An endoglycosidase which cleaves heparin and heparan sulfate was isolated from outdated human platelets by freeze-thaw solubilization, heparin-Sepharose chromatography, DEAE-cellulose chromatography, hydroxylapatite chromatography, octyl-agarose chromatography, concanavalin A-Sepharose chromatography, and Sephacryl S-200 gel filtration. The overall extent of purification of the platelet heparitinase is about 240,000-fold and the overall yield of the enzyme is about 5.8% as compared to the initial freeze-thaw solubilization preparation. The final product is physically homogeneous as judged by disc gel electrophoresis at acidic pH as well as gel filtration chromatography and exhibits an apparent molecular weight of approximately 134,000. Furthermore, our results indicate that the above enzyme is present within platelet lysosomes.

The biologic potency of the endoglycosidase was examined as a function of pH. The data show that the platelet heparitinase is maximally active from pH 5.5 to pH 7.5. However, the enzyme possesses minimal ability to cleave heparin at pH less than 4.0 or greater than 9.0. The substrate specificity of the platelet endoglycosidase was determined by identifying susceptible linkages within the heparin molecule that can be cleaved by the above component. Our studies indicate that this enzyme is only able to hydrolyze glucuronsyl-glucosamine linkages. Furthermore, investigation of the structure of the disaccharide which lies on the nonreducing end of the cleaved glucuronic acid residue suggests that N-sulfation of the glucosamine moiety or ester sulfation of the adjacent iduronic acid groups are not essential for bond scission.

A series of specific exoglycosidases are responsible for the degradation of heparin and heparan sulfate within mammalian species (1). These enzymes sequentially release inorganic sulfates and monosaccharide residues from the nonreducing ends of the above mucopolysaccharides (2). This process is facilitated by the action of endoglycosidases that cleave internal glycosidic bonds and thereby increase the number of reducing terminals available to the exoglycosidases (3).

Recent studies have demonstrated that one of these exoglycosidases is present within human platelets (4, 5). In this communication, we provide the first method for isolating the above enzyme in homogeneous form. In addition, we report several important physical and biologic properties of the platelet heparitinase. Furthermore, we describe the subcellular site of localization of the mucopolysaccharide-cleaving enzyme within the human platelet. The subsequent paper in this issue outlines the critical role of the above endoglycosidase in modulating smooth muscle cell proliferation.

**MATERIALS AND METHODS**

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Platelet heparitinase was isolated from outdated human platelets by freeze-thaw solubilization of the enzyme (Step I), heparin-Sepharose chromatography (Step II), DEAE-cellulose chromatography (Step III), hydroxylapatite chromatography (Step IV), octyl-agarose chromatography (Step V), concanavalin A-Sepharose chromatography (Step VI), initial Sephacryl S-200 gel filtration (Step VII), and final Sephacryl S-200 gel filtration (Step VIII).

The individual steps have been performed 10 to 20 times and have not deviated markedly from the average specific activity and recovery estimates provided in Table I. All buffers contained 1 mM sodium azide as a bactericidal agent.

Step I: Solubilization of Platelet Heparitinase—Approximately 150 units of outdated human platelets stored at −20 °C were rapidly thawed at 37 °C, acidified to pH 5.0 with glacial acetic acid, and centrifuged at 8000 × g for 30 min at 4 °C. Direct measurement of the resultant supernatant solution revealed that significant amounts of heparin-cleaving activity had been lost from the above cellular elements. Homogenization of the platelet pellet with a Waring blender for 1 min in the presence or absence of 0.1% (w/v) Triton X-100 detergent released less than 10% additional heparin-cleaving activity.

RESULTS AND DISCUSSION

Purification of Platelet Heparitinase

Platelet heparitinase was isolated from outdated human platelets by freeze-thaw solubilization of the enzyme (Step I), heparin-Sepharose chromatography (Step II), DEAE-cellulose chromatography (Step III), hydroxylapatite chromatography (Step IV), octyl-agarose chromatography (Step V), concanavalin A-Sepharose chromatography (Step VI), initial Sephacryl S-200 gel filtration (Step VII), and final Sephacryl S-200 gel filtration (Step VIII).

The individual steps have been performed 10 to 20 times and have not deviated markedly from the average specific activity and recovery estimates provided in Table I. All buffers contained 1 mM sodium azide as a bactericidal agent.
Human Platelet Heparitinase

Table I

Summary of purification of human platelet endoglycosidase

These data represent average results obtained employing 900 units of outdated human platelets.

<table>
<thead>
<tr>
<th>Step and procedure</th>
<th>Protein Activity</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Freeze-thaw</td>
<td>mg</td>
<td>milliunits</td>
<td>milliunits/mg</td>
<td>%</td>
</tr>
<tr>
<td>II. Heparin-Sepharose</td>
<td>2,469.960</td>
<td>112,947</td>
<td>0.046</td>
<td>100</td>
</tr>
<tr>
<td>III. DEAR-cellulose</td>
<td>3,721</td>
<td>84,710</td>
<td>15.9</td>
<td>75</td>
</tr>
<tr>
<td>IV. Hydroxylapatite</td>
<td>2,640</td>
<td>59,129</td>
<td>22.4</td>
<td>52.4</td>
</tr>
<tr>
<td>V. Octyl-agarose</td>
<td>427</td>
<td>46,236</td>
<td>108.3</td>
<td>40.9</td>
</tr>
<tr>
<td>VI. Concanavalin A-Sepharose</td>
<td>1.75</td>
<td>36,545</td>
<td>290</td>
<td>32.4</td>
</tr>
<tr>
<td>VII. First Sephacryl S-200</td>
<td>3.96</td>
<td>22,484</td>
<td>5,678</td>
<td>19.9</td>
</tr>
<tr>
<td>VIII. Second Sephacryl S-200</td>
<td>1.84</td>
<td>16,539</td>
<td>10,085</td>
<td>14.6</td>
</tr>
</tbody>
</table>

The above fluid was usually found to contain significant amounts of platelet endoglycosidase and was therefore combined with enzyme initially eluted from the concanavalin A-Sepharose column. The Step VI preparation was concentrated by ultrafiltration from 12 ml to about 10 ml utilizing PM-10 membranes, and stored at -80 °C prior to use. Under these conditions, the above products were stable for at least 2 weeks.

**Step VII: Initial Sephacryl S-200 Gel Filtration**—The Step VI preparation isolated from 450 units of outdated platelets and containing about 2.0 A units of protein was further purified by gel filtration. To this end, 700 μl of this material which represented an entire Step VI product were chromatographed on a Sephacryl S-200 column (0.6 x 128 cm) that had been equilibrated at 4 °C with 0.15 M NaCl in 0.01 M sodium phosphate, pH 6.0. The filtration procedure was conducted at a flow rate of 3 ml/h. A typical Sephacryl S-200 chromatogram is shown in Fig. 3. Two discrete protein peaks were observed with the component of lower molecular size exhibiting the bulk of the mucopolysaccharide-cleaving activity. As indicated, fractions with specific activities of 9500 milliunits/mg or greater were pooled. These products were stored at -80 °C prior to use and were stable for at least 3 weeks.

**Step VIII: Final Sephacryl S-200 Gel Filtration**—In order to remove trace amounts of impurities, Step VII preparations were rechromatographed on Sephacryl S-200. To this end, two of the Step VII products obtained from 900 units of outdated platelets and containing about 1.6 A units of protein were concentrated by ultrafiltration from 12 ml to about 700 μl with PM-10 membranes. This material was chromato-

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**Fig. 1 (left).** Heparin-Sepharose chromatography of the Step I product. The platelet heparitinase was eluted with a linear salt gradient. Fractions of 12 ml were collected and subsequently assayed for heparin-cleaving activity after a 2-fold dilution in 0.15 M NaCl with 0.2 mg/ml of bovine serum albumin added. See "Materials and Methods" as well as "Results and Discussion" for additional experimental detail.

**Fig. 2 (center).** Hydroxylapatite chromatography of the Step III product. The platelet heparitinase was eluted with a linear salt gradient. Fractions of 11 ml were collected and subsequently assayed for heparin-cleaving activity after a 5-fold dilution in 0.15 M NaCl with 0.2 mg/ml of bovine serum albumin added. See "Materials and Methods" as well as "Results and Discussion" for additional experimental detail.

**Fig. 3 (right).** First Sephacryl S-200 gel filtration of the Step VI product. The platelet heparitinase was chromatographed on a Sephacryl S-200. Fractions of 1.2 ml were collected and subsequently assayed for heparin-cleaving activity after a 20-fold dilution in 0.15 M NaCl with 0.2 mg/ml of bovine serum albumin added. See "Materials and Methods" as well as "Results and Discussion" for additional experimental detail.

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0.50 m potassium phosphate, pH 6.0. The columns were washed with the above buffer at the same flow rate and temperature until the absorbance readings reached baseline levels. Under these conditions, the platelet heparitinase does not bind to the octyl-agarose and can be recovered in the initial column wash. The three separate octyl-agarose preparations were pooled, concentrated by ultrafiltration from about 120 to about 10 ml utilizing PM-30 membranes, and stored at -80 °C prior to use. In this state, products were stable for at least 8 weeks.

**Step VI: Concanavalin A-Sepharose Chromatography**—The Step V preparation obtained from 450 units of outdated platelets and consisting of about 63.0 A units of protein was filtered at 2 ml/h through a concanavalin A-Sepharose column (1.25 x 2.5 cm) that had been equilibrated at 4 °C with 0.15 M NaCl in 0.01 M sodium phosphate, pH 6.0. The lectin-Sepharose matrix was washed with an additional 10 to 15 ml of the above solution at the same flow rate as well as temperature and bound platelet heparitinase was harvested with 6 ml of a 1 M glucose and 0.5 M NaCl in 0.01 M sodium phosphate, pH 6.0. Subsequently, 10 ml of the above eluting solution were mixed with the chromatographic matrix and the resultant suspension was maintained at 4 °C for 12 h. At the end of this period of time, the supernatant liquid was carefully drawn off and then examined for mucopolysaccharide-cleaving activity. The above fluid was usually found to contain significant amounts of platelet endoglycosidase and was therefore combined with enzyme initially eluted from the concanavalin A-Sepharose column.
Physical Properties of Platelet Heparitinase

The physical homogeneity of a Step VIII preparation with respect to charge was assessed by disc gel electrophoresis at an acidic pH utilizing a system adapted from Kresse and Neufeld (14). Since this protein stains poorly with Coomassie blue and only a relatively small amount of the component is available, the platelet endoglycosidase was labeled with Na\textsuperscript{125}I utilizing chloramine-T according to the method of Greenwood et al. (21). Free isotope was removed by filtering the above mixture on a Sephadex G-25 column (0.8 × 30 cm) equilibrated with 0.05 M sodium phosphate, pH 7.5, containing 1 mg/ml of bovine serum albumin. The radiolabeled protein was then electrophoresed as outlined under "Materials and Methods." The polyacrylamide matrices were sectioned into 2-mm slices and the various segments were counted for \textsuperscript{125}I counts. Results obtained from a typical examination of the platelet heparitinase are depicted in Fig. 4b. Counts present in segment 1 probably represent material trapped at the interface between the stacking gel and the running gel. On two occasions, 20 \textmu g of the unlabeled enzyme were electrophoresed as outlined above, and the acrylamide disc gels were stained with Coomassie blue as well as destained as previously described (6). A single faint band was observed in the same area of the electrophoretic matrix as the peak of \textsuperscript{125}I counts. The above data suggest that Step VIII preparations of the platelet endoglycosidase are essentially homogeneous with respect to charge.

The apparent molecular weight of platelet heparitinase was established by gel filtration chromatography. To this end, the \textsuperscript{125}I-labeled enzyme (see above) and proteins of known molecular weight were filtered at 2 ml/h on a Sephacryl S-200 column (0.6 × 128 cm) equilibrated with 0.5 M NaCl in 0.01 M Tris-HCl, pH 7.5. The molecular weight markers utilized were catalase (\textit{M}_r = 240,000), aldolase (\textit{M}_r = 158,000), bovine serum albumin (\textit{M}_r = 67,000), ovalbumin (\textit{M}_r = 45,000), and ribonuclease (\textit{M}_r = 13,700). Under these conditions, the logarithm of the molecular weight of proteins with similar shape is linearly related to the partition coefficient, \textit{K}_\text{av} (22).

\[ \text{\textit{K}}_{\text{av}} = A + B \log M, \]

Data obtained from two gel filtrations of the five standard proteins were fitted to the latter equation by linear least squares analyses (22). The results indicate that the values of \textit{A} and \textit{B} are 1.63 and -0.294, respectively, with a correlation coefficient of 0.979. The \textit{K}_\text{av} of the radiolabeled platelet endoglycosidase was determined within the above chromatographic system and suggests that this enzyme has an apparent \textit{M}_r = 134,000 (Fig. 4c).

Specific Enzymatic Properties of Platelet Heparitinase

The mucopolysaccharide-cleaving potency of the Step VIII preparation was analyzed as a function of pH with the final enzyme concentration set at 10 milliunits/ml diluted in 0.1 mg/ml of albumin and the final ionic strength maintained at 0.1. The results of this study are depicted in Fig. 5 and indicate that the platelet endoglycosidase is maximally active over a broad pH ranging from 5.5 to 7.5. Furthermore, the above enzyme possesses minimal ability to cleave heparin at pH less than 4.0 or greater than 9.0. These data are somewhat at variance with those reported by Wasteson et al. (4) who claim that the pH optima of platelet endoglycosidase lies between 5.0 and 5.6. However, in agreement with the above authors, we have observed that the platelet enzyme is virtually inactive at pH 3.6 and retains ~90% of its activity at physiologic pH. The discrepant findings of Wasteson et al. (4) may be due to the fact that these investigators utilized crude platelet lysates as a source of enzyme and soluble heparan sulfate as substrate.

The proteolytic activity of the Step VIII product was also examined with the \textsuperscript{125}I-labeled a-casein assay (see, "Materials and Methods"). At concentrations as high as 50 \textmu g/ml, the platelet enzyme exhibited no capacity to cleave radiolabeled casein.

The substrate specificity of the Step VIII material was determined by identifying susceptible linkages within the
heparin molecule that can be cleaved by the platelet component. To this end, the pre-existing reducing terminals of anticoagulantly active heparin were blocked by reduction with 140-fold molar excess of unlabeled sodium borohydride at pH 8.8. The above mucopolysaccharide was then incubated for 40 h with the platelet enzyme at final concentrations of 1000 and 15 μg/ml, respectively. The environmental conditions utilized were 0.1 M sodium acetate, pH 5.4, and 24 °C. At the end of this time period, the reaction mixture was heated for 2 min at 100 °C, evaporated to dryness, and then dissolved in 0.6 M NaHCO₃.

The cleavage of heparin by platelet heparitinase exposed new reducing terminals that were labeled by reduction for 30 min at 50 °C with 0.05 M sodium [³H]borohydride at pH 8.8. To destroy traces of residual reducing agent, the above solution was initially acidified and then neutralized. Labeled oligosaccharides were separated from free isotope by Whatman paper chromatography utilizing Solvent System I and gel filtration at 2.4 ml/h on a P2-polyacrylamide column (0.6 × 200 cm) equilibrated with 0.5 M NH₄HCO₃. All tritium tagged fragments were pooled, lyophilized to dryness, and then subjected to nitrous acid treatment at pH 1.5 (23). The resultant molecular species were analyzed by filtration at 2.5 ml/h on a P2-polyacrylamide column (0.6 × 200 cm) equilibrated with 0.5 M NH₄HCO₃. Results obtained during a typical examination are depicted in Fig. 6. Comparison with markers of known molecular size revealed that trisaccharides and monosaccharides had been formed in relatively equal amounts during the degradation procedure. The presence of odd numbered labeled fragments suggested that cleavage of mucopolysaccharide by platelet heparitinase involves hydrolysis of uronic acid linkages.

To demonstrate that the above interpretation was correct, labeled trisaccharide was subjected to total acid hydrolysis and nitrous acid degradation at pH 4.2 (23). The labeled monosaccharide generated by this treatment as well as that obtained from the initial enzymatic cleavage and nitrous acid degradation were identified by cellulose-PO₄ paper chromatography utilizing Solvent System II. The results of these analyses are depicted in the inset to Fig. 6. It is apparent from the above data that the monosaccharide exposed by the action of platelet endoglucuronidase migrates in a manner identical with the gulonic acid marker but is clearly distinguished from the idonic acid marker. The presence of nitrous acid degradation fragments of radiolabeled glucosaminitol was excluded by Whatman paper chromatography utilizing Solvent System I (not shown).

The labeled trisaccharide was also examined to determine the uronic acid type located at its nonreducing terminal. To accomplish this goal, about 800,000 cpm of the above oligosaccharide were mixed with various exoglycosidases that liberate specific uronic acid moieties from the desired position. Three kinds of enzymatic degradation were utilized. (a) The trisaccharide was exposed for 18 h at 37 °C to 16,000 units/ml of purified β-glucuronidase. The environmental conditions were established with 0.1 M sodium acetate, pH 4.3. (b) The trisaccharide was exposed for 18 h at 24 °C to 40 μg/ml of purified a-iduronidase. The environmental conditions were established with 0.175 M NaCl in 0.05 M sodium formate, pH 4.0. (c) The trisaccharide was exposed for 9 h at 37 °C to 33 μg/ml of purified iduronate sulfatase. The environmental conditions were established with 0.1 M NaCl in 0.125 M sodium acetate, pH 4.5. After completion of the above procedure, the resultant species were then treated as described in b. Subsequently, the various reaction mixtures were filtered at 2.5 ml/h on a P2-polyacrylamide column (0.6 × 200 cm) equilibrated with 0.5 M NH₄HCO₃ to determine the extent to which the trisaccharide had been converted to disaccharide. The results obtained by averaging two independent experiments revealed that the nonreducing terminal of the trisaccharide consisted of sulfated iduronic acid (22.3%) and nonsulfated iduronic acid (73.7%).

Thus, our studies demonstrate that the platelet heparitinase is only able to hydrolyze glucuronyl-glucosamine linkages. Furthermore, our investigations of the structure of the disaccharide which lies immediately on the nonreducing end of the cleaved glucuronic acid residue indicate that N-sulfation of the glucosamine moiety or ester sulfation of the iduronic acid group are not essential for bond scission. These results confirm as well as extend observations on substrate specificity made...
of purification of platelet heparitinase is about 240,000-fold and the overall yield of the enzyme is about 16% as compared to the initial solubilized preparation. Furthermore, the final product of the above procedure is physically homogeneous as judged by disc gel electrophoresis at acidic pH as well as by gel filtration chromatography and exhibits an apparent $M_r = 134,000$.

It is of interest to note that human platelets possess relatively small amounts of this enzyme. If one takes into account the numbers of these cellular elements employed as starting material as well as the final yield of the heparitinase obtained, it is possible to calculate that each platelet must contain about 1000 molecules of this endoglycosidase. This level of enzyme is considerably less than that noted for other platelet constituents such as $\beta$-thromboglobulin, platelet factor 4, or thrombospondin (24-26), but is similar to the quantities of growth factor found within the $\alpha$-granules of these cellular elements (27).

Prior attempts to isolate platelet endoglycosidase have met with limited success. The best results have been reported by Oldberg et al. (5). These investigators chromatographed human platelet lysates on columns of heparan sulfate-Sepharose 4B. The products obtained were purified about 200-fold with an overall yield of about 20%. Further purification of the platelet heparitinase was difficult because of a rapid decline in the biologic potency of this enzyme preparation.

In large measure, the success of our isolation technique is dependent upon utilizing substantial amounts of platelet concentrates as starting material as well as identifying environmental conditions that preserve the activity of the endoglycosidase. Indeed, we suspect that the instability of the heparan sulfate-Sepharose 4B products reported by Oldberg et al. (5) was due to the very low concentrations of protein eluted from the affinity matrix as well as the relatively high pH utilized during the above separation procedure. Once the critical nature of these two parameters was appreciated, little difficulty was experienced in designing an isolation technique capable of producing mucopoly saccharide-cleaving enzyme in homogeneous form.

Previous subcellular fractionation studies of platelets have revealed that characteristic components such as $\beta$-thromboglobulin, thrombospondin, platelet factor 4, growth factor, etc. are located within $\alpha$-granules (28), acid hydrolases such as $\beta$-glucuronidase, $\beta$-N-acetylhexosaminidase, etc. are found within lysosomes (28, 29), and serotonin as well as metabolically inert adenine nucleotides are contained within the dense bodies (30). Data presented in this communication demonstrate that the platelet heparitinase and the acid hydrolases are housed within the same subcellular structures. The lysosomal localization of the above enzyme suggests that release of the platelet endoglycosidase will demand a higher concentration of platelet agonists than is required for species found within the $\alpha$-granules of these cellular elements. This hypothesis is compatible with our observation that addition of small amounts of thrombin to platelet-rich plasma results in the discharge of only $\sim 10\%$ of the available endoglycosidase but permits virtually complete liberation of the $\alpha$-granule component thrombospondin (data not shown). Furthermore, the above supposition is also strengthened by the data of Oldberg et al. (5) which indicate that release of platelet endoglycosidase is more readily attained with thrombin or collagen than with adrenaline or ADP.

It should be noted that liberation of the above enzyme into the circulatory system as part of the platelet release action could lead to the cleavage of heparan sulfates bound to the endothelium. Indeed, an in vitro analogue of this process has been observed with crude fractions of the platelet heparitinase.

**Subcellular Localization of Platelet Heparitinase**

In order to establish the subcellular site of localization of the above enzyme, washed human platelets were homogenized using the nitrogen decompression technique, and membranes, as well as organelles, were separated by sucrose density gradient centrifugation (see "Materials and Methods"). The various fractions obtained by this procedure were comparable to those previously described by Brockman et al. (20). The results of a typical experiment are depicted in Fig. 7. It is apparent that the relative specific activities of $\beta$-glucuronidase and $\beta$-N-acetylhexosaminidase were maximal in fraction 4 (mitochondria and lysosomes), whereas that of platelet factor 4 was highest in fraction 7 ($\alpha$-granules). The distribution profile of the relative specific activity of platelet heparitinase was virtually identical with that of the acid hydrolases but distinct from that of platelet factor 4. Two additional experiments yielded similar results.

The particulate origin of the above enzyme was also confirmed by determining the latency of an acid hydrolase with the detergent Triton X-100 and comparing these results to that obtained with the platelet endoglucuronidase. The $\beta$-glucuronidase showed latencies of 80 to 126% in fractions 3 to 8, while the platelet heparitinase exhibited latencies of 48 to 96% in similarly designated regions of the sucrose density gradient.

In summary, we have isolated a heparin or heparan sulfate degrading enzyme from human platelets. The overall extent of purification of platelet heparitinase is about 240,000-fold and the overall yield of the enzyme is about 16% as compared to the initial solubilized preparation. Furthermore, the final product of the above procedure is physically homogeneous as judged by disc gel electrophoresis at acidic pH as well as by gel filtration chromatography and exhibits an apparent $M_r = 134,000$.

It is of interest to note that human platelets possess relatively small amounts of this enzyme. If one takes into account the numbers of these cellular elements employed as starting material as well as the final yield of the heparitinase obtained, it is possible to calculate that each platelet must contain about 1000 molecules of this endoglycosidase. This level of enzyme is considerably less than that noted for other platelet constituents such as $\beta$-thromboglobulin, platelet factor 4, or thrombospondin (24-26), but is similar to the quantities of growth factor found within the $\alpha$-granules of these cellular elements (27).

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and cultured endothelial cells (31). This event might culmi-
nate in the removal of the anticoagulantly active heparin-like
molecules from specific areas of the vascular surface as well
as in the discharge of these potent substances into the blood.
Dramatic alterations of local or systemic blood coagulability
could ensure. Furthermore, the liberated mucopolysaccha-
rides might be responsible for other important physiologic
effects. In the next communication of this issue, we shall
demonstrate that heparan sulfates released from endothelial
cells by the action of platelet endoglycosidase are capable of
suppressing smooth muscle proliferation initiated by platelet-
derived growth factor. Thus it would appear that this muco-
polysaccharide-cleaving enzyme may represent a critical reg-
ulatory element that is involved in the development of throm-
botic and atherosclerotic lesions.

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