The inhibition of thrombin-dependent positive-feedback reactions is critical to the expression of the anticoagulant effect of heparin

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Heparin catalyses the inhibition of two key enzymes of blood coagulation, namely Factor Xa and thrombin, by enhancing the antiproteinase activities of plasma antithrombin III and heparin cofactor II. In addition, heparin can directly inhibit the activation of Factor X and prothrombin. The contributions of each of these effects to the anticoagulant activity of heparin have not been delineated. We therefore performed experiments to assess how each of these effects of heparin contributes to its anticoagulant activity by comparing the effects of heparin, pentosan polysulphate and D-Phe-Pro-Arg-CH₂Cl on the intrinsic pathway of coagulation. Unlike heparin, pentosan polysulphate catalyses only the inhibition of thrombin by plasma. D-Phe-Pro-Arg-CH₂Cl is rapid enough an inhibitor of thrombin so that when added to plasma no complexes of thrombin with its inhibitors are formed, whether or not the plasma also contains heparin. Heparin (0.66 µg/ml) and pentosan polysulphate (6.6 µg/ml) completely inhibited the intrinsic-pathway activation of ¹²⁵I-prothrombin to ¹²⁵I-prothrombin fragment 1 + 2 and ¹²⁵I-thrombin. On the addition of thrombin, a good Factor V activator, to the plasma before each sulphated polysaccharide, the inhibition of prothrombin activation was demonstrable only in the presence of higher concentrations of the sulphated polysaccharide. D-Phe-Pro-Arg-CH₂Cl also completely inhibited the intrinsic-pathway activation of prothrombin in normal plasma. The inhibitory effect of D-Phe-Pro-Arg-CH₂Cl was reversed if thrombin was added to the plasma before D-Phe-Pro-Arg-CH₂Cl. The inhibition of the activation of prothrombin by the three agents was also abolished with longer times with re-added Ca²⁺. Reversal of the inhibitory effects of heparin and pentosan polysulphate was associated with the accelerated formation of ¹²⁵I-thrombin–antithrombin III and ¹²⁵I-thrombin–heparin cofactor complexes respectively. These results suggest that the anticoagulant effects of heparin and pentosan polysulphate are mediated primarily by their ability to inhibit the thrombin-dependent activation of Factor V, thereby inhibiting the formation of prothrombinase complex, the physiological activator of prothrombin.

INTRODUCTION

Catalysis of the multiple antiproteinase actions of antithrombin III is considered to be the principal mode of action of heparin, both as an anticoagulant and an antithrombotic agent (Rosenberg, 1979; Rosenberg & Rosenberg, 1984). The relative sensitivity of the various serine proteinases of coagulation to inhibition by the heparin/antithrombin III system has been the subject of several recent studies (Jordan *et al.*, 1980; Scott *et al.*, 1982; Nesheim, 1983; Buchanan *et al.*, 1985; McNeely & Griffith, 1985; Ofosu *et al.*, 1985; Pixley *et al.*, 1985). Some of these studies suggest that, compared with Factor Xa and thrombin, heparin is a poor catalyst for the inactivation of Factors XIIa, XIa and IXa (Scott *et al.*, 1982; McNeely & Griffith, 1985; Ofosu *et al.*, 1985; Pixley *et al.*, 1985). We have demonstrated that both the anticoagulant and antithrombotic effects of heparin and other sulphated polysaccharides appear to be critically dependent on their ability to enhance the inactivation of thrombin and/or their ability to inhibit the generation of thrombin activity (Buchanan *et al.*, 1985; Ofosu *et al.*, 1985). Furthermore, it has been demonstrated that

the ability of sulphated polysaccharides to inhibit the generation of Factor Xa activity and/or to enhance the inactivation of Factor Xa as such is less important than the antithrombin activity for their antithrombotic and anticoagulant properties (Barrowcliffe *et al.*, 1984; Buchanan *et al.*, 1985; Ofosu *et al.*, 1985). Antithrombotic effect is defined as the inhibition of thrombus growth and/or the inhibition of thrombus formation. Anticoagulant activity is defined as the inhibition of the formation of enzymically active thrombin in plasma.

There are three ways in which sulphated polysaccharides could inhibit the formation of enzymically active thrombin in plasma and thus express their anticoagulant effects: the inhibition of the activation of prothrombin and/or the catalysis of the inhibition of the thrombin as it is generated *in situ*. The contributions of each of the three possibilities to the anticoagulant effects of sulphated polysaccharides have not been clearly delineated. A consideration of the requirements for the efficient activation of prothrombin suggests how the inhibition of the activation of prothrombin could occur. The complex of Factor Xa and Factor Va on a coagulant surface (prothrombinase complex) is the physiological

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activator of prothrombin (Nesheim & Mann, 1979; Rosing *et al.*, 1980; Nesheim *et al.*, 1981a,b; Tracy *et al.*, 1981; Kane & Majerus, 1982). The binding of prothrombin to this complex results in the efficient activation of the former. Thus inhibition of the activation of either Factor X or Factor V, or the inhibition of the Ca^{2+} -dependent interactions of prothrombin, Factor Xa or Factor Va with coagulant phospholipids (Walker & Esmon, 1979), could result in the inhibition of the activation of prothrombin.

The present paper describes studies that explore the mechanisms by which heparin inhibits the formation of enzymically active thrombin in contact-activated plasma. Experiments were also performed with the thrombin inhibitor D-Phe-Pro-Arg-CH₂Cl and pentosan polysulphate. D-Phe-Pro-Arg-CH₂Cl is a specific inhibitor of thrombin at low concentrations (Kettner & Shaw, 1979). Experiments with D-Phe-Pro-Arg-CH₂Cl allowed us to compare the relative importance of the thrombin-mediated and Factor-Xa-dependent activation of Factor V with normal prothrombin activation. Pentosan polysulphate inhibits the activation of Factor X by an antithrombin-III-independent mechanism (Fischer *et al.*, 1982a). Unlike heparin, pentosan

polysulphate catalyses the inhibition of thrombin but not that of Factor Xa by plasma (Scully & Kakkar, 1984; Ofori *et al.*, 1985). Use of pentosan polysulphate thus made it possible to assess the importance of the inhibition of Factor X activation (without catalysis of the inhibition of Factor Xa) and the catalysis of thrombin inhibition to the expression of anticoagulant effects.

The activation of prothrombin was assessed by three methods. The appearance of active thrombin in normal human plasma and in human plasma depleted of both antithrombin III and heparin cofactor II was determined with a thrombin chromogenic substrate. The latter plasma is referred to subsequently as depleted plasma. Since neither heparin nor pentosan polysulphate catalysed the inhibition of thrombin added to depleted plasma, inhibition of the formation of enzymically active thrombin in that plasma could be attributed directly to the inhibition of prothrombin activation. Use of normal plasma allowed us to estimate the contributions of the antithrombin-III-dependent and heparin-cofactor-II-dependent catalytic effects of heparin and pentosan polysulphate respectively to the expression of their anticoagulant properties. The third method assessed the

Fig. 1. Effects of heparin (33 $\mu\text{g}/\text{ml}$) and dermatan sulphate (150 $\mu\text{g}/\text{ml}$) on the formation in human plasma of ^{125}I -thrombin-antithrombin III and ^{125}I -thrombin-heparin cofactor II complexes 1 min after the thrombin had been added to an equal volume of the plasma at 37 °C

(a) Effects of dilution of pooled human plasma on the formation of complexes of ^{125}I -thrombin with its plasma inhibitors. Lanes 1–3, plasma diluted 1:100; lanes 4–6, plasma diluted 1:50; lanes 7–9, plasma diluted 1:10. Plasmas in lanes 1, 4 and 7 contained dermatan sulphate; plasmas in lanes 2, 5 and 8 contained heparin; plasmas in lanes 3, 6 and 9 had no glycosaminoglycan added. (b) Formation of ^{125}I -thrombin-inhibitor complexes in undiluted human plasma depleted of both antithrombin III and heparin cofactor II compared with undiluted pooled normal plasma. Lane 1, depleted plasma; lane 2, depleted plasma + heparin; lane 3, depleted plasma + dermatan sulphate; lane 4, normal plasma + heparin; lane 5, normal plasma + dermatan sulphate. Abbreviations: HCII, heparin cofactor II; ATIII, antithrombin III.

conversion of ^{125}I -prothrombin into ^{125}I -thrombin and ^{125}I -prothrombin fragment 1+2 and the effects of heparin, pentosan polysulphate or D-Pro-Phe-Arg-CH₂Cl on the preceding reaction. The results obtained suggest that the inhibition of the thrombin-dependent activation of Factor V and Factor VIII are the steps in the intrinsic-pathway coagulation sequence that are most sensitive to inhibition by catalytic amounts of heparin and pentosan polysulphate.

MATERIALS AND METHODS

Materials

Fatty acid-free bovine serum albumin and pig skin dermatan sulphate (lot no. 109C-2314) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Pig mucosal heparin was obtained from Diosynth BV, Oss, The Netherlands. Pentosan polysulphate was a gift from Bene-Chemie, Munich, Germany. APTT (automated partial thromboplastin time) reagent was obtained from General Diagnostics, Toronto, Ont., Canada. D-Phe-Pro-Arg-CH₂Cl and Dns-Glu-Gly-Arg-CH₂Cl were obtained from Behring-Calbiochem, San Diego, CA, U.S.A. Human α -thrombin was generously provided by Dr. J. W. Fenton, New York Department of Health, Albany, NY, U.S.A. S-2238 (D-Phe-pipecolyl-Arg *p*-nitroanilide) and S-2222 (benzyloxycarbonyl-Ile-Glu-Gly-Arg *p*-nitroanilide) were obtained from Maynard Scientific, Toronto, Ont., Canada.

Methods

Preparation and partial characterization of plasma depleted of heparin cofactors. Citrated platelet-poor plasma was depleted of both antithrombin III and heparin cofactor II by immunoaffinity chromatography (Sie *et al.*, 1986). Neither antithrombin III nor heparin cofactor II antigen was detectable in the depleted plasma. Heparin or pentosan polysulphate failed to enhance the inhibition of Factor Xa and thrombin by the depleted plasma. Formation of ^{125}I -thrombin-inhibitor complexes in normal plasma and depleted plasma were assessed as previously described (Tollefsen *et al.*, 1983; Ofosu *et al.*, 1984b). The results are summarized in Fig. 1. No complexes of thrombin with antithrombin III or heparin cofactor II could be detected when ^{125}I -thrombin was added to an equal volume of the undiluted depleted plasma, even in the presence of heparin or dermatan sulphate. These complexes of thrombin were detected in pooled normal plasma diluted 1:100.

Effects of heparin and pentosan polysulphate on the activities of the prothrombinase and tenase complexes in normal plasma and depleted plasma. The effects of heparin and pentosan polysulphate on intrinsic-pathway activation of Factor X and prothrombin in normal plasma and depleted plasma were assessed by using the APTT reagent as activator and S-2222 and S-2238 as substrates for Factor Xa and thrombin activity respectively (Ofosu *et al.*, 1980, 1981, 1984a,b, 1985). In some experiments the plasma contained 1.0 μM -D-Phe-Pro-Arg-CH₂Cl in order to evaluate the contribution of the thrombin-dependent and the Factor Xa-dependent activation of Factor VIII-to-Factor X activation (Ofosu *et al.*, 1980; Vehar & Davie, 1980). As noted below, thrombin activity could not be detected with S-2238 in

plasma containing D-Phe-Pro-Arg-CH₂Cl up to 5 min after the re-addition of Ca²⁺.

Activation of prothrombin to thrombin and prothrombin fragment 1+2 was also determined in pooled normal plasma to which ^{125}I -prothrombin (2 $\mu\text{g}/\text{ml}$ of plasma) had been added. This was done in order to relate the activation of prothrombin to the appearance of active thrombin, which was detected with S-2238. Prothrombin was isolated from Factor IX concentrates (Modi *et al.*, 1984) and was labelled with Na¹²⁵I (Tollefsen *et al.*, 1983). The initial specific radioactivity was 2×10^6 – 5×10^5 c.p.m./ μg of prothrombin. When ^{125}I -prothrombin was added to normal plasma, portions of the contact-activated plasma to which Ca²⁺ had been re-added were subsampled after various incubation times into 5 mM-EDTA/0.15 M-NaCl/0.03 M-sodium barbiturate buffer, pH 7.4, and assayed for thrombin activity with S-2238 as the substrate (Ofosu *et al.*, 1980, 1981). Portions of the plasma to which Ca²⁺ had been re-added were simultaneously subsampled into 5 mM-EDTA/0.15 M-NaCl/0.03 M-sodium barbiturate buffer, pH 7.4, containing 1.0 μM -D-Phe-Pro-Arg-CH₂Cl and 1.0 μM -Dns-Glu-Gly-Arg-CH₂Cl. This latter buffer served to prevent both further prothrombin activation and the reaction of the thrombin generated *in situ* with its plasma inhibitors.

^{125}I -prothrombin activation was then assessed after electrophoresis in 7.5% polyacrylamide gels containing 0.1% SDS (Tollefsen *et al.*, 1983; Ofosu *et al.*, 1984b). In addition to the reduced samples used previously, non-reduced samples of the prothrombin activation suspension were also subjected to electrophoresis. The effects of various concentrations of heparin, pentosan polysulphate and 1 μM -D-Phe-Pro-Arg-CH₂Cl on the activation of ^{125}I -prothrombin in normal plasma were also determined. The activation of ^{125}I -prothrombin was also initiated in defibrinated plasma to which thrombin (1.0 NIH unit/ml, i.e. 10 nM) was added for 1 min during the third minute of contact activation. The thrombin was added to initiate the activation of Factor VIII and Factor V before re-addition of Ca²⁺. D-Phe-Pro-Arg-CH₂Cl (1 μM final concentration) was then added just before re-addition of Ca²⁺ to inhibit the added thrombin and any thrombin that was generated *in situ* as a result of the re-addition of Ca²⁺.

RESULTS

Effect of heparin on the appearance of thrombin and Factor Xa activities in plasma

When Ca²⁺ was re-added to contact-activated plasma, maximum thrombin activity (measured with S-2238) was best detected 45 and 60 s after the re-addition. The results summarized in Fig. 2 show that more thrombin activity was detected in depleted plasma than in normal plasma. Heparin completely inhibited the formation of enzymically active thrombin in the two plasmas, but was more effective in this capacity in normal plasma than in depleted plasma (Fig. 2). Since heparin failed to catalyse the inhibition of thrombin added to the depleted plasma (Fig. 1), the results in Fig. 2 suggest that heparin can inhibit the activation of prothrombin in plasma without catalysing the antiproteinase activities of two plasma heparin cofactors, namely antithrombin III and heparin cofactor II. The greater inhibitory effect of heparin on

Fig. 2. Effects of increasing concentrations of heparin or pentosan polysulphate on the appearance of thrombin activity in normal plasma or in plasma depleted of both antithrombin III and heparin cofactor II

The data shown are those obtained 1 min after the re-addition of Ca^{2+} to contact-activated plasma. ■, Thrombin activity in normal plasma containing heparin; □, thrombin activity in normal plasma containing pentosan polysulphate; ●, thrombin activity in the depleted plasma containing heparin; ○, thrombin activity in depleted plasma containing pentosan polysulphate.

the expression of thrombin activity in normal plasma was thought to represent the additional effects due to its ability to catalyse the inhibition of the enzymically active thrombin by the antithrombin III and/or heparin cofactor II that are also present in the plasma. This possibility was investigated further and the results are summarized below.

Heparin also inhibited the formation of enzymically active Factor Xa in normal plasma and depleted plasma (Fig. 3), and caused greater inhibition in normal plasma than in depleted plasma. In contrast with the effects on the formation of thrombin activity, heparin failed to inhibit completely the formation of Factor Xa activity in the two plasmas. Inhibition of the formation of Factor Xa activity in the depleted plasma represented inhibition of Factor X activation, whereas inhibition of Factor Xa activity in normal plasma represented both inhibition of Factor X activation and catalysis of the inhibition of the Factor Xa as it was generated *in situ*. The results summarized in Fig. 3 are those obtained 2 min after the re-addition of Ca^{2+} , as maximum Factor Xa activity was detectable after this incubation period.

Effect of pentosan polysulphate on the appearance of thrombin and Factor Xa activities in plasma

Pentosan polysulphate inhibited the formation of enzymically active thrombin in normal plasma and depleted plasma (Fig. 2). Unlike heparin, however, pentosan polysulphate was equally effective in inhibiting the formation of active thrombin in the two plasmas, as complete inhibition of the formation of thrombin

Fig. 3. Effects of increasing concentrations of heparin and pentosan polysulphate on the appearance of Factor Xa activity in normal plasma and in plasma depleted of both antithrombin III and heparin cofactor II

The data shown are those obtained 2 min after the re-addition of Ca^{2+} to contact-activated plasma, as the maximum Factor Xa activity was observed at this time in both plasmas. See the legend to Fig. 2 for an explanation of the symbols used.

activity was achieved with 6.6 μg of pentosan polysulphate/ml of either plasma. Thus the anticoagulant activity of pentosan polysulphate results from its heparin-cofactor-independent effects on inhibiting the activation of prothrombin. The inhibitory effects of pentosan polysulphate on the formation of enzymically active Factor Xa are summarized in Fig. 3. Pentosan polysulphate was marginally better able to inhibit the appearance of Factor Xa activity in normal plasma than in depleted plasma. Pentosan polysulphate also failed to completely inhibit the appearance of Factor Xa activity in normal plasma.

The pooled normal plasma used in the experiments of Figs. 2 and 3 had been incubated at 4 °C for approx. 7 h, the duration of procedures required for the immunodepletion of both heparin cofactor II and antithrombin III from plasma. This led to an increase in the amount of heparin required to inhibit completely the appearance of thrombin activity in this plasma compared with freshly frozen normal plasma. The presence of 0.66 μg of heparin/ml of this control plasma caused a 50% decrease in the thrombin activity detected, whereas this concentration of heparin completely inhibited the formation of active thrombin in freshly frozen pooled normal plasma.

Mechanisms for the inhibition of Factor X activation

Heparin and pentosan polysulphate inhibited the formation of enzymically active Factor Xa in normal plasma (Fig. 3). This effect could have resulted from the inhibition of the activation of Factor X, and, for heparin, also from the catalysis of the inhibition of the Factor Xa formed in normal plasma. Further studies

were therefore carried out to determine the mechanisms by which the two sulphated polysaccharides inhibited the expression of Factor Xa activity in normal plasma. These studies also evaluated the relative contributions of the thrombin-dependent and Factor-Xa-dependent pathways for Factor VIII activation (Vehar & Davie, 1980; Ofosu *et al.*, 1980) to the intrinsic activation of Factor X.

Effects of D-Phe-Pro-Arg-CH₂Cl. The effect of D-Phe-Pro-Arg-CH₂Cl on the formation of Factor Xa activity in normal plasma is summarized in Table 1. D-Phe-Pro-Arg-CH₂Cl (1 μ M) delayed the appearance of maximum Factor Xa activity. Since this concentration of D-Phe-Pro-Arg-CH₂Cl did not inhibit Factor Xa, it must have inhibited the expression of Factor Xa activity by inhibiting the intrinsic-pathway activation of Factor X. The inhibitory effect of D-Phe-Pro-Arg-CH₂Cl on the generation of Factor Xa activity in normal plasma was completely reversed if thrombin (10 nM) was added to the plasma for 1 min during contact activation before D-Phe-Pro-Arg-CH₂Cl was added to the plasma (results not shown in Table 1). Thus inhibition of the thrombin-dependent activation of Factor VIII (by D-Phe-Pro-Arg-CH₂Cl) prolongs the time required to optimize Factor X activation.

Effects of heparin. The results obtained with two concentrations (0.66 and 6.6 μ g/ml of plasma) of heparin are summarized in Table 1. Both concentrations of heparin suppressed significantly the appearance of Factor Xa activity in normal plasma. In addition, the lower concentration of heparin initially delayed the appearance of Factor Xa activity. At 2 min after the re-addition of Ca²⁺, however, increased Factor Xa activity was evident in plasma that contained 0.66 μ g of heparin/ml. Thus, like D-Phe-Pro-Arg-CH₂Cl, the lower concentration of heparin inhibits the activation of Factor X, probably by diminishing the thrombin-dependent activation of Factor VIII. But, unlike D-Phe-Pro-Arg-CH₂Cl, when this inhibition is reversed, heparin then catalyses the inhibition of the Factor Xa then formed by anti-thrombin III. This possibility is supported by the results obtained when the plasma contained both D-Phe-Pro-Arg-CH₂Cl and 0.66 μ g of heparin/ml (Table 1).

Effects of pentosan polysulphate. The lower concentration of pentosan polysulphate (0.66 μ g/ml) had no

effect on the formation of enzymically active Factor Xa (Table 1). Thus the combination of D-Phe-Pro-Arg-CH₂Cl and 0.66 μ g of pentosan polysulphate/ml gave results that were similar to those obtained with D-Phe-Pro-Arg-CH₂Cl alone. The higher concentration of pentosan polysulphate (6.6 μ g/ml) both delayed the appearance of and decreased the maximum amount of Factor Xa activity detected (Table 1). When the plasma contained both D-Phe-Pro-Arg-CH₂Cl and 6.6 μ g of pentosan polysulphate/ml, Factor Xa activity was detected only 10 min after the re-addition of Ca²⁺ (results not shown in Table 1). These results confirm that pentosan polysulphate does not catalyse the inhibition of Factor Xa by normal plasma. The delay in the appearance of Factor Xa activity in normal plasma containing pentosan polysulphate therefore results primarily from the inhibition of Factor X activation.

Mechanisms for the inhibition of prothrombin activation

The results summarized in Fig. 2 show that heparin and pentosan polysulphate can inhibit completely the activation of prothrombin in the depleted plasma. Experiments were therefore carried out with normal plasma containing ¹²⁵I-labelled human prothrombin to determine whether the absence of thrombin activity in normal plasma containing heparin or pentosan polysulphate also resulted from the inhibition of prothrombin activation.

Prothrombin activation in the absence of sulphated polysaccharides. On the re-addition of Ca²⁺ to contact-activated plasma, the activation of ¹²⁵I-prothrombin to prothrombin fragment 1 + 2 and thrombin could readily be demonstrated. Inactivation of the thrombin (generated *in situ*) by plasma antithrombin III, α_2 -macroglobulin and heparin cofactor II could also be demonstrated. The activation of prothrombin was associated with a decrease in the intensity of the band that corresponds to prothrombin. These results are summarized in Fig. 4, lanes 1–4. Reduction of the samples with 2-mercapto-ethanol before electrophoresis altered the electrophoretic mobilities of prothrombin, prothrombin fragment 1 + 2, thrombin–antithrombin III complex and thrombin– α_2 -macroglobulin complex (Fig. 4, lanes 5–8). As shown in Fig. 4, lanes 2–4, residual prothrombin could be distinguished more clearly from the thrombin–antithrombin III complex when non-reduced samples were

Table 1. Effects of D-Phe-Pro-Arg-CH₂Cl, heparin and pentosan polysulphate on the appearance of Factor Xa activity in normal plasma up to 10 min after the re-addition of Ca²⁺ to contact-activated plasma

The concentration of D-Phe-Pro-Arg-CH₂Cl (PPACK) used was 1 μ M. Two concentrations of heparin and pentosan polysulphate are reported, namely 0.66 μ g/ml (A) and 6.6 μ g/ml (B). Each datum is the mean of six determinations. The standard deviation was 5% and 10% for values greater than and less than 20 pmol/ml respectively.

Time after re-addition of Ca ²⁺ (min)	Concn. of Factor Xa (pmol/ml)								
	Control	PPACK	Heparin		Heparin + PPACK	Pentosan polysulphate		Pentosan polysulphate + PPACK	
			A	B	A	A	B	A	
1	37	8	10	9	8	32	9	8	
2	55	17	20	9	12	50	18	24	
5	35	35	9	7	8	35	25	34	
10	19	17	7	5	5	19	11	18

Fig. 4. Effect of different times with re-added Ca^{2+} on the intrinsic-pathway activation of ^{125}I -prothrombin and the formation of ^{125}I -thrombin-inhibitor complexes in pooled normal plasma

Samples in lanes 1–4 were not reduced before electrophoresis and autoradiography. Lane 1, unactivated plasma; lanes 2–4, contact-activated plasma to which Ca^{2+} had been re-added. The times of the samples with re-added Ca^{2+} in lanes 2–4 were 45 s, 1 min and 5 min respectively. Samples in lanes 5–8 were reduced with 2-mercaptoethanol before electrophoresis. Lane 5, unactivated plasma to which Ca^{2+} had been re-added; the times of the contact-activated plasmas with re-added Ca^{2+} in lanes 6–8 were 45 s, 1 min and 5 min respectively. Key: A, ^{125}I -thrombin; B, ^{125}I -prothrombin fragment 1+2; C, ^{125}I -prothrombin; D, ^{125}I -thrombin-antithrombin III complex; E, ^{125}I -thrombin- α_2 -macroglobulin complex; F, ^{125}I -thrombin-heparin cofactor II complex.

subjected to electrophoresis. Because of the increase in the difference of their electrophoretic mobilities after reduction, prothrombin fragment 1+2 and thrombin could be distinguished more clearly with reduced samples (Fig. 4, lanes 6–8).

Effect of heparin on prothrombin activation. The effects of increasing concentrations of heparin on prothrombin activation, 1 min after the re-addition of Ca^{2+} to contact-activated plasma, are shown in Fig. 5. The addition of heparin to the plasma (Fig. 5, lanes 3–5) resulted in the complete inhibition of the activation of prothrombin, hence the absence of the electrophoretic bands that represent prothrombin fragment 1+2 and thrombin. This effect coincided with the near absence of thrombin activity (less than 5% of control; Table 2) in the respective plasmas containing heparin. The absence of thrombin activity from heparin-treated plasma was therefore due to the inhibition of the activation of

Fig. 5. Effect of D-Phe-Pro-Arg- CH_2Cl or heparin on the intrinsic-pathway activation of ^{125}I -prothrombin in normal plasma

The data shown are those obtained 1 min after the re-addition of Ca^{2+} to contact-activated plasma. Lane 1, 1 μM -D-Phe-Pro-Arg- CH_2Cl ; lane 2, no inhibitor; lanes 3–5, 0.66, 6.6 and 66.0 μg of heparin/ml of plasma respectively. The samples were not reduced with 2-mercaptoethanol before electrophoresis. Key: A, ^{125}I -thrombin; B, ^{125}I -prothrombin fragment 1+2; C, ^{125}I -prothrombin; D, ^{125}I -thrombin-antithrombin III complex; E, ^{125}I -thrombin- α_2 -macroglobulin complex.

prothrombin. The activation of prothrombin could not be demonstrated when D-Phe-Pro-Arg- CH_2Cl (1 μM) was added to contact-activated plasma before the addition of CaCl_2 (Fig. 5, lane 1).

Effect of pentosan polysulphate on prothrombin activation. The addition of a low concentration of pentosan polysulphate to pooled normal plasma (0.66 $\mu\text{g}/\text{ml}$ of plasma) was accompanied with normal activation of prothrombin. As shown in Fig. 6, lane 3, a small proportion of thrombin generated was inactivated primarily by heparin cofactor II. This corresponded to a 20% decrease in the thrombin activity measured with S-2238. When the concentrations of pentosan polysulphate were increased to 6.6 or 66.0 $\mu\text{g}/\text{ml}$ of plasma (Fig. 6, lanes 4 and 5 respectively), no activation of prothrombin could be demonstrated. This also coincided with near absence of detectable thrombin activity (less than 5% of control, Table 2) in the plasma. These results suggest that the anticoagulant effects of higher concentrations of pentosan polysulphate result from the inhibition of prothrombin activation.

Role of thrombin-dependent feedback reactions of prothrombin activation. The ability of heparin and pentosan polysulphate to inhibit the activation of prothrombin in plasma containing Factor VIIIa and Factor Va was determined in order to estimate the contribution of the two thrombin-dependent positive-feedback reactions to prothrombin activation. To do this, Factor VIIIa and Factor Va were generated *in situ* by the addition of thrombin (10 nM) to plasma during the

Table 2. Summary of the effects of heparin and pentosan polysulphate on the appearance of thrombin activity up to 10 min after the re-addition of Ca^{2+} to contact-activated normal plasma

Two concentrations of each sulphated polysaccharide are reported, namely $0.66 \mu\text{g/ml}$ (A) and $6.6 \mu\text{g/ml}$ of plasma (B). Each datum is the mean of six to eight determinations. The standard deviation was approx. 5%.

Time after re-addition of Ca^{2+} (min)	Concn. of thrombin (pmol/ml)				
	No inhibitor	Heparin		Pentosan polysulphate	
		A	B	A	B
0.75	750	30	20	660	40
1	700	30	10	570	40
2	290	60	10	200	260
5	140	50	10	100	60
10	130	50	10	100	60

third minute of contact activation. Under these conditions complete inhibition of prothrombin activation was observed only with $66.0 \mu\text{g}$ of either heparin or pentosan polysulphate/ml (results not shown). The inhibition of the activation of prothrombin obtained with D-Phe-Pro-Arg- CH_2Cl was completely reversed in plasma similarly pretreated with thrombin (results not shown). The added thrombin alone did not activate prothrombin in plasma. These results suggest that the inhibition of the thrombin-dependent positive-feedback reactions is a key step that results in the inhibition of the activation of prothrombin.

Effects of longer times with re-added Ca^{2+} on the abilities of D-Phe-Pro-Arg- CH_2Cl , heparin and pentosan polysulphate to inhibit the activation of prothrombin

Since Factor Xa has been demonstrated to activate Factor V (Foster *et al.*, 1983), we reasoned that the inhibition of the activation of prothrombin by D-Phe-Pro-Arg- CH_2Cl , heparin and pentosan polysulphate may be diminished with contact-activated plasma to which Ca^{2+} had been re-added for longer than 1 min (see Table 1).

D-Phe-Pro-Arg- CH_2Cl . Normal activation of prothrombin in contact-activated plasma containing $1 \mu\text{M}$ -D-Phe-Pro-Arg- CH_2Cl and ^{125}I -prothrombin was

Fig. 6. Effect of pentosan polysulphate on the intrinsic-pathway activation of ^{125}I -prothrombin in normal plasma 1 min after CaCl_2 was added to contact-activated plasma

Lane 1, control plasma; lane 2, contact-activated plasma; lanes 3–5, pentosan polysulphate added to the contact-activated plasma in concentrations of 0.66 , 6.6 and $66.0 \mu\text{g/ml}$ respectively. The samples were not reduced with 2-mercaptoethanol before electrophoresis. Key: A, ^{125}I -thrombin; B, ^{125}I -prothrombin fragment 1+2; C, ^{125}I -prothrombin; D, ^{125}I -thrombin-antithrombin III complex; E, ^{125}I -thrombin- α_2 -macroglobulin complex; F, ^{125}I -thrombin-heparin cofactor II complex.

Fig. 7. Effect of increasing times with re-added Ca^{2+} on the intrinsic-pathway activation of ^{125}I -prothrombin in pooled normal plasma containing $0.66 \mu\text{g}$ of heparin/ml

The samples were not reduced before electrophoresis. The times with re-added Ca^{2+} were: lane 1, 0 s; lane 2, 30 s; lane 3, 45 s; lane 4, 60 s; lane 5, 120 s; lane 6, 300 s; lane 7, 600 s. See the legends to Figs. 4–6 for an explanation of the key.

Fig. 8. Effects of increasing times with re-added Ca^{2+} on the activation of ^{125}I -prothrombin in pooled normal plasma containing $6.6 \mu\text{g}$ of pentosan polysulphate/ml

The samples were not reduced before electrophoresis. The times with added Ca^{2+} were: lane 1, 0 s; lane 2, 30 s; lane 3, 45 s; lane 4, 60 s; lane 5, 120 s; lane 6, 300 s; lane 7, 600 s. See the legends to Figs. 4–6 for an explanation of the key.

observed when the times with re-added Ca^{2+} were 2 min or longer (results not shown). When the subsamples to which Ca^{2+} had been re-added were assayed for thrombin activity with S-2238, however, thrombin was detected only in the 10 min subsample (results not shown). Thus the suppression of thrombin activity with D-Phe-Pro-Arg- CH_2Cl delays prothrombin activation.

Heparin. Normal activation of prothrombin was observed 2 min after the re-addition of Ca^{2+} to contact-activated plasma containing $0.66 \mu\text{g}$ of heparin/ml of plasma. These results are summarized in Fig. 7. The heparin catalysed the inhibition of the thrombin generated *in situ* by antithrombin III (Fig. 7). This increased formation of the thrombin–antithrombin III complex explains the decrease in the amount of thrombin activity detected with S-2238 in the presence of heparin (Table 2). Normal activation of prothrombin in plasma containing $0.66 \mu\text{g}$ of heparin/ml coincided with the appearance of the maximum amount of Factor Xa activity detectable in this plasma (Table 1).

On increasing the concentration of heparin to $6.6 \mu\text{g}/\text{ml}$, the activation of prothrombin could not be demonstrated up to 10 min after the re-addition of Ca^{2+} . This coincided with the near absence of thrombin activity (Table 2). Thus low (i.e. catalytic) concentrations of heparin initially inhibit the activation of prothrombin. When this inhibition is relieved on longer times with re-added Ca^{2+} , the thrombin formed is rapidly inactivated by antithrombin III.

Pentosan polysulphate. The inhibitory effect of pentosan polysulphate ($6.6 \mu\text{g}/\text{ml}$ of plasma) on the intrinsic activation of prothrombin 1 min after the re-addition of Ca^{2+} (Fig. 6) was reversed if the times with re-added Ca^{2+} were prolonged to 2 min or longer (Fig. 8). Formation of thrombin–heparin cofactor II complex was accelerated in this plasma, thus causing a decrease in the amount of thrombin activity detectable with S-2238 relative to the control plasma (Table 2). The activation of prothrombin in normal plasma that contained pentosan polysulphate coincided with increased generation of Factor Xa activity (Table 1). Thus initially pentosan polysulphate ($6.6 \mu\text{g}/\text{ml}$ of plasma) inhibits the activation of prothrombin. When this inhibition is relieved on longer times of the plasma with re-added Ca^{2+} , pentosan polysulphate catalyses the inhibition of the thrombin generated *in situ* by heparin cofactor II.

DISCUSSION

Heparin catalyses the antithrombin-III-dependent inhibition of several serine proteinases of blood coagulation (Rosenberg, 1979; Jordan *et al.*, 1980; Scott *et al.*, 1982; Rosenberg & Rosenberg, 1984; McNeely & Griffith, 1985; Pixley *et al.*, 1985). The catalysis of thrombin inhibition is more important than catalysis of Factor Xa inhibition for the expression of antithrombotic and anticoagulant effects of sulphated polysaccharides (Buchanan *et al.*, 1985; Ofofu *et al.*, 1985). The present study shows that the major action of heparin in inhibiting coagulation appears to be the inhibition of the thrombin-dependent positive-feedback reactions. We hypothesize that the catalytic effect of heparin on the inhibition of the initial thrombin by antithrombin III limits the thrombin-dependent activation of Factor V and Factor VIII, as indicated in Scheme 1. The catalysis

Scheme 1. Scheme indicating the two steps in intrinsic-pathway activation of prothrombin that are most sensitive to inhibition by catalytic concentrations of heparin and pentosan polysulphate

The scheme postulates that catalysis of thrombin inhibition by heparin or pentosan polysulphate (indicated by the hatched rectangles), which leads to the formation of thrombin–antithrombin III and thrombin–heparin cofactor II complexes respectively, decreases the thrombin-dependent activation of Factor V and Factor VIII. The symbols V and Va represent Factor V and Factor Va respectively; IXa represents Factor IXa; VIII and VIIIa represent Factor VIII and Factor VIIIa respectively; X and Xa represent Factor X and Factor Xa respectively; F_{1+2} indicates prothrombin fragment 1 + 2.

of the heparin-cofactor-II-dependent inhibition of the initial thrombin by pentosan polysulphate similarly limits the activation of Factor V and Factor VIII by thrombin. A direct consequence of the catalysis of thrombin inhibition is the delay of the activation of both Factor X and prothrombin.

In support of this hypothesis, the addition of the thrombin-specific inhibitor D-Phe-Pro-Arg-CH₂Cl to plasma before the re-addition of Ca²⁺ completely inhibits the intrinsic-pathway activation of prothrombin under conditions where maximum thrombin activity and prothrombin fragment 1+2 would be detected in the absence of D-Phe-Pro-Arg-CH₂Cl. The addition of thrombin to contact-activated plasma before the addition of D-Phe-Pro-Arg-CH₂Cl reverses the inhibitory effect of D-Phe-Pro-Arg-CH₂Cl. Thrombin similarly diminishes the ability of heparin and pentosan polysulphate to inhibit the activation of prothrombin. The inhibitory effects of D-Phe-Pro-Arg-CH₂Cl, heparin and pentosan polysulphate are also reversed when the times of contact-activated plasma with re-added Ca²⁺ exceed 1 min. These results suggest that thrombin is probably the enzyme that efficiently activates Factor V and Factor VIII during intrinsic-pathway activation of prothrombin. When thrombin activity is inhibited in plasma containing one of the three inhibitors, Factor V is then activated by the Factor Xa generated *in situ* (Table 1, and Foster *et al.*, 1983).

Several investigations have shown the important catalytic effect of Factor Va on the activation of prothrombin when purified clotting factors were used (Nesheim & Mann, 1979; Rosing *et al.*, 1980; Nesheim *et al.*, 1981a,b; Tracy *et al.*, 1981; Kane & Majerus, 1982). The importance of the enhancement of the activity of antithrombin III for the complete inhibition of prothrombin activation by low concentrations of heparin was suggested in previous studies, where it was shown that as little as 2 µg of antithrombin III added to 1 ml of antithrombin III-depleted plasma completely inhibited thrombin generation initiated by the addition of either Factor IXa or Factor Xa (Ofosu *et al.*, 1981).

Pentosan polysulphate, a polysaccharide with anticoagulant and antithrombotic properties (Bergquist *et al.*, 1980; Fischer *et al.*, 1982a,b; Scully & Kakkar, 1984), was also evaluated. The primary anticoagulant effect of pentosan polysulphate appears to result from the limitation of the activation of Factor V and Factor VIII by thrombin. This effect is probably mediated by the enhancement of the inhibition of thrombin by heparin cofactor II, as pentosan polysulphate catalysed the formation of the thrombin-heparin cofactor II complex when Ca²⁺ was re-added to the contact-activated plasma (Figs. 6 and 8). It is possible that the concentration of pentosan polysulphate that completely inhibits the activation of prothrombin also inhibits the activation of Factor V by thrombin whether or not heparin cofactor II is present (Baruch *et al.*, 1986; Sie *et al.*, 1986).

Heparin has been shown (1) to inhibit the formation of the prothrombinase complex (Walker & Esmon, 1979), (2) to inhibit the activity of the tenase complex (Ofosu *et al.*, 1980), and (3) to inhibit the activation of Factor VIII and Factor V by thrombin in the absence of heparin cofactors (Baruch *et al.*, 1986). Tenase complex is the physiological activator of Factor X and consists of Factor IXa and Factor VIII—Factor VIIIa bound to coagulant surfaces. Therefore we used the depleted

plasma to determine the contribution of these heparin-cofactor-independent effects to the inhibition of prothrombin activation. Heparin was a weaker inhibitor of the activation of prothrombin in depleted plasma than in normal plasma (Fig. 2). Baruch *et al.* (1986) have shown that in the absence of antithrombin III the concentration of unfractionated heparin required to halve the initial rate of Factor V activation by thrombin is 10 µg/ml. This concentration is one order of magnitude greater than the amount needed to inhibit completely prothrombin activation in normal plasma. The primary anticoagulant effect of heparin therefore results from the suppression of the thrombin-dependent positive-feedback reactions. Catalysis of the formation of thrombin-antithrombin III complex by heparin explains why low concentrations of heparin completely inhibit prothrombin activation.

At concentrations below 6.6 µg/ml pentosan polysulphate was more able to inhibit the activation of prothrombin in the depleted plasma than in normal plasma (Fig. 2). However, 6.6 µg of pentosan polysulphate/ml completely inhibited the activation of prothrombin in both plasmas. These observations support the possibility that a major portion of the anticoagulant effect of pentosan polysulphate is due to its ability to inhibit the activation of Factor V by thrombin, a process that may occur without the catalysis of the activities of plasma heparin cofactors (Baruch *et al.*, 1986).

Although these studies do not permit the direct quantification of the extent of Factor V and Factor VIII activation required for the optimal activation of prothrombin, our evidence strongly suggests that the principal anticoagulant action of low (i.e. catalytic) concentrations of heparin is through its ability to limit the activation of Factor V and Factor VIII, thus delaying the activation of prothrombin. The inhibitory effects of higher concentrations of heparin and pentosan polysulphate are proposed to occur independently of antithrombin III and heparin cofactor II catalysis (Walker & Esmon, 1979; Ofosu *et al.*, 1980, 1982; Baruch *et al.*, 1986).

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