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The molecular-weight dependence of the rate-enhancing effect of heparin on the inhibition of thrombin, Factor X*a*, Factor IX*a*, Factor XI*a*, Factor XII*a* and kallikrein by antithrombin

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Heparin fractions of different molecular weight and with high affinity for antithrombin were studied with respect to their ability to potentiate the inhibition of activated clotting factors by antithrombin. Inhibition of thrombin, Factor IX*a* and Factor XI*a* showed similarities in the dependence on the molecular weight of heparin and was found to decrease with decreasing molecular weight. Inactivation of Factor X*a*, Factor XII*a* and kallikrein was, however, less dependent on the size of the polysaccharide and, to a great extent, was potentiated even by low-molecular-weight heparin fractions that had virtually no effect on the inhibition of thrombin, Factor IX*a* and Factor XI*a*.

The anticoagulant action of heparin is generally believed to involve interaction with antithrombin III (heparin cofactor). Antithrombin III, a serine proteinase inhibitor, inhibits thrombin (Abildgaard, 1968), Factor IX*a*, Factor X*a* (Kurachi *et al.*, 1976; Østerud *et al.*, 1976), Factor XI*a* (Damus *et al.*, 1973), Factor XII*a* (Stead *et al.*, 1976) and kallikrein (Venneröd *et al.*, 1976) by forming a very stable complex, possibly a covalent complex, with them (Owen, 1975). Heparin strongly affects all these reactions by increasing the rate at which they proceed. There is a widespread opinion that this is caused by the binding of heparin to antithrombin III so as to induce a conformational change that makes the antithrombin III become a more rapidly acting inhibitor. The molecular basis of the anticoagulant action of heparin is, however, still a matter of controversy.

Only a portion of the heparin in clinically employed preparations has affinity for antithrombin III and displays anticoagulant activity. Thus, by using affinity chromatography on matrix-bound antithrombin III, heparin can be divided into one fraction (about one-third of the total amount) with high anticoagulant activity (high-activity heparin) and another almost inactive fraction (low-activity heparin) (Höök *et al.*, 1976; Lam *et al.*, 1976; Andersson *et al.*, 1976). The difference in biological activity between the two fractions has been shown to be paralleled by structural differences (Rosenberg & Lam, 1979; Lindahl *et al.*, 1979). Digestion of high-activity heparin by bacterial heparinase has shown that a fragment with molecular weight 3000–4000 comprises all structures essential for high-affinity binding to antithrombin III (Hopwood *et al.*, 1976). A structural characterization of a similar antithrombin-binding fragment, obtained by partial cleavage of heparin with nitrous acid, has been published (Lindahl *et al.*, 1979).

Heparin is also heterogeneous with regard to molecular size. Normal heparin preparations consist of components with molecular weights ranging from 5000 to 30000 and with an average molecular weight of about 10000–15000 (Johnson & Mulloy, 1976). Studies on heparin fractions of different molecular weights obtained by gel filtration have demonstrated a molecular-size-dependency of the anticoagulant activity (Andersson *et al.*, 1976, 1979; Laurent *et al.*, 1978). Measurements by multiple-role clotting assays such as the activated partial thromboplastin time assay (APTT assay) or the British Pharmacopoeia (B.P. assay) have shown a markedly diminished specific activity with decreasing molecular weight. The same relation has also been found in specific systems measuring the inhibition of thrombin by heparin and purified antithrombin III. However, the potentiation of the inhibition of Factor X*a* by antithrombin III produces another pattern in that low-molecular-weight heparin fractions, with low ability to potentiate

Abbreviations used: symbols for amino acids (which are of the L-configuration if not stated otherwise) and their derivatives follow the Recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature [*Biochem. J.* (1972) **126**, 773–780]; additional symbols are: pNA, *p*-nitroanilide; Pip, pipecolic acid.

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thrombin inhibition, retain high anti-(Factor X*a*) activity. The properties of a heparin fragment composed of 10–16 monosaccharide units (mol.wt. 3000–4000), which includes the antithrombin-binding sequence, confirm these observations; it strongly enhances Factor X*a* inhibition, whereas the clotting time (APTT assay) and thrombin inhibition are practically unaffected (Holmer *et al.*, 1980).

In the present study we have investigated the effects of different molecular-weight fractions of high-activity heparin, including the minimal-size antithrombin-binding fragment, on antithrombin III inhibition of each of all the serine proteinases known to occur in the intrinsic pathway of the coagulation cascade.

Materials

Heparin fractions

Pig mucosal heparin was fractionated according to molecular size by gel filtration on Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) in a 5.0cm × 170cm column. A load of 4g in 40ml was eluted with 0.3M-NaCl at a flow rate of 100ml/h. Six subfractions were utilized and submitted to affinity chromatography on matrix-bound antithrombin III (Andersson *et al.*, 1976). The high-affinity material was adsorbed, eluted, desalted and used in the experiments described in the present study. The average molecular weights of the fractions were found to be 17000, 13500, 10500, 8200, 6300 and 4800.

Antithrombin-binding heparin fragment

This fragment was prepared by partial degradation of pig mucosal heparin with nitrous acid followed by affinity chromatography on matrix-bound antithrombin III as described by Lindahl *et al.* (1979). The high-affinity material was recovered and used in the present study. The elution position of the fragment on analytical gel filtration corresponded to a molecular weight of about 3400, which is similar to that of a fragment claimed to comprise the antithrombin-binding sequence of heparin. The anticoagulant properties of the fragment used in the present study, with respect to thrombin and Factor X*a* inhibition, were found to be essentially the same as those of a corresponding antithrombin-binding heparin fragment kindly prepared and donated by Dr. U. Lindahl, Swedish University of Agricultural Sciences, Uppsala, Sweden (Lindahl *et al.*, 1979; Holmer *et al.*, 1980).

Human antithrombin III

This was prepared as previously described (Miller-Andersson *et al.*, 1974) and diluted to the desired concentration in Tris/BSA buffer (see below).

Clotting factors

Bovine Factor X was purified and activated by Russell's-viper-venom proteinase by the method described by Fujikawa *et al.* (1972). Bovine Factor IX was purified by the method of Fujikawa *et al.* (1973) and activated by Factor XI*a* in compliance with the conditions described by DiScipio *et al.* (1978). Bovine Factor XI was purified and activated by Factor XII*a* as described by Kurachi *et al.* (1980). Bovine Factor XII*a* was prepared and kindly donated by Dr. K. Fujikawa of the Department of Biochemistry, University of Washington. Bovine kallikrein was prepared and kindly donated by Dr. R. Heimark of the Department of Biochemistry, University of Washington. Bovine thrombin was kindly donated by Dr. W. Kisiel of the Department of Biochemistry, University of Washington.

All clotting factors were diluted in Tris/BSA buffer to the desired concentration, by using $A_{280}^{1\%}$ values of 19.5, 10.0, 14.3, 12.6, 14.2 and 10.9 for thrombin, Factor Xa_β, Factor IX*a*, Factor XI*a*, Factor XII*a* and kallikrein respectively.

Chromogenic substrates

Compounds S-2238 (D-Phe-Pip-Arg-pNA), S-2222 (Bz-Ile-Glu-Gly-Arg-pNa) and S-2302 (D-Pro-Phe-Arg-pNA) were obtained from Kabi Diagnostica, Stockholm, Sweden. The recently developed substrates compounds S-2511 and S-2512 with sensitivity for Factor XI*a* and Factor XII*a* respectively were prepared and kindly donated by Kabi AB, Peptide Research, Mölndal, Sweden. Each substrate was dissolved to a concentration of 1mM in water containing 0.33mg of Polybrene (EGA-Chemie, Steinheim, W. Germany)/ml.

Tris/BSA buffer and Tris buffer

The buffers referred to as Tris/BSA buffer and Tris buffer were 50mM-Tris/HCl/0.15M-NaCl, pH7.8, containing 0.1mg of bovine serum albumin/ml and 50mM-Tris/HCl/0.15M-NaCl, pH7.8, respectively.

Heparin standard

The heparin standard was the 3rd International Standard for Heparin, obtained from the National Institute for Biological Standards and Control, London, U.K.

Methods

The carbazole/H_2SO_4 method (Bitter & Muir, 1962) was used to determine the heparin content of the subfractions, and the average molecular weight was determined by analytical gel filtration (Johnson & Mulloy, 1976) on Ultrogel AcA-44 (LKB,

Bromma, Sweden). The heparin fractions were diluted as required for anticoagulant assays in Tris buffer.

Influence of heparin fractions on the inhibition of thrombin, Factor Xa, Factor XIa, Factor XIIa and kallikrein by antithrombin III

This was studied as indicated in the following general outline.

Solutions of antithrombin III and heparin fraction were mixed and pre-warmed at 37°C for 3 min. Enzyme was then added. After a certain reaction time the chromogenic substrate solution (1 mM, 0.33 mg of Polybrene/ml) was added. (Control experiments showed that heparin fractions, including the fragment, were neutralized on the addition of Polybrene.) The reaction was stopped after exactly the desired period of time by adding 50% (v/v) acetic acid. The absorbance at 405 nm was read. Under the conditions used the absorbance changes were found to be linear with time and the absorbance values decreased linearly with increasing heparin concentration. The details of the assays are listed in Table 1.

Effect of heparin fractions on the inhibition of Factor IXa by antithrombin III

This was studied by using the following method.

A 50 μl volume heparin fraction (0–8 units/ml) was mixed with 50 μl of antithrombin III (1 unit/ml). After pre-warming of the mixture for 3 min at 37°C, 10 μl of Factor IX*a* (15 μg/ml) was added. Exactly 180 s later the reaction was stopped by the addition of 50 μl of Polybrene (0.33 mg/ml) followed by immediate freezing of the reaction mixture by placing the test tube in ethanol/solid CO_2.

The remaining Factor IX*a* was then determined for a series of samples by using a clotting assay in citrated bovine plasma as described previously (Fujikawa *et al.*, 1974). The frozen samples were thawed and diluted to an appropriate concentration by adding 4.0 ml of Tris/BSA buffer to the test tube. Then 100 μl of this mixture was used immediately in the clotting assay.

Calculation of specific activity

The specific activity of the heparin fractions was calculated from standard curves plotted for each assay system by making dilutions of the 3rd International Standard for Heparin.

Results

The effect of antithrombin III and heparin on activated clotting factors was studied in systems consisting of enzyme, antithrombin III and various amounts of heparin or heparin fraction. Residual enzyme activity after a certain reaction time was measured by using chromogenic peptide substrates, except with Factor IX*a*, for which a clotting technique was used. In conformity with previous reports, it was demonstrated that heparin enhances the rate of the inhibition of these proteinases by antithrombin III. Within the concentration range and time period studied, the fraction of enzyme inhibited was found to be proportional to the heparin concentration. A notable observation is that the reaction rate seems to vary considerably among enzymes. To inhibit a certain fraction of kallikrein and Factor XI*a* activity, for instance, a much higher concentration of antithrombin and heparin and a much longer reaction time were required than for the inhibition of thrombin and Factor X*a* (see Table 1).

The ability of the isolated heparin fractions to accelerate the inhibition of enzyme by antithrombin III was compared in each system with that of the 3rd International Standard for Heparin and expressed as specific activity (i.u./mg). The results shown in Fig. 1(*a*) confirm previous reports on molecular-weight–specific-activity relationships for heparin found for the inhibition of thrombin and Factor X*a*. The ability to potentiate thrombin inhibition decreases

Table 1. *Influence of heparin fractions on the inhibition of thrombin, Factor Xa, Factor XIa, Factor XIIa and kallikrein by antithrombin III*

Details of the assay procedures are outlined in the text.

	Antithrombin III		Heparin		Enzyme		Reaction time (s)	Substrate		Reaction time (s)	50% acetic acid
	(μl)	(units/ml)	(μl)	(units/ml range)	(μl)	(μg/ml)		(compound)	(μl)		(μl)
Thrombin	200	0.1	200	0–0.8	100	6.2	30	S-2238	300	30	300
Factor X*a*	100	0.2	100	0–0.2	100	15	30	S-2222	300	30	300
Factor XI*a*	100	2.0	100	0–12	100	38	300	S-2511	300	120	300
Factor XII*a*	50	2.0	50	0–2.0	100	26	60	S-2512	150	300	150
Kallikrein	50	3.0	50	0–6.0	50	6.0	300	S-2302	150	120	150

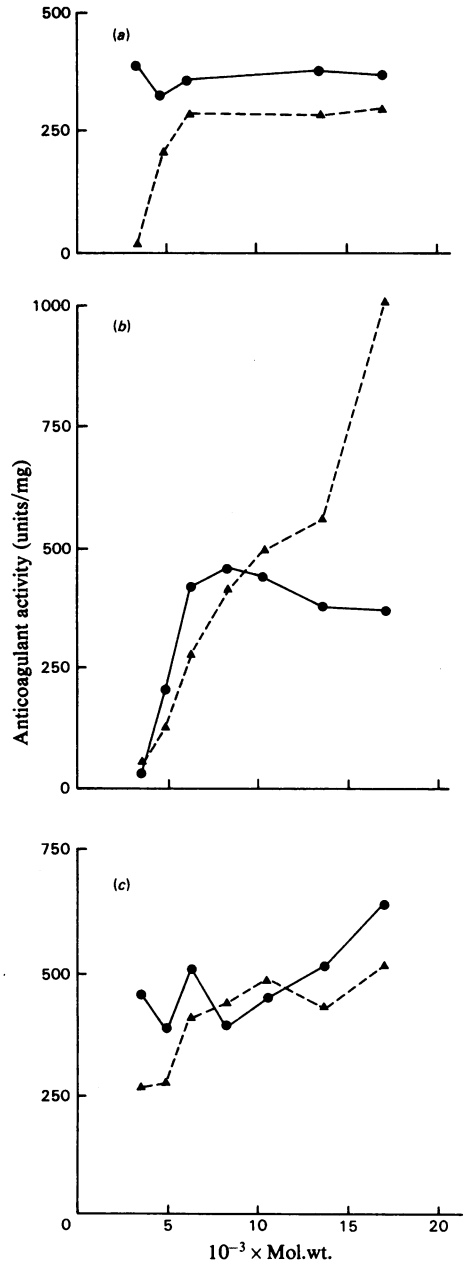

Fig. 1. *Effect of heparin fractions of various molecular weights on the inactivation of coagulation enzymes by antithrombin III*

The fraction of lowest molecular weight corresponds to the antithrombin-binding fragment obtained by chemical degradation of heparin. The specific activities of the heparin subfractions were compared with that of the 3rd International Standard for Heparin and expressed in units/mg. (*a*) Inactivation of Factor X*a* (●) and thrombin (▲). (*b*) Inactivation of Factor IX*a* (●) and Factor XI*a* (▲). (*c*) Inactivation of Factor XII*a* (●) and kallikrein (▲).

with decreasing molecular weight, whereas Factor X*a* inhibition is fully potentiated even by low-molecular-weight heparin fractions.

Inhibition of Factor IX*a* and Factor XI*a* (Fig. 1*b*) follows a molecular-weight–specific-activity profile similar to that of thrombin, characterized by no or low activity in the heparin fractions of lowest molecular weight. The molecular-weight-dependence is especially accentuated in the inhibition of Factor XI*a*, with very high specific activities in the high-molecular-weight fractions.

It can be seen from Fig. 1(*c*) that the molecular-weight-dependence of the inhibition of Factor XII*a* and kallikrein is distinctly different from that of thrombin, Factor IX*a* and Factor XI*a*. Relatively high specific activities are found even in the low-molecular-weight fractions, a phenomenon essentially similar to that found in Factor X*a* inhibition.

Discussion

Heparin fractions of various molecular sizes have been assayed for their ability to potentiate the inhibition of clotting factors by antithrombin III. In previous studies it has been demonstrated that there is a difference in the molecular-weight anticoagulant-activity-dependence of thrombin and Factor X*a* inhibition (Andersson *et al.*, 1979). Inactivation of Factor X*a* is highly potentiated by low-molecular-weight heparin fractions that have limited ability to affect the inhibition of thrombin. Recent studies on a small heparin fragment (10–16 monosaccharide units) capable of binding antithrombin III definitely established this difference (Holmer *et al.*, 1980).

The findings in the present study show that the heparin molecular-weight–activity profiles obtained for clotting factors mainly follow either of the two patterns characteristic of Factor X*a* or thrombin inhibition. Factor IX*a* and Factor XI*a* together with thrombin constitute a group with an apparently similar behaviour: in the lower-molecular-weight range the specific activity of heparin falls dramatically as its molecular weight decreases. The fragment with a molecular weight of about 3400 has very little potentiating effect on the inhibition of these enzymes.

These findings can be seen in relation to the mechanism for heparin action. Induction of a conformational change in the antithrombin III molecule by heparin binding has been suggested to be the major cause of the potentiation of the inhibitor reactions. All the heparin fractions used in the present study bind tightly to antithrombin III, as they were fractionated with regard to affinity for antithrombin III. Nevertheless the fractions of low molecular weight show very low activity. Thus heparin binding to antithrombin III does not seem to

be a sufficient condition for full activity in the case of inhibition of thrombin, Factor IX*a* and Factor XI*a*. As regards thrombin, a tentative model involving simultaneous binding of both enzyme and inhibitor to the polysaccharide has been suggested (Laurent *et al.*, 1978; Holmer *et al.*, 1979). Statistical considerations of such a model indicated that the anticoagulant-activity–molecular-weight-dependence could be related to the probability of finding adjacent binding sites for thrombin and antithrombin III on the heparin molecule (Laurent *et al.*, 1978).

The similarities in behaviour make it reasonable to believe that the same model can also be adapted to Factor IX*a* and Factor XI*a* inhibition. Heparin activity, as measured by Factor XI*a* inactivation, is more highly dependent on its molecular weight than is so with thrombin and Factor IX*a* inhibition. This is in agreement with the suggested model if it is taken into consideration that simultaneous binding of Factor XI*a* (with mol.wt. 124000) and antithrombin III requires a longer heparin chain than is needed for the corresponding attachment of thrombin (mol.wt. 39000) and Factor IX*a* (mol.wt. 44000).

Inactivation of kallikrein and Factor XII*a* shows patterns that mainly coincide with those found for Factor X*a*. Inhibition of these enzymes seems to be less dependent on the length of the polysaccharide chain. Their inhibition by antithrombin III is thus characteristically potentiated to a great extent by the low-molecular-weight heparins that have very little effect on the inhibition of thrombin, Factor IX*a* and Factor XI*a*. This disparity between the two groups of enzymes may point to differences in the mechanism of their inactivation. Such differences have been postulated for thrombin and Factor X*a* inhibition (Andersson *et al.*, 1979; Thunberg *et al.*, 1979). The findings in the present paper make it reasonable to extend this postulate to the two groups of enzymes. Thus the 'thrombin group' of enzymes seems to require a certain length of the heparin chain that enables simultaneous binding of enzyme and inhibitor for full potentiation. For the 'Factor X*a* group', on the other hand, the attachment of antithrombin III alone seems to be a sufficient condition.

The ability of heparin preparations to prevent thrombus formation has long been assumed to be reflected by their anticoagulant activity, i.e. their ability to prolong clotting time as measured by the pharmacopoeial assays (B.P. or U.S.P.) or the APTT assay. This relationship has been disputed, however, and it has been suggested that the antithrombotic property is rather due to the anti-(Factor X*a*)-potentiating property of heparin (Yin *et al.*, 1971). Clinical investigations with a low-molecular-weight semi-synthetic heparin analogue tend to support this theory (Kakkar *et al.*, 1978). This analogue displayed antithrombotic properties, but only slightly affected the clotting time. The anti-(Factor X*a*) activity was relatively high, however. No assays were performed to investigate possible effects on the earlier stages of the coagulation cascade. The present study demonstrates similarities in the inhibition of Factor X*a*, Factor XII*a* and kallikrein. This suggests the possibility that the Factor X*a*-inhibiting property of the analogue is accompanied by anti-kallikrein and anti-(Factor XII*a*) properties, which in turn implies that the antithrombotic properties of the analogue may be related to the ability to potentiate inactivation of serine proteinases in the initial stages of the coagulation cascade. The amplification effect during the generation of proteinases in the different steps of the coagulation cascade is in accord with this suggestion, since it would be easier to inhibit coagulation in the initial stages.

According to this hypothesis, low-molecular-weight heparin would possess antithrombotic properties with small effects on the clotting time, and, possibly, without causing bleeding. A confirmation or rejection of this hypothesis must await further clinical investigations. However, knowledge of the mechanism of action of heparin in the different stages of the coagulation cascade are probably of great importance for the understanding of its anticoagulant and antithrombotic nature. Such insights can be expected to be useful for the improvement of antithrombotic agents and therapy.

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