The advent of low-molecular-weight heparins (LMWH) made it surprisingly clear how little we know about the connection between the antithrombotic potency of heparin and its (bio)chemical properties. Classical unfractionated heparin (UFH) has been shown to be an acceptable antithrombotic, although the bleeding risk that it may induce is not negligible. More than half a century of clinical trial and error, often of a type that would not stand up to modern ethical and scientific standards, have been necessary to find an acceptable dosage scheme. We simply cannot think of repeating the same procedure with each new type of heparin that is introduced. Therefore, we have to compare new heparins with the classical preparations on the basis of laboratory tests. Given this situation, it is slightly awkward that we do not know what the laboratory properties of heparin are that indicate its antithrombotic or hemorrhagic activities. The search for the proper laboratory correlate to in vivo antithrombotic action has occupied us for the last few years. We feel that this subject is a central one in heparin research. Only if we know what tests in the laboratory reflect the action of heparins in a patient, can we attempt to answer the three crucial questions that are posed by these drugs:

1. What tests are suitable for controlling the quality of heparin therapy in a patient?
2. How can different types of heparin be standardized, i.e., what amount of LMWH is the functional equivalent of 1 U of standard heparin?
3. What laboratory tests indicate the antithrombotic potency of an unknown heparin?

The ultimate answer to the question of the ex vivo correlate to in vivo action cannot come from the laboratory alone, because the proof of the heparin pudding will always remain in the clinical eating. Yet on the basis of laboratory studies, we can exclude certain possibilities. If, for instance, it can be demonstrated that the anti-factor Xa action of UFH does not play a role in the thrombin generation in plasma, then it is highly unlikely that anti-factor Xa activity could relate to antithrombotic activity. Therefore, on the basis of laboratory results one can make an educated guess and put forward hypotheses on the mode of action of heparin in vivo and hence on the laboratory correlate to that.

Is the Correlate To Be Found in the Coagulation System?

One may well argue that the antithrombotic action of heparins does not necessarily have anything to do with its action on blood coagulation. In fact, many actions of heparin are known apart from its effects on the blood coagulation
It has, for instance, been described that heparin fractions with a low affinity to antithrombin III (ATIII) and consequently with a limited effect on blood coagulation potentiate the effect of high-affinity fractions. Such findings need not automatically indicate a mode of action of heparin that bypasses the coagulation system. As soon as plasma forms part of the reaction medium, especially when it contains or has contained activated platelets, various heparin-neutralizing proteins are present. Heparins with a low affinity to ATIII may still have a high affinity to heparin-neutralizing proteins. If such heparins bind to neutralizing factors, they will automatically cause an increased availability of heparins with a high ATIII affinity.

One argument pretty well excludes the possibility that the anticoagulant properties of heparins have nothing to do with their antithrombotic action: a completely different family of anticoagulant drugs, the vitamin-K-antagonist coumarin congeners, are also good antithrombotics. These oral anticoagulants, like heparin, have several effects that are not related to blood coagulation, but these are completely different from the noncoagulation effects of heparin (for a review, see ref. 9).

The only action that, to our knowledge, coumarin congeners have in common with heparin is their anticoagulant action. Against this argument one can make the point that a third recognized antithrombotic, viz., aspirin, has no anticoagulant properties. This is certainly true if one investigates platelet-poor plasma. In platelet-rich plasma, however, the procoagulant action of platelets can be inhibited by aspirin. This leaves open the possibility that in this case also inhibition of thrombin formation is the essential mode of action of the drug, whether it be mediated by inhibition of cyclooxygenase or not.

We conclude that for the moment it is reasonable to assume that the antithrombotic action of heparin is related to its antithrombin action.

Is Factor Xa Inhibition the Laboratory Correlate to Antithrombotic Action?

When evidence was produced suggesting that LMWH might be as good an antithrombotic agent as UFH is and at the same time induce a lesser bleeding risk, it was logical to think that the yardstick for the safety of a heparin was to be found in a property that was different in both types of heparin. Such a property was found to be the relative anti-factor Xa potency. LMWHs with decreasing chain length gradually lose their antithrombin action, but to a certain extent retain their anti-factor Xa action. It was therefore thought that the anti-factor Xa action indicated the antithrombotic properties and that antithrombin activity parallels the bleeding risk.

For some time it therefore has been thought that anti-factor Xa action was a crucial property of all heparins. In the last few years this proposition has been doubted on various grounds. It is surprising to see that in fact the ratio between the antithrombin potency of LMWH and its antifactor Xa potency does not differ significantly from that of UFH in a purified system (Table 1). On the other hand, the difference does appear when anti-factor Xa units are determined by the standard procedure, i.e., by measuring the decay of added factor Xa in plasma. The setup that uses whole plasma probably approaches the in vivo situation better than the purified system does, but still the addition of a large amount of isolated factor Xa far from mimicks physiological conditions.

We approached the question by measuring the functional factor Xa activity in clotting plasma in a situation as close as possible to the situation in vivo. We
TABLE 1. Breakdown Constants of Various Clotting Proteases with Different Heparins in a Purified System

<table>
<thead>
<tr>
<th>Heparins</th>
<th>IIa</th>
<th>IXa</th>
<th>Xa</th>
<th>XIa</th>
<th>IIa/Xa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>38.7</td>
<td>0.180</td>
<td>4.1</td>
<td>0.021</td>
<td>9.4</td>
</tr>
<tr>
<td>PK 10169</td>
<td>7.3</td>
<td>0.109</td>
<td>0.8</td>
<td>0.002</td>
<td>9.1</td>
</tr>
<tr>
<td>CY 216</td>
<td>2.87</td>
<td>0.101</td>
<td>0.27</td>
<td>0.001</td>
<td>10.6</td>
</tr>
<tr>
<td>CY 222</td>
<td>1.91</td>
<td>0.058</td>
<td>0.45</td>
<td>0.002</td>
<td>4.2</td>
</tr>
<tr>
<td>Pentasaccharide</td>
<td>0</td>
<td>0.032</td>
<td>11.4</td>
<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td>EMT 966</td>
<td>14.8</td>
<td>0.158</td>
<td>1.3</td>
<td>0.006</td>
<td>11.4</td>
</tr>
<tr>
<td>EMT 967</td>
<td>0</td>
<td>0.020</td>
<td>0.4</td>
<td>0.001</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values are in min⁻¹ (μg heparin/ml)⁻¹

developed a method that allows calculation of the activity of prothrombinase in clotting plasma. Prothrombinase is the complex of factor Xa and factor Va adsorbed onto procoagulant phospholipid, only in this complex factor Xa acquires kinetic properties that enable it to activate prothrombin at physiologically relevant velocities.

It is known that factor Xa, when functioning in prothrombinase, is protected from antithrombin III and heparin action by phospholipid and factor Va. The burst of prothrombinase activity in plasma is short because the available prothrombin in clotting plasma exhausts rapidly. We wanted to know whether prothrombinase in clotting plasma during the time that it actually produces thrombin is inhibited by heparin. We reasoned that at any moment the velocity of observed thrombin generation is the sum of the velocities of prothrombin conversion and of thrombin breakdown (Fig. 1). We showed that thrombin breakdown is a pseudo-first-order process with a breakdown constant that does not change during the time course of the thrombin generation curve. This means that at any point of the curve we can calculate the velocity of thrombin breakdown if we know the concentration of free thrombin. The formation of the amidolytically active α2-macroglobulin–thrombin complex complicates the algorithm but does not invalidate the approach. The calculations are readily carried out by a personal computer. By applying this method it is possible to obtain the time course of prothrombinase activity from the thrombin generation curve. This curve gives more important information than the course of factor Xa activity, in the sense that it gives the factor Xa activity only insomuch as it is involved in prothrombin conversion.

When we computed prothrombinase activity in the presence of various concentrations of heparin, it appeared that heparin concentrations inhibited the observed velocity of thrombin generation for up to 85%, but would not inhibit prothrombinase (Fig. 2). This means that, for all practical purposes heparin does not inhibit factor Xa, at least as far as it is active in prothrombin conversion but scavenges the thrombin formed. This already clearly indicates that anti-factor Xa action cannot be the ex vivo property we are looking for, because heparin is a good antithrombotic and yet has no anti-prothrombinase activity to speak of. Clotting factors activated in situ tend to have properties that differ from isolated clotting proteases. This holds not only for factor Xa but also for thrombin. In general, the breakdown constants of endogenous thrombin in plasma are lower than those found with the purified protease.

To our surprise, two low-molecular-weight heparins were found to act primarily on thrombin, just like UFH. Two very low molecular weight heparins; the
FIGURE 1. The analysis of the thrombin generation curve. Line A is the experimentally observed amidolytic thrombin activity (T). Line B represents the activity of the α2-macroglobulin thrombin complex (M). At any given moment (S), the amount of free thrombin (F) can be calculated \[ F = T - M \]. Then the breakdown velocity of thrombin can be found \[ \frac{dT}{dt} = -(k_1 + k_2)F \] and the prothrombin conversion velocity obtained \[ \frac{dP}{dt} = \frac{dT}{dt} + \frac{dF}{dt} \].

synthetic pentasaccharide that represents the high-affinity ATIII binding site of heparin and the very low molecular weight fraction of enoxaparine (fraction B), were shown to be completely devoid of anti-IIa activity. The inhibition of the generation of thrombin by these heparin fractions, therefore, is solely caused by their inhibitory action on prothrombinase (Fig. 3 and Table 2).

It thus appears that there are two entirely different types of heparin. Those that act virtually only on thrombin and those that act only on prothrombinase. The first we called S-type heparins after their prototype, standard heparin. They comprise both UFH and different LMWHs. The second type, the P-type heparins, of which the above-mentioned pentasaccharide is the prototype, acts on prothrombinase, but not on thrombin. Only very low molecular weight heparins belong to this group.

Inspection of prothrombinase activity curves in the presence of heparin shows that, although the peak activity is not inhibited, the velocity with which the peak is attained is lower in the presence of heparin (Fig. 2B). This must be attributed to inhibition of the generation of either factor Xa or factor Vα. Direct measurement of the activity of these two factors in clotting plasma shows that both are inhibited but both in a different way. Because the main activator of factor V is thrombin, it stands to reason that the inhibition of factor V activation is a result of thrombin scavenging, whereas factor Xa is scavenged directly. This does not contradict our earlier conclusion that factor Xa in prothrombinase is not inhibited by heparin. The quantity of prothrombinase necessary to activate all the prothrombin present is exceedingly small (<0.1 nM). Factor Xa is generated in large excess (peak
FIGURE 2. The influence of unfractionated heparin on (A) thrombin generation and (B) prothrombinase activity in plasma triggered via the extrinsic pathway. Coagulation was started by the addition of 60 μl of human brain thromboplastin diluted 1:40 in Tris buffer containing 100 mM CaCl₂ to 240 μl of platelet-poor plasma. The heparin concentrations were none (○); 0.02 U/ml (○); 0.04 U/ml (■); and 0.1 U/ml (Δ).
amount = 12 nM). The first traces of factor Xa formed therefore suffice to obtain all the prothrombinase that is necessary. The activation of factor V seems to be the rate-limiting step (Fig. 4).

When we start coagulation via the intrinsic system (Fig. 5), e.g., with kaolin, we then see a clearcut lag time before thrombin generation starts. In the presence

TABLE 2. IC_{50} Values of Different Heparins in Platelet-Poor Plasma^a

<table>
<thead>
<tr>
<th></th>
<th>Thrombin Generation</th>
<th>PTase Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFH</td>
<td>0.25</td>
<td>1.2</td>
</tr>
<tr>
<td>Fraxiparine</td>
<td>2.5</td>
<td>&gt;&gt;10</td>
</tr>
<tr>
<td>CY 222</td>
<td>2.0</td>
<td>&gt;&gt;10</td>
</tr>
<tr>
<td>Pentasaccharide</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Enoxaparine</td>
<td>0.6</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>Fraction A</td>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>Fraction B</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

^a Values are in μg/ml. One unit of unfractionated heparin (UFH) roughly equals 6 μg.
FIGURE 4. Generation of activated clotting factors in thromboplastin triggered plasma. Coagulation was started by the addition of 60 μl of human brain thromboplastin diluted 1:10 in Tris buffer containing 100-mM CaCl₂ to 240 μl of platelet-poor plasma. Samples were removed at different time intervals and assayed for factor Xa (△), factor Va (■), and thrombin (○). Prothrombinase activity (○) was calculated from the thrombin generation curve as described in the legend to FIGURE 1.
FIGURE 5. The influence of the unfractionated heparin on (A) thrombin generation and (B) thrombin-enhanced activity in intrinsically buffered platelet poor plasma. Experimental conditions: A) 0.5 ml of 500 U/ml of heparin in 0.5 ml of PBS + 0.01 M KCl. The heparin concentrations were none ( ○ ), 0.02 U/ml (●), 0.04 U/ml (■), and 0.1 U/ml ( ● ).
of heparin these lag times are prolonged. Addition of preactivated factor VIII or addition of nanomolar amounts of thrombin to the plasma prior to starting the reaction abolishes this lag time. This is a direct indication that heparins also can inhibit prothrombinase formation by inhibiting the positive feedback activation of factor VIII by thrombin.

It is interesting to see that in intrinsic coagulation the amount of prothrombinase decreases when heparin is added. This cannot be attributed to factor Xa inactivation to a larger extent than in the extrinsic system because, as soon as the system is started with the factor X activating complex, the inhibition disappears. It must therefore be due to a decrease of the amount of tenase that is available. The formation of tenase depends on factor IXa and factor VIIIa generation. It has been demonstrated that the activation of factor IX is not inhibited by heparin. Thus, like the formation of the prothrombinase complex, it is reasonable to as-

FIGURE 6. The influence of unfractionated heparin on thrombin generation in platelet-rich plasma. Experimental conditions as in FIGURE 2 but for the use of platelet-rich plasma (3 x 10^6 platelets/ml) and human brain thromboplastin in a dilution of 1:400. The heparin concentrations were none (△); 0.1 U/ml (○); and 0.3 U/ml (△). The low-molecular-weight heparin CY 216 (■) was present at 0.07 anti-IIa U/ml.

sume that formation of the factor-X-activating complex largely depends on the generation of the cofactor, factor VIIIa. Because thrombin is the main activator of factor VIII, low tenase concentrations might be explained by the heparin-mediated scavenging of thrombin. Alternatively, inactivation of noncomplexed factor IXa might also explain the reduced generation of tenase when heparin is present.

The Action of Heparins in Platelet-Rich Plasma

Thrombin generation in platelet-rich plasma (PRP) is an in vitro system that is one step nearer to physiological reality than thrombin generation in platelet-poor plasma (PPP). As we did not find essential differences between the mode of action of UFH and several LMWHs in platelet-poor plasma, it was a logical step to study platelet-rich plasma next. Thrombin generation in PRP differs essentially from that in PPP in that it always shows a burst of thrombin formation after a lag time (Fig. 6). If no external
trigger is added the lag phase lasts 10–12 minutes. Trace amounts of thromboplastin (final dilution in the system, 1:2400) shorten this lag by 3–5 minutes. These concentrations of thromboplastin are without effect on PPP. We could show that the thrombin burst is caused by the production of procoagulant phospholipids by the platelets. Nanomolar amounts of thrombin generated during the lag time are instrumental in triggering the platelets.

All substances that inhibit thrombin formation or lower the ambient thrombin level by inactivation will prolong the lag time.39 This phenomenon therefore is produced by UFH, S-type LMWHs, P-type LMWHs, and hirudin alike. Once the platelets are activated, a curious difference of behavior appears among the various heparins. On the one hand unfractionated heparin appears almost completely neutralized by products from the activated platelets, i.e., platelet factor 4 (PF 4). As a consequence, the peak of the thrombin burst is only retarded but is not inhibited by UFH (Fig. 6). The same concentrations of UFH, however, greatly inhibited thrombin appearance in PPP (Fig. 2).

On the other hand, the low-molecular-weight heparins are less subject to PF 4 neutralization than UFH. A typical example is shown in Figure 6. Fraxiparine, which is equally effective on the basis of anti-IIa U/ml in platelet-poor plasma when compared with UFH, does inhibit the thrombin generation in platelet-rich plasma much more effectively than UFH.

Summary, Conclusion, and a Hypothesis

The low-molecular-weight heparins that we have tested and that are available for treatment of patients act upon the coagulation of platelet-poor plasma in essentially the same way as standard heparin. Most, like UFH, do have a slight anti-prothrombinase action that becomes evident at higher concentrations. Very low molecular weight heparins that lack antithrombin activity retain their antiprothrombinase action, however.

The fact that most heparins have primarily an antithrombin action does not mean that they would not work on thrombin generation too. Both from our work and from that of others it appears that the product activation by thrombin is an essential feature of the natural clotting mechanism at least at three levels: platelet activation, factor V activation, and factor VIII activation. Both UFH and LMWH act primarily by inhibiting these feedback loops. We found only quantitative differences between the mode of action of commercially available LMWHs and UFH on this mechanism.

However, an essential difference between LMWH and UFH is their susceptibility to inactivation by PF 4. Fully activated platelets at 3 × 10^8 ml^-1 are capable of neutralizing UFH concentrations that can almost completely inhibit thrombin formation in platelet-poor plasma. LMWH is neutralized to a much lesser extent. We hypothesize that this difference in PF 4 susceptibility explains much of the difference between LMWH and UFH. Thrombin generation in platelet-rich plasma is putatively the best predictor of in vivo antithrombotic action.

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REFERENCES


