The Binding of Low Molecular Weight Heparin to Hemostatic Enzymes*

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A low molecular weight preparation of porcine heparin (specific anticoagulation activity = 125 units/mg) was fractionated to obtain a mucopolysaccharide product of 8500 daltons (specific anticoagulant activity = 373 units/mg) that is homogeneous with respect to its interaction with antithrombin. This material was treated with fluorescamine in order to introduce a fluorescent tag into the mucopolysaccharide. Initially, we showed that the fluorescamine-heparin conjugate and the unlabeled mucopolysaccharide interacted with antithrombin in a virtually identical fashion. Subsequently, we demonstrated that labeled heparin could be utilized in conjunction with fluorescence polarization spectroscopy to monitor the binding of mucopolysaccharide to thrombin, factor IXa, factor Xa, and plasmin. The interaction of this complex carbohydrate with thrombin exhibited a stoichiometry of 2:1 with \( K_{D}^{th} = 8 \times 10^{-7} \text{ M} \). The formation of mucopolysaccharide-factor Xa complex is characterized by a stoichiometry of 1:1 with \( K_{D}^{mX} = 2.58 \times 10^{-7} \text{ M} \). The binding of heparin to factor Xa or plasmin occurred with low avidity. Therefore, the stoichiometries of these processes could not be established. However, our experimental data were compatible with a single-site binding residue with \( K_{D}^{m} = 8.73 \times 10^{-8} \text{ M} \) and \( K_{D}^{H} = -1 \times 10^{-7} \text{ M} \), respectively.

Several enzymes of the hemostatic mechanism bind tightly to heparin-Sepharose (1, 2). On this basis, it has been tacitly assumed that these proteins would exhibit a significant avidity for this mucopolysaccharide in solution. Numerous attempts have been made to characterize these interactions by indirect means (3, 4). Unfortunately, these studies have yielded confusing and disparate results (3, 4).

In this communication, we describe a simple technique for incorporating a fluorescent label into a well defined preparation of heparin that does not alter the functional properties of this complex polysaccharide. In addition, we demonstrate that the labeled mucopolysaccharide can be utilized in conjunction with fluorescence polarization spectroscopy to monitor the binding of heparin to various hemostatic enzymes. Furthermore, we provide direct estimates of the stoichiometries and dissociation constants of these processes.

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Materials and Methods

Chemicals—L-Aminocaproic acid was purchased from Sigma. Fluorescamine (Fluram) was obtained from Roche. All other chemicals utilized were reagent grade or better.

Heparin—Heparin of porcine origin was obtained from the Wilson Chemical Co. at an early stage in the manufacturing process and prior to treatment of the mucopolysaccharide with oxidizing agents. It was further purified by cetylpyridinium chloride precipitation. Low molecular weight species were prepared from this material by filtering 4 g of the mucopolysaccharide at flow rates of 40 ml/h through a column of Sephadex G-100 (5 x 190 cm) equilibrated with 0.15 \( \text{ M} \) NaCl in 0.01 \( \text{ M} \) Tris-HCl, pH 7.5, and pooling fractions with \( M_{r} = 6000 \) to 8000. This product was concentrated by rotary evaporation and extensively dialyzed against 0.15 \( \text{ M} \) NaCl in 0.01 \( \text{ M} \) Tris-HCl, pH 7.5, prior to use.

Proteins—Streptokinase was obtained from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N. J. Bovine serum albumin (radioimmunoassay grade) was purchased from Sigma. Purified Russell’s Viper venom was generously provided by Dr. Bruce Furie, Boston, Mass. Human factor Xa was a gift from Dr. Allan Kaplan, Stony Brook, N. Y.

Human antithrombin was isolated by heparin-Sepharose chromatography and DEAE-cellulose fractionation according to techniques established in our laboratory (6). Human thrombin was prepared in physically homogeneous form by methods previously reported (7).

Human plasminogen was isolated by lysine-Sepharose chromatography (8). Human plasmin was obtained by addition of the above zymogen to streptokinase at final concentrations of 3 mg/ml and 3000 units/ml, respectively. Subsequently, the reaction mixture was incubated for 15 min at 37°C. The solvent utilized was 25% glycerol and 0.05 \( \text{ M} \) lysine in 0.02 \( \text{ M} \) Tris-HCl, pH 8.1. The resultant enzyme preparation exhibited a single band when examined by sodium dodecyl sulfate-gel electrophoresis in the absence of reducing agents.

Human factor IX was isolated as previously described (9). Human factor IXa was prepared by the addition of purified factor Xa to the above zymogen at final concentrations of 11 \( \mu \text{g/ml} \) and 330 \( \mu \text{g/ml} \), respectively. The resulting mixture was incubated for 1 h at 37°C. The solvent utilized was 25% glycerol and 0.05 \( \text{ M} \) lysine in 0.02 \( \text{ M} \) Tris-HCl, pH 7.5. The final product exhibited a single band when analyzed by disc gel electrophoresis and sodium dodecyl sulfate-gel electrophoresis in the absence of reducing agents.

Human factor X was isolated according to a previously reported technique (10). Human factor Xa was generated from this zymogen by admixture of purified Russell’s Viper venom with this material at final concentrations of 4.5 \( \mu \text{g/ml} \) and 178 \( \mu \text{g/ml} \), respectively. The reaction mixture was incubated for 1 h at 37°C. The solvent was 0.50 \( \text{ M} \) NaCl and 0.01 \( \text{ M} \) CaCl\(_2\) in 0.01 \( \text{ M} \) Tris-HCl, pH 7.5. The enzyme produced was homogeneous when examined by disc gel electrophoresis and sodium dodecyl sulfate-gel electrophoresis in the absence of reducing agents.

Measurements of Protein and Mucopolysaccharide Concentrations—Protein concentrations were determined by absorbance measurements at 280 nm. The extinction coefficients of human thrombin, human antithrombin, human factor IX, human factor X, and human plasminogen were assumed to be 18.2 (11), 6.5 (12), 13.2 (13), 11.6 (14), and 17.9 (15), respectively. Heparin concentrations were estimated colorimetrically by assay of uronic acid according to the method of Bitter and Muir (16) or by measurement of hexosamine as...
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described by Balazs et al. (17). The relationship between these two parameters and the dry weight of heparin fractions was determined experimentally.

Assays of Biologic Activity—Factor IX (18), factor Xa (18), factor X (19), factor Xa (19), factor Xla (18), plasminogen (20), plasmin (20), thrombin (21), and antithrombin (22, 23) were assayed by minor modifications of standard techniques.

Gel Electrophoresis—The disc gel electrophoretic procedure of Davis (24) as modified by Rosenberg and Waugh (25) was used to ascertain whether protein preparations were homogeneous with respect to charge. The sodium dodecyl sulfate-gel electrophoretic system of Laemmli (26) was employed to determine whether protein preparations were homogeneous with respect to size. In the latter case, the composition of the matrix was established utilizing 0.27% bisacrylamide and 10% acrylamide.

Fluorescence Spectroscopy—Fluorescence measurements were performed with a Perkin-Elmer MFP-44A spectrophotometer equipped with thermostated sample compartment, polarization accessory, and differential corrected spectra module. The binding of unlabeled heparin to antithrombin was quantitated by measuring the enhancement in fluorescence of tryptophan residues within the protease inhibitor that were excited at 280 nm and emitted at 350 nm. The dissociation constants for the mucopolysaccharide-protein complexes with associated standard errors were calculated by nonlinear least squares fits of the data as previously described (1).

The interactions of fluorescamine-heparin with hemostatic system enzymes or antithrombin were examined by monitoring the enhancement in polarization (ΔP) of labeled mucopolysaccharide as a function of protein concentration. These measurements were conducted at excitation and emission wavelengths of 390 nm and 500 nm to minimize contributions from high levels of protein. Furthermore, a 430-nm cutoff filter was employed in front of the emission monochromator to suppress transmission of stray or scattered light. Polarization values were computed from the following formula:

\[ P = \frac{V_t - L_v}{V_t + L_v} \]  (I)

The symbols \( V_t \) and \( L_v \) refer to the magnitude of the vertically and horizontally polarized emission beams after excitation by horizontally polarized light. The symbols \( V_t \) and \( L_v \) designate the magnitude of the corresponding beams after excitation by vertically polarized light. The parameter, ΔP, was obtained by calculating the difference in polarization of fluorescamine-heparin in the presence and absence of various levels of serum proteases or antithrombin.

The dissociation constants for mucopolysaccharide-protein complexes with 1:1 stoichiometries were estimated by nonlinear least squares fit of the data to the equation:

\[ \Delta P = \frac{K_{biss}}{2[H]} + \frac{(H) + (E)}{2[H]} \]  (II)

Where (H) and (E) are the total concentrations of heparin and protein, respectively, and the adjustable parameters \( K_{biss} \) and \( K_{biss} \) are the polarization of the fluorescamine-heparin saturated with protein and the dissociation constant of this complex, respectively.

Data from mucopolysaccharide-protein interactions which exhibit stoichiometries of 1:1 as well as 1:1 were initially analyzed to obtain estimates of the projected maximal increase in polarization (ΔPmax). This was accomplished by fitting our observations to the following equation:

\[ \Delta P = \frac{K[E]}{1 + K[E]} \]  (III)

Where \( K \) is an adjustable parameter and \( E \) is the concentration of protein. The multiple \( r \) squares values for the curve fit averaged 0.99. The polarization measurements were subsequently utilized as plots of \( \Delta P/\Delta P_{max} \) versus [E].

The two dissociation constants for this interaction cannot be directly computed by fitting the above data to an appropriate equation. This difficulty is caused by our inability to obtain a simple algebraic expression which relates the magnitude of spectral signals to the concentrations of reactants and their respective dissociation constants. For this reason, we employed an indirect procedure to extract these parameters from experimental observations. To this end, we derived a series of equations which accurately describe the binding of mucopolysaccharide to protein (see below). Subsequently, these expressions were numerically evaluated for assumed values of the respective dissociation constants and specified concentrations of reactants.

The data obtained were used to construct theoretical plots of polarization for given sets of dissociation constants. Finally, these families of curves were compared to the actual observations by appropriate statistical procedures. In this fashion, we were able to choose that pair of dissociation constants which best fit our polarization data.

The theoretical profiles of polarization were computed by employing the following equation:

\[ \frac{\Delta P}{\Delta P_{max}} = \frac{[E]}{[H]} + \frac{2[H][E]}{[H]} \]  (IV)

Where \( [H] \) and \( [E] \) represent the concentrations of heparin and heparin enzyme complexes with stoichiometries of 1:1 and 2:1, respectively, and \( [H] \) signifies the initial concentration of mucopolysaccharide.

The algebraic expressions were provided below define \( [H] \) and \( [E] \) as functions of the dissociation constants \( K_{biss} \) and \( K_{biss} \) for given values of \([E]\) and \([H]\). These relationships were derived from standard mass action and conservation equations.

\[ 
\begin{align*}
[H][E] = & (H) [E] - [H][E]^2 \\
K_{biss} = & 2[H][E] + [H][E] \\
- & 4[H][E] \cdot [H][E] \\
& (H)[K_{biss} + [H][E] - [H][E]^2] \\
& - [H][K_{biss} + [H][E] - [H][E]^2] \\
& + [H][E][K_{biss} + [H][E] - [H][E]^2] \nonumber
\end{align*} 
\]  (V)

Theoretical profiles of polarization (ΔP/ΔPmax as a function of [E]) were calculated from Equation IV at \([H] = 5.57 \times 10^{-7} \text{ M} \) varying from 4.14 \times 10^{-8} \text{ M} to 1.5 \times 10^{-7} \text{ M} and for given sets of \( K_{biss} \) and \( K_{biss} \). This was accomplished by obtaining estimates of \( [H] \) and \( [E] \) from Equations V and VI at reactant concentrations provided above and for given values of \( K_{biss} \) and \( K_{biss} \) that ranged from 10^{-5} to 10^{-4} \text{ M}. The numerical evaluations were initiated by setting \( K_{biss} = 1 \times 10^{-5} \text{ M} \) and \( K_{biss} = 1 \times 10^{-6} \text{ M} \). \( [H] = 5.57 \times 10^{-7} \text{ M} \), and \([E] = 4.14 \times 10^{-8} \text{ M} \), respectively. Potential values of \( [H] \) and \( [E] \) were obtained by solving Equation VI via the iterative procedure of Newton and Horner as described by Southworth and DeLeeuw (27). Inspection of these estimates indicated that only one root of this equation represented a physically appropriate solution. Substitution of this parameter into Equation IV yielded a corresponding value for \( [H] \) and \( [E] \).

The concentrations of the two interaction products were introduced into Equation IV in order to obtain a value for \( \Delta P/\Delta P_{max} \). The entire estimation technique was repeated with \([E]\) varying from 4.14 \times 10^{-8} \text{ M} to 4.96 \times 10^{-8} \text{ M}. At the conclusion of this cycle of numerical analysis, a profile of \( \Delta P/\Delta P_{max} \) versus \([E]\) was constructed.

Subsequently, different values of \( K_{biss} \) and \( K_{biss} \) were utilized to calculate alternate theoretical plots of polarization. This was accomplished by holding \( K_{biss} = 1.0 \times 10^{-5} \text{ M} \) and advancing \( K_{biss} \) from 1.1 \times 10^{-5} \text{ M} to 1 \times 10^{-5} \text{ M} in steps of 0.1 \times 10^{-5}, where \( n = 1 \) is the order of magnitude of the inserted \( K_{biss} \), while repeating the estimation procedure as outlined above. Finally, \( K_{biss} \) was varied from 1.1 \times 10^{-5} \text{ M} to 1 \times 10^{-5} \text{ M} in increments of identical magnitude, while the various cycles of numerical evaluation were completed as previously described. In this fashion, theoretical plots of \( \Delta P/\Delta P_{max} \) versus \([E]\) were obtained for all combinations of \( K_{biss} \) and \( K_{biss} \) between 1 \times 10^{-5} \text{ M} and 1 \times 10^{-8} \text{ M}.

These theoretical profiles were compared to the actual polarization measurements by weighted least squares analyses (27). The plot that exhibited minimal deviation from our binding data was selected. This allowed us to establish the optimal set of \( K_{biss} \) and \( K_{biss} \) which fitted our experimental determinations.

RESULTS AND DISCUSSION

Preparation and Characterization of Heparin

Porcine heparin was chromatographed on Sephadex G-100 as described under "Materials and Methods" and mucopolysaccharide species of approximate \( M_r = 6000 \) to 8000 were isolated with specific anticoagulant activities that averaged...
125 ± 10 units/mg. This material was fractionated by a previously reported technique that is based upon the affinity of heparin species for antithrombin (1). The first cycle of this process was initiated by adding protease inhibitor to the mucopolysaccharide pool at a molar ratio of 0.05. Heparin bound to antithrombin, H(B1), was separated from mucopolysaccharide present in solution, H(U1). The H(B1)-inhibitor complex was subsequently processed to obtain heparin free of protein. The second cycle of fractionation was initiated by admixing antithrombin with H(U1) at a molar ratio of 0.05. The resultant solution was handled as described above to isolate H(B2) and H(U2). The third, fourth, fifth, sixth, and seventh cycles of fractionation were subsequently conducted in a like manner. In each instance, the protease inhibitor was added to the pool of heparin generated during the preceding stage of fractionation, the mucopolysaccharide-antithrombin interaction product was isolated, and complex sugar was obtained free of extraneous protein. The molar ratio of antithrombin to heparin utilized during these five cycles were 0.05, 0.05, 0.10, 0.15, and 0.15, respectively.

After completing this procedure, the various heparin species were assayed to determine their mucopolysaccharide masses and biologic potencies. The relative abundances and specific anticoagulant activities of H(B1), H(B2), H(B3), H(B4), H(B5), H(B6), H(U1), and H(U2) are provided in Table 1. Results obtained for intermediate fractions H(U) through H(U6) have been omitted. As expected from similar fractionations conducted with mucopolysaccharide derived from other sources (1), “active” heparin species appear to exhibit differing affinities for antithrombin and varying biologic potencies. Statistical analyses revealed that H(B1), H(B2), and H(B3) possess specific anticoagulant activities which are indistinguishable. However, a significant reduction in this parameter was observed for H(B4), H(B5), H(B6), and H(U2). Based upon these data, we believe that H(B4) through H(B6) are homogeneous with respect to their ability to bind to and activate antithrombin.

Subsequently, a preparative technique was designed to isolate this homogeneous form of “active” heparin. To this end, antithrombin was added to the mucopolysaccharide pool at a molar ratio of 0.08, heparin bound to the inhibitor was harvested, and the complex carbohydrate was obtained free of contaminating protein. The specific anticoagulant activity of this material is 373 ± 15 units/mg (average of 6 large scale fractionations). These preparations were filtered at flow rates of 4 ml/h through a column of Sephadex G-100 (0.55 × 180 cm). The chromatographic matrix was previously equilibrated with 0.5 m NaCl in 0.01 m Tris-HCl, pH 7.5, and calibrated with heparin standards of known molecular weight. Various products emerged at an elution volume that corresponded to an approximate M, = 6500 (not shown). This material was utilized in all subsequent experiments.

### Table 1

**Affinity fractionation of low molecular weight heparin species**

| Cycle | Fraction | Initial sample mass | Specific anticoagulant activity |
|-------|----------|---------------------|---------------------------------|
| 1     | H(B1)    | 4.6                 | 383                             |
| 2     | H(B2)    | 3.7                 | 384                             |
| 3     | H(B3)    | 5.2                 | 364                             |
| 4     | H(B4)    | 5.8                 | 348                             |
| 5     | H(B5)    | 6.2                 | 322                             |
| 6     | H(B6)    | 16.5                | 182                             |
| 7     | H(U1)    | 4.9                 | 98                              |
| Total |          | 53.1                | 4.9                             |

*These estimates were calculated from the relative amounts of mucopolysaccharide partitioning at each cycle of fractionation.

The incorporation of a fluorescent tag into the heparin molecule and the evaluation of the functional properties of the labeled mucopolysaccharide

In order to introduce a fluorescent label into the heparin molecule, we treated the mucopolysaccharide with fluoresceamine. To this end, 1 volume of a solution consisting of fluoresceamine dissolved in acetone at a concentration of 0.3 mg/ml was rapidly admixed with 3 volumes of a second solution composed of mucopolysaccharide dissolved in water at a level of 7.5 × 10⁻⁵ M. After incubation of the resultant mixture for 2 min at 24°C, it was concentrated by rotary evaporation to reduce the content of acetone and then dialyzed against 0.15 m NaCl in 0.01 m Tris-HCl, pH 7.5.

The fluorescence intensity of this heparin conjugate was quantitated by exciting the mucopolysaccharide at 380 nm and measuring the level of emission at 475 nm. Comparisons were made with unlabeled compounds, such as O-methylserine, suggest that approximately 0.4 group of fluoresceamine are incorporated per molecule of heparin. These estimates were confirmed by radiolabeling the mucopolysaccharide with [¹⁴C]acetic anhydride prior to and immediately after treatment with the fluorescent dye. The difference in ¹⁴C content between the two samples indicated that approximately 0.3 group of fluoresceamine is incorporated per molecule of heparin. The labeled mucopolysaccharide was subsequently degraded with nitrous acid at pH 1.5 and the resultant fragments were filtered on columns of P-2 polyacrylamide (28, 29). Preliminary data obtained by this technique suggest that the α-amino group of serine, within the linkage region, represents a major site for incorporation of tag (not shown).

To demonstrate that our labeling procedure had not altered the functional characteristics of the complex sugar, we compared the interactions of fluoresceamine-heparin and unlabeled mucopolysaccharide with antithrombin.

Firstly, we have analyzed the binding of unlabeled heparin to antithrombin with respect to the stoichiometry and avidity of this process. This examination was initiated by admixing varying concentrations of mucopolysaccharide which ranged from 8 × 10⁻⁷ to 5 × 10⁻⁵ M with antithrombin at a constant level of 1 × 10⁻⁵ M. The solvent was 0.15 m NaCl in 0.01 m Tris-HCl, pH 7.5, and 37°C. Subsequently, the various solutions were excited at 280 nm and their enhancements in fluorescence emission at 330 nm were recorded. It was noted that the maximal observed increase in this parameter was attained at a heparin level of 1.3 × 10⁻⁵ M. Given that the concentration of inhibitor utilized in these studies was approximately 100-fold greater than the K_diss for this interaction (see below), the maximal observed level of fluorescence represented virtually complete saturation of antithrombin with mucopolysaccharide.

In Fig. 1A, we have plotted the fluorescence enhancement of the various samples of heparin and antithrombin normalized to the maximal observed increase in this parameter (ΔF/
The binding of heparin to antithrombin as monitored by fluorescence spectroscopy. A, estimate of reaction stoichiometry. Antithrombin was admixed with varying concentrations of heparin as described in the text. The extent of fluorescence enhancement of antithrombin at 380 nm, \( \Delta F \), was determined in duplicate by quantitating this parameter in the presence and absence of mucopolysaccharide. This value normalized to the observed maximal increase (\( \Delta F/\Delta F_{\text{max}} \)) is plotted versus the molar ratio of heparin to antithrombin employed. \( \Delta F/\Delta F_{\text{max}} \) signifies the maximal observable enhancement of fluorescence. B, determination of \( K_{dts} \). The experimental conditions are similar to A except that the concentration of antithrombin was reduced to approximate the \( K_{dts} \) (see text for details). The changes in fluorescence were determined in duplicate as previously described. This parameter normalized to the projected maximal increase (\( \Delta F/\Delta F_{\text{max}} \)) is plotted versus the concentration of heparin added. \( \Delta F/\Delta F_{\text{max}} \) represents a nonlinear least squares computer fit of our data to a one-binding site model. The multiple \( r^2 \) squared value for the curve fit is 0.99.

**Fig. 1.**

The avidity of fluorescamine-heparin for antithrombin was established by measuring the \( \Delta P \) of labeled mucopolysaccharide at varying levels of added inhibitor. The concentrations of reactants were similar to those employed above except that the level of antithrombin was reduced to \( 1 \times 10^{-7} \) M. This latter value approximates the \( K_{dts} \) of this interaction (see below) and is, therefore, optimal for estimating this parameter (30). Figure 1B shows the results of a typical experiment in which the fluorescence enhancement of various mixtures of mucopolysaccharide and antithrombin normalized to the projected maximal increase in this parameter (\( \Delta F/\Delta F_{\text{max}} \)) are plotted versus the molar concentrations of heparin utilized. The solid curve represents a direct computer fit of our data to a single binding site model with \( K_{dts} = 4.14 \times 10^{-8} \) M. Similar examinations of the mucopolysaccharide-inhibitor interaction were conducted on 10 separate occasions and \( K_{dts} \) averaged 5.74 \( \pm 0.72 \times 10^{-8} \) M.

Secondly, we have studied the binding of fluorescamine-heparin to antithrombin with regard to stoichiometry and avidity. This analysis was initiated by admixing varying concentrations of inhibitor that ranged from \( 1 \times 10^{-9} \) to \( 2.5 \times 10^{-7} \) M with labeled mucopolysaccharide at a constant level of 9.23 \( \times 10^{-6} \) M. The solvent was 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5, and 37°C. Subsequently, the various solutions were excited at 390 nm and their enhancement in fluorescence polarization at 500 nm (\( \Delta P \)) was quantitated. The maximal observed change in this parameter (\( \Delta P_{\text{max}} \)) represents an augmentation of approximately 100% and is reached at an inhibitor concentration of \( 1.4 \times 10^{-5} \) M or greater. Under these conditions, the protein contributes less than 5% to the total emission signal. Since the concentration of fluorescamine-heparin employed in these titrations was approximately 100-fold greater than the \( K_{dts} \) of this interaction (see below), the maximal level of polarization fluorescence corresponds to the virtually complete saturation of labeled mucopolysaccharide by antithrombin.

In Fig. 2A, we have plotted the enhancement in fluorescence polarization of the various samples normalized to the observed maximal increase in this parameter (\( \Delta P/\Delta P_{\text{max}} \)) versus the molar ratio of antithrombin to fluorescamine-heparin utilized. The stoichiometry of this interaction was calculated as outlined above. The value obtained is equivalent to a molar stoichiometry of inhibitor to labeled mucopolysaccharide of 0.96, which is virtually identical with that observed with unlabeled heparin.

The avidity of fluorescamine-heparin for antithrombin was calculated by measuring the \( \Delta P \) of labeled mucopolysaccharide at varying levels of added inhibitor. The concentrations of reactants were similar to those employed above except that the level of fluorescamine-heparin was reduced to \( 4.62 \times 10^{-7} \) M. Figure 2B represents the average results of several experiments in which the \( \Delta P \) of various mixtures normalized to the projected maximal increase in this parameter (\( \Delta P/\Delta P_{\text{max}} \)) are plotted versus the molar concentration of inhibitor utilized. The dashed curve depicts a direct computer fit of our data to a single-site model with \( K_{dts} = 1.06 \pm 0.17 \times 10^{-8} \) M. This elevation in the \( K_{dts} \) of fluorescamine-heparin as compared to that of unlabeled mucopolysaccharide may have resulted from conducting experiments at reactant concentrations which are almost 5-fold higher than the dissociation constant. Unfortunately, this is necessary given the fluorescent intensity of the labeled heparin. Alternatively, the incorporation of an intrinsic label into the mucopolysaccharide may minimally distort the binding of heparin to antithrombin.

**The Interactions of Heparin with Enzymes of the Hemostatic Mechanism**

The data provided above demonstrate that fluorescamine-heparin and unlabeled mucopolysaccharide interact in a virtually identical manner with antithrombin. Thereafter, flu-
to an increase of approximately 80% and represents virtually complete saturation of fluorescamine-heparin with antithrombin, for reasons outlined above. In Fig. 3A, we have plotted \( \Delta P/\Delta P_{\text{max}} \) of the various reaction mixtures versus the molar ratios of factor IXa to heparin utilized. Appropriate extrapolation of the linear portion of the binding isotherm as previously described suggests that the stoichiometry of this process corresponds to a molar ratio of enzyme to mucopolysaccharide of 1.05.

The avidity of heparin-factor IXa interactions was determined by measuring the \( \Delta P \) of labeled mucopolysaccharide at varying levels of added inhibitor. The concentrations of reactants were similar to those utilized above except that the level of fluorescamine-heparin was decreased to 4.29 \( \times \) \( 10^{-7} \) \( \text{M} \), which approximates \( K_{\text{IIIXa}} \) (see below). Fig. 3B depicts the average results of several experiments in which \( \Delta P \) of the various reaction mixtures normalized to the projected \( \Delta P_{\text{max}} \) of this process (\( \Delta P/\Delta P_{\text{max}} \)) are plotted versus the molar concentrations of factor IXa employed. The dashed curve represents a direct computer fit of the above data to a single-binding site model with \( K_{\text{IIIXa}} = 2.58 \pm 0.35 \times 10^{-7} \) \( \text{M} \).

**Thrombin-Mucopolysaccharide Interactions**—The binding of thrombin to fluorescamine-heparin was studied under the same conditions and in a manner identical with that described in the previous section. The stoichiometry of this interaction was determined by adding varying concentrations of thrombin, which ranged between 5 \( \times \) \( 10^{-7} \) \( \text{M} \) and 3 \( \times \) \( 10^{-5} \) \( \text{M} \), to a constant level of fluorescamine-heparin maintained at 8.57 \( \times \) \( 10^{-6} \) \( \text{M} \) and measuring the \( \Delta P \) of each of these reaction...

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**Fig. 2.** The binding of fluorescamine-heparin to antithrombin as monitored by fluorescence polarization spectroscopy. A, estimate of reaction stoichiometry. Fluorescamine-heparin is added to varying levels of antithrombin as outlined in the text. The extent of fluorescence polarization enhancement of the labeled mucopolysaccharide at 500 nm, \( \Delta P \), was determined in duplicate by quantitating this parameter in the presence and absence of antithrombin. This value normalized to the observed maximal increase (\( \Delta P/\Delta P_{\text{max}} \)) is plotted versus the molar ratio of antithrombin to fluorescamine-heparin utilized. —represents a linear least squares fit of the binding data contained within the ascending limb of the isotherm (\( r = 0.99 \)). \( \Delta P \) signifies the maximal observable enhancement in fluorescence. B, determination of \( K_{\text{IIIXa}} \). The experimental conditions are similar to A except that the concentration of fluorescamine-heparin is lowered to approximate the \( K_{\text{IIIXa}} \) (see text for details). The changes in fluorescence polarization were determined in duplicate as outlined above. This parameter normalized to the projected maximal increase (\( \Delta P/\Delta P_{\text{max}} \)) is plotted versus the concentration of antithrombin added. —represents a nonlinear least squares computer fit of our data to a one-binding site model. The multiple \( r \) squared value for the curve fit is 0.99.

**Factor IXa-Mucopolysaccharide Interactions**—The binding of factor IXa to fluorescamine-heparin was measured under conditions identical with those employed for examining the interaction of antithrombin with labeled mucopolysaccharide. The stoichiometry of this process was evaluated by admixing varying concentrations of factor IXa that ranged from 5.0 \( \times \) \( 10^{-8} \) \( \text{M} \) to 7.5 \( \times \) \( 10^{-6} \) \( \text{M} \) with labeled mucopolysaccharide at a constant level of 5.0 \( \times \) \( 10^{-5} \) \( \text{M} \) and quantitating the \( \Delta P \) of the various mixtures as previously described. Added enzyme contributed less than 5% of the total emission signal at the highest concentrations employed. The \( \Delta P_{\text{max}} \) of this interaction was experimentally established by noting that \( \Delta P \) attained a constant value for inhibitor concentrations of 6.43 \( \times \) \( 10^{-7} \) \( \text{M} \) or higher. This maximal enhancement corresponds to an increase of approximately 80% and represents virtually complete saturation of fluorescamine-heparin with antithrombin, for reasons outlined above. In Fig. 3A, we have plotted \( \Delta P/\Delta P_{\text{max}} \) of the various reaction mixtures versus the molar ratios of factor IXa to heparin utilized. Appropriate extrapolation of the linear portion of the binding isotherm as previously described suggests that the stoichiometry of this process corresponds to a molar ratio of enzyme to mucopolysaccharide of 1.05.

The avidity of heparin-factor IXa interactions was determined by measuring the \( \Delta P \) of labeled mucopolysaccharide at varying levels of added inhibitor. The concentrations of reactants were similar to those utilized above except that the level of fluorescamine-heparin was decreased to 4.29 \( \times \) \( 10^{-7} \) \( \text{M} \), which approximates \( K_{\text{IIIXa}} \) (see below). Fig. 3B depicts the average results of several experiments in which \( \Delta P \) of the various reaction mixtures normalized to the projected \( \Delta P_{\text{max}} \) of this process (\( \Delta P/\Delta P_{\text{max}} \)) are plotted versus the molar concentrations of factor IXa employed. The dashed curve represents a direct computer fit of the above data to a single-binding site model with \( K_{\text{IIIXa}} = 2.58 \pm 0.35 \times 10^{-7} \) \( \text{M} \).

**Fig. 3.** The binding of fluorescamine-heparin to factor IXa as monitored by fluorescence polarization spectroscopy. See Fig. 2 and text for experimental details. A, estimate of reaction stoichiometry. —represents a linear least squares fit of binding data contained within the ascending limb of the isotherm (\( r = 0.99 \)). B, determination of \( K_{\text{IIIXa}} \). —represents a nonlinear least squares computer fit of our data to a one-binding site model. The multiple \( r \) squared value for the curve fit is 0.99.
mixtures. The $\Delta P_{\text{max}}$ of this process was experimentally defined by observing that $\Delta P$ reached a constant level when thrombin concentrations of $1.0 \times 10^{-7}$ M or greater were employed. This value represents an augmentation in fluorescence polarization of approximately 150%. During these titrations, thrombin contributed less than 5% of the total emission signal at the highest concentration of enzyme used. In Fig. 4A, we have plotted $\Delta P/\Delta P_{\text{max}}$ of the various mixtures versus the molar ratio of thrombin to fluorescamine-heparin employed. As noted, a line drawn through the initial linear portion of the binding isotherm intersects the horizontal asymptote at a molar ratio of thrombin to fluorescamine-heparin of 1.8. This suggests that a single molecule of enzyme is capable of binding two molecules of mucopolysaccharide (see below). It should be emphasized that the extrapolation technique utilized a horizontal asymptote which corresponds to the enhancement in fluorescence polarization generated by complexes formed between single molecules of fluorescamine-heparin and thrombin. These species are expected to predominate as the concentration of enzyme becomes large relative to that of mucopolysaccharide.

Subsequently, we sought to determine the avidity of this complex polysaccharide for thrombin. To this end, we quantified the $\Delta P$ of fluorescamine-heparin at varying levels of added enzyme as outlined earlier. However, the concentration of labeled mucopolysaccharide was reduced to $8.57 \times 10^{-7}$ M, which is optimal for evaluating the dissociation constants of this interaction. Fig. 4B shows the average results of several experiments in which the $\Delta P$ of various mixtures, normalized to the projected $\Delta P_{\text{max}}$ of this process, are plotted versus the molar concentrations of thrombin employed. Our polarization data are most compatible with a two-binding-site model. The best statistical fit was achieved by utilizing $K_{1,\text{M}}^{\text{LW}} = 8.0 \times 10^{-7}$ M (Fig. 4B). However, our analyses also revealed that variations in the magnitude of $K_{1,\text{M}}^{\text{LW}}$ and $K_{2,\text{M}}^{\text{LW}}$ of ~40% had an insignificant effect upon this statistical fit provided that the sum of these two parameters was held constant at twice their initial value ($1.6 \times 10^{-6}$ M).

We have employed the dissociation constants and interaction model provided above to examine the validity of our procedure for estimating reaction stoichiometry. To this end, a theoretical binding curve was constructed at reactant concentrations identical with those utilized for evaluating this parameter. Extrapolation of the linear portion of this isotherm intersected the horizontal asymptote at a molar ratio of heparin to thrombin of 1.8. Thus, the experimentally defined reaction stoichiometry is identical with that predicted from the two-binding-site model.

Our analyses of the heparin-thrombin interaction are based upon the assumption that the binding of each molecule of mucopolysaccharide to enzyme will generate an equivalent spectral signal. This appears quite reasonable in view of the fact that an extrinsic probe is utilized to monitor these events and in light of simple physical considerations which govern the enhancement of fluorescence polarization signals. The validity of this assumption is further bulwarked by the close approximation of our mucopolysaccharide-enzyme stoichiometric ratio to 2. However, complex models in which two or more heparins bind to thrombin and contribute in a differential fashion to the resultant fluorescence polarization signal cannot be completely excluded.

**Factor Xa-Mucopolysaccharide Interactions**—The binding of factor Xa to fluorescamine-heparin was studied under identical conditions and in a manner similar to other hemo-static enzyme-mucopolysaccharide interactions. Given the low avidity of factor Xa for labeled mucopolysaccharide (see below), enzyme levels in excess of $5.0 \times 10^{-7}$ M would be required to saturate the fluorescamine-heparin and unambiguously establish the molar stoichiometry of this process. Unfortunately, it proved impossible to conduct studies under these conditions because high levels of factor Xa contributed significantly to our spectral signal.

Therefore, we assumed that this process exhibited a molar stoichiometry of 1 and attempted to define the $K_{1,\text{M}}^{\text{LW}}$ of this interaction. To this end, we admixed varying concentrations of factor Xa which ranged from $1 \times 10^{-7}$ M to $2 \times 10^{-7}$ M with fluorescamine-heparin maintained at a constant level of $4.29 \times 10^{-6}$ M and determined the $\Delta P$ of the various solutions. Fig. 5 depicts the average results of several experiments in which $\Delta P$ normalized to the projected $\Delta P_{\text{max}}$ is plotted versus the molar concentration of enzyme employed. The dashed curve represents a direct computer fit of the data to a single-binding site model with $K_{1,\text{M}}^{\text{LW}} = 8.79 \pm 1.20 \times 10^{-6}$ M. Our fluorescence polarization studies to enzyme also analyzed in terms of the more complex two-binding site model employed for thrombin-mucopolysaccharide interactions. In this case, the two dissociation constants extracted from our data were similar in magnitude to that provided by the single-binding site model.

**Plasmin-Mucopolysaccharide Interactions**—We attempted to investigate the binding of plasmin to fluorescamine-heparin in a fashion similar to that described for the factor Xa-mucopolysaccharide interaction. However, this serine protease is known to exhibit limited solubility in aqueous media unless small amounts of $\varepsilon$-amino acids are present (31). Therefore, the binding of protein to mucopolysaccharide was examined in 0.15 M NaCl and 0.005 M $\varepsilon$-aminocaproic acid in 0.01 M Tris-HCl, pH 7.5. Utilizing experimental protocols outlined in the

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**Fig. 4.** The binding of fluorescamine-heparin to thrombin as monitored by fluorescence polarization spectroscopy. See Fig. 2 and text for experimental details. A, estimate of reaction stoichiometry. --- represents a linear least squares fit of binding data contained within the ascending limb of the isotherm ($r = 0.99$). B, determination of $K_{1,\text{M}}^{\text{LW}}$ and $K_{2,\text{M}}^{\text{LW}}$. --- represents theoretical values calculated from the two-binding-site model that best fits our experimental data. These estimates were obtained by numerical evaluation of Equations V and VI with $K_{1,\text{M}}^{\text{LW}} = K_{2,\text{M}}^{\text{LW}} = 8 \times 10^{-7}$ M. $LMW$, low molecular weight.
previous section we were able to detect modest alterations in the fluorescence polarization of heparin.

For example, varying concentrations of plasmin that ranged from 0 to 2.6 x 10^{-5} M were admixed with labeled mucopolysaccharide at a constant level of 5 x 10^{-6} M. The various solutions were examined as previously described in order to determine the DP of heparin. This parameter exhibited a small increase at very high concentrations of added enzyme (Table II). If these data are employed in conjunction with the average DP max of other mucopolysaccharide-enzyme interactions, $K_{D,HSA}$ is estimated as $10^{-4}$ M. For comparison, similar titrations were conducted with factor Xa and bovine serum albumin (Table II). Appropriate calculations suggest that $K_{D,HSA}$ = 8.7 x 10^{-4} M and $K_{D,BSA}$ = 4.2 x 10^{-6} M, respectively. The former constant is in excellent agreement with that previously determined (see above), whereas the latter parameter indicates that albumin binds more tightly to heparin than plasmin. These data suggest that the interaction of mucopolysaccharide with plasmin is probably "nonspecific."

In summary, we have utilized a highly discriminating but simple isolation technique based upon the affinity of heparin for antithrombin to fractionate a low molecular weight pool of mucopolysaccharide. This approach allowed us to isolate a subpopulation of heparin with a $M = ~6500$ that is homogeneous with respect to its interactions with protease inhibitor. The availability of this well defined preparation of mucopolysaccharide prompted us to initiate a detailed examination of the binding of this component to the various enzymes of the hemostatic mechanism.

Prior to this study, quantitative techniques were not available to directly monitor these interactions. On the one hand, the aromatic amino acid residues of hemostatic enzymes are not significantly perturbed when these proteins bind to heparin. On the other hand, no spectral transitions have been defined within the mucopolysaccharide that might be employed to detect complex formation. Therefore, we decided to covalently attach an extrinsic label to one of the reactants and utilize this moiety to analyze these processes.

To this end, our carefully fractionated heparin preparation was treated with fluorescamine so that a spectral tag could be incorporated into the mucopolysaccharide. Subsequently, we were able to demonstrate that the presence of this extrinsic label did not significantly alter the interaction of this component with antithrombin. Thereafter, fluorescamine-heparin was employed in conjunction with fluorescence polarization spectroscopy to characterize the binding of mucopolysaccharide to thrombin, factor Xa, factor Xa, and plasmin.

The $K_{D,HSA}$ of these processes varied from $10^{-4}$ M (plasmin) to 2.58 x 10^{-5} M (factor Xa) but never attained a value equivalent to $K_{D,HSA}$ of 5.72 x 10^{-5} M. Thus our results contradict previous claims that heparin may bind more tightly to heparin than antithrombin (32, 33). Two of these interactions also exhibited a relatively high $K_{D,BSA}$ that was greater than the similarly designated parameter obtained with albumin. Given the high concentrations of the latter component normally present within blood, it seems unlikely that mucopolysaccharide-serine protease complex formation are required for the anticoagulant action of heparin. However, the binding parameters established during the present investigation will be utilized in the subsequent communication (34) to conduct a detailed evaluation of the kinetic importance of the various heparin-protein complexes.

The approach described in this report should also prove useful in examining other biologic interactions of this type. It is thought that mucopolysaccharides bind tightly to proteins such as fibronecin (35), lipoprotein lipase (36), lipoproteins, collagen, $\beta$-thromboglobulin, platelet factor four (37), etc. Unfortunately, it has not been possible to characterize the stoichiometries, specificities, and avidities of these processes. Studies of several of these events utilizing the fluorescence polarization technique outlined above are currently underway in our laboratory.

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**FIG. 5.** The binding of fluorescamine-heparin to factor Xa as monitored by fluorescence polarization spectroscopy. Determination of $K_{D,HSA}$ represents a nonlinear least squares computer fit of our data to a one-site binding model. The multiple $r$ squared value for the curve fit is 0.99.

**TABLE II**

Interactions of fluorescamine-heparin with Factor Xa, plasmin, and bovine serum albumin as monitored by polarization fluorescence spectroscopy

| Protein             | Concentration of component | Change in fluorescence polarization of heparin |
|---------------------|----------------------------|-----------------------------------------------|
| Factor Xa           | $10^{-6}$                  | 51.0                                          |
|                     | $10^{-7}$                  | 60.0                                          |
|                     | $10^{-8}$                  | 60.0                                          |
|                     | $10^{-9}$                  | 60.0                                          |
|                     | $10^{-10}$                 | 60.0                                          |
|                     | $10^{-11}$                 | 60.0                                          |
| Plasmin             | $10^{-6}$                  | 4.0                                           |
|                     | $10^{-7}$                  | 4.0                                           |
|                     | $10^{-8}$                  | 4.0                                           |
|                     | $10^{-9}$                  | 6.0                                           |
|                     | $10^{-10}$                 | 9.0                                           |
|                     | $10^{-11}$                 | 9.0                                           |
| Bovine serum albumin| $10^{-6}$                  | 20                                            |
|                     | $10^{-7}$                  | 20                                            |
|                     | $10^{-8}$                  | 27                                            |
|                     | $10^{-9}$                  | 27                                            |
|                     | $10^{-10}$                 | 28                                            |
|                     | $10^{-11}$                 | 32                                            |

$a$ The extent of fluorescence polarization was determined in triplicate as described under "Materials and Methods."
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