Distribution of Acid Mucopolysaccharides in Inner and Outer Layers of Bovine Aorta

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Bovine aortas were separated into 2 layers, an inner layer composed chiefly of intima and an outer layer composed of adventitia and most of the media. A greater concentration of acid mucopolysaccharides, especially of the chondroitin sulfuric acid group, was found in the inner layer of the aorta.

The presence of acid mucopolysaccharides (MPS) in the ground substance of the aorta, the matrix in which cellular and fibrous structures are embedded, has been known for some time. Principally, 4 MPS, hyaluronic acid (HA), chondroitin sulfate (CSA) A and B, and more recently, heparitin sulfate have been isolated from bovine aorta and identified.1-3 Because the different MPS vary considerably in their chemical structures, compositions, and potential physiologic functions, it was of further interest to investigate the distribution of the compounds in layers of the aorta. These experiments describe the isolation of the MPS from bovine aortas after a division into 2 layers had been achieved and, in addition, present the results of isolation of heparitin sulfate by a method not previously used in studies of this compound.

METHODS

Division of Aortas into Layers. Bovine aortas, the arch and thoracic segment, were collected fresh and dissected free of extraneous tissue. The aortic tissue was separated into 2 layers at a division near anatomic intima. The inner layer ("intima"), about 1 mm. thick, consisted of the intima and approximately one-fifth of the media and the remaining outer layer ("externa"), about 3 mm. thick, consisted of the major part of the media and the adventitia. The division was achieved by freezing the outer surface of aortas to the flat surface of a metal container filled with acetone and dry ice and planing off the intimal layer; or, after repeated freezing and thawing the rubbery intima was dissected free from the remaining aorta. The separated tissues were ground, defatted with acetone and air dried.*

Isolation of MPS from Layers of Bovine Aorta. The methods for isolating preparations of MPS from bovine aorta were the same as described previously.2 In brief, the defatted tissues were extracted with 2 per cent NaOH, protein-digested with trypsin and precipitated with trichloroacetic acid, and the MPS precipitated with alcohol. A crude preparation was obtained which consisted of a mixture of MPS.

The scheme for resolving the individual compounds is summarized in table 1. Zone electrophoresis of the mixture allows separation into two groups of compounds, a glucosamine-containing group (HA and heparitin sulfate) and the chondroitin sulfate group (CSA-A and CSA-B). Electrophoresis was repeated on pooled samples from each group as an additional purification prior to further fractionation by means of chromatography. The homogenous material isolated as CSA by electrophoresis was separated into CSA-A and CSA-B by a cellulose column using a mixture of zinc acetate solution and propanol as the solvent system.

In the previous study2 the electrophoretic, homogenous material isolated as HA deviated from theoretic analyses by containing traces of

*The authors appreciate the histologic sections performed by Dr. J. Geer, Louisiana State University School of Medicine.

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FIG. 1 Top. Enzymatic hydrolysis of acid mucopolysaccharides (MPS) from bovine aorta by testicular hyaluronidase. The assays were performed by a turbidimetric method. 0.1% Hydrolysis of a crude preparation of MPS from umbilical cord is shown for comparison. The hyaluronic acid (HA) material isolated after electrophoresis contained a small quantity of material resistant to hyaluronidase. Further fractionation by chromatography resulted in isolation of heparitin sulfate. Also shown are the results of hydrolysis of chondroitin sulfate (CSA) A and B obtained by means of electrophoresis and chromatography. The incomplete resistance to hydrolysis by CSA-B suggests the presence of CSA-A in this preparation.

FIG. 2 Middle. Chromatography of the glucosamine-containing MPS of bovine aorta after separation from the CSA group by zone electrophoresis. The method uses a reverse-phase celite column incorporating lauryl amine and butanol and elution by increasing concentrations of NaCl. Determinations of MPS content of each fraction were performed by carbazole color reaction. The major peak is HA and the other identified as heparitin sulfate. The approximate content of the 2 materials in the mixture is consistent with observations of enzymatic hydrolysis by testicular hyaluronidase as shown in figure 1.

FIG. 3 Bottom. Zone electrophoresis of MPS from 2 layers of bovine aorta compared to materials isolated from the full thickness of the aorta. The "intima," inner layer composed of intima and small part of media attached, and the "externa," outer layers composed of the major part of media and adventitia, yielded considerable differences in content of MPS. The concentrations of MPS are indicated by uronic acid determinations by carbazole. The material with positive migration is composed of both CSA-A and B and because of a decreased intensity of color for l-iduronic acid, the actual concentrations are somewhat greater than shown. The recoveries and analyses of materials isolated from the electrophoretic studies are summarized in table 2. The pattern obtained for "intima" suggests an additional middle peak of heparitin sulfate.
MUCOPOLYSACCHARIDES IN BOVINE AORTA

acids,12 (8) infra-red studies* which were performed with a Perkin-Elmer spectrophotometer, model 21, using potassium bromide discs.13 More details of the analytic methods are given in the earlier study.2

RESULTS

The results are summarized in tables 2 and 3 and figure 3.

The 2 layers of the aorta differ considerably in content of MPS. Although the "intima" represented one-fifth of the weight of the full-thickness of the aorta it contained approximately 35 per cent of the total extractable MPS. Twice the amount of MPS was isolated from the inner layer than could be obtained from equal quantities of the outer layer. A further difference between the 2 layers was observed by electrophoretic separations of the MPS fractions obtained from each layer. Figure 3 demonstrates a greater concentration of the CSA-group and possibly heparitin sulfate occurring in the "intima," while the outer layers contain more of the glucosamine-containing MPS.

Further study of materials separated by electrophoresis failed to show significant differences for the two layers either by analytic methods or by attempts at chromatographic resolution. Although the comparison of extractable MPS from the 2 layers and relative proportions isolated by means of electrophoresis should be valid, the amounts of the individual compounds recovered in these studies should not be considered strictly quantitative. Errors are inherent in the methods of isolation and in extensive fractionations and, as one compound, heparitin sulfate obtained from urine, has been stated to dialyze slowly,14 it is possible that unequal losses of the individual compounds could occur. The over-all results do indicate quantitative and qualitative differences—a greater concentration of MPS in the "intima" and, particularly, of the CSA compounds.

The analyses of the hyaluronic acid which was isolated by chromatography from the

*These studies were performed by R. T. O'Connor, Southern Regional Research Laboratory, New Orleans, La. The authors are grateful for his assistance.

Table 1.—Plan of Separation of the Acid Mucopolysaccharides from Aorta

<table>
<thead>
<tr>
<th>Macropolysaccharide mixture</th>
<th>Zone electrophoresis, PO, buffer, pH 6.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid (HA) (mixture)</td>
<td>Chromatography (lauryl amine)</td>
</tr>
<tr>
<td>Chondroitin sulfate (CSA) (mixture)</td>
<td>Chromatography (zinc acetate-propanol)</td>
</tr>
</tbody>
</table>

lauryl amine columns agreed with known analyses. The heparitin sulfate has an optical rotation [a]D23 of +38°, equimolar amounts of nitrogen, glucosamine and glucuronic acid but a low sulfate content (2 per cent as ester sulfur), and was resistant to hydrolysis by testicular hyaluronidase. These results are similar to those reported for this compound from other sources.10 Variations in sulfate content of this compound have been noted.16 The results of analyses of the CSA-A and B from the aorta have been described previously.2

DISCUSSION

Gross and histologic divisions of the aorta into layers with structural differences have been described,17 and as would be expected, the two layers used in these studies appeared different even upon gross examination. The intima was slightly yellow and more resilient. Because of anatomic differences, variation in concentration of acid MPS within the aorta might be expected, but cannot be predicted from present knowledge concerning the MPS.

Information gained by actual isolation may complement and possibly extend that made by numerous histochemical studies.18-21 Reference to early studies of MPS-stainable material suggested the presence of heparin in the aorta, but the staining quality was recognized later as probably due to a CSA.22
TABLE 2.—Analyses of Mucopolysaccharides Isolated from Inner and Outer Layers of Bovine Aorta

<table>
<thead>
<tr>
<th></th>
<th>Fraction of whole aorta (%)</th>
<th>Dry defatted weight (% wet weight)</th>
<th>MPS isolated by electrophoresis (mg./mg. MPS mixture)</th>
<th>MPS isolated by chromatography of electrophoretically separated groups (mg./mg. MPS mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HA-group</td>
<td>CSA-group</td>
</tr>
<tr>
<td>Intima</td>
<td>21.0</td>
<td>31.3</td>
<td>0.01</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.15-0.25)</td>
<td>(0.30-0.43)</td>
</tr>
<tr>
<td>Externa</td>
<td>79.0</td>
<td>28.3</td>
<td>0.37</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.30-0.43)</td>
<td>(0.10-0.20)</td>
</tr>
<tr>
<td>Whole aorta§</td>
<td>12.4</td>
<td></td>
<td>0.10</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.05-0.20)</td>
<td>(0.18-0.25)</td>
</tr>
</tbody>
</table>

* Averages of triplicate experiments.
† Averages of duplicate chromatographic separations.
§ Infra-red studies demonstrated this material to be like that of bovine cartilage.
§ Based on previous studies of a number of preparations.

TABLE 3.—Analyses of Mucopolysaccharides Resolved by Electrophoresis*

<table>
<thead>
<tr>
<th></th>
<th>Optical rotation ([α]D D)</th>
<th>N (%)</th>
<th>Ester (%)</th>
<th>Hexosamine (%)</th>
<th>Hexuronic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intima</td>
<td>-18.6</td>
<td>4.1</td>
<td>1.2</td>
<td>30.2</td>
<td>34.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(glucosamine)</td>
<td>(glucuronioic)</td>
</tr>
<tr>
<td>Externa</td>
<td>-24.1</td>
<td>3.8</td>
<td>1.0</td>
<td>31.5</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(glucosamine)</td>
<td>(glucuronioic)</td>
</tr>
<tr>
<td>CSA-group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intima</td>
<td>-28.6</td>
<td>3.7</td>
<td>6.4</td>
<td>20.4</td>
<td>29.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(galactosamine)</td>
<td>(glucuronioic and 1-iduronioic)</td>
</tr>
<tr>
<td>Externa</td>
<td>-33.3</td>
<td>2.8</td>
<td>5.1</td>
<td>24.0</td>
<td>21.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(galactosamine)</td>
<td>(glucuronioic and 1-iduronioic)</td>
</tr>
</tbody>
</table>

* Pooled fractions were re-electrophoresed and analyzed. All fractions demonstrated small amounts resistant to hydrolysis by testicular hyaluronidase.

which was the first MPS isolated from the aorta. Unfortunately, the histochemical methods currently employed lack specificity for the different MPS, although CSA seems to be more easily demonstrated, particularly by methods using metachromasia. These studies indicate the presence of MPS throughout the interstices of the aorta, but concentrated within and near the intima. An appreciable increase of stainable MPS occurs in the intima in early atherosclerosis. The observations made by histologic means seem consistent with findings by chemical isolation and identification of the MPS, but at present isolation is necessary to distinguish the individual compounds.

Although it would be speculation to assign a specific importance to the MPS in the aorta because of a variation in distribution, the findings are of interest in the study of the relationship to other structural or chemical elements within the aorta and the physio-
logic roles which the MPS must perform. A relationship of MPS to other elements of connective tissue has been suggested by the isolation of certain MPS from different types of connective tissue, yet the relationship and functional counterpart remain largely unknown. In general CSA, especially A, can be isolated from tissue formed by more differentiated fibroblastic activity, i.e., cartilage and bone, and HA from tissue of less structural identity, i.e., young tissue, synovial fluid, and vitreous humour. Chondroitin sulfate B, which under certain conditions in vitro is a potent anticoagulant, has a relatively high concentration in the aorta. From such observations alone it is difficult to establish their physiologic importance; however, information is gradually accumulating which suggests that the MPS contribute to multiple functions, for example, in growth, aging, repair and inflammation, and anticoagulation. These activities are obviously necessary for maintenance of intact blood vessels and other cardiovascular tissues. The accumulation of data from anatomic and biochemical studies is slowly contributing to the understanding of the function of these substances.

The presence of heparitin sulfate and CSA-B in the aorta may be briefly considered because of recent interest in these compounds. Both materials have anticoagulant properties in vitro and may be of importance as anticoagulants in vivo. The former substance, presumably the same compound obtained from liver as a heparin monosulfate, has also been isolated from amyloid tissue and urine. Both compounds have been obtained from the urine of patients with the Hurler syndrome (gargoylism). Interestingly, children with gargoylism have a metabolic error of connective tissue and develop severe cardiovascular disease including atherosclerosis. One case which we observed (a 4-year-old white female) demonstrated extensive atheroma of the coronary arteries. The relation of these compounds, however, to cardiovascular disease awaits future clarification.

**Summary**

A comparison of the amount of four acid mucopolysaccharides (MPS) that could be isolated from 2 layers of bovine aortas revealed considerable differences. The "intimal" layer, consisting of the anatomic intima and a part of the media attached, contained a greater concentration of extractable MPS than did the remaining outer layer. Qualitative differences were also observed in that larger amounts of galactosamine-containing compounds, chondroitin sulfate A and B, were obtained from the inner layer of the aorta.

**Summario in Interlingua**

Le comparation del quantitates de quatro mucopolysaccharidos acide isolabile ab duo stratos de aorta bovin revelava considerebile differentias. Le strato "intimal," consistente del intima anatomic e de un parte del media attachate a illo, conteneva un plus grande concentration de extrahibile mucopolysaccharidos acide que le strato exterior del aorta. Differentias qualitative esseva etiam observe in tanto que plus grande quantitates de compositos continente galactosamina, sulfato de chondroitina A e B, esseva obtenite ab le strato interior del aorta.

**REFERENCES**

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