The effect of Ca^{2+} , phospholipid and Factor V on the anti-(Factor Xa) activity of heparin and its high-affinity oligosaccharides

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The influence of Ca^{2+} , phospholipid and Factor V was determined on the rate of inactivation of Factor Xa by antithrombin III, in the absence and in the presence of unfractionated heparin and of three high-affinity heparin oligosaccharides in the M_r range 1500–6000. In the absence of heparin the addition of Ca^{2+} , phospholipid and Factor V caused a 4-fold decrease in rate of inactivation of Factor Xa. As concentrations of unfractionated heparin were increased the protective effect of Ca^{2+} /phospholipid/Factor V was gradually abolished, and at a concentration of 2.4 nM there were no differences in rates of neutralization of Factor Xa in the presence or absence of Ca^{2+} , phospholipid and Factor V. In contrast, heparin decasaccharide (M_r 3000) and pentasaccharide (M_r 1500) fragments were unable to overcome the protective effect of Ca^{2+} /phospholipid/Factor V; in the presence of these components their catalytic efficiencies were 16-fold and 40-fold less respectively than that of unfractionated heparin. A heparin 20–22-saccharide fragment (M_r approx. 6000) gave similar inactivation rates in the presence and in the absence of Ca^{2+} /phospholipid/Factor V. Human and bovine Factor Xa gave similar results. These results indicate that in the presence of Ca^{2+} /phospholipid/Factor V optimum inhibition of Factor Xa requires a saccharide sequence of heparin additional to that involved in binding to antithrombin III. The use of free enzyme for the assessment of anti-(Factor Xa) activity of low- M_r heparin fractions could give misleading results.

INTRODUCTION

The anticoagulant activity of heparin, as measured by global assays such as the activated partial thromboplastin time, declines with decreasing M_r , whereas its activity when measured by anti-(Factor Xa) assays is maintained down to very low- M_r oligosaccharides (Andersson *et al.*, 1976; Lane *et al.*, 1978; Laurent *et al.*, 1978; Barrowcliffe *et al.*, 1979). The reason for this is that anti-(Factor Xa) activity requires only the presence of a specific pentasaccharide sequence that binds with high affinity to antithrombin III (AT III) (Thunberg *et al.*, 1982; Petitou, 1984), whereas for neutralization of thrombin an additional polysaccharide sequence is required for binding to the enzyme (Pomerantz & Owen, 1978; Holmer *et al.*, 1981; Lane *et al.*, 1984).

However, experimental thrombosis studies in animals showed that high-affinity heparin oligosaccharides of M_r 3000, 4800 and 6000 were less effective antithrombotic agents than was unfractionated heparin, despite their much higher anti-(Factor Xa) activities (Thomas *et al.*, 1982; Barrowcliffe *et al.*, 1984): this lack of correlation between anti-(Factor Xa) activity and antithrombotic action of very-low- M_r heparin fragments was also found in other laboratories (Holmer *et al.*, 1982; Ockelford *et al.*, 1982).

In anti-(Factor Xa) assays the ability of heparin/AT III to accelerate the neutralization of free Factor Xa in the absence of Ca^{2+} is measured, whereas *in vivo* Factor Xa is bound to platelets via Ca^{2+} , phospholipid (PL) and Factor Va (Miletich *et al.*, 1978; Dahlbäck & Stenflo, 1978) and in this condition is protected from inhibition

by AT III (Walsh & Biggs, 1972). The comparative inhibitory actions of heparin and its oligosaccharides on this more physiological form of Factor Xa could be quite different from those obtained with the free enzyme.

In the present study we have therefore examined the effects of addition of Ca^{2+} , PL and Factor V on rates of inhibition of Factor Xa by AT III in the presence of unfractionated heparin and of high-affinity heparin oligosaccharides in the M_r range 1500–6000.

MATERIALS AND METHODS

Heparin

Unfractionated heparin was the 3rd and 4th International Standard (N.I.B.S.C.). High-affinity and low-affinity heparin were prepared by affinity chromatography of bulk heparin (Diosynth BV, Oss, The Netherlands) on a column of human AT III covalently bound to Sepharose 4B. Low-affinity material was collected in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.05 M-NaCl. Medium-affinity material was eluted from the column in the same buffer containing 0.15 M-NaCl, and discarded, and high-affinity heparin was eluted at 1.5 M-NaCl. The high-affinity heparin had a specific activity of 350 i.u./mg, and the low-affinity heparin 5 i.u./mg. High-affinity heparin constituted 40% of the bulk material.

High-affinity heparin oligosaccharides were prepared by nitrous acid depolymerization, AT III affinity chromatography and gel chromatography, as previously described (Thunberg *et al.*, 1982). The two oligo-

Abbreviations used: PL, phospholipid; AT III, antithrombin III.

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saccharides used in the present study were the 10–12-saccharide fragment, containing predominantly the decasaccharide, and a >18-saccharide fragment, containing mainly 20–22-saccharide fragments. Some studies were also carried out with a high-affinity pentasaccharide prepared by chemical synthesis (Petitou, 1984); this was kindly given by Dr. Petitou, Laboratoires Choay, Paris, France.

AT III

Human AT III concentrate for immobilization on Sepharose was kindly supplied by Dr. Mazurier, C.R.T.S., Lille, France. Purified AT III for kinetic studies was prepared from this concentrate by chromatography on heparin-Sepharose, and had a specific activity of 8 i.u./mg. Addition of Polybrene did not affect its rate of neutralization of Factor Xa, indicating that there was no contamination with heparin.

Factor Xa

A highly purified reference preparation (N.I.B.S.C. 75/590) of bovine Factor Xa was used for most experiments. For some experiments this was replaced by bovine Factor Xa obtained from Diagnostic Reagents Ltd. (Thame, Oxon, U.K.). These preparations contained albumin, and 0.1% bovine serum albumin (Sigma Chemical Co., Poole, Dorset, U.K.) was added to all dilution buffers.

Human Factor Xa was prepared by activation of human Factor X with purified Russell's-viper-venom protein (Williams & Esnouf, 1962), followed by chromatography on QAE- (quaternary aminoethyl-) Sephadex. Human Factor X was purified from a Factor II/Factor IX/Factor X concentrate (Blood Products Laboratory, Elstree, Herts., U.K.) by chromatography on DEAE-Sephacel, dextran sulphate-Sepharose and heparin-Sepharose as described by Modi *et al.* (1984).

Factor V

Bovine Factor V was purified from freshly collected bovine blood as described by Nesheim *et al.* (1979), involving adsorption on barium citrate, precipitation with poly(ethylene glycol) and chromatography on QAE-Sephadex and octyl-Sepharose. Proteinase inhibitors were included throughout the preparation. Analysis by SDS/polyacrylamide-gel electrophoresis showed one major band of M_r approx. 300 000, with minor bands visible on silver staining of M_r 100 000 and 60 000. For some experiments Factor V was activated with thrombin (1 i.u./ml at 37 °C for 3 min) and the Factor Va was used immediately without further purification. The increase in activity on thrombin activation was approx. 11-fold, compared with 15–20-fold for thrombin activation of Factor V in normal human plasma.

PL

N.I.B.S.C. preparation 83/555 was extracted from bovine brain by drying with acetone, extraction with light petroleum (b.p. 40–60 °C), evaporation, redissolution in diethyl ether and precipitation with acetone as described by Barrowcliffe *et al.* (1982). It consisted of 60% phospholipids and 40% neutral lipids, mainly cholesterol and cerebrosides. The phospholipid composition was phosphatidylcholine 22%, phosphatidylethanolamine 30%, phosphatidylserine 26% and other phosphatides 22%.

Determination of molar concentrations of AT III, Factor V and Factor Xa

Molar concentrations of AT III and Factor V were estimated by assays of biological activity against normal plasma and use of published values for plasma concentrations and M_r values. For AT III the International Reference Preparation (N.I.B.S.C. 72/1) was used as a standard (1 i.u. = AT III in 1 ml of normal plasma), and values of 125 µg/ml of plasma (Murano *et al.*, 1980) and M_r 55 000 (Kurachi *et al.*, 1976) were assumed. For Factor V a frozen pool of 20 donors' plasma was used as standard, and values of 7 µg/ml (Tracy *et al.*, 1982) and M_r 330 000 (Nesheim *et al.*, 1979) were assumed. Molar concentrations of Factor Xa were estimated by using the manufacturer's data for K_m and K_{cat} for S-2222 (benzoyl-Ile-Glu-Gly-Arg-p-nitro-anilide).

Amidolytic assay of Factor Xa

Samples (0.6 ml) from the incubation mixture were subsampled into 0.5 ml of 0.4 mM-S-2222 (Kabi, Stockholm, Sweden) in Tris/NaCl buffer (0.05 M-Tris/HCl buffer, pH 8.4, containing 0.15 M-NaCl) at 37 °C, and $\Delta A_{405}/\text{min}$ was measured. Measurements were converted into percentage Factor Xa from a dilution curve. In experiments in which Factor Xa was incubated with Ca^{2+} , PL and Factor V, these components were included in the dilutions to take account of the changes in ΔA_{405} when these components are present (Nesheim *et al.*, 1981).

Measurement of anti-(Factor Xa) activity

Factor Xa (8 nM) was pre-incubated at 37 °C for 5 min with either Tris/NaCl buffer or one or more of the following components: CaCl_2 (3 mM), PL (40 µM) and Factor V (30 nM). AT III (260 nM), pre-mixed with either Tris/NaCl buffer, heparin or heparin oligosaccharide, was added and the rate of inhibition of Factor Xa was monitored by subsampling at intervals into S-2222 as described above. In some experiments the AT III and Factor Xa concentrations were different, but the ratio of AT III to Factor Xa concentration was always greater than 30:1. For experiments involving activated Factor V (Factor Va), the concentration of this factor was decreased to 10 nM. All concentrations cited are those in the final incubation mixture.

Calculation of kinetic data

Pseudo-first-order rate constants were calculated from a plot of $\log(\text{residual Factor Xa})$ against time. A least-squares regression line was fitted by a computer program, and the rate constant $k_{app.}$ was calculated from the half-life of Factor Xa, $t_{1/2}$, by the following equation:

$$k_{app.} = \frac{\ln 2}{t_{1/2}}$$

It has been shown in an international collaborative study (G. van Dedem, personal communication) that at a given AT III concentration there is a linear increase in $k_{app.}$ with increasing heparin concentration, which can be described by the following equation:

$$k_{app.} = k_1 + k_c[\text{AT III}][\text{heparin}]$$

where k_1 is the rate constant measured in the absence of heparin.

The single parameter k_c , which describes the catalytic behaviour of any given heparin, is referred to in our studies as the catalytic constant, and is derived from graphs of k_{app} plotted versus heparin concentration, the slope being divided by the AT III concentration.

RESULTS

Effects of Ca²⁺/PL/Factor V in the absence of heparin

The effects of addition of each component separately, and the whole complex, on rates of inhibition of Factor Xa by AT III were initially studied by using both clotting and amidolytic assays for Factor Xa. Concentrations of each component were varied independently and the concentrations chosen (see the Materials and methods section) were those that had maximum effect. Results obtained with the clotting and amidolytic systems were very similar (results not shown), and subsequent studies were carried out with the amidolytic system only. Fig. 1 shows the results in the absence of heparin. The addition of Ca²⁺ and PL gave a slight decrease in inhibition rate, whereas the whole complex gave marked protection from AT III inhibition to the Factor Xa.

The pseudo-first-order rate constants for these reactions are summarized in Table 1; as can be seen, there was an approximately 4-fold decrease when Factor Xa was complexed with Ca²⁺, PL and Factor V, compared with the rate of inhibition of free Factor Xa. Results with Factor Va were similar to those with Factor V, except that the optimum Factor V concentration was lowered to 10 nM when Factor Va was used.

Results with unfractionated heparin

The rates of Factor Xa inhibition by AT III at a low heparin concentration are shown in Fig. 2 for both free and complexed enzyme. As can be seen there is still some

Table 1. Effect of Ca²⁺, PL and Factor V on rate of inhibition of bovine Factor Xa by AT III (no heparin)

For experimental details see the text. k_{app} is the pseudo-first-order rate constant.

Addition	$10^3 \times k_{app}$ (s ⁻¹)
None	2.3
Ca ²⁺	2.2
Ca ²⁺ /PL	1.8
Ca ²⁺ /PL/Factor V	0.6

protection of complexed Factor Xa from inhibition by AT III at this concentration. However, the increase in reaction rate over the uncatalysed reaction is greater for complexed Factor Xa (2.7-fold) than for free Factor Xa (1.5-fold), and as the heparin concentration increases this results in a gradual loss of the protective effect of Ca²⁺/PL/Factor V. Fig. 3 shows a plot of pseudo-first-order rate constants for inhibition of free and complexed Factor Xa versus heparin concentration. The relationships were both slightly curved when the values at zero heparin concentration were taken into account. However, for free Factor Xa the curvature is not statistically significant by analysis of variance. For complexed Factor Xa there is significant curvature, but the relationship is linear between 0.48 and 2.4 nM: this is consistent with the fact that the protective effect is still apparent at the lowest heparin concentration, but is lost as the heparin concentration increases. At a heparin concentration of 2.4 nM there is no significant difference in reaction rates between the free and complexed enzymes, i.e. no protection of Factor Xa by Ca²⁺/PL/Factor V.

Results with heparin oligosaccharides

The effects of increasing concentrations of the decasaccharide fragment on rates of inhibition of free

Fig. 1. Effects of addition of Ca²⁺, PL and Factor V on rate of inhibition of Factor Xa by AT III in the absence of heparin

For experimental details see the text. ◇, AT III; ▲, + Ca²⁺; □, + Ca²⁺/PL; ●, + Ca²⁺/PL/Factor V.

Fig. 2. Effect of addition of Ca²⁺, PL and Factor V on rate of inhibition of Factor Xa by AT III in the presence of unfractionated heparin (0.48 nM)

For experimental details see the text. ◇, AT III; ▲, + Ca²⁺; □, + Ca²⁺/PL; ●, + Ca²⁺/PL/Factor V.

Fig. 3. Effects of increasing concentrations of unfractionated heparin on pseudo-first-order rate constants for inhibition of Factor Xa by AT III

For experimental details see the text. ○, Free Factor Xa, (no additions); △, complex of Factor Xa with Ca^{2+} /PL/Factor V. Data are means $\pm$ S.E.M. for eight experiments.

Fig. 4. Effects of increasing concentrations of heparin decasaccharide fragment on pseudo-first-order rate constants for inhibition of Factor Xa by AT III

For experimental details see the text. ○, Free Factor Xa (no additions); △, complex of Factor Xa with Ca^{2+} /PL/Factor V. Data are means $\pm$ S.E.M. for eight experiments.

and complexed Factor Xa by AT III are shown in Fig. 4. Unlike the situation with unfractionated heparin, the rate enhancement with the complex of Factor Xa with Ca^{2+} /PL/Factor V was less than with the free enzyme, so that the decasaccharide, even at high concentrations, was unable to overcome the protective effect of Ca^{2+} /PL/Factor V. The pentasaccharide gave similar results to the decasaccharide; as shown in Fig. 5, it was relatively ineffective as a catalyst for the inhibition of complexed Factor Xa. The slopes of the lines in Figs. 3–5 are a measure of the catalytic efficiency of each heparin material and, by taking into account the AT III concentration, a single parameter, the catalytic constant, is obtained. Table 2 summarizes the catalytic constants for heparin and these two oligosaccharide fragments. With free Factor Xa, the oligosaccharides are 2–4-fold less active than heparin, but when Factor Xa is complexed to Ca^{2+} /PL/Factor V the decasaccharide and pentasaccharide fragments become 16-fold and 40-fold less active respectively. However, as also shown in Table 2, the 20–22-saccharide fragment, like unfractionated heparin, has similar catalytic constants for free and complexed enzyme.

Results with high-affinity and low-affinity heparin

Calculations of molar activities for unfractionated heparin were based on the assumption that only the molecules with high affinity for AT III, which constituted 40% of the unfractionated heparin, contributed to anti-(Factor Xa) activity. This assumption was tested directly by measuring anti-(Factor Xa) activity of high-affinity heparin at the same concentration as that calculated to be in unfractionated heparin. At two

Fig. 5. Effects of increasing concentrations of synthetic heparin pentasaccharide on pseudo-first-order rate constants for inhibition of Factor Xa by AT III

For experimental details see the text. ○, Free Factor Xa (no additions); △, complex of Factor Xa with Ca^{2+} /PL/Factor V. Data are means $\pm$ S.E.M. for eight experiments.

Table 2. Catalytic constants of unfractionated heparin and oligosaccharide fragments

For experimental details see the text. 'Free Factor Xa' refers to Factor Xa + buffer, and 'Complexed Factor Xa' to Factor Xa + Ca²⁺/PL/Factor V. k_c , the catalytic constant, was derived from the slopes of the graphs of k_{app} , plotted versus heparin concentration, as described in the Materials and methods section.

Polysaccharide	$10^{-12} \times k_c \text{ (M}^{-2} \cdot \text{s}^{-1}\text{)}$	
	Free Factor Xa	Complexed Factor Xa
Heparin	7.9	9.9*
Pentasaccharide	2.0	0.25
Decasaccharide	4.5	0.6
20–22-saccharide	4.7	4.2

* Slope derived from linear portion of the curve, excluding the zero heparin point.

heparin concentrations, 0.5 nM and 2 nM, there were no significant differences in rates of inhibition of Factor Xa, either free or complexed, between unfractionated and high-affinity heparin.

In separate experiments the effect of the addition of low-affinity heparin on the anti-(Factor Xa) activity of the decasaccharide fragment was studied. The addition of low-affinity heparin at concentrations up to 2-fold those of the decasaccharide fragment had no effect on rates of inhibition of free or complexed Factor Xa.

Human Factor Xa

Some experiments were carried out with human Factor Xa, at a similar concentration, instead of the bovine enzyme. Because of the lower rate of inhibition of human Factor Xa, the AT III concentration was increased by 75%. As shown in Table 3, results were similar to those with bovine factor Xa: unfractionated heparin was able to overcome the protective effect of Ca²⁺/PL/Factor V, whereas Factor Xa was still protected even when the decasaccharide fragment was used at high concentrations.

Effect of Ca²⁺ alone

It was noticeable that, in experiments with human Factor Xa, which were carried out at higher AT III

Table 3. Effects of Ca²⁺/PL/Factor V on rates of inhibition of human Factor Xa by AT III and heparin

For experimental details see the text.

Polysaccharide	$10^3 \times k_{app} \text{ (s}^{-1}\text{)}$	
	Free Factor Xa	Complexed Factor Xa
None	1.63	0.54
Unfractionated heparin (2.4 nM)	3.02	5.62
Decasaccharide (6.2 nM)	4.76	1.4
Decasaccharide (17 nM)	13.74	5.24

concentrations, the rate of neutralization of complexed Factor Xa in the presence of unfractionated heparin actually exceeded that of the free enzyme (Table 3). This was due to the fact that Ca²⁺ alone accelerated the neutralization of Factor Xa by AT III in the presence of unfractionated heparin, the rate constants being approx. 20% higher with Ca²⁺. The accelerating effect seen with unfractionated heparin was not found with any of the oligosaccharides tested.

DISCUSSION

Our initial studies confirmed the findings by Marciniak (1973) that, in the absence of heparin, Factor Xa clotting activity was protected from inhibition by AT III when Ca²⁺, PL and Factor V were added. Detection of Factor Xa by clotting assay gave very similar results to the amidolytic assay (results not shown), but the amidolytic system was thought to be more reliable for studies involving heparin. The decrease in inhibition rate of Factor Xa in the presence of Ca²⁺/PL/Factor V of approx. 4-fold (Fig. 1 and Table 1) agrees closely with the data of Nesheim & Mann (1983), who used a similar amidolytic system.

On addition of unfractionated heparin, the protective effect of Ca²⁺, PL and Factor V was maintained at low concentrations (Fig. 2). However, as its concentration was increased, unfractionated heparin was able to overcome the protective effect and, at a concentration of 2.4 nM, there were no significant differences in the inhibition rates of free and complexed Factor Xa.

The high-affinity heparin decasaccharide fragment behaved quite differently from unfractionated heparin. Rates of inhibition of complexed Factor Xa were always markedly lower than those of free Factor Xa (Fig. 4), even at high concentrations. Thus the decasaccharide was a relatively ineffective catalyst of inhibition of complexed Factor Xa. The pentasaccharide fragment was even less effective (Fig. 5); the catalytic constants for the decasaccharide and pentasaccharide fragments were some 16-fold and 40-fold less than that of unfractionated heparin for inhibition of complexed Factor Xa. However, the 20–22-saccharide fragment was more effective than the decasaccharide fragment in inhibiting complexed Factor Xa; its catalytic constant was similar for the free and the complexed enzyme (Table 2), though it was still a less effective catalyst than unfractionated heparin. These results indicate that, for optimum neutralization of Factor Xa complexed with Ca²⁺/PL/Factor V, a polysaccharide sequence in addition to the pentasaccharide AT III-binding sequence is required. The size of this sequence cannot be determined with certainty from the current studies, but is likely to be between five and 15 residues.

Previous studies have shown that low-affinity heparin enhances the inhibition of Factor Xa generation in plasma by high-affinity heparin oligosaccharides (Barrowcliffe *et al.*, 1984). Low-affinity heparin has been shown to inhibit prothrombin activation, possibly by disruption of the complex of Factor Xa with Ca²⁺/PL/Factor V (Ofosu *et al.*, 1982). It was thought that such disruption might make available more free Factor Xa, and hence promote its inhibition by high-affinity heparin molecules. However, the results of the present study do not support this hypothesis, since

the presence or absence of low-affinity heparin had no effect on inhibition rates of either free or complexed Factor Xa.

Miletich *et al.* (1978) found that Factor Xa binds with high affinity to platelets, and a number of studies have shown that this binding requires the presence of Factor Va (Dahlbäck & Stenflo, 1978; Kane *et al.*, 1980; Tracy *et al.*, 1981) and PL (Bevers *et al.*, 1982; Rosing *et al.*, 1985). However, the effect of heparin/AT III on platelet-bound Factor Xa is not clear. Miletich *et al.* (1978) found that platelet-bound Factor Xa was completely protected from AT III/heparin at a heparin concentration of 1 i.u./ml. Teitel & Rosenberg (1983) also found that heparin at 2 i.u./ml was unable to suppress prothrombin activation in whole blood, as measured by assay of F_2/F_{1+2} fragments of prothrombin. These observations are difficult to reconcile with the fact that heparin is nonetheless an effective anticoagulant in whole blood, preventing thrombin formation at a concentration of 0.5 i.u./ml (Krachmalnicoff & Thomas, 1983).

Our own studies indicate that, if platelet-bound Factor Xa is protected against AT III/heparin, this protection cannot be due entirely to binding to Ca^{2+} /PL/Factor V. It is possible that initial binding of Factor Xa to platelets occurs via PL and Factor Va, with subsequent tighter binding involving other membrane components. This is supported by other studies indicating that platelet-bound Factor Xa is a more effective prothrombin activator (Dahlbäck & Stenflo, 1978) and is more protected against inhibition by AT III (Ellis *et al.*, 1984) than is Factor Xa bound to Ca^{2+} /PL/Factor Va.

The effects of heparin oligosaccharides on platelet-bound Factor Xa have not been extensively studied. Teitel & Rosenberg (1983) found that a 6500- M_r fraction gave similar results to unfractionated heparin with regard to protection of Factor Xa. Ellis *et al.* (1986) found that the anti-(Factor Xa) activity of an 8–10-saccharide fragment was less affected by Factor Xa binding to platelets than was the activity of unfractionated heparin, a result apparently at variance with our own findings. However, closer examination of the data of Ellis *et al.* (1986) shows that, if the catalytic efficacies of the 8–10-saccharide fragment and unfractionated heparin for inhibition of platelet-bound factor Xa are compared directly, unfractionated heparin is a more effective catalyst, giving a steeper increase in rate constant with increasing concentration, and a higher maximum rate ($4.1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ compared with $9.8 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ for the 8–10-saccharide fragment). Therefore our results with Factor Xa complexed to Ca^{2+} /PL/Factor V are not inconsistent with these data. However, Ellis *et al.* (1986) found that the 8–10-saccharide fragment was some 16–23-fold less active in inhibition of free Factor Xa than was unfractionated heparin, whereas in our studies the decasaccharide was only 2-fold less active than unfractionated heparin.

Part of the reason for these differences may be that the experiments of Ellis *et al.* (1986) were conducted in 5 mM- $CaCl_2$, whereas in our experiments inhibition of free Factor Xa was measured in the absence of Ca^{2+} , since all current assays of the anti-(Factor Xa) activity of heparin are carried out in the absence of Ca^{2+} . In our studies we found that 3 mM- $CaCl_2$ promoted the inhibition of free Factor Xa by unfractionated heparin but not by the oligosaccharide fragments, and hence

inclusion of $CaCl_2$ would accentuate the difference between heparin and the oligosaccharides.

The effects of $CaCl_2$ may also account for some differences between our results and those obtained by Ofosu *et al.* (1984), since all experiments were carried out by those authors in 10 mM- $CaCl_2$. Marciniak (1973) found that increases in $CaCl_2$ concentration beyond the physiological range of 1–3 mM markedly enhanced the rate of inhibition of Factor Xa by AT III, and high $CaCl_2$ concentrations could have similar effects on inhibition rates in the presence of heparin. Ofosu *et al.* (1984) found that both 'cephalin' and activated platelets protected Factor Xa even at high concentrations of heparin. Our own results agree with these findings if inhibition of complexed Factor Xa is compared with that of free Factor Xa in the presence of 3 mM- $CaCl_2$, though in our studies the differences are smaller than those found by Ofosu *et al.* (1984), probably owing to the differences in $CaCl_2$ concentration. Unlike our own data, and also those of Yin (1975), Walker & Esmon (1979) and Ellis *et al.* (1984), Ofosu *et al.* (1984) found no protective effect of 'cephalin' or activated platelets in the absence of heparin. This, together with the fact that gel-filtered platelets were as effective as activated platelets, and that thrombin was protected as well as Factor Xa, led these authors to postulate a 'sequestering' effect of 'cephalin' and platelets on heparin, rather than high-affinity binding of Factor Xa as the explanation for their observations; platelet factor 4 was also considered to play a role in the effects of platelets.

Although the relevance of our results to activities of heparin and its oligosaccharides *in vivo* remains uncertain, the anti-(Factor Xa) potencies of the oligosaccharides in comparison with standard heparin, particularly the decasaccharide, were much more related to their antithrombotic action *in vivo* when measured in the presence of Ca^{2+} /PL/Factor V than when measured with the free enzyme. Thus assays of anti-(Factor Xa) activity with complexed rather than free enzyme may be more relevant to the physiological situation, and further studies on a variety of low- M_r fractions are warranted.

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