hexuronate
Per cent sialate
Per cent hexosamine
Per cent hexose (corrected)
Limiting viscosity
Phosphate (0.05 M)
Tris (0.01 M)
Potassium acetate
Calcium chloride
Phosphate buffer at pH 7.0, containing 0.1
KCl. Or-

From the yield of each fraction and its uronic acid content. This
Absorbance, l'$&, 280 rnp
Per cent sedimented in:
0.05
0.01
m

Amino Acid Profile—Amino acid analyses on the final products
of the fractionation are given in Table IV. Included are figures
from earlier work with PP-L2 (3). Since the protein contents of
these products cover a wide range (9 to 36%), all figures are given
as amino acid residues per 1000 residues. Study of these figures
shows a rough parallelism in the amino acid profile, with glycine
and glutamate always highest, glycine, histidine, cystine, and
methionine always lowest. So nearly parallel are the values that
the averages for each amino acid are listed in the last column to
facilitate comparisons. The product which most frequently
deviates most widely from the average is PP-L6 which has the
lowest values for glycine, glutamate, and proline, and the highest
values for aspartate, valine, arginine, and isoleucine. PP-L6 has
extreme values in several other respects: It is obtained in the
from the yield of each fraction and its uronic acid content. This
summarized in Table II from which it appears that 90% of the
uronic acid is accounted for, 86% is in the five PP-L products and
4% is in the final insoluble cartilage residue, CRR.

In Table III are collected analytical data for each of the new
products into which PP-L has been separated as well as data for

PP-L taken from a previous study (2) for comparison. The
analytical methods used were: protein (5), hexuronate (4),
hexosamine (6), galactosamine (7), hexose (8), corrected for color
produced by uronate and hexosamine (2), sialate (9), hydroxy-
proline (10), and chain weight (11). The method used to measure
the percentage sedimented in 2.5 N salt has been described earlier in this paper. The dinitrophenyl derivatives were prepared by the usual method (12), allowing reaction times of 4 and 20 hours. The limiting viscosity number (dl/g) was obtained by extrapolation to zero concentration of the curves of Fig. 2. Relative viscosities were measured in a phosphate buffer (0.05 M, pH 7.0) containing KCl (0.1 M) using a single Ostward viscosimeter.

In this buffer PP-L6 does not dissolve, but it does dissolve in a Tris buffer (0.01 M, pH 7.0, with no added KCl). In this buffer viscosities of both PP-L5 and PP-L6 were measured and are also recorded in Fig. 2 with the use of an expanded abscissa scale and a contracted ordinate. PP-L5 and PP-L6 in the Tris buffer give nearly identical viscosity values.

Of the five products of Table III, three (PP-L2, PP-L3, and
PP-L4) do not show any significant analytical differences. Their
significance as separate entities lies in the stage (Diagram 1) at
which each becomes separable. The other two products, PP-L5 and
PP-L6, are clearly distinct both from each other and from the
first three.


table III
Results of analyses and other procedures on products isolated

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<tr>
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<tr>
<td>Absorbance, 1%, 980 mP</td>
<td>1.10</td>
<td>0.65</td>
<td>0.90</td>
<td>2.50</td>
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</tr>
<tr>
<td>Per cent protein</td>
<td>12.0</td>
<td>8.5</td>
<td>10.0</td>
<td>27.0</td>
<td>36.0</td>
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<tr>
<td>Per cent hexuronate</td>
<td>24.8</td>
<td>27.0</td>
<td>25.8</td>
<td>19.9</td>
<td>15.5</td>
</tr>
<tr>
<td>Per cent hexosamine</td>
<td>23.2</td>
<td>25.5</td>
<td>27.5</td>
<td>23.2</td>
<td>15.0</td>
</tr>
<tr>
<td>Per cent galactosamine</td>
<td>20.3</td>
<td>22.5</td>
<td>21.1</td>
<td>16.5</td>
<td>9.3</td>
</tr>
<tr>
<td>Per cent hexose (corrected)</td>
<td>4.4</td>
<td>2.2</td>
<td>3.8</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Per cent sialate</td>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>1.2</td>
<td>1.3</td>
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<tr>
<td>Per cent hydroxy proline</td>
<td>0.15</td>
<td>0.0</td>
<td>0.0</td>
<td>0.18</td>
<td>0.32</td>
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<tr>
<td>Chain wt × 10⁻⁸</td>
<td>63</td>
<td>90</td>
<td>91</td>
<td>109</td>
<td>100</td>
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<td>Limiting viscosity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate (0.05 M), KCl (0.1 M)</td>
<td>1.1</td>
<td>1.4</td>
<td>1.5</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Tris (0.01 M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Per cent sedimented in:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 N potassium acetate</td>
<td>15.3</td>
<td>6.0</td>
<td>8.0</td>
<td>95.0</td>
<td>96.0</td>
</tr>
<tr>
<td>2.5 N calcium chloride</td>
<td>2.0</td>
<td>1.4</td>
<td>6.0</td>
<td>28.0</td>
<td>86.0</td>
</tr>
<tr>
<td>Absorbance (0.1%) of dinitrophenyl derivative at 355 mP after:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4-hr treatment</td>
<td>0.42</td>
<td>0.18</td>
<td>0.34</td>
<td>1.25</td>
<td>2.14</td>
</tr>
<tr>
<td>20-hr treatment</td>
<td>0.38</td>
<td>0.23</td>
<td>0.35</td>
<td>1.23</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 2. Reduced viscosity number as a function of PP-L concentration for each form of PP-L. Viscosities for 1 (PP-L), 2
(PP-L2), 3 (PP-L3), 4 (PP-L4), and 5 (PP-L5) were measured in 0.05 M phosphate buffer at pH 7.0, containing 0.1 M KCl. Ordinates are at the left, abscissas are at the bottom. Viscosities for 5' (PP-L5) and 6' (PP-L6) were measured in 0.1 M Tris buffer at pH 7.0. Ordinates are at the right, abscissas are at the top.
smallest yield; it has the highest protein content; it has the highest hydroxyproline content; and it is the most difficult to purify. Contamination with collagen could not account for most of its extreme amino acid values. With the possible exception of PP-L6, the products show a similarity in their amino acid pattern indicating a common protein component.

**Dinitrophenyl Derivatives**—Having the lysine content of each product and the absorbance of its dinitrophenyl derivative, the number of moles of DNP introduced per mole of lysine can be calculated (Table V). To be sure that the introduction of DNP groups had reached an end, the reaction was allowed to go for 4 and for 20 hours. The results show no great differences (Table III), and mean values are used in Table V. For each product the moles of DNP introduced per mole of lysine present lies in the range 0.5 to 0.8. Even disregarding amino end group of the protein that may react, it appears that not more than two-thirds of the lysine reacts to form DNP derivative.

**Sedimentation Profiles**—A comparison of the behavior of four of the forms of PP-L was made in the analytical ultracentrifuge. PP-L3 at a series of concentrations (0.2% to 0.6%) sedimented as a single sharp peak that showed no tendency to separate into components after 80 min at 56,000 rpm; PP-L2 and PP-L4 showed a clear separation of components; PP-L5 showed a single sharp peak, but at the same time there was a considerable accumulation of heavy sediment at the bottom. Fig. 3 shows tracings of the sedimentation patterns made by these four products at the same concentration (5 mg per ml), in the same buffer, and under identical centrifugal conditions. The sedimentation patterns of some of these forms of PP-L are sharply dependent on their concentration. For example at 8 mg per ml, PP-L2 shows a single sharp peak in contrast to the pattern shown in Fig. 3. PP-L3 gave no evidence of dissociation of its peak even at 2 mg per ml and long centrifugal time.

**TABLE V**

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>PP-L2</td>
<td>29</td>
<td>0.120</td>
<td>35</td>
<td>0.40</td>
<td>0.69</td>
<td></td>
<td></td>
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<tr>
<td>PP-L3</td>
<td>31</td>
<td>0.085</td>
<td>24</td>
<td>0.21</td>
<td>0.54</td>
<td></td>
<td></td>
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<tr>
<td>PP-L4</td>
<td>31</td>
<td>0.100</td>
<td>30</td>
<td>0.35</td>
<td>0.70</td>
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<tr>
<td>PP-L5</td>
<td>31</td>
<td>0.27</td>
<td>112</td>
<td>1.24</td>
<td>0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP-L6</td>
<td>44</td>
<td>0.36</td>
<td>152</td>
<td>2.14</td>
<td>0.85</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*From Table IV.
1. 104,000 divided by the moles of lysine in Column 2 (104,000 is the weight of 1000 amino acid residues from Table IV).
2. From Table III.
3. Column 4 divided by Column 3 X 10^4.
4. Absorbance at 555 nm of DNP-PP-L from Table III.
5. Column 6 divided by 0.0184 (16,400 is the average molar absorbance of several DNP-amino acids).
6. Column 7 divided by Column 5.

**DISCUSSION**

A major aim of this laboratory has been to try to determine the forms or forms in which the polysaccharides of cartilage exist in the native state so we may study their interactions with each other and with collagen fibrils and approach the problem of accounting for the mechanical properties of living cartilage in health and disease. Just as the collagen of connective tissue may exist in soluble and insoluble forms, it now begins to appear likely that the polysaccharides of cartilage occur in different forms of PP more or less readily extractable as soluble products. The use of physicochemical methods to determine molecular size and shape is dependent on having water-soluble products and we want them to be as little degraded as possible.

The procedure of Diagram 1 achieves, by chemically mild means without the use of alkali or proteases, an essentially complete separation of all the chondroitin sulfate of bovine nasal cartilage in the form of a series of fractions all of which are easily soluble in water. Although we may hope that the products are in approximately their native state, it is unlikely that they all exist in the cartilage in the form in which they are isolated. Yet by further study of changes that might occur at each step of Diagram 1, we may hope to learn how these different forms of PP-L are associated with collagen fibers to produce the native elastic tissue from which these soluble products cannot simply be leached. An attempt at such a study has already been made with whole PP-L before it was realized that PP-L could be fractionated.

After Step 1 of Diagram 1, it is not possible to extract any further significant amount of PP-L from the cartilage residue.
(CR) with water or dilute salt solution. Yet nearly one-third of the total chondroitin sulfate of the original cartilage remains associated with or attached to CR (3). Most of this chondroitin sulfate can be extracted in the form of PP-L2 by treatment of CR by Step 2, leaving the residue, CRR, with only 1% each of hexosamine and hexuronate. It is not clear what NH₂OH does to release PP-L2 from CR. The fact that Step 2 gives as good a yield of PP-L2 when carried out at pH 7 as at pH 9 makes it questionable whether an ester bond is broken. The determination of hydroxamic acid in the products is of no help since treatment of some proteins with NH₂OH yields small amounts of hydroxamic acid, perhaps by reaction with amide groups of asparagine. From OR (1 g), 2 μmoles of hydroxamic acid were found in CRR (0.6 g) and 2.5 μmoles in PP-L2 (0.2 g) (3). Even if a molecule of PP-L2 had a molecular weight as low as 100,000, and were attached to collagen by a single ester bond, 2 μmoles of hydroxamic acid could account for the amount of PP-L2 released from OR. The method of separation of PP-L2 raises a doubt that it exists as such in the cartilage. It might exist attached to some insoluble component in CR, perhaps the collagen.

Step 1 readily separates PP-L from cartilage powder, and Step 3 readily separates PP-L3 from PP-L. It is quite possible that PP-L3 exists free in the original cartilage.

PP-L4 is not sedimented in 2.5 N potassium acetate, yet it could not be extracted from the residue, PP-LR, in Step 3. Only after PP-LR had been treated with CaCl₂ (Step 4) did PP-L4 resist sedimentation in 2.5 N potassium acetate in Step 5. These puzzling observations suggest that PP-LR is, or contains, a more complex molecular species of which PP-L4 is a part, and that this more complex species can be altered by calcium at a high concentration in a way to release PP-L4 which then turns out to be similar analytically to PP-L3.

Although PP-L3 may exist in the cartilage ground substance in the form isolated, the other four products may not. PP-L2 and PP-L4 are analytically closely similar to PP-L3, yet to release PP-L2 requires treatment of CR with NH₂OH at pH 7, and to release PP-L4 requires treatment of PP-LR with CaCl₂. Neither treatment can be explained in chemical terms. PP-L2 and PP-L4 might be components split off from larger molecular structures. PP-L2 and PP-L4 in the analytical ultracentrifuge do not behave as individual compounds.

PP-L5 and PP-L6 differ from the other forms of PP-L in their high protein contents and in the high values of their limiting viscosity numbers. Because of the great ease with which PP-L6 is sedimented, it was not studied in the analytical ultracentrifuge. PP-L5, although sedimenting as a single sharp peak, also shows the presence of some very rapidly sedimenting material, perhaps similar to PP-L6. Though most closely related to each other, PP-L5 and PP-L6 are also related to PP-L2, PP-L3, and PP-L4 in their similar amino acid profiles.

The particular need to work at low PP-L concentrations and high salt concentration to fractionate PP-L may be related to the highly diffuse nature of these molecules. Evidence that in solution they occupy large domains has been discussed elsewhere (14). In solution at a concentration of about 1%, such molecules are not merely adjacent but probably interpenetrate. Higher dilution and high ionic strength would tend to minimize such intermolecular interaction. This might also account for the great dependence of sedimentation patterns on dilution.

From human costal cartilage a product has been isolated by a method similar to Step 1 of Diagram 1 (15). To distinguish it from the product from bovine nasal cartilage, we may call one BN-PP-L and the other HC-PP-L. HC-PP-L has a much higher content of both protein and keratan sulfate, and a lower content of chondroitin sulfate than BN-PP-L. By the use of lanthanum HC-PP-L was fractionated into three products, all easily soluble in water, each containing chondroitin sulfate, keratan sulfate, and protein. Thus, two cartilages are now known from which PP-L of widely different compositions can be prepared, and which can in each case be separated into a series of fractions of differing composition and properties.

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