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The Molecular-Weight-Dependence of the Anti-Coagulant Activity of Heparin*

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It is proposed that the anti-coagulant activity of heparin is related to the probability of finding, in a random distribution of different disaccharides, a dodecasaccharide with the sequence required for binding to antithrombin. It is shown that this probability is a function of the degree of polymerization of heparin. The hypothesis has been tested with a series of narrow-molecular-weight-range fractions ranging from 5600 to 36000. The fractions having mol.wts. below 18000 (comprising 85% of the original preparation) followed the predicted probability relationship as expressed by the proportion of molecules capable of binding to antithrombin. The probability that any randomly chosen dodecasaccharide sequence in heparin should bind to antithrombin was calculated to 0.022. The fraction with mol.wt. 36000 contained proteoglycan link-region fragments, which may explain the deviation of the high-molecular-weight fractions from the hypothetical relationship. The relationship between anti-coagulant activity and molecular weight cannot be explained solely on the basis of availability of binding sites for antithrombin. The activity of high-affinity heparin (i.e. molecules containing high-affinity binding sites for antithrombin), determined either by a whole-blood clotting procedure or by thrombin inactivation in the presence of antithrombin, thus remained dependent on molecular weight. Possible explanations of this finding are discussed. One explanation could be a requirement for binding of thrombin to the heparin chain adjacent to antithrombin.

When polydisperse heparin preparations have been fractionated according to molecular weight it has been found that the anti-coagulant activity of the fractions increases with increasing degree of polymerization of the heparin (Laurent, 1961; Lasker & Stivala, 1966; Liberti & Stivala, 1967; Stivala & Liberti, 1967; Cifonelli, 1974; McDuffie *et al.*, 1975; Andersson *et al.*, 1976; Riesenfeld *et al.*, 1977). This phenomenon may now be partly explained.

The biosynthesis of heparin is initiated by polymerization of alternating *N*-acetyl-D-glucosamine and D-glucuronic acid residues. Various modifications are introduced at the polymer level (Lindahl *et al.*, 1977). Most *N*-acetyl groups are replaced by *N*-sulphate groups. Some D-glucuronic acid residues are epimerized to L-iduronic acid residues. Sulphate is also inserted at the C-6 and C-2 positions of some of the glucosamine and iduronic acid units respectively. The concerted modification reactions thus yield a polymer, heparin, composed of a number of different disaccharide units.

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Abbreviations used: HA-heparin, high-affinity heparin; LA-heparin, low-affinity heparin.

Antithrombin III (also called heparin cofactor) is a protein (Abildgaard, 1968), which binds to thrombin in a stable stoicheiometric complex, thereby inhibiting the enzymic activity of the latter (Rosenberg & Damus, 1973). Heparin acts as an anti-coagulant by catalysing this complex formation (Abildgaard, 1968; Björk & Nordenman, 1976). It has been demonstrated that heparin has affinity both for thrombin (Li *et al.*, 1974; Machovich, 1975; Nordenman & Björk, 1978*a*) and for antithrombin (Rosenberg & Damus, 1973). The antithrombin–heparin system also inactivates several other factors in the coagulation cascade such as IX_a, X_a, XI_a and XII_a (Biggs *et al.*, 1970; Damus *et al.*, 1973; Rosenberg *et al.*, 1975; Stead *et al.*, 1976).

Recent reports (Lam *et al.*, 1976; Höök *et al.*, 1976; Andersson *et al.*, 1976) have shown that only a fraction of all molecules in heparin preparations have high affinity for antithrombin III. These HA-heparin molecules account for most of the anti-coagulant activity of the preparations. Hopwood *et al.* (1976) digested a HA-heparin–antithrombin complex with bacterial heparinase and were then able to isolate oligosaccharides that could be released from the protein by increasing ionic strength. The

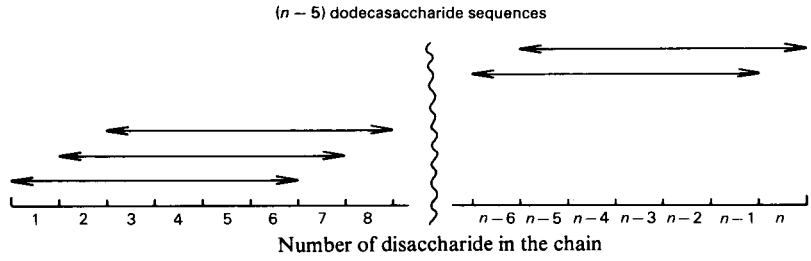

Fig. 1. *Illustration of the number of dodecasaccharide sequences that can be found in a chain containing n disaccharides*

oligosaccharides had a mol.wt. of about 4000 [i.e. approximately corresponding to that of a dodecasaccharide (six disaccharides)] and retained a high affinity for antithrombin; they obviously contained the sequence of disaccharides required for binding of heparin to antithrombin. This sequence is not yet known.

The results of Hopwood *et al.* (1976) imply that antithrombin binds to a specific site on the heparin molecule and that the anti-coagulant activity of heparin should be related to the probability of finding this site in the molecules of the preparation. We will assume that the binding site for antithrombin in heparin is a dodecasaccharide. The number of dodecasaccharide sequences in a heparin molecule containing n disaccharides is $n-5$ (see Fig. 1). The total number of dodecasaccharide sequences per unit weight of heparin is thus proportional to $(n-5)/n$. Assuming that the probability of binding to antithrombin is the same everywhere along the heparin chain it follows that also the anti-coagulant activity expressed per unit weight should be a function of $(n-5)/n$ and thus of the molecular weight of heparin. The relationship could be more complex if reactions in the anti-coagulation mechanism, other than the binding of antithrombin to heparin, also are dependent on the molecular weight of the polysaccharide. The aim of the present investigation has been to test whether the probability of antithrombin binding to heparin is proportional to $(n-5)/n$ and if so, whether this probability is reflected in the anti-coagulant activity of heparin.

Materials and Methods

Materials

A commercial sample of heparin from pig intestinal mucosa was kindly given by AB Vitrum (Stockholm, Sweden) (batch 6514). It has an anti-coagulant activity of 137.5 units/mg dry weight.

A separate batch of pig mucosal heparin and a radioactively labelled heparin from mouse mastocytoma (Bengtsson *et al.*, 1977) was used in auxiliary experiments. The mucosal heparin (stage 14) was purchased from Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A., and purified by repeated precipitation with cetylpyridinium chloride from 1.2M-NaCl as described (Lindahl *et al.*, 1965).

Bovine antithrombin was a gift from Dr. Ingemar Björk, Uppsala, Sweden, and human thrombin was a gift from AB Kabi, Stockholm, Sweden.

Analytical methods

Uronic acid was determined by the carbazole method of Bitter & Muir (1962) with glucuronolactone as standard. Appropriate corrections for the effects of salt on the colour yield were made when uronic acid was determined in chromatograms eluted with NaCl gradients. Heparin concentrations were determined by assuming a uronic acid content of 30%. Glucosamine and galactosamine were determined by high-pressure ion-exchange chromatography (Lohmander, 1972) of the amino sugar monosaccharides on Aminex A5 (Bio-Rad Laboratories, Richmond, CA, U.S.A.); before analysis polysaccharides were hydrolysed in 8M-HCl for 3h at 100°C. Amino acid analyses were performed with a Beckman model 121-M amino acid analyser, after hydrolysis of samples *in vacuo* with 6M-HCl at 110°C for 24h. Electrophoresis of polysaccharides was carried out on cellulose acetate strips in 0.1M-HCl by the method of Wessler (1971).

Analytical gel chromatography was performed with a 2cm×100cm column of Sephadex G-100. The elution conditions were the same as in the preparative runs (see below). $K_{av.}$ was determined as described (Laurent & Killander, 1964).

Molecular weights were determined by ultracentrifugation at 20°C in a Spinco model E Analytical Ultracentrifuge equipped with RTIC-unit and electronic speed control. Interference optics and the long-column meniscus-depletion method of Chervenka (1970) were used. The runs were made in 12mm double-sector capillary synthetic boundary cells at speeds between 24000 and 44000rev./min. The

solvent was 1M-NaCl. Each fraction was analysed at several concentrations varying from 0.17 to 0.9mg/ml. Equilibrium conditions were obtained after 18h. The interference pattern was analysed in a microcomparator (Nikon model 6CT2); the fringe displacement was measured on two separate fringes in each run. Molecular weights were calculated from plots of log(fringe displacement) versus r^2 (r, distance from centre of rotation) using the least-squares method. The value for the partial specific volume of heparin was taken to be 0.47 (Lasker & Stivala, 1966) and the density of the solvent 1.0385g/cm^3. The apparent molecular weights obtained for at least three different concentrations were used to extrapolate to the value at infinite dilution.

Anti-coagulant activities were determined by the British Pharmacopoeia (1968) whole-blood assay (B.P.) or by an antithrombin-activation assay based on the inactivation of thrombin in the presence of heparin and antithrombin (Björk & Nordenman, 1976). Each assay was carried out with 10ng of heparin, 2.7μg of bovine antithrombin and 2 N.I.H. units (0.8μg) of thrombin. Thrombin activity was determined with D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride (S-2238; AB Kabi Diagnostica, Stockholm, Sweden) as substrate.

Digestion of heparin fractions with chondroitinase ABC

Galactosaminoglycan contaminants in heparin fractions (see below) were eliminated by digestion with chondroitinase ABC (Yamagata *et al.*, 1968). The digestion mixture contained per ml of 0.05M-Tris/HCl (pH8.0)/0.03M-sodium acetate, 0.1mg of bovine serum albumin, a total of 1mg of polysaccharide and 0.1 unit of enzyme (Miles Laboratories Inc., Kankakee, IL, U.S.A.). The digestion was carried out at 37°C for 15h and was then terminated by heating to 100°C for 3min. The digest was concentrated by evaporation to 4ml and chromatographed on a 2cm×73cm column of Sephadex G-100 with 0.5M-NH_4HCO_3 as a solvent. The high-molecular-weight fractions containing enzyme-resistant polysaccharide were evaporated to 5ml in a rotary evaporator; the polysaccharide was precipitated with cetylpyridinium chloride (Scott, 1960), dissolved in an equal volume of 2M-NaCl, precipitated with 3vol. of ethanol, dissolved in water and freeze-dried.

Fractionation of heparin

Gel chromatography. Heparin was fractionated on a 5cm×90cm (1850ml) column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). The void volume and the total volume of the column were determined by chromatography of dextran (mol.wt. 2×10^6) and ^3H_2O respectively. Approx. 3g of heparin, dissolved in 10ml of 0.2M-NaCl, was applied to the column; the eluting medium was 0.2M-NaCl and the temperature 4°C. The elution rate was approximately 60ml/h and 15ml fractions were collected. The fractions were analysed for uronic acid by the carbazole method (Bitter & Muir, 1962) and pooled as indicated in Fig. 2. The pooled fractions were extensively dialysed against water and freeze-dried; corresponding fractions from six separate chromatographic runs were pooled. Rechromatography of the pooled samples under identical conditions yielded sharp peaks for each fraction.

Amino sugar analysis showed that fractions 1–5 contained considerable amounts of galactosamine, presumably due to contamination with dermatan sulphate. These fractions were therefore digested with chondroitinase ABC, as described above.

Affinity chromatography on antithrombin–Sepharose. Bovine antithrombin was purified and coupled to Sepharose 4B as described by Höök *et al.* (1976), yielding a gel with a protein content of 7mg/ml. The coupled gel was packed into a 75ml column in 0.05M-Tris/HCl, pH7.4, and 0.05M-NaCl. Fraction 1 (12mg), 16mg of fraction 2 and 20mg of the other fractions were separately chromatographed on the column at 4°C. The material was applied at a concentration of 1mg/ml and followed by 200ml of the starting buffer. Elution of the column was carried out at a flow rate of 45ml/h with a linear 1000ml salt gradient (0.05–3M-NaCl in 0.05M-Tris/HCl buffer, pH7.4). Fractions (7.5ml) were collected and analysed for uronic acid. Each of the LA- and HA-heparin fractions (see below) were pooled, concentrated in a rotary evaporator, desalted on columns of Sephadex G-25 and freeze-dried. Fraction 9-HA was concentrated by adsorption to DEAE-cellulose and elution with 2M-NaCl before the chromatography on

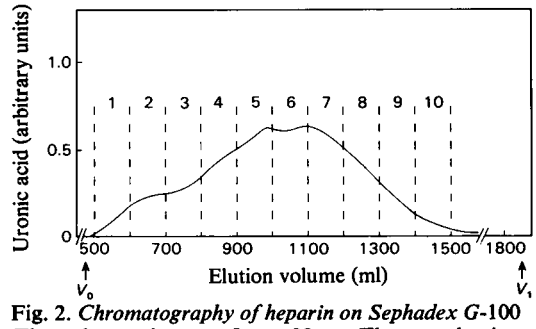

Fig. 2. *Chromatography of heparin on Sephadex G-100*
The column size was 5cm×90cm. The sample size was 3g and it was eluted with 0.2M-NaCl at a flow rate of 60ml/h. The material was fractionated in ten fractions as indicated in the Figure.

Sephadex G-25. All the preparations were then redissolved in 1ml of 0.5M-NaCl, precipitated with cetylpyridinium chloride (Scott, 1960), dissolved in 1ml of 2M-NaCl and precipitated with 3vol. of ethanol. Finally the fractions were dissolved in water for determination of heparin content (uronic acid) and anti-coagulant activity.

Two unfractionated samples of heparin of different origin were also chromatographed on antithrombin–Sepharose as described above. The HA- and LA-heparin fractions were then analysed by gel chromatography as described in the legend to Fig. 6.

Results and Discussion

Analysis of data from the literature

Literature values showing a molecular-weight dependence of the anti-coagulant activity of heparin have been collected in Fig. 3. There is a considerable spread of the values, presumably due to structural differences between preparations and different means of estimating the molecular weights and the anti-coagulant activities in the various investigations. There is, however, an indication that preparations with mol.wts. below 5000 lack activity. The activity then rises to about 150 units/mg at a mol.wt. of 12000. Also shown in Fig. 3 is a curve showing the relationship:

$$\text{Anti-coagulant activity} = \text{constant} \cdot (n-5)/n \quad (1)$$

The proportionality constant was chosen so that the curve would pass through the average of all points. In spite of the scatter of the points one can notice a deviation of the hypothetical function from the experimental results in the lower-molecular-weight range, where the anti-coagulant activity is lower than predicted.

In view of the diverse results demonstrated in Fig. 3 it was deemed necessary to carry out a careful fractionation of a large batch of heparin and analyse the fractions under identical conditions.

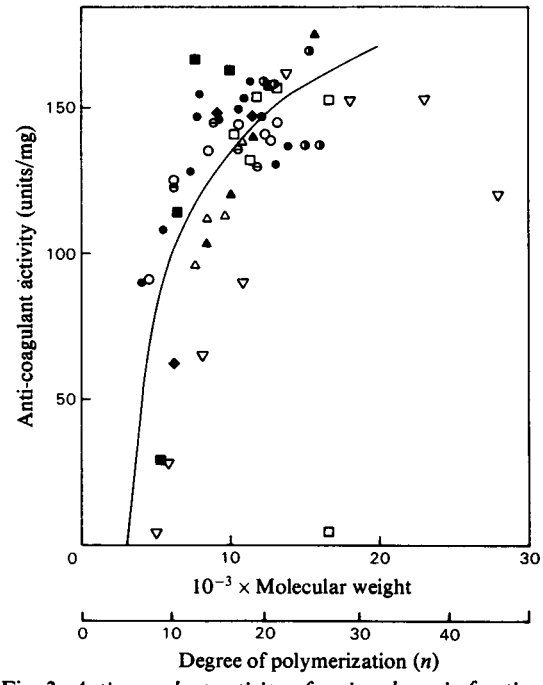

Fig. 3. *Anti-coagulant activity of various heparin fractions described in the literature plotted versus molecular weight and degree of polymerization*

The values are taken from the following references: △, Laurent (1961); ■, fractions BL, 3 I–V and, ◆, fractions HM, 3 II–IV of Cifonelli (1974); ●, ethanol fractions and, ▲, ECTEOLA-cellulose fractions of Lasker & Stivala (1966); ○, fractions P79-1 to P79-7 and, ⊖, dioxan fractions D79-1 to 79-4 of Liberti & Stivala (1967); □, Barlow *et al.* (1961); ◑, Barlow *et al.* (1964); ▽, Andersson *et al.* (1976). The line follows the relationship described in eqn. (1) and the constant was chosen for closest fit.

Molecular-weight fractionation

Heparin fractions prepared by gel chromatography and treated with chondroitinase ABC (fractions 1–5) were analysed for hexosamine. In all fractions glucosamine was the major hexosamine component; the amount of galactosamine invariably accounted for less than 5% of the total hexosamine. Electrophoresis in 0.1M-HCl showed single components that had migrated like fully sulphated heparin, the only exception being fraction 1, which showed, in addition, a faint band corresponding to a monosulphated polysaccharide. Although it was not possible to determine the relative amounts of the different polysaccharides in fraction 1 it may be concluded that all fractions contained heparin as the major constituent.

The results of the molecular-weight determinations, the analytical gel chromatography and the assay for anti-coagulant activity are presented in Table 1. The amount of material in fraction 10 was not sufficient for analysis, presumably due to losses during the dialysis step. For the remaining fractions molecular weights between 5600 and 36000 were recorded and the weight-average molecular weight for the whole preparation was calculated to be 14200. The $K_{av.}$ values of the fractions obtained by analytical gel chromatography at low concentrations did not correspond to their elution volumes in the preparative fractionation (Fig. 2), indicating that there was a strong concentration dependence in the gel chromatography.

Table 1. *Analytical data for fractions obtained by gel chromatography of heparin*

The percentage of total material was calculated from the chromatogram in Fig. 1 after corrections for galactosamine content. $K_{av.}$ values were based on peak positions obtained in analytical gel chromatograms on Sephadex G-100. —, Insufficient material for analysis. The molecular-weight averages (M_w and M_n) are for the unfractionated material.

Fraction no.	Percentage of total material	Molecular weight	$K_{av.}$ on Sephadex G-100	Anti-coagulant activity (units/mg)
1	0.8	36000	0.05	139
2	3.8	27000	0.09	188
3	7.6	25000	0.14	184
4	12.4	19200	0.21	179
5	16.3	16500	0.24	181
6	19.1	13000	0.27	159
7	17.4	10900	0.35	119
8	13.0	7800	0.47	109
9	6.9	5600	0.59	4
10	2.7	—	0.68	—
	100.0	$M_w = 14200$ $M_n = 11200$		

The molecular-weight-dependence of the anti-coagulant activity of four fractions is shown in Fig. 4. The experimental values are compared with the function given by eqn. (1), which has been adjusted to fit the experimental point at mol.wt. 16500. As in Fig. 3, the values at lower molecular weights do not conform to this function. Furthermore, the fraction with mol.wt. 36000 has a much lower activity than expected. It is also demonstrated in Fig. 4 that the experimental points up to mol.wts. of 17000 are better represented by the function: activity $\propto (n-9)/n$; corresponding to a required heparin sequence of ten disaccharides for activity.

In view of these results it appeared important to reconsider the size of the antithrombin-binding site on heparin.

Chromatography of heparin fractions on anti-thrombin-Sepharose

The work of Hopwood *et al.* (1976) indicated that the binding region for antithrombin in the heparin molecule corresponds to a sequence of approximately a dodecasaccharide size. As shown in Fig. 1, a heparin molecule with n disaccharides contains $(n-5)$ possible dodecasaccharide sequences. Let α be the probability that any given dodecasaccharide sequence has the structure required for binding to antithrombin; $(1-\alpha)$ is then the probability that the dodecasaccharide cannot bind. The probability that no dodecasaccharide in the heparin molecule contains the binding sequence is $(1-\alpha)^{n-5}$. From this it

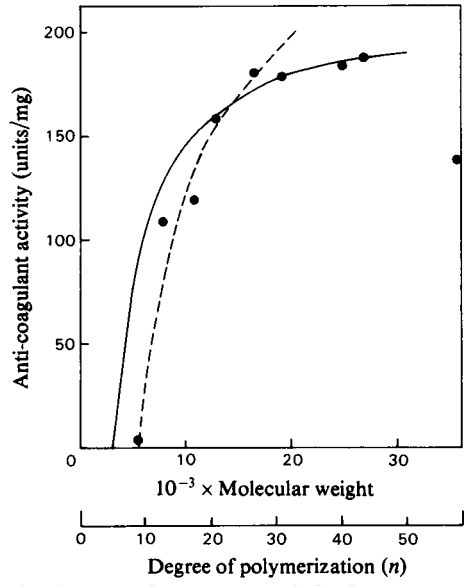

Fig. 4. *Anti-coagulant activity of the heparin fractions described in the present paper plotted versus molecular weight and degree of polymerization*

The continuous line follows the relationship described in eqn. (1), and has been adjusted to the value at the mol.wt. 16500. The broken line follows the relationship: activity = constant $\cdot (n-9)/n$; and has been adjusted to the experimental points below 13000.

follows that the fraction of molecules in a heparin sample that contains one or more binding sites for antithrombin (F_{HA}) is:

$$F_{HA} = 1-(1-\alpha)^{n-5} \qquad (2)$$

As F_{HA} is a function of the degree of polymerization (n), eqn. (2) can be tested experimentally by determining the percentage of each heparin fraction that binds to antithrombin–Sepharose. [The probability that a molecule should contain a specified number of binding sites if these are randomly distributed can be calculated from the following expression:

$$\alpha^0(1-\alpha)^{n-5} + \frac{(n-5)}{1}\cdot\alpha\cdot(1-\alpha)^{n-6} + \frac{(n-5)(n-6)}{1\cdot 2}\cdot\alpha^2\cdot(1-\alpha)^{n-7}\ldots + \frac{(n-5)(n-6)\ldots(3\cdot 2\cdot 1)}{1\cdot 2\cdot 3\ldots(n-5)}\cdot\alpha^{n-5}\cdot(1-\alpha)^0 = 1$$

The first term is the probability that no sequence is correct, the second that one sequence is correct, the third that two binding sites exist and the last term that every sequence is a correct sequence.]

Each heparin fraction was chromatographed separately. Chromatograms of one high-molecular-weight and one low-molecular-weight fraction are shown in

Fig. 5. One can define three peaks in the chromatograms. Part of the material in the low-molecular-weight fractions 6–9 did not adsorb to the column in the starting buffer (0.05M-Tris+0.05M-NaCl). Another portion of all the fractions (denoted LA-heparin) was eluted at approx. 0.5M-NaCl. The remaining material (HA-heparin) was eluted at NaCl concentrations above 1M. The proportions of the three types of heparin are given in Table 2. The overall average yield of uronic acid in the chromatographies was about 85% (range 74–99%).

The anti-coagulant activities of the HA- and LA-heparin fractions are also given in Table 2. In agreement with previous findings (Höök *et al.*, 1976) the anti-coagulant activity resides essentially in the HA-heparin fractions. It is therefore reasonable to assume that only the HA-heparin material contains appropriate binding sites for antithrombin. The residual activity in the LA-heparin material may conceivably be ascribed to sequences that bind less strongly and therefore result in partial activation of the anti-thrombin.

The fraction of the total material that is found in the HA-heparin peak (Table 2) has been plotted

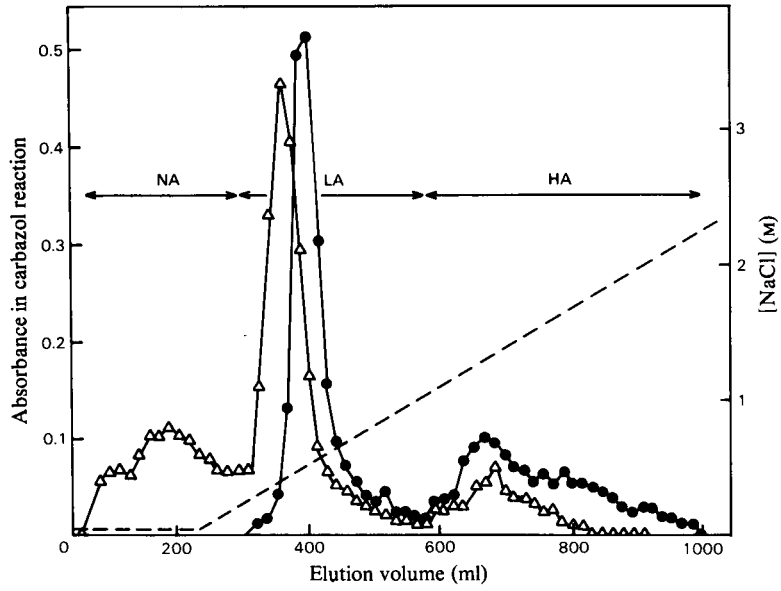

Fig. 5. *Chromatography of heparin fractions on antithrombin–Sepharose*
Fractions 2 (16mg) (●) and 8 (20mg) (△) were chromatographed on a 75ml column of antithrombin–Sepharose. The column was first washed with 0.05M-Tris/HCl, pH7.4, containing 0.05M-NaCl and then eluted with a linear gradient of NaCl (as shown in the Figure) containing 0.05M-Tris buffer. The material was fractionated into a non-adsorbed fraction (NA), low-affinity material (LA) and high-affinity material (HA).

Table 2. *Separation of heparin fractions into high- and low-affinity material (HA- and LA-heparin) on an antithrombin–Sepharose column*

Fraction no.	Total recovery of uronic acid (%)	Fraction size			Anti-coagulant activity (units/mg of heparin)		Thrombin inactivation (HA) (nkat/μg of heparin)
		Non-adsorbed	LA	HA	LA	HA	
1	76		61	39	23	209	154
2	99		55	45	23	228	184
3	83		60	40	19	273	220
4	74		59	41	23	308	219
5	99		61	39	17	323	233
6	84	4	63	33	9	303	221
7	88	6	68	26	7	274	228
8	85	28	55	17	6	258	190
9	84	46	46	8	<2	33	98

versus molecular weight (and n) in Fig. 6. The continuous line corresponds to the theoretically expected relationship (eqn. 2); α was set to 0.022 to provide the closest possible fit with the experimental values. Fractions 4–9 follow the predicted line, whereas fractions 1–3 deviate considerably. It should be stressed that fractions 4–9 represent 85% of the original heparin preparation and it is within their molecular-weight range that heparin fractions usually are found (compare Fig. 3). Molecular weights above 20000 are rarely observed.

The electrophoretic analysis indicated that fraction 1 could be contaminated with a polysaccharide of lower degree of sulphation, thus explaining the deviation from the hypothetical relationship. However, a more probable reason for this deviation is that the high-molecular-weight fractions represent molecules composed of polysaccharide fragments bound to the core component of a heparin proteoglycan (Horner, 1971). Such fragments are known to have a chemical composition different from that of the more peripheral portions of the heparin chains (Lindahl, 1966). In order to investigate this possibility, fraction 1 was hydrolysed and its amino acid composition determined. The protein content was approx. 10%. Serine and glycine were the predominating amino acids and occurred in equimolar amounts, in agreement with the composition tentatively ascribed to the protein core of a heparin proteoglycan. [Recent studies on rat skin heparin have indicated that it is a proteoglycan with a polypeptide core consisting largely of serine and glycine residues, probably in alternating sequence. At least two out of three serine residues are substituted with polysaccharide chains (Robinson *et. al.*, 1978). Owing to the presence of endoglycosidases the heparin proteoglycan in most tissues is converted into single polysaccharide chains of the kind recovered in commercial heparin preparations (Horner, 1972; Ögren & Lindahl, 1975, 1976).] All other amino acids occurred in less than half the amount of either of these. The molar ratio of serine to glucosamine was 1:8.33 corresponding to approximately 7 serine residues per molecule in fraction 1. These results indicate that a major portion of fraction 1 consists of a polysaccharide-substituted peptide core; it seems probable that similar components occur, although in lower proportions, also in the other fractions that deviate from the curve in Fig. 6. Fragments containing the polysaccharide–protein linkage region are expected to behave differently from other heparin fragments since they have a different chemical composition and, in addition, a branched structure; they are therefore not applicable to the statistical approach that we have used. The presence of fragments from the linkage region in fraction 1 also explains its unexpectedly low anti-coagulant activity (Table 1 and Fig. 4).

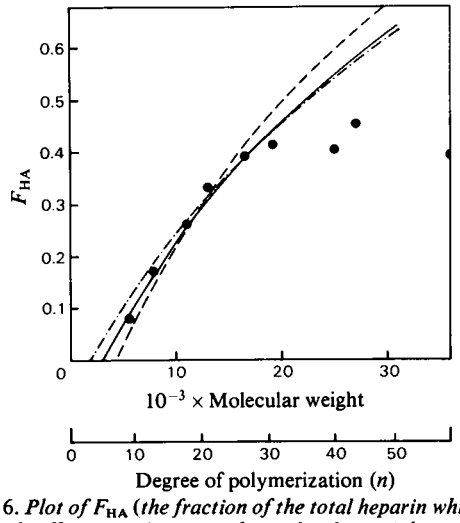

Fig. 6. *Plot of F_{HA} (the fraction of the total heparin which is of high-affinity type) versus the molecular weight and the degree of polymerization*

n is the number of disaccharide units per chain. ——, Theoretically expected relationship (eqn. 2) assuming a binding site for antithrombin composed of six disaccharide units; the probability (α) of finding a correct binding sequence has been set to 0.022 in order to give the closest possible fit with the experimental data. The two other curves represent the relationships expected for binding sites corresponding to four (-·-·-) and eight (– – – –) disaccharide units respectively. To obtain these curves the exponent in eqn. (2) was changed to $(n-3)$ and $(n-7)$ respectively and α was chosen so that all curves should pass through the same point at $n = 20$.

The data shown in Fig. 6 clearly conform to the dodecasaccharide size postulated for the antithrombin binding site in heparin (Hopwood *et al.*, 1976). Functions corresponding to binding sites containing four and eight disaccharides have also been incorporated in the Figure and fall on either side of the experimental points in the low-molecular-weight region.

The fact that heparin fractions, which represent the bulk of the material, follow a relationship of the type described in eqn. (2) is a strong indication that the binding of heparin to antithrombin can be explained on a probability basis. The probability that any given dodecasaccharide sequence represents a high-affinity binding site is 1:45 as calculated from the value 0.022 for the factor α.

Chromatography of unfractionated heparin on antithrombin–Sepharose

Another qualitative test of the hypothesis that the binding between heparin and antithrombin is dependent on the molecular weight of the polysac-

charide can be obtained by chromatography of unfractionated heparin on antithrombin–Sepharose, followed by an examination of molecular weight by gel chromatography. Owing to the increased probability of binding to antithrombin with increasing molecular weight one should find an enrichment of high-molecular-weight heparin in the HA-heparin fraction and of low-molecular-weight material in the LA-heparin fraction. This experiment was performed using two heparins from different sources; the resulting HA- and LA-heparin fractions were analysed by gel chromatography. In both cases the elution profiles of the HA- and LA-heparin fractions differed in accordance with expectations (Fig. 7).

Anti-coagulant activity of HA-heparin as a function of molecular weight

The experiments discussed above clearly indicate that the molecular-weight dependence of the anti-coagulant activity may be correlated to the probability of finding a specific dodecasaccharide sequence (i.e. the binding site for antithrombin) in a heparin molecule. However, this probability cannot be the only determining factor, as the anti-coagulant activity of HA-heparin (in which every molecule must contain at least one binding site for antithrombin) is still dependent on molecular weight (Table 2). Furthermore, if antithrombin binding to heparin were the only factor there should be an agreement between the relationship described by eqn. (1) and the experimental data in the low-molecular-weight regions in Figs. 3 and 4.

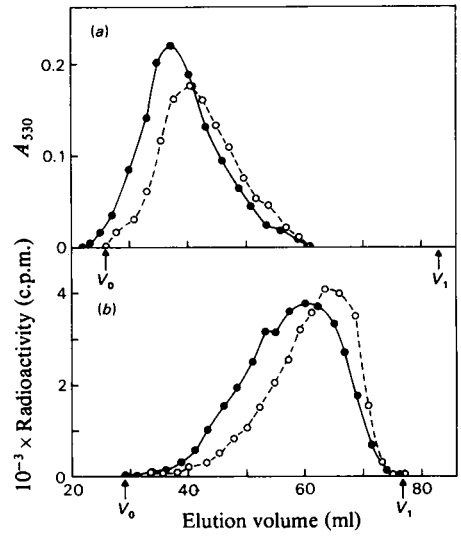

Fig. 7. *Demonstration that separation of polydisperse heparin into high- and low-affinity material also gives a molecular-weight fractionation*

(*a*) A sample of pig mucosal heparin was fractionated into HA- and LA-heparin by affinity chromatography on antithrombin–Sepharose. A 1 mg sample of each fraction was applied separately on a column (1 cm × 98 cm) of Sephadex G-100 and eluted with 1 M-NaCl in 0.05 M-Tris/HCl buffer, pH 8.0. Fractions of about 2.5 ml were collected and analysed for uronic acid. ●, HA-heparin; ○, LA-heparin. (*b*) [^{3}H]HA-heparin and [^{3}H]LA-heparin prepared from mouse mastocytoma were applied separately on a column (1 cm × 90 cm) of Sepharose 4B. The column was eluted with the same buffer as in (*a*) and the symbols are the same.

Since the whole-blood B.P. assay reflects the effect of heparin on a number of the reactions in the coagulation cascade the fractions were also analysed for antithrombin-activating activity (Table 2). The two assays gave essentially similar results with regard to molecular weight of the heparin with the exception that fraction 9 (mol.wt. 5600) showed a ratio of antithrombin-activating activity to B.P. activity higher than that of the other fractions. A somewhat related observation has been made by Andersson *et al.* (1976), who found that their heparin fractions of lowest molecular weight had a relatively higher anti-(Factor X_a) activity than anti-coagulant activity.

Fig. 8 shows the biological activities of the HA-heparin fractions as a function of molecular weight. The activity has been expressed both on a weight basis and a molar basis. At mol.wts. below 5000 to 6000 the activity is negligible. When it is expressed in units per weight an activity maximum is reached at a mol.wt. of 15000, whereafter it drops. From a mechanistic point of view it is more appropriate to express the activity in units per mol. The molar activity increases rapidly with degree of polymerization. At present one can only give a speculative explanation of the results described in Fig. 8.

Fragments obtained as described by Hopwood *et al.* (1976) by heparinase digestion of a complex of HA-heparin and antithrombin have a residual antithrombin-activating activity, which, expressed on a molar basis, is approximately 10% of that of the parent HA-heparin (M. Höök, L. Thunberg, F. Linker & U. Lindahl, unpublished observation). This is approximately the same value as measured for our fraction 9. The mere binding of antithrombin to the specific site of heparin can therefore account for at most 10% of the biological activity; obviously a heparin chain longer than a dodecasaccharide is required for full activity. A possible explanation could be that interactions between heparin and antithrombin outside the specific binding site contribute to the induction of a conformational change in the latter molecule (Villanueva & Danishefsky, 1977; Nordenman & Björk, 1978*b*), which may be a pre-

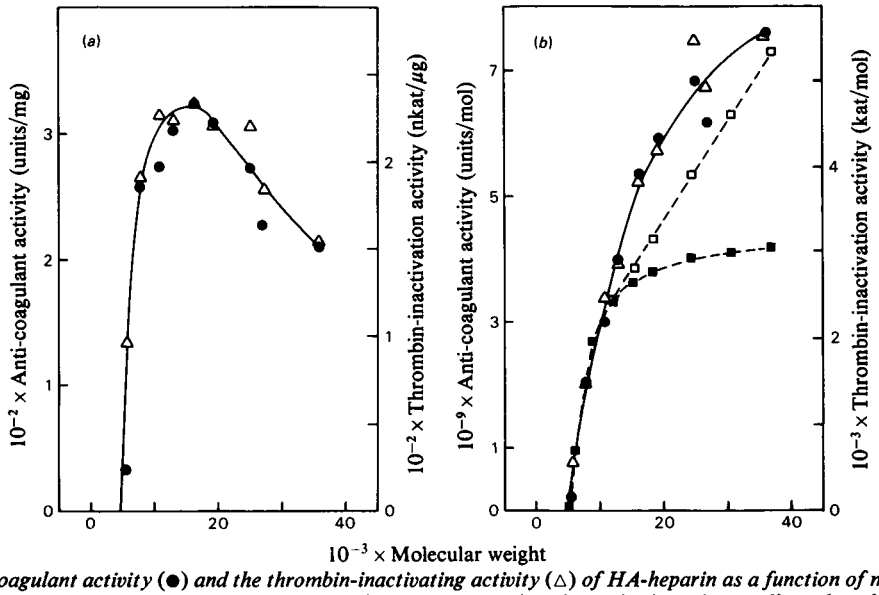

Fig. 8. *Anti-coagulant activity* (●) *and the thrombin-inactivating activity* (△) *of HA-heparin as a function of molecular weight*
The activities are given in units per weight (*a*) and units per mol (*b*). The scales have been adjusted so that the curves should overlap. Two theoretical curves have also been incorporated in (*b*). One has been drawn according to eqn. (3) and adjusted to the experimental points in the low-molecular-weight region (■). It shows the probability of finding a thrombin-binding site adjacent to a single antithrombin-binding site on each heparin molecule. The second curve (□) has been obtained by multiplying the first curve with the probable number of antithrombin-binding sites per heparin molecule. In these calculations it has been assumed that the probability for a dodecasaccharide to bind to antithrombin is 0.022; that each antithrombin-binding site is furnished with an adjacent octasaccharide for thrombin-binding; and that two complete thrombin–antithrombin-binding sequences should not overlap. This was accomplished by placing the first thrombin–antithrombin-binding sequence in every possible position along the heparin chain, and then calculating the number of remaining available sequences of ten disaccharides for each position. The average of these values was multiplied with 0.022 to give the probability for a second site. The probability for a third site is negligible.

requisite for its binding to thrombin. Another possibility implies that the reaction between thrombin and antithrombin might be facilitated were they both simultaneously bound to the same heparin chain, adjacent to each other. Also this situation may be analysed on the basis of probability.

It is well documented that thrombin binds to heparin (Li *et al.*, 1974; Machovich, 1975; Nordenman & Björk, 1978*a*); the interaction does not block the catalytic site on thrombin, which is inactivated by antithrombin. Furthermore, prevention of the interaction between heparin and thrombin, by modification of arginine residues in the protein, retards the inactivation of the enzyme in the antithrombin–heparin system. We may thus assume that thrombin binds to heparin adjacent to one side of antithrombin before the two proteins react (Fig. 9). As heparin is required to have a mol.wt. of approx. 6000 for appreciable activity we will assume that thrombin binds to an octasaccharide, which, together with the antithrombin-binding site (a dodecasaccharide), gives a mol.wt. of 6150. The length of an octasaccharide is 4nm, which is of the same order as the size of thrombin. In a heparin molecule of n disaccharides there are $(n-5)$ possible antithrombin-binding sequences, but only $(n-9)$ sequences that allow thrombin to bind adjacent to antithrombin. The thrombin-inactivating activity (β) of a heparin molecule with one antithrombin-binding site should therefore follow the equation:

$$\beta = \text{constant} \cdot (n-9)/(n-5) \qquad (3)$$

If thrombin requires a specific disaccharide sequence at the binding site, as in the case for antithrombin, the probability of finding this sequence adjacent to antithrombin is included in the constant. A curve has been plotted in Fig. 8(*b*) according to eqn. (3) and adjusted to fit the experimental points in the low-molecular-weight region. It is evident that it does not describe the entire range of experimental points. However, as the heparin chain increases in length, there is an increasing probability that the chain contains a second antithrombin–thrombin-binding site. A second function has been drawn in Fig. 8(*b*), which corresponds to the first function multiplied with the probable number of antithrombin–

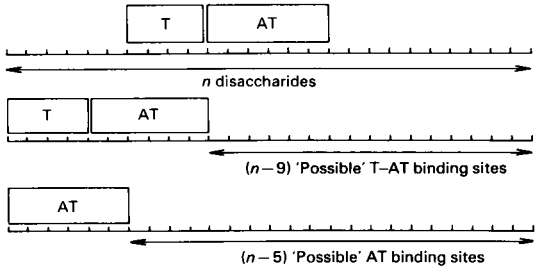

Fig. 9. *Illustration of the hypothesis that thrombin (T) should bind to an octasaccharide residue of heparin adjacent to antithrombin (AT)*

The two proteins are assumed to occupy together ten disaccharides. There are $(n-9)$ sequences containing ten disaccharides, but $(n-5)$ to which antithrombin possibly could bind. The probability of finding a complete sequence for the two proteins compared with finding a binding site for antithrombin is therefore given by eqn. (3).

thrombin-binding sites per heparin molecule. Such a correction apparently closes the gap between experimental and predicted relationships.

The need for a segment of ten disaccharides for full activity of heparin is also in agreement with the data on the anti-coagulant activity of unfractionated heparin presented in Fig. 4.

Final comments

The calculations presented above have been based on simple assumptions such as random distribution of disaccharides in heparin and a specific dodecasaccharide sequence for binding to antithrombin. These assumptions may have to be modified when more refined structural data are available for heparin; in fact a number of observations indicate that the heparin structure is not entirely random (Lindahl, 1976). It should be emphasized, however, that complete structural randomness is not a prerequisite to our hypothesis. Furthermore, the term 'specific dodecasaccharide sequence' should not be interpreted as if there is only one possible binding sequence. There may be several dodecasaccharide sequences which fit antithrombin; as a matter of fact, the common character of these sequences may be as limited as to the correct position of a single (unique) sugar unit.

The concept of specific sequences, related to biological activity, in a partly random polymer has interesting consequences. Heparin has other biological activities than anti-coagulant activity, which may be due to other binding sequences; the LA-heparin, essentially lacking anti-coagulant activity, thus retains full lipase-releasing activity (Bengtsson *et al.*, 1977). The heparinase from mouse mastocytoma described by Ögren & Lindahl (1975), which degrades macromolecular heparin to fragments with a mol.wt. of 14500, can obviously find only one sequence out of about 25 to bind and to cleave. This sequence must be different from at least some of the antithrombin and lipoprotein lipase binding sites, otherwise the enzyme would have destroyed the biological activities of heparin. Different degrees of modifications of the heparin molecules on the polymer level can therefore have different effects on its various biological functions. The recognition properties of heparin may be explained as a statistical phenomenon.

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