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On the Molecular-Weight-Dependence of the Anticoagulant Activity of Heparin

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The inactivation of thrombin and factor X_a by antithrombin was determined in the presence of heparin fractions of different molecular weights and with high affinity for antithrombin. The ability to potentiate the inactivation of either coagulation factor increased with increasing length of the polysaccharide chain.

The plasma protein antithrombin (often referred to as antithrombin III) inhibits a number of serine proteinases involved in blood coagulation. In the presence of heparin, the rate of inactivation is greatly accelerated (Rosenberg, 1977). This is due to the tight binding between antithrombin and a segment of the polysaccharide molecule corresponding to 12–16 monosaccharide units (Hopwood *et al.*, 1976). Only a fraction of the molecules in heparin preparations contain the appropriate sequence (or sequences) of sugar units required for such binding (Hopwood *et al.*, 1976), and therefore only a fraction of the molecules possesses significant anticoagulant activity (Lam *et al.*, 1976; Höök *et al.*, 1976; Andersson *et al.*, 1976).

It is well established that the anticoagulant activity of heparin is correlated with its molecular weight [see Laurent *et al.* (1978) for references]. The molecular-weight-dependence could be partly explained in terms of the statistical probability of finding a particular dodecasaccharide sequence in a polysaccharide molecule made up of various randomly or quasi-randomly distributed disaccharide units (Laurent *et al.*, 1978). This probability, as reflected by the fraction of molecules binding with high affinity to antithrombin, was found to increase with increasing molecular weight of the heparin preparations in a predictable manner. However, other factors must be involved, since heparin fractions already selected for by their high affinity for antithrombin retained a positive correlation between molecular weight and anticoagulant activity. The same results were obtained whether the biological

activity was measured by the British Pharmacopoeia whole-blood assay or by a less complex assay system based on heparin, antithrombin, thrombin and a synthetic tripeptide thrombin substrate. It was suggested that the anticoagulant mechanism of heparin might involve binding of the polysaccharide not only to antithrombin but also to the target proteinase, thrombin (Laurent *et al.*, 1978; see also Pomerantz & Owen, 1978; Holmer *et al.*, 1979).

It would obviously be of interest to study the inactivation of other serine proteinases in the coagulation cascade in relation to the molecular weight of heparin. Andersson *et al.* (1976) thus found that, unlike thrombin, factor X_a was preferentially inactivated by heparin fractions of low molecular weight. The interpretation of these results was complicated by the fact that blood plasma was used instead of purified antithrombin in the assay mixtures, thus introducing additional components with potential ability to interact with heparin. The present investigation was undertaken to determine the effects of heparin fractions of different molecular weights on the antithrombin-mediated interaction of thrombin and factor X_a, in well-defined and simple assay systems.

Materials and Methods

The heparin preparations used have been described elsewhere (Laurent *et al.*, 1978). Pig mucosal heparin was fractionated according to molecular size by gel chromatography, and the resulting subfractions were subjected to affinity chromatography on anti-

thrombin-Sepharose. Molecules with high affinity for antithrombin were recovered and used in the present study. In addition, heparin fragments essentially corresponding to the actual antithrombin-binding site were isolated after digestion of heparin-antithrombin complexes with bacterial heparinase. The procedure described by Hopwood *et al.* (1976) was scaled up and applied to pig mucosal heparin and to bovine lung heparin having high affinity for antithrombin (Lindahl *et al.*, 1979). The products had mol.wts. of 3500–4000, as determined by gel chromatography on Sephadex G-100, with appropriate reference standards. The preparations of antithrombin and thrombin have been described (Laurent *et al.*, 1978). Factor X_a was prepared as described by Jackson & Hanahan (1968).

The ability of heparin fractions to potentiate the inactivation of thrombin in the presence of antithrombin was measured as described previously (Laurent *et al.*, 1978). The inactivation of factor X_a was determined in a similar manner; each assay was carried out with 30 ng of heparin, 2.5 μ g of bovine antithrombin and 10 μ g of factor X_a . Factor X_a activity was determined with *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S-2222; A.B. Kabi Diagnostica, Stockholm, Sweden) as substrate.

Results and Discussion

The isolated heparin fractions were analysed for their ability to inhibit thrombin and factor X_a in the presence of purified antithrombin. Both the thrombin- and the factor X_a -inactivating potencies clearly decreased with decreasing molecular weight of the polysaccharide, when expressed on a molar basis (Fig. 1*a*). This finding suggests that the molecular-weight-dependence of heparin-induced anticoagulation applies to the inactivation of either coagulation factor. However, when anti- X_a activity was expressed on a weight basis the trend was reversed for the smallest heparin fragment (Fig. 1*b*). This observation may be explained in terms of our previous hypothesis (Laurent *et al.*, 1978) that maximal activity of antithrombin, i.e., maximal inactivation of the target serine proteinase, may require binding of both protein molecules to the same heparin chain. The probability of such dual binding will increase with increasing length of the heparin chain, above a certain minimal length, corresponding to a mol.wt. of about 6000. Below this limiting size the heparin chain can accommodate one protein molecule only; such fragments, ranging down to the minimal size (about 4000 daltons) required for antithrombin binding, will all have about the same anti- X_a activity on a molar basis and hence an increasing activity with decreasing size on a weight basis (see Fig. 1*b*). Owing to the low number of experimental points in

Fig. 1. Inactivation of thrombin (closed symbols) and Factor X_a (open symbols) by antithrombin in the presence of high-affinity heparin fractions (mucosal heparin, $\circ$, $\bullet$; lung heparin, $\triangle$, $\blacktriangle$) of different molecular weights

The anticoagulant activities of the heparin preparations were related to that of the Third International Heparin Standard and expressed on a molar (a) as well as on a weight (b) basis.

the low-molecular-weight region, it is not clear whether a similar relationship applies to the inactivation of thrombin. In any case, the smallest heparin fragments have a relatively higher capacity to inactivate factor X_a than to inactivate thrombin, in the presence of antithrombin. This difference may indicate that the inactivation of thrombin is more dependent on actual binding of the proteinase to the heparin chain than is the inactivation of factor X_a .

The anti-X_a activity of the heparin fragments, as related to that of the Third International Heparin Standard was 200–250 units/mg. Similar comparison, based on an assay system with blood plasma substituted for purified antithrombin, gave a specific activity of about 1600 units/mg (E. Holmer, L.-O. Andersson, L. Thunberg & U. Lindahl, unpublished work). These data suggest that the exceedingly high anti-X_a activities recorded for low-molecular-weight heparin in plasma systems (Andersson *et al.*, 1976) primarily reflect neutralization of the high-molecular-weight reference standard, owing to interaction with other plasma macromolecules. Binding to plasma proteins would be expected to preferentially involve heparin species of high molecular weight (Scott, 1968). Data consistent with this interpretation have been obtained recently, by directly comparing the anti-X_a activities of heparin fractions of different molecular weights in the absence and presence of blood plasma respectively (Andersson *et al.*, 1979.)

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