Mode of action of low-molecular-weight heparins in plasma and its consequences for the clinical laboratory

Low-Molecular-Weight Heparins in Prophylaxis and Therapy of Thromboembolic Diseases

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Mode of Action of Low-Molecular-Weight Heparins in Plasma and Its Consequences for the Clinical Laboratory

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I. INTRODUCTION

The questions to be posed in heparin pharmacology are the same as those in any other field of pharmacology: what are the relevant concentrations of the acting substance at the relevant site and what are the relevant functions of the body that are influenced by these substances? Then, what is the relationship between these two; i.e., what is the dose-effect relationship? If we admit that heparin acts in the blood and on the clotting system, then physiological chemistry can help pharmacology to answer the following questions: (1) How can we determine the *concentrations* of heparin (or heparins if there appears to be more than one relevant substance)? (2) What is the relevant laboratory parameter to determine the *effect* of these concentrations in a sample of the patient's blood?

Simple and straightforward as this may seem, in the case of heparins the solutions are not immediately apparent. For more than half a century the necessity to treat patients has induced a pragmatic approach, based on the state of the art of many years ago. The introduction of low-molecular-weight heparins (LMWHs) renewed the interest in these matters and clearly showed that the traditional approach was in need of reworking. In recent years, the work of many groups slowly outlined the contours of what might be a rational approach to heparins (1). In this chapter we discuss these contours, aware that we suggest solutions for some of the problems existing in the field, but that have not as yet been extensively tested in practice.

The anticoagulant action of crude tissue extracts had already been observed in the 19th century. It is difficult to decide whether the substances under investigation have been heparins. Many inhibitory substances can be obtained from tissue apart from heparin, such as certain phospholipids (2), excess of procoagulant phospholipid (3), different types of peptides, including fibrin degradation products (4), thrombomodulin (5), Annexin V (6), and so forth. Usually McLean is cited as having discovered heparins, but chances are that he purified a phospholipid and that Howell, in modifying McLean's procedure, may at a given moment have found a polysaccharide with anticoagulant properties (7). Those interested in the early history of heparin are referred to the intriguing article of Jaques (8), who explains how the attempts of Howell to fit his observations in his theory of blood coagulation retarded progress in this field. Best ameliorated heparin production and purification to the extent of making it available as a drug (for the history of heparin discovery and the heparin units see Ref. 9).

Unfractionated heparin (UFH) thus has been available since the 1920s. There consequently is quite some established practice in dealing with these drugs. Howell, for example, defined a unit of anticoagulant material (heparin or not) as that amount of material that would retard coagulation of 1 ml of cat blood so much that it would only half-clot after one night in the refrigerator. Best adopted a similar unit for quantifying his heparin preparations and called it the "Howell unit." This is not only of historical interest because the U.S. Pharmacopoeia Unit (USP unit), which also is used as the international unit (IU) and in many national pharmacopoeias, is barely different from the Howell unit, being defined as that amount of heparin that retards coagulation of 1 ml of sheep plasma enough to make it half-clot after 1 hr at 37°C (8).

Heparin was used as an anticoagulant in the late 1930s. Thus the problems of clinical dose finding have by now been solved by trial and error. The different types of heparin used (e.g., bovine lung, pig mucosa) may indeed be different in many respects. They are, however, sufficiently similar to be compared by the global clotting test used for the determination of the USP unit. When LMWH were introduced as a drug by a trial in 1976 (10), it was natural that they were measured according to the principles established for UFH. These preparations contain material that, with regard to its action of blood coagulation, is comparable to UFH, but also other material that acts essentially different from the classical heparins. Different LMWHs contain these two materials in different proportions. It is therefore not surprising that the convenient correlation between in vitro retardation of coagulation of sheep plasma and in vivo pharmacological action in the human disappeared.

Research in the last few years has made it clear that the traditional way to approach heparin potency is not the most adequate one for this more complicated situation. Indeed, there is a considerable amount of confusion arising from attempts to define LMWHs in terms that were adequate to deal with UFH. In

this chapter we propose a more logical approach to the clinical chemistry of heparins based on recent biochemical data. We will indicate what the differences are with the existing practice and we will show how existing practice can be brought in line with the new approach.

II. TYPES OF HEPARIN

We recall some of the structural data that are important for the understanding of the mode of action of heparin. Heparin is a mixture of linear polysaccharides consisting of alternating iduronic acid or glucoronate units and *N*-acetylglucosamine units joined by glycosidic 1–4 linkages. Most of the *N*-acetyls of the glucosamine are replaced by *N*-sulfo groups. Most iduronates are *O*-sulfated in their 2-position, most glucosamines in their 6-position [Fig. 1, from Barrowcliffe et al. (11)]. This results in a wide variety of different possible monosaccharides. They occur in almost random order, and the chain lengths can vary from 5 to 100 or more. This chemical diversity is reflected in a diversity of biological activities. There is one sequence known to date with a specific function: the pentasaccharide that causes the heparin to bind with high affinity to AT III (12, and references therein). This binding importantly increases the biological activity of the inhibitor. The structure of the active pentasaccharide has been confirmed by organic synthesis (13, 14). Heparins that contain this sequence we call high-affinity material (HAM).* Heparins that do not contain this sequence consequently do not bind specifically to AT III. This low-affinity material (LAM) as such is not active in AT III-dependent reactions but may influence the action of HAM by replacing it in nonspecific reactions (see below).

It appears (15–17) that 17 sugar units is a critical chain length: All the HAM heparins catalyze AT III-dependent factor Xa inactivation, but only the HAM longer than 17 units is capable of catalyzing thrombin inactivation. Heparin thus contains two subfractions: on the one hand, material with a chain length of 5–17 monosaccharide units (MW 1700–5400), which only catalyzes anti-factor Xa inactivation and which we call below critical chain length material (BCLM); on the other hand, material that is longer than 17 monosaccharide units (MW > 5400), which catalyzes both thrombin and factor Xa inactivation and which we call above critical chain length material (ACLM) (Fig. 2). See also Figure 3 for a schematic representation of the MW distribution of different heparins.

As far as its anticoagulant properties are concerned, every heparin preparation, although always highly heterogeneous, can be regarded as composed of the

*1 USP unit of standard heparin is 6 μg of crude product, i.e., about 2 μg of HAM, or 0.15 nmol. 1 U/ml = 0.15 μmol. Due to the uncertainties about the molecular weight and the distribution of the molecular weight of HAM14, these are only approximate values.

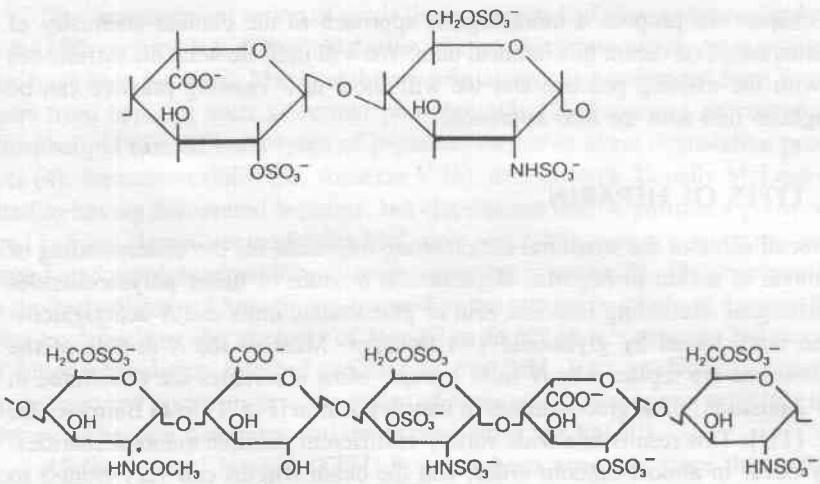

Figure 1 Structure of heparin subunits. (Top) The main repeating disaccharide subunit of heparin. (Bottom) The pentasaccharide sequence with high affinity for AT III. (From Ref. 11.)

three functionally different fractions LAM, ACLM, and BCLM (HAM is ACLM plus BCLM insofar as present). Much of the confusion existing in the field can be avoided if we realize that unfractionated heparin consists of ACLM and LAM, whereas LMWH contains a good proportion of BCLM as well (Fig. 3). It is also essential to recognize that with diminishing molecular weight, the proportion of BCLM and ACLM shifts in favor of BCLM, but also the HAM (= ACLM + BCLM) content decreases (18). As a rule, HAM represents a subpopulation of heparin molecules with a higher mean molecular weight than the total material (19).

Rendering heparin diversity by three different fractions is, of course, both a simplification of the real situation and a complication of current practice. For a logical description of the anticoagulant activity of heparin it seems to be necessary and sufficient.

Apart from anticoagulant action, two other important properties of heparin are dependent on its molecular weight: its affinity for platelet factor 4 (pf4) and its pharmacological behavior. The latter will be treated in a separate paragraph.

Platelet factor 4 is a protein contained in the α-granules of blood platelets and released when platelets are activated. Its physiological function is unknown. It binds to heparin molecules, HAM and LAM alike, with an affinity dependent on the chain length (20). From 10 to 16 saccharide units, the affinity gradually increases; above 16 units (MW ~4800) the affinity is constant and high. This

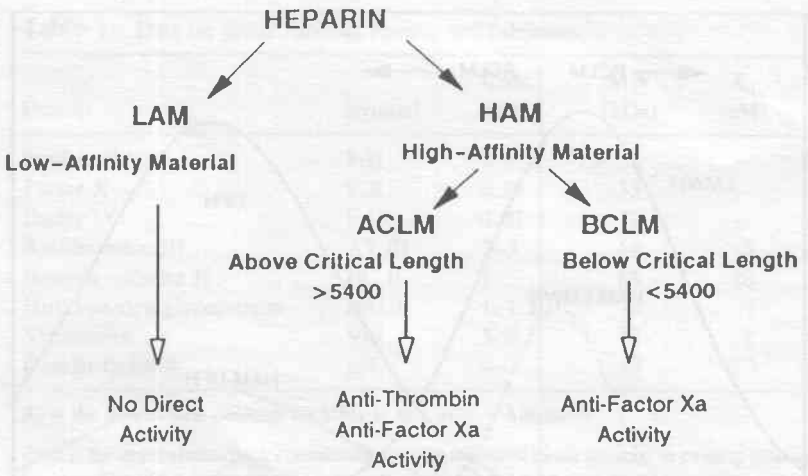

Figure 2 Subdivision of heparin species.

means that all ACLM binds maximally and is effectively neutralized. Half-neutralization requires less than 1 mol/mol, and full neutralization is observed when the ratio of pf4 to heparin molecules is 3:1 (21). For all practical purposes, the binding to ACLM may be considered irreversible; it is so strong that AT III cannot effectively compete with pf4.

BCLM escapes neutralization to a great extent because it binds to pf4 much less tightly than ACLM does. It should be stressed that pf4 binding is not dependent on the specific pentasaccharide sequence; i.e., heparin molecules of the LAM family with a sufficiently high chain length bind as tightly as ACLM (20). One of the ways in which LAM can influence heparin activity in situations where platelets are activated is precisely by their binding to pf4, which then will no longer be able to bind the catalytically active species ACLM. This may explain the potentiating effect of LAM on HAM (22).

The platelet activation that inevitably accompanies venipuncture and handling of blood samples for the clinical laboratory will always cause some pf4 to be released, which then will neutralize a certain amount of ACLM in the sample. Van Putten showed that 0.185 ± 0.072 U/ml of UFH activity on average is lost in plasma samples in the clinical laboratory (23). Apart from that, some pf4 is released from the endothelium after injection of heparin (24).

As heparin is a highly charged molecule, it will bind with a certain affinity to different positively charged plasma proteins, such as histidine-rich glycoprotein (25) and vitronectin, especially after the latter has been modified by thrombin (26–28). This binding is of comparable strength as that of AT III but yet not

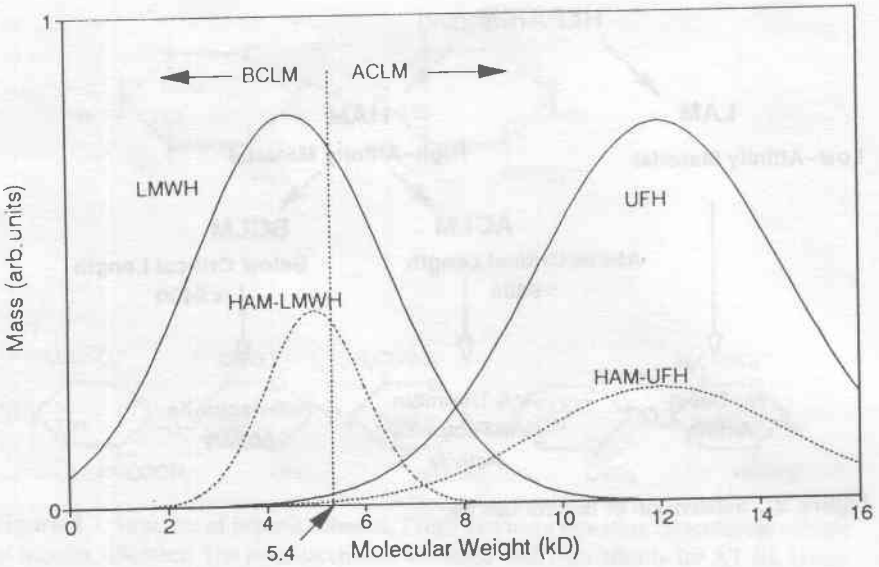

Figure 3 Schematic representation of the molecular weight distribution of different heparin species. (From Ref. 64.)

dependent on the presence of the active pentasaccharide. The HAM in a given heparin will distribute over AT III and other heparin-binding proteins in plasma. The LAM present in that heparin will bind to the other proteins. Both AT III and the other proteins occur in molar excess over heparin in therapeutic and prophylactic dosages (Table 1). The amount of HAM that binds to AT III determines the observed heparin activity. It depends on the partition of the heparin between AT III and the other proteins, which in its turn is determined by (1) the amount and the heparin affinity of AT III as compared to the amount and affinity of the other proteins, and (2) the number of heparin-binding sites on the other proteins that are occupied by LAM. This has two important consequences. First, the amount of heparin bound to AT III will be proportional to the AT III concentration, even though far from all AT III molecules will bind a heparin molecule. This is because the concentration of AT III relative to the concentration of other heparin-binding proteins will determine the probability of a free heparin molecule being bound to AT III (29). Second, LAM will occupy a certain number of binding sites for heparin on the other proteins and so make HAM material available for binding to AT III. This situation may again explain the observation that addition of LAM to HAM increases the effect of the latter (22).

Table 1 Data on Some Clotting Factors and Inhibitors

Protein	Symbol	Conc. (μM)	MW (kDa)	K_d (nM)	Peak (nM)
Prothrombin	F.II	2	66	—	200
Factor X	F.X	0.18	55	—	12
Factor IX	F.IX	0.07	70	—	2
Antithrombin III	AT III	2–3	58	5	—
Heparin cofactor II	HC II	1	65	10	—
Histidine-rich glycoprotein	HRGP	1–1.5	75	7	—
Vitronectin	Vitr.	5–5.5	75	7	—
Platelet factor 4	pf4	—	40	0.3	60

K_d is the dissociation constant for UFH in nM; kDa = kilodalton.

Peak is the approximate peak concentration of the activated factor attained in clotting plasma without heparin. For pf4 it is the amount released into platelet-rich plasma during coagulation.

III. SPECIFIC ACTIONS OF HEPARINS

It is essential to distinguish between *specific* and *composite* biological effects of heparin. The specific effects are its catalytic action on a number of well-defined biochemical reactions. The composite effects are the consequences of the specific effects in composite reaction systems. Enhancement of thrombin inactivation by AT III is one of the specific effects of heparin; prolongation of the APTT and prevention of thrombosis are two examples of composite effects.

The relative contributions of the different specific activities to the various composite effects are not exactly known as yet. There is no reason to assume that one composite action (e.g., the bleeding tendency) will depend on the specific effects (antithrombin activity, anti-thrombin Xa activity) in the same manner as another (e.g., the antithrombotic effect). This is the rationale behind the search for heparins with a more favorable distance between therapeutically active and potentially dangerous doses.

The set of specific activities of heparins is relatively large. All clotting proteases are involved (Kallikrein, Factors XIIa, XIa, Xa, IXa, and thrombin) (30–33) and two different inhibitors (AT III, HC II (34 & 35)). In practice, we do not have to deal with all of them. The contribution of HC II must be considered to be negligible (36,37) except at high concentrations of heparin, such as are seldom encountered (>5 U/ml). The effect of heparin on the inactivation of clotting factors XIa, XIIa, and kallikrein is much smaller than that on the other factors and probably without consequences for heparin action (38). Also, contact activation presumably is only of minor importance in hemostasis and thrombosis (39). These inhibitions therefore need not be considered further. We are thus left

with the inactivation of thrombin, factor Xa, and factor IXa as the important specific actions of heparin.

Not all specific actions are catalyzed by the same type of heparin. There are two different families. Factors IXa and XIa follow the same pattern as thrombin inactivation (40); i.e., they are catalyzed by ACLM. Factor XIIa and kallikrein, like factor Xa, can also be catalyzed by BCLM (40). This division into two groups makes it possible to conveniently discuss the effects of ACLM and BCLM in terms of anti-factor Xa activity and antithrombin activity. A given concentration of ACLM can cause a composite effect because of its action on one or more of any of the clotting factors. It will cause an increase of the decay velocity of thrombin. Therefore, its concentration can be determined by its action on thrombin, and the magnitude of the composite effect will always correlate with the thrombin effect. In a comparable way, any global effect of BCLM will correlate with the anti-factor Xa activity.

This convenient parallelism between specific and composite actions, and therefore between composite actions among one another, has been the basis of the assessment of heparin action. It will, however, not hold as soon as mixtures of ACLM and BCLM appear. Reduction of thrombosis or induction of a bleeding tendency, for example, is an overall effect to which no doubt both ACLM and BCLM contribute, but to a different extent. Also, tests like the APTT are influenced by both ACLM and BCLM. It seems logical to assess the heparin effect in a patient by an overall test such as the APTT. This is tacitly based on the concept that the APTT would be sensitive to ACLM and to BCLM in the same way as thrombosis and bleeding are. This, however, is an unproven assumption. In fact, some substances, such as pentasaccharide and dermatan sulfate, hardly influence the APTT in doses that have a significant antithrombotic effect. Also, the prolongation of the APTT at a fixed dose of heparin is dependent on the individual plasma in which the heparin happens to be present (23).

A fixed prolongation of the APTT can be obtained by *a* ng/ml of ACLM or by *b* ng/ml of BCLM or by an infinity of mixtures of both substances (½*a* of ACLM plus ½*b* of BCLM, etc.). A given antithrombotic effect may again be obtained by *a* ng/ml of heparin in the patient's plasma, but then is not necessarily also obtained by *b* ng/ml of BCLM, but, e.g., by *c* ng/ml of BCLM. There is again an infinity of mixtures that will have the same antithrombotic effect, but this series will *not* necessarily give an identical prolongation of the APTT. This shows that the APTT can be used without problems if we deal with ACLM only (i.e., in UFH) but is no longer useful as soon as mixtures with BCLM appear, i.e., in LMWH. The only way to understand what is indicated by a given prolongation of the APTT is to determine *a* and *b* as in the example above. They will probably differ even between different types of APTT. One can imagine an ideal test that reacts to ACLM, BCLM, and all other anticoagulants in the same

way as the thrombotic tendency does. For the moment we can only prove that the existing overall tests are not suitable candidates.

There is a way out of this dilemma: On basis of the specific actions on thrombin and factor Xa, we can determine the *concentrations* of ACLM and BCLM separately. This opens the possibility of studying pharmacokinetics of heparin directly, without the confusing need to compare one composite effect (e.g., APTT) to the other (e.g., bleeding).

In order to understand the quantitation of heparins on the basis of this catalytic action, we must briefly discuss the kinetics that govern this type of reactions.*

The inactivation of clotting enzymes by AT III in plasma is a bimolecular reaction ($E + A \xrightarrow{k} I$). According to standard chemical kinetics, its reaction velocity is given by $v_t = kA_tE_t$. The concentration of AT III in plasma is much higher than the highest concentration of thrombin, the most abundant enzyme in clotting plasma (AT III: 2.5 μM, thrombin about 0.2 μM at its peak). Therefore, A_t can thus be considered constant during a decay experiment, so that the decay velocity of the enzyme can be given by $v_t = kA_0E_t$, or $v = k_{dec}E_t$, where $k_{dec} = kA_0$.

The time course of E can than be given by $E_t = E_0e^{-kdec\,t}$; i.e., the enzyme decays semilogarithmically. The half-life time is inversely proportional to k_{dec}; therefore, $t_{1/2} = 0.693/k_{dec}$.† The dimension of k_{dec} thus is inverse time, usually expressed in min^{-1}. Heparin catalyzes the above reaction. Although the mechanism of catalysis may be complex (41), its effect is simple: decrease of the half-life time and increase of k_{dec}. The decay constant increases proportionally to the AT III concentration and to the concentration of heparin: $k_{dec} = kAH$. This proportionality can be used to define a unit of heparin activity (see below). It should be noticed that H indicates the concentration of *active* heparin. For the decay of factor Xa, the HAM molecules count; for the decay of thrombin, only the ACLM fraction counts.

Heparins also have a number of biological activities that are not related to their anticoagulant action. They influence the growth of endothelial (42) and smooth muscle (43) cells. They also cause the release of lipoprotein lipase and tissue factor pathway inhibitor (TFPI) (44) from the vessel wall. They are neither specific nor composite effects in the sense discussed above. Here we will restrict ourselves to the effect of heparin on the blood coagulation process in plasma, because that is the basis for the assessment of the heparin effect in the clinical

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*In the reaction equations E stands for enzyme (thrombin, factor Xa, etc.), A is AT III, I is inactive product. In the kinetic equations the same letters stand for the concentrations of these substances. The subscripts denote the time; E_0 is the enzyme concentration at zero time, and so forth.

†Because the natural logarithm of ½ equals −0.639.

laboratory. Also, the effect of nonanticoagulant actions of heparin will be difficult to establish unless the anticoagulant action can be precisely quantified. Insofar as the release effect influences overall thrombin generation, as it surely does via TFPI, it should be measured in tests that are meant to determine the overall effect and it should not influence tests that are used to measure heparin levels (see below).

IV. THE MODE OF ACTION OF HEPARINS ON THROMBIN GENERATION

Anticoagulation is the most effective way of preventing thrombosis (39). All anticoagulants have one thing in common: they diminish the amount of thrombin that is generated in clotting plasma. Occasionally an antithrombotic is announced that has no influence on blood coagulation, such as dermatan sulfate. Invariably, in our experience, such substances do not or hardly influence the APTT, but still they significantly influence the course of thrombin concentration in clotting plasma. Even aspirin need not be an exception (45). It does not seem to matter *how* the appearance of active thrombin is prevented. In principle there are two ways. Either prothrombin conversion is inhibited or thrombin inactivation is accelerated. Pentasaccharide AT III is a pure inhibitor of factor Xa, i.e., of prothrombin conversion (46). Dermatan sulfate, by enhancing the action of HC II, acts only on thrombin decay. Usually, however, the mode of action is mixed. Oral anticoagulation acts because less prothrombin is available for conversion into thrombin and conversion itself is retarded. Heparins act on all clotting enzymes, and until recently it was not clear which of its actions was the essential one. On the basis of the cascade theory of blood coagulation, it has been surmised that inhibition of factor Xa would be a more adequate way to inhibit thrombin occurrence than scavenging the thrombin by enhancing the action of AT III (47). This idea has been motivating the development of the LMWHs. Enhancing the inhibition of factor Xa relative to that of thrombin by decreasing the molecular weight of heparin was thought to be the aim. As we will see, it turned out to be false, and we will see why. Whatever the reasons for the superiority of LMWHs over UFH may be, they are not to be found in their action on the clotting mechanism per se.

The mechanism of thrombin formation is not rendered adequately by a cascade mechanism. It is a very complicated mechanism in which a number of positive and negative feedback mechanisms interact to produce a thrombin explosion that is limited both in place and in time (Fig. 4). The reader is referred to Ref. 39 for details of this mechanism. It is essential to recognize here that the product of the reaction mechanism, thrombin, both enhances and inhibits its own production. The enhancement is immediate by a direct action of thrombin on the accelerating factors V and VIII, whereas the inhibition is dependent on the

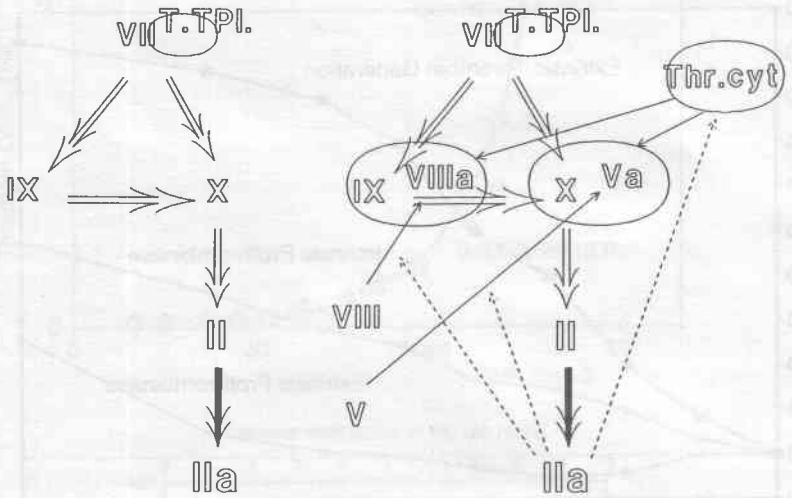

Figure 4 The clotting mechanism. (Left) The proteolytic interactions. (Right) The accelerators and feedback reactions included. Ovals indicate a phospholipid surface. (Open arrows) Proteolytic activation. (Closed arrows) Chemical conversion. (Dashed arrows) Enzymatic action. (Modified from Ref. 65.)

binding of thrombin to thrombomodulin and the activation of protein C. The important consequence for the mode of action of heparin is that accelerated thrombin disappearance in the earlier phases of the reaction will prevent the feedback activation of factors V and VIII (48–53).

We devised methods to obtain thrombin generation curves and to determine the half-life of thrombin with great precision. Via a mathematical procedure we could then, from these data, calculate the time course of prothrombin conversion (29, 54). To our surprise, it appeared that in the extrinsic system the bulk of heparin action must be attributed to its antithrombin activity (Fig. 5). This was true not only for UFH, but also for most LMWHs! It thus appeared that the enhanced inactivation of factor Xa that is undoubtedly caused by UFH and is even more important in LMWHs did not influence the coagulation mechanism very much. The reason for this unexpected finding is that factor Xa does not act as such, but as a member of the prothrombinase complex, a tripartite complex of factors Xa and Va and phospholipid. The member with the lowest concentration determines the amount of prothrombinase that can be formed. Full prothrombin conversion is observed at very low concentrations of prothrombinase: 0.3 nM. The peak concentration of factor Xa is about 12 nM, but factor Va is rate limiting in clotting plasma (51, 55). Factor Xa therefore is present in large excess,

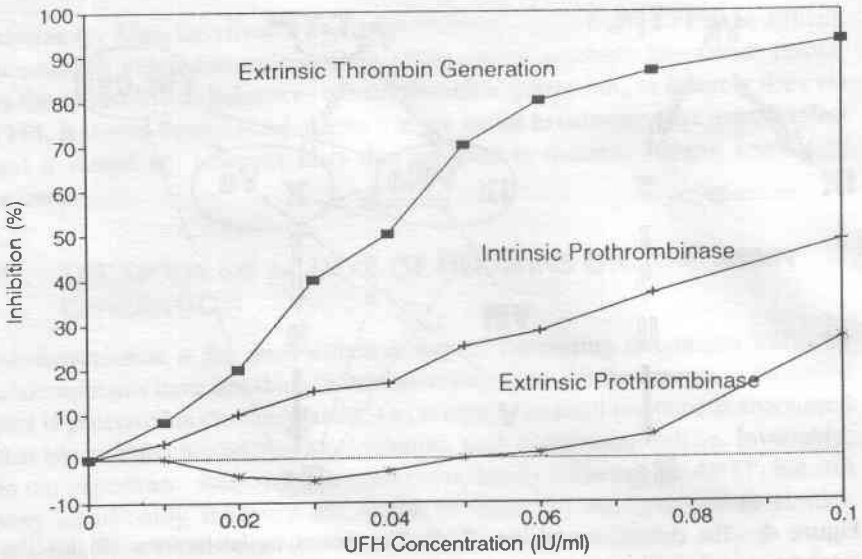

Figure 5 Inhibition of the extrinsic and the intrinsic thrombin and prothrombinase formation by heparin. (Modified from Ref. 53.)

and quite a lot of factor Xa inhibition can take place before prothrombinase is inhibited.

In the intrinsic system the inhibition of prothrombin conversion is more important than in the extrinsic system. This cannot be attributed to inhibition of factor Xa because the role of that factor is the same in the two systems. It is explained by the fact that the feedback activation of factor VIII is more sensitive to inhibition of the thrombin involved than that of factor V. The tissue factor–factor VII(a) complex of the extrinsic system is not inhibited by heparin-AT III. So in the intrinsic system the generation of factor Xa is inhibited but in the extrinsic system it is not. Further studies showed that the lag time of thrombin generation in the intrinsic system is dependent on the amount of thrombin available for the feedback activation of factor VIII and thus is prolonged in the presence of heparin (56). The amount of prothrombin eventually converted into thrombin in the intrinsic system is determined by the action of heparin on factor IXa (56). But the majority of the heparin effect also in the intrinsic system is due to direct inhibition of thrombin via AT III. From these results it is also clear why the APTT is susceptible to heparin influence: This test measures the lag time of thrombin generation in the intrinsic system. It therefore measures the time necessary for enough factor VIIIa to be formed. In other words, it is influenced by the inhibition of minute amounts of thrombin that are formed during the lag phase (Fig. 6).

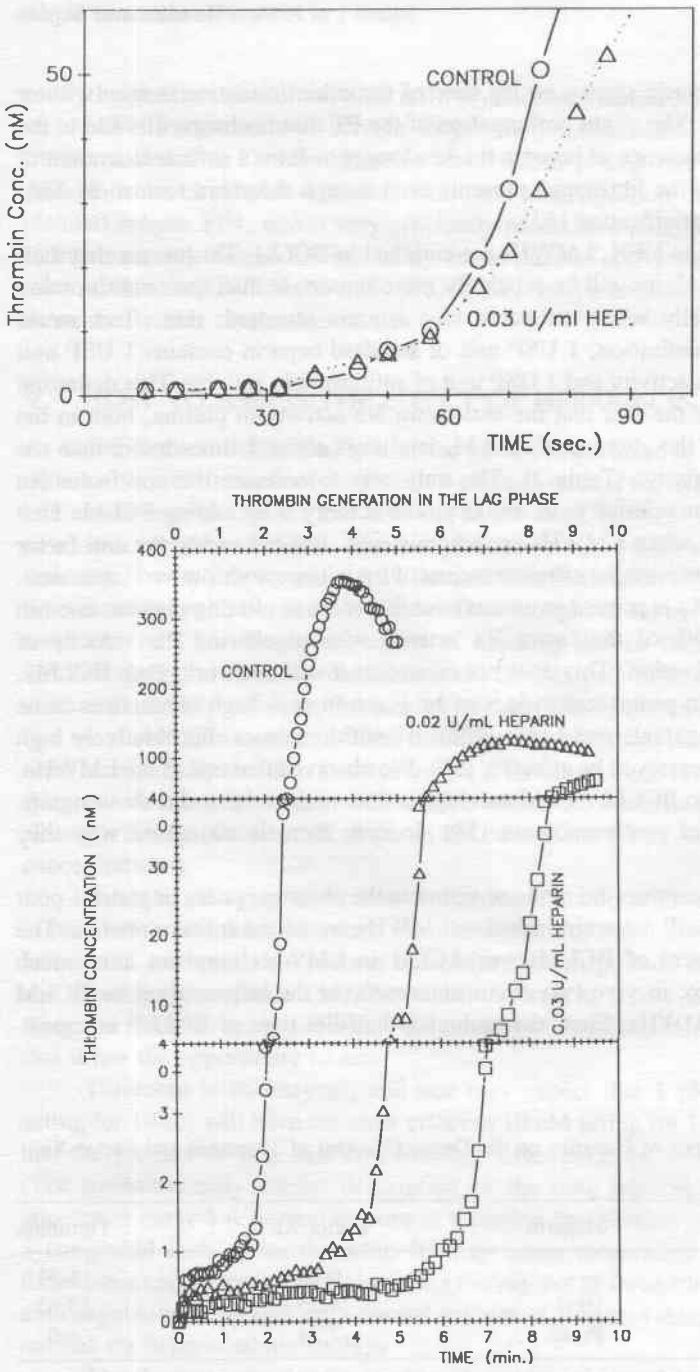

Figure 6 Early phase of thrombin formation in plasma. (Top) Extrinsic system (thromboplastin time). (Bottom) Intrinsic system (APTT). (From Ref. 66.)

In the extrinsic system no lag time of thrombin formation is seen with or without heparin. The slight prolongation of the PT that is observed is due to the fact that in the presence of heparin it takes longer to form a sufficient amount of thrombin to clot the fibrinogen present, even though thrombin formation starts directly after recalcification (53).

Compared to UFH, LMWHs are enriched in BCLM. This means that their anti-factor Xa activity will be relatively more important than their antithrombin activity. Especially when compared to a heparin standard, this effect seems impressive. By definition, 1 USP unit of standard heparin contains 1 USP unit of anti-factor Xa activity and 1 USP unit of antithrombin activity. This definition tends to obscure the fact that the anti-factor Xa activity in plasma, both in the presence and in the absence of ACLM, is always about 3 times lower than the antithrombin activity. (Table 2). The only way to enhance the anti-factor Xa activity in plasma relative to its antithrombin activity is by adding BCLM. That is what happens when LMWHs are administered. But enhancing the anti-factor Xa activity in plasma is an efficient means of inhibiting prothrombin conversion. Because factor Xa is present in excess over factor Va in clotting plasma, one can inhibit some 90% of the factor Xa activity without altering the velocity of prothrombin activation. This does not mean that it will not work. Pure BCLMs, like the synthetic pentasaccharide, can be given in such high amounts as to be efficient anticoagulants and hence efficient antithrombotics, but relatively high amounts are necessary to be effective (57). We observed that one of the LMWHs, in which the ratio BCLM/ACLM was higher than in the others, did show significant inhibition of prothrombinase (58). Indeed, there is no reason why they should not.

Other effects than the mode of action in the clotting system in platelet-poor plasma in vitro will determine whether LMWHs are useful antithrombotics. The relative enrichment of BCLM over ACLM in LMWHs may not have much influence in vitro; in vivo two circumstances favor the influence of the BCLM injected with LMWHs. First, the biological half-life time of BCLMs is signifi-

Table 2 Influence of Heparins on the Decay Constant of Thrombin and Factor Xa in Plasma

	Heparin	Factor Xa	Thrombin
No heparin	—	0.49	1.51
ACLM	UFH	4.25	13.65
BCLM	Penta	7.24	~0

The figures are in min^{-1} per μM AT III (no heparin) or in min^{-1} per μM AT III per μg heparin per ml plasma.

cantly longer than that of ACLMs (60). By this mechanism the BCLM fraction of a LMWH injected will survive longer in the circulation than the ACLM fraction, so that a "fractionation in vivo" takes place. The ratio of BCLM/ACLM in the circulation increases with time after injection. Second, activated platelets release PF4, which very efficiently neutralizes ACLM but not BCLM. Hemostasis and thrombosis inevitably entail platelet activation, hence PF4 release. In this way BCLM, but not ACLM, may escape neutralization at precisely those sites where thrombosis occurs.

V. HOW TO ASSESS THE EFFECT OF HEPARIN IN A PATIENT

As stated in the Introduction, it is of the utmost importance to dispose of a test that ex vivo, on a sample of the patient's plasma, reflects the extent of anticoagulant action in vivo. Such a test does not as yet exist, but here we would like to point to a possible candidate. Current practice provides only indirect measures that may or may not correlate with the antithrombotic effect. The prothrombin time correlates with the antithrombotic effect brought about by oral anticoagulation. The APTT indicates the effect of heparin, and so forth. The fact that different drugs, at effective concentrations, have largely different influences on these tests already indicates that neither of them can be the universal parameter that we should like to obtain. The reason for this is easy to imagine. A clotting time is essentially the time it takes before a thrombin burst takes place (60). It does not necessarily contain information about the magnitude of that burst, although it may of course do so (as in the case of the APTT at varying heparin concentrations).

It can be safely assumed that the enzymatic action of thrombin is essential in hemostasis and thrombosis and that the amount of thrombin that can act in a hemostatic or thrombotic process determines the magnitude of the hemostatic and thrombotic response (39). So what we want to know is the *amount* of thrombin formed—more precisely, the amount of thrombin formed and the time that it has the opportunity to act.

Thrombin is an enzyme, and one may expect that 1 nM of an enzyme acting for 10 sec will have the same effect as 10 nM acting for 1 sec. This means that the product of time and concentration determines the effect of thrombin. (The mathematically correct description is: the time integral of the thrombin generation curve.) A normal amount of thrombin that persists longer because of a congenital lack of antithrombin III may cause thrombosis. A tendency to thrombosis can be remedied by lowering the amount of thrombin formed via oral anticoagulation or by making a normal amount of thrombin disappear faster than normal via heparin administration.

This demonstrates that the product of concentration and time is an important variable. We called it the *thrombin potential (TP)* (61). It is not a new one; the

area under the thrombin generation curve, introduced before 1950 (62), is precisely the same thing. The TP has the dimension of time multiplied by (thrombin) concentration; its value in normal plasma is around 500 nM min (61). It may depend on the experimental conditions, such as intrinsic or extrinsic clotting, but varies little if at all with thromboplastin concentration.

In our experience, all antithrombotics, including those that have little influence on the APTT (e.g., dermatan sulfate), in clinically relevant doses invariably bring about a significant decrease of the TP.

One can think of thrombin-mediated antithrombotic medication as the administration of a drug to decrease the TP value under the threshold limit of thrombogenesis but remaining above the threshold of hemostasis. It remains an open question whether the nature of the drug has an influence on the thresholds. Those who are looking for the ideal anticoagulant, one that prevents thrombosis without inhibiting hemostasis, tacitly assume that this is possible. In our opinion, this is not very likely. We think rather that the differences between drugs such as UFH and LMWH are largely due to different pharmacological properties, which means that they can be maintained more easily in the required range of plasma levels. Secondary circumstances may also intervene. Drugs that act via heparin cofactor II (dermatan sulfate, lactobionic acid) can hardly be overdosed because the plasma level of heparin cofactor II is lower than that of prothrombin, so the TP cannot be inhibited beyond a certain level.

We recently developed an easy way to determine the TP in a single spectrophotometric test (63). In this test an *inefficient* chromogenic substrate for thrombin is added to the plasma, before coagulation is triggered. The thrombin that generates and disappears will continuously convert some of the substrate, but never more than about 10% of the total amount added. In this way the final amount of chromogenic substrate converted is proportional to the concentration-time integral of thrombin.

VI. HOW TO MEASURE HEPARIN LEVELS

It is current practice to express the potency of a given heparin by comparing it to that of a standard preparation in a given clotting test. When heparin is expressed in USP units, it is assumed to be compared to the effect of a standard preparation on the clotting of sheep plasma. The outcome of any clotting test is a *composite* effect of heparin. Different combinations of ACLM and BCLM may add up to the *same final effect* on such a test. A pure BCLM, like the synthetic AT III–binding pentasaccharide, will, just like UFH, prolong the APTT or any other clotting test (except the thrombin time) to any desired length when added in high enough concentrations. Yet a given amount of pentasaccharide, which in a given clotting test is equivalent of a defined amount of standard heparin, will not automatically, in vivo, have the same antithrombotic effect as that amount of

UFH. An equivalent effect of two doses of different LMWHs in one test will not necessarily mean an equivalent effect in another test or in an in vivo situation, because LMWHs are mixtures of two fundamentally different heparins (ACLM and BCLM) that cannot be measured with the same yardstick.

Therefore, the potency of a heparin, assessed by comparison via a clotting test, is ill defined. It depends both on the nature of the standard and on the method of comparing the activities. We calculated, for example, that the activities given on the labels of LMWH vials, which are based on the current method of estimating anti-factor Xa activity (in the absence of Ca^{2+} ions), overestimate the real heparin activity in plasma 1.37–1.76 times. It is therefore necessary to define a new type of unit that allows one to express heparin activity in a standard, method-independent way.

Also, the clotting of sheep blood cannot be used to determine the heparin effect in a sample of plasma from a patient. This makes it compulsory to compare the effect in the patient's plasma to that of a standard preparation. This gives rise to the bizarre situation that LMWH in a plasma sample from a patient is compared via some type of test (e.g., an anti-factor Xa activity test) to the action of UFH in sheep blood in a quite different test. The conditions in these tests can vary so much that the behavior of heparins becomes quite different in each. The presence or absence of Ca^{2+} is often at the basis of confounding results.

The recognition of the specific actions of ACLM and BCLM on anti-factor Xa activity (both) and antithrombin activity (ACLM only) opens the possibility of a more rigorous approach. If a sample contains ACLM, then the decay of thrombin in that sample will be enhanced, or in other words, the k_{dec} of thrombin will be increased. This increase can be measured directly in min^{-1}, independent of any standard. The increase per micromole of AT III in the sample will, independent of any standard or of any method of measurement, be proportional to the amount of ACLM in the sample. The proportionality factor is the specific activity of the ACLM injected. Analogously, HAM activity can be unequivocally quantified by its anti-factor Xa activity and expressed in $min^{-1}/\mu M$ AT III, and again, via the specific anti-factor Xa activity of the HAM injected, in μg of material.

Because the decay constant, k_{dec}, is proportional to the AT III concentration and because heparin increases k_{dec} proportionally to the HAM concentration, we can define a standard, method-independent unit as: *One unit of HAM (or anti-factor Xa) activity is that amount of HAM that, in plasma with* n *μM AT III increases* k_{dec} *of factor Xa by* n min^{-1}.

Analogously we define: *One unit of ACLM (or antithrombin) activity is that amount of ACLM that, in plasma with* n *μM AT III increases* k_{dec} *of thrombin by* n min^{-1}.

It is important to note that the reaction conditions are defined to be as close as possible to the conditions in which heparin acts in vivo: normal plasma, diluted

as few as the circumstances allow, and a normal concentration of free Ca^{2+} (1.15–1.32 mM).

Any LMWH preparation now can be assigned two *specific activities* (S_{ham} *and* S_{aclm}), which are simply the number of (HAM or ACLM) activity-units per unit weight (μg). The specific activity allows the determination of heparin concentrations in plasma. If we encounter a plasma sample in which we measure an anti-factor Xa activity of Z units/ml, then we can calculate the amount of HAM material as being Z/S_{ham} μg/ml. Analogously, from the antithrombin activity (say Q), we may calculate the ACLM content as Q/S_{aclm}. Finally, we can obtain BCLM from HAM minus ACLM.

For the adoption of the new unit, no important changes in everyday laboratory practice are required. Any well-designed antithrombin test is dependent on thrombin breakdown velocity only, hence will measure ACLM. Likewise, good antifactor Xa activity tests will only measure factor Xa breakdown velocity.

The results of such *monospecific* tests can be expressed directly in standard independent units. To this end one should calibrate the test against a k_{dec} ($t_{1/2}$) determination or against a heparin preparation whose potency is known in terms of SI units. This brings back the heparin standard, but this time it is no longer the standard that defines the unit. The standard is now a tool for calibration that can be abandoned by those who prefer to measure k_{dec} ($t_{1/2}$) directly. Hence the definition of the unit is no longer dependent on the standard or on the method by which the standard and the sample are compared.

A suitable ACLM preparation can be used to calibrate both anti-factor Xa tests in terms of SIU Xa and antithrombin tests in terms of SIU IIa. To determine wether the test under observation is indeed monospecific, a BCLM standard can be used. A monospecific antithrombin test will not react to BCLM at all. A monospecific anti-factor Xa test will give the same result with an ACLM and a BCLM standard.

Once the suitable calibrations are carried out, monospecific anti-factor Xa tests can then be used to determine the HAM level in samples (in μg/ml of plasma, if the specific activity of the HAM material injected is known), and monospecific anti-thrombin tests can be used to determine ACLM levels (again in μg/ml of plasma, if the specific activity of the ACLM material injected is known).

VII. CONSEQUENCES FOR HEPARIN PHARMACOKINETICS AND PHARMACODYNAMICS

The most compelling question about LMWHs was whether they were better drugs than UFHs. A number of clinical studies, referred to in other chapters, seem to point in that direction. The next question is how to explain this phenomenon. The bottom line of our biochemical studies on the mechanism of action of

heparins in plasma is that LMWHs are rather more like UFHs than one would like to admit at first sight. Anyhow, as discussed above, the fact that thrombin is inhibited seems to be more important for thrombosis and hemostasis than the mechanism responsible for this inhibition.

Rather than in the biochemistry per se, we think that the difference between LMWHs and UFHs is to be found in their different pharmacological behavior. This makes it compulsory to have exact information about this behavior. A large amount of data is available in this field. Interpretation, however, sometimes poses a problem. It is not always possible to distinguish between effects on the blood levels of active materials and the consequences of these levels for functional clotting tests.

Often the effect of heparin on a blood coagulation test has been investigated rather than the amount of heparin present. The existing data on antithrombin and anti-factor Xa activity levels after heparin injection contain the relevant information. When expressed in international (= USP) (antithrombin or anti-factor Xa) units, they give levels that are proportional to, respectively, the ACLM and the HAM levels. The proportionality factor is different for the two substances, and the BCLM levels cannot immediately be calculated from the difference of the two curves. This is regrettable because the presence of BCLM constitutes the essential difference between UFH and the LMWHs, and the most important differences between the LMWHs are in the ratio ACLM/BCLM. The use of standard independent units and the determination of specific heparin activities enable us to assess separately the levels of ACLM and BCLM in the circulation. This means that we can discuss heparin pharmacokinetics in terms of concentrations of active molecules. Only then can we in a meaningful way discuss the effects of these concentrations on composite phenomena such as clotting times, thrombin formation, thrombus growth, and bleeding tendency. In this way it is possible to separate heparin pharmacokinetics from its pharmacodynamics.

It may be possible that the specific activity, when expressed per mole of active material (i.e., HAM for anti-factor Xa activity), is very similar for highly different heparin preparations. In six UFH and LMWH preparations of different origin, in which the specific activities ranged from 0.71 to 4.25 SIU Xa/μg crude material, the mean specific activity was 87 ± 13 SIU Xa per nM HAM.

In a completely analogous way, we can determine the specific antithrombin activity of a heparin. Again, from the specific activity of the crude material, one can calculate the specific activity per mole of active heparin species, i.e., of the ACLM fraction. It again seems that the specific antithrombin activity of ACLM molecules could be similar for different types of heparin (305 ± 54 SIU IIa per nM ACLM).

The constant specific activity at first sight may seem to contradict earlier findings that reported a gradual change of potency of heparin preparations with

mean molecular weight (see Ref. 11 for a detailed discussion). However, in these publications the activities were expressed per unit weight of crude material, which does not take into account that the amount of active material decreases proportional to the mean molecular weight of the preparation (18). If our conjecture on the identical specific activity of all HAM is true, then the SIU Xa value would indicate the number of active HAM molecules in the sample independent of the type of heparin injected. Analogously, the ACLM level can be obtained from the SIU IIa value.

For this approach it is necessary that the heparin that is injected is well characterized as to its biochemical actions on the clotting system ex vivo.

The following data are minimally required:

1. Content of high-affinity material (% HAM).
2. Distribution of the high-affinity material around the 5400-Da limit, i.e., % ACLM and % BCLM in the HAM.
3. Specific anti-factor Xa activity of the HAM fraction.
4. Specific antithrombin activity of the ACLM fraction. Optionally the specific anti-factor Xa activity of the ACLM and the BCLM fractions may be given.

When these data are known, it is possible to express the results of common antithrombin and anti-factor Xa activity tests in terms of circulating concentrations of active (ACLM and BCLM) heparin. Also, these data will give insight in the as-yet-unsolved question of whether the different LMWH preparations have essentially different activities or differ only in their relative contents of ACLM and BCLM.

Only when a precise description is given of the pharmacology of the anticoagulant substances in UFH and the LMWHs can we start to answer the question of the importance of nonanticoagulant actions of heparin for its antithrombotic effects.

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