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Anticoagulantly Active Heparin-like Molecules from Vascular Tissue†

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ABSTRACT: Mucopolysaccharides were isolated from calf cerebral microvasculature and calf aorta. The only complex carbohydrates that exhibited anticoagulant activity were heparin-like components. The biologic potencies of calf cerebral and aortic heparin-like species were 2.92 units/mg of anti-factor Xa activity and 2.85 units/mg of anti-factor IIa activity, as well as 0.56 unit/mg of anti-factor Xa activity and 0.19 unit/mg of anti-factor IIa activity, respectively. Additional experiments revealed that the anticoagulantly active aortic components were significantly present only within the intima. The above populations of heparin-like species were affinity fractionated with antithrombin. The highly active component obtained from calf cerebral microvasculature exhibited an anti-factor Xa activity of 40.7 units/mg as well as an anti-factor IIa activity of 36.8 units/mg, constituted about 4.2% of the initial mass of the starting material, and represented about 75% of the biologic potency of the starting material. The highly active component derived from calf aorta exhibited an anti-factor Xa activity of 55.4 units/mg as well as an anti-factor IIa activity of 11.3 units/mg, constituted about 0.3% of the initial mass of the starting material, and represented about 60% of the biologic potency of the starting material. The highly active cerebral microvascular species possessed a molecular weight and charge density similar to that of heparan sulfate whereas the highly active aortic species displayed a molecular weight and charge density equivalent to that of a hexadecasaccharide fragment of heparin.

Heparin is known to be synthesized by mast cells and is capable of accelerating the neutralization of hemostatic enzymes by antithrombin (Metcalfe et al., 1979; Damus et al., 1973; Stead et al., 1976; Rosenberg et al., 1975b). Heparan sulfate, a heparin-like substance, is found on various cell surfaces as well as in basement membranes and is known to exhibit small amounts of anticoagulant activity (Kanwar & Farquhar, 1979; Kraemer, 1971; Buonassisi & Root, 1975; Teien et al., 1976; Thomas et al., 1979; Radhakrishnamurthy et al., 1977; Oegema et al., 1979). The present investigation was undertaken to determine whether heparan sulfate associated with vascular tissue contains a small population of anticoagulantly active mucopolysaccharide chains that function in a biochemically identical manner with heparin.

To this end, heparin-like molecules were isolated from calf cerebral microvessels and aortas. The ability of these components to enhance the rate of inactivation of thrombin or factor Xa by antithrombin was established by standard chromogenic assays. Affinity fractionation of the above preparations with protease inhibitor revealed that only a small portion of these products could bind to antithrombin. Fur-

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thermore, mucopolysaccharide species that interacted with the protease inhibitor were responsible for most of the biologic potency of the starting material. Thus, our results indicate that the anticoagulant activity of heparan sulfate of vascular origin is due to a small population of glycosaminoglycan chains that behave in a fashion analogous to heparin.

Materials and Methods

Isolation of Vascular Tissue. The aortas were obtained from yearling calves at a local abbatoir and immediately placed in an iced solution of 0.15 M NaCl in 0.01 M sodium phosphate, pH 7.4 (PBS). A single aorta represented ~15 cm of tissue taken immediately after bifurcation of the aortic and carotid arteries, and ~30–100 aortas were utilized for each preparation. The adventitial layers were removed with employment of forceps and scissors. The aortas were either utilized in this state or mechanically separated into intimal, inner medial, and outer medial layers. In the latter instance, the contaminating blood elements were removed from the aortas by gentle washing with iced PBS, the intima was surgically isolated with a scalpel, and inner as well as outer media were mechanically separated with tissue forceps along the external elastic lamina. The above products were pooled until sufficient tissue was available to initiate isolation of mucopolysaccharide components (see below).

Brains were also obtained from yearling calves at a local abbatoir and immediately placed in iced PBS. Meninges were removed, and gray matter was mechanically separated from white matter. Microvessels were isolated according to the method of Gimbrone et al. (1979). Approximately 50–150 brains were used for each preparation. To this end, gray matter was homogenized in a specially modified glass apparatus (Meezan et al., 1974) and filtered through a 155-μm nylon sieve. The material retained on this first sieve was collected with iced PBS, rehomogenized, and filtered through a 100-μm nylon sieve. The material retained on the second sieve was again collected as described above, rehomogenized, and filtered through a 215-μm nylon sieve. The material retained on the third sieve was collected as previously outlined. The filtrate from the third sieve was passed through a 85-μm nylon sieve. Products obtained from the 215- and 85-μm sieves were pooled until sufficient tissue was available to initiate isolation of mucopolysaccharide components (see below).

Histologic Examination. The vascular preparations were routinely examined by light microscopy. Samples were fixed in 2.0% paraformaldehyde, 2.5% gluteraldehyde, and 0.025% $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4, and processed to obtain 1 μm thick Giemsa-stained epon-embedded sections as described by Dvorak et al. (1977). The above procedure is extremely effective in identifying small dispersed populations of extraneous cell types in comparison to techniques such as electron microscopy. Moreover, the above approach possesses sufficient resolution to allow classification of these cellular elements into categories such as mast cells, basophils, and leukocytes (Galli & Dvorak, 1979).

Vascular preparations were also analyzed by immunofluorescence microscopy to unambiguously establish the presence of endothelial cells. Samples were fixed overnight in 10% formaldehyde, centrifuged at 100*g* for 5 min at 4 °C, and suspended in 0.14 M NaCl, 0.01 M KCl, 0.012 M $NaHCO_3$, and 5 mM glucose, pH 7.4. The above procedure was repeated twice with the final cell pellets suspended for 45 min at 24 °C in 0.1 mL of a 1:20 dilution of goat antisera directed against human von Willebrand's protein (Atlantic Antibodies, Westbrook, ME). The specificity of the antisera was enhanced prior to use by extensive adsorption against plasma obtained from a patient severely deficient in von Willebrand's protein. After incubation with the antisera, the cells were washed with the above buffer and pelleted at 100*g* for 5 min at 24 °C. The cells were washed 3 additional times and resuspended in 0.1 mL of a 1:20 dilution of fluorescein-conjugated rabbit antisera directed against goat immunoglobulin G (IgG) (Cappel Laboratory) for 45 min at 24 °C. Samples were washed, mounted, and examined with a Leitz Ortholux II microscope by epifluorescent illumination. Nonspecific immunofluorescence was judged to be minimal by utilizing smooth muscle cells as control.

Isolation of Mucopolysaccharides from Vascular Tissue. Tissue was resuspended in a buffer titrated to pH 7.4 containing 0.1 mM KCN, 10 mM cysteine hydrochloride and 10 mM ethylenediaminetetraacetic acid (EDTA). Papain was added to the mixture at a ratio of 600:1 (units of enzyme to grams of tissue), and the resultant suspension was incubated at 60 °C for 24 h. Pronase P was subsequently introduced at a ratio of 0.005:1 (grams of enzyme to grams of tissue), calcium acetate was added at a final concentration of 1 mg/mL, and the pH was titrated to 8.0. After incubation of the above solution at 40 °C for 24 h, the resultant material was centrifuged, and the supernate was extensively dialyzed against 0.15 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.5 at 4 °C.

The tissue digestates of either brain microvessels or aortas were chromatographed on DEAE-Sepharose A-25 previously equilibrated with 0.15 M NaCl in 0.01 M Tris, pH 7.5. The mucopolysaccharides were eluted with an ascending linear salt gradient constructed by employing 0.15 M NaCl in 0.01 M Tris, pH 7.5, in the mixing chamber and 2.0 M NaCl in 0.01 M Tris, pH 7.5, in the reservoir. Column effluents were monitored for protein and DNA/RNA content by adsorption at 280 and 260 nm, respectively, and for uronic acid content by the carbazole reaction at 530 nm (Bitter & Muir, 1962). Peak fractions were pooled, dialyzed against distilled water, and lyophilized to dryness. Contaminating DNA/RNA was removed by treatment of the samples with deoxyribonucleotidase I and ribonucleotidase A and subsequent filtration on Sepharose 4B (10 cm × 115 cm). DNA/RNA and uronic acid were determined as outlined above. Peak fractions of mucopolysaccharides free of peptide and polynucleotide contamination were pooled, dialyzed against distilled water, and lyophilized to dryness.

Preparation and Quantitation of Proteins. Thrombin, factor Xa, and antithrombin were isolated from human plasma according to methods outlined by Rosenberg et al. (1975a). The final products were homogeneous as judged by polyacrylamide gel electrophoresis and sodium dodecyl sulfate (SDS) gel electrophoresis.

Histamine methyl transferase was isolated from rat kidneys according to the method of Shaff & Beaven (1979). A crude preparation of *Flavobacterium* heparinase, provided by Dr. Robert Langer, Boston, MA, was chromatographed on columns of hydroxylapatite, cellulose–phosphate, and Sephacryl S-200 as outlined in a prior paper from this laboratory (Castellot et al., 1981). The final product exhibited a heparin cleaving potency of 3000 units/mg and was unable to degrade chondroitin sulfate, dermatan sulfate, or hyaluronic acid at enzyme concentrations as high as 250 μg/mL.

Chondroitinase ABC or AC and bovine testicular hyaluronidase were purchased from Miles Laboratories. Papain, Pronase P, deoxyribonucleotidase I, annd ribonucleotidase A were obtained from Sigma. Protein concentrations were quantitated by adsorbance measurements at 280 nm with the

appropriate extinction coefficients (Rosenberg et al., 1975a) or by the method of Lowry et al. (1951).

Quantitation of Mucopolysaccharides. Mucopolysaccharide concentrations were estimated by the carbazole reaction of Bitter & Muir (1962). Color values for the various glycosaminoglycans were obtained by examining known amounts (dry weight) of the NIH standard mucopolysaccharides.

Identification of Vascular Mucopolysaccharides. The vascular mucopolysaccharide isolated by anion-exchange chromatography were identified by specific enzymatic degradation. To this end, peak fractions of glycosaminoglycan (50 μg) were incubated either with 0.5 unit of purified *Flavobacterium* heparinase for 1 h at 30 °C, with 0.1 unit of chondroitinase ABC or AC for 4 h at 37 °C, or with 325 units of testicular hyaluronidase for 24 h at 37 °C. The environmental conditions utilized were either 0.0025 M calcium acetate in 0.25 M sodium acetate, pH 7.0 (*Flavobacterium* heparinase), or 0.0025 M Tris in 0.18 M sodium acetate, pH 7.0, with 0.5 mg/mL of bovine serum albumin added (chondroitinase ABC or AC) or 0.15 M NaCl in 0.01 M Tris, pH 7.5 (testicular hyaluronidase). Samples were obtained prior to as well as immediately after treatment with the above enzymes and were filtered at flow rates of 5.0 mL/h through columns of Sephadex G-25 (0.5 cm × 27 cm) equilibrated with 0.15 M NaCl in 0.01 M Tris, pH 7.5. Column effluents were monitored with the carbazole reaction. Degradation of mucopolysaccharides was ascertained by comparing the levels of complex carbohydrate present in the excluded volume of the column (polysaccharide) vis á vis those located in the included volume of the column (oligosaccharides). The NIH standard mucopolysaccharides provided by A. J. Cifonelli were utilized to check all assignments.

The single dimensional electrophoretic technique of Hata & Nagai (1973) was also employed to confirm the identification of vascular mucopolysaccharides obtained by anion-exchange chromatography. To this end, cellulose–acetate sheets (15 cm × 15 cm) were fixed on a glass plate (20 cm × 20 cm), and samples of the glycosaminoglycan (1.5 μg in 3.0 μL) were applied. Electrophoresis was carried out at 180 V for 1 h in 0.10 M pyridine containing 0.46 M formic acid, pH 3.1, with the cathode at the top of the sheet. After completion of the run, the sheet was placed in 1% alcian blue for 20 min and destained with 0.1% acetic acid. The various products were identified by comparison of their respective electrophoretic mobilities to those of the NIH standard mucopolysaccharides described above.

Assay Procedures. The assay for factor Xa–antithrombin–mucopolysaccharide interactions was initiated by adding a predetermined amount of the complex carbohydrate to purified antithrombin at a final concentration of 2.27×10^{-7} M. Subsequently, equimolar amounts of purified human factor Xa were admixed, and the resulting mixture was incubated for 45 s at 37 °C. Thereafter, polybrene and S-2222 synthetic substrate dissolved in distilled water were added at final concentrations of 0.31 and 3.75 mg/mL, respectively, and incubated for 90 s at 37 °C. Enzymatic activity was quenched with acetic acid at a final concentration of 6.7 M. Substrate amidolysis was determined by quantitating absorbance at 405 nm. Measurements were performed in triplicate.

The assay for thrombin–antithrombin–mucopolysaccharide interactions was initiated by admixing a predetermined amount of the complex carbohydrate with purified and antithrombin at a final concentration of 2.72×10^{-7} M. Subsequently, purified thrombin was added at a final concentration of 1.25×10^{-7} M, and the resulting mixture was incubated at 37 °C for 12 s. Thereafter, polybrene and S-2160 synthetic substrate dissolved in distilled water were added at final concentrations of 1.0 mg/mL and 26 μg/mL, respectively, and incubated for 96 s at 37 °C. Enzymatic activity was quenched with acetic acid at a final concentration of 6.7 M. Substrate amidolysis was determined by quantitating absorbance at 405 nm. All measurements were performed in quadruplicate.

An unfractionated heparin preparation with known USP potency was employed as a reference standard. Direct action of the mucopolysaccharides on factor Xa or thrombin amidolysis was determined in the absence of antithrombin. If significant, the latter kinetic effect was subtracted from the initial assay result in order to obtain the antithrombin-dependent activity of the mucopolysaccharides.

The histamine content of vascular tissue was quantitated according to the method of Shaff & Beaven (1979). Briefly, [^{14}C]methyl groups were transferred from an *S*-adenosyl-[methyl^{14}C]methionine carrier to histamine in the presence of a partially purified histamine methyl transferase at final concentrations of 1.4 and 280 μg/mL, respectively. Values obtained were compared to a standard reference curve constructed with known amounts of histamine ranging from 15.5 to 50 ng/mL. The specificity of the above assay was confirmed by demonstrating that pretreatment of samples for 60 min at 37 °C with diamine oxidase (Sigma) at a final concentration of 50 μg/mL would completely prevent transfer of [^{14}C]methyl groups into vascular tissue.

Angiotensin-converting enzymatic activity of vascular tissue was quantitated according to the method of Friedland & Silverstein (1976). The level of L-histidyl-L-leucine generated from the substrate hippuryl-L-histidyl-L-leucine (Sigma) at final concentration of 5 mM was determined in the presence and absence of 10 μM pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (Sigma, SQ20881), a specific inhibitor of the above enzyme. The level of angiotensin-converting enzymatic activity was measured by comparison of sample fluorescence with appropriate standards after subtraction of nonspecific fluorescence obtained in the presence of SQ20881.

Results

The examination of the cerebral microvessels by light microscopy with 1-μm sections revealed that the above preparations were composed predominantly of arterioles, venules, and capillaries with occasionally trapped erythrocytes. The examination of surgically isolated aortic intima by light microscopy with 1-μm sections revealed the presence of endothelial cells with rare smooth muscle cells or elastic fibrils. The occurrence of endothelial cells in the cerebral microvessel and aortic intimal preparations was established by immunofluorescent microscopy employing antisera directed against von Willebrand's protein (data not shown) and by high angiotensin-converting enzyme activities (cerebral microvessel sp act. = 4353 units/mg of protein and aortic intimal sp act. = 9142 units/mg of protein). Closer histologic examination of both preparations with 1 μm thick Giemsa-stained sections revealed the presence of a small population of metachromatically staining cells (<1% of nucleated cells of both samples). The assignment of these cellular elements as mast cells was supported by histamine determinations of 2.89 and 3.57 μg of diamine/g of wet tissue for cerebral microvessels and aortic intimal preparations, respectively.

The two preparations were enzymatically digested, and the resultant complex carbohydrates were separated by DEAE-Sepharose A-25 chromatography with a linear salt gradient as outlined under Materials and Methods. As shown in Figure 1, two peaks of mucopolysaccharide were observed for cerebral

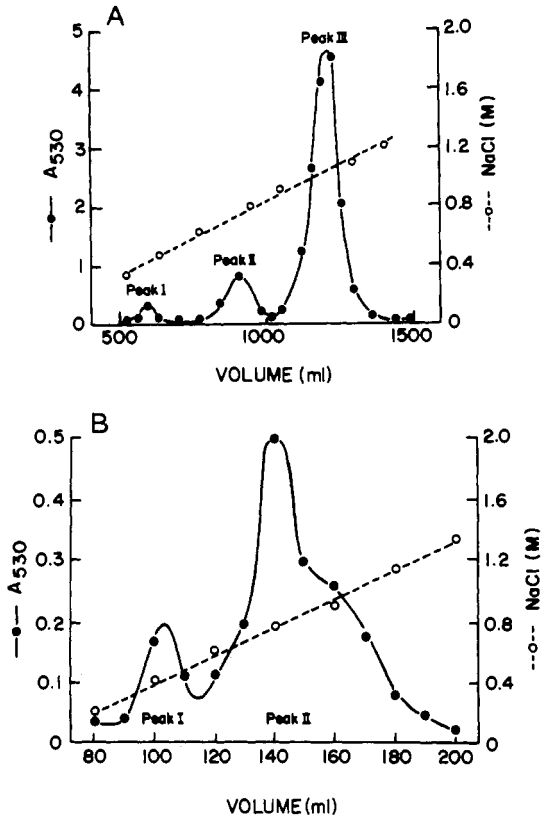

FIGURE 1: Isolation of mucopolysaccharides by DEAE-Sepharose A-25 chromatography. (A) Mucopolysaccharides were isolated from calf aortas by employing a DEAE-Sepharose A-25 column (110 cm × 2.5 cm) developed with a linear gradient of 1 L of 0.1 M NaCl–0.01 M Tris, pH 7.5, in the mixing chamber and 1 L of 2.0 M NaCl–0.01 M Tris, pH 7.5, in the reservoir. Mucopolysaccharide concentrations were estimated by the carbazole assay. (B) Mucopolysaccharides were isolated from calf cerebral microvessels by utilizing a similar ion-exchange column (1.6 cm × 47 cm) developed with a linear gradient of 0.1 L of 0.1 M NaCl–0.01 M Tris, pH 7.5, in the mixing chamber and 0.1 L of 2.0 M NaCl–0.1 M Tris, pH 7.5, in the reservoir. Mucopolysaccharide concentrations were determined by the carbazole assay.

microvessels, whereas three peaks of mucopolysaccharide were noted for whole aortas. Chromatograms of the initimal and medial layers of the aortas were similar to those of whole aortas (data not shown).

Complex carbohydrates within the above peaks were identified by specific degradatory enzymes and cellulose–acetate paper electrophoresis as outlined under Materials and Methods. Peak 2 from both preparations was cleaved by purified *Flavobacterium* heparinase, while peak 1 from both preparations and peak 3 from calf aortas were unaltered by this enzyme. It should be emphasized that our *Flavobacterium* heparinase product had previously been shown to scission NIH standard heparan sulfate or heparin whereas the same enzyme had no detectable effect upon NIH standard hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, or dermatan sulfate. Peak 3 from aortas was completely degraded by chondroitinase ABC, while ~70% of this peak was sensitive to chondroitinase AC. Approximately 5–10% of peak 2 from both preparations was sensitive to the above mucopolysaccharidases, whereas peak 1 from both preparations was totally degraded with these enzymes. Examination of chondroitinases ABC and AC with NIH standard mucopolysaccharides revealed that these enzymes are able to cleave hyaluronic acid as well as chondroitin sulfates. The peak assignments were also assessed by cellulose–acetate electrophoretic analyses, which showed that peak 1 from both

Table I: Anticoagulant Activity of Vascular Mucopolysaccharides[a]

component	biological activity (USP units/mg) anti-factor IIa act.	anti-factor Xa act.
Calf Aorta		
hyaluronic acid	<0.01 (n = 3)	<0.01 (n = 3)
chondroitin sulfate	<0.01 (n = 3)	<0.01 (n = 3)
heparin-like molecules	0.19 ± 0.06 (n = 6)	0.56 ± 0.06 (n = 6)
Calf Cerebral Microvasculature		
hyaluronic acid	<0.01 (n = 3)	<0.01 (n = 3)
heparin-like molecules	2.85 ± 0.48 (n = 3)	2.92 ± 0.32 (n = 3)

[a] The anticoagulant activities of the different mucopolysaccharides from calf aortas and calf cerebral microvessels were determined with specific amidolytic assays by employing purified human human proteins (see text for details). Values are expressed as mean ± SD. No biological activity was observed after pretreatment of samples with purified *Flavobacterium* heparinase or when buffer was substituted for antithrombin.

preparations comigrated with NIH standard hyaluronic acid, whereas peak 3 from aortas comigrated with NIH standard chondroitin sulfate and dermatan sulfate (data not shown). Peak 1 from both preparations was completely degraded with testicular hyaluronidase, while ~70% of peak 3 from the aortas was sensitive to this enzyme.

On the basis of the above data, the yields of mucopolysaccharides from the aorta per gram of wet tissue were 0.09 μg of hyaluronic acid, 0.3 μg of heparin-like molecules, 1.6 μg of chondroitin sulfate, and 0.7 μg of dermatan sulfate. The yields of mucopolysaccharides from the cerebral microvessels per gram of wet tissue were 13.8 μg of hyaluronic acid, 69.5 μg of heparin-like molecules, and 7.8 μg of chondroitin sulfate.

The anticoagulant activity of mucopolysaccharide fractions was determined by utilizing purified human proteins and well-defined synthetic substrates. As shown in Table I, only cerebral microvascular and whole aortic heparin-like species exhibit appreciable anti-factor IIa (anti-thrombin) or anti-factor Xa activities. These components display no anticoagulant potency when treated with purified *Flavobacterium* heparinase or if buffer is substituted for protease inhibitor in our assay system.

The cerebral microvascular and whole aortic heparin-like components were affinity fractionated by employing purified human antithrombin and concanavalin A–Sepharose. To this end, six separate aliquots of ~2.65 mg of the calf aortic glycosaminoglycans or eight separate aliquots of ~0.7 mg of the cerebral microvascular glycosaminoglycans were added to antithrombin at a molar ratio of 1.5. The resulting mixtures were chromatographed on concanavalin A–Sepharose columns (0.4 cm × 5.0 cm) previously equilibrated with 0.15 M NaCl in 0.01 M Tris, pH 7.5, in the presence of dextran sulfate at a final concentration of 3.8×10^{-6} M. The columns were then washed with four column volumes of equilibrating buffer containing dextran sulfate at a final concentration of 3.8×10^{-6} M and rewashed with four column volumes of equilibrating buffer in the absence of dextran sulfate. Bound mucopolysaccharides were harvested with 1.0 M NaCl in 0.01 M Tris, pH 7.5. The resultant products were pooled, dialyzed against distilled water, lyophilized to dryness, and resuspended in a minimum volume of equilibrating buffer.

On the basis of data obtained from these experiments, approximately 0.3% of the chemical mass of the calf aortic mucopolysaccharide and approximately 4.2% of the chemical mass of the cerebral microvascular mucopolysaccharide were eluted from the affinity matrix whereas approximately 94.5%

Table II: Affinity Fraction of Heparin-like Molecules[a]

component	biological activity (USP units/mg) anti-factor IIa act.	anti-factor Xa act.
Aortic Heparin-like Molecules		
prior to affinity fractionation	0.14 ± 0.01 (n = 3)	0.46 ± 0.03 (n = 3)
after affinity fractionation	11.3 ± 1.36 (n = 3)	55.4 ± 4.81 (n = 5)
Cerebral Microvascular Heparin-like Molecules		
prior to affinity fractionation	2.81 ± 0.04 (n = 3)	2.79 ± 0.10 (n = 3)
after affinity fractionation	36.8 ± 0.86 (n = 6)	40.7 ± 0.61 (n = 3)

[a] The anticoagulant activities of the various mucopolysaccharides were determined as described in Table I and under Materials and Methods. Values are expressed as mean ± SD. No biological activity was observed after pretreatment of samples with purified *Flavobacterium* heparinase or when buffer was substituted for antithrombin.

of the chemical mass of the calf aortic mucopolysaccharide and approximately 94.2% of the chemical mass of the cerebral microvascular mucopolysaccharide were recovered in the initial column filtrate. The specific anticoagulant activities of the starting preparations and the eluted components are provided in Table II. The cerebral microvascular and aortic mucopolysaccharides that complexed with the protease inhibitor were responsible for about 75% and about 60%, respectively, of the anticoagulant activities of the starting materials. Given the above data, we estimate that about 5.9×10^{-4} and about 9.0×10^{-3} USP unit of heparin-like activity (both activities refer to factor Xa inhibition) were isolated from single aorta and cerebral microvascular product from the gray matter of a single brain, respectively. This would correspond to about 11 ng of anticoagulantly active heparin-like component per single aorta and 180 ng of anticoagulantly active heparin-like component per cerebral microvessel product from a single brain, respectively.

The whole aortas were also separated into intimal, inner medial, and outer medial layers as outlined under Materials and Methods. Heparin-like components were isolated by ion-exchange chromatography and were then subjected to affinity fractionation with antithrombin. The anti-factor IIa and anti-factor Xa activities of intimal heparin-like species that bound to protease inhibitor were 2.67 and 8.75 USP units/mg, respectively, whereas the similarly designated activities of inner medial or outer medial heparin-like species that bound to protease inhibitor were less than 0.2 and less than 0.8 USP unit/mg, respectively (data not shown). In all of the above cases, biologic activity was not observed when buffer was substituted for antithrombin or when the products were treated with purified *Flavobacterium* heparinase prior to assay.

The charge density and molecular size of heparin-like species from cerebral microvessels were examined by cellulose–acetate electrophoresis and gel filtration on Sepharose 4B. As shown in Figure 2B, the cerebral microvascular components either before (track 3) or after affinity fractionation (track 5) migrated about 40% behind NIH standard heparin (track 1) and slightly behind NIH standard heparin sulfate (track 2). As depicted in Figure 3B, the cerebral microvascular heparin-like component prior to affinity fraction exhibited a molecular weight that averaged about 12000. The anticoagulant activity of this product when measured by an anti-factor Xa amidolytic assay chromatographed in a virtually identical position. Small amounts of the affinity-fractionated cerebral microvascular

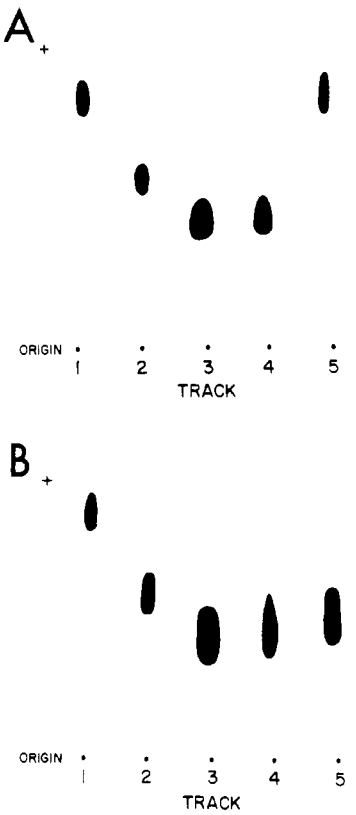

FIGURE 2: Cellulose–acetate electrophoresis of heparin-like molecules. (A) Heparin-like molecules isolated from calf aortas were electrophoresed on cellulose–acetate sheets according to the method of Hata & Nagai (1973) (see text for details). Tracks 1 and 2 represent NIH standard heparin and heparan sulfate, respectively. Track 3 represents heparin-like molecules from calf aortas prior to affinity fractionation with purified human antithrombin. Track 4 represents heparin-like molecules that are depleted of anticoagulant activity after affinity fractionation with antithrombin. Track 5 represents heparin-like molecules that exhibit anticoagulant activity after affinity fractionation with antithrombin. (B) Heparin-like molecules isolated from calf cerebral microvessels were electrophoresed as above. Tracks 1 and 2 represent NIH standard heparin and heparan sulfate, respectively. Track 3 represents heparin-like molecules from calf cerebral microvessels prior to affinity fractionation with purified human antithrombin. Track 4 represents the mucopolysaccharide depleted of anticoagulant activity after affinity fractionation with antithrombin. Track 5 represents mucopolysaccharide that exhibits anticoagulant activity after affinity fractionation with antithrombin.

heparin-like component were then added to the above product, and the resultant mixture was again examined by Sepharose 4B gel filtration. Although the anti-factor Xa potency of the sample was greatly augmented, no shift in the position of its inhibitory activity was apparent. However, more careful examination of the above mixture revealed a secondary peak of biologic activity with a molecular weight of about 60000–80000. This may represent a contribution from trace amounts of an anticoagulantly active heparin sulfate proteoglycan.

The charge density and molecular size of heparin-like species from whole aortas were also analyzed by cellulose–acetate electrophoresis and gel filtration on Sepharose 4B. As shown in Figure 2A, the aortic component before affinity fractionation (track 3) migrated 40% behind NIH standard heparin (track 1) and only slightly slower than NIH standard heparan sulfate (track 2). However, the aortic component after affinity fractionation (track 5) migrated with NIH standard heparin (track 1), which suggested that this anticoagulantly active species is more highly charged than the similarly designated species isolated from cerebral microvessels. As depicted in Figure 3A, the aortic heparin-like component prior to affinity

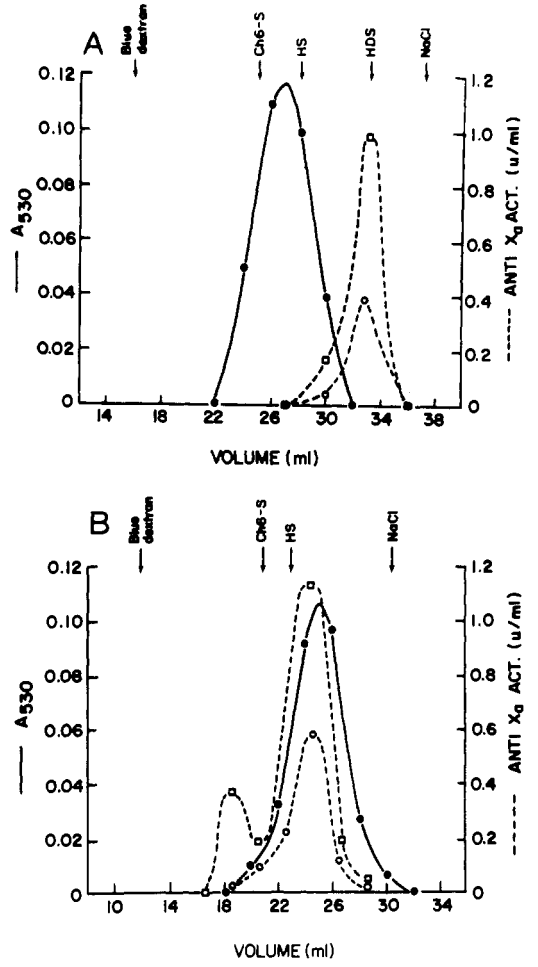

FIGURE 3: Molecular weight estimates of heparin-like molecules. (A) Heparin-like molecules isolated from calf aortas were examined on Sepharose 4B columns (0.6 cm × 110 cm) equilibrated with 0.25 M NaCl–0.01 M Tris, pH 7.5 (see text for details). The molecular weight standards utilized were the NIH standard mucopolysaccharides Ch6-S (chondroitin 6-sulfate, M_r 29 000), HS (heparan sulfate, M_r 15 000), and HDS (a hexadecasaccharide obtained by nitrous acid degradation of commercial heparin with a M_r about 5000). Chromatography of the calf aortic mucopolysaccharide revealed a single peak by uronic acid determination (●) with a molecular weight of approximately 22 000 and a single peak by anti-factor Xa assay (○) with a molecular weight of approximately 5000. The addition of an excess of the anticoagulantly active species isolated by affinity fraction augmented the activity peak by 3-fold without changing its elution position (□). (B) Heparin-like molecules isolated from cerebral microvasculature were also examined as described above. Chromatography of the aortic mucopolysaccharide revealed a single peak by uronic acid assay (●) and anti-factor Xa activity (○). The molecular weight of this species is approximately 12 000. The addition of the anticoagulantly active species from affinity fractionation with purified human antithrombin augmented the activity peak almost 2-fold and revealed the presence of a higher molecular weight component of about 60 000–80 000 (□).

fractionation exhibited a molecular weight that averaged about 22 000. However, the anticoagulant activity of this product when detected by the anti-factor Xa amidolytic assay chromatographed at a molecular weight of about 5000. Small quantities of the affinity-fractionated aortic heparin-like component were then added to the above preparation, and the mixture was again analyzed by Sepharose 4B gel filtration. The anti-factor Xa potency of the above sample was significantly enhanced, and no alteration in its position of inhibitory activity was noted. This observation indicates that the anticoagulantly active aortic species is considerably smaller than the similarly designated species isolated from cerebral microvessels. Moreover, the low molecular weight of this species may, in part, account for its elution prior to chondroitin sulfate during ion-exchange chromatography.

Discussion

Our studies suggest that heparin-like species found within calf cerebral microvessels or aortas exhibit significant anticoagulant activity. These findings are in reasonable accord with the observations of Teien et al. (1976) and Thomas et al. (1979), who have isolated heparan sulfate from the human aorta as well as porcine intestinal mucosa and have noted that this glycosaminoglycan possessed the ability to accelerate the inhibition of hemostatic enzymes.

However, our investigation also indicates that hyaluronic acid, chondroitin sulfate, or dermatan sulfate present within the above vascular tissues cannot enhance the rate of neutralization of thrombin or factor Xa by antithrombin. These latter conclusions are at variance with experimental data reported by other investigators (Izake & Murata, 1972; Vijayagopal et al., 1980). This discrepancy may be due to the inadequate identification of mucopolysaccharides and the questionable validity of the assay systems employed.

It is well established that heparin functions as an anticoagulant by binding to antithrombin and thereby accelerating hemostatic enzyme–protease inhibitor interactions (Rosenberg & Damus, 1973). Furthermore, this mucopolysaccharide is known to be synthesized by mast cells (Metcalfe et al., 1979). Previous studies (Teien et al., 1976; Thomas et al., 1979) as well as this paper have shown that heparan sulfate, a heparin-like species, exhibits anticoagulant activity and is found within the vascular tree. However, the manner by which the above component expresses this biologic property and its probable location within vascular tissue remain unresolved.

To examine these issues, we have isolated heparan-like molecules from calf cerebral microvessels and aortas. Our studies of these purified mucopolysaccharides indicate that anti-factor IIa and anti-factor Xa potencies of heparin-like species from cerebral microvessels were 2.85 and 2.92 units/mg, respectively, whereas the similarly designated potencies of heparin-like species from whole aortas were 0.19 and 0.56 unit/mg, respectively. The substitution of buffer for antithrombin in the above assays totally eliminated the biologic potency of the mucopolysaccharides, which suggests that these species express their anticoagulant activity via the protease inhibitor.

Affinity fractionation of the two types of heparin-like molecules with antithrombin supports the latter hypothesis. It has been demonstrated that heparin can be separated by this technique into two subpopulations of mucopolysaccharide with and without anticoagulant activity (Lam et al., 1976; Hook et al., 1976; Jordan et al., 1979). The affinity fractionation of heparin-like molecules from whole aortas and cerebral microvessels revealed the presence of two distinct subpopulations of glycosaminoglycans. Aortic heparin-like species that bound to anti-thrombin exhibited specific anti-factor IIa and anti-factor Xa activities of 11.3 and 55.4 USP units/mg, respectively. This subpopulation constitutes ~0.3% of the chemical mass of the complex carbohydrate and accounts for ~60% of the anticoagulant activity of the starting material. Cerebral microvascular heparin-like species that interact avidly with protease inhibitor exhibited specific anti-factor IIa and anti-factor Xa activities of 36.8 and 40.7 USP units/mg, respectively. This subpopulation constitutes ~4.2% of the chemical mass of the complex carbohydrate and is responsible for ~75% of the anticoagulant activity of the starting material.

Our data demonstrate that vascular heparin-like molecules express their anticoagulant activities via interactions with

antithrombin. Prior to affinity fractionation, aortic and cerebral microvascular components clearly differ in their anti-factor Xa potency per milligram because of varying levels of anticoagulantly active species found within the starting heparin-like preparations. This finding is most likely secondary to the large quantities of heparin-like molecules present within the medial layers of the aortas that possess insignificant amounts of anticoagulant activity (see below). After affinity fractionation, the aortic and cerebral microvascular heparin-like species exhibit significant disparities in the ratio of anti-factor II activity per milligram vis á vis anti-factor Xa activity per milligram. This phenomenon may be due to the inherent molecular properties of the two heparin-like preparations. Partial characterization of the anticoagulantly active heparin-like molecules from cerebral microvessels revealed a charge density and molecular weight distribution of approximately that of classical heparan sulfate. Preliminary examination of the anticoagulantly active heparin-like molecules from aortas indicated a charge density and molecular weight distribution similar to that of hexadecasaccharide or octadecasaccharide fragments of heparin. Heparin-like molecules of greater than approximately 7000 usually possess a binding domain that accelerates factor Xa–anti-thrombin interactions as well as "activation" and "approximation" sites that enhance the rate of thrombin–antithrombin interactions (Oosta et al., 1981). Oligosaccharides of 16 residues contain the binding domain outlined above but do not usually include the activation and approximation sites previously described (Stone et al., 1982). These structure–function relationships are probably responsible for the differing biologic potencies of the affinity-fractionated aortic and cerebral microvascular heparin-like species.

The non-thrombogenic characteristics of the vascular endothelium have been attributed to the presence of anticoagulantly active heparin-like molecules (Damus et al., 1973). However, no data are currently available to place these components at or near the surface of endothelial cells. To examine this proposition, we have subdivided calf aortas into intimal, inner medial, and outer medial layers. Mucopolysaccharides located within these regions of the vasculature were isolated by ion-exchange chromatography, and heparin-like species were affinity fractionated with antithrombin. Approximately 75% of the total heparin-like activity is present in the intima (4.41×10^{-4} USP unit/aorta), whereas only ~20% of the total activity is in the inner media (1.22×10^{-4} USP unit/aorta) and ~5% is in the outer media (0.32×10^{-4} USP unit/aorta). These values correspond to anti-factor Xa activity. However, an identical distribution of heparin-like activity was observed when calculations were based upon anti-factor IIa activity. Thus, our results demonstrated that the anticoagulantly active components were predominantly restricted to the intimal layer of the aortas. The lower specific anticoagulant activity of the affinity-fractionated material from the intima with respect to the whole aorta may reflect combination of the highly active species with inactive species. We have observed that the inactive mucopolysaccharide can bind nonspecifically to the concanavalin A–Sepharose matrix under certain conditions and coelute with the active species. Given the relatively small amount of heparin-like molecules from the intima (5 times less than that available from the whole aorta) that were subjected to affinity fractionation vis á vis the amount of matrix employed for chromatography, it is conceivable that inactive species could have bound nonspecifically to the matrix, coeluted with the highly active species, and thereby reduced our estimation of its specific anticoagulant activity. However, we would note that the intimal mucopolysaccharide preparation was augmented in its specific activity ~10-fold by affinity fractionation.

The occurrence of endothelial cells within the aortic intimal preparation was confirmed by measurement of angiotensin-converting enzyme and visualization of surface-bound von Willebrand's protein. However, additional examination of these products revealed the existence of a small population of metachromatically staining granulated cells (<1% of the total nucleated cells), which resembled mast cells. Further study of these tissue samples by histamine radioisotopic assay confirmed the presence of these cellular elements. It is conceivable that the anticoagulantly active oligosaccharides found within the intimal region of the calf aortas are synthesized by mast cells located just beneath the endothelium.

In similar fashion, we have also demonstrated that cerebral microvessels contain anticoagulantly active heparin-like species. The latter components exhibit the structural characteristics of heparan sulfate rather than heparin fragments. Microscopic examination of the above cellular preparations revealed that endothelial cells predominated as judged by angiotensin-converting enzyme and von Willebrand's protein. However, careful analyses of these products also showed a small subpopulation of mast cells (<1% of nucleated cells) as judged by histologic studies and histamine radioisotopic assay. Given the heparan sulfate like nature of the above components, it would appear less likely that mast cells are responsible for synthesizing this anticoagulantly active fraction. Despite these complexities, our demonstration that anticoagulantly active heparin fragments and heparan sulfate like species are present in close proximity to endothelial cells lends credence to the view that these components may function to maintain the non-thrombogenic characteristics of blood vessels.

Registry No. Factor IIa, 9002-04-4; factor Xa, 9002-05-5; heparin, 9005-49-6; hyaluronic acid, 9004-61-9; chondroitin sulfate, 9007-28-7; dermatan sulfate, 24967-94-0.

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Isolation and Identification of the Phosphorylated Species of Rhodopsin†

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ABSTRACT: Rhodopsin is phosphorylated in a light-dependent manner by a kinase intrinsic to the rod outer segment. We have used chromatofocusing to separate six phosphorylated species of rhodopsin and have recovered in the pH gradient fractions 60–80% of the initial phosphorylated sample loaded on the column. The isolated species of rhodopsin coincide with the species that are observed in isoelectric focusing gels in the pH range 6.1–4.7. Unphosphorylated rhodopsin focuses at a p*I* of 6.0. Two species having two phosphates per rhodopsin with isoelectric points of 5.45 and 5.40 have been isolated. The phosphate to rhodopsin ratios for the remaining species are 3.8, 5.0, 6.1, and 8.2 with isoelectric points of 5.16, 4.99, 4.85, and 4.73, respectively. The chromatofocusing profile suggests that there may be multiple forms of rhodopsin with the same number of phosphates among some of the other phosphorylated forms of rhodopsin.

Rhodopsin, the photosensitive pigment found in the disk membrane of the rod outer segment (ROS)[1] of the retina, is phosphorylated after photon absorption by an intrinsic rhodopsin kinase (Kuhn & Dryer, 1972; Bownds et al., 1972; Kuhn et al., 1973). There are 7 serine and threonine sites in the last 15 amino acids of the carboxyl terminus of rhodopsin as well as other sites more interior in the protein (Virmaux et al., 1975; Hargrave & Fong, 1977) which can serve as potential phosphorylation sites. The localization of the phosphorylation sites, the order of phosphorylation, and also the role of this phosphorylation in the visual process are not known. The half-times of phosphorylation and dephosphorylation are approximately 2 (Kuhn & Bader, 1976) and 13 min (Kuhn, 1974), respectively. Since these rates are much slower than those associated with the processes of visual transduction, it has been proposed that the phosphorylation is involved in light–dark adaption (Kuhn & Bader, 1976). On the other hand, when ATP is included in a continuous assay of the light activation of the ROS cGMP-specific phosphodiesterase, there is a rapid drop in the enzyme activity initiated by a flash of light (Liebman & Pugh, 1980). This observation has lead to the proposal that phosphorylation of bleached rhodopsin terminates its ability to participate in the phosphodiesterase activation cycle. Phosphorylated disks, containing a mixture of the phosphorylated species of rhodopsin, have been shown to exhibit a reduced ability to light activate the ROS phosphodiesterase as compared to unphosphorylated disks (Aton & Litman, 1983), further suggesting that the phosphorylation process is involved in the regulation of the ROS phosphodiesterase activity.

The various forms of phosphorylated rhodopsin can be differentiated by their isoelectric points. This property has been exploited in this study to separate the phosphorylated forms of rhodopsin by employing chromatofocusing, a column chromatographic method for separating proteins on the basis of differences in their isoelectric points. Using the nonionic detergent octyl glucoside, we have isolated seven species of rhodopsin with little contamination from adjacent species. Our experience suggests that this technique will be a valuable preparative technique for the separation of the phosphorylated species of rhodopsin as well as other phosphorylated proteins.

Materials and Methods

Rhodopsin Concentration Determination. The rhodopsin concentration was determined throughout by the difference in absorbance at 500 nm before and after bleaching a rhodopsin sample solubilized in 70–100 mM OG using an ϵ_{500} of 42000. Aliquots of Con A purified rhodopsin solubilized in 70 mM OG, 50 mM Tris–acetate, pH 7.0, diluted 1:5 with either 100 mM OG, 50 mM Tris–acetate, pH 7.0, or 1.2% Emulphogene BC-720, 66.7 mM potassium phosphate, pH 6.5,

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[1] Abbreviations: ROS, rod outer segments; OG, octyl β-D-glucoside; Con A, concanavalin A–Sepharose 4B; IEF, isoelectric focusing; *P*/*R*, moles of phosphate per mole of rhodopsin; CB, Coomassie Brilliant Blue R250; Tris, tris(hydroxymethyl)aminomethane; ϵ_{500}, molar extinction coefficient at 500 nm; EDTA, ethylenediaminetetraacetic acid.

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