Heparin in molluscs: chemical, enzymatic degradation and $^{13}$C and $^1$H n.m.r. spectroscopical evidence for the maintenance of the structure through evolution

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The structural features and anticoagulant activity of heparins isolated from three species of molluscs (Anomalocardia brasiliana, Donnax striatus and Tivela mactroides) are reported. It is shown by chemical analysis, type of products formed by action of heparinase and heparitinase II, anticoagulant activity, $^{13}$C and $^1$H n.m.r. spectroscopy, that the mollusc heparins are virtually indistinguishable from heparins present in mammalian tissues. These data, taken as a whole, suggest that heparin has maintained its main structural features through evolution. The implications of these findings are discussed.

Keywords: Heparin; molluscs; n.m.r. spectroscopy

Introduction
Heparin, a sulphated glycosaminoglycan, present in several mammalian and other vertebrate tissues has been widely used in medicine for more than 40 years because of its anticoagulant, antithrombotic and antilipaemic activities.

Previous studies have shown that heparin-like compounds are present in some invertebrates. A substance denoted mactin, with anticoagulant activity and structural similarities to mammalian heparins was isolated from the molluscs Cyprinia islandica and Mactrus pussula. Another compound from the clam Mercenaria mercenaria also exhibited several structural similarities to heparin.

A heparin-like compound with high anticoagulant activity has been isolated as well from the mollusc Anomalocardia brasiliana. Enzymatic and nitrous acid degradation have shown that the compound has a disaccharide composition similar to that of mammalian heparins. A few significant quantitative differences were observed for the mollusc heparin when compared with the ones of mammalian origin, namely, a higher degree of binding with antithrombin III, higher molecular weight and higher anticoagulant activity.

Due to the proposed biological role of heparin as an anticoagulant it became extremely important to ascertain if the heparin-like compounds present in the mollusc, are indeed, heparins indistinguishable from those obtained from mammalian sources.

The present paper reports the characterization of heparin from Anomalocardia brasiliana, Donnax striatus and Tivela mactroides by n.m.r. spectroscopy, chemical analyses, disaccharide composition and anticoagulant activity.

Experimental
Enzymes and mammalian heparins
Heparin from bovine intestinal mucosa was a gift from Dr P. Bianchini (Opocrin Research Laboratories, Modena, Italy) and heparin from bovine lung was obtained by courtesy of Dr L. L. Coleman (Upjohn Co., Kalamazoo, MI). Heparinase and heparitinase II were prepared from induced F. heparinum cells by methods previously described.

Extraction and purification of mollusc heparins
Anomalocardia brasiliana, Donnax striatus and Tivela mactroides were collected in different regions of the beach of Tibau, Mossoró, Rio Grande do Norte, Brazil at different time intervals over a period of 4 years. Immediately after each collection, the molluscs were
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heated in water for 15 min at 100°C for removal of the shells; 15–20 kg of meat were ground with 2 vol of 0.9 M NaCl in a Waring blender. The pH of the mixture was adjusted with NaOH and 10 g of Superase (Pfizer Laboratory, New York, NY) was added. After 24 h of incubation with agitation and periodical adjustments of pH at 60°C the mixture was filtered through cheese-cloth. To the filtrate 500 g of Amberlite IRA 900, ion-exchange resin (Rohm and Haas, São Paulo, Brazil) were added and the resulting mixture was agitated for 24 h at room temperature under a layer of toluene. The suspension was then filtered again through the same cheese-cloth. The resin retained in the cloth was washed with 10 litres of water at 50°C and subsequently washed with 10 litres of 0.85 M NaCl at room temperature. The washed resin was suspended in 1 litre of 2 M NaCl and agitated for 4 h and filtered. This operation was repeated twice. The filtrates were combined and maintained for 48 h at 5°C after addition of 2 vol of methanol. The precipitate formed was collected by centrifugation at 5000 rev/min for 30 min, washed with 80% methanol and dried. The yields of these crude heparins per kg of meat were: 2.2–2.8 g for Anomalocardia brasilianga, 1.8–2.5 g for Donnax striatus and 2.7–3.8 g for Tivela macrodora in different preparations. These crude heparins were then purified by barium fractionation as follows: 10 g of the mollusc heparin was dissolved in 400 ml of water. To the solution, 20 g of barium acetate was added slowly with stirring and the pH was adjusted to 6.5. The mixture was heated at 60°C and then left at 5°C for 48 h. The precipitate formed was collected by centrifugation at 5°C and suspended in 250 ml of 0.1 M disodium hydrogen phosphate and the pH was adjusted to 8.8. The mixture was heated to 60°C and then left at 5°C for 48 h. The precipitate formed was washed twice with barium fractionation as follows: 10 g of the mollusc heparin was dissolved in 400 ml of water. To the solution, 20 g of barium acetate was added slowly with stirring and the pH was adjusted to 6.5. The mixture was heated at 60°C and then left at 5°C for 48 h. The precipitate formed was collected by centrifugation at 5°C and suspended in 250 ml of 0.1 M disodium hydrogen phosphate and the pH was adjusted to 8.8. The mixture was heated to 60°C and filtered at this temperature. To the filtrate, 2 vol of methanol were added and the mixture was maintained in the cold for 2 h. The precipitate formed was washed twice with methanol and dried under vacuum. The yield of the heparins purified with barium was in the order of 50–60% from the crude preparations. Heparan sulphate and chondroitin sulphate, which are also present in the molluscs, remained in the supernatant after precipitation of the heparins with barium.

Enzymatic degradation of the mollusc heparins

A typical incubation mixture contained 0.1 U of heparinase + glycuronidase or heparitinase II, 100 μg of heparins and other additions as indicated in 0.05 M ethylenediamine acetate buffer, pH 7.0 in a final volume of 30 μl. The incubation mixtures were spotted on Whatman no. 1 paper and subjected to chromatography in isobutyric acid: 1 M NH₃, 5/3, v/v for 48 h. Electrophoresis of the degradation products was performed on Whatman no. 3MM paper in 0.25 M (NH₄)₂HCO₃ buffer, pH 8.5. The unsaturated products formed were detected by short wave u.v. lamp, eluted from the paper with water and quantitated by the hexosamine content (see below) and characterized as described previously. For the identification of high molecular weight products formed from the heparins by action of heparinase, gel filtration was used as follows: 5 mg of each heparin was incubated with 5 U of heparinase as described above. The incubation mixtures were then applied in a Sephadex G-50 column (1 x 120 cm) previously equilibrated with 1 N acetic acid and eluted with the same solution. The products were identified in the fractions (1 ml) by the carbazole reaction (see below) and absorption at 232 nm.

N.m.r. spectroscopy

All spectra were recorded on a Varian XL-300 spectrometer. The 1H-n.m.r. spectra were recorded at 300 MHz with solutions of the polysaccharides (1–2%) in deuterium oxide, following deuterium exchange, at 72°C, and were referenced to external TSP. The 13C spectra (75 MHz) were acquired with solutions of the polysaccharides (5–10%, w/v) in deuterium oxide, at 72°C, and were referenced to external tetramethylsilane. Each solution was given a preliminary treatment with Chelex ion-exchange resin, to remove possible traces of paramagnetic ions.

Other methods

Hexosamine was determined after acid hydrolysis (4 M HCl, 100°C, 6 h) by the Rondle–Morgan procedure and uronic acid by the carbazole reaction. Total sulphate was measured by a method previously described. Agarose gel electrophoresis was performed in three different buffer systems. Anticoagulant activity was determined according to the United States Pharmacopeia assay. Molecular weight was estimated by polyacrylamide gel electrophoresis as described elsewhere.

Results

Analytical data from heparins

Figure 1 shows the electrophoretic migration of the three mollusc heparins compared with two distinct mammalian heparins in barbital buffer, diaminopropane acetate buffer and in the discontinuous barium/diaminopropane system. All the heparins have similar electrophoretic migrations in these systems and are distinguished from heparan sulphate in barbitane buffer and the other sulphated glycosaminoglycans in diaminopropane buffer. As previously shown the mammalian heparins show two different components in the discontinuous system. This is also observed for the three mollusc heparins (Figure 1).

The ratios of hexosamine, uronic acid and sulphate of the mollusc heparins compared with those of the two mammalian heparins from different sources are shown in Table 1. All the heparins show similar ratios, except for the sulphate content which is slightly lower in the mollusc heparins. Significant differences were found in the average molecular weight and anticoagulant activity. The mollusc heparins have a higher molecular weight and higher anticoagulant activity when compared with the mammalian heparins.

Enzymatic degradation of the heparins

The products formed from a mollusc and a mammalian heparin by action of heparinase are illustrated in Figure 2. Both heparins are extensively degraded by the enzyme to small molecular weight products with a similar elution profile in Sephadex. The different peaks shown in Figure 2 were pooled, concentrated and analysed. Peaks III and IV had the same chromatographic and electrophoretic migrations of unsaturated tri- and di-sulphated disaccharides respectively. Peaks I and II with the elution profiles of hexa- and tetra-saccharides were totally degraded by heparitinase II. Figure 3 shows the different
Figure 1 Electrophoretic migration of mollusc and mammalian heparins in three different buffer systems. Aliquots of 5 µl containing 10 µg of the different heparins as indicated were applied in agarose gel slabs prepared with different buffers and subjected to electrophoresis in the conditions described in ref. 1. After fixation and drying the gels were stained with toluidine blue. CHS, chondroitin sulphate; DS, dermatan sulphate; HTS, heparan sulphate; HEP, heparin, OR, origin.

Table 1 Analytical data and anticoagulant activity of mollusc and mammalian heparins

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Molar ratios of hexosamine (x 10^-3)</th>
<th>Average M,</th>
<th>Anticoagulant activity (i.u./mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uronic acid</td>
<td>Sulphate acid</td>
<td></td>
</tr>
<tr>
<td>Anomalocardia</td>
<td>1.5</td>
<td>2.5</td>
<td>32</td>
</tr>
<tr>
<td>Tivela</td>
<td>1.4</td>
<td>2.3</td>
<td>25</td>
</tr>
<tr>
<td>Donnax</td>
<td>1.6</td>
<td>2.4</td>
<td>20</td>
</tr>
<tr>
<td>Bovine mucosa</td>
<td>1.6</td>
<td>2.6</td>
<td>13</td>
</tr>
<tr>
<td>Bovine lung</td>
<td>1.4</td>
<td>2.8</td>
<td>12</td>
</tr>
</tbody>
</table>

Disaccharide products formed from a mammalian and a mollusc heparin by action of the heparinase and heparitinase II. Although in different proportions, the same types of disaccharides are produced by the action of the two enzymes on both heparins.

The relative proportions of the degradation products formed from the five heparins analysed are shown in Table 2. The heparin from Anomalocardia brasiliana and that from bovine intestinal mucosa are very similar regarding the relative amounts of disaccharides formed either by heparinase or heparitinase II, whereas bovine lung heparin gave higher amounts of products with heparinase. Conversely, the heparins prepared from Donnax striatus and Tivela mactroides were more extensively degraded by heparitinase II. Since heparinase is specific for iduronic acid-glucosaminido linkages and heparitinase II acts preferentially upon glucuronic acid-glucosaminido linkage,11,12 it can be concluded that the Donnax and Tivela heparins contain higher amounts of glucuronic acid residues in their structures than do the other heparins.

N.m.r. spectra

The foregoing conclusions based on the analysis of the enzymolysis products, are confirmed by n.m.r. spectroscopic data for the intact mollusc heparins. As shown by the proton spectrum of a mollusc heparin in Figure 4B, there is an obviously close affinity between the structure of this polymer and that of bovine mucosal heparin (Figure 4A), because the overall patterns of signals are so similar. In both spectra, signals I-1 and I-5, which are attributable to protons-1 and -5 of the \( \alpha \)-iduronic acid 2-sulphate residues, are far stronger than signal G-1. The latter corresponds in chemical shift to glucuronic acid residues having the \( \beta \)-d-configuration, and the relative intensities of these signals indicate that the iduronic:glucuronic ratios in these heparins is approximately 5:1, which accords with the data on mucosal
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Heparin, in Table 2. Residues of 2-deoxy-2-sulphamino-\(\alpha\)-d-glucose produce signals A-1 (proton-1) and A-2 (proton-2), whereas the relatively minor signal A-1a is probably due to the small proportion (Table 2) of the glucosamine residues in which C-6 is not sulphated.

These spectra provide direct evidence for the presence of residues of 2-acetamido-2-deoxy-\(\alpha\)-d-glucose. That is, the methyl protons of the acetamido group produce signal Ac\(^{18,19}\), the intensity of which in the mucosal spectrum (Figure 4A) corresponds to \(~15\%\) of such residues. (In the spectrum of bovine lung heparin\(^{18,19}\) the Ac signal is barely detectable.)

There are some notable differences between these two spectra and the spectrum (Figure 4C) of the mollusc heparin from *Tivela mactroides*, and also of that from *Anomalocardia*.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Percentage amounts of the main unsaturated products formed from mollusc, bovine lung and bovine intestinal mucosa heparins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>Products heparinase</td>
</tr>
<tr>
<td></td>
<td>(\Delta)Tetra</td>
</tr>
<tr>
<td></td>
<td>(-2S)-GlcNS,6S</td>
</tr>
<tr>
<td>Lung</td>
<td>40.8</td>
</tr>
<tr>
<td>Mucosa</td>
<td>24.0</td>
</tr>
<tr>
<td><em>Anomalocardia</em></td>
<td>18.2</td>
</tr>
<tr>
<td><em>Donnax</em></td>
<td>15.1</td>
</tr>
<tr>
<td><em>Tivela</em></td>
<td>16.2</td>
</tr>
</tbody>
</table>

nd, Not detected
residue in these heparins at moderate and high levels, acid (G-1) confirms the expected presence of the latter 2-deoxy-2-sulphamino-α-D-glucose 6-sulphate (A-1). In evident that the lung heparin contains little glucuronic acid, because the only prominent signals are attributable to residues of α-L-iduronic acid 2-sulphate (I-1) and characteristic 2° of the anomeric (C-1) carbons, it is 5B examination of 13C-n.m.r. spectra. Those illustrated in Figure 5 are due, respectively, to a bovine lung heparin (A), and the two mollusc heparins represented by Figures 5B and 5C. From the region close to δ100, which is characteristic of the anomeric (C-1) carbons, it is evident that the lung heparin contains little glucuronic acid, because the only prominent signals are attributable to residues of α-L-iduronic acid 2-sulphate (I-1) and 2-deoxy-2-sulphamino-α-D-glucose 6-sulphate (A-1). In Figures 5B and 5C, a peak attributable to β-D-glucuronic acid (G-1) confirms the expected presence of the latter residue in these heparins at moderate and high levels, respectively. In addition, these spectra furnish direct evidence to the effect that there is less 6-sulphation of the hexosamine residues of the mollusc heparins. This is shown by the presence in Figures 5B and 5C of two signals of comparable intensities—the one at δ67 (A-6s) is attributable to carbon-6 of the 6-sulphated residue, whereas that at δ62 (A-6) must represent a non-sulphated carbon-6 of hexosamine residues. By contrast, and consistent with the high degree of sulphation of the lung heparin, only the δ68 C-6 signal (A-6s) is observable in Figure 5A.

Another noteworthy feature of spectrum 5C is its overall greater complexity, especially in comparison with 5A which, of course, is consistent with the much lower degree of structural homogeneity among the disaccharide sequences in the mollusc heparin than the lung heparin. To a lesser degree, this comparison applies also for the other mollusc heparin (Figure 5B). With an increase in the proportion of β-glucuronic acid, not only are carbon signals due to the latter increasingly more evident, but also the magnetic shielding of the carbon nuclei of nearby hexosamine residues is altered. Consequently, such signals as A-1, A-2, A-6s become split into components differing slightly in chemical shift. In addition, one finds in spectra 5B and 5C the occurrence of other signals, e.g. at δ81 and δ77 which, tentatively, are assigned to carbons-4 and -5, respectively, of the β-glucuronic acid residues themselves.

In conclusion, both the 1H and 13C-n.m.r. spectra of the mollusc heparins are fully compatible in their detailed characteristics, with those of heparins of mammalian origin. This evidence provides additional justification, therefore, for designating the mollusc polymers as ‘true’ heparins.

Discussion

The three sulphated glycosaminoglycans obtained from molluscs described in this paper meet all of the criteria for defining them as heparins. Thus, their anticoagulant activity, chemical composition, and susceptibility to specific enzymes, as well as their 13C and 1H n.m.r. spectroscopy, are very similar to those of mammalian heparins.

The differences found in anticoagulant activity for the three mollusc heparins and mammalian heparins seem to be related to the different molecular weight of the compounds. Thus, the heparins with higher molecular weight have higher anticoagulant activity. These do not seem to be relevant differences, since mammalian heparin populations with these features have been isolated from the commercial preparations by a variety of methods. Other quantitative structural differences, namely, the relative content of glucuronic and iduronic acid, revealed by the susceptibility to heparitinase II and heparinase and 13C n.m.r. spectroscopy, have also been observed between different mammalian heparin preparations.

These data, taken as a whole, indicate that heparins in molluscs are virtually indistinguishable from those present in mammalian tissues, and suggest that they have maintained their main structural features through evolution.

In addition, the present findings pose some questions regarding the real biological function of heparin. It is unlikely that these molecules function as anticoagulants.
or antithrombotics in molluscs, because these species are devoid of such coagulation systems as the one present in mammals.

Acknowledgements

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References

2 Gomes, P. B. and Dietrich, C. P. Comp. Biochem. Physiol. 1982, 73B, 857
5 Cassaro, C. M. F. and Dietrich, C. P. J. Biol. Chem. 1977, 252, 2254
7 Cifonelli, J. A. and Mathews, M. B. Connect. Tissue. Res. 1972, 1, 121
8 Jordan, R. E. and Marcun, J. A. Arch. Biochem. Biophys. 1986, 248, 690
11 Silva, M. E. and Dietrich, C. P. J. Biol. Chem. 1974, 250, 6841
21 Perlin, A. S. and Hamer, G. K. ACS Symposium No 103 1979, 123
24 Huckerby, T. N. and Nieduszynski, I. A. Carbohyd. Res. 1982, 103, 141