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Binding of platelet factor 4 to heparin oligosaccharides

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Heparin fractions of differing M_r (7800–18 800) prepared from commercial heparin by gel filtration and affinity chromatography on immobilized anti-thrombin III had specific activities when determined by anti-Factor Xa and anti-thrombin assays that ranged from 228 to 448 units/mg. The anti-Factor Xa activity of these fractions could be readily and totally neutralized by increasing concentrations of platelet factor 4 (PF_4). That these fractions bound to immobilized PF_4 was indicated by the complete binding under near physiological conditions of ^3H-labelled unfractionated commercial heparin. An anti-thrombin III-binding oligosaccharide preparation (containing predominantly eight to ten saccharide units), prepared by degradation of heparin with HNO_2 had high (800 units/mg) anti-Factor Xa, but negligible anti-thrombin, specific activity. The anti-Factor Xa activity of this material could not be readily neutralized by PF_4, and the ^3H-labelled oligosaccharides did not completely bind to immobilized PF_4. A heterogeneous anti-thrombin III-binding preparation containing upwards of 16 saccharides had anti-thrombin specific activity of just less than one-half the anti-Factor Xa specific activity. This material was completely bound to immobilized PF_4 and was eluted with similar concentrations of NaCl to those that were required to elute unfractionated heparins from these columns. Furthermore, increasing concentrations of PF_4 neutralized the anti-Factor Xa activity of this material in a manner similar to that of unfractionated heparin. It is concluded that heparin oligosaccharides require saccharide units in addition to the anti-thrombin III-binding sequence in order to fully interact with PF_4.

Heparin acts as an anti-coagulant by tightly binding to the circulating proteinase inhibitor anti-thrombin III and enhancing its neutralization of the serine proteinases of the blood-coagulation cascade (Rosenberg & Damus, 1973). It has been demonstrated that heparin with high affinity for anti-thrombin III (HA heparin) had at least ten times the anti-coagulant potency of heparin that binds with low affinity (LA heparin) to the inhibitor (Hook *et al.*, 1976; Lam *et al.*, 1976; Andersson *et al.*, 1976). A specific pentasaccharide sequence present in about one-third of commercial heparin molecules is required for anti-thrombin III binding (Thunberg *et al.*, 1980, 1982; Lindahl *et al.*, 1980).

The anti-coagulant activity of heparin can be determined by broad-spectrum non-specific assays or by assay methods that reflect the ability of heparin to accelerate the inhibitory action of anti-thrombin III against individual coagulation proteinases. With unfractionated or gel-filtered heparins of different M_r measurable differences in specific activities may be observed when assays employing different proteinases are used. Thus, the ratio of anti-Factor Xa to anti-thrombin activity increases with decreasing M_r of the heparin (Andersson *et al.*, 1976; Lane *et al.*, 1978; MacGregor *et al.*, 1979). This difference in activity is accentuated in assay systems containing blood plasma, due to the preferential binding of high-M_r heparin (including the reference standard) to plasma proteins other than anti-thrombin III (MacGregor *et al.*, 1979, 1980). Nevertheless, with systems based on purified components, very-low-M_r oligosaccharides with high affinity for anti-thrombin III have high anti-Factor Xa activity but negligible anti-thrombin activity and this is probably caused by the structural

Abbreviation used: PF_4, platelet factor 4.

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requirements of the heparin-inhibitor–proteinase interactions (Thunberg *et al.*, 1979; Jordan *et al.*, 1980; Holmer *et al.*, 1981). The most potent inhibitor of heparin's anti-coagulant activity found in blood is the platelet α-granule protein, PF_4. It is a secretable low-molecular-weight (7800) protein that has a high affinity for heparin (Handin & Cohen, 1976). It has been shown that both HA and LA heparin bind to PF_4 with equal affinity (Niewiarowski *et al.*, 1979) and that heparin can simultaneously bind to both PF_4 and antithrombin III (Piepkorn, 1981).

The purpose of the present work was to study the interaction between PF_4 and heparins of different molecular sizes, in particular the effect on the anti-Factor Xa activity of small oligosaccharides with high affinity for anti-thrombin III.

Materials and methods

Heparin was supplied by Leo Laboratories, Copenhagen, Denmark (lot no. A63A 180065).

Heparin fractions of different molecular weights were prepared by gel filtration on a column (2.6 cm × 95 cm) of Ultrogel AcA44 ($V_0 = 170$ ml, $V_t = 450$ ml in 0.5 M-NaCl, pH 7.0). Fractions were assayed for heparin as uronic acid by the orcinol reaction (Brown, 1946). Pooled fractions were recovered after dialysis against 10 litres of water and freeze-drying. HA heparin was prepared by affinity chromatography on anti-thrombin III bound to a column of concanavalin A–Sepharose as described by Denton *et al.* (1981).

Oligosaccharides with high affinity (HA oligosaccharides) for anti-thrombin III were prepared by affinity chromatography on immobilized anti-thrombin III after partial random depolymerization of heparin with HNO_2 (Lindahl *et al.*, 1979; Thunberg *et al.*, 1982). Preparations of lower (D1) and higher (D2) average M_r were isolated respectively from more or less extensively degraded polysaccharide.

Radioactive heparins were prepared by reduction with NaB^3H_4 (Amersham International, Amersham, Bucks., U.K.) in 0.1 M-$NaHCO_3$ buffer, pH 8.0. Free isotope was removed by dialysis or by desalting on a column (2.4 cm × 30 cm) of Sephadex G-10. Oligosaccharides with high affinity for anti-thrombin III were rechromatographed on immobilized anti-thrombin III after labelling, and only materials that bound to the column in a salt concentration of greater than 0.9 M-NaCl were recovered for further study.

Molecular weights of gel-filtered and unfractionated (undergraded) heparins were determined by analytical ultracentrifugation using an M.S.E. Centriscan 75 analytical ultracentrifuge. A four-place rotor fitted with 20 mm single-sector cell housings was employed. Heparin solutions (2–5 mg/ml) in 0.5 M-NaCl, pH 7.0, were dialysed overnight against this buffer to achieve Donnan equilibrium. Molecular weights were determined by using the low-speed sedimentation equilibrium method. A value of 0.45 for the partial specific volume of heparin was taken from Laurent (1961).

The chain lengths of heparin oligosaccharides were determined by Sephadex G-50 chromatography (Thunberg *et al.*, 1980).

Heparin potencies were determined with respect to the 3rd International Heparin standard. Inhibition of human α-thrombin-mediated hydrolysis of the chromogenic substrate S2238, or Factor Xa-mediated hydrolysis of the chromogenic substrate S2222, were measured by an end-point method (Teien *et al.*, 1977; Larsen *et al.*, 1978) using either purified anti-thrombin III or plasma as a source of enzyme inhibitor. Briefly, assay mixtures (0.5 ml) contained 1.6 μg of enzyme, 25 μg of purified anti-thrombin III (or 200 μl of normal human citrated plasma) and 0–1.0 μg of heparin. These were incubated in 0.15 M-NaCl/0.01 M-EDTA/0.05 M-Tris/HCl buffer, pH 8.4, for 60 s before addition of 200 μl of 0.75 mM-S2238 (KabiVitrium) (or 1.0 mM-S2222 for the anti-Factor Xa assay). Hydrolysis was allowed to proceed for 60 s (S2238) or 240 s (S2222) before the reaction was terminated with acetic acid. The extent of substrate hydrolysis was measured by absorbance at 405 nm.

Purified human anti-thrombin III was prepared by a modification (Denton *et al.*, 1981) of the method described by Miller-Andersson *et al.* (1974). Purified human α-thrombin (sp. activity 2500 units/mg) was a gift from Dr. M. Griffiths, Department of Pathology, University of North Carolina at Chapel Hill, NC, U.S.A. Bovine Factor Xa was obtained from Diagnostic Reagents, Thame, Oxon, U.K.

Purified human PF_4 isolated as described by Moore *et al.* (1975) was a gift from Dr. D. Pepper, S.E. Scotland Blood Transfusion Service, Edinburgh, Scotland, U.K. Matrix-bound PF_4 was prepared by coupling 5 mg of purified protein to 10 ml of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) in 0.3 M-NaCl/0.1 M-$NaHCO_3$ buffer, pH 8.0, for 4 h at room temperature.

The neutralization of heparin with purified PF_4 was examined by adding increasing concentrations (0–100 μg/ml) of PF_4 into tubes containing 1.0 μg of heparin in 1.0 ml 0.15 M-NaCl/0.05 M-Tris/HCl buffer, pH 8.4. The samples were pre-incubated for at least 2 min and then processed to determine residual anti-Factor Xa activity, as described above. A solution of 1.0 μg of heparin/ml without added PF_4 was used as a 0% neutralization standard and a solution of 10 μg of PF_4/ml containing no heparin was used to calculate 100% neutralization.

Affinity chromatography of heparin on matrix-bound PF_4 was carried out at 4°C. The column

(1.5cm × 7.0cm) was pre-equilibrated with 0.15M-NaCl/0.10M-Tris/HCl buffer, pH 7.4. Then 1ml of a solution of radioactive heparin in this buffer (concentration less than 1mg/ml) was loaded on to the column and washed through with approx. 8 column vol. of buffer. Elution of the column was carried out with either 2.0M-NaCl in the Tris/HCl buffer, pH 7.4, or a gradient of 0.15M-NaCl to 2.0M-NaCl in the Tris/HCl buffer, pH 7.4, was used. Buffers used for gradient elution contained trace amounts of $Na^{36}Cl$ (Amersham International) to facilitate gradient estimation. Alternatively, the gradient was determined by measuring the conductivity of the eluted samples. Fractions collected were assayed for radioactivity by liquid-scintillation counting using an Inter-technique ABAC SL 40 liquid-scintillation counter. Samples were diluted to 2ml and mixed with 3ml of scintillation cocktail (Packard Instagel) before counting. Alternatively, heparin concentrations were measured with the dye-binding method of Jaques *et al.* (1949).

Results

The molecular weight of HNO_2-depolymerized HA oligosaccharides were determined by gel chromatography on a Sephadex G-50 column. Elution profiles of the two preparations are shown in Fig. 1, together with the elution positions of reference oligosaccharides. The preparation of lowest M_r (HA D1) consisted of chains of mainly 8 and 10 saccharide units. The oligosaccharides of the higher-M_r preparation HA D2 were more heterogeneous and the upper limit of the chain size was difficult to estimate on this chromatographic column. However, it is reasonable to conclude that the chain size was predominantly upwards of 16 saccharide units.

The anti-coagulant potencies of these two preparations and the gel-filtered heparins are listed in Table 1. Gel-filtered heparins of mean M_r ranging from 7800 to 18800 had an appreciable range of

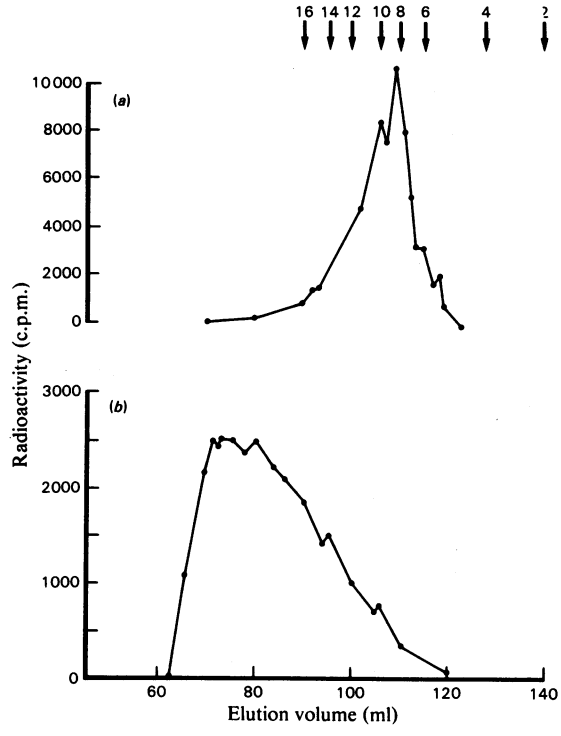

Fig. 1. *Gel filtration of (a) HA D1 oligosaccharides and (b) HA D2 oligosaccharides on a Sephadex G-50 column (1.0cm × 196cm) equilibrated with 1M-NaCl*
The elution positions of reference saccharides are also indicated.

Table 1. *Molecular size, anti-Factor Xa and anti-thrombin activities of three heparin fractions prepared by gel filtration (fractions 1, 3 and 6) and two fragments isolated after HNO_2 depolymerization (D1 and D2)*
Specific activities were determined by chromogenic substrate assays for Factor Xa and thrombin before (unfractionated) and after (high affinity) chromatography on immobilized anti-thrombin III. The relatively low specific activities of the unfractionated 18800-M_r material (fraction 1) may reflect the occurrence of a polysaccharide contaminant (presumably dermatan sulphate). Specific activities are expressed as means ± S.E.M. ($n = 5$), except for those of the D1 preparation, where very low activities were obtained and a mean could not be accurately computed. Abbreviation: nd, not determined.

		Specific activity (units/mg)			
		Unfractionated		High affinity	
Fraction	Molecular size	Factor Xa	Thrombin	Factor Xa	Thrombin
1	18800 Daltons	31 ± 1.3	30 ± 3.2	379 ± 9.8	288 ± 18.9
3	12400 Daltons	172 ± 3.8	168 ± 4.2	420 ± 11.9	448 ± 17.5
6	7800 Daltons	52 ± 0.86	89 ± 6.2	448 ± 9.8	368 ± 9.6
D1	8–10 saccharide units	<1	nd	800 ± 5.6	<10
D2	Upwards of 16 saccharide units	58 ± 5.0	19 ± 3.6	244 ± 11.5	94 ± 11.8

specific activities (30–172 units/mg) when determined by the anti-Factor Xa and thrombin chromogenic substrate methods. When these fractions were further purified by affinity chromatography on immobilized anti-thrombin III, the specific activities increased, as might be expected. The HNO_2 degradation fragments had very low activities before purification on anti-thrombin III. Material that was eluted with greater than 0.9 M-NaCl from immobilized anti-thrombin III had comparable or greater specific activity than the gel-filtered HA heparins when determined by anti-Factor Xa assay, but greatly reduced specific activity determined by anti-thrombin assay. The HA D1 preparation had no detectable anti-thrombin and very high anti-Factor Xa (800 units/mg) activity, whereas the anti-thrombin activity (94 units/mg) of the larger HA D2 oligosaccharide material was less than half its anti-Factor Xa activity (244 units/mg). The specific activities of the HA D1 preparation are comparable with those reported by other investigators (see the Discussion section), who have used human rather than bovine Factor Xa. This suggests no significant species specificity in the heparin-accelerated inhibition of Factor Xa by anti-thrombin III when oligosaccharides are compared with standard heparins.

The anti-Factor Xa activity of heparin fractions of high, intermediate and low M_r was neutralized by the addition of PF_4, with approx. 10 μg of purified PF_4 totally neutralizing 1 μg of heparin. Similar results were obtained before and after isolation of HA gel-filtered heparins by affinity chromatography on immobilized anti-thrombin III. The sigmoid neutralization curves are shown in Fig. 2, which also shows that 50% of heparin activity was neutralized by approx. 3 μg of PF_4.

Neutralization of the HA D1 oligosaccharides had to be examined at a heparin concentration of 10% (by weight) of that of non-deploymerized heparin because of the very high anti-Factor Xa potency of this material. It was found that only 50%

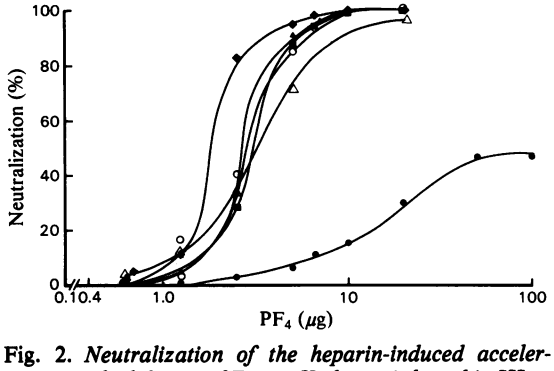

Fig. 2. *Neutralization of the heparin-induced acceleration of inhibition of Factor Xa by anti-thrombin III*
To incubation mixtures of anti-thrombin III, Factor Xa and substrate S2222 (see the Materials and methods section) were added heparin and increasing amounts of PF_4. A concentration of 1 μg of heparin/ml was studied, except for the experiments with the HA D1 oligosaccharide, where 100 ng/ml was used. ■, Unfractionated (on anti-thrombin III) fraction 3; ♦, HA fraction 1; O, HA fraction 3; ▲, HA fraction 6; △, HA D2 oligosaccharides; ●, HA D1 oligosaccharides.

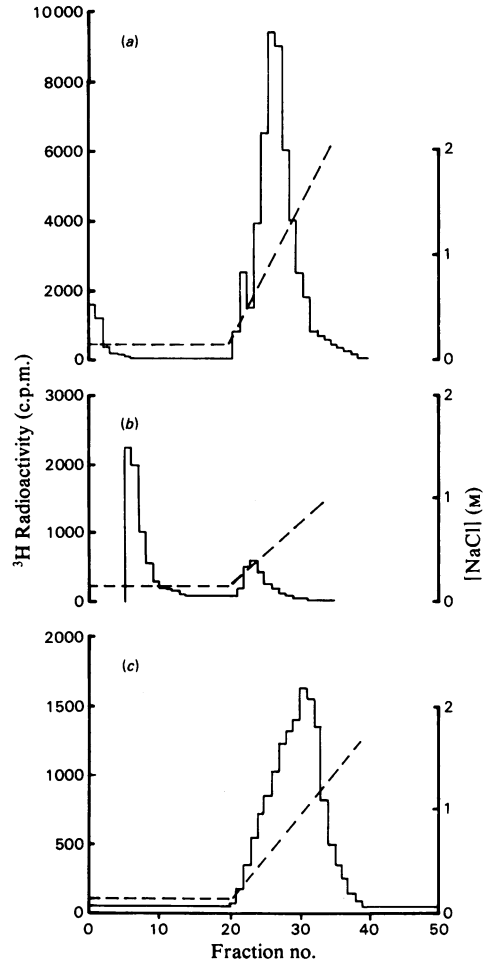

Fig. 3. *Affinity chromatography of ^3H-labelled heparins on immobilized PF_4*
The heparins were applied to the column in 0.1 M-Tris/HCl buffer, pH 7.4, that contained 0.15 M-NaCl. The fraction volume was 2 ml. Elution was achieved with a gradient of NaCl as indicated by the broken line. (*a*) Unfractionated commercial heparin; (*b*) HA D1 oligosaccharides; (*c*) HA D2 oligosaccharides.

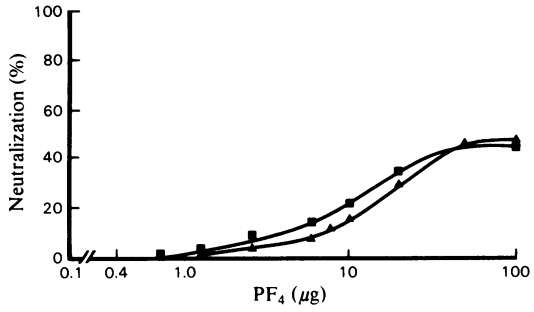

Fig. 4. *Neutralization of the HA D1 oligosaccharides before (■) and after (▲) chromatography on immobilized PF_4*

The peak of ^3H isolated from fractions 20–30 in Fig. 3(*b*) was examined in parallel with the parent HA D1 oligosaccharides in the anti-Factor Xa inhibition assay.

of the activity of these fragments could be neutralized by PF_4 and that this neutralization required a PF_4 to heparin ratio of about 500:1. However, neutralization of the larger HA D2 oligosaccharides proceeded almost identically with the neutralization of the non-depolymerized heparin fractions, with 50% neutralization occurring at a PF_4 to heparin ratio of 3:1.

Results of affinity chromatography experiments on immobilized PF_4 are shown in Fig. 3. Unfractionated heparin was almost completely retained by the column and was eluted by an increasing-ionic-strength gradient with peak elution at 0.9 M-NaCl (Fig. 3*a*). Of the HA D1 oligosaccharide 60% emerged as an initial peak and the remaining 40% of material eluted as a broad peak with a maximum at the salt concentration of 0.4 M-NaCl (Fig. 3*b*). The retarded component was neutralized with increasing concentrations of PF_4 in a manner similar to that of the parent fraction (Fig. 4). The difference in chemical composition between these two oligosaccharide peaks derived from the HA D1 oligosaccharides were not identified but were probably subtle, resulting in a small difference in charge.

On affinity chromatography on immobilized PF_4 the larger HA D2 oligosaccharide material was completely bound to the gel in the application buffer (Fig. 3*c*). Its elution with increasing salt concentration exhibited a single peak at 0.8–0.9 M-NaCl.

Discussion

Heparin inhibits the proteinases of the blood-coagulation system by binding to and potentiating the action of the naturally occurring inhibitor anti-thrombin III (Rosenberg & Damus, 1973). Additionally, proteinases such as thrombin may bind with high affinity to heparin and various authors have suggested that this interaction is essential for proteinase inhibition (Machovich *et al.*, 1975; Smith, 1977; Laurent *et al.*, 1978; Griffith, 1979; Holmer *et al.*, 1979; Oosta *et al.*, 1981). Oligosaccharides of very low M_r with high affinity for anti-thrombin III may be prepared by chemical or enzymic degradation followed by affinity chromatography on anti-thrombin III. Such material may have very different inhibitory activities towards different proteinases. The results of specific-activity determinations on the HA D1 preparation indicate that oligosaccharides containing essentially the anti-thrombin III-binding region (Thunberg *et al.*, 1982) have very high anti-Factor Xa activity, and yet minimal anti-thrombin activity. This confirms results of previous investigations of oligosaccharide material that was similarly prepared by chemical and/or enzymic degradation with HNO_2 (Thunberg *et al.*, 1979; Holmer *et al.*, 1980, 1981; Casu *et al.*, 1981; Oosta *et al.*, 1981).

PF_4 is a tetrameric protein contained in platelet α-granules and is extruded during the platelet release reaction as a complex with a chondroitin sulphate carrier (Barber *et al.*, 1972). Heparin can displace the carrier and bind with high affinity to PF_4 (Handin & Cohen, 1976; Luscombe *et al.*, 1981). Addition of purified PF_4 to solutions containing heparin, anti-thrombin III and proteinases neutralizes the anti-coagulant action of heparin. The mechanism of neutralization is uncertain but a specific interaction with the anti-thrombin III-binding pentasaccharide is unlikely as PF_4 binds to both HA and LA heparins (Niewiarowski *et al.*, 1979).

The interaction of PF_4 and heparin fractions of differing M_r has been the subject of a recent study. Bock *et al.* (1980) have demonstrated that heparin fractions prepared by gel filtration can form a number of distinct complexes with PF_4. Heparins of M_r in excess of 9000 are capable of simultaneously binding to two PF_4 tetramers, whereas each PF_4 tetramer can bind two or more heparins of M_r less than 9000. The present study has shown that PF_4 neutralizes heparin (mean M_r 7800–18800) on a weight basis with 10 μg of PF_4 completely neutralizing 1 μg of heparin. The stoichiometry of heparin–PF_4 complexes and their role in the neutralization of the anti-coagulant activity of heparin is uncertain. However, heterogeneous commercial heparin was shown here to bind completely to immobilized PF_4 and this indicates that the structural requirements for binding and neutralization were present on the non-depolymerized heparins used in the present study. That this is not the case for oligosaccharides containing 8–10 sugar units, i.e. little more than the anti-thrombin III-binding sequence, was demon-

strated by the weak binding of the HA D1 preparation to immobilized PF_4 and the inability of the protein to completely neutralize the anti-Factor Xa activity of this material. Because the somewhat larger fragments of the more heterogeneous HA D2 oligosaccharides behaved in a similar manner to unfractionated heparin with respect to PF_4 binding and neutralization it seems that oligosaccharides only slightly larger than those of the D1 preparation, containing upwards of about 16 sugar units per molecule, contain the necessary structural features for an efficient interaction.

In summary, an octasaccharide sequence of the heparin molecule, including the anti-thrombin III-binding region, is insufficient to support efficient PF_4 interaction with the polysaccharide. The heterogeneity of our D2 anti-thrombin III-binding preparation precludes a definition of exactly which additional sequences are required, but it does seem that an additional sequence of eight to ten saccharides may be sufficient for the neutralizing agent to bind with high affinity. It is significant that the HA D2 preparation shows appreciable anti-thrombin activity, whereas the HA D1 preparation does not. Affinity chromatography experiments have been performed (results not shown) in which the ^3H-labelled heparin and oligosaccharides were applied to and eluted from an immobilized thrombin column. These experiments demonstrated that the HA D1 preparation barely bound to the immobilized thrombin but that the HA D2 preparation bound completely and could be eluted with a comparable NaCl concentrated used to elute the unfractionated commercial heparin. The interesting possibility therefore arises from this work that both thrombin and PF_4 may interact with essentially similar domains on the heparin molecule. This possibility can best be studied by using oligosaccharides with well defined and restricted chain length. Results of such a study may provide insight into the mechanism of PF_4 neutralization of heparin.

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