The antiheparin effect of a heparinoid, pentosan polysulphate

Investigation of a mechanism

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A pentosan polysulphate [a fully sulphated (1-4)- β -D-xylopyranose with a single laterally positioned 4-O-methyl- α -D-glucuronic acid] has been shown to inhibit the anticoagulant activity of high-affinity heparin as observed in plasma and when using purified enzyme and inhibitor. The activity was shown to be concentration-dependent with an apparent K_i of approx. 2 μ M. The antiheparin property was not shown by a number of other anionic carbohydrates when tested. The rate of thrombin inhibition at 0.33 μ M-heparin was reduced from $7.1 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ in the absence of pentosan polysulphate to $2.3 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ at 2 μ M-pentosan polysulphate and to $0.3 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ at 20 μ M. Using the random bireactant model of heparin action [Griffiths (1982) *J. Biol. Chem.* **257**, 13899–13902] it was observed that the pentosan polysulphate had no effect on the K_m for antithrombin III (150 nM) but increased the K_m for thrombin from 25 nM to 450 nM. A reduction in the inhibition rate by 17.3-fold predicted by substitution of these values into the general two-substrate reaction-rate equation was confirmed experimentally.

The principal mechanism by which heparin exerts its anticoagulant effect is by acceleration in a catalytic manner of the rate of interaction between the coagulation serine proteinases and the plasma proteinase inhibitor, antithrombin III (for review see Barrowcliffe & Thomas, 1981; Rosenberg & Oosta, 1981).

A number of other sulphated polysaccharides have been shown to have anticoagulant properties *in vitro* and *in vivo* (Pulver, 1961; Thomas *et al.*, 1977; Coccheri *et al.*, 1979) and are grouped loosely under the heading of heparinoids. The mechanism of the anticoagulant effect of heparinoids has only recently come under investigation in evaluating whether these compounds act by combination with coagulation proteinases directly or by acceleration of the effect of antithrombin III (Walker & Esmon, 1978; Czapek *et al.*, 1980; Vinazzer *et al.*, 1980; Soria *et al.*, 1980; Thomas *et al.*, 1980; Nilsson *et al.*, 1982).

One of such heparinoids is a pentosan polysulphate prepared by sulphation of carbohydrate extracted from the bark of the beech tree, *Fagus sylvatica*. The structure of the parent polysaccharide has been elucidated as chains of (1-4)- β -D-xylo-

pyranose with laterally positioned 4-O-methyl- α -D-glucuronic acid at C-2 of xylose (Aspinall, 1959). All available hydroxyl groups are occupied upon sulphation (Raveux *et al.*, 1966). The degree of polymerization corresponds to a molecular mass in the region of 6500–10000 Da. It has been shown to be a low-potency heparin (Soria *et al.*, 1980), about 10 times less effective *in vitro* than heparin on a weight-to-weight basis (Scully *et al.*, 1983).

In investigation of these anticoagulant properties it was observed that upon addition of the heparinoids at low concentrations to heparinized plasma it completely inhibited the prolongation of clotting time caused by heparin. When this property was examined in purified systems it appeared to be due to inhibition of the heparin potentiation of the rate of inhibition of thrombin by antithrombin III. In this present study we have investigated the mechanism by which the antiheparin property may be occurring and conclude that it is related to lowering of the binding affinity of thrombin for the heparin-antithrombin III complex.

Materials and methods

Materials

Triethanolamine and polyethylene glycol 6000 were obtained from BDH. Chondroitin sulphates

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A, B, C and hyaluronic acid (grade I) were obtained from Sigma. Pentosan polysulphate was obtained from Clin Midi, Paris, France as a solution of 50 mg/ml. Antithrombin III and heparin were coupled to Sepharose 4B (Pharmacia) after activation by CNBr according to March *et al.* (1974). Pooled normal plasma was prepared by centrifugation of blood from ten healthy individuals at 3000g for 15 min at 4°C. Plasma was then pooled and stored frozen at -25°C.

Human antithrombin III was obtained from Kabi Vitrum, Stockholm, Sweden. This material contained albumin as bulking agent and was purified by chromatography on heparin-Sepharose (Miller-Andersson *et al.*, 1974), any contaminating heparin being removed by further chromatography on DEAE-cellulose (Griffiths, 1982a). The protein was stored in 0.05M-Tris/0.15M-NaCl, pH 7.4, frozen at -20°C. The protein was homogenous when tested by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and titration with stoichiometric amounts of thrombin. A molecular mass for antithrombin of 67000 Da (Miller-Andersson *et al.*, 1974) and absorption coefficient of $6.1 \text{ dl} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ ($A_{1\%}^{1\text{cm}, 280}$) (Jesty *et al.*, 1974) were used in all calculations. Bovine thrombin (Thrombostat, Parke Davis) was purified and the α -thrombin fraction prepared by the method of Lundblad *et al.* (1975). This material was stored in 0.75M-NaCl frozen at -20°C and had a specific activity by clotting assay of 2000 NIH units/mg and was homogenous on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. A molecular mass of 39000 Da (Mann *et al.*, 1971) and absorption coefficient of $19.5 \text{ dl} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ ($A_{1\%}^{1\text{cm}, 280}$) (Windsor & Scheraga, 1964) were used in all calculations.

High- and low-affinity fractions of average molecular mass were prepared from pure unfractionated heparin by the method of Danielsson & Bjork (1981). Mucosal heparin (500 mg; Leo) in 15 ml of 0.02M-imidazole/HCl/0.3M-NaCl, pH 7.4, were placed on a column (90 cm $\times$ 2.6 cm) of Ultrogel AcA44 (LKB) equilibrated with the same buffer. Chromatography was carried out at 30 ml/h. Fractions were examined for heparin by metachromatic assay using Azure A (Lam *et al.*, 1976) and those containing peak concentrations were pooled. These corresponded to an average molecular mass of 13200 Da (Danielsson & Bjork, 1981) and were subjected to chromatography on antithrombin III-Sepharose. The high- and low-affinity fractions (eluted at approx. 0.8M-NaCl) were pooled separately, dialysed against distilled water and lyophilized. The high-affinity material was found to have a specific activity of 220 i.u./mg when compared with unfractionated heparin. Heparin concentration was measured by meta-

chromatic assay in relationship to a known heparin standard; a molecular mass of 13200 Da was used in all calculations. This fractionated high-affinity heparin was used throughout.

Assay methods

Thrombin clotting time was measured in an automated coagulometer (Burkard Instruments, Uxbridge, U.K.) after addition of 0.2 ml of thrombin (1 NIH unit/ml in water) to 0.1 ml of pre-warmed (37°C) pooled citrated human plasma. Heparin or the pentosan polysulphate were added in 5–10 μ l aliquots to achieve the concentrations described in the text.

Kinetics of interactions between antithrombin III and thrombin were followed using a chromogenic substrate for thrombin, H-D-Phe-Pip-Arg-p-nitroanilide HCl (S2238, Flow Laboratories) (Scully & Kakkar, 1977). The buffer systems of Griffiths *et al.* (1980) were used. Antithrombin III-thrombin interaction was carried out in 0.1M-triethanolamine/HCl/0.15M-NaCl/0.1% polyethylene glycol 6000, pH 7.8. At intervals aliquots were withdrawn and added to the same buffer but containing 0.2% Polybrene (Sigma) and 0.2 mM with respect to S2238. The dilution at this stage was varied according to the thrombin concentration employed. The initial rate of substrate hydrolysis was measured in an Abbott Biochromatic Analyzer 100 using the 380/450 nm filter and related to enzyme concentration from curves prepared with thrombin standards.

Results

Gel filtration

In Fig. 1 are shown the results of gel filtration of 500 mg of the pentosan polysulphate on a column (90 cm $\times$ 2.6 cm) of AcA44 equilibrated with 0.02M-imidazole/HCl/0.3M-NaCl, pH 7.4, and a flow rate of 30 ml/h. The molecular mass distribution of heparin chromatographed on the same column is shown for comparison. A skewed distribution is seen, eluting with an apparent molecular mass between 5000 and 13000 Da approx., using heparin as a standard (Danielsson & Bjork, 1981). The bulk of the pentosan polysulphate was eluted at a molecular mass corresponding to fraction V of Danielsson & Bjork (1981), i.e. 6000 Da. The fractions containing this material were pooled and used in further studies.

Antiheparin effect

In Fig. 2 is shown the effect of an increasing concentration of the pentosan polysulphate upon the prolongation of the thrombin clotting time of

Fig. 1. Gel chromatography of pentosan polysulphate and heparin

Elution profile of pentosan polysulphate and unfractionated heparin from a column of AcA44 equilibrated with 0.02M-imidazole/HCl/0.3M-NaCl, pH7.4. The column was eluted at a rate of 30ml/h and 6ml fractions were collected. The concentration of pentosan polysulphate (x) or heparin (●) in each fraction was measured by metachromatic assay with Azure A or by observed antiheparin activity (◇).

plasma by high-affinity heparin at a final concentration of $0.166\mu\text{M}$. The results show clearly the reversal of the heparin anticoagulation, such that at $15\mu\text{g}$ of pentosan polysulphate the prolongation is completely nullified. This confirms our previous results using unfractionated heparin (Scully *et al.*, 1983). A number of other sulphated glycosaminoglycans and hyaluronic acid were tested for ability to reverse the prolongation of the thrombin clotting time by heparin and the results are shown in Table 1. None of these compounds (apart from chondroitin sulphate A which had a slight effect) was observed to have the antiheparin property of pentosan polysulphate and tended instead to increase the heparin anticoagulation.

Effect of concentration of pentosan polysulphate on the time course of antithrombin III-thrombin interaction in the presence of heparin

In Fig. 3 is shown the time course of inactivation of thrombin by antithrombin III in the presence of a single concentration of heparin and of increasing concentrations of the pentosan polysulphate. Under the conditions used the inhibition of thrombin is very rapid in the presence of heparin alone and is essentially complete within 15s. The rate is reduced in the presence of increasing concentrations of the pentosan polysulphate, the maximum effect being observed at greater than

Fig. 2. Effect of pentosan polysulphate on the prolongation of the thrombin clotting time of plasma

Pentosan polysulphate was added to plasma (0.1 ml) at increasing concentrations in the presence (●) and absence (○) of $0.166\mu\text{M}$ -high-affinity heparin. Clotting was initiated by the addition of 0.2ml of thrombin (1 NIH unit/ml).

$100\mu\text{g/ml}$ of reaction mixture. Initial rates of thrombin inhibition were calculated from these data and the inverse of rate plotted against inhibitor concentration gave an apparent K_i value of

Table 1. *Effect of various anionic carbohydrates on the heparin-induced prolongation of the thrombin clotting time*
To 0.1 ml of prewarmed plasma (37°C) was added 0.2 ml of thrombin (1 NIH unit/ml) and the clotting time was measured using an automated coagulometer. Heparin was present at a final concentration of 50 nM and various anionic carbohydrates at a final concentration of 25 µg/ml. Control plasma (without additives) had a clotting time of 29.2 ± 1.25 (S.E.M.). Heparinized plasma had an average clotting time of 123.4 ± 10 s.

Carbohydrate	Clotting time (s) with carbohydrate present	
	- Heparin (n = 2)	+ Heparin (n = 3 or 4)
Pentosan polysulphate	34.0 ± 0.03	36.2 ± 2.7
Chondroitin sulphate A	27.6 ± 0.41	81.9 ± 6.5
Chondroitin sulphate B	42.1 ± 0.14	172.5 ± 49
Chondroitin sulphate C	28.5 ± 0.05	122 ± 12.7
Hyaluronic acid	28.8 ± 0.59	176 ± 38

Fig. 3. *Time course of antithrombin III-thrombin interaction in the presence or absence of heparin and increasing concentrations of pentosan polysulphate*

Antithrombin III (8.8 nM) and thrombin (10 nM) were allowed to interact at 37°C in the presence of 0.33 µM-heparin and increasing concentrations of pentosan polysulphate: (●) 11 µg/ml, (△) 21 µg/ml, (■) 42 µg/ml, (□) 85 µg/ml, (○) 127 µg/ml, (◆) 170 µg/ml, (×) 0. Aliquots were removed at intervals into a Polybrene/buffer/substrate mixture (see the Materials and methods section) and residual thrombin was measured. Time course in the absence of heparin and pentosan polysulphate (▽) and the mean of observations made in the absence of heparin but with increasing concentrations of pentosan polysulphate (11–170 µg/ml) (▲).

13 µg/ml, equivalent to approx. 2 µM (Fig. 4). When the relationship between the concentration of pentosan polysulphate and antiheparin activity (Fig. 3) was used to calculate the concentration of pentosan polysulphate in each of the column fractions described in Fig. 1 there was a close correspondence to the concentration determined meta-chromatically. This suggests that the antiheparin

activity is a characteristic of the carbohydrate sulphate alone and is not related to polymer size. In Fig. 3 is also shown the effect of increasing concentrations of pentosan polysulphate in the absence of heparin. The effect was similar across the concentration range used and was characterized by an initial rapid fall in thrombin concentration equivalent to approx. 8% of the total followed by a

Fig. 4. Effect of pentosan polysulphate on the inverse of the rate of thrombin inhibition by antithrombin III in the presence of heparin

Data are taken from those shown in Fig. 3.

Fig. 5. Effect of pentosan polysulphate on the rate of inhibition of thrombin by antithrombin III in the presence of increasing heparin concentrations

A volume of prewarmed (37°C) thrombin solution was added to an equal volume of an antithrombin III/pentosan polysulphate/heparin mixture, and at timed intervals aliquots were removed into polybrene/buffer/substrate and the residual thrombin concentration was measured. Curves are shown for the variation in rate according to heparin concentration in the presence of 0 (○), 1.0 μg/ml (×), 10 μg/ml (□) and 100 μg/ml (▲) of pentosan polysulphate. The final concentration of thrombin and antithrombin III was for each 10 nM.

Fig. 6. Effect of increasing thrombin concentration upon the rate of antithrombin III-thrombin interaction in the absence (a) and presence (b) of pentosan polysulphate (80 μg/ml)

Mixtures of antithrombin III (final concentration 400 nM), heparin (final concentration 0.3 nM) and various concentrations of thrombin were sampled at time intervals and residual thrombin was measured. In (a) the final concentrations of thrombin were 5 nM (■), 10 nM (●), 20 nM (▲), 40 nM (□), 80 nM (×) and 160 nM (○). In (b) the final concentrations of thrombin were 53 nM (●), 107 nM (Δ), 215 nM (□), 430 nM (○) and 860 nM (×).

Increasing concentrations of heparin showed an increase in the rate of interaction between antithrombin III and thrombin, reaching a maximum of $7.1 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ at $0.33 \mu\text{M}$ (Fig. 5) as has been shown previously by other workers. Above this concentration there was a decrease in the observed rate (Jordan *et al.*, 1980; Griffiths *et al.*, 1980), which is thought to reflect non-contributory binding between thrombin and heparin. Pentosan polysulphate at increasing concentrations did not affect the occurrence of the ascending and descending part of the peak but did reduce the maximal rate observed. At the highest concentrations (100 μg/ml) no activity was observed under the

slower phase with a rate equivalent to that of antithrombin III-thrombin alone ($10 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$).

conditions of measurement, and the rate was equivalent to that in the absence of heparin.

Effect of pentosan polysulphate on the enhanced antithrombin III-thrombin reaction in the presence of low concentrations of heparin

Measurement of the effect of the thrombin and antithrombin III concentration upon the rate of interaction observed in the presence of low concentrations of heparin (0.3 nM) was carried out as des-

cribed by Griffiths (1982b). Under these conditions, the reaction has been described as a two-substrate reaction (heparin being treated as the enzyme) of either random (Griffiths, 1982b) or ordered (Pletcher & Nelsestuen, 1983) sequence, and values for K_m for thrombin and antithrombin III may be calculated. Figs. 6(a) and 6(b) show the effect of increasing concentrations of thrombin on

Fig. 7. Effect of increasing antithrombin III concentration upon the rate of antithrombin III-thrombin interaction in the absence (a) and presence (b) of pentosan polysulphate (80 µg/ml)

Mixtures of thrombin (final concentration 50 nM), heparin (final concentration 0.3 nM) and various concentrations of antithrombin III were sampled at timed intervals and residual thrombin was measured. The final concentration of antithrombin III was 50 nM (○), 100 nM (×), 200 nM (□), 400 nM (●) and 800 nM (■) in the absence of pentosan polysulphate and 50 nM (×), 100 nM (△), 200 nM (□), 400 nM (▲) and 800 nM (○) in the presence of pentosan polysulphate.

Fig. 8. Estimation of the apparent K_m in the antithrombin III-thrombin/heparin reaction for thrombin (a) and antithrombin (b) in the presence or absence of pentosan polysulphate

Initial rates of thrombin inactivation taken from the time course plots shown in Figs. 6(a), 6(b), 7(a) and 7(b) are plotted here according to $[S]_i/v_i = [S]_i/V_{max} + K_m/V_{max}$. In (a) the variation is plotted according to thrombin concentration in the absence (×) and presence (□) of pentosan polysulphate (80 µg/ml). Likewise in (b) the plots are according to antithrombin concentration in the absence (○) and presence (●) of pentosan polysulphate.

the time course of reaction with antithrombin III in the presence and absence of pentosan polysulphate respectively. Initial reaction velocities were determined from the data and permitted calculation of a value for K_m from plots of $[S]/v$ versus $[S]$. Similar experiments were carried out by varying the concentration of antithrombin III (Figs. 7a and 7b). Levelling off of the rate of loss of activity in these experiments, at low thrombin and antithrombin concentrations, is presumably due to the effect of the product inhibition by the antithrombin III-thrombin complex (Griffiths, 1982b). The plots of these data, Figs. 8(a) and 8(b), gave a K_m value of 25 nM for thrombin and 150 nM for antithrombin III. These values can be compared with those found by Griffiths (1982b) of 36 nM and 100 nM respectively, and by Pletcher & Nelstuen (1983) of <2 nM and 120 nM respectively. In the presence of near-saturation concentrations of pentosan polysulphate (80 µg/ml), no change in the K_m for antithrombin III was observed (Fig. 8b), but the K_m for thrombin increased to 450 nM (Fig. 8a).

Discussion

In a previous paper, it was observed that the addition of pentosan polysulphate to heparinized plasma inhibited the prolongation of the clotting time caused by heparin (Scully *et al.*, 1983). The use of fractionated high-affinity heparin has confirmed this phenomenon when investigated in heparinized plasma (Fig. 2), and also in a purified system (Fig. 3). Furthermore, the property has been found to be a characteristic of the compound rather than other anionic carbohydrates, perhaps due to its high sulphate content (15%) (Table 1). Concentrations of pentosan polysulphate up to 30 µg/ml have no effect on the thrombin time of normal plasma (Scully *et al.*, 1983), and clotting times are reduced by 15 µg of pentosan polysulphate/ml to those seen without heparin. The amidolytic activity of thrombin is not susceptible to significant inhibition by pentosan polysulphate, even at high concentrations if incubation is carried out in appropriate buffers which prevent non-specific loss of thrombin (Fig. 3). This suggests that the anticoagulant effect in the clotting system is primarily related to binding to fibrinogen, or interference with the fibrinogen-binding site on thrombin. In purified systems, although a concentration-dependent inhibition by pentosan polysulphate of amidolytic activity was not seen when thrombin was incubated with antithrombin III, an early rapid loss of activity (equivalent to 7–8% of initial concentration) was observed (Fig. 3). After this event, the loss of activity ran parallel to the

blank; although the reasons for this early fall are not clear, it may be a non-specific effect.

Identification of a possible mechanism for the antiheparin activity of pentosan polysulphate has been carried out in the light of current concepts of the mechanism by which heparin exerts its effect of accelerating the rate of interaction between antithrombin III and thrombin. Three mechanisms have been proposed: (a) the increased reactivity of the antithrombin III is due to a conformational change induced by heparin (Rosenberg & Damus, 1973; Li *et al.*, 1976; Villanueva & Danishefsky, 1977; Nordenman *et al.*, 1978); (b) the primary interaction of heparin and thrombin renders it more susceptible to the inhibitor (Machovich, 1975; Machovich *et al.*, 1975; Smith, 1977; Hatton & Regoeczi, 1977); (c) binding of both inhibitor and enzyme to the same heparin molecule is necessary for enhanced inhibition to occur (Laurent *et al.*, 1978; Pomerantz & Owen, 1978; Machovich & Arányi, 1978). This latter hypothesis, the template hypothesis, has received support from investigation of the kinetics of inhibition of several haemostatic proteinases by antithrombin in the presence of heparin (Jordan *et al.*, 1980; Griffiths, 1982c). More recently Griffiths (1982b) has shown that in the presence of low concentrations of heparin, the general rate equation for a random bi-reactant enzyme-catalysed reaction may be applied (heparin substituting for the enzyme in the reaction). On the other hand, the work of Pletcher & Nelstuen (1983) suggests that the mechanism may be compared with an ordered sequential, bi-reactant enzyme-catalysed reaction in which antithrombin is the first substrate and the proteinase the second substrate.

Pentosan polysulphate does not affect the binding of antithrombin III to heparin as might be expected since it is of low affinity, being eluted at 0.3 M-NaCl from antithrombin III-Sepharose (Scully *et al.*, 1983). Furthermore, we have observed that even a high concentration (100 µg/ml) of pentosan polysulphate has no effect on the heparin-induced change of the fluorescent spectrum of antithrombin III (Nordenmann *et al.*, 1978) in the presence of stoichiometric concentrations of high affinity heparin (M. F. Scully & V. V. Kakkar, unpublished work). Pentosan polysulphate itself was found to have a negligible effect on antithrombin III fluorescence. Passage of pentosan polysulphate through heparin-agarose columns did not reduce the antiheparin effect.

The work carried out here suggests that the mechanism by which the pentosan polysulphate acts is through binding to thrombin, effectively altering the K_m of thrombin in the antithrombin III-thrombin interaction. Corroborative evidence for the mechanism comes from the observation of

Jordan *et al.* (1980) who suggested that the decrease in rate seen at high concentrations of heparin was due to binding to thrombin. Pletcher & Nelsestuen (1983) have further identified this as due to an increase in the observed K_m for thrombin of <2 nM in this system to about 100 nM at high concentrations of heparin.

The primary action in the acceleration of the rate by heparin has been shown to be an increase in the affinity of the initial complex with minimal effect on the rate limiting step, the transition of the antithrombin-thrombin to an essentially irreversible complex (Olson & Shore, 1982). Assuming this also to be the case in the presence of pentosan polysulphate, the rate equation for the two-substrate reaction may be applied to estimate the relative velocity which will be seen in the presence and absence of pentosan polysulphate.

$$v = \frac{Ke \frac{ab}{K_a K_b}}{\left(1 + \frac{a}{K_a}\right) \left(1 + \frac{b}{K_b}\right)}$$

K_a and K_b are the estimated K_m for thrombin and antithrombin III, and a and b the initial concentrations of each. Heparin concentration is set as e , and is constant, and K is assumed as constant as noted above. Calculation predicts the relative rate using K_m values for thrombin and antithrombin in the presence or absence of pentosan polysulphate, and using the initial concentration of reactants described in the legend to Fig. 3, to be 17.3-fold slower in the presence of saturating amounts of pentosan polysulphate (12.3 μM , apparent K_i , 2 μM). A 15.6-fold difference in rate was observed, (Fig. 4).

In determining the K_m values for thrombin-antithrombin III interaction in the presence and absence of pentosan polysulphate, it was observed that the absolute rate of thrombin inhibition was much higher in mixtures containing the heparinoid. Calculating the V_{max} values from Figs. 8(a) and 8(b) with respect to thrombin and antithrombin III, a value of $2.5 \times 10^8 \text{ M} \cdot \text{min}^{-1}$ for $V_{\text{max}}^{\text{T}}$ and $6.0 \times 10^8 \text{ M} \cdot \text{min}^{-1}$ for $V_{\text{max}}^{\text{AT}}$ was observed in the absence of pentosan polysulphate and $6.0 \times 10^7 \text{ M} \cdot \text{min}^{-1}$ for $V_{\text{max}}^{\text{T}}$ and $1.5 \times 10^7 \text{ M} \cdot \text{min}^{-1}$ for $V_{\text{max}}^{\text{AT}}$ in the presence of pentosan polysulphate. The first order rate constant, K_i , can be calculated (Griffiths, 1982b) to be 312 min^{-1} in the absence of pentosan polysulphate (compared with 324 min^{-1} reported by Olson & Shore, 1982), and 2750 min^{-1} in the presence of pentosan polysulphate, an apparent 9-fold increase in rate. As noted previously, it has been found that heparin has a negligible effect on the rate-limiting step, suggesting that faster rates observed are due to increase in the

binding affinity of the thrombin-pentosan polysulphate complex for the heparin-antithrombin III complex. Since the approximation effect has been shown to have little effect (2–3%; Jordan *et al.*, 1980), on the observed acceleration of the antithrombin III-thrombin interaction, and is presumably nullified in the presence of pentosan polysulphate, the observed increase in reactivity may be an indication of the increase in susceptibility of thrombin to the inhibitor upon binding to heparin (Machovich, 1975; Machovich *et al.*, 1975; Smith, 1977; Hatton & Regoeczi, 1977). When thrombin and antithrombin III are bound to the same heparin molecule, the increased reactivity is effected at low concentrations of thrombin. When thrombin and antithrombin III are not bound to the same molecule (high heparin concentration or in the presence of pentosan polysulphate), much higher concentrations of thrombin are required. Binding of heparin or pentosan polysulphate to thrombin may therefore increase V_{max} , while binding on the same heparin molecule as antithrombin III decreases the K_m for thrombin. Pentosan polysulphate may therefore be useful to elucidate the mechanisms of heparin, and further studies are in progress.

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