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Some of the biological properties of factor X-fractionated heparin

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Summary

Heparin and heparin molecular fractions have been chromatographed using bovine factor X immobilised to Biogel A15. The eluted fractions were assayed chemically using azure A dye, and biologically using the activated partial thromboplastin time (APTT), and prothrombin activation inhibition (PAI) assays. On the basis of these assays, low-affinity (LA-FX) and high-affinity (HA-FX) fractions, with respect to factor X, were isolated and the anticoagulant activities (B.P., APTT and antifactor Xa) determined. Several of the heparins could be used as antithrombotic agents and showed similar characteristics to those previously reported for low-molecular-weight heparin preparations.

Introduction

Heparin is a heterogeneous glycosaminoglycan extracted from mammalian tissues and is used extensively by clinicians as an anticoagulant. Although heparin was discovered in the early part of this century, it is only in the past decade that advances have been made to understand the essentials of its structure–function relationship. It is now generally agreed that the major effect of heparin is its interaction with antithrombin III (AT III), a natural inhibitor of the serine proteases (thrombin and the activated forms of coagulation factors IX, X, XI, XII) involved in the intrinsic coagulation process. However, only some heparin molecules are capable of interacting with AT III and many studies have focussed on identifying and describing other interactions of heparin in relation to their physiological significance.

In a previous paper (Morton et al., 1984), this group has shown that significant differences exist between heparin fractions of different molecular weights in their ability to release lipoprotein lipase from endothelium, adipose tissue and liver sites. It has also been shown (Andersson et al., 1976; Morton et al., 1981) that heparin interacts with factor Xa and that low-molecular-weight heparin fractions have an enhanced anti-factor Xa activity (Johnson et al., 1976; Thomas and Merton, 1982) in contrast to a decrease in anticoagulant activity when assays such as the British Pharmacopoeia (B.P.) and APTT have been used. The role of

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factor Xa in blood clotting appears to be more important than originally thought and clinicians have realised the potential advantages of heparin preparations possessing enhanced anti-factor Xa activity. Walker and Esmon (1979a) have shown that heparin fractions with high affinity for factor X whilst being poor anticoagulants of plasma were effective inhibitors of prothrombin activation by factor Xa, calcium and phospholipid.

In this study, factor X binding profiles of several different heparins and heparin fractions have been examined in order to gain a better understanding of the interaction between heparin and this blood protein.

Materials and Methods

Sodium heparin (Diosynth, Morden, U.K.) was chromatographed on Ultrogel AcA 44 (LKB, Cambridge, U.K.) to produce 5 distinct heparin fractions (Morton et al., 1981). The starting material (Diosynth heparin; 164 U/mg), fraction I (high molecular weight; 178 U/mg) and fraction IV (medium–low molecular weight; 121 U/mg) together with three other commercial heparins were examined. These additional heparins were obtained from Glaxo Laboratories, Runcorn, U.K. (102 U/mg) and Abbott Laboratories, IL, U.S.A. (low potency; 68 U/mg and high potency; 157 U/mg).

Molecular weight estimations of heparin fractions were carried out by high-pressure liquid chromatography (Harenberg and De Vries, 1983) (HPLC, Perkin-Elmer, U.S.A.), using a column (Ultrapac TSK G3000 SW, 600 × 7.5 mm i.d., particle size 10 ± 2 μm; LKB No. 2135-360), a detector (Differential Refractometer R401, Waters) and a two-channel recorder. A pre-column (Ultrapac TSK GSWP, 75 × 7.5 mm i.d., 10 μm LKB) was connected between the pump and the main column. The mobile phase was 0.1 M phosphate buffer (pH 7.0; filtered (0.45 μm) and degassed before use). The column was calibrated using heparins of defined molecular weights (Abbott Labs., IL, U.S.A., provided by Dr. Grant Barlow). The degassed and filtered 0.1 M phosphate buffer was used to prepare a solution (3% w/v) of heparin which was passed through a 0.45 μm filter before 60 μl was injected onto the column. The molecular weight parameters of heparin were calculated using a Perkin-Elmer Basic program (GPC 2L).

Bovine blood (35 litres) and brains (~ 100 g) were obtained from a local abattoir. The blood was collected into plastic vessels (5 litres) containing anhydrous sodium oxalate (13.4% w/v) and centrifuged at 2000 *g* for 2 h at 4 °C to obtain platelet-poor plasma. The bovine brain free from all vascular and connective tissues was liquidised and progressively dehydrated with acetone. Factor X was extracted (Fig. 1) according to the method of Jackson et al. (1968) and detected by reaction with the chromogenic substrate S-2222 (Kabi, Stockholm) using the method of Aurell et al. (1977).

Factor X was activated by the Cyanogen Bromide method (Cuatrecasas, 1970) and bound to Biogel A15 (BioRad, Watford, U.K.). Heparin (15,000 U (B.P.) in 3 ml) was passed down an affinity column (0.9 × 14 cm) of FX-Biogel A15

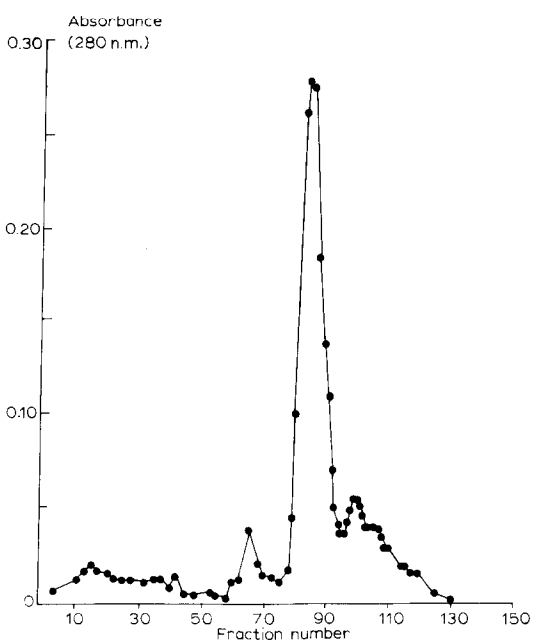

Fig. 1. Gel filtration of factor X on Sephacryl S-200. Column, 2.5 × 70 cm; buffer, 0.01 M Tris-HCl-0.50 M NaCl (pH 7.5); sample, factor X from DEAE-cellulose, 20 ml; flow rate, 15 ml/h; fraction volume, 2.5 ml; temp. 20–25 °C.

and eluted with a linear gradient (0.01–0.2 M) of sodium chloride. The flow was maintained at 11 ml/h and 1.1-ml fractions were collected. Each fraction was stored at ca. −28 °C until assayed.

The anticoagulant activities of the heparin fractions were determined firstly using the APTT (Thomson [1980] as modified in 1983) and a modified prothrombin activation inhibition (PAI) assay (Walker and Esmon, 1979a).

These two assays were performed in duplicate for all the fractions analysed. The modification to the PAI assay involved adding prothrombin (100 μl of 720 μl/ml in 0.02 M Tris-HCl, 0.1 M NaCl) to 100 μl of heparin (1.0 U/ml) plus fibrinogen (100 μl, Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) and a FXa/phospholipid mixture (100 μl; 28% phosphatidylcholine; 26% phosphatidylserine). The phospholipid (1.0 mg/ml) was extracted from bovine brain according to the method of Bligh and Dyer (1959) and mixed 1 : 1 with factor Xa (76 μg/ml) in 0.02 M Tris-HCl, 0.1 M NaCl). The reactants were incubated at 37 °C for 10 min and the clotting reaction was initiated by the addition of calcium chloride (100 μl of 10 mM). The tube was left undisturbed for 30 s and tilted gently until fibrin strands formed a tight clot. Duplicate tests (±2 s) were performed on each heparin sample and the time for clot formation after recalcification was recorded.

Heparin concentration was chemically determined by its reaction with azure A dye according to the method of Jaques et al. (1949), as modified elsewhere (Lam et al., 1976). The increase in absorbance at 520 nm was measured. All assays were found to be linear in the range 0.1–0.8 U/ml of heparin.

The (W.H.O.) Third International Reference Preparation of Heparin was used as a standard for all assays and the reagents were of Analar grade. Distilled water was used as solvent and sodium chloride (0.9% w/v) as a reference in each assay.

On the basis of the PAI assay, fractions were separated (Figs. 2 and 3) into those with low affinity for factor X (LA-FX) and high affinity for factor X (HA-FX). The active fractions were pooled, dialysed (against distilled water for 24 h with changes at 2, 5 and 20 h) and lyophilised.

The anticoagulant activities of the pooled preparations were subsequently determined using the B.P. (1973 method), APTT and activated factor X assay (anti-factor Xa; Teien et al., 1976) using the synthetic substrate B_Z-Ile-Glu-Gly-Arg-pNA (S-2222, Kabi, Stockholm). The factor Xa test was performed as follows: Bovine factor Xa (Diagnostic Reagents, Thame, Oxon, U.K.) (100 μl of a solution prepared by dissolving one ampoule [3 mg] of factor Xa in 0.8 ml of distilled water) was allowed to react for 30 s at 37 °C with the heparin (20 μl) and human plasma (200 μl). Afterwards the substrate solution (200 μl of 1 mM) was added and incubated for a further 3 min. The reaction was terminated by the addition of acetic acid (300 μl of 50% v/v). The absorbance was measured at 405 nm against a normal plasma blank (200 μl distilled water; 300 μl plasma; 300 μl acetic acid) within 1 h of assay.

Results

In this study several heparins have been chromatographed on factor X covalently bound to Biogel A15, in order to examine the distribution profile of different heparins and to determine some of the biological characteristics of the fractions collected. The 6 heparins selected varied in commercial source, anticoagulant activity and molecular weight distribution (Table 1). Diosynth 164 heparin had a polydispersity (Q) of 1.22 and a weight-average molecular weight (M_w) of 12,470. Following gel chromatographic fractionation of Diosynth 164, two distinct fractions were selected; one with a mol. wt. of 18,020 (Diosynth 178) and a Q of 1.10 and the other with a M_w of 9,560 (Diosynth 121) with a Q of 1.11. The changes in M_w of these two fractions from the parental source (Diosynth 164) are also shown in their M_n and M_z values (Table 1). The two most polydisperse samples were those with the lowest anticoagulant (B.P.) activities (Glaxo 102 and Abbott 68) and these samples were characterised by two peaks using HPLC. These peaks are possibly produced by glycosaminoglycan impurities such as dermatan and heparan sulphate associated with the heparin molecules which could be attributed to the lower anticoagulant activities obtained.

TABLE 1

Physical and biological properties of the heparin starting material

Type of heparin	Activity (BP) U/mg	M_w	M_n	M_z	Q
Diosynth 164	164	12,470	10,190	15,150	1.22
Diosynth 178	178	18,020	16,430	19,890	1.10
Diosynth 121	121	9,560	8,650	10,560	1.11
Glaxo 102	102	9,120	6,980	13,220	1.31
Abbott 68	68	15,840	11,210	20,530	1.41
Abbott 157	157	11,660	9,630	13,830	1.21

Anticoagulant activity was assayed using the B.P. (1973 method) and molecular weights were determined using high performance liquid chromatography.

The heparins were chromatographed on a factor X affinity column (Figs. 2 and 3) and the fractions assayed using azure A dye, APTT and PAI assays. In all the elution profiles there seemed to be a close relationship between the anticoagulant (APTT) activity of the heparin and the azure A chemical assay with a very high proportion eluted in the first 12–26 fractions. For example, Diosynth 164 and 121 (Fig. 2a, c) had two heparin peaks and Glaxo 102 and Abbott 68 (Fig. 3a, c) have "shoulders" or extended peaks. These features were apparent for both the chemical and APTT assays. Most of the heparin added binds loosely to the factor X and can be eluted with low concentrations of sodium cloride.

The prothrombin activation inhibition assay was used in order to determine the ability of the heparin fractions to interact with specific clotting factors (FXa, prothrombin, thrombin and fibrinogen) in the absence of AT III (heparin cofactor). This test required the presence of phospholipid and calcium to produce a clearly visible clot and reproducibility of less than 2 s was obtained. The heparin samples varied in their interaction with prothrombin but no clear pattern emerged. Indeed for some heparins, the fractions eluted into two or more ranges. However, it is apparent that the fractions requiring a higher concentration of sodium chloride for elution, possessed a higher affinity for factor X than those which were eluted earlier. It was on this basis that the heparins were pooled into those with a high affinity for factor X (HA-FX) and those with low affinity for factor X (LA-FX).

After this pooling of fractions, the anticoagulant activities were measured (Table 2). With the B.P. assay, only a slight decrease in anticoagulant activity of the HA-FX fractions occurred. With the APTT assay, a more pronounced decrease in activity was obtained for the HA-FX samples. In contrast to the clotting assays, antifactor Xa activity generally increases with affinity for factor X (with the exception of Abbott 157).

The anticoagulant activities have also been presented as test ratios (Table 2) in order to clearly determine the trends as a result of factor X frac-

TABLE 2

Properties of heparin fractionated on a factor X affinity column

Heparin type	Biological tests (U/mg)			Test ratio Anti-FXa/APTT
	B.P.	APTT	Anti-FXa	
Diosynth 164 LA-FX	167	194	131	0.68
Diosynth 164 HA-FX	166	174	145	0.84
Diosynth 178 LA-FX	185	222	109	0.49
Diosynth 178 HA-FX	181	116	169	1.49
Diosynth 121 LA-FX	129	169	115	0.68
Diosynth 121 HA-FX	112	54	122	2.25
Glaxo 102 LA-FX	101	112	102	0.91
Glaxo 102 HA-FX	105	102	110	1.07
Abbott 68 LA-FX	51	63	59	0.95
Abbott 68 HA-FX	59	58	54	0.94
Abbott 157 LA-FX	156	385	283	0.73
Abbott 157 HA-FX	151	129	133	1.03

Anticoagulant activity (B.P., APTT and antifactor Xa) of heparin fractions with low and high affinities for bovine factor X.

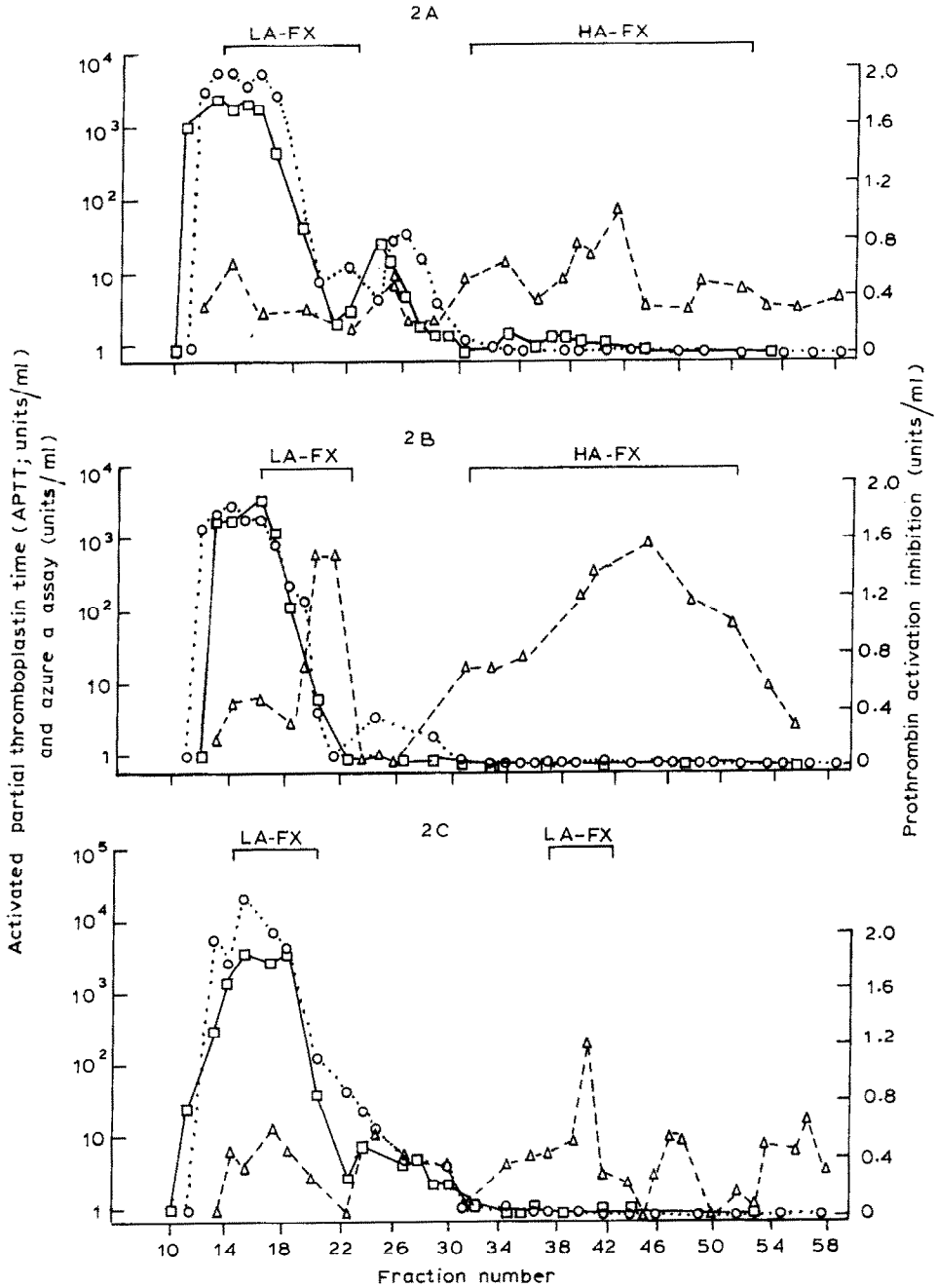

Fig. 2. Chromatography of heparin on factor X-Biogel A 15. a: Diosynth 164 U/mg. b: Diosynth 178 U/mg. c: Diosynth 121 U/mg Fractions were assayed for heparin by the azure A method (□———□). Anticoagulant activity was measured by the APTT (○ · · · · · ○) and prothrombin activation inhibition assays (△- - - - -△).

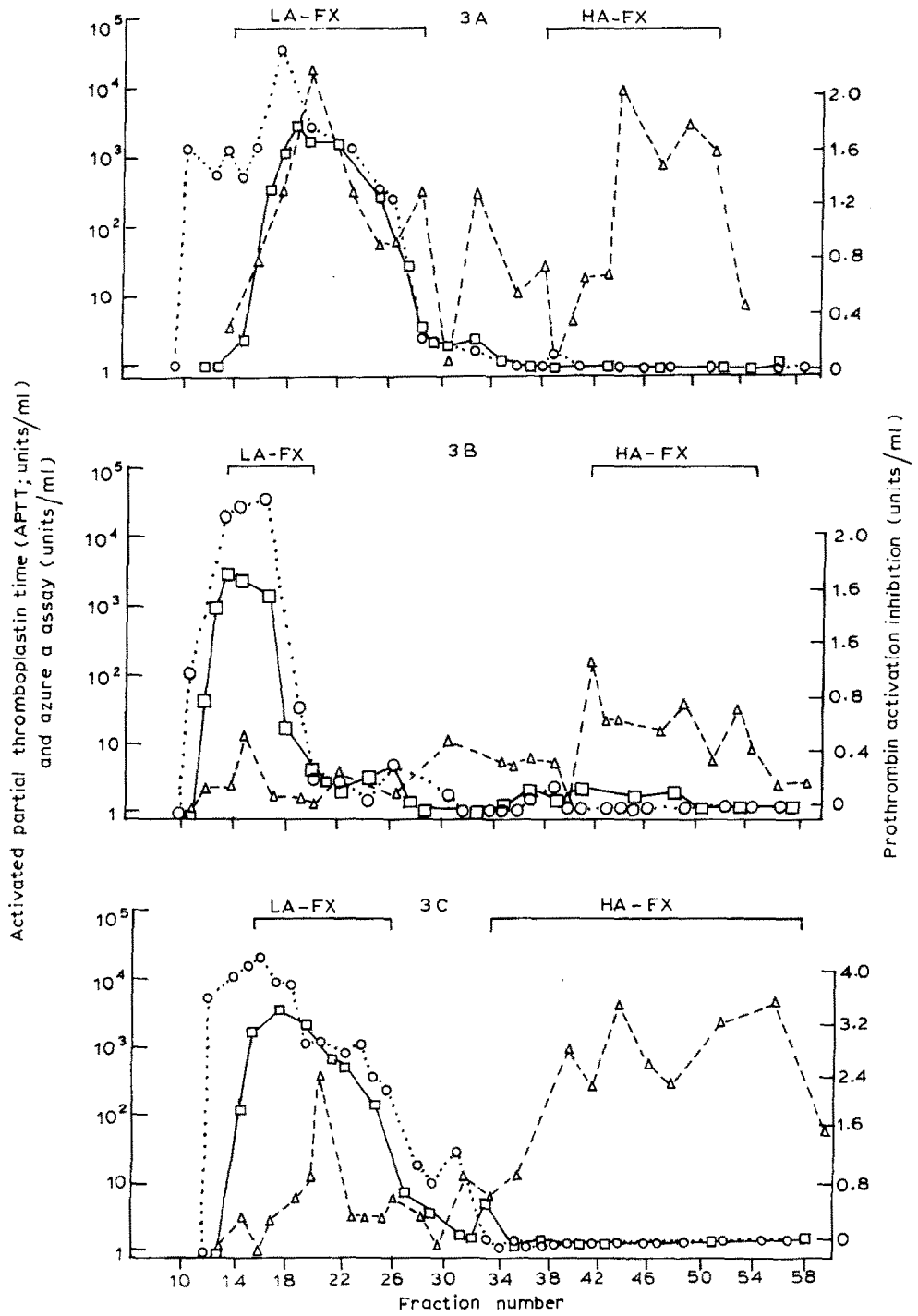

Fig. 3. Chromatography of heparin on factor X-Biogel A 15. a: Glaxo 102 U/mg. b: Abbott 157 U/mg. c: Abbott 68 U/mg. Fractions were assayed for heparin by the azure A method (□———□). Anticoagulant activity was measured by the APTT (○ · · · · · ○) and prothrombin activation inhibition assays (△- - - - - -△).

tionation. The anti-FXa/APTT ratio generally increases for the heparin fractions with affinity for factor X (with the exception of Abbott 68). It was noted that the HA-FX values for the molecular weight fractions (Diosynth 178 and 121) increased threefold when compared to the LA-FX values. These differences were considerably higher than the corresponding ratios in the other 4 heparins examined.

Discussion

One of the aims of this study was to investigate the biological properties of unfractionated and fractionated heparin in terms of factor X binding. Numerous investigations using low-molecular-weight heparin have shown that such preparations possess a high antifactor Xa/APTT activity (Barrowcliffe et al., 1979; Thomas et al., 1981; Thomas and Merton, 1982; Bergquist et al., 1983; Aiach et al., 1983). In a previous study by this group (Morton et al., 1984), the same trend was reported using the B.P. and antifactor Xa assays. Low-molecular-weight heparin, therefore, has been shown to be an effective antithrombotic (anti-factor Xa) agent with potential clinical use (Kakkar et al., 1982; Holm et al., 1986), although some groups have reported bleeding complications (Schmitz-Huebner et al., 1984).

It was shown using the HPLC method that the heparins differed from one another in terms of number-average (M_n), weight-average (M_w), Z-average (M_z) molecular weights and polydispersity (Table 1). This method overcomes the disadvantages of other techniques used to determine the molecular weight profiles of heparin as highly purified heparin oligosaccharides are used as calibration standards for HPLC.

Although heparin binds to factor X (Walker and Esmon, 1979a), another important effect on coagulation occurs by its interaction with factor Xa. It is well established that heparin binds to factor Xa (Yin et al., 1974), which is the primary serine protease of the prothrombin activation system. However, the major interaction of heparin in blood is with AT III (heparin cofactor), and it is known that less than half the molecules in a commercial preparation bind to AT III with high affinity (Andersson et al., 1976; Hook et al., 1976; Gitel et al., 1973). Heparin-AT III interactions have been the subject of many investigations. In contrast, the binding of heparin to factor X has received less attention and its contribution to the overall antithrombotic action of heparin remains to be fully established.

This study shows that different heparins bind to factor X and can be eluted on an affinity basis by increasing the ionic strength of the mobile phase. However, not all heparins bind to factor X with equal strength and as a consequence the biological activity of the fractions varies considerably. A common feature of all the heparins, however, is the similar elution pattern of the chemical (azure A) and anticoagulant activities. The fact that a low potency heparin (Abbott 68) also shows this trend indicates that the dermatan sulphate and heparan sulphate components which are possibly present, do not bind to factor X.

The prothrombin activation inhibition assay data shows further differences among the heparin preparations. It is noted that the heparins eluted at the latter end of the sodium chloride gradient, possess both an affinity for factor X and an ability to inhibit prothrombin activation. In contrast the fractions which bind less strongly to factor X, although having a comparable effect on the inhibition of prothrombin activation are better anticoagulants of plasma.

The PAI assay is performed in a plasma-free or AT III-free environment. Hence the assay measures the ability of heparin to prevent prothrombin activation in the absence of cofactor. It is well established that both factor Xa and prothrombin are linked to the phospholipid surface via calcium strands (Walker and Esmon, 1979b). The heparin could act by preventing the binding of factor Xa and/or prothrombin to the phospholipid surface. This mechanism has been supported by studies showing that heparin can reverse the lipid protection of factor Xa (Owen et al., 1974) by the formation of a [heparin factor Xa] complex.

It is known that factor X, factor IXa and prothrombin do not compete with factor Xa for the factor Xa binding sites on human platelet phospholipid (Walker and Esmon, 1979b). In this

study, although LA-FX and HA-FX heparins bind to factor X in different degrees the inhibition of prothrombin activation is similar since the factor Xa sites of both fractions are presumably free for factor Xa binding. This result per se shows that factor X binds to a different section of the heparin molecule.

When the antifactor Xa activity is measured, it is found that this activity generally increases with affinity for factor X (Table 2). In the plasma system, this assay measures the ability of heparins to enhance the capacity of AT III to inactivate factor Xa. Since the HA-FX fractions are most potent in this respect, it seems that the factor X and AT III binding regions of the heparin molecule are associated with one another and that fractionation on the basis of an affinity for factor X could be related to AT III fractionation.

When the antifactor Xa activity is expressed as a ratio of the APTT value (Table 2), the ratio is highest in the HA-FX fractions. This effect has been reported extensively for low-molecular-weight heparins (Barrowcliffe et al., 1979; Thomas et al., 1981; Thomas and Merton, 1982; Begquist et al., 1983). The present study shows that this property also occurs with commercial and both low- and high-molecular-weight fractions of heparin when fractionated on a factor X affinity column. It appears that heparins which bind to factor X are effective inhibitors of factor Xa whilst being poor anticoagulants of plasma. This activity is most pronounced in the lower and high molecular weight Diosynth samples. A possible explanation for this result could be that when heparins are fractionated into distinct molecular weight fractions, the molecules responsible for factor X binding are isolated and retain the ability to bind AT III and thereby serve to inactivate factor Xa.

These in vitro results suggest that HA-FX Diosynth 178 and Diosynth 121 could have a clinical potential as antithrombotic agents. However this suggestion needs to be confirmed by in vivo experiments. The results currently show that antifactor Xa activity is not confined exclusively to low-molecular-weight heparin.

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